EFFECTS OF LOW DENSITY LIPOPROTEIN AND OXIDIZED LOW DENSITY LIPOPROTEIN ON THE MYOCYTE

BY

KAN-ZHI LIU

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Physiology
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

May, 1993

ISBN 0-315-85908-3

Canada

Name _____

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

0	5	6	6

SUBJECT CODE

U·M·I

_____

SUBJECT TERM

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology ... 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology .. 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics ... 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

EFFECTS OF LOW DENSITY LIPOPROTEIN AND OXIDIZED LOW DENSITY LIPOPROTEIN ON THE MYOCYTE

BY

KAN-ZHI LIU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENTS

I want to express my sincere gratitude and appreciation to Dr. Grant N. Pierce, my supervisor, not only for his excellent guidance and encouragement during the course of this investigation, but also for his model example of a perfect scientist, i.e. his great enthusiasm and attitude towards research, his sincere desire to help his fellow man and his harmonious relations with his colleagues. Grant, I owe you too much, I wish I can repay you some day.

Also, I feel that I have received an excellent doctoral training from this division, and would like to acknowledge my graduate committee: Drs. E. Kardami, W.C. Cole, N. Leblanc, and P. Choy for their continuous encouragement and guidance. In addition, I wish to thank the staff in the Div. of Cardiovascular Science for their scientific contributions and friendly support throughout my Ph.D. study. Special thanks and gratitude are due to the members of my lab (past and present), with whom many years of technical help and personal interactions has given me a wealth of memories: Thane Maddaford, Bram Ramjiawan, Dr. Michael J.B. Kutryk, Dr. He-ping Meng, Hamid Massaeli.

The work presented in this thesis was supported by and, therefore, great appreciation is due to the St. Boniface General Hospital Research Foundation, the Medical Research Council of Canada and the Manitoba Health Research Council.

Finally, I would like to express my sincere gratitude to my parents, their efforts and cooperation have certainly made my work easier. A special, sincere heartful thanks to my wife, Bong-Sun, for her emotional support and sustained encouragement throughout my study. Without her consistent support, this work could not have been completed.

TABLE OF CONTENTS

SUMMARY

Despite the importance of cholesterol in heart dysfunction and disease and the critical role of Ca^{2+} in heart function, little is known about the effects of cholesterol on Ca^{2+} movements in the heart. Nothing is known about the effects of low density lipoprotein (LDL), the primary carrier of cholesterol in the circulation, on Ca^{2+} movements in heart muscle cells. Thus, the effects of LDL and oxidized LDL (oxLDL) on Ca^{2+} transients of isolated rabbit cardiomyocytes were investigated. It was found that LDL can induce an increase in the magnitude of the Ca^{2+} transient in isolated cardiomyocytes. This is a relatively slow process. The mechanism appears to involve a stimulation of a transsarcolemmal Ca^{2+} transport pathway. It was also found that oxLDL can induce relatively rapid alterations in the Ca^{2+} transients in isolated cardiomyocytes. The mechanism appears to involve a modification of Ca^{2+} entry into the cell, possibly via the L-type Ca^{2+} channel. Furthermore, fatty acyl chain peroxidation in the LDL moiety appears to be more important than oxidized cholesterol in the generation of oxLDL-induced increases in Ca^{2+} transients in isolated cardiomyocytes. These findings have important implications for cardiac contractile function in hypercholesterolemic and drug-treated hypercholesterolemic subjects. Furthermore, our data suggest that LDL oxidation induced by free radicals may play an important role in influencing cardiac contractile function during pathological conditions like ischemia/reperfusion or hypoxia/reoxygenation challenge.

OxLDL is known to be an important factor in atherogenesis. The oxidative status of isolated LDL and VLDL were investigated in 23 patients with proven coronary disease

and 23 healthy asymptomatic control subjects. Our results demonstrate that LDL from patients with coronary disease have an elevated oxidized cholesterol content and are more susceptible to peroxidative modification. Conversely, the LDL apoprotein does not appear to have been oxidatively modified in these patients. The data are consistent with a role for oxidized LDL in coronary artery disease and indicate that the LDL lipid may be an important oxidation site. Since vascular smooth muscle cells (VSMC) are a source of foam cells in the atherosclerotic plaque, the effects of cholesterol oxidase and oxLDL on cultured VSMC was also investigated. Our results indicate that oxidation of LDL cholesterol can alter lipid deposition in the cell and change VSMC morphology to resemble foam cells. The oxidation of LDL cholesterol in vivo may play an important role in the modification of LDL which could contribute to the generation of the lipid-laden foam cells and an atherogenic plaque.

LIST OF FIGURES

Figure 11. Representative recordings of calcium transients in control cardiomyocytes (A), cardiomyocytes treated with 0.1 mg cholesterol/ml oxidized LDL (oxLDL) (B) or 1 mg oxLDL cholesterol/ml (C).

Figure 12. Representative recordings of calcium transients in the cardiomyocytes after treatments with 0.3 mM DHF (A) or 3 mM DHF (B) where $FeCl_3$-ADP was maintained at 1 mM and 0.5 mM respectively.

Figure 13. Representative recordings of calcium transients after exposure to 4 μM nicardipine (arrow) in a control cardiomyocyte (A) or a cell treated for 20 minutes with 0.1 mg oxLDL cholesterol/ml (B).

Figure 14. The effect on calcium transients (systolic $[Ca^{2+}]$) of nicardipine in the absence (■) or presence (o) of 0.1 mg/ml oxLDL pretreatment of cardiomyocytes.

Figure 15. The effects of varying the oxLDL concentration on the alteration of calcium transient and MDA production.

Figure 16. The effects of varying $FeCl_3$ concentrations on the MDA production and change of systolic calcium concentration in cardiomyocytes.

Figure 17. The effects of lazaroid on MDA production in oxLDL and the change of systolic calcium concentration in cardiomyocytes.

Figure 18. Representative recordings of calcium transients in cardiomyocytes treated with LDL oxidized by C.O. ± catalase

Figure 19. The effects of varying the oxLDL concentration on the cellular calcium transient and the LDL MDA content.

Figure 20. The effects of catalase and other antioxidants on MDA production in oxLDL.

Figure 21. Representative recordings of calcium transients in the cardiomyocytes after treatment with C.O. ± catalase.

Figure 22. Representative HPLC recordings of cholesterol species in cardiomyocytes ± cholesterol oxidase treatment.

Figure 23. Representative recordings of calcium transients in myocytes after exposure to different H_2O_2 concentration and oxLDL oxidized by 0.1 mM H_2O_2.

Figure 24. Average time to contracture in myocytes exposed to varying H_2O_2 treatments.

Figure 25. Representative HPLC recording of cholesterol species in LDL from a healthy, asymptomatic subject (A) and a coronary disease patient (B).

LDL pre-incubated with cholesterol oxidase.

LIST OF TABLES

INTRODUCTION

Cholesterol is a common but important constituent of all membrane systems within the myocardial cell. It alters the general physical properties of the membrane by conferring structural stability and rigidity to the phospholipid bilayer (139). However, its function in the membrane does not appear to be a simple or static one. Strong evidence exists that cholesterol is arranged in membranes asymmetrically (315,316) and can form cholesterol-rich domains around proteins embedded in the membrane (139,258). These cholesterol interactions with membrane proteins can alter ion movements in various tissues. For example, in vitro methods of altering membrane cholesterol levels have been shown to affect activities of the kidney fibroblast Na^+ pump (317), skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} pump (274) and erythrocyte Ca^{2+} channels (318).

Because Ca^{2+} is critical in determining the contractile state of the heart, the role of any potential modifier of myocardial Ca^{2+} flux (like cholesterol) is worthy of extensive investigation. Previous studies (258,291,319) have employed liposomes to enrich or deplete cholesterol levels in isolated sarcolemmal membranes. The effects of cholesterol varied depending upon the transport protein examined. For example, cardiac Na^+/Ca^{2+} exchange was stimulated by cholesterol enrichment (258) whereas the sarcolemmal Ca^{2+} pump was inhibited (258) and K^+-pNPPase activity and passive Ca^{2+} binding were unaffected (258). Despite this information, we still do not know the net effect that cholesterol incorporation has on cellular Ca^{2+} transients in whole ventricular cells. Further, we have no information on its effect on ion transport when the lipid is presented to the cell in the more physiologically relevant form of low density lipoprotein (LDL). Thus,

the purpose of the first part of my study was to determine the effect of the primary carrier of cholesterol, LDL, on cellular Ca^{2+} transients.

It is now clear that LDL can be oxidized through non-enzymatic (proteoglycans,immune complexes, free radicals) as well as enzymatic (lipase, oxygenase) modification (88,112,329). Under these circumstances, the three major parts of the lipoprotein moiety (apoprotein, fatty acyl chain and cholesterol) can be oxidized. (88,329). If the fatty acyl chain of phospholipids and triglycerides present in the LDL is oxidized extensively, a broad spectrum of shorter-chain aldehydes (conjugated diene, malondialdehyde and 4-hydroxynonenal) is generated (88,330,331). The cholesterol in LDL can also be oxidized into several cholesterol oxide derivatives (114,332,344). OxLDL has been identified in the subendothelial space of artery (106,326,327,334). A growing body of evidence suggests that the oxidative modification of LDL which occurs in vivo may play an important role in the process of atherogenic disease (88,320,99). For example, oxidized LDL is able to recruit monocytes and induce foam cell formation through an accelerated lipid influx into the cell (88,321,322). The effects of oxLDL are relevant to cells other than just those in the vascular system. It is clear that a relatively high concentration of LDL exists in the interstitial space in the heart and, therefore, cardiomyocytes would be exposed to this LDL concentration (323). It is also increasing evident that oxygen-derived free radicals are present in this extracellular space (324,325). When free radical generation increases under conditions like ischemia/reperfusion or hypoxia/reoxygenation (324,333), the potential increases for the generation of oxLDL in the extracellular fluid in contact with cardiomyocytes. OxLDL has been identified in the subendothelial space (106,320, 326,327).

Thus, the potential exists for an effect of oxLDL on some aspect of the function of cardiomyocytes. The effects of oxLDL on the Ca^{2+} transient in isolated cardiomyocytes are not known. However, it has been shown that cholesterol, when it is oxidized in situ in myocardial sarcolemmal membrane vesicles, has significant effects on both passive and active transarcolemmal Ca^{2+} flux (282). Furthermore, one recent study demonstrated that perfusion of isolated arterial segments with oxLDL resulted in enhanced agonist-induced vasoconstrictions which were the result of a direct action on the vascular smooth muscle (328). The authors suggested (but did not test) that oxLDL may be modulating vascular contractility via an effect on transsarcolemmal Ca^{2+} flux.

Since oxLDL concentration may increase in the heart during ischemia/reperfusion or hypoxia/reoxygenation when free radical production increases, it is important to determine the effect of oxLDL on the myocardial Ca^{2+} transient. The Ca^{2+} transient is a cellular reflection of cardiac contractile performance. However, it is important to note that although there is a close relationship between the Ca^{2+} transient and force generation in a myocardial muscle cell, in some pathological some cases this relationship can dissociate. Ca^{2+} overload is a common pathological event during ischemic/reperfusion injury and other disease states in the heart. The mechanism responsible is not clear but may involve a complex combination of enzymatic alterations and membrane structural changes. The second part of my study was, therefore, undertaken to examine the effect of oxLDL on the Ca^{2+} transient of isolated rabbit cardiomyocytes.

At present, it is difficult to evaluate which lipid in the oxLDL moiety plays the most important role in altering the calcium transient in isolated cardiomyocytes. In the third part of this study, cholesterol oxidase (3-ß-hydroxy-steroid oxidase), which catalyses the oxidation of cholesterol to 4-cholesten-3-one and other oxidized cholesterol derivatives, was chosen

to specifically oxidize the cholesterol in the LDL. Thus, our objective was to examine if oxidized cholesterol within the modified LDL is critical for producing the effects of oxLDL on the cardiomyocyte Ca^{2+} transient.

If oxidized LDL is an important factor in atherogenesis, it is reasonable to postulate that its circulating concentration may be higher in patients suffering from coronary artery disease. The oxidative status of circulating lipoprotein in patients with coronary artery disease is still unknown. The purpose of the fourth part of this study was to investigate if oxidized lipoproteins were more prevalent in patients with severe coronary artery disease as compared to asymptomatic, healthy individuals. In addition, it was of interest to examine which of the three major parts of the lipoprotein moiety (apoprotein, cholesterol, fatty acid chains) may be more sensitive to oxidative modification during atherosclerotic heart disease.

In order to determine the mechanism for the atherogenic effects of oxidized LDL in the vasculature, it has become important to identify the site of oxidation on the LDL moiety. The oxidized site in the LDL may be the apoprotein or the fatty acid moiety or the cholesterol (15,88,101,320). Peng and colleagues (335,336) examined the effect of oxidized cholesterol on cultured smooth muscle cells by adding oxidized derivatives of cholesterol directly to the incubation medium. Their results demonstrated that autooxidation products of cholesterol were strongly cytotoxic whereas purified cholesterol at the same concentration produced no such effects. On the basis of these cytotoxic effects of oxidized cholesterol, the authors concluded that oxidized cholesterol may be involved in atherosclerosis (335,336). However, because the cholesterol derivatives were presented freely in suspension in these studies (335,336), it was likely to be interacting in a non-specific manner with the cell membrane, which makes it impossible to determine if the effects were due to an intracellular action of the oxidized cholesterol (337) or due to a general disordering of the

cell plasma membrane (338). Thus, the cytotoxicity as well may be a function of the mode of presentation of the oxidized cholesterol to the cell. Therefore, to avoid some of these uncertainties, cholesterol oxidase was chosen in the fifth part of my study to investigate the effect of oxidation of in situ cholesterol on viability, morphology and lipid deposition in cultured aortic smooth muscle cells. The sixth part of my study was designed to observe if LDL cholesterol could be oxidized and, if so, what its in vitro effects were on cultured vascular smooth muscle cells. Cholesterol oxidase was employed to investigate the effect of oxidized LDL cholesterol on vascular smooth muscle cells.

B. REVIEW OF THE LITERATURE

PART ONE: LDL, OXIDIZED LDL AND ATHEROSCLEROSIS

I. The Pathogenesis of Atherosclerosis

Cardiovascular disease is still the major cause of death in North America and western Europe. The majority of these deaths are due to myocardial or cerebral infarction, atherosclerosis being the principal cause (1).

The lesions of atherosclerosis have been observed in humans for centuries and were known to the 19th century European pathologists, who speculated about its origin. The generic term "arteriosclerosis" for scarring and calcification of arteries was coined by Lobstein in 1829, and the distinctive type of arteriosclerosis characterized by lipid-rich deposits was named "atherosclerosis" by Marchand in 1904. The clinical syndrome of angina pectoris had been described by Heberden in 1772, and myocardial infarction by Weigart in 1880 (2). James Herrick, a Chicago cardiologist, linked the clinical signs and symptoms of myocardial infarction with atherosclerosis and thrombosis of the coronary arteries in 1912 (3). Coronary artery disease was introduced into the international list of causes of death in 1930 (4).

a. Pathology of atherosclerosis.

Atherosclerosis, a specific form of arteriosclerosis, is primarily an intimal disease characterized by fibrous (fibrolipid, atheromatous) plaques. The major types of atherosclerosis lesions are: a) fibromuscular intimal thickening; b) fatty streaks; c) fibrous streak; and d) complicated lesions (5-7).

Fibromuscular intimal thickening. Diffuse fibromuscular intimal thickening of

arteries that develops with age has been viewed as an integral part of the atherosclerotic process or at least a requisite change for the development of atherosclerosis (8). There has been considerable debate about whether this thickening represents a normal anatomic structure peculiar to the coronary arteries, or whether it is an early stage of atherosclerosis. Most evidence suggests that it is a normal structure, because it appears in the coronary arteries of persons from all population groups, regardless of their disposition to atherosclerosis in later life (9-11).

The fatty streak. The first clearly recognizable changes of atherosclerosis appear in the intima and inner media of affected arteries as scattered deposits of lipid in smooth muscle cells, interstitial spaces, and macrophages (4,7). Most often the lipids, being predominantly cholesterol and its esters, are present in closely packed foam cells immediately beneath the endothelium or in elongated fat-containing cells scattered within the intima. In both human and experimental models, the foam cells have been shown to be derived from either smooth muscle cells (12) or macrophages (13). It is not surprising that not all fatty streaks necessarily progress into advanced atherosclerotic plaques (14,15). Since the intimal macrophage system protects the arterial wall against the cytotoxic effects of the anionic oxLDL particles, theoretically, the fatty streak can be considered an almost physiologic event (16). When the level of plasma LDL is elevated, the amounts of intimal LDL available for oxidation are increased, and the oxLDL is taken up relentlessly by the non-down-regulating macrophage scavenger receptor. Therefore, the rate of foam cell formation and the genesis of the fatty streak, are dependent upon the dynamic balance among plasma and intimal LDL concentration, monocyte-macrophage recruitment, the oxidative (free radical) potential of the arterial intima, and the HDL mediated reverse cholesterol transport system (17,18).

The fibrous plaque. The term fibrous plaque refers to the gross morphologic appearance of the lesion that is the hallmark of atherosclerosis (19,20). This is the lesion that causes narrowing of the artery, predisposes to thrombosis, calcifies, and leads to weakening of the muscle and aneurysmal dilation. The fibrous or atheromatous plaques are rounded, raised lesions, usually off-white to white in colour superficially, and perhaps a centimetre in diameter (4,7,21,22). The centre of larger plaques may exude a yellow, grummous fluid, hence the name atheroma (Greek for gruel). Plaques exhibit histologic variability, but a typical cellular plaque consists of: i) a fibrous cap, composed mostly of smooth muscle cells with a few leukocytes, and relatively dense connective tissue containing elastin, collagen fibrils, proteoglycans, and basement membrane (23,24); ii) a cellular area beneath and to the side of the cap consisting of a mixture of macrophages, smooth muscle cells, and T lymphocytes (25); and iii) a deeper "necrotic core" which contains cellular debris, extracellular lipid droplets, cholesterol crystals, and calcium deposits. This necrotic core often contains numerous large foam cells of both the macrophage and smooth muscle origin.

One of the most controversial aspects of the pathogenesis of atherosclerosis is the relationship of childhood fatty streaks to adult fibrous plaques. Some hypotheses of etiology and pathogenesis, such as the mutagenic hypothesis (32), suggest that fibrous streaks arise by a process independent of fatty steaks. In support of the relationship, however, many lesions in the arteries of young adults are intermediate between fatty streaks and fibrous plaques in their histologic and chemical characteristics (25-28). Furthermore, within the coronary arteries, the sites of preference for fatty streaks in children also are the sites where fibrous plaques form in adults (28).

Complicated lesions. Complicated plaques develop from preexisting fibrous plaques

as a result of one or a combination of several pathologic changes including calcification, ulceration, thrombosis, and hemorrhage. The complicated lesion is the most common type of atherosclerotic lesion that produces significant circulatory change and clinical disease (5-7).

b. Current concepts of pathogenesis.

Because atherosclerosis is a slowly progressive disease which begins in childhood and does not become manifest until middle-age or later, its etiology and pathogenesis have been difficult to elucidate. Yet in the past decade or two, cellular and molecular approaches to the study of cells of the vascular wall, as well as innovative methods for examination of abnormalities in lipid metabolism, have provided numerous insights into the pathogenesis of atherosclerosis (21,22,29-36).

There are several hypothesis concerning pathogenesis of atherosclerosis currently, such as the inflammatory hypothesis (21,22), response to injury hypothesis (29-31), monoclonal hypothesis (32) and lipid infiltration hypothesis (33-36). Each recognizes the roles of lipids and lipoproteins, inflammatory cells (monocytes/macrophages), arterial smooth muscle cells, endothelial cells, and platelets.

1. The inflammatory hypothesis (21,22) of atherogenesis is based upon the demonstrated attachment of mononuclear cells to the endothelium (recruitment) and the accumulation of plasma constituents in the intima. In support of this hypothesis, many important aspects of the inflammation process have been identified. For examples, chemoattractants for mononuclear cells have been identified from different sources, monocyte/macrophage migration into (or out of) the intima is proven, the monocyte origin of some plaque foam cells is established, the oxLDL receptor in macrophage has been

identified and growth factors that stimulate smooth muscle cell proliferation can been secreted in many different cells.

2. **The response to injury hypothesis,** based upon the early proposals made by Virchow (29), was formulated by Ross and Glomset (30-31). This hypothesis was based on the following important experimental observations: (a) that smooth muscle proliferation and lesions resembling atheromatous plaques can be induced in experimental animals by endothelial denudation (such as with a balloon catheter). The development of these lesions can be accentuated by hypercholesterolemia, or by subtle changes in function (endothelial dysfunction); and (b) PDGF (platelet-derived growth factor) and other growth promoting (and growth inhibitory) factors derived from different cell types such as macrophages and endothelial cells, may regulate smooth muscle growth. The initial hypothesis postulated that injury to arterial endothelium by mechanical, chemical, toxic, viral, or immunologic agents caused endothelial denudation, and was followed by platelet adhesion and aggregation, with consequent release of PDGF and other growth factors, in turn leading to migration into and proliferation of smooth muscle cells in the intima and secretion of connective tissue components.

3. The proliferative nature of atherosclerosis also is stressed in the **monoclonal (mutagenic) hypothesis** proposed by Benditt (32). Benditt suggested that each fibrous plaque begins with proliferation of a single, genetically transformed smooth muscle cell. The hypothesis is based on the finding that fibrous plaques from black females who were heterozygotes for glucose-6-phosphate dehydrogenase (G6PD) frequently contained only one of the two G6PD isoenzymes. In these women, normal arterial and other tissues should be heterozygous for G6PD isoenzymes (mosaicism) because of random inactivation of one X chromosome in embryonic life. The finding of monotypism of the smooth muscle cells of the

plaques indicated their probable origin from a single cell. This is similar to the known monoclonal nature of uterine leiomyomas, which presumably arise by mutation. Known mutagenic agents include radiation, some viruses, some chemicals in cigarette smoke, and some cholesterol metabolic products that are carried by the lipoproteins.

4. Very recently, a large body of evidence has supported the important role of LDL and oxLDL in the pathogenesis of atherosclerosis (33-36). Therefore, **the lipid infiltration hypothesis**, modified from the response to injury theory, was proposed upon the following studies: (a) that the fatty streak lesion, which antedates the fibrous plaque, actually develops under a structurally intact endothelial surface (37,38); and (b) the dominant fat-laden cells in the early lesions of experimental atherosclerosis, once thought to be primarily of smooth muscle cell origin, are now recognized to be mostly of monocyte/macrophage origin (39,40). This is also true for some human lesions, as shown using monoclonal antibodies specific for monocyte-associated antigens (41). Therefore, in the lipid infiltration hypothesis, key initial participants in the focal arterial lesion-prone sites were emphasized at the focal intimal influx and accumulation of LDL and a preferential recruitment of blood monocytes. Both are further enhanced in the presence of hyperlipidemia, when the quantity of intimal LDL and the oxidative potential of the intima exceed the capacity of macrophages to remove, via the non-down-regulating scavenger receptor, cytotoxic anionic oxLDL macromolecules. Foam cells, pathognomonic of the fatty streak, form during the receptor-mediated uptake of oxLDL by the macrophages and/or smooth muscle cells. Interstitial free radicals and the excess of oxLDL particles injure and kill cells, including the foam cells, with the formation of the necrotic extracellular lipid core, a key transitional step in lesion progression.

A summary of pathogenesis of atherosclerosis could be drawn from the above concepts (21,22,29-36). Hyperlipidemia, or some component of hyperlipidemic serum, as well

as other risk factors, are thought to cause endothelial injury, resulting in adhesion of platelets and/or monocytes and release of PDGF (and other growth factors), which leads to smooth muscle migration and proliferation. It is clear that endothelial injury need not be denuding, and in fact may consist of altered endothelial function. Adhesion of monocytes, increased permeability of endothelium, and disturbances in growth control can occur without morphologically obvious endothelial injury. Hyperlipidemia, hypertension, oxLDL, free radicals, immune injury, and other risk factors may contribute to this endothelial dysfunction in different ways and sometimes in combination. Smooth muscle cells produce large amounts of collagen, elastin, and proteoglycans and these form part of the atheromatous plaque. Foam cells of atheromatous plaques are derived from macrophages and from smooth muscle cells. The mechanisms of foam cells formation from macrophages may be via the LDL receptor and more possibly, by way of LDL modification, recognized by the acetyl-LDL receptor (such as oxLDL). However, the mechanisms of how smooth muscle cells become foam cells are less certain. Smooth muscle cell proliferation may occur without endothelial injury but through other several postulated mechanisms such as loss of growth control, direct smooth muscle injury (by LDL or oxLDL), and autonomous proliferation by the mechanisms suggested by Benditt.

c. Risk Factors for Atherosclerosis.

Over the last decade it has become clear that alleviation of certain risk factors leads to a reduction in the morbidity and mortality from atherosclerosis (42). There is also evidence from the treatment of hypercholesterolaemia that regression of an atherosclerotic lesion may occur once those conditions which favoured its development are removed (43,44). Therefore, it is important to clarify the existing risk factors for atherosclerosis and it should

be kept in mind that the important risk factors - hyperlipidemia, hypertension, and smoking affect more than one step in atherogenesis, including the function of any of the cell types involved and the constituents of the plasma which interact with the vessel wall (45).

1. Age, male sex, family history and homocystinuria are **unmodifiable risk factors** (6). Although the natural history of atherosclerosis is most often an age-related phenomenon (46), atherosclerosis is not simply the result of unmodified, intrinsic, biologic aging processes. Some populations age without showing clinical evidence of atherosclerosis, and most mammalian species age without spontaneously developing atherosclerosis. Although aging appears to play a role, particularly in relation to the changes in cells of the arterial wall, atherosclerosis can best be considered an age-related disease that can be influenced by both environmental and genetic factors.

The greater risk of coronary heart disease in men than in women is well established, but the reason for it is poorly understood (47,48). Atherosclerotic coronary heart disease is predominantly a disease of men, especially at younger ages; the prevalence in men at the fourth decade is three times that in women (47). Possible explanations for the sex differences include levels of estrogenic hormones and higher levels of high-density lipoprotein, which is known to be antiatherogenic (49,50), in premenopausal women than in men.

Premature atherosclerosis is often familial. This is frequently due to the inheritance of risk factors such as hypertension, diabetes mellitus and hyperlipidaemia, but some families can be found where no known risk factor is apparent. Homocystinuria, when due to cystathionine beta-synthase deficiency, is associated with premature atherosclerosis (51).

2. Partially/potentially correctable risk factors are hypercholesterolaemia, hypertriglyceridaemia, low HDL level, hypertension, and diabetes mellitus.

A directly proportional relationship between the level of serum cholesterol and the risk of atherosclerotic coronary heart disease (CHD) has been repeatedly established in epidemiologic, clinical, and pathologic studies (52-54). There is a positive correlation between the level of cholesterol carried by LDL and the risk of CHD (55). This relationship between cholesterol level and risk of atherosclerosis holds for all values of cholesterol including those within the so-called "normal range". The risk rises steadily with increasing cholesterol until the total cholesterol level is 6.0-6.5 mmol/litre and then rises sharply (53,54).

The status of plasma triglyceride concentrations as a risk factor for atherosclerotic disease remains uncertain (50). Hulley et al concluded that hypertriglyceridemia was not an independent risk factor after reviewing the epidemiological evidence (56). However, subsequent evidence indicated that this conclusion was not shared by all investigators. The concentrations of triglyceride in plasma might be important in subsets of the population, as indicated by a National Heart, Lung, and Blood Institute Workshop (57). Evans et al suggested that hypertriglyceridaemia is positively correlated with an increased risk of CHD (6). However, it is negatively associated with HDL-C (high density lipoprotein cholesterol) level and if this is taken into account the correlation between hypertriglyceridaemia and CHD usually disappears (6).

Many epidemiologic investigations reveal a strong negative correlation between rates of CHD and the HDL-C level both in humans and experimental animals (58,59). This may be related to the postulated role of certain HDL subfractions in " reverse cholesterol transport", i.e. the transport of cholesterol from the peripheral tissues to the liver for excretion (60).

Hypertension emerged from the early epidemiologic studies of CHD as a strong

predictor of CHD, and it has been shown repeatedly to be associated with accelerated progression of atherosclerosis in both humans and experimental animals (58,61). Blood pressure tends to increase with age. This might be related to changes in physical activity or to dietary factors. The atherogenic mechanism of hypertension may be related to increased workload on arterial smooth muscle, mechanical injury to the arterial wall, or increased filtration pressure and increased permeability of the endothelium.

Diabetes mellitus contributes importantly to all manifestations of atherosclerotic diseases, including CAD, peripheral disease, and cerebrovascular disease. Although other risk factors, particularly elevated blood cholesterol levels, high blood pressure, obesity, and low plasma levels of HDL, are significantly related to diabetes mellitus, these factors do not completely explain the added risk of diabetes mellitus (7,62,63).

3. Totally correctable risk factors are cigarette smoking, obesity and physical inactivity. The increased risk of atherosclerosis is proportional to the amount smoked (64-66). Given that cigarette smoke is a very complex mixture of chemicals, and given the many physiological responses to cigarette smoke inhalation, probably several mechanisms will affect the cardiovascular and hemostatic system (65,66). For example, cigarette smokers have lower concentrations of HDL cholesterol in plasma than do nonsmokers. Smokers have increased leukocyte counts and fibrinogen content in their plasma, both of which may predispose to thrombosis on the surface of an atherosclerotic plaque. In examining peroxidation of LDL in cigarette-smoking subjects, Harats et al demonstrated that cigarette smoking renders plasma LDL more susceptible to subsequent in vitro peroxidative modification by a free radical generating system (132).

Life insurance data suggested that marked obesity (greater than 130% ideal body weight) is a risk factor for atherosclerosis (67). Physical activity appears to have a protective

influence. This might be brought about in several ways. Acute exercise has little effect on HDL levels, but prolonged training leads to an elevation in HDL and a decline in total cholesterol, LDL-C and triglyceride levels (67). Training also has beneficial effects on carbohydrate metabolism and on blood pressure. It will also reduce the tendency for obesity itself (67).

II. The Involvement of Lipoproteins in the Atherogenesis

To understand the complexity of lipoprotein metabolism and its influence on atherosclerosis, one must be aware of the physiologic characteristics and function of the different lipoprotein classes, apo-lipoproteins and enzymes.

a. Lipoprotein classes.

There are five major classes of lipoproteins in the blood-stream carrying water-insoluble lipids (68,69). These lipoproteins are named either by their density or by their electrophoretic mobility. Generally speaking, all the lipoproteins have 2 major components: the inside core, including triglycerides and cholesterol esters, and the surrounding surface of more polar lipids - unesterified cholesterol and phospholipids -apolipoproteins. The surface coat, whose lipids provide a covering structure, serves as an interface between the aqueous plasma and the inner nonpolar lipid core. It is this polar surface that makes possible the transport of the highly insoluble cholesterol esters and triglycerides in plasma.

Chylomicrons, with a density of approximately 0.98 g/ml, ranges from 1000 to 4000 Å in diameter. Several apoproteins are found in the surface covering such as Apo A series (A-I, A-II, and A-IV), Apo B-48, Apo C and Apo E. The function of chylomicron is to transport exogenous lipid from the intestine to the liver and peripheral cells. Being

synthesized in intestinal mucosal cells, these lipoproteins are composed largely of triglycerides derived from dietary fat. When chylomicrons enter the peripheral circulation, they come into contact with the enzyme lipoprotein lipase, which is located on the surface of capillary endothelial cells of those tissues which either store or oxidize lipids. This enzyme hydrolyzes the triglycerides of chylomicrons to free fatty acids, which are then taken up by adipose tissue and muscle cells. Then, the chylomicron remnant, formed after most of the triglycerides in the chylomicron have been hydrolyzed, returns to the circulation and is removed rapidly by the liver.

Very low-density lipoproteins (VLDL), produced primarily in the liver, are responsible for the transport of endogenously produced lipids. The diameter of this triglyceride - rich lipoprotein ranges from 400 to 700 Å with a major structural apoprotein B-100, but it also contains apo C's (C-I, C-II, C-III) and apo E. Mature VLDL interact with lipoprotein lipase on the surface of capillary endothelial cells, and fatty acids are released into the circulation. Moreover, phospholipids, most apo C's, and some apo E's leave the surface coat of VLDL and are transferred to HDL. The VLDL remnants can have two fates: they can be taken up by the liver or transformed into LDL. Normally, 60% to 70% of VLDL remnants are removed directly from the circulation by liver cells, through the LDL receptors (72). 30% to 40% of all VLDL remnants are converted to LDL.

Intermediate-density lipoprotein (IDL), normally are not synthesized directly but are the metabolic by-products of chylomicron and VLDL metabolism. Its density is between 1.019 - 1.063 g/ml. The function of IDL is not well known.

Low-density lipoprotein (LDL), the major cholesterol-transporting lipoproteins, transport cholesterol and phospholipid to the peripheral cells. LDL cholesterol also serves as a precursor for steroid production in the endocrine organs. They have a density of 1.019

- 1.063 g/ml. They are not normally synthesized directly but are the metabolic end products of VLDL catabolism. The surface coat of LDL contains unesterified cholesterol and the phospholipids, together with a single apoprotein, apo-B-100, which can be recognized by the LDL receptor. The diameter of the LDL particle ranges from 225 to 275 Å.

VLDL and IDL have a short half life and are removed from the circulation within hours. However, LDL has a rather long life and circulates in the blood for about 2 days before it is cleared. Especially, this LDL carries about 60% of the total serum cholesterol (73). The uptake of LDL by cells occurs via a receptor-mediated pathway (B/E receptor) and by nonspecific endocytosis (74).

High-density lipoprotein (HDL), produced mainly in the liver and intestine, can also be derived as metabolic by-products of chylomicron and VLDL catabolism. HDL is the smallest in terms of size among all the lipoproteins with the density of > 1.063. HDL is the major lipoprotein class associated with decreased incidence of atherosclerosis. The major function of HDL is the transport of cholesterol from the peripheral cells to the liver, i.e., "reverse cholesterol transport" (70), but HDL also serves to carry cholesterol to the endocrine organs to provide precursor cholesterol for steroid synthesis in some species.

b. Lipoproteins and Atherogenesis.

Lipoproteins that promote the deposition of plasma lipids in the artery wall and elicit the formation of a fatty streak and/or an atherosclerotic plaque may be referred to as atherogenic lipoproteins (34).

Lipoproteins may contribute to the process of atherogenesis in at least three distinct ways. First, they are the carriers of lesion lipids, especially cholesterol and perhaps slowly metabolized phospholipids like sphingomyelin. Secondly, they may transport dietary fatty

acid of various levels of saturation as raw materials for membrane synthesis affecting membrane fluidity and function, as well as providing the precursors for bioactive lipid "second" messengers. Thirdly, they may in other ways function as modulators or mediators of cell biological phenomena associated with atherogenesis. For example, lipoproteins are involved in lipid deposition in the cell, monocyte recruitment and secretion of various growth factors (35,88).

LDL is the prototype of atherogenic lipoproteins. LDL has long been implicated in the development of atherosclerosis. The evidence implicating LDL as the major atherogenic lipoprotein is based on following observations: a) LDL is the major lipoprotein in plasma and carries the bulk of the plasma cholesterol and its ester, which is a major source of lesion atherosclerosis; b) In the genetic disorder, familial hypercholesterolemia, homozygous patients develop massive LDL concentrations and frequently die within the second decade of life from complications of coronary artery atherosclerosis (71); c) Aside from this genetic caricature of human atherogenesis, in the large human population there is a well established correlation between plasma concentrations of LDL and the risk of atherosclerotic heart disease (75); d) The induction of atherosclerosis in experimental animal models is also invariably associated with elevations of plasma LDL concentrations (76); e) Examination of human and animal atherosclerotic plaques reveals the presence of receptors of LDL - apoprotein B, the protein of LDL and the associated cholesteryl ester. In some cases LDL-like lipoproteins have been extracted from the plaque (77).

Other atherogenic lipoproteins rather than LDL. VLDL is at least an atherogenic lipoprotein precursor. In animal models of experimental atherosclerosis fed cholesterol-rich diets and in type III hyperlipoproteinemia in man, ß-VLDL, a VLDL with ß rather than pre-ß mobility that is enriched in both cholesteryl ester and apoprotein E, was detected (78).

Therefore, this lipoprotein is thought to be atherogenic. Subsequent studies have revealed that ß-VLDL can promote cholesteryl ester storage in macrophages in culture and is a potential source of lipid for foam cell formation entering the macrophages by the LDL receptor using apoprotein E as a high affinity ligand (79). Such VLDL may also promote monocyte adhesion to endothelial cells (80,81) and possible trans endothelial transport of LDL (82).

As was first noted by Gofman et al (83), IDL may be potentially atherogenic. IDL concentration was increased in the Watanabe and the fat-fed rabbit, as well as in the mutant pigs who have apoprotein B mutations associated with atherosclerosis (84-86). In type III hyperlipoproteinemia, IDL concentration was also increased (78). The concentration of IDL appears to be predictive of the progression of atherosclerosis in men with established atherosclerotic heart disease (87).

III. Role of Oxidized LDL in Atherosclerosis

One cell type which is unique to the atherosclerotic plaque is the "foam cell". It is characterized by large lipid inclusions which are composed of cholesteryl esters (88). Evidence from morphologic and immunohistologic chemical studies have shown that foam cells in early lesions of atherosclerosis are predominantly from macrophages, while in more advanced lesions, they can derive form smooth muscle cells as well (86, 89,90). Although elevated LDL cholesterol levels are unquestionably an important risk factor for atherosclerosis, the mechanism by which increased LDL leads to cholesterol accumulation by macrophages within the intima-foam cell formation, remains unclear.

Under normal physiologic circumstances, the LDL particle is internalized by most cells through the classic LDL receptor (74). This receptor is down-regulated when the

cellular need for cholesterol is met. Several lines of evidence would support the hypothesis that the lipid deposition into the atherosclerotic foam cells may occur through an LDL receptor independent pathway. First, patients genetically deficient in functional LDL receptors, still develop severe atherosclerosis with characteristic macrophage-derived foam cells (86,91). Second, cultured macrophages do not accumulate cholesterol ester even when incubated with very high concentrations of native LDL (92). Finally, recent in situ hybridization studies on atherosclerotic lesions, using probes for the LDL receptor, showed the absence of LDL receptor expression in macrophage foam cells (93). Therefore, it was proposed that certain chemical modifications of LDL, for example, acetylation, resulted in rapid uptake in macrophages via a specific receptor termed the scavenger or acetyl-LDL receptor (94,95). Because the activity of this receptor is not regulated by cellular cholesterol, massive accumulation of cholesterol ester occurs in macrophages exposed to such modified LDL in vitro (96). Recent work has implicated oxLDL in the atherogenic process (33,88,96).

a. Production of oxLDL in Vitro and in Vivo.

Endothelial cells, smooth muscle cells, monocyte-macrophages, and even fibroblasts are capable of promoting the oxidative modification of LDL in tissue culture (96-98). The mechanism for the cell-induced modification of LDL may be through free radical peroxidation of LDL (101,102). Similar oxidative modification induced by cells can be achieved by simply incubating LDL in a serum-free medium in the presence of a sufficiently high concentration of copper, iron or free radical generating systems (103,104, 292). The rate at which modification of LDL occurs correlates directly with the rate of superoxide secretion by the cells and is inversely related to the cellular content of superoxide dismutase and catalase (99). Furthermore, superoxide dismutase can inhibit the initiation of LDL oxidation

by cultured monocyte-macrophages (100). Thus, it was proposed that superoxide was required for initiation of oxidative modification but that subsequent propagation and amplification of the oxidative modification involved other free radicals, most likely lipid oxyradicals rather than superoxide (99).

There are several lines of evidence to show that oxLDL is generated in vivo: a) oxLDL has been identified in the atherosclerotic plaques from humans and WHHL rabbits by using anti-malondialdehyde (MDA)-LDL antibodies (105,106). A recent study provided direct evidence for the existence in vivo of oxLDL within arterial lesions (107); b) a modified LDL fraction was isolated in the plasma of Lp(a)-negative normolipidemic healthy male subjects (108), and low levels of MDA-LDL immunoreactivity have been detected in plasma LDL in some individuals (109); c) LDL that is gently eluted from aortic lesions in WHHL rabbits and subjected to polyacrylamide-gel electrophoresis and Western blotting shows cross-reactivity with antibodies specific to MDA-conjugated LDL (110).

b. Properties of oxLDL.

The initial interest in oxLDL focused on its ability to promote cholesterol accumulation in macrophages. Subsequently a wide array of other unique and potentially atherogenic properties have been identified (Table A) (35,88,111, 133).

Table A. Properties of oxLDL which are not shared with native LDL

Chemical and Physicochemical Properties

1. oxLDL has an increased negative charge, an increased lysolecithin and oxidized cholesterol content, and a decreased content of polyunsaturated fatty acids because of oxidation.
2. oxLDL has a decreased histidine, lysine and proline content as well as a fragmentation of apoprotein B_{100}.
3. oxLDL has a decreased content of endogenous antioxidants and has an increased content of conjugated dienes, MDA, hexanal, HNE and other aldehydes.
4. oxLDL has an increased density and electrophoretic mobility, an increased tendency to aggregate, and a heterogeneity in size.

Biological Properties Related to Atherogenesis

1. oxLDL is rapidly taken up by macrophages through the scavenger receptor and leads to cholesterol accumulation.
2. oxLDL is chemotactic for circulating monocytes but inhibits macrophage motility.
3. oxLDL is cytotoxic to endothelial cells and smooth muscle cells.
4. oxLDL inhibits endothelium-derived vasodilator activity and promotes platelet aggregation.
5. oxLDL can alter production of growth factors and inflammatory mediators by cultured cells.
6. oxLDL can alter gene expression in arterial cells, for instance, by inducing endothelial cells to express colony-stimulating factors and monocyte chemotactic protein-1 (MCP-1).

c. Antioxidants and oxLDL.

LDL oxidation involves the peroxidation of the polyunsaturated fatty acids on the surface phospholipids. The peroxidation was initiated when free radicals extract protons from polyunsaturated fatty acids to form fatty-acid radicals. Fatty acid peroxyl radicals were further developed when the radicals react with oxygen. Final results of polyunsaturated fatty acids peroxidation include bond rearrangement to form diene conjugates or degradation products, such as malonyldialdehyde (MDA). In addition to the LDL fatty acids, the cholesterol on the surface (unesterified cholesterol) and in the core of the LDL molecule (cholesteryl ester) can be also oxidized (112). Many factors may affect the susceptibility of LDL to undergo lipid peroxidation such as intrinsic and extrinsic factors (35,88,112). Intrinsic factors include the composition and location of its polyunsaturated fatty acids. Also, LDL contains endogenous antioxidants (vitamin E, ß-carotene and ubiquinol). Extrinsic factors are the extracellular content of copper ions, vitamin E concentration and the cellular oxidative systems (oxygenases, superoxides) (35,88,111,112).

Since oxidative modification of LDL mediated by cells or occurring in cell-free medium results from lipid peroxidation, water- and lipid-soluble antioxidants should have a prominent effect in retarding or preventing the modification. Recent epidemiological studies indicate that relative deficiencies in vitamins E and C may contribute to high incidences of ischemic heart disease (99,113), suggesting that the prevention of LDL oxidation by antioxidants could diminish the risk of developing atherosclerosis. It is evident that endogenous antioxidants contained in LDL particles are consumed during LDL oxidation (114) and addition of exogenous antioxidants can protract the lag period or even prevent the LDL oxidation as shown with tocopherol, butylhydroxytoluene, urate, ascorbate, vitamin E, flavonoid, ubiquinol-10, quercetin, glutathione, lazaroid and probucol (96,115-118,

292).

Among many antioxidants, probucol was studied first both in vitro and in vivo. Probucol, 4,4'-(isopropylidenedithio)bis(2,6-di-tert-butylpheno), was introduced in the early 1970s as a cholesterol-lowering drug (119). In 1986, Parthasarathy et al (115) reported that probucol inhibited both cell-mediated and copper-catalyzed oxidative modification of LDL. In addition, the LDL isolated from the plasma of patients treated with probucol was resistant to oxidative modification. In WHHL rabbits, an animal model for familial hypercholesterolemia, probucol was found to be able to reduce the formation of atherosclerotic lesions (120). This effect was more than expected by the degree of plasma cholesterol level lowering. When probucol and lovastatin were administered independently to WHHL rabbits at doses designed to keep their cholesterol level comparable, probucol more effectively reduced atherosclerotic lesion development (121). Moreover, LDL from control rabbits had a 10-fold increase in TBARS compared with probucol-treated rabbits (120). In addition, LDL in the controls was 7.4-fold more susceptible to copper-induced oxidative modification than LDL from probucol-treated rabbits containing 308 μg/ml probucol (120). Therefore, these authors and others proposed that probucol's antiatherogenic effect was related to limited LDL oxidation (120,121). There is at this time no direct evidence that probucol inhibits oxidation of lipoprotein in arterial walls, where oxLDL is believed to be generated (122). Steinbrecher et al showed that inclusion of a high content of vitamin E into the culture medium prevented oxidative modification of LDL by endothelial cells, as measured by TBARS and macrophage uptake (103). The protective effect of vitamin E against cell-mediated oxidation has been repeatedly found in cultures of endothelial cells (102,123), smooth muscle cells (102) and in monocyte-macrophage cultures (114,124). Daily doses of 108 IU of vitamin E/day significantly reduced symptoms of

atherosclerosis in primates fed an atherogenic diet (125). Chronic deficiency of vitamin C or vitamin E was found to be associated with atherosclerosis-like lesions in rodents, pigs, and primates (126,127).

In inhibiting aqueous radical-mediated lipid peroxidation in human plasma, ascorbate (the reduced form of vitamin C) and, in a site-specific manner, bilirubin are more effective than all of the other endogenous antioxidants (128-129). Among lipid-soluble antioxidants, ubiquinol-10 was found to be much more efficient in inhibiting LDL oxidation than either lycopene, ß-carotene, or α-tocopherol (118). In a recent report that a comparison of the protective effect of three antioxidants (probucol, catechin, vitamin E) against cell injury due to LDL oxidation was investigated (130). These results revealed that: a) probucol (25 mμmol/l) is very effective in preventing lipid peroxidation of LDL and its subsequent 'cytotoxicity', but it cannot protect cells against the 'cytotoxicity' of previously oxidized LDL; b) vitamin E (100 mμmol/l) poorly inhibits the ultraviolet-induced lipid peroxidation of LDL, but is able to block simultaneously the cellular oxidative stress and the 'cytotoxicity' induced by previously oxidized LDL; c) catechin (10 mμmol/l) exhibited two types of protective effects: it inhibits the lipid peroxidation of LDL (and their subsequent 'cytotoxicity') and very effectively protects the cells against the 'toxicity' of previously oxidized LDL (130). Various flavonoids have been shown to prevent LDL oxidation induced by copper, UV-radiations or by macrophages (114,116,117). The flavonoids, like quercetin or rutin, are able to prevent the cytotoxicity of oxLDL at the cellular level since cells preincubated with polyphenolic flavonoids were protected against the cytotoxic effect of previously oxidized LDL (117). The protection against a cytotoxic effect was well correlated with an inhibition of TBARS formation.

Another study showed that an orally active antioxidant, DPPD (N,N'-dipheynyl-1,4-

phenylenediamine), can inhibit in vitro LDL oxidation at a concentration much lower than other reported antioxidants (131). Further, DPPD slowed the progression of atherosclerosis in cholesterol-fed rabbits without affecting plasma cholesterol levels (131). This study supported the hypothesis that oxLDL may play an important role in the genesis of atherosclerosis.

PART TWO. THE MODULATION OF MEMBRANE ION MOVEMENTS BY CHOLESTEROL

I. THE STRUCTURE AND CHARACTERISTICS OF CHOLESTEROL IN MEMBRANE

a. Chemical Structure of Cholesterol

Cholesterol structure can be broken down into three important regions: 1) a relatively rigid steroid nucleus composed of four fused rings, referred to as the A,B,C and D rings. The conformation of the A ring can alter its orientation in the crystal state permitting more flexibility for the whole molecule (134); 2) in ring A, an alcoholic hydroxyl group is covalently attached at carbon 3; and 3) a freely movable, branched hydrocarbon side chain attached to carbon 17 of the D ring (Figure 1). It is important to point out that all three of the features of cholesterol are important for characteristic cholesterol-like performance.

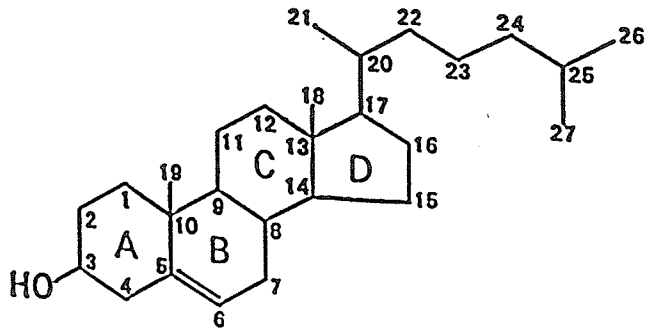


Figure A. The chemical structure of cholesterol.

Cholesterol is a weak amphiphile due to the hydrophobic rings, the hydrocarbon side chain and the polar 3ß-hydroxyl group. Being an amphiphile, cholesterol is able to fit into a phospholipid bilayer with its polar hydroxyl group protruding into the polar surface region, and the hydrophobic steroid rings oriented parallel to, and buried in, the hydrocarbon chains of the phospholipids. Cholesterol creates a physical state which is intermediate between the liquid crystal and gel phases in the surrounding lipids of the membrane, possibly by a close fit contact between the steroid and specific lipid chains (135-138). From its specific conformation in the membrane, cholesterol confers to the membrane a state of "intermediate fluidity", enabling motion of hydrocarbon chains in the gel phase while restricting motion in the liquid crystalline phase. Cell membranes usually work best when the lipid bilayer membrane is in the liquid crystalline state (139).

b. Distribution and Movement of Cholesterol in the Cell

1. Distribution of Cholesterol in Cell Membranes. The content of cholesterol in eukaryotes is entirely different among the various membranes of the cell (140). By using the enzyme cholesterol oxidase, which can oxidize plasma membrane cholesterol without affecting intracellular cholesterol, plasma membrane cholesterol has been studied as a percentage of total cellular cholesterol. In fibroblasts and Chinese hamster ovary cells, more than 90% of total cell cholesterol was found in the plasma membrane (139). Studies in different cells also showed that 80% of total cellular cholesterol is in the plasma membrane of hepatocytes (140) and 82% of unesterified cholesterol was associated with the plasma membrane in rat ovarian granulosa cells (141). It has been noted that the distribution of the cholesterol among the membranes of mammalian cells is very uneven. Higher concentrations of cholesterol are found in the plasma membranes and secretory vesicles,

moderate concentrations in the Golgi apparatus and secondary lysosomes, and lower concentrations of cholesterol in the endoplasmic reticulum and mitochondria (142).

More recently, another method has been developed that allows a determination of cholesterol content in biological membranes without isolating the membranes (143). Filipin, an antibiotic that binds specifically to membrane cholesterol, was used to treat the cell membrane and then viewed by freeze-fracture electron microscopy. The density of large pits, which bind filipin, is representative of the cholesterol concentration in the membrane (143). Using this method, cholesterol concentration in the basolateral membranes was found to be higher than in brush-border membranes in the rat intestinal mucosa (144,145). Furthermore, the cholesterol concentration in the plasma membrane of the same colonic cells is different according to the region (146). In the colonic epithelium of the guinea pig, the cholesterol concentration in the plasma membrane in the proximal colon was higher than in the distal colon. Therefore, there is a greater fluidity in the membranes of the absorbing cells in the distal colon which can be correlated to the higher absorption rates of short chain fatty acids characteristic of this region (146).

In the cardiomyocyte, filipin-cholesterol complexes were observed mainly in the sarcolemma where they occurred in patches or, sometimes, they densely populated the entire cell surface (147). Clusters of filipin-induced lesions can be seen in plasma membrane, which indicates the presence of cholesterol-rich domains surrounded by regions of much lower cholesterol content (148). Some can be seen in the sarcoplasmic reticulum and in the mitochondria, but they were very rare in the sarcoplasmic reticulum (149). A non-homogeneous distribution of cholesterol in SR was observed. The filipin-induced deformations occurred more commonly in free SR at or near the Z-region of the sarcomere than in other parts of the free SR or the junctional SR (150). Few existed in the nuclear

envelope in cardiac muscle. By studying filipin-sterol complexes in the nuclear membrane, a heterogeneous distribution of cholesterol was observed (149). The outer nuclear membrane was more homogeneously distributed than the inner membrane (149).

There have been suggestions that cholesterol is not uniformly distributed between the inner and outer leaflets of a membrane as well. In the erythrocyte, the external surface of the membrane was significantly richer in cholesterol than the cytoplasmic surface (18). This observation was further confirmed by several other studies in red cell ghosts and in erythrocytes where there is a very slight enrichment of cholesterol in the outer membrane leaflet (152,153). This unequal distribution of cholesterol may be due to the difference in phospholipid composition between the two halves of the erythrocyte membranes (154). Since cholesterol had a preferential affinity for sphingolipid and phosphatidylcholine, and the phospholipids having the higher affinity for cholesterol are concentrated at the outer membrane leaflet (155,156), it is reasonable to hypothesize that this side of the membrane should contain more cholesterol than the cytoplasmic leaflet (138,155).

2. Cholesterol Movement in the Cell. Membrane cholesterol may come from two sources: plasma lipoproteins and cellular biosynthesis. Low density lipoprotein, a major carrier of cholesterol in the blood, enters cells through both receptor mediated (157) and non-receptor-mediated pathways (158). Mammalian cells, cultured in the presence of serum lipoproteins, acquire cholesterol necessary for growth from the uptake and lysosomal hydrolysis of LDL.

Newly synthesized cholesterol is made in the endoplasmic reticulum of cells but at short time intervals it can be detected in the Golgi apparatus, lysosomes, mitochondria and plasma membranes using radioactively labelled sterol precursors (159). Therefore, cholesterol appears to be transported very quickly from its site of synthesis to the other

membranes of the cell. Several studies have traced intracellular cholesterol movement in various cell types. Transport of newly synthesized cholesterol to the cell surface seems to be vesicular and energy and temperature dependent (160). The transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane ceased at 15 ^{0}C, whereas cholesterol synthesis continued in Chinese hamster ovary cells (160). When synthesis occurred at 15 ^{0}C, the newly synthesized cholesterol was found to accumulate in the endoplasmic reticulum and in a low density, lipid-rich vesicular fraction (160). Also in Chinese hamster ovary cells, the transfer of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane occurred with a half-time of 10 minutes (161). Human skin fibroblasts and Chinese hamster ovary cells require a half-time of about 1 hour to transfer cholesterol between plasma membrane and internal membranes (162). In rat ovarian granulosa cells, newly synthesized cholesterol can reach the plasma membrane in less than 20 minutes (t1/2) (141). Cholesterol movement to the plasma membrane appears to be regulated by gonadotropins in these cells. Cholesterol movement was much slower (t1/2 approximately 2 h) in cells stimulated by FSH and androstenedione (141).

It has been proposed that the movement of cholesterol in the cell may be through several mechanisms: a) diffusion through the cytoplasm; b) protein-mediated transport, and c) vesicular transport (138). The movement of intracellular cholesterol might be unidirectional since radio-labelled cholesterol introduced into the plasma membrane did not subsequently move to the cell interior (162). One exception is that zymosterol, the precursor of cholesterol, can move rapidly and in both directions between the rough endoplasmic reticulum (a sterol-rich intracellular membrane bearing nascent cholesterol) and the plasma membrane (163). It is possible that the cholesterol transfer occurs via a pool of sterol molecules in the aqueous medium since cholesterol can readily transfer between structures

such as liposomes, lipoproteins, and erythrocytes (164). However, studies in vitro have demonstrated that when membranes, lipoproteins or vesicles containing little sterol are incubated with similar structures containing a high concentration of sterol, there is a net transfer of sterol from the latter to the former so that the concentration differences are reduced (165). The large differences in the cholesterol content of mammalian cell membranes would suggest that either this transfer process does not occur intracellularly in vivo, or that it occurs too slow to equalize the concentration differences, or that its effects are outweighed by other factors. Therefore, it is unlikely that cholesterol transfer occurs by simple diffusion within the cell.

Another possible way of cholesterol transport is via an active transport in which newly synthesized cholesterol is transported against its concentration gradient to the plasma membrane and then diffuses back down the gradient to the cell interior. However, this possibility is excluded by experiments showing that for many hours at 37^0C, exogenous [^{14}C]-cholesterol inserted into the plasma membrane did not mix with newly synthesized [^{3}H] cholesterol (162).

The existence of sterol carrier proteins is suggested by the observation that the rate of movement of cholesterol between mitochondria and microsomal vesicles could be increased by rat liver supernatant (166). Several small soluble, cytoplasmic sterol-binding proteins have been identified in cells: a) a sterol-carrier protein (167); b) the 'fatty acid binding protein', which is identical to the sterol-carrier protein described by Dempsey et al. (168); c) ligandin (169). There are several roles of these sterol-carrier proteins in the regulation of intracellular cholesterol movement such as: a) they promote the transfer of intermediates in the final stages of cholesterol biosynthesis between microsomes (170); b) they can increase the rate of transfer of cholesterol to microsomal vesicles containing acyl-

CoA:cholesterol acyltransferase (167,171); c) they may be involved in the movement of cholesterol both from plasma membrane lipid droplets to mitochondria and from the outer to the inner mitochondria membrane (172,173); d) in some pathologic conditions such as hepatoma, the low abundance of the high molecular weight form of sterol carrier protein-2 from hepatoma peroxisomes may be related to the accumulation of cholesterol in the cells (174). Sterol carrier proteins may be involved in cholesterol transport between membranes not only in steroidogenic cells but also in non-steroidogenic cells (175).

The transfer of newly synthesized cholesterol to the plasma membrane may also be mediated by membrane vesicles. Various types of vesicles were present in cells such as those that appear during endocytosis or as part of the transport of proteins from the rough endoplasmic reticulum via the Golgi apparatus to the cell surface for insertion in the plasma membrane (176,177). There are some differences between vesicles involved in cholesterol transport to the plasma membrane and those involved in bulk protein transport to the cell surface (177). However, this type of membrane flux is probably too slow to account for the transfer of newly-made cholesterol to the plasma membrane (161).

The cellular mechanisms of cholesterol efflux via high density lipoprotein (HDL) receptor-mediated transport have been studied in cultured human fibroblasts and bovine aortic endothelial cells (178). HDL3 induced a rapid movement of [3H] cholesterol from a preplasma membrane compartment to the plasma membrane preceding [3H] cholesterol efflux. It was suggested that receptor binding of HDL3 could facilitate removal of cellular cholesterol from specific intracellular pools by initiation of translocation of intracellular cholesterol to the plasma membrane (178).

3. Pathological Alterations in Distribution & Movement of Cholesterol in Membranes. Abnormalities of membrane cholesterol have been observed in some diseases.

In liver disease, an increased cholesterol/phospholipid ratio in erythrocyte membrane resulted from abnormalities in the metabolism and composition of plasma lipoproteins. Ultimately, this produced a decreased membrane fluidity (179). In obese children with either abnormal and normal plasma cholesterol levels, the fluidity of erythrocyte membranes decreased significantly which may indicate that the erythrocyte membrane responds very quickly to a modification of plasma cholesterol levels (180). In rats fed an atherogenic diet (cholesterol supplemented), the membrane fluidity of liver microsomes was also decreased (181). In the erythrocyte plasma membrane from insulin-dependent diabetes mellitus patients, the total cholesterol and phospholipid content was lowered, and the cholesterol-to-phospholipid molar ratio was significantly decreased as well (182). In the chronically diabetic rat, the cholesterol content of sarcolemmal membranes from heart was significantly increased (183). This was membrane specific because cardiac mitochondria (184) and SR (185) membrane cholesterol content was unaffected during chronic diabetes.

In neoplastic disease, a decreased membrane fluidity was observed (186). More rigid membranes produced by the decreased membrane fluidity, accompanied by increases of cholesterol, was observed in solid tumours like hepatomas (186). The synthesis of cholesterol was deregulated and control was lost early after exposure to carcinogenic events and much earlier than the appearance of malignant growth in the malignant cells (187). The ratio of cholesterol/phospholipid increases whereas the total lipid content and the degree of fatty acid unsaturation decreases with increasing growth rate of a tumour (188). In hepatomas, the regulation of cholesterol metabolism has been altered (189). For example, the content of free cholesterol in hepatomas was about twice as much as that in control liver. Esterified cholesterol increased about 2-5 times in hepatomas (189). More striking, the rates of in vivo hepatoma cholesterol synthesis were 1.5-4 times higher than those in the control liver (189).

The activity of the LDL receptor was also found to decrease in renal carcinoma (190) and to increase in leukemia (191). LDL receptor expression in experimental murine tumours may be related to the in vivo uptake of lipoprotein (192). The authors suggested that the relative high in vivo uptake of LDL was determined by the elevated LDL-receptor expression in the tumours (192).

The effects of ischemia and anoxia-induced alterations in cholesterol content of sarcolemmal membranes in rat heart cells has been studied (193). Cholesterol content in cultured heart cells was significantly decreased after 1 hour anoxia. This change in sarcolemmal cholesterol content preceded the release of cytoplasmic proteins from the cells. Therefore, the authors concluded that anoxia in cardiac cells destroyed sarcolemmal structure and function secondary to the loss of cholesterol (188). An effect of ischemia on membrane cholesterol content has also been observed. The cholesterol content of sarcolemma decreased and that of mitochondria rose in the ischemic myocardium (194,195). It was suggested that cholesterol is redistributed within the ischemic heart cell. Longer durations of ischemia (15-60 minutes) in isolated perfused rat heart caused a progressive loss of cholesterol from sarcolemma and sarcoplasmic reticulum, concomitant with a significant increase in mitochondrial cholesterol content (196). Following these compositional changes, a marked increase in sarcolemmal and mitochondrial microviscosity were noticed. In contrast, others reported no change in cardiac sarcolemmal membrane cholesterol content during an in vitro ischemic challenge (197). In reperfused ischemic canine myocardium, the normally random distribution of free membranous particles underwent a dramatic change to patches of aggregation. This may have been due to the lateral movement of intramembranous proteins to lipid phases which were nonhomogeneously distributed over the membrane area (198). The mechanism may involve

sarcolemmal cholesterol depletion, which can introduce semicrystalline phases, forcing protein to move to patchy areas with normal fluidity.

A decrease in membrane cholesterol content has also been observed in hereditary cardiomyopathy in hamsters (199). In contrast, others report that in genetic cardiomyopathy (200) and muscular dystrophy in hamsters, the contents of cholesterol from the heart homogenate, mitochondria and microsomal vesicles (201) are increased. However, although pretreatment of the dystrophic hamster for 20 days with verapamil prevented cardiac necrosis, it had no effect on membrane cholesterol (201).

In erythrocyte membranes from guinea pigs fed diets supplemented with 1% cholesterol, the cholesterol content increases up to 2-fold whereas the phospholipid content and the fatty acid composition of these membranes remains essentially unchanged (202). In cultured rabbit aortic smooth muscle cells (SMCs) and isolated SMC plasma membrane microsomes, incubation with medium containing LDL and cholesterol enriched phospholipid liposomes resulted in an increased cholesterol mass without affecting PL mass (203).

Niemann-Pick disease type C (NPC) is characterized by substantial intracellular accumulation of unesterified cholesterol. In the cultured NPC fibroblasts, the transport of LDL-derived [3H] cholesterol to the plasma membrane is slower than control (204). However, the rate of appearance of [3H] acetate-derived, endogenously synthesized [3H] cholesterol at the plasma membrane is the same for normal and NPC cells (204).

c. The Effect of Cholesterol on the Biophysical Properties of Membranes

1. Cholesterol Effects on Cellular Membrane Fluidity. Lipid fluidity is defined as the motional freedom of lipid molecules or lipid soluble probes within a membrane bilayer. Free cholesterol is thought to participate in the regulation of fluidity in mammalian plasma

membranes in general. The molar ratio of free cholesterol to phospholipid (FC/PL) in the membrane correlates with lipid microviscosity (205). There is ample evidence that cholesterol decreases the fluidity of the plasma membrane (202-212). For example, enrichment of membrane cholesterol resulted in an increase in the local viscosity of the membranes compared to the controls in guinea pig erythrocyte (202). It is also believed that cholesterol is able to condense and rigidify the plasma membrane by restricting the random motion and mean cross-sectional area occupied by the neighbouring phospholipid acyl chain (206,207). Very recently, more data have been available to support the view that increases in membrane cholesterol content are associated with decreased membrane fluidity. This relationship has been identified in arterial smooth muscle cells (203), leukaemia cells (208), erythrocytes (179,209), rat intestinal microvillus (205,210), liver surface membranes (211) and fibroblasts (212).

2. Cholesterol Effects on Permeability of the Cell Membrane. Cholesterol affects the permeability of both artificial phospholipid membranes and biological membranes (213-224). Pure lipid bilayer membranes are characterized by an extremely low permeability to diffusible ions, which is principally mediated by channels or pores. However, an alteration of the passive permeability produced by the amount of membrane cholesterol has been observed. Low cholesterol content in the membrane can increase membrane permeability whereas higher cholesterol concentrations can strongly suppress the permeability (214). The effect of cholesterol on membrane permeability may be due to the effect of cholesterol on phospholipid hydrocarbon chain ordering in membranes (215,216).

In artificial phospholipid membranes, the permeability of many solutes such as glycerol, glucose and erythritol, decreases considerably upon incorporation of cholesterol into the membrane (217,218). In biological membranes, cholesterol also appears to affect

permeability. Enrichment of membrane cholesterol in rat liver mitochondria decreases the passive proton permeability (219). In erythrocytes from cholesterol-fed guinea pigs, the permeability to both hydrophillic and amphiphilic non-electrolytes and to both active and the passive Na^+ efflux was decreased (220). The possible mechanism may be a tighter packing of the lipid components which in turn decreases the mobility of the permeants within the membrane matrix. A recent study also demonstrated that enrichment of cholesterol in renal cortical brush-border membranes can decrease membrane fluidity and stimulate Na^+-dependent D-glucose uptake by increasing the affinity of the carrier (221). Other studies have shown that in human erythrocytes, partial removal of membrane cholesterol led to an increase in the permeability of the membrane to glycerol (222), although the permeability to water was not changed (223). However, some results conflict with the above observations. For example, oxidation of membrane cholesterol did not produce alterations in the permeability of red blood cell membranes to cations (224). Furthermore, in control and ischemic hearts, loss of myocardial membrane cholesterol did not affect cell permeability (196). It could be that membrane permeability is unaffected until some threshold for membrane cholesterol content is passed.

II. THE EFFECT OF CHOLESTEROL ON MEMBRANE PHYSIOLOGICAL FUNCTIONS

a. Membrane-Bound Proteins

Cholesterol may affect the activity of a membrane-bound enzyme via an indirect route, a special molecular ordering effect on the membrane, or bind directly to the enzyme, and thereby alter its behaviour. The effect upon a membrane protein by cholesterol is dependent upon the particular membrane protein. Cholesterol may inhibit, stimulate, or

have no effect at all (215, 225-232).

As an example of the former possibility, when human leukaemic cells were incubated with cholesterol, Na^+, K^+-ATPase activity was reduced significantly compared to that of untreated cells (225). Similar inhibitory effects of cholesterol have been observed on alkaline phosphatase of rat intestinal microvillus membrane (226), UDP-glucuronosyltransferase in the lipid bilayer (227) and bovine rhodopsin (228). Cholesterol may also stimulate membrane protein function. For example, in Novikoff hepatoma cells, a five- to sixfold increase in gap junction assembly between reaggregated cells and a 50% increase in cellular cholesterol content were observed with 20 μM added cholesterol (229). A similar effect has been observed on carrier mediated lactate transport (230) and sodium coupled glucose uptake in renal proximal tubular cells (231). However, some membrane proteins seem to be insensitive to cholesterol alterations in the membrane. It has been observed that the susceptibility of the band 3 protein of the human erythrocyte membrane to proteolytic digestion at either surface of the membrane was not altered when the membrane cholesterol was increased by 65-103% (232). Sucrase and lactase activities of the rat intestinal microvillus are also not affected by alterations in the membrane cholesterol levels (205).

b. Membrane Receptors and Cell Growth

Membrane receptors and cell growth are also subject to control by membrane cholesterol levels. In liver cirrhosis patients, impairments in insulin receptor processing was associated with an increase in the erythrocyte membrane cholesterol to phospholipid molar ratio (233). Furthermore, similar changes in insulin receptor processing were observed when the molar ratio of cholesterol to phospholipid in normal erythrocytes was modified in vitro by incubation with cholesterol-rich liposomes (233). Another study has also shown that low

membrane cholesterol decreased the number of insulin receptors in erythrocytes without altering the affinity (234). The possible mechanism may be that membrane cholesterol affects insulin receptor behaviour through an alteration of membrane fluidity, depending on the phase state of the membrane (234). The author further demonstrated that the increased cholesterol content in the erythrocyte membrane was responsible for the increased receptor number and insulin binding to the erythrocytes from patients suffering from alcoholic liver cirrhosis (234). The requirement for membrane cholesterol has also been observed in the acetylcholine receptor (235), ß-adrenergic receptor density (236), adenylate cyclase activity stimulated by receptors (237) and the glycophospholipid-anchored membrane receptor for 5-methylterahydrofolate (238).

Recent studies have demonstrated that cholesterol plays a central role in the control of membrane biosynthesis and cell growth. For example, myeloma cells (239) and Mycoplasma (240) have an absolute growth requirement for exogenous sterol. Most animal tissues are capable of synthesizing cholesterol de novo from acetate, and, perhaps because of this, there are relatively few mammalian cells which express an absolute requirement for an exogenous source of cholesterol in vitro. For example, rat and mouse myeloma cells which had been cultivated in serum-free medium containing cholesterol for more than 6 months still required cholesterol in vitro for growth in serum -free medium. This requirement for cholesterol could be replaced by human low density lipoprotein but not by mevalonic acid lactone (239). Similarly, in Mycoplasma, sterol starvation inhibited both lipid and protein synthesis, which suggested an important link between sterols and cell growth (240). Furthermore, cholesterol prolonged the survival of adult rat hepatocytes in a serum-free primary culture (241). The effect appeared to be due to stabilization of the plasma membrane. Cholesterol (at low levels) also stimulated unsaturated phospholipid synthesis,

which was followed by RNA and protein synthesis in the same cells (242).

III. MODULATION OF ION MOVEMENT BY MEMBRANE CHOLESTEROL

a. Na^+-K^+ ATPase

Na^+-K^+ ATPase or the Na^+ pump, which is present in the plasma membrane of all cells of the body, is responsible for pumping sodium ions out of the cell in exchange for extracellular potassium ions against their respective concentration gradients. This is an energy consuming process. Therefore, this pump is responsible for maintaining the sodium and potassium concentration differences across the cell membrane as well as for establishing a negative electrical potential inside the cells (243). Na^+-K^+ ATPase activity is electrogenic because it creates an electrical potential along the cell membrane as it pumps 3 Na^+ ions to the outside of the cell for every 2 K^+ ions pumped to the interior. These properties place this enzyme in a vital role in a number of cellular processes, including maintaining normal cell volume, establishing electrical potentials along the plasma membrane and contributing to sodium cotransport systems.

Since Na^+-K^+ ATPase is an important membrane transport system which plays an essential role in cellular function and is affected by changes in membrane composition, extensive studies have been carried out to investigate the effect of membrane cholesterol on the function of Na^+-K^+ ATPase. Recent study has demonstrated that Na^+-K^+ ATPase activity from cardiac sarcolemmal membranes strongly required cholesterol (244). This sterol requirement was highly specific for cholesterol in that several cholesterol analogues with minor structural changes were unable to support Na^+-K^+ ATPase activity. These data suggested the importance of cholesterol structure in the interaction with the Na^+ pump and

was suggestive of a direct interaction of cholesterol with the Na^+-K^+ ATPase in the membranes. Others have shown that the stimulatory effects of cholesterol were dependent upon the initial membrane cholesterol content. For example, when the membrane cholesterol content is lower than that found in the native membranes of bovine kidney, cholesterol could stimulate the activity of Na^+-K^+ ATPase (245). This effect had structural specificity in that some cholesterol analogues such as ergosterol and lanosterol were not as effective as cholesterol at stimulating the activity of Na^+-K^+ ATPase (245). It is very possible that cholesterol may interact directly with Na^+-K^+ ATPase as an essential positive effector rather than influence the motional order of the membrane (245,246). In other studies, depletion of membrane cholesterol had a biphasic effect in human erythrocytes. Depletion by 5-25% increased Na^+-K^+ ATPase activity by a mean of 16.1%, whereas depletion by 35-50% decreased Na^+-K^+ ATPase activity by a mean of 14.8% (253). Similar biphasic responses of Na^+-K^+ ATPase to cholesterol depletion were also found in rabbit renal medullar membrane vesicles (245) and human red blood cells (248). It was assumed that the effect of cholesterol depletion was mediated through a redistribution of the phospholipids located in the microenvironment of the protein which have been shown to play a role in the activation of the Na^+-K^+ ATPase (248). The biphasic response of Na^+-K^+ ATPase to cholesterol depletion supports the idea of an 'optimum' membrane cholesterol content for maximal activity of a membrane transport system (246). In certain pathological conditions, Na^+-K^+ ATPase activity is stimulated with increasing cholesterol content. For example, in sarcolemmal membranes from hearts of rats fed a 2% cholesterol diet for 12 weeks, Na^+-K^+ ATPase activity was increased as was the sarcolemmal cholesterol/phospholipid molar ratio (249). A similar result was found in sarcolemma from genetically myopathic hamsters which display increased membrane cholesterol (251) and

enhanced Na^+-K^+ ATPase activity (250).

However, several other studies dispute these findings to varying degrees. Purified Na^+-K^+ ATPase, appears to be relatively insensitive to cholesterol content (252) or could be depressed in artificial membranes by cholesterol (253). Several groups have studied cholesterol modulation of the Na^+-K^+ ATPase in membrane vesicles and intact cells. Early work noted that cholesterol inhibits delipidized and soluble Na^+-K^+ ATPase activity from rabbit kidney outer medulla (254). The Na^+-K^+ ATPase activity is only partially inhibited by cholesterol in the presence of unsaturated phospholipids, while complete inhibition can be achieved in the presence of saturated phospholipid which may indicate the interaction of cholesterol with phospholipids of different chain length and unsaturation (254). Subsequently, other experiments have shown an inhibitory effect of high membrane cholesterol content on Na^+-K^+ ATPase from human red blood cells membranes (246), rabbit erythrocyte membranes (255,259), normal clear bovine and human lens (257), canine ventricular sarcolemmal vesicles (258,259), rabbit myocardium after cholesterol rich diet (260), rat liver membranes (261) and kidney basolateral membranes (245).

Several mechanisms have been proposed for the inhibitory effect on the Na^+-K^+ ATPase by membrane cholesterol. The rigid sterol structure of cholesterol may lead to an increase in the anisotropic motional ordering of the lipid bilayer of the membrane, which in turn may lead to an increase in the ordering of the conformation of the Na^+-K^+ ATPase (218,246). Therefore, the capability of the Na^+-K^+ ATPase to undergo conformational change will be reduced, and as a result, would inhibit its function. A second possibility may involve a direct interaction between cholesterol and the Na^+-K^+ ATPase which will modulate its activity (139). Direct binding of cholesterol to various membrane proteins (such as the band 3 protein from human erythrocytes) has been reported (262). A third

mechanism may be the lipid peroxidation that may occur after the enrichment of cholesterol content in the cell membrane (255). Membrane lipid peroxidation would certainly explain the decreased Na^+-K^+ ATPase activity (255).

c. Calcium Pumps

Calcium pumps appear to be ubiquitous components of eukaryotic cells and their major function is to translocate Ca^{2+} across membranes against a Ca^{2+} gradient. Biochemically, calcium pumps are expressed as a Ca^{2+}-ATPase since they require energy supplied from ATP hydrolysis. The best characterized Ca^{2+}-ATPases are located in the sarcoplasmic reticulum and plasma membrane.

Sarcoplasmic Reticulum Ca^{2+}-ATPase. The Ca^{2+}-ATPase is responsible for sequestering Ca^{2+} which has been released in muscle cells to stimulate myofilament contraction. Two moles of Ca^{2+} are translocated for each mole of ATP hydrolysed (263). Amongst all the proteins in the sarcoplasmic reticulum (SR), 80% are ATPase proteins responsible for calcium uptake (264). This enzyme requires a hydrophobic environment or fluid membrane to sustain its activity (265). It has been proposed that about 30 phospholipid molecules interact directly with the hydrophobic surface of the ATPase spanning the bilayer, and form the minimal lipid environment required to maintain full Ca^{2+}-ATPase activity (266).

Earlier study suggested that cholesterol does not affect the activity of the sarcoplasmic reticulum calcium-ATPase from rabbit muscle because the protein in the Ca^{2+}-ATPase specifically excludes cholesterol from the immediate vicinity of the protein (267). If cholesterol replaces phospholipids in the annulus, a potent inhibition of activity of Ca^{2+}-ATPase was observed and this inhibition can be abolished by sufficient phospholipid to

reform a complete annulus (267). Therefore, the authors proposed that cholesterol may be inhibitory because it does not meet the requirement of the protein for specific chemical structures in the annulus (267). More studies agreed that cholesterol is normally excluded from direct contact with Ca^{2+}-ATPase enzyme (268-270) and excluded from other membrane enzymes as well (271). Using a reconstituted system, another study showed that a high membrane cholesterol content in proteoliposomes (cholesterol/phospholipid molar ratio = 0.3) reduced the Ca^{2+}-ATPase activity (272). However, this decline in Ca^{2+}-ATPase activity induced by cholesterol is quite small which agrees reasonably well with results derived from a reconstitution study undertaken by Warren et al (267). In rabbit skeletal muscle, the SR Ca^{2+}-ATPase was notably unaffected by cholesterol in reconstituted phosphatidylcholine : phosphatidylserine vesicles (273).

In reconstitution experiments in which phosphatidylethanolamine was used as a dominant lipid component in the membrane, cholesterol enhanced the Ca-transport function of the Ca^{2+}-ATPase (272). Therefore, under certain circumstances, cholesterol may stimulate Ca^{2+}-ATPase in the reconstituted proteoliposomes via, possibly, direct binding of cholesterol to Ca^{2+}-ATPase (272).

Nevertheless, some studies have disagreed with the above conclusion that Ca^{2+}-ATPase was not sensitive to cholesterol. Warren et al showed that Ca^{2+}-ATPase activity is decreased in direct proportion to the cholesterol content of the membrane. They proposed the effect of cholesterol on Ca^{2+}-ATPase is consistent with an effect on the fluidity of the SR membrane (273-275). Recovery of Ca^{2+}-ATPase activity was achieved upon the removal of cholesterol from the SR membrane, which suggested that cholesterol does reversibly inactivate the enzyme (274). Similar qualitative results have been interpreted quite differently by other investigators. In bovine arterial smooth muscle cells, increased

cholesterol content of the cells through liposome fusion resulted in a reduction of SR Ca^{2+}-ATPase activity (276). Since the alteration of membrane cholesterol content was paralleled by a decrease in the Ca^{2+}-ATPase activity of SR (275), and the inhibition was accompanied by a loss in enzyme sensitivity to calmodulin (277), they proposed that the change of Ca^{2+}-ATPase activity was caused by a direct effect of cholesterol on the enzyme (276). Other studies have had similar conclusions that the inhibitory effect of cholesterol on the Ca^{2+}-ATPase was due to the direct interaction of cholesterol on the enzyme (278) and not a change of membrane fluidity induced by cholesterol (279,280).

The effect on Ca^{2+}-ATPase activity of an atherogenic (1% cholesterol) diet fed to swine (281) or rabbits (283) has been studied. In swine fed an atherogenic diet, total Ca^{2+} uptake was decreased as was the altered cholesterol/phospholipid ratio without having effect on Ca^{2+}-ATPase activity (281). In rabbits fed an atherogenic diet for 12 weeks, the rate of SR calcium binding was unchanged but calcium uptake was increased (283).

Plasma Membrane Ca^{2+}-ATPase. This enzyme acts to pump Ca^{2+} out of the cell to regulate the intracellular [Ca^{2+}]. Two calcium ions are transported across the membrane for each ATP hydrolysed. Membrane fluidity might be an important determinant of Ca^{2+}-ATPase activity. A 50% increase in Ca^{2+}-ATPase activity was observed concomitant with increased membrane fluidity (284).

There has been a great controversy for several years concerning an indirect versus a direct effect of cholesterol upon the muscle Ca^{2+}-ATPase. In a cardiac sarcolemmal preparation, cholesterol has an inhibitory effect upon the Ca^{2+}-ATPase activity (158,277,278). The effect of cholesterol enrichment on the sarcolemmal Ca^{2+}-pump has been examined (158). The Ca^{2+} accumulating ability of the Ca^{2+}-ATPase is inhibited 40% by cholesterol enrichment at all incubation times (158). The same inhibitory effect has been

observed in sarcolemmal Ca^{2+}-ATPase activity in rats fed a 2% cholesterol diet for 24 weeks (249). Not only can the incorporation of cholesterol into the sarcolemma inhibit Ca^{2+}-ATPase activity, depletion of cholesterol can induce a much more pronounced, stimulatory effect (277). This may indicate that there is a critical concentration of cholesterol in the sarcolemma which normally modulates ATPase activity (277). Since the slight changes in E_a (Arrhennius activation energy) and transition temperatures produced by cholesterol did not have an important effect upon the fluidity of the membranes, the authors suggested that cholesterol may have a direct effect upon the sarcolemmal Ca^{2+}-ATPase (277). The authors also suggested that there is a possibility of a slow exchange of cholesterol between the tightly bound lipid surrounding the Ca^{2+}-ATPase and the bulk lipid of the sarcolemma (277). Another study demonstrated that cholesterol enrichment of the sarcolemmal membrane was associated with a loss of calmodulin sensitivity of Ca^{2+} ATPase (278).

Ca^{2+} uptake via the sarcolemmal Ca^{2+} pump was also inhibited if the endogenous membrane cholesterol was oxidized with cholesterol oxidase (279). Cholesterol oxidase inhibited ATP-dependent Ca^{2+} uptake by 22%, 36%, and 52% at 15, 30, and 60 seconds of reaction time, respectively (279). However, it was not clear if the inhibitory effect was a direct action on the Ca^{2+} pump or if it was primarily a reflection of a non-specific increase in membrane permeability.

c. The Calcium Channel

The Ca^{2+} channel is a protein which facilitates the selective movement of Ca^{2+} across a membrane down its concentration gradient. There are at least two types of Ca^{2+} channels: one is potential dependent and the other is receptor-operated (285). The first report of the

effect of membrane cholesterol on the calcium channel was carried out in human red blood cells. Calcium influx was strongly influenced by the cholesterol content of the plasma membrane (286). Calcium influx through the calcium channel increased when the membrane was enriched with cholesterol using liposomes, and conversely, cholesterol depletion decreased influx (286,287).

Arterial smooth muscle cells are the primary cell type which are enriched with unesterified cholesterol in an atherosclerosis plaque (288). The entry of extracelluar calcium is a major regulatory factor in a number of vascular smooth muscle functions. Vascular smooth muscle Ca^{2+} content may also be important in plaque development. Therefore, it is important to understand the interactions of membrane cholesterol and intracellular calcium in arterial smooth muscle cells. Calcium channel activity in arterial smooth muscle cells was increased upon the enrichment of membrane cholesterol by exposure to either cholesterol-rich liposomes or LDL (203,273,276,289,290). For example, in cultured rabbit aortic smooth muscle cells, cholesterol enrichment of cell membranes was associated with a significant increase in the unstimulated calcium influx rate which is sensitive to channel blockers (203). A similar observation was reported for intact carotid arterial segments after perfusion with cholesterol-rich liposomes (290). The cholesterol-induced calcium influx was reversible after exposure to medium containing 10% serum (no liposome) for 4 days (203). Since lipid dynamics in the arterial smooth muscle cells membrane can be altered by cholesterol enrichment, the authors proposed that cholesterol enrichment in smooth muscle cell membranes unmasked a new, or otherwise silent calcium channel (present, but inactive under normal conditions) with pharmacological characteristics similar to L-type channels, i.e. dihydropyridine sensitivity (203).

The mechanism by which cholesterol-rich liposomes and cholesterol enrichment

increase calcium currents in vascular smooth muscle cells was investigated by using whole-cell patch clamp recording (273). Enrichment of membrane free cholesterol in arterial smooth muscle cells resulted in a gradual increase in the L-type current over 20 hours and a plateau (73±7% increase over basal) between 20 and 32 hours (273). The authors demonstrated that the effect of cholesterol enrichment was not due to either an increase in L-type channel expression (i.e. increased synthesis, decreased degradation) or availability (e.g., recruitment), since there was no increase in the maximum L-type current stimulated by (+)-PN-202-791 in cholesterol-enriched cells (273). Instead, a shift in the inactivation potential and a decrease in sensitivity to dihydropyridine antagonists (273), suggested that cholesterol enrichment may be associated with a change in the functional properties of the L-type calcium channel. Therefore, altered L-type channel function might be a result of a change in membrane physical properties such as fluidity and thickness which are modulated by cholesterol (273). Cholesterol enrichment of the membrane may also slow or inhibit drug interactions with membrane receptors (293).

d. Na^+-H^+ exchange

The plasma membrane Na^+-H^+ exchanger is a membrane-bound glycoprotein which exists in a wide range of cell types. It catalyses the coupled transmembrane exchange of extracellular sodium (Na^+) for intracellular H^+ without consuming any ATP (295). The main physiological roles of the Na^+-H^+ exchanger are regulating intracellular pH, controlling cell growth and proliferation, and adjusting cell volume (296,297). In some pathological states, including essential hypertension, ischemia/reperfusion, diabetic cardiomyopathy and diabetic nephropathy, Na^+-H^+ exchange also plays an important role (298-301).

Earlier studies have shown that the fluidity of rat colonic brush-border membranes can influence Na^+-H^+ exchange activity. As discussed previously, fluidity changes commonly occur following alterations in membrane cholesterol (302,303). When cultured human lymphoblasts were incubated with liposomes of varying cholesterol concentration, alterations of Na^+-H^+ exchange were observed (304). Enrichment of lymphoblast membrane cholesterol resulted in an inhibition of antiporter activity, whereas depletion of membrane cholesterol enhanced the activity of Na^+-H^+ exchange (304). The mechanism of the cholesterol effect on Na^+-H^+ exchange remains unclear. One recent study on the effect of cholesterol on human platelets found cholesterol enrichment resulted in an increase in phospholipase A_2 (305). They proposed that membrane-modulated phospholipase A_2 may act as a molecular-switch for the regulation of Na^+-H^+ exchange in cholesterol-enriched platelets (305).

e. Na^+-Ca^{2+} exchange

Another pathway contributing to trans-plasma membrane Ca^{2+} flux is Na^+-Ca^{2+} exchange. It has been found in various cells including epithelia, nerves and muscles. The Na^+-Ca^{2+} exchanger was first hypothesized by Reuter and Seitz twenty years ago (306), and was then identified in isolated cardiac sarcolemmal membranes by Reeves and Sutko (307). This is a carrier-mediated process coupling the flux of Na^+ in one direction to the movement of Ca^{2+} in the opposite direction (308). The stoichiometry of Na^+-Ca^{2+} exchange is three Na^+ for each Ca^{2+} transported, making it electrogenic and sensitive to membrane potential (309). One of putative roles of Na^+-Ca^{2+} exchange is to maintain the intracellular concentration of Ca^{2+} in cells at a resting level (310).

All studies concerning the influence of membrane cholesterol on Na^+-Ca^{2+} exchange

have used cardiac sarcolemmal vesicles (244,258,282,312,313). Cholesterol enrichment of sarcolemmal vesicles resulted in a significant increase in the initial rate of Na^+-Ca^{2+} exchange (258). There is a very good general correlation between the cholesterol content of sarcolemmal membranes and Na^+-Ca^{2+} exchange (258). It was suggested that a cholesterol-rich annulus surrounding the Na^+-Ca^{2+} exchange protein may modulate its function (258). This was confirmed later in a study investigating the thermotropic transition points of Na^+-Ca^{2+} exchange in proteoliposomes reconstituted with varying cholesterol concentrations (314).

In situ oxidation of membrane cholesterol with cholesterol oxidase resulted in an inhibition of Na^+-Ca^{2+} exchange (258,282). This inhibition was particularly prominent (67%) during the early linear part of the reaction (0.5 seconds) (282). Oxysterols are distributed quite differently in the membrane. They are less polar than cholesterol and occupy a higher molecular area in the membrane (311). The effect on Na^+-Ca^{2+} exchange produced by oxysterols may be due to the disturbance of cholesterol-rich domains in the membrane by the oxidation reaction (282). This would further support the previous conclusions that a cholesterol rich annulus may be associated with the Na^+-Ca^{2+} exchange protein (258). If large amounts of cholesterol were oxidized in the membrane, Na^+-Ca^{2+} exchange could be completely eliminated. However, this was primarily due to an elimination of any effective permeability barrier in the membrane (282).

In a study of reconstituted phosphatidylserine/phosphatidylcholine vesicles, no exchange was observed unless significant amounts (20% by weight) of cholesterol were also present (312). It is interesting to note that this amount of cholesterol is similar to endogenous levels of sarcolemmal cholesterol (312). This observation was confirmed by another study (313). This sterol requirement for Na^+-Ca^{2+} exchange is highly specific for

cholesterol. Several cholesterol analogues with minor structural modifications were unable to substitute for cholesterol in supporting exchange activity (244).

C. MATERIALS AND METHODS

I. Materials

Cholesterol oxidase (pseudomonas fluorescens) and catalase (bovine liver) were purchased from Sigma Chemical Co. The cholesterol oxidase was purified as a lyophilized powder. Other preparations of cholesterol oxidase (nocardia erythropolis) which were chromatographically purified yielded similar results in preliminary experiments. The fluorescence probes, nile red and dimethyl aminostyryl-methylpyridiniumiodine (DASPMI) were obtained from Molecular Probes Inc. (Eugene, OR). Some selected cholesterol derivatives which were used as standards for the high performance liquid chromatography (HPLC) analysis were purchased from Steraloids Inc., Wilton, NH. Dil and fura-2 were obtained from Molecular Probes Inc., Junction City, Oregon. Collagenase was obtained from Worthington Biochemicals (Freehold, New Jersey). The 2% cholesterol supplemented rabbit chow was purchased from ICN Biochemicals Inc. (Cleveland, OH). All other chemicals were of standard reagent grade and were purchased from Sigma.

II. Lipoprotein Isolation

Male albino New Zealand white rabbits weighing ~ 2.5 kg were placed on a 1% cholesterol supplemented diet for at least 1 month prior to blood collection. Animals were used for only a 4 month period for the collection of blood. Blood samples were collected from the central artery of the ear in the presence of 1 mg/ml EDTA and plasma separated and collected immediately by centrifugation at 1500 xg for 10 minutes at 4°C. Blood

samples of human's were collected in test tubes containing ethylenediamine tetraacetic acid (EDTA; 1 mg/ml) and immediately centrifuged at 3,000 rpm at 4^0C. LDL and VLDL (very low density lipoprotein) were isolated by single spin density gradient ultracentrifugation in the presence of 0.18 μM thimerosal, 1.5 μM DTNB and 2 μM PMSF (339,340). Dithiobisnitrobenzoic acid (1.5 mM), phenylmethylsulphonyl fluoride (2 mM), thimerosal (0.08 mg/ml) were added to the plasma to inhibit lecithin:cholesterol acyl transferase, proteolysis and bactericides, respectively (341). Vitamin C (50 μM) was also included to inhibit oxidation (341). Low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions were isolated from plasma by the ultracentrifugal method of Lindgren (342). The fractions were identified according to their buoyant density and the percentage composition of triglycerides, cholesterol and phospholipid. The LDL fraction was not oxidized during isolation or prior to their use in experiments as assessed by an absence of malondialdehyde reactive products (343,344). Cholesterol (free and esterified) and phospholipid were biochemically quantitated as described (258,345) after lipid extraction in chloroform:methanol (2:1) (258).

III. Cardiomyocyte Isolation

Calcium tolerant, contractile single cells were isolated from control rabbits not used for lipoprotein isolation. The procedure, which involves retrograde perfusion of an excised rabbit heart with a Ca^{2+}-free then minimal Ca^{2+} containing solution supplemented with 1.3 mg/ml collagenase and 0.5 mg/ml hyaluronidase, has been described in detail previously (346). After ~ 30 minutes perfusion, the heart was removed and the cells gently teased free in a petri dish. Cells were separated at 1 xg and viable myocytes adhered to coverslips which had been previously coated for 20 minutes with 0.2 mg/ml laminin. The cells were

used the same day that they were isolated. This procedure yielded cells which were 70 - 80% in a rod-like shape, did not beat spontaneously and excluded trypan blue. The resting membrane potential of the cells was -80 mV (measurement carried out by Drs. W.C. Cole and N. Leblanc).

IV. Smooth Muscle Cell Isolation and Treatment

Smooth muscle cells were isolated from the aorta of adult male albino rabbits as described by Pierce et al (347). The cells were plated on 60 x 15 mm Primaria culture dishes (Falcon Plastics) in Medium 199 which contained 10% fetal calf serum, 2.0 mM glutamine, 100 U penicillin/ml and 100 ug streptomycin/ml. For the histochemical fluorescence studies (see below), cells were grown on gelatin-coated glass coverslips (22 RD, Propper). The cells were treated (unless otherwise indicated) with 1.0 mg cholesterol oxidase (4.9 U/mg) and a 75 fold higher catalase concentration (U:U) in 4 ml of the above medium at 37°C. Cells which were plated on 60 x 15 mm Primaria culture dishes were used for cholesterol and protein assays as well as HPLC analysis. For the histochemical fluorescence studies (see below), cells were grown on gelatin-coated glass coverslips (22 RD, Propper) and then treated with LDL with or without cholesterol oxidase preincubation. Photomicrographs were taken using Kodak film at ASA 100 with a Nikon 2020 camera mounted on a Nikon phase contrast microscope with a 20x objective.

V. Labelling with Fluorescent LDL

Rabbit LDL was labelled with the fluorescent probe Dil as described (348). Dil-labelled LDL is an effective moiety to observe LDL/receptor interactions on a cell (349,350). Freshly isolated cardiomyocytes were incubated with varying concentrations of

Dil-LDL in Medium 199 for 30 - 120 minutes at 37°C, then fixed at 22°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by mounting in 70% glycerol in PBS (351,352). Cells were photographed on a Nikon Labphot microscope excited at 520-550 nm and detected through a 580 nm barrier filter. Fluorescence intensity was quantified with photomultiplier tubes and the data collected and stored on computer (353).

VI. Histochemical Methods

1). Nile Red: a) LDL (0.1ml) was pre-incubated with or without 0.05 mg/ml cholesterol oxidase for varying durations, then added to the confluent cells for 3 hours at 37 ^{0}C; b) Confluent cells were treated with varying concentrations of cholesterol oxidase and different exposure times. Both cells from a) and b) were then washed briefly (2x4ml) with HEPES solution (140 mM NaCl, 6.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM dextrose, 6.0 mM HEPES (pH 7.4)). The cells were then stained with 1 μg/ml nile red in acetone for 15 minutes, rinsed with HEPES solution again, air dried and mounted (354,355). Fluorescence was monitored with a Spex spectrofluorometer by setting the excitation wavelengthat 480 nm, slit width at 4 mm and using a Nikon DM510 epi-fluorescence filter without the B-2A excitation filter. Fluorescence intensity was quantified with photo multiplier tubes and the data collected and stored on computer. Ten randomly chosen fields on the coverslip were examined for fluorescence intensity from each sample. Fluorescence photomicrographs were taken with a Nikon Labphot microscope equipped with epi-fluorescence optics using a 20x fluorescence objective and DM510 filter. Fuji colour transverse films at ASA 400 were used.

2). DASPMI: Confluent cells were treated with 1 mg/ml cholesterol oxidase for 3 hours at 37 °C. In a separate series of experiments, cells were treated with 1 mM

2,4-dinitrophenol (DNP) for 15 hours at 37°C, 0.1 mg/ml 4-cholesten-3-one (4-3-one) for 24 hours at 37 °C, or 75% ethanol for 20 minutes at room temperature. Samples were stained with DASPMI (9μg/ml in HEPES) for 30 minutes at 25 °C (356-359), and then rinsed briefly with HEPES (2x4ml). The fluorescence intensity was detected with a Spex spectrofluorometer under the conditions described above for the nile red study. The fluorescence photographs were taken with a Nikon Labphot microscope as described above.

3). Trypan blue: Trypan blue was used to assess cell viability ± treatment with cholesterol oxidase, DNP, 75% ethanol or 4-cholesten-3-one (4-3-one) under the experimental conditions described above. After the treatments, all the samples were stained with 20% trypan blue for 2 minutes at 25 °C, then rinsed with HEPES briefly to remove excess dye. Photomicrographs were taken on the Nikon phase contrast microscope described above.

VII. HPLC (High Performance Liquid Chromatography)

HPLC was used to identify and quantify the oxidized cholesterol content in cells after treatment with cholesterol oxidase. Vascular smooth muscle cells were taken from the incubator after treatment ± varying concentrations of cholesterol oxidase for 24 hours(unless otherwise indicated) and washed with 1 ml of HEPES solution at 37°C. The cells were scraped from the culture dish into a glass homogenizer containing 2 ml of 2:1 chloroform : methanol and then homogenized with a glass pestle. The fragmented cells were poured into glass conical tubes and the homogenization vessel washed with 2 ml + 1 ml of the chloroform : methanol solution. The conical tubes were stored at 0 - 5 °C overnight for extraction of lipids as described (360). The solvent was evaporated under a stream of N_2 gas, then the lipids were suspended in 1 ml of methylene chloride and filtered through a 25 micron filter attached to a Hamilton syringe. The filtrate was then exposed to nitrogen gas

to evaporate the solvent and suspended again in 100 μl of methylene chloride of which 20 μl was used for HPLC analysis. A modification of the technique of Sevanian and McLeod (361) was employed to detect the oxidized cholesterol species. The HPLC used was a Beckman Instruments model 166 detector, a 116 solvent delivery system and a Beckman Ultrasphere silica column (4.6 mm x 15 cm). Flow rate was maintained at 1.0 ml/min and the mobile phase was 95:5 hexane:isopropanol. UV detection of the peaks was carried out at a wavelength of 208 nm. HPLC was also used to identify and quantify the oxidized cholesterol species in LDL and VLDL from the coronary artery disease patients and the control group.

VIII. Preparation and Measurement of Lipid Peroxidation

Malondialdehyde (MDA) content of lipoprotein after treatment with cholesterol oxidase ± catalase was determined by the thiobarbituric acid reactive substances (TBARS) method (343,362). Briefly, lipoproteins (150 μg protein) were suspended in 1 ml of 140 mM KCL, 20 mM MOPS (3-[N-Morpholino]propane-sulfonic acid) (pH 7.4), followed by the addition of 2 ml of 15% TCA (trichloroacetic acid), 0.375% TBA (thiobarbituric acid) and 0.25 N HCL. The mixture was boiled for 15 minutes, then cooled in ice and subsequently centrifuged at 1500 rpm for 20 minutes. The absorbance of the supernatant was determined immediately using a Beckman Du-62 spectrophotometer at a wavelength of 535 nm. A free radical generating system was employed to induce lipid peroxidation of lipoprotein. Reactions were initiated by the addition of freshly prepared Fe^{3+}-ADP (50 uM $FeCl_3$, chelated by 0.5 mM ADP) and 3 mM DHF (dihydroxyfumaric acid) (363). Incubations were carried out at 37^0C for 60 minutes. Freshly diluted malondialdehyde bis (dimethyl acetal) (1,1,3,3-tetramethoxypropane) was used as a reference standard. TBARS were expressed

as MDA equivalent content (nanomoles of MDA per milligram of lipoprotein protein).

IX. Analysis of Lysine Content

Free amino acid groups in LDL were measured using trinitro- benzenesulfonic acid (TNBS) as described (104,364). Lipoprotein samples (30 -60 ug protein) were suspended in 1 ml of 140 mM KCl, 20 mM MOPS (pH 7.4), and mixed with 1 ml of 4% $NaHCO_3$ (pH 8.4) and 50 ul 0.1% TNBS. The mixture was incubated at 37 ^{0}C in a water bath for 90 minutes, and then the absorbance at 340 nm was recorded. The oxidation of lysine was produced by incubating the lipoprotein sample with 50 uM $CuSO_4$ in 1 ml of 140 mM KCl, 20 mM MOPS (pH 7.4) for 60 minutes at 37^{0}C, then the TNBS reactivity was estimated as described above. The amino acid concentration was determined by reference to a lysine standard. The lysine reactivity was expressed as μmol per milligram lipoprotein.

X. Measurement of Cellular Ca^{2+} Transients

Intracellular [Ca^{2+}] was measured spectrofluorometrically using the Ca^{2+} indicator dye fura-2 (365). Cardiomyocytes were incubated for 15 minutes at 22°C with 2 μM of the acetoxymethylester of fura-2 (366,367). In a typical experiment, a cover slip containing adherent myocytes loaded with fura-2 was mounted in a Leiden chamber (368) heated to 37°C with a Medical Systems PDMI-2 Open Perfusion Micro-Incubator (Greenvale, NY) in Kreb's perfusion solution containing (in mM): NaCl 120; $NaHCO_3$ 25; KCl 4; KH_2PO_4 1.2; $MgSO_4$ 1.2; dextrose 5.5, $CaCl_2$ 1.8 (pH 7.4). This system was fixed on the stage of a Nikon Diaphot epifluorescent microscope which was attached to a SPEX Fluorolog spectrofluorometer (SPEX Industries, Edison, NJ) (366). Myocytes were initially viewed

under phase contrast bright field microscopy to optimize cell placement and focus. To monitor cellular fluorescence, excitation light was switched via a rotating chopper blade from 340 to 380 nm which was derived from the SPEX dual wavelength spectrofluorometer. Cell epifluorescence was emitted at 505 nm and recorded and quantitated with photomultiplier tubes coupled to a computer. The fluorescent signal from the cells was calibrated with the in situ method of Grynkiewicz et al (365) using 20 uM 4-bromo-A23187 to obtain maximal fluorescence and 10 mM EGTA to determine the minimum fluorescence (365,369).

For each experiment, a single cell was selected and stimulated by passing currents via two platinum electrodes spaced ~ 8 mm apart. The cell was stimulated to contract at 0.4 Hz with 8 - 12 V (in air) and a 3 ms duration. These cells were capable of contracting in response to these stimuli under these conditions for over one hour.

XI. Subjects Studied

Twenty three patients (21 men and 2 women) with coronary artery disease and proven myocardial infarction were studied. They were 44 to 75 years old (mean: 60.8 ± 1.8 years). All patients had at least one myocardial infarction and eight had two or more previous myocardial infarctions. Coronary angiograms carried out in thirteen patients revealed significant stenoses or occlusions of the right anterior descending and circumflex arteries. The ECG (electrocardiogram) on 12 patients revealed an anterior or an anterolateral infarction, and 10 showed an inferior or inferiolateral location of the infarction. Three ECGs had anterior and inferior sites of infarctions. One patient had a left bundle branch block on the ECG, but an anterior wall motion abnormality was confirmed. Radioventriculograms were carried out in all 23 patients and demonstrated a diminished left

ventricular ejection fraction (LVEF) averaging 42% and ranging from 15-73%. Echocardiograms in 5 patients revealed regional or regional and global reduction in left ventricular systolic motion. Twenty of the 23 patients had extra heart sounds including both S_3 and S_4. The medications in these patients included one or more of the following: ASA EC (enteric-coated aspirin), captopril, persantine, diltiazem, or nitroglyceride. At the time of blood collection, the patients were following no special dietary restrictions. The control group consisted of healthy, asymptomatic men (n=16) and women (n=7), aged between 36 and 66 years old (mean 48.4 ± 1.9 years). Their histories were negative for diabetes, hypertension, cancer, heart disease, alcohol or drug abuse, and their serum cholesterol and triglycerides were within the normal level. Only 3 of the patients and 2 of the control subjects were cigarette smokers.

XII. Statistical Analysis:

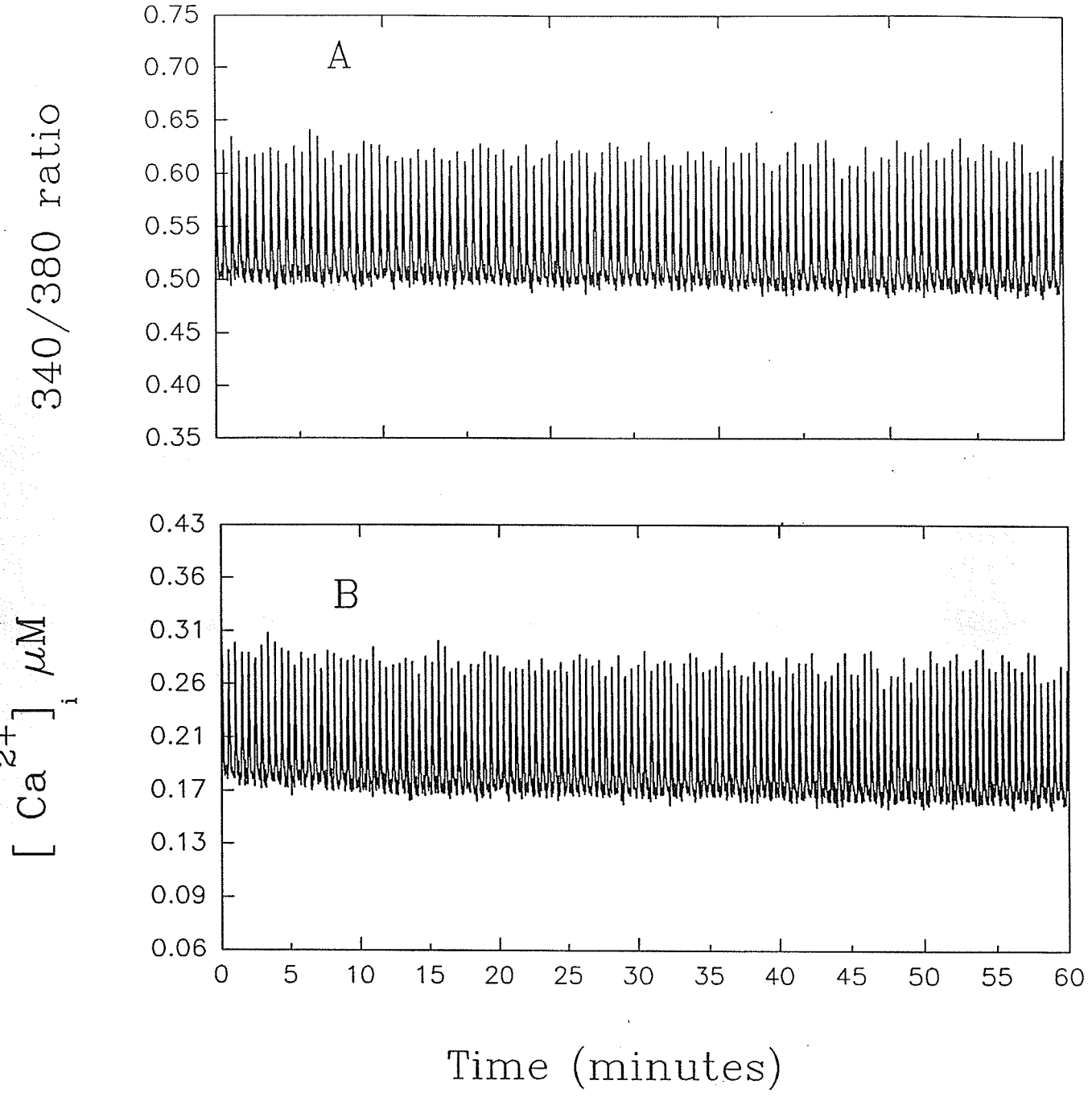
Data were analyzed statistically with a student's t-test or, where appropriate, a one-way analysis of variance test followed by Duncan's new multiple range post hoc test (341). The level of significance was arbitrarily set at < 0.05.

D. RESULTS

I. The Effects of Low Density Lipoprotein on Calcium Transients in Isolated Rabbit Cardiomyocytes.

The effects of adding LDL to the solution bathing the cardiomyocytes were investigated. The effects of LDL on the cellular Ca^{2+} transient were studied over a 60 minute time period (Figure 1). The cardiomyocyte could be stimulated and displayed a stable Ca^{2+} transient for this length of time (Figure 1A, B). Recordings were interrupted after 30 seconds every 5 minutes to avoid photo bleaching the dye which would have occurred during continuous periods of light exposure. Incubation of the cardiomyocyte with LDL induced a relatively slow but potent increase in the Ca^{2+} transient (Figure 1C, D). It is important to note that these cells appeared structurally unchanged upon microscopic examination during the 60 minute exposure to LDL and responded appropriately to the electrical stimulation. These effects of LDL on the Ca^{2+} transient of cardiomyocytes were carried out as a function of the LDL cholesterol concentration and duration of incubation in a series of experiments (Table 1). Peak systolic Ca^{2+} levels in cardiomyocytes were significantly increased by 1.0 mg LDL/ml after 30 or more minutes of incubation. A higher LDL cholesterol concentration (3.0 mg/ml) also induced a significant increase in peak systolic Ca^{2+} levels in cardiomyocytes and this tended to be slightly larger than the effects with 1.0 mg/ml. Concentrations below 1.0 mg/ml were ineffective in significantly changing the transient. Diastolic Ca^{2+} concentration was unaffected by LDL treatment over the time course and concentration range studied.

The relationship between the effects of LDL on the Ca^{2+} transient and lipid


A
B
340/380 ratio
[Ca2+]i μM
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.43
0.36
0.31
0.26
0.21
0.17
0.13
0.09
0.06
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (minutes)

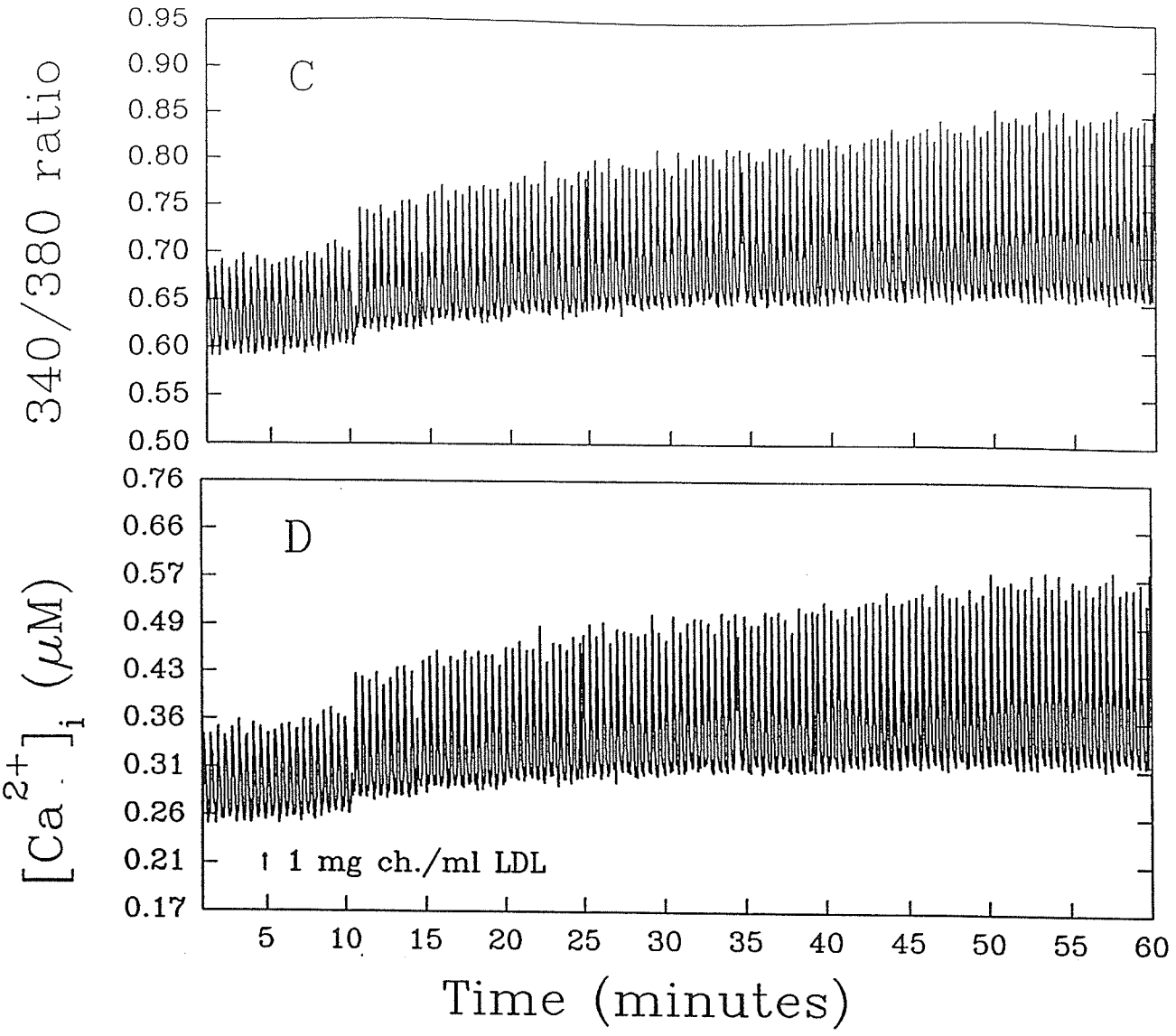


Figure 1. Representative recordings of calcium transients in control cardiomyocytes (A,B) or cardiomyocytes treated with 1 mg LDL cholesterol/ml perfusion medium (C,D). If present, the LDL was added at the time point indicated by the arrow. The recordings are shown as the ratio of the fura-2 fluorescent signal at excitation wavelengths of 340 nm to 380 nm (A,C) or after conversion to intracellular Ca^{2+} concentration (B,D) as described in the Methods section. The recordings here are not continuous but only for approximately 30 seconds then stopped. At the fifth minute, another 30 second recording was taken, followed by another 30 second recording at the tenth minute, etc. This stop-time recording allowed us to avoid the problems associated with photo bleaching but still monitor Ca^{2+} transients in the same cell over a 60 minute time period. Note the stability in the Ca^{2+} transient recording in the control cell (A,B) over the entire measurement period and the increase in the size of the Ca^{2+} transient induced in another cell after treatment with LDL (C,D).

Table 1. Time course of varying concentrations of LDL on intracellular calcium concentration of rabbit ventricular myocytes

LDL	Time (minutes)				
	0	15	30	45	60
0 mg/ml					
Systole	273.2 ± 35.4	264.3 ± 42.6	268.5 ± 31.8	281.5 ± 48.6	299.5 ± 31.7
Diastole	102.8 ± 28.7	104.5 ± 25.7	107.6 ± 19.3	105.3 ± 26.6	101.4 ± 19.5
0.1 mg/ml					
Systole	265.7 ± 54.3	301.2 ± 51.6	314.8 ± 66.2	307.5 ± 46.1	311.5 ± 42.1
Diastole	125.5 ± 31.2	125.7 ± 31.9	131.4 ± 37.4	129.5 ± 34.8	133.9 ± 35.1
0.3 mg/ml					
Systole	217.8 ± 49.7	221.8 ± 79.4	297.3 ± 53.6	289.5 ± 46.2	295.6 ± 45.8
Diastole	109.3 ± 31.6	140.9 ± 31.5	159.2 ± 47.3	154.7 ± 32.1	155.6 ± 41.6
0.6 mg/ml					
Systole	275.2 ± 44.5	317.8 ± 74.4	325.1 ± 79.5	333.2 ± 58.9	341.6 ± 62.1
Diastole	166.2 ± 30.9	217.4 ± 50.3	210.4 ± 38.5	216.8 ± 41.2	205.3 ± 33.2
1 mg/ml					
Systole	242.3 ± 64.5	325.7 ± 64.6	398.5 ± 45.7*	437.5 ± 36.2*	485.6 ± 51.2*
Diastole	126.4 ± 28.5	170.3 ± 28.5	189.3 ± 34.6	179.5 ± 26.5	194.6 ± 38.4
3 mg/ml					
Systole	189.4 ± 36.1	357.2 ± 39.6	413.9 ± 49.2*	442.5 ± 65.3*	568.6 ± 67.3*
Diastole	144.7 ± 25.3	193.4 ± 57.2	223.7 ± 37.5	231.2 ± 46.2	234.3 ± 30.1

*: $P<0.05$ VS control. (n = 4-8). Calcium concentration is in nM.

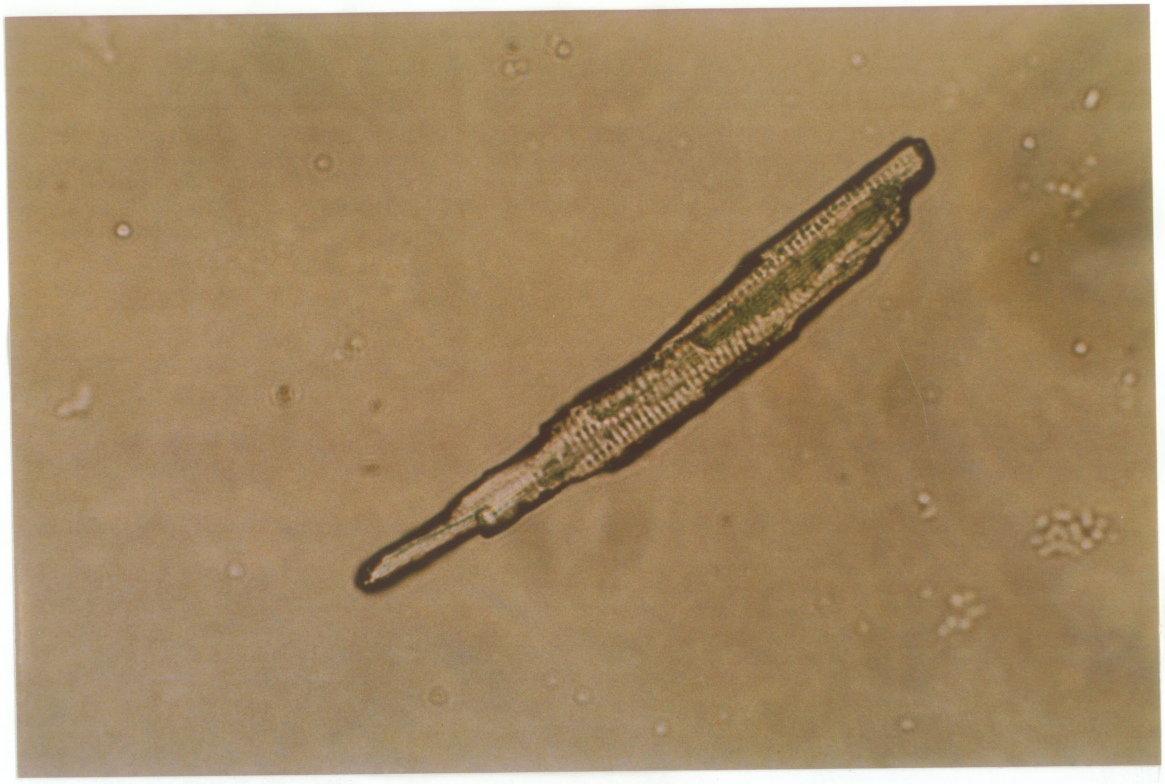
alterations within the cardiomyocyte were of interest. Thus, cardiomyocytes were treated with 3 mg LDL cholesterol/ml for varying times, washed briefly with LDL free media to remove any LDL which was loosely associated with the cell surface, then scraped from the dishes and cell cholesterol and phospholipid content measured (Table 2). Incubation of cardiomyocytes with LDL induced a significant increase in cellular free cholesterol, cholesterol esters and total cholesterol within 30 minutes. Longer times of incubation induced a further, modest increase. Cell phospholipid content did not change significantly after LDL treatment. This observation, together with the time dependency of the reaction would strongly suggest that the LDL was being internalized and does not simply represent a cell surface association of the LDL.

To obtain further information regarding the nature of the association of LDL with the cardiomyocyte and to determine if the reaction was receptor mediated, a fluorescent label was incorporated into isolated rabbit LDL and its interactions with the cardiomyocyte observed. As shown in Figure 2, cardiomyocytes were stained well after incubation with the fluorescently labelled Dil-LDL. As the concentration of Dil-LDL was increased, fluorescent-staining of the cell became more intense (Figure 2C). This response was quantitated in a number of experiments. In agreement with the biochemical work shown in Table 2, a significant increase in fluorescent staining intensity of cardiomyocytes was observed at an LDL cholesterol concentration $\geq$ 1 mg/ml (Figure 3). Concentrations below this showed no significant effect. The reaction was also dependent upon the duration of incubation of the Dil-LDL with the cardiomyocytes (Figure 4). Dil-LDL interactions were also temperature dependent (37°C was greater than 4°C) (Figure 5). Competitive inhibition of the reaction by pre-incubation of the cell with an unlabelled LDL probe at a ten-fold higher concentration is frequently employed to detect the presence of a specific LDL receptor

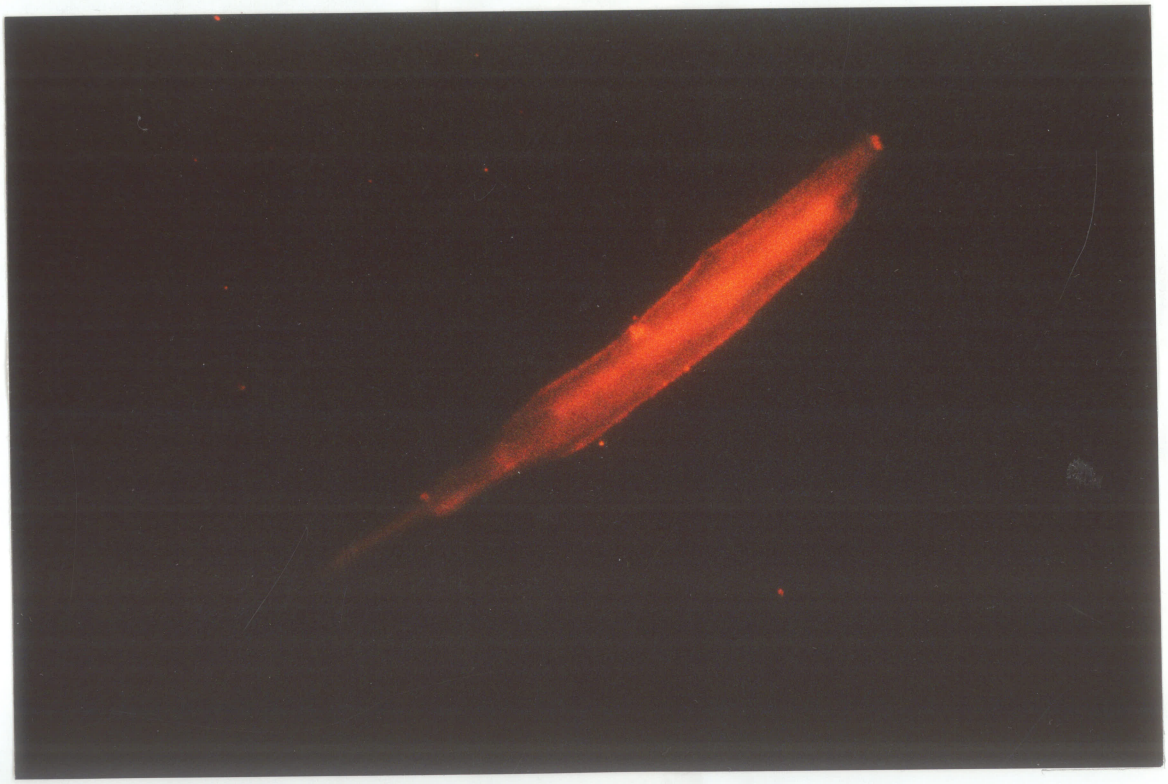
Table 2. Cellular lipid alterations in cardiomocytes treated with LDL for varying times

LDL incubation time	Free chol.	Chol. ester	Total chol.	Phospholipid
min		nmol/mg protein		
0	w7.49± 1.97	6.24± 1.10	13.73± 1.33	97.76± 15.48
5	10.63± 1.97	11.14± 1.68	21.74± 3.45	72.06± 10.79
30	13.82± 0.37*	16.66± 2.35 *	29.13± 1.11 *	122.36± 16.77
60	16.65± 1.76*	18.43± 1.67 *	34.99± 2.96 *	109.18± 15.41

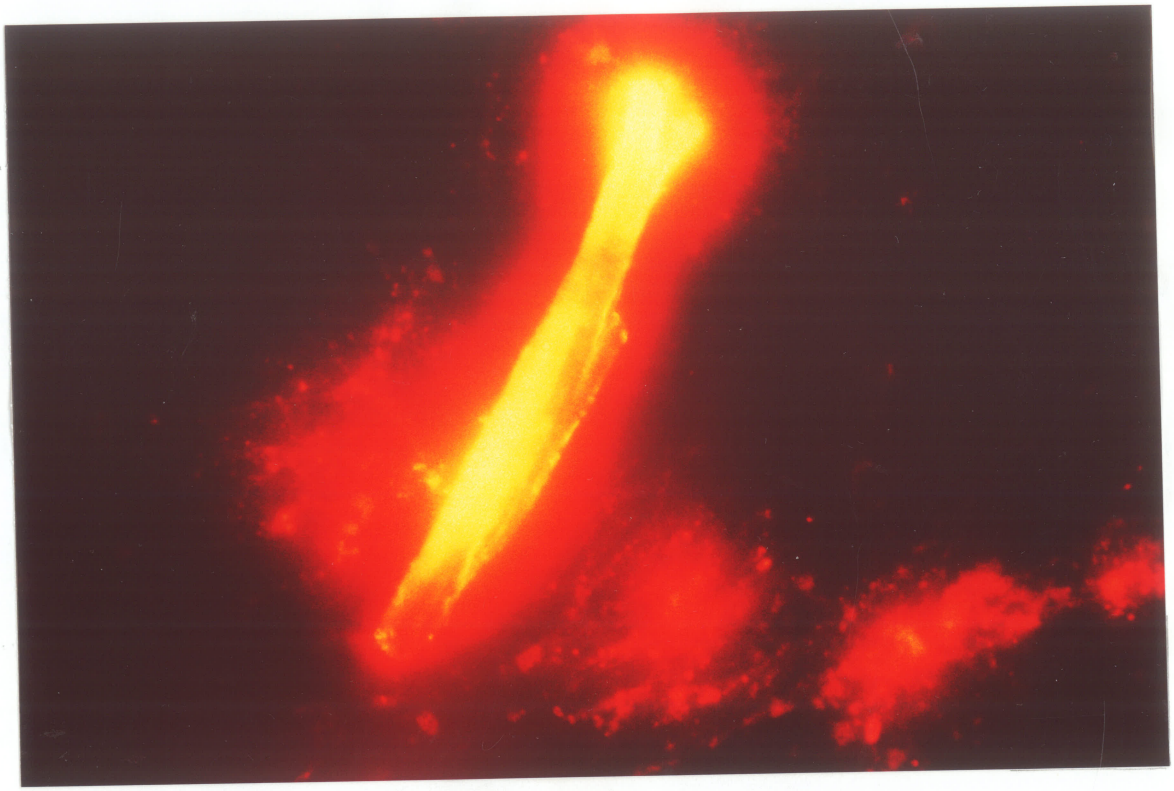
Free chol.: free cholesterol; Chol. ester: cholesterol ester; Total chol.: total cholesterol. In the 0 minute time group, cells were exposed to 3 mg LDL/ml, then immediately washed free of the LDL and lipids extracted. Data are means ± SE. * Significantly different from 0 minute group ($P < 0.05$). (n=4).



A.



B.



C.

Figure 2. Representative phase contrast photomicrograph of a cardiomyocyte (A) and the same cell with fluorescent photomicrography after exposure for 60 minutes at 37°C to 1 mg/ml Dil-labelled LDL (B). More intense fluorescent staining was exhibited by cardiomyocytes after incubation for 60 minutes at 37°C with 3 mg/ml Dil-labelled LDL (C).

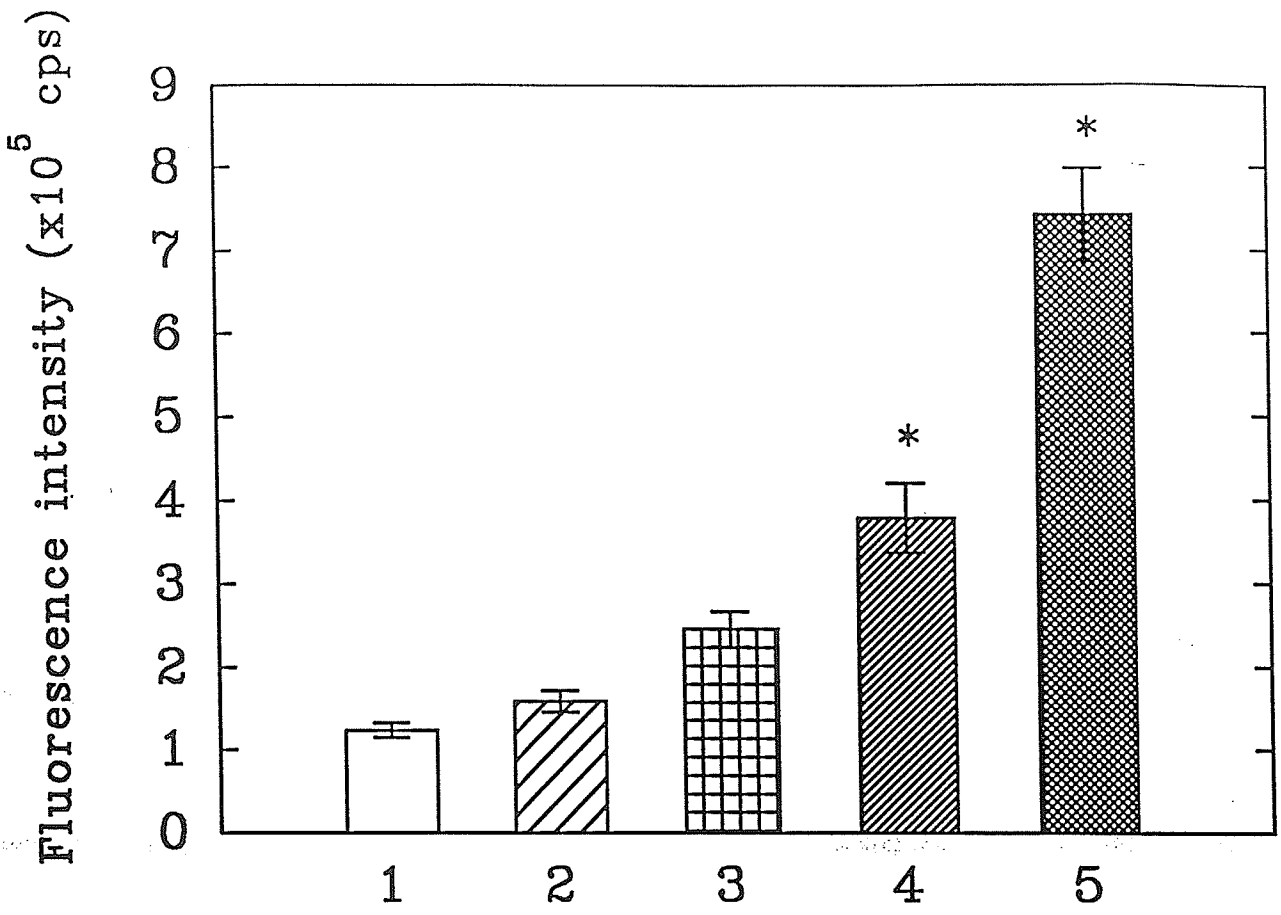


Figure 3. Fluorescent intensity of cardiomyocytes was quantitated with photomultiplier tubes after (1) no treatment, or treatment for 60 minutes at 37°C with (2) 0.05, (3) 0.1, (4) 1.0, or (5) 3.0 mg Dil-labelled LDL/ml perfusion medium (mg LDL cholesterol). Fluorescent intensity is expressed in arbitrary units of counts per second. Values represent mean ± S.E. of separate 30 determinations from 5 experiments. * $P < 0.05$ versus control values.

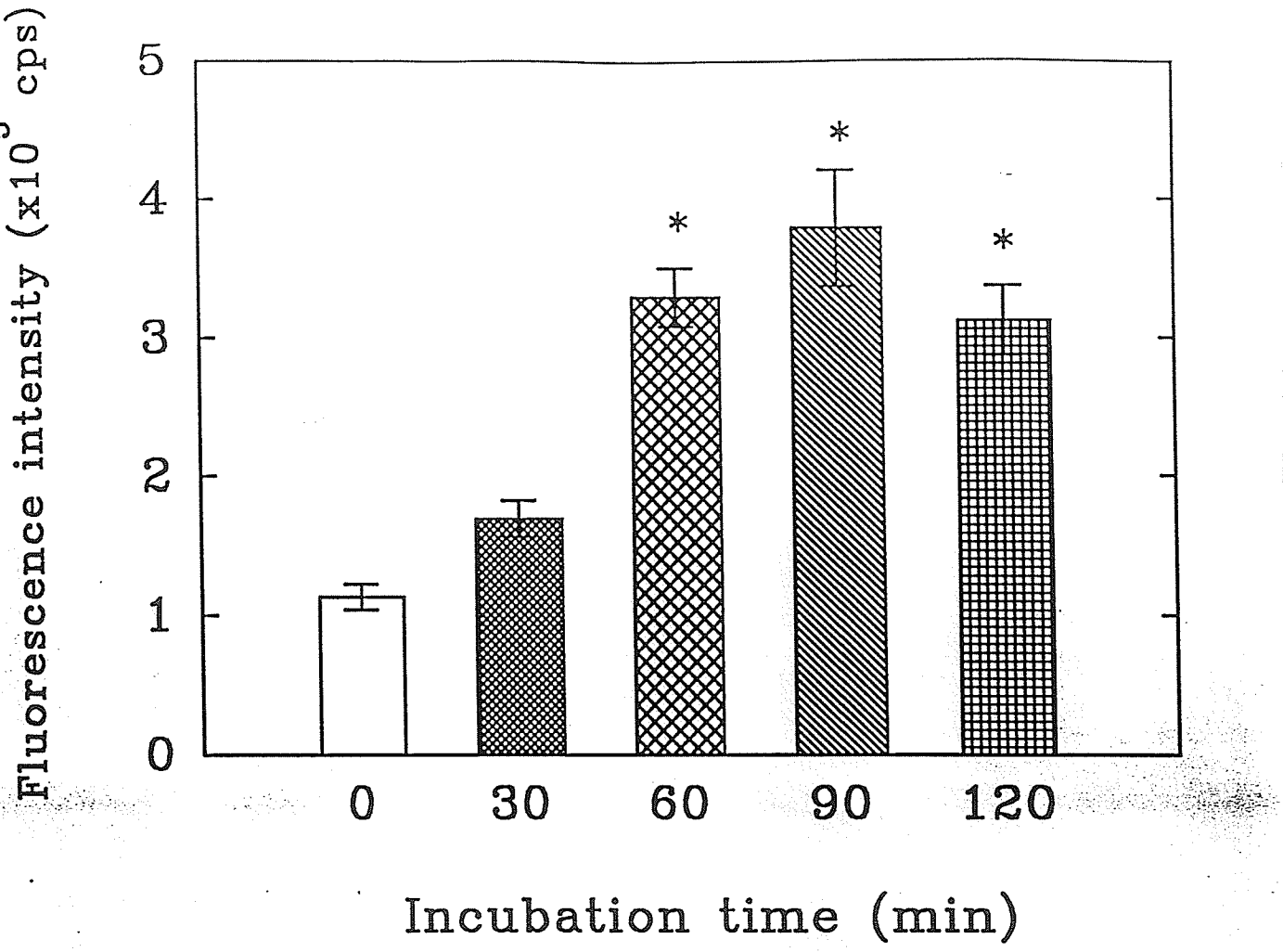


Figure 4. Fluorescence intensity of cardiomyocytes after varying incubation times of incubation with 1 mg/ml Dil-labelled LDL (mg cholesterol). Values represent mean ± S.E. of 30 separate determinations from 5 experiments. * $P < 0.05$ versus control (O time) values.

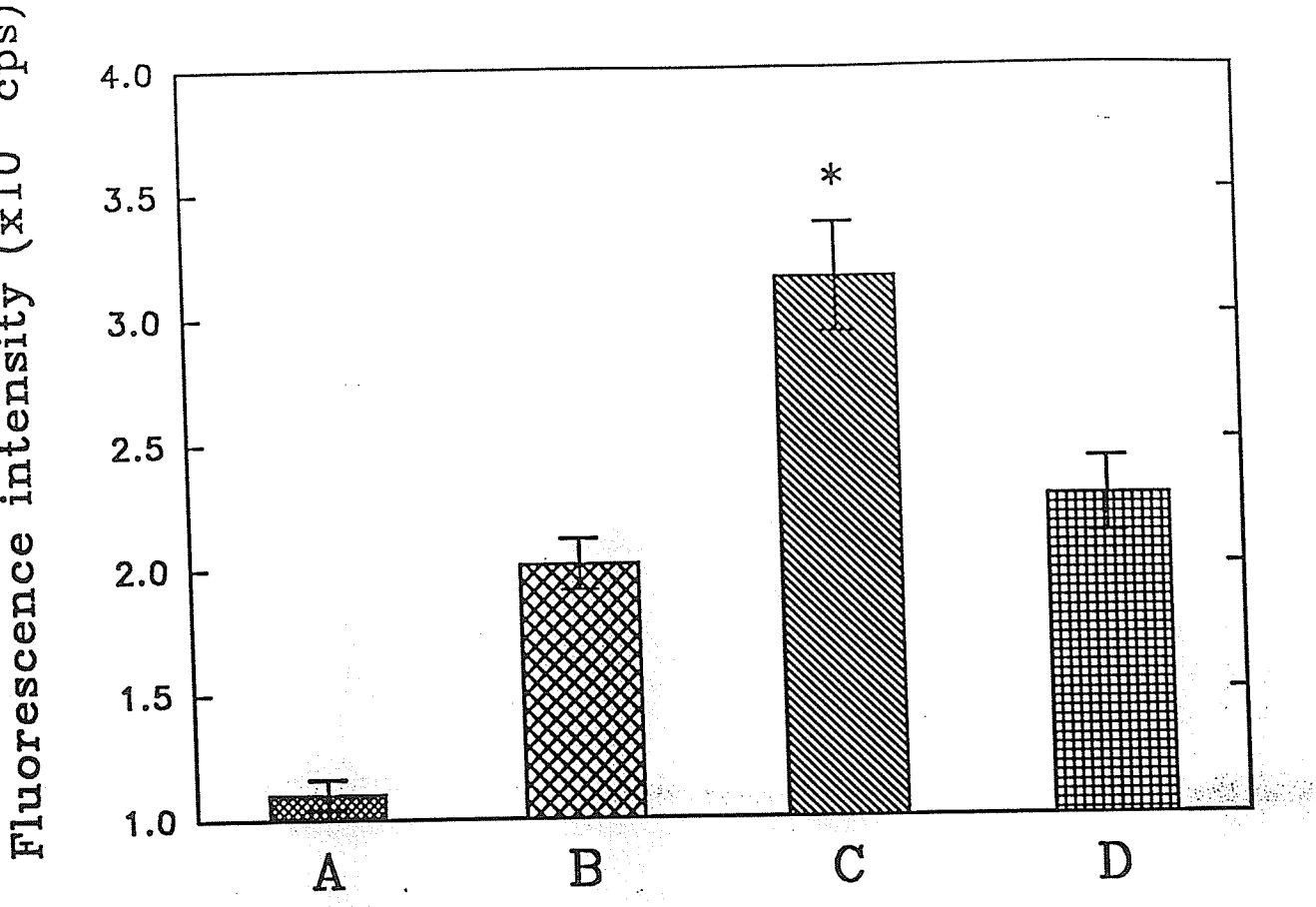


Figure 5. Fluorescence intensity of untreated, control cardiomyocytes (A) or cells after incubation with 1 mg/ml Dil-labelled LDL for 2 hours at 4°C (B) or 37°C (C) or as in (C) except that the cells were pre-incubated for one hour at 37°C with 10 mg/ml native, unlabelled LDL followed by the Dil-labelled LDL. Values represent the mean ± S.E. of 30 separate measurements from 4 experiments. * $P < 0.05$ versus control values.

mediated action (348-351). Native unlabelled LDL was pre-incubated with cardiomyocytes at a concentration of 10 mg/ml prior to the addition of 1 mg/ml Dil-labelled LDL. The native LDL significantly reduced the Dil-labelled LDL interactions with the cardiomyocyte (Figure 5).

The effects of LDL on the Ca^{2+} transient in cardiomyocytes was specific to this lipoprotein. As shown in Figure 6, HDL did not induce an increase in the Ca^{2+} transient as did LDL. In a series of experiments (n = 4), HDL caused no change in the Ca^{2+} transient in comparison to control ($P > 0.05$).

The mechanism responsible for the LDL-induced increase in the cardiomyocyte Ca^{2+} transient was investigated with the use of several pharmacological agents which block different ion transport pathways in the cardiomyocyte. Because the LDL effects on the transient were relatively slow to induce ($\geq$ 30 minutes), the drugs were introduced after or simultaneously with the LDL. Ryanodine is a plant alkaloid which is frequently used to probe the role of Ca^{2+} release from the sarcoplasmic reticulum in supporting intracellular Ca^{2+} transients and contractile activity in the heart (370,371). Thirty minutes perfusion of the cardiomyocytes with 10 μM ryanodine strongly depressed the Ca^{2+} transient in control cardiomyocytes (Figure 7). This effect could not be washed out. Ryanodine failed to have this effect in LDL-treated cardiomyocytes. In a series of experiments (n = 5), 10 μM ryanodine inhibited the Ca^{2+} transient by 81 ± 6.5% and 2 ± 0.8% in control and LDL-treated cardiomyocytes, respectively ($P < 0.05$). This effect of 10 μM ryanodine on the Ca^{2+} transient in control cardiomyocytes is in agreement with previous studies (370,371). It is important to note that the LDL-treated cardiomyocytes were less sensitive to ryanodine but not absolutely resistant to it. A much higher ryanodine concentration (100 μM) inhibited the Ca^{2+} transient completely in both LDL-treated and control cardiomyocytes.

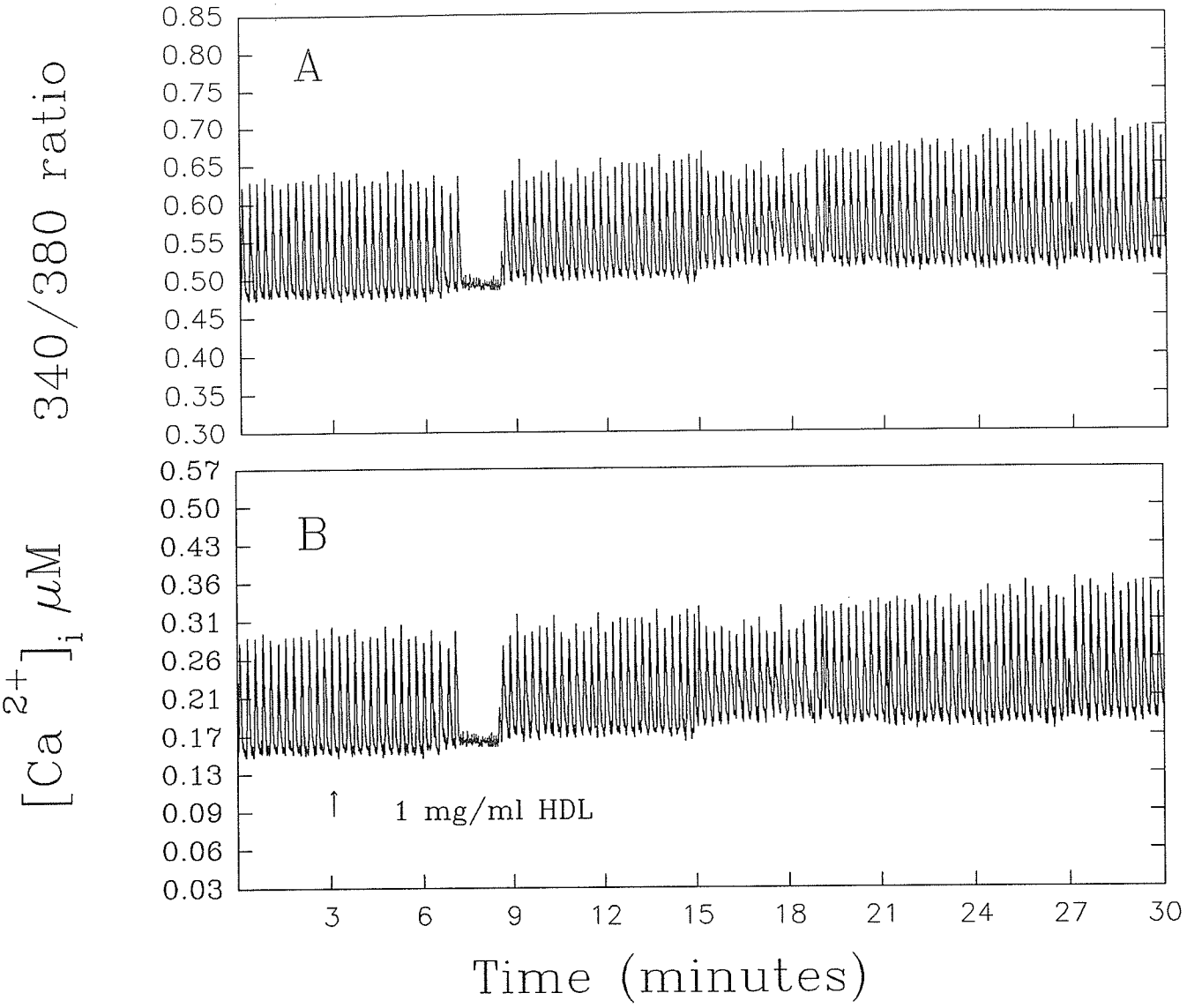


Figure 6. Representative recording of the 340/380 nm ratio of the fura-2 fluorescent signal (A) or the calibrated value of intracellular Ca^{2+} concentration (B) of transients in cardiomyocytes after treatment with 1.0 mg HDL cholesterol/ml. Recordings were only for ~ 30s, then stopped and the data saved before re-initiating recording again for ~ 30s three minutes later. The HDL was added at the time point indicated by the arrow. Note the lack of an effect on the Ca^{2+} transient. The spontaneous loss of signal (here observed at 7 minutes) was consistently observed in other cells treated with HDL although the time it occurred varied.

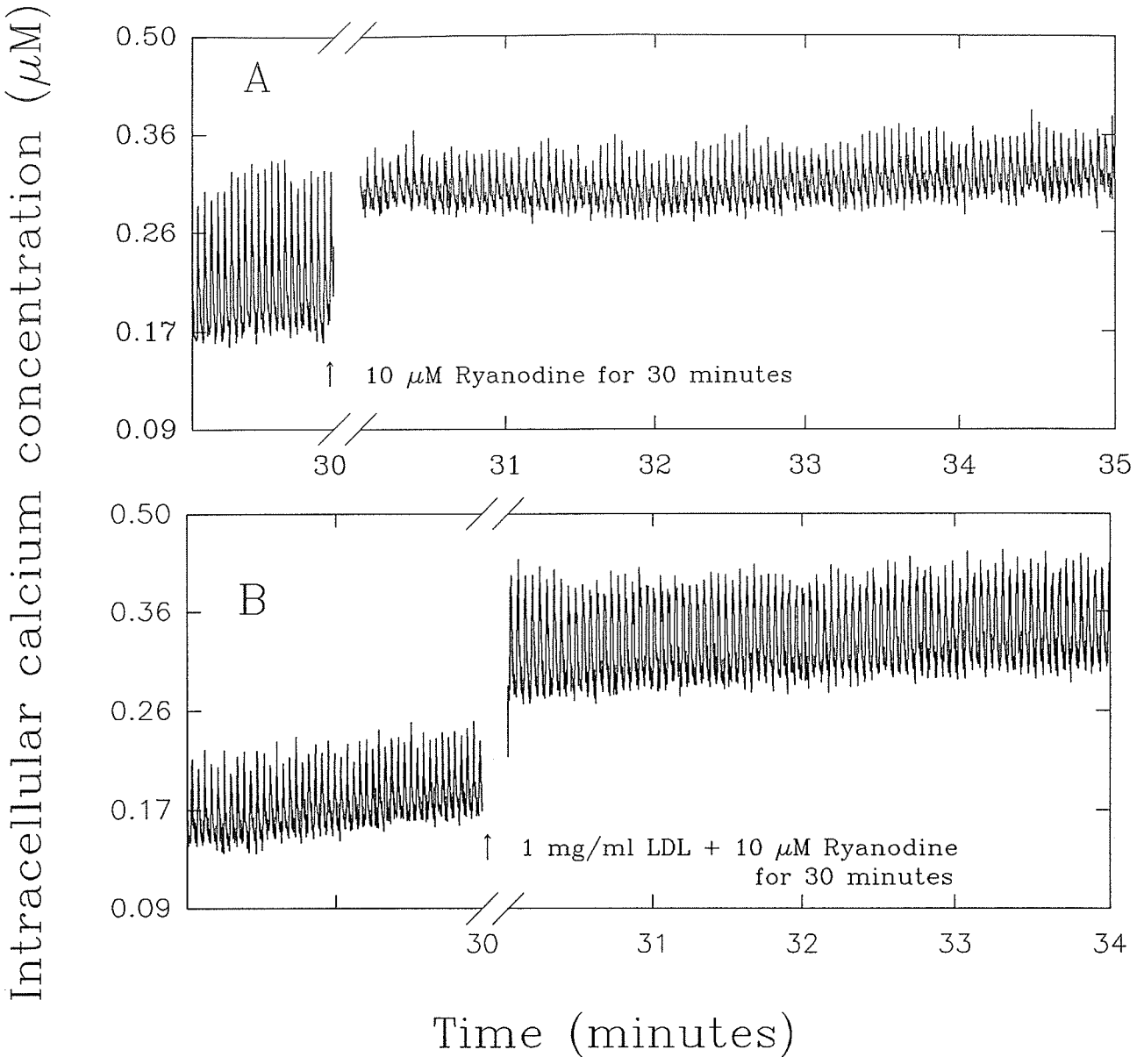


Figure 7. Effect of 10 μM ryanodine on intracellular Ca^{2+} transients in a control cardiomyocyte (A) or a cardiomyocyte treated simultaneously with 1 mg LDL cholesterol/ml perfusion medium (B). Ryanodine or LDL, if present, was added at the time point indicated by the arrow. The Ca^{2+} transient was not recorded until 30 minutes later and then continuously recorded for 4-5 minutes. Note the increase in Ca^{2+} transient induced by the 30 minute incubation with LDL which was not affected by ryanodine. This is in striking contrast to the effects of ryanodine on control cardiomyocytes.

Nicardipine is a potent antagonist of the L-type Ca^{2+} channel (372,373). The effects of 4 μM nicardipine on the Ca^{2+} transient in control and LDL-treated cells were investigated. Although the drug inhibited the transient in both cell types, the effect of the drug appeared slower in LDL-treated cell (Figure 8). A similar, although more exaggerated response to administration of the drug dichlorobenzamil (DCB) was also observed (Figure 9). DCB is a potent inhibitor of Na^+/Ca^{2+} exchange but also exhibits Ca^{2+} and K^+ channel blocker activity in the micromolar concentration range (374). DCB was a more potent inhibitor of the Ca^{2+} transient in control cells than in the LDL-treated cells. This was repeated in a series of experiments and the results presented in Figure 10. The cardiomyocytes treated with LDL showed a lower sensitivity ($P < 0.05$) to nicardipine and DCB than the control cardiomyocytes.

BAYK 8644 acts to stimulate Ca^{2+} entry into the myocyte through slow Ca^{2+} channels (375). Administration of 0.5 μM BAYK 8644 enhanced the Ca^{2+} transient by 20 $\pm$ 4% in control myocytes within 4 minutes. However, in cardiomyocytes treated for 30 minutes with 1 mg/ml LDL, 0.5 μM BAYK 8644 increased the Ca^{2+} transient by only 4 $\pm$ 2% (n=5) which was significantly different than the response in control cardiomyocytes ($P < 0.05$) (data not shown).

The Ca^{2+} transient in the cardiomyocyte, and thus, myocardial contractility, is extremely sensitive to changes in extracellular Ca^{2+} concentration. The effect of LDL on the Ca^{2+} transient in cardiomyocytes was examined as a function of the perfusate Ca^{2+} concentration (Table 3). As demonstrated previously (Figure 1, table 1), LDL induced an increase in the cellular Ca^{2+} transient within 30 minutes. If the $[Ca^{2+}]$ was reduced to 1.0 mM, the effect on the Ca^{2+} transient was less potent and required 45 minutes to become

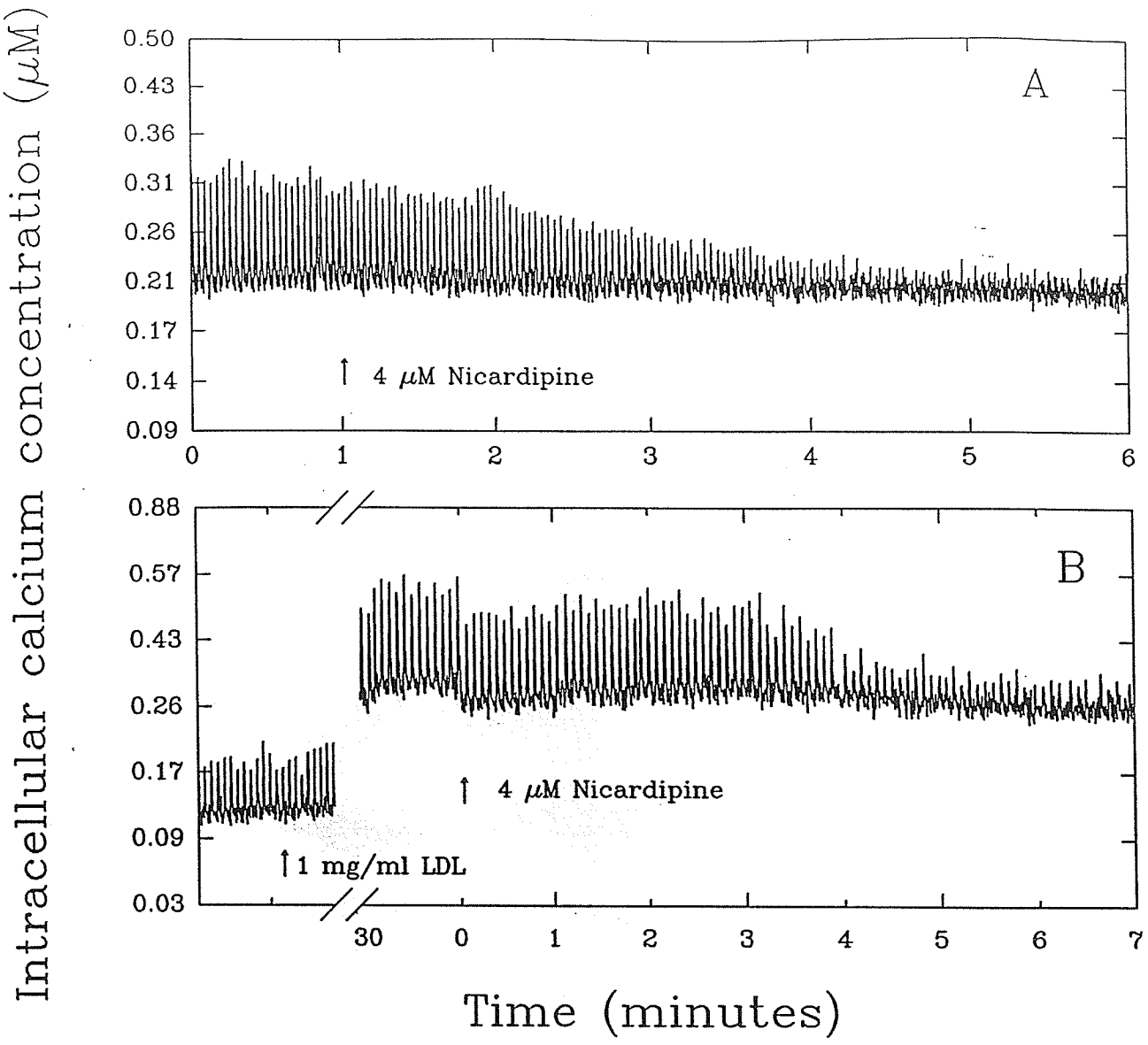


Figure 8. Representative recordings of Ca^{2+} transients after exposure to 4 μM nicardipine (arrow) in a control cardiomyocyte (A) or after 30 minute exposure to 1 mg LDL cholesterol/ml (B). In the former recording, the measurements were continuous for the entire 6 minute nicardipine treatment period. In the lower tracing (B), the recording was for ~ 30 seconds, interrupted for 30 seconds, etc. during the > 6 minute nicardipine treatment period.

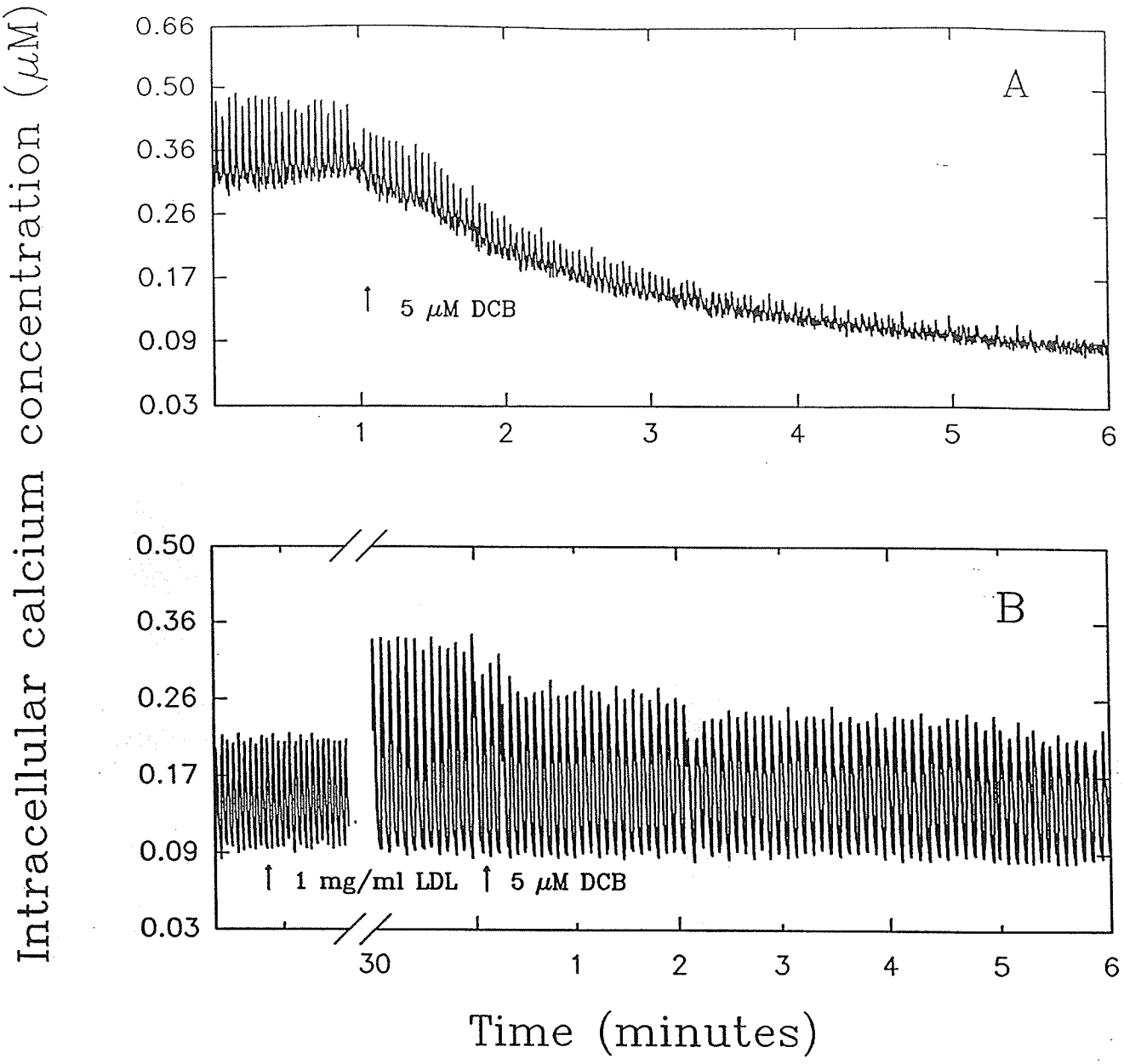


Figure 9. Representative recordings of Ca^{2+} transients after exposure to 5 μM dichlorobenzamil (DCB) (added at the arrow) in a control cardiomyocyte (A) or a cell after 30 minutes of treatment with 1 mg LDL cholesterol/ml (B). The recordings were continuous in A but interrupted for ~ 30 seconds every minute in B.

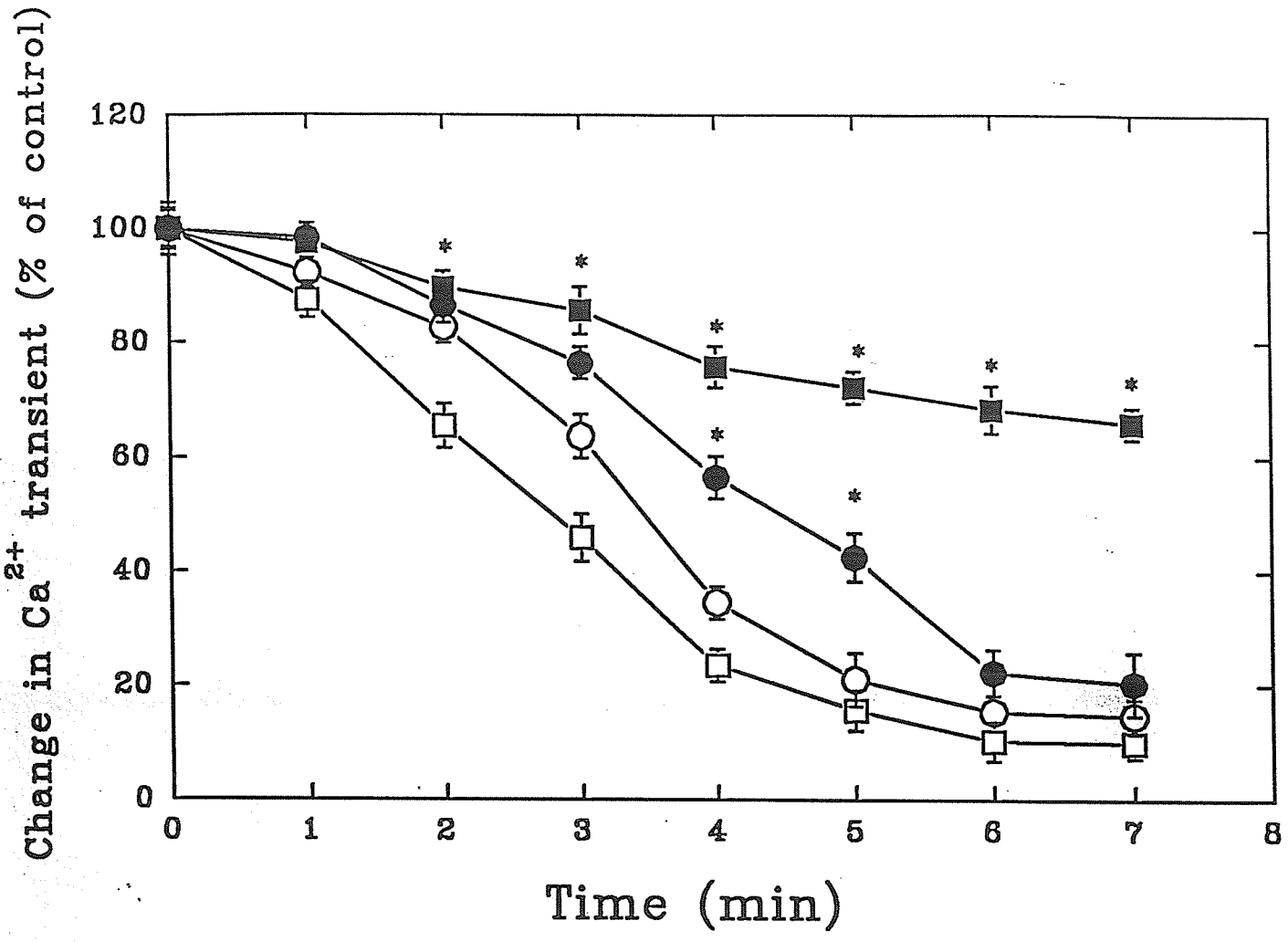


Figure 10. The effect on Ca^{2+} transients of nicardipine (o) or dichlorobenzamil (DCB) (□), in the absence (open symbols) or presence (filled symbols) of LDL pretreatment of cardiomyocytes. Values represent the mean ± S.E. from 5-7 experiments using different cells. * $P < 0.05$ vs. same time point for respective LDL-untreated groups.

Table 3. Influence of extracellular calcium concentrations on the effects of LDL enhancing calcium transients in rabbit ventricular myocytes

LDL 1 mg/ml at varying $[Ca^{2+}]_o$	Time (minutes)				
	0	15	30	45	60
0.7 mM					
Systole	203.2 ± 43.4	224.8 ± 51.6	235.5 ± 51.9	261.6 ± 32.7	289.5 ± 45.7
Diastole	112.7 ± 27.8	115.5 ± 27.8	117.2 ± 29.3	135.5 ± 34.6	142.4 ± 23.5
1.0 mM					
Systole	236.7 ± 35.3	267.2 ± 51.6	326.8 ± 54.2	377.5 ± 44.1*	399.5 ± 52.1*
Diastole	129.5 ± 25.2	122.7 ± 25.9	132.5 ± 29.4	128.1 ± 34.8	136.7 ± 35.9
1.8 mM					
Systole	279.8 ± 34.7	332.8 ± 65.4	397.3 ± 43.6*	459.2 ± 56.9*	495.6 ± 55.9*
Diastole	135.3 ± 26.6	139.9 ± 23.5	159.2 ± 47.3	173.7 ± 27.1	185.4 ± 31.6

*: $P<0.05$ VS control. (n=4). Calcium concentration is in nM.

significant. At 0.7 mM Ca2+, LDL was unable to significantly increase the cellular Ca^{2+} transient within the 60 minute time period studied.

II. The Action of Oxidized Low Density Lipoprotein on Calcium Transients in Isolated Rabbit Cardiomyocytes.

The effect of oxidized LDL on the intracellular calcium transient of isolated cardiomyocytes was investigated. Figure 11 shows representative results of calcium transients over time in both control and oxLDL treated cardiomyocytes. In the control myocyte (Fig. 11A), the Ca^{2+} transient was stable during stimulation for 24 minutes. Recordings were interrupted after 25 seconds every 2 minutes to avoid photo bleaching the dye which could have occurred during long exposure times to light. This protocol was used for all cells. When 0.1 mg oxLDL cholesterol/ml was incubated with the cardiomyocyte, a slow and steady increment in the Ca^{2+} transient was observed (Fig. 11B). The peak systolic [Ca^{2+}] during the transient was noticeably augmented during the treatment whereas the diastolic Ca^{2+} level was unchanged. However, when the concentration of oxLDL was increased to 1 mg cholesterol/ml, the amplitude of the Ca^{2+} transient was decreased not increased. Furthermore, the diastolic [Ca^{2+}] was increased with time (Fig. 11C). A series of experiments were carried out to determine the effects of varying concentrations of oxLDL on the Ca^{2+} transients in cardiomyocytes (Table 4). The systolic level of the Ca^{2+} transients was not significantly increased until the oxLDL concentration reached 100 μg oxLDL cholesterol/ml. This effect required at least 16 minutes before statistical significance was achieved. Doubling the [oxLDL] further enhanced the effect and shortened the length of time required to produce the effect. A toxic effect was observed when the concentration

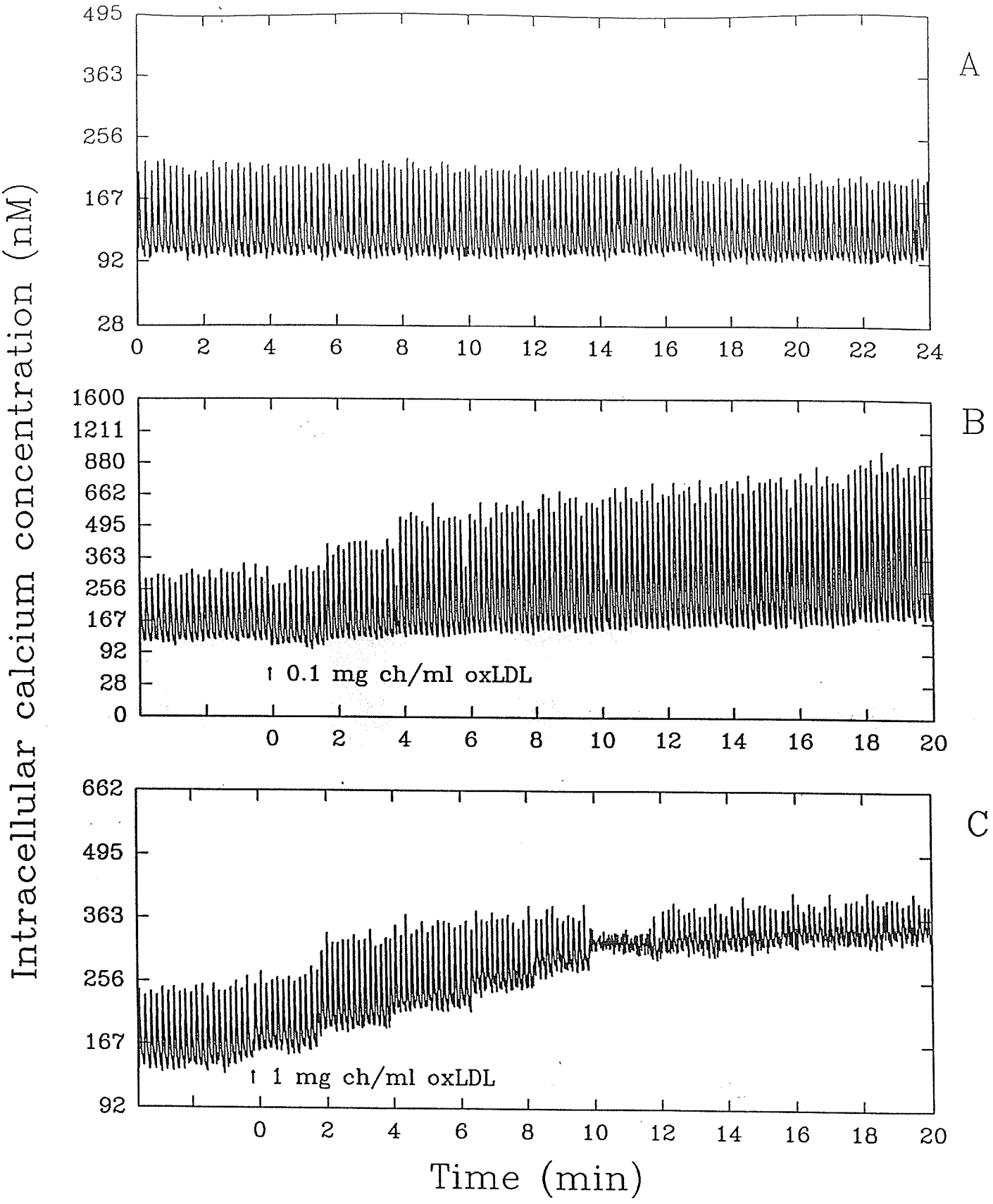

Intracellular calcium concentration (nM)
495
363
256
167
92
28
0 2 4 6 8 10 12 14 16 18 20 22 24
A
1600
1211
880
662
495
363
256
167
92
28
0
↑ 0.1 mg ch/ml oxLDL
0 2 4 6 8 10 12 14 16 18 20
B
662
495
363
256
167
92
↑ 1 mg ch/ml oxLDL
0 2 4 6 8 10 12 14 16 18 20
C
Time (min)

Figure 11. Representative recordings of calcium transients in control cardiomyocytes (A), cardiomyocytes treated with 0.1 mg cholesterol oxLDL/ml (B) or 1 mg cholesterol oxLDL/ml (C). The recordings here are not continuous but for about 25 seconds then stopped. At the second minute, another 25 second recording was resumed, followed by another 25 second recording at the fourth minute, etc. Note the stability in the calcium transient recording in the control cell (A) over the entire measurement period and different patterns of the increment in the cardiomyocytes treated with 0.1 mg oxLDL/ml and 1 mg oxLDL/ml.

Table 4. The effects of varying concentrations of oxidized LDL on calcium transients in rabbit ventricular myocytes

Oxidized [LDL]	Time (minutes)					
	0	4	8	12	16	20
10 μg/ml						
Systolic	482 ± 121	647 ± 175	701 ± 167	719 ± 191	753 ± 212	776 ± 233
Diastolic	189 ± 39	203 ± 64	234 ± 77	256 ± 87	277 ± 91	302 ± 123
$[Ca^{2+}]$						
25 μg/ml						
Systolic	277 ± 56	314 ± 62	407 ± 83	445 ± 181	481 ± 193	516 ± 227
Diastolic	128 ± 24	159 ± 34	163 ± 39	203 ± 39	233 ± 54	238 ± 80
$[Ca^{2+}]$						
50 μg/ml						
Systolic	339 ± 119	480 ± 138	555 ± 192	650 ± 213	650 ± 227	675 ± 251
Diastolic	179 ± 49	205 ± 52	248 ± 72	281 ± 88	329 ± 87	356 ± 95
$[Ca^{2+}]$						
100 μg/ml						
Systolic	426 ± 64	670 ± 98	787 ± 117	866 ± 156	894 ± 165*	915 ± 206*
Diastolic	211 ± 40	236 ± 40	272 ± 45	301 ± 53	311 ± 56	315 ± 49
$[Ca^{2+}]$						
200 μg/ml						
Systolic	413 ± 123	695 ± 189	998 ± 296	1027 ± 281*	1102 ± 340*	1195 ± 380*
Diastolic	185 ± 59	198 ± 69	215 ± 95	281 ± 89	332 ± 79	349 ± 92
$[Ca^{2+}]$						
1000 μg/ml						
Systolic	234 ± 35	250 ± 39	282 ± 41	304 ± 49	320 ± 49	321 ± 37
Diastolic	132 ± 18	168 ± 21	204 ± 18*	236 ± 23*	261 ± 23*	286 ± 19*
$[Ca^{2+}]$						

Values represent mean ± S.E. *: $P<0.05$ vs control. (n=4-8). Calcium concentration is in nM.

of oxLDL was raised to 1000 μg/ml. The diastolic [Ca^{2+}] was significantly increased after only 8 minutes of treatment.

Free radicals themselves may have a strong influence on the cardiomyocyte Ca^{2+} transient (369,376,377). Therefore, in order to exclude the possibility that free radicals may be primarily producing the effect rather than the oxLDL, the effect on the Ca^{2+} transient of varying the concentration of DHF was examined in the absence of LDL (Fig. 12). When the concentration of $FeCl_3$-ADP was maintained at 1 mM and 0.5 mM respectively, 0.3 mM DHF did not produce a noticeable increment in the myocyte Ca^{2+} transient (Fig. 12A). However, when the DHF concentration was increased to 3 mM, a striking increment in the diastolic component of the Ca^{2+} transient of treated myocyte was observed (Fig. 12B).

The mechanism responsible for oxLDL-induced increment in the cardiomyocytes Ca^{2+} transient was of great interest. Nicardipine is a potent antagonist of the L-type Ca^{2+} channel (372,373). The effect of 4 μM nicardipine on the Ca^{2+} transient in control and oxLDL-treated cells were investigated. Nicardipine blocked the Ca^{2+} transients from both cell groups, however, the drug was more potent in oxLDL treated cardiomyocytes (Fig. 13). In a series of experiments, the effect of nicardipine on the peak systolic [Ca^{2+}] was evident within 100 seconds, whereas in the control cells nicardipine did not cause a significant decrease until 150 seconds after application (Fig. 14).

The effect of oxLDL on the Ca^{2+} transient in cardiomyocytes was examined as a function of the perfusate Ca^{2+} concentration (Table 5). As observed earlier (Figure 11B and Table 4), oxLDL produced a significant elevation of systolic Ca^{2+} transient within 16 minutes when the extracellular [Ca^{2+}] was 1.8 mM. However, when the extracellular Ca^{2+} concentration was lowered to 0.5 and 1 mM, oxLDL failed to produce a significant increment in the Ca^{2+} transient during 20 minutes of treatment.

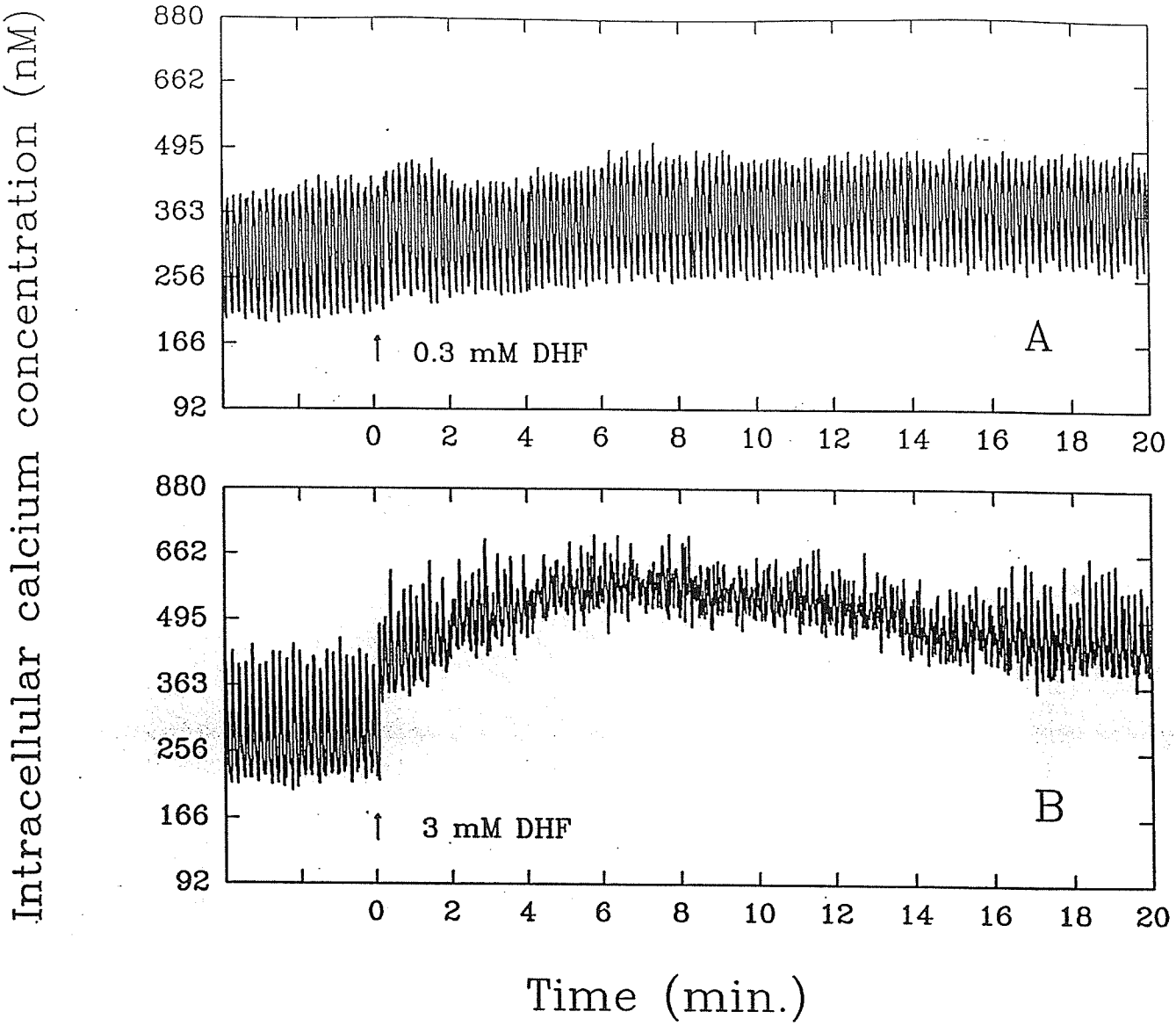


Figure 12. Representative recordings of calcium transients in the cardiomyocytes after treatments with 0.3 mM DHF (A) or 3 mM DHF (B) where $FeCl_3$-ADP was maintained at 1 mM and 0.5 mM respectively. Recordings were only for ~25s, then stopped and the data saved before re-initiating the recording again for ~25s two minutes later. The DHF was added at the time point indicated by the arrow. Note the stability in the calcium transient recorded from cells treated with 0.3 mM DHF (A) and the striking elevation in the diastolic component of the calcium transient induced by 3 mM DHF treatment (B).

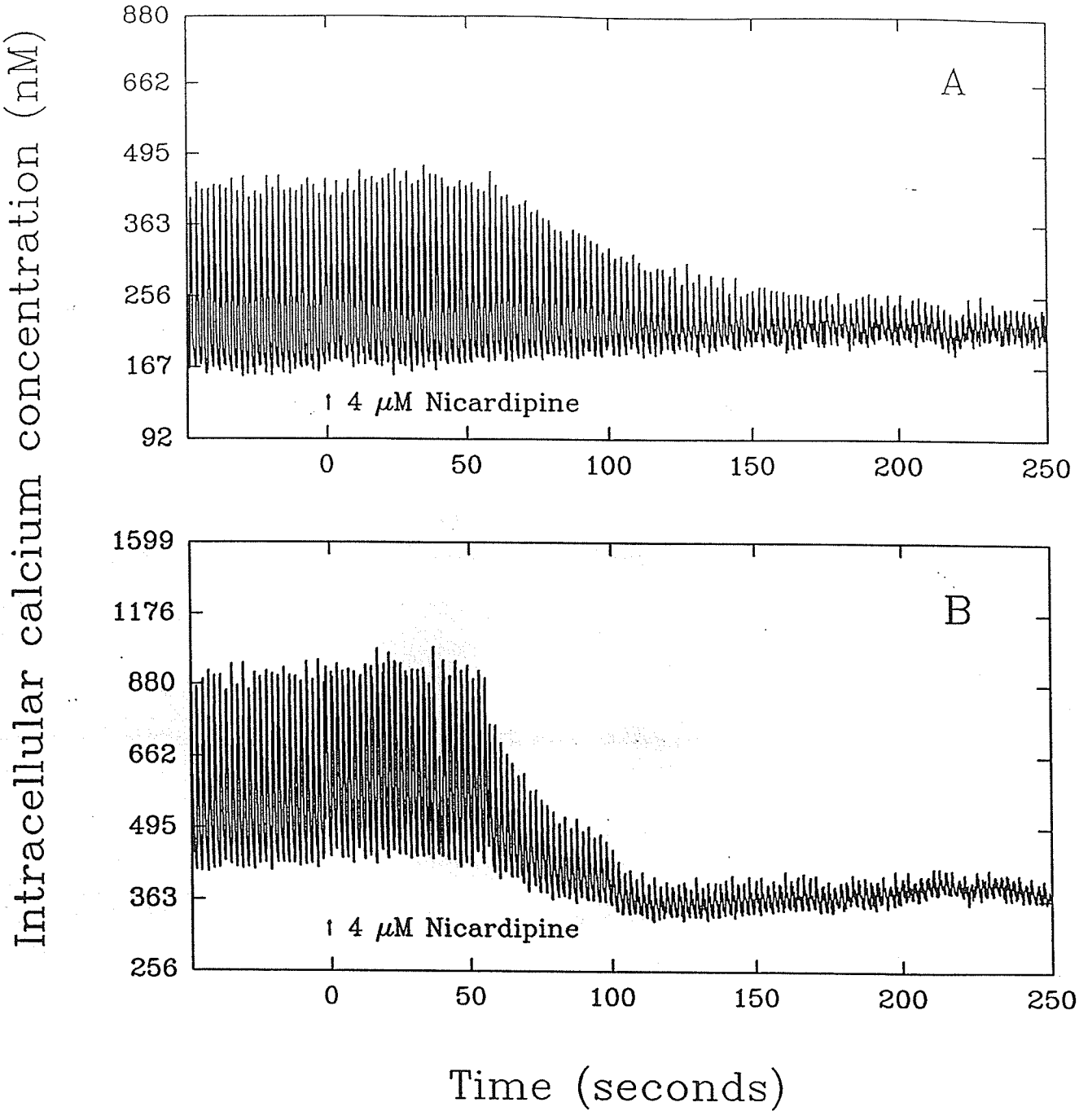


Figure 13. Representative recordings of calcium transients after exposure to 4 μM nicardipine (arrow) in a control cardiomyocyte (A) or a cell treated for 20 minutes with 0.1 mg oxLDL cholesterol/ml (B). Both recordings were continuous for the entire nicardipine treatment period (250 second).

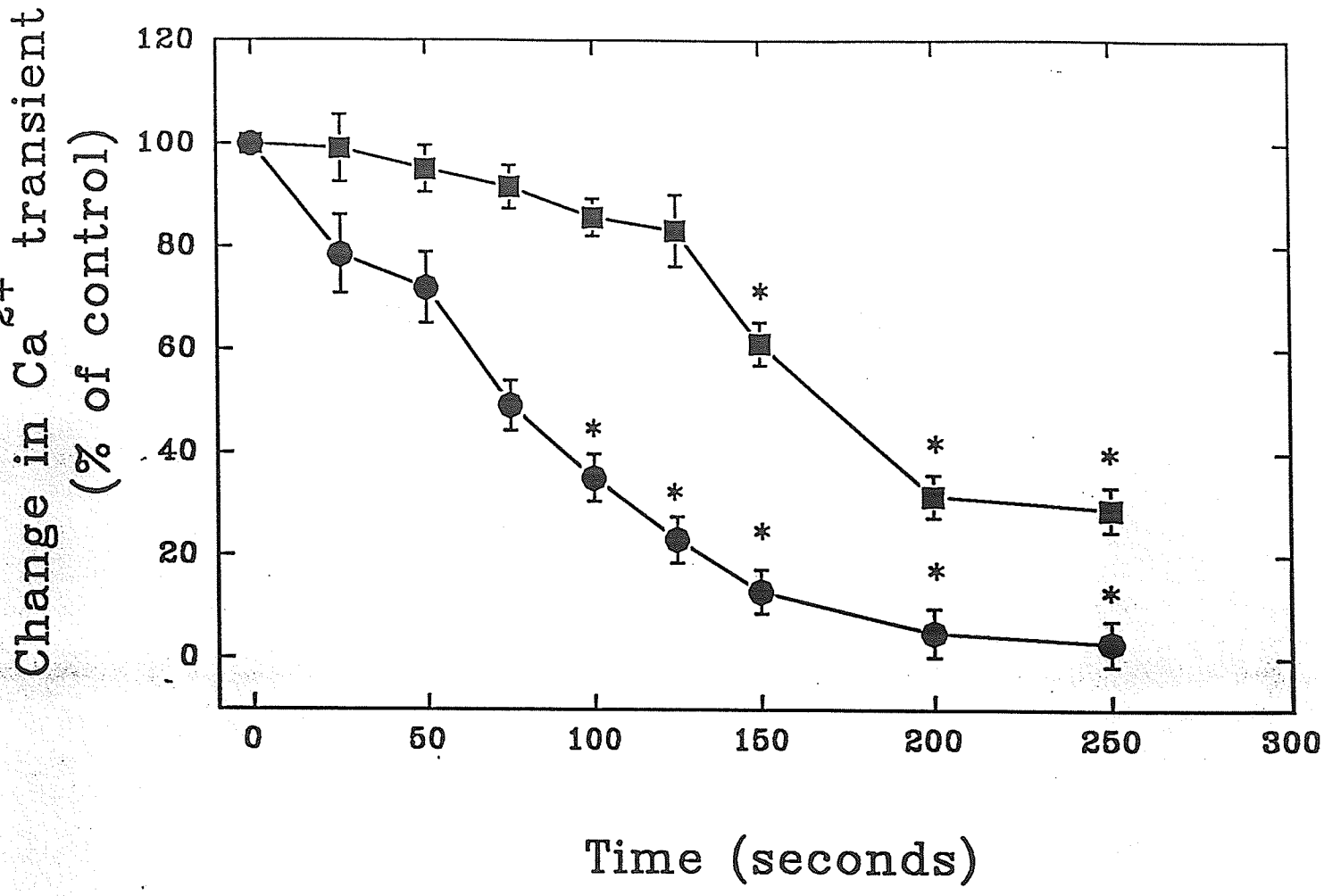


Figure 14. The effect on calcium transients (systolic [Ca^{2+}]) of nicardipine in the absence (■) or presence (o) of 0.1 mg/ml oxLDL pretreatment of cardiomyocytes. Values represent the mean ± S.E. from 5-7 experiments using different cells. * $P<0.05$ vs. 0 second for both groups.

Table 5. Influence of extracellular calcium concentrations on the effects of oxidized LDL on calcium transients in rabbit ventricular myocytes

$[Ca^{2+}]_o$	Time (minutes)					
	0	4	8	12	16	20
0.5 mM						
Systolic	159 ± 28	176 ± 35	182 ± 37	184 ± 37	199 ± 37	202 ± 29
Diastolic	108 ± 31	122 ± 30	130 ± 33	136 ± 33	142 ± 34	157 ± 31
$[Ca^{2+}]$						
1.0 mM						
Systolic	226 ± 33	234 ± 38	310 ± 74	351 ± 93	355 ± 103	355 ± 78
Diastolic	151 ± 37	222 ± 55	252 ± 79	286 ± 88	300 ± 87	300 ± 89
$[Ca^{2+}]$						
1.8 mM						
Systolic	426 ± 64	670 ± 98	787 ± 117	866 ± 156	894 ± 165*	915 ± 206*
Diastolic	211 ± 40	236 ± 40	272 ± 45	301 ± 53	311 ± 56	315 ± 49
$[Ca^{2+}]$						

All the experiments were carried out at 0.1 mg cholesterol oxLDL/ml. *: $P<0.05$ vs control. (n=4-8). Calcium concentration is in nM.

To further assess the mechanism for the effect of oxLDL on the Ca^{2+} transient of treated cardiomyocytes, the relationship of lipid peroxidation within the LDL to the change in the cellular Ca^{2+} transient was examined. Lipid peroxidation was assessed as MDA products within the LDL. There was a good correlation between the oxLDL MDA content and the percentage increase in the systolic Ca^{2+} transient (Fig. 15A-C).

The amount of MDA produced in the oxLDL was also dependent upon the concentration of $FeCl_3$ present in the free radical generating system. 0.5 mM $FeCl_3$ in the presence of 0.3 mM DHF and 0.5 mM ADP initiated a significant increase in the MDA content of the oxLDL (Fig. 16A). However, there was no significant alteration in the peak systolic $[Ca^{2+}]$ until the $FeCl_3$ concentration reached 1.0 mM (Fig. 16B). The correlation coefficient between oxLDL MDA content and percentage change in systolic $[Ca^{2+}]$ was 0.78 (Fig. 16C).

The efficacy of an antioxidant in preventing the effects of free radicals on LDL and subsequently the changes in the cellular Ca^{2+} transient was examined. Lazaroid (U-74500A), a novel antioxidant (378-380), completely inhibited MDA formation in the oxLDL (Fig. 17A). If preincubated with 2.5 - 10 μM lazaroid, LDL failed to produce any significant increment in the systolic Ca^{2+} transient of treated cardiomyocytes (Fig. 17B). A good correlation ($r=0.86$) was displayed between the MDA production in oxLDL (as modified by varying the preincubation concentration of lazaroid) and the percentage change of the systolic $[Ca^{2+}]$ in the transient (Fig. 17C).

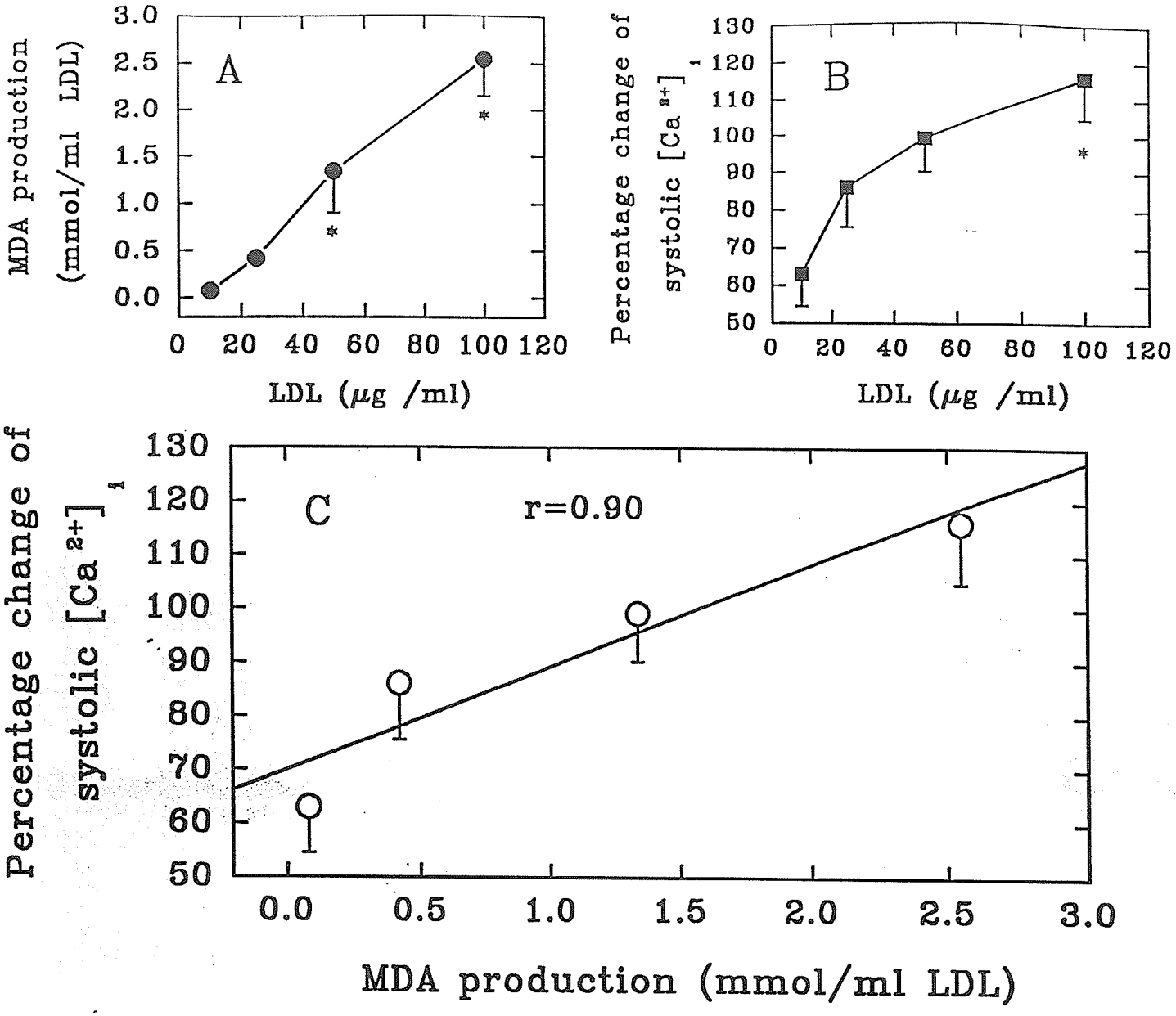


Figure 15. The effects of varying the oxLDL concentration on the alteration of calcium transient and MDA production. Panel A shows the MDA production as a function of the LDL concentration. * $P<0.05$ vs. MDA value at 10 μg/ml LDL. Panel B shows the percentage change of systolic calcium transient vs. oxLDL concentration. * $P<0.05$ vs. control untreated LDL. Values represent the mean ± S.E. from 4-6 experiments using different cells in both panels. Panel C shows the correlation between the MDA content of oxLDL and the percentage change of systolic calcium transients in cardiomyocytes treated with oxLDL.

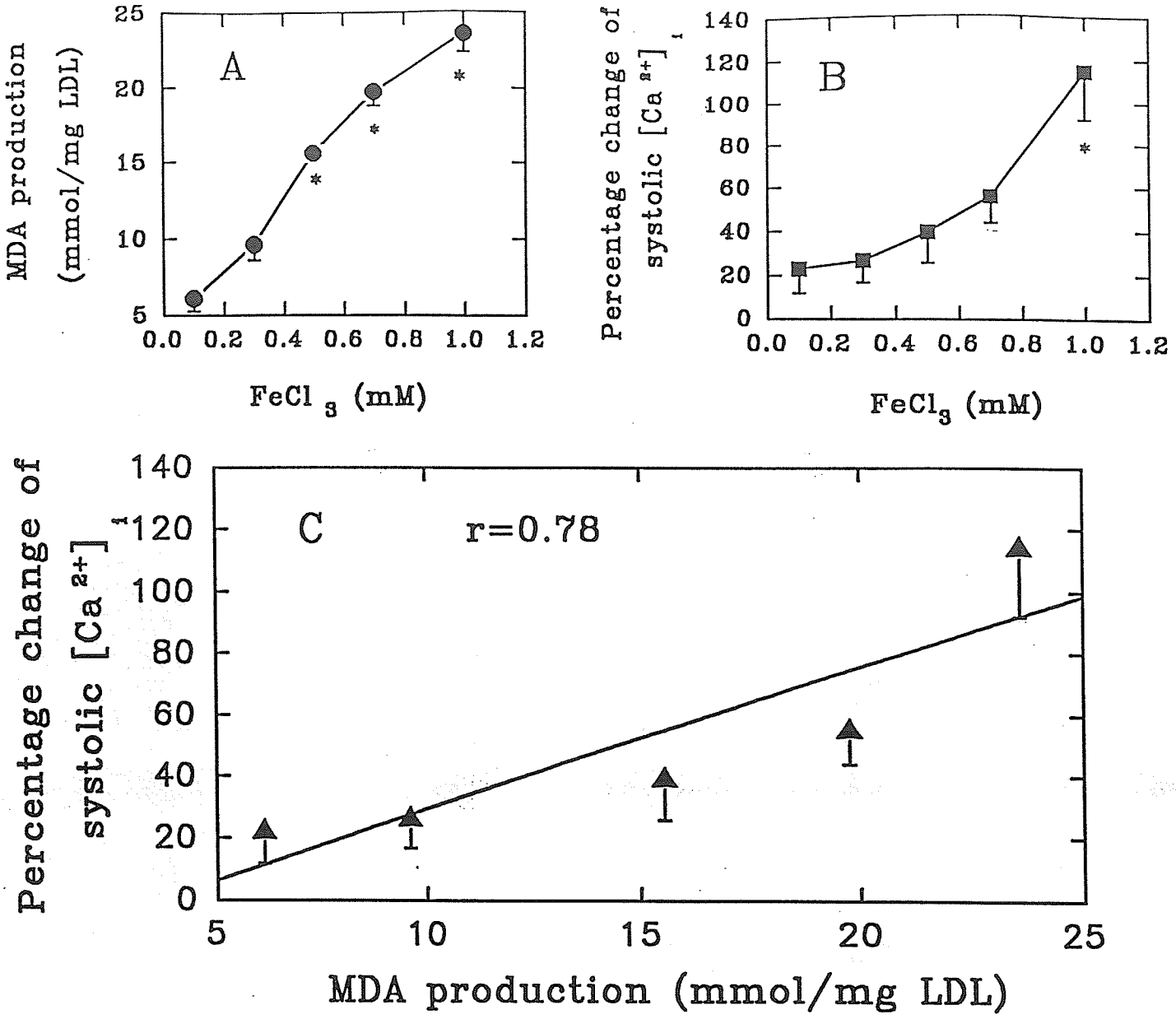


Figure 16. The effects of varying $FeCl_3$ concentrations on the MDA production and change of systolic calcium concentration in cardiomyocytes. Panel A depicts the MDA production in LDL as a function of $FeCl_3$ concentration in the free radical generating system. * $P<0.05$ vs. 0.1 mM $FeCl_3$. Panel B presents the percentage change of systolic $[Ca^{2+}]_i$ at different $FeCl_3$ concentrations. * $P<0.05$ vs. control untreated LDL. Values represent mean ± S.E. from 4-7 experiments. Panel C displays the correlation between the MDA production in LDL and the percentage change of systolic $[Ca^{2+}]_i$.

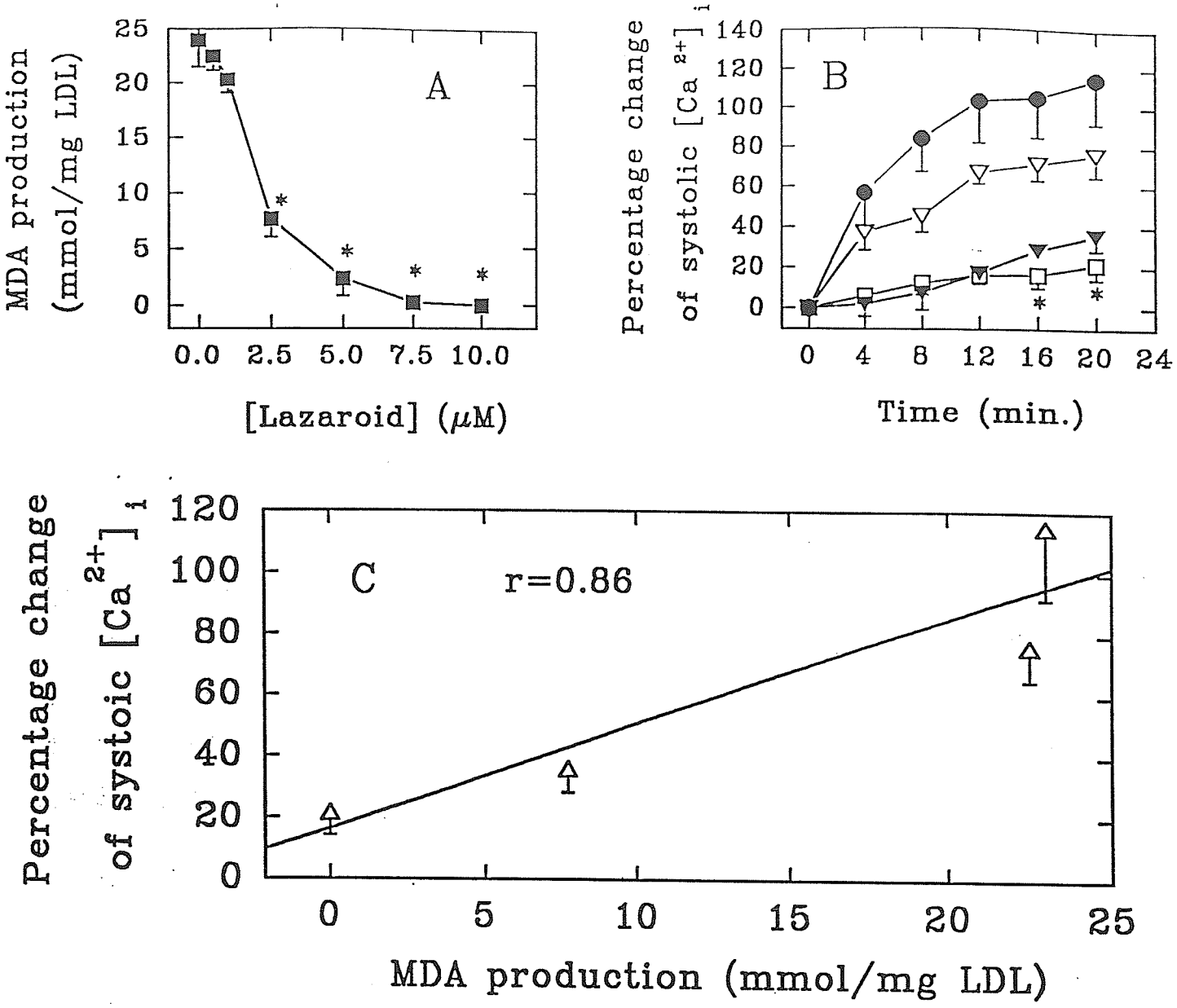


Figure 17. The effects of lazaroid on MDA production in oxLDL and the change of systolic calcium concentration in cardiomyocytes. Panel A shows the influence of lazaroid on the MDA production. * $P<0.05$ vs. 0 μM lazaroid. Values are mean ± S.E. from 4-8 different experiments. Panel B shows the % change in Ca^{2+} transients in cardiomyocytes after incubation with 0.1 mg cholesterol oxLDL/ml ± 0 (●), 0.5 μM (▽), 2.5 μM (▼), 10 μM (□) lazaroid. * $P<0.05$ vs. control group (●). Panel C presents the correlation between the MDA content in LDL and the percentage change of systolic $[Ca^{2+}]_i$.

III. Oxidation of selected lipids in LDL: Effects on calcium transients in isolated rabbit cardiomyocytes.

LDL was pre-incubated with 0.1 mg /ml C.O. in the absence of catalase. The incubation of LDL with C.O. alone will result in an oxidation of LDL cholesterol and the generation of H_2O_2. An aliquot was removed and placed in a solution bathing the cardiomyocytes and the intracellular calcium transient of isolated cardiomyocytes was measured. In the control cells, the Ca^{2+} transient is stable for > 60 minutes of stimulation (292). Figure 18 shows representative results of calcium transients over time in cardiomyocytes incubated with C.O. treated LDL ± catalase. Recordings were paused after 25 seconds every 2 minutes in order to avoid photo bleaching the dye which could have occurred during long exposure times to light. This protocol was used throughout the experiments. A slow and steady increment in the Ca^{2+} transient was observed after oxLDL was incubated with the cardiomyocyte (Fig. 18A). A striking increment in peak systolic $[Ca^{2+}]$ during the transient was noticeable during the treatment whereas the diastolic Ca^{2+} level was unaltered. An decrease in excitability of treated cells was consistently observed after ~16 minutes treatment with oxLDL (Fig. 18A). When the above treatment was applied to cells but in the presence of 0.1 mg/ml catalase to inactivate the H_2O_2 (Fig. 18B), the amplitude of the Ca^{2+} transient was not significantly increased. A number of experiments were carried out to investigate the effects of varying concentrations of oxLDL ± catalase on the Ca^{2+} transients in cardiomyocytes (Table 6). The systolic level of the Ca^{2+} transients was not significantly increased until the oxLDL concentration reached 50 μg cholesterol/ml oxLDL. This effect required at least 16 minutes before statistical significance was achieved. This effect was not observed when catalase was present during the treatment of oxLDL. The diastolic $[Ca^{2+}]$ was not significantly altered at any of the concentrations applied.

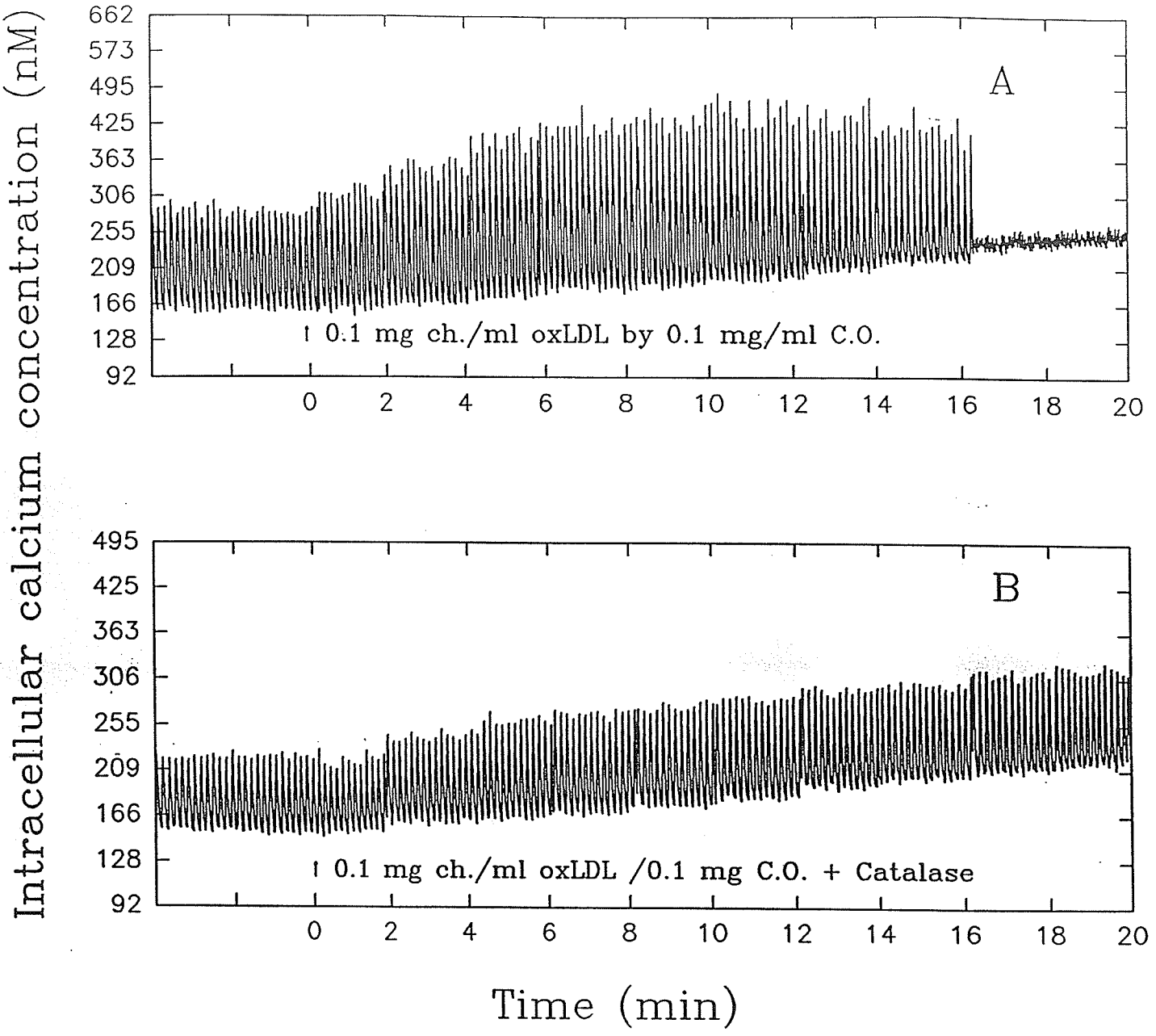


Figure 18. Representative recordings of calcium transients in cardiomyocytes treated with 0.1 mg cholesterol/ml LDL oxidized by 0.1 mg/ml C.O. (A) or 0.1 mg cholesterol/ml LDL oxidized by 0.1 mg/ml C.O. in the presence of 0.1 mg/ml catalase (B). The recordings here are not continuous but for 25 seconds then stopped. At the second minute, another 25 second recording was resumed, followed by another 25 second recording at the fourth minute, etc. Note the increase in the size of calcium transient induced after the cell was exposed to 0.1 mg/ml oxLDL treatment (A) and the relative stability in the calcium transient recording in the cell treated with 0.1 mg/ml oxLDL but in the presence of 0.1 mg/ml catalase (B).

Table 6. The effects of varying concentrations of oxidized LDL ± catalase on calcium transients in rabbit ventricular myocytes

Oxidized [LDL] ± catalase	Time (minutes)				
	0	4	8	12	16
			nM $[Ca^{2+}]$		
10 µg/ml					
Systolic	207 ± 13	209 ± 11	205 ± 14	208 ± 11	208 ± 12
Diastolic	117 ± 8.9	117 ± 7.5	121 ± 6.8	119 ± 11	124 ± 9.4
50 µg/ml					
Systolic	224 ± 25	243 ± 30	276 ± 20	310 ± 38	355 ± 52 *
Diastolic	114 ± 16	114 ± 16	128 ± 24	132 ± 27	133 ± 28
50 µg/ml +catalase					
Systolic	238 ± 25	242 ± 27	252 ± 26	256 ± 25	260 ± 21
Diastolic	155 ± 25	156 ± 24	162 ± 23	172 ± 27	181 ± 28
100 µg/ml					
Systolic	235 ± 49	287 ± 62	326 ± 61	355 ± 73	383 ± 39 *
Diastolic	147 ± 42	169 ± 45	183 ± 46	207 ± 52	227 ± 51
100 µg/ml +catalase					
Systolic	169 ± 23	181 ± 20	202 ± 21	208 ± 29	221 ± 27
Diastolic	98 ± 6	103 ± 7	118 ± 14	119 ± 15	127 ± 24

Values represent mean ± S.E. (n=4-8) *: $P<0.05$ vs control. If included, catalase was present at ratio of 1:1 (mg/mg) with cholesterol oxidase. Calcium concentration is in nM.

To understand the mechanism of action of oxLDL on the Ca^{2+} transient of treated cardiomyocytes, the association of lipid peroxidation resulting from C.O. treatment within the LDL to the change in the intracellular Ca^{2+} transient was investigated. MDA products within the LDL were determined as an index of lipid peroxidation. A good correlation between the oxLDL MDA content and the percentage increase in the systolic Ca^{2+} transient was observed (Fig. 19A-C).

The efficacy of antioxidants in preventing the effects of cholesterol oxidase on LDL was compared. Catalase in the ratio 1:1 (w:w) to cholesterol oxidase completely inhibited MDA formation in the oxLDL (Fig. 20A). Lazaroid, a novel antioxidant, and vitamin E also prevented the production of MDA in the oxLDL but less efficiently compared to catalase (Fig. 20B).

Cholesterol oxidase itself may have a strong influence on the cardiomyocyte Ca^{2+} transient. Therefore, in order to exclude the possibility that C.O. itself may be inducing a direct effect rather than the oxLDL, the effect on the Ca^{2+} transient of varying the concentration of C.O. ± catalase was examined in the absence of LDL (Fig. 21, n=4~6). When 1 mg/ml C.O. was applied to cardiomyocytes, no noticeable alteration in the myocyte Ca^{2+} transient was observed except a decrease in excitability appeared at 18 minutes (Fig. 21A). However, when the C.O. concentration was increased to 1.5 mg/ml, a striking increment in the diastolic component of the Ca^{2+} transient of treated myocytes was observed (Fig. 21B). An immediate increment in the diastolic component of the Ca^{2+} transient appeared when the C.O. concentration applied to the cardiomyocyte was increased to 2 mg/ml (Fig. 21C). The possible protective role of catalase against this effect produced by C.O. was also examined. The Ca^{2+} transient of cardiomyocytes remained stable after 0.5 mg/ml catalase was incubated with cardiomyocytes for 20 minutes (Fig. 21D). However,

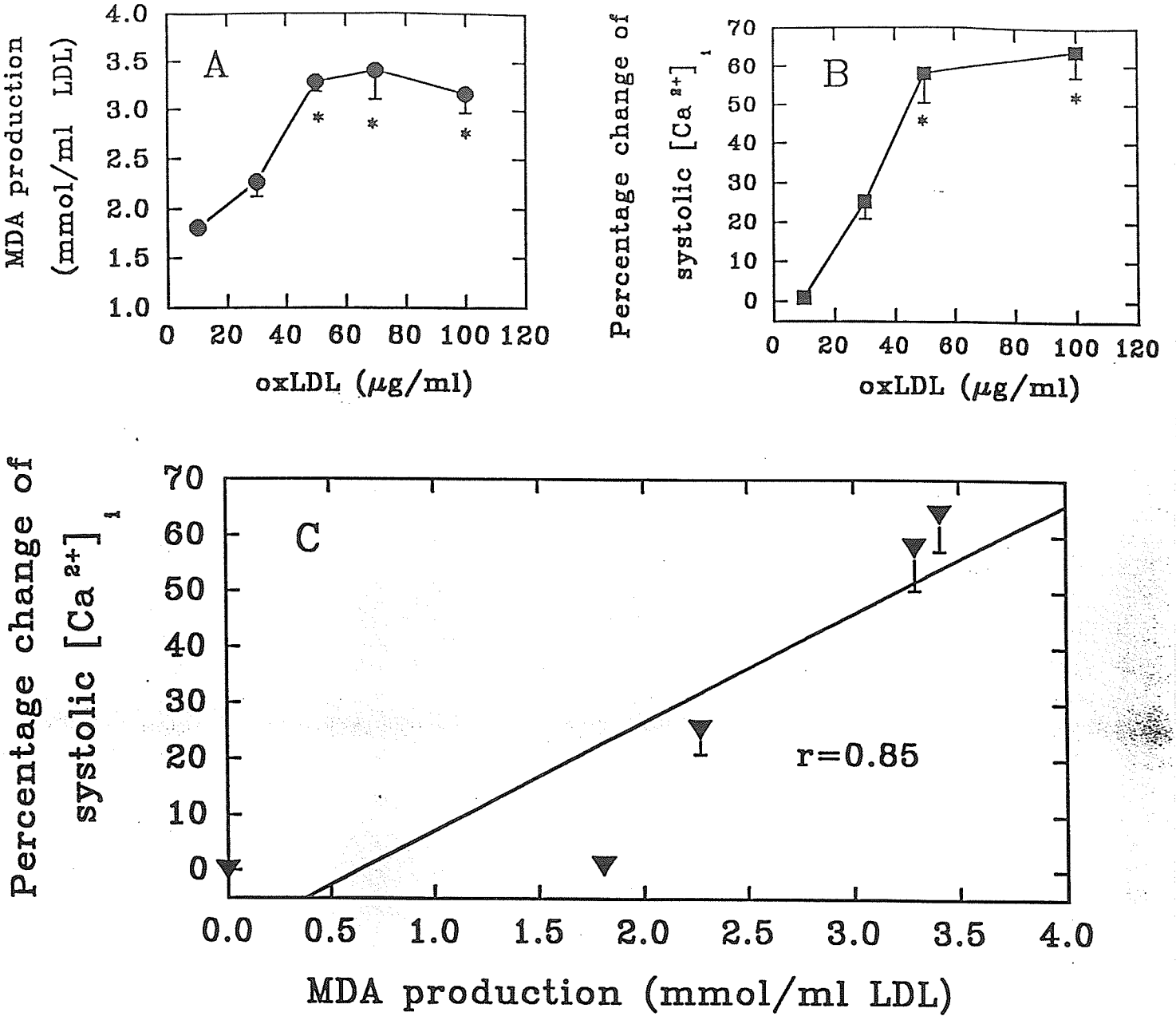


Figure 19. The effects of varying the oxLDL concentration on the cellular calcium transient and the LDL MDA content. Panel A shows the MDA production as a function of the oxLDL concentration. * $P<0.05$ vs. MDA value at 10 μg/ml LDL. Panel B shows the percentage change in the systolic calcium transient vs. oxLDL concentration. * $P<0.05$ vs. control untreated LDL. Values represent the mean ± S.E. from 4-6 experiments using different cells in both panels. Panel C presents the correlation between the MDA content of oxLDL and the percentage change of systolic calcium transients in cardiomyocytes treated with oxLDL.

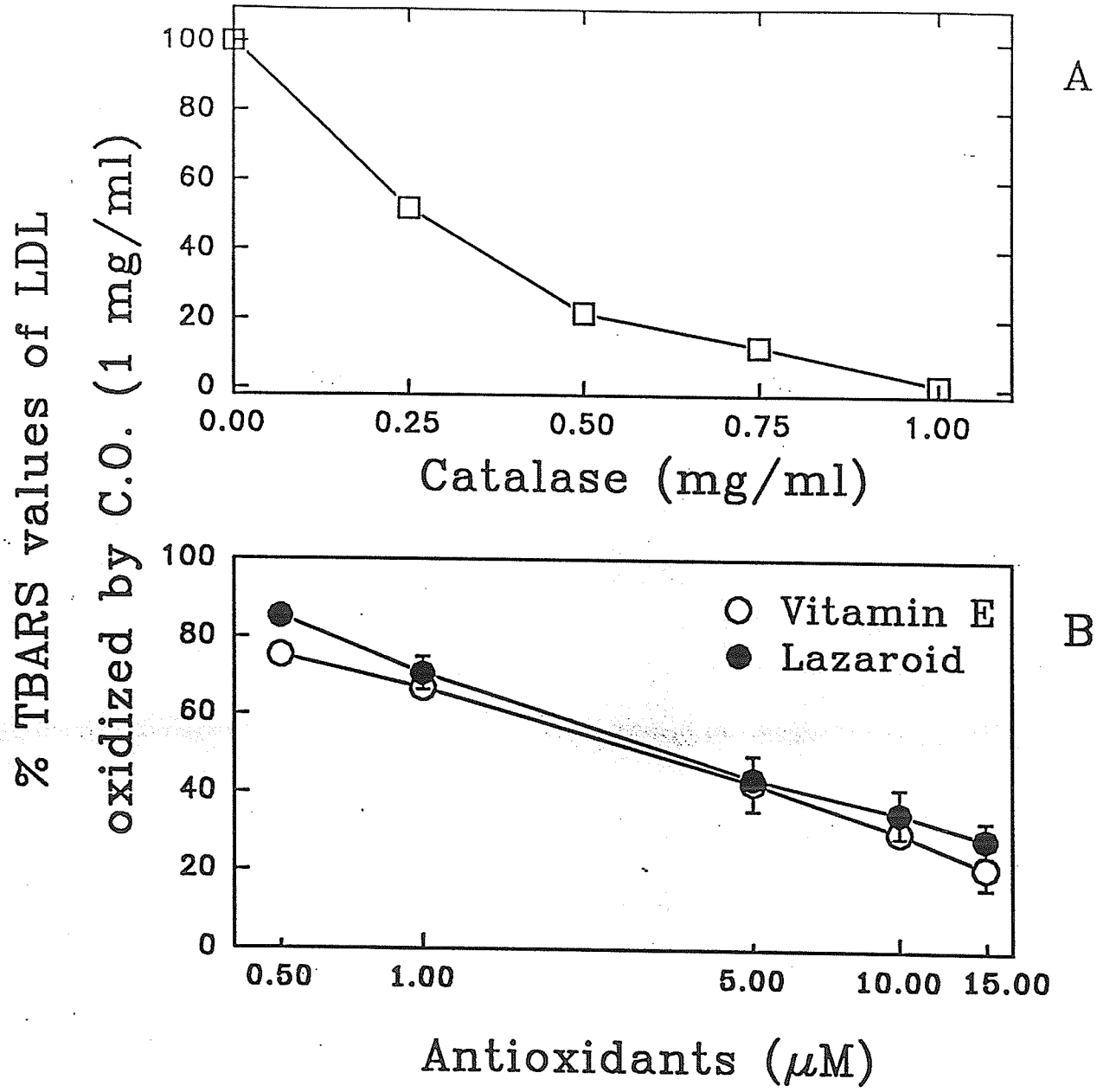


Figure 20. The effects of catalase and other antioxidants on MDA production in oxLDL. Panel A presents the effect of catalase on the MDA production. Panel B shows the influence of vitamin E and lazaroid on the MDA production. Values are the mean ± S.E. from 4-6 different experiments. If standard error bars are not present, then the symbol size was greater than the standard error of the mean.

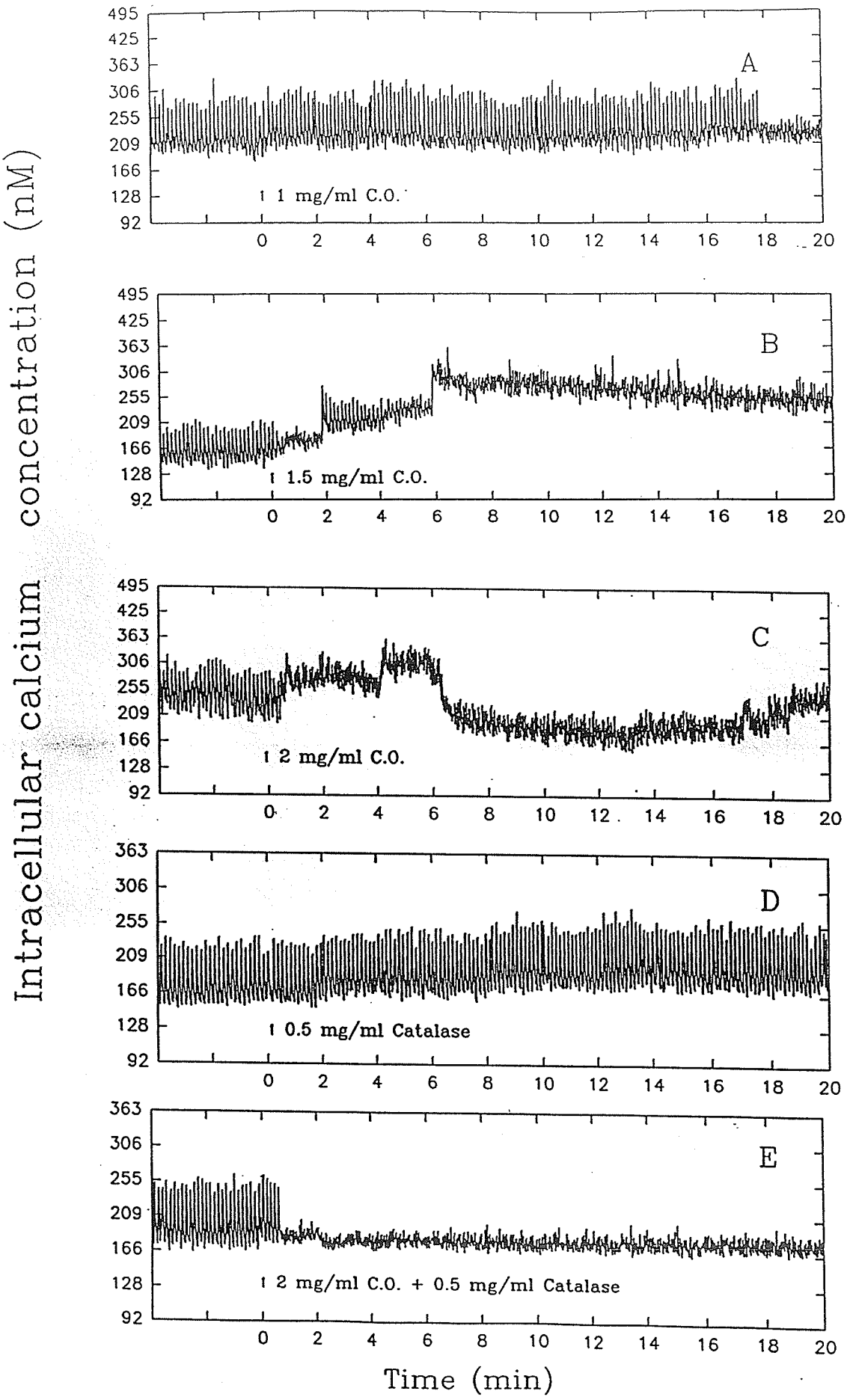

Intracellular calcium concentration (nM)
A
↑ 1 mg/ml C.O.
B
↑ 1.5 mg/ml C.O.
C
↑ 2 mg/ml C.O.
D
↑ 0.5 mg/ml Catalase
E
↑ 2 mg/ml C.O. + 0.5 mg/ml Catalase
Time (min)

Figure 21. Representative recordings of calcium transients in the cardiomyocytes after treatment with 1 mg/ml C.O. (A), 1.5 mg/ml C.O. (B), 2 mg/ml C.O. (C), 0.5 mg/ml catalase (D) and 2 mg/ml C.O. + 0.5 mg/ml catalase (E). Recordings were only for 25s, then stopped and the data saved before re-initiating the recording again for 25s two minutes later. The C.O. was added at the time point indicated by the arrow. Note the stability in the calcium transient recorded from cells treated with 1 mg/ml C.O. (A) and the striking elevation in the diastolic component of the calcium transient induced by 1.5 and 2 mg/ml C.O. treatment (B & C). Note the steadiness of calcium transient in the cells treated with 0.5 mg/ml catalase (D) and the complete loss of excitability in the cells treated with 2 mg/ml C.O. + 0.5 mg/ml catalase (E).

catalase at this concentration failed to protect against the effects of 2 mg/ml C.O. on the cell Ca^{2+} transient (Fig. 21E).

HPLC was used to determine if C.O. was indeed oxidizing cell membrane cholesterol (Fig. 22). In the control cardiomyocytes, cholesterol is the major component of cell membrane (Fig. 22B). One oxidized cholesterol, 20 α-OH was also detectable in the control cardiomyocyte. This may be due to the presence of some necrotic cells in the preparation. Our cell isolation yield is about 70 - 80% rod shape, live, viable cells and 20-30% rounded, necrotic cells (Fig. 22B). The most striking change in membrane cholesterol after C.O. treatment was the appearance of 4-cholesten-3-one (Fig. 22C & D). 4-cholesten-3-one became very prominent when the C.O. concentration increased to 2 mg/ml and cholesterol disappeared (Fig. 22D).

The by-product of cholesterol oxidation by cholesterol oxidase is hydrogen peroxide (381). The possibility exists, therefore, that the effects on the cardiomyocyte Ca^{2+} transient produced by oxLDL may be due to the presence of H_2O_2 rather than oxLDL itself. Therefore, the effect of varying the H_2O_2 concentration on the Ca^{2+} transient of cardiomyocytes was investigated (Fig. 23). A significant increment in the diastolic Ca^{2+} concentration of treated cardiomyocytes was observed. The greater the $[H_2O_2]$ that was applied to the cell, the faster the diastolic $[Ca^{2+}]$ increased (Fig. 23A-C). 10 mM H_2O_2 treatment produced a rapid (4 ± 1 minutes, n=4) increment in the diastolic $[Ca^{2+}]$ of treated cardiomyocytes (Fig. 23A). 1 mM and 0.1 mM H_2O_2 also produced an increment in diastolic $[Ca^{2+}]$ in treated cardiomyocytes but it required a longer time to develop (10 ± 2 and 28 ± 2 minutes, respectively, n=4-6) (Fig. 23 B&C). However, the effects of H_2O_2 on the cell Ca^{2+} transient were very different if LDL was present. If LDL was pre-incubated with 0.1 mM H_2O_2 and then this oxLDL was incubated with cardiomyocytes, an increment

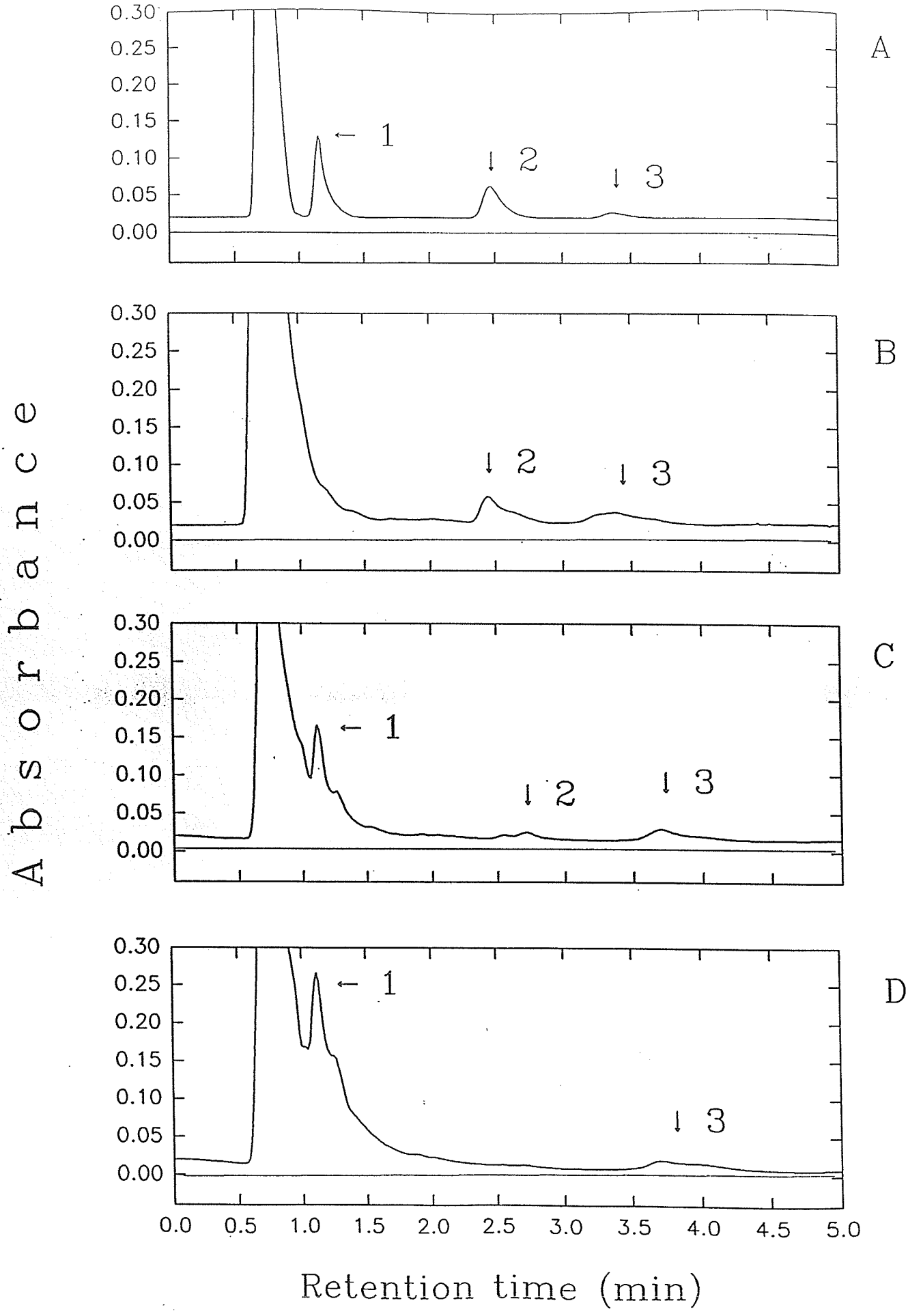

A
B
C
D
0.30
0.25
0.20
0.15
0.10
0.05
0.00
← 1
↓ 2
↓ 3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Absorbance
Retention time (min)

Figure 22. Representative HPLC recordings of cholesterol species in cardiomyocytes ± cholesterol oxidase treatment. A. Standard cholesterol and oxidized cholesterol species, peak #1: 4-cholesten-3-one; peak #2: cholesterol; peak #3: 20 α-OH cholesterol. B. Control cardiomyocyte. C. Cardiomyocyte was incubated with 1 mg/ml C.O. for 60 minutes at 37°C. D. Cardiomyocyte was incubated with 2 mg/ml C.O. for 60 minutes at 37°C. Catalase was present at ratio of 1:1 (mg/mg) with C.O. in C & D.

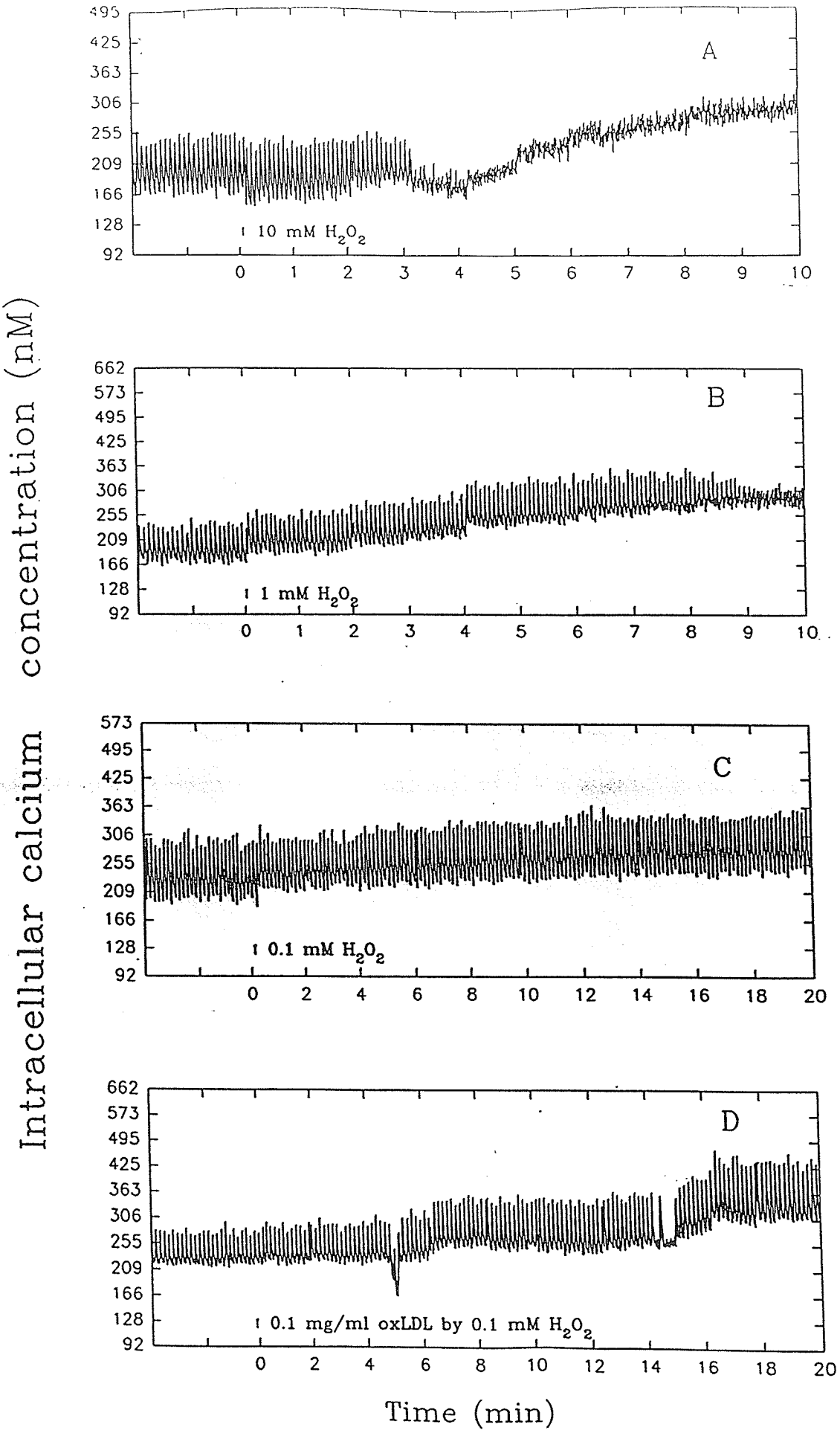

A
B
C
D
↑ 10 mM H_2O_2
↑ 1 mM H_2O_2
↑ 0.1 mM H_2O_2
↑ 0.1 mg/ml oxLDL by 0.1 mM H_2O_2
Intracellular calcium concentration (nM)
Time (min)

Figure 23. Representative recordings of calcium transients in myocytes after exposure to 10 mM H_2O_2 (A), 1 mM H_2O_2 (B), 0.1 mM H_2O_2 (C) or 0.1 mg/ml oxLDL oxidized by 0.1 mM H_2O_2 (D). Note the difference in the time to a change in diastolic [Ca^{2+}] after addition of various concentrations of H_2O_2 (A-C) and the increment in the calcium transient after cells were treated with 0.1 mg/ml LDL oxidized by 0.1 mM H_2O_2 (D).

(54.4 ± 4%) of the systolic Ca^{2+} transient of cardiomyocytes was observed (Fig. 23D) (n=4).

The time to contracture of cardiomyocytes after treatment with varying concentrations of H_2O_2 was also measured. The cells first lost excitability and then suddenly shortened to a rounded state indicative of contracture. A very rapid contracture (2.4 ± 0.3 minutes) of cardiomyocytes was produced by 20 mM H_2O_2 treatment (Fig. 24). When the concentration of H_2O_2 was decreased to 0.1 mM, a much longer time was required for cardiomyocytes to develop a contracture (28 ± 2 minutes).

IV. Oxidative status of lipoproteins in coronary disease patients.

Clinical data from both groups are shown in Table 7. The level of serum cholesterol in patients was significantly elevated in comparison to that of the control group ($P<0.05$). The LDL cholesterol in patients was also higher than control. The increased LDL cholesterol in patients included total, free and esterified cholesterol as well. The LDL triglyceride from patients was also notably higher compared to control.

The oxidized species of cholesterol in LDL and VLDL from both groups were assessed using HPLC. The oxidized species of cholesterol identified were 4-cholesten-3-one and 20 α-OH cholesterol (Figure 25). The content of 4-cholesten-3-one in LDL from patients was significantly elevated over that of control (Table 8). The nonoxidized cholesterol in the patients' LDL was also elevated in the HPLC measurements, thus confirming the enzymatic analyses in Table 7. There was no significant difference between the two groups with regard to the quantity of cholesterol and oxidized cholesterol in VLDL.

MDA was barely detectable in the LDL and VLDL of the two groups (Figure 26). The average value of MDA in LDL and VLDL was 0.22 - 0.30 nmol/mg of lipoprotein

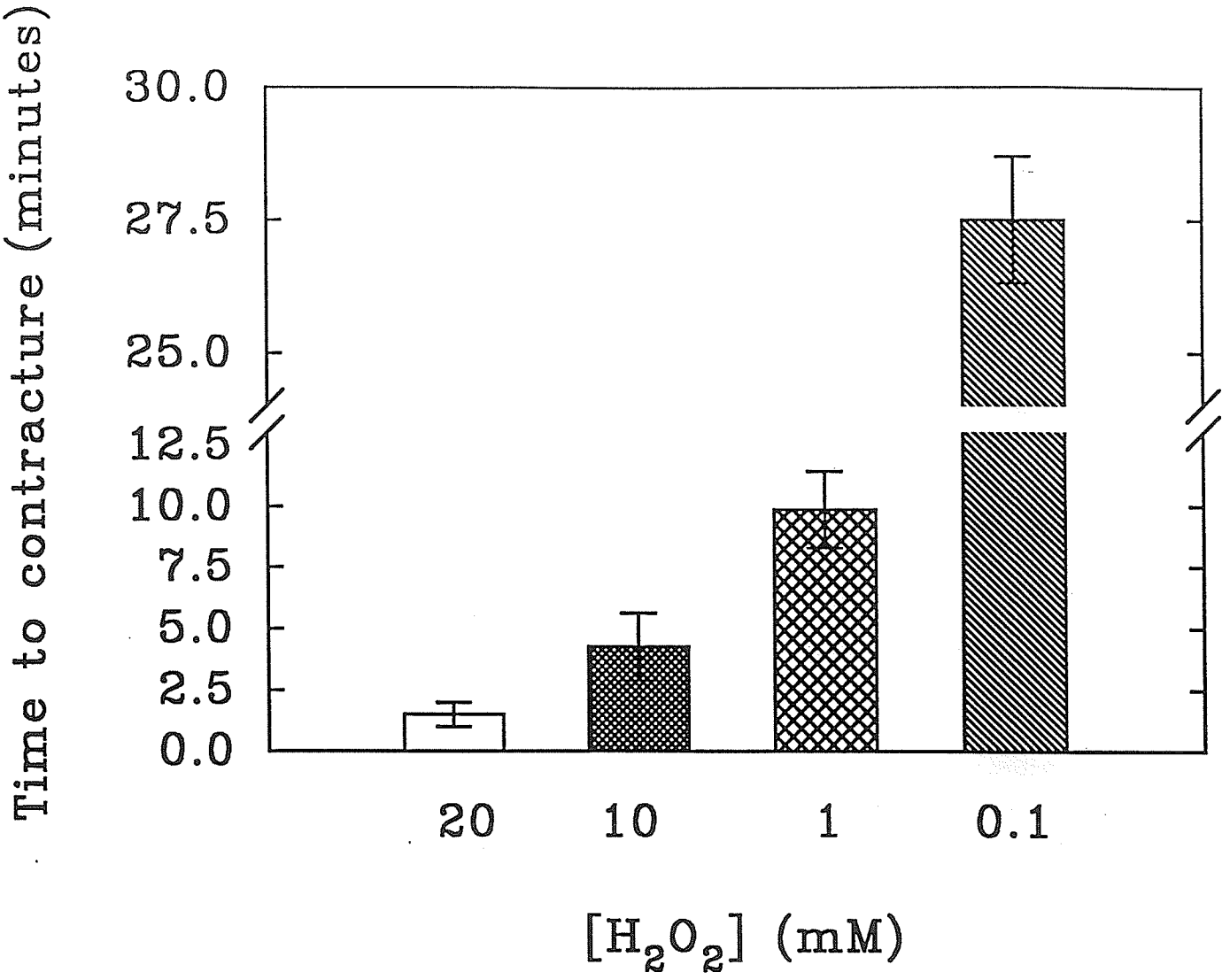


Figure 24. Average time to contracture in myocytes exposed to varying H_2O_2 treatments. Values represent mean ± S.E. of 4-6 separate cell measurements.

Table 7. Clinical data of control and patients

	Control	Patients
Age (years)	48.4 ± 1.9	60.8 ± 1.8
Sex (M/F)	16/7	21/2
Serum chol. (mg/dl):		
Total	159.9 ± 6.8	211.5 ± 6.6 *
Free	71.9 ± 3.1	ND
Ester	88.1 ± 4.7	ND
LDL chol. (mg/dl):		
Total	89.6 ± 7.2	161.5 ± 20.6 *
Free	49.2 ± 7.3	82.4 ± 11.2 *
Ester	40.8 ± 6.6	79.2 ± 4.7 *
Triglyceride (mg/dl):		
VLDL	96.3 ± 10.2	95.4 ± 8.3
LDL	42.2 ± 8.8	92.1 ± 7.8 *

All the data are means ± SE of 23 samples. Chol: cholesterol; ND: not determined.
* significantly different from control ($P < 0.05$).

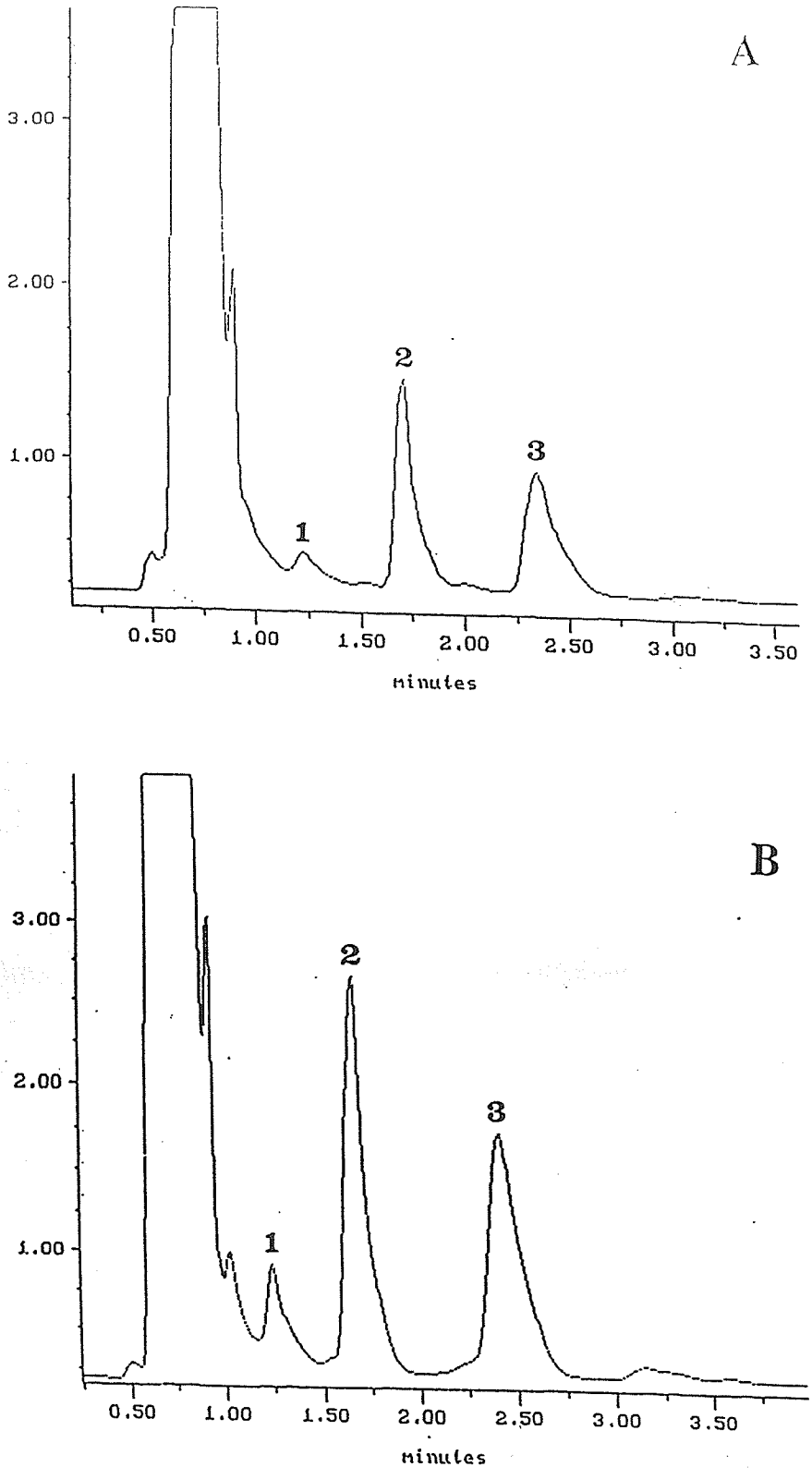


Figure 25. Representative HPLC recording of cholesterol species in LDL from a healthy, asymptomatic subject (A) and a coronary disease patient (B). Peak #1: 4-cholesten-3-one; Peak #2: cholesterol; Peak #3: 20 α-OH cholesterol.

Table 8. HPLC data of LDL and VLDL cholesterol

		chol	4-3 one	20-α OH	total chol
Control	(μg/mg LDL protein)	98.52 ± 7.22	0.98 ± 0.22	79.86 ± 16.52	180.07 ± 17.65
	(mg/dl)	51.23 ± 3.7	0.51 ± 0.11	41.52 ± 8.59	93.63 ± 9.18
Patients	(μg/mg LDL protein)	163.84* ± 15.11	4.38* ± 1.62	138.71 ± 26.76	306.45* ± 37.33
	(mg/dl)	90.44* ± 8.34	2.42* ± 0.89	76.57 ± 14.77	169.11* ± 20.61
Control	(μg/mg VLDL protein)	76.46 ± 7.96	1.22 ± 0.65	123.32 ± 23.36	210.11 ± 20.49
	(μg/dl)	16.51 ± 1.71	0.26 ± 0.14	26.64 ± 5.01	43.25 ± 4.33
Patients	(μg/mg VLDL protein)	75.23 ± 4.79	1.35 ± 0.43	112.31 ± 21.96	189.23 ± 25.33
	(μg/dl)	16.25 ± 1.04	0.29 ± 0.11	24.26 ± 4.74	40.87 ± 5.41

Values are means ± SE of 23 samples. chol = cholesterol; 4-3 one = 4-cholesten-3-one; 20 α-OH = 20-α hydroxy cholesterol; total chol = total cholesterol. * Significantly different from the value of control value ($P<0.05$).

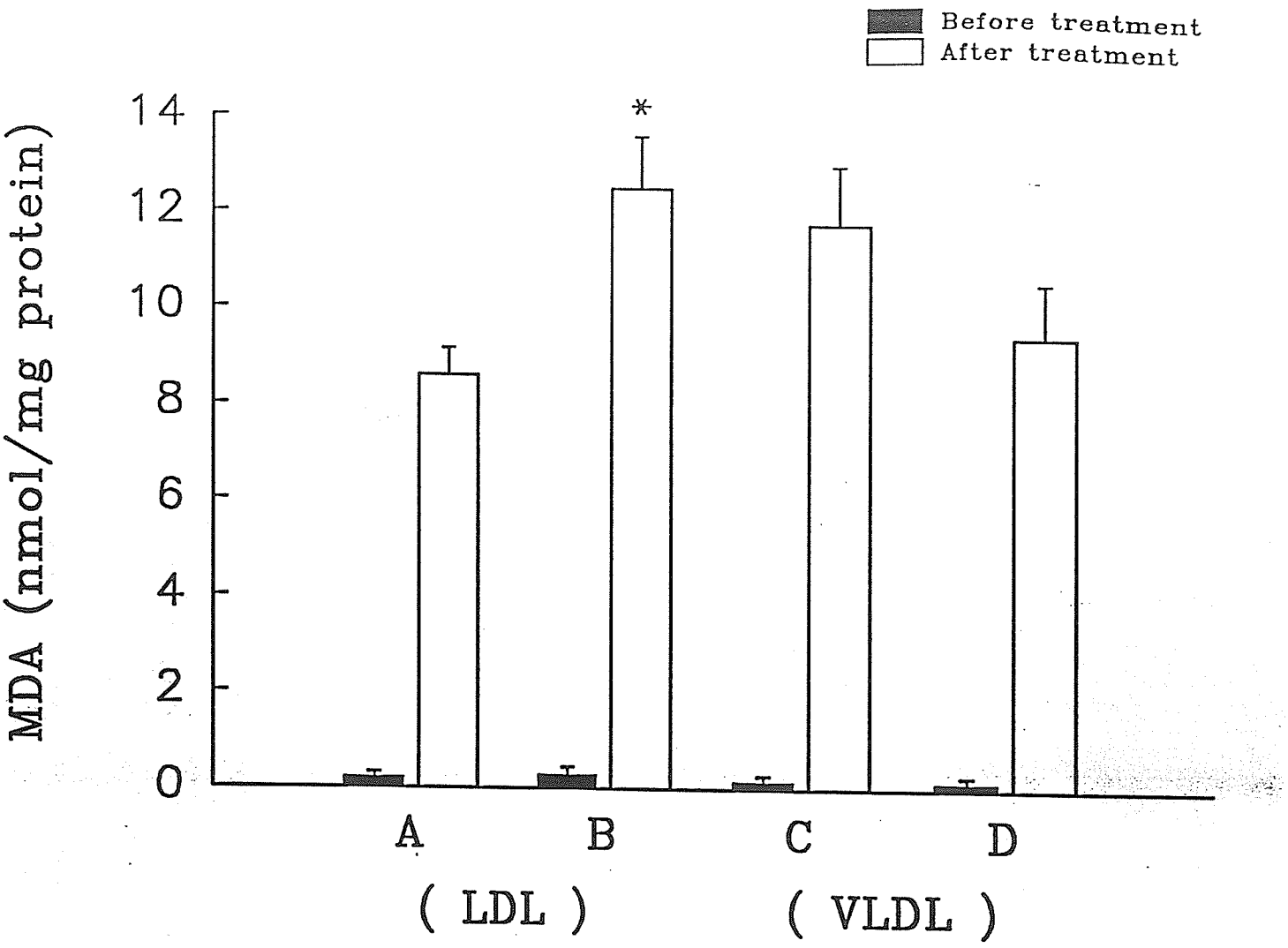


Figure 26. Malondialdehyde levels in LDL and VLDL before and after treatment with Fe^{3+}-ADP-DHF, a free radical generating system. A: Control LDL; B: LDL from coronary disease patients; C: Control VLDL; D: VLDL from coronary disease patients. * significantly different from respective control sample ($P<0.05$).

protein. A free radical generating system was utilized to determine the sensitivity of these fractions to oxyradical attack. MDA production in LDL from coronary patients was significantly higher than that from control samples after LDL incubation with the DHF-Fe^{3+}-ADP system. The MDA generated by this system in VLDL was not significantly different between the two groups.

Basal LDL lysine content from the two groups was similar (Figure 27). However, VLDL lysine reactivity was significantly higher in control samples. As expected from previous data (382), lysine reactivity of LDL was significantly decreased after $CuSO_4$ treatment for 60 minutes. This response was qualitatively and quantitatively similar between the two groups. However, lysine reactivity after $CuSO_4$ treatment in patients' VLDL was significantly decreased whereas control VLDL samples were unaffected by the treatment.

V. Effects of cholesterol oxidase on cultured vascular smooth muscle cells.

Treatment of vascular smooth muscle cells with cholesterol oxidase resulted in morphological changes. Two major morphological alterations were observed, one of which was the appearance of numerous transparent vacuolized droplets in the cytoplasm without a change in cell shape. The other major change observed was that the cells became round and transparent in the cytoplasm, under moderate treatment, with the centre of the cell appearing to be more dense (Fig.28). The extent of morphological change of the cells was dependent upon the time of exposure and the concentration of cholesterol oxidase applied (Fig.29). After 3 hours treatment with 1 mg/ml cholesterol oxidase, the number of affected cells increased significantly relative to untreated cells.

Trypan blue staining of cells was used to determine cell viability after cholesterol oxidase treatment. Control cells excluded trypan blue (Fig 30A). Cells treated with

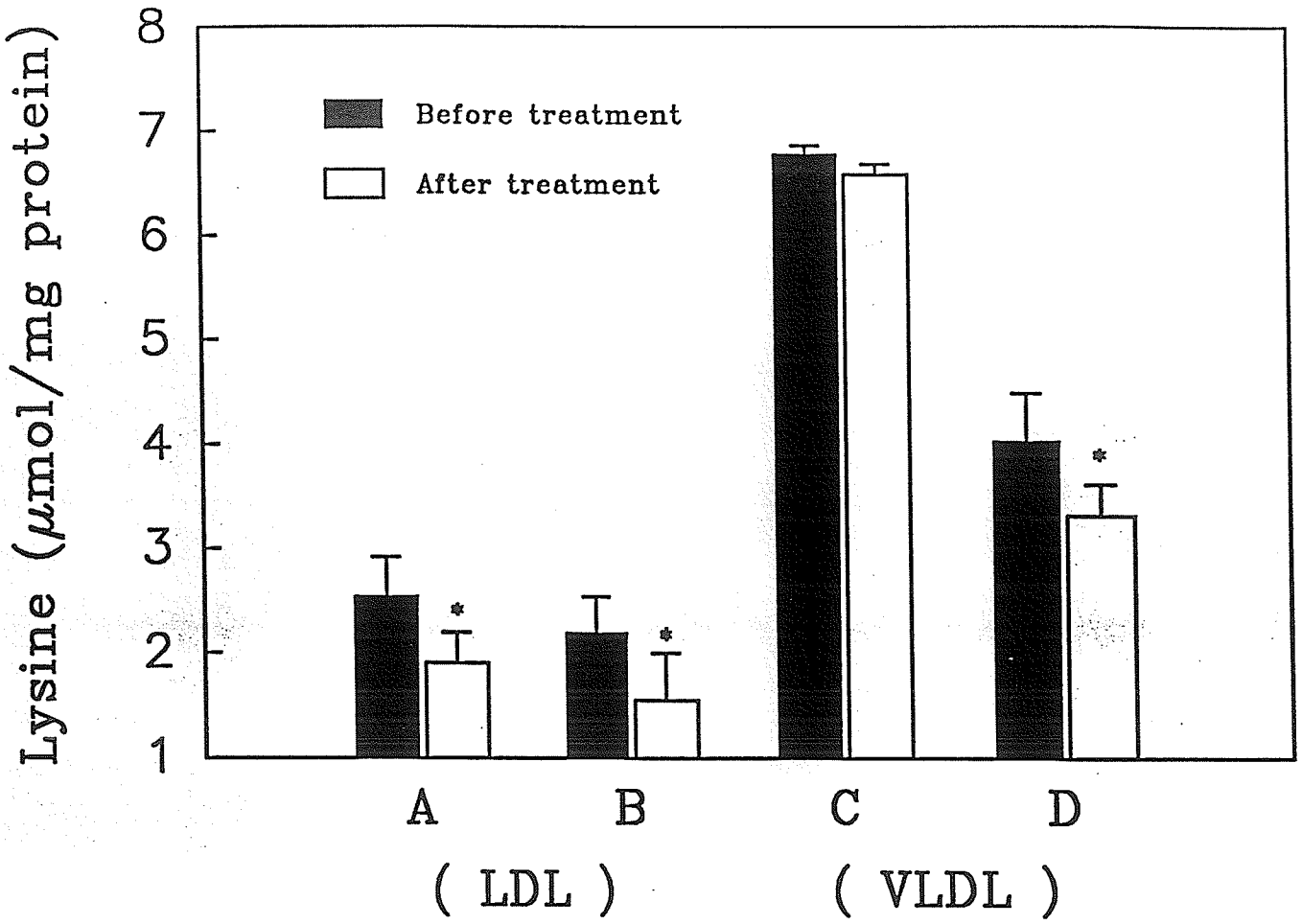
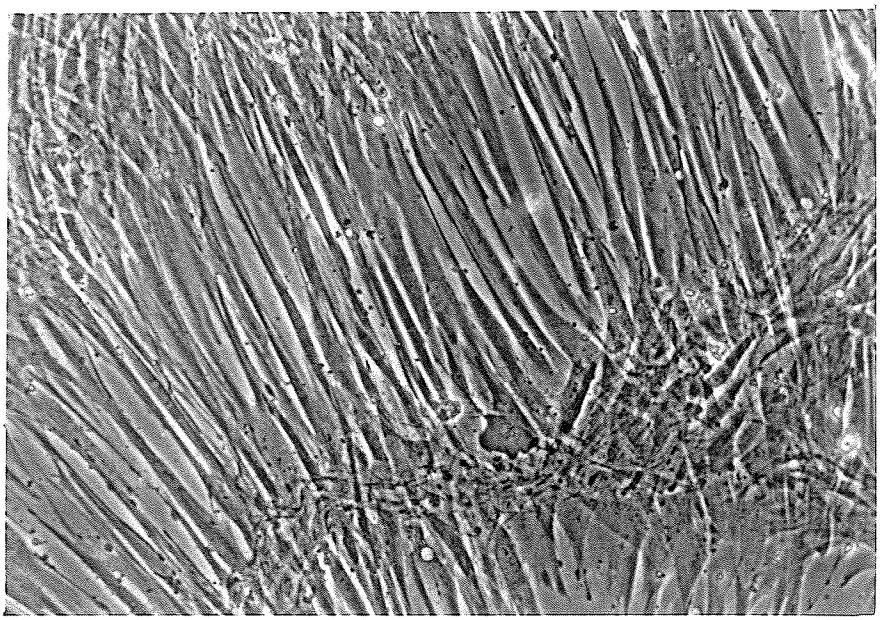
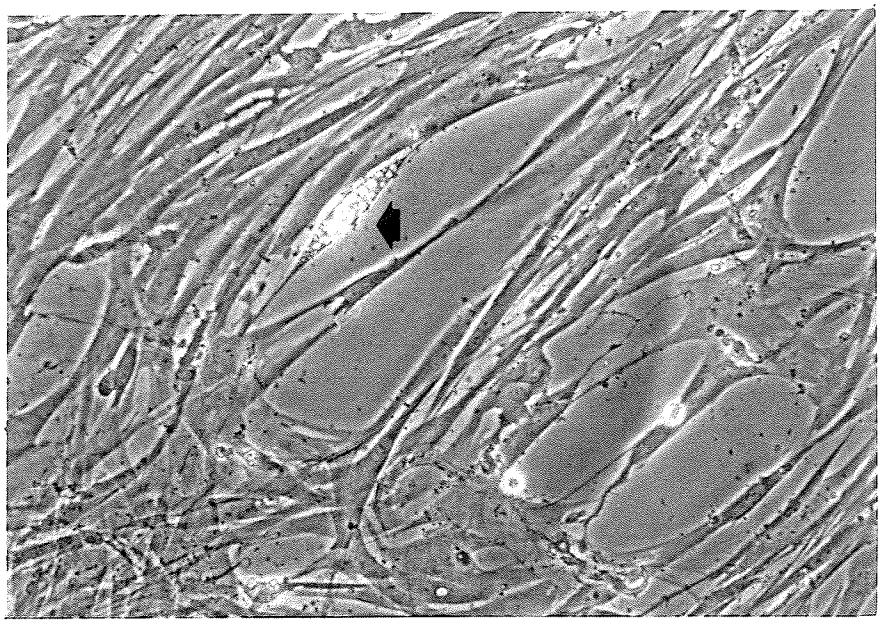


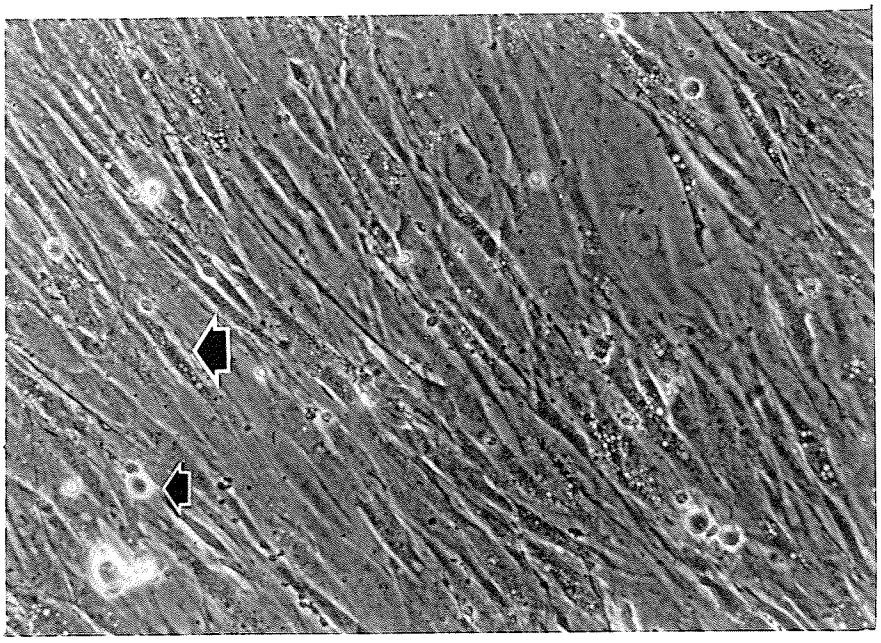
Figure 27. Lysine reactivity of LDL and VLDL before and after $CuSO_4$ treatment. A: Control LDL; B: LDL from coronary disease patients; C: Control VLDL; D: VLDL from coronary disease patients. * significant change in lysine reactivity of lipoproteins due to $CuSO_4$ treatment. ** significantly different from control ($P<0.05$).



A.



B.



C.

Figure 28. Effects of cholesterol oxidase on cell morphology. A. Normal smooth muscle cells showing typical, elongated spindle shape. B. After 1 hour treatment with 1mg/ml cholesterol oxidase, cells become transparent and many vacuoles appear inside the cytoplasm (arrows) but the shape is unchanged. C. After treatment with 1 mg/ml cholesterol oxidase for 3.5 hours, two changes can be observed. Some cells are spindlelike but contain transparent vacuoles in the cytoplasm (large arrow), whereas other cells are rounded and transparent (small arrow).

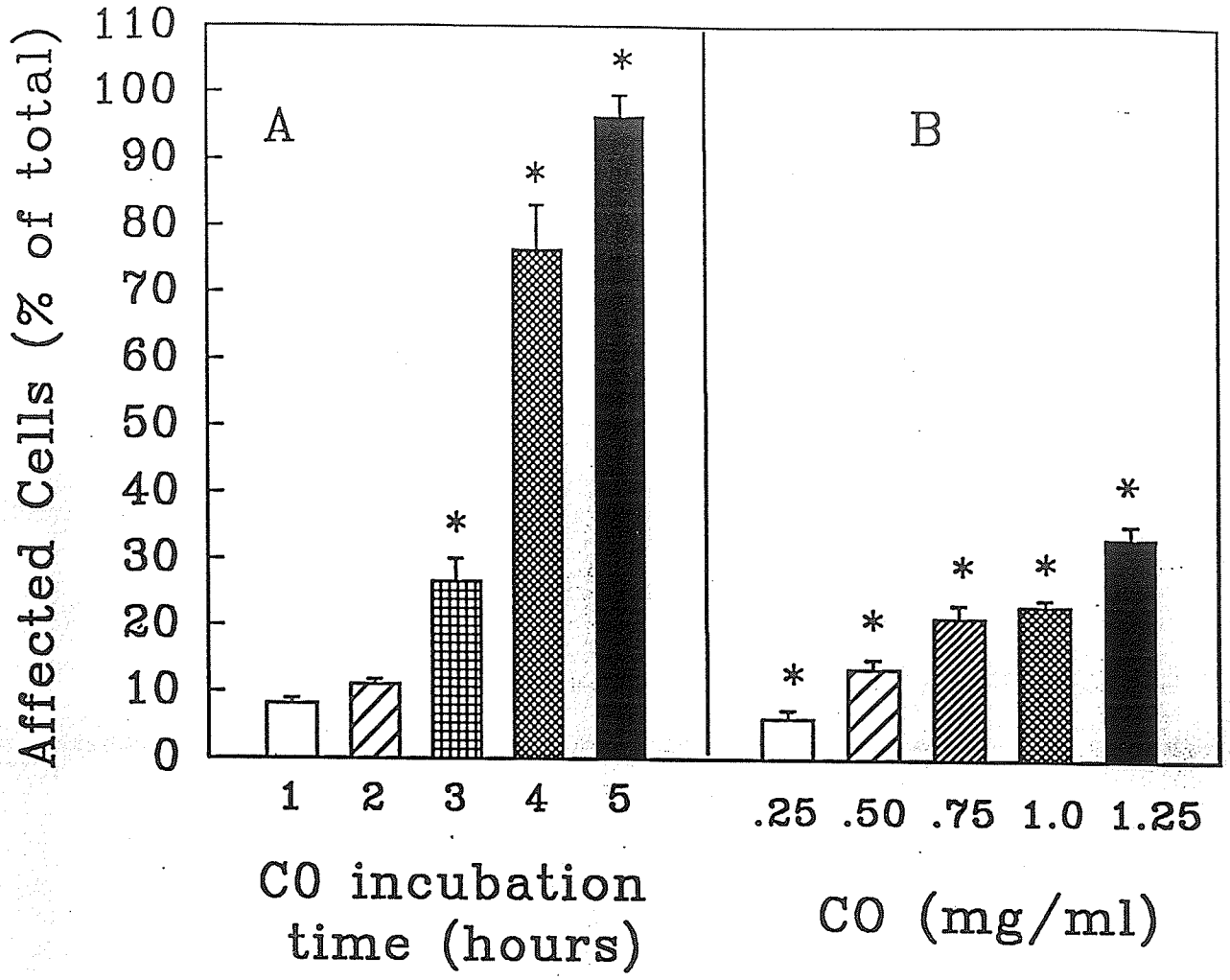
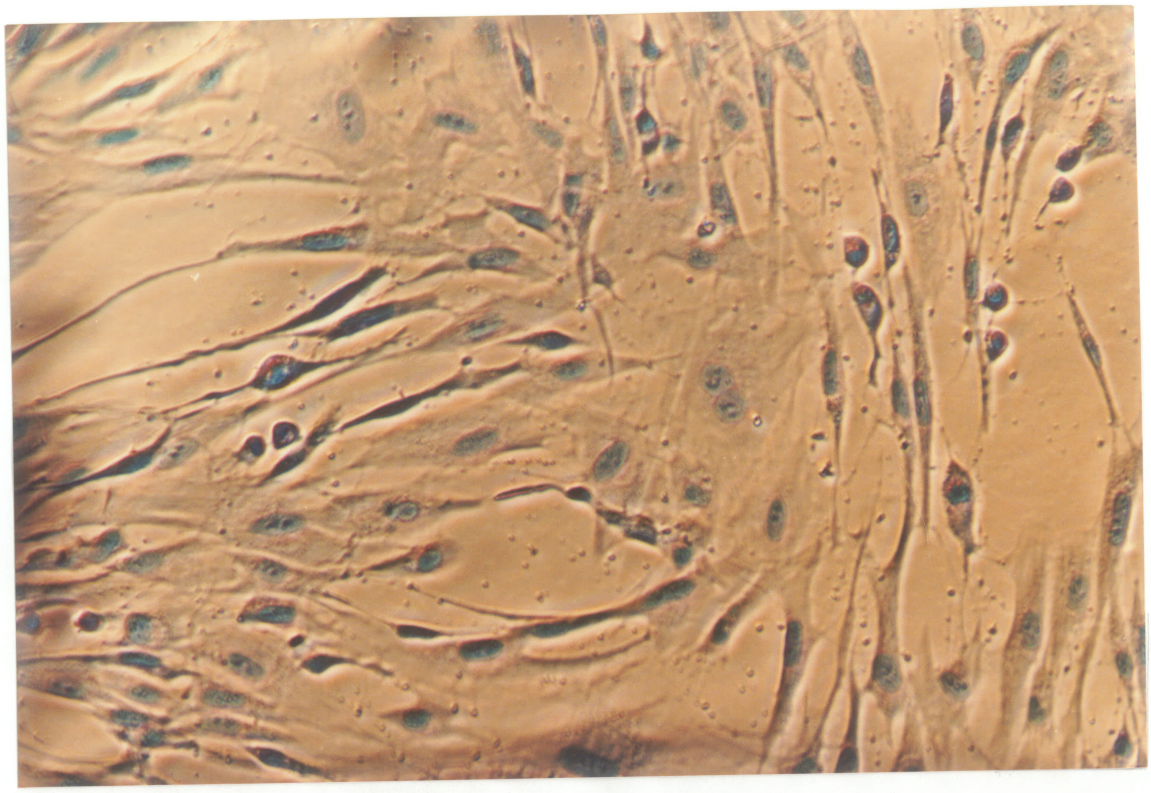
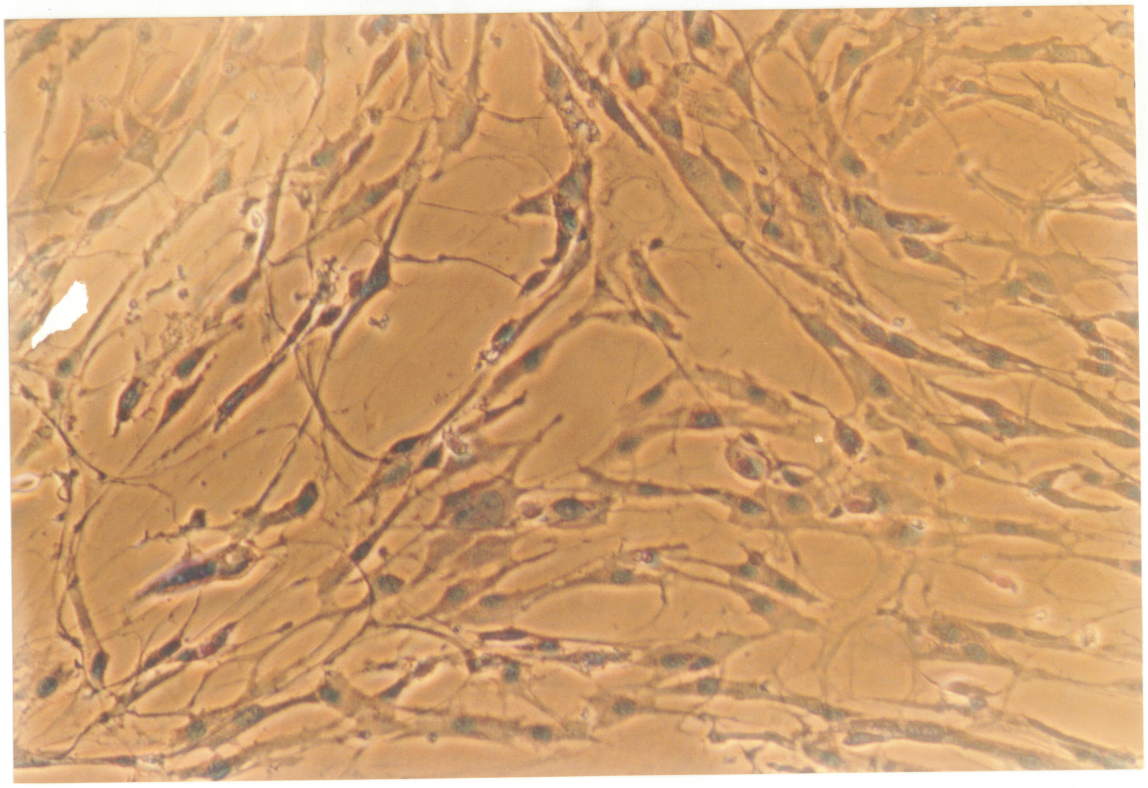


Figure 29. Quantitation of the effects of cholesterol oxidase on cell morphology. A. The cells were treated with 1 mg/ml cholesterol oxidase for 1-5 hours then 5 photomicrographs were randomly taken for each sample and affected cells counted versus normal cells. Affected cells were identified on the basis of features as depicted in Figure 1C. * $p<0.05$. B. The cells were treated for 3 hours with different concentrations of cholesterol oxidase (0.25-1.25 mg/ml), then the data were collected as in A. * $p<0.05$ vs untreated cells.

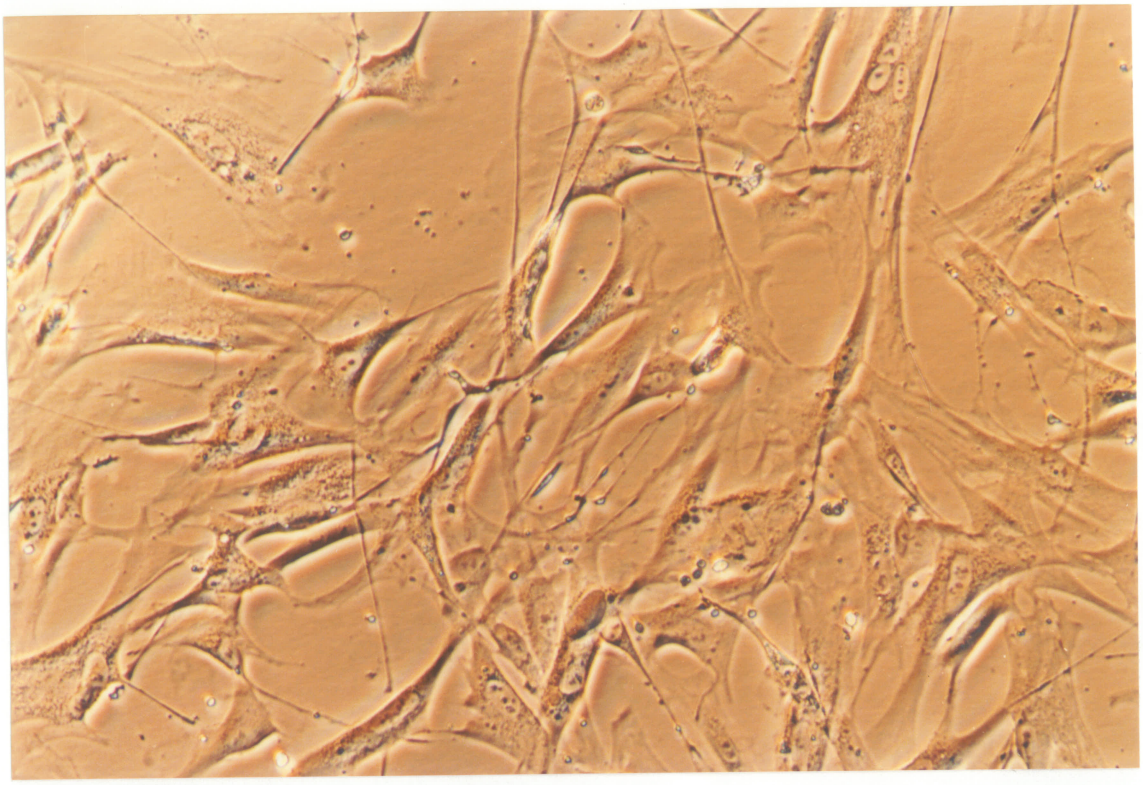


C.

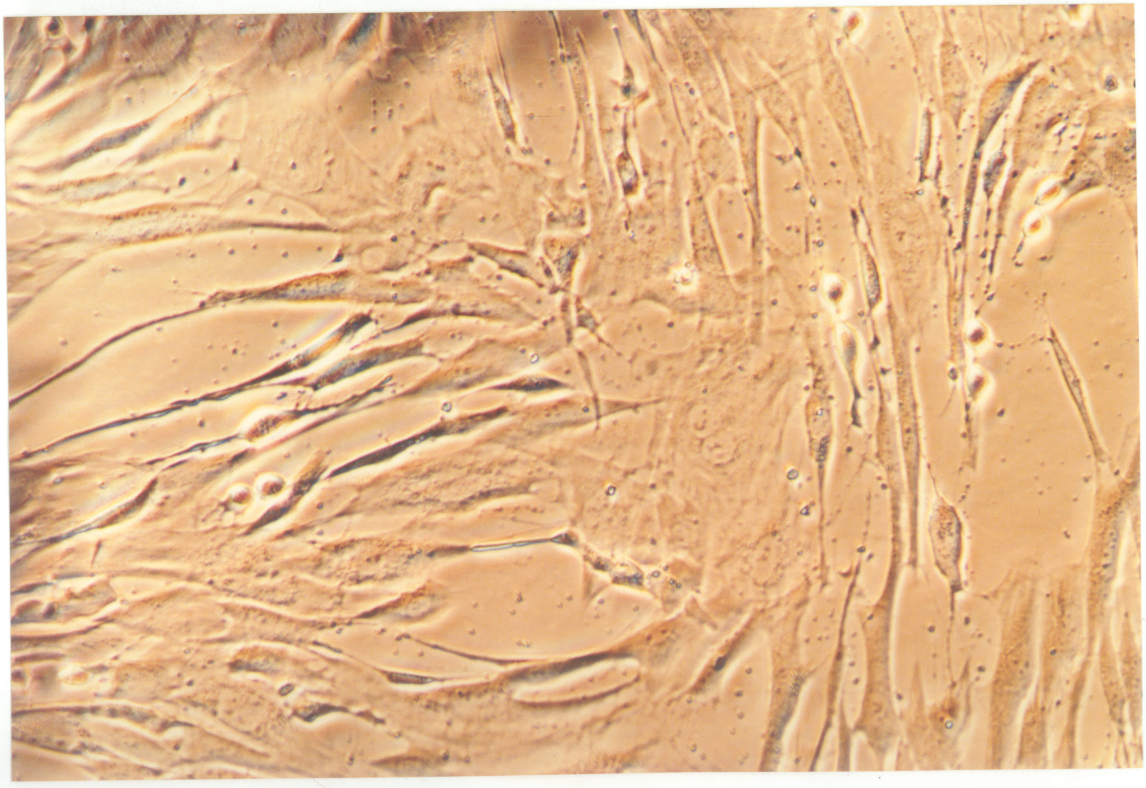


D.

Figure 30. Cell viability as detected by the trypan blue stain. A. Normal cells. B. Cells treated with 1mg/ml cholesterol oxidase for 3 hours. C. The same cells that were treated with cholesterol oxidase in B were then exposed to 75% ethanol for 20 minutes. Note the strong staining with trypan blue. D. Cells treated with 1 mM DNP, a mitochondrial poison, for 15 hours.



A.

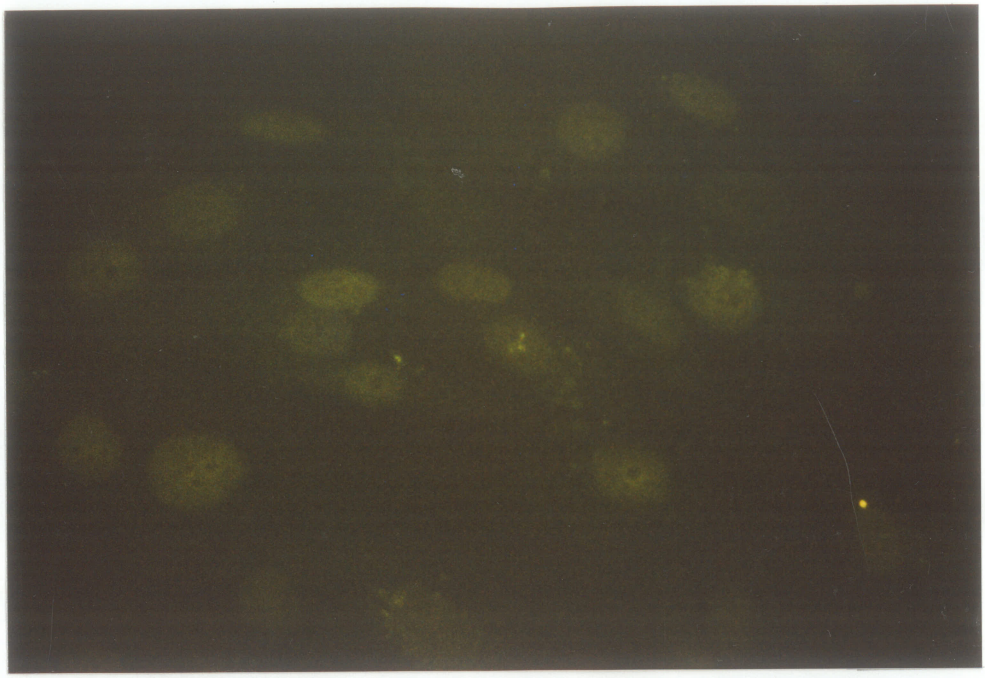


B.

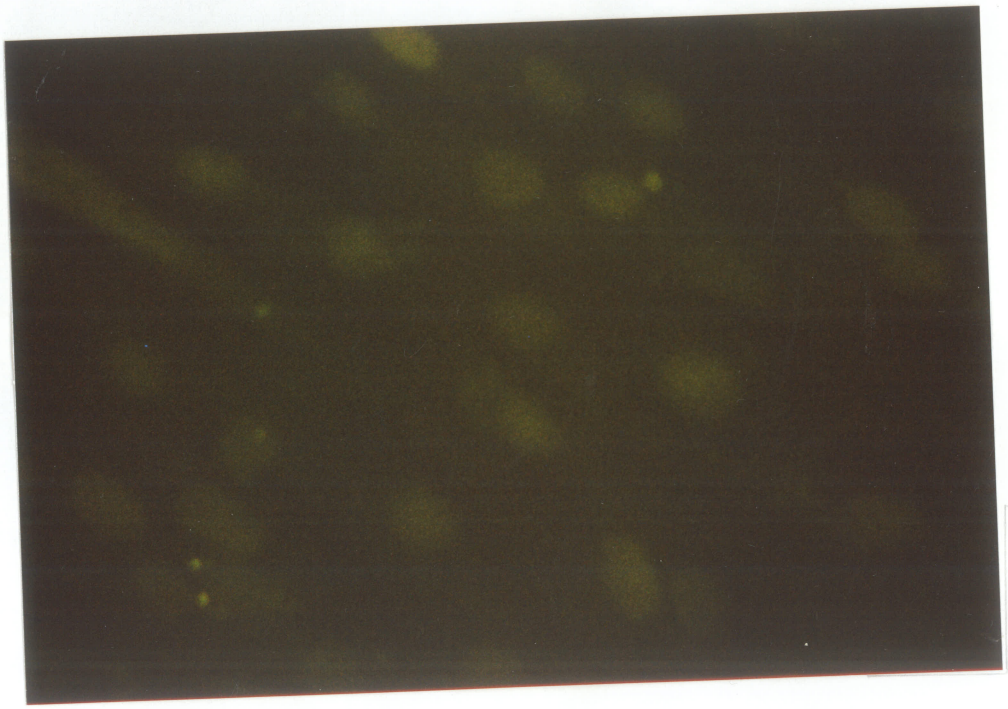
cholesterol oxidase also excluded trypan blue (Fig.30 B). In order to determine that this response was not artifactual, the same cells were then exposed to 75% ethanol for 20 minutes. These cells were now strongly stained with trypan blue (Fig.30 C). In separate experiments, cell viability was compromised by treating the cells with 1 mM DNP, a mitochondrial poison (359), for 15 hours. These cells exhibited strong staining with trypan blue (Fig.30 D). Cells treated with 0.1 mg/ml 4-cholesten-3-one for 24 hours exhibited strong staining with trypan blue as well (data not shown).

DASPMI stains nuclei and energized mitochondria and can be used to detect the integrity of mitochondrial energy production (356-359). Thus, DASPMI is a reliable fluorescent indicator of cell viability (356-359). The fluorescence photomicrographs showed that control cells were evenly and brightly stained, and the cells which were treated with cholesterol oxidase were similar to control (Fig.31 A&B). DASPMI fluorescence in cells after ethanol or DNP treatment appeared less bright (Fig.31 D&E). The fluorescence intensity from a number of experiments was quantified. The fluorescence intensity of cholesterol oxidase treated cells was not changed in comparison to control, but the intensity of the cells treated with ethanol, DNP and 4-cholesten-3-one was significantly decreased (Fig.32).

Nile red was used as a sensitive fluorescent stain for the detection of cytoplasmic lipid droplets (354,355). The cells showed brilliant droplets of yellow-gold fluorescence which is indicative of neutral lipid deposits (383). As demonstrated in the photomicrographs, normal smooth muscle cells possessed a few fluorescent droplets sparsely spread throughout the cells, whereas the fluorescence of cholesterol oxidase treated cells was more brightly and densely distributed in the cytoplasm (Fig.33 A&B). Fluorescence intensity of treated cells was quantified and demonstrated to be significantly increased which was

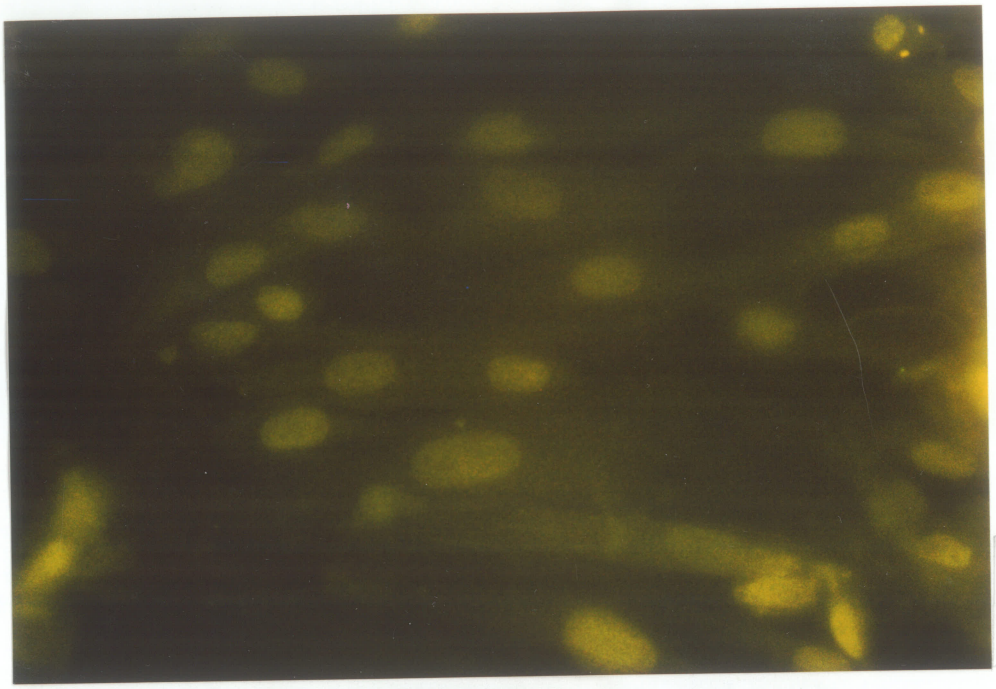


C.

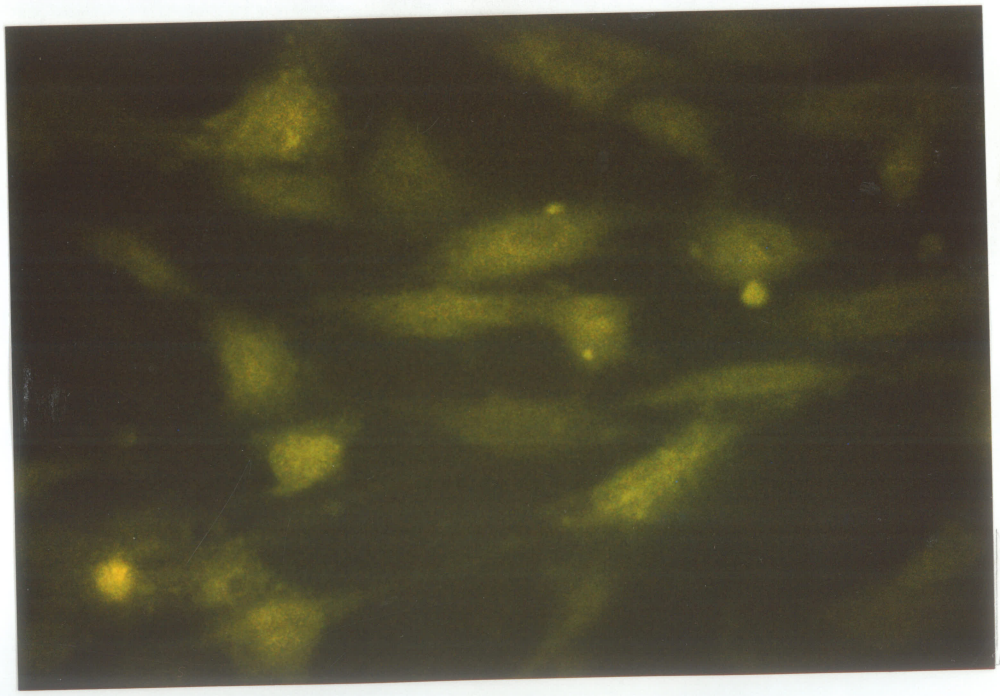


D.

Figure 31. Energy status of the cells as determined with the DASPMI stain. A. Control cells showing even and bright staining. B. Cells were treated with 1mg/ml cholesterol oxidase for 3 hours and then stained. C. Cells were treated with 75% ethanol for 20 minutes, then stained. D. Cells treated for 15 hours with 1 mM DNP.



A.



B.

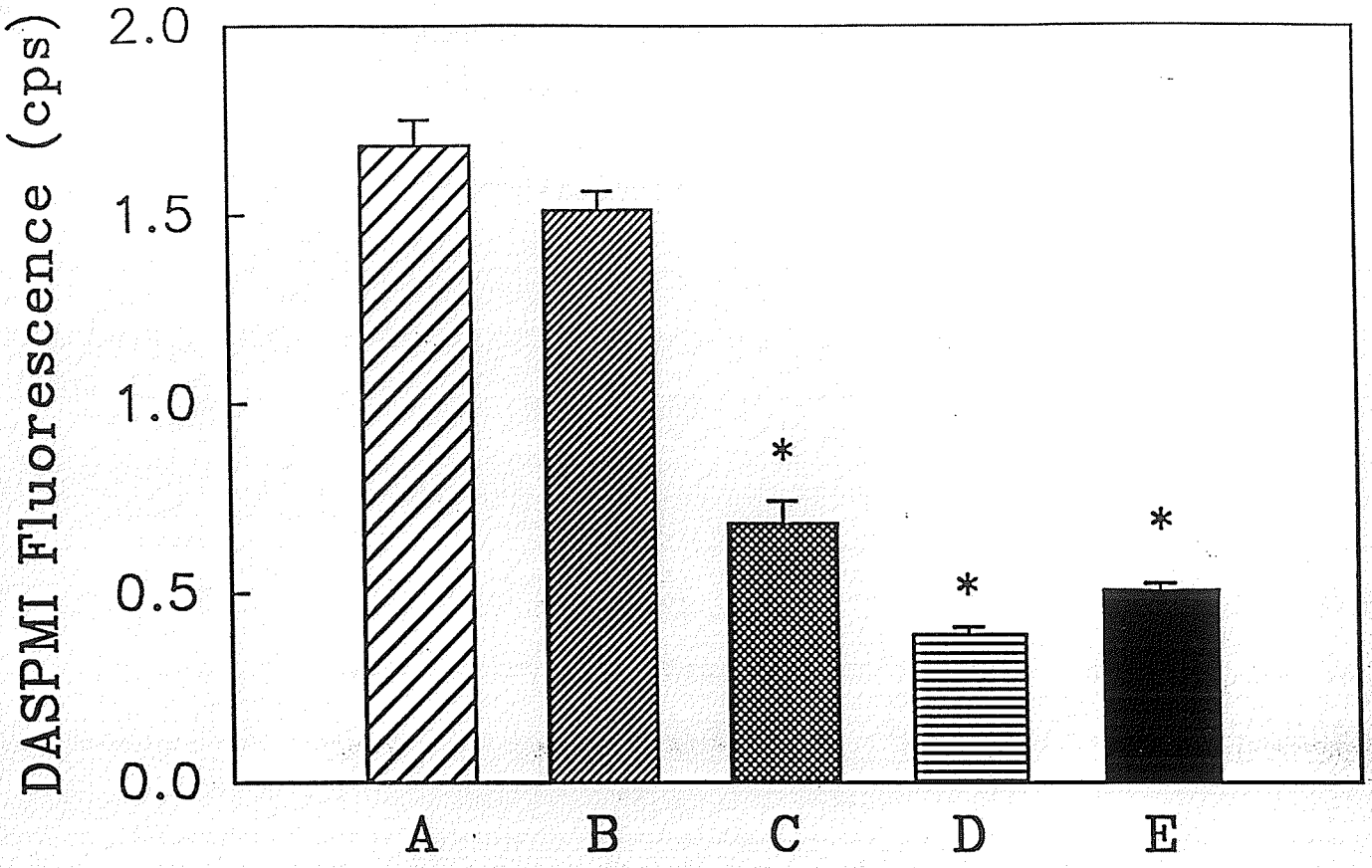
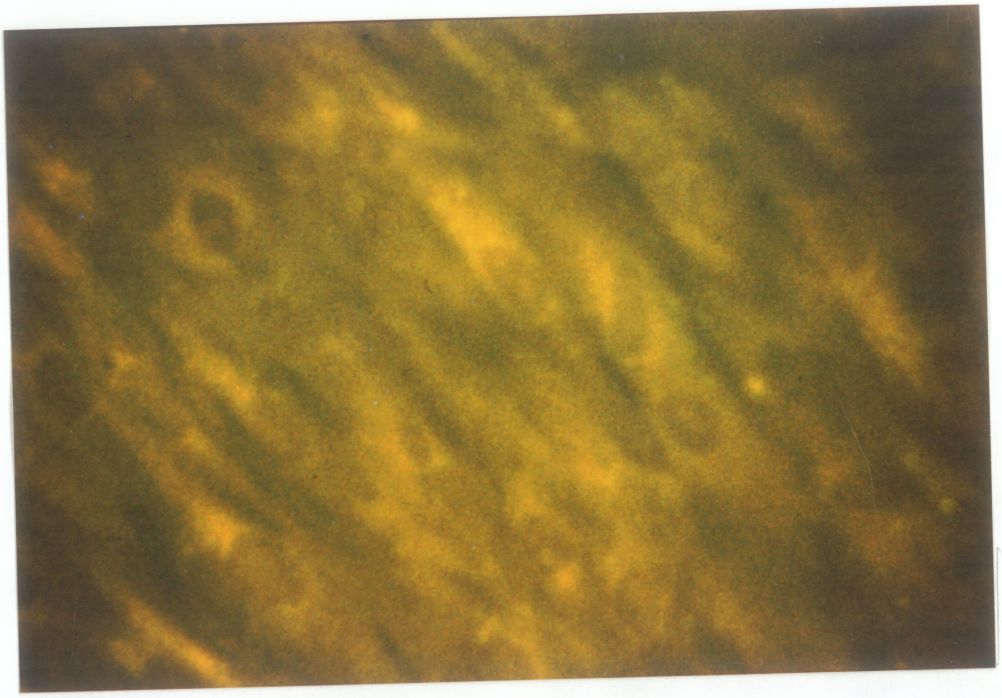
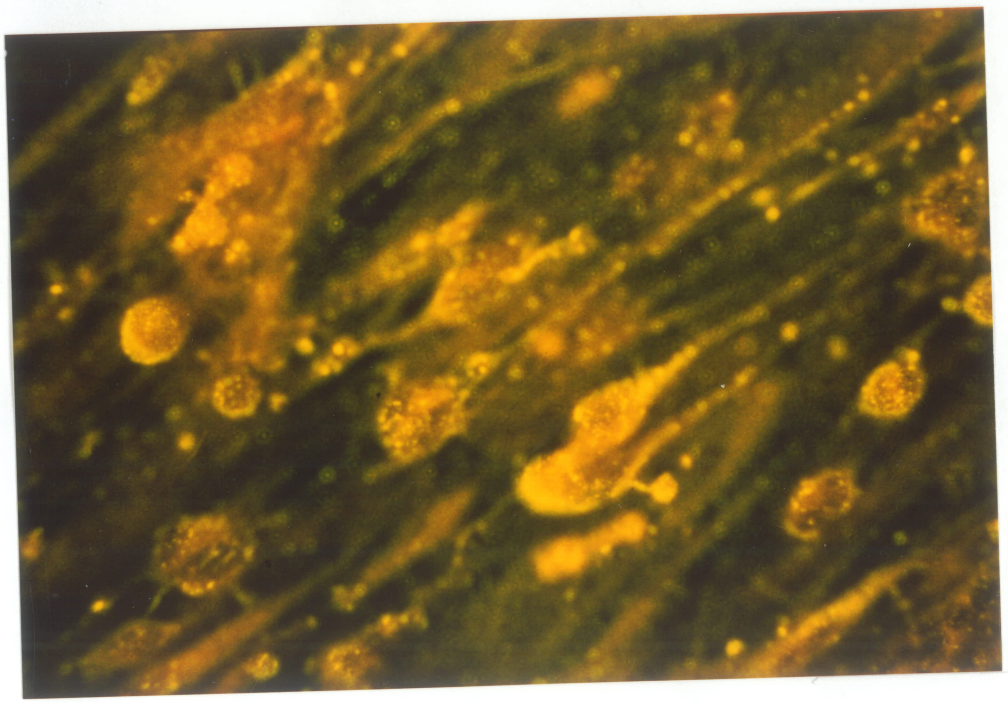


Figure 32. Quantitation of the effects of cholesterol oxidase on fluorescent intensity of DASPMI staining of smooth muscle cells. Cells were untreated (A), or treated with 1 mg/ml cholesterol oxidase for 3 hours (B), or treated with 1 mM DNP for 15 hours (C), or treated with 75% ethanol for 20 minutes (D), or treated with 0.1 mg/ml 4-cholesten-3-one for 24 hours (E). * $p<0.05$ vs untreated cell. (n=3).



A.



B.

Figure 33. Nile red stain for intracellular lipids in untreated and cholesterol oxidase-treated cells. A. Normal smooth muscle cells. Note the few fluorescent droplets sparsely spread throughout the cells. B. Cells treated wtih 1mg/ml cholesterol oxidase for 3 hours showed more brightly and densely distributed fluorescence droplets in the cytoplasm.

dependent not only upon the concentration of cholesterol oxidase used but also the time of exposure of the cells to the enzyme (Fig. 34&35).

Oxidized cholesterol was not detectable by HPLC analysis in control cells. However, oxidized species of cholesterol were generated after cholesterol oxidase treatment. The oxidized cholesterol species which appeared were 4-cholesten-3-one and 20-a-OH cholesterol. These species were significantly increased after 30 minutes exposure of the cells to cholesterol oxidase treatment (Table 9). The content of 4-cholesten-3-one and 20-a-OH cholesterol increased as the concentration of cholesterol oxidase applied to the cells increased whereas the cholesterol content decreased correspondingly (Fig.36).

Two possible mechanisms of action of cholesterol oxidase can be considered. The enzyme may be acting via: 1) a direct effect on the cell membrane itself, or 2) an indirect action on some component of the medium surrounding the treated cells. Because the only source of lipid content in the treatment medium is from the fetal calf serum which was always present at 10% (V:V) in the cholesterol oxidase experiments, the effect of cholesterol oxidase on cells in the absence of fetal calf serum was examined. The cells were treated with 1 mg/ml cholesterol oxidase for 4 hours with or without fetal calf serum. The cells treated in the presence of fetal calf serum showed the same morphological change as described above (Fig.28) whereas the cells treated without fetal calf serum demonstrated no changes in comparison to untreated cells (data not shown). Further, if these same cells (+ cholesterol oxidase treatment but in the absence of fetal calf serum) were washed, then incubated for 18 hours in a medium which contained fetal calf serum, no morphological changes were observed.

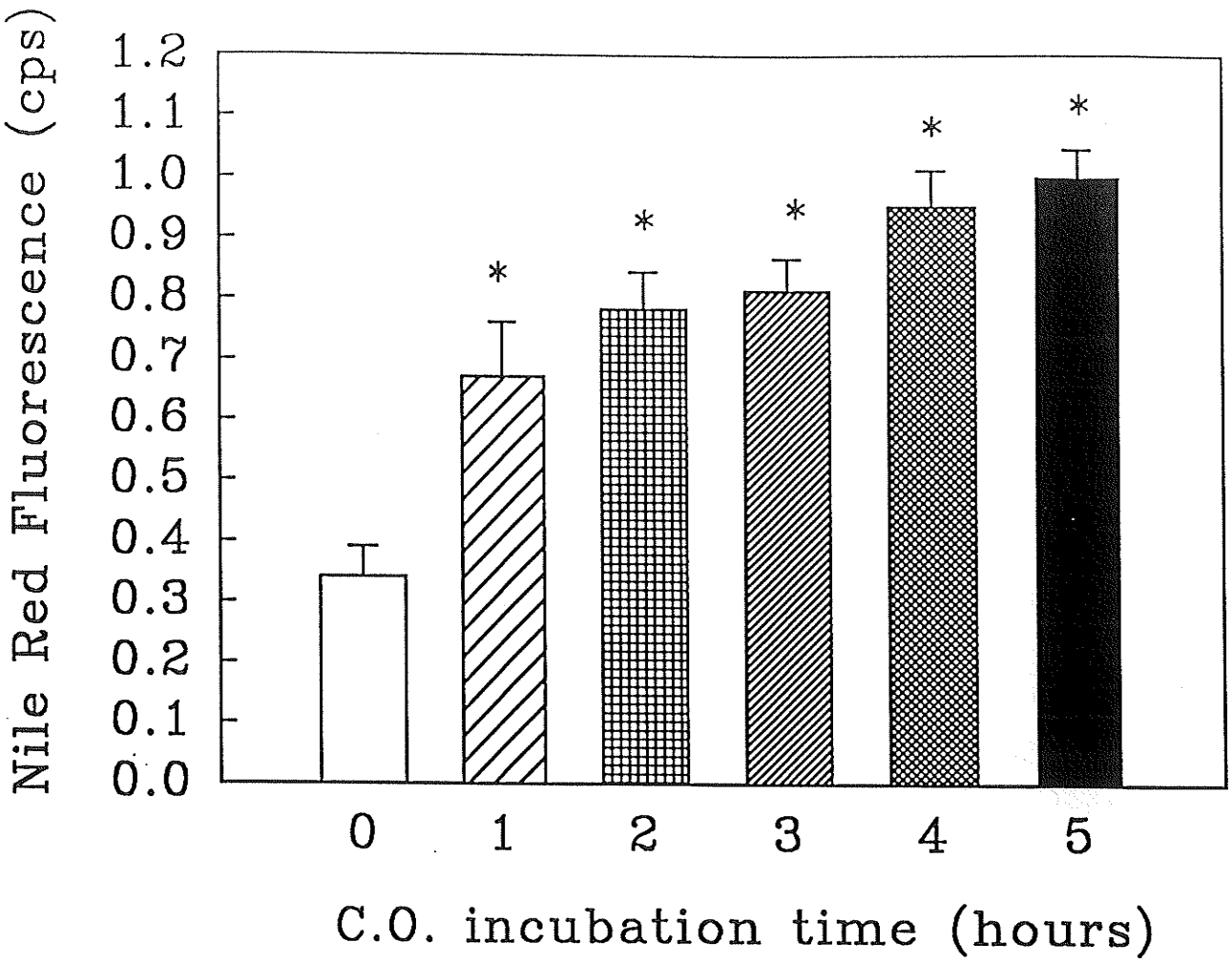


Figure 34. Quantitation of nile red fluorescence after cholesterol oxidase treatment for different times. The fluorescence intensity of nile red was significantly increased after 1-5 hours treatment. * $p<0.05$ vs control. (n=3).

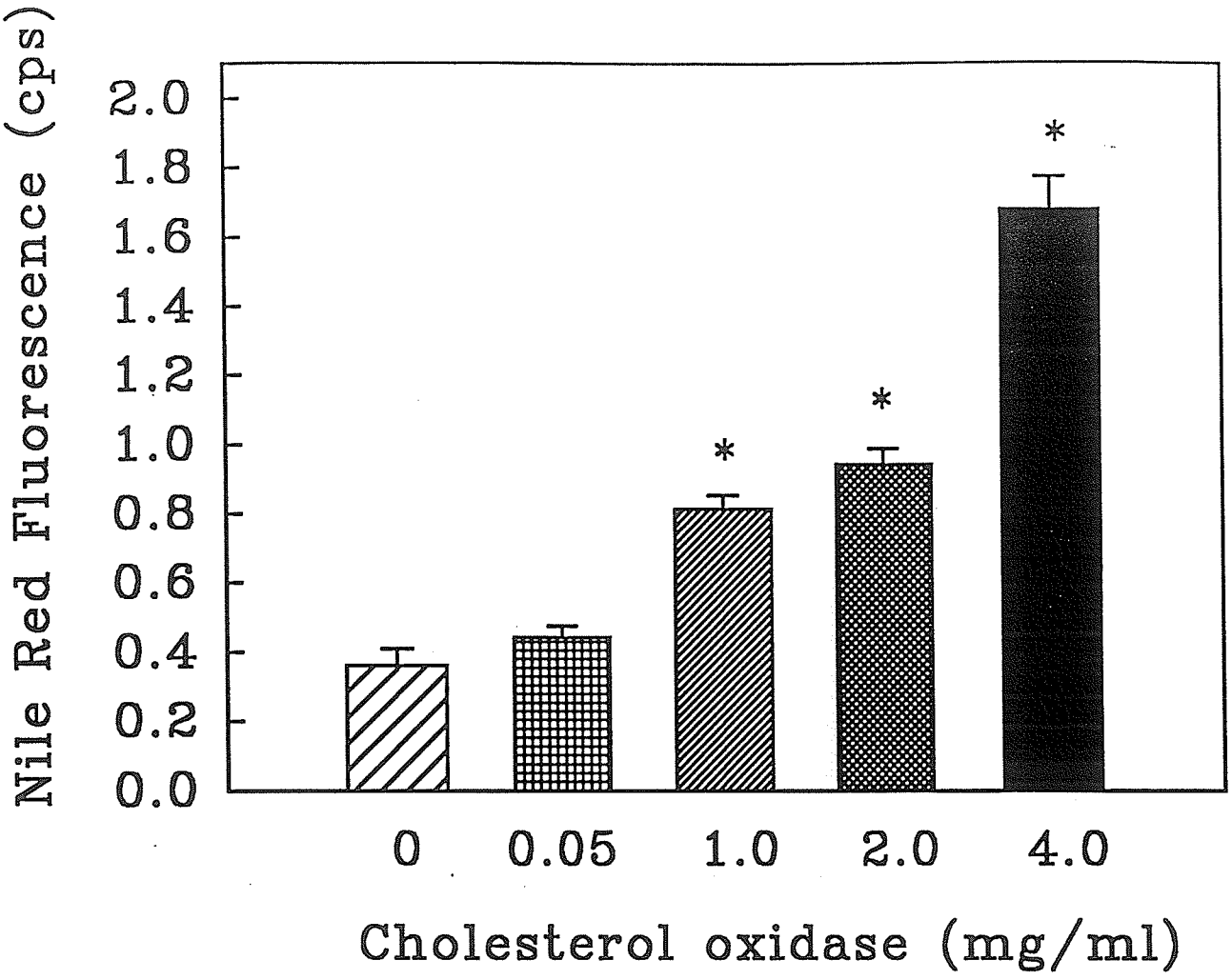


Figure 35. Quantitation of nile red fluorescence intensity as a function of varying concentrations of cholesterol oxidase. Incubation time was for 2 hours. * $p<0.05$. vs untreated cells. (n=3).

Table 9. Generation of oxidized cholesterol in vascular smooth muscle cells after varying times of exposure with cholesterol oxidase

Time (min)	(% of total cholesterol species)		
	Cholesterol	4-3 one	20 α-OH
0	100	0.0	0.0
15	95.3 ± 2.4	4.2 ± 1.4	0.6 ± 1.8
30	86.4 ± 2.4*	10.0 ± 1.5*	3.8 ± 1.8*
60	84.7 ± 2.7*	8.5 ± 1.7*	6.7 ± 1.9*
300	56.4 ± 2.4*	15.2 ± 1.5*	28.4 ± 1.8*

Values are means ± SE of 4-6 experiments. Cholesterol oxidase at a concentration of 1.4 mg/ml was used to treat the cells. Lipids were extracted after treatment and cholesterol species analyzed via HPLC. * Significantly different ($P<0.05$) from control (time=0).

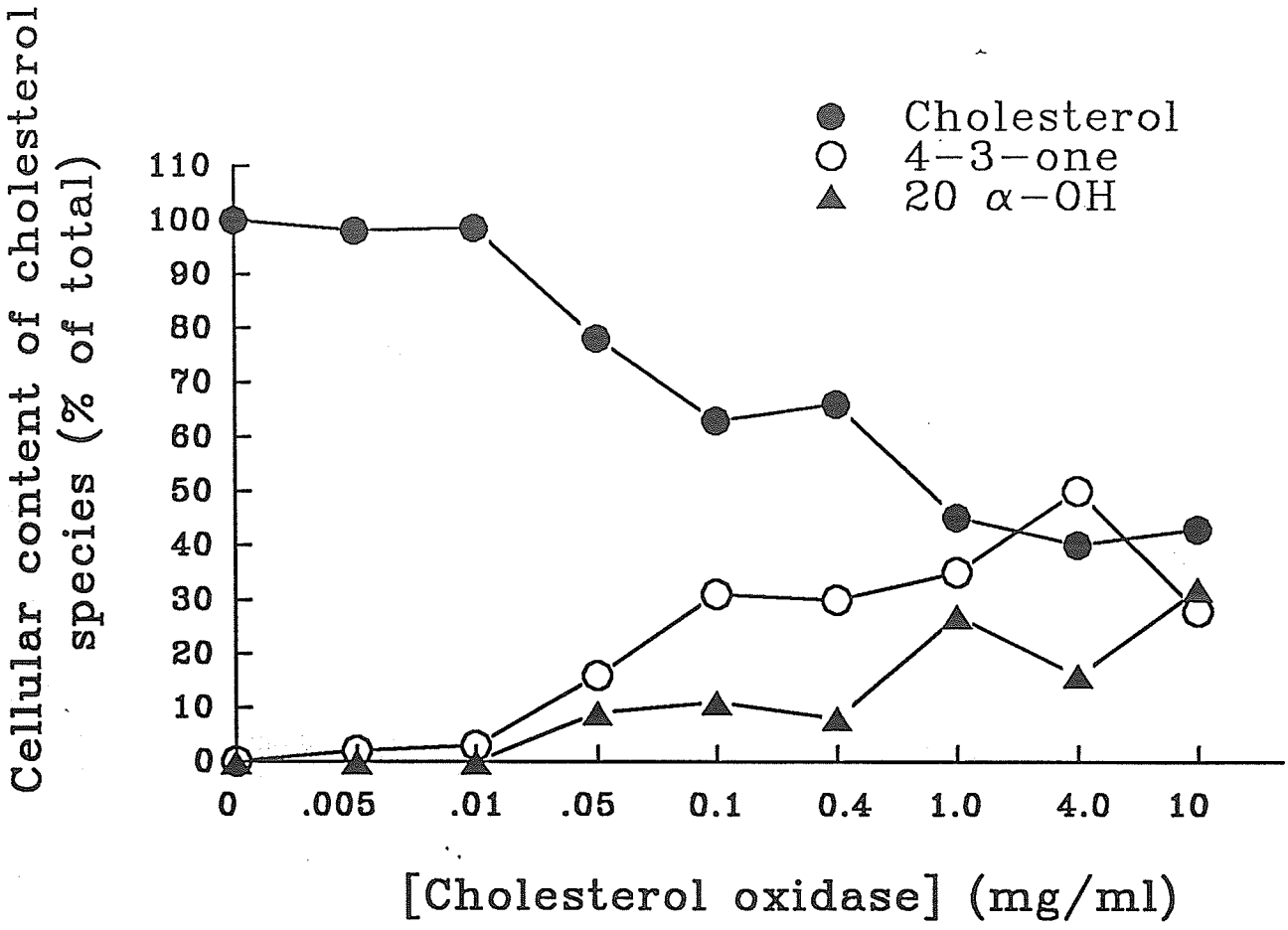


Figure 36. Representative results for the quantitation by HPLC of the cellular content of cholesterol species after treatment with different concentrations of cholesterol oxidase for 24 hours.

VI. Effects of oxidative modification of cholesterol in isolated low density lipoproteins on cultured smooth muscle cells.

Oxidized cholesterol was not detectable in untreated isolated LDL fractions from rabbit. After LDL was incubated with cholesterol oxidase, oxidized cholesterol species were detected by HPLC (Fig 37). The oxidized cholesterols generated were 4-cholesten-3-one, 20-α-OH cholesterol and 25-OH cholesterol after LDL was incubated with cholesterol oxidase for 20 minutes or more. The results were quantified and are shown in Figure 38. The major oxidized cholesterol species at 20 minutes was 4-cholesten-3-one. The content of non-oxidized cholesterol in LDL progressively decreased as the time of incubation with cholesterol oxidase increased. Approximately 55% of the LDL cholesterol was oxidized after 5 hours incubation with cholesterol oxidase.

Lipoprotein peroxidation in LDL after the treatment of cholesterol oxidase was determined by MDA formation using the TBARS method (Fig. 39). The amount of MDA produced from the lipoprotein after treatment with cholesterol oxidase was dependent upon the concentration of the enzyme applied. The production of MDA from lipoprotein was completely prevented by including catalase during the treatment with cholesterol oxidase.

Treatment of vascular smooth muscle cells with cholesterol-oxidized LDL resulted in morphological changes. The morphological changes were similar to the cells treated with cholesterol oxidase in a previous study (353). Briefly, two major alterations were observed, one of which was the appearance of numerous transparent vacuolized droplets in the cytoplasm without a change in cell shape. The other was that the cells became round and transparent in the cytoplasm with the centre of the cell appearing to be more dense under moderate treatment (data not shown but identical to that described in a previous study (353)). The latter change was more prevalent.

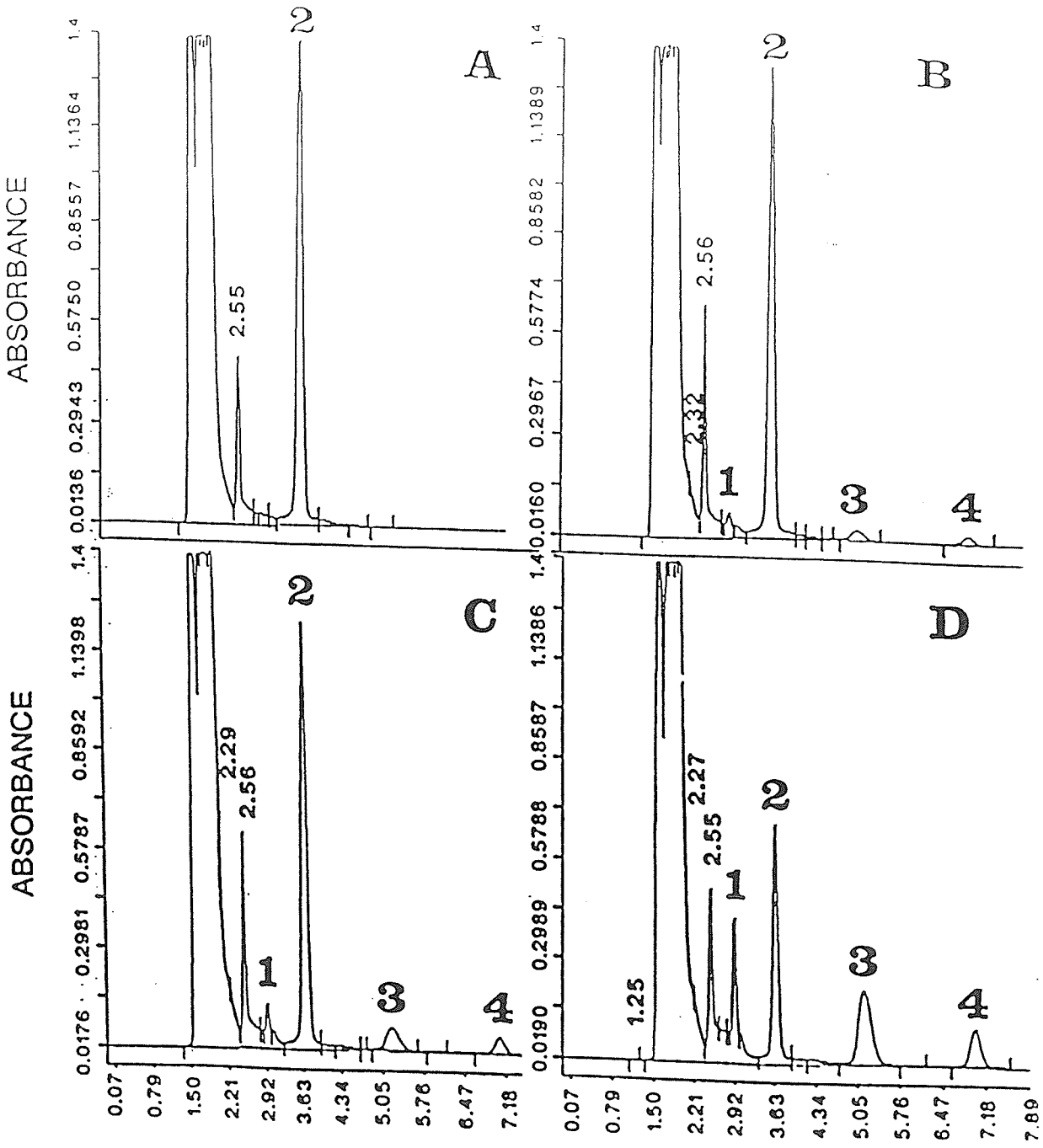


Figure 37. Representative HPLC recordings of cholesterol species in LDL after incubation with cholesterol oxidase for different times. A. Control, peak#2 is identified as cholesterol. LDL (0.1 ml) was incubated with 0.1 mg cholesterol oxidase for 20 minutes (B), 40 minutes (C), or 300 minutes (D). Peak #1: 4-cholesten-3-one; Peak #3: 20 α-OH cholesterol; Peak #4: 25-OH cholesterol.

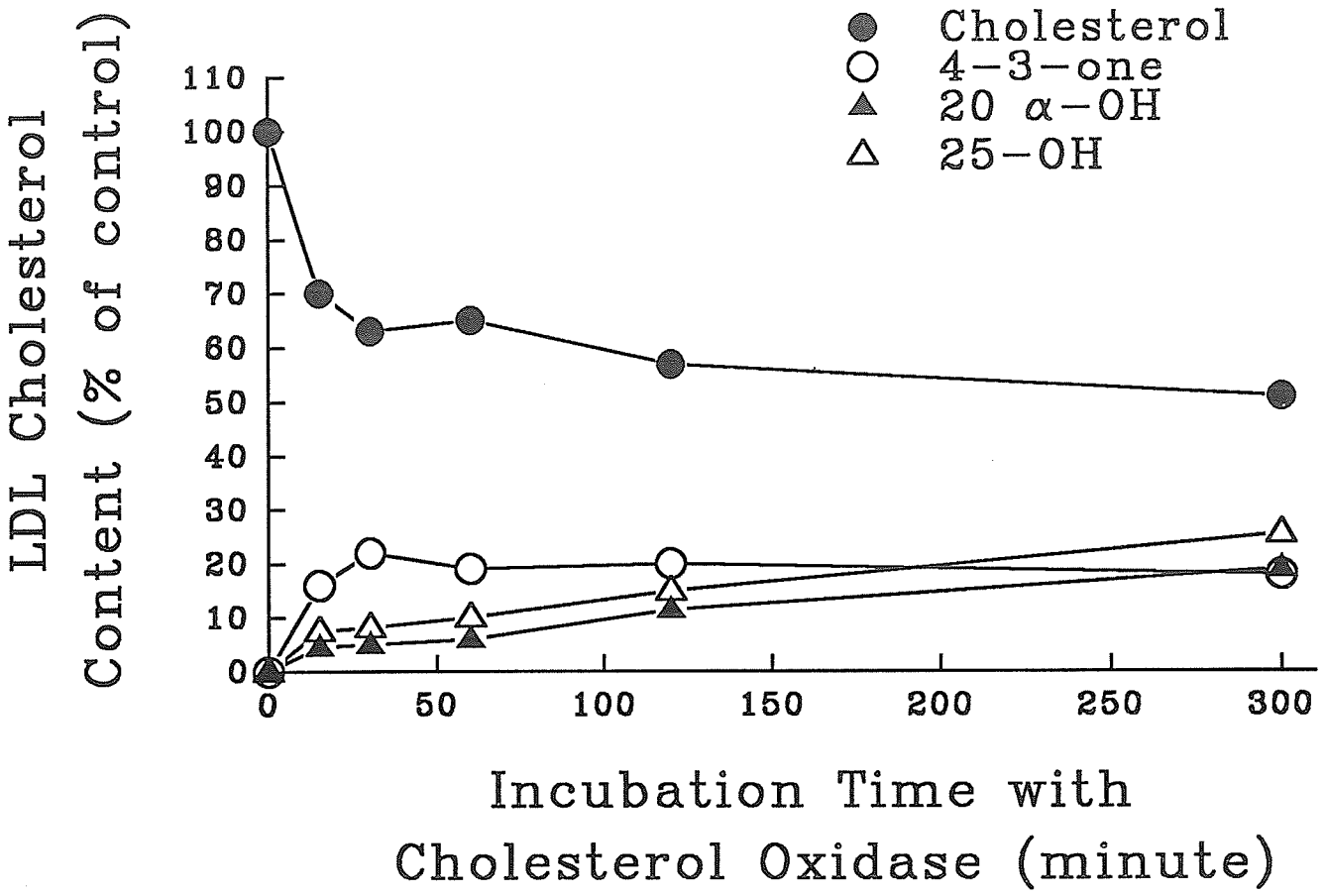


Figure 38. LDL cholesterol species detected by HPLC after incubation with cholesterol oxidase for varying times. The cholesterol content decreased whereas oxidized cholesterol species increased (n=3). 4-3-one: 4-cholesten-3-one, 20 α-OH: 20 α-OH cholesterol, 25-OH: 25-OH cholesterol.

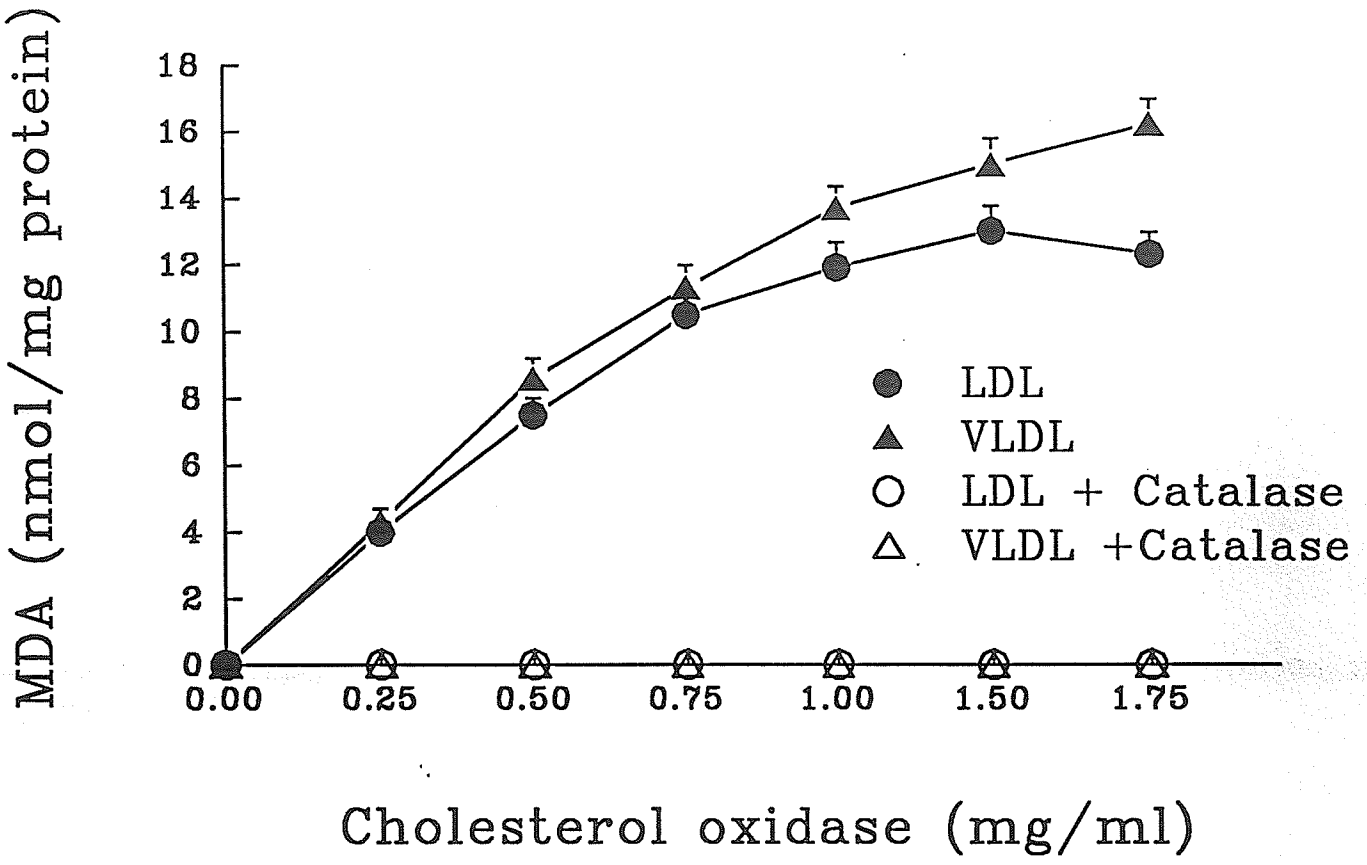


Figure 39. Lipoprotein peroxidation produced by cholesterol oxidase as detected by TBARS / MDA formation. If present, the amount of catalase (mg/ml) used here was four-fold less than the cholesterol oxidase utilized in the experiments. 150 μg of LDL or VLDL protein in 1 ml of 140 mM KCL, 20 mM MOPS (pH 7.4) was incubated with cholesterol oxidase ± catalase for 60 minutes at 37 ^{0}C.

Nile red was used as a sensitive fluorescent stain for the detection of cytoplasmic lipid droplets (383). Cells were exposed to the various compounds for 3 hours at 37 ^{0}C. The fluorescence intensity of cells increased significantly after treatment with LDL pre-incubated with cholesterol oxidase, whereas the fluorescence intensity of cells treated with cholesterol oxidase alone, or LDL alone, or LDL and cholesterol oxidase but without pre-incubation was not significantly changed (Fig 40).

The effect on the cells of different pre-incubation times of cholesterol oxidase with LDL was investigated (Fig 41). After LDL underwent 2 or more hours of pre-incubation with cholesterol oxidase and was then incubated with the cultured cells, the fluorescence intensity of the treated cells was significantly increased. This increase in nile red fluorescence intensity indicates an elevated lipid content in the cells. This observation is corroborated by biochemical measurements of cellular cholesterol content after treatment with cholesterol oxidase. LDL (0.1 ml) and cholesterol oxidase (0.1 mg/ml) pre-incubated for 3 hours and then applied to the cells for 5 hours resulted in a significant increase in the cholesterol content in the cells (control: 39.52 ± 2.61 nmol/mg; treated: 55.18 ± 3.71 nmol/mg, $P < 0.05$ ($n=3$)). The cholesterol species detected by this method include the oxidized species 20-α-OH cholesterol and 25-OH cholesterol but do not include 4-cholesten-3-one.

The oxidized cholesterol species in the cells were identified by HPLC after treatment of the cells with LDL pre-incubated with cholesterol oxidase (Fig 42). The non-oxidized cholesterol content gradually decreased as the pre-incubation time increased whereas the % content of oxidized cholesterol species in the cells increased. The species of oxidized cholesterol identified were 4-cholesten-3-one, 20-α-OH cholesterol and 25-OH cholesterol. The predominant oxidized cholesterol species present was 20 α-OH cholesterol.

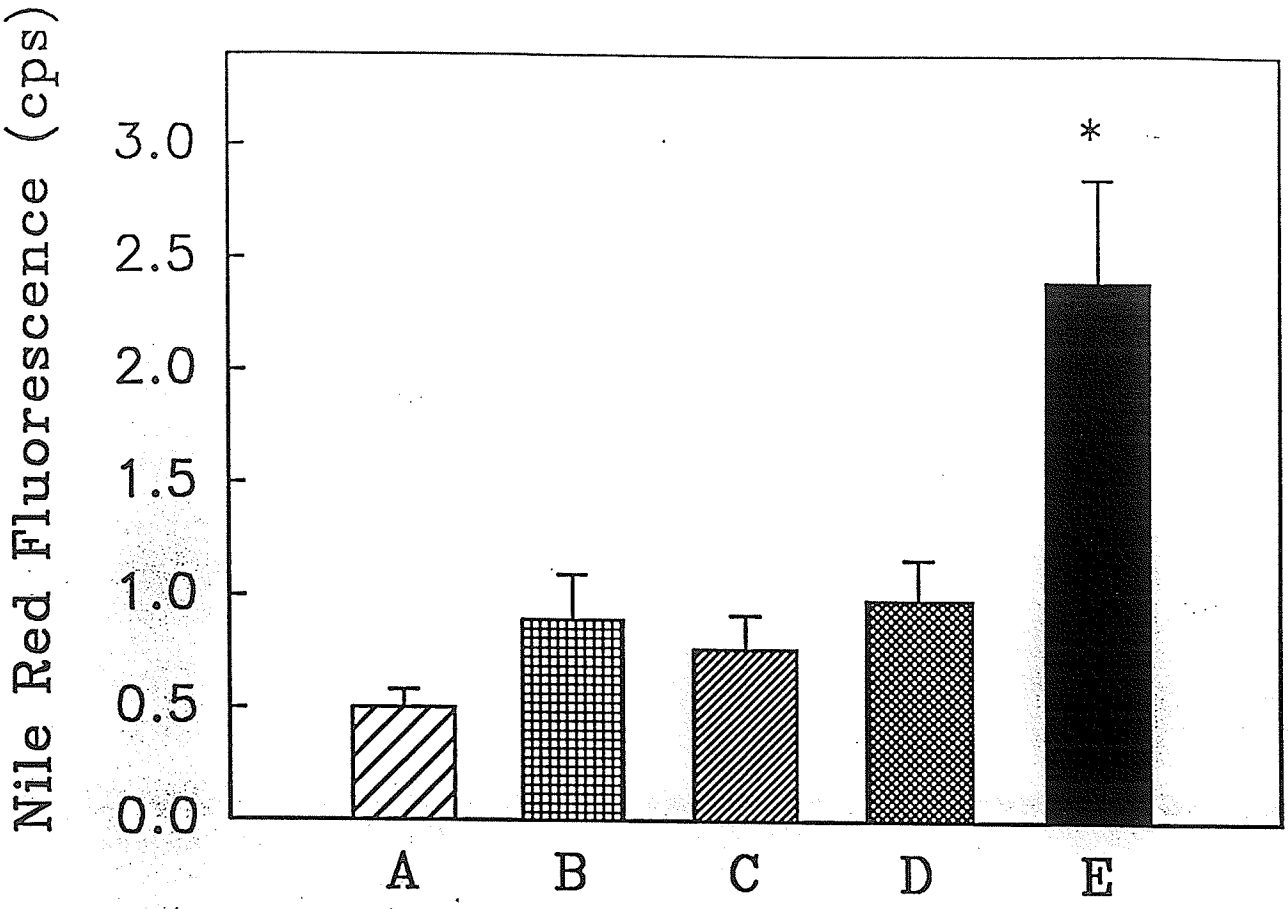


Figure 40. Quantitation of cellular nile red fluorescence after 3 hours treatment at 37 ^{0}C with LDL and/or cholesterol oxidase. A: control; B: cholesterol oxidase (0.1 mg/ml). C. LDL (0.1 ml); D. LDL (0.1 ml) and cholesterol oxidase (0.05 mg/ml) without pre-incubation. E. LDL (0.1 ml) and cholesterol oxidase (0.05 mg/ml) after pre-incubation for 24 hours. The nile red fluorescence intensity of cells was significantly increased only after LDL was pre-incubated with cholesterol oxidase. * $p<0.05$ vs untreated cells. (n=5).

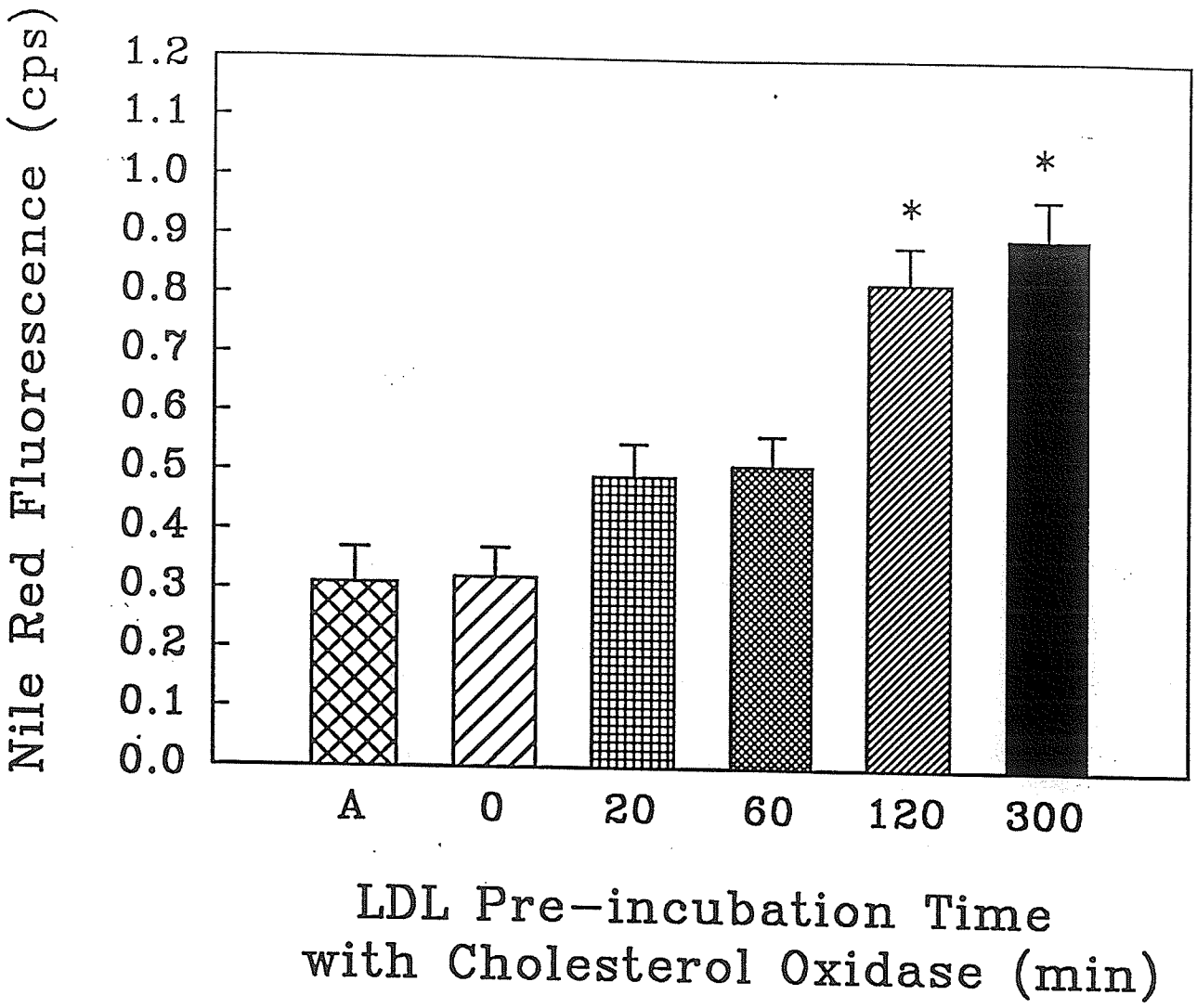


Figure 41. Nile red fluorescence intensity was quantitated after incubation of cells for 5 hours with LDL which had been treated with cholesterol oxidase for varying periods of time. The nile red fluorescence intensity of cells was significantly increased after LDL was pre-incubated with cholesterol oxidase for 120 or more minutes. A: control. * $p<0.05$, ($n=3$).

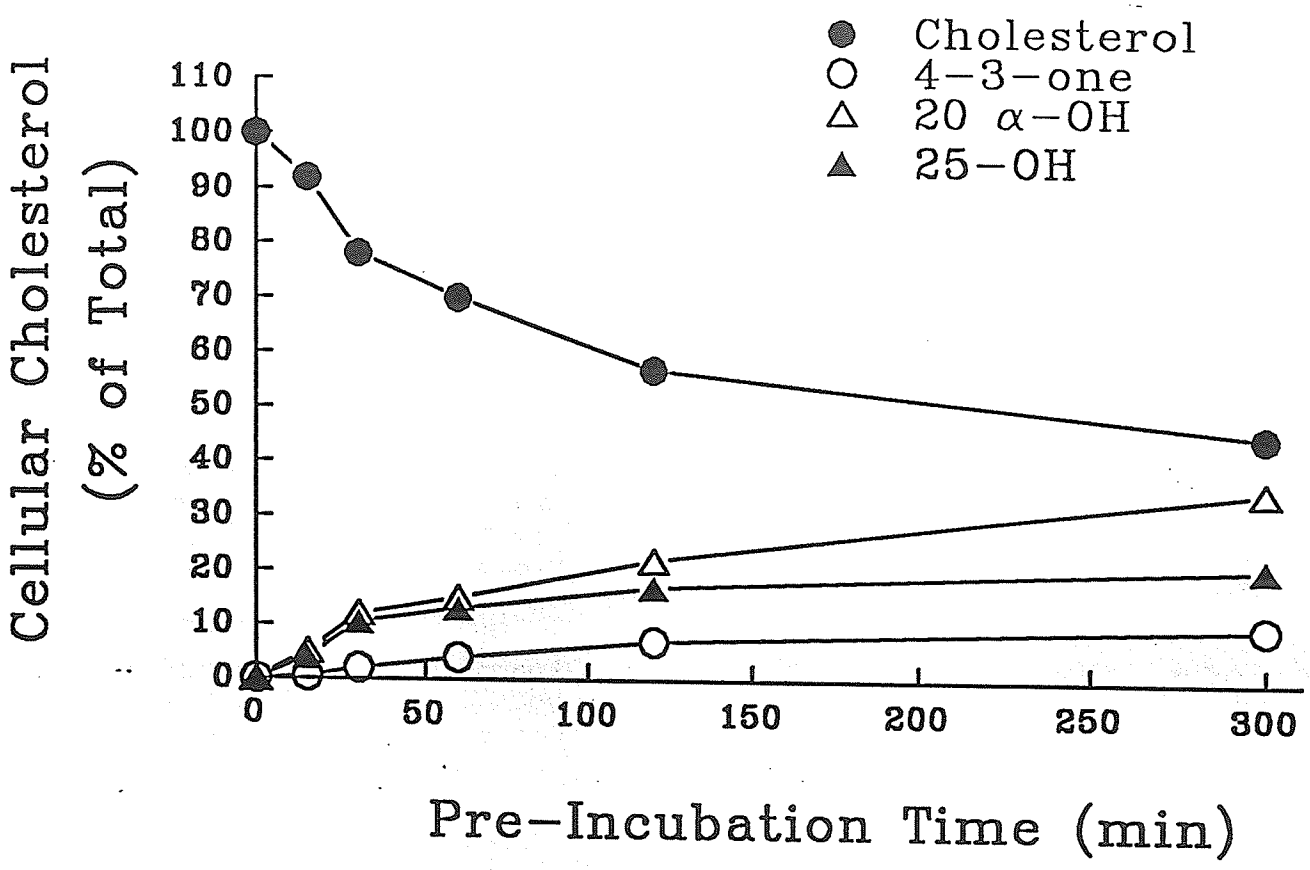


Figure 42. Cellular cholesterol composition as detected by HPLC after treatment with LDL pre-incubated with cholesterol oxidase. LDL (0.1ml) and cholesterol oxidase (0.1mg/ml) were pre-incubated for different times, then the pre-incubated LDL was used to treat the cells for 5 hours.

E. DISCUSSION

The results of my first study demonstrated that LDL has the capacity to alter Ca^{2+} transients in isolated cardiomyocytes. This finding was not dependent upon a defect in the cardiomyocyte. The cardiomyocyte preparation employed was capable of following regular electrical stimulation and maintaining a stable Ca^{2+} transient for over 1 hour. The resting membrane potential of the cells was maintained at -80 mV for over 1 hour under experimental stimulation. The LDL did not induce an elevation in resting [Ca^{2+}] which would have suggested that the membrane integrity of the cell had become compromised. Furthermore, no change in cell shape or morphology could be detected visually upon microscopic evaluation. The relative slowness of the effect ($\geq$ 30 minutes) would also argue against a direct, artifactual modification of the fura signal itself by the LDL. The amplitude of calcium transients are in an well agreement with other studies (range 110 - 400 nM) (366,369,434).

The effects of LDL on the Ca^{2+} transient were probably due to a deposition of cholesterol within the cardiomyocyte. The time dependency of the lipid deposition and the change in Ca^{2+} transient was similar. The length of time required to produce this effect on the transient would argue strongly that the lipid had to be internalized and was not due to a simple, non-specific association of LDL with the cell. However, it is less clear if the reaction occurred via an LDL receptor mediated event. The competitive inhibition of the Dil-LDL interaction with the cell by non-labelled LDL and the lack of an affect by HDL argue in favour of a receptor mediated action. The increase in cellular fluorescence with Dil-LDL at 37°C as opposed to 4°C is consistent with LDL internalization as well (384). Furthermore, the time course of the lipid deposition ($\geq$ 30 minutes) is consistent with lipid

deposition via LDL receptors in other cell types (384). The increase in cellular cholesteryl esters (Table 2) would also suggest LDL internalization took place, however, free cholesterol esterification within the cell may also have produced this result. Conversely, the requirement for such relatively high LDL concentrations to induce both lipid deposition and the changes in the Ca^{2+} transient would suggest that cholesterol may enter the cell via a non-receptor pathway. An LDL concentration of 1 mg cholesterol/ml is far higher than saturating levels for LDL receptors in any other cell type (348,350,352). Furthermore, others have found in preliminary studies that it was difficult to observe active LDL receptors in cardiomyocytes (385).

The effect of LDL on the Ca^{2+} transient was dependent upon extracellular Ca^{2+} (Table 3). This would strongly suggest that LDL is inducing an increase in the intracellular Ca^{2+} transient by stimulating transsarcolemmal Ca^{2+} influx. The pathway that LDL may be affecting is unclear but both Na^{+}/Ca^{2+} exchange and the Ca^{2+} channel (two pathways involved in Ca^{2+} entry in the myocardium (386,387)) are sensitive to alterations in sarcolemmal cholesterol content (258,318). Although the drug interactions with the cardiomyocyte suggest that LDL may be influencing specific ion transport pathways, we do not believe these data are interpretable in this manner in the present study. LDL induced a generalized subsensitivity to all of the drugs tested. It was surprising to us that both an antagonist (nicardipine) and an agonist (BAYK 8644) of the same pathway (Ca^{2+} channel) would exhibit depressed responses after LDL treatment. It is likely that cholesterol deposition in the membrane may be making it more difficult for the drug to exert its effect on Ca^{2+} movements. Experimental evidence has shown that drugs may interact with the membrane lipid domain prior to binding to specific receptors (388). It has been suggested that cholesterol deposition may reduce the fluidity characteristics of the membrane and

make it more difficult for drugs to reach their receptor sites (389). Our data are consistent with this hypothesis.

The findings of the present study have significant pathophysiological implications. The concentration of LDL cholesterol in serum from normal and hypercholesterolemic patients is in the range of < 1 mg/ml and > 1 mg/ml, respectively (69). The LDL concentration in the interstitial space which is in contact with cardiomyocytes is about 50% of the plasma concentration (323). Under atherosclerotic conditions when the endothelial barrier may be compromised (390), one would expect this percentage to be far higher than 50% (391). Thus, the LDL concentration (1 mg/ml) which was shown to induce an increase in the cardiomyocyte Ca^{2+} transient may not have physiological relevance but is well within the range expected in hypercholesterolemic patients. Out data would also suggest that lower concentrations than 1 mg/ml may induce changes in the Ca^{2+} transient under more chronic incubation conditions than one hour. The data, therefore, may partially explain the alterations in cardiac contractile performance observed under conditions of high circulating cholesterol or atherosclerosis (392-395). The present results also suggest the potential for hypercholesterolemic patients to possess a general insensitivity to drug therapy.

The second part of my study demonstrates that oxLDL ($\leq$200 μg/ml) can increase the peak systolic [Ca^{2+}] without affecting the diastolic [Ca^{2+}] in isolated cardiomyocytes. Three confounding factors should be addressed before discussing these results. First, it is possible that the integrity of the cells was compromised during isolation which may have predisposed them to the effects of oxLDL. However, several observations would argue against this. The cells appeared viable, undamaged and functionally intact based upon their rod-like shape and regular rhythmic contraction which followed the electrical stimulation for > 60 minutes. Furthermore, the resting membrane potential of the cells was maintained at -

80 mV for over 60 minutes under our experimental conditions (292). The cells in the present study were employed for much less than 60 minutes. Therefore, the cells did not appear to be compromised prior to oxLDL exposure. Secondly, at low concentrations (100 μg/ml), oxLDL did not induce an elevation in resting [Ca^{2+}] which would have suggested that the membrane integrity of the cell had become compromised during the treatment of the cells. Finally, the possibility also exists that the effects on the transient were not produced by the oxLDL but instead caused by the free radicals which accompanied the oxLDL when it was applied to the cells. However, the concentration of DHF present with 100 μg oxLDL/ml (0.3 mM) did not produce any alteration in the Ca^{2+} transient on its own. Thus, the alteration in the Ca^{2+} transient was a result of an action by the oxLDL.

The mechanism by which oxLDL altered the Ca^{2+} transient was investigated. The effect of oxLDL on the Ca^{2+} transient was dependent upon extracellular Ca^{2+}. This would strongly imply that oxLDL has the ability to produce an increment in the intracellular Ca^{2+} transient by stimulating transsarcolemmal Ca^{2+} influx. Opening of the Ca^{2+} channel is primarily responsible for Ca^{2+} entry in the myocardium (387). The cardiomyocytes treated with oxLDL were more sensitive to the L-type Ca^{2+} channel antagonist nicardipine than control cells. This would suggest that oxLDL may be altering the Ca^{2+} channel characteristics in some way. This interpretation agrees with the work of Josephson and colleagues who recently reported that oxidant-induced calcium loading in cardiac myocytes was due to an increased Ca^{2+} influx through the voltage-gated Ca^{2+} channels (396).

The free radical generating system in our study is capable of producing several species of free radicals, such as superoxide anions, hydrogen peroxide and hydroxyl radicals (373,397). These free radicals interact with LDL and cause rapid peroxidation in vitro and in vivo (88,97,398). MDA content is a relatively good indicator of lipid peroxidation (399,400).

During the process of lipid peroxidation in LDL, a variety of highly reactive aldehyde products (including MDA) are generated, which, in turn, can form covalent bonds with protein, and oxidize cholesterol (88,401). Our results demonstrate a good correlation between the MDA content in the oxLDL and the increment of systolic [Ca^{2+}] in the treated cells. The observation that lazaroid effectively protected the LDL from lipid peroxidation and also protected against the change in Ca^{2+} transients in the treated cells would further support the conclusion that an interaction of the lipid peroxidation products in the oxLDL with the cardiomyocyte may play an important role in modifying the Ca^{2+} transient. It is unclear at present precisely how it does this. Two possibilities exist. Receptor mediated endocytosis of oxLDL has been reported in other cell types (352,402,403). However, using a fluorescently labelled oxLDL probe (dil-oxLDL), we could not detect any evidence of oxLDL receptors in cardiomyocytes (data not shown). An alternative transport process would involve passive, concentration dependent diffusion of the lipid from the LDL into the cell (72). Oxidized lipids are known to enter cells at a much faster rate than native lipid species (404,405). In this regard, the oxLDL produced an effect on the Ca^{2+} transient in cardiomyocytes at a much faster rate (~2 fold) and at a much lower concentration (~10 fold) than native LDL (292).

The findings of the current study have significant pathological implications. Iron-dependent lipid peroxidation is thought to play a central role in in vivo situations where oxygen radical mediated tissue damage is important (406). Free radical induced cardiac dysfunction and damage may be important in ischemia/reperfusion or hypoxia/reoxygenation conditions (324,325). Previous studies with these experimental models (324,396,407,408) have used buffer perfused hearts to examine the effects of free radicals. Our work suggests that these previous studies may have underestimated the effects of free radicals by excluding

lipids from the circulating buffer system. Our work shows LDL potentiates the effects of oxygen derived free radicals. There were no alterations in the Ca^{2+} transient after the cardiomyocyte was exposed to a free radical generating system containing 0.3 mM DHF. However, in the presence of LDL, 0.3 mM DHF treatment produced significant increment in both systolic and diastolic [Ca^{2+}]. Therefore, our results suggest that oxLDL may play a role in the alteration of cardiac contractile performance during ischemia/reperfusion and hypoxia/ reoxygenation conditions, particularly in patients with higher serum LDL cholesterol concentration. Our work has relevance to the in vivo state. The concentration of LDL cholesterol in the interstitial space which is in contact with cardiomyocytes is $\leq$ 500 μg/ml or > 500 μg/ml in normal subjects and hypercholesterolemic patients, respectively (69,323). This is well in excess of what was required to produce an effect on the cardiomyocyte in the present study. LDL has also been previously demonstrated to be oxidized in the subendothelial space (106,320,326,327). Thus, these data are consistent with the possibility that LDL can be oxidized and cardiomyocytes may be in contact with this oxLDL. In addition, the protective effect of lazaroid against both lipid peroxidation by free radicals and changes in [Ca^{2+}] in the oxLDL treated cells, may also have an important clinical implication.

The third component of my study demonstrated that LDL oxidized by C.O. ($\leq$100 μg/ml) could increase the peak systolic [Ca^{2+}] without affecting the diastolic [Ca^{2+}] in isolated cardiomyocytes. It is possible that the effects on the Ca^{2+} transient were not produced by the oxLDL but were instead due to the C.O. which accompanied the oxLDL when it was applied to the cells. However, the amount of C.O. present with 0.1 mg oxLDL/ml (0.1 mg/ml) did not generate any change in the Ca^{2+} transient on its own (data was not shown). Similarly, it is also possible that H_2O_2, a by-product of the effects of C.O.

on cholesterol, may also have a direct effect on the Ca^{2+} transient. Since 1 mol of H_2O_2 can be generated per mol of cholesterol oxidized, oxidation of all cholesterol in 0.1 mg cholesterol/ml LDL moiety will yield 0.26 μmol H_2O_2 or an $[H_2O_2]$ of 0.26 mM. A 0.1 mM $[H_2O_2]$ did not produce alterations in Ca^{2+} transients during the $\leq$ 20 minutes time course of the experiments in the present study. Furthermore, generation of 0.26 mM H_2O_2 is dependent upon all cholesterol becoming oxidized which is unlikely at C.O. concentrations $\leq$ 1.0 mg/ml (332). Therefore, the $[H_2O_2]$ generated by the C.O. action on LDL in the absence of catalase would more likely be much lower than 0.26 mM. Thus, the change in the cellular Ca^{2+} transient was a result of an action exerted directly by the oxLDL.

The treatment of LDL by C.O. alone can produce an oxidation of cholesterol in LDL and the generation of H_2O_2, which in turn can oxidize lipids in the LDL moiety (332,381). By including catalase in the incubation of LDL with C.O., we can negate the effects of H_2O_2 and separately define the roles of cholesterol oxidation and fatty acyl chain peroxidation in the effects of oxLDL on Ca^{2+} transients. Several lines of evidence suggest that it is fatty acyl chain peroxidation rather than oxidized cholesterol in the LDL moiety which plays the primary role in the oxLDL-induced alteration of Ca^{2+} transients. First, a good correlation was observed between the MDA content in the oxLDL and the increase of systolic $[Ca^{2+}]$ in the treated cells. MDA is an indicator of lipid peroxidation (400,401). Secondly, catalase effectively protected LDL from lipid peroxidation and also protected against the change in Ca^{2+} transients in the treated cells. This would further support the conclusion that an interaction of the lipid peroxidation products in the oxLDL with the cardiomyocyte may play an important role in modifying the Ca^{2+} transient. The protective effect of catalase against the MDA production in the oxLDL was more efficient than that of lazaroid and vitamin E. This would suggest that LDL peroxidation was primarily induced by H_2O_2 since catalase is

a more efficient at inactivating H_2O_2 (409,410). Lazaroid and vitamin E are good general free radical scavengers (102,123,133,379). Third, H_2O_2 treatment of LDL induced the same increase in systolic [Ca^{2+}] in the cell that C.O. treatment of LDL (- catalase) did (Fig. 23D). Together, these data strongly suggest that H_2O_2 induced fatty acyl chain peroxidation in the LDL and it was these products which caused the change in systolic Ca^{2+} levels. Conversely, when LDL cholesterol was selectively oxidized by C.O. in the presence of catalase, no change in systolic [Ca^{2+}] was observed. This clearly rules out oxidized LDL cholesterol as a mechanistic factor.

The effects of C.O. treated LDL ± catalase on the cell [Ca^{2+}] were very different than the effects of C.O. or H_2O_2 on cellular Ca^{2+} in the absence of LDL. Exposure of cells to C.O. without LDL produced a decrease in excitability, an increase in diastolic [Ca^{2+}] and eventually cell contracture. Systolic [Ca^{2+}] was not increased as was the case when cells were exposed to oxLDL. It is very likely that oxidation of cell membrane cholesterol by C.O. produced nonspecific membrane damage resulting in a leakage of Ca^{2+} into the cell. HPLC data showed the generation of 4-cholesten-3-one in the membrane with a corresponding decrement of membrane cholesterol after the cardiomyocytes were treated with C.O. In isolated cardiac sarcolemmal membranes, oxidation of membrane cholesterol resulted in significant changes in Ca^{2+} flux and an increase in passive Ca^{2+} permeability characteristics (282). It is possible as well that cholesterol oxidation by C.O. may have increased cell Ca^{2+} via a direct inhibition of sarcolemmal ion transport protein activities (282). Catalase, the specific chelator of H_2O_2, did not provide any protection against the effects of C.O. on cell contracture.

At higher concentrations, H_2O_2 also has its own effect on the intracellular diastolic Ca^{2+} level and can cause cardiomyocyte contracture. Our results agree with other studies

that this effect of H_2O_2 is time and concentration dependent (396,411). There are several mechanisms for the increase in the diastolic [Ca^{2+}] caused by H_2O_2. These include: 1) an inhibition of sarcolemmal Na^+-K^+ ATPase, resulting in intracellular Ca^{2+} overload via Na^+-Ca^{2+} exchange (363); 2) nonspecific membrane damage caused by sarcolemmal lipid peroxidation which renders the sarcolemma leaky to Ca^{2+} (412); 3) H_2O_2 might depress the sarcoplasmic reticulum Ca^{2+} pump function which may lead to Ca^{2+} overload in the treated cardiomyocytes; 4) accelerated calcium influx through voltage gated calcium channels (294,396).

The findings of the current study have significant pathological implications for the heart. Cardiac dysfunction and damage produced by oxygen derived free radicals may be important in ischemia/reperfusion or hypoxia/reoxygenation conditions (324,333). Previous work has demonstrated that LDL can potentiate the effects on Ca^{2+} transients of oxygen derived free radicals on their own (294). Lipid peroxidation is thought to play a critical role in in vivo situations where oxygen radical mediated tissue damage is important (88,330,399). Our study suggests the fatty acyl groups found in LDL phospholipids, triglycerides and cholesteryl esters are the most important component in oxLDL for stimulating the Ca^{2+} transient of isolated cardiomyocytes. Oxidation of LDL cholesterol does not account for the effect on the Ca^{2+} transient produced by oxLDL. Protection against LDL peroxidation by antioxidants like lazaroid, vitamin E and especially catalase may have important clinical applications. Clearly, in view of the differences we observed in the effects of H_2O_2 on cell function in the absence or presence of LDL, the present data demonstrate the importance of studying free radical effects in a medium which contains circulating lipids.

Oxidatively modified LDL has attracted much attention because of a potentially important role in the atherosclerosis process (430-433). In vitro oxidation of LDL results

in an increased content of oxidized forms of cholesterol, derivatization of lysine amino groups, decreased content of polyunsaturated fatty acids and an increased negative charge and density (88,99). Unfortunately, no study to date has identified which of the oxidation sites within the LDL moiety is most affected in patients suffering from coronary artery disease. In the forth part of my study, the most striking change in the oxidative profile of the LDL in coronary disease patients was in the cholesterol component. Two oxidized LDL cholesterol species, 4-cholesten-3-one and 20 α-OH cholesterol, were present in both control and coronary disease patients. Other studies have identified 7 α-OH cholesterol, 7-ketocholesterol (413) and 26-OH cholesterol (414) in plasma from healthy individuals. Thus, the present study represents the first identification of 4-cholesten-3-one and 20 α-OH cholesterol in plasma from coronary disease patients. Our HPLC method for separating the oxidized cholesterol species is ideal to detect these species, particularly 4-cholesten-3-one (361). This species was not resolved from the solvent peak using previous methods. The presence of oxidized cholesterol in the sample is unlikely to be a result of oxidative modification of the sample during isolation. Antioxidant agents like EDTA and propyl gallate were included throughout the lipoprotein isolation, during dialysis and storage to prevent lipoprotein autooxidation (415). Thus, the results are unlikely to be generated during isolation and processing and instead reflect an accurate in situ condition.

Our results revealed that very low levels of MDA were present in LDL and VLDL from both coronary heart patients and healthy control subjects. The low MDA level indicates that little lipid peroxidation of circulating lipoprotein occurs in vivo. Although some controversy exists concerning the specificity of the TBARS-MDA methodology used in the present study, the assay remains a useful tool in monitoring relative lipid peroxidation events in vitro (88,132,399). The in vitro free radical generating system used here was Fe^{3+}

-ADP-DHF. Auto-oxidation of DHF produces large steady state levels of superoxide anions which may then form additional active oxygen radicals capable of inducing lipid peroxidation (363,397). In the presence of Fe^{3+} -ADP with DHF, an active oxygen radical (i.e., hydroxyl) can be produced which further promotes the peroxidative reaction in lipoprotein (397,416). When LDL was incubated with this free radical generating system, MDA production from coronary heart disease patients was significantly higher than control (Figure 26). This suggests that LDL from coronary heart patients was more vulnerable to peroxidative modification by free radicals. Our results are qualitatively similar to the work of Harats et al (132) who examined peroxidation of LDL in cigarette smoking subjects. They demonstrated that cigarette smoking renders plasma LDL more susceptible to subsequent in vitro peroxidative modification by a free radical generating system despite similar low levels of LDL peroxidation in native plasma or LDL of smokers and nonsmokers (132). Smoking by the subjects in either group was unlikely to be a confounding factor in our study. Only 3 of the patients and 2 of the control subjects were cigarette smokers. They ranged from 0.25-1.0 packs/day. The remaining subjects in this study had never smoked or had quit smoking for at least three years prior to the study.

LDL contains polyunsaturated fatty acids that can be converted to reactive aldehydes and other oxidized lipids which then react with the protein in LDL (88,99). Oxidation of the LDL apoprotein has been detected previously by demonstrating an alteration in the lysine reactivity (104,364). In its native state, LDL lysine reactivity was not different between the groups indicating that apoprotein oxidation was similar between the groups. This observation is consistent with the LDL lipid peroxidation data which demonstrated no significant difference between groups with respect to native LDL peroxidation. The greater reactivity of the patients' VLDL lysine to oxidative modification by $CuSO_4$ is difficult to

explain, particularly since it was the only change observed in the VLDL fraction.

It is possible that the larger percentage of women in the control group may have affected the results. In examining the data from the women in comparison to the total data, we could find no outlying data that would suggest to us that the women in either group were skewing the data one way or another. Thus, we do not believe that the smaller proportion of women in the patient group independently accounted for any of the observed differences. However, the small sample size for the women in this study limits the relevance of the findings to the female population.

The results may have pathological significance. Oxidized LDL cholesterol has been demonstrated to have important effects on lipid deposition and morphology of cultured vascular smooth muscle cells (353). In addition, generation of the 4-cholesten-3-one species of oxidized cholesterol in cell membranes has been shown to result in significant changes in ion movements (282). LDL which has had its lipid peroxidized, for example by monocytes (343), smooth muscle cells (97) or endothelial cells (405), has a greater capacity to enter the macrophage and induce foam cell formation (88,99). Therefore, the higher content of oxidized cholesterol in LDL and the greater sensitivity of the LDL to peroxidative challenge in patients with coronary artery disease may provide an explanation for the accelerated atherosclerotic changes in the vasculature of these patients. Our data suggest that the subendothelial space may not be the only location where lipoprotein oxidation is evident. Our results are consistent with a role for oxidized LDL in the coronary disease process in humans.

Much attention has been given recently to the oxidatively modified derivatives of cholesterol with regard to their potential in the atherosclerosis process. In the fifth part of my study, cholesterol oxidase, an enzyme which catalyzes the oxidation of cholesterol to

4-cholesten-3-one and other oxidized cholesterol derivatives, was used to treat cultured rabbit aortic smooth muscle cells. The results demonstrated that cholesterol oxidase altered lipid deposition in the cell and changed cell morphology.

The cell viability was not changed after moderate treatment with cholesterol oxidase. This conclusion is based upon several observations. First, the treated cells excluded trypan blue but cells treated with other agents known to compromise cell viability demonstrated intense staining. Secondly, DASPMI, a mitochondrial marker which was used as a fluorescent indicator of cell viability, demonstrated that cholesterol oxidase treatment did not alter mitochondrial energetics whereas other agents like DNP and ethanol did affect the cellular energy status. Further, cells were monitored visually on a daily basis for up to 1 week after cholesterol oxidase treatment. The affected cells (as in Fig 28B) did not exhibit further morphological changes nor were they released from the culture dish surface. Together, these observations would strongly suggest that in spite of a significant accumulation of oxidized cholesterol, morphological changes and elevated lipid deposition, cell viability was maintained.

This conclusion would appear to be in contrast to earlier results of Peng and coworkers. Peng et al (335,336) demonstrated that smooth muscle cells were necrotic after adding 200 ug/ml of cholesterol autooxidation products to the culture medium for 24 hours. The dead cells were small, round and stained darkly. Ultrastructurally, the cells were totally devoid of intracellular organelles and were replaced by granular structures, whirled membranes, and dilated vacuoles (335,336). In order to compare the effect of cholesterol oxidase and oxidized cholesterols in cultured cells, we repeated Peng's experimental protocol. Our experiments using 4-cholesten-3-one treatment of cells demonstrated similar results to those of Peng et al (335,336). The reason for the difference in results between

adding 4-cholesten-3-one to the medium and exposing the cells to cholesterol oxidase, therefore, appears to be the method of introducing oxidized cholesterol to the smooth muscle cells.

The effect of cholesterol oxidase on smooth muscle cells was dependent upon the fetal calf serum which was present in the culture medium. If fetal calf serum was absent, cholesterol oxidase had no effect. Thus, the effects of cholesterol oxidase were not due to an action on the cell membrane itself but due to some factor in the fetal calf serum. Many factors in the serum could be responsible for the effects, but low density lipoprotein is a major carrier of cholesterol in the blood. Therefore, we have hypothesized that the most likely factor of importance is low density lipoprotein. Further work with isolated LDL fractions has confirmed this hypothesis (see the sixth part of thesis). The oxidized LDL may be interacting with the cell via a specific receptor pathway (417,418). Conversely, when Peng and colleagues (335,336) examined the effect of oxidized cholesterol on cells by adding oxidized cholesterol directly to the incubation medium, it is highly improbable that it was interacting with the cell through this receptor pathway. Instead, it may have been absorbing to the membrane or inserting itself in a non specific manner into the membrane to elicit local disordering effects. It is difficult from the present data to conclude what receptor the modified LDL may be interacting with. It may be a "scavenger receptor" or, because only the cholesterol and not the apoprotein was being oxidized, the modified LDL may be acting through the conventional LDL receptor.

The present results may have pathological significance in the atherogenic process. Foam cells occur in early atherosclerotic lesions (fatty streaks), as well as in mature plaques. The foam cells are derived from either intimal smooth muscle cells or from wandering blood monocytes (419). The cells treated with cholesterol oxidase exhibit a

similar morphological appearance and excessive lipid deposition as foam cells. Fowler and colleagues used electron microscopy to study ultrastructural features of arterial smooth muscle cells in varying stages of atherosclerotic lesions and demonstrated varying degrees of lipid accumulation (419). Foam cells are enriched in cholesterol and lysosomal enzymes (419,420). Foam cells are not necrotic . Our data would suggest that cholesterol oxidation is not necessarily toxic to smooth muscle cells. Instead, these cells can accumulate lipids and transform in a manner similar to that observed in atherosclerotic lesions. Oxidized cholesterol, therefore, may play an important role in atherogenesis and lipid deposition.

Considerable experimental evidence now implicates oxidatively modified LDL as a highly atherogenic factor (88,326). The site of oxidation within the LDL is currently under investigation. The sixth part of this study has focused on the oxidative modification of cholesterol in LDL and the effect of these modified lipoprotein particles on cultured vascular smooth muscle cells. The results demonstrate that oxidized cholesterol is not present in isolated, native LDL fractions from rabbit and this LDL does not affect smooth muscle cells when incubated with these cells under our experimental conditions. However, cholesterol-oxidized LDL has the potential to influence intracellular lipid deposition and induce morphological changes of vascular smooth muscle cells.

Hydrogen peroxide is a by-product of cholesterol oxidation by cholesterol oxidase (381). The possibility exists, therefore, that the effects observed were due to the presence of H_2O_2 and not due to the cholesterol oxidation. However, catalase was included in the medium to scavenge the H_2O_2 whenever cholesterol oxidase was used in the present experiments. We have calculated that even if all the LDL cholesterol were oxidized, the amount of H_2O_2 produced would still be three-fold less than the catalase activity present which would be required to scavenge it completely. The results from MDA determination of lipoprotein

peroxidation (Fig. 39) are consistent with our hypothesis that H_2O_2 produced by the cholesterol oxidase reaction has the ability to peroxidize the lipoprotein. However, in the presence of catalase, the peroxidative effect of H_2O_2 on lipoprotein was completely prevented. Therefore, the results reflect an effect of cholesterol oxidation on LDL and not an action of hydrogen peroxide.

Biochemical assays, HPLC analysis and the fluorescence data all suggest that total cellular lipid content is elevated after the cell is exposed to LDL which contains oxidized cholesterol. This is likely to be as a result of accelerated deposition of the different cholesterol species from the LDL into the cell. However, it is curious that the non-oxidized cholesterol content in the treated cells actually decreased not increased (Figure 42). This is consistent with previous results (353). The reason that non-oxidized cholesterol decreased remains unclear. Several possibilities exist. First, endogenous cellular cholesterol synthesis may be inhibited by exposure to oxidized cholesterol. Previous data support this possibility (419). Secondly, the free cholesterol on the cell surface may be oxidized by the cholesterol oxidase (282,421); therefore, cholesterol content may correspondingly decrease. Third, the cholesterol oxidase enzyme may enter the cell and oxidize in situ cholesterol. This is unlikely unless the membrane is very leaky (421). Fourth, cholesterol oxidation may proceed from H_2O_2 production or via self propagation. There is no evidence currently for the latter possibility and, as discussed above, it is highly unlikely that hydrogen peroxide had any effect on the cells. Thus, the decrease in cellular unoxidized cholesterol is probably due to a combination of inhibition of cholesterol synthesis by the entry of oxidized LDL cholesterol and some oxidation of surface membrane cholesterol at the cell surface. This oxidation of membrane cholesterol, however, is not sufficient to alter membrane permeability and cell viability (353,421).

The present results confirm and extend earlier observations (353) on the effects of cholesterol oxidase on smooth muscle cells. The effects of the enzyme are not due to a direct action on the cell. The observation (Figure 40) that pre-incubation of LDL with cholesterol oxidase was necessary to elicit changes clearly demonstrates that cholesterol oxidation of LDL, not the cells, was central for the effect. It also confirms the hypothesis (353) that the effects of cholesterol oxidase which were dependent upon the medium surrounding the cells was likely due to LDL particles in the medium. However, it does not rule out the possibility that other factors in the serum may also play a role. The present finding that incubation of cells in the presence of cholesterol oxidase and serum did not elicit significant changes in nile red fluorescence (Fig. 40B) would appear to be in contrast to our previous results (353). However, the cholesterol oxidase concentration selected in the present study (0.1 mg/ml) is below the range required to elicit significant changes (353).

The physiological significance of oxidized cholesterol is unclear. Several possibilities exist. Cholesterol oxidase is known to be present in liver where it is involved in the metabolism of cholesterol (422). Also, the enzyme is found in some bacteria in the colon (421,422). The possibility exists that the enzyme could leak from the liver or transfer through the colon from bacteria to the blood where it could modify LDL in vivo. Alternatively, oxidized cholesterols which are present in various foods (423) may also be absorbed through our diet and enter the bloodstream. It is also possible that cholesterol oxidation may proceed in vivo by free radical mechanisms (101,424,425). In pathological conditions which result in a stimulation of free radical production (ischemia, inflammation),cholesterol oxidation may occur (426).

Since our results demonstrated that oxidation of cholesterol in LDL can alter lipid deposition and cell morphology in a manner similar to that commonly exhibited in

atherosclerotic plaques (353,419), the present results may have implications in atherogenesis. Free radical scavenging systems (vitamin E, butylated hydroxytoluene, probucol) have been shown to prevent the formation of oxidized LDL and inhibit atherogenesis (422,427). The present study supports the hypothesis (88,353) that one of the important sites of oxidation within the LDL molecule may be cholesterol.

REFERENCES

1. Report of the Working Group of Arteriosclerosis of the National Heart, Lung, and Blood Institute (1981) Vol.2. Washington DC Government Printing Office (DHEW publication no. (NIH) 82-2035)

2. Long, E.R. (1933) The development of our knowledge of arteriosclerosis. In: Cowdry, E.V. ed. Arteriosclerosis: a survey of the problem. New York: The Macmillan Co., 19-52

3. Herrick, J.B. (1912) Clinical features of sudden obstruction of the coronary arteries. J. Am.Med. Assoc. 59, 2015-20

4. McGill Jr, H.C. (1988) The pathogenesis of atherosclerosis. Clin. Chem. 34, B33-B39

5. McGill Jr, H.C. (1984) Persistent problems in the pathogenesis of atherosclerosis. Arteriosclerosis. 4: 443-451

6. Evnas, G.R. and Taylor, K.G. (1988) The paediatric origins of atherosclerosis. Br. J. Hosp. Med. 39(2): 132-137

7. Kissane, J.M. (1990) Anderson's Pathology. Ninth Edition. Vol.1. (The C.V. Mosby Company, 757-766)

8. Haust, M.D. (1978) Atherosclerosis in childhood. in Rosenberg, H.S., and Bolande, R.P., editors: Perspectives in Paediatric Pathology, vol. 4, Chicago. Year Book Medical Publishers.

9. Stary, H.C. (1987) Macrophages, macrophage foam cells, and eccentric intimal thickening in the coronary arteries of young children. Atherosclerosis 64, 91-108

10. Geer, J.C., McGill Jr, H.C., Robertson, W.B. and Strong, J.P. (1968) Histologic characteristics of coronary artery fatty streaks. Lab. Invest. 18, 565-70

11. Stary, H.C. (1987) Evolution and progression of atherosclerosis in the coronary arteries of children and adults. In: Bates SR, Gangloff EC, eds. Atherogenesis and aging. New York: Springer-Verlag. 20-36

12. Geer, J.C. and Haust, M.D. (1972) Smooth muscle cells in atherosclerosis: monograph on atherosclerosis, vol. 2, S. Karger A.G.

13. Gerrity, R.G. (1981) The role of monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am. J. Pathol. 103, 181-90

14. Mitchell, J.R.A. and Schwartz, C.J. (1965) Arterial Disease. Blackwell Scientific Publications, Oxford. 411, XV

15. Schwartz, C.J., Kelley, J.L., Nerem, R.M., Sprague, E.A., Rozek, M.M., Valente, A.J., Edwards, E.H., Prasad, A.R.S., Kerbacher, J.J. and Logan, S.A. (1989) Pathophysiology of the atherogenic process. Am. J. Cardiol. 64, 23G-30G

16. Hessler, J.R., Morel, D.W., Lewis, L.J. and Chisolm, G.M. (1983) Lipoprotein oxidation and lipoprotein-induced cytotoxicity. Arteriosclerosis. 3, 215-22

17. Glomset, J.A. (1968) The plasma lecithin: Cholesterol acyltransferase reaction. J. Lipid Res. 9, 155-61

18. Fielding, P.E., Fielding, C.J., Havel, R.J., Kane, J.P. and Tun, P. (1983) Cholesterol net transport, esterification, and transfer in human hyperlipidemic plasma. J. Clin. Invest. 71,449-60

19. Wissler, R.W. (1984) Principles of the pathogenesis of atherosclerosis. In Braunwald, E., editor: Heart disease: a textbook of cardiovascular medicine, Philadelphia, W.B. Saunders Co.

20. Haust, M.D. (1978) Light and electron microscopy of human atherosclerotic lesions. Adv. Exp. Med. Biol. 104, 33-59

21. Majno, G., Joris, I. and Zand, T. (1985) Atherosclerosis: new horizon. Hum. Pathol. 16, 3-5

22. Munro, J.M. and Cotran, R.S. (1988) Biology of diseases: the pathogenesis of atherosclerosis: atherogenesis and inflammation. Lab. Invest. 58, 249-261

23. Murata, K., Motayama, T. and Kotake, C. (1986) Collagen types in various layers of the human aorta and their changes with the atherosclerotic process. Atherosclerosis 60, 251-62

24. Kramsch, D.M., Franzblau, C. and Hollander, W. (1971) The protein and lipid composition of arterial elastin and its relationship to lipid accumulation in the atherosclerotic plaque. J. Clin. Invest. 50, 1666-77

25. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. and Hansson, G.K. (1986) Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 6, 131-38

26. Robertson, W.B., Geer, J.C., Strong, J.P., McGill Jr, H.C. (1963) The fate of the fatty streak. Exp. Mol. Pathol. (Suppl 1), 28-39

27. Katz, S.S. (1981) The lipids of grossly normal human aortic intima from birth to old age. J. Biol. chem. 256, 12275-80

28. Montenegro, M.R. and Eggen, D.A. (1968) Topography of atherosclerosis in the coronary arteries. Lab. Invest. 18, 586-93

29. Virchow, R. (1856) Der atheromatose prozess der arterien. Wien. Med. Wochenschr. 6, 825-831

30. Ross, R. and Glomset, J.A. (1976) II. The pathogenesis of atherosclerosis. N. Engl. J. Med. 295, 420-25

31. Ross, R. (1986) The pathogenesis of atherosclerosis-an update. N. Engl. J. Med. 314, 488-500

32. Benditt, E.P. (1978) The monoclonal theory of atherosclerosis. In Paoletti, R. and gotto, A.M., Jr., editors: Atherosclerosis reviews, vol. 3, New York, Raven Press.

33. Schwartz, C.J., Valente, A.J., Sprague, E.A., Kelley, J.L. and Nerem, R.M. (1991) The pathogenesis of atherosclerosis: an overview. Clin. Cardiol. 14, I-1-16

34. Getz, G.S. (1990) The involvement of lipoproteins in atherosclerosis: evolving concepts. Ann.N.Y.Acad.Sci. 598, 17-28

35. Parthasarathy, S., Steinberg, D. and Witztum, J.L. (1992) The role of oxidized low density lipoproteins in the pathogenesis of atherosclerosis. Annu. Rev. Med. 43, 219-25

36. Steinberg, D. and Witztum, J.L. (1990) Lipoproteins and atherogenesis: current concepts. JAMA 264, 3047-3052

37. Poole, J.C.F. and Florey, H.W. (1958) Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits. J. Pathol. Bacterial. 75, 245-251

38. Davies, P.F., Reidy, M.A., Goode, T.B. and Bowyer, D.E. (1976) Scanning electron microscopy in the evaluation of endothelial integrity of the fatty lesion in atherosclerosis. Atherosclerosis. 25, 125-130

39. Fowler, S., Shio, H. and Haley, N.J. (1979) Characterization of lipid-laden aortic cells from cholesterol-fed rabbits, IV: investigation of macrophage-like properties of aortic cell populations. Lab. Invest. 41, 372-378

40. Gerrity, R.G., Natio, H.K., Richardson, M. and Schwartz, C.J. (1979) Dietary induced atherogenesis in swine. Am. J. Pathol. 95, 775-792

41. Agel, N.M., Ball, R.Y., Waldman, H. and Mitchinson, M.J. (1984) Monocytic origin of foam cells in human atherosclerotic plaques. Atherosclerosis. 53, 265-301

42. Glueck, C.J. (1986) Paediatric primary prevention of atherosclerosis. N. Engl. J. Med. 314, 175-177.

43. Malinow, M.R. (1981) Regression of atherosclerosis in humans: fact or myth?

Circulation 64, 1-3

44. Blankenhorn, D.H. (1978) Progression and regression of femoral atherosclerosis in man. In Atherosclerosis Reviews 3 (edited by Paoletti, R, Gotto, A.M.). Raven Press, New York. pp. 169-181

45. Pooling Project Research Group (1978): Relationship of blood pressure, serum cholesterol, smoking habit, relative weight and ECG abnormalities to incidence of major coronary events: Final report of pooling project. J. Chron. Dis. 31,201-11

46. Strong, J.P., Restrepo, C. and Guzman, M. (1978) Coronary and aortic atherosclerosis in New Orleans. II. Comparison of lesions by age, sex, and race. Lab. Invest. 39, 364-69

47. Dawber, T.R. (1980) The Framingham study: the epidemiology of atherosclerotic disease, Cambridge, Mass., Harvard University Press.

48. McGill Jr. H.C. (1979) Sex and atherosclerosis (Review). Atherosclerosis Rev. 4, 157-242

49. McGill, Jr H.C. and Stern, M.P. (1977) Sex and atherosclerosis. In Paoletti, R. and Gotto, A.M., Jr., editors: Atherosclerosis reviews, vol. 2, New York, Raven Press

50. Gotto, Jr. A.M. (1979) Status report: plasma lipids, lipoproteins, and coronary artery disease. In Paoletti, R. and Gotto, A.M. Jr., editors: Atherosclerosis reviews, vol. 4, New York, Raven Press

51. Wilken, DEL, Reddy Sri, G. and Gupta, V.S. (1983) Homocysteinaemia, ischaemic heart disease, and the carrier state for homocystinuria. Metabolism. 32, 363-370

52. Oalmann, M.C., Malcom, G.T., Toca, V.T., Guzman, M.A. and Strong, J.P. (1981) Community pathology of atherosclerosis and coronary heart disease: post-mortem serum cholesterol and extent of coronary atherosclerosis. Am. J. Epidemiol. 113, 396-403

53. Rose, G. and Shipley, M. (1986) Plasma cholesterol concentration and death from coronary heart disease: 10 years results of the Whitehall study. Brit. Med. J. 293, 306-307

54. International Collaborative Study Group (1986) Metabolic epidemiology of plasma cholesterol: mechanisms of variation of plasma cholesterol within populations and between populations. Lancet ii, 91-95

55. Freedman, D.S., Scrinvasan, S.R., Shear, C.L., Franklin, F.A., webber, L.S. and Berenson, G.S. (1986) The relation of apolipoproteins A-I and B in children to parental myocardial infarction. N. Eng. J. Med. 315, 721-726

56. Hulley, S.B., Rosenman, R.H., Bawol, R.D. and Brand, R.J. (1980) Epidemiology as a guide to clinical decisions. The association between triglyceride and coronary heart disease [Review]. N. Engl. J. Med. 302, 1383-89

57. Lipppel, K., Tyroler, H., Eder, H., Gotto, Jr. A. and Vahouny, G. (1981) Relationship of hypertriglyceridemia to atherosclerosis. Arteriosclerosis. 1, 406-17

58. Solberg, L.A. and Strong, J.P. (1983) Risk factors and atherosclerotic lesions. A review of autopsy studies [Review]/ Arteriosclerosis 3, 187-98

59. McGill Jr, H.C., McMahan, C.A., Kruski, A.W. and Mott, G.E. (1981) Relationship of lipoprotein cholesterol concentrations to experimental atherosclerosis in baboons. Arteriosclerosis 1, 3-12

60. Reichl, D. and Miller, N.E. (1986) The anatomy and physiology of reverse cholesterol esterol transport. Clin. Sci. 70, 221-31

61. Roberston, W.B. and Strong, J.P. (1968) Atherosclerosis in persons with hypertension and diabetes mellitus. Lab. Invest. 18, 538-51

62. Cowell, J.A., Franz, M.J. and Ginsberg, H. et al. (1989) Consensus statement, American Diabetes Association. Role of cardiovascular risk factors in the prevention and treatment of macrovascular disease in diabetics. Diabetes Care 12, 573-9

63. Colwell, J.A., Lopes-Virella, M.L. and Halushka, P.V. (1981) Pathogenesis of atherosclerosis in diabetes mellitus. Diabetes Care 4, 121-3

64. Lewis, B. (1984) Risk factors for coronary heart disease-assessment in airline pilots. European Heart J. 5, (suppl A)17-24

65. McGill Jr. H.C. (1988) The cardiovascular pathology of smoking. Am. Heart J. 115, 250-57

66. McGill Jr. H.C. (1979) Potential mechanisms for the augmentation of atherosclerosis and atherosclerotic disease by cigarette smoking [Review]. Prev. Med. 8, 390-403

67. Bierman, E.L. (1983) Atherosclerosis and other forms of arteriosclerosis. In Harrison's Principles of Internal Medicine, 10th edition edited by Petersdorf, R.G., Adams, R.D., Braunwald, E., Isselbacher, K.J., Martin, J.B., Wilson, J.D.). Raven Press, New York. pp. 169-181

68. Roheim, P.S. (1986) Atherosclerosis and lipoprotein metabolism: role of reverse cholesterol transport. Am. J. Cardiol. 57, 3C-10C

69. Grundy, S.M. (1990) Cholesterol and atherosclerosis. Diagnosis and treatment. Editor: Gabello, W.J. Copyright by Gower Medical Publishing, 101 Fifth Ave, New York, NY. 10003

70. Golmset, J.A. (1968) The plasma lecithin:cholesterol acyltransferase reaction. J.Lipid Res. 9, 155-167

71. Fredrickson, D.S., Gotto, A.M. and Levy, R.I. (1972) Familial lipoprotein deficiency. In the metabolic basis of inherited disease. J.B. Stanbury, J.B. Wyngaarden, and D.S. Fredrickson, editor. McGraw-Hill New York, NY. 493-530

72. Brown, M.S. and Goldstein, J.L. (1983) Lipoprotein receptors in the liver control signals for plasma cholesterol traffic. J. Clin. Invest. 72, 743-747

73. Myant, N.B. (1990) Cholesterol metabolism, LDL and the LDL receptor. San Diego: Academic Press; 99-111

74. Brown, M.S., Kovanen, P.T. and Goldstein, J.L. (1981) Regulation of plasma cholesterol by lipoprotein receptors. Science 212, 628-635

75. Lipid Research Clinics Program (194): The Lipid Research Clinics Coronary Primary Prevention Trial Results II. J. Am. Med. Assoc. 251, 365-374

76. Wissler, R.W. and Wesselinovitch, D. (1968) Experimental models of human atherosclerosis. Ann. N.Y. Acad. Sci. 149, 907-922

77. Smith, E.B. & Slater, R.S. (1973) In Atherogenesis: Initiating Factors (Ciba Foundation Symposium No. 12). 39. Elsevier Publishing Co. Amsterdam.

78. Mahley, R.W. (1982) Atherogenic hyperlipoproteinemia. The cellular and molecular biology of plasma lipoproteins altered by dietary fat and cholesterol. Med. Clin. North Am. 66, 375-402

79. Koo, C., Wernette-Hammond, M.E. and Innerarity, T.L. (1986) Uptake of canine beta-very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. J. Biol. Chem. 261, 11194-201

80. Territo, M.C., Berliner, J.A., Almada, L., Ramirez, R. and Fogelman, A.M. (1989) Beta-very low density lipoprotein pretreatment of endothelial monolayer increases monocyte adhesion. Arteriosclerosis 9, 824-8

81. Alderson, L.M., Endemann, G. Lindsey, S., Pronczuk, A., Hoover, R.L. and Hayes, K.C. (1986) LDL enhances monocyte adhesion to endothelial cells in vitro. Am. J. Pathol. 123, 334-42

82. Navab, M., Hough, G.P., Berliner, J.A., Frank, J.A., Fogelman, A.M., Haberland, M.E. and Edwards, P.A. (1986) Rabbit beta-migrating very low density lipoprotein increase endothelial macromolecular transport without altering electrical resistance. J. Clin. Invest. 78, 389-97

83. Gofman, J.W., Lindgren, F., Elliot, H., Mantz, W., Hewitt, J. Strisower, B., Herring,

V. and Lyon, T.P. (1950) Role of lipids and lipoproteins in atherosclerosis. Science 111, 166-171

84. Havel, R.J.N., Yamada, N. and Shames, D.M. (1989) Watanabe heritable hyperlipidemic rabbit. Animal model of familial hypercholesterolemia. Arteriosclerosis 9, 33-38

85. Checovich, W.J., Fitch, W.L., Krauss, R.M., Smith, M.P., Rapacz, J., Smith, C.L. and Attie, A.D. (1988) Defective catabolism and abnormal composition of low density lipoproteins from mutant pigs with hypercholesterolemia. Biochemistry 27, 1934-41

86. Rosenfeld, M.E., Tsukada, T., Gown, A.M. and Ross, R. (1987) Fatty streak initiation in Watanabe heritable hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 7, 9-23

87. Krauss, R.M., Williams, P.T., Brensike, J., Detre, K.M., Lindgren, F.T., Kelsey, S.F., Vranizan, K. and Levy, R.I. (1987) Intermediate-density lipoproteins and progression of coronary artery disease in hypercholesterolemic men. Lancet 2, 62-66

88. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. N. Engl. J. Med. 320, 915-24

89. Gerrity, R.G. (1981) The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am. J. Pathol. 103, 181-90

90. Agel, N.M., Ball, R.Y., Waldman, H. and Mitchinson, M.J. (1984) Monocytic origin of foam cells in human atherosclerotic plaques. Atherosclerosis 53, 265-271

91. Buja, L.M., Kita, T., Goldtein, J.L., Watanabe, Y. and Brown, M.S. (1983) Cellular pathology of progressive atherosclerosis in the WHHL rabbit: an animal model of familial hypercholesterolemia. Arteriosclerosis 3, 87-101

92. Goldstein, J.L. and Brown, M.S. (1977) The low density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 46, 897-930

93. Yla-Herttuala, S., Rosenfeld, M.E., Parthasrathy, S., Sigal, E. and Sarkioja, T. (1991) Gene expression in macrophage-rich human atherosclerotic lesions: 15-lipoxygenase and acetyl LDL receptor mRNA colocalize with oxidation-specific lipid-protein adducts. J. Clin. Invest. 87, 1146-52

94. Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980) The scavenger cell pathway for lipoprotein degradation: specificity of the binging site that mediates the uptake of negatively charged LDL by macrophages. J. Supramol. Struct. 13, 67-81

95. Brown, M.S. and Goldstein, J.L. (1983) Lipoprotein metabolism in the macrophage:

implications for cholesterol deposition in atherosclerosis. Ann. Rev. Biochem. 52, 223-61

96. Henriksen, T., Mahoney, E.M. and Steinberg, D. (1983) Enhanced macrophage degradation of biologically modified low density lipoprotein. Arteriosclerosis 3, 149-159

97. Heinecke, J.W., baker, L., Rosen, L. and Chait, A. (1986) Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. J. Clin. Invest. 77, 757-61

98. Steinbrecher, U.P. (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. Biochim. Biophys. Acta 959, 20-30

99. Steinbrecher, U.P., Zhang, H. and Lougheed, M. (1990) Role of oxidatively modified LDL in atherosclerosis. Free Rad. Biol. Med. 9, 155-168

100. Hiramatsu, K., Rosen, H., Heinecke, J.W., Wolfbauer, G. and Chait, A. (1987) Superoxide initiate oxidation of low density lipoprotein by human monocytes. Arteriosclerosis 7, 55-60

101. Morel, D.W., Hessler, J.R. and Chisolm, G.M. (1983) Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J. Lipid Res. 24, 1070-76

102. Morel, D.W., DiCorleto, P.E. and Chisolm, G.M. (1984) Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis 4, 357-64

103. Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. U.S.A. 83, 3883-7

104. Steinbrecher, U.P., Witztum, J.L., Parthasarathy, S. and Steinberg, D. (1987) Decreased in reactive amino groups during oxidation or endothelial cell modification of LDL: correlation with changes in receptor-mediated catabolism. Arteriosclerosis 1,135-43

105. Yla-herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L. and Steinberg, D. (1989) Evidence of the presence of oxidatively modified LDL in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84, 1086-95

106. Haberland, M.E., Fong, D. and Cheng, L. (1988) Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. Science 241, 215-8

107. Haberland, M.E., Fong, D. and Cheng, L. (1990) Malondialdehyde, modified

lipoproteins, and atherosclerosis. European Heart J. 11 suppl E, 100-4

108. Avogaro, P., Bon, G.B. and Cazzolato, G. (1988) Presence of a modified low density lipoprotein in humans. Arteriosclerosis 8, 79-87

109. Salmon, S., Maziere, C., Theron, L., Beucler, I., Ayrault-Jarrier, M., Goldstein, S. and Polonovski, J. (1987) Immunological detection of low-density lipoproteins modified by malondialdehyde in vitro or in vivo. Biochim. Biophys. Acta 920, 215-220

110. Palinski, W., Rosenfeld, M.E., Yla-herttuala, S., Gurtner, G.C., Socher, S.S., Butler, S., Parthasarathy, S., Carew, T.E., Steinberg, D. and Witztum, J.L. (1989) Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. U.S.A. 86, 1372-76

111. Steinbrecher, U.P. (1991) Role of lipoprotein peroxidation in the pathogenesis of atherosclerosis. Clin. Cardiol. 14, 865-7

112. Aviram, M. (1993) Modified forms of low density lipoprotein and atherosclerosis. Atherosclerosis 98, 1-9

113. Gey, K.F. and Puska, P. (1989) Plasma vitamins E and A inversely correlated to mortality from ischemic heart disease in cross-cultural epidemiology. Ann. N.Y. Acad. Sci. 570, 268-82

114. Jessup, W., Rankin, S.M., de Whalley, C.V., Hoult, J.R.S., Scott, J. and Leake, D.S. (1990) α-tocopherol consumption during LDL oxidation. Biochem. J. 265, 399-405

115. Parthasarathy, S., Young, S.G., Witztum, J.L., Pittman, R.C. and Steinberg, D. (1986) Probucol inhibits oxidative modifications of LDL. J. Clin. Invest. 77, 641-44

116. De Whalley, C.V., Rankin, S.M., Hoult, J.R.S., Jessup, W. and Leake, D.S. (1990) Flavonoids inhibit the oxidative modification of LDL by macrophages. Biochem. Pharmacol. 39, 1743-50

117. Negre-Salvayre, A. and Salvayre, R. (1992) Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cell lines. Free Rad. Biol. Med. 12, 101-6

118. Stocker, R., Bowry, V.W. and Frei, B. (1991) Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol. Proc. Natl. Acad. Sci. USA 88, 1646-50

119. Barnhart, J.W., Sefranka, J.A., and McIntosh, D.D. (1970) Hypocholesterolemic effect of 4,4'-(isopropylidenedithio)bis(2,6-di-tert-butylpheno) (probucol). Am J. Clin. Nutr. 23, 1229-33

120. Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H. and Kawai, C. (1987) Probucol prevents the progression of atherosclerosis in Watanabe

heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc. Natl. Acad. Sci. USA 84, 5928-31

121. Carew, T.E., Schwenke, D.C. and Steinberg, D. (1987) Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc. Natl. Acad. Sci. USA 84, 7725-29

122. Kuzuya, M. and Kuzuya, F. (1993) Probucol as an antioxidant and antiatherogenic drug. Free Radic. Biol. Med. 14, 67-77

123. Van Hinsbergh, V.W.M., Van Scheffer, M., Havekes, L. and Kempen, H.J.M. (1986) Role of endothelial cells and their products in the modification of low density lipoproteins. Biochim. Biophys. Acta 878,49-64

124. Cathcart, M.K., Morel, D.W. and Chisolm, G.M. (1985) Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. J. Leukocyte Biol. 38,341-50

125. Verlangieri, A.J. and Bush, M. (1990) Prevention and regression of primate atherosclerosis by d-alpha-tocopherol. Free Rad. Biol. Med. 9(Suppl. 1), 73

126. Liu, S.K., Dolensek, E.P. and Tappe, J.P. (1984) Cardiomyopathy associated with vitamin E deficiency in seven gelada baboons. J. Am. Vet. Med. Assoc. 185, 1347-50

127. Nelson, J.S. (1980) Pathology of vitamin E deficiency. In: Machlin, L.J., ed. Vitamin E, a comprehensive treaties. New York: Dekker, 397-428

128. Frei, B., England, L. and Ames, B.N. (1989) Ascorbate is an outstanding antioxidant in human blood plasma. Proc. Natl. Acad. Sci. USA 86, 6377-81

129. Stocker, R., Glazer, A.N. and Ames, B.N. (1987) Antioxidant activity of albumin-bound bilirubin. Proc. Natl. Acad. Sci. USA 84, 5918-22

130. Negre-Salvayre, A., Alomar, Y., Troly, M. and Salvayre, R. (1991) Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. III. the protective effect of antioxidants (probucol, catechin, vitamin E) against the cytotoxicity of oxidized LDL occurs in two different ways. Biochim. Biophys. Acta 1096, 291-300

131. Sparrow, C.P., Doebber, T.W., Olszewski, J., Wu, M.S., Ventre, J., Stevens, K.A. and Chao, Y.S. (1992) Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N'-Diphenyl-Phenylenediamine. J. Clin. Invest. 89, 1885-91

132. Harats, D., Ben-Naim, M., Dabach, Y., Hollander, G., Stein, O. and Stein, Y. (1989) Cigarette smoking renders LDL susceptible to peroxidative modification and

enhanced metabolism by macrophages. Atherosclerosis 79, 245-52

133. Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic. Biol. Med. 13, 341-90

134. Duax, W.L., Griffin, J.F., Rohere, D.C., and Weeks, C.M. (1980) Conformational analysis of sterols: comparison of X-ray crystallographic observations with data from other sources. Lipids 15, 783-792

135. Small, D.M., Bourges, M. and Dervichian, D.G. (1966) The biophysics of lipidic associations. I. The ternary systems: Lecithin-bile salt-water. Biochim. Biophys. Acta 125, 563-80

136. Rand, R.P. and Luzatti, V. (1968) X-ray diffracting study in water of lipids extracted from human erythrocytes: the position of cholesterol in the lipid lamellal. Biophys. J. 8, 125-37

137. Chapman, D. (1982) in: Biological Membranes, Vol.4, Academic Press, New York.

138. Lange, Y. (1986) Membrane Cholesterol. in Biochemistry and Biology of Plasma Lipoproteins. edited by Scanu, A.M. and Spector, A.A. Marcel Dekker, INC. New York.

139. Yeagle, P.L. (1989) Lipid regulation of cell membrane structure and function. FASEB J. 3, 1833-42

140. Lange, Y. and Ramos, B. (1983) Analysis of the distribution of cholesterol in the intact cell. J. Biol. Chem. 258, 15130-4

141. Lange, Y., Schmit, V.M. and Schreiber, J.R. (1988) Localization and movement of newly synthesized cholesterol in rat ovarian granulosa cells. Endocrinology 123, 81-6

142. Green, C. (1977) in Biochemistry of Lipid II (Goodwin, T.W., ed.), pp. 101-152, University Park Press, Baltimore

143. Miller, R.G. (1984) The use and abuse of filipin to localize cholesterol in membranes. Cell Biol. Int. Rep. 8, 519-35

144. Chapelle, S. and Gilles-Baillien, M. (1983) Phospholipids and cholesterol in brush border and basolateral membranes from rat intestinal mucosa. Biochim. Biophys. Acta 753, 269-71

145. Luciano, L, Reale, E., Konitz, H., Boseck, U. and Boseck, S. (1989) Alignment of cholesterol in the membrane bilayer. J. Histochem. Cytochem. 37, 1421-25

146. Luciano, L., Konitz, H. and Reale, E. (1989) Localization of cholesterol in the

colonic epithelium of the guinea pig: regional differences and functional implications. Cell Tissue Res. 258, 339-47

147. Sommer, J.R., Dolber, P.C. and Taylor, I. (1982) Fillipin-sterol complexes in the membranes of cardiac muscle. J. Ultras. Res. 80, 98-103

148. Severs, N.J. (1981) Plasma membrane cholesterol in myocardial muscle and capillary endothelial cells. Distribution of filipin-induced deformations in freeze-fracture. Europ. J. Cell Biol. 25, 289-99

149. Alroy, J., Merk, F.B., Goyal, V. and Ucci, A. (1981) Heterogeneous distribution of filipin-sterol complexes in nuclear membranes. Biochim. Biophys. Acta 649, 239-43

150. Severs, N.J. (1982) Cholesterol distribution and structural differentiation in the sarcoplasmic reticulum of rat cardiac muscle cells. Cell Tissue Res. 224,613-24

151. Fisher, K.A. (1976) Analysis of membrane halves: cholesterol. Proc. Natl. Acad. Sic. U.S.A. 73, 173-7

152. Lange, Y., Culter, H.B. and Steck, T.L. (1980) The effect of cholesterol and other intercalated amphipaths on the contour and stability of the isolated red cell membrane. J. Biol. Chem. 255, 9331-7

153. Lange, Y. and Slayton, J.M. (1982) Interaction of cholesterol and lysophosphatidylcholine in determining red cell shape. J. Lipid. Res. 23, 1121-7

154. Op den Kamp, J.A.F. (1979) Lipid asymmetry in membranes. Annu. Rev. Biochem. 48, 47-71

155. Demel, R.A., Jansen, J.W.C.M., Van Dijick, P.W.M. and Van Deenen, L.L.M. (1977) The preferential interaction of cholesterol with different classes of phospholipids. Biochim. Biophys. Acta 465, 1-10

156. Slotter, J.P. and Bierman, E.L. (1988) Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts. Biochem. J. 250, 653-8.

157. Brown, M.S. Kovanen, P.T. and Goldstein, J.L. (1981) Regulation of plasma cholesterol by lipoprotein receptors. Science 212, 628-635

158. Attie, A.D., Pittman, R.C. and Steinberg, D. (1982) Hepatic catabolism of low density lipoprotein: mechanisms and metabolic consequences. Hepatology 2, 269-81

159. Lange, Y. and Matthies, H.J.G. (1984) Transfer of cholesterol from its site of synthesis to the plasma membrane. J. Biol. Chem. 259, 14624-30

160. Kaplan, M.R. and Simoni, R.D. (1985) Transport of cholesterol from the endoplasmic

reticulum to the plasma membrane. J. Cell Biol. 101, 446-53

161. DeGrella, R.F. and Simoni, R.D. (1982) Intracellular transport of cholesterol to the plasma membrane. J. Biol. Chem. 257, 14256-62

162. Huang, C.H. (1977) A structural model for the cholesterol-phosphatidylcholine complexes in bilayer membranes. Lipids 12, 348-56

163. Lange, Y., Echevarria, F. and Steck, T.L. (1991) Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblasts. J. Biol. Chem. 266, 21439-43

164. McLean, L.R. & Phillips, M.C. (1981) Mechanism of cholesterol and phosphatidylcholine exchange or transfer between unilamellar vesicles. Biochemistry 20, 2893-900

165. Cooper, R.A., Arner, E.C., Wiley, J.S. & Shattil, S.J. (1975) Modification of red cell membrane structure by cholesterol-rich lipid dispersions. A model for the primary spur cell defect. J. Clin. Invest. 55, 115-126

166. Bell, F.P. (1975) Cholesterol exchange between microsomal, mitochondrial and erythrocyte membranes and its enhancement by cytosol. Biochim. Biophys. Acta 398, 18-27

167. Gavey, K.L., Noland, B.J. & Scallen, T.J. (1981) The participation of sterol carrier protein2 in the conversion of cholesterol to cholesterol ester by rat liver microsomes. J. Biol. Chem. 256, 2993-2999

168. Dempsey, M.E., McCoy, K.E., Baker, H.N., Vafiadou-Dimitriadou, A., Lorsbach, T. and Howard, J.B. (1981) Large scale purification and structural characterization of squalene and sterol carrier protein. J. Biol. Chem. 256, 1867-73

169. Tiltaman, A.J. (1984) Ligandin in the human ovary. J. Pathol. 142, 61-6

170. Ishibashi, T. and Bloch, K. (1981) Intermembrane transfer of 5 alpha-cholest-7-en-3 beta-ol. Facilitation by supernatant protein (SCP). J. Biol. Chem. 256, 12962-67

171. Trzaskos, J.M. and Gaylor, J.L. (1983) Cytosolic modulators of activities of microsomal enzymes of cholesterol biosynthesis. Purification and characterization of a non-specific lipid-transfer protein. Biochim. Biophys. Acta 751, 52-65

172. Chanderbhan, R., Noland, B.J., Scallen, T.J. and Vahouny, G.V. (1982) Sterol carrier protein2. Delivery of cholesterol from adrenal lipid droplets to mitochondria for pregenolone synthesis. J. Biol. Chem. 257, 8928-34

173. Conneely, O.M., Headon, D.R., Olson, C.D., Ungar, F. and Dempsey, M.E. (1984) Intramitochondrial movement of adrenal sterol carrier protein with cholesterol in response to corticotrophin. Proc. Natl. Acad. Sci. U.S.A. 81, 2970-4

174. Lyons, H.T., Kharroubi, A., Wolins, N., Tenner, S., Chanderbhan, R.F., Fiskum, G. and Donaldson, R.P. (1991) Elevated cholesterol and decreased sterol carrier protein-2 in peroxisomes from AS-30D hepatoma compared to normal rat liver. Arch. Biochem. Biophys. 285, 238-45

175. van Haren, L., Teerds, K.J., Ossendorp, B.C., van Heusden, G.P., Orly, J., Stocco, D.M., Wirtz, K.W. and Rommerts, F.F. (1992) Sterol carrier protein 2 (non-specific lipid transfer protein) is localized in membranous fractions of Leydig cells and Sertoli cells but not in germ cells. Biochim. Biophys. Acta 1124, 288-96

176. Steinman, R.M., Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96, 1-27

177. Voelker, D.R. (1991) Organelle biogenesis and intracellular lipid transport in eukaryotes. Microbiol. Rev. 55, 543-60

178. Slotte, J.P., Oram, J.F. and Bierman, E.L. (1987) Binding of high density lipoproteins to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. J. Biol. Chem. 262, 12904-7

179. Cooper, R.A. (1977) Abnormalities of cell membrane fluidity in the pathogenesis of disease. N. Engl. J. Med. 297, 371-7

180. Ferretti, G., Curatola, G., Bertoli, E., Catassi, C. and Giorgi, P.L. (1991) Erythrocyte membrane fluidity and changes in plasma lipid composition: a possible relationship in childhood obesity. Biochem. Med. Metab. Biol. 46, 1-9

181. Muriana, F.J., Ruiz-Gutierrez, V. and Vazquez, C.M. (1992) Influence of dietary cholesterol on polyunsaturated fatty acid composition, fluidity and membrane-bound enzymes in liver microsomes of rats fed olive and fish oil. Biochimie. 74, 551-6

182. Baldini, P., Sandra, I., Lambert-Gardini, S., Spinedi, A. and Luly, P. (1989) Membrane lipid alterations and Na^+-pumping activity in erythrocytes from IDDM and NIDDM subjects. Diabetes 38, 825-31

183. Pierce, G.N. and Dhalla, N.S. (1983) Sarcolemmal Na^+-K^+ ATPase activity in diabetic rat heart. Am. J. Physiol. C241-C247

184. Pierce, G.N. and Dhalla, N.S. (1985) Heart mitochondrial function in chronic experimental diabetes in rats. Can. J. Cardiol. 1, 48-54

185. Ganguly, P.K., Pierce, G.N., Dhalla, K.S. and Dhalla, N.S. (1983) Defective sarcoplasmic reticular calcium transport in diabetic cardiomyopathy. Am. J. Physiol. 244, E528-E535

186. Shinitzky, M. and Inbar, M. (1976) Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta 433, 133-49

187. Coleman, P.S. and Lavietes, B.B. (1981) Membrane cholesterol, tumorigenesis and the biochemical phenotype of neoplasia. Crit. Rev. Biochem. 11, 341-93

188. Bartoli, G.M., Bartoli, S., Galeotti, T. and Bertoli, E. (1980) Superoxide dismutase content and microsomal lipid composition of tumours with different growth rates. Biochim. Biophys. Acta 620, 205-211

189. Erickson, S.K., Cooper, A.D., Barnard, G.F., Havel, C.M., Watson, J.A., Feingold, K.R., Moser, A.H., Hughes-Fulford, M. and Siperstein, M.D. (1988) Regulation of cholesterol metabolism in a slow-growth hepatoma in vivo. Biochim. Biophys. Acta 960, 131-138

190. Clayman, R.V., Bilhartz, L.E., Buja, L.M., Spady, D.K. and Dietschy, J.M. (1986) Renal cell carcinoma in the Wistar-Lewis rat: A model for studing the mechanisms of cholesterol acquisition by a tumour in vivo. Cancer Res. 46, 2958-2963

191. Vitols, S., Gahrton, G., Bjorkholm, M. and Peterson, C. (1985) Hypocholesterolnemia in malignancy due to elevated low-density-lipoprotein-receptor activity in tumour cells: Evidence from studies in patients with leukaemia. The Lancet Nov. 23, 1150-53

192. Lombardi, P., Norata, G., Maggi, F.M., Canti, G., Franco, P., Nicolin, A. and Catapano, A.L. (1989) Assimilation of LDL by experimental tumours in mice. Biochim. Biophys. Acta 1003, 301-06

193. Altona, J.C. and Laarse, van der A. (1982) Anoxia-induced changes in composition and permeability of sarcolemmal membranes in rat heart cell cultures. Cardiovasc. Res. 16, 138-43

194. Laarse, van der A. (1987) Cholesterol and myocardial membrane function. in: Lipid metabolism in the normoxic and ischaemic heart. (eds. by Stam, H. and Vusse van der G.J.) pp: 137-145 CIP-Kurztitelaufnahme der Deutschen Bibliothek.

195. Rouslin, W., Macgee, J., Gupte, S., Wesselman, A. and Epps, D.E. (1982) Mitochondrial cholesterol content and membrane properties in porcine myocardial ischemia. Am. J. Physiol. 242, H254-H259

196. Venter, H., Genade, S., Mouton, R., Huisamen, B. Harper, I.S. and Lochner, A. (1991) Myocardial membrane cholesterol: effects of ischaemia. J. Mol. Cell. Cardiol. 23, 1271-86

197. Bersohn, M.M., Philipson, K.D. and Fukushima, J.Y. (1982) Sodium-calcium exchange and sarcolemmal enzymes in ischemic rabbit hearts. Am. J. Physiol. 242, C288-C295

198. Ashraf, M. and Halverson, C.A. (1977) Structural changes in the freeze-fractured sarcolemma of ischemic myocardium. Am. J. Pathol. 88, 583-588

199. Owens, K. and Weglicki, W.B., Sonnenblick, E.H. and Gertz, E.W. (1972) Phospholipid and cholesterol content of ventricular tissue from the cardiomyopathic Syrian hamster. J. Mol. Cell. Cardiol. 4, 229-236

200. Szollar, L., Pucsok, J., Szelenyi, I. and Sos, J. (1973) Lipid composition of serum, heart, and liver of hereditary myocardiopathic hamsters (Bio 14.6). In: Bajusz E. and Rona, G. (eds). Recent Advances in Studies on Cardiac Structure and Metabolism. Vol. 2. University Park Press, Baltimore, pp 313-320

201. Slack, B.E., Boegman, R.J. Downie, J.W. and Jasmin, G. (1980) Cardiac membrane cholesterol in dystrophic and verapamil-treated hamsters. J. Mol. Cell. Cardiol. 12, 179-185

202. Kroes, J., Ostwald, M. and Keith, A. (1972) Erythrocyte membrane-compression of lipid phase by increased cholesterol content. Biochim. Biophys. Acta 274, 71-4

203. Gleason, M.M., Medow, M.S. and Tulenko, T.N. (1991) Excess membrane cholesterol alters calcium movements, cytosolic calcium levels, and membrane fluidity in arterial smooth muscle cells. Circ. Res. 69, 216-27

204. Liscum, L., Ruggiero, R.M. and Faust, J.R. (1989) The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-pick type C fibroblasts. J. Cell Biol. 108, 1625-36

205. Brasitus, T.A., Dahiya, R., Dudeja, P.K. and Bissonnette, B.M. (1988) Cholesterol modulates alkaline phosphatase activity of rat intestinal microvillus membranes. J. Biol. Chem. 263, 8592-97

206. Myant, N.B. (1981) The biology of cholesterol and related steroids, in Myant, N.B. (ed): Steroids in Biological Membranes. London, William Heimnemann Medical Books Ltd. pp 161-396

207. Shinitzky, M. and Inbar, M. (1976) Microviscosity parameters and protein mobility in biological membranes. Biochim. Biophys. Acta 433, 133-149

208. Wainberg, R.H., Walden, T.L.Jr., Stebler, B.A. and Yatvin, M.B. (1991) Effect of diet on hyperthermia-induced cell lethality and prostaglandin release. Prostaglandins 41, 501-13

209. Cantin, B., Brun, L.D., Gngne, C., Murthy, M.R., Lupien, P.J. and Julien, P. (1992) Alterations in erythrocyte membrane lipid composition and fluidity in primary lipoprotein lipase deficiency. Biochim. Biophys. Acta 1139, 25-31

210. Schwarz, S.M., Bostwick, H.E. and Medow, M.M. (1988) Estrogen modulates ileal basolateral membrane lipid dynamics and Na^+/K^+-ATPase activity. Am. J. Physiol. 254, G687-G694

211. Davis, R.A., Kern, F., Showalter, R., Sutherland, E., Sinensky, M. and Simon, F.R. (1978) Alterations of hepatic Na^+/K^+-ATPase and bile flow by estrogen: Effects on liver surface membrane lipid structure and function. Proc. Natl. Sci. U.S.A. 75, 4130-4134

212. Incerpi, S., Jefferson, J.R., Wood, W.G., Ball, W.J. and Schroeder, F. (1992) Na pump and plasma membrane structure in L-cell fibroblasts expressing rat liver fatty acid binding protein. Arch. Biochem. Biophys. 298, 35-42

213. Sommer, J.R., Dolber, P.C. and Taylor, I. (1982) Filipin-sterol complexes in the membranes of cardiac muscle. J. Ultrastruct. Res. 80, 98-103

214. Corvera, E., Mouritsen, O.G., Singer, M.A. and Zuckermann, M.J. (1992) The permeability and the effect of acyl-chain length for phospholipid bilayer containing cholesterol: theory and experiment. Biochim. Biophys. Acta 1107, 261-70

215. Yeagle, P.L. (1985) Cholesterol and the cell membrane. Biochim. Biophys. Acta 822, 267-87

216. Yeagle, P.L. (1992) Modulation of membrane function cholesterol. In Cholesterol and Coronary Heart Disease. (edited by: Gold,P., Grover, S. and Roncari, D.A.K.), Parthenon Publishing Group Ltd. Park Ridge, New Jersey.

217. de Gier, J., Mandersloot, J.G. and van Deenen, L.L. Van (1968) Lipid composition and permeability of liposomes. Biochim. Biophys. Acta 150, 666-75

218. Demel, R.A., Bruckdorfer, K.R. and Deenen, L.L.M.v. (1972) The effect of sterol structure on the permeability of liposomes to glucose, glycerol and Rb+. Biochim. Biophys. Acta 255, 321-30

219. Baggetto, L.G., Clottes, E. and Vial, C. (1992) Low mitochondrial proton leak due to high membrane cholesterol content and cytosolic creatine kinase as two features of the deviant bioenergetics of Ehrlich and AS30-D tumour cells. Cancer Res. 52, 4935-41

220. Kroes, J. and Ostwald, R. (1971) Erythrocyte membranes-effect of increased cholesterol content on permeability. Biochim. Biophys. Acta 249, 647-650

221. Bruckdorfer, K.R., Demel, R.A., de Gier, J. and van Deenen, L.L.M. (1969) The effect of partial replacements of membrane cholesterol by other steroids on the osmotic fragility and glycerol permeability of erythrocytes. Biochim. Biophys. Acta 183, 334-45

222. Shaafi, R.I., Gary Bobo, C.M. and Solomon, A.k. (1969) Cholesterol effect on hydraulic conductivity of red cell membranes. Biochim. Biophys. Acta 173, 141-2

223. Imai, Y., Scoble, J.E., McIntyre, N. and Owen, J.S. (1992) Increased Na^+-dependent

D-glucose transport and altered lipid composition in renal cortical brush-border membrane vesicles from bile duct-ligated rats. J. Lipid Res. 33, 473-83

224. Cardenas, A.M., Cortes, M.P., Fernandez, E. and Pena, W. (1992) Lipid peroxidation and loss of potassium from red blood cells produced by phototoxic quinolones. Toxicology 72, 145-51

225. Diomede, L., Bianchi, R., Modest, E.J., Piovani, B., Bubba, F. and Salmona, M. (1992) Modulation of ATPase activity by cholesterol and synthetic ether lipids in leukaemic cells. Biochem. Pharmacol. 43, 803-7

226. Brasitus, T.A., Dahiya, R., Dudeja, P.K. and Bissonnette, B.M. (1988) Cholesterol modulates alkaline phosphatase activity of rat intestinal microvillus membranes. J. Biol. Chem. 263, 8592-7

227. Rotenberg, M. and Zakim, D. (1991) Effects of cholesterol on the function and thermotropic properties of pure UDP-glucuronosyltransferase. J. Biol. Chem. 266, 4159-61

228. Mitchell, D.C., Straume, M., Miller, J.L. and Litman, B.J. (1990) Modulation of metarhodopsin formation by cholesterol-induced ordering of bilayer lipids. Biochemistry 822, 9143-9

229. Meyer, R., Malewicz, B., Baumann, W.J. and Johnson, R.G. (1990) Increased gap junction assembly between cultured cells upon cholesterol supplementation. J. Cell Sci. 96, 231-238

230. Grunze, M., Forst, B. and Deuticke, B. (1980) Dual effect of membrane cholesterol on simple and mediated transport process in human erythrocytes. Biochim. Biophys. Acta 600, 860-8

231. Vrtovsnik, F., el-Yandouzi, E.H., Le-Grimellec, C. and Friedlander, G. (1992) Sphingomyelin and cholesterol modulate sodium coupled uptake in proximal tubular cells. Kindey Int. 41, 983-91

232. Kirby, C.J. and Green, C. (1980) Erythrocyte membrane cholesterol levels and their effects on membrane proteins. Biochim. Biophys. Acta 598, 422-425

233. Peterson, S.W., Angelico, M., Masella, R., Foster, K., Gandin, C. and Cantafora, A. (1992) Altered insulin receptor processing and membrane lipid composition in erythrocytes of cirrhotic patients. Ital. J. Gastroenterol. 24, 65-71

234. Meahara, K. (1991) Membrane cholesterol and insulin receptor in erythrocytes. Fukuoka. Igaku. Zasshi. 82, 586-602

235. Criado, M., Eibl, H. and Barrantes, F.J. (1982) Effects of lipids on acetylcholine receptor. Essential need of cholesterol for maintenance of agonist-induced state

transitions in lipid vesicles. Biochemistry, 21, 3622-3629

236. O'Connor, S.W., Scarpace, P.J. and Abrass, I.B. (1984) The effect of age and cholesterol on the rat lung beta-adrenergic system. Biochim. Biophys. Acta 778, 497-502

237. Whetton, A.D., Gorden, L.M. and Houslay, M.D. (1983) Elevated membrane cholesterol concentrations inhibit glucagon-stimulated adenylate cyclase. Biochem. J. 210, 437-49

238. Rothberg, K.G., Ying, Y.S., Kamen, B.A. and Anderson, R.G. (1990) Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. J. Cell Biol. 111, 2931-8

239. Li, J.L., Li, Y.J., Chao, Y.J., Lin, L.X., Ouyang, M.H. Peng, Y.B. and Chang, W.S. (1991) Cholesterol requirement for growth of IR983F and P3X63-Ag8-U1 meyloma cells in serum-free medium. Cytobios. 68, 15-22

240. Dahl, J.S., Dahl, C.E. and Block, K. (1981) Effect of cholesterol on macro molecular synthesis and fatty acid uptake by Mycoplasma capricolum. J. Biol. Chem. 256, 87-91

241. Miyazaki, M., Bai, L. and Namba, M. (1991) Extending effects of phospholipids, cholesterol and ethanolamines on survival of adult rat hepatocytes in serum-free primary culture. Res. Exp. Med. Berl. 191, 77-83

242. Dahl, J.S. and Dahl, C.E. (1983) Coordinate regulation of unsaturated phospholipid, RNA, and protein synthesis in Mycoplasma capricolum by cholesterol. Proc. Natl. Acad. Sci. U.S.A. 80, 692-696

243. Wallick, E.T., Lane, K.L. and Schwartz, A. (1979) Biochemical mechanism of the sodium pump. Annu. Rev. Physiol. 41, 397-411

244. Vemuri, R. and Philipson, K.D. (1989) Influence of sterols and phospholipids on sarcolemmal and sarcoplasmic reticular cation transporters. J. Biol. Chem. 264, 8680-85

245. Yeagle, P.L., Young, J. and Rice, D. (1988) Effects of cholesterol on (Na^+,K^+)-ATPase ATP hydrolysing activity in bovine kidney. Biochemistry 27, 6449-6452

246. Yeagle, P.L. (1983) Cholesterol modulation of Na^+-K^+ ATPase, ATP hydrolysing activity in the human erythrocyte. Biochim. Biophys. Acta 727, 39-44

247. Lucio, F.J., Hendry, B.M. and Ellory, J.C. (1991) The effects of cholesterol depletion on the sodium pump in human red cells. Exp. Physiol. 76, 437-43

248. Giraud, F., Claret, M., Bruckdorfer, R. and Chailley, B. (1981) The effects of membrane lipid order and cholesterol on the internal and external cationic sites of

the Na^+-K^+ pump in erythrocytes. Biochim. Biophys. Acta 647, 249-258

249. Moffat, M.P. and Dhalla, N.S. (1985) Heart sarcolemmal ATPase and calcium binding activities in rats fed a high cholesterol diet. Can. J. Cardiol. 1, 194-200

250. Sulakhe, P.V., Dhalla, N.S. (1973) Alterations in the activity of cardiac Na^+-K^+ stimulated ATPase in congestive heart failure. Exp. Mol. Pathol. 18, 100-111

251. Slack, B.E., Boegman, R.J. Downie, J.W. and Jasmin, G. (1980) Cardiac membrane cholesterol in dystrophic and verapamil-treated hamsters. J. Mol. Cell. Cardiol. 12, 179-185

252. De Pont, J.H.H.M., Peters, W.H.M. & Bonting, S.L. (1983) Role of cholesterol and other neutral lipids in Na^+-K^+ ATPase. Current Topics in Membranes and Transport 19, 163-166

253. Anner, B.M. (1985) Interaction of (Na^+ + K^+)-ATPase with artificial membranes. I. Formation and structure of (Na^+ + K^+) -ATPase-liposomes. Biochim. Biophys. Acta 822, 319-34

254. Kimelberg, H.K. and Papahadjopoulos, D. (1974) Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^+ + K^+)-stimulated adenosine triphophatase. J. Biol. Chem. 249, 1071-80

255. Uysal, M. (1986) Erythrocyte lipid peroxidation and (Na^+ + K^+) ATPase activity in cholesterol fed rabbits. Int. J. Vitam. Nutr. Res. 56, 307-10

256. Kamber, E. and Kopeikina-Tsiboukidou, L. (1986) Alterations in the activities of rabbit erythrocyte membrane-bound enzymes induced by cholesterol enrichment and depletion procedure. Z. Naturforsch. C. 41, 301-9

257. Baghieri, S. and Garner, M.H. (1992) Na^+-K^+ ATPase and phospholipid degradation in bovine and human lenses. Curr. Eye Res. 11, 459-67

258. Kutryk, M.J.B. and Pierce, G.N. (1988) Stimulation of sodium-calcium exchange by cholesterol incorporation into isolated cardiac sarcolemmal vesicles. J. Biol. Chem. 263, 13167-72

259. Grupp, I., Lee, W.B., Lee, C.O., Pecker, M.S. and Schwartz, A. (1985) Relation of sodium pump inhibition to positive inotropy at low concentrations of ouabain in rat heart muscle. J. Physiol. Lond. 360, 149-160

260. Kutscherskij, E., Gunther, J. and Methley, E. (1983) K-p-Nitrophenylphosphsphatase activity, Na and K content, Na permeability and membrane lipid composition in rabbit myocardium after cholesterol rich diet. Experientia 40, 812-15

261. Field, F.J., Albright, E. and Mathur, S.N. (1986) Effect of dietary cholesterol on

biliary cholesterol content and bile flow. Gastroenterology 91, 297-304

262. Klappauf, E. and Schubert, D. (1977) Band 3-protein from human erythrocyte membranes strongly interacts with cholesterol. FEBS Lett. 80, 423-25

263. Hasselblach, W. and Makinose, M. (1962) ATP and active transport. Biochem. Biophys. Res. Commun. 7, 132-6

264. Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Reconstitution of a calcium pump using defined membrane components. Proc. Natl. Acad. Sci. U.S.A. 71, 622-6

265. Hidalgo, C., Thomas, D.D. and Ikemoto, N. (1978) Effect of the lipid environment on protein motion and enzymatic activity of the sarcoplasmic reticulum calcium ATPase. J. Biol. Chem. 253, 6879-87

266. Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Reversible lipid titrations of the activity of pure adenosine triphosphatase-lipid complexes. Biochemistry 13, 5501-7

267. Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. Nature 255, 684-7

268. Johannson, A., Keithly, C.A., Smith, B.A. and Metcalfe, J.C. (1981) Cholesterol in sarcoplasmmic reticulum and the physiological significance of membrane fluidity. Biochem. J. 196, 505-511

269. Simmonds, A.C., East, J.M., Jones, O.T., Rooney, E.K., McWhirter, J. and Lee, A.G. (1982) Annular and non-annular binding sites on the $(Ca^{2+}+Mg^{2+})$-ATPase. Biochim. Biophys. Acta 693, 398-406

270. Almeida, L.M., Vaz, W.L.C., Zachariasse, K.A. and Madeira, V.M.C. (1984) Modulation of sarcoplasmic reticulum Ca^{2+}-pump activity by membrane fluidity. Biochemistry 23, 4714-20

271. Houslay, M.D. and Stanley, K.K. (1982) in Dynamics of Biological Membranes: influence on synthesis, structure, and function. Publisher: Chichester. Wiley, New York

272. Cheng, K.H., Lepock, J.R., Hui, S.W. and Yeagle, P.L. (1986) The role of cholesterol in the activity of reconstituted Ca^{2+}-ATPase vesicles containing unsaturated phosphatidylethanolamine. J. Biol. Chem. 261, 5081-87

273. Sen, L., Bialecki, R.A., Smith, E., Smith, T.W. and Colucci, W.S. (1992) Cholesterol increases the L-type voltage-sensitive calcium channel current in arterial smooth muscle cells. Circ. Res. 71, 1008-14

274. Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Cholesterol modulates activity of calcium-dependent ATPase of the sarcoplasmic reticulum. Nature 279, 538-541

275. Madden, T.D., King, M.D. and Quinn, P.J. (1981) The modulation of Ca^{2+}-ATPase of sarcoplasmic reticulum by membrane cholesterol. Biochim. Biophys. Acta 641, 265-269

276. Zhou, Q., Jimi, S., Smith, T.L. and Kummerow, F.A. (1991) The effect of cholesterol on the accumulation of intracellular calcium. Biochim. Biophys. Acta 1085, 1-6

277. Ortega, A. and Mas Oliva, J. (1984) Cholesterol effect on enzyme activity of the sarcolemmal ($Ca^{2+}+Mg^{2+}$)-ATPase from cardiac muscle. Biochim. Biophys. Acta 773, 231-36

278. Ortega, A. and Mas Oliva, J. (1986) Direct regulatory effect of cholesterol on the calmodulin stimulated calcium pump of cardiac sarcolemma. Biochim. Biophys. Res. Commun. 139, 868-74

279. Abeywardena, M.Y., McMurchie, E.J., Russell, G.R., Sawyer, W.H. and Charnock, J.S. (1984) Response of rat heart membranes and associated ion-transporting ATPases to dietary lipid. 776, 48-59

280. East, J.M., Jones, O.T., Simmonds, A.C. and Lee, A.G. (1984) Membrane fluidity is not an important physiological regulator of the (Ca^{2+}-Mg^{2+})-dependent ATPase of sarcoplasmic reticulum. 259, 8070-71

281. Jacobson, M.S., Ambudkar, I.S., Young, E.P., Naseem, S.M., Heald, F.P. and Shamoo, A.E. (1985) Cardiac sarcoplasmic reticulum: Effects of an atherogenic diet during the neonatal and juvenile period. Atherosclerosis 55, 81-91

282. Kutryk, M.J.B., Maddaford, T.G., Ramjiawan, B. and Pierce, G.N. (1991) Oxidation of membrane cholesterol alters active and passive transsarcolemmal calcium movement. Circ. Res. 68, 18-26

283. Gunther, J. and Will, H. (1979) Zur Funktion des sarkoplasmatischen Retikulums. Acta Biol. Med. Germ. 38, 1467-72

284. Strittmatter, W.J., Hirata, F. and Axelrod, J. (1979) Increased Ca^{2+}-ATPase activity associated with methylation of phospholipids in human erythrocytes. Biochim. Biophys. Res. Commun. 88, 147-53

285. Triggle, D.J. and Swamy, V.C. (1983) Calcium antagonists. Some chemical-pharmacologic aspects. Circ. Res. 52, 1-17

286. Locher, R., Neyses, L., Stimpel, M., Kuffer, B. and Vetter, W. (1984) The cholesterol content of the human erythrocyte influences calcium influx through the channel. Biochem. Biophys. Res. Commun. 124, 822-28

287. Rosier, F., M'Zali, H. and Giraud, F. (1986) Cholesterol depletion affects the Ca^{2+} influx but not the Ca^{2+} pump in human erythrocytes. Biochim. Biophys. Acta 863, 253-263

288. Shio, H., Haley, N.J. and Fowler, S. (1978) Characterization of lipid-laden aortic cells from cholesterol-fed rabbits: III. Intracellular localization of cholesterol and cholesteryl esters. Lab. Invest. 39, 390-97

289. Bialecki, R., Tulenko, T.N. and Colucci, W.S. (1991) Cholesterol enrichment increases calcium influx and intracellular content in rat vascular smooth muscle cells. J. Clin. Invest. 88, 1894-1900

290. Sachinidis, A., Locker, R. and Vetter, W. (1990) Induction of cell proliferation and vasoconstriction by low density lipoprotein. Klin. Wochenschr. 68, (suppl 20), 65-67

291. Bialecki, R.A. and Tulenko, T.N. (1989) Excess membrane cholesterol alters calcium channel activity in smooth muscle of rabbit carotid arteries. Am. J. Physiol. 257, C306-C314

292. Liu, K.Z. and Pierce, G.N. (1993) The effects of low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes. J. Biol. Chem. 268, 3767-75

293. Mason, R.P., Moisey, D.M. and Shajenko, L. (1992) Cholesterol alters the binding of Ca^{2+} channel blockers to the membrane lipid bilayer. Mol. Pharmacol. 41, 315-21

294. Liu, K.Z, Massaeli, H. and Pierce, G.N. (1993) The action of oxidized low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes. J. Biol. Chem. 268, 4145-51

295. Mahnensmith, R.L. and Aronson, P.S. (1985) The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ. Res. 57, 773-788

296. Roos, A. and Boron, W.F. (1983) Intracellular pH. Physiol. Rev. 61, 296-434

297. Parker, J.C. (1983) Volume-responsive sodium movements in dog red blood cells. Am. J. Physiol. 244, C324-C330

298. Livne, A., Balfe, J.W., Veitch, R., Marquez-julio, A., Grinstein, S. and Rothstein, A. (1987) Increased platelet Na^+-H^+ exchange rates in essential hypertension: application of a novel test. Lancet 1, 533-36

299. Meng, H., Lonsberry, B.B. and Pierce, G.N. (1991) Influence of perfusate pH on the postischemic recovery of cardiac contractile function: Involvement of sodium-hydrogen exchange. J. Pharmacol. Exp. Ther. 258, 772-77

300. Ng, L.L., Simmons, D., Frighi, V., Garrido, M.C., Bomford, J. and Hockaday, T.D.R.

(1990) Leukocyte Na^+-H^+ antiport activity in type 1 (insulin-dependent) diabetic patients with nephropathy. Diabetologia 33, 371-77

301. Pierce, G.N., Ramjiawan, B., Dhalla, N.S. and Ferrari, R. (1990) Na^+-H^+ exchange in cardiac sarcolemmal vesicles isolated from diabetic rats. Am. J. Physiol. 258, H255-H261

302. Brasitus, T.A., Dudeja, P.K., Worman, H.J. and Foster, E.S. (1986) The lipid fluidity of rat colonic brush-border membrane vesicles modulates Na^+-H^+ exchange and osmotic water permeability. Biochim. Biophys. Acta 855, 16-24

303. Dudeja, P.K., Foster, E.S. and Brasitus, T.A. (1987) Modulation of rat distal colonic brush-border membrane Na^+-H^+ exchange by dexamethasone: role of lipid fluidity. Biochim. Biophys. Acta 905, 485-493

304. Poli De Figueiredo, C.E., Ng, L.L., Davis, J.E., Lucio-cazana, F.J., Ellory, J.C. and Hendry, B.M. (1991) Modulation of Na^+-H^+ antiporter activity in human lymphoblasts by altered membrane cholesterol. Am. J. Physiol. 261, C1138-42

305. Kochhar, N. and Kaul, D. (1992) Molecular link between membrane cholesterol and Na^+-H^+ exchange within human platelets. FEBS. Lett. 299, 19-22

306. Reuter, H. and Seitz, N. (1968) The dependence of calcium efflux from cardiac music on temperature and external ion composition. J. Physiol. Lond. 195, 451-470

307. Reeves, J.P. and Sutko, J.L. (1979) Sodium-calcium ion exchange in cardiac membrane vesicles. Proc. Ntal. Acad. Sci. U.S.A. 76, 590-94

308. Reuter, H. (1982) Na-Ca countertransport in cardiac muscle. In: Martonosi, A.N. eds. Membranes and transport. Vol. 1. N.Y.: Plenum Press, pp 623-31

309. Bridge, J.H.B. and Bassingthwaighte, J.B. (1983) Uphill sodium transport driven by an inward calcium gradient in heart muscle. Science 219, 178-180

310. Crespo, L.M., Grantham, C.J. and Cannell, M.B. (1990) Kinetics, stoichiometry and role of the Na-Ca exchange mechanism in isolated cardiac myocytes. Nature 345, 618-21

311. Theunissen, J.J.H., Jackson, R.L., Kempen, H.J.M. and Demel, R.A. (1986) Membrane properties of oxyterols: Interfacial orientation, influence on membrane permeability and redistribution between membranes. Biochim. Biophys. Acta 860, 66-74

312. Vemuri, R. and Philipson, K.D. (1987) Phospholipid composition modulates the Na^+-Ca^{2+} exchange activity of cardiac sarcolemma in reconstituted vesicles. Biochim. Biophys. Acta 937, 258-68

313. Dalla-serra, M., Pederzolli, C., Antolini, R., Cusinato, F., Luciani, S. and Menestrina, G. (1991) Activation energy of the cardiac Na^{+}-Ca^{2+} exchanger in sarcolemmal vesicles and reconstituted proteoliposomes. Cardioscience 2, 193-7

314. Luciani, S., Bova, S., Cargnelli, G., Cusinato, F. and Debetto, P. (1991) Modulation of sodium-calcium exchange by lipids. In: Sodium-calcium exchange, proceedings of the second international conference. Ann. N. York Acad. Sci. 639,156-165

315. Nemecz, G. and Schroeder, F. (1988) Time-resolved fluorescence investigation of membrane cholesterol heterogeneity and exchange. Biochemistry 27, 7740-9

316. Wood, W.G., Schroeder, F., Hogy, L., Rao, A.M. and Nemecz, G. (1990) Asymmetric distribution of a fluorescent sterol in synaptic plasma membranes: effects of chronic ethanol consumption. Biochim. Biophys. Acta 1025, 243-246

317. Alivisatos, S.G.A., Deliconstantinos, G. and Theodosiadis, G.P. (1981) Specificity of binding of cholesterol, steroid hormones and other compounds in synaptosomal plasma membranes, and their effect on ouabain-sensitive ATPase. Biochim. Biophys. Acta 643, 650-58

318. Locker, R., Neyses, L., Stimpel, M., Kuffer, B. and Vetter, W. (1984) The cholesterol content of the human erythrocyte influences calcium influx through the channel. Biochim. Biophys. Res. Commun. 124, 822-28

319. Yokoyama, M. and Henry, P.D. (1979) Sensitization of isolated canine coronary arteries to calcium ions after exposure to cholesterol. Circ. Res. 45, 479-86

320. Palinski, W., Rosenfeld,M.E., Yla-Herttuala,S., Gurtner, G. C., Socher, S.S., Butler, S.W., Parthasarathy, S., Carew, T. E., Steinberg, D., Witztum,J.L. (1989) Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. USA 86, 1372-76

321. Quinn MT, Parthasarathy S, Fong LG, Steinberg D. (1987) Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc. Natl. Acad. Sci. USA 84, 2995-98

322. Aviram, M. (1992) Low density lipoprotein modification by cholesterol oxidase induces enhanced uptake and cholesterol accumulation in cells. J. Biol. Chem. 267, 218-25

323. Julien, P., Downer, E. and Angel, A. (1981) Lipoprotein composition and transport in the pig and dog cardiac lymphatic system. Circ. Res. 49, 248-54

324. Burton, K.P. (1988) Evidence of direct toxic effects of free radicals on the myocardium. Free Rad. Biol. Med. 4, 15-24

325. McCord, J.M. (1985) Oxygen-derived free radicals in postischemic tissue injury. New Engl. J. Med. 312, 159-63

326. Ylä-Herttuala, S. Palinski, W., Rosenfeld, M.E., Parthasarathy, S. Carew, T.E., Butler, S., Witztum, J.L. and Steinberg, D. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84, 1086-1095

327. Rosenfeld, M.E., Palinski, W., Ylä-Herttuala, S. Butler, S., and Witztum, J.L. (1990) Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. Arteriosclerosis 10, 336-349

328. Galle, J., Bassenge, E. and Busse, R. (1990) Oxidized low density lipoproteins potentiate vasoconstrictions to various agonists by direct interaction with vascular smooth muscle. Circ. Res. 66, 1287-1293

329. Parthasarathy, S., Carew, T.E., Steinberg, D. and Witztum, J.L. (1992) The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. Annu. Rev. Med. 43,219-25

330. Heinecke, J.W., Rosen, H. and Chait, A. (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. J. Clin. Invest. 74, 1890-94

331. Esterbauer, H., Jurgens, G., Quehenberger, O. and Koller, E. (1987) Autooxidation of human low density lipoprotein: loss of polyunsaturated fatty acid and vitamin E and generation of aldehydes. J. Lipid Res. 28,495-509

332. Liu, K.Z., Ramjiawan, B., Kutryk, M.J.B. and Pierce, G.N. (1991) Effects of oxidative modification of cholesterol in isolated low density lipoproteins on cultured smooth muscle cells. (1991) Mol. Cell. Biochem. 108, 49-56

333. McCord, J.M., Roy, R.S. and Schaffer, S.W. (1985) Free radicals and myocardial ischemia. The role of xanthine oxidase. Adv. Myocardiol. 5, 183-9

334. Yla-Herttuala, E., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J.L., Steinberg, D. (1989) Arteriosclerosis: modified lipoproteins/receptors. Suppl. II Circ 80: II-160

335. Peng, S.K., Bruce,T., Philip, T. Werthessen, N.T. (1978) Effect of auto-oxidation products from cholesterol on aortic smooth muscle cells. Arch. Pathol. Lab. Med. 102, 57-61

336. Peng, S.K., Tham, P., Taylor, C.B., Mikkelson, B. (1979) Cytotoxicity of oxidation derivatives of cholesterol on cultured aortic smooth muscle cells and their effect on cholesterol biosynthesis. Am. J. Clin. Nutr. 32, 1033-42

337. Jacobson,M.S., Nair, P.P., Naseem, S.M., Smith, T.D., Heald, F,P. (1981) Oxygenated cholesterols: new risk factors for atherosclerosis? Ped Res 15, 535

338. Ben-Yashar, V., Barenholz, Y. (1989) The interaction of cholesterol and cholest-4-en-3-one with dipalmitoylphosphatidyl-choline. Comparison based on the use of three fluorophores. Biochim. Biophys. Acta 985, 271-78

339. Chapman, M.J., Goldstein, S., Lagrange, D., Laplaud, P.M. (1981) A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. J Lipid Res 22, 339-58

340. Radding, C.M., Steinberg, D. (1960) Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. J. Clin. Invest. 39, 1560-69

341. Kutryk, M.J.B. and Pierce, G.N. (1991) Effects of low-density lipoprotein on calcium ion movements in cultured vascular smooth muscle cells. Coronary Artery Dis. 2, 1093-1101

342. Lingren, F.T. In: Analysis of lipids and lipoproteins. (Perkins, E.G., Ed.) Chemists Soc. 1975, pp. 204-225.

343. Fogelman AM, Shechter I, Seager J, Hokon M, Child JS, Edwards PA. (1980) Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. Proc Natl Acad Sci USA 77:2214-8.

344. Liu, K.Z., Cuddy, T.E. and Pierce, G.N. (1992) Oxidative status of lipoproteins in coronary disease patients. Am. Heart J. 123, 285-90

345. Sale, F.O., Marchesini, S., Fishman, P.H., Berra, B. (1984) A sensitive enzymatic assay for determination of cholesterol in lipid extracts. Anal. Biochem. 142, 347-50

346. Langer, G.A., Frank, J.S. and Orner, F.B. (1987) Calcium exchange, structure, and function in cultured adult myocardial cells. Am. J. Physiol. 252, H314-H324

347. Pierce, G.N., Langer, G.A., Wright,G.B., Kutryk,M.J.B. Calcium is rapidly exchangeable in cultured vascular smooth muscle cells from rabbit aorta. J Mol Cell Cardiol 21: 889-899, 1989.

348. Reynold, G. and St. Clair, R.W. (1985) A comparative microscopic and biochemical study of the uptake of fluorescent and 125I-labeled lipoproteins by skin fibroblasts, smooth muscle cells, and peritoneal macrophages in culture. Am. J. Pathol. 121, 200-211

349. Pitas, R.E., Innerarity, T.L. and Mahley, R.W. (1983) Foam cells in explants of atherosclerotic rabbit aortas have receptors for beta-very low density lipoproteins and modified low density lipoproteins. Atherosclerosis 3, 2-12

350. Berg, K.A., Berry, M.L., Sapareto, A.S. and Petty, H.R. (1986) Fluorescence studies of macrophage recognition and endocytosis of native and acetylated low-density lipoprotein. Biochim. Biophys. Acta 887, 304-14

351. Barak, L.S. and Webb, W.W. (1981) Fluorescent low density lipoprotein for observation of dynamics of individual receptor complexes on cultured human fibroblasts. J. Cell. Biol. 90, 595-604

352. Pitas, R.E. (1990) Expression of the acetyl low density lipoprotein receptor by rabbit fibroblasts and smooth muscle cells. Up-regulation by phorbol esters. J. Biol. Chem. 265, 12722-27

353. Liu, K.Z., Maddaford, T.G., Ramjiawan, B., Kutryk, M.J.B., Pierce, G.N. (1991) Effects of cholesterol oxidase on cultured vascular smooth muscle cells. Mol. Cell. Biochem. 108, 39-48

354. Greenspan, P., Fowler, S. D. (1985) Spectrofluorometric studies of the lipid probe, nile red. J. Lipid Res. 26, 781-89

355. Greenspan, P., Mayer, E.P., Fowler, S.D. (1985) Nile red: A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965-73

356. Miyakawa, I., Aoi, H., Sando, N. (1984) Fluorescence microscopic studies of mitochondrial nucleoids during miosis and sporulation in the yeast, saccharomyces cerevisiae. J. Cell Sci. 66, 21-38

357. Fuchs, J., Zimmer, G., Bereiter, Hahn. J. (1987) A multiparameter analysis of the perfused rat heart: responses to ischemia, uncouplers and drugs. Cell Biochem. Funct. 5, 245-53

358. Eng, J., Lynch, R.M., Balaban, R.S. (1989) Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging of isolated cardiac myocytes. Biophys. J. 55, 621-30

359. Kennedy, C.H., Winston,G.W., Church, D.F. and Pryor,W.A. (1989) Benzoyl peroxide interaction with mitochondria: inhibition of respiration and induction of rapid, large-amplitude swelling. Arch Biochem Biophys 271, 450-70

360. Panagia, V., Pierce, G.N., Michiel, D.F. and Dhalla, N.S. (1984) Methods for studying biochemical structure of heart membranes. In: Methods in Studying Cardiac Membranes. Vol II, CRC Press, Boca Raton. 45-58

361. Sevanian, A. and McLeod, L.L. (1987) Cholesterol autooxidation in phospholipid membrane bilayer. Lipids 22, 627-36

362. Lee, D.M. (1980) Malondialdehyde formation in stored plasma. Biochem. Biophys. Res. Commun. 95, 1663-72

363. Kramer,J.H., Tong, Mak. I. and Weglicki, W.B. (1984) Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipid peroxidation. Circ. Res. 55, 120-4

364. Habeeb, A.F.S.A. (1966) Determination of free amino groups in proteins by trinitrobenzene sulphonic acid. Anal. Biochem. 14, 328-36

365. Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440-50

366. Cheung, J.Y., Tillotson, D.L. and Yelamarty, R.V. (1989) Cytosolic free calcium concentration in individual cardiac myocytes in primary culture. Am. J. Physiol. 256, C1120-C1130

367. Hohl, C.M., Li, Q. (1991) Compartmentation of cAMP in adult canine ventricular myocytes. Relation to single-cell free Ca^{2+} transients. Circ. Res. 60, 1369-79

368. Ince, C., Van-Dissel, J.T. and Diesselhoff, M.M. (1985) A teflon culture dish for high-magnification microscopy and measurements in single cells. Pflug. Arch 403, 240-44

369. Burton, K.P., Morris, A.C., Massey, K.D., Buja, L.M. and Hagler, H.K. (1990) Free radicals alter ionic calcium levels and membrane phospholipids in cultured rat ventricular myocytes. J. Mol. Cell. Cardiol. 22, 1035-47

370. White, E. and Terrar, D.A. (1990) The effects of ryanodine and caffeine on Ca-activated current in guinea-pig ventricular myocytes. Br. J. Pharmacol. 101, 399-405

371. Wimsatt, D.K., Hohl, C.M., Brierley, G.P. and Altschuld, R.A. (1990) Calcium accumulation and release by the sarcoplasmic reticulum of digitonin-lysed adult mammalian ventricular cardiomyocytes. J. Biol. Chem. 265, 14849-57

372. Razszetti, R., Bongrani, S. and Schiantarelli, P. (1984) In vitro effects of nicardipine on vascular and cardiac muscle preparations. Pharmacol. Res. Commun. 16, 795-808

373. Sanguinette, M.C. and Kass, R.S. (1984) Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. Circ. Res. 55, 336-348

374. Bielefeld, D.R., Hadley, R.W., Vassilev, P.M. and Hume, J.R. (1986) Membrane electrical properties of vesicular Na-Ca exchange inhibitors in single atrial myocytes. Circ. Res. 59, 381-9

375. Schramm, M., Thomas, G., Towart, R. and Franckowiak, G. (1983) Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. Nature 303, 535-7

376. Geeraerts, M.D., Ronveaux-Dupal, M.F., Lemasters, J.J. and Herman, B. (1991) Cytosolic free Ca^{2+} and proteolysis in lethal oxidative injury in endothelial cells. Am. J. Physiol. 261, C889-C96

377. Kobayashi, A., Hayashi, H., Watanabe, H, Miyata, H., Kurata, C. and Yamazaki, N. (1991) The role of free radicals in myocardial cell injury. Bratisl. Lek. Listy. 92, 59-65

378. Braughler, J.M. and Pregenzer, J.F. (1989) The 21-aminosteroid inhibitors of lipid peroxidation: reactions with lipid peroxyl and phenoxy radicals. Free Rad. Biol. Med. 7, 125-130

379. Fisher, M., Levine, P. H., Doyle, E.M., Arpano, M.M. and Hoogasian, J.J. (1991) A 21-aminosteroid inhibits oxidation of human low density lipoprotein by human monocytes and copper. Atherosclerosis 90, 197-202

380. Carrea, F.P., Lesnefsky, E.J., Kaiser, D.G. and Horwitz, L.D. (1992) The lazaroid U74006F, a 21-aminosteroid inhibitor of lipid peroxidation, attenuates myocardial injury from ischemia and reperfusion. J. Cardiovasc. Pharmacol. 20, 230-5

381. Thurnhofer, H., Gains, N., Mutsch, B. and Hauser, H. (1986) Cholesterol oxidase as a structural probe of biological membranes: its application to brush-border membrane. Biochim. Biophys. Acta 856, 174-81

382. Steinbrecher, U.P. (1987) Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotien B by lipid peroxide decomposition products. J. Biol. Chem. 262, 3603-8

383. Brown, W.J., Warfel, J. and Greenspan, P. (1988) Use of nile red stain in the detection of cholesteryl ester accumulation in acid lipase-deficient fibroblasts. Arch. Pathol. Lab. Med. 112, 295-97

384. Brasaemle, D.L. and Attie, A.D. (1990) Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts. J. Lipid Res. 31, 103-112

385. Dietschy, J.M., Spady, D.K. and Stange, E.F. (1983) Quantitative importance of different organs for cholesterol synthesis and low-density-lipoprotein degradation. Biochem. Soc. Trans. 11, 639-41

386. Leblanc, N. and Hume, J.R. (1990) Sodium current-induced release of calcium from cardiac sarcoplsmic reticulum. Science 248, 372-376

387. Triggle, D.J. (1990) Calcium, calcium channels, and calcium channel antogonists. Can. J. Physiol. Pharmacol. 68, 1474-81

388. Herbette, L.G., Vant, Erve, Y. and Rhodes, D.G. (1989) Interaction of 1,4

dihydropyridine calcium channel antagonists with biological membranes: lipid bilayer partitioning could occur before drug binding to receptors. J. Mol. Cell. Cardiol. 21, 187-201

389. Mason, R.P., Moisey, D.M. and Shajenko, L. (1992) Cholesterol alters the binding of Ca^{2+} channel blockers to the membrane lipid bilayer. Mol. Pharmacol. 41, 315-321

390. Moore, S. Lab. Invest. (1973) Thromboatherosclerosis in normolipemic rabbits. A result of continued endothelial damage. 29, 478-87

391. Rutledge, J.C., Curry, F.R.E., Lenz, J.F. and Davis, P.A. (1990) Low density lipoprotein transport across a microvascular endothelial barrier after permeability is increased. Circ. Res. 66, 486-95

392. Tilton, R.G., Cole, P.A., Zions, J.D., Daugherty, A., Larson, K.B., Sutera, S.P., Kilo, C. and Williamson, J.R. (1987) Increased ischemia-reperfusion injury to the heart associated with short-term, diet-induced hypercholesterolemia in rabbit. Circ. Res. 60, 551-559

393. Peterson, D.W., Griffith, D.W. and Napolitano, C.A. (1979) Decreased myocardial contractility in papillary muscle from atherosclerotic rabbits. Circ. Res. 45, 338-46

394. Peterson, D.W., Napolitano, C.A. and Griffith, D.W. (1980) Spontaneous mechanical alterations in papillary muscles from atherosclerotic rabbits. Am. J. Physiol. 239, H674-H80

395. Senges, J., Weihe, E., Brachmann, J., Petzer, D., Nimmrich, H. and Kubler, W. J. (1981) Effects of hypercholesterolemia without ischemia on some electrophysiological and ultrastructural properties of the rabbit heart. J. Mol. Cell. Cardiol. 13, 253-64

396. Josephson, R.A., Silverman, H.S., Lakatta, E.G., Stern, M.D. and Zweier, J.L. (1991) Study of the mechanisms of hydrogen peroxide and hydroxyl free radical-induced cellular injury and calcium overload in cardiac myocytes. J. Biol. Chem. 266, 2354-2361

397. Halliwell B. (1977) Generation of hydrogen peroxide, superoxide and hydroxyl radicals during the oxidation of dihydroxyfumaric acid by peroxidase. Biochem. J. 163, 441-48

398. Ambrosio, G., Flaherty, J.T., Duilio,C., Tritto, I., Santoro, G., Elia, P.P., Condorelli, M. and Chiariello, M. (1991) Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. J. Clin. Invest. 87, 2056-66

399. Bird R.P., Draper, H.H. (1984) Comparative studies on different methods of malonaldehyde determination. Meth. Enzymol. 105, 299-305

400. Janero, D.R. (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Rad. Biol. Med. 9, 515-40

401. Steinberg, D. (1988) in Atherosclerosis Reviews, eds. Stokes, J., III, & Mancini, M. (Raven, New York), Vol. 18, pp. 1-23

402. Sparrow, C.P., Parthasarathy, S. and Steinberg, D. (1989) A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. J. Biol. Chem. 264, 2599-604

403. Haberland, M.E., Rasmussen, R.R., Olch, C.L. and Fogelman, A.M. (1986) Two distinct receptors account for recognition of maleyl-albumin in human monocytes during differentiation in vitro. J. Clin. Invest. 77, 681-9

404. Henrriksen, T., Mahoney, E.M. and Steinberg, D. (1983) Enhanced macrophage degradation of biologically modified low density lipoprotein. Arteriosclerosis 3, 149-59

405. Henriksen, T., Mahoney, E.M. and Steinberg, D. (1981) Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by the receptor for acetylated low density lipoproteins. Proc. Natl. Acad. Sci. USA 78, 6499-503

406. Halliwell, B. and Gutteridge, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1-14

407. Takemura, G., Onodera, T. and Ashraf, M. (1992) Quantification of hydroxyl radical and its lack of relevance to myocardial injury during early reperfusion after graded ischemia in rat hearts. Circ. Res. 71, 96-105

408. Yamada, M., Hearse, D.J. and Curtis, M.J. (1990) Reperfusion and readmission of oxygen. Pathophysiological relevance of oxygen-derived free radicals to arrhythmogenesis. Circ. Res. 67, 1211-24

409. Roos, D., Weening, R.S., Wyss, S.R. and Aebi, H.E. (1980) Protection of human neutrophils by endogenous catalase: Studies with cells from catalase-deficient individuals. J. Clin. Invest. 65, 1515-22

410. Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxides metabolism in mammalian organs. Physiol. Rev. 59, 527-605

411. Hayashi, H., Miyata, H., Watannabe, H., Kobayashi, A. and Yamazaki, N (1989) Effects of hydrogen peroxide on action potentials and intracellular Ca^{2+} concentration of guinea pig heart. Cardiovasc. Res. 23, 767-73

412. Tones, M.S. and Poole-Wilson, P.A. (1985) Alpha-adrenoceptor stimulation,

lysophosphoglyceride, and lipid peroxidation in reoxygenation induced calcium uptake in rabbit myocardium. Cardiovasc. Res. 19, 228-36

413. Addis, P.B., Emanuel, H.A., Bergmann, S.D. and Zavoral, J.H. (1989) Capillary GC quantification of cholesterol oxidation products in plasma lipoproteins of fasted humans. Free Radical. Biol. Med. 7, 179-82

414. Javitt, N.B, Kok, E., Burstein, S., Cohen, B. and Kutscher, J. (1981) 26-hydroxycholesterol: identification and quantitation in human serum. J. Biol. Chem. 256, 12644-6

415. Schuh, J., Fairclough, G.F. Jr. and Haschemeyer, R.H. (1978) Oxygen-mediated heterogeneity of apo-low-density lipoprotein. Proc. Natl. Acad. Sci. USA 75, 3173-7

416. Tong, Mak. I., Weglicki, W.B. (1988) Protection by ß-blocking agents against free radical-mediated sarcolemmal lipid peroxidation. Circ. Res. 63, 262-66

417. Brown, M.S., Goldstein, J.L. (1990) Scavenging for receptors. Atherosclerosis 343, 508-509

418. Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira P. and Krieger, M. (1990) Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. Nature 343, 531-535

419. Fowler, S.D., Mayer, E.P. and Greenspan, P. (1985) Foam cells and atherogenesis. Ann. N.Y. Acad. Sci. 454, 79-90

420. Lewis, J.C., Taylor, R.G., Jerome, W.G. (1985) Foam cell characteristics in coronary arteries and aortas of white carneau pigeons with moderate hypercholesterolemia. Ann. N.Y. Acad. Sci. 454, 91-100

421. Ogiso, T., Iwaki, M., Kurata, A., Saito, H. (1988) Effect of phospholipase A_2 and cholesterol oxidase on perazine and promethazine penetration into human erythrocytes and on fluidity of the membrane. Chem. Pharm. Bull. 36, 2168-75

422. Salen, G., Shefer, S., Berginer, V.M. In the metabolic basis of inherited disease (J.B. Stanbury et al., eds.), Ed. 5, pp. 713-730 (McGraw-Hill, New York)., 1983.

423. Hubbard, R.W., Ono, Y., Sanchez, A. (1989) Atherogenic effect of oxidized products of cholesterol. Prog. Food Nutr. Sci. 13, 17-44

424. Redwell, S., Dean, R.T., Jessup, W. (1989) The action of defined oxygen-centred free radicals on human low-density lipoprotein. Biochem. J. 262, 707-12

425. Terao, J., Sugino, K., Matsushita, S. (1985) Fe^{2+} and ascorbic acid induced oxidation of cholesterol in phosphatidylcholine liposomes and its inhibition by α-tocopherol.

J Nutr Sci Vitaminol 31, 499-508

426. McCord, J.M. Free radicals and heart disease. In: Nutritional Impact of Food Processing. Somogyi JC, Muller HR (eds). Bibl Nutr Dieta, Karger, 43: 327-337, 1989.

427. Steinberg, D., Prathasarathy, S., Carew, T.E. (1988) In vivo inhibition of foam cell development by probucol in Watanabe rabbits. Am. J. Cardiol. 62, 6B-12B

428. Kritchevsky, D. Cholesterol, John Wiley and Sons, Inc., New York, P.132, 1958.

429. Linder, R. (1984) Alteration of mammalian membranes by the cooperative and antagonistic actions of bacterial proteins. Biochem. Biophys. Acta 779, 423-35

430. International Collaborative Study Group. (1986) Metabolic epidemiology of plasma cholesterol: mechanisms of variation of plasma cholesterol within populations and between populations. Lancet 2 (8514), 991-6

431. Martin,M.J., Hulley, S.B., Browner, W.S., Kuller, L.H., Wentworth, D. (1986) Serum cholesterol, blood pressure and mortality: implications from a cohort of 361,662 men. Lancet 2 (8513), 933-6

432. Quinn, M.T., Parthasarathy, S., Fong, L.G., Steinberg,D. (1987) Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc. Natl. Acad. Sci. USA 84, 2995-98

433. Jiala, I., Chait, A. (1989) Differences in the metabolism of oxidatively modified low density lipoprotein and acetylated low density lipoprotein by human endothelial cells: inhibition of cholesterol esterification by oxidatively modified low density lipoprotein. J. Lipid Res. 30, 1561-68

434. O'Rourke, B., Reibel, D.K. and Thomas, A.p. (1990) High-speed digital imaging of cytosolic Ca^{2+} and contraction in single cardiomyocytes. Am. J. Physiol. 259, H230-H242

G. Appendix

The results of this thesis research have been published in the following journals:

1. K.Z. Liu, T.G. Maddaford, B. Ramjiawan, M.J.B.Kutryk, G.N.Pierce. Effects of cholesterol oxidase on cultured vascular smooth muscle cells.
   Molecular and Cellular Biochemistry 108: 39-48, 1991

2. K.Z. Liu, B. Ramjiawan, M.J.B.Kutryk and G.N.Pierce. Effects of oxidative modification of cholesterol in isolated low density lipoproteins on cultured smooth muscle cells.
   Molecular and Cellular Biochemistry 108:49-56, 1991

3. K.Z. Liu, T.E. Cuddy and G.N.Pierce. Oxidative status of lipoproteins in coronary disease patients.
   American Heart Journal 123: 285-290, 1992

4. K.Z. Liu and G.N. Pierce. The effects of low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes.
   J. Biol. Chem. 268:3767-75, 1993

5. K.Z. Liu, H. Massaeli and G.N. Pierce. The action of oxidized low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes.
   J. Biol. Chem. 268:4145-51, 1993

6. K.Z. Liu and G.N. Pierce. Oxidation of selected lipids in LDL: Effects on calcium transients in isolated rabbit cardiomyocytes. (Submitted)