

**β -Adrenoceptor Signal Transduction
in High-Output Heart Failure Due to Aortocaval Shunt in Rat**

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for the Degree of Doctor of Philosophy

By

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Aortocaval Shunt in Rat**

BY

Xi Wang

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree
of
Doctor of Philosophy**

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Dedicated to
my mom, Yunxin,
my husband, Zhiyu, my daughter, Deede
and my two sisters, Peipei and Lili

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ABSTRACT

The failing heart is characterized by blunted β -adrenoceptor (β -AR) responses associated with a number of changes in the signal transduction pathway including downregulation of β -ARs, increased expression of inhibitory G-proteins and G-protein coupled receptor kinases (GRKs) and impaired adenylyl cyclase (AC) activities. However, a careful review of the literature reveals that most types of heart failure in human and experimental models used to study β -AR signaling are associated with low cardiac output heart failure with significant fibrosis in the myocardium. No information is available regarding the regulation of β -AR signal transduction in heart failure with a high-output status without fibrosis. Therefore, the objective of this work is to use a high-output heart failure model without fibrosis and examine the status of β -AR signaling. To reach this aim, we first characterized a high-output model induced by an aortocaval shunt from several aspects including general features, morphology and histology of the heart, circulatory congestion, hemodynamics and contractile function in vivo and in vitro. The results demonstrated that the development of hypertrophy and heart failure typically occurred in three stages: developing hypertrophic stage (first two weeks after the induction of shunt), established hypertrophic stage (2-8 weeks) and decompensated hypertrophic or failing stage (8-16 weeks). The failing stage is characterized by dramatic hypertrophy of both left (LV) and right (RV) ventricles with signs of circulatory congestion, decreased in vivo and in vitro cardiac performance and shift of myosin heavy chain isoforms. However, the response of the failing heart to β -AR stimulation was not blunted but instead showed stimulation of contractile function.

To investigate the mechanisms underlying the enhanced β -AR response in aortocaval shunted animals, we examined changes at the receptor level. Agonist binding experiments

showed a selective increase in density of β_1 -AR but not β_2 -AR, without any changes in the affinities. Western blot experiments confirmed that the increase in β_1 -AR density is due to an enhanced expression of protein levels rather than an increased externalization of receptors. However, the steady state mRNA level of β_1 -AR was not altered indicating an increased translational rate or decreased protein degradation underlying the increased β_1 -AR protein expression. In view of the important role of GRKs, β -arrestins and $G_{\beta\gamma}$ in regulating β -AR signaling, we examined changes in these parameters in order to determine their contribution to the enhanced β -AR signaling in this model. Measurement of total GRK activity revealed a decrease in membranous fraction and increase in cytosolic fraction in the shunted rats as compared to controls. Western blot analysis of three cardiac isoforms of GRKs (GRK2, 3, 5) suggested that changes in GRK activity were associated with similar changes in the protein expressions of these three isoforms. However, determination of GRK activity and protein content in the homogenate fraction showed no significant difference between the shunted and control rats indicating that the observed changes in the activity and protein content might be a result of redistribution of proteins rather than an upregulation of activity or protein expression. Although both β -arrestins and $G_{\beta\gamma}$ act synergistically with GRKs (especially GRK2 and GRK3) in regulating β -AR signaling in vitro, we detected changes in β -arrestin1 but not $G_{\beta\gamma}$ similar to GRKs indicating $G_{\beta\gamma}$ may not be the major factor in modifying β -adrenergic signaling in this model.

To examine alterations in AC and its contribution to enhanced signaling in the failing heart due to aortocaval shunt, we measured AC activities in the presence and absence of different stimulants. The results showed a significant increase in basal and isoproterenol-, Gpp(NH)p-, NaF-, forskolin-stimulated AC activities. When the data were converted to fold-stimulation over the basal level, only isoproterenol- and forskolin-stimulated AC activities were increased

indicating enhanced signaling at receptor and effector levels. Since Gpp(NH)p- and NaF-stimulated AC activities were not altered, it is suggested that signaling through G-proteins may not contribute significantly to the observed changes in β -AR signaling in this model. Further examination of AC protein content revealed that the enhanced AC activities in basal and under forskolin stimulation were due to increased protein expression of AC type V/VI in the myocardium after aortocaval shunt. In order to further verify the contribution of G-proteins to the enhanced β -AR signaling observed in this model, we examined the bioactivity, protein content and mRNA expression of both $G_{s\alpha}$ - and $G_{i\alpha}$ -proteins. The results showed a significant decrease in $G_{s\alpha}$ -bioactivity with no change of $G_{i\alpha}$ -bioactivity as measured by toxin-catalyzed ADP-ribosylation. However, the protein contents for both $G_{s\alpha}$ - and $G_{i\alpha}$ -proteins were not altered. On the other hand, a significant reduction of mRNA levels for $G_{s\alpha}$ and an elevation of mRNA levels for $G_{i\alpha}$ were detected indicating changes at the G-protein level may not contribute to the enhanced function of β -AR signaling. $G_{s\alpha}$ has been shown to regulate L-type Ca^{2+} -channel in a cAMP independent manner, the decreased bioactivity of $G_{s\alpha}$ may contribute to the decreased contractile function observed in the basal state.

Renin-angiotensin system is another important signal transduction pathway that contributes to the pathogenesis of heart failure and there is evidence for its interaction with β -adrenergic system in health and disease. In addition, blockade of renin-angiotensin system by angiotensin converting enzyme inhibitor (ACEI) or angiotensin II receptor type I (AT1) blocker has demonstrated beneficial effects in the failing heart. Therefore, we examined the effect of a long acting ACEI, imidapril, and AT1 receptor blocker, losartan, on β -AR signaling in this model. The results showed that in addition to their effects in attenuating hemodynamic and hypertrophic responses due to aortocaval shunt, imidapril and losartan interact with the β -AR signaling

pathway by partially reversing changes in β_1 -AR density and protein content, GRK activity and protein expression, cholera toxin mediated ADP-ribosylation of $G_{s\alpha}$, mRNA levels of $G_{s\alpha}$ and $G_{i\alpha}$. However, these agents did not modify changes which occurred in AC activity and protein content. Thus, it seems that β -AR and G-protein are the direct or indirect targets for these drugs.

In summary, we have demonstrated that there is an enhancement of β -AR signaling pathway due to an upregulation of function at the receptor and effector levels in this study in a high-output heart failure model without fibrosis. The impaired $G_{s\alpha}$ -bioactivity may not cause any significant reduction in the β -AR signal but may contribute to contractile dysfunction by modulating the L-type Ca^{2+} -channel in a cAMP independent manner. The interaction of two drugs that antagonize the renin-angiotensin system at the receptor and G-protein levels indicate crosstalk between these two systems and the beneficial effect of the pharmacological intervention in this model may at least in part be due to their modulation of the β -AR signal pathway.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT	iii
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
I. LITERATURE REVIEW	1
1. Introduction	1
2. Biochemical aspect of β -adrenergic signal transduction	3
a. β -adrenoceptors	3
b. G-proteins	7
c. Adenylyl cyclase	9
d. Regulation of β -adrenoceptors	9
3. Manipulation of gene expression for β -adrenergic signaling	15
a. Genetic manipulation of β -ARs	15
b. Genetic manipulation of GRKs and β -arrestins	19
c. Genetic manipulation of G-proteins and adenylyl cyclase	22
4. β -Adrenergic signaling in heart failure	26
a. Alterations in β -adrenoceptors in heart failure	27
b. Alterations of G-proteins in heart failure	38
c. Alterations of adenylyl cyclase in heart failure	42
II. STATEMENT OF THE PROBLEM AND HYPOTHESES TO BE TESTED.	45

III. METHODS AND MATERIALS	48
1. Experimental model	48
2. Experimental design and experimental groups	49
3. General characteristics	50
4. <i>In vivo</i> hemodynamic assessment	50
5. <i>In vitro</i> cardiac function	51
6. Myosin heavy chain isoenzyme analysis	52
7. Isolation of crude membrane	53
8. Isolation cytosolic and membranous proteins	53
9. Isolation of total RNA	54
10. β_1 - and β_2 -adrenoceptors binding assay	55
11. G-protein coupled receptor kinase assay	55
12. Toxin-catalyzed ADP-ribosylation	56
13. Determination of adenylyl cyclase activity	57
14. SDS-PAGE electrophoresis and Western blot assay:	58
15. Plasmid amplification and preparation of cDNA probes	59
16. Northern blot analysis	59
17. Statistical analysis	59
IV. RESULTS	61
1. Characterization of the experimental model	61
a. General characteristics of the rat model	62
b. Morphological and histological changes	63
c. Circulatory congestion	67

d. <i>In vivo</i> cardiac performance and arterial hemodynamics	67
e. Biochemical changes	71
f. Alterations in intrinsic contractile function	75
g. Contractile response to isoproterenol stimulation	75
2. Alterations in β -adrenoceptors and β -adrenergic receptor kinase in heart failure induced by aortocaval shunt	75
a. Modification of general characteristics and hemodynamic changes	78
b. Modification of changes in β -ARs	84
c. Alterations of GRKs, β -arrestins and $G_{\beta\gamma}$ -subunit	87
3. Alterations of adenylyl cyclase and G-proteins in heart failure induced by aortocaval shunt	98
a. General and hemodynamic features	101
b. Alterations of adenylyl cyclase in volume overload induced heart failure	101
c. Alterations of G-proteins in volume overload induced heart failure	106
V. DISCUSSION	112
1. Development of cardiac hypertrophy and heart failure in rat due to aortocaval shunt	112
2. β -adrenoceptors and G-protein coupled receptor kinases in heart failure induced by aortocaval shunt	118
a. Alterations of β -ARs in aortocaval shunt induced heart failure	118
b. Alterations of GRKs in aortocaval shunt induced heart failure	122
3. Adenylyl cyclase and G-proteins in heart failure induced by aortocaval shunt	127
a. Alterations of adenylyl cyclase in aortocaval shunt induced heart failure	127
b. Alterations of G-proteins in aortocaval shunt induced heart failure	130

4. Effect of imidapril and losartan on β -adrenergic signaling in heart failure induced by aortocaval shunt	132
VI. CONCLUSIONS	135
VII. REFERENCES	136

LIST OF TABLES

Table 1: General characteristics of β -adrenergic receptors	4
Table 2: Transgenic models of β -adrenergic signal transduction pathway	24
Table 3: Knockout models of β -adrenergic signal transduction pathway	25
Table 4: Human heart diseases and animal models used for studying β -adrenergic signal transduction pathway	28
Table 5: Alterations in G-protein-coupled receptor kinases in diseased hearts of human and animal models	36
Table 6: General characteristics of rats with or without inducing aortocaval shunt for different time intervals	64
Table 7: Lung and liver weights in rats with or without inducing aortocaval shunt for different time intervals	68
Table 8: Myosin heavy chain isoenzyme composition of rats at 4, 8 and 16 weeks after inducing the aortocaval shunt	74
Table 9: Effect of isoproterenol on cardiac function in sham control and aortocaval shunt in rats for 4, 8 and 16 weeks	77
Table 10: General characteristics of rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril and losartan treatment	79
Table 11: <i>In vivo</i> hemodynamic changes in rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment	81
Table 12: Effect of bolus isoproterenol on <i>in vivo</i> hemodynamics of rats with heart failure induced by aortocaval shunt for 16 weeks with and without treatment treatment of imidapril or losartan	82

Table 13: Effect of isoproterenol on <i>in vitro</i> cardiac performance of rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment	83
Table 14: Kinetic parameters of β -adrenoceptor binding in rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment	85
Table 15: General and hemodynamic characteristics of sham and experimental rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment	102
Table 16: Folds increase of adenylyl cyclase activities under different stimulants in rat with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment	104

LIST OF FIGURES

Figure 1: Morphological studies showing development of eccentric hypertrophy in rat heart at 4, 8 and 16 weeks after inducing the aortocaval shunt	65
Figure 2: Histological changes of LV myocardium at 4, 8 and 16 weeks after inducing the aortocaval shunt in rat	66
Figure 3: Histological changes in the rat lung and liver at 4, 8 and 16 weeks after inducing the aortocaval shunt	69
Figure 4: <i>In vivo</i> left ventricular hemodynamic changes in rats after inducing the aortocaval shunt for different time intervals	70
Figure 5: Changes in arterial pressures in rats after inducing the aortocaval shunt for different time intervals	72
Figure 6: Time course changes in myosin heavy chain isoform composition in rats after inducing the aortocaval shunt	73
Figure 7: <i>In vitro</i> left ventricular hemodynamic changes in rats after inducing the aortocaval shunt for different time intervals	76
Figure 8: Alterations of protein content in β -ARs in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan	86
Figure 9: Typical Northern blots showing β_1 -adrenoceptors, GRK2 mRNA levels in LV and RV of chronic failing heart induced by aortocaval shunt with or without treatment of imidapril and losartan	88
Figure 10: Alterations of mRNA levels in β_1 -adrenergic receptors in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan	89

- Figure 11: Alterations of GRK activity in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan 91
- Figure 12: Alterations of protein content of GRK2 in both membranous and cytosolic fractions in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan 92
- Figure 13: GRK activities and GRK2 and GRK3 protein content in LV and RV homogenate preparations in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan 94
- Figure 14: Alterations of mRNA level of GRK2 in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan 95
- Figure 15: Alterations of protein content of GRK3 in both membranous and cytosolic fractions in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan 96
- Figure 16: Alterations of protein content of GRK3 in both membranous and cytosolic fractions in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan 97
- Figure 17: Alterations of β -arrestins in the LV and RV chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan 99
- Figure 18: Alterations of protein content of $G_{\beta\gamma}$ -subunit in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan 100

Figure 19: Alterations of AC activities in the LV and RV of chronic failing heart induced by aortocaval shunt with or without treatment of imidapril and losartan	103
Figure 20: Alterations of protein content of AC type V/VI in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan	105
Figure 21: Cholera toxin and pertussis toxin catalyzed ADP-ribosylation in LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan	107
Figure 22: Alterations of protein content of G_{sa} - and G_{it} -subunit in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan	108
Figure 23: Typical Northern blots showing the mRNA level of G_{sa} , G_{it2} , GAPDH	110
Figure 24: Alterations of mRNA levels in G_{sa} and G_{it} subunit in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan	111

I. LITERATURE REVIEW

1. Introduction

The cardiac β -adrenoceptor-linked signal transduction pathway mediates the powerful effects of neuronally released and circulating catecholamines on the heart. The overall effect of β -adrenergic receptor stimulation on the heart includes an increase in force of cardiac contraction (inotropism), rate of cardiac relaxation (lusitropism), heart rate (chronotropism), and conduction (dromotropism) (1). The classic paradigm of biochemical and molecular events leading to these biological responses involve triggering the interaction of guanine nucleotide-binding proteins (G-proteins) with adenylyl cyclases (ACs) to synthesize the second messenger cAMP from ATP after binding of catecholamines to the β -adrenoceptors (β -ARs). The increase in intracellular cAMP levels in turn activates cAMP-dependent protein kinase (PKA), which then stimulates the phosphorylation of target proteins such as cardiac Ca^{2+} -channels, phospholamban, troponin I and troponin C, leading to various physiological responses (2).

Congestive heart failure, a common clinical condition and the final outcome of different types of heart diseases, is associated with a number of alterations in the β -adrenergic signal transduction pathway due to the sustained activation of the sympathetic nervous system (3). Studies with failing hearts from human and animal models have identified such alterations as changes in the expression and function of β -ARs, G-proteins, AC and G-protein coupled receptor kinases (GRKs). Due to its physiological and pathological importance as well as therapeutic implications, this system probably is the most extensively studied and best understood signaling pathway; however, it continues to attract the attention of numerous investigators, particularly with respect to its mode of signal transduction in cardiac disease. It should be mentioned that

although there are several receptor-signaling systems that are capable of exerting positive inotropic effects on the heart through different mechanisms, the β -adrenergic pathway remains the most powerful tool by which heart rate and contractile force development are physiologically regulated and maintained (4). Since β -ARs represent important pharmacological targets for the treatment of congestive heart failure, ischemic heart disease and hypertension, analysis of the mechanisms leading to alterations of β -AR responsiveness may lead to the identification of new means to normalize the receptor function (5). Furthermore, it is not known whether alterations in β -AR system during heart failure are a contributing factor to or a result of ventricular dysfunction. In view of such issues and the availability of modern molecular biological and transgenic technologies, a plethora of information has been collected in the past two decades regarding the physiological and pathological functions as well as regulatory mechanisms of the β -AR system. These include: a) the discovery of the G-protein coupled receptor kinases (GRKs), especially GRK2 (also known as β -adrenergic receptor kinase 1) and β -arrestins, which play a critical role in regulating β -AR system by involving phosphorylation, internalization (sequestration) and resensitization (for review, see Ref. 6); b) by using transgenic animals in which a specific component or regulator was overexpressed or disrupted, the *in vivo* role of each component and regulator has become more clear than previously defined by using subtype-specific ligands. At the same time, the pathological role of each component has started to be unraveled by inducing various pathological conditions such as pressure overload, myocardial infarction in the transgenic mice or by crossbreeding them with genetic cardiomyopathic models and subsequently examining the cardiac function by applying microhemodynamic techniques (for recent reviews see (7-10); and c) the identification of a cAMP-independent pathway of β_2 -AR mediated response, cardiodepressant effect of β_3 -AR in cardiac tissue as well as the putative β_4 -

AR reveal the diversity and complexity and provide new insights for differential regulation and functionality of β -AR subtypes in normal and diseased heart (for review see Ref. 11). It has become clear that β -AR signaling is not an isolated entity but is one among a complex network of signal pathways well controlled by the needs of the heart. It is the purpose of this article to review the physiological functions and pathological alterations of the key components and their regulation in β -AR signal transduction pathway based on the newly emerged information.

2. Biochemical aspect of β -adrenergic signal transduction

Since the β -AR signal transduction pathway is known to consist of three components, namely, β -ARs, G-proteins and AC, it is proposed to discuss these components individually. Furthermore, it is planned to deal with the potential mechanisms involved in the regulation of β -AR signal transduction in this section.

a. β -adrenoceptors

β -ARs belong to the large superfamily of G-protein coupled receptors (GPCRs), which are heptahelical, membrane localized receptors for drugs, neurotransmitters, and hormones. The structural, biochemical and physiological features of β -ARs are outlined in Table 1. Although four types of β -ARs are considered to be present in human heart, only three genes have been identified (12-15). The possibility for the presence of a fourth β -AR is mainly based on pharmacological and physiological data; the confirmation of its existence awaits its cloning and sequencing. The three known genes are characterized by an extracellular glycosylated amino (N)-terminus, an intracellular carboxyl (C)-terminal region and seven transmembrane domains

Table 1. General characteristics of β -adrenergic receptors

	β_1 -AR	β_2 -AR	β_3 -AR	β_4 -AR
Chromosomal location	10	5	8	NA
mRNA size (kb)	2.6	2.2	2.3	NA
Protein length (amino acids)	477	413	402	NA
Prototypic tissue distribution	Heart	Lung	Fat	NA
Cardiac tissue distribution	Whole heart	Ventricles and atria	Coronary vascular beds	SA node, atria, ventricles
Coupler	Gs	Gs and Gi	Gi	Gs
Effector	AC	AC	AC	AC
Catecholamine preference	Noradrenaline	Adrenaline	Noradrenaline	NA
Selective agonist	Xamoterol	Procaterol	CGP12177A, CL316,243	(-)CGP12177, Cyanopindolol
Selective antagonist	CGP 20712A	ICI 118551	Bupranolol	Bupranolol
General function	Vasodepression	Brochodilation	Lipolysis	NA
Cardiac function	stimulation	stimulation	depression	stimulation

For detailed information and literature, see text under literature review. NA: information is not available at present.

(TM1-7) linked by three extracellular and three intracellular loops. Such a structure of β -ARs has been indicated to be responsible for a high activity, stability and resistance to proteases due to its compact core arrangement (16). The β_1 - and β_2 -ARs show 48.9% homology, whereas β_3 -AR exhibits 50.7% and 45.5% homology with the other two β -ARs, respectively (17). The functional role of the hydrophilic N-terminal is not currently defined whereas the amino acid composition of the transmembrane regions has shown structural and functional specificity (14). Experiments using the prototypic β_2 -AR involving mutant receptors in which certain amino acids or regions were deleted or substituted, as well as chimeric receptor subtypes provide valuable information on the β -AR functional domains. It has been suggested that the catecholamine-binding domain is a pocket lined by residues belonging to the hydrophobic TMs, which are also essential for protein folding involved in the receptor-ligand interaction (18). The site of covalent incorporation to the antagonist was found to be located in the second transmembrane spanning domain (19). The amino acid residues essential for agonist binding are different from their interactions with antagonist (18,19). Interestingly, the C-terminus and the cytoplasmic loops, specially the third intracellular loop, were found to be responsible for G-protein coupling (20,21), phosphorylation (13,22,23), desensitization and receptor cycling (21,24-26). The extracellular loops are of low functional activity, although there is evidence to support the importance of the cysteine residues within these domains in stabilizing the ligand-binding pocket via disulfide linkages (27).

All four β -AR subtypes are integral membrane proteins present in the human heart. The β_1 -AR is a protein of 477 amino acids and is located on chromosome 10 (13); it is expressed in all parts of the heart (28). The β_1 -AR subtype couples G_s protein and leads to an increase in heart rate and contractile force development upon stimulation. The β_2 -AR subtype consists of 413

amino acids and is located on chromosome 5 (14). It is concentrated mainly in the ventricles and atria (29). In both atria and ventricles, the β_1 - and β_2 -subtypes exist in a ratio of approximately 3:1. High proportions of the β_2 -AR are apparently found in the pacemaker and conducting regions, indicating they may be important in controlling heart rate and rhythm. β_3 -AR contains 402 amino acids and is located on chromosome 8 (17). Although majority of β_3 -ARs are distributed in fat tissue, they are also found in the coronary vascular beds (26). In addition to its major role in regulating energy metabolism and thermogenesis, Gauthier *et al.* (30) have shown the β_3 -AR mediated cardiodepressant effect in human ventricular preparation by using β_3 -selective agonists or isoproterenol in the presence of both β_1 - and β_2 -antagonists. The β_3 -AR mRNA was also detected in human ventricle and this further confirmed its presence in cardiac tissue (31). The cardiac effects mediated through β_4 -AR have been detected *in vivo* in several species as well as *in vitro* in both atrial and ventricular preparations of human and animals (32-35). All four β -ARs have been suggested to be involved in the regulation of energy expenditure and lipolysis (36).

It is commonly held that β -agonists promote the activation of G_s -protein, which in turn increases the activity of AC. The different physiological responses induced by specific β_1 and β_2 agonist stimulation (smaller positive effect and no lusitropic effect in β_2 as compared to β_1) have promoted the research for identifying couplers other than Gs for β_2 -ARs. It has now been demonstrated that β_2 -AR is also coupled to pertussis toxin sensitive G-proteins, G_i/o in different aspects indirectly and directly (for a recent review, see Ref. 37). Recent experiments using COS7 cells and reconstituted β_2 -AR have shown that PKA phosphorylation of β_2 -AR induces the switch of its coupling from G_s -protein to G_i -protein and subsequently leads to the activation of another signal transduction pathway (38); the results have revealed the diversity and specificity of β -AR

and G-protein interaction. The fact that cardiodepressant effect of β_3 -AR on human ventricular tissue can be blocked by treatment with pertussis toxin (30) indicated the coupling of β_3 -AR to G_i -protein; this effect was also observed in the adipocytes (39,40). The proposal of a patent β_4 -AR is based on the cardiostimulant effect of non-conventional partial agonists *in vitro* in a variety of species (33). Non-conventional partial agonists are blockers with high affinity for β_1 - and β_2 -AR which cause cardiostimulant effects at considerably higher concentrations than those that antagonize the effects of catecholamines (33,41). The β_4 -AR mediated sinoatrial tachycardia (35,42) and increase in atrial contractile force as well as ventricular contractile force suggested its coupling to stimulatory G-proteins.

b. G-proteins

The heterotrimeric G-proteins are composed of α , β and γ subunits with molecular weights ranging from 45 to 52, 35 to 37 and 8 to 10 kDa, respectively. These heterotrimeric proteins function as transducers in various transmembrane signaling pathways (for review, see Ref. 43). The specificity of G-proteins in terms of receptor interactions and possibly effector interactions resides in the α subunit because the $\beta\gamma$ subunits appear relatively interchangeable among the various α subunits (44,45). The α subunit contains the guanine triphosphate (GTP) binding site as well as the intrinsic guanine triphosphatase (GTPase) activity that is responsible for deactivating the GTP-liganded α subunit by promoting the hydrolysis of GTP to guanine diphosphate (GDP) (43). The α subunits are also the substrates for various toxin-catalyzed adenosine diphosphate (ADP)-ribosylation reactions. To date, 20 α subunits, 5 β -subunits and 11 γ -subunits have been identified (46-48). At least seven α -subunits, four G_{α} gene splice variants and one each for $G_{\alpha-1}$, $G_{\alpha-2}$ and $G_{\alpha-3}$, are involved in the regulation of AC signaling mechanisms (49). While four G_{α} -subunits are abundantly expressed in cardiac tissue, $G_{\alpha-2}$ -

protein is predominant in the heart with a little $G_{i\alpha-3}$ -protein (50,51). Of the various β and γ subunits, protein expression of $\beta 1$, $\beta 2$, $\gamma 3$, $\gamma 5$ and $\gamma 7$ was detected in cardiac tissue by using subunit specific antibodies (52). The possibilities of different combinations between the three subunits display a large potential for diversity and complexity in the receptor-G-proteins and G-protein-effector interactions.

The mechanisms of stimulation of AC by G_s -protein are relatively well understood. The binding of an agonist to β -AR catalyzes the exchange of GDP bound to $G_{s\alpha}$ -protein to GTP, with resultant dissociation of GTP- $G_{s\alpha}$ complex from the $\beta\gamma$ subunit and a direct interaction of GTP- $G_{s\alpha}$ complex with the catalytic unit of AC activates the enzyme. Termination of the AC activation by $G_{s\alpha}$ -GTP complex is rapidly achieved by the intrinsic GTPase activity of α subunits initiating the hydrolysis of $G_{s\alpha}$ -bound GTP to GDP (43). On the other hand, inhibition of AC activity by G_i -proteins is mediated by direct interaction of AC with GTP- $G_{i\alpha}$ complex or $\beta\gamma$ subunit that is dissociated from $G_{i\alpha}$ -protein depending on the isoform of the AC and the G_i -proteins involved (53,54). Direct interaction of GTP- $G_{i\alpha}$ complex with AC type V and VI, the dominant isoforms in cardiac tissue, has been detected by using purified AC and $G_{i\alpha}$ -protein in reconstituted membranes (54,55).

Although the $G_{\beta\gamma}$ subunit is made of two polypeptides, G_{β} and G_{γ} , it is functionally a monomer because the two subunits cannot be dissociated except with denaturants (56). Only recently has the $G_{\beta\gamma}$ subunit been recognized as a signal transduction molecule in its own right that regulates as many protein targets as G_{α} -protein (for recent review, see Ref. 56). In addition to its direct regulation on several isoforms of AC (53), the $G_{\beta\gamma}$ subunit has been shown to directly stimulate several effector systems, including phospholipase C, a cardiac potassium channel, a retinal phospholipase A2, and specific receptor kinase (56). Particularly relevant to the β -AR

system is its synergistic enhancement of ARK1 (GRK2) which participates directly in regulating the β -AR function (57).

c. Adenylyl cyclase

AC serves as the effector of β -AR signal pathway and amplifies the agonist-initiated signal by catalyzing the conversion of ATP to second messenger cAMP. Biochemical, immunological, and molecular cloning studies suggested the existence of at least 9 isoforms of AC named as types I to IX (for review see, Ref. 58). They are all membrane associated proteins with molecular weights ranging from 115-150 kDa (58-60). These different isozymes not only have different tissue distributions, but also type-specific regulatory features. Accordingly these AC isoforms are divided into three broad categories: the first category, including type I, III and VIII are synergistically activated by $G_{s\alpha}$ and Ca^{2+} /calmodulin (61,62). The second category, consisting of type II, IV, VII, is activated synergistically by $G_{s\alpha}$ and $G_{\beta\gamma}$ whereas the third category, including type V and VI is inhibited by $G_{i\alpha}$ and Ca^{2+} (58,63-65). Out of the different isoforms of AC, the presence of types II to VII and IX has been detected in heart tissues (58,66,67) with the abundance of type V and VI (63,68,69). The enzyme is stimulated by its coupling with $G_{s\alpha}$ -protein as well as by Mn^{2+} and forskolin, whereas NaF, cholera toxin and pertussis toxin are known to stimulate the enzyme by interacting with G-proteins (63,68). On the other hand, activation of α_2 -ARs and M_2 -muscarinic receptors interact with G_i -protein leading to inhibition of the enzyme activity (70).

d. Regulation of β -adrenoceptors

The regulation of β -ARs requires a coordinated and delicate balance between the process governing receptor activation, desensitization and sensitization. While some information has been provided about activation of β -AR system in the previous section, this section mainly discusses

the processes of desensitization and sensitization in regulating the β -AR signal transduction.

Desensitization refers to the attenuation of receptor signaling despite continued presence of agonist stimulation. Many of these events involve regulation of GPCRs, while others occur down stream of the receptors. By using β_2 -AR as a model, three events namely, *uncoupling*, *internalization*, and *down regulation* were found to be involved in the process of desensitization at the receptor level (71).

Uncoupling of receptors from G-proteins results from receptor phosphorylation, which rapidly desensitize the receptor. At least in the case of β_2 -AR, receptor phosphorylation can occur by the actions of two distinct classes of serine-threonine kinases. One class is the second messenger-dependent kinases, namely, cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), which phosphorylate and directly uncouple β_2 -AR from $G_{s\alpha}$ -protein. Because the different types of agonists and receptors can use the same second messengers, phosphorylation by this class of kinase can provide a basis for cross-talk in the regulation of different receptors simultaneously. The second class of kinases, the G-protein coupled receptor kinases, play a highly specialized role in receptor desensitization because only agonist occupied receptors serve as substrates for these enzymes (72,73). GRK phosphorylation of receptors does not directly inhibit receptor G-protein interaction but rather the GRK phosphorylated receptor serves as a binding site for certain cytosolic proteins, members of the arrestin family. Binding of arrestin protein sterically blocks β_2 -AR mediated G-protein activation.

At least six GRKs sharing 53-93% overall sequence homology have been identified and sequenced to date (73). They have been subdivided into three groups according to their sequence homology and functional similarities. Group 1 consists of the rhodopsin kinase (GRK1), which is predominantly localized to the retina. Group 2 consists of the β -AR kinase 1 and 2 (GRK2-3),

which exhibit more ubiquitous tissue distribution and the third subfamily is composed of GRK4, 5 and 6. GRK 4 is localized primarily to the testis, whereas GRK5 and GRK6 are more ubiquitously expressed. Three subtypes, GRK2, GRK3 and GRK5 are largely expressed in cardiac tissue. While GRK 5 is thought to be a membrane-associated protein, GRK2 and GRK3 are mainly cytosolic protein. As they all phosphorylate membrane-incorporated receptor substrate, efficient receptor phosphorylation thus requires membrane association of the kinase. Experiments using purified and reconstituted β_2 -AR have shown G-protein $\beta\gamma$ subunit greatly enhanced β ARK1 mediated phosphorylation (74). The addition of $G_{\beta\gamma}$ subunit does not directly activate the kinase but by virtue of the γ subunit, which is isoprenylated, promotes membrane association of the enzyme. Mapping of the $G_{\beta\gamma}$ subunit binding domain defined a 125 amino acid residue domain at the carboxyl terminus of β ARK1 which coincides with the pleckstrin homology (PH) domain of this enzyme (74,75). PH domains are approximately 100 amino acid residue regions of protein homology found in numerous proteins involved in signal transduction processes (71). Later experiments by reconstitution of β_2 -AR to vesicles containing purified phosphatidylcholine (PC) in either the absence or presence of phosphatidylinositol 4, 5-bisphosphate (PIP2) revealed that PIP2 is another important factor in translocating β ARK to the membrane. In the absence of PIP2, no $G_{\beta\gamma}$ subunit-mediated enhancement of β ARK activity was observed; significant β ARK mediated β_2 -AR phosphorylation was only observed in the presence of PIP2 and $G_{\beta\gamma}$ subunit. The amino-terminal PH domain of the β ARK was thought to interact with PIP2. Because β ARK mediated phosphorylation of soluble substrates is unaffected in the presence of PIP2 and/or $G_{\beta\gamma}$ subunit, membrane targeting of the kinase appears to account for the enhanced receptor phosphorylation observed in the presence of these two PH domain-binding ligands (74). It should be pointed out that the relative contribution of PKs and

GRKs is still unclear. Essential questions relating to determinants of the decision by the cell as to which pathway and under what conditions the desensitization process proceeds have not been answered.

Once the receptor has been phosphorylated and uncoupled from the signaling pathway, a process called *sequestration* or *internalization* will remove and translocate the plasma membrane receptor to an intracellular compartment into endosomes. Although the exact mechanisms which control this process are still unclear, some work carried out in this regard have shown several important factors that control and regulate the process of receptor sequestration (76-79). Several lines of evidence using mutant receptors lacking the GRK-phosphorylation sites and dominant negative mutant of β -arrestin1 have demonstrated that GRK mediated phosphorylation was not an absolute requirement for sequestration, rather it serves to stabilize the membrane and enhance the binding of β -arrestins. On the other hand, β -arrestins play the major role in mediating receptor sequestration (76,77). Thus β -arrestins play a dual role in regulation of β_2 -AR responsiveness in which they not only bind and uncouple the agonist activated GRK-phosphorylated β_2 -AR from its G-protein but also participate in directing the receptor for agonist-promoted sequestration. Several series of experiments by examining the interaction of β -arrestins with clathrin, a major structural protein in coated pits and dynaminI, a large guanine triphosphatase (GTPase) which is required to pinch off clathrin coated vesicles from the plasma membrane suggested that agonist dependent receptor phosphorylation, arrestin binding, and subsequent endocytosis may serve as the general mechanism for receptor sequestration (78,79).

Another process associated with desensitization is the downregulation of β -ARs, in which there is a net decrease in the total number of receptors after persistent agonist stimulation. Down

regulation of β -ARs as a result of prolonged agonist exposure has been widely described in cardiovascular diseases and is well known to be a side effect of long-term treatment with drugs that act as receptor agonists. However, molecular events involved in down regulation of β -ARs are poorly understood. Recent evidence indicates lysosomal degradation of internalized receptor may be one of the reasons for receptor down regulation and the fact that down regulation of β -ARs is inhibited by dominant-negative constructs of arrestin and dynamin suggests that arrestin-mediated internalization of receptor is required for this process (80). In addition, reduced mRNA levels have been observed in association with β -ARs down regulation in disease conditions and in cells with decreased receptor synthesis (81) indicating decreased mRNA and protein synthesis (82-84) may also be involved in the process of β -ARs downregulation.

After internalization, the sequestered receptors usually have two fates; one fate is being degraded by the lysosomes and this may contribute to down regulation of the receptors. Alternatively and probably the major part of the sequestered receptors are dephosphorylated and recycled back to the plasma membranes as fully functional receptors, this contributes to a process of receptor *resensitization*. While the mechanisms underlying receptor activation and desensitization have been fairly well characterized, the molecular mechanisms by which β -AR responsiveness is re-established have remained unknown. However, recent studies have identified the phosphatase that dephosphorylates the phosphorylated and sequestered receptors as a membrane associated PP-2A class of serine-threonine phosphatase (83-85). Receptor sequestration appears to be required for the resensitization since sequestration deficient mutant of receptor or inhibitors of sequestration process have been demonstrated to block the receptor sequestration (86). The fact that vesicle-associated β_2 -ARs are less phosphorylated than their plasma membrane counterparts indicate that receptor dephosphorylation may occur during the

process of sequestration of receptors into a vesicular population. Indeed, the findings that acidification promotes and alkalization inhibits dephosphorylation have suggested that resensitization occurs through an endosomal dependent pathway (86).

While most studies dealing with β -ARs regulation were conducted on the β_2 -AR subtype, Bouvier *et al.* (87) have examined desensitization of the β_1 - and β_3 -subtypes. The β_3 -AR exhibits fewer of the regulatory processes than does β_2 -subtype. The findings for the β_3 -AR appear to result at least partly from absence of phosphorylation sites for PKA or β ARK in the third cytoplasmic loop and carboxyl-terminal tail of the receptor. Studies of chimeras indicated that domains within these regions and in the second cytoplasmic loop contribute to uncoupling but do not appear to enable sequestration; this implies that the molecular mechanisms of the two processes differ from each other. β_3 -ARs also differ from the other subtypes of β -ARs with respect to long-term regulation, as these show slower and less extensive down regulation and without agonist induced endocytosis or receptor degradation; findings in β_1 -ARs are intermediate between the other two subtypes. It should also be noted that most of these results regarding regulation of β -ARs were carried out *in vitro* in several cell lines or in reconstituted membrane systems, whereas the environment surrounding the β -ARs *in vivo* is much more complex. Thus some caution needs to be exercised while interpreting this information in terms of the *in vivo* regulation of β -ARs.

In addition to receptor modification, alterations of other proteins involved in regulating cAMP levels may also contribute to the loss of β -AR responsiveness following prolonged agonist treatment. Although not well documented in cardiac cells, prolonged activation of β -AR pathway in many other systems redistributes G_{src} out of the membrane and promotes enhanced G_{src} turnover (88,89). Other mechanisms which may contribute to the deactivation of the β -AR signal include

decreases in the activities of AC and PKA, increased metabolism of cAMP and increased expression of $G_{\alpha s}$.

3. Manipulation of gene expression for β -adrenergic signaling

In the past several years, the study of β -AR signaling pathway has been greatly facilitated by the development of transgenic technology. The ability to manipulate the expression of almost each component of this system *in vivo* has led to a better understanding of the specific roles that each component plays in the signaling mechanisms and during the subsequent physiological events. In addition, creating heart failure in the transgenic mice or crossbreeding them with genetic cardiomyopathic models has provided the investigator with an extremely powerful approach to examine the pathogenic or therapeutic role of these components, thereby improving the prospects for therapeutic advances directed at this system.

a. Genetic manipulation of β -ARs

The first demonstration of β -AR transgenesis was carried out by fusing the human β_1 -AR coding sequence with human ANF promoter sequences (90). One line of transgenic mice selected for study expressed approximately eightfold more atrial β_1 -AR sites than wild-type controls. When assessed by spectral analysis these mice show decreased heart rate variability, and atrial strips derived from these mice displayed increased basal contractile force development which cannot be further stimulated by isoproterenol. This suggests that elevated β_1 -AR levels resulted in spontaneous and maximal activation of the signal transduction pathway. Interestingly, the basal as well as Gpp(NH)P stimulated atrial AC activity was depressed in these animals (91) indicating the degree of positive inotropic mediated through the β_1 -AR can be dissociated from effects on

cAMP generation, possibly through direct coupling of receptor to L-type Ca^{2+} -channel activation (92,93). Most recently, transgenic models overexpressing β_1 -AR under the control of α -myosin heavy chain promoter have been reported (94). In both cases, the transgenic mice are born and develop normally. The increased expression of β_1 -AR resulted in increased sensitivity of transgenic mice to catecholamines; however, these mice exhibited increased cardiac contractility at young age only as they were found to develop left ventricular dilation with decreased contractile function at the age about 9 months of age (94). These results seem to indicate that down-regulation of β_1 -AR in different types of heart failure *in vivo* might be a protective mechanism and overexpression of β_1 -AR in the myocardium may not be the way to counteract the desensitized β -adrenergic signaling in heart failure.

Transgenic mice have also been created to overexpress β_2 -AR. Using α -myosin heavy chain promoter, cardiac β_2 -AR expression in transgenic mice was elevated by about 200-fold of the controls values (95). In contrast to the β_1 -AR overexpressing mice, the β_2 -AR overexpressing mice do not develop myocardial dysfunction with age. Since the duration of these observations was approximately 4 months, this period may not be sufficient to conclude that an adverse effect will not occur with time. Nevertheless, these mice demonstrate a two-fold greater AC activity, enhanced heart rate and force and hastened relaxation compared to normal mice at the basal state; but isoprenaline failed to cause additional stimulation (95). The lack of response to β -stimulation in this transgenic line suggests that β -AR signaling pathway was maximally activated. This finding is consistent with the model of receptor activation proposed by some investigators (96,97). It has been indicated that at any given time, a fixed percentage of receptors are in the active state and are coupled to G-protein in the absence of agonist. While overexpression of β -ARs does not necessarily affect this percentage, the excessive number of β_2 -AR leads to a

greater absolute number of receptors coupled to G-protein which results in a saturation of β -AR signaling capacity in the absence of agonist. Since that contractile enhancement during β_2 -AR overexpression, unlike β_1 -AR overexpression, is well correlated to cAMP levels, it has been suggested that the behaviors of that β_1 - and β_2 -AR coupling are distinctly different from each other *in vivo*. β_1 -AR may preferentially modulate cardiac function through second messengers other than cAMP. Pharmacological evidence exists for a different coupling efficiency between β_1 - and β_2 -ARs and support the idea that cAMP may not be the sole regulator of β -AR stimulated cardiac function (98).

The fact that β_2 -AR overexpression leads to enhanced cardiac performance without deleterious effect has led to the hypothesis that β_2 -AR overexpression may prevent the development of cardiac dysfunction in heart failure. This issue was first tested by crossbreeding the β_2 -AR overexpression transgenic line with a murine model of dilated cardiomyopathy and heart failure caused by disruption of muscle LIM protein (MLP) (99). The resultant mice, however, had significantly reduced survival rather than improved ventricular function (100). Most recently this issue was again tested by challenging the β_2 -AR overexpression mice with pressure overload induced by aortic stenosis (101). Instead of preventing the heart from deleterious changes, β_2 -AR overexpression exacerbated the development of heart failure after aortic stenosis (101). Thus β_2 -AR overexpression was confirmed deleterious at least in two models of congestive heart failure and this is consistent with adverse outcomes from clinical trials that treatment with β -agonist or phosphodiesterase inhibitors lead to increased cardiovascular mortality and worsening of clinical symptoms in heart failure patients (102, 103).

Gene disruption of β_1 -, β_2 - or β_3 -ARs and gene disruption of both β_1 - and β_2 -ARs have been reported; these knockout mice have been informative with respect to the specific functions

of β -ARs subtype *in vivo*. Disruption of the β_1 -AR gene in mice was shown to have effects on both embryonic development and cardiovascular function. Approximately 90% of mice homozygous for the β_1 -AR knockout die in uterus between day 10.5 and 18.5 when the disruption is carried out on a congenic background of the 129Sv mouse strain. Mortality is still evident but reduced to 70% when the disruption is done on a mixed strain background (129Sv, C57B1/6J, and DBA2/J) (104). These experiments suggest that β_1 -AR is critical for proper development; β_1 -AR knockouts which survive are fertile and appear normal. In terms of cardiovascular function, loss of β_1 -AR expression was not associated with alterations in either baseline blood pressure and heart rate, although the ability of β -AR agonists to directly stimulate heart rate or contractility in these mice were completely lacking. This insensitivity was observed despite β_2 -AR was expressed in the hearts of β_1 -AR knockout (104). These results suggest that β_1 -AR are selectively coupled to cardiac chronotropic and inotropic responses. Interestingly, the expression of β_2 -AR in β_1 -AR knockout mice was modestly but significantly reduced compared to controls. Although the mechanisms for this phenomenon are not clear, desensitization induced by sympathetic activation or compensatory down-regulation intending to decrease its coupling through G_i could be the reasons (104). In contrast to β_1 -AR knockout, disruption of β_2 -AR gene has no effect on mouse viability or survival. These mice display normal resting heart rate and blood pressure. However, the relaxant effect of β -agonist in both vasculature and uterus is attenuated in these animals (67,105).

β_1 -/ β_2 -AR double knockouts were generated by simple crossbreeding of β_1 -AR and β_2 -AR knockouts. These animals have been developed on a mixed strain mouse background, and thus do not display an increase in mortality relative to wild type littermates. Whereas resting heart rate and blood pressure are normal in β_1 -/ β_2 -AR double knockouts, classic β -AR-mediated effects are

lacking in these animals (106). This model could may be helpful in identifying the physiological function of β_3 -AR in vasculature and heart since both β_1 - and β_2 -ARs are absent in these tissues. Although β_3 -AR knockout mice have also been developed and a thorough study has been carried out to identify the effects of β_3 -AR disruption on lipolysis and energy metabolism (107), the impact of β_3 -AR disruption on cardiovascular function has not been reported.

b. Genetic manipulation of GRKs and β -arrestins

Molecular and biochemical information gathered *in vitro* suggests that three of the six cloned GRKs, namely, GRK2 (also known as β ARK1), GRK3 (also known as β ARK2) and GRK5 are expressed in cardiac tissue. These kinases are considered to regulate cardiac β -AR signaling and cardiac contractility *in vivo*. In the past several years, different lines of transgenic mice and knockout mice have been produced to test the specific functions of GRKs. Transgenic mice with cardiac specific overexpression of GRK2 (β ARK1) exhibit attenuated isoproterenol-stimulated left ventricular contractile force development *in vivo*, these animals show dampening of myocardial AC activity and reduced coupling of β -AR (108) suggesting β -ARs are *in vivo* substrates for GRK2 and enhanced GRK2 activity can lead to cardiac dysfunction. Further evidence of the critical role of GRK2 in cardiac function was found in mice that overexpressed a peptide inhibitor of GRK2 (the peptide corresponding to the carboxyl terminus of GRK2 which *in vitro* competes for $G_{\beta\gamma}$ binding). These mice had enhanced cardiac contractility under baseline conditions and demonstrated supersensitivity to β -stimulation (108). The reciprocal nature of the physiology of these two transgenic lines of mice indicates a critical role for GRK2 in normal cardiac regulation and function.

GRK3 overexpression transgenic mice were also created (109) but these animals demonstrated no attenuation of β -AR inotropic reserve indicating that β -ARs are not the *in vivo*

substrates for GRK3; this is in contrast to the previous belief that GRK2 and GRK3 are isoenzymes. On the other hand, GRK3 overexpression induced desensitized signaling through the thrombin receptor suggesting its role in regulating this pathway. Similar to the findings with GRK2 overexpressing mice, transgenic mice with cardiac-specific overexpression of GRK5 showed severe attenuation in β -AR signaling and loss of β -AR-mediated inotropic effect (110). Thus at least, two GRKs appear to modulate β -AR signaling in the mouse heart. In contrast, animal overexpressing GRK2 had attenuated angiotensin II induced increase in left ventricular contractile force development, whereas those overexpressing GRK5 had normal responses to angiotensin II, indicating that GRK2 but not GRK5 may modulate responsiveness to occupation of angiotensin II receptors in the mouse heart (110). This finding is inconsistent to the *in vitro* studies which demonstrated in a heterologous overexpression cell system, both β ARK and GRK5 could phosphorylate and desensitize angiotensin II receptors (111). These results not only demonstrated the role of GRKs in regulating myocardial function but also provide valuable information for the *in vivo* GRK substrates selectivity.

In addition to transgenic mice, GRKs knockout mice were also created to further understand their *in vivo* physiological roles. Homozygous GRK2 knockouts were found to be embryonic lethal at age 15 day (112). Embryos at this age showed severe defects in cardiac development and most likely died of cardiac failure, indicating an important role for GRK2 in early cardiac development. Heterozygous GRK2 knockouts are viable and have 50% of less GRK2 globally. These animals have increased β -AR signaling and *in vivo* contractility, which is essentially identical to the transgenic mice overexpressing β ARK1 inhibitor (113). Thus increased cardiac contractility seems to be proportional to decreased β ARK1 activity in heart. This is again confirmed by the hybrid gene-targeted animals generated as a result of crossbreeding between

GRK2 inhibitor transgenic and GRK2 heterozygous knockout which demonstrated the highest cardiac contractility and lowest cardiac levels of GRK2 activity (113). These results provide further evidence to support the critical role of GRK2 in the regulation of myocardial function.

Since increased GRK2 levels were found to be associated with down regulation of β -ARs and cardiac dysfunction in failing hearts of both human and animal models, and since inhibition of GRK2 function or reduced GRK2 expression in heart leads to enhanced cardiac function, it is assumed that decreased GRK2 activity may prevent β -adrenergic desensitization in the diseased heart. One attempt to test this issue was to crossbreed mice overexpressing the β -ARK inhibitor with the previous mentioned MLP mice and examine the cardiac function of the resultant hybrid. Strikingly, the hybrid showed normal left ventricular function and chamber size. Moreover, the desensitized β -adrenergic signaling in MLP mice was completely reversed to normal indicating that overexpression of this GRK2 inhibitor prevents the development of cardiomyopathy in this murine model of heart failure. In addition, adenoviral-mediated gene transfer of GRK2 inhibitor to cardiomyocytes from a pacing induced heart failure model in rabbit restored the attenuated β -AR signaling (114). These findings imply that GRK2 might be an important target for the treatment of heart failure and point the way toward development of agents to inhibit GRK2 as a novel mode of therapy.

Due to a critical role of β -arrestins in mediating desensitization and resensitization of β_2 -AR *in vitro*, β -arrestin1 knockout mice have also been generated in order to test its physiological function *in vivo*. Homozygous knockout were overtly normal indicating that β -arrestin1 may not be essential for mouse development or viability. Resting cardiovascular parameters modulated by β -ARs such as heart rate, blood pressure, and left ventricular ejection fraction were not altered as compared to their wild type littermates. However, homozygotes

were more sensitive to β -AR agonist-stimulated increases in the ejection fraction, this is consistent with a role of β -arrestin1 in β -AR receptor desensitization (115).

c. Genetic manipulation of G-proteins and adenylyl cyclase

To assess whether the amount of G-proteins expressed in cardiomyocytes can affect the signaling pathway, transgenic mice with targeted overexpression of $G_{\alpha s}$ were generated (116). *In vitro* studies revealed no alteration in AC activity under the basal state and isoproterenol stimulation, although a lag time necessary for enzyme activation was reduced. *In vivo* studies showed infusion of catecholamines lead to enhanced heart rate and contractility as compared to control mice, indicating an increase in β -AR signaling (117). However, by 16 months of age, these mice demonstrated myocardial degeneration and atrophy, replacement fibrosis and hypertrophy of cardiomyocytes associated with chamber dilation, reduced ejection fraction and increased mortality (117). These results suggested that chronic stimulation of $G_{\alpha s}$ may lead to deterioration of the heart, and the down regulation of $G_{\alpha s}$ and upregulation of $G_{\alpha i}$ in heart failure might be an adaptive rather than a pathogenetic mechanism. This issue is consistent with the recent finding in this transgenic line in which chronic treatment with a non-specific β -blocker propranolol has been shown to prevent cardiac dilation and depressed left ventricular function as well as premature mortality, indicating that β -AR blockade may be salutary in chronic β -stimulation induced cardiomyopathy (118).

Unlike the other components of β -AR system, cardiac-specific manipulation of AC genes was not available until recently (119,120). Overexpression of AC type VI transgene resulted approximately 20-fold increase in AC type VI protein content without affecting β -AR and G-protein content. The mice showed normal basal cAMP and cardiac function but enhanced cardiac function and increased cAMP production upon β -stimulation. Furthermore, long-term

transgene expression was not associated with abnormal histological findings or deleterious changes in cardiac function. Thus unlike β -AR or G-protein overexpression which yields continuous activation and has detrimental consequences, the overexpression of AC does not alter transmembrane signaling except when β -ARs are activated. Together with the fact that the estimated molar proportions of the elements of the β AR/Gs/AC complex in cardiac myocytes are 1:200:3 (121); increasing β -AR number by 20-200 fold in cultured cardiac myocytes and transgenic mice (100) was achieved by only a 2-fold increase in cAMP production, and overexpression of G_{sc} in transgenic mice increased cardiac adrenergic responses minimally (122,123), it was suggested that the amount of AC sets a limit on cardiac signaling *in vivo* and increase of AC, independent of β -AR number and G-protein content, may provide a safe means to increase cardiac responsiveness to β -AR stimulation. This issue was soon confirmed in another study in which transgenic mice overexpressing AC type VI was crossbred with the cardiomyopathic murine model induced by cardiac-directed G_q overexpression (124). The resultant G_q /AC mice showed improved basal cardiac responsiveness *in vivo* and *in vitro* and enhanced cardiac response and increased cAMP production upon β -stimulation. These findings set a new therapeutic target to improve β -AR mediated cardiac response.

Although valuable information has been obtained through the mice models with genetic modification of the key components or regulators of β -AR signaling pathway, some caution should be exercised in terms of interpreting these data and comparing them with those obtained from non-transgenic animals. The above-mentioned transgenic (Table 2) and knockout (Table 3) murine models are generated by using classic gene modification methods in which a particular gene was modulated from the early embryonic stage (125). In this case, the modulation failed to mimic the normal expression patterns or diseases process in which alterations occur mostly in

Table 2. Transgenic models of β -adrenergic signal transduction pathway

Mouse	Transgene	Biochemical phenotype	Physiological phenotype
Atrial β_1 -AR (90)	ANF-human β_1 -AR	\uparrow 8-fold of atrial β_1 -AR number	\uparrow Atrial contractility, no response to isoproterenol
Cardiac β_1 -AR (94)	α -MHC- human β_1 -AR	\uparrow 5-15 fold of β_1 -AR number	\uparrow Basal and dobutamine-stimulated contractility at young age, \downarrow contractility at 16 wks. Hypertrophy and fibrosis at all ages, failure at older age (35 wks)
Cardiac β_2 -AR (95)	α -MHC- human β_2 -AR	\uparrow 200-fold of β_2 -AR number, \uparrow AC activity	\uparrow Basal HR; \uparrow basal contractility; \uparrow myocardial relaxation; insensitive to β -stimulation, no deleterious effect (4 months period)
GRK2 (108)	α -MHC-bovine β ARK1	\uparrow GRK2 mRNA, protein and activity; \downarrow AC activity	Normal basal HR and contractility; \downarrow inotropic and chronotropic response to β -AR and AII stimulation
β ARK1 inhibitor (108)	α -MHC-carboxy terminal 194 AA of β ARK1	\downarrow G $\beta\gamma$ stimulated β ARK1 activity	\uparrow Basal and isoproterenol stimulated contractility, unaltered basal and isoproterenol stimulated HR
GRK3 (109)	α -MHC-bovine GRK3	\uparrow 12-fold of GRK3 expression; \uparrow activity	Unaltered β -AR mediated signaling; \downarrow myocardial thrombin signaling
GRK5 (110)	α -MHC-bovine GRK5	\uparrow 30-fold of GRK5 expression; \uparrow activity	\downarrow Basal and isoproterenol stimulated contractility; unaltered HR
G α (116)	α -MHC-canine G α	\uparrow G α mRNA, protein expression; unaltered AC activity	Normal basal HR and contractility; \uparrow inotropic and chronotropic responses at younger age; hypertrophy and fibrosis occur at later stage (16 mths);
AC type VI (120)	α -MHC-murine AC type VI	\uparrow 20-fold of type VI AC expression; unaltered basal AC activity	Normal basal HR and contractility; \uparrow inotropic and chronotropic responses to β -stimulation

See text for further details. α -MHC: α -myosin heavy chain; HR: heart rate; BP: blood pressure; \uparrow indicates increases; \downarrow indicates decreases.

Table 3. Knockout models of β -adrenergic signal transduction pathway

Mouse	gene targeted	Biochemical phenotype	Physiological phenotype
β_1 -AR knockout (104)	Knock out of β_1 -AR	Absence of β_1 -AR, \downarrow AC activity	70% embryonic lethality; unaltered basal HR and contractility; insensitive to β -stimulation
β_2 -AR knockout (105)	Knockout of β_2 -AR	Absence of β_2 -AR,	No embryonic lethality; normal HR and BP; normal chronotropic response to β -stimulation; reduced vasodilation to β -stimulation
β_1/β_2 double knockout (106)	Knock out of both β_1 - and β_2 -ARs	Absence of both β_1 and β_2 -ARs	Normal basal HR and BP, blunted inotropic and chronotropic responses to β -stimulation
β_3 -AR knockout (107)	Knockout of β_3 -AR	Absence of β_3 -AR	Moderate increase in fat stores, absent of β_3 -agonist stimulated AC activity and lipolysis; cardiac performance has not been assessed.
GRK2 knockout (112)	Knockout of β ARK1 gene	Absence of β ARK1 expression and GRK activity at age 13 days of embryo	Embryonic lethality in homozygous mice, defects in cardiac development and impaired myocardial function in embryos
β -arrestin 1 knockout (115)	Knockout of β -arrestin 1 gene	Absence of β -arrestin1	Normal basal HR, BP, contractility; \uparrow inotropic responses to β -stimulation

See text for further details. α -MHC: α -myosin heavy chain; HR: heart rate; BP: blood pressure; \uparrow indicates increases; \downarrow indicates decreases.

adulthood. In addition, the expression of a transgene or deletion of an endogenous gene at unwanted time not only influence the proper development of these animals but also raise the potential problem that compensatory mechanism may develop and interfere with the physiological and pharmacological characteristic of the phenotype. Nevertheless, some advanced gene modification techniques are now available to enable the induction of transgenic expression (126) or repression of an endogenous gene (127,128) at any particular time and in particular tissue. The application of these advanced techniques in the study of β -AR signaling may circumvent the problems and adverse effects induced by the classic gene modification techniques and thus greatly facilitate our understanding of β -AR signaling *in vivo* and in turn lead to more effective and specific therapeutic treatments in humans.

4. β -Adrenergic signaling in heart failure

Heart failure is a pathophysiological state in which the heart is unable to pump enough blood to meet metabolic needs of the body (129). It describes an end stage syndrome of various types of cardiac diseases (cardiac muscle diseases or workload induced heart diseases) that can be acute or chronic in terms of time course. Although several compensatory processes are activated during the development of heart failure, the activation of sympathetic system is the most prominent one which at the early stage results in the stimulation of β -AR-G-protein-AC complex for maintaining heart function (130-132). However, it has become apparent that the extent of elevation in circulating catecholamines, particularly norepinephrine, was associated with the severity of heart failure, the highest catecholamine level was associated with the poorest prognosis (132-134). This notion has led to a great number of studies examining changes in β -

AR transmembrane signaling in both human heart failure and a variety of experimental models of congestive heart failure (for reviews, see Refs. 135-137). Table 4 summarizes the human heart diseases and animal models that have been used to study the β -adrenergic signaling in diseased conditions. It can be observed from Table 4 that basically every kind of human heart disease and all experimental animals of cardiac hypertrophy and heart failure have been used to examine alterations of β -AR signaling. It should also be mentioned that most types of heart failure are preceded by a compensatory hypertrophic stage. Although functional states between cardiac hypertrophy and heart failure are completely different, a desensitization of β -AR signaling pathway usually occurs in most types of cardiac hypertrophy which is similar to alterations observed in heart failure (for review see Refs. 138,139). Since cardiac hypertrophy and heart failure appear to be two stages of the same disease, the changes related to desensitization of β -AR signaling in cardiac hypertrophy will not be discussed separately but will rather be considered as a specific stage of heart failure. Although there is a general agreement based on information obtained from failing hearts of human and animal models that decreased responsiveness to β -agonist stimulation is associated with a selective down regulation of β_1 -AR, uncoupling of β_2 -AR, increase of $G_{\alpha s}$ expression and GRK2 expression, and/or decreased AC activity, variations exist depending on etiologies and stages of the underlying diseases (135-137,139,140). It is therefore the purpose of this section to review the literature on changes and mechanisms of each of these components and their regulation during the development of heart failure in both human and animals with different etiologies and at different stages.

a. Alterations in β -adrenoceptors in heart failure

A decrease in the actual number of β -ARs in failing human myocardium with dilated

Table 4. Human heart diseases and animal models used for studying β -adrenergic signal transduction pathway

Human heart diseases	Experimental models
Cardiomyopathy Idiopathic cardiomyopathy Dilated cardiomyopathy Ischemic cardiomyopathy	Cardiomyopathy Genetic cardiomyopathy Diabetic cardiomyopathy Adriamycin-induced cardiomyopathy Pacing-induced cardiomyopathy
Hypertension Essential hypertension Acquired hypertension	Hypertension Spontaneous hypertensive rat Dahl salt-sensitive hypertensive rat DOCA salt-sensitive hypertensive rat
Valvular diseases Mitral insufficiency Mitral stenosis Aortic insufficiency Aortic stenosis	Cardiac hypertrophy Aortic banding Mitral Insufficiency Catecholamine-induced hypertrophy Hyperthyroxine-induced hypertrophy
Coronary artery disease	Ischemic diseases Myocardial infarction Global ischemia
Congenital heart disease	Genetic models

cardiomyopathy was first reported by Bristow *et al.* (141). This phenomenon was subsequently confirmed by several other investigators both in human and animal models (142-147). In subsequent years, Bristow and his coworkers (148-153) extended their studies to gain an in-depth information on this subject. Although there is a general agreement that β -ARs are down-regulated in heart failure, the receptor subtypes, and the extent of changes may differ slightly or completely due to different stages and etiologies of the heart failure. Earlier investigations showed that mainly total β -ARs were downregulated in heart failure but with the recognition of specific antagonists for both β_1 - and β_2 -ARs, such as ICI 118,551 and CGP-20712A, respectively, subtype specific evaluation was made possible. Thus a selective reduction in the β_1 -receptor density with an unaltered or even increased β_2 -receptors density was detected in most types of heart failure. This was confirmed by Altschuld (154) in both tachycardia-induced failing canine cardiomyocytes and failing human cardiomyocytes. Both canine and human cardiomyocytes showed positive response to a highly selective β_2 -receptor agonist, zinterol, indicating that these cell types contained functional β_2 receptors. In fact, the ventricular responses to β_2 -AR stimulation was increased in heart failure due to tachycardia in dogs. Studies from our laboratory using cardiomyopathic hamster (UM-X7.1) have also shown that although there was no change in the affinities for β_1 - and β_2 -ARs, the number of β_1 -ARs, unlike that of β_2 -ARs, was markedly decreased in cardiac membranes from failing hearts (155).

The possibility of down-regulation of β -ARs being related to the degree of heart failure was also investigated. A decrease in myocardial β -AR density was associated with an increase in symptoms and the decrease of left ventricular function in patients with chronic mitral regurgitation with or without aortic regurgitation (156). Subsequently Ohsuzu *et al.* (157) also demonstrated that there was good correlation between β -ARs numbers and the stages of heart

failure in the failing human heart despite its wide range of etiology. Similar results were obtained from different animal species with different types of heart failure. Sethi *et al.* (158) studied the status of β -adrenergic mechanisms in the myocardium of cardiomyopathic hamster (UM-X7.1) during different stages of heart failure. They reported that the number of β -ARs was increased at prefailure and early failure, unchanged at moderate failure, and decreased at severe stage of congestive heart failure. Simultaneously, there was an increase in norepinephrine turnover in all cardiomyopathic hearts except those at severe stages of heart failure. In another study, Witte *et al.* (159) showed that there was a lower efficiency of coupling of β_1 -AR compared to the β_2 subtype in ventricles from healthy Syrian hamster and as the disease progressed there was a further reduction in β_1 -adrenergic function in the cardiomyopathic animals. In dog model of pacing induced heart failure, the β -AR density was not significantly different in early stage heart failure, but was decreased by 63% at the peak stage of heart failure and was restored to control value after recovery from heart failure; this indicated a good relationship of severity of heart failure with the changes in the density of β -ARs (160). However, similar work carried out by another group showed that changes in myocardial β -adrenergic signal transduction shortly (1 day) after the initiation of pacing in the dog heart which occurred even before the development of heart failure (161).

There is also evidence showing that alterations of both β_1 -ARs and β_2 -ARs are related to type and etiology of the disease (162). Brodde *et al.* (163) reported in an extensive study that the number of β_1 -receptors was reduced in all types of chronic heart failure whereas that of the β_2 -receptors decreased in mitral valve disease, tetralogy of Fallot and end stage ischemic cardiomyopathy, but remained unaltered in the end stage of dilated cardiomyopathy and aortic valve disease. Therefore the changes in the β_1 - and β_2 -subtype distribution may be related to the

etiology rather than to the clinical degree of heart failure (146). In another study of patients with end stage heart failure due to idiopathic cardiomyopathy (ICD) and ischemic cardiomyopathy, the total β -AR density was reduced by 50-60% in all regions but the β_1 -AR and β_2 -AR distribution differed with the cause of heart failure. In idiopathic cardiomyopathic patients, the β_1 -ARs were not reduced significantly while in patients with ischemic cardiomyopathy, both β_1 - and β_2 -ARs were decreased significantly in all regions (164). It seems that β -ARs are regionally regulated in heart failure. Although the β -AR density was decreased in atrial muscle strips, the isoproterenol-mediated increase in force of contraction was unaffected in the failing human heart. On the other hand, increasing the degree of heart failure was accompanied with progressive reduction in isoproterenol-mediated increase in force of contraction as well as a progressive decrease in the number of β -ARs in papillary muscle strips (165). A transmural heterogeneity of norepinephrine uptake (measured by specific [3 H] norepinephrine accumulation) has been reported in human heart with the uptake in the subendocardial regions of failing left ventricles being five-fold lower than that in the subepicardial regions. These findings support the hypothesis that a subendocardial defect in norepinephrine uptake may chronically elevate local interstitial catecholamine levels and thereby down-regulate β -ARs in a spatially heterogeneous distribution (166). Similarly, a significant transmural gradient in the density of myocardial β_1 -AR exists in hearts of patients with ischemic and idiopathic dilated cardiomyopathy, resulting in a markedly altered ratio of β_1 : β_2 receptor densities in the subendocardium (167). This is also the case in some animal models. A chamber specific decrease in β -AR density was found in the early stages of right ventricular hypertrophy in monocrotaline-treated rats whereas β -AR density and AC activity in the interventricular septum were decreased in the advanced stages of heart failure (168). Similar heterogeneity of β -ARs distribution was found in failing cardiomyopathic hamsters

(BIO 14.6) with an increase in β -ARs density in septal and subendocardial regions showing increased interstitial fibrosis; this indicated that an increase in β -AR density may be associated with the development of cardiomyopathy in hamster (169). However, in another study, it was showed that in human severe failing ventricles, β -ARs were evenly down-regulated and with no regional variations (170), indicating that changes in regional distribution of β -ARs may be dependent upon stage and type of heart failure.

Despite general reduction of β -ARs reported in studies on human and several animal models of heart failure, there are some reports showing that β -ARs are either unaltered or increased. In the spontaneous hypertensive rat model of heart failure, impaired intrinsic left ventricular myocardial function and depressed inotropic responsiveness to β -AR stimulation were not associated with down-regulation of the β -ARs (171). On the other hand, the β -AR density was found to be increased in these failing hearts. No alteration in β -AR number and site specific stimulated AC activity was found in the heart failure due to myocardial infarction in rat (172), indicated that this system might not be responsible for altered inotropic responsiveness to catecholamine seen in this model. In the early stage of hereditary cardiomyopathy in hamster, isoproterenol-stimulated AC activity was significantly decreased, while NaF- and forskolin-stimulated AC activity remained unchanged (173). This suggested that the decrease in isoproterenol effect was receptor-mediated. However, neither the density nor the subcellular distribution of the β -ARs were affected, but the agonist binding properties of the β -ARs showed some evidence for receptor uncoupling (173). Another study reported that there was an increase instead of a decrease in the number of β -ARs in hereditary cardiomyopathy in hamsters (BIO 14.6) (169).

Desensitization of myocardial β -ARs may result both from impairment of the

norepinephrine neuronal uptake and from an increase in circulating norepinephrine concentration as a consequence of increased concentration of norepinephrine at the receptor site (174). In human heart failure, there was a presynaptic defect in the sympathetic nervous system leading to a reduction in the uptake of norepinephrine (175). Significant inverse correlation between β -AR density and interstitial norepinephrine was found in pacing-induced heart failure (176). Norepinephrine uptake was assessed by [125 I]-metaiodobenzylguanidine (MIBG) scintigraphy in 18 patients with congestive heart failure due to idiopathic dilated cardiomyopathy and the results showed that cardiac norepinephrine uptake was significantly decreased as compared with control subjects. This decrease was related to severity of the disease based on hemodynamic index (177). In rabbits, the β -AR density and norepinephrine concentration were lower in left ventricle but not in right ventricle with acute heart failure induced by aortic regurgitation but this was not the case in chronic heart failure induced by adriamycin treatment. These findings suggested that a local abnormality in symptoneuronal regulation may depend upon the stage of failing myocardium (178). In adriamycin-induced heart failure in rats, Tong *et al.* (179) also showed that norepinephrine levels in the myocardium varied with the stage of heart failure indicating a significant increase at 3 weeks and a decrease at 6 weeks. In addition, chronic blockade of β -AR prevented the β -adrenergic subsensitivity in a dog model with right-sided congestive heart failure (180) indicating that an increase in cardiac catecholamines plays an important role in abnormality of β -ARs. On the other hand, the β -ARs were down-regulated and their function was impaired in hypertrophic obstructive cardiomyopathy; this down-regulation was not caused by catecholamines as the plasma levels were either normal or lower than the normal (181).

In addition to examining *in vivo* relationships between β -AR alterations and catecholamine levels, efforts have also been made to examine the effect of external β -agonists on β -ARs.

Several studies using *in vivo* infusion of isoproterenol in rats found down regulation of both β_1 - and β_2 -ARs with greater reduction in β_2 -AR in crude myocardial membrane preparations (182,183). For this inconsistency, it was argued that the endogenous transmitter norepinephrine can induce greater downregulation of β_1 -AR than is induced by the synthetic agonist isoproterenol, either directly because of higher affinity of norepinephrine for β_1 -AR or indirectly by stimulation of α -ARs. In support of this hypothesis, when a norepinephrine secreting pheochromocytoma was implanted in rats for 3-4 weeks, selective down-regulation of β_1 -AR was observed in myocardial membrane preparations (184). These results suggest that chronic stimulation by β -agonists may lead to downregulation of β -ARs and the selective downregulation of β_1 -AR in heart failure may be due to elevated levels of norepinephrine.

It has been shown that alterations in gene expression may be another mechanism of β -AR downregulation (185,186). The abundance of β_1 -AR mRNA was reported to be decreased in the failing human left ventricle as compared with nonfailing control (187). Engelhardt *et al.* (188) showed that β_1 -AR mRNA levels in the human ventricular tissue were decreased by 7% in mild, 26% in moderate and 50% in severe heart failure, and there was a good correlation between hemodynamic indicators and β_1 -AR mRNA levels. In contrast, β_2 -AR mRNA levels were unaffected by heart failure, which is consistent with no changes in the density of β_2 -AR under these conditions.

Since the GRKs play an important role in regulating β -AR function, especially in β -AR desensitization and downregulation, it has been hypothesized that the increased expression of one or more GRKs is related to desensitization of β -AR signaling and downregulation of β -ARs. Data supporting this hypothesis has been obtained from both human and animal models of heart failure. Ungerer *et al.* were the first to report that increases in the expression and activity of

β ARK1 (GRK2) accompanied the downregulation of β_1 -AR and uncoupling of β_2 -AR in patients with end-stage congestive heart failure (189). Subsequently, enhanced expression of GRK(s) has been detected in several animal models, including cardiomyopathic Syrian hamster (190), pacing induced congestive heart failure in porcine (66) and rabbit (114); myocardial infarction in rats (191); spontaneous hypertensive rats (192) and aortic constriction induced pressure overload in mouse (193) as well as catecholamine induced cardiac hypertrophy in mouse (194) (see Table 5 for a summary). The increase in GRK (particularly β ARK1)-mediated phosphorylation of both β_1 - and β_2 -ARs in heart failure may contribute to the loss of their responsiveness, leading to impairment of their function through receptor uncoupling. Although the mechanisms of enhanced GRK expression in heart failure is still not clear, activation of the sympathetic system seems to play a role. It has been shown that β -stimulation in mice induced the expression of GRK2 (194,195) whereas β -blockers inhibited the expression of GRK2 and enhanced β -AR signaling in mice and pig (195,196). This reciprocal regulation of GRK2 by β -agonist and blocker suggest that GRK2 may be affected by sympathetic tone. In fact, enhanced contractility and decreased GRK2 has been recently observed in transgenic mice lacking endogenous norepinephrine and epinephrine without changes in β AR density or G-protein levels (197). This not only reflects sustained activation by norepinephrine as a primary stimulus that promotes deterioration of cardiac signaling but also demonstrates the key role of GRK2 in regulating the β -AR signaling in both normal and abnormal situations. Due to the intimate relationship of GRK2 with both β -arrestins and $G_{\beta\gamma}$, the increase in GRK2 seems to imply that these two factors may also be influenced in a similar fashion in heart failure. However, both β -arrestin protein content and mRNA expression remained unaltered in heart failure (198) and until now, no significant alteration has been obtained regarding changes in G_{β_1} subunit in heart failure. Thus it appears that

Table 5. Alterations in G-protein-coupled receptor kinases in diseased hearts of human and animal models

Models	Activity	Protein content	mRNA	References
Human end stage heart failure	↑	ND	↑ GRK2; -GRK3	(189)
Pacing				
Dog 4 days	↑	-GRK2; ↑GRK5	-GRK2; ↑GRK5	(66)
28 days	↑	-GRK2; ↑GRK5	↑GRK2; ↑GRK5	(66)
Rabbit	↑	↑GRK2; -GRK3	ND	(114)
Cardiomyopathic Syrian hamster			↑GRK2	(190)
Myocardial infarction in rat	↑	↑GRK2	ND	(191)
Spontaneous hypertensive rats	↑	↑GRK2, -GRK5	ND	(192)
Pressure overload in mice (aortic constriction)	↑	↑GRK2	ND	(193)
Catecholamine-induced cardiac hypertrophy in mice	↑	↑GRK2	ND	(194)

See text for more details. ↑ Indicates increases; - indicates unaltered; ND: not determined.

the events leading to β -AR desensitization in heart failure are probably regulated primarily at the level of receptor phosphorylation by GRKs, without affecting the level of β -arrestins and $G_{\beta\gamma}$. Theoretically, PKA phosphorylation induced receptor desensitization may also contribute to desensitized β -AR signaling observed in heart failure, however, very little information is available regarding possible changes in PKA function and how it may regulate β -AR signaling in heart failure (199). Nevertheless, we have recently shown that PKA activity and protein level in both cardiac and skeletal muscles were significantly increased in the failing heart from cardiomyopathic hamster (200), indicating an active role of PKA in altering β -AR signaling in heart failure.

On the basis of the foregoing discussion, it is difficult to propose a unifying explanation concerning desensitization of β -AR mechanisms in the failing heart. However, the attenuated response of the failing heart to catecholamines appears to be caused by two mechanisms: down-regulation of β -ARs and/or β -AR uncoupling. Preferential down-regulation of β_1 - compared with β_2 -receptors is probably due to closer proximity of the former to sympathetic nerve endings, being in direct line of the neuronal signal transduction process. Internalization of β -ARs does not seem to play a significant role in chronic heart failure since several studies have shown no difference in the quantity of β -ARs in a light vesicular fraction of non-failing and failing human hearts (170,201,202). Emerging information collected from both human and animal models of heart failure as well as from genetic modulated mice models in recent years suggest that GRKs, especially GRK2, may play an important role in desensitization of β -AR signaling observed in heart failure. However, the exact mechanisms regarding up-regulation of GRKs and its implication in heart failure are still largely unknown. Future studies in this area by applying inducible gene modification to heart failure models will give more accurate information of this enzyme in regulating β -AR signaling in diseased conditions.

b. Alterations of G-proteins in heart failure

Defects in the β -AR signal transduction system in heart failure may be due to alterations in both stimulatory (G_s) and inhibitory (G_i) G-proteins (49,203,204). Quantitative and functional changes in G-proteins have been described in various forms of β -AR desensitization (205). A growing number of studies suggest that the quantities of G_s - and G_i -proteins are altered by chronic agonist-induced activation of either stimulatory or inhibitory receptor systems coupled to AC (206-210). AC could also become less sensitive to catecholamine stimulation either by decreasing G_s -protein (206,208) or by increasing G_i -protein contents (171). Alterations ...: G-proteins in terms of function, protein content, and mRNA levels in different types of heart failure in human and various animal species have been reported (206-220). The majority of studies have found that cardiac expression of α -subunit of G_i was increased. This has been initially demonstrated by pertussis toxin-catalyzed ADP-ribosylation which measures all forms of G_i - as well as G_o -proteins (217-219,221). Myocytes isolated from failing human heart showed a reduced ratio of isoproterenol/calcium-induced contractile response in comparison to control preparations. However treatment of the failing myocytes with pertussis toxin increased the isoprenaline/calcium ratio significantly, while cholera toxin had no effect. These observations support the hypothesis that increased G_i -protein levels or activities contribute to the attenuated responses to β -AR stimulation in human heart failure (222). Northern blot experiments with ^{32}P -labeled cDNA probes in transplanted human hearts showed that $G_{i\alpha-2}$ - and $G_{i\alpha-3}$ -protein are the predominant $G_{i\alpha}$ -mRNA subtypes in ventricles, whereas $G_{i\alpha-1}$ -mRNA was not detectable (223). Two different groups have demonstrated that both the pertussis toxin substrate and $G_{i\alpha}$ -protein content, as assessed by Western blot, were increased in myocardial tissue from patients with congestive heart failure (218,221). To determine the effects of species and etiology

of heart failure, changes in pertussis toxin substrate and G_i -protein content were investigated in rats and hamsters with cardiomyopathy induced by adriamycin, as well as in coronary artery ligation-induced ischemic heart failure in rats. It was found that G_i -content, as determined by Western blot, was increased by 15-35% in all groups; assessment of G_i -protein function by pertussis toxin-catalyzed ADP-ribosylation revealed an increase of 24-44% in G_i -proteins. In addition, G_i -protein function, as assessed by the acetylcholine-induced inhibition of AC, was also increased (224). Alterations of $G_{i\alpha}$, like β -ARs, may be also regionally regulated. In this regard, Bohm *et al.* (214) quantitated $G_{i\alpha}$ -proteins by a fast radioimmunoassay technique using the iodinated synthetic peptide ^{125}I -KENLKDCGLF in human heart. It was shown that immunodetectable $G_{i\alpha}$ -protein was increased in failing left ventricle but not in the right ventricle, in dilated cardiomyopathy and ischemic cardiomyopathy, while downregulation of β -AR occurred in both ventricles. These findings indicate that the ability of the left ventricle but not the right ventricle to express $G_{i\alpha}$ -proteins may be increased in left ventricular heart failure, suggesting regional regulation of G_i -proteins in heart failure. In contrast to G_i -proteins, G_s -proteins were either increased or unchanged in heart failure of both human and several animal species with different etiologies (225-228).

There are a number of studies in failing heart with respect to changes in myocardial G_s -proteins that form the integral part of β -adrenergic signal transduction system. In studies with various strains of cardiomyopathic Syrian hamsters (BIO 14.6 and TO), it was demonstrated that there was a substantial decrease in functional activity of $G_{s\alpha}$ -proteins at 28 days of age (before the onset of phenotype changes); however, in older animals (154 days), steady state levels of the mRNA encoding $G_{s\alpha}$ -proteins were decreased (229). In contrast, the BIO 53.58 strain of cardiomyopathic hamsters, which do not develop hypertrophy, were found to have a functional

defect in G_{src} -protein only at the time of cardiac dilation (230). Ransnas *et al.* (231) demonstrated a substantial decrease in G_{src} -protein content of cardiac membranes from idiopathic dilated cardiomyopathic patients. In a rat model of ischemic heart failure produced by coronary artery ligation, no significant alterations in G_{src} -, G_{ir} -mRNA expression and protein content, β -AR binding and basal and MnCl_2 -stimulated AC activities were observed. However, the NaF- and forskolin-stimulated AC activities were depressed, indicating a functional change in G_{src} -proteins (227). Kaura *et al.* (155) examined the status of β -adrenergic signal transduction system in severe cardiomyopathic hamster (UM-X7.1) hearts and found that the functional activity of G_s -proteins (measured by cholera toxin stimulation of AC) was depressed whereas that of G_i -proteins (measured by pertussis toxin stimulation of AC) was increased, in the failing heart. These results indicated that the loss of adrenergic support at severe stages of congestive heart failure in cardiomyopathic hamsters may involve a reduction in the number of β_1 -ARs, an increase in G_i -protein content and bioactivity in addition to an uncoupling of G_s -proteins from the catalytic site of AC in cardiac membrane. There is a general consensus that in heart failure no marked or consistent change occurs in myocardial G_s -protein content, but the uncoupling of G_{src} -protein may be another mechanism that contributes to the depressed function of β -adrenergic system in heart failure (156).

The mechanisms underlying alterations of G-proteins are far from clear; however, it has been hypothesized that an increase in G_i -proteins in the failing heart is due to the increased activity of sympathetic nervous system. This is based on the observation that chronic exposure of guinea pig cardiomyocytes (222) and rat neonatal cardiomyocytes (232) to norepinephrine resulted in increased G_i -proteins. The chronic treatment of the rats with isoproterenol increased G_i -proteins (233,234), as well as $G_{\text{ir-2}}$ and $G_{\text{ir-3}}$ mRNA levels (233,235) in addition to increasing

the pertussis toxin catalyzed ADP-ribosylation. The mRNA level of $G_{i\alpha-2}$ -proteins was also increased in failing hearts with idiopathic dilated cardiomyopathy and ischemic cardiomyopathy when compared to nonfailing hearts whereas the levels of $G_{i\alpha-3}$ - and $G_{s\alpha}$ -mRNA were not altered (236). An infusion of isoproterenol in rats for a period of 4 days induced an increase in $G_{i\alpha-2}$ - and $G_{i\alpha-3}$ -mRNA levels in the heart by about 50% and 30%, respectively. While no changes in the level of $G_{s\alpha}$ -mRNA were recorded (237), the treatment with isoproterenol resulted in a significantly hypertrophied heart. Therefore, it was suggested that isoproterenol may induce transcriptional increase of the genes via cAMP-dependent activation of transcription factors; this is considered as a self-adaptative mechanism to increased cAMP levels due to prolonged adrenergic stimulation.

There are possibilities that changes in protein level and mRNA expression of G_i -proteins may be differentially regulated in various regions of the heart (214). In porcine heart, topographical differences in G-proteins and β -ARs have been demonstrated (196,238). It was shown that due to physiological and anatomic differences between the right atrium and the left ventricle, the right atrium of the porcine heart had 58% less AC activity, 74% less $G_{s\alpha}$ -mRNA and 83% less $G_{i\alpha-2}$ -mRNA without any difference in β -AR numbers as compared to left ventricle. However, when the hearts were rapidly paced to produce heart failure, the left ventricle showed significant reduction in mRNA expression for both $G_{s\alpha}$ - and $G_{i\alpha-2}$ -proteins, whereas the right atrium showed increased $G_{s\alpha}$ - and $G_{i\alpha-2}$ -mRNA expression. Despite divergent mRNA expression, $G_{s\alpha}$ - and $G_{i\alpha-2}$ -proteins in both chambers were downregulated in heart failure. On the other hand, both the chambers had similar β_1 - and β_2 -AR mRNA contents, but there was a selective reduction of β_1 -AR protein and mRNA levels in response to heart failure (238). It was suggested that elevated levels of circulating catecholamines in heart failure may lead to the alterations in the level

of G_i -mRNA which consequently caused changes in G_i -protein concentration and influenced function of β -adrenergic system (239).

c. Alterations of adenylyl cyclase in heart failure

Compared with the other two components in β -AR system, relatively less information is available about changes in AC in chronic heart failure. This may be due to several reasons including the fact that the enzyme is regulated by stimulatory G- and inhibitory G-proteins. Furthermore, the enzyme is extremely unstable and difficult to assess under *in vivo* and *in vitro* conditions. In addition, there are no specific agonists or antagonists available at present for this enzyme. However, the amount of AC may set a limit on β -AR transmembrane signaling since the estimated molar proportions of β -AR: G_s :AC is 1:200:3 (121). In addition, increased cardiac β -AR or G_s expression does not yield proportional increase in transmembrane signaling (95, 116, 240) whereas increased expression of AC type VI in neonatal cardiomyocytes has been shown to increase β -AR stimulated production of cAMP proportionally. This evidence indicates the important role of AC in mediating, regulating and controlling the signals imposed on β -ARs. One approach that has been used to examine AC function in tissues utilizes forskolin, an activator of AC. Reduced forskolin stimulated AC activity has been demonstrated in cardiac tissue of several experimental models. (143, 152, 161, 168, 171, 185, 206, 207, 210, 213, 217, 228, 241, 241-246). In heart failure initially induced by volume overload and later by pressure overload in rabbits, the forskolin-stimulated AC activity was markedly impaired (242). Rapid pacing-induced heart failure caused a reduction in basal and forskolin-stimulated AC activities, as well as the steady state mRNA for AC types V and VI in dogs (245). Reduced high affinity binding of β -ARs and modest decrease of AC activity were also reported in the canine model of rapid-pacing induced heart failure (247). In studies with cardiomyopathic hamsters with severe heart failure, basal AC

activity of cardiac membranes was unaltered, but the activation of the enzyme by different concentrations of isoproterenol, forskolin, NaF, or Gpp(NH)p was depressed significantly. However, no change in basal or stimulated AC activity was observed at prefailure or early failure stages (155,248). Using a rat model with myocardial infarction, we have also reported that the attenuated response to isoproterenol stimulation of the LV was associated with significantly depressed basal as well as forskolin-stimulated AC activities whereas enhanced basal as well as forskolin-stimulated AC activity was observed in the hypertrophied and compensated RV with an augmented levels of cAMP with or without isoproterenol stimulation (249,250). An analysis of the existing literature indicates that the basal AC activity and alterations in stimulatory effects of one or more interventions at the catalytic/regulatory sites of the enzyme may contribute towards the desensitization of the β -adrenergic system in heart failure.

In summary, the down-regulation of β_1 -AR and uncoupling of β_2 -ARs are associated with increased activity and gene expression of GRK2 and G_{in} with little changes in AC catalytic subunit and G_{sa} as well as G_{py} . However, it should be noticed that most of these changes were detected in myocardial samples either from human end stage heart failure or experimental models with congestive heart failure. These samples contained several tissue types, including blood vessels, muscle, connective tissue and nerves, so routine membrane preparation from myocardium represent several cell types. It is also known that significant fibrosis occurred in almost each human heart disease and experimental models of heart failure. The occurrence of fibrosis has the potential of changing normal composition of myocardium, since cardiac myocytes would be replaced by fibroblasts, inflammatory cells, and collagen. Due to the fact that β -ARs, G-proteins, GRKs and AC are ubiquitously expressed in fibroblasts, smooth muscle cells and nerve cells in addition to cardiomyocytes and different cell type have different percentage of β -AR subtype

expression, myocardial fibrosis could alter the density as well as the subtype composition in myocardial homogenate. In addition, most types of heart failure are also associated with hypertrophy, which makes it difficult to distinguish actual downregulation (internalization and degradation) of receptor from a dilution of the receptor on the plasma membrane. Thus the current information on changes in β -adrenergic signaling obtained from myocardial samples needs to be interpreted carefully. Further studies by isolating and separating different cell types from failing myocardium followed by examination of their alterations in each component of the β -AR signaling pathway can be seen to circumvent these problems. Alternatively, animal models of heart failure with no fibrosis will also provide a useful tool to examine changes in β -AR signaling pathway.

II. STATEMENT OF THE PROBLEM AND HYPOTHESES TO BE TESTED

From the foregoing discussion, it is clear that β -AR signal transduction pathway is a complex system consisting of several components including β -AR, G-proteins, AC and regulatory mechanisms. Limitations of studying such a system in healthy and diseased hearts include species differences, multiple etiological factors, problems with experimental, biochemical and molecular biological techniques, and inability to examine a single component in isolation. Many investigators in this area have used the human failing heart while the transplanted donor heart served as a control. In these studies, the patients at the end stage heart failure may have received various drug interventions and the control hearts were usually from those individuals, who were either suffering from severe non-cardiac disease or died from a sudden accident and were used after different durations. There are thus insurmountable problems in using the human material. Although animals are considered to provide a mean of circumventing some of these problems, a careful review of the literature has revealed that most of the studies on the β -AR signaling were done on low cardiac output heart failure models. No information is available regarding the status and regulation of β -AR signal transduction in high-output heart failure. High-output state is associated with several clinical situations such as congenital and valvular heart diseases, arteriovenous fistulas, severe anemia, beriberi and hyperthyroidism. This study was undertaken to examine the changes in the β -AR signal transduction pathway in a high cardiac output model induced by aortocaval shunt in rat.

It is now generally held that a series of abnormalities including downregulation of β_1 -AR, uncoupling of β_2 -ARs, enhanced activity and gene expression of GRK2 and $G_{i\alpha}$ -protein with little

changes in AC catalytic subunit and $G_{\alpha s}$ -protein accompanies the development of heart failure. Several lines of evidence have shown changes in β -AR signal transduction in myocardial samples from failing hearts of humans and animal models, which were confounded with significant fibrosis. Since β -ARs, G-proteins, GRKs and AC are ubiquitously expressed in fibroblasts, smooth muscle cells, nerve cells as well as in cardiomyocytes, and different cell types have different percentage of subtype expression, myocardial fibrosis can be seen to alter the density as well as the composition of these components in myocardial samples. However, cardiac hypertrophy and heart failure due to aortocaval shunt have been demonstrated to exhibit no fibrosis in the myocardium. It is thus expected that information regarding changes in β -adrenergic signaling in the fibrosis-free hypertrophied and failing hearts will provide some valuable information regarding the influence of connective tissue on cardiac muscle in terms of changes in β -AR signaling.

The important role of GRKs, β -arrestins and $G_{\beta\gamma}$ -protein in regulating β -AR signaling has been recognized; however, most of these studies are carried out *in vitro* by using cultured cell lines rather than cardiomyocyte. In fact the information regarding the significance of these factors in regulating cardiac function *in vivo* through the modulation of β -AR signaling is limited. While studies on the role of GRK2 in regulating cardiac function in health and disease are just emerging, little significant information regarding the role of other two isoforms of GRKs, namely GRK3 and GRK5, which are expressed in heart is known. Such a case is also similar with β -arrestins and $G_{\beta\gamma}$ -protein. It is thus proposed that examination of changes in the regulatory factors in heart failure will yield the required information for their *in vivo* role under pathological conditions.

There is an increasing degree of evidence showing the interaction between adrenergic and renin-angiotensin systems. For example, renin release was observed to be under the control of β_1 -AR (251), and angiotensin II may modulate adrenergic activity by facilitating synaptic release

of norepinephrine (252-256). In addition, both protein kinases and GRKs are known to phosphorylate β_2 -ARs and angiotensin II type one (AT1) receptor *in vitro*. Furthermore, *in vivo* overexpression of GRK2 was found to blunt β -AR as well as AII mediated cardiac responses. These were considered to form the molecular basis for crosstalk between these two systems. Thus it is reasonable to assume that ACE inhibitors and AII receptors antagonists may exert some beneficial effect on presynaptic adrenergic activity and postsynaptic β -adrenergic signaling. Because very little information is known regarding this issue, it is proposed to use one long acting ACE inhibitor, imidapril, and one AT1 receptor blocker, losartan, in the aortocaval shunted rat model to examine their effect on the β -adrenergic signaling. It is hoped that the effect of these AII antagonistic drugs on β -adrenergic signaling will improve our understanding of crosstalk between AII and β -adrenergic receptors.

III. METHODS AND MATERIALS

1. Experimental model

Male Sprague-Dawley rats weighing 250-300 g were kept in temperature-controlled rooms with a 6:00 am to 6:00 pm light/dark cycle. Tap water and rat chow were provided ad libitum. Experiments were conducted in accordance with the *Guidelines to the Care and Use of Experimental Animals* issued by *Canadian Council on Animal Care*. The aortocaval shunt was produced according to the method described by Garcia and Diebold (257) with some modifications. Briefly, after anesthetizing the animal with isoflurane, a ventral abdominal laparotomy was performed. The intestine was placed laterally using sterilized gauze and kept moist with normal saline. The aorta and vena cava between the renal arteries and iliac bifurcation were then exposed by blunt dissection. Both vessels were temporarily occluded proximal and distal to the intended puncture site, and an 18-gauge needle held on a plastic syringe was inserted into the exposed abdominal aorta and advanced through the medial wall into the vena cava to create the shunt. The needle was inserted and withdrawn across the medial wall several times before it was finally withdrawn from the aorta to ensure the size and presence of the shunt. The puncture site was immediately sealed with a drop of isocyanate (Krazy glue) after withdrawing the needle. Creation of a successful shunt was visualized by the pulsatile flow of oxygenated blood into the vena cava from abdominal aorta. The intestine was repositioned and the abdominal musculature and the skin incisions were closed by standard techniques with absorbable suture and autoclips. Sham operated animals, which served as control, were subjected to the same surgical procedures except for creation of the shunt. During the whole process, the animal was ventilated by positive-pressure inhalation of 95% O₂ and 5% CO₂ mixed with isoflurane.

2. Experimental design and experimental groups

Three series of experiments were designed in this study. The first series was devoted to characterization of the model as well as establishing the time course for development of cardiac hypertrophy and heart failure in aortocaval shunted rats. For this purpose, aortocaval shunted rats and sham-operated animals were studied at 1, 2, 4, 8 and 16 weeks after the operation. The second series of experiments was carried out to determine the responses of the failing heart to β -AR stimulation *in vivo* and *in vitro* in order to find alterations of β -AR signaling in failing heart. Changes of β -ARs and GRKs and β -arrestins in terms of functionality, protein and mRNA were examined in order to define their roles in altering the cardiac responses to β -AR stimulation. In order to find the interaction between β -adrenergic system and renin-angiotensin system, the animals were treated with two drugs, imidapril and losartan. Sham-operated animals served as control (sham) and shunted rats were randomly divided into three groups, aortocaval shunt (AV), aortocaval shunt with the treatment of imidapril (AV+Imp) and aortocaval shunt with the treatment of losartan (AV+Los). Imidapril (1 mg/kg/day) and losartan (20 mg/kg/day) were dissolved in tap water and given orally by a gastric tube starting at 3 days after surgery. Tap water was given to sham and AV groups. Animals were used at 16 weeks after surgery. We did not include the sham treatment groups because our preliminary studies showed that there was no significant difference between sham and sham with treatment of either imidapril or losartan. The third series of experiments was designed to determine the role of G-proteins and AC in the β -adrenergic signaling in heart failure. For this purpose, similar groups and time point as that of the second set of experiments were used, and G-proteins and AC functionality as well as protein and mRNA expression were determined. Imidapril was kindly supplied by Tanabe Seiyaku Co.,

Osaka, Japan whereas Losartan is a kind gift from Merck.

3. General characteristics

For the determination of general characteristics, rats were weighed and then sacrificed; the heart was removed and immediately placed in the ice-cold saline to wash out the blood. Total heart, LV and RV weights were measured after removal of connective tissue. The lung and liver were also taken out and their wet weights were assessed. Lung and liver tissues (about 0.5 g each) were dried at 100°C overnight and the wet/dry weight ratio was then calculated. Gross morphology of the heart was determined at 4, 8 and 16 weeks after surgery when hypertrophy was visually obvious. The hearts from sham and AV shunt groups were taken out, washed and fixed in formaline buffer for 1 week. The hearts were then dried and vertically cut to show the size of the cavity and thickness of LV and RV wall as well as that of the septum. For histological studies, heart, lung and liver tissue were formaline-fixed and paraffin-embedded, sliced and stained with both hematoxylin-eosin and Masson's trichrome stain. Pictures for the same organ were taken under the same magnification.

4. *In vivo* hemodynamic assessment

In vivo hemodynamic parameters were measured after the rat was anesthetized with an intraperitoneal injection of mixed ketamine (60 mg/kg) and xylazine (10mg/kg). The right carotid artery was isolated and an ultraminiature catheter connected to a pressure transducer (model SPR-249, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the LV. The left ventricular systolic pressure (LVSP), left ventricular end diastolic

pressure (LVEDP), heart rate and rate of pressure development (+dP/dt) and pressure decay (-dP/dt) were recorded in a computer program of Biopac data Acquisition System (Biopac System Inc., Goleta, CA). The catheter was subsequently withdrawn to the aorta and the arterial systolic pressure (ASP) and arterial diastolic pressure (ADP) were measured. The arterial pulse pressure (APP) was then calculated by the difference of ASP and ADP, whereas the mean arterial pressure (MAP) was calculated as the sum of ADP and one-third of APP ($MAP = ADP + \frac{1}{3}APP$).

5. *In vitro* cardiac function

Isolated hearts were perfused as reported previously (258). Briefly, after anesthetizing the animals with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) and heparination (1000 U), the thorax was opened and the heart was dissected out and immediately placed in the ice-cold saline. The adherent connective tissue was removed and the heart was perfused by the Langendorff technique at a constant flow (10ml/min) with Krebs-Henseleit solution containing (in mmol/L) NaCl 120; KCl 4.74, KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; $NaHCO_3$ 25, $CaCl_2$ 1.25 and glucose 11. This solution (pH 7.4) was continuously oxygenated with a 95% O_2 and 5% CO_2 mixture and maintained at 37°C. The atrioventricular node conduction was surgically blocked and the heart was paced at 300 beats/min by a square wave of 1.5 milliseconds duration at twice the threshold voltage throughout the experiments using the Philips & Bird stimulator (Richmond, VA). The left ventricular developed pressure (LVDP), as well as the maximum rate pressure development (+dP/dt) and the maximum rate of pressure decay (-dP/dt) were measured using a transducer connected to a latex balloon inserted into the left ventricle. The size of the balloon

was adjusted to the size of left ventricular cavity of each individual heart and the initial end diastolic pressure was adjusted to about 10 mm Hg by inflating the balloon with water. The data were recorded and stored in the Biopac data Acquisition System (Biopac System Inc. Goleta, CA) after a 30 min period of stabilizing the heart. For estimating β -AR stimulation, 1 μ M isoproterenol was infused to the heart during the perfusion and changes in LVDP and \pm dP/dt were recorded.

6. Myosin heavy chain isoenzyme analysis

The composition of myosin heavy chain isoenzymes was determined by polyacrylamide gel electrophoresis in the presence of pyrophosphate (259). Portions of left and right ventricle (\approx 50 mg) were cut into small pieces and incubated for 15 min by gentle agitation at 0°C with 3 volumes (v/w) of 40 mM $\text{Na}_4\text{P}_2\text{O}_7$ (pH 8.8, adjusted with HCl at 2°C), 1 mM 1,4-dithioerythritol, 5 mM ethylene glycol bis(β -aminoethyl ether)-N,N-tetraacetic acid (EGTA). Following centrifugation at 2,000 g for 15 min, the supernatant was collected and diluted 1:10 (v/v) with ice-cold glycerol and immediately loaded on the gel. The gel contained 3.8% acrylamide and 0.12% N,N'-methylene-bis-acrylamide. Electrophoresis buffer was 20 mM $\text{Na}_4\text{P}_2\text{O}_7$ (pH 8.8), 10% glycerol (v/v). Electrophoresis was carried out at 2°C for approximately 16 hr at a voltage gradient of 10 V/cm. Gels were stained with Coomassie brilliant blue R250 for 2 hr and were destained with 7% acetic acid by diffusion. Relative amounts of isoenzymes were estimated from densitometric tracings using Quick Scan densitometer (Desaga, Heidelberg, Germany). The isoenzymes V_1 , V_2 , V_3 were quantitated by measuring peak heights and values were expressed as percentage of total isoenzymes.

7. Isolation of crude membrane

Crude membranes were used for biochemical studies and relative protein content determination of β -ARs, G-proteins and AC. Briefly, the left and right ventricular tissues were minced and then homogenized separately in 50 mM Tris-HCl, pH 7.4 (15 ml/g tissue) with a PT-3000 Polytron (Brinkman Instruments, Westbury, NY), twice for 20 s each at a setting of 6. The resulting homogenate was centrifuged at $1000 \times g$ for 10 min and the pellet was discarded. The supernatant was centrifuged at $48,000 \times g$ for 20 min. The resulting pellet was resuspended and centrifuged twice in the same buffer at the same speed, and the final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 25 mM sucrose and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at -70°C for further experiments.

8. Isolation cytosolic and membranous proteins

Cytosolic and membranous proteins were isolated according to the method described by Benovic *et al.* (260). Briefly, about 100 mg tissue samples were taken from LV and RV and placed in 1 ml of the lysis buffer containing: 25 mM Tris-HCl, pH 7.5, 5mM EDTA, 5 mM EGTA, 20 mg leupeptin, 20 mg benzamidine and 40 mg phenylmethylsulfonyl fluoride. The tissue was homogenized for 30 sec with a PT-3000 Polytron (Brinkman Instruments, Westbury, NY, USA) at setting 6 and centrifuged for 30 min at $400,000 g$. The supernatant was collected and labeled as the cytosolic fraction. The pellet was resuspended in the lysis buffer with 250 mM NaCl and homogenized again. The pellet suspension was centrifuged again and this supernatant was labelled as the membrane fraction. NaCl was added to both cytosolic and membranous fractions to a concentration of 50 mM, and the 0.5 ml of samples were equilibrated with 0.5 ml

of 50% (vol/vol) diethylaminoethyl Sephacel, pH 7.0, for 15 min on ice. This slurry was then placed into small columns and eluted with 0.5 ml of the lysis buffer. The volume of this elute was reduced by filtration in a microconcentrator Centricon 30 (Amicon) to yield 100 μ l. These fresh preparations were used immediately to determine GRK activity. At the same time Western blot analysis was used to determine protein expression of GRKs.

9. Isolation of total RNA

Total RNA was isolated from the frozen LV and RV by the method of acid guanidinium thiocyanate/phenol/chloroform extraction described by Chomczynski and Sacchi (261). Samples about 500 mg each were quickly minced under liquid nitrogen in a mortar. The minced tissue was placed in 4 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) and homogenized with a Polytron device twice for 10 sec each. Subsequently, 0.1 vol of 2 M sodium acetate, pH 4.0, 0.1 vol of water saturated phenol, pH 4.0, and 0.2 vol of chloroform/isoamyl alcohol (49:1) were added; the mixture was shaken vigorously after each addition and was centrifuged at 14,000 g for 20 min at 4°C, the aqueous phase was transferred into a new tube and 1 vol of isopropanol was added. After incubating at -20°C for at least 2 hr, the sample was centrifuged at 3,500 g for 10 min. The resulting pellet was resuspended in 0.5 ml of solution D and precipitated by incubating with 0.5 ml isopropanol at -20°C for 1 hr followed by centrifugation at 11,000 g for 15 min. The resulting pellet was washed and centrifuged twice by using 0.8 ml of 75% ethanol. After drying with speed vacuum, the resultant pellet (total RNA) was dissolved in DEPC-treated water in a concentration around 1 μ g/ μ l and stored at -70°C for later use. The amount of RNA present was determined by UV

absorption. The ratio of optical density ($OD_{260nm} \cdot OD_{280nm}$) was 1.8-2.0 in all preparations used in this study.

10. β_1 - and β_2 -adrenoceptors binding assay

β_1 - and β_2 -AR bindings were determined as described previously (262). Briefly, 100 μ g of membranes were incubated in Tris-HCl buffer (pH 7.4) for 60 min at 37°C with different concentrations (6.25 - 400 pM) of [125 I]cyanopindolol ([125 I]ICYP) in the absence (non-specific) or presence of either 200 μ M CGP-20712A (a highly selective β_1 -antagonist) or 10 μ M ICI-118,551 (a highly selective β_2 antagonist). The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters and the membranes were washed three times with 5 ml of cold water. The radioactivity was counted by a Beckmann gamma-counter. Specific binding to β_1 -receptors was calculated as the difference between [125 I]ICYP binding values in the presence and absence of CGP-20712A, whereas β_2 -receptors specific binding was the difference between [125 I]ICYP binding values in the presence and absence of ICI-118,551. The kinetic parameters, maximal binding (B_{max}) and dissociation constant (K_d), were calculated from the Scatchard plot analysis according to the interactive LIGAND program.

11. G-protein coupled receptor kinase assay

The GRK activity was determined according to the method described by Benovic *et al.* (260). The reaction is based on the light-dependent phosphorylation of rhodopsin. The purified cytosolic or membranous preparations containing 50 μ g of protein were incubated with 500 pmol rhodopsin, 10 mM $MgCl_2$, and 0.3 mM [γ - ^{32}P]ATP, in a total volume of 30 μ l lysis buffer at 30

degree for 15 min. Reactions were terminated by addition of 30 μ l ice-cold lysis buffer. All vials were centrifuged for 15 min at 11,000 g. Free radioactivity in the supernatant was discarded, and the pellet was resuspended in 30 μ l of 2 \times Laemmli buffer by vigorous shaking for 20 min. The samples were electrophoresed on 10% SDS-PAGE gels. Gels were stained with Coomassie blue and autoradiographed. In the next step, rhodopsin bands were cut out from the gel, and Cerenkov radiation was counted in a beta scintillation counter. Kinetic assay showed that the reaction was linear up to 10 minutes and depended upon the amount of heart preparations used. The phosphorylating activity was completely inhibited by 1 μ M heparin, an inhibitor of GRK, but not in the presence of 1 μ M protein kinase A inhibitor (PKI). Rhodopsin purified from dark-adapted bovine rod outer segments was purchased from Calbiochem-Novabiochem Corporation.

12. Toxin-catalyzed ADP-ribosylation

ADP-ribosylation of G_i - and G_s -proteins was studied by treating membranes with pertussis toxin (PT) and cholera toxin (CT) according to the methods described earlier, respectively (158). To determine ADP-ribosylation of G_i proteins, 100 μ g of membranes were incubated for 60 min at 37°C in 100 μ l of 100 mM Tris-HCl (pH 7.4) containing 2 mM ATP, 2 mM GTP, 10 mM thymidine, 15 μ M of ovalbumin, 0.5% of lubrol PX, 3 μ M of 32 P-NAD (5 Ci/mmol) and activated pertussis toxin (5 μ g/ml). For ADP-ribosylation of G_s protein, 100 μ g of membranes were incubated for 90 min at 37°C in 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 10 mM arginine, 10 mM thymidine, 1 mM NADP⁺, 0.5% lubrol PX, 3 μ M [32 P]NAD (15 Ci/mmol) and activated cholera toxin (200 μ g/ml). The reactions were stopped by the addition of 20% cold trichloroacetic acid. The membranes were

then precipitated by centrifugation at 3000 rpm for 5 min and suspended in Laemmli buffer. The proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) which was dried and subjected to autoradiography using Kodak X-AR5 film at -80°C for 10-24 hr.

13. Determination of adenylyl cyclase activity

The AC activity was determined by measuring the formation of [^{32}P]cAMP from [α - ^{32}P]ATP as described earlier (250). Unless otherwise indicated, the incubation assay medium contained 50 mM glycylglycine (pH 7.5), 0.5 mM cAMP, 0.5 mM Mg-ATP, 100 mM NaCl, 5 mM MgCl_2 , 0.1 mM EGTA, 0.5 mM 3-isobutyl-1-methylxanthine, 10 U/ml adenosine deaminase, [^{32}P]ATP ($1-1.5 \times 10^6$) and an ATP-regenerating system comprising of 2 mM creatine phosphate, 0.1 mg/ml creatine kinase, 36 U/ml myokinase in a final volume of 200 μl . The reaction was initiated by addition of 40-60 μg of crude membranes to the reaction mixture, which had been equilibrated for 3 min at 37°C . The incubation time was 10 min at 37°C and the reaction was terminated by addition of 0.6 ml of 120 mM zinc acetate containing 0.5 mM unlabelled cAMP. [^{32}P]cAMP formed during the reaction was determined upon co-precipitation of other nucleotides with ZnCO_3 by the addition of 0.5 ml 144 mM Na_2CO_3 and subsequent chromatography by a double column system as described earlier (250). The unlabelled cAMP served to monitor the recovery of [^{32}P]cAMP by measuring absorbance at 259 nM. The AC activity was expressed as pmol cAMP/mg protein/10 min. The AC activity was linear with respect to protein concentration and time of incubation under the assay conditions used. When G-protein stimulated AC was estimated, different doses of Gpp(NH)p or NaF were added to the reaction mixture.

14. SDS-PAGE electrophoresis and Western blot assay

The relative amounts of protein content of β_1 -, β_2 -ARs, GRK2 (β ARK1), GRK3 (β ARK2), GRK5, β -arrestin1, $G_{s\alpha}$, $G_{i\alpha-2}$, G_{β} , G_{γ} and AC type V/VI were determined by separation of the protein in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunostaining by Western blot assay. Briefly the membranes were dissolved in SDS-PAGE sample buffer and denatured by boiling for 5 min. The samples were separated on 6-12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) by employing a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol. The membranes were incubated in 20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl (TBS) and non-fat dry milk for 2 hr at room temperature. After one wash with TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated with TBS-T diluted specific antibody for 1 hr at room temperature on a shaker. The primary binding of the antibody was recognized by incubation of the membranes with biotinated anti-rabbit IgG (Amersham-Pharmacia Biotech, 1:3000-1:5000). The antigen-antibody complex was then detected by using horseradish peroxidase (Amersham-Pharmacia Biotech, 1:3000-1:5000). Each incubation was followed by three washes with TBS-T (10 min each). Blots were made visible by the ECL system (Amersham-Pharmacia Biotech) according to the instructions of the manufacturer. Polyclonal antibodies for β -ARs, GRKs, β -arrestins, AC were purchased from Santa Cruz Biotechnology, Inc.; those for $G_{s\alpha}$ and $G_{i\alpha-2}$ were purchased from NEN Life Science Products and antibodies for $G_{\beta 2}$ and $G_{\gamma 7}$ were purchased from Calbiochem-Novabiochem Corporation.

15. Plasmid amplification and preparation of cDNA probes

Plasmids containing cDNAs for β_1 -AR, β_2 -AR, β ARK1, β ARK2, G_{src} and $G_{\text{int-2}}$ respectively were purchased from American Type Culture Collection (ATCC). The plasmid DNA was amplified and collected by using a kit from Qiagen according to the manufacturer's handbook. The cDNA insert was then cut out by using ATCC suggested restriction enzyme(s). These cDNA probes were used for detecting mRNA signals for β_1 -AR, β_2 -AR, β ARK1, β ARK2, G_{src} and $G_{\text{int-2}}$ by using Northern blot. Due to the small amount of β_2 -AR and β ARK2 contained in the myocardium, their signals can not be detected in both LV and RV by using Northern blot. The other probes worked fine for detecting the respective signal by using Northern blot.

16. Northern blot analysis

Steady state mRNA levels of β -ARs, GRKs, G_{src} and $G_{\text{int-2}}$, were detected by Northern blotting. Briefly, 20 μg of total RNA was electrophoresed in a 1.2 % agarose/formaldehyde gel and the fractionated RNA was transferred to a Nytran Plus membrane (Schleicher & Schuell, Keene, NH, USA). The membrane then was hybridized with the specific cDNA probes labeled with ^{32}P for β -ARs, GRKs, G_{src} and $G_{\text{int-2}}$, respectively. Results of autoradiographs from Northern blot analysis was quantified by densitometry. The signals of specific mRNAs was corrected by both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S to normalize for difference in loading and transfer of mRNA.

17. Statistical analysis

All values were expressed as mean \pm SEM. Western blot, Northern blot data were

expressed as percentage of control. One-way analysis of variance (ANOVA) was used for multigroup comparisons and unpaired student t-test was used for comparisons between two groups. Differences were considered statistically significant at a level of $P < 0.05$.

IV. RESULTS

1. Characterization of the experimental model

In order to study β -adrenergic signaling in heart failure with a high-output state and without fibrosis, we choose the rat model of aortocaval shunt. Although opening an arteriovenous shunt in rat and several other species has long been used as a model of hypertrophy due to volume overload, the changes in cardiac hypertrophy and hemodynamic parameters caused by the arteriovenous shunt are quite variable. Increase of cardiac mass has been reported to range from 20-100% (263-268), whereas cardiac contractility was reported increased (264), decreased (269,270) and unchanged (265,266). Circulatory congestion was also reported absent (271) or present (270). This may be due to the techniques that were applied to make the model. Conventionally, arteriovenous fistula in rats were made by side to side anastomosis of the aorta and vena cava (263) or by end-to-side anastomosis of left iliolumbar vein (266). Both conditions required microvascular surgery, and the circulation system was obstructed for 15 min (263) or 30 min (266), respectively. The entire surgical procedure needs about 40 min to complete. Due to the technical difficulties and prolonged time of the procedures, the mortality rate was as high as 76% (263) and 47% (266) respectively. In addition, the shunt size and thus hypertrophic and hemodynamic characteristics were inconsistent (263,266) which makes this model less valuable.

In 1990, Diebold and Garcia (257) described a simple, rapid and effective method in which an 18-gauge needle was used for making a shunt between abdominal aorta and inferior vena cava. The puncture site was then sealed by a drop of cyanoacrylate glue (Krazy glue). The circulation system was only occluded for 1-2 min and the entire procedure can be finished within 10 min. The mortality was limited under 10% (257). Subsequently, Huang et al. (272) have evaluated

the needle technique by using three different sizes of needle and confirmed that this technique can control the size of the shunt and provide consistent changes in hypertrophic growth and some hemodynamic parameters. However, these investigators only provided limited information at 4 and 5 weeks after creating the shunt. The dynamic development of cardiac hypertrophy and its transition to heart failure has never been carefully defined in this experimental model. Therefore, the main purpose of this series of the experiments was to examine the time course changes regarding the general characteristics, morphological and histological features, hemodynamic performance as well as biochemical changes after aortocaval shunt in order to establish the time course of the development of cardiac hypertrophy and heart failure.

a. General characteristics of the rat model

Out of 60 rats that underwent aortocaval shunt surgery, 5 (8.3 %) died within 24 to 72 hr after the surgery. Autopsy examination revealed no bleeding from the puncture site and no obstruction of either aorta or vena cava in these animals; however, the hearts were dramatically dilated. The size of right atrium was as large as the size of a normal heart and both LV and RV were greatly enlarged. The heart stopped in the diastolic phase suggesting that these animals may have died from acute heart failure rather than the surgical procedure itself. While no further mortality occurred between 1 wk to 8 wks, 3 rats (5%) died between 10 to 16 wks after the aortocaval shunt surgery. Autopsy examination of these animals demonstrated hypertrophied heart, congested liver and lung, presence of ascities and pleural effusion as well as edema of the limbs suggesting these animals died of the end stage of congestive heart failure. In addition, rats which survived 16 wks after aortocaval shunt surgery were sluggish in movements, and the hairs around their face and neck area were stained with blood that most probably came from bloody sputum due to lung congestion. A significant number (30%) of rats sacrificed at 16 weeks

demonstrated ascities. The sham control group had no mortality during 16 wks after the operation.

The time course changes in general characteristics in rats with and without aortocaval shunt are shown in Table 6. Although there was no significant difference in body weight (BW) between sham and AV groups at any time interval, heart weight of the AV group increased progressively throughout 1 to 16 wks. Accordingly, heart weight to body weight ratio was also significantly increased at all time intervals. While examining LV and RV weight separately, we found that cardiac hypertrophy in RV developed more dramatically than in LV (152, 193 and 249% of sham for RV and 112, 147 and 188% of sham for LV) at 1, 2 and 16 wks after the aortocaval shunt. However, the extent of hypertrophy in LV and RV were similar at 4 and 8 wks (181%, 171% of sham for LV vs 187%, 171 of sham in RV). Similar to findings of other investigators (263,265,266,273,274), no significant difference was found in heart rate between sham and AV groups throughout the observation period indicating.

b. Morphological and histological changes

Morphological changes in the heart are shown in Figure 1. The left panel shows the progressive enlargement of the heart from 4 wks to 16 wks in AV group whereas the right panel shows the dilation of the left and right ventricular cavities and increase of wall thickness in the vertically cut heart indicating eccentric hypertrophy occurred in this model. The hearts were also embedded, sliced and stained for examination of histological changes in the myocardium. Figure 2 shows the HE staining of myocardium from sham and AV groups at 4, 8, and 16 wks after surgery. In the AV group, the myofibrils were thickened and nuclei were enlarged and condensed. At 8 and 16 wks after inducing aortocaval shunt, disarray of the left myocardium was also seen in some hearts. Masson's trichrome staining showed no significant fibrosis at each time

Table 6. General characteristics of rats with or without an aortocaval shunt for different time intervals

	BW (g)	LVW (mg)	RVW (mg)	HW (mg)	HW/BW (mg/100g)	HR (beats/min)
1 week:						
Sh	294 ± 5	730 ± 43	134 ± 7	870 ± 40	296 ± 24	311 ± 10
AV	288 ± 8	815 ± 35*	204 ± 9*	1021 ± 32*	355 ± 14*	318 ± 16
2 weeks:						
Sh	346 ± 15	751 ± 60	166 ± 6	917 ± 70	265 ± 9	321 ± 16
AV	351 ± 18	1100 ± 67*	320 ± 15*	1420 ± 70*	404 ± 16*	309 ± 23
4 weeks:						
Sh	418 ± 14	764 ± 57	206 ± 10	970 ± 71	232 ± 8	325 ± 20
AV	424 ± 18	1380 ± 65*	385 ± 31*	1715 ± 62*	405 ± 27*	320 ± 18
8 weeks:						
Sh	513 ± 25	875 ± 74	238 ± 10	1107 ± 88	215 ± 10	316 ± 13
AV	530 ± 31	1493 ± 82*	408 ± 48*	1898 ± 95*	358 ± 34*	314 ± 20
16 weeks:						
Sh	612 ± 31	1003 ± 80	247 ± 20	1240 ± 90	202 ± 6	326 ± 15
AV	636 ± 28	1890 ± 90*	615 ± 60*	2500 ± 100*	393 ± 28*	333 ± 21

Data expressed as mean ± SEM were obtained from 8-12 rats in each group. HR: heart rate; BW: body weight; LVW: left ventricular weight, RVW: right ventricular weight; HW: heart weight; HW/BW ratio: heart weight to body weight ratio, HR: heart rate, Sh: sham control, AV: aortocaval shunt. *P<0.05 vs sham control group.

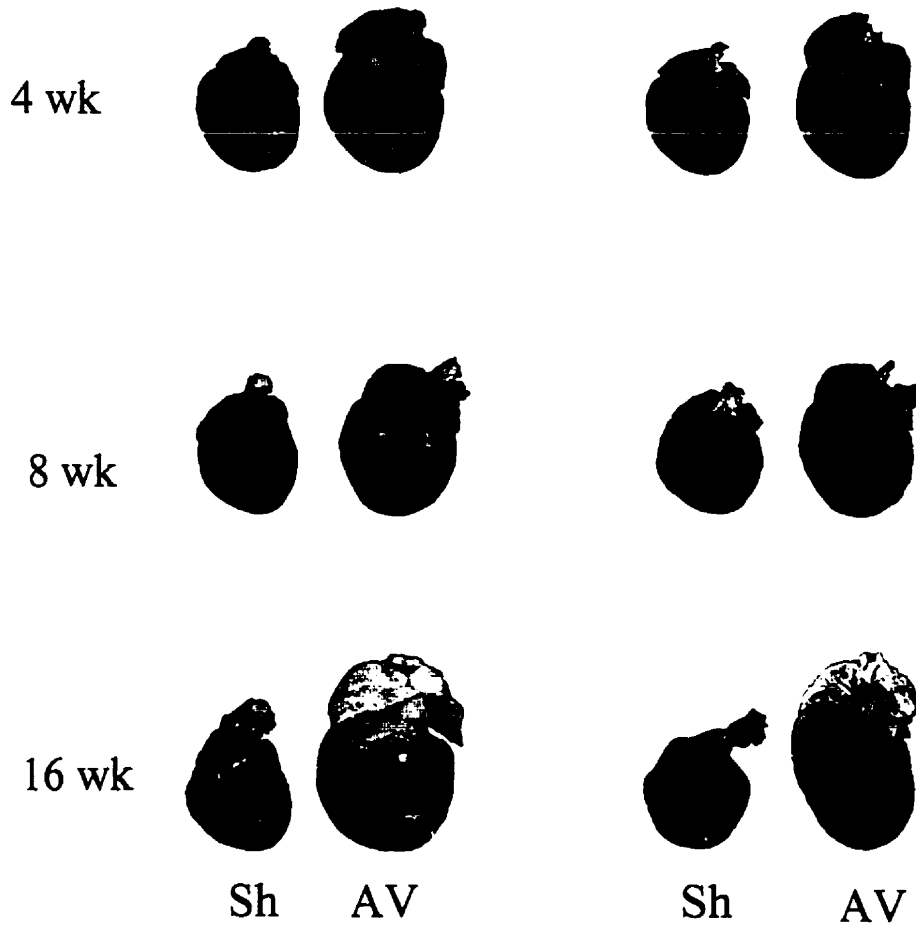


Figure 1. Morphological studies showing development of eccentric hypertrophy in rat heart at 4, 8 and 16 wks after inducing the aortocaval shunt. Sh: sham group, AV: aortocaval shunt.

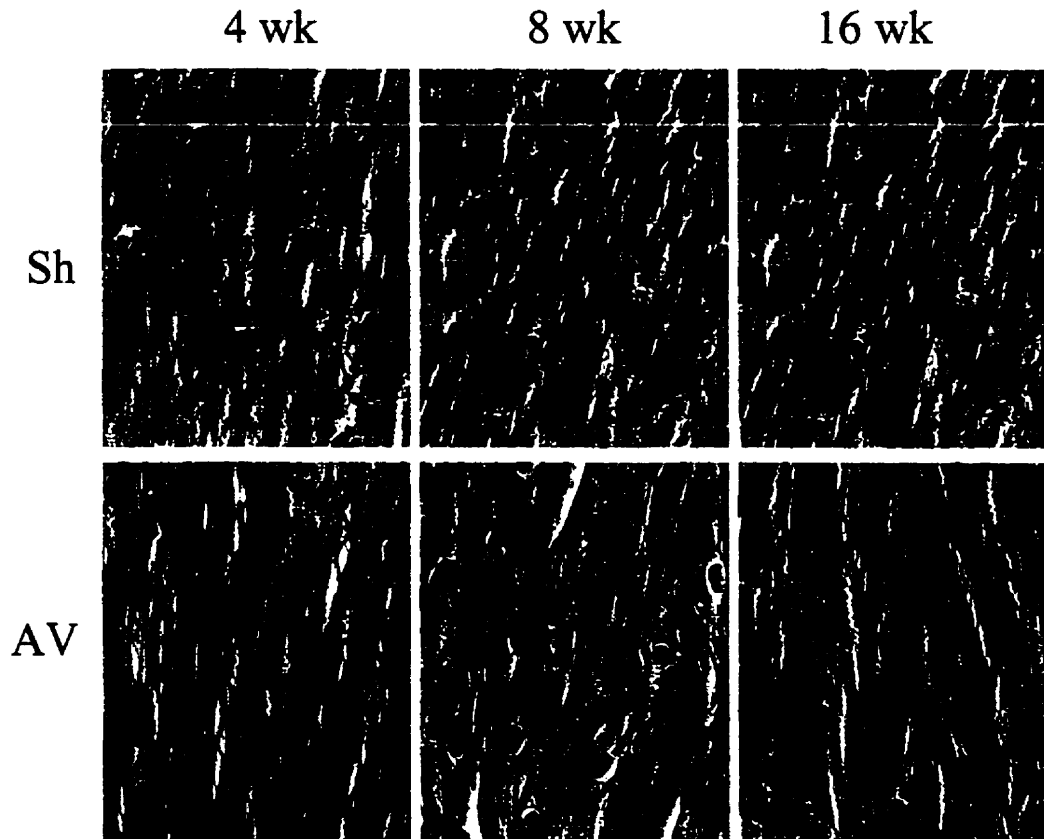


Figure 2. Histological changes of LV myocardium at 4, 8 and 16 wks after inducing the aortocaval shunt in rat. Sh: sham control, AV: aortocaval shunt. The myocardium was stained hematoxylin-eosin (HE), Magnification: $\times 400$

point examined (data not shown).

c. Circulatory congestion

In order to determine if congestive heart failure occurred in this model, we examined the circulatory congestion in the rats at different time intervals by measuring and determining the dry to wet weight ratio for lung and liver. Table 7 shows that lung wet weight in AV group was significantly increased at each time interval, however, the dry/wet ratio was not altered indicating a fluid-independent increase in lung mass after AV surgery. Flaim *et al.* (263) suggested that the fluid-independent character might be related to increased hematocrit in this model because no effort, rather than gentle surface blotting, was made to squeeze blood from the organ. However, our histological data of the lung tissue suggested that the increase in actual organ weight of lung may be due to the progressive thickening of pulmonary interstitial tissue with dilated capillaries and fine fibrosis indicating chronic edema in the lung (Figure 3, upper panel) after the aortocaval shunt. Liver wet weight and dry/wet ratio was not altered until 8 and 16 wks after the surgery indicating the occurrence of hepatic congestion at later stages of inducing aortocaval shunt. The lower panel in Figure 3 shows the changes occurring in the liver after inducing an aortocaval shunt for 4, 8 and 16 wks, respectively. The distended central veins and fibrosis of the vessel wall in the liver are the signs of long-term congestion in the liver.

d. *In vivo* cardiac performance and arterial hemodynamics

The time course of changes in cardiac performance is shown in Figure 4. Although a significant elevation of LVEDP was detected throughout the observation period (panel A), the change is biphasic. The first peak occurred at 1 wk, the elevation was then reduced somewhat at 2 and 4 wks whereas it started to increase at 8 wks and reached another peak at 16 wks. On the other hand, the LVSP was not decreased until 8 wks (panel B). While no change was

Table 7. Lung and liver weights in rats with or without inducing aortocaval shunt for different time intervals

	Lung wet wt (g)	Lung dry/wet wt (ratio)	Liver wet wt (g)	Liver dry/wet wt (ratio)
<u>1 week:</u>				
Sh	1.40 ± 0.04	19.8 ± 0.8	14.1 ± 0.48	29.8 ± 0.5
AV	1.59 ± 0.04*	18.2 ± 0.7	15.8 ± 0.67	30.1 ± 0.8
<u>2 weeks:</u>				
Sh	1.45 ± 0.01	21.9 ± 0.5	16.5 ± 0.70	30.1 ± 0.7
AV	1.81 ± 0.14*	19.0 ± 0.7	17.1 ± 1.18	29.1 ± 1.2
<u>4 weeks:</u>				
Sh	1.48 ± 0.16	21.5 ± 0.6	17.4 ± 1.7	31.4 ± 0.7
AV	1.82 ± 0.12*	20.3 ± 0.8	18.5 ± 0.6	30.5 ± 0.6
<u>8 weeks:</u>				
Sh	1.79 ± 0.08	21.9 ± 1.0	20.1 ± 0.09	30.7 ± 0.2
AV	2.04 ± 0.06*	20.9 ± 0.7	23.6 ± 0.91*	27.9 ± 0.5*
<u>16 weeks:</u>				
Sh	1.90 ± 0.08	20.3 ± 0.4	20.2 ± 1.53	30.2 ± 0.5
AV	2.59 ± 0.06*	21.7 ± 0.9	26.2 ± 1.12*	27.3 ± 0.4*

Data expressed as mean ± SEM were obtained from 8-12 separate organs in each group. Dry/wet ratios for lung and liver were obtained as described in Materials and Methods. Sh: sham control; AV: aortocaval shunt; *P<0.05 vs sham control group.

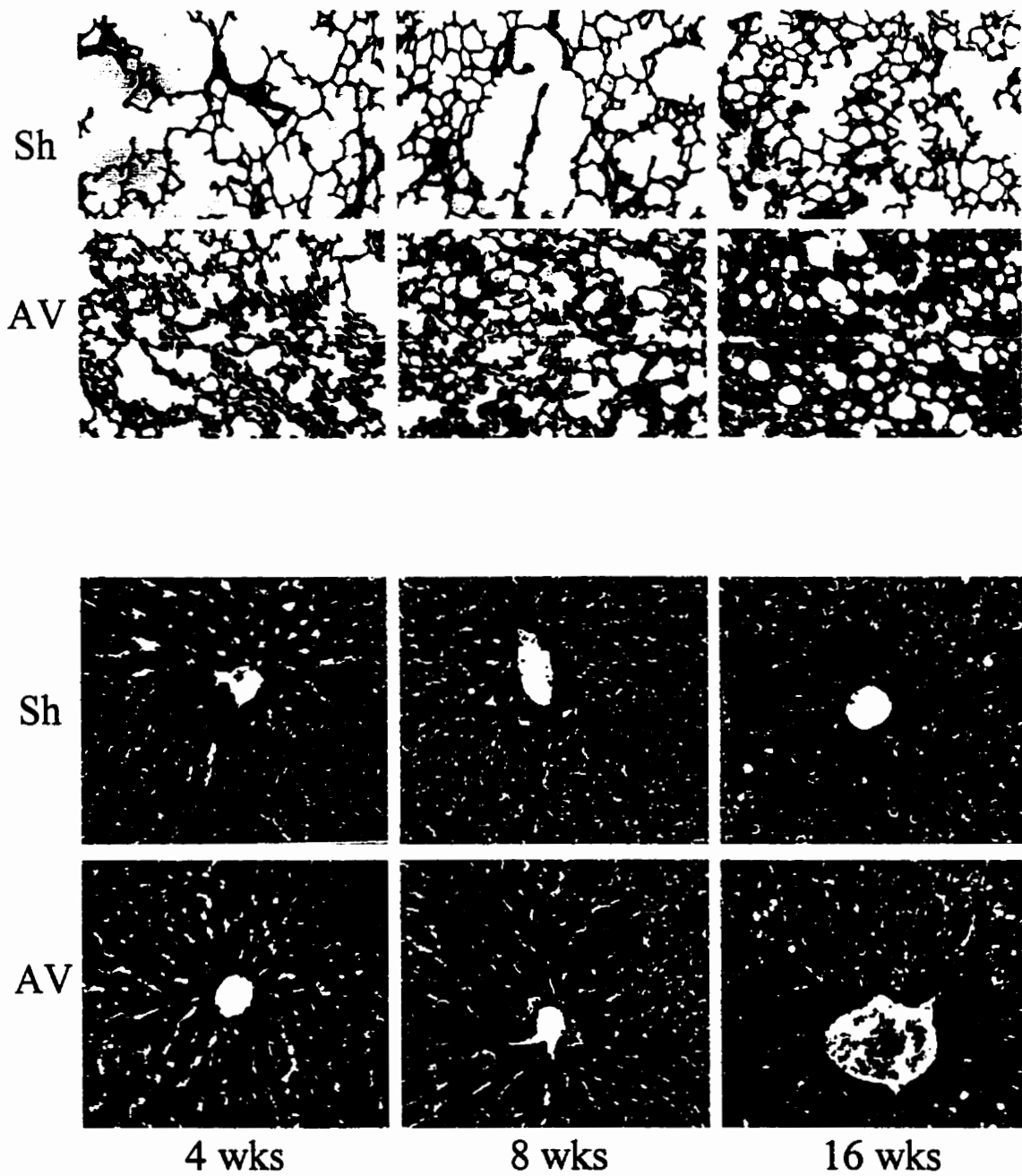


Figure 3. Histological changes in the rat lung (upper panel) and liver (lower panel) at 4, 8 and 16 wks after inducing the aortocaval shunt. Sh: sham control, AV: aortocaval shunt. The lung was stained with hematoxylin-eosin (HE) whereas the liver was stained with Masson's Trichrome. Magnification for lung: $\times 100$; liver: $\times 200$.

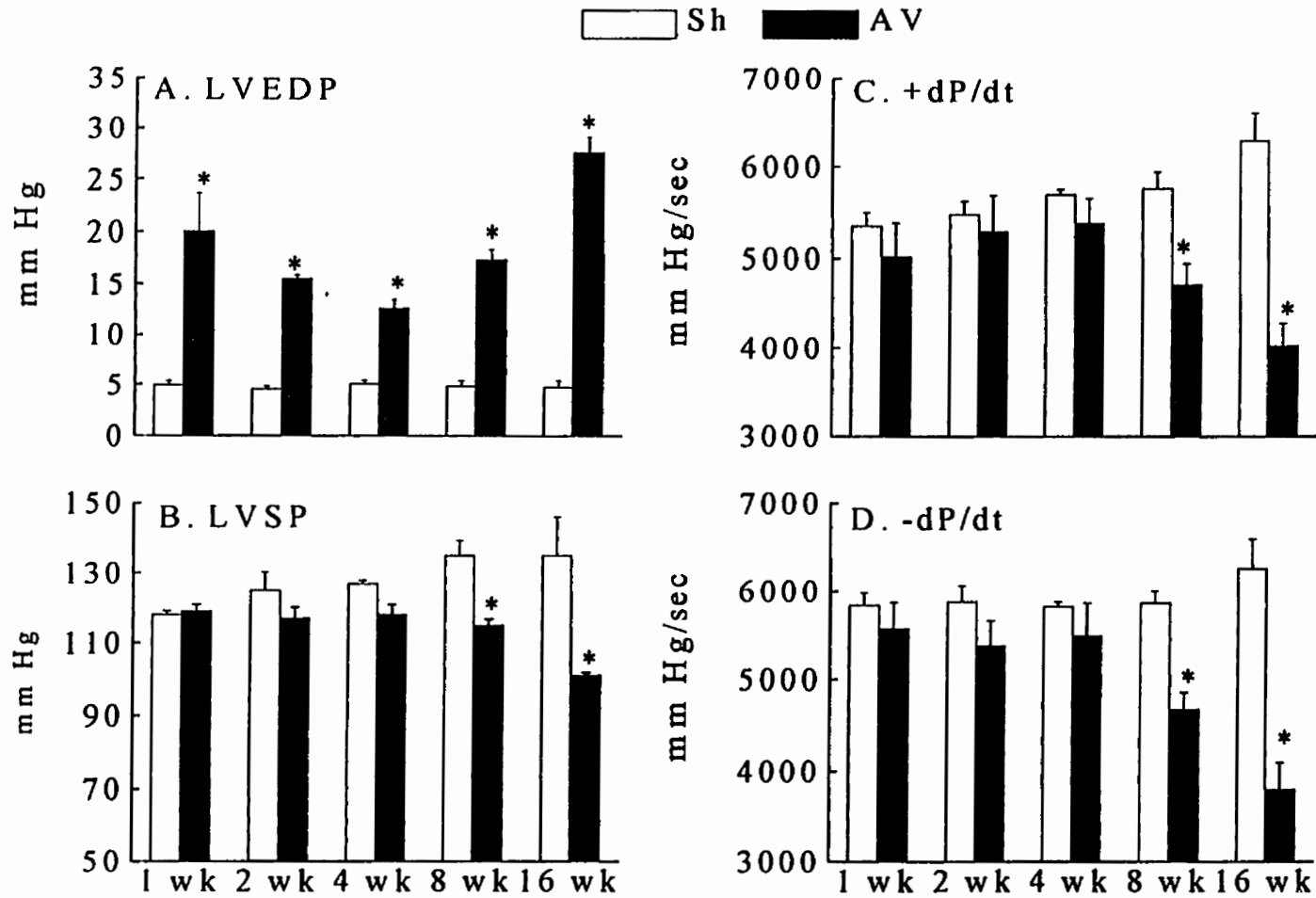


Figure 4. *In vivo* left ventricular hemodynamic changes in rats after inducing the aortocaval shunt for different time intervals. Panel A. LVEDP: left ventricular end diastolic pressure; Panel B. LVSP: left ventricular systolic pressure; Panel C +dP/dt: maximum rate of pressure development; Panel D. -dP/dt: maximum rate of pressure decay. *P<0.05 vs sham.

detected for $+dP/dt$ and $-dP/dt$ at 1, 2 and 4 wks after the surgery, significant depressions were seen at 8 and 16 wks (panels C and D). Figure 5 shows the time course of changes in arterial hemodynamics. A biphasic change in both arterial systolic pressure (ASP, panel A) and mean arterial pressure (MAP, panel B) were detected with significant depression at earlier stages (1 and 2 wks) and later stages (16 wks) but normal values in between (4 and 8 wks). The arterial diastolic pressure (ADP) was significantly lower in the AV group in comparison to the sham group although the extent of decrease is milder at 4 and 8 weeks (panel C). The pulse pressure (PP), which is the difference between the ASP and ADP, was significantly increased through out the observation period indicating the presence of shunt in the AV group (panel D).

e. Biochemical changes

We also examined the time course of changes in myosin heavy chain isozyme composition in the AV group. The upper panel of Figure 6 shows the positions of V_1 , V_2 , and V_3 on the gel electrophoresis. The lower panels show the time course changes of myosin heavy chain isozyme composition in both RV and LV after inducing aortocaval shunt in rats. There was a slight age-dependent increase in V_3 in both LV and RV which is similar to those observed by Mercadier *et al.* (267). However, no significant shift of myosin heavy chain V_1 to V_3 was detected until 8 wks and 16 wks in LV and RV, respectively. Table 8 shows the actual values of V_1 , V_2 and V_3 (in percentage) in RV and LV at 4, 8 and 16 wks after inducing aortocaval shunt. The values at 1 and 2 weeks were not shown in Table 8 because these were similar to those at 4 wks and no change was detected between sham and AV groups. The time course of changes in the isozyme composition of myosin heavy chain in the LV corresponded to the time course of changes in the left ventricular performance (Figure 4).

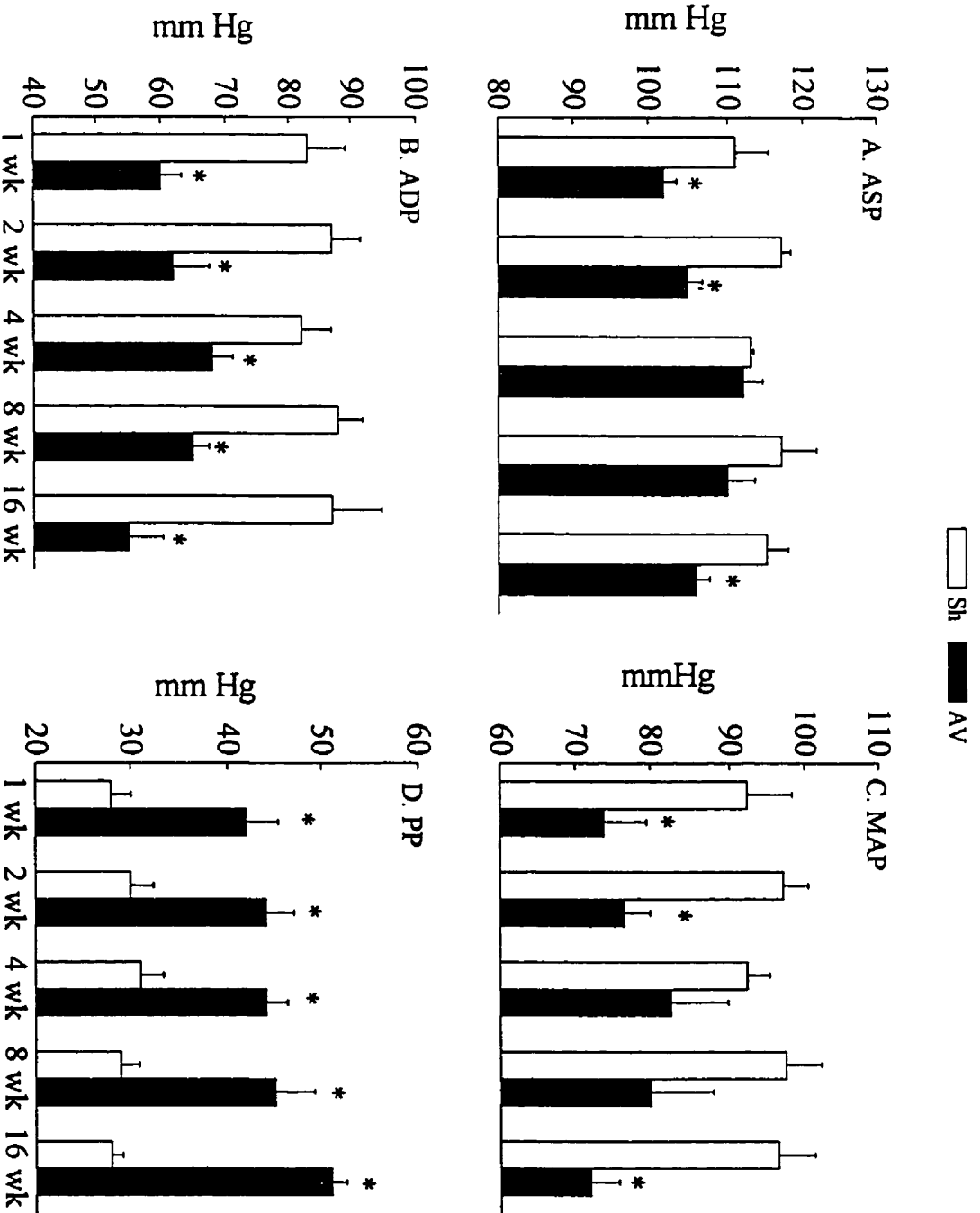


Figure 5. Changes in arterial pressures in rats after inducing the aorticocaval shunt for different time intervals. ASP: arterial systolic pressure; ADP: arterial diastolic pressure; MAP: mean arterial pressure; PP: pulse pressure. * P<0.05 vs sham.

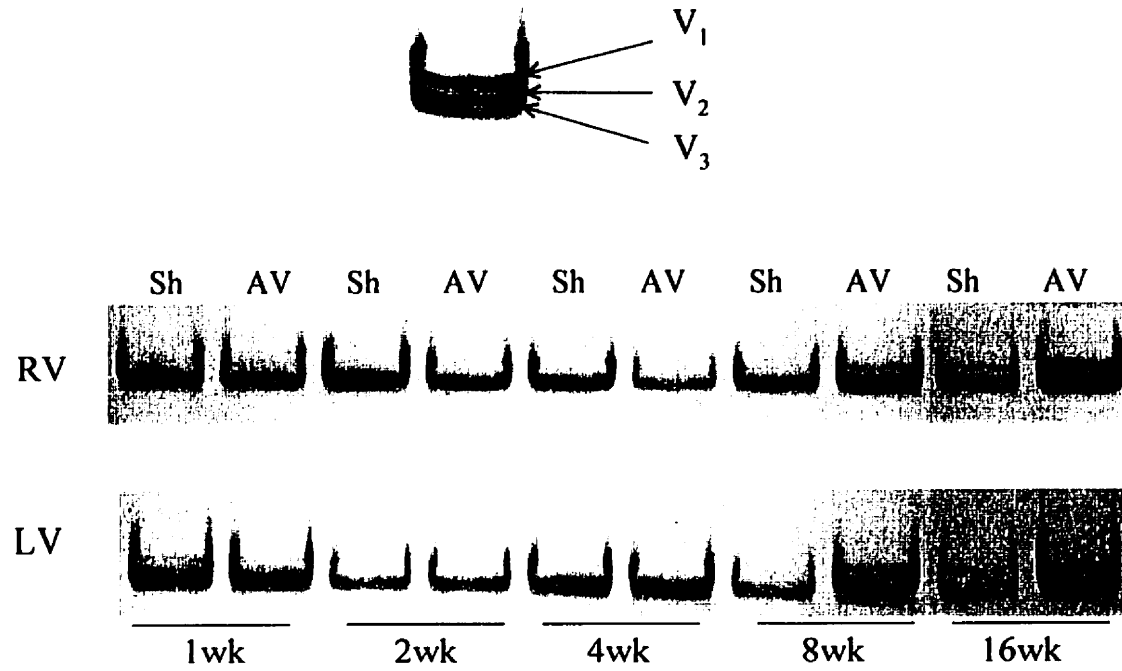


Figure 6. Time course changes in myosin heavy chain isoform composition in rats after inducing the aortocaval shunt. RV, right ventricle; LV, left ventricle; Sh: sham control; AV: aortocaval shunt.

Table 8. Myosin heavy chain isoenzyme composition of rats at 4, 8 and 16 weeks after inducing the aortocaval shunt

Myosin heavy chain Isozymes (%)	LV		RV	
	Sh	AV	Sh	AV
<i>4 weeks:</i>				
V ₁	71.2 ± 2.3	72.0 ± 3.3	75.3 ± 4.1	72.9 ± 4.2
V ₂	18.3 ± 1.2	16.3 ± 2.1	18.1 ± 2.5	19.6 ± 2.1
V ₃	10.5 ± 1.8	11.7 ± 0.9	6.6 ± 0.8	7.5 ± 0.8
<i>8 weeks:</i>				
V ₁	67.4 ± 2.3	52.9 ± 3.0*	76.5 ± 3.7	72.9 ± 4.2
V ₂	19.2 ± 1.2	23.3 ± 1.0	16.1 ± 1.5	19.6 ± 2.1
V ₃	13.2 ± 2.0	23.8 ± 2.7*	7.4 ± 0.6	7.5 ± 0.8
<i>16 weeks:</i>				
V ₁	65.6 ± 1.4	33.9 ± 4.0*	73.5 ± 1.9	42.8 ± 3.8*
V ₂	18.4 ± 1.9	26.3 ± 3.0	18.1 ± 3.1	25.4 ± 1.2
V ₃	16.1 ± 1.9	39.9 ± 3.0*	8.5 ± 0.9	31.6 ± 2.9*

Myosin heavy chains were extracted from left (LV) and right ventricle (RV) and separated on gel electrophoresis as described in Materials and Methods. Data expressed as mean ± SEM were obtained from 4 separate sample preparation in each group. Sh: sham control; AV: aortocaval shunt. * P < 0.05 vs sham control group.

f. Alterations in intrinsic contractile function

Since *in vivo* performance of the heart was largely influenced by a number of neurohormonal factors, we measured the contractile function in the isolated perfused heart to determine if an intrinsic myocardial dysfunction was present in the heart of shunted rats. Figure 7 shows that LVDP, +dP/dt and -dP/dt were significantly decreased at 4, 8 and 16 wks intervals. Compared to the *in vivo* hemodynamic parameters, the myocardial contractile dysfunction occurred earlier (4 wks vs 8 wks) and was more dramatic (75, 63 and 48% of sham vs 94, 82 and 64% of sham for +dP/dt, 82, 60 and 50% of sham vs 94, 79 and 61% of sham for -dP/dt at 4, 8 and 16 wks, respectively) *in vitro* indicating the effect of compensatory mechanisms under *in vivo* condition. The cardiac performance of hearts from 1 and 2 wks AV group was not different from the sham control group (data not shown).

g. Contractile response to isoproterenol stimulation

The responses of isolated perfused heart to the infusion of 1 μ M of isoproterenol are shown in Table 9. The contractile responses to isoproterenol stimulation expressed as percentage increase were greatly enhanced at 4 wks but maintained at 8 and 16 wks after inducing aortocaval shunt as reflected by the values for LVDP, +dP/dt, and -dP/dt.

2. Alterations in β -adrenoceptors and β -adrenergic receptor kinase in heart failure induced by aortocaval shunt

In view of the enhanced inotropic responses of the hypertrophied and failing heart to isoproterenol, the next series of experiments were undertaken to examine changes in β -ARs and

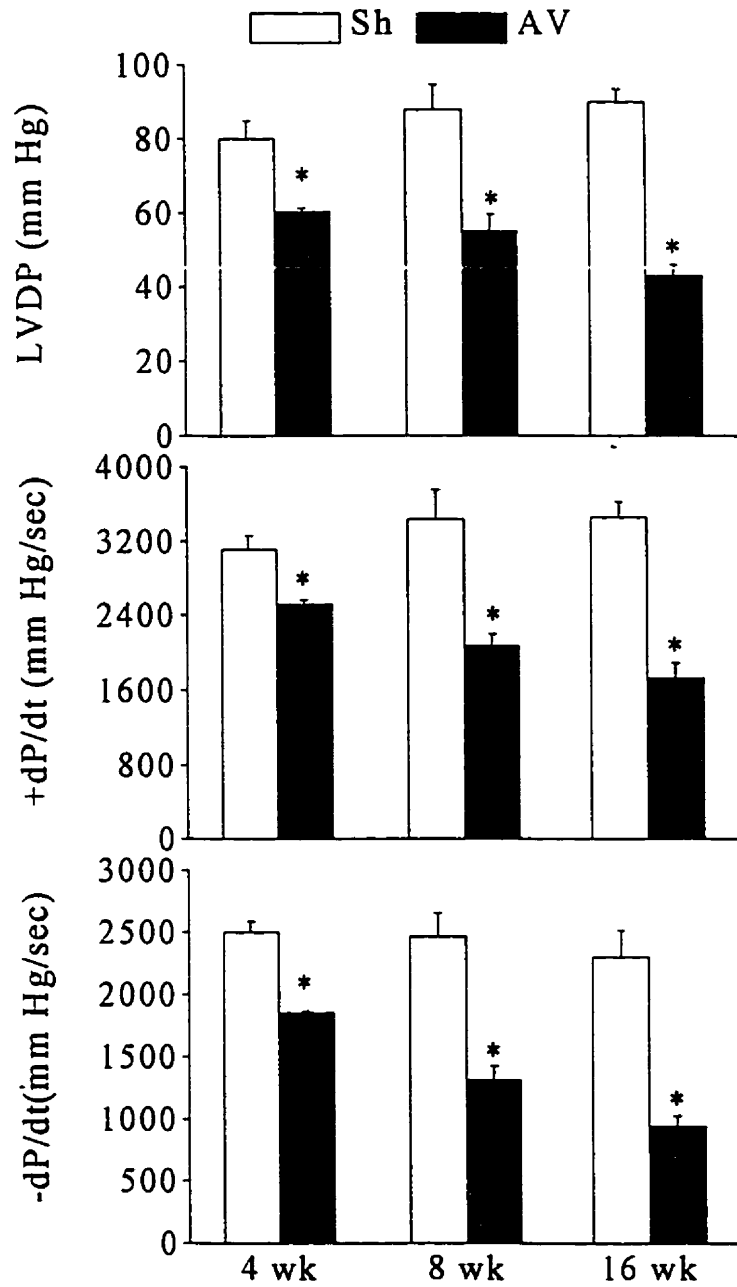


Figure 7. *In vitro* left ventricular hemodynamic changes in rats after inducing the aortocaval shunt for different time intervals. LVDP: left ventricular developed pressure; +dP/dt: maximum rate of pressure development; -dP/dt: maximum rate of pressure decay. *P<0.05 vs sham.

Table 9. Effect of isoproterenol on cardiac function in sham control and aortocaval shunt in rats for 4, 8 and 16 weeks

	LVDP	+dP/dt	-dP/dt
4 weeks			
Sh	271 ± 11	300 ± 16	320 ± 10
AV	539 ± 19*	640 ± 29*	814 ± 29*
8 weeks			
Sh	269 ± 14	288 ± 13	304 ± 11
AV	436 ± 22*	431 ± 18*	579 ± 25*
16 weeks			
Sh	160 ± 5	170 ± 9	200 ± 12
AV	307 ± 17*	378 ± 25*	510 ± 26*

Data in the table were percentage of increase after isoproterenol stimulation. They were expressed as mean±SEM and obtained from 4-6 hearts for each group. Sh: sham control; AV: aortocaval shunt; LVDP: left ventricular developed pressure. +dP/dt: maximum rate of pressure development; -dP/dt: maximum rate of pressure decay. Isoproterenol (1 µM) was used to infuse the heart after basal values were recorded. *-Significant (P<0.05) as compared with sham group.

β ARK in the aortocaval shunted hearts. Furthermore, in view of the relationship between β -adrenergic system and renin-angiotensin system, we investigated the effects of ACE inhibitors and angiotensin II receptor antagonists on the changes in β -AR signaling in heart failure due to AV shunt. In this regard, four experimental groups, namely, sham control (Sh), aortocaval shunt (AV), aortocaval shunt with imidapril treatment (AV+Imp) and aortocaval shunt with losartan treatment (AV+Los) were used. The treatment was started three days after surgery and continued for 16 weeks before the animals were sacrificed. It should be pointed out that our preliminary data showed that sham control with treatment for either losartan or imidapril at the dose employed in this study did not alter the general and hemodynamic characteristics as well as biochemical compositions of the myocardium. One group of the animals were used for assessment of *in vivo* cardiac performance and hemodynamics as well as their response to isoproterenol stimulation followed by examination of general characteristics. Hearts from another group of animals were taken for *in vitro* perfusion and isoproterenol stimulation. A separate group of animals were sacrificed for collecting tissue from both left and right ventricles for the experiments on receptor binding, biochemical assay as well as Western and Northern blot analysis.

a. Modification of general characteristics and hemodynamic changes

Table 10 shows the general characteristics of 16 wks aortocaval shunted rats with or without imidapril or losartan treatments. Although body weight was not altered, the increased heart weight and HW/BW ratio in the untreated AV group were attenuated by both imidapril and losartan. Treatment of imidapril attenuated the hypertrophy response in LV and RV by about 22% and 32%, respectively. Losartan treatment reduced the hypertrophic response by 21 % and 22% in LV and RV, respectively. The increased lung and liver weight in the untreated AV group

Table 10. General characteristics of rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril and losartan treatment

	Sh	AV	AV+Imp	AV+Los
BW (g)	627 ± 31	616 ± 28	609 ± 25	617 ± 30
HW (mg)	1300 ± 85	2580 ± 130*	1950 ± 80 [#]	2037 ± 88 [#]
HW/BW (×100)	207 ± 7.6	418 ± 28*	320 ± 26.3 [#]	330 ± 30.2 [#]
LVW (mg)	1017 ± 46	1887 ± 58*	1477 ± 54 [#]	1492 ± 69 [#]
RVW (mg)	280 ± 13	694 ± 37*	472 ± 23 [#]	545 ± 33 [#]
Lung wt (g)	1.91 ± 0.08	2.49 ± 0.09*	1.94 ± 0.11 [#]	2.03 ± 0.10 [#]
Liver wt (g)	19.8 ± 1.7	27.0 ± 1.2*	23.4 ± 1.0 [#]	21.7 ± 1.3 [#]

Data expressed as mean ± SEM were obtained from 8-12 rats in each group. BW: body weight; HW: heart weight; HW/BW ratio: heart weight to body weight ratio. LVW: left ventricular weight; RVW: right ventricular weight. Sh: sham control group; AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment; AV+Los: aortocaval shunt with losartan treatment. Imidapril (1 mg/kg/day) and losartan (20 mg/kg/day) were given daily by gastric lavage. *P<0.05 vs Sh group, [#]P<0.05 vs AV group.

were also attenuated by treatment with imidapril or losartan.

Table 11 shows the *in vivo* hemodynamic changes obtained from different groups by using carotid artery catheterization. Neither losartan nor imidapril treatment induced any change in the heart rate. Unlike the hypertrophic responses, the depressed ASP and ADP were not reversed significantly by the treatment with imidapril or losartan. On the other hand, increased LVEDP and depressed LVSP, as well as $\pm dP/dt$ were partially reversed by treatment with imidapril or losartan. It seems that treatment of losartan improved the contractile function more than imdidapril since both $+dP/dt$ and $-dP/dt$ reversed to a greater extent than that treated by imidapril. In a preliminary study we examined the β -adrenergic response *in vivo* by injecting different doses of isoproterenol (0.5-20 $\mu\text{g}/\text{kg}$) into femoral artery and recording the hemodynamic responses; 0.3% of ascorbic acid was included in the isoproterenol solution to prevent its oxidation. It was observed that the dose of 10 $\mu\text{g}/\text{kg}$ isoproterenol was optimal and thus was used as a bolus dose for studying its effect in control and experimental groups. The data in Table 12 indicate that although the basal values for LVDP, $+dP/dt$ and $-dP/dt$ were significantly depressed in aortocaval shunt as well as in the treatment groups, their stimulatory responses to isoproterenol were similar. Thus the fold increases calculated by values after isoproterenol stimulation over the basal values were actually greater in these groups.

Since the heart under *in vivo* conditions is controlled by numerous neurohormonal factors in addition to the endogenous catecholamines, we eliminated the possibilities of interferences of these factors by using the isolated perfused heart model *in vitro* to evaluate the contractile responses to isoproterenol stimulation. The results in Table 13 show attenuated LVDP, $+dP/dt$ and $-dP/dt$ in the aortocaval shunt group and partial improvement in the two treatment groups.

Table 11: *In vivo* hemodynamic changes in rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment

	Sh	AV	AV+Imp	AV+Los
Heart Rate (beats/min)	336 ± 15	340 ± 12	330 ± 16	326 ± 10
ASP (mm Hg)	120 ± 3	103 ± 5*	107 ± 4	106 ± 6
ADP (mm Hg)	84 ± 5	55 ± 5*	61 ± 3	62 ± 5
LVSP (mm Hg)	135 ± 7	95 ± 4*	114 ± 2 [#]	119 ± 7 [#]
LVEDP (mm Hg)	4.7 ± 0.7	28.0 ± 1.6*	15.3 ± 2.3 [#]	16.8 ± 1.0 [#]
+dP/dt (mm Hg/sec)	6283 ± 214	4026 ± 149*	4809 ± 159 [#]	5331 ± 140 [#]
-dP/dt (mm Hg/sec)	6257 ± 239	3791 ± 199*	4585 ± 157 [#]	5162 ± 198 [#]

Data expressed as mean±SEM were obtained from 4-6 hearts for each group. Sh: sham control, AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment, AV+Los: aortocaval shunt with losartan treatment, Imidapril (1mg/kg/day) and losartan (20 mg/kg/day) were given daily by gastric lavage. ASP: arterial systolic pressure, ADP: arterial diastolic pressure, LVSP: left ventricular systolic pressure, LVEDP: left ventricular end diastolic pressure, +dP/dt: maximum rate of pressure development, -dP/dt: maximum rate of pressure decay. *P<0.05 as compared with Sh group, [#]P<0.05 as compared with AV group.

Table 12. Effect of bolus isoproterenol on *in vivo* hemodynamics of rats with heart failure induced by aortocaval shunt for 16 weeks with and without treatment of imidapril or losartan

	LVDP	+dP/dt	-dP/dt	HR
<u>Sh:</u> Basal	133 ± 10	6450 ± 250	6377 ± 327	354 ± 21
Isoproterenol	259 ± 18	13780 ± 947	12959 ± 857	663 ± 19
<u>AV:</u> Basal	65 ± 7*	4051 ± 206*	3675 ± 155*	362 ± 28
Isoproterenol	263 ± 21	13997 ± 1042	13046 ± 905	674 ± 33
<u>AV+Imp:</u> Basal	101 ± 10 [#]	4733 ± 186 [#]	4508 ± 277 [#]	349 ± 17
Isoproterenol	254 ± 16	14526 ± 973	12880 ± 974	654 ± 31
<u>AV+Los:</u> Basal	104 ± 9 [#]	4832 ± 219 [#]	4632 ± 303 [#]	352 ± 20
Isoproterenol	270 ± 14	13025 ± 760	12554 ± 671	660 ± 29

Data expressed as mean±SEM were obtained from 4-6 hearts for each group. Sh: sham control, AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment, AV+Los: aortocaval shunt with losartan treatment, Imidapril (1mg/kg/day) and losartan (20 mg/kg/day) were given daily by gavage. LVDP: left ventricular developed pressure, +dP/dt: maximum rate of pressure development, -dP/dt: maximum rate of pressure decay. Isoproterenol was given as a bolus dose (10 µg/kg) through femoral artery. *P<0.05 as compared with Sh group, [#]P<0.05 as compared with AV group.

Table 13. Effect of isoproterenol on *in vitro* cardiac performance of rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment

	Sh	AV	AV+Imp	AV+Los
A. LVDP				
Basal	90 ± 3.6	43 ± 2.9*	68 ± 8.1 [#]	79 ± 6.2 [#]
Isoproterenol	147 ± 5.2	132 ± 12.3	124 ± 10.1	148 ± 4.3
Fold of stimulation	1.63	3.07	1.82	1.87
B. +dP/dt				
Basal	3501 ± 163	1956 ± 153*	2639 ± 190 [#]	2829 ± 211 [#]
Isoproterenol	6637 ± 613	6833 ± 541	5195 ± 472	6545 ± 630
Fold of stimulation	1.90	3.49	1.97	2.31
C. -dP/dt				
Basal	2450 ± 207	938 ± 94*	1433 ± 139 [#]	1612 ± 124 [#]
Isoproterenol	4723 ± 328	4883 ± 246	4231 ± 369	5005 ± 481
Fold of stimulation	1.93	5.21	2.95	3.10

Data expressed as mean±SEM were obtained from 4-6 hearts for each group. Sh: Sham control, AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment, AV+Los: aortocaval shunt with losartan treatment. Imidapril (1 mg/kg/day) and losartan (20 mg/kg/day) were given once daily by gavage. LVDP: left ventricular developed pressure. +dP/dt: maximum rate of pressure development; -dP/dt: maximum rate of pressure decay. Isoproterenol (1 μM) was infused to the heart after basal values were recorded. *P<0.05 as compared with Sh group, [#]P<0.05 as compared with AV group.

However, no difference was detected in these parameters after infusion of isoproterenol. Accordingly, the fold increase was greater in the AV group, reduced a bit but still stay higher in the treatment groups. This indicated no desensitization of β -adrenergic system was present in this model but instead, it seems there is an upregulation of this system.

b. Modification of changes in β -ARs

In order to examine the possibility of up-regulation of β -adrenergic system, we examined the functional changes at the receptor level by using the binding assay. The results in Table 14 show the values for K_d and B_{max} for β_1 - and β_2 -receptors, respectively. In both left and right ventricle, there was a significant increase in B_{max} of β_1 -receptors in the AV shunt group indicating an increase in β_1 -receptor density. Treatment with imidapril or losartan partially reversed this increment. No change in K_d thus affinity of the β_1 -receptors was detected among all the groups in both LV and RV. For β_2 -ARs, no change was detected for K_d and B_{max} in both LV and RV with or without drug treatments.

Since receptor-binding assay is considered to detect the functionally active receptors which are located outside the membrane, it is difficult to pin-point the location of the observed changes in β_1 -receptor density. Accordingly, we used Western blot analysis to examine the changes in total receptor number which may be located either inside or outside the membrane. The results in Figure 8 show the typical Western blot bands and statistical data for β_1 - and β_2 -receptors in both LV and RV. In agreement with the data from the binding assay, we found a dramatic increase in β_1 -receptor protein content in both LV and RV in the AV group. Treatment with imidapril or losartan partially reversed this increment in the two ventricles. On the other hand, protein content of β_2 -receptors in both LV and RV were not altered in any group;

Table 14: Kinetic parameters of β -adrenoceptor binding in rats with heart failure induced by aorticaval shunt for 16 weeks with and without imidapril or losartan treatment

	β_1 -AR		β_2 -AR	
	K_d	B_{max}	K_d	B_{max}
A. Left ventricle:				
Sh	43 ± 2.8	70 ± 6.2	15.1 ± 0.9	28.0 ± 2.1
AV	48 ± 4.7	110 ± 8.0*	16.3 ± 0.7	29.4 ± 1.9
AV+Imp	45 ± 4.3	95 ± 6.4 [#]	15.4 ± 0.3	27.6 ± 2.3
AV+Los	47 ± 4.0	89 ± 5.1 [#]	14.9 ± 0.4	27.3 ± 2.4
B. Right ventricle:				
Sh	40 ± 1.8	68 ± 4.7	14.7 ± 1.0	26.3 ± 1.8
AV	42 ± 2.0	105 ± 3.9*	15.9 ± 0.8	25.9 ± 2.0
AV+Imp	42 ± 1.6	84 ± 1.3 [#]	14.9 ± 0.6	24.9 ± 2.7
AV+Los	41 ± 2.3	89 ± 2.4 [#]	15.3 ± 2.2	25.5 ± 1.7

Data expressed as mean ± SEM were obtained from 4-6 hearts. K_d : dissociation constant, B_{max} : maximal binding. Sh: sham control, AV: aorticaval shunt, AV+Imp: aorticaval shunt with imidapril treatment; AV+Los: aorticaval shunt with losartan treatment. Imidapril (1 mg/kg/day) and losartan (20 mg/kg/day) were given daily by gavage. *P<0.05 as compared with Sh group, [#] P<0.05 as compared with AV group.

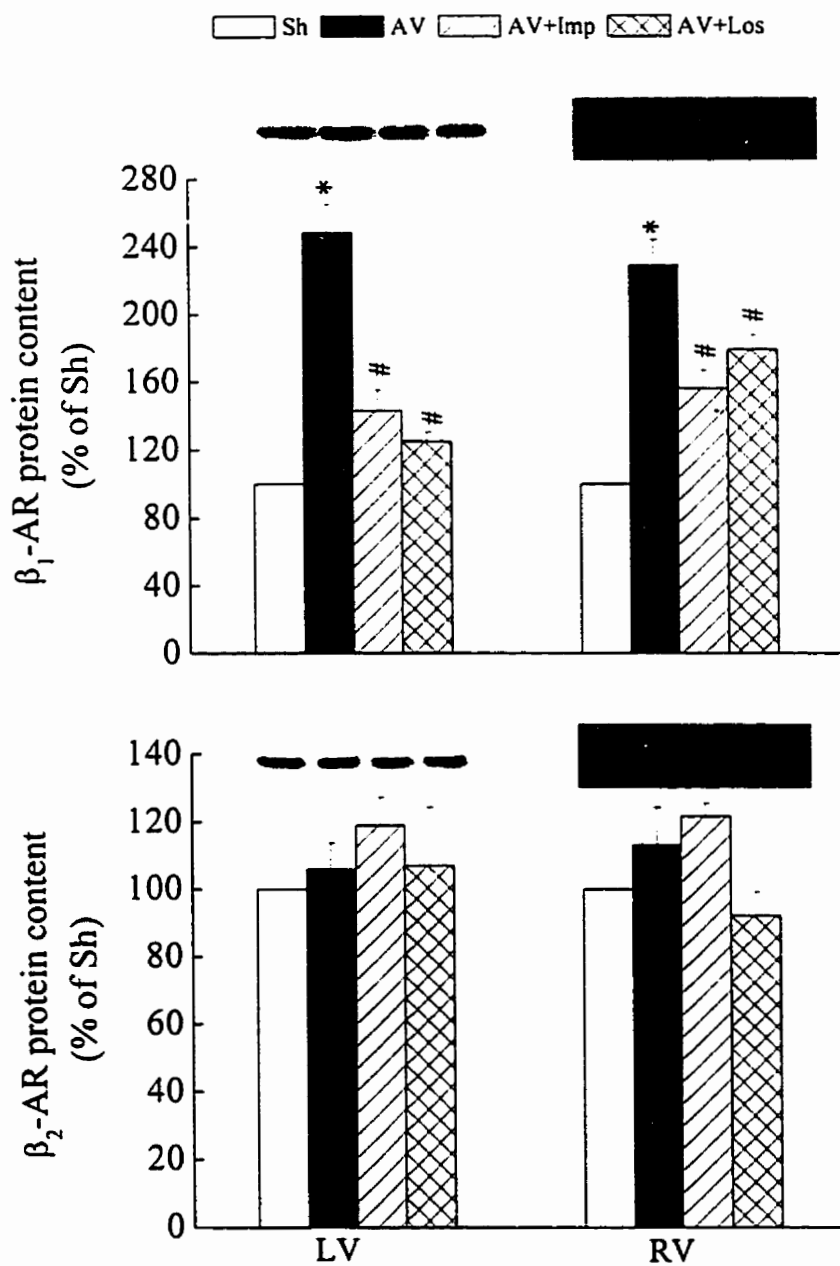


Figure 8. Alterations of protein content in β -ARs in the chronic heart failure induced by aorticaval shunt with and without treatment of imidapril and losartan

this is also in agreement of the results in the receptor binding experiments. It is obvious that the extent of increase in protein content is much higher than that of the receptor density (148% and 129% vs 57% and 54% in LV and RV, respectively). It is possible that there was a homogenous increase of β_1 -receptors including functionally active and inactive receptors. Another possibility may be due to differences in the sensitivity of these two methods applied in this study.

To find out the molecular basis of increased β_1 -receptor protein content, we prepared a cDNA probe to detect the mRNA levels of β_1 -AR in the myocardium by using Northern blot analysis. Figure 9 shows the RNA loading and typical bands for two internal controls, 18S and GAPDH, as well as bands for β_1 -AR in both LV and RV. Figure 10 shows the statistical data calculated from the density of the bands and corrected by internal controls. It should be pointed out that no significant changes were observed among the groups for either 18S or GAPDH. In both LV and RV, there was a slight but insignificant decrease of mRNA level for β_1 -AR in AV group indicating that the increase of protein level may not be associated with an increase of transcription but instead is due to a decreased degradation of the β_1 -AR protein.

c. Alterations of GRKs, β -arrestins and $G_{\beta\gamma}$ -subunit

Although three isoforms of GRKs have been found abundantly expressed in cardiac tissue, their roles in normal and diseased conditions are still largely unknown. Recent studies have shown that an increase of GRK2 (β ARK1) expression was associated with the desensitization of β -adrenergic system in human and a few animal models with heart failure. However, no information was available regarding its changes in a high-output heart failure model. In addition, little information is available regarding to the changes of GRK3 and GRK5 in cardiac diseases. Although *in vitro* studies have shown that β -arrestins and $G_{\beta\gamma}$ facilitate GRK2 and GRK3

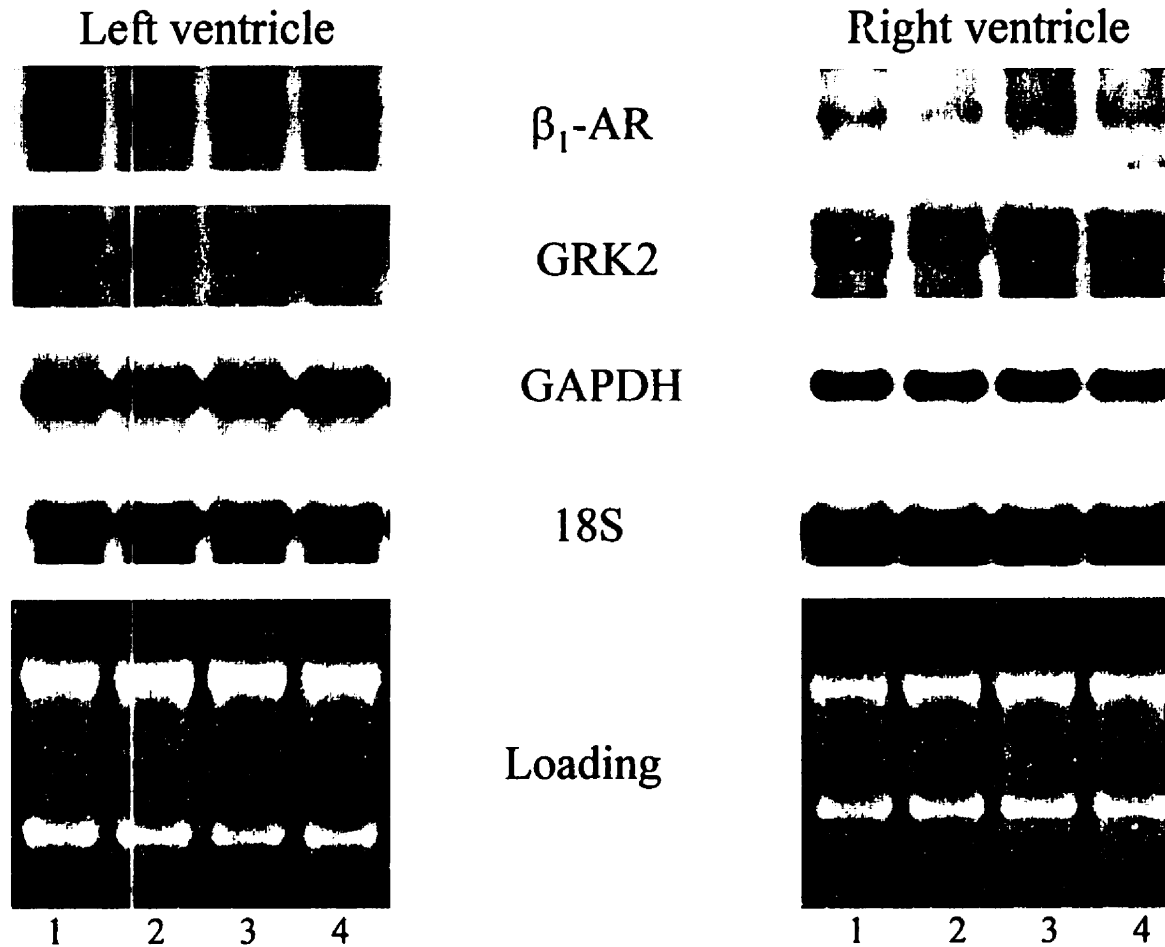


Figure 9. Typical Northern blots showing β_1 -adrenoceptors, GRK2 mRNA levels in LV and RV of chronic failing heart induced by aorticaval shunt with or without treatment of imidapril and losartan. Signals for GAPDH and 18S were used as internal controls. Loading sequence lane 1 for Sh, lane 2 for AV, lane 3 for AV+Imp, lane 4 for AV+Los

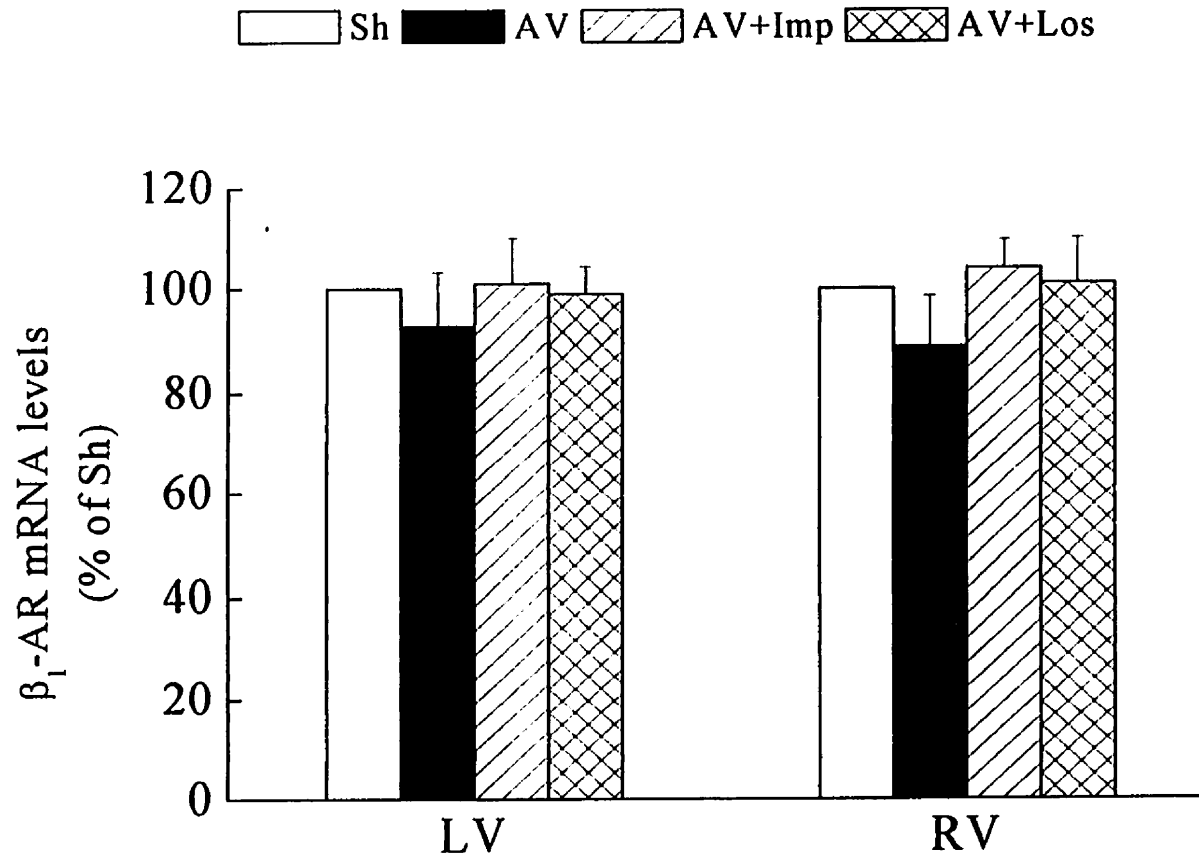


Figure 10. Alterations of mRNA levels in β_1 -adrenoceptors in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan.

phosphorylation of β -ARs, *in vivo* changes for these factors in response to cardiac overload are not known. Therefore this series of experiments were undertaken to test the changes in GRKs and β -arrestins as well as $G_{\beta\gamma}$ in the high-output heart failure model in order to examine their contribution towards the altered β -adrenergic signaling.

Figure 11 shows that the aortocaval shunt for 16 weeks resulted in a significant reduction of GRK activity in the membranous fraction and a significant increase in the cytosolic fraction. It should be noted that GRK2 and GRK3 are cytosolic proteins and require a membrane translocation event before their activation whereas GRK5 is believed to be a membrane-associated protein. Thus the cytosolic activity represents mainly the sum of GRK2 and GRK3 activity whereas membranous activity represents the sum of the three cardiac GRKs. A decrease in membranous activity indicates less phosphorylation of β -ARs and subsequently less desensitization of β -ARs. However, it is difficult to determine at this point which GRK isoform may contribute to this decrease in the activity. It is also hard to imagine what underlies this decrease – an altered membrane translocation event or an absolute down regulation of GRKs on the membrane? To gain some information on this point, we examined the GRK protein expression individually. Figure 12 shows that GRK2 protein expression in both LV and RV was decreased in the membranous fraction but increased in the cytosolic fraction, which patterns of changes is similar when compared that for the GRK activity. Since GRK2 needs to be translocated to the membrane in order to phosphorylate the receptors, a decrease in membrane and simultaneous increase in cytosol suggested that the changes in activity and protein content might be a result of GRK2 translocation rather than a net change in the respective fractions. To confirm this point, we used myocardium homogenate rather than membranous or cytosolic

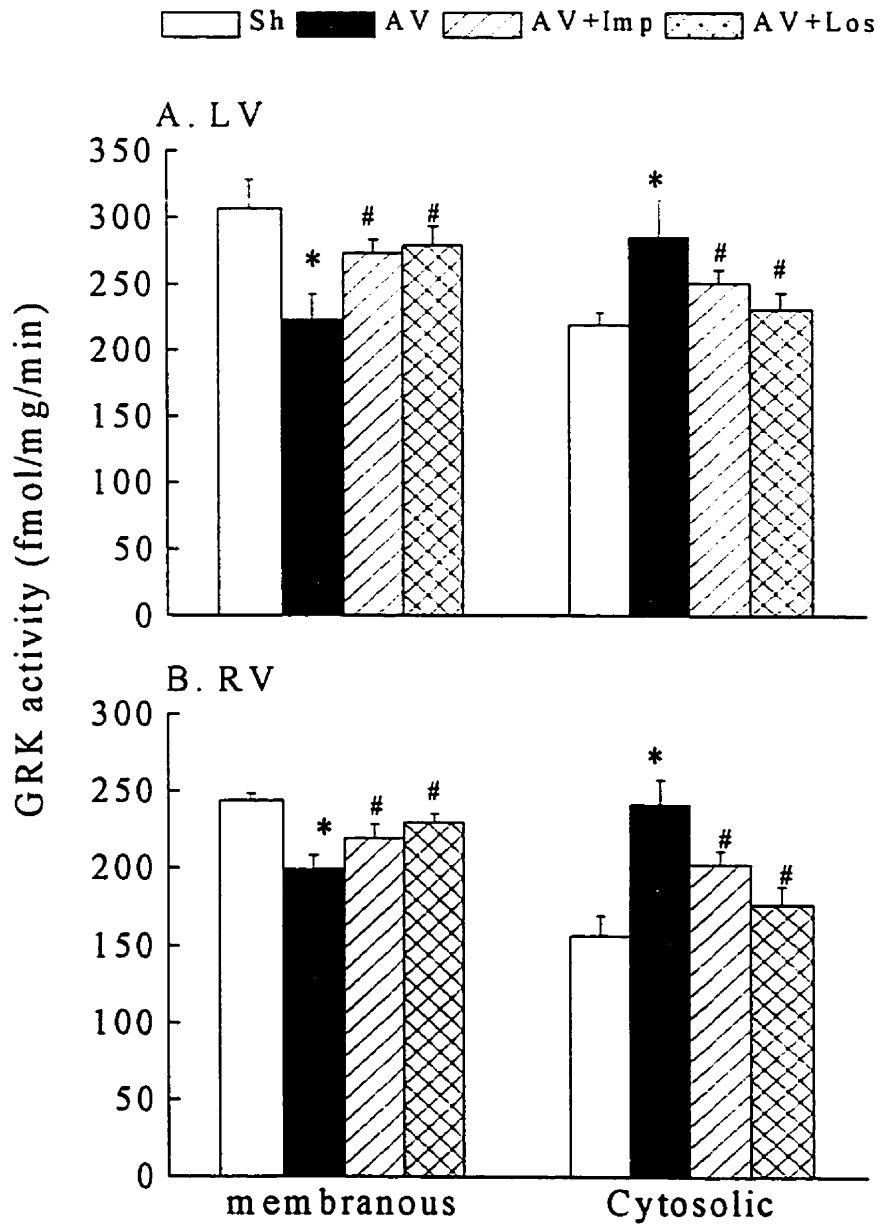


Figure 11. Alterations of GRK activity in the chronic heart failure induced by aorticaval shunt with and without treatment of imidapril and losartan.

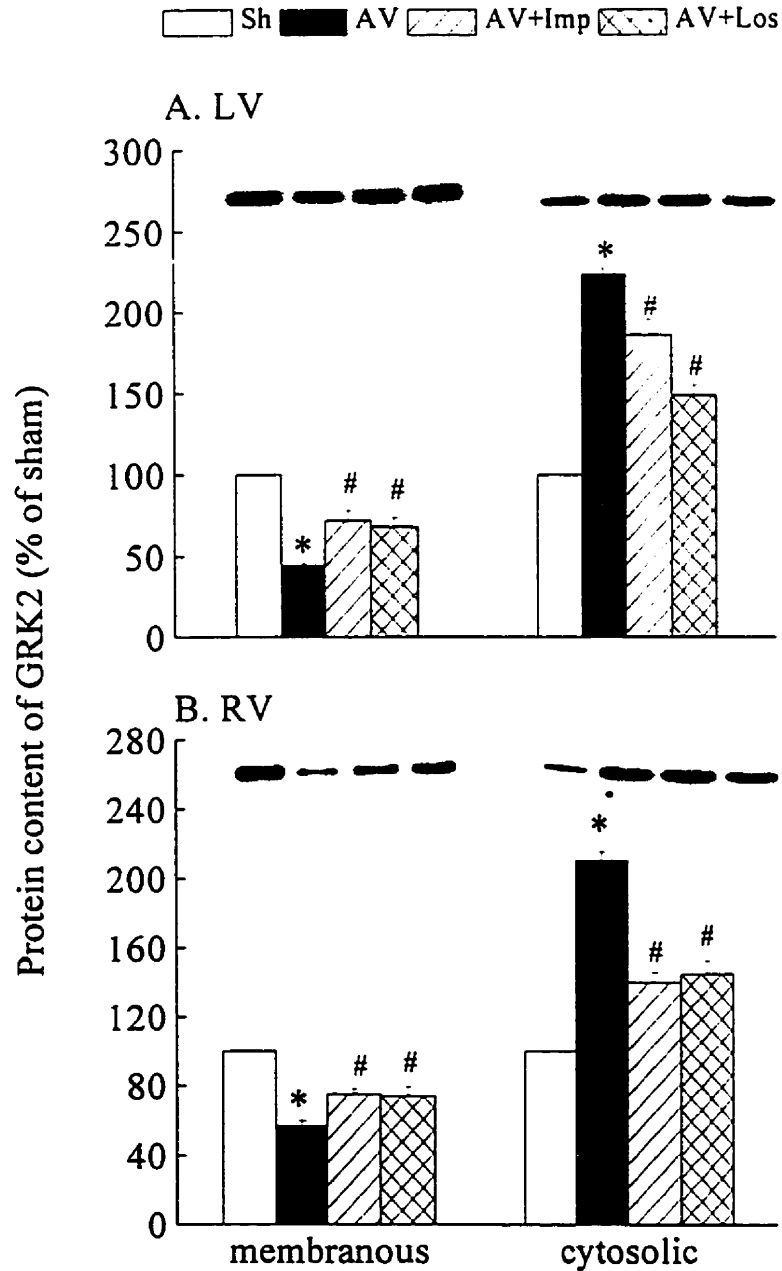


Figure 12. Alterations in protein content of GRK2 in both membranous and cytosolic fractions in the chronic heart failure induced by aorticaval shunt with and without treatment of imdapril and losartan.

fraction to determine the activity and protein content of GRK2. Results in panels A and B of Figure 13 show no change in activity and protein content in the homogenate preparation in all groups in both LV and RV. Furthermore, we measured the mRNA level of GRK2 which also showed no significant alteration in both LV and RV (see Figure 14) indicating that the observed changes in activity as well as protein content reflects a change in the translocation of the GRK2 molecules between membrane and cytosol. It should be noted from Figures 11 and 12, that treatment with either imidapril or losartan partially reversed the cellular redistribution of GRK2 in both LV and RV, suggesting that these drugs might be able to modify the membrane translocation event.

We have also examined protein content of GRK3 (also known as β ARK2 previously) in both cytosol and membranous preparation. Results in Figure 15 indicates that GRK3 underwent changes similar to that for GRK2 in terms of protein expression in these animals which is an increase in cytosolic and decrease in membranous fractions. We also measured total GRK3 protein expression in the homogenate preparation and panel C in Figure 13 shows no alterations for GRK3 protein content for all the groups. Although GRK5 is believed to be a membrane-associated isoform, Ping *et al.* (66) have detected its expression in the cytosolic preparation. Thus we also determined its expression in the both fractions. Figure 16 demonstrated that GRK5 antibody recognized a band at approximately 68 kDa which is at a similar position as reported by Ping *et al.* (66). Both LV and RV GRK5 protein expression was significantly decreased in the membranous fraction but increased in the cytosolic fraction. We also determine mRNA levels for GRK3 and GRK5, however, with the Northern blot method we were not able to detect these signals. This may be due to the quality of the probe used in this

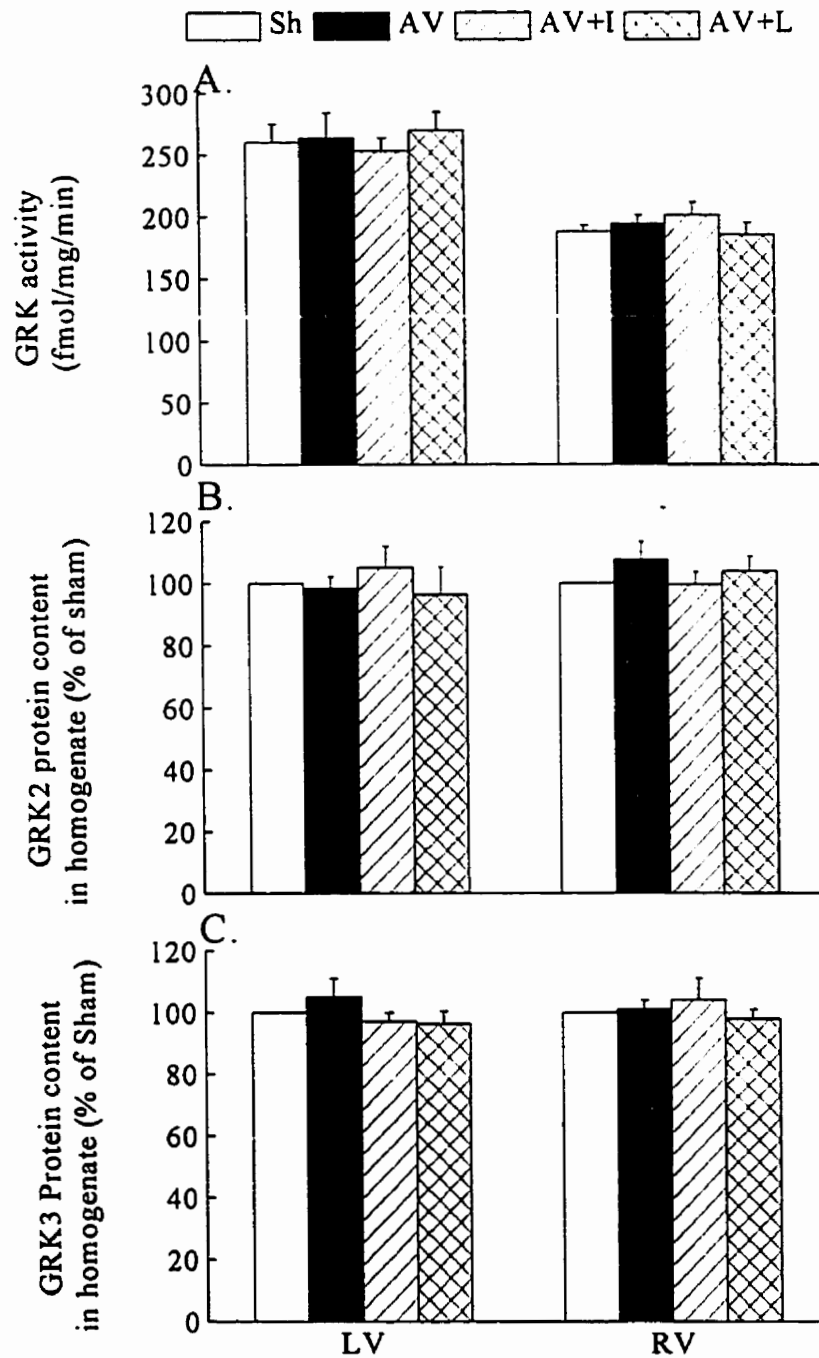


Figure 13. GRK activities and GRK2 and GRK3 protein content in LV and RV homogenate preparations in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan.

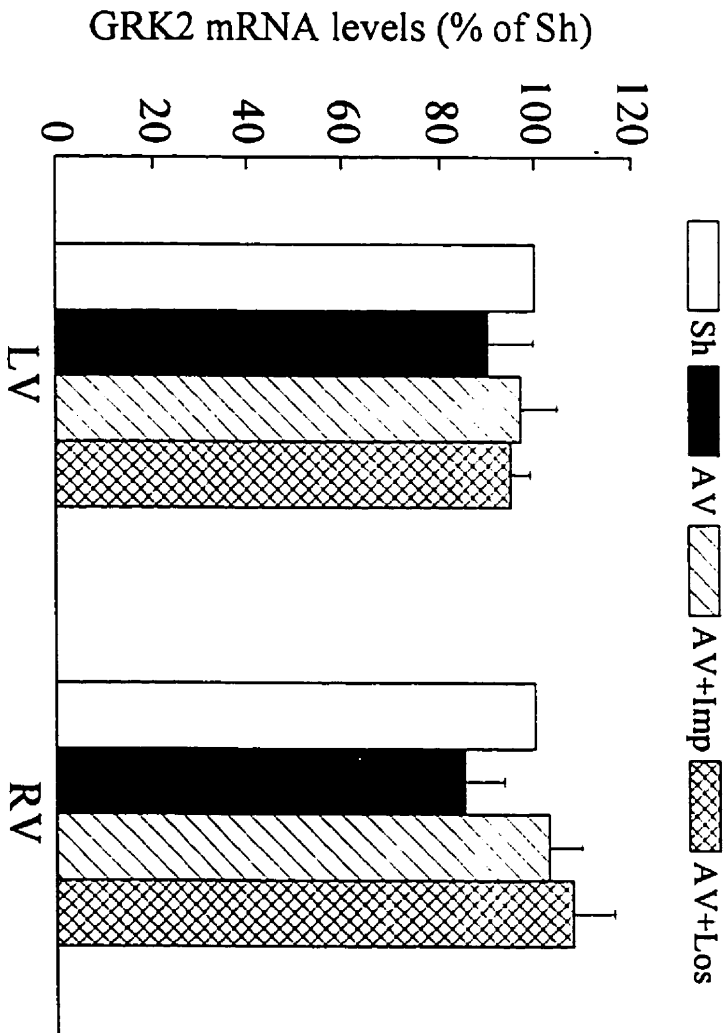


Figure 14. Alterations of mRNA level of GRK2 in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan.

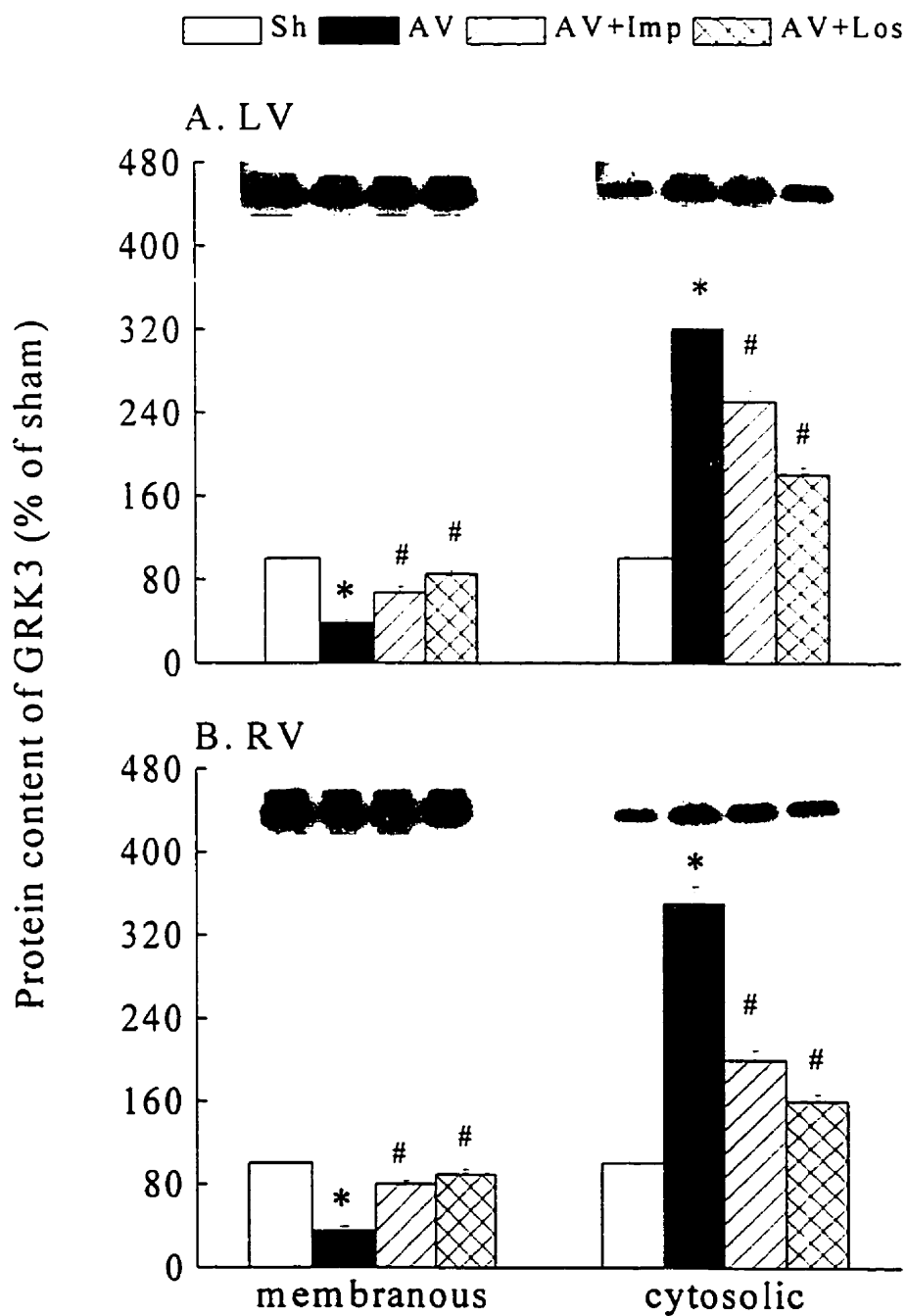


Figure 15. Alterations of protein content of GRK3 in both membranous and cytosolic fractions in the chronic heart failure induced by aorticaval shunt with and without treatment of imidapril and losartan.

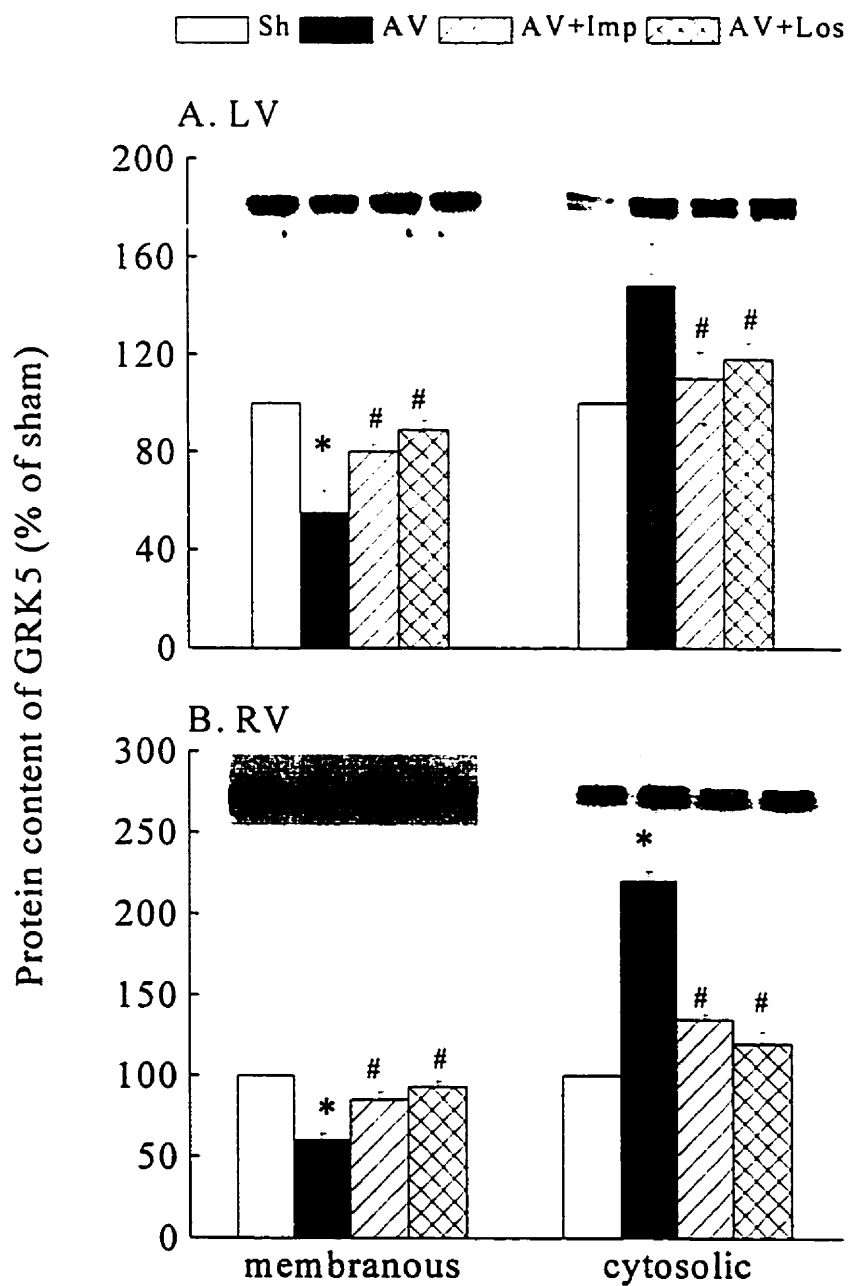


Figure 16. Alterations of protein content of GRK3 in both membranous and cytosolic fractions in the chronic heart failure induced by aorticaval shunt with and without treatment of imidapril and losartan.

study or the low levels of expression of these two isoforms in heart. Further studies using more sensitive method may be able to determine if the changes observed for GRK3 and GRK5 related to the gene expression.

Due to the important role of β -arrestins in receptor desensitization, we investigated the most abundant isoform of β -arrestin, β -arrestin1. Figure 17 reveals that the β -arrestin1 antibody recognized a band approximately at the level of 50 kDa. In both LV and RV, β -arrestin1 showed pattern of changes in membranous and cytosolic preparations similar to that for the GRK2; however, the extent of changes was not as dramatic as that for GRK2. Since $G_{\beta\gamma}$ helps in translocation of the GRK2 and GRK3 to the membrane and thus facilitate the phosphorylation of receptors, the altered distribution of GRK2 and GRK3 in the membranous and cytosolic fraction may be related with the availability of $G_{\beta\gamma}$. Accordingly we measured the cardiac isoforms of $G_{\beta\gamma}$, the β_2 and γ_7 . Results in Figure 18 reveal no change of both G_{β_2} and G_{γ_7} in the LV and RV after 16 wks of inducing the aortocaval shunt, indicating that changes in the GRK activity are not related to any alteration of $G_{\beta\gamma}$.

3. Alterations of adenylyl cyclase and G-proteins in heart failure induced by aortocaval shunt

By using a separate group of animals, the third series of experiments was carried out to evaluate the role of two important components of the β -adrenergic signal transduction pathway, namely, the effector: AC and the coupler: G-proteins in altering the response to β -adrenergic stimulation in aortocaval shunt induced heart failure with or without treatment with imidapril or

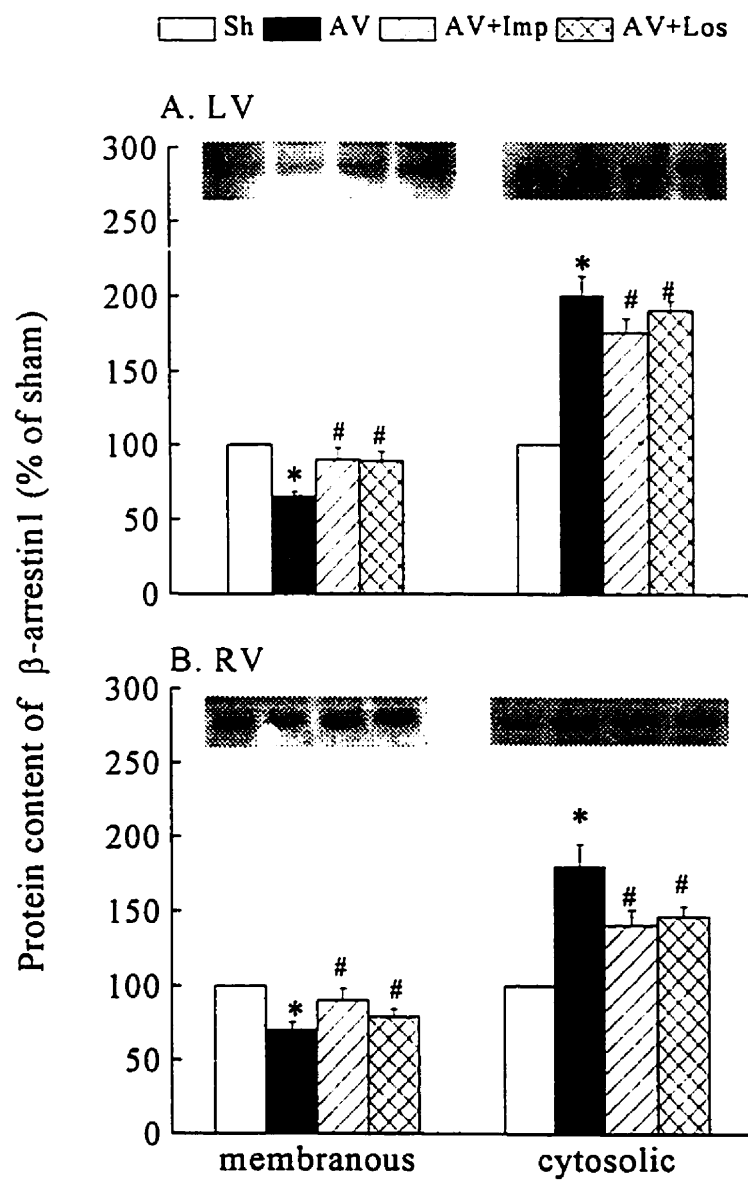


Figure 17. Alterations of β -arrestins in the LV and RV chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan.

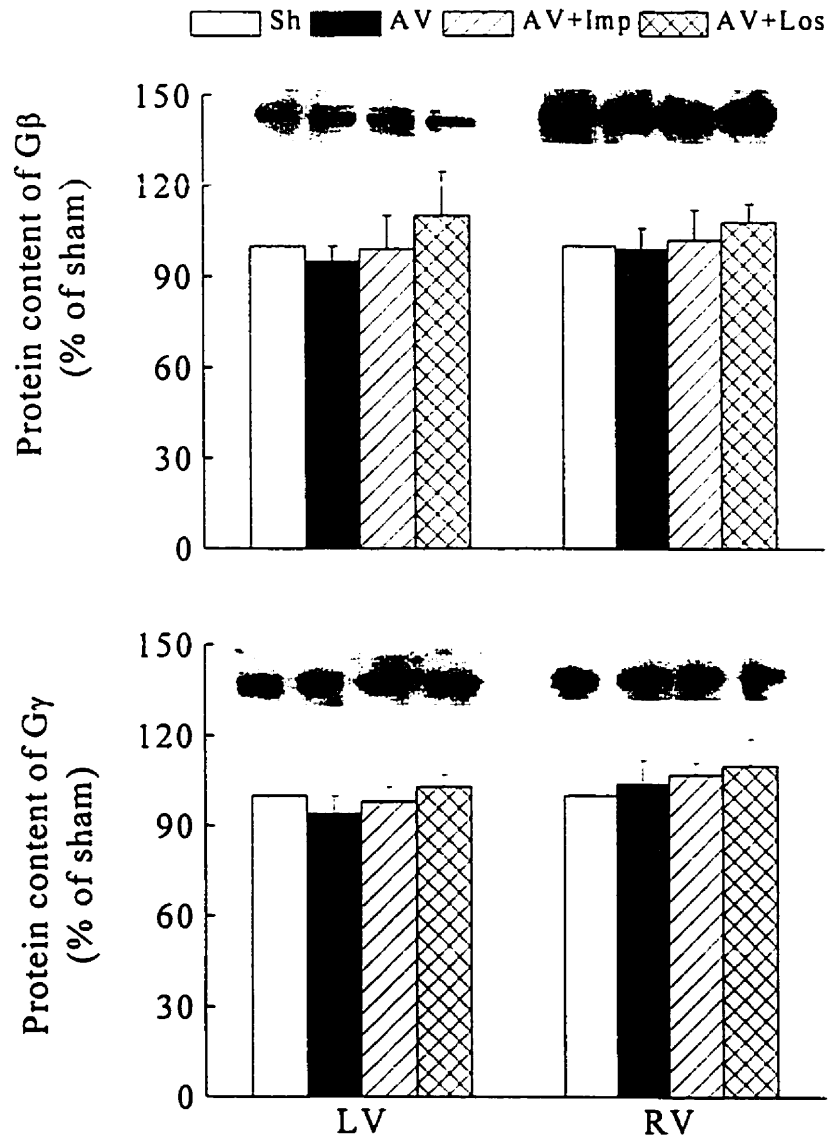


Figure 18. Alterations of protein content of G β _y subunit in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan.

losartan.

a. General and hemodynamic features

Table 15 indicates that the general hemodynamic characteristics of this group of animals were similar to those observed earlier. No change of body weight was seen among all the groups; however, dramatic hypertrophy of both LV and RV occurred. Although heart rate was not altered, the LVSP, +dP/dt and -dP/dt were significantly depressed while the LVEDP was dramatically increased indicating both systolic and diastolic dysfunction at this stage of heart failure. All these changes in the AV were attenuated by treatment with imidapril or losartan.

b. Alterations of adenylyl cyclase in volume overload induced heart failure

Figure 19 shows changes of AC activities in both LV and RV in the absence or presence of different stimulants. A significant increase of AC activity was observed in basal as well as in the presence of isoproterenol, Gpp(NH)p, NaF and forskolin. When the data were converted to fold stimulation over the respective basal values (Table 16), only isoproterenol- and forskolin-stimulated AC activities were increased whereas Gpp(NH)p- and NaF-stimulated AC activities were unaltered. Neither imidapril nor losartan reversed these changes. In order to examine if increased basal AC activity was associated with an increase in the protein content, we used AC type V/VI antibody to determine the protein content of AC. Figure 20 shows that AC type V/VI antibody recognized a band approximately at the level of 130 kDa. There is a dramatic increase in AC protein content in both LV and RV in AV group. The treatment of AV animals with imidapril or losartan did not reverse the changes in protein content indicating that these drugs may not be able to modify AC.

Table 15: General and hemodynamic characteristics of sham and experimental rats with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment

	Sh	AV	AV+Imp	AV+Los
BW (g)	609 ± 29	598 ± 19	595 ± 21	607 ± 25
LVW (mg)	1011 ± 51	1859 ± 70*	1500 ± 43 [#]	1472 ± 66 [#]
RVW (mg)	281 ± 11	694 ± 26*	472 ± 27 [#]	545 ± 35 [#]
Heart Rate (beats/min)	350 ± 18	348 ± 14	339 ± 18	356 ± 20
LVSP (mm Hg)	133 ± 9	92 ± 6*	110 ± 4 [#]	115 ± 6 [#]
LVEDP (mm Hg)	4.5 ± 0.6	26.8 ± 2.0*	14.7 ± 2.3 [#]	15.4 ± 0.9 [#]
+dP/dt (mm Hg/sec)	6330 ± 206	3980 ± 152*	4665 ± 182 [#]	5297 ± 166 [#]
-dP/dt (mm Hg/sec)	6292 ± 315	3705 ± 184*	4577 ± 139 [#]	5033 ± 206 [#]

Data expressed as mean±SEM were obtained from 4-6 hearts for each group. Sh: sham control, AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment, AV+Los: aortocaval shunt with losartan treatment. Imidapril (1mg/kg/day) and losartan (20 mg/kg/day) were given daily by gavage. BW: body weight, LVW: left ventricular weight, RVW: right ventricular weight. LVSP: left ventricular systolic pressure, LVEDP: left ventricular end diastolic pressure, +dP/dt: maximum rate of pressure development, -dP/dt: maximum rate of pressure decay. *P<0.05 as compared with Sh group, [#]P<0.05 as compared with AV group.

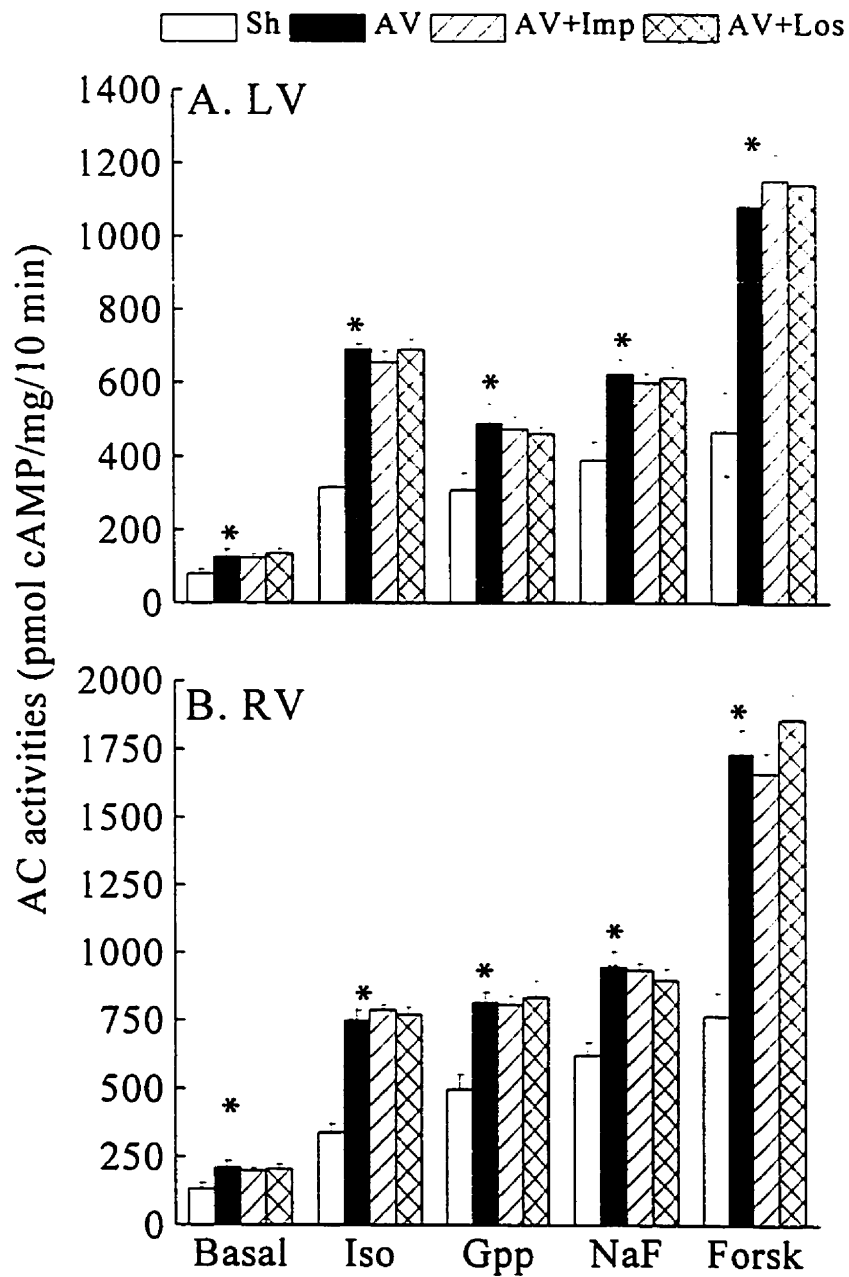


Figure 19. Alterations of AC activities in the LV and RV of chronic failing heart induced by aortocaval shunt with or without treatment of imidapril and losartan.

Table 16. Folds increase of adenylyl cyclase activities under different stimulants in rat with heart failure induced by aortocaval shunt for 16 weeks with and without imidapril or losartan treatment

	isoproterenol	Gpp(NH)p	NaF	Forskolin
<u>A. LV:</u>				
Sham	3.89 ± 0.21	3.81 ± 0.29	4.80 ± 0.22	5.75 ± 0.13
AV	5.48 ± 0.36*	3.87 ± 0.14	4.93 ± 0.36	8.58 ± 0.50*
AV+Imp	5.27 ± 0.19	3.81 ± 0.15	4.82 ± 0.28	9.28 ± 0.29
AV+Los	5.05 ± 0.22	3.39 ± 0.24	4.50 ± 0.27	8.38 ± 0.34
<u>B. RV:</u>				
Sham	2.55 ± 0.16	3.75 ± 0.18	4.69 ± 0.16	5.78 ± 0.33
AV	3.57 ± 0.18*	3.89 ± 0.30	4.51 ± 0.29	8.24 ± 0.52*
AV+Imp	3.98 ± 0.11	4.09 ± 0.23	4.73 ± 0.35	8.40 ± 0.12
AV+Los	3.77 ± 0.31	4.08 ± 0.22	4.39 ± 0.30	9.06 ± 0.36

Data expressed as mean±SEM were obtained from 4-6 hearts for each group. Sh: sham control, AV: aortocaval shunt, AV+Imp: aortocaval shunt with imidapril treatment, AV+Los: aortocaval shunt with losartan treatment. Imidapril (1mg/kg/day) and losartan (20 mg/kg/day) were given daily by gavage. Doses for isoproterenol, Gpp(NH)p, NaF and forskolin are 100 µM, 30 mM, 10 mM, and 100 µM respectively.

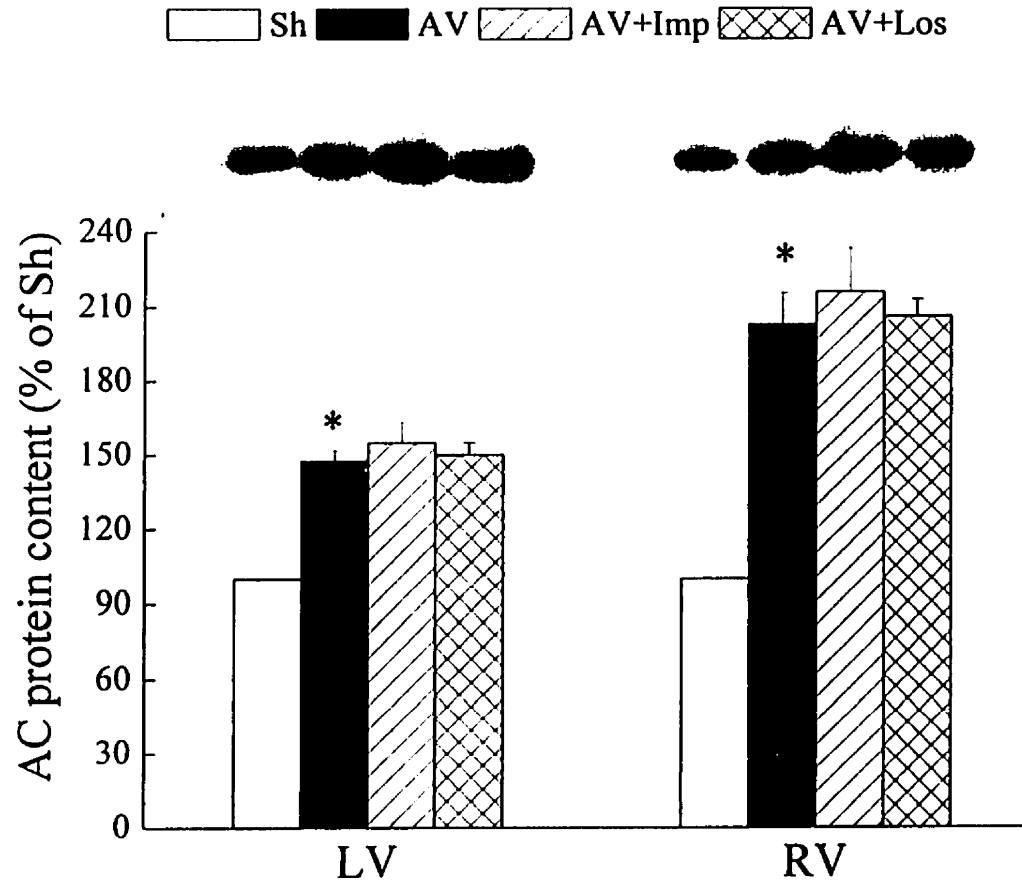


Figure 20. Alterations of protein content of AC type V/VI in the chronic heart failure induced by aortocaval shunt with and without treatment of imidapril and losartan.

c. Alterations of G-proteins in volume overload induced heart failure

The cardiac AC function is predominantly under the dual stimulatory and inhibitory regulation by receptors through the G_s - and G_i -proteins, respectively. Thus in order to gain further information regarding the status of β -adrenergic transduction system in the AV group, we determined changes in the G-protein levels. Since G-protein α subunit have ADP ribosylation sites for cholera toxin (G_{sc} -protein) or pertussis toxin (G_{ic} -protein), the cholera-toxin and pertussis toxin catalyzed ADP-ribosylation activities were measured in the experimental groups. Figure 21 reveals two bands of ribosylation substrates catalyzed by cholera toxin, one at 52 and the other at 45 reflecting the protein products from spliced G_{sc} -protein gene whereas one band at 40 kDa was detected as ribosylation substrate for G_{ic} . A significant decrease in cholera toxin-catalyzed ADP-ribosylation was observed in AV group in both LV and RV; imidapril or losartan treatment partially reversed this change (Figure 21, upper panel). On the other hand, no change was found in the pertussis toxin-catalyzed ADP-ribosylation among all the groups. Since the accuracy of this methodology depends on a number of factors such as biophysical membrane properties, posttranslational modifications of G_{α} -proteins and several cofactors required for the ADP ribosylation reaction, the changes detected may reflect a change in one of the factors rather than a real change in the protein expression. To eliminate the possibility, we used Western blot method to detect relative protein content of both G_{sc} and G_{ic} subunit. The results in Figure 22 show no change in both G_{sc} (upper panel) and G_{ic} (lower panel) protein content among all the groups in both LV and RV. This seems to suggest that the decreased CT-catalyzed ADP-ribosylation may be due to either the biophysical properties of the membrane or post-transcriptional modulation of G_{sc} subunit rather than a decreased expression of the G_{sc}

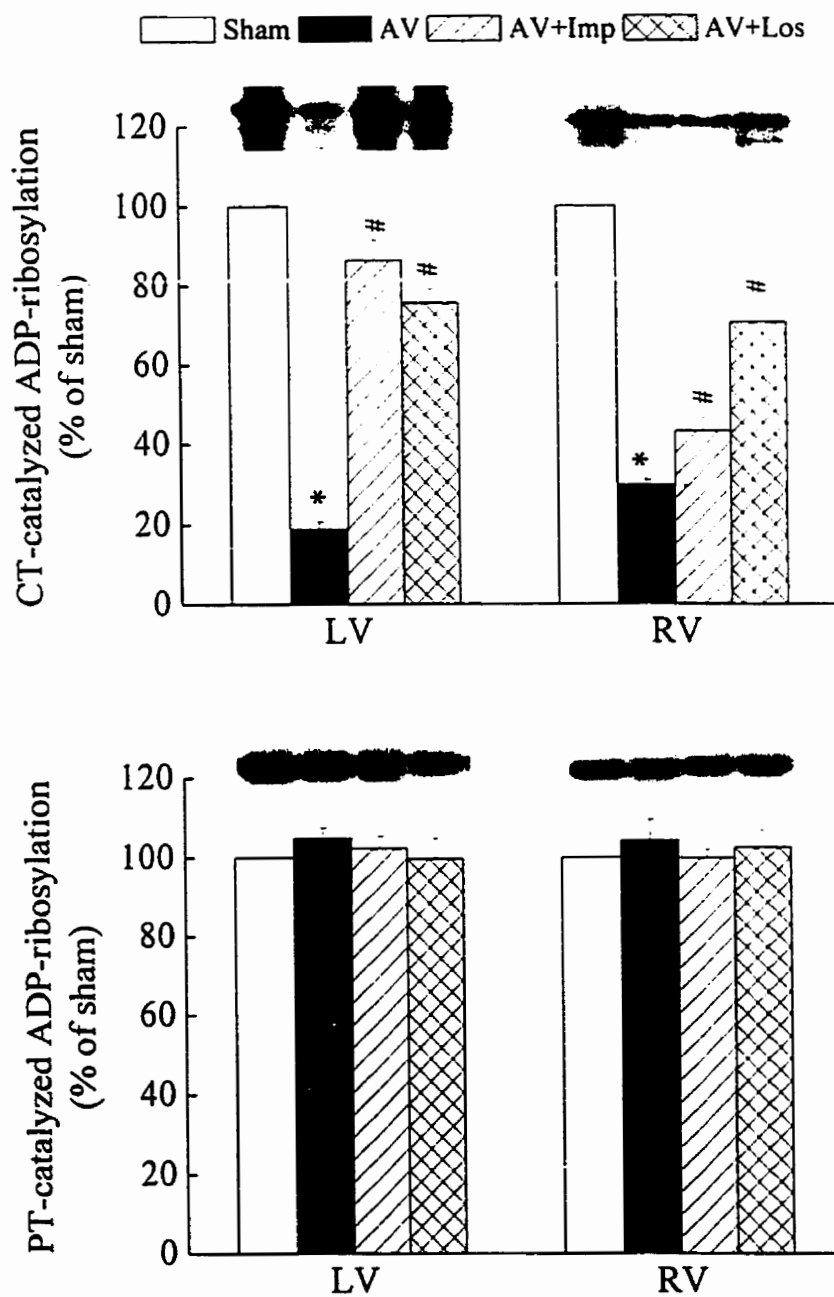


Figure 21. Cholera toxin and pertussis toxin catalyzed ADP-ribosylation in LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan.

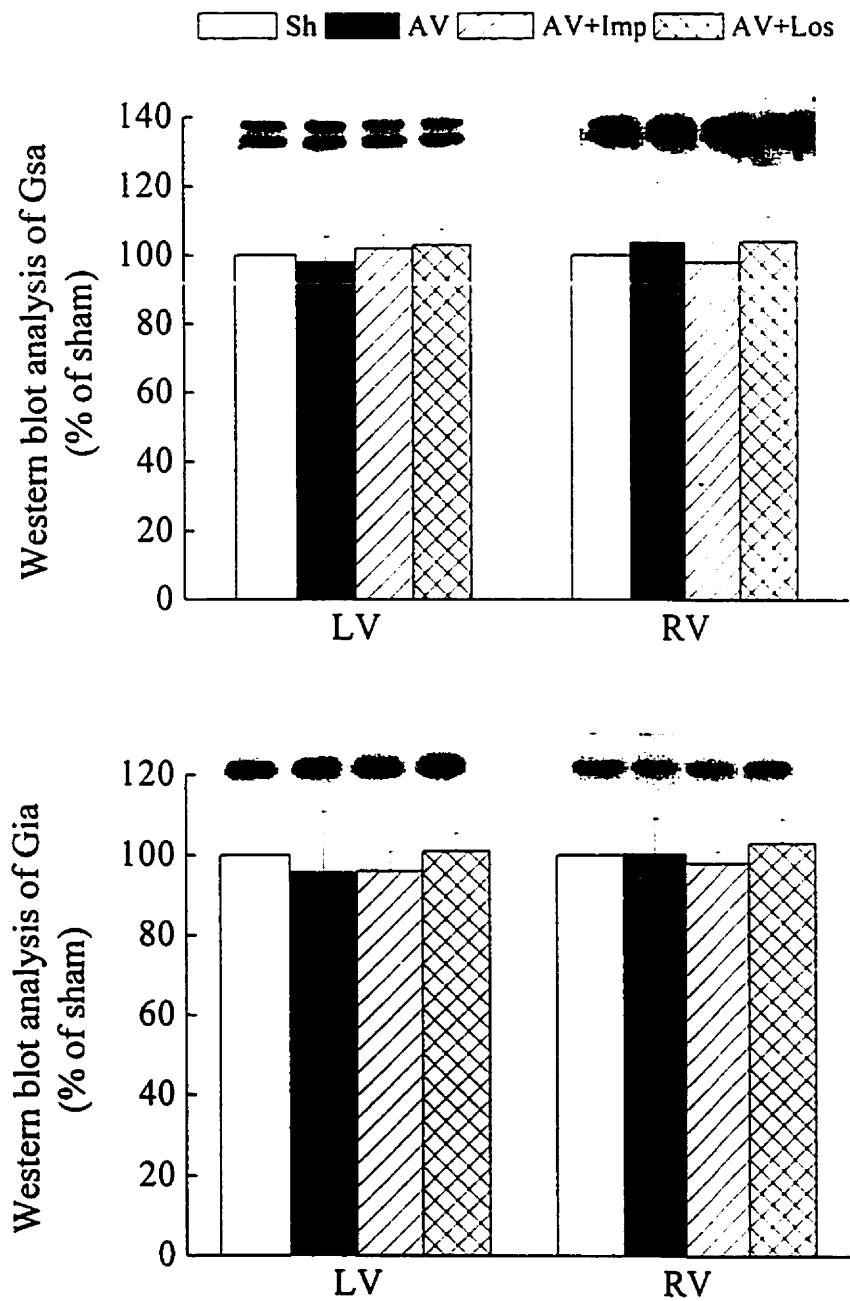


Figure 22. Alterations of protein content of G_{sa} and G_{ia} subunit in the LV and RV of chronic failing heart induced by aortocaval shunt with and without treatment of imidapril and losartan.

protein content. We also measured the mRNA level of G_{sa} - and G_{ic-2} -proteins and found a small (20%) but significant decrease for G_{sa} -protein and increase for G_{ic-2} -protein; RNA loading and typical bands for G_{sa} and G_{ic} are shown in Figure 23 and the statistical data are shown in Figure 24.

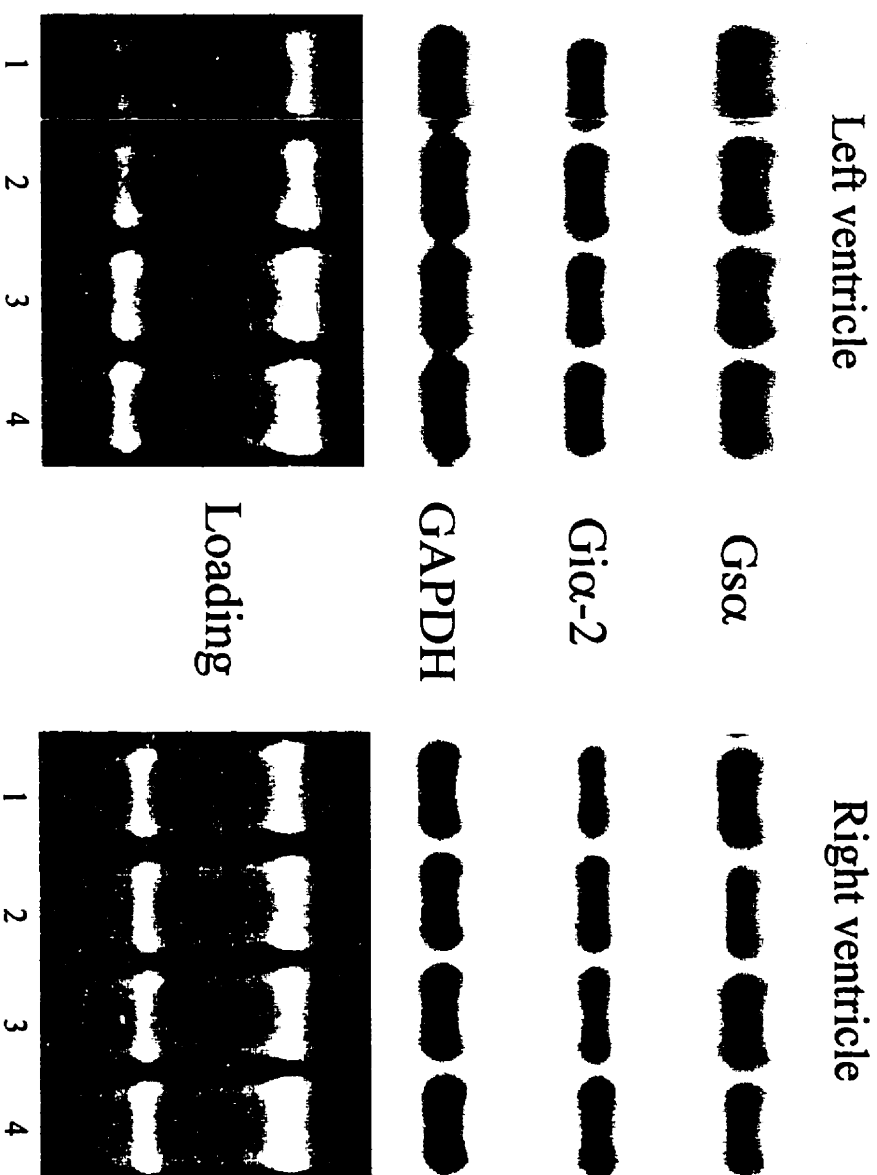


Figure 23. Typical northern blot bands showing the mRNA level of $Gs\alpha$, $Gi\alpha-2$, GAPDH. Loading sequence is lane 1 for sham control, lane 2 for aorticaval shunt group, lane 3 for aorticaval shunt with the treatment of imidapril, lane 4 for aorticaval shunt with the treatment of losartan.

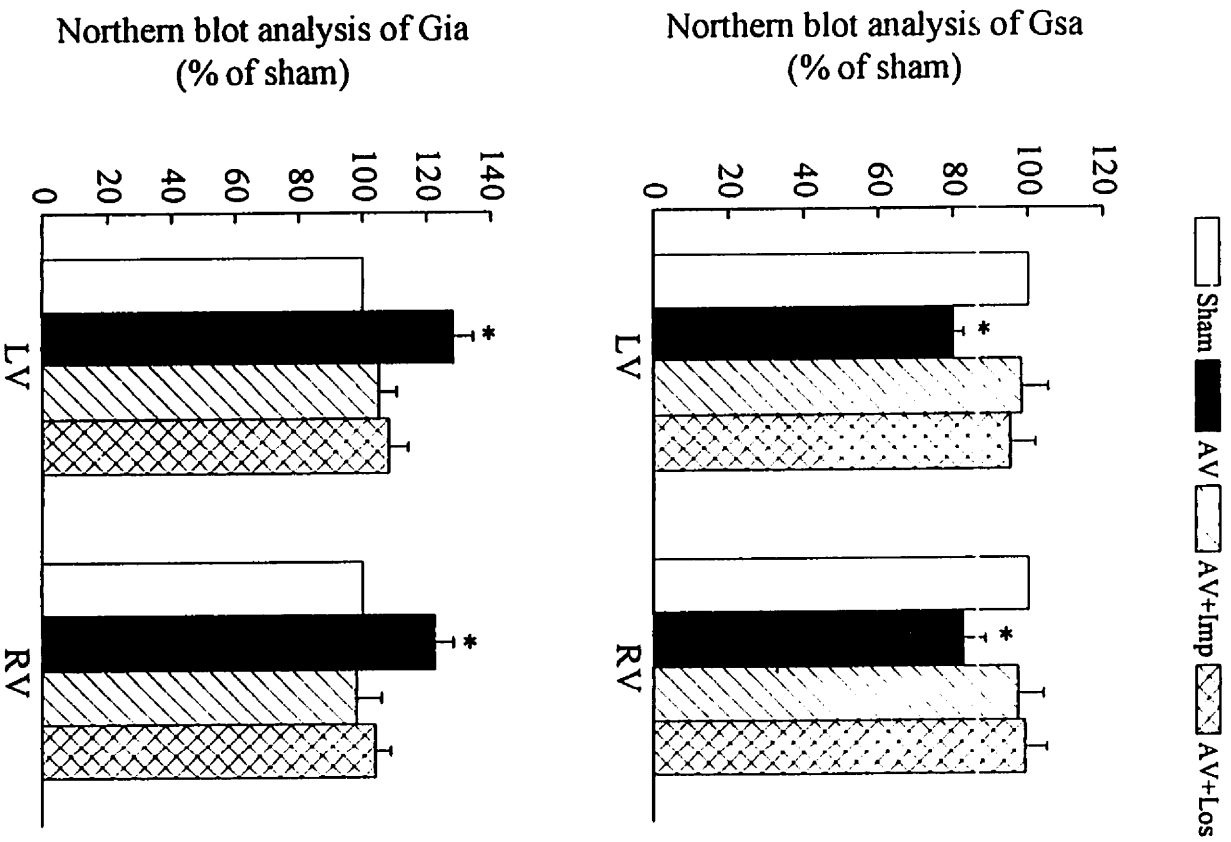


Figure 24. Alterations of mRNA levels in G_{sa} and G_{ia} subunit in the chronic heart failure induced by aorticaval shunt with and without treatment of imidapril and losartan.

V. DISCUSSION

1. Development of cardiac hypertrophy and heart failure in rat due to aortocaval shunt

In this study, we characterized the volume overload model induced by aortocaval shunt in rats at different time points with respect to several criteria. We examined the general characteristics of the animals including increases in cardiac mass, changes in morphology and histology of the heart, signs and symptoms of circulatory congestion as well as alterations of myosin heavy chain isozyme composition (a biochemical marker of hypertrophy). Second, we determined the *in vivo* arterial and cardiac hemodynamic responses after establishing the aortocaval shunt. Third, we examined the *in vitro* contractile function and its response to the inotropic agent, isoproterenol. The time course changes in these criteria suggested that the development of cardiac hypertrophy and heart failure in this model is consistent with three stages defined by Meerson (275) for cardiac overloading: developing hypertrophy, established hypertrophy and decompensated hypertrophy or heart failure. The data obtained during the first two weeks after aortocaval surgery indicate that the animals were at the developing hypertrophic stage during this period. This is based on the dramatic increase in cardiac mass (17 and 39% at 1 and 2 wks, respectively) and unchanged contractile function of the heart both in *in vivo* and *in vitro* conditions. While the dramatic increase in LVEDP indicates sudden increase of wall stress due to the volume overload after opening of the AV shunt, the significant decrease in LVEDP from 1 wk to 2 wks suggests the developing cardiac hypertrophy and dilation of the cardiac chamber. Although MAP was decreased in the first 2 wks, it mainly reflects the presence of the shunt and a significant flow of cardiac output through it (274,276). The slightly increased lung

weight at 1 and 2 wks also indicates the increased capacity of lung circulation after the AV shunt. The changes occurring during 2 to 8 wks period of AV shunt reflect an established hypertrophic stage. The cardiac mass growth slowed down from 39 at 2 wks to 21% at 4 wks and the rate of cardiac growth at 8 wks was comparable to that in the sham group indicating that the hypertrophic growth in the experimental animals reached a stable level. The reversal of MAP to normal level suggested the activation of compensatory mechanisms and subsequently volume expansion (273,277). The LVEDP reached its lowest level after the AV shunt at 4 wks indicating fully activated compensated status at this time point. The myosin heavy chain isozyme composition was not altered until 8 wks when a slight increase of myosin isozyme V₃ was seen only in the LV. Comparison between the *in vivo* and *in vitro* contractile performance at 4 and 8 wks suggested that although an intrinsic myocardial dysfunction was present at 4 and 8 wks, it was compensated *in vivo*. On the other hand, changes which occurred after 8 wks indicated the start of decompensated hypertrophic or failing stage. Although there is another burst of cardiac mass growth from 11% at 8 wks to 32% at 16 wks, the hemodynamic functions of both arterial system and heart were not maintained. The increase of LVEDP and contractile dysfunction at 8 and 16 wks suggested a deteriorating process in which both diastolic and systolic function gradually failed and thus resulting in circulatory congestion of the body and biochemical alterations in the heart. The time course changes in all the parameters examined also suggest that 8 wks of aortocaval shunt may represent a transition turning point from compensated hypertrophic stage to failing stage. The development of these different stages in response to the an aortocaval shunt makes this model suitable for research in several areas such as cardiac hypertrophy, transition of compensated hypertrophy to decompensated hypertrophy and heart failure.

It should be pointed out that during the process of development of cardiac hypertrophy in this model the gain of weight in RV was more pronounced than that in LV. This may be due to the difference in hemodynamic challenge which the LV and RV faced upon inducing the AV shunt. By using radioactive microsphere techniques, several studies have shown dramatically increased cardiac output after inducing an arteriovenous fistula (266,274,276) and in fact about 75% of the cardiac output was shunted through the fistula (272). In addition, pulmonary hypertension has long been recognized as a consequences of aortocaval fistula in dogs and humans (278,279). Accordingly, the RV may be under a great challenge of both volume and pressure overload thus resulting in more rapid and greater extent of hypertrophy. The greater hypertrophic response in RV was also observed by several other investigators (265,266,273). By measuring the length and width of isolated cardiomyocytes, Liu *et al.* (265,266) found that the cell volume increase in RV was more prominent than that in LV and interventricular septum at 1 wk (266) as well as 1 and 5 months after creating the aortocaval fistula in rats (265). Similarly, Ruzicka *et al.* reported an increase of 40% and 76% in LV and RV 4 wks after inducing an aortocaval shunt, respectively (273).

Since the hypertrophic response observed in this study was comparable with those applying the same needle technique to create the AV shunt (271,273,274,277) it appears that the experimental model is highly reproducible. In comparison, those applying microvascular surgery to create AV shunt provided more variable extents of cardiac hypertrophy despite the long duration for the procedure and high mortality (263,264,266,273,274,277). As shown by histological data of the heart, the increase of HW was mainly due to the enlargement of cardiomyocytes characterized by thickening of myofibers and appearance of enlarged nucleus.

Similarly, Liu *et al.* (265) and Hatt *et al.* (280) have demonstrated that the increase in heart weight was consistent with the increase of cell volume in aortocaval shunted rats. In addition, studies of collagen accumulation in this model revealed that no fibrosis occurred due to chronic volume overload (281,282). Thus hypertrophy of the cardiomyocytes is the major contributor to the gain of cardiac mass which is different from other models such as pressure overload (283) and myocardial infarction (284) in which fibrosis was commonly seen. This unique feature enables this model extremely useful to single out of the role of cardiomyocyte hypertrophy in altering the performance of whole heart. Another advantage of this model lies on the extent of hypertrophy in the AV group which doubled at 16 wks in comparison to the of the sham control. This is comparable to that occurring in human hypertrophic hearts, which often doubled or tripled the normal control heart (285,286), whereas the extent of hypertrophy in most other types of experimental models for hypertrophy and heart failure can not attain this level.

Although chronic heart failure is easily detected in human with arteriovenous fistula (287-289) as well as in large animals with aortocaval shunts (270,290,291), heart failure in rats with aortocaval shunts has been a subject of considerable controversy. However, our results from several different criteria and at different time intervals indicated that the long term volume overload finally leads the heart to the failing stage characterized by cardiac hypertrophy, depressed contractile function, occurrence of circulatory congestion and altered biochemical composition of the myocardium. Although Liu *et al.* (265) found that LVSP, +dP/dt and -dP/dt were not altered in LV but enhanced in RV five months after inducing a large shunt in rat by end to side anastomosis of left iliolumbar vein and aorta, they did detect the presence of an increased renal and hepatic fluid content suggesting the rats may be approaching the congestive heart failure

stage. By using the slope of the linear peak isovolumetric pressure-volume (P_{\max} -V) relationship, Brower *et al.* (271) have demonstrated a decrease in contractility in the isolated rat heart at 1, 3, 5 and 8 wks after inducing an aortocaval shunt suggesting an intrinsic myocardial dysfunction in the shunted rats. However, the number of rats which progressed to clinical overt heart failure was less than 3% during their 8 wks observation period. Since half of the AV shunted rats at 8 wks showed an increased lung weight in their study, it is evident that ventricular remodeling secondary to chronic volume overload in the AV shunt model would ultimately result in a gradual progression to heart failure. By using left ventriculography to evaluate cardiac function, Yang *et al.* (292) also reported that cardiac dysfunction was minimal in rat with aortocaval shunt 12 wks after the surgery. Such controversial results for the occurrence of heart failure after creating an arteriovenous shunt may be due to different techniques applied to making the shunt, sizes of the shunt, duration of overload as well as sex, body weight and different strains of the rats. For example, high mortality (>47%) was observed in making shunt with microvascular surgery (263,266), it is possible that rats with large fistula were more vulnerable to surgical trauma and thus died after a prolonged surgical procedure and the results obtained from the rats that survived may reflect a population with smaller fistula. In fact, two separate groups have reported that different sizes of the fistula can induce different hypertrophic and hemodynamic responses (272,273). Similarly in the case of two other frequently used volume overload models, such as mitral regurgitation and aortic insufficiency, severity of the lesions made on the valves are the major determinant of the subsequent severity of the disease (293-295).

Myosin heavy chain isozyme composition has long been known as an indicator of myosin Ca^{2+} -ATPase and myocardial contractility (296,297). While V_1 myosin heavy chain exhibits high

Ca²⁺-ATPase activity and fast velocity of contraction, V₃ myosin heavy chain shows low Ca²⁺-ATPase activity and slow velocity of contraction (296,297). A correlation of cardiac hypertrophy and myosin heavy chain isozyme shift from V₁ to V₃ has also been obtained from several hemodynamic overload models (267). However, our results demonstrated that myosin heavy chain isozyme shift occurred only at the decompensated hypertrophic stage after the aortocaval shunt where heart weight doubled that of the control. Although at earlier stages, significant hypertrophy was present, the V₃ myosin heavy chain expression was not increased. This is consistent with the findings of Mercadier *et al.* (267) who showed that increased expression of V₃ myosin heavy chain isozyme was not correlated significantly with the extent of cardiac hypertrophy in rats with aortocaval fistula whereas in the model of aortic stenosis and aortic insufficiency, a well correlated relationship of V₃ expression and hypertrophy was observed. It is difficult to explain this phenomenon; however, this discrepancy may be due to differences in the hemodynamic loads on the experimental models. Nonetheless, our results also demonstrate a difference in the time course of the V₃ shift between LV and RV. Theoretically, the shift should have occurred earlier in the RV because rapid and more prominent hypertrophy was observed there. On the contrary, the shift occurred first in the LV at 8 wks whereas it was only observed at 16 wks in RV. One possibility may be due to the different hemodynamic function between LV and RV. The other may be due to the fact that RV normally contains less V₃ isozyme than the LV (267) and thus may be less susceptible to changes under the experimental conditions employed in this study. Nevertheless, our results indicated that one of the molecular basis of the observed contractile dysfunction in LV may be due to the alteration of myosin heavy chain isozyme composition, because the time course of myosin heavy isozyme shift from V₁ to V₃ corresponded to the changes in LV function. Another unique feature of this model is that

although the failing stage is achieved at 16 wks, the inotropic response of the heart to isoproterenol was not depressed but instead the fold of increase was augmented. Since the heart rate was not altered in this model, enhanced response to β -stimulation may be one of the mechanisms to maintain the high-output status of the AV shunted animal. However, the molecular mechanisms for this phenomenon deserve further examination.

2. β -adrenoceptors and G-protein coupled receptor kinases in heart failure induced by aortocaval shunt

Although rats 16 wks after inducing aortocaval shunt were in a failing stage as characterized by cardiac hypertrophy, depressed contractile function *in vivo* and *in vitro*, circulatory congestion and shift of myosin heavy chain isoforms, the positive inotropic response to isoproterenol both *in vivo* and *in vitro* conditions was not attenuated. This is in contrast to most other types of heart failure, in which depressed response to β -stimulation is usually evident. The results presented in this study reveal an up-regulation of β_1 -AR number, redistribution of GRK2 and GRK3, as well as an decrease of GRK5 in the membrane and these alterations in the β -AR signal transduction may underlie the unusual response to β -stimulation in this experimental model of heart failure.

a. Alterations of β -ARs in aortocaval shunt induced heart failure

At the β -AR level, we detected a selective increase in β_1 -AR density by binding experiments. In view of the inherent problems of the binding assay, which can only detect the functionally active receptors on the outside of the membrane and which is also largely dependent

on the agonist and antagonist selectivity, it is difficult to distinguish this increase from an upregulation of the total receptor numbers on the membrane or the increased externalization of normally inactive receptors (spare receptors). Further results from Western blot by using specific antibodies for β_1 - and β_2 -AR, respectively, suggested that a selective increase of β_1 -AR protein content occurred in the heart 16 wks after aortocaval shunt. It is surprising to observe an upregulation of β_1 -AR in a failing heart, several reasons may contribute to this atypical change. First of all, a dramatic difference between this model and other models of congestive heart failure including cardiomyopathy, ventricular pacing, myocardial infarction and pressure overload, is the lack of fibrosis in the heart. Since most studies for receptor downregulation measured the receptor binding using crude membranes prepared from myocardial tissue, which contains blood vessels, connective tissue, muscle and nerves, a significant change of the cell type may alter the density and subtype distribution of β -ARs. Thus the altered β -AR density observed in a heart with significant fibrosis could be confounded by fibroblasts which have different total and subtype composition of β -ARs compared to cardiomyocytes (298). In fact, several investigations have either indirectly or directly demonstrated that fibrosis may contribute significantly to alter the β -AR density. In the cardiomyopathic hamster model, the β -AR density was increased at prefailure and early failure, unchanged at moderate failure, and decreased at severe stage of congestive heart failure heart (158) when measured by using binding assays in crude membranes. Since fibrosis is a progressive process which is related with the severity of the cardiomyopathy, it is reasonable to assume that the dynamic changes in β -ARs reflect a gradual increase in interference by fibrotic growth. In accordance with this hypothesis, Tawarahara *et al.* (169) examined the β -AR density by using a quantitative autoradiographic technique with the same ligand [125 I]ICYP which can detect the β -ARs in situ in myocardial slice. Surprisingly, an increase

(instead of a decrease) in the number of β -ARs was detected in the failing cardiac myocytes of the cardiomyopathic hamster. Interestingly, the regions with increased β -AR density corresponded to the sites of increased interstitial fibrosis (169). Another indirect evidence supporting that fibrosis may interfere with the results obtained from a fibrotic myocardium is the fact that an up-regulation of β -ARs was detected in acute ischemia in which fibrosis has not occurred but impaired intrinsic myocardial contractile function and massive released catecholamines were present (for review, see Ref. 299). Since no data regarding changes of β -ARs in the isolated and purified cardiomyocytes from a failing heart with fibrosis are available, the previous results showing the down regulation of β -ARs from the failing heart with fibrosis deserve some caution before extrapolating these data to the cardiomyocytes.

Another factor which may alter the expression of β -ARs could be the catecholamine levels in this model. Several studies using *in vivo* infusion of isoproterenol in rats found downregulation of β -ARs (182,183). On the other hand, using chronic β -blockade *in vivo* upregulated the β -ARs density (196,300). These findings suggest that chronic stimulation by β -agonist leads to downregulation whereas chronic inhibition of β -adrenergic signaling results in upregulation of β -ARs. Therefore, it is possible that up-regulated β_1 -AR observed in the aortocaval shunt model is related to a decrease in the catecholamine level. In fact, Communal *et al.* (301) have examined the plasma and ventricular noradrenaline and adrenaline levels and observed a decreased concentration of catecholamines in the ventricle without any change in plasma levels in rats 4 weeks after inducing aortocaval shunt in rat. The selective up-regulation of β_1 -ARs without a change in β_2 -AR may be due to a depression in the neuronally released norepinephrine, which has a selective affinity of β_1 -AR. Since it has been shown in human heart failure (185,187-189) and

some animal models (238) that decreased β_1 -ARs density was associated with decreased mRNA expression, we speculated that a parallel relationship between mRNA/protein may also exist in this model. However, by using a specific cDNA probe and Northern blot technique, we found no increase of β_1 -AR mRNA in the AV group. Thus the increased β_1 -AR density and protein content may rather be due to a decreased degradation of protein or an increased translational efficiency than an increased transcriptional change in the message.

Two studies have previously examined the β -ARs density in this experimental model. Cartagena *et al.* (144) determined total β -AR density and affinity at 2, 7, 21 and 56 days after creating an aortocaval shunt in rat. While no alteration of affinity was detected at all the time points, a significant increase of total receptor density from 7 to 21 days followed by a decrease on day 56 was found in the myocardium. On the other hand, a sustained increase of β -ARs was observed in adipocytes throughout the examination periods. The discrepancy of their results to ours may on one hand be due to different time point examined (2, 7, 21 and 56 days versus 16 weeks) and the other different parameters examined (total receptor versus specific subtypes). Communal *et al.* (301) have examined β_1 -AR density at different regions 4 weeks after inducing aortocaval shunt in rat and demonstrated an increased density in epicardium and decreased density in endocardium of the LV with no alteration in total receptor density and β_1/β_2 ratio. Despite that they choose a very early time point with compensated hypertrophy, the β -AR density was determined on ventricular homogenate and ventricular slices rather than membrane preparation. In addition to these two studies on rat, Hammond *et al.* studied the effect of aortocaval shunt on β -adrenergic signaling on porcine (185). In their study congestive heart failure occurred 3-4 wks after the aortocaval fistula, which is much shorter than the time required for the occurrence of

heart failure in rat. In contrast to the data obtained from the rat, they demonstrated a significant reduction in β_1 -AR density. Thus the diverse results of changes in β -ARs due to aortocaval shunt seem to be related to the species and technique used as well as stages of the disease.

b. Alterations of GRKs in aortocaval shunt induced heart failure

Although 6 isoforms of GRKs have been identified to date, 3 isoforms, namely GRK2, GRK3 and GRK5, were found to be abundantly expressed in cardiac tissue (73). GRK2 has been implicated to play an important role in β -ARs desensitization both *in vitro* and *in vivo*. In cultured cells with β_2 -ARs, GRK2 translocates from the cytosol to the membrane upon agonist stimulation, phosphorylate the receptors and thereby uncouples the β_2 -ARs from G_i -proteins to terminate the signal (71,72). Evidence for GRK2 phosphorylation of β_1 -ARs *in vivo* has also been presented in recent years. Transgenic mice overexpressing GRK2 showed attenuation of the isoproterenol-stimulated left ventricular contractility *in vivo*, dampening of cardiac AC activity and reduced functional coupling of β -ARs. On the other hand, mice expressing the GRK2 inhibitor displayed enhanced cardiac contractility *in vivo* with or without isoproterenol (108). Some studies have also shown that chronic β -AR stimulation in normal mice by infusing isoproterenol leads to upregulation of GRK2 and downregulation of β -AR (194). Conversely, chronic reduction of β_1 -AR activation in porcine by using bisoprolol treatment resulted in downregulation of GRK and enhanced β -adrenergic signaling (196). These investigations reveal the important role of GRK2 in modulating *in vivo* myocardial function. GRK2 has also been implicated in pathological conditions such as heart failure where a desensitization of β -adrenergic signaling often occurs. Ungerer *et al.* have observed that GRK2 activity, protein content and mRNA levels were significantly increased in human failing heart with decreased expression of

β_1 -AR mRNA and attenuated response to β -AR stimulation (189,198). Subsequently, similar pattern of changes were detected in several animal models including cardiomyopathic hamster (190), paced porcine and rabbit (66,114), myocardial infarction in mice (191), spontaneous hypertensive rats (192) and pressure overload in mice (193). However, most of these studies only examined GRK2 activity or protein expression in the cytosolic fraction. Since GRK2 is known to phosphorylate β -ARs only when it is physically associated with the membrane, an increase of protein content and activity in cytosol does not necessarily indicate an enhanced function of GRK2 on the membrane. In fact, a recent study has shown that a substantial portion (40%) of the total GRK activity could be attributed to the membrane (196). In addition, one common feature of human end stage heart failure and all the animal models used for study GRK2 are low cardiac output heart failure with significant fibrosis, no information is available regarding regulation of GRK in an high output heart failure model without fibrosis. Thus in this study we measured GRK activity, and GRK2 protein content and mRNA expression in aortocaval shunt induced heart failure in rat.

Since all three cardiac forms of GRKs have the potential to phosphorylate rhodopsin (the substrate we used to measure the GRK activity), the values should reflect the total activity of all cardiac GRKs. However, Choi *et al.* (193) have shown that increased GRK activity in the heart extract from pressure overload induced heart failure in mice was mainly attribute to GRK2 by using monoclonal antibodies specific for different GRK subtypes. Although we detected an increase of GRK activity in the cytosolic preparation, the GRK activity in the membranous preparation was significantly decreased. Since GRK2 is a cytosolic protein and has to be translocated from the cytosol to the membrane in order to phosphorylate the receptor, we predicted that this reciprocal change might be due to a redistribution of GRK2 rather than a real

alteration of the activity. To confirm this, we first examined the GRK activity in myocardial homogenate and found no change in the activity between sham and AV groups. We then determined the GRK2 protein content by using a specific antibody in three fractions, cytosol, membrane and homogenate. The results showed similar pattern as that of the activity which is an increase in cytosolic preparation and a decrease in membranous preparation and no change in the homogenate preparation. Furthermore, Northern blot analysis of GRK2 mRNA expression in the total RNA showed no significant change between Sham and AV groups. Taken together, these results indicate that a redistribution rather than a downregulation or upregulation of GRK2 protein occurred after the induction of aortocaval shunt. Since only those GRK2 proteins which are associated with the membrane can phosphorylate β -ARs and lead to desensitization, a decrease in membrane and increase in cytosol would favour sensitization of the β -adrenergic signaling. This is in accordance with our data obtained from the β -ARs study in which we detected a selective increase of β_1 -AR and an enhanced cardiac response to β -stimulation both *in vivo* and *in vitro*.

Although some recent studies have examined GRK activity and protein level in membranous fraction from failing heart, the results are variable. Ping *et al.* (66) showed in paced porcine heart that both cytosolic and membranous GRK activities were increased in heart failure without disturbance of its cellular distribution. However, this increase of GRK activity was not associated with an increase of GRK2 protein content in either cytosolic or membranous fraction and in fact GRK2 content was not altered in the two fractions in mild heart failure (4 days pacing) and decreased significantly in the supernatant but not significantly in the membranous fraction in severe heart failure (28 days). On the other hand, Ishgai *et al.* (191) demonstrated that myocardial infarction in rat induced an increased activity and protein content in both cytosol and

membrane. Although a similar abnormality in β -adrenergic signaling was observed in these two studies, divergent changes of GRK2 concentration was found indicating that alteration of GRK2 may be dependent on the etiologies and stages of the disease. Although GRK3 has been shown to have similar function as GRK2 *in vitro*, information regarding its changes *in vivo* due to heart failure is quite limited. Ungerer *et al.* (198) have shown a slight increase of GRK3 mRNA level in human end stage heart failure in the cytosolic preparation of myocardium. Recent studies in transgenic mice indicated that β -ARs may not be the *in vivo* substrates for GRK3 because overexpressing GRK3 does not affect the β -adrenergic signaling, rather the cardiac thrombin signaling is greatly impaired (109). Nevertheless, we have also determined GRK3 protein content and showed similar changes as that of GRK2. Based on this information, it is possible that the cardiac thrombin signaling is also enhanced in this experimental model of heart failure.

While the role of GRK2 has become relatively clear in recent years after extensive studies *in vitro* and *in vivo*, the physiological and pathological roles of GRK5 are hardly explored. However, GRK5 has started to attract the attention of investigators in heart research in recent years because it is most abundantly expressed in heart (GRK2 is most abundantly expressed in brain) (302,303). GRK5 overexpressing mice showed blunted response to β -AR stimulation indicating that its role in regulating the β -AR signaling *in vivo* (110). We have in this study determined GRK5 expression in membranous and cytosolic fractions and found similar changes as that of GRK2 between sham and AV group. It is believed that GRK5 is a membrane associated protein, however, we also detected its expression in the cytosol. The presence of GRK5 in cytosol has also been detected previously by one study (66). It has been suggested that cytosol preparation may be somewhat contaminated by membrane and therefore the cytosolic and membrane preparations may not be suitable to detect changes in GRKs. However, in our study

we used $400,000 \times g$ centrifugation to precipitate the membrane fraction without using any detergent, thus eliminating the possibility of contamination of membranes in the cytosol. In addition, since the membranous fraction demonstrated a decreased change of GRK5, contamination by membranes will show a decreased trend in the cytosol but we have actually detected an increase in the cytosolic preparation. It is possible that GRK5 normally stays very close to the membrane or just lightly attaches to the membrane by some unknown mechanisms. Nevertheless, we found a decrease in GRK5 protein expression at membrane fraction and an increase in the cytosolic fraction whereas no change was detected in the homogenate. This result is different to those demonstrated by Anderson *et al.* (192) who showed no change of GRK5 in heart from spontaneous hypertensive rats although a significant upregulation of GRK2 was observed. In addition, two studies have used specific subtype antibodies to inhibit one or more subtype of GRKs in order to test the contribution of the specific GRK to up-regulation of GRK activity in heart failure, and GRK5 was found to contribute mainly to the basal GRK activity whereas the increases in cardiac diseases is mainly due to GRK2 (114,193). On the other hand, Ping *et al.* (66) found that persistent increase in total GRK activity in paced porcine is associated with increased protein content and mRNA level of GRK5 but not GRK2. Due to the limited number of studies on GRK5, it is difficult to draw any meaningful conclusion regarding the alteration of GRK5 in heart failure. However, it is possible that regulation of GRK5 may vary with different species, different etiologies and stages of the disease. This study demonstrated no change in the $G_{\beta\gamma}$ -subunit in the AV group indicating that $G_{\beta\gamma}$ -subunit may not be the major factor that modifies β -adrenergic signaling in this model. However, we detected a change for β -arrestin1 showing a decrease in the membrane and an increase in the cytosol, suggesting its synergistical actions in regulating β -adrenergic signaling with GRK2. The only study regarding

changes in β -arrestins is reported by Ungerer *et al.* in human end congestive heart failure in which no alteration of β -arrestin I in terms of mRNA and protein expression was observed (198). The discrepancy between their and our results may be mainly due to the differences in the model of heart failure.

3. Adenylyl cyclase and G-proteins in heart failure induced by aortocaval shunt

a. Alterations of adenylyl cyclase in aortocaval shunt induced heart failure

AC plays a pivotal role in regulating myocardial contractility due to its function to generate cAMP and its dual regulation by G_s - and G_i -proteins. Nine isoforms of mammalian AC have been reported and seven AC (II, III-VII and IX) mRNA isoforms have been detected in heart tissue (58,66,67), with types V and VI being the most abundant ones (63,68,69). Due to the fact that AC is a very minute component of the cell membrane (constituting only approximately 0.001% of total membrane protein) with a very fragile enzymatic activity *in vitro* (incubation at 37°C for 30 min readily inactivates the enzyme when it is uncoupled from G_{sa}) (304,305), the information regarding its regulation in heart failure is rather limited. Most studies have measured AC activity in crude membrane preparations by determining its capability to convert α -[32 P]ATP to [32 P]cAMP whereas studies regarding mRNA expression of types V and VI in cardiac development and heart failure have just started to appear in the literature. No information on AC protein expression is available due to the absence of specific antibodies. In addition, most studies regarding changes of AC in heart failure were carried out in low cardiac-output heart failure, and virtually nothing is known regarding changes in its activity and protein expression in a high-output failure status. Thus in this study, we measured AC activity

as well as protein expression of type V/VI in the cardiac membranes in LV and RV from different experimental groups.

The AC activity is activated in different ways, from the receptor, G-proteins as well as catalytic unit. Activities measured in the presence of different stimulants reflect contribution of the respective locations in regulating AC. While isoproterenol is known to activate AC through the β -ARs, Gpp(NHP) and NaF are used to activate G_s -protein. Gpp(NH)p is a less hydrolysable analogue of GTP, it binds to G_{sa} and fails to be converted to GDP thus it keeps the G_{sa} protein in a constant active state. On the other hand, NaF is known to directly interact with the G_{sa} subunit and activate its function. Forskolin and Mn^{2+} are known to interact with the catalytic unit of AC directly and exert stimulating effects. Our results showed increased AC activities in basal, isoproterenol-, Gpp(NH)p-, NaF-, as well as forskolin-stimulated AC in both LV and RV at 16 wks of inducing aortocaval shunt, indicating a generalized activation of β -adrenergic signal transduction in this model. This is in accordance with the enhanced response to β -stimulation *in vivo* and *in vitro* by isoproterenol. In view of the fact that the basal AC activity is significantly increased in both LV and RV of shunted rats, it is possible that the actual increase observed under different stimulants reflects amplification based on the basal values rather than an increase due to stimulation. To confirm that, we calculated the data with respect to fold stimulation by each agent for the respective basal level. The results indicated that isoproterenol- and forskolin-induced more activation of AC in AV group whereas the fold of stimulation due to Gpp(NH)p, NaF were similar between sham and control groups. This suggests enhanced signals at the receptor and effector level. Since β_1 -ARs are upregulated in AV group, the enhanced stimulation by isoproterenol reflects that more receptors are activated. On the other hand, increased basal AC activities and increased stimulation by forskolin suggest more AC

protein may be responsible. By using an antibody that recognizes both type V and type VI isoforms, we confirmed that an increased protein content of AC was induced in both LV and RV 16 wks after aortocaval shunt; this can be seen to account for the increased basal and forskolin-stimulated activities.

The upregulation of AC may be the major mechanism underlying the enhanced response of the heart to β -AR stimulation. Several studies have shown that the stoichiometry of β -AR: G_s :AC is about 1:200:3 (121), suggesting that the amount of AC may set a limit on β -AR-mediated transmembrane signaling. In fact, it has been shown that increased expression of cardiac β -AR or G_s does not yield proportional increase in transmembrane signaling (95,116,240). On the other hand, increased expression of AC type VI in neonatal cardiomyocytes has been shown to increase β -AR stimulated production of cAMP proportionally (123). Accordingly the increased activity and protein expression of AC observed in this study may contribute significantly to the sensitized β -adrenergic signaling in this model. The inverse relationship between impaired AC and desensitized β -AR signaling has also been reported by our laboratory (155,249,250). In a rat model with myocardial infarction, the attenuated response to isoproterenol stimulation of the LV was associated with significantly depressed basal as well as forskolin-stimulated AC activities. On the other hand, enhanced basal as well as forskolin-stimulated AC activity was observed at the hypertrophied and compensated RV with an augmented levels of cAMP with or without isoproterenol stimulation (249,250). Similarly in the cardiomyopathic hamsters with severe heart failure, forskolin-stimulated AC activity was depressed significantly (155).

Different studies have shown that downregulation of AC mRNA levels are associated with decreased AC in some experimental models. Ishikawa *et al.* have shown in a paced dog model of heart failure, the basal as well as forskolin-stimulated AC activities decreased significantly in

the LV; these were accompanied by a reduction in the steady state mRNA levels of AC types V and VI (245). Other studies using paced pig model of heart failure found that AC activities and mRNA levels were not altered at mild stage of failure (4 days pacing) but the activities were significantly depressed at severe stage of failure (28 days pacing) with decreased mRNA expression for type VI and unaltered for type V and type II (66). Recently, Espinasse *et al.* determined AC type V and VI mRNA levels in the LV of rats after myocardial infarction for two months and six months (306). While no changes were observed at two months after myocardial infarction, a significant reduction of type VI mRNA level without alteration of type V was detected, indicating impaired type VI gene expression in severe heart failure; these studies suggested that AC isoforms may be differentially regulated. In fact, some studies have shown that AC type V is greater in mature adult rat ventricles than in fetal and neonatal stages whereas the level of AC type VI decreases with age (307,308). However, due to the lack of antibody for type V or type VI specifically, we were unable to measure the AC protein expression of type V and VI individually. Thus several possibilities may exist, such as an increase in one and no change in the other, increase for both isoforms or one increase and the other decrease. Nevertheless, the basal and forskolin-stimulated AC activity are dramatically increased indicating a up-regulation at the effector level in the heart failure due to aortocaval shunt.

b. Alterations of G-proteins in aortocaval shunt induced heart failure

Since the functional studies have shown an enhanced response of the AV-shunted rat hearts upon β -stimulation *in vivo* and *in vitro*, it can be argued that the changes in G-proteins may also favor an enhanced signaling in this model. To our surprise, we detected no changes in protein expression for both $G_{\alpha s}$ - and $G_{\alpha i}$ -subunits in the experimental model. On the other hand, functional studies using toxin-mediated ribosylation showed a significant reduction in cholera

toxin-mediated $G_{\alpha s}$ ribosylation without change in pertussis toxin-mediated ribosylation for $G_{\alpha i}$. Furthermore, a slight but significant decrease of $G_{\alpha s}$ mRNA level and increase of $G_{\alpha i}$ mRNA level were found. These changes seem to suggest the desensitization of signals through G-proteins which contradict the findings with respect to myocardial functional responses to β -AR stimulation in this model. Since the estimated molar proportions of the elements of the β -AR/ $G_{\alpha s}$ /AC complex in cardiac myocytes are 1:200:3 (121), it seems that a large amount of G_s -proteins is not coupled with the β -AR system. In fact, studies have shown $G_{\alpha s}$ -proteins can regulate myocardial contractility in a cAMP-independent mechanism by direct regulating L-type Ca^{2+} -channel on the membrane (92,93). Thus the decreased $G_{\alpha s}$ -protein functionality may be a good explanation for the decreased basal myocardial contractility observed in this model. Regarding the contribution of G-proteins to the altered β -AR signaling, the increase in AC activity by NaF and Gpp(NH)p was not altered in the AV group; this indicated that the β -adrenergic signaling mediated by G-proteins is not altered in this model.

Toxin-catalyzed ADP-ribosylation of G_{α} -protein and immunoblotting by using specific antibody are two common methods used to detect protein content of G_{α} -subunits. However, in our study, the amount detected by cholera-toxin ribosylation is different from that by using specific $G_{\alpha s}$ antibody. Similar situations have been observed regarding $G_{\alpha s}$ -proteins content by using pertussis toxin and specific antibody for $G_{\alpha i}$ -protein by other investigators (309). This may be due to the intrinsic character of these two techniques. While immunoblotting can detect all the G_{α} -proteins, ADP-ribosylation is influenced by many factors such as biophysical properties of the membrane, posttranslational modifications of G_{α} -proteins. The function of a membrane protein can be modulated at the level of gene transcription, by the transcriptional efficiency of the mRNA encoding the protein, or by posttranslational modification. Alterations in both transcription and

translational efficiency usually modify protein function by changing absolute levels of a protein content, while posttranslational modification alters protein function independent of the protein content. In fact, there is evidence to show posttranslational events of G_{α} -proteins, such as myristoylation (310) and phosphorylation (311). Although we have observed a decrease of G_{α} mRNA and an increase of G_{β} mRNA which are similar to many previous study of G-proteins in heart failure model with a downregulation of β -AR and desensitized β -AR stimulation, the change in mRNA level may not lead to a change in protein expression. However, the mechanisms why gene expression at G-protein level tends to downregulate the β -AR signaling is unclear. One possibility may be a feedback response to inhibit the enhanced signaling. The discordance of mRNA expression and protein expression may on one hand be due to the fact that we only examined the steady state mRNA level whereas alteration of the rates of mRNA degradation, transcriptional rate, and protein degradation will influence the gene and protein expression. On the other hand, it is possible that the duration of experimental period after the aortocaval shunt used in this study may be not long enough to produce the required changes.

4. Effect of imidapril and losartan on β -adrenergic signaling in heart failure induced by aortocaval shunt

It has been reported that plasma renin activity and cardiac renin activity were increased shortly after the induction of the aortocaval shunt in rats (273), and treatment with ACEI and AT1 receptor blocker attenuated the hemodynamic and hypertrophic responses induced by the AV shunt (312-314). It is also known that the renin release is under β_1 -adrenergic control (251) and angiotensin II may modulate adrenergic activity by facilitating synaptic release of

norepinephrine (252-256), suggesting cross-talk between renin-angiotensin system and β -adrenergic system. In addition, several studies have shown that ACEI alters the β -adrenergic signaling through modifying one or more components of this system. Therefore in this study, we applied two drugs, imdapril; a long acting ACEI, and losartan; a well-known AT1 receptor blocker to examine their interaction with β -adrenergic signaling pathway in this model. Our results showed that imidapril and losartan have similar effects in attenuating hemodynamic and hypertrophic responses to the aortocaval shunt. In addition, these agents seem to interact with the β -AR signaling pathway by partially reversing changes in β_1 -AR density and protein content, GRK activity and protein expression, cholera toxin mediated ADP-ribosylation of $G_{\alpha s}$, mRNA levels of $G_{\alpha s}$ and $G_{\alpha i}$; however, these agents did not modify changes in AC with respect to its activity or protein content. Thus, it seems that receptors and G-proteins are the direct or indirect targets for these drugs. This view is similar to that put forward by Horn *et al.* (315), who demonstrated that the beneficial effects of ACEIs, such as captopril and lisinopril, for the treatment of congestive heart failure in human are associated with reversal of the decreased β -AR density as well as decreased level of $G_{\alpha s}$ -proteins in the lymphocytes. Maisel *et al.* (316) examined the effect of captopril on β -AR signaling in normal and isoproterenol-induced hypertrophy in guinea pigs and showed that while captopril treatment led to an up-regulation of cardiac β_1 -AR density and an increase in isoproterenol-stimulated AC activity in the normal heart, it attenuated the decreased β_1 -AR number and responsiveness of cardiac β -AR signaling in the isoproterenol-induced cardiac hypertrophy. A later study by Gilbert *et al.* (317) revealed that an ACEI, lisinopril, lowered the cardiac adrenergic drive and increased the β -AR density in heart failure patients with increased cardiac drive but had no effects in those with normal cardiac drive; this indicated that at least some of the beneficial effects of ACEI were mediated through their

optimal regulation of β -adrenergic signaling pathway. Our results provide the evidence that AT1 receptor blocker has effects similar to that of the ACEIs.

VI. CONCLUSIONS

In this study, we characterized a cardiac hypertrophy and heart failure model with high cardiac output induced by aorticaval shunt and examined extensively the β -adrenergic signaling pathway at the failure stage in the AV groups and sham as well as upon treatment with either imidapril or losartan. Based on the results obtained, the following conclusions are drawn:

1. The development of cardiac hypertrophy and heart failure induced by aorticaval shunt shows three stages: developing hypertrophy (the first two wks after the induction of aorticaval shunt); established hypertrophy (4-8 wks), and decompensated hypertrophy or heart failure (16 wks).
2. Although the myocardial contractility in failing stage was significantly attenuated both *in vivo* and *in vitro* conditions, the contractile response to β -stimulation was enhanced rather than blunted.
3. The mechanisms for the enhanced β -stimulation in the failing stage were:
a) Increased β_1 -AR density and protein content; b) Decreased GRK activity and increased GRK2, and GRK5 protein expression in the membrane; c) Increased activity and protein content of cardiac AC isoforms.
4. The mechanism for decreased basal myocardial contractility may involve the dramatically decreased bioactivity of $G_{\alpha s}$ subunit which may modulate the L-type Ca^{2+} -channel independent of cAMP production.
5. Treatment of imidapril or losartan can modify changes at the receptor and G-protein level but not at the effector level. The improved hemodynamic function may at least in part be through their abilities to reverse the decreased $G_{\alpha s}$ bioactivity in the failing heart due to aorticaval shunt.

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