Effects of Conjugated Linoleic Acid Isomers in the Phospholipase C γ_1 Response to Oxidative Stress in Isolated Cardiomyocytes.

By

Rabban Mangat

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

MASTER OF SCIENCE IN HUMAN NUTRITIONAL
SCIENCES

Department of Human Nutritional Sciences
Faculty of Human Ecology
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
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Of

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ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

BHT..Butylated hydroxy-toluene

CLA...Conjugated linoleic acid

c9,t11 CLA.....................................cis-9, trans-11 conjugated linoleic acid

DAG..*sn*-1,2-Diacylglycerol

DR...Dietary record

+dP/dt...Rate of pressure development

-dP/dt...Rate of pressure decay

ERK..Extracellular signal regulated protein kinase

FFQ...Food frequency questionnaire

GAPDH...Glyceraldehyde-3-phosphate dehydrogenase

HDL...High density lipoprotein

H_2O_2...Hydrogen peroxide

I-R...Ischemia-reperfusion

IP_3..Inositol 1,4,5-trisphosphate

JNK...Janus Kinase

KDa...Kilodalton

MDA..Malondialdehyde

MAP...Mean arterial pressure

MAPK...Mitogen activated protein kinase

MEF...Mouse embryonic fibroblast

Mn-SOD...............................Manganese-Superoxide dismutase

PIP_2...................................Phosphatidylinositol 4,5-bisphosphate

PKC...................................Protein kinase C

PLC...................................Phospholipase C

PLD...................................Phospholipase D

PI.....................................Phosphatidylinositol

PUFA.................................Polyunsaturated fatty acid

RACK..................................Receptors for activated C kinase

ROS...................................Reactive oxygen species

RA.....................................Rumenic Acid

SL.....................................Sarcolemma

SR.....................................Sarcoplasmic reticulum

SOD...................................Superoxide dismutase

t10,c12 CLA..........................trans-10, cis-12 conjugated linoleic acid

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ABSTRACT

The present study was conducted to examine the role of a major cardiac phospholipase C (PLC) isozyme, PLC γ_1, in cardiomyocytes during oxidative stress and thereafter to examine the role of CLA isomers on the PLC γ_1 response to oxidative stress. Left ventricular cardiomyocytes were isolated by collagenase digestion from adult male Sprague-Dawley rats (250-300 g) and treated with 20, 50 and 100 μM H_2O_2 for 15 minutes. A concentration-dependent increase in the mRNA level and membrane protein content of PLC γ_1 was observed with H_2O_2 treatment. Furthermore, PLC γ_1 was activated in response to H_2O_2, as revealed by an increase in the phosphorylation of its tyrosine residues. However, catalase prevented the H_2O_2 induced activation of PLC γ_1. There was also a marked increase in the phosphorylation of the anti-apoptotic protein, Bcl-2 by H_2O_2; a PLC inhibitor, U73122, attenuated this change. Furthermore, while both PKC δ and ϵ protein contents were increased in the cardiomyocyte membrane fraction in response to H_2O_2 only PKC ϵ activation was almost completely prevented by the PLC inhibitor, U73122 (2 μM). Trypan blue exclusion revealed a loss of cardiomyocyte viability when cells were pretreated with U73122 (2 μM) and treated with H_2O_2 (50 μM). Our results suggest that PLC γ_1 may play a role in cardiomyocyte survival during oxidative stress via PKC ϵ and phosphorylation of Bcl-2. Isolated adult ventricular cardiomyocytes were incubated overnight with CLA isomers c9,t11 CLA and t10,c12 CLA (5, 50,100 μM) and were subjected to

oxidative stress (50 μM H_2O_2) for 15 minutes and PLC γ_1 activity was assessed. No change in PLC γ_1 activity was seen in response to oxidative stress with t10, c12 CLA isomer, whereas c9,t11 CLA isomer treatment lowered the cardioprotective elevation of PLC γ_1 in isolated cardiomyocytes. These data suggest that the treatment of cardiomyocytes with c9,t11 CLA isomer may be deleterious for people recovering from ischemia-reperfusion injury. More studies need to be concluded to evaluate the efficacy and safety of CLA isomers as well as side effects before its use in the normal healthy individuals and during pathophysiological situations can be recommended.

I. INTRODUCTION

The phosphoinositide-specific phospholipase C (PLC) isozymes associated with the cardiac sarcolemma (SL) membrane play an important role in activating intracellular signal transduction pathways for the regulation of various cell functions (209). PLC γ_1 is a predominant PLC isozyme expressed in the heart, is known to be activated by tyrosine phosphorylation (192) and to convert its substrate, phosphatidylinositol 4,5-bisphosphate into two messenger molecules, inositol 1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG). While IP$_3$ may serve to enhance the sarcoplasmic reticulum (SR) Ca^{2+} release (225), DAG functions as a potent activator of protein kinase C (PKC) isozymes, which in turn phosphorylate several cardiac proteins (10), that influence cardiomyocyte function (226).

Ischemia-reperfusion (I-R) injury is known to occur during clinical procedures such as coronary bypass surgery, angioplasty, thrombolytic therapy and cardiac transplantation (109, 108) resulting in myocardial abnormalities (151, 227). We have previously shown that I-R of the isolated rat heart is associated with changes in PLC isozymes (208). Specifically, PLC γ_1 was activated in the first minute of reperfusion of heart subjected to a 30-minute period of global ischemia. Recent evidence has suggested an anti-apoptotic role of PLC γ_1 activation in oxidative stress in mouse embryonic fibroblasts (228, 214). Furthermore, PKC has been implicated in the PLC γ_1 mediated

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survival signaling in these cells (214). However, the functional significance of PLC γ_1 activation in the heart during oxidative stress is not known.

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (18:2) that have beneficial effects in atherosclerosis, hypertension and cardiac arrhythmias. It is also known that CLA gets incorporated into the phospholipids of the heart membranes (53). Yet nothing is known about the effect of this fatty acid change in the phospholipids and subsequently the activity of PLC isozymes.

The present study was therefore undertaken to determine the role of PLC γ_1 mediated signal transduction processes in isolated adult rat left ventricular cardiomyocytes exposed to different concentrations of hydrogen peroxide, a major oxidant molecule known to be generated during cardiac I-R (229, 230) and the effect of CLA incorporation on these changes.

II. LITERATURE REVIEW

A. Conjugated Linoleic Acid

1. Introduction

Literature referring to CLA dates back to 1950's and 1960's (1, 2). However, it became the hub of research interest after the pioneering work of Michael Pariza and colleagues in 1980's when they reported mutagenic inhibitory activity from both cooked and uncooked ground beef (3-5) and later showed that applying both crude extracts and synthetically prepared CLA inhibited chemically induced epidermal papilomas and decreased tumor incidence. This discovery paved the way for research on the biological functions and health benefits of CLA from various perspectives including cancer, immune function, atherosclerosis, food/energy intake, as well as body composition (6-9).

CLA refers to a group of positional and geometrical isomers of the essential fatty acid, linoleic acid. In linoleic acid, the double bonds are separated by more than one single bond; making the double bonds isolated or non-conjugated. In CLA, the double bonds are conjugated and occur in several positions in the carbon chain: 7,9; 8,10; 9,11; 10,12; or 11,13. Each double bond can exist in either the cis or trans form (10). The most biologically active CLA isomers are cis-9, trans-11 (c9,t11 CLA) or rumenic acid, and trans-10,cis-12-CLA (t10,c12 CLA). The major sources of CLA in the human diet

are meat and dairy products derived from ruminants. In these products the predominant CLA isomer (>90%) is c9,t11. Recent studies have suggested that the biological effects of CLA are isomer specific and that of all the isomers the c9,t11 and t10,c12 isomers of CLA have important potential biological activities (11).

2. Biological effects of conjugated linoleic acid

a. Free radical scavenging properties of conjugated linoleic acid

Free radical scavenging capacities of CLA have been detected (12, 13) and might contribute to its multifactorial biological effects. It is becoming increasingly evident that c9,t11 CLA and t10,c12 CLA might act differently in biological systems and respond to the different biological and physiological effects (14). To understand the beneficial effects of individual isomers, it is important to investigate whether these isomers differ in their reactions with free radicals.

The t10,c12 CLA isomer is said to be a kinetically preferred substrate for free radicals. This is supported by a study (13) according to which t10,c12 CLA demonstrated a stronger oxyradical scavenging capacity than c9,t11 CLA.

In another study (15), a series of combinations of c9,t11 CLA and t10,c12 CLA resulted in a more rapid initial velocity of CLA-DPPH· reaction. Changing the ratios of c9,t11 CLA/t10,c12 CLA from 100:0 to 75:25, 50:50,

and 25:75 corresponded to a respective increase in the initial reaction velocity. The mixtures at all testing ratios of the CLA isomers had greater initial velocity than either of the pure isomers, thus indicating that individual CLA isomers might have synergistic effects in their reactions with free radicals. Likewise, in an another study (16), c9,t11 CLA, t10,c12 CLA and a mixture of CLA isomers showed their inhibitory activities on human platelet thromboxane B2 formation from exogenously added [^{14}C]arachidonic acid. It was inferred from this study that t10,c12 CLA might be a stronger inhibitor for the human platelet cyclooxygenase than c9,t11 CLA, and that the isomer mixture of CLA may be a stronger inhibitor of cycloxygenase than any single CLA isomer including c9,t11 CLA and t10,c12 CLA.

In spite of these studies, CLA is not yet considered as an antioxidant because for CLA to act as an antioxidant it must be more reactive than the substrate it should protect. A study (17) of the oxidation of linoleic acid and CLA following 24h of exposure to air showed that, CLA incubated on its own was oxidized much faster than linoleic acid, and when incubated as a mixture with linoleic acid, the oxidation rate of linoleic acid increased while the oxidation rate of CLA decreased. In another set of experimental conditions in which oxygen uptake by canola oil was monitored after heating to 90° C in the presence of CLA (0.1-1.0%), a dose dependent pro-oxidant activity of CLA was observed (18). A more direct measure of assessing antioxidant potential involved is comparing the antioxidant activities of CLA, vitamin E, and

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butylated hydroxy-toluene (BHT) using unsaturated phospholipid model membranes that were exposed to a stream of oxyradicals. After an hour of oxidative stress in the presence of CLA, oxidation was only marginally lower than in the absence of CLA (17). These results indicated that vitamin E and BHT are excellent radical-scavenging anti-oxidants, while CLA is not effective in this respect.

The mechanisms of anticarcinogenic effects of CLA isomers are not clear, so it has also been suggested that increased lipid oxidation might be contributing to the cytotoxic effects of this agent in cancer cell lines. Uptake of poly unsaturated fatty acid (PUFA) by tumor cells provides the potential to alter the membrane composition of a tumor cell. Such changes might alter phospholipid distribution or may activate phospholipid hydrolysis and affect activity of membrane bound enzymes (12, 13).

b. CLA and plasma lipids

Animal studies in hamsters (19-22) and rats (23, 24) have reported a significant cholesterol-lowering effect. On the other hand, the human studies (25, 26, 27, 28, 29, 30, 31, 32) did not show any significant effect of CLA on plasma cholesterol concentrations or on LDL-cholesterol concentrations. In the study by Smedman and Vessby (30), CLA significantly increased total and LDL-cholesterol concentrations, but this increase was not significant in comparison with that seen in the control group. In another study (30) it was found that, relative to the change in HDL-cholesterol concentrations in the

control group, HDL-cholesterol concentrations decreased significantly when the t10,c12 isomer was administered, but not when a mixture of the t10,c12 and c9,t11 isomers was administered (32,33). In another study, (29) a significant HDL-cholesterol-lowering effect of CLA was reported, but this change in HDL cholesterol concentration was not significant when compared with that in the control group. Unlike this result, another study (30) found that, CLA significantly increased HDL-cholesterol concentrations, but this increase was smaller than that in the control group, as a result, the net effect of CLA on HDL was negative. Plasma triacylglycerol concentrations decreased significantly after CLA administration in two of these studies (34, 35), yet this decrease was not significant when compared with changes in the control group. This effect of triacylgycerol concentrations on plasma was seen only when a mixture of the two isomers was administered, but not when the c9, t11 isomer was given (35).

c. Effect on atherosclerosis

There are no epidemiological studies demonstrating the effect of CLA on the prevention of atherosclerosis. Animal studies have suggested that CLA supplementation decreases the development of early atherosclerotic lesions. All of the published studies have used a mixture of CLA isomers. Therefore, there is no clear consensus on the beneficial effects of CLA on atherosclerosis (37, 38).

In rabbits fed a high fat diet (14% by weight) containing 0.1% cholesterol supplemented with 0.5 g/d of an isomeric mixture of CLA (~ 40% c9,t11 CLA and 40% t10,c12 CLA) significantly reduced aortic atherosclerosis after 22 weeks. The results were based on the extent of aortic surface with lipid deposition and connective tissue involvement (39). In another study (40), it was demonstrated that low doses of CLA (0.1-1% of calories) could actually reverse early atherosclerotic lesions in the rabbit model. Even as little as 0.05% CLA in the diet reduced the severity of the lesions (41).

In hamsters (42), a mixed isomer CLA preparation was provided in 10% of saturated fat, 0.12% cholesterol diet. After 11 weeks of supplementation, atherosclerosis was reduced. In this study the unconjugated form of linoleic acid also reduced the fatty accumulations. However, a direct comparison of the effects of linoleic acid and CLA (as mixed isomers) in hamsters on an higher-fat-diet (i.e. 20% hydrogenated coconut oil, 0.12% cholesterol), showed that CLA was more effective than linoleic acid in reducing the extent of atherosclerotic lesions (43).

Unlike the rabbit and hamster studies, a study in mice showed that CLA has no effect on reducing, and may even promote atherosclerotic lesion development. In C57BL/6 mice fed an atherogenic diet, CLA supplementation at 2.5-5 g/d increased the fatty streak lesion in the aorta (44).

Due to differences in diets, methods of assessment, and CLA mixtures being used, it has become difficult to assess the potential of CLA in the management of atherosclerosis.

d. Direct cardiac effects of CLA

Although CLA isomers have been reported to exhibit antioxidant properties (12, 45), anticarcinogenic effects via induction of apoptosis and cytotoxic activity (46-51), modulate fatty acid composition (52-55), effect prostanoid formation (52), reduce body fat in animals (56, 57) and possibly humans (36), as well as affect the expression of cytokines and growth factors (58-60), there is no information available on the impact of CLA isomers or mixtures on heart function. Such baseline information is important in order to assess the therapeutic applications as well as the potential risks and benefits of CLA isomers on heart function during different pathophysiological conditions.

Although some studies have shown that CLA could exert cardiovascular benefits through its hypolipidemic and antiatherosclerotic effects (61, 62, 40, 63, and 64), a blood pressure lowering effect of t10,c12 in obese rats (65) and in spontaneously hypertensive rats (66) has been reported. It is interesting to note that several studies have shown increases in the amounts of CLA isomers incorporated into myocardial membrane phospholipids of experimental animals fed different CLA isomers (52-55). In addition, while CLA has been reported to depress the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neonatal

cardiomyocytes (67), the type and proportion of the CLA isomers was not identified and contractile activity was not measured.

Data from our unpublished study on CLA shows that while the overall response to dietary CLA was a reduction in the rate of contraction and relaxation of the heart and a reduction in mean arterial pressure (MAP), with either single CLA isomers or as a free fatty acid mixture in equal proportion, the hemodynamic response was further characterized by gender differences and was influenced by the form of the CLA supplementation. In this regard, the triglyceride form of the mixed isomer CLA produced a decrease in the heart rate of male rats, and an increase in both +dP/dt (rate of pressure development) and -dP/dt (rate of pressure decay) is suggesting that the heart is working more efficiently at a slower rate, which could be viewed as a beneficial effect of CLA on heart function. In female rats, the triglyceride CLA mixture induced no significant changes in cardiac function. On the other hand, with the t10,c12 CLA isomer, a specific increase in the heart rate of the female rats was observed. This may be seen as a compensatory mechanism in response to the decrease in systolic pressure, +dP/dt and MAP, in order to maintain the cardiac output; however, this might have long-term adverse effects that could eventually lead to cardiac fatigue, resulting in cardiac hypertrophy and ultimately heart failure. It is pointed out that no cardiac hypertrophy was observed in either male or female rats following the 16-week supplementation of the diet with CLA isomers. Nonetheless, a hypertrophic response cannot be

excluded, as it is possible that a longer feeding period of CLA isomers could further depress cardiac performance, which in turn could result in a compensatory hypertrophy. From these results it could also be inferred that CLA isomers as single or as a fatty acid mix act as cardiodepressants. Such cardiac effects in the female rats may be a concern, since there is a strong promotion for the use of CLA supplements to aid in reducing body fat, and women are often consumers of commercial weight loss products. It is therefore apparent that several notable differences exist between male and female rats in regard to heart function following dietary supplementation with the different forms of CLA, (triglyceride versus fatty acid form).

The therapeutic potential of CLA isomers in different pathophysiological conditions should also include an evaluation of the benefits, interactions and potential side effect(s) of each isomer to ensure that food products that may be enriched with CLA isomers as well as CLA supplements (nutraceuticals) are safe. For instance, although it has been shown that CLA isomers may improve the glucose handling during diabetes (68), depression of an already failing heart during diabetes (69-72) could result in an increase in the occurrence of congestive heart failure.

Another area of investigation should include the effect of CLA isomer incorporation into the cardiomyocyte SL membrane on the phospholipid-mediated signaling pathways and cardiac function. In this regard, it is conceivable that the incorporation of CLA isomers as well as their metabolites

into the SL membrane could be accompanied by changes in the products generated by SL PLC and PKC activities, resulting in changes in downstream signaling events, and ultimately altered $[Ca^{2+}]_i$ and cardiac contractile performance.

Table: 1 Biological effects of conjugated linoleic acid.

Physiological effects	Reference
Provides anti-carcinogenic effect	3, 5, 75
Enhances immune function	77, 78
Reduces inflammation	82
Reduces catabolic effects of immune stimulation	80
Reduces asthma in animal models	81
Reduces atherosclerosis	76, 40-43
Reduces body fat gain	83
Reduces symptoms of diabetes	73,74
Reduces hypertension	66
Inhibits eicosanoid synthesis	79
Exerts antioxidant properties	12, 13, 15
Alters lipid profiles	86, 19-22 25-32
Enhances bone formation	84
Enhances growth of young rodents	85

3. Dietary Sources of CLA

According to data collected by dietary records, total CLA distributions of food sources have been similar between genders (11). The major dietary sources of CLA are animal products. Meat derived from ruminants contains more CLA than the meat derived from non-ruminants. Dairy products contribute 60% of CLA followed by beef at 32%. Among dairy products, cheese is the major source, contributing about 30% and 33% of the total CLA (11). Among meat sources of CLA, with 2.7 mg CLA/g fat, veal has the least CLA while lamb has the highest amount of CLA at 5.6 mg CLA/g fat. Seafood contains low amounts of CLA, i.e. 0.3 to 0.6 mg CLA/g fat (87). CLA concentration in vegetable oil ranges from 0.6 to 0.9 mg/g fat. Processed, canned, and infant foods are comparable in CLA content to similar unprocessed foods. Of the total CLA present in dairy products, 73% to 93% is the biologically active isomer c9,t11. Similarly 57% to 85% of the total CLA found in beef is c9,t11 isomer (87-89).

CLA content in milk varies with the type of feed given to cows and also varies with season (90). Processing conditions also influence CLA content of dairy foods (75, 88, 89). For instance aged cheeses have lower amounts of CLA than cheeses with a shorter ripening time (11). In processed cheeses, higher processing temperatures, the addition of sodium caseinate, use of hydrogen donors (butylated hydroxytoluene, propyl gallate or ascorbic acid), addition of whey powder, non-fat dry milk or iron all increase the content of

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CLA (88). Storage or processing of dairy products such as low- fat yogurt, regular-fat yogurt, low-fat and regular ice cream, sour cream or cheeses at low temperatures do not produce any change in CLA content (3). According to Ha et al (75), grilling beef can increase CLA content by about four fold while Shantha et al (89) did not suggest an increase in CLA content due to cooking on beef, but did suggest that CLA is stable and not destroyed by cooking or storage.

Table 2: Conjugated linoleic acid content of various foods. Adapted from Chin et. al. (87):

Dairy products	mg/g fat	Meats/Fish	mg/g fat
Homogenized milk	5.5	Fresh ground beef	4.3
2% milk	4.1	Veal	2.7
Butter fat	6.1	Lamb	5.8
Condensed milk	7.0	Pork	0.6
Cultured butter milk	5.4	Chicken	0.9
Butter	4.7	Fresh ground turkey	2.6
Sour cream	4.6	Salmon	0.3
Ice cream	3.6	Egg yolk	0.6
Low-fat yogurt	4.4	**Vegetable Oil**	
Medium Cheddar	4.1	Safflower oil	0.7
American processed	5.0		

4. Safety of CLA isomers

In a study, (91) which involved a 36-wk feeding trial of Fischer 344 rats, the rats were fed either a control diet or a diet supplemented with 1.5% CLA, a level ~30 times greater than humans would ingest at 3 g CLA/d. No adverse effects were observed in the food disappearance, body weights, cageside examinations, or hematologic and histopathologic analyses done on 15 major organs. In a 90-d oral rat toxicity study, accompanied by a battery of in vitro genotoxicity studies typical for assessment of food ingredient safety, it was concluded that the *"no observed adverse effect levels"* for male and female rats were 2433 and 2728 mg $\cdot$ kg body wt^{-1} $\cdot$ d^{-1} respectively (92).

A number of human clinical trials looking at the safety and efficacy of CLA have also been conducted. A number of human studies have been conducted with high –quality CLA preparations that consist almost entirely (~, >90%) of the 2 most biologically active isomers in approximately equal amounts (~45% of each). In numerous human studies, when ~ 90% pure CLA was administered at 3-6 g/d, no adverse effects were observed (86, 77, 93, 94).

In spite of these studies, there are concerns regarding the safety of CLA isomers (95-97). These concerns include the induction of fatty liver, insulin resistance, and lipodystrophy observed in mice fed with CLA-supplemented diets. In human trials, enhanced C-reactive protein, lipid peroxidation, unfavourable changes in serum lipids, and reduced milk fat have emerged as safety concerns.

Hamsters fed CLA, and female rats fed diet supplemented with 15% CLA also exhibit enlarged livers, but this enlargement has been reported to be liver hypertrophy as opposed to fat accumulation (92). Toxicologists consider neither fatty liver nor liver hypertrophy to be toxic effects (98). The liver hypertrophy observed in female rats fed a diet supplemented with 15% CLA was completely reversible when the animals were switched to a CLA free diet (92).

Concerns about the elevations in oxidative stress and unfavourable changes in blood lipids also exist. One study (97) investigated the effects of CLA supplementation in men with metabolic syndrome. This study compared a typical high quality CLA preparation consisting of equal amounts of c9,t11 and t10, c12 CLA, with a supplement that was enriched for t10,c12 CLA but contained very little c9,t11 CLA. Enhanced lipid peroxidation, enhanced C-reactive protein in serum, and elevated VLDL coupled with reduced HDL were significant relative to placebo for the patients taking t10,c12 CLA supplements. These parameters were reduced in patients taking the typically commercially available CLA supplement relative to placebo. Hence it was concluded from this study that CLA could enhance inflammation and the risk of cardiovascular disease. However, other studies have demonstrated that CLA reduces inflammation (99).

It is also documented that t10,c12 CLA reduces milk fat. This has been concluded from studies in cows and lactating women (100, 101), and may

mean that less energy is available for the nursing infant. However, in a study with rats, the pups nursing dams fed CLA-supplemented diet actually grew to a larger size (85).

CLA has also been shown to decrease linoleic acid in liver cardiolipin. The decrease in linoleic acid in cardiolipin of heart mitochondrial membrane resulted in diminished heart cytochrome C oxidase activity that required cardiolipin as an activator (102).

It is apparent that a number of valid safety concerns remain in regard to CLA intake; clearly more extensive research is needed so that the benefits of CLA intake can be more knowledgably weighed against the risks.

5. CLA intakes in humans

Various methodologies have been used to quantify intakes of CLA, including the use of disappearance data, dietary recalls, food frequency questionnaires (FFQ), weighed food records and biochemical analysis of food duplicates. However, all these techniques have inherent limitations. According to a study (11) done on US college aged males, total CLA intake estimated using 3-day food duplicates was found to be 212 ± 14 and 151± 14 mg/d for men and women respectively. Rumenic acid (RA) (i.e. the c9, t11 isomer of CLA) was estimated to be 193 ± 13 and 140 ± 14 mg/d for men and women respectively. Typical human consumption of CLA estimated from 3-day written dietary records was reported as 139 mg/d in young men and women

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(103). In another study (104) dealing with lactating women during periods of low and high dairy consumption, mean CLA intake was 20 and 290 mg/d, respectively. FFQ was used in this study and the mean chronic CLA intake in lactating women was reported as 277 ± 180 mg/d.

CLA intakes in other countries appear to be comparable to that of the US population. As estimated by 7-day weighed dietary record (DR) and 24-h recalls (105), mean CLA intakes in older Swedish men was 160 mg/d. According to a study (11) using a national consumption survey, German men and women consume approximately 430 and 350 mg RA/day respectively. These estimated intakes are approximately twice those for the US population. However, Germans consume 10% more energy from fats than most Americans (11).

Average c9,t11 linoleic acid intake estimated by 7-day diet records in a small group of young Canadians (106) was determined to be 94.9 ± 40.6 mg/day, but ranged between 15-174 mg/day. Intake of the c9, t11 isomer of CLA, when expressed as mg CLA per unit of energy consumed significantly correlated with the intake of saturated fat (r =0.62, P<0.002), but not with intake of total fat (r = 0.39, P<0.08).

According to animal studies, consumption of a diet containing as little as 0.1g CLA/100g dry weight is sufficient to significantly reduce tumors, atherosclerosis and increase HDL-cholesterol concentrations (107). If this is extrapolated to humans, RA intake would have to be 620 and 441 mg/day for

men and women respectively in order to exhibit cancer protective properties and exert an anti-atherosclerotic effect. It must be noted that existing literature suggests that current as well as chronic total intakes of CLA and RA in men and women do not exceed 500mg/day. Due to lack of and inconsistency in experimental human data, caution must be excercised before increasing dietary CLA levels.

B. Cardiac Oxidative Stress

A decrease in the blood supply to the heart due to atherosclerosis, thrombosis or coronary artery spasm is well known to induce myocardial ischemia (108). Although reperfusion of the ischemic myocardium during early stages is essential to prevent cardiac damage, reperfusion of the ischemic heart after a certain critical period has been reported to have deleterious effects due to the generation of reactive oxygen species (ROS) (109-114). This phenomenon, known as "ischemia-reperfusion injury" is considered to occur during different clinical procedures, such as angioplasty, coronary bypass surgery, thrombolytic therapy and cardiac transplantation, particularly after a prolonged period of ischemia (108, 115, 116). Ischemia reperfusion injury may also occur after the termination of an anginal attack, whether there is vasospasm, platelet aggregation or collateral blood flow perfusion. Since myocardial ischemia has been shown to serve as an initial signal for the development of acute and chronic heart failure at later stages, it is believed that

oxidative stress plays a significant role in different types of cardiac diseases (117-124). In fact, the involvement of ROS in ischemia-reperfusion injury has been shown directly by employing electron para-magnetic resonance spectroscopy (125, 126), spin trap [alpha]-phenyl-*N-tert*-butylnitrone (127) and luminal-enhanced ter-butyl-initiated chemiluminescence (128). This view is further substantiated upon observing the beneficial effects of antioxidants in hearts subjected to ischemia-reperfusion (129, 130, 132). This oxidative stress-induced cellular damage has been estimated by measuring the levels of lipid peroxidation through different detection methods involving malondialdehyde (MDA) or thiobarbituric acid (133, 134). Moreover, exposure of the heart or subcellular organelles to oxyradical generating systems has been reported to produce effects similar to those observed in hearts subjected to ischemia-reperfusion (129, 135-139).

It should be noted that small amounts of ROS are normally formed during mitochondrial respiration. On the other hand, during ischemia, the mitochondrial carriers are in a reduced state due to the degradation of the adenine nucleotide pool (140). The increase in electron leakage from the respiratory chain leads to increased formation of $O_2^{\cdot-}$ due to the interaction with molecular oxygen trapped within the inner membrane of the mitochondria. During reperfusion, there will be further leakage of electrons due to the lack of ADP resulting in increased production of $O_2^{\cdot-}$. Additionally, during ischemia, activated neutrophils secrete $O_2^{\cdot-}$ as well as oxidative and

hydrolytic enzymes (141), and when plugged in the capillary bed, exacerbate the ischemic injury (142). The auto-oxidation of catecholamines in the ischemic myocardium also participates in the increased levels of ROS due to the formation of amino-chromes (143).

In ischemic-reperfused hearts, the increase in oxidative stress was observed to correlate well with cardiac dysfunction (129, 116), a decrease in the antioxidant defense mechanisms (144, 145) and an increase in lipid peroxidation (145, 146), leading to increased membrane permeability. An increase in the levels of MDA and decreased activities of superoxide dismutase (SOD) and catalase have been reported in hearts exposed to 30 min of ischemia (128). Similar increases in the oxidative stress level were observed in the ischemic-reperfused hearts with normal levels of antioxidant activities. Regional differences were observed in the glutathione peroxidase levels, which were normal in the left ventricle of the ischemic and reperfused heart, but were increased in the right ventricle (128). Hearts treated with SOD plus catalase showed a decrease in infarct size (130), an improvement in cardiac function (72) and sarcoplasmic reticular regulatory function associated with Ca^{2+}/calmodulin protein kinase (132). The depressed SL Ca^{2+}-pump, Na^+–Ca^{2+} exchange (147) and Na^+–K^+ ATPase activities in the ischemia-reperfused hearts were also prevented with a combination of SOD plus catalase (148). This treatment was also

found to prevent the ischemia-reperfusion induced depression in the protein levels and the gene expression of the SR proteins (129).

Overexpression of Mn-SOD in transgenic mice demonstrated a decrease in the ischemia-reperfusion injury as reflected by improved cardiac performance and decreased lactate dehydrogenase release (149). A similar protection was also reported in conscious rabbits genetically treated with adenovirus-mediated SOD transfer (150). Hearse et al (152) suggested a link between reperfusion ventricular fibrillation and the generation of free radicals. SR dysfunction due to excessive amounts of free radicals generated during reperfusion leading to Ca^{2+}-overload has been suggested to be a possible mechanism for the arrhythmias (151). Depression in the L-type Ca^{2+}-channel density, Na^+–Ca^{2+} exchange and Ca^{2+}-pump ATPase activities have also been reported in ischemic heart disease (152, 153). Other abnormalities include defects in the SL superficial stores of Ca^{2+}, depressed SL Na^+–K^+ ATPase (154, 155), and decreased myofilament responsiveness to Ca^{2+} due to thiol group oxidation (137) and inhibition of the myofibrillar creatine kinase activity (136). These results are consistent with the view that oxidative stress may result in the occurrence of intracellular Ca^{2+}-overload and subsequent arrhythmias, myocardial cell damage and cardiac dysfunction due to ischemia-reperfusion injury (108).

C. Redox Signaling

a. Introduction

ROS are produced endogenously as a consequence of normal cell functions or derived from external sources. They pose a threat to cells living in an aerobic environment because they can result in DNA, protein and lipid damage. As mentioned previously, ROS also play an important role in the pathophysiology of many diseases, including ischemic heart disease. All cells contain a number of antioxidant defense mechanisms to minimize fluctuations in ROS, however, when ROS generation exceeds a cell's antioxidant capacity, the result is a condition known as oxidative stress. A host's survival then depends upon the ability of it's cells and tissues to either adapt to or resist this stress. A number of stress response mechanisms have evolved to protect cells from oxidative insult and these mechanisms are rapidly activated. Some of these pathways are preferentially linked to enhanced survival, while others are associated with cell death. In ischemic heart disease, ROS, along with abnormal lipid metabolism and calcium homeostasis, gives rise to the "death signal" resulting in apoptotic cell death that leads to an infracted heart. The mammalian heart is also protected against ischemic injury by several lines of defense. The first line of defense consists of intracellular antioxidants such as superoxide dismutase, catalase and other protective enzyme systems. Recently, it has become apparent that the heart produces oxidative stress-inducible proteins in an attempt to counteract the invading ROS and that these proteins

can also function as a defense system. I-R injury is likewise associated with the induction of a number of both pro- and anti- apoptotic genes and transcription factors (156).

The finding of the production of ROS during the agonist-induced activation of NF-κB provided the first concrete evidence for the role of ROS as a second messenger. NF-κB regulates the inducible expression of a number of genes involved both in cell survival and death. For example NF-κB has been found to regulate the anti-apoptotic gene, Bcl-2, and the pro-apoptotic factors, bax and p53, in the ischemic/reperfused myocardium (157).

At the cellular level oxidative stress gives rise to a variety of responses, which range from proliferation to growth arrest to cell death. The final outcome varies depending on the cell type, as well as the dose of the ROS. The final observed effect is a balance between a variety of intracellular stress signaling pathways that have been activated in response to oxidative stress. These pathways modulate transcription factors and enzymes which then induce changes in gene expression (Scheme 1). Some of these pathways are linked directly to enhanced survival while others are known to produce cell death. A variety of other pathways produce an effect depending on the circumstances.

Scheme1. Redox signaling at the cellular level. [Adapted from Martindale et al (156)].
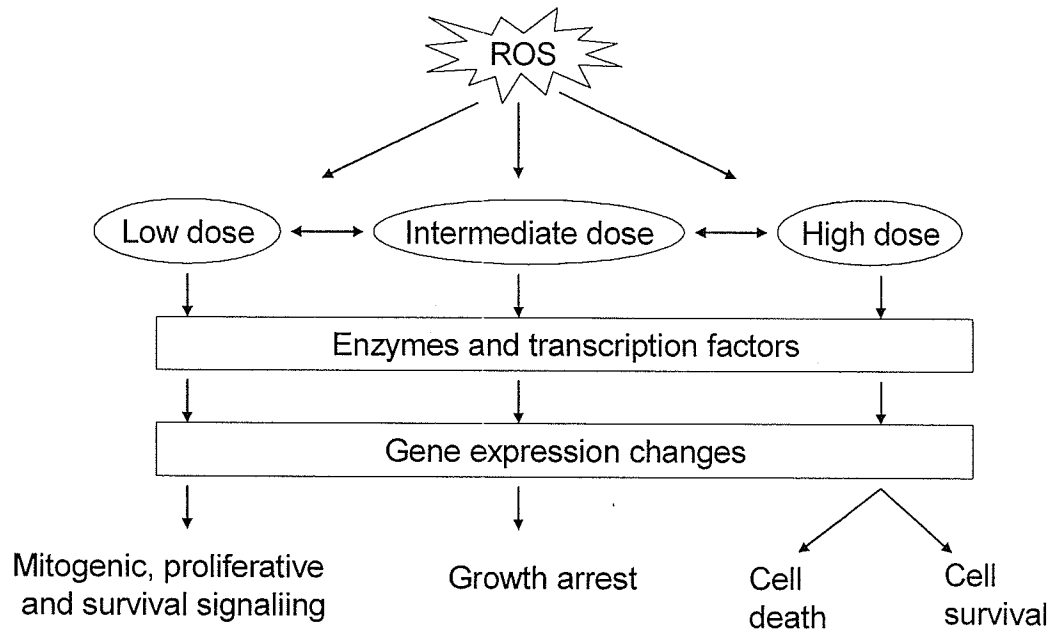
b. Death and survival machinery

Bcl-2 is an anti-death gene that functions as an intracellular antioxidant. Recent analysis of the Bcl-2 gene family reveals an intricate network that regulates apoptosis. Within this Bcl-2 gene family, some members suppress apoptosis while others can induce apoptosis (158). Among the proteins coded by the genes of this family, Bcl-2 and Bcl-x_L act as cell death repressors (159), while Bax and an alternatively sliced Bcl-x product, Bcl-xs, promote cell death (160). When in excess of Bcl-2, Bax counteracts the repressive action of Bcl-2 on apoptosis. Likewise, excess Bcl-xs antagonizes the function of BCL-x_L.

Therefore, a critical balance between the Bcl-2, Bax, and Bcl-$x_{L/S}$ molecules may determine the fate of cells in response to cytotoxic agents, or environmental or oxidative stress. It has also been reported that p53 activates the transcription of the Bax gene via p53-response elements while downregulating the expression of Bcl-2 (161).

Overexpression of Bcl-2 can block apoptosis initiated by several stimuli. For instance, the activation of Bcl-2 was associated with the inhibition of apoptosis in the adapted myocardium (162, 163). By inducing Bcl-2 expression and reducing Bax expression, preconditioning converts the death signal triggered by ischemia/reperfusion (which decreases Bcl-2 and increases Bax) into survival signal.

c. PKC signaling

PKC is a family of at least 11 phospholipid-dependent serine-threonine kinases. There is enough evidence that a variety of PKC isoforms also act as major modulators of the myocyte death machinery, having both pro- and anti-apoptotic effects. A great majority of these have been identified in the mammalian heart (164-166). PKC are susceptible to redox regulation and various antioxidants can inhibit PKC-dependent cellular responses. Both pro-survival and apoptotic functions for PKC during oxidative stress have been described (171-182). The 11 enzymes of the PKC family are often broken

down into three distinct subgroups: classical PKCs (α, βI, βII, γ), which are activated by calcium and diacylglycerol (DAG); novel PKCs (δ, ϵ, η, θ), which are activated by DAG but not calcium; and finally the atypical PKCs (λ/ι, ζ), which are insensitive to both DAG and calcium, but are activated by distinct lipids (164). The classical pathway for PKC activation is by G-protein coupled receptors, which through hydrolysis of phospholipids elevate intracellular levels of DAG and calcium (164). However, PKC activation is not limited to seven transmembrane receptors, as growth factor receptors, nitric oxide, and ROS can also activate select PKC isoforms in the myocardium (167-169). PKC activation is associated with translocation to different subcellular compartments, mediated by interaction of the kinases with proteins termed as receptors for activated C kinase or RACKs (170). The discovery that each PKC isoform binds to its own specific RACK has proved extremely useful as it has allowed the generation of small peptides that either facilitate or abrogate this interaction, and therefore the activation of individual PKC isoforms is possible.

Select PKC isoforms have been shown to regulate cardiac hypertrophy, calcium handling, and contractile protein function. In this regard, most attention has focused upon two PKC isoforms, specifically PKC δ and PKC ϵ, which although exhibiting very similar structures, have disparate effects on myocyte survival and death. Studies in non-cardiac cell lines have demonstrated a pro-death action for PKC δ (171), and the same seems to be

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true in cardiac myocytes. In one study (172), it has been demonstrated that adenoviral infection with constitutively active PKC δ, but not PKC ε, directly induced apoptosis in rat ventricular myocytes. Consistent with this observation, inhibition of PKC δ translocation attenuated hyperglycemia-induced DNA laddering and TUNEL (Terminal deoxy nucleotidyl transferase Biotin-dUTP Nick End Labeling) staining (173). Therefore activation of PKC δ appears to contribute to ischemic injury in cardiac myocytes. For example, using small peptides that specifically regulate PKC δ, it has been demonstrated that activation of PKC δ exacerbates ischemia-reperfusion-induced injury in rat myocytes (174), whereas inhibition of PKC δ translocation blocks ischemia-induced apoptosis in isolated rat heart (175). A similar study reported that PKC δ inhibition blocked simulated ischemia-induced mitochondrial permeability transition and apoptosis (176). Conversely, PKC ε appears to exert an anti-apoptotic effect in myocytes. Inhibition of PKC ε translocation attenuated both hypoxia and phorbol-induced protection against simulated ischemia in neonatal myocytes (177). Likewise, activation of PKC ε was associated with the ability of ischemic preconditioning to block ischemia-induced apoptosis (178). Therefore the majority of the *in vitro* data implicate PKC δ as being pro-apoptotic and PKCε as being anti-apoptotic in myocytes, especially in the context of ischemia-reperfusion injury.

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The hypothesis that PKC δ is pro-apoptotic while PKC ε is anti-apoptotic is also supported by a number of *in vivo* reports. Intra-coronary administration of a PKC δ translocation inhibitor peptide was able to significantly attenuate ischemia-reperfusion-induced infarction and caspase-3 cleavage in the pig (179). Transgenic expression of the same PKC δ inhibitory peptide also blocked ischemia-reperfusion-induced injury in the mouse, whereas overexpression of a PKC δ activator peptide augmented ischemic injury (174,180). In contrast, transgenic expression of a PKC ε activator peptide was able to blunt apoptosis and therefore heart failure in Gα$_q$-overexpressing transgenic mice (181), while expression of a PKC ε inhibitory peptide had the opposite effect. Moreover, transgene-mediated activation of PKC ε protected the myocardium against ischemia-reperfusion injury (174,182).

The potential mechanisms by which two such structurally similar kinases could have opposing effects on the apoptotic process are still unclear. One possibility is that signal specificity lies in the signaling events downstream of each PKC isoform. For example, in myocytes, adenoviral PKC δ was found to selectively activate the JNK and p38 MAPK pathways, whereas PKC ε activated the ERK pathway (172). Given the proposed pro-apoptotic actions of JNK/p38 versus the anti-apoptotic actions of ERK, such differential signaling may account for the different survival/death effects of each PKC isoform.

Another potential protective mechanism was suggested by the observation that PKCε-dependent cardioprotection was associated with activation of the anti-apoptotic kinase Akt (183). Differences may also exist as to how each kinase might affect members of the pro-death and pro-survival pathways that directly impact the mitochondria. Both PKC δ and PKC ε have been localized to the mitochondria in the heart (184, 183).

Indeed, translocation of PKC δ to the mitochondrion has been shown to be essential for apoptosis in a variety of cell lines and is associated with mitochondrial permeability transition and activation of the mitochondrial death pathway (171, 176, 185, 186). In contrast, PKC ε can prevent mitochondrial-mediated cell death (187, 188) through a mechanism that appears to involve association with and inhibition of mitochondrial pore opening, and is associated with protection against ischemic injury in mice expressing active PKC ε (189).

Whether each PKC isoform can directly affect the pore itself, potentially through direct phosphorylation, or whether it is through secondary effectors such as JNK or ERK remains to be tested. The ability of PKC isoforms to affect mitochondrial-dependent apoptosis may also be related to modulation of Bcl-2-family proteins. PKC ε has been reported to bind to and inhibit the pro-apoptotic protein Bax (187), and PKC ε transgenesis is associated with phosphorylation of Bad (184). On the other hand, ischemia-reperfusion-

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induced dephosphorylation, and presumably activation of Bad was prevented by PKC δ inhibition (175).

In summary, according to recent investigations, specific mechanisms have been identified whereby PKC δ and PKC ε might antithetically regulate cardiac myocyte apoptosis.

D. Phospholipase C isozymes

a. Introduction

PLC is a modular monofunctional enzyme, which is involved in numerous transmembranal signals (190). Its most common physiological substrate, PIP_2, is synthesized in the SL membrane by the coordinated and successive action of two key enzymes, phosphatidylinositol 4 kinase and phosphatidylinositol phosphate 5 kinase. PIP_2 is converted into two messenger molecules, IP_3 and DAG, which participate in many different physiological signaling processes (190). PLC enzymes have been characterized into four immunologically distinct PLC superfamilies, designated β, δ, γ and ε, that are expressed in adult ventricular cardiomyocytes (191, 192). The β, γ and δ families have been well characterized but there is some controversy over the ε families. PLCs of the β, γ, and δ classes display differences in terms of structure, activating mechanisms and functions (193). The diversity in primary structure together with different regional and cellular expression of the

isozymes suggests that each isozyme has a defined function in processing the physiological response to different cell types to a variety of external stimuli (191). For example, the mechanisms of recruitment to the plasma membrane also differ among the different PLC classes (194).

PLC γ is cytosolic and is activated by growth factor receptor tyrosine kinases. Binding of polypeptide growth factors (195) to their receptors with intrinsic or associated tyrosine kinase activates PLC γ and PLC β isoenzymes (190). Tyrosine kinases can activate a number of different intracellular signaling pathways, including tyrosine phosphorylation in the case of PLC and PLD (196). Binding of polypeptide growth factors to their receptors with intrinsic or associated tyrosine kinase activity activates PLC γ. A non-tyrosine-kinase mediated activation as well as G protein coupled receptor via non-receptor tyrosine kinase activation of PLC γ isoenzymes has also been reported (197). Phosphatidic acid was found to stimulate SL PLC γ_1 as well as PIP_3; however, PIP_3 cannot stimulate PLC β_1 and δ_1 isoenzymes (193, 198). Defects in the thiol 2 and 3 domains of PLC $\gamma1$ may impair the enzymes association with and phosphorylation by activated growth factor receptors and its subsequent localization to the cytoskeleton. This exemplifies the irreplaceable role of PLC γ_1 in mammalian growth and development.

Activation of PLC generates various lipid-derived second messengers such as IP_3 and sn-1,2 DAG. IP_3 causes Ca^{2+} release from intracellular Ca^{2+}

storage sites, and DAG activates PKC, which plays an important role in stretch-induced immediate early gene expression, such as c-*fos* and Erg-1 (199). Recently it has been reported that PI-PLC activity increases in cardiomyopathic hamster hearts and SHR (200-203). PLC-dependent PIP_2 breakdown in the SL of failing hearts is unknown. However, it is known that the two signaling molecules (IP_3 and DAG) produced by the activation of PLC are critical in increasing contractile force development, and it is likely that changes in PLC may be responsible in altering cardiac contractile force in coronary heart failure (204).

b. Ischemia-Reperfusion Induced Changes in Phospholipase C isozymes

At present, a few studies have reported alterations in the metabolism of the inositol phospholipids in the setting of myocardial ischemia-reperfusion. It has been reported that a 30 min global ischemia results in a decrease in IP_3, whereas reperfusion results in a rapid increase in the release of IP_3. This observation was inferred from assessing the inositol phosphate content of whole ventricular tissue. On the other hand, an increase in IP_3 has been reported in both ischemia and reperfusion (205). These contrasting findings could be due to differences in ^{3}H-inositol labeling of cardiac membranes, which is questionable given the fact that distinct membrane phospholipid pools and microenvironments exist. Ischemia has also been shown to elicit an α_1

adrenoceptor mediated increase in the mass of total myocardial DAG, and was suggested to be due to activation of myocardial PLC. A role for PLC is further suggested through the observation that the reperfusion as well as norepinephrine-induced rise in IP_3 could be prevented by neomycin, a PLC blocker. I-R induced activation of PLC activity has been evidenced; however, this was observed in a microsomal fraction isolated from whole ventricular tissue (205). Other investigators have reported a decrease in PLC activity in ischemia and an increase in reperfusion (206); however, this was conducted in a total membrane preparation from whole ventricular tissue. Similarly, although a recent study has reported that myocardial ischemia induces an increase in PLC activity, measurement of the activity was conducted in left ventricular (LV) total homogenate. Clearly, studies that do not use pure SL membrane preparations risk conflict from combining many subcellular organelles (SL, SR, mitochondria and nuclei), which may have distinct or unique PLC pathways (207). Although exposing SL membranes to oxidants results in a derangement of the PLC signaling pathway, very little information is available with respect to the mechanisms responsible for the I-R induced changes in PLC isozymes as well as their functional consequences.

Recently, (208) work from our laboratory has shown that I-R induces differential changes in PLC isozymes. I-R was associated with marked increases in sarcolemmal PLC γ_1 and PLC δ_1 activities, with peak activation occurring at 1 and 5 min of reperfusion, (253% and 200%, respectively). Also,

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recently a role for PLC γ_1 in protection against oxidative stress induced damage in mouse embryonic fibroblasts has been reported. In addition, the effect on PLC in hearts perfused with H_2O_2, peroxynitrite or HOCl, which are major oxidants in the myocardium, is not known.

c. PLC- γ_1 signaling

PLC γ_1 and PLC γ_2 are essential components of a growth factor receptor-mediated signaling pathway that is activated in response to oxidant injury. PLC γ_1 and PLC γ_2 belong to the phospholipase C family. All of the PLC isozymes catalyze the hydrolysis of PIP_2 to IP_3 and DAG, which act as intracellular second messengers to mobilize Ca^{2+} and activation of protein kinase C, respectively (209, 210). However, only the PLC γ subtypes of PLC isozymes are activated by the receptor tyrosine kinases while others are activated by a variety of other agonists including various hormones. While the PLC γ_2 isoform is expressed selectively in hematopoietic cells, the PLC γ_1 form is ubiquitously expressed. PLC γ_1 and PLC γ_2 are substrates for tyrosine kinases and tyrosine phosphorylation is an essential step in their activation. Present in the cytoplasm of unstimulated cells, growth factor stimulation results in the translocation of the PLC γ_1 and PLC γ_2 isoforms to the membrane, allowing their interacion with and phosphorylation by receptor and non-receptor tyrosine kinases.

A number of studies have demonstrated that PLC γ_1 undergoes phosphorylation in response to treatment with H_2O_2 (211-213) but until now the mechanisms involved in the activation process, as well as their functional significance, have been unclear. In most studies, phosphorylation of PLC γ_1 either required or was enhanced by co-treatment of cells with vanadate, suggesting that oxidant-mediated inactivation of a phosphatase contributed to the effect. Studies using pharmacologic inhibitors of various kinases in mouse embryonic fibroblasts (MEF) have implicated both Src family tyrosine kinases and Epithelial growth factor receptor (EGFR) tyrosine kinase in hydrogen peroxide-stimulated PLC γ_1 phosphorylation (214).

Only a few studies have addressed the functional consequences of PLC γ_1 phosphorylation during oxidative stress. A study that employed PC12 cells in which PLC γ_1 was overexpressed suggested that elevated PLC γ_1 expression suppressed UVC-induced apoptosis (215). However, a second study by the same people found no protective effect of PLC γ_1 overexpression in NIH3T3 cells subjected to several different oxidative stress-inducing agents (i.e., hydrogen peroxide, tert-butylhydroperoxide, and cadmium chloride) (216), although in that study PLCβ1 exerted a protective effect. In another study it has been observed that MEF derived from mice rendered deficient for PLC γ_1 by targeted disruption of both *plc-gamma1* alleles are much more sensitive to hydrogen peroxide treatment relative to normal fibroblasts (214). In the same

study reconstitution of PLC γ_1 protein expression in PLC γ_1 deficient MEF restored cell survival following hydrogen peroxide treatment, thus suggesting a protective function of PLC γ_1 activation during cellular response to oxidative stress. A study done in human intestinal epithelial (Caco-2) cells showed that EGF treatment protected the cells against oxidants through PLC γ_1 dependent signaling (217). Further studies will be needed to address the extrapolation of this effect for other cell types and to identify the downstream targets involved in mediating the protective effects.

There is no information in literature about the effect of ROS specifically H_2O_2 on PLC γ_1 in isolated adult cardiomyocytes and the related survival signaling (Scheme 2). There is also no information about the effect of fatty acid incorporation in the phospholipids membrane and its effect on PLC γ_1 signaling. These are some of the questions that this research project has tried to investigate and have been summarized in scheme 2.

Scheme2. Proposed redox signaling pathway:
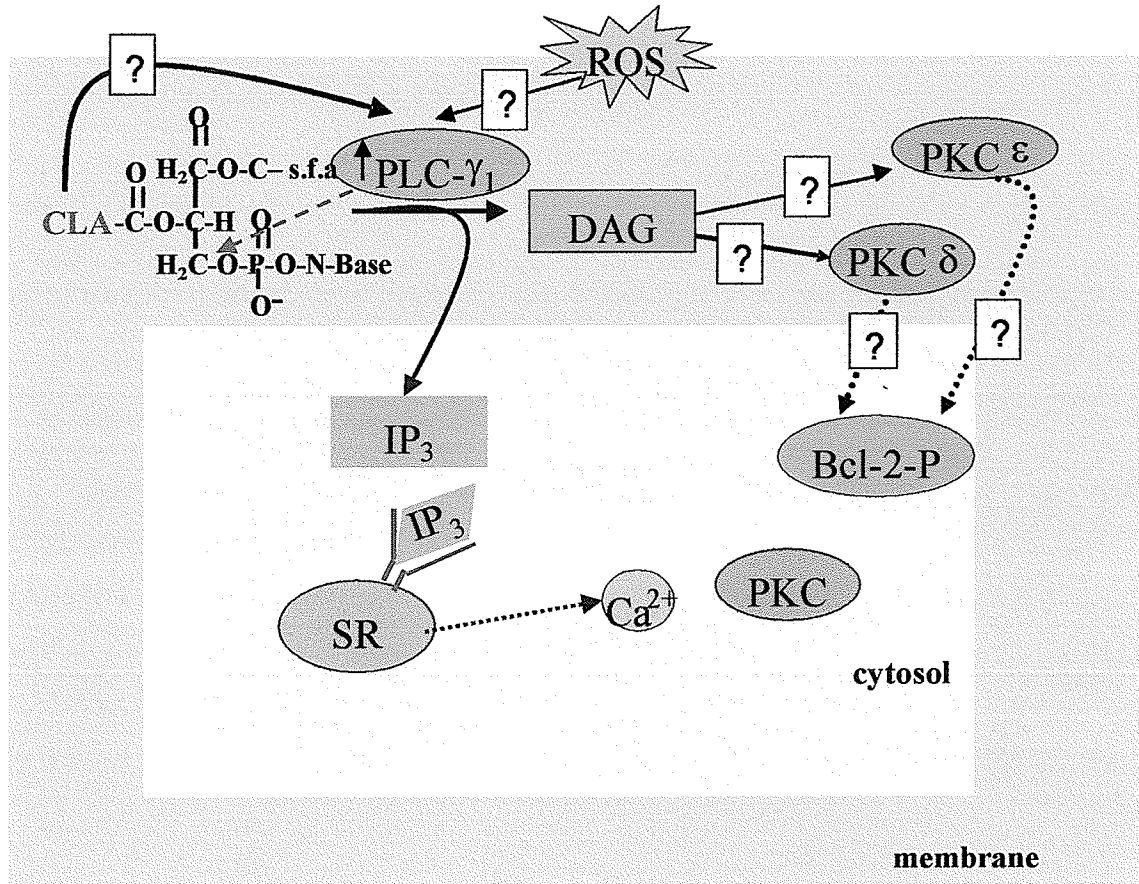
d. Effects of fatty acid incorporation in cardiomyocytes

PUFAs have structural roles in phospholipids of all cell membranes in the body, influencing membrane viscosity and permeability and, associated herewith, the function of membrane proteins. A common mechanism of n-3 and n-6 PUFAs in these biological responses may be by interference with the functioning of the phosphatidylinositol (PI) cycle. The PI cycle is a signaling

pathway involved in receptor-mediated biological responses such as platelet aggregation, smooth muscle contraction, and formation of relaxing and contracting factors by endothelial cells. The PI cycle involves the receptor-mediated activation phospholipase C-β (PLC β) and phospholipase C-γ (PLC γ), resulting in the formation of the Ca^{2+}-releasing compound IP_3 and DAG, the activator of the PKC isoenzymes (218).

It has been reported that cultured cardiomyocytes that were pretreated with linoleic acid or eicosapentaenoic acid (EPA) showed less PLC-β activity in response to α_1-adrenoceptor stimulation (219). In another study it was shown that feeding rats with n-3 and n-6 PUFAs caused a depression of total release of inositol phosphates in left atrial tissue in the presence or absence of norepinephrine (220). Accordingly, it has been demonstrated that dietary n-6 and n-3 PUFAs attenuated the α_1-adrenergic-mediated positive inotropy in perfused rat hearts. It has also been shown that dietary n-6 and n-3 PUFAs could prevent or terminate isoproterenol-induced arryhthmias (221).

Modification of the fatty acid composition of the membrane phospholipids can influence many steps of the PtdIns signaling cascade. It has been shown that incorporation of n-3 and n-6 PUFAs in the membrane phospholipids was associated with a decreased affinity of the α_1-adrenoceptors for their antagonist ligand in heart muscle (222). Furthermore, the function of PKC isozymes may also be affected by either free PUFAs or by alteration of

the molecular species of 1,2 DAG due to changed fatty acid composition of the phospholipids source (223).

We need to learn more about the effects of the incorporation of n-3 and n-6 PUFAs in membrane phospholipids on the structure and function of the SL, in terms of the various steps of the transmembrane signaling by the PI cycle.

III. STATEMENT OF THE PROBLEM

Objective 1: To examine PLC isozymes in isolated cardiomyocytes treated with an oxidant H_2O_2 .

Hypothesis 1: Hydrogen peroxide activates PLC γ_1 in cardiomyocytes and mediates cardiomyocyte survival via PKC and Bcl-2 activation.

To test this hypothesis isolated adult cardiomyocytes will be treated with H_2O_2 (20, 50, 100 μM) for 15 minutes in the presence and absence of catalase and changes in the PLC isozyme mRNA level, protein content and activities will be determined. To determine the significance of PLC γ_1 activation, cardiomyocyte-survival pathway will be examined by PKC activation, Bcl-2 phosphorylation and cell viability by trypan blue exclusion method.

Objective 2: To examine the role of CLA on PLC γ_1 response to oxidative stress.

Hypothesis 2: The c9,t11 CLA and t10,c12 CLA isomers augments the PLC γ_1 response to hydrogen peroxide.

To test this hypothesis isolated adult ventricular cardiomyocytes will be treated with different concentrations of H_2O_2 (20, 50, 100 μM) in the presence of CLA isomers cis-9, trans-11 CLA and trans-10, cis-12 CLA (5, 50, 100 μM).

IV. MATERIALS AND METHODS

A. Materials

PLC γ_1 and Bcl-2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). PKC δ and PKC ϵ antibodies were from Santa Cruz Biotechnology (California, USA). Secondary antibodies: Goat anti-mouse and Goat anti-rabbit, IgG (H + L)–HRP conjugate, blotting grade affinity purified, TEMED-N,N,N,N'-tetramethylethylenediamine, ammonium persulfate, 30% acrylamide/bis solution were obtained from BioRad Labs (Hercules, CA. USA).

Benchmark prestained protein ladder was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Biosciences (Little Chalfont Buckinghamshire, England).

Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Medium M199 and Penicillin/Streptomycin were purchased from Gibco (Grand Island, NY, USA).

CLA isomers were a gift from Lipid Nutrition, a division of Loders Croklaan (Channon, IL, USA). The CLA isomers were 84% t10,c12 plus 16% c9,t11 CLA or 84% c9,t11 plus 16% t10,c12 CLA.

Access RT-PCR kits and Trizol reagent were purchased from Promega Corp. (Madison, WI, USA). Protein G sepharose 4 fast flow was purchased

from Amersham Pharmacia Biotech (Uppsala, Sweden). Instant skim milk powder was a product of Nestle Carnation (North York, ON, USA). All other solvents used were purchased from Fischer Biotech, (Fair Lawn, NJ, USA) and all chemicals were purchased from SIGMA-ALDRICH Co. (St. Louis, MO, USA). All the reagents were of analytical grade or of the highest grade available.

B. METHODS

Cardiomyocyte isolation

After proper anesthesia, the heart is rapidly excised and cannulated immediately by the aorta on the Langendorff. After five minutes of preliminary perfusion at 37.5° C (to washout the blood and stabilize the heart) with calcium free Krebs- Henseleit bicarbonate buffer gassed with 95%O_2; 5%CO_2 (without recirculation), the atria are trimmed and the heart is perfused with Collagenase at a concentration 1mg/ml, dissolved in Krebs Henseleit bicarbonate buffer with 0.1% Bovine Serum Albumin (BSA) and 25μM $CaCl_2$ and gassed with 95%O_2; 5% CO_2. When the digestion is complete (when heart becomes soft), the heart is removed and placed in Krebs Henseleit bicarbonate buffer with 0.1% BSA and 25μM $CaCl_2$.

The heart is then dissolved using foreceps and pippeting the tissue suspension. Cell suspension is collected in a 50 mL tube and centrifuged at 200 rpm for 2 minutes. The supernatant containing dead cells is removed and the cell pellet is resuspended first in Krebs buffer containing 1%BSA and

50 µM $CaCl_2$, 1%BSA and 200 µM $CaCl_2$ for the second time, and 1%BSA and 500 µM $CaCl_2$ for the third time. Each time it is centrifuged at 100 rpm for 2 min. Finally the cell pellet is resuspended in a Krebs buffer containing 4% BSA and 1mM $CaCl_2$ and centrifuged at 200 rpm for 2 min.

Finally the cell pellet is resuspended in a certain amount (according to the number of plates coated) of medium M199 containing 0.2%BSA and 4% fetal calf serum and 1 ml Penicillin/Streptomycin. It is then plated out in the laminin coated dishes which were coated at least 30 minutes before with laminin dissolved in medium M199. And finally they are incubated in a humidified incubator. After an hour the medium is changed to M199 containing 0.2% BSA and 4% fetal calf serum and the cells are left for overnight incubation in the humidified incubator (232).

Cardiomyocyte treatment and fractionation

After 24 hrs the cells are treated with different concentrations of H_2O_2 (20, 50,100µM). The cells are be incubated for 15 minutes. After the incubation is completed, the medium is removed by aspiration and the dishes are placed on ice. Cardiomyocytes are scraped off in 2 ml of phosphate-buffered saline (PBS) and collected by centrifugation at $27g$ for 1 minute. Briefly, cells are homogenized in 1 ml of the above-mentioned buffer using a glass homogenizer, followed by centrifugation at 280 000 $\times g$ for 25 min. The resultant pellet (total membranes) is resuspended and homogenized in Pitt's 4

solution (250mM sucrose, 10mM histidine) and stored at -80°C until use. Protein concentrations are determined by the Lowry method (192).

Rationale for various treatments given to cardiomyocytes

To simulate conditions of I-R in vitro the cardiomyocytes were treated with increasing concentrations of H_2O_2 i.e 20, 50, and 100 µM. These concentrations employed in our study are compatible with those detected in vivo during myocardial I-R (236). Although other oxidant molecules are generated during I-R, such as HOCl, superoxide anion and peroxynitrite, H_2O_2 was chosen for study because it is readily available and easy to handle.

The myocytes were incubated overnight with different concentrations of CLA isomers. They were incubated with 5, 50 and 100 µM of t10,c12 and c9,t11 CLA isomers. Except the study (67), in which in vitro antiarrhythmic effects of PUFA's were being studied on cardiomyocytes, there are no other studies with CLA and cardiomyocytes. In this study CLA was used at a concentration of 5 µM. In other in vitro studies involving cancer cells (250) and CLA it has been used at 250 to 100 µM range. There is evidence in literature that when myocytes are incubated overnight with different fatty acids in the culture medium in ethanol vehicle, they get around 10 times enriched in the heart lipids as compared to controls.

Solubilisation of membrane proteins and immunoprecipitation

Membrane proteins are extracted from the membrane, by sonication (probe sonicator for 10secs at amplitude of 80Hz) in a buffer containing 1% w/v sodium cholate, 50 mmol/L HEPES (pH 7.2), 200 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L PMSF, and 10 μg/mL each of aprotinin, soya bean trypsin inhibitor, and leupeptin and further agitated by rotation in the same buffer for 2 h at 4°C. The sample is then centrifuged (280 000 × g for 25 min) and the supernatant recovered as the solubilized membrane fraction. Protein concentrations will be determined by the Lowry method of protein assay according to the manufacturer's instructions (192).

Solubilized membrane proteins are incubated overnight at 4°C with monoclonal antibodies to phosphotyrosyl residues (25 μl of the antibody per 850 μg of membrane protein). The immunocomplex is captured with 100 μL (50 μL packed beads) of washed (3 times with 30 mM HEPES; pH 6.8) protein-G-sepharose slurry at 4°C by rotation for 2 h. The beads are collected by pulse centrifugation (5 s) at 10 000 × g, washed with HEPES buffer and then used for determination of phosphotyrosyl PLC γ_1 by western blotting using monoclonal antibodies against PLCγ_1.

RNA isolation and semi-quantitative PCR

Total RNA was isolated from LV cardiomyocytes using RNA isolation kit (Life Technologies, ON, Canada) according to the manufacturer's

procedures. Reverse transcription (RT) was conducted for 45 min at 48 ^{0}C using the Superscript Preamplification System for first strand cDNA synthesis (Life Technology, ON, Canada) as previously described (208, 231). Primers used for amplification were synthesized as follows:

PLC γ_1: 5'- CCTCTATGGAATGGAATTCCG-3' (forward) and 5'-CTAGGGAGGACTCGCTGGAGAACT-3' (reverse). Temperatures used for PCR were as follows: denaturation at 94 ^{0}C for 30 s, annealing at 62 ^{0}C for 60 s, and extension at 68 ^{0}C for 120 s, with a final extension for 7 min; 25 amplification cycles for each individual primer sets was carried out. For the purpose of normalization of the data, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5'-TGAAGGTCGGTGTCAACGGAT TTG-3' (forward) and 5'-GCATGTCAGATCCACAACGGATAC-3' (reverse) were used to amplify GAPDH gene as a multiplex with the target genes. The PCR products were analyzed by electrophoresis in 2% agarose gels. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) as a ratio of a target gene over GAPDH.

Western blot of PLC isozymes

High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 20 µg total membrane proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described. (208, 231) Separated proteins were transferred onto 0.45-µm polyvinylidene

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difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with mouse monoclonal primary PLC γ_1 antibody (Upstate Biotechnology, NY, USA). Primary PLC γ_1 antibodies were diluted in TBS-T (1:2000 according to the manufacturer's instructions). Horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad, CA, USA) was diluted 1:3000 in TBS-T and used as a secondary antibody. PLC γ_1 was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim, Laval, PQ).

Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). The linearity of the Western blot procedure used for the quantification of PLC γ_1 has been previously determined. (208) In subsequent blotting experiments 20 μg membrane protein was used because it is in the linear range. The the time of exposure used was 5 min. In some experiments, Western blotting with PLC γ_1 was performed with immunoprecipitated SL phosphotyrosyl proteins as previously described (231). Immunoprecipitation was performed with anti phosphotyrosyl monoclonal antibodies (PY99, Santa Cruz Biotechnology, CA, USA); 5 μg of antibody to 855 μg membrane extract. The solubilization of membrane proteins was conducted as described above.

Assessment of cardiomyocyte viability by trypan exclusion

For cell survival assays, cardiomyocytes were pretreated with the PLC inhibitor, U73122 (0.5, 1 and 2 μM) for 30 min and then exposed to H_2O_2 (50 μM) for 15 min. Following treatment, cardiomyocytes were harvested and stained with 0.25% trypan blue for 2 min and live cells were counted using a hemocytometer as described elsewhere (214). The percentage of viable cardiomyocytes in the treated cells was determined from cell counts in treated cardiomyocytes divided by the number of cardiomyocyte counts in untreated cells. The reduction in the number of viable cardiomyocytes reflected cell death (214).

Statistical analysis

All values are expressed as mean ± SEM. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. A probability of 95% or more ($P<0.05$) was considered significant.

V. RESULTS

A. Effect of H_2O_2 on PLC γ_1 in cardiomyocytes

A concentration-dependent increase in the PLC γ_1 mRNA level and protein content were observed in cardiomyocytes treated with H_2O_2 (20, 50 and 100μM) for 15 minutes (Figures 1 and 2). To examine whether H_2O_2 induced an activation of PLC γ_1, tyrosine phosphorylation of PLC γ_1 was also determined by immunoblotting. It can be seen in Figure 3 that there is a H_2O_2-dependent increase in phosphotyrosyl-PLC γ_1 protein content in the cardiomyocyte membrane fraction, when cells were treated with 20, 50 and 100μM of H_2O_2 with a peak effect occurring at 50 μM H_2O_2. To examine whether the observed changes were due to H_2O_2, the same experiment was performed with catalase (243U) being added simultaneously with H_2O_2 (Figure 4). The increase in phosphorylation of PLC γ_1 tyrosine residues, which was observed in the presence of H_2O_2, was completely attenuated.
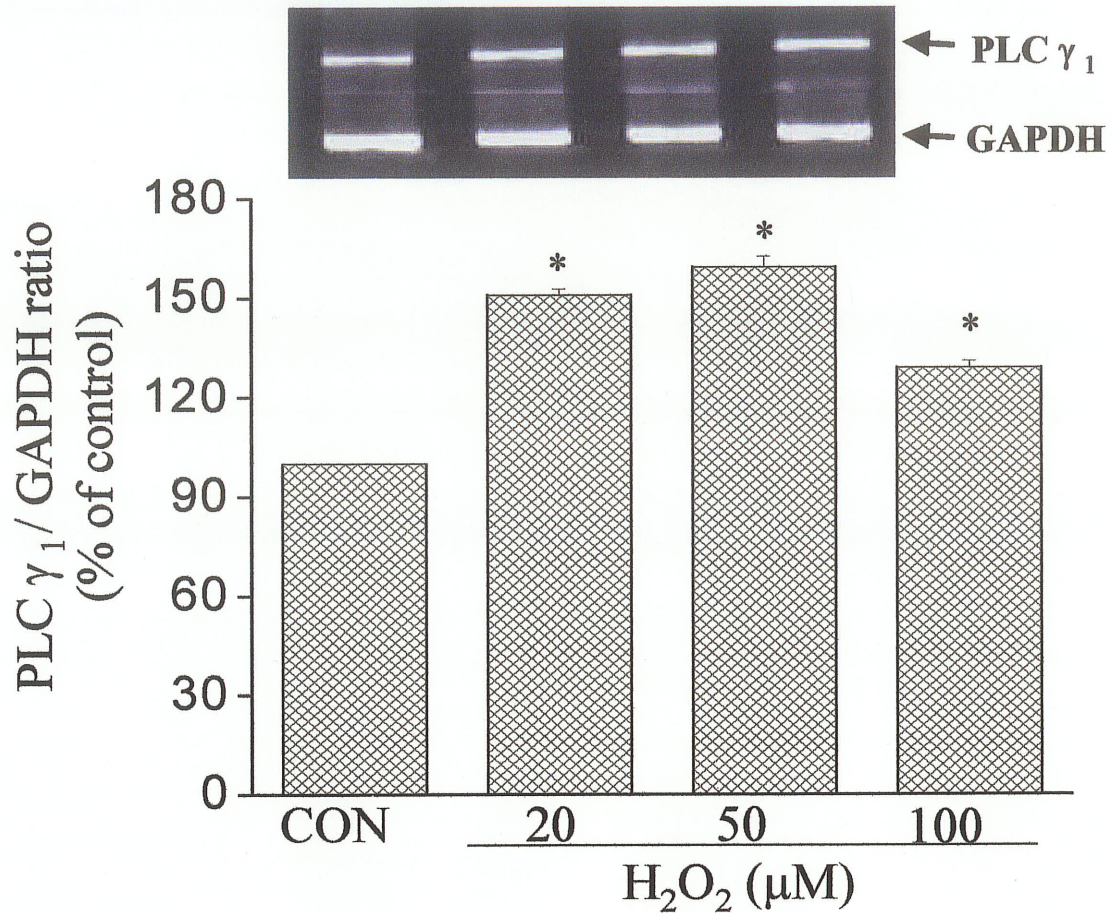
Figure 1. **Phospholipase C γ_1 mRNA levels in cardiomyocytes treated with hydrogen peroxide.**

Representative blot showing (arrow) the PLC γ_1 mRNA (724 b.p.) level and ratio (% of control) *vs.* GAPDH (800 b.p.). Isolated cardiomyocytes were treated with H_2O_2 (20, 50 and 100 µM) for 15 min as described in the Materials and Methods. Values are means ± S.E. of 5 experiments. * $P < 0.05$ *vs.* control.
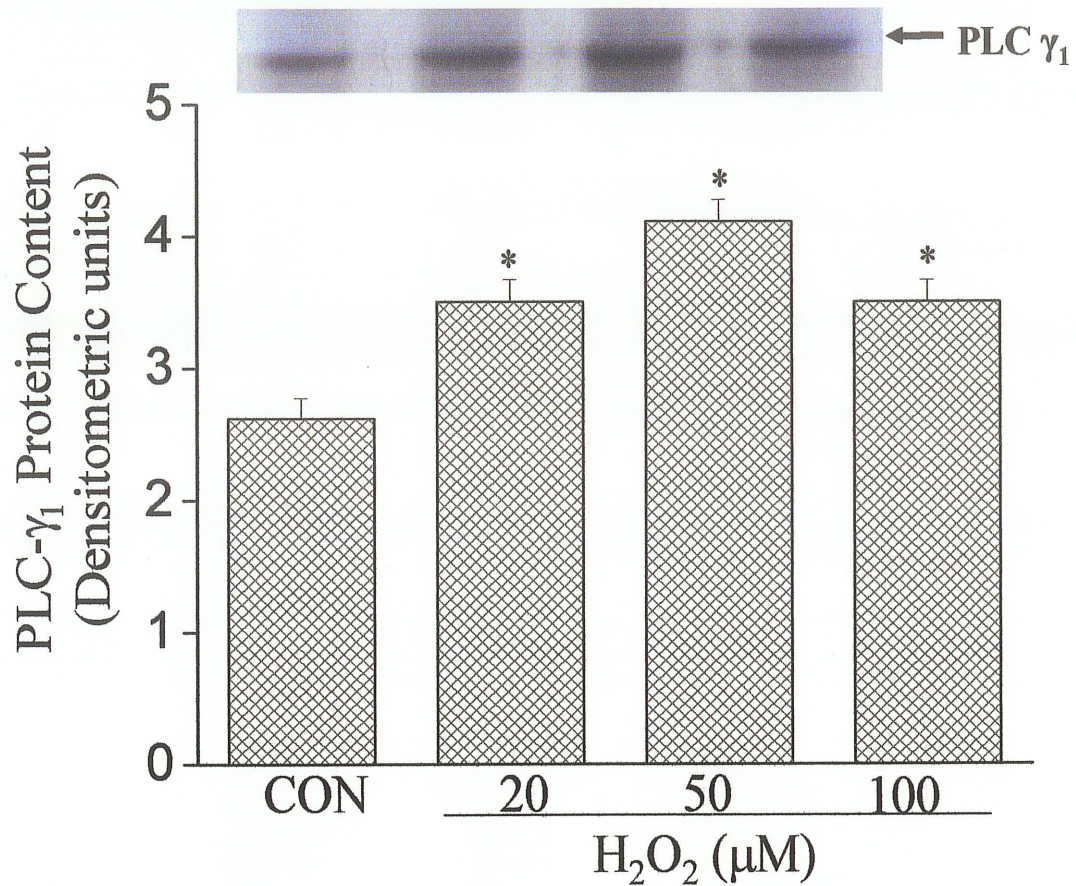
Figure 2. **Phospholipase C γ_1 protein content in cardiomyocytes treated with and without hydrogen peroxide.**

Representative Western blot and quantified data of PLC γ_1 protein content. Western blot shows (arrow) the PLC γ_1 protein (135 kDa). Isolated cardiomyocytes were treated with H_2O_2 (20, 50 and 100 µM) for 15 min as described in the Materials and Methods. Values are means ± S.E. of 5 experiments. $*P < 0.05$ vs. control.
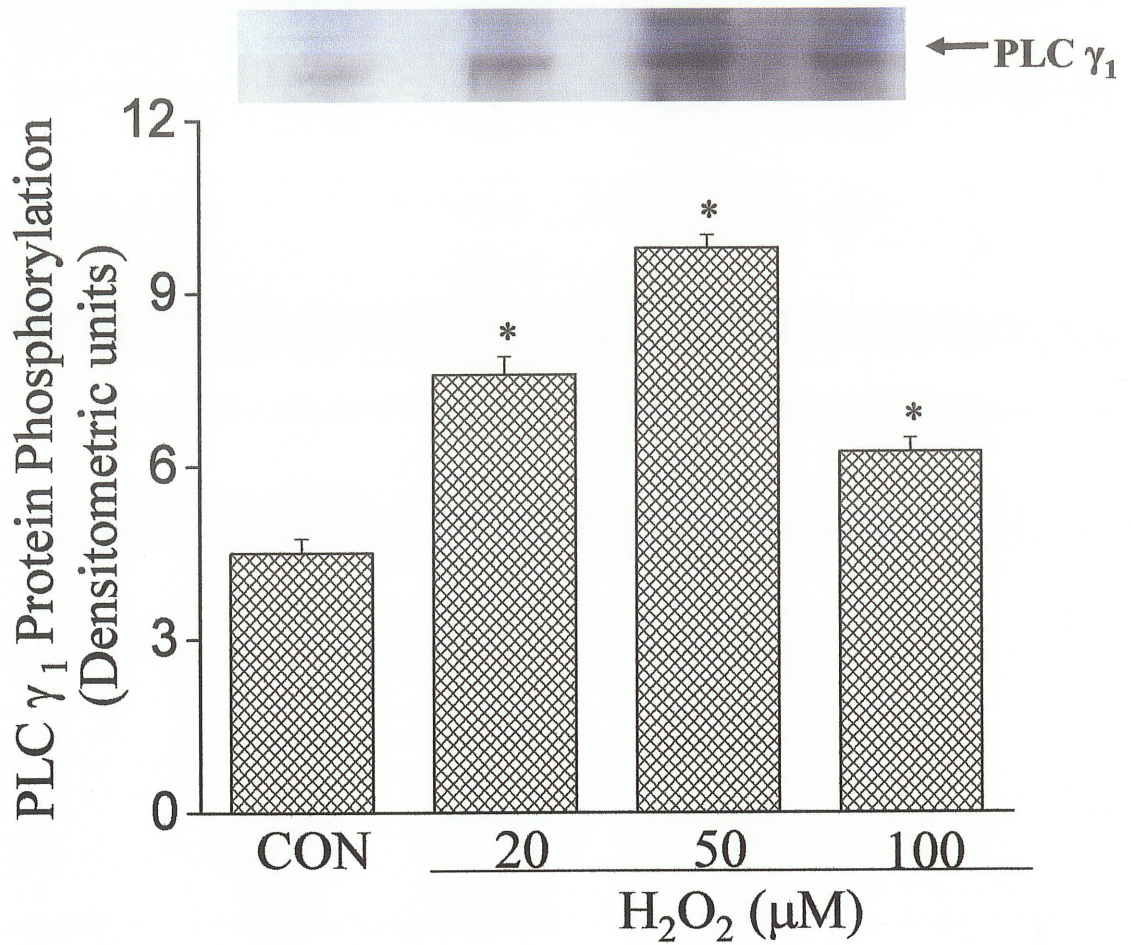
Figure 3. **Phospholipase C γ_1 protein phosphorylation in cardiomyocytes treated with hydrogen peroxide.**

Representative immunoblot and quantified data showing (arrow) phosphotyrosyl-PLC γ_1 protein (135 kDa) content in cardiomyocytes treated with H_2O_2 (20, 50 and 100 μM) for 15 min. $*P < 0.05$ *vs.* control.

Figure 4. **Phospholipase C γ_1 protein phosphorylation in cardiomyocytes treated with hydrogen peroxide in the presence of catalase.**

Representative immunoblot and quantified data showing (arrow) phosphotyrosyl-PLC γ_1 protein (135 kDa) content in cardiomyocytes treated with H_2O_2 (20, 50 and 100 μM) for 15 min in the and presence of catalase. *$P < 0.05$ vs. control.

B. H$_2$O$_2$ induced phosphorylation of Bcl-2 mediated by PLC γ_1

In order to examine the signal transduction processes associated with PLC γ_1 activation as well as to investigate if PLC γ_1 has a protective role during cardiac oxidative stress, the relationship between PLC γ_1 activation and the anti-apoptotic protein, Bcl-2 in cardiomyocytes treated with different concentrations of H$_2$O$_2$ was investigated. It can be seen from Figure 5 that H$_2$O$_2$ induced a concentration-dependent increase in the phosphorylation of Bcl-2 and that this increase was prevented by catalase (Figure 6). Moreover, the phosphorylation of Bcl-2, induced by H$_2$O$_2$, was almost completely prevented by the PLC inhibitor, U73122 (1 μM) (Figure 7). To identify the mediator of the PLC γ_1 signal to phosphorylation of Bcl-2, the activation of PKC isozymes (δ and ε) was investigated in cardiomyocytes treated with H$_2$O$_2$ (50 μM) in the presence and absence of U73122 (1 μM) (Figures 8 and 9). Figure 8 shows that PKC δ protein content was increased in the membrane fraction, isolated from cardiomyocytes after H$_2$O$_2$ treatment, and that this activation of PKC δ was not prevented by U73122 (1 μM). In contrast, while the PKC ε protein content (Figure 9) was increased in the membrane fraction in response to H$_2$O$_2$, this activation of PKC ε was almost completely prevented by U73122 (1 μM).
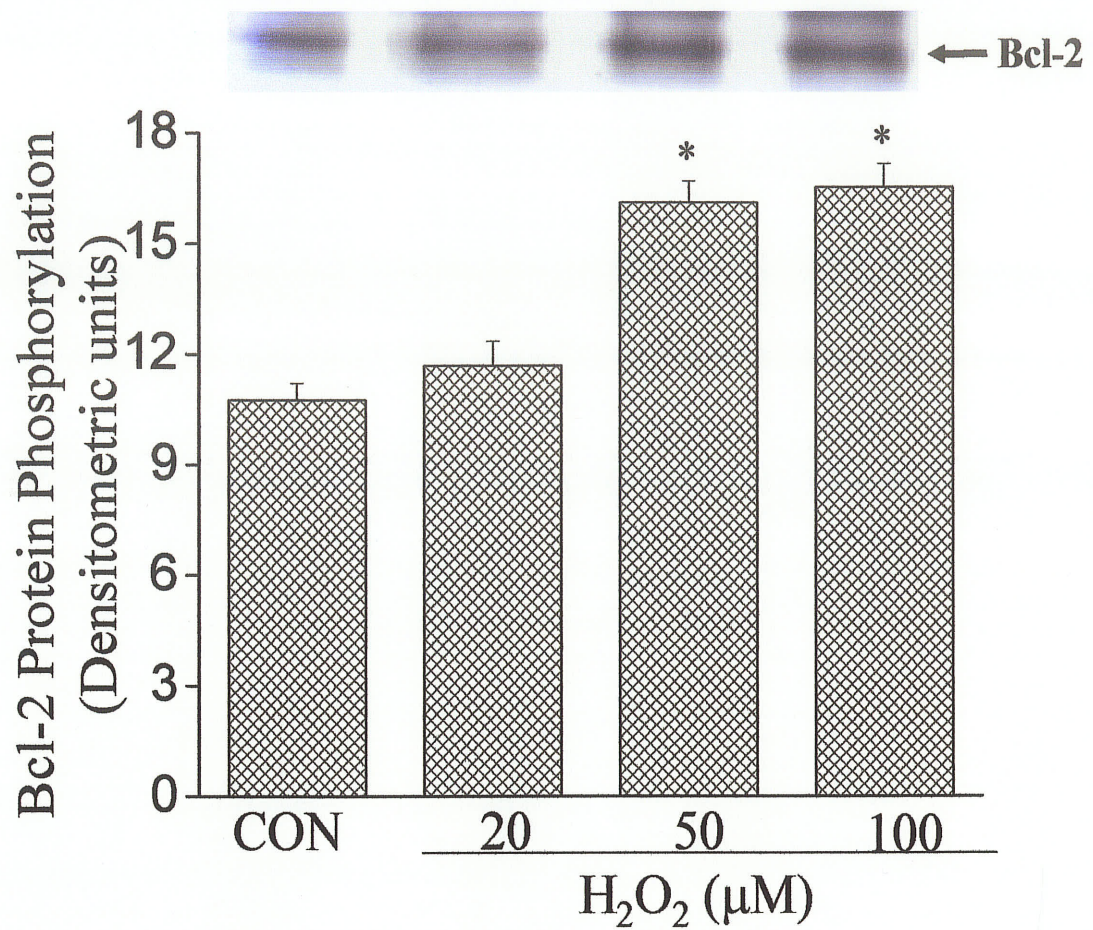
Figure 5. **Bcl-2 protein phosphorylation in cardiomyocytes treated with hydrogen peroxide.**

Quantified data showing phospho-Bcl-2 protein contents in cardiomyocytes treated with H_2O_2 (20, 50 and 100 µM) for 15 min. Values are means ± S.E. of 5 experiments. * P < 0.05 *vs.* control.

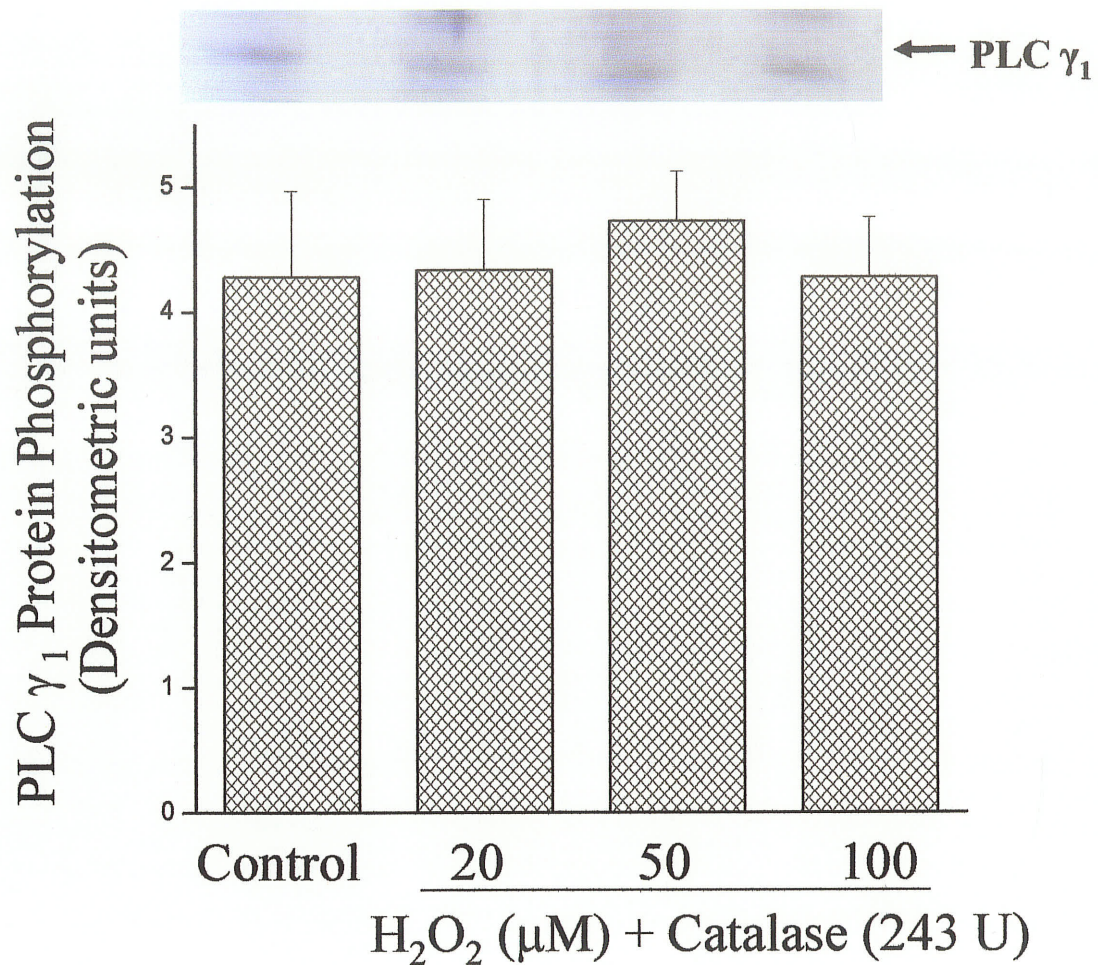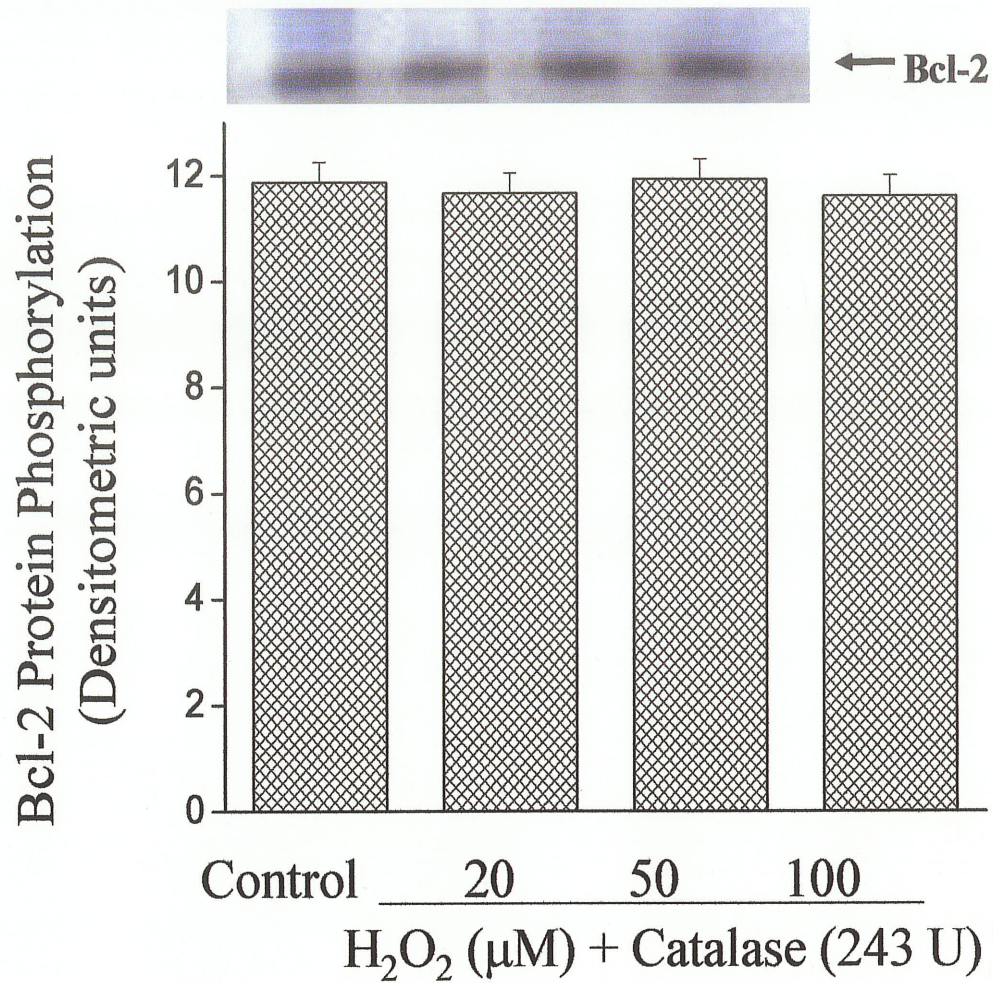**Figure 6.** **Bcl-2 protein phosphorylation in cardiomyocytes treated with hydrogen peroxide in presence of catalase.**

Representative Western blot and quantified data of phophorylated Bcl-2. Western blot shows (arrow) the phosphorylated Bcl-2 protein (28 kDa). Isolated cardiomyocytes were treated with H_2O_2 (20, 50 and 100 μM) for 15 min in the presence of catalase as described in the Materials and Methods. Values are means ± S.E. of 5 experiments.
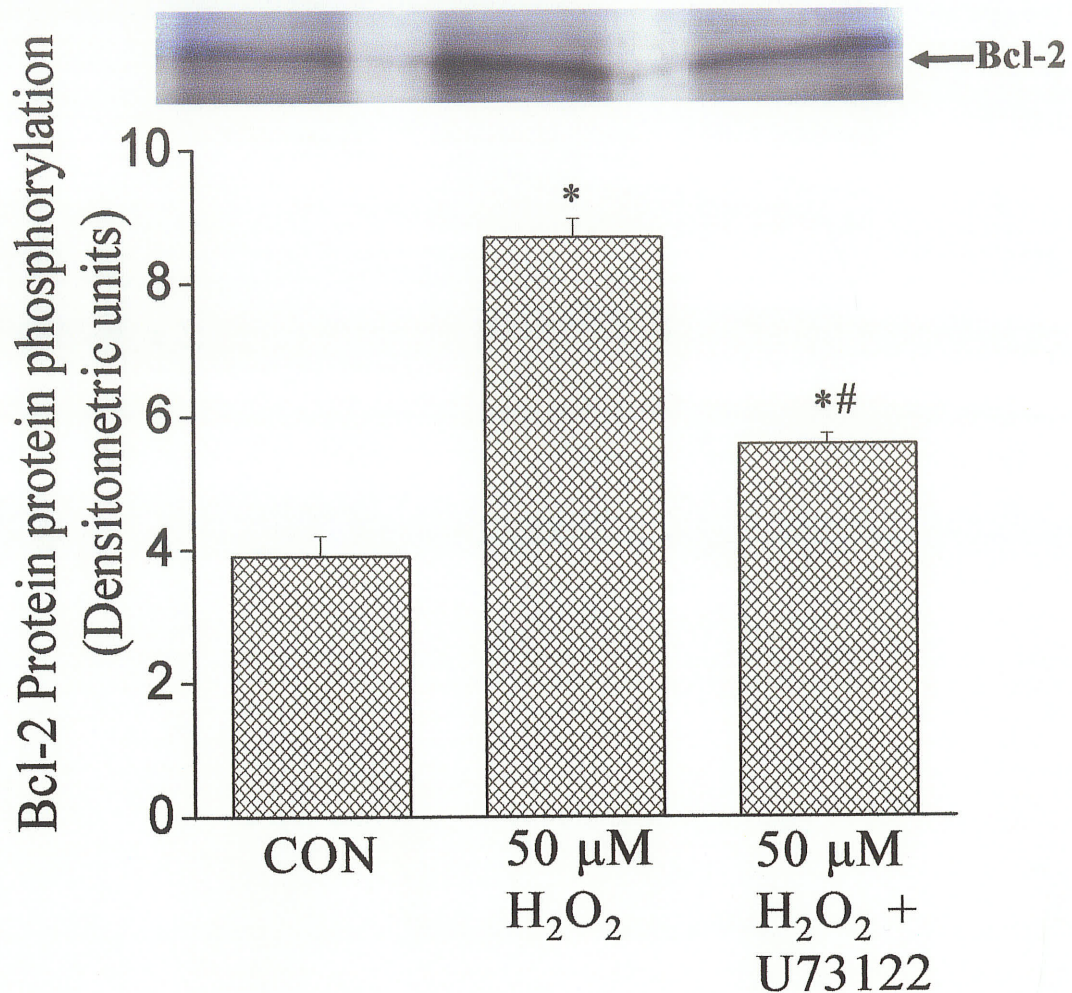
Figure 7. **Bcl-2 protein phosphorylation in cardiomyocytes treated with hydrogen peroxide in the absence and presence of U73122.**

Quantified data showing phospho-Bcl-2 protein contents in cardiomyocytes treated with H_2O_2 (50 µM) for 15 min in the absence and presence of U73122 (1 µM). Values are means ± S.E. of 5 experiments. Corresponding representative blots (arrow) showing phospho-Bcl-2 protein contents. * P< 0.05 vs. control, # P < 0.05 *vs.* H_2O_2 value in the absence of U73122.
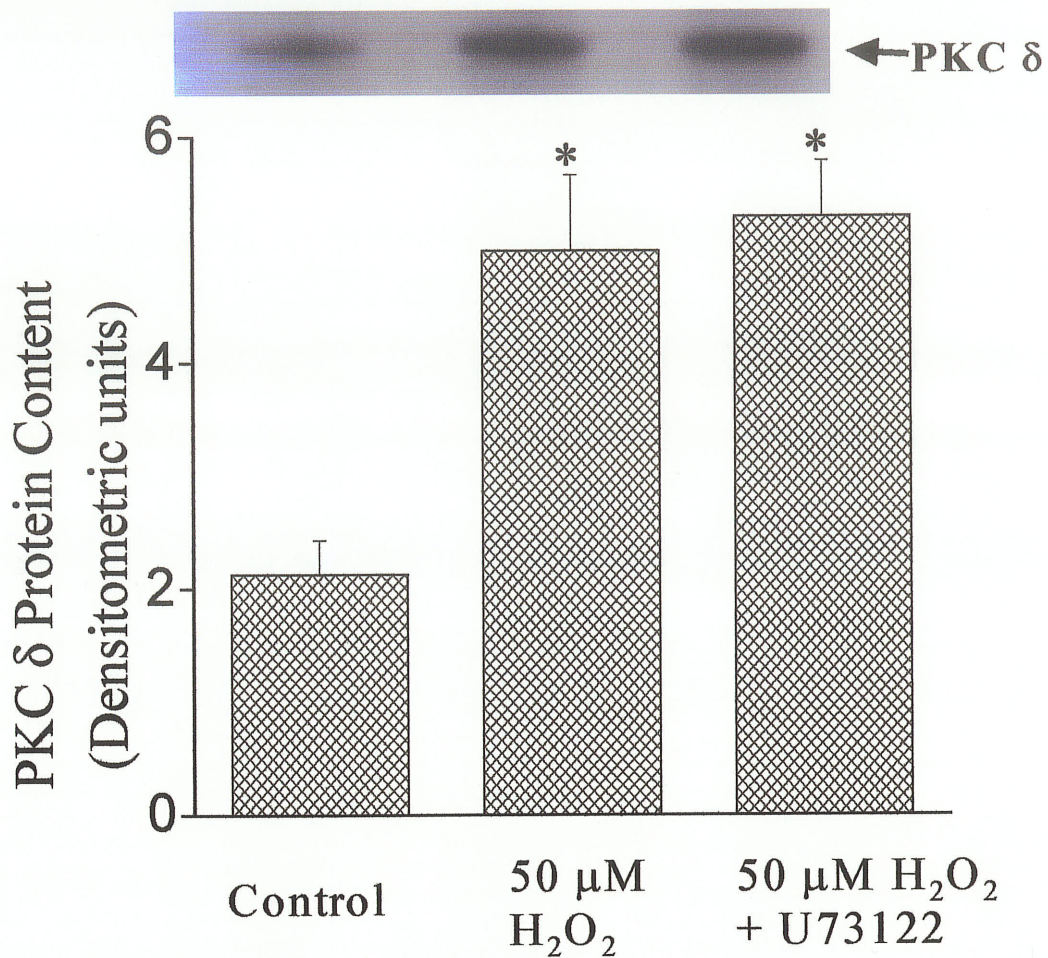
Figure 8. **Protein kinase C isozyme protein content in cardiomyocytes treated with and without hydrogen peroxide in the presence and absence of U73122.**

Representative Western blot and quantified data of PKC δ protein contents. Western blot shows (arrow) the PKC δ protein (77 kDa). Isolated cardiomyocytes were treated with H_2O_2 (50 μM) for 15 min in the absence and presence of U73122 (2 μM) as described in the Materials and Methods. Values are means ± S.E. of 5 experiments. *P < 0.05 vs. control.
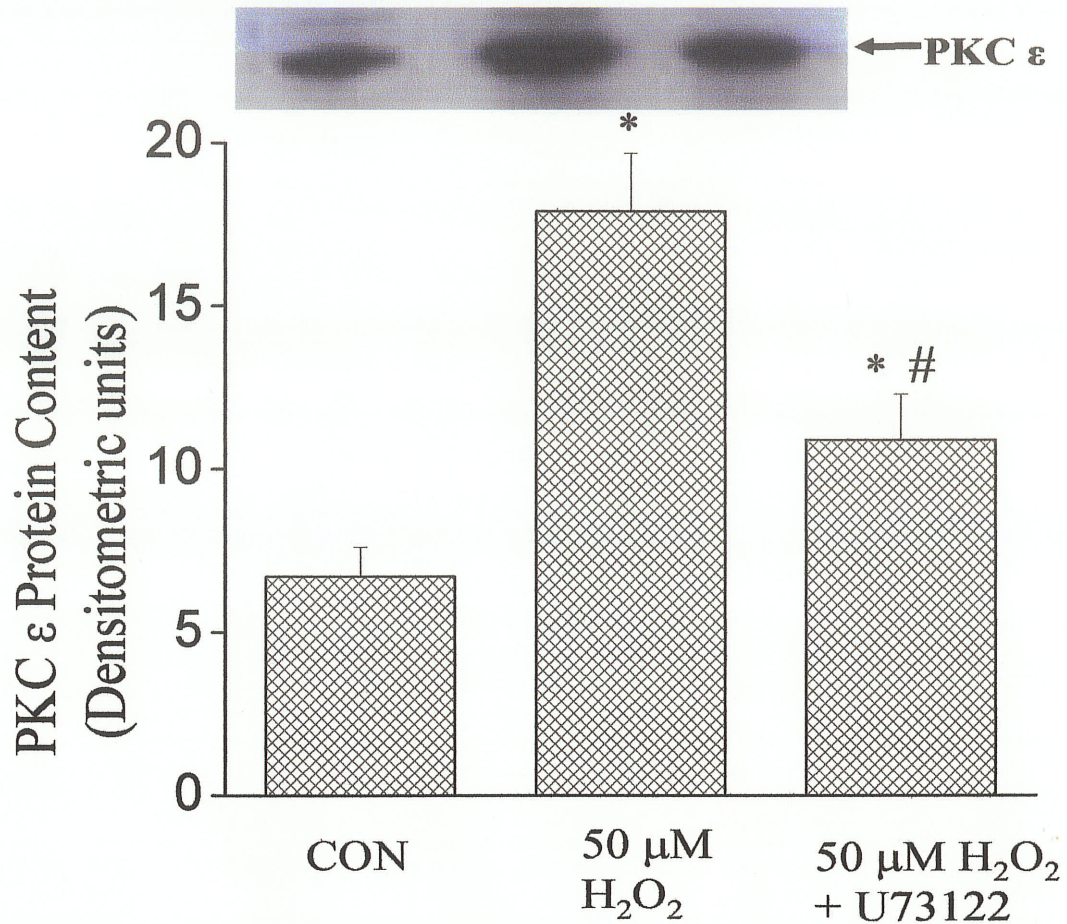
Figure 9. Protein kinase C isozyme protein content in cardiomyocytes treated with and without hydrogen peroxide in the presence and absence of U73122.

Representative Western blot and quantified data of PKC ε protein contents. Western blot shows (arrow) the PKC ε (83 KDa). Isolated cardiomyocytes were treated with H_2O_2 (50 μM) for 15 min in the absence and presence of U73122 (2 μM) as described in the Materials and Methods. Values are means $\pm$ S.E. of 5 experiments. *P<0.05vs.Control, # P< 0.05 vs. H_2O_2 value in the absence of U73122.

C. Inhibition of PLC γ_1 attenuates cardiomyocyte viability in the presence of H_2O_2

To establish the cardioprotective nature of PLC γ_1 activation, cardiomyocyte viability was determined by the trypan blue exclusion method. While H_2O_2 (50 μM) reduced cardiomyocyte viability to 57%, a progressive decrease (55, 33 and 19%) in the number of viable cardiomyocytes treated with H_2O_2 in the presence of different concentrations (0.5, 1 and 2 μM) of U73122 was seen (Figure 10).
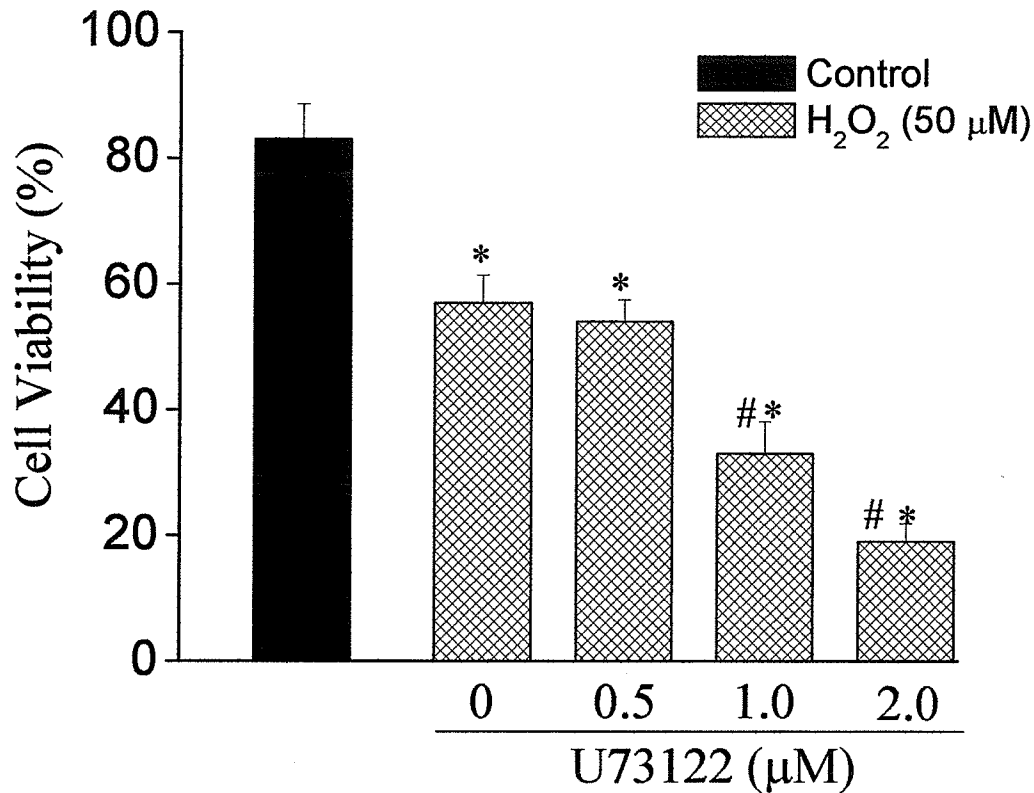
Figure 10. **Cardiomyocyte viability after exposure to hydrogen peroxide in the absence and presence of U73122.**

Values are means ± S.E. of 5 experiments. Cell viability was determined by trypan blue exclusion as described in the Materials and Methods. Cardiomyocytes were pretreated for 30 min with different concentrations (0.5, 1.0 and 2.0 μM) of U73122 before exposure to 50 μM H_2O_2 for 15 min. * P < 0.05 *vs.* control, # P < 0.05 vs. H_2O_2 value in the absence of U73122.

D. Effect of CLA incorporation on the PLC γ_1 activity in response to oxidative stress.

To determine the effect of CLA incorporation on the PLC γ_1 activity in response to oxidative stress, cardiomyocytes were preincubated with CLA isomers c9,t11 CLA and t10,c12 CLA at 5, 50, and 100 μM concentration. After the overnight incubation the cardiomyocytes were subjected to oxidative stress by 15 minutes incubation with H_2O_2 (50 μM) treatment. Thereafter the phosphorylation of PLC γ_1 at tyrosyl residues was examined with immunoblotting. While no effect on the H_2O_2 induced phosphorylation of PLC γ_1 seen when the cardiomyocytes were treated with t10,c12 CLA isomer (Figure 11), a significant reduction in the phosphorylation of PLC γ_1 in response to H_2O_2 when the cardiomyocytes were treated with c9, t11 CLA isomer was observed (Figure 12).
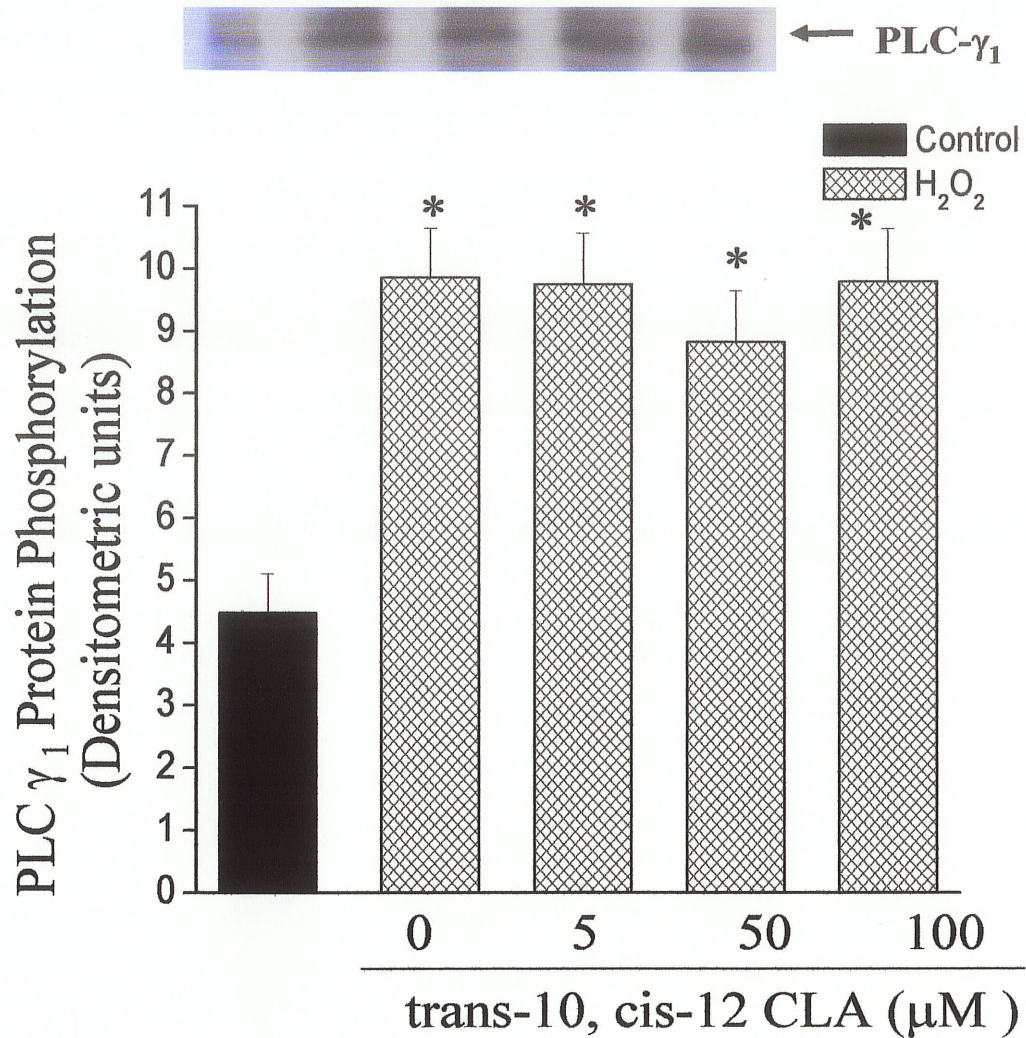
Figure 11. **Phospholipase C γ_1 protein phosphorylation in cardiomyocytes treated with and without hydrogen peroxide in the presence and absence of t10,c12 CLA isomer.**

Representative immunoblot and quantified data showing (arrow) phosphotyrosyl-PLC γ_1 protein (135 kDa) content in cardiomyocytes pretreated with t10,c12 CLA and then subjected to H_2O_2 (50 µM) for 15 min. *$P < 0.05$ vs. control.
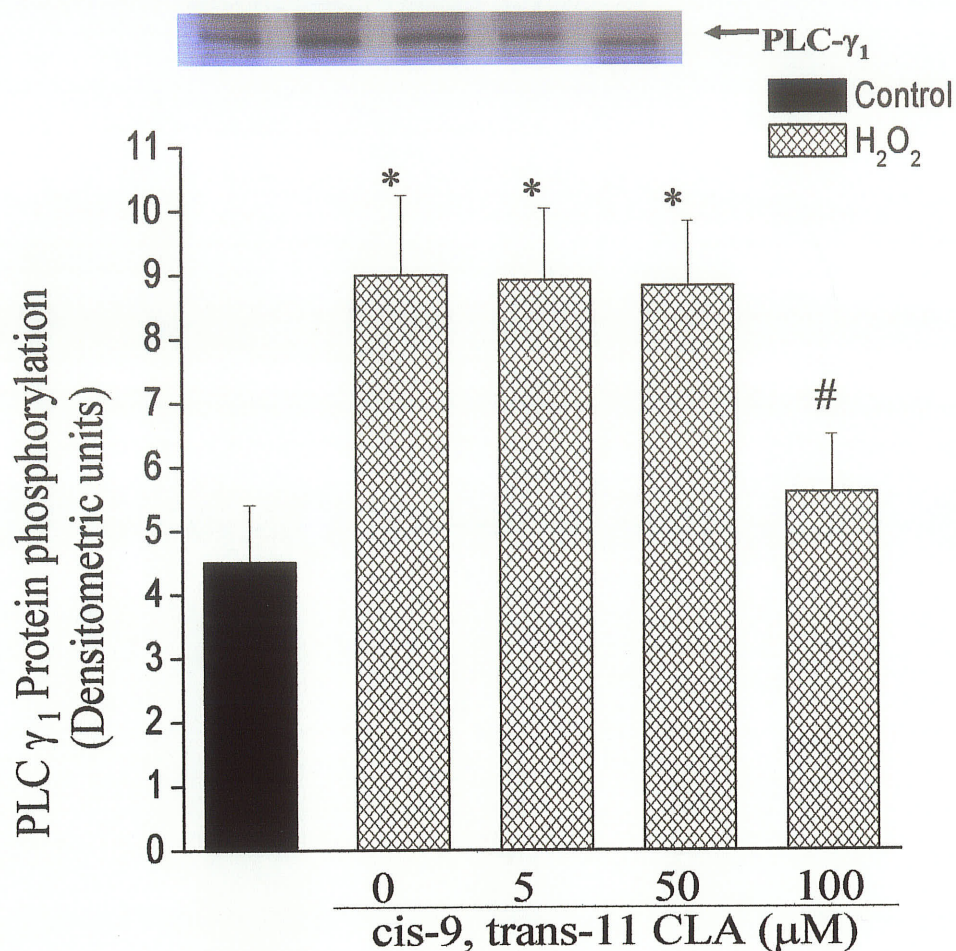
Figure 12. **Phospholipase C γ_1 protein phosphorylation in cardiomyocytes treated with and without hydrogen peroxide in the presence and absence of c9,t11 CLA isomer.**

Representative immunoblot and quantified data showing (arrow) phosphotyrosyl-PLC γ_1 protein (135 kDa) content in cardiomyocytes pretreated with c9,t11 CLA isomer and then subjected to H_2O_2 (50 µM) for 15 min. * $P < 0.05$ vs. control, # $P < 0.05$ vs. value of H_2O_2 without c9t11 CLA.

VI. DISCUSSION

Action of CLA on the heart

Although some studies have shown that CLA could exert cardiovascular benefits through its hypolipidemic, antiatherosclerotic (61,62,40,63,64) and hypotensive effects (66), yet there is no information on the direct cardiac effects of CLA. Data from the unpublished work of Tappia et al shows that while the overall response to dietary CLA was a reduction in the rate of contraction and relaxation of the heart and a reduction in mean arterial pressure (MAP), with either single CLA isomers or as a free fatty acid mixture in equal proportion, the hemodynamic response was further characterized by gender differences and was influenced by the form of the CLA supplementation. Results from this study indicate that CLA isomers could exert direct cardiac effects on the contractility and the hemodynamic parameters of the heart. With a lot of research being focused on CLA and the anticarcinogenic and other beneficial effects it is important to ascertain the effect of CLA on the heart. In addition there is evidence that CLA isomers get incorporated in the heart lipids however what would be the effect of this fatty acid incorporation is not known. Therefore in this study we investigated for the first time, the effects of CLA incorporation in the SL phospholipids and its effect on SL enzyme PLC-γ_1.

Intakes of CLA over the years

Though there is no data on CLA consumption over the years, but it is speculated that CLA consumption has come down. The rationale behind such a statement is because the overall fat consumption in human populations has been on a downward slope over the two decades. Main contribution for this change is because of negative press associated with fats. According to a report of the *Center for Nutrition Policy and Promotion*, an organization of the US Department of Agriculture, adult Americans have dramatically lowered the percent of caloric intake from fats over the last three decades. The reduction is about 45% of calories from fat in 1965 to about 34% in 1995 (although total caloric intake and intake of refined sugars have increased). The main source of CLA is dairy fat or animal fat whereas most people prefer to take skimmed milk and lean meat thereby decreasing their CLA intakes.

Incidence of ischemic heart disease

According to a report, 'The growing burden of heart disease and stroke in Canada 2003' published by the Heart and Stroke Foundation , the greatest number of prescriptions are for the treatment of cardiovascular diseases. From 1979 to the mid 1990's, the number of hospitalizations for cardiovascular diseases is increasing. In 2000/01, hospitalization rates for ischemic heart disease were much higher for men than women for all age groups. While the actual number of hospitalizations for ischemic heart disease is projected to

increase for both men and women in the next 20 years, the increase is projected at a much higher rate for men. It is estimated that there would be an approximately 22% increase in the number of hospitalizations for ischemic heart disease for women and approximately 24% increase in the number of hospitalizations for ischemic heart disease for men in the next 10 years. In 1998, costs attributable to cardiovascular diseases were the largest among all diagnostic categories. Therefore a lot of research is being put in the management, and prevention of cardiovascular diseases, including ischemic heart disease.

Mechanisms of PLC γ_1 activation in respose to H_2O_2

PLC γ_1 is known to get activated by phosphorylation at the tyrosine residues and also by mechanisms independent of tyrosine phosphorylation (209). Phosphorylation of the tyrosyl residues is catalysed by protein tyrosine kinases (PTKs) and when PTK is activated it provides docking site for PLC γ_1 and then catalyses its phosphorylation. PTKs are activated by growth factors such as platelet derived growth factor, epidermal growth factor and insulin receptor (209). Furthermore it has been shown that oxidants including H_2O_2 mimic the action of epidermal growth factor and perhaps other growth factors (248,249).

The phosphorylation state, which is also called the activation state of a cellular protein, is the net effect of tyrosine kinase activity (if that protein is

phosphorylated by tyrosine kinase), which phosphorylates the protein, and corresponding protein tyrosine phosphatase PTP activity, which reverses this reaction. Furthermore PTP activity has been demonstrated to be downregulated by H_2O_2 (Scheme 3) If PTP activity is inhibited, the basal level of tyrosine kinase activity is sufficient to initiate signal pathways in the absence of receptor stimulation by growth factors (247).

Scheme 3. Role of H_2O_2 in a reaction catalysed by protein tyrosine kinase (247).
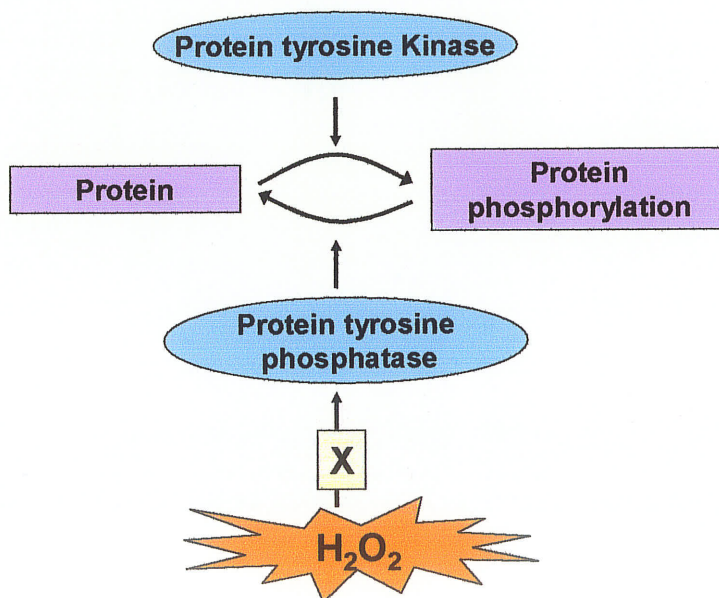
Effect of H_2O_2 induced oxidative stress on PLC γ_1 activity

It has been previously reported that cardiac I-R induces differential changes in PLC isozyme activities, SL protein contents and mRNA expression levels. Specifically, PLC γ_1 was activated in the first minute of reperfusion of heart subjected to a 30-minute period of global ischemia (208). Recently we have shown that inhibition of PLC either indirectly with verapamil or directly with U73122 improves post-ischemic recovery of the heart (69). While this demonstrates the importance of PLC in the setting of I-R, the distinct functions of each PLC isozyme in the adult cardiomyocyte has yet to be completely understood. The present study was therefore undertaken to determine some of the mechanisms responsible for the activation of PLC γ_1 as well as to investigate the functional significance of this change under conditions of oxidative stress. Although other mechanisms also exist for activation of PLC γ_1, our findings are the first to demonstrate that cardiac PLC γ_1 is activated in response to H_2O_2 treatment in cardiomyocytes in a tyrosine kinase-dependent mechanism.

It is pointed out that although a variety of reactive oxygen species as well as oxidant molecules are generated during reperfusion of the ischemic heart, post-ischemic reperfusion has been shown to lead to the production of H_2O_2 (231). In a clinical setting in which the heart is exposed to transient ischemia followed by coronary reflow, infiltrating polymorphonuclear leukocytes (neutrophils and eosinophils) can also produce H_2O_2 (233, 234).

Some studies have shown mitochondria-dependent production of H_2O_2 during ischemia (144) and reperfusion (235). The concentrations of H_2O_2 employed in our study are compatible with those detected *in vivo* during myocardial I-R (236). On this basis it is likely that our present *in vitro* observations in response to H_2O_2 may occur at the level of the cardiomyocyte during I-R.

The present study has also shown that PLC γ_1 mRNA level during oxidative stress was increased. In this regard, it is known that genes may be sensitive to regulatory elements or up-regulated by other transcription factors, which are activated during the ischemic phase and that some transcription factors are expressed acutely and chronically in response to hypoxia and I-R (237), which have numerous targets, including, possibly, PLC γ_1. Since our data represent the steady-state level of the PLC γ_1 gene, the enhanced expression could also be due to either increased rate of transcription and/or increased mRNA stability. Therefore, caution should be exercised in the interpretation of the mRNA data. Also it is pointed out that the increase in the membrane contents of PLC γ_1 and PKC δ and ϵ isozymes in response to H_2O_2 most likely represents translocation from the cytosol to the membrane compartment (208,238).

Role of PLC γ_1 in cell survival

Bcl-2 is an antideath gene that functions as an intracellular antioxidant. We have provided evidence for the first time that stimulation of PLC γ_1 is

required for cardiomyocyte survival during oxidative stress for Bcl-2 phosphorylation. A significant decrease in Bcl-2 phosphorylation was observed when the cardiomyocytes were pretreated with a PLC inhibitor U73122 and then subjected to oxidative stress. Cell viability was assessed using trypan blue exclusion method and the observations suggested that H_2O_2 reduced cardiomyocyte viability to 57% however a further decrease in cardiomyocyte viability was observed (55, 33 and 19%) when they were pretreated with PLC inhibitor U73122. Therefore suggesting that PLC γ_1 activation might play a role in cell survival.

It should be mentioned that in view of our earlier observation that inhibition of PLC improves post-ischemic recovery of the heart (69), it could be difficult to reconcile this with the findings of the present study and the suggestion that PLC γ_1 may be protective of cardiomyocyte viability during oxidative stress.

However, this can be explained on the basis that in hearts subjected to I-R, there is a specific increase in PLC β_1 activity during the ischemic phase (208,69), which may be more deleterious for post-ischemic recovery. In these whole heart experiments the inhibitor blocked both the PLC β_1 activation in the ischemic phase and PLC γ_1 activation in the reperfusion phase which results in an overall better recovery, whereas the conditions in our experiments mimicked only the reperfusion phase. Thus blocking the PLC γ_1 activation

during the reperfusion phase in the absence of PLC β_1 activation (which is in the ischemic phase) results in a reduced cell viability. Therefore although inhibition of PLC β_1 and PLC γ_1 improved the recovery of the heart, it is reasonable to assume, given the findings of the present study, that a better recovery would have been observed if PLC γ_1 activity was not inhibited, which is activated in the first minute of reperfusion (208, 69). In this regard, the fibrosis which occurs in I-R (175) may be mediated by PLC β_1 (176). Furthermore, it is interesting to note that prazosin, an α_1-adrenoceptor blocker, has been reported to attenuate myocardial injury in I-R (176).

Role of PKC isozymes as a mediator in transmission of cell survival signal

The role of PKC in cardiac I-R is well documented (166, 169, 179, 180, 239, 238). PKC ε activation is considered to be crucial to cardioprotection during I-R, since isozyme-specific inhibitory peptides are able to abolish protection in response to ischemic preconditioning (239). Furthermore, the targeted discruption of PKC ε gene abolishes the infarct size reduction that follows ischemic preconditioning (180). In addition, inhibition of PKC δ during reperfusion provides protection from I-R injury (238,179).

While a similar activation of PLC γ_1 and Bcl-2 phosphorylation has been reported in mouse embryonic fibroblasts in response to H_2O_2 (214,228), it has also been demonstrated that the mediator of the signal from PLC γ_1 to Bcl-2

phosphorylation is PKC (228), but the identity of the PKC isoform involved was not determined.

Our results show that PKC ε and PKC δ are activated during cardiomyocyte oxidative stress; however, it appears that PKC ε may be activated by PLC γ_1, since inhibition of PLC γ_1 with U73122 almost completely prevented the activation of PKC ε, whereas PKC δ activation was not prevented by U73122. These data strongly support a functional link between PLC γ_1 and PKC ε and a protective role during cardiac oxidative stress. The question arises as to how the activation of PKC δ is not prevented by U73122.

It is possible that PKC δ activation is independent of PLC γ_1 under our experimental conditions. It is pointed out that while the present study has focused on the role of PLC γ_1 under conditions of oxidative stress, another predominant PLC isozyme, PLC δ_1, is also activated during reperfusion; however, we have recently suggested that the activation of this PLC isozyme during I-R is due to Ca^{2+} (69).

It has recently been reported that the activities of the major cardiac sarcolemmal phospholipase D (PLD) isozyme, PLD2, and phosphatidate phosphohydrolase (PAP) are increased during I-R (240), suggesting that DAG derived from the PLD-PAP pathway may be involved in activating PKC δ. This possibility warrants further investigation, as PKC isozymes have been suggested to be activated specifically by PLC-derived DAG (241,242), and the *in vivo* significance of PLD-PAP derived DAG remains to be determined

(243). Another possibility is that PKC δ activation in cardiomyocytes may occur through a tyrosine phosphorylation, DAG-independent mechanism similar to that reported in other cell types in response to H_2O_2 (244,245,174).

Effect of CLA incorporation on PLC γ_1 response to oxidative stress

Although some studies have reported that CLA could exert cardiovascular benefits through its hypolipidemic and antiatherosclerotic effects (40, 61, 62, 63, 64), there is no information available on the impact of CLA isomers or mixtures on the contractile function of the heart or the effect of CLA isomers incorporation on the sarcolemmal enzymes.

Our results suggest that after the overnight incubation of cardiomyocytes with t10,c12 CLA isomer there was no effect on the activation of PLC-γ_1 in response to oxidative stress induced by H_2O_2 (50 μM for 15 minutes).Unlike this, when the cardiomyocytes were pretreated with c9,t11 CLA isomer (5, 50, 100 μM), there was a decrease in the activity of PLC-γ_1 in response to oxidative stress induced by H_2O_2 (50 μM for 15 minutes). These results clearly suggest that the c9,t11 isomer would be detrimental for the heart during ischemia-reperfusion. These results also suggest that incorporation of CLA isomers into the phospholipid membrane is capable of causing changes in the sarcolemmal enzyme systems.

There is evidence that differential accumulation of CLA isomers takes place in the major heart lipid classes (53). c9,t11 CLA isomer gets incorporated significantly more as compared to the t10,c12 CLA isomer. This differential accumulation of CLA isomers could be a possible reason for no observed effect of t10,c12 CLA isomer on PLC γ_1 response to oxidative stress.

The observed effect of c9,t11 CLA isomer could be a direct or an indirect effect on the activity of PLC γ_1. Incubation with CLA isomers could result in incorporation of CLA isomers into various lipid classes of SL including phosphatidylcholine which on hydrolysis by phospholipase D gives phosphatidic acid and choline (Scheme 4). Phosphatidic acid has been shown to activate both tyrosine-phosphorylated as well as unphosphorylated forms of PLC γ_1 (190).Therefore a depression in phospholipase D activity in the membrane as a result of CLA incorporation into phosphatidylcholine can also possibly result in depression in activity of PLC γ_1. In addition to this arachidonic acid also stimulates the activity of PLC γ_1 and CLA isomers have been shown to inhibit arachidonic acid production (190).

Scheme 4. Activation of PLC γ_1 by phospholipase D (209).
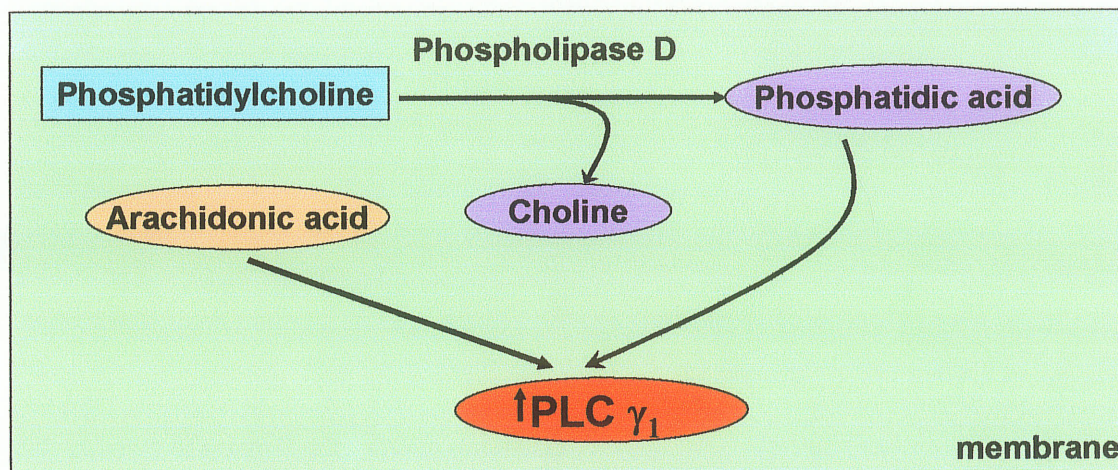
There is evidence in literature (246) that when cardiomyocytes are incubated overnight with different fatty acids in the culture medium in ethanol vehicle, they get incorporated into the heart lipids. Therefore in this study it has been assumed that when cardiomyocytes were incubated with CLA isomers in ethanol vehicle they got incorporated into the heart lipids.

Concentration of CLA isomers used was based on a study (A Leaf) in which in vitro antiarrhythmic effects of PUFA's were being studied on cardiomyocytes. Though CLA was not studied but linoleic acid was used at 50 and 100μM concentrations. In other in vitro studies involving cancer cells and CLA, it has been used at 5, 50 and 100 μM.

VII. CONCLUSIONS

1. H_2O_2 induced an increase in PLC γ_1 mRNA and protein expression in isolated adult cardiomyocytes.

2. H_2O_2 increases phosphorylation of tyrosyl residues of PLC γ_1 indicating PLC γ_1 activation.

3. Increased phosphorylation of the anti apoptotic Bcl-2 protein occurs in cardiomyocytes exposed to H_2O_2.

4. Pretreatment of the cardiomyocytes with PLC inhibitor U73122 attenuated the increase in phosphorylation of Bcl-2 expression.

5. Inhibiting the PLC- γ_1 in cardiomyocytes using the PLC inhibitor U73122 reduces the viability of cardiomyocytes during oxidative stress. Thus, PLC γ_1 may protect the cardiomyocytes from oxidative induced cell death.

6. PKC-ε seems to be the mediator of the PLC- γ_1 signal to phosphorylation of Bcl-2.

7. t10,c12 CLA isomer seems to have no effect on PLC γ_1 activation during oxidative stress.

8. CLA isomer c9,t11 seems to decrease the activation of PLC γ_1 during oxidative stress. Thus, incorporation of this isomer may be detrimental for the recovery of the heart during oxidative stress.

9. Change in the fatty acid composition of sarcolemma may change activity of sarcolemmal enzymes and thereafter their downstream signaling pathways.

10. Since PLC γ_1 dependent PKC ε activation and subsequent phosphorylation of Bcl-2 in cardiomyocytes may be involved in cardiomyocyte survival during oxidative stress and therefore PLC γ_1 could be a potential target not only for the pharmaceutical intervention and clinical management of ischemic heart disease but could constitute a target for novel nutritional strategies.

VIII. SUMMARY OF THE MAJOR FINDINGS
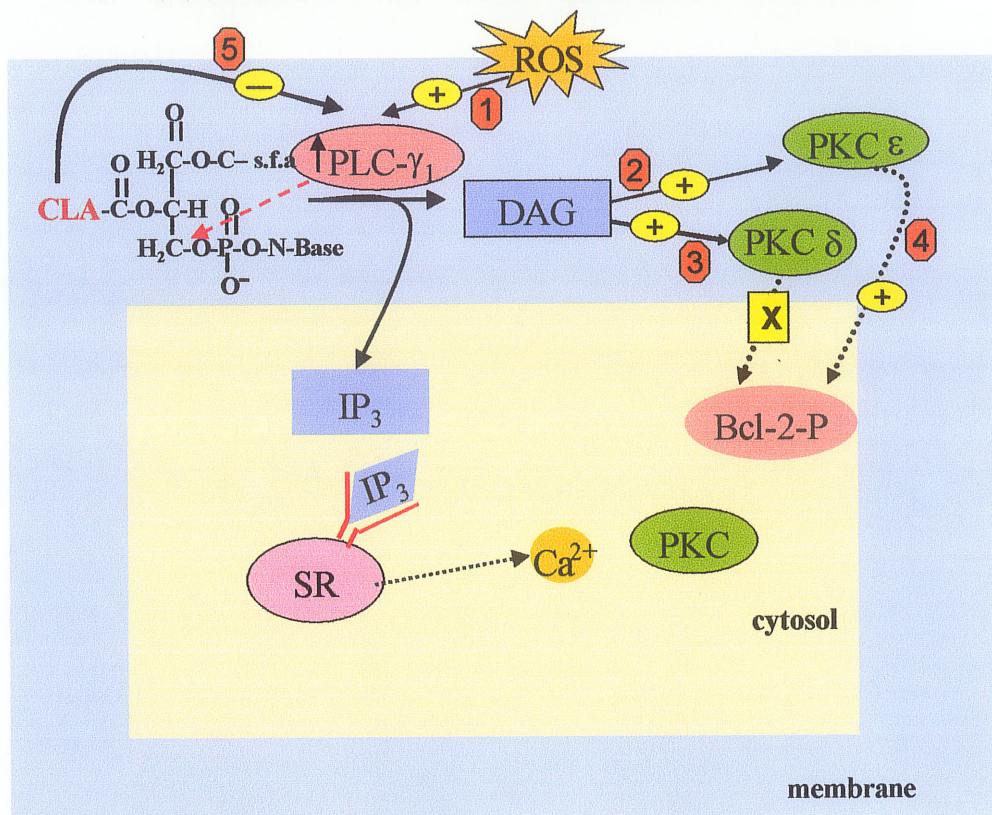
+ activation,   − inhibitory,   X no effect

1. ROS, specifically H_2O_2 causes activation in the activity of PLC γ_1.

2. ROS, specifically H_2O_2 causes an activation of PKC ε. This activation of PKC ε is through PLC γ_1.

3. ROS, specifically H_2O_2 causes an activation of PKC δ. This activation of PKC δ is not through PLC γ_1.

4. PKC ε phosphorylates anti-apoptotic protein Bcl-2.

5. Incorporation of c9,t11 CLA isomer in the SL membrane of the cardiomyocytes decreases the activity of PLC γ_1 during H_2O_2 induced oxidative stress.

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IX. FUTURE DIRECTIONS

1. Different fatty acids can be used to understand the specificity of the CLA effects. The specificity of the effects of H_2O_2 on PLC γ_1 can be obtained by examining the effects of H_2O_2 on other PLC isozymes located in cardiomyocytes.

2. The results of this in vitro study can be checked by planning an in vivo study. An appropriate animal model can be used and after feeding different CLA isomers to the animals, I-R on a working or isolated heart model can be conducted to examine how the heart the heart recovers from I-R injury.

3. Adenovirus technique can be used to create PLC γ_1 knock out cells and then their viability can be checked by subjecting them to oxidative stress.

4. The activation of PLC γ_1 was examined only in the presence of a single reactive oxygen species and scavenging system. Therefore in order to examine the specificity of the activation of PLC γ_1 in response to oxidative stress, and the related downstream signaling, different ROS generating and scavenging systems should be used. For instance, HOCl, hydroxyl radical, xanthine + xanthine oxidase can be used with superoxide dismutase, catalase or glutathione peroxidase.

X. LIMITATIONS

1. The response of only one oxidant molecule, H_2O_2 was examined.

2. The incorporation of CLA isomers into cardiomyocyte membrane was not determined.

3. Although CLA affects the phosphorylation of PLC γ_1, the response of the downstream signaling events i.e Bcl-2 phosphorylation or PKC-ε activation were not examined.

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