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This project explores how β_1 adrenoceptor (β_1-AR) stimulation affects cellular hypertrophy and apoptosis in PAC-1, a cultured rat pulmonary artery cell line. Insights into these responses may further the current understanding of vascular remodeling. Promoter-reporter activity for the hypertrophy-specific gene smooth muscle myosin heavy chain decreased as measured by a luciferase assay when PAC-1 cells were treated with the selective β_1-AR agonist dobutamine (DOB) in 0.4% fetal bovine serum (FBS) supplemented media. However, activity of a β_1-gal control vector also decreased, and neither response was attenuated by pre-treatment with a β_1-AR selective antagonist metoprolol. A MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt] viability assay shows that while there is a loss of cells with decreasing amounts of serum, this effect is not exacerbated by DOB in 0.4% FBS. DNA fragmentation assays were inconclusive as to the mode of cell death occurring. However, an increase in Bax/Bcl-2 ratio suggest that apoptosis is induced with DOB treatment in 10% FBS, but that this DOB treatment in 0.4% and serum-free media does not increase this apoptotic index compared to control.

Both similar and conflicting cellular responses have been documented in rat neonate cardiomyocytes as well as in murine transgenic models selectively over-expressing adrenergic receptors in the heart. Comparison of the vascular smooth muscle cell response to the cardiomyocyte response may lead to a more tailored use of adrenergic agents for treatment during different stages of cardiovascular disease.

HYPERTROPHIC VERSUS APOPTOTIC RESPONSE OF VASCULAR SMOOTH MUSCLE TO β_1 ADRENERGIC RECEPTOR STIMULATION

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CHAPTER I

INTRODUCTION

Hypertrophy in the Cardiovascular System

The heart has traditionally been considered to be a terminally differentiated organ, although this convention has been challenged as of late[1]. During development, the muscle cells of the heart (cardiomyocytes) have the ability to grow and divide as needed. The cells of the adult heart, however, have a very limited ability to proliferate. To maintain homeostasis during increased demand, the cardiomyocytes instead increase in size without dividing[1]. This process, known as hypertrophy, can successfully maintain cardiac function for a limited time but will inevitably lead to decompensated pathologies such as dilated cardiomyopathy[2]. In cardiomyocytes, the hypertrophy response is characterized by a transition from a fully differentiated state to an embryonic gene program and is marked by the up-regulation of genes for fetal isoforms of contractile proteins. These proteins include atrial natriuretic factor, β-myosin heavy chain, skeletal α-actin and atrial myosin light chain-1[3].

Vascular smooth muscle cells can also undergo a hypertrophy response similar to that seen in the heart. VSMC hypertrophy is characterized by an increase in cell mass due to both an up-regulation in protein synthesis and an inhibition of protein degradation[4,5]. In particular, the contractile proteins smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α-actin are specific for differentiated smooth muscle cells, and are up-

regulated in hypertrophied VSMCs[6,7]. VMSC hypertrophy may also be accompanied by the development of polyploidy, where the DNA content increases without cell division[8,9].

Unlike cardiomyocytes, VSMCs retain the ability to de-differentiate to a proliferative phenotype characterized by decreasing levels of α-actin and extracellular matrix proteins, and low levels of contractile myosin[10]. Excessive VSMC proliferation, or hyperplasia, can play a key role in vascular remodeling in atherosclerosis, restenosis, and after vascular injury (Figure 1)[11,12]. While hyperplasia and hypertrophy can both occur, vascular hypertrophy is found to be the predominant growth response in resistant vessel vascular disease and often preceeds cardiac hypertrophy in a number of other cardiovascular disease states such as secondary hypertension[13,14].

Apoptosis in the Cardiovascular System

Programmed cell death, known as apoptosis, is a normal part of maintaining tissue homeostasis in many organs, including the vasculature. Whereas necrosis involves loss of membrane integrity and random DNA degradation, apoptosis is a programmed event that requires energy to maintain cellular function until late in the process [15]. A number of cellular and molecular markers have been identified in apoptotic cells. Early events include condensation of nuclear chromatin along with cell shrinkage and membrane blebbing. Phosphatidylserine, a phospholipid normally found only in the inner leaflet of the lipid bilayer, is subsequently exposed on the outer surface of an apoptotic cell. This allows apoptotic cells to be recognized by phogocytes[16]. Specific cysteine proteases known as caspases are also expressed during apoptosis, leading to the cleavage of cellular proteins, and to DNA fragmentation by caspase activated DNase[12,17].

A wide range of stimuli can induce apoptosis in the cardiovascular system. Hypoxia during ischemia is the major cause of myocyte death in the heart, while VSMC apoptosis may be triggered by flow dependent stimuli such as nitric oxide and shear stress. Inflammatory cells also play a role in VSMC apoptosis in atherosclerosis and aneurysm formation via the expression of surface death ligands or the release of pro-apoptotic cytokines[12].

Two major pathways are involved in the regulation of apoptosis. Particularly significant to the immune system are death receptors belonging to the gene superfamily of tumor-necrosis factor (TNF) death receptors including Fas (CD95) and TNF-R1 (Figure 2). These receptors can be activated by ligands or cytokines released from

inflammatory cells such as tumor necrosis factor-α, whose production is increased during cardiovascular injury[18]. Death receptor activation leads to the oligomerization of pro-caspase 8 to produce caspase 8, which in turn can activate downstream effector caspases to promote apoptosis[19].

The mitochondrial pathway accounts for the second mode of apoptosis and is regulated by the Bcl-2 family of proteins (Figure 3). Pro-apoptotic factors such as Bax and Bid are translocated to the mitochondria upon activation. Here they interact with anti-apoptotic factors such as Bcl-2, leading to the release of the mitochondrial mediator of apoptosis, cytochrome *c*. Altough the mechanism of cytochrome *c* release is still in debate, is has been shown to involve the opening of a mitochondrial permeability transition pore[20]. This leads to the cleavage of pro-caspase 9 to its active form, which activates caspase 3 and the downstream caspase cascades [12].

Dysregulation of vascular smooth muscle cell (VSMC) growth and death has been implicated in a number of vascular pathologies. Excessive growth coupled with a deficiency of appropriate VSMC death contributes to neointimal thickening as well as atherosclerotic plaque formation. Conversely, excessive apoptosis is associated with plaque rupture and thrombosis as well as vessel wall thinning in aortic aneurysm [12,21]. Apoptosis also occurs in the diseased heart, particularly following post-ischemic reperfusion [22], and is associated with the transition from compensated to decompensated cardiac hypertrophy [23].

Adrenergic Receptor Control of Cell Growth and Death in the CV System

Adrenergic receptors play a major role in cardiovascular regulation via their influence on contractility in the heart and vasomotor tone in the vasculature [24]. The two major classes of adrenoceptors, α and β are found throughout the cardiovascular system. However, receptor subtype density and distribution varies greatly from cardiac to vascular tissue and within the vascular tree itself [24,25]. Each group of adrenergic receptors elicits a different effect depending on which guanosine nucleotide binding protein they are coupled to (Table 1, Figure 4). For example, β_1-ARs are coupled to the stimulatory G_s complex, which activates the synthesis of 3′5′-cyclic adenosine monophosphate (cAMP) by adenylate cyclase (AC). This second messenger system is responsible for the activation of protein kinase A (PKA), which in turn regulates many pathways and receptors. For example, in cardiac myocytes PKA is responsible for the phosphorylation of L-type Ca^{2+} channels, phospholamban, and myofibrillar proteins to cause a positive inotropic effect [26]. PKA can also modulate gene expression through the cAMP-responsive element and the Ras/Raf/MAPK pathway [27]. The stimulatory effects of β_1-AR receptor activation can be counterbalanced by α_2-ARs, which couple to the inhibitory G_i complex that down-regulates AC activity. This inhibits the opening of voltage-gated Ca^{2+} channels and the activation of PKA, meanwhile activating K^+ channels [25]. The G_q family, which is coupled to α_1-ARs, has a very different mode of action, than the G_i and G_s proteins. This receptor activates phospholipase C which is responsible for the hydrolysis of phosphatidylinositol-4,5,-bisphosphate (PIP_2) to produce inositol-(1,4,5)-

trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers lead to intracellular calcium release and the activation of protein kinase C (PKC). α_1-AR stimulation can also effect gene expression through the mitogen-activated protein kinase (MAPK) pathway. This response has been reported to be partially to completely dependent on the activation of PLC [28].

Both cell growth and death can be influenced by stimulation of adrenergic receptors. In the heart, both α_1-AR and β_1-AR up-regulate expression of hypertrophy-related proteins [29,30]. In contrast, over-expression of β_2-AR not only prevents but reverses hypertrophy associated with $G_{q\alpha}$ over-expression [31]. Intriguingly, β_2-AR stimulation can protect against β_1-AR induced apoptosis [32].

The effect of adrenergic stimulation on vascular smooth muscle hypertrophy and apoptosis remains unclear. It has been established that the α_1-AR signaling cascade can induce transcription of hypertrophy-sensitive contractile proteins in arterial myocytes [33,34]. However, the transcriptional consequences of β_1- and β_2-AR signaling have yet to be fully explored in VSMCs. It is unknown how stimulation of these receptors affects hypertrophic gene transcription or programmed cell death. One may hypothesize that there will likely be dissimilar responses between VSM and cardiomyocytes due to differences in the relative adrenergic receptor densities in each cell type. In the heart, beta-adrenergic receptors are more abundant than alpha-adrenergic receptors. Among these, the β_1-ARs predominate over β_2-ARs. In VSMCs however, α-ARs are more abundant than β-ARs, but the β_2-ARs predominate over the β_1-ARs. The purpose of this study is to determine the effects of adrenergic signaling on VSMC hypertrophy and apoptosis.

Figure 1- Vascular remodeling may occur in response to changes in flow, pressure, and atherosclerosis. For example, increased flow or atherosclerosis may cause an increase in the vessel diameter (outward), while the media does not change in cell mass (eutrophic). However, the chronic increase in blood pressure caused by hypertension can cause an increase in vessel mass (hypertrophic), while decreasing the lumen diameter (inward) (adapted from Berk, 2001[11])

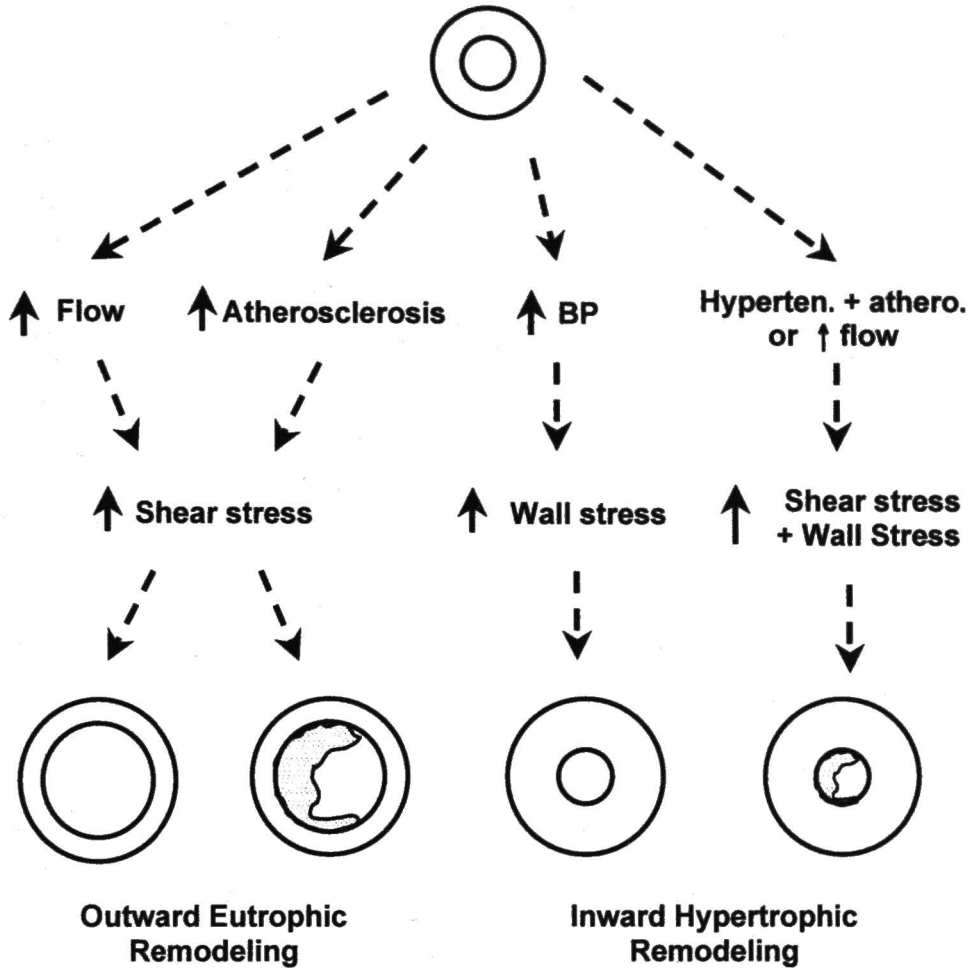

Flow
Atherosclerosis
BP
Hyperten. + athero.
or ↑ flow
Shear stress
Wall stress
Shear stress
+ Wall Stress
Outward Eutrophic
Remodeling
Inward Hypertrophic
Remodeling

Figure 2- Activation of Fas death receptor family may occur in response to cardiovascular injury. This leads to the cleavage of pro-caspase 8 to active caspase 8, leading to further downstream caspase activation and apoptosis (adapted from Bennett, 2002[12]).

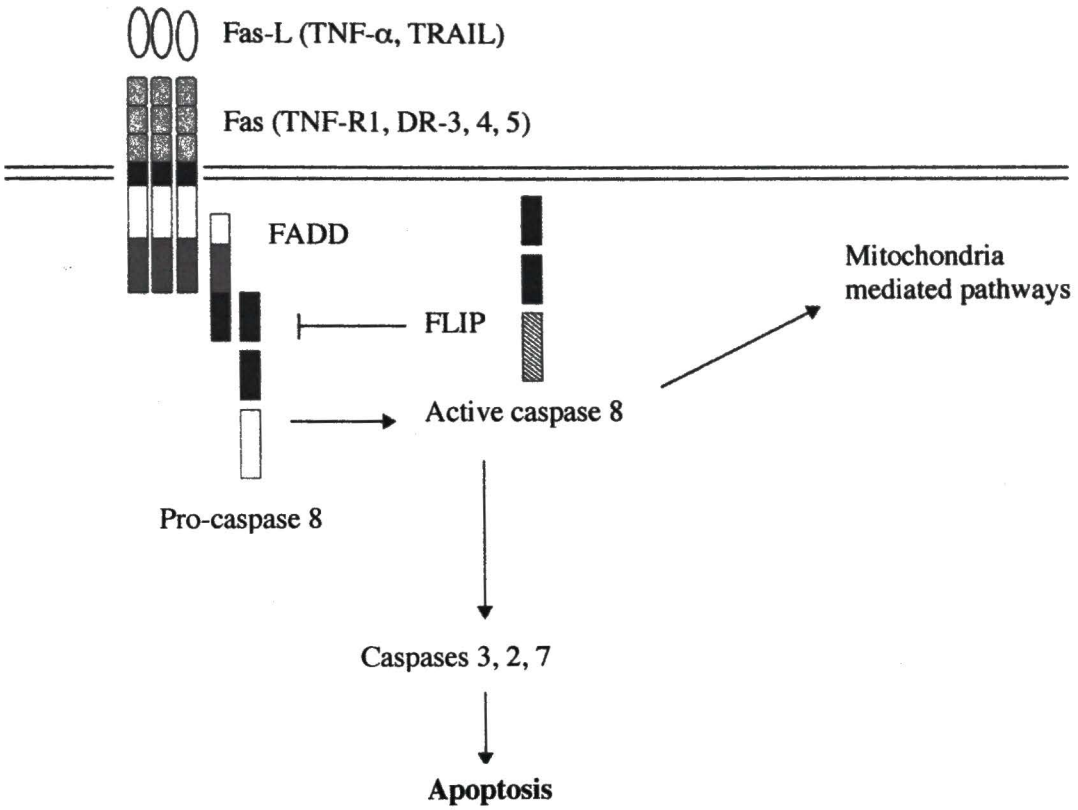

Fas-L (TNF-α, TRAIL)
Fas (TNF-R1, DR-3, 4, 5)
FADD
FLIP
Mitochondria
mediated pathways
Active caspase 8
Pro-caspase 8
Caspases 3, 2, 7
Apoptosis

Figure 3- A number of stress factors can induce mitochondrial-mediated apoptosis in the cardiovascular system including mechanical stress, ischemia-reperfusion, and survival factor withdrawal. Activation of the Bcl-2 family of proteins can induce the release of cytochrome C from the mitochondria, leading to the activation of the downstream caspase cascade and apoptosis (adapted from Bennett, 2002[12]).

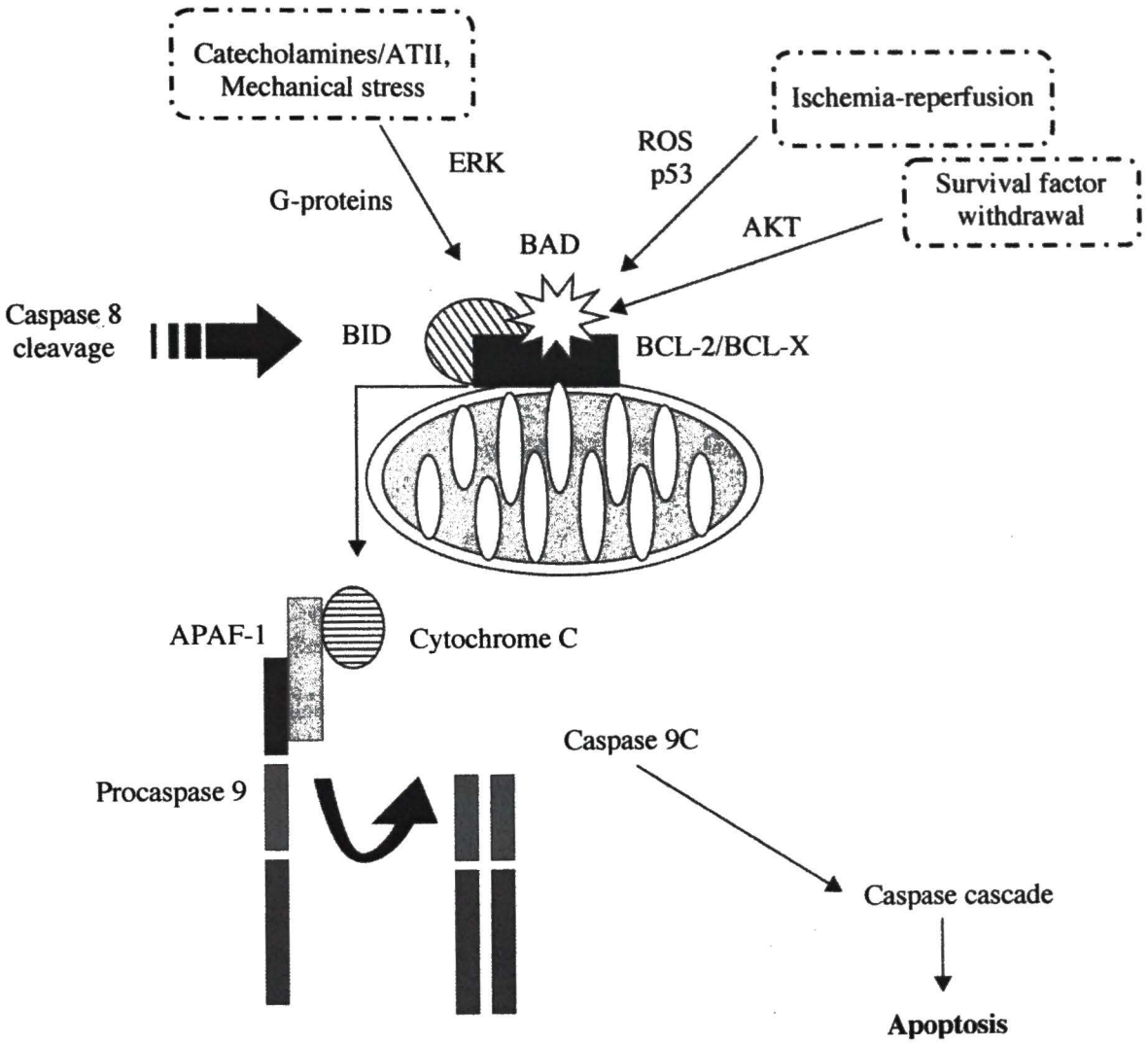
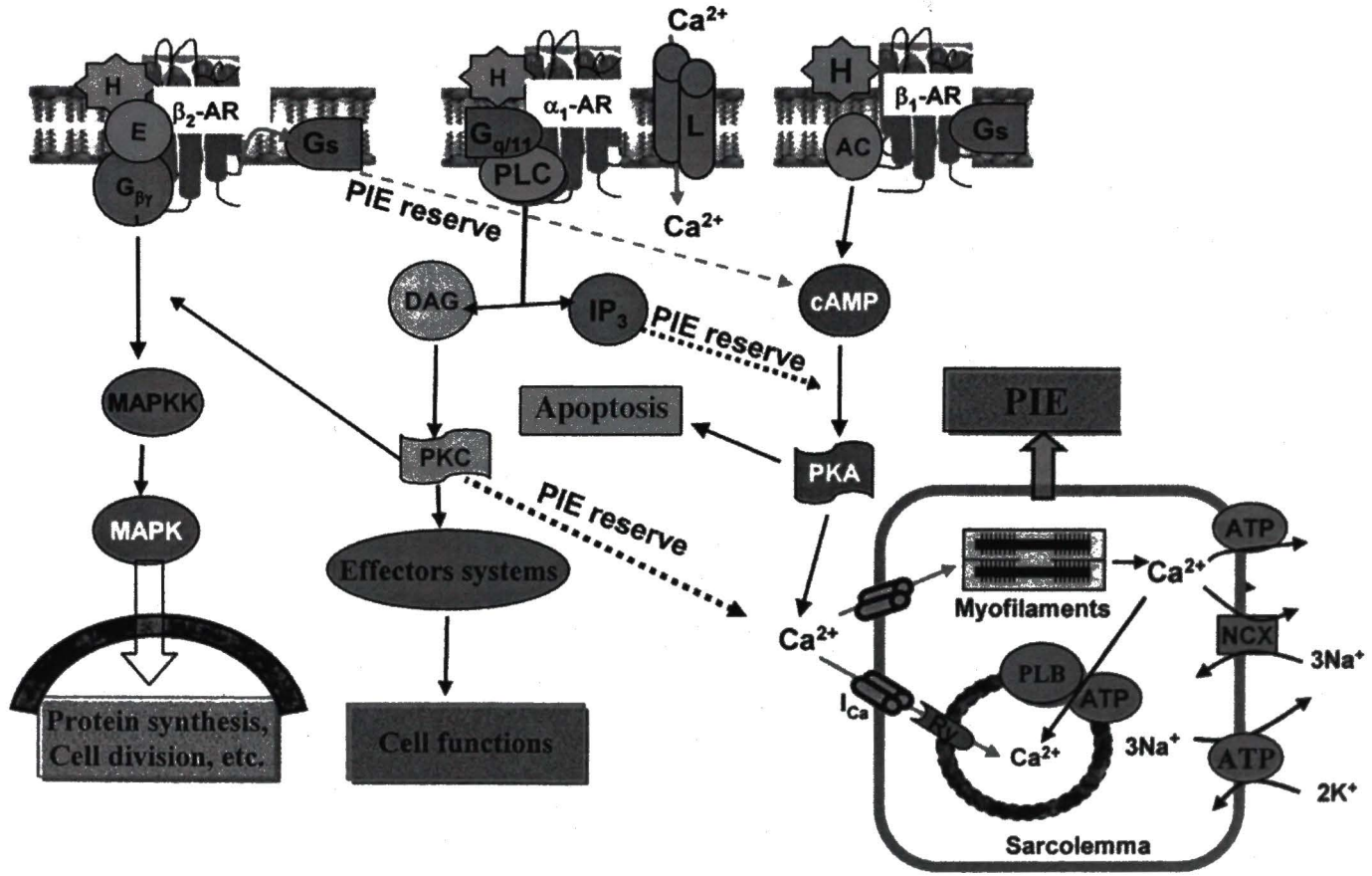

Catecholamines/ATII,
Mechanical stress
Ischemia-reperfusion
ROS
p53
ERK
G-proteins
Survival factor
withdrawal
AKT
BAD
Caspase 8
cleavage
BID
BCL-2/BCL-X
APAF-1
Cytochrome C
Caspase 9C
Procaspase 9
Caspase cascade
Apoptosis

Table 1- Cardiovascular adrenoceptor distribution and function

Class	Subtype	Localization	Function	Coupler	Mechanism of Action
α-AR	α_1	Heart, blood vessels	VSM, myocardial contraction	G_q	PLC-mediated $[Ca^{2+}]_v$ activation, inhibition of cAMP accumulation
	α_2	Heart	Vasoconstriction	$G_{i/o}$	Ca^{2+}-dependent $[K^+]$ activation, $[Ca^{2+}]_v$ inhibition
β-AR	β_1	Myocardium	PIE	G_s	AC-mediated cAMP synthesis
	β_2	Cardiac chambers	Heart rate control; VSM relaxation	G_s; G_q, $G_{i/o}$	AC-mediated cAMP synthesis AC inhibition

Abbreviations:; AC, adenylate cyclase; cAMP, 3′5′-cyclic adenosine monophosphate; PIE, positive inotropic effect; PLC, phospholipase C; $[Ca^{2+}]_v$, voltage-gated Ca^{2+} channel; VSM, vascular smooth muscle

Figure 4– G-protein signaling via adrenergic receptor can affect a number of cellular functions in myoctyes. Among them are contractility, mitogenesis and cell survival (adapted from Dzimiri, 2002[20]). *Abbrev.: L, L-type Ca^{2+} channel; PIE, positive inotropic effect; PLB, phospholamban; R, receptor; Ry, ryadine receptor; X, crosstalk regulatory switch; V, voltage gated Ca^{2+} channel..*


H
E
β_2-AR
Gs
$G_{\beta\gamma}$
H
α_1-AR
$G_{q/11}$
PLC
L
Ca^{2+}
H
AC
β_1-AR
Gs
PIE reserve
Ca^{2+}
DAG
IP_3
PIE reserve
cAMP
MAPKK
Apoptosis
PIE
PKC
PKA
PIE reserve
MAPK
Effectors systems
Myofilaments
Ca^{2+}
ATP
Ca^{2+}
NCX
$3Na^+$
PLB
ATP
I_{Ca}
Ca^{2+}
$3Na^+$
ATP
$2K^+$
Protein synthesis,
Cell division, etc.
Cell functions
Sarcolemma

CHAPTER II

METHODS

Cell Culture

Both the hypertrophic and apoptotic response of VSMCs to adrenergic stimuli was measured using PAC-1, a rat pulmonary arterial smooth muscle cell model. This is a stable cell line that retains its ability to switch between proliferative and contractile phenotypes. In the differentiated phenotype, PAC-1 cells express normal smooth muscle-specific contractile proteins and maintain functional cell surface receptors for neuorhormonal stimuli[35].

PAC-1 cells were maintained in media 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 50 μg/ml gentimicin in an incubator at 95% relative humidity, 37 C, 5% CO_2. Cells were passed by trypsinization.

Promoter-Reporter Assay

To assess the expression of hypertrophy-related proteins in this cell line, a luciferase-based promoter-reporter assay system was used to measure the expression of the smooth muscle myosin heavy chain (SM-MHC). In this system, a plasmid construct containing the promoter region of the gene of interest upstream from an expression sequence for firefly luciferase is transiently transfected into cultured PAC-1 cells. After the specified treatments, cells are lysed and the whole cell lysate is combined with a luciferase substrate. Luciferase activity can then be quantified by luminometry. Promoter activity is measured as a function of the amount of luciferase being expressed, i.e. the amount of luciferase activity.

The SM-MHC promoter-reporter construct contains a 1,249 base pair truncation of the promoter region of the rat hypertrophy-related gene SM-MHC (truncation from −1161 to +88, Figure 5) ligated to pGL2-Basic (Promega, Madison, WI), a luciferase expression vector[6] (gift of S. White, University of Vermont, Burlington) (Figure 6). White *et al.* determined that this truncation of the promoter excludes upstream negative regulatory elements and is therefore the most active region of the promoter. This plasmid was amplified by transformation of a competent *Escherichia coli* cell line, DH5α (Invitrogen, Carlsbad, CA), followed by plasmid purification. 10 ng of SM-MHC 1.2 plasmid DNA was incubated on ice with 50 µl competent cells for 30 minutes (500 pg of pUC19 was transformed separately as a positive control). Transformations were placed on ice for 2 minutes after heat-shocking cells for 20 seconds at 37 C. 1 mL of room temperature luria broth including 100 µg/ml of ampicillin (LB-Amp^{100}) (Sigma, St.

Louis, MO) was added to each transformation and incubated for 1 hour at 37 C in a shaker at 225 cycles/min. 100 µl of each transformation as well as 100 µl of a 1:10 dilution of each transformation was spread onto LB agar plates made with 100 µg/ml ampicillin, which were then incubated overnight at 37 C. Two colonies were chosen from each SM-MHC 1.2 plate to inoculate 2 ml of LB-Amp^{100}. These cultures were incubated for approximately 6 hours at 37 C and 225 cycles/min before adding 500 µl to 400 ml of LB-Amp^{100}. Cultures were grown overnight before lysis and isolation of plasmid DNA using a standard Qiagen Maxi-prep protocol (Qiagen, Valencia, CA).

PAC-1 cells were cultured as described. Passes 3-12 were used for promoter-reporter assays. For each assay, 2.8 x 10^5 cells were plated per 12-well plate and grown for 48 hours to 70-80% confluency before transfection.

PAC-1 cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA). This is a liposomal transfection reagent composed of a polycationic lipid (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate, DOSPA), and the neutral lipid (dioleoyl phosphatidylethanolamine, DOPE). 2 µl of reagent was diluted with 50 µl of serum/antibiotic-free media 199 per well of a 12-well plate to be transfected. 30 ng of SM-MHC 1.2 plasmid DNA was also diluted in 50 µl of media 199 per well then combined with the diluted transfection reagent and incubated at room temperature for 30 minutes. This concentration of promoter-reporter has been previously determined by this laboratory to sufficiently report promoter activity without negatively affecting the health of the cell. In some trials, 30 ng of pSV-β-Gal (Promega, Figure 7) reporter was added to the transfection mixture to be used as a transfection

control. This vector contains the *lac Z* gene downstream of a SV40 promoter and lacks any known mammalian promoter sequences. This allows for stable expression which is independent of cellular signaling or metabolism.

Meanwhile, growth media was removed from the tissue culture plates and cells were washed twice with 0.5 mL of media 199 per well. 0.5 ml of media 199 per well was then added to the transfection mixture and 0.5 ml of this dilution was aliquoted to each well. Transfections were then incubated for approximately 12 hours prior to drug treatments.

Transfected cells were then treated with specific adrenergic agonists (see Table 2 for activity of each agonist and antagonist). Each drug was freshly diluted prior to treatment. All drugs were initially diluted in water then serially diluted to desired concentration in serum-free media, with the exception of dobutamine and prazosin, which were initially diluted in DMSO due to their poor solubility in water. Final dilutions to be added to transfected cultures were in 1 ml of 0.4% FBS-supplemented media 199 per well to be treated. It has been previously determined by this laboratory that this low concentration of serum is sufficient to maintain healthy PAC-1 cell cultures without significantly stimulating proliferation. This prevents the cellular growth response from indirectly affecting the promoter-reporter expression.

Dose responses were initially carried out for each agonist using a range of 10^{-9} to 10^{-5} M concentrations to determine the approximate half-maximal doses to be used with antagonist. Time course trials were also carried out from 6 to 48 hours to determine optimal time for maximal response, which was determined to be 24 hours. To verify

specific receptor activation, various concentrations of antagonist were added to wells 1 hour prior to addition of the agonist. Since α_1-AR stimulation has been previously shown to induce VSMC hypertrophy, it was used as a positive control for this assay [33,34].

After incubation with continuous exposure of cell cultures to the drug treatment for 24 hours, media was removed from each well and cells were washed twice with approximately 0.5 ml of phosphate-buffered saline per well. 25 µl of reporter lysis buffer (Promega, Madison, WI) was added to each well and incubated for 5-10 min at room temperature prior to vigorously scraping with a cell scraper to remove lysed cells from plates. Lysates were transferred to individual disposable luminometer cuvettes. 100 µl of Luciferase Assay Reagent (Promega, Madison, WI) was added to each lysate and vortexed briefly prior to measuring luciferase activity in a Turner TD-20e Luminometer (Turner designs, Sunnyvale, CA).

Applied Biosystem's (Foster City, CA) Galacto-Star™ Assay System was used to measure β-galactosidase activity in cultures co-transfected with the pSV-β-gal reporter. This system uses a light-emitting substrate, 1,2-dioxetane, to measure enzyme activity. To perform both the luciferase and β-Gal activity simultaneously, cells were lysed in 50 µl of lysis buffer per well as previously described. 20 µl aliquots were then added to separate tubes for each assay. The Galacto-Star® substrate was diluted 1:50 with reaction buffer diluent; 300 µl of reaction buffer was then added to the cell lysates and incubated of 60 min. β-Gal activity was measured with a luminometer in the same manner as the luciferase assay.

Each treatment was quadruplicated in 12-well plates and repeated a minimum of three individual times. Data was expressed graphically as relative Turner units versus drug dosage. Results were analyzed by one-way ANOVA, followed by the Tukey *post hoc* test using the GraphPad Prism 4 software (GraphPad, San Diego, CA).

Viability Assay

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) was used to measure cellular viability in response to drug treatments that decreased promoter-reporter activity. This colorimetric assay uses a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] combined with an electron-coupling reagent, phenazine ethosulfate (PES), which enhances the stability of the compound. NADPH or NADH in metabolically active cells reduces the MTS tetrazolium compound to produce a soluble colored product formazan, which can be measured at an absorbance of 490 nm (Figure 8). The amount of reduced product is directly proportional to the amount of viable cells in the culture.

For this assay, 1.66×10^5 cells were plated in a 96-well plate with 100 µl of media 199 + 10% FBS per well and incubated for 48 hours as described previously. Growth media was then replaced with media 199 + 0.4% FBS or serum-free media, with or without drug, and incubated for an additional 24 hours, with each treatment carried out in 8 wells of the 96-well plate. 20 µl of CellTiter 96® AQ_{ueous} One Solution reagent was added to each well and incubated for 5 hours at 37 C in a humidified, 5% CO_2 incubator. Formazan production was measured at 490 nm in a 96-well plate reader (BIO-TEK FL600). Results were analyzed using GraphPad Prism 4 software as previously described.

DNA fragmentation

During apoptosis, caspase activation leads to degradation of DNA into internucleosomal fragments containing multiples of approximately 200 base-pairs in length. This DNA fragmentation produces a 200 bp laddered pattern when visualized by agarose gel electrophoresis. Selective extraction of low molecular weight DNA from PAC-1 cells was used to determine if DNA laddering could be seen after activation of β_1-AR by the selective agonist dobutamine.

2.8×10^5 PAC-1 cells were plated per 6-well plate and incubated for 48 hours as previously described. Plates were washed twice with serum-free media 199 before addition of freshly prepared dilutions of the stated doses of the β_1-AR agonist, dobutamine in media 199 supplemented with 0.4% FBS. Drug treated cells were then incubated for 24 hours. Serum-withdrawal was demonstrated by Gibbons *et al.* to stimulate DNA fragmentation in vascular smooth muscle cells[36]. Hence, incubation in serum-free media was used as a positive control and media 199 supplemented with 10% FBS media as a negative control.

To isolate low-molecular weight DNA, cells were first washed twice with 0.5 mL PBS. Cells were then lysed in 150 µl of NP-40 lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris·HCl) per well for 5 minutes; lysates were collected by scraping and transferred to 1.5 ml tubes. Wells were washed once with 350 µl of PBS, which was then pooled with cell lysates. Cellular debris was pelleted by centrifugation at 14,000 rpm for 5 minutes; supernatants were then transferred to fresh 1.5 ml tubes. After addition of

sodium-dodecyl sulfate to a final concentration of 5%, any residual protein or RNA was digested with proteinase K and RNase A respectivly. 5 µl of 2 mg/ml RNase A was added to each supernatant and incubated at 42 C for 1 hour, followed by additional incubation with 5 µl of 1 mg/ml proteinase K at 37 C for 1 hour. After the addition of 1/2 volume of 3 M sodium acetate and 2 1/2 volumes of ethanol, DNA was precipitated overnight at -80 C. DNA was then pelleted by centrifugation for 10 minutes at 14,000 rpm and re-suspended in 15 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), to which 3 µl of 6x gel loading buffer was added (12% ficoll, 0.06 M EDTA, pH 8, 0.15% bromophenol blue, 0.15% xylene cyanol). The entire volume was analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide for UV visualization of DNA. Results were documented with a UVP BioDoc-It™ System.

RT-PCR of Bax/Bcl-2

As discussed earlier, a change in the ratio of the Bcl-2 family of proteins can also be used to measure apoptosis. RT-PCR was used to measure the relative expression of the pro-apoptotic Bax and the anti-apoptotic Bcl-2. An increase in the Bax/Bcl-2 ratio is indicative of apoptosis.

To determine whether the Bax/Bcl-2 ratio was affected by adrenergic receptor stimulation, 2.8×10^5 PAC-1 cells were plated per 100 mm dish and incubated for 48 hours as previously described. Cultures were then treated with freshly prepared dilutions of the stated concentrations of agonists, also as previously described.

After 24 hours of continuous exposure to the drug treatments, total cellular RNA was harvested with TRIzol™ reagent (Invitrogen, Carlsbad, CA). This reagent contains phenol and guanidinium isothiocyanate, which inhibits RNases. Media was first removed from the treated plates and transferred to 15 ml polypropylene tubes. The remaining cells were then gently scraped with a cell scraper in 1 ml of PBS, followed by washing the plates with additional PBS, and pooled with the media. Cells were pelleted by centrifugation at 5,000 rpm, room temperature, for 5 minutes and the supernatant was removed. Cells were re-suspended in 1 ml of TRIzol, transferred to a 1.5 ml polypropylene tube, and incubated at room temperature for 5 minutes. 200 µl of chloroform was then added, mixed by inversion, and incubated at room temperature for 3 minutes. The aqueous phase containing the cellular RNA was separated by centrifugation at 12,000 × *g*, 4 C, for 15 minutes and transferred to a fresh tube. 500 µl of isopropanol was added, mixed by inversion and incubated at room temperature for 10

minutes. Samples were then centrifuged again for 10 minutes at 12,000 × *g*, 4 C, and the supernatant removed and discarded. RNA pellets were washed once with ice cold 75% ethanol. After careful removal of as much ethanol as possible, RNA pellets were air-dried then re-suspended in RNase-free water. RNA samples were stored at -80 C or used immediately for reverse transcription.

SuperScript II™ (Invitrogen, Carlsbad, CA) reverse transcriptase was used to synthesize cDNA from the isolated total cellular RNA. 1 μg of each RNA sample was combined with 1 μl 50 μM Oligo dT, 1 μl 10 mM dNTP mix and RNase-free water to a final volume of 12 μl in 0.5 ml tubes. Samples were mixed gently and heated at 65 C for 5 minutes in a thermocycler (Eppendorf Mastercycler® Gradient, Hamburg, Germany) to denature RNA and anneal Oligo dT primers then chilled on ice. 4 μl of 5X first-strand buffer, 1 μl of 1M DTT, and 1 μl of RNaseOUT™ (Invitrogen, Carlsbad, CA) was added to the samples, which were then mixed and incubated at 42 C for 2 minutes. 1 μl of SuperScript II was then added and mixed by pipetting; reverse transcription was carried out for 50 minutes at 42 C then terminated by heating at 70 C for 15 minutes.

Forward and reverse primer sets for Bax and Bcl-2 as well has the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as a control) were acquired from IDT (see Table 3 for primer sequences). A PCR Master Mix (Promega, Madison, WI) containing 50 units/ml *Taq* DNA polymerase, 400 μM each of dATP, dGTP, dCTP and dTTP, and 3 mM $MgCl_2$ was used for DNA synthesis. For each sample, 12.5 μl of 2X PCR Master Mix was combined with 100 ng of cDNA, 2 μl each of 1 μM forward and reverse GAPDH primers, 1 μl each of 10 μM Bax or Bcl-2 forward

and reverse primers, and water to a final reaction volume of 25 μl. Duplexing the gene of interest with GAPDH allowed for the evaluation of changes in expression levels of Bax or Bcl-2 as compared to GAPDH, which should be stably expressed under these treatment conditions. The polymerase chain reaction was carried out in the thermocycler with the following temperature cycles:

1. Denature- 94 C, 2 minutes
2. Anneal- 60 C, 1 minute
3. Extension- 72 C, 1 minute
4. Final extension- 72 C, 5 minutes
5. Hold- 4 C

Initially, steps 1-3 were repeated for 25, 30, 35, and 40 cycles to determine the linear range for the amplification of each gene. 30 cycles was found to be sufficient for both Bax and Bcl-2. After PCR was complete, 10 μl of each sample was combined with 2 μl of 6X gel loading dye and analyzed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide for UV visualization of PCR product. Scanned images of each gel were analyzed using NIH ImageJ software to measure band intensity (http://rsb.info.nih.gov/ij/). Relative band intensities were normalized as a ratio to GAPDH.

Table 2- Adrenergic drugs and receptor specificity	
Drug	Receptor Specificity
Dobutamine	β_1 agonist
Metoprolol	β_1 antagonist
Phenylepherine	α_1 agonist
Prazosin	α_1 antagonist

Figure 5- SM-MHC promoter sequence. The 1.2 kb truncation from -1161 to +88 is outlined[6].

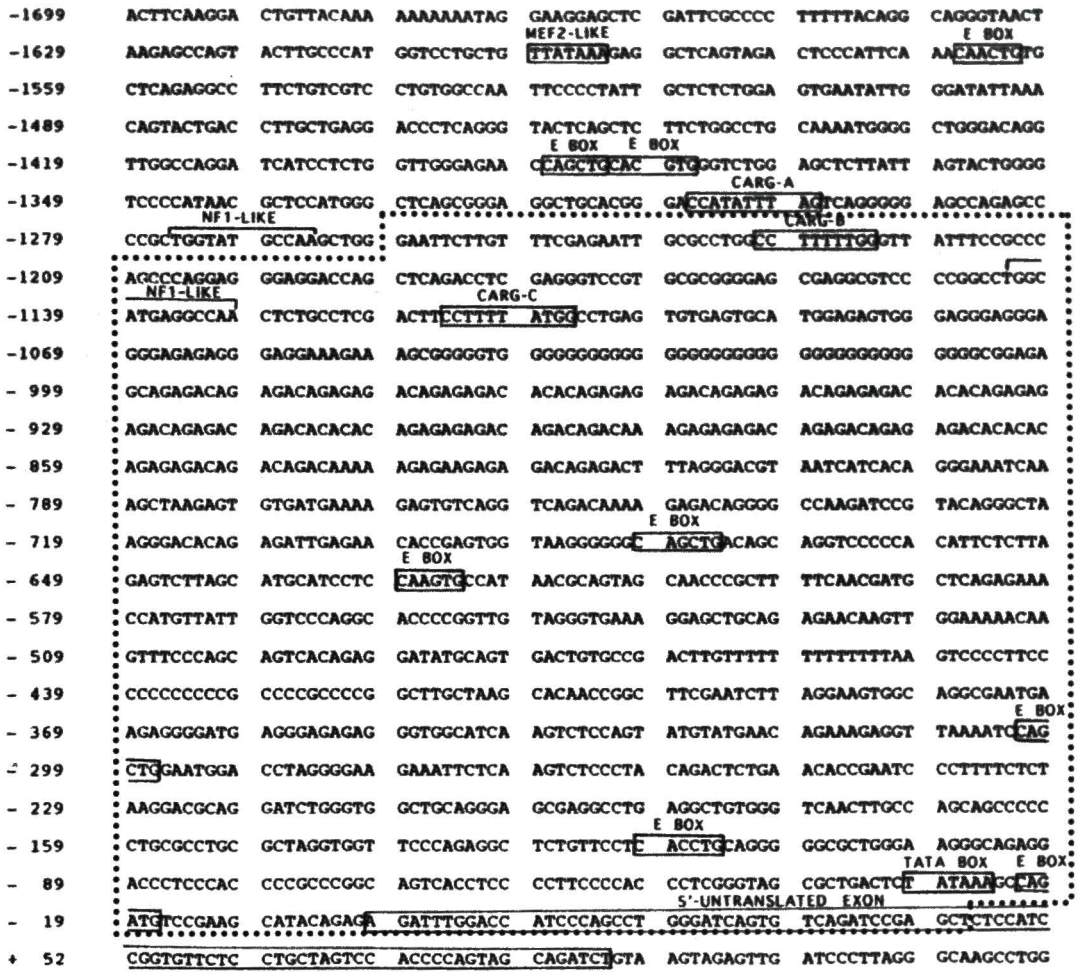

MEF2-LIKE
E BOX
E BOX
E BOX
CARG-A
NF1-LIKE
CARG-B
NF1-LIKE
CARG-C
E BOX
E BOX
E BOX
E BOX
TATA BOX
E BOX
5'-UNTRANSLATED EXON

Figure 6- The pGL2-Basic vector contains multiple cloning regions upstream and downstream of the *luc* gene. Bacterial origin of replication and an ampicillin resistance gene allows for bacterial amplification and selection[37].

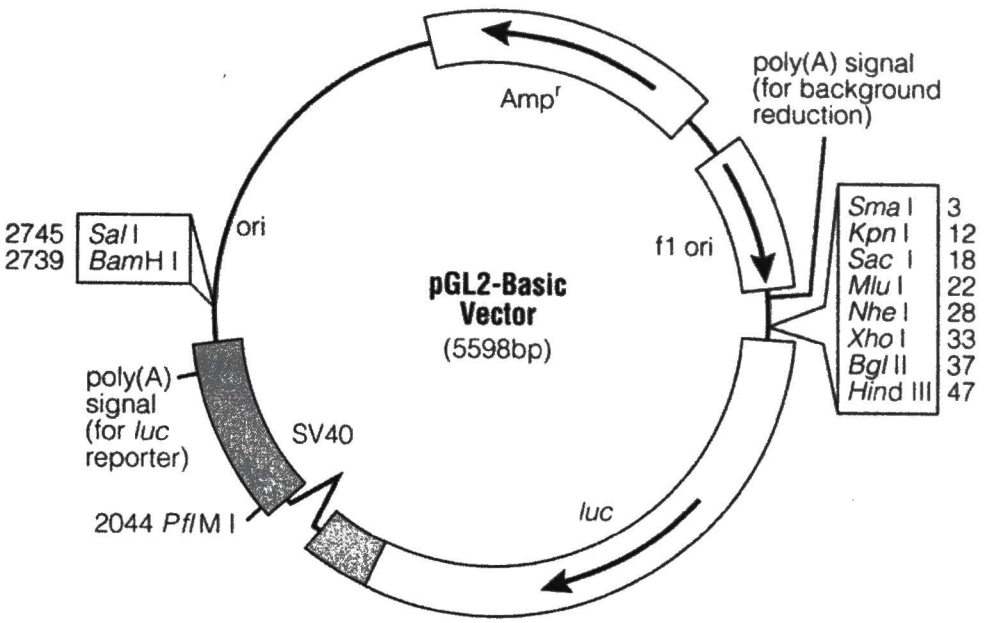

Amp^r
poly(A) signal
(for background
reduction)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Hind III 47
f1 ori
2745 Sal I
2739 BamH I
ori
pGL2-Basic
Vector
(5598bp)
poly(A)
signal
(for luc
reporter)
SV40
2044 PflM I
luc

Figure 7- The pSV-β-Galactosidase vector is driven by an SV40 promoter/enhancer upstream of the *lacZ* gene for β-galactosidase expression. Bacterial origin of replication and an ampicillin resistance gene allows for bacterial amplification and selection[38].

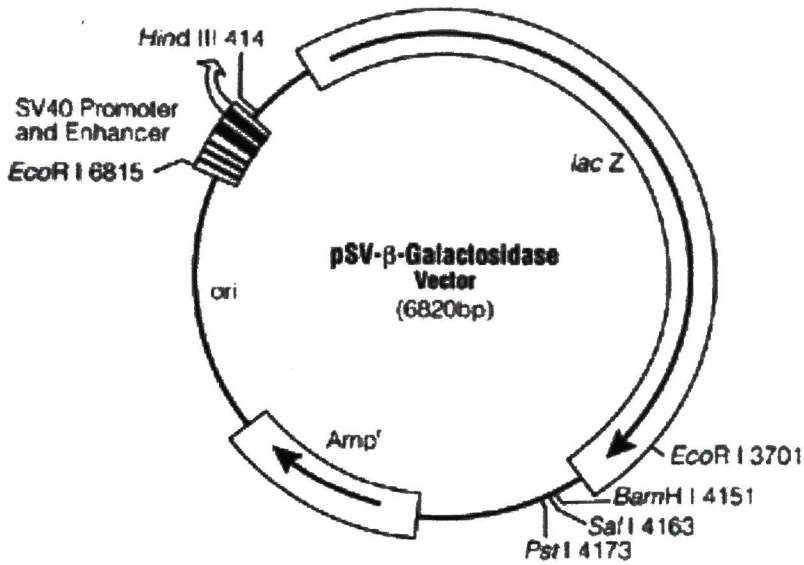

Hind III 414
SV40 Promoter
and Enhancer
EcoR I 6815
lac Z
pSV-β-Galactosidase
Vector
(6820bp)
ori
Amp'
EcoR I 3701
BamH I 4151
Sal I 4163
Pst I 4173

Figure 8- The MTS tetrazolium conversion to formazan requires NAD(P)H and the presence of an electron transfer reagent, in this case phenozine ethosulfate (PES).

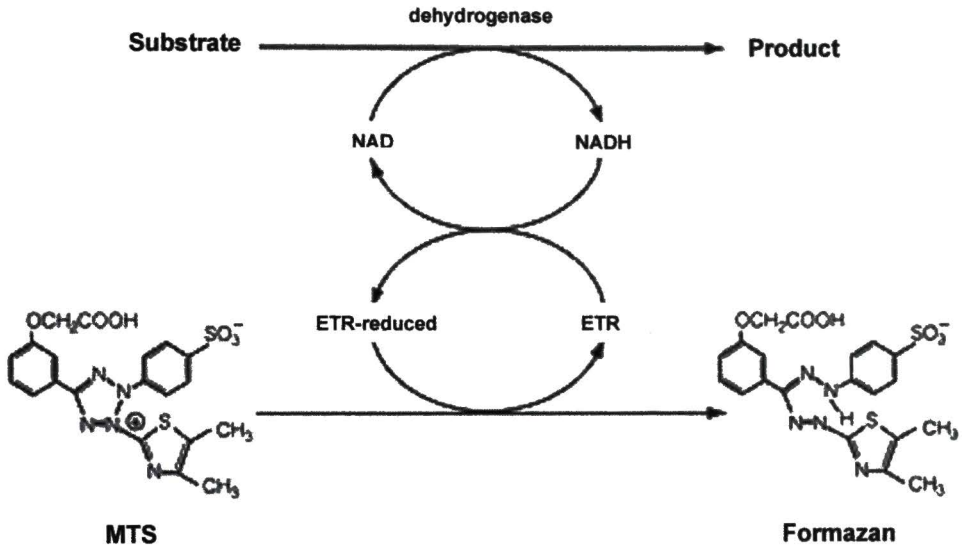

dehydrogenase
Substrate
Product
NAD
NADH
ETR-reduced
ETR
MTS
Formazan

Table 3- PCR primer sequences	
Primer	Sequence
GAPDH (F)	5'- GTG TGA ACG GAT TTG GCC GTA TTG -3'
GAPDH (R)	5'- TCA TAC TTG GCA GGT TTC TCC AGG -3'
Bax (F)	5'- GTT TCA TCC AGG ATC GAG CAG -3'
Bax (R)	5'- CTT CCA GAT GGT GAG CGA GG -3'
Bcl-2 (F)	5'- AGC TGC ACC TGA CGC CCT T -3'
Bcl-2 (R)	5' CAG CCA GGA GAA ATC AAA CAG AGG -3'

CHAPTER III

RESULTS

It has been shown in both cultured rat aortic smooth muscle cells as well as intact rat aorta that α_1 adrenergic receptor stimulation with the selective agonist phenylephrine can induce cellular hypertrophy as measured by protein content[33,34]. In this study, the smooth muscle myosin heavy chain promoter was activated by PE stimulation in a dose-dependent manner in cultured PAC-1 cells (Figure 9). This indicates that α_1 receptor activation also has the ability to induce cellular hypertrophy in this cell line in a similar manner to primary aortic smooth muscle cells. This response was blocked by the α_1-AR specific antagonist prazosin (Figure 10). One-way analysis of variance between the non-drug treated control, 1 μM PE stimulated and 10 μM PZ+ 1 μM PE treated groups shows a statistically significant variance of means, with a P value of 0.0008. Agonist treatment with PE produced a 2.2 fold increase in promoter-reporter activity compared to control ($P<0.01$). Pre-treatment with PZ significantly reduced the response to PE back to baseline values ($P<0.001$).

In contrast to the α_1-AR response, stimulation of SM-MHC transfected PAC-1 cells with the β_1-AR agonist dobutamine caused a decrease in promoter-reporter activity in a dose-dependent manner (Figure 11A). One-way analysis of variance between the non-drug treated control, 10 μM metoprolol, 1 μM DOB, and 10 μM MET + 1 μM DOB

treated groups shows a strongly significant difference with agonist treatment ($P<0.0001$, Figure 12A). Agonist treatment with DOB caused a 6-fold decrease in promoter-reporter activity ($P<0.001$ compared to control). However, pre-treatment with the antagonist MET was unable to inhibit this response, although MET alone had no effect. This response conflicts with that seen in the hearts of transgenic mice where over-expression of β_1-AR causes an induction of cardiac myocyte hypertrophy that progresses to heart failure[29].

Further analysis of this response with the pSV-β-Gal control vector suggests that the decrease in promoter-reporter activity may not be entirely due to transcriptional regulation. β-Gal activity also decreased in DOB treated cultures in a dose-dependent manner (Figure 11B). The greater than 1.5-fold decrease of β-Gal activity in response to 1 μM DOB was not as strong as the decrease in promoter-reporter activity, but was still significant ($P<0.001$). Pre-treatment with MET was also unable to inhibit this decrease in β-Gal activity (Figure 12B).

Taken together, these assays suggest that β_1-AR stimulation does cause a decrease in SM-MHC promoter transcriptional activity, however this may be accompanied by a decrease in cellular viability. This explains the decrease in β-Gal activity, which should not be changed in response to cellular metabolic activity.

A MTS viability assay was performed to determine if this is indeed the case (Figure 13). Cultures were treated in 96-well plates in media containing high (10%), low (0.4%), or no FBS to determine if serum-withdrawal may play a role in cell death. 24 hours in low and serum-free media alone caused a significant decrease in cell viability

($P<0.001$). When cells were treated with 1 μM DOB in high serum, there was also a significant decrease in viability ($P<0.001$). When treated in low serum, the decrease in viablility was no greater than the untreated low serum group. However, DOB treatment in serum-free media did cause an additional decrease in viability compared to the untreated serum-free group ($P<0.05$).

These data demonstrate that serum-withdrawal does play a role in DOB induced cell death. However, the fact that DOB treatment does not exacerbate cell death in the presence of low serum does not support the hypothesis that the decrease in SM-MHC promoter-reporter and β-gal reporter activity is due to a decrease in cellular viability as previously discussed.

Further studies were carried out to determine if the decrease in cell viability seen in the MTS assay is due to apoptosis or necrosis. Since serum-withdrawal has been shown by Gibbons *et al.* to induce apoptosis in VSMCs[36], DNA fragmentation was measured in serum-free as well as 0.4% and 10% FBS supplemented media 199, with and without addition of 1 μM DOB (Figure 14). As expected, no DNA degradation was seen in cultures maintained with 10% FBS. Low serum and serum-free groups showed increased amounts of DNA degradation. When cells were treated with 1 μM DOB in 10% FBS supplemented media, some DNA degradation was also seen. However, DOB treatment with 0.4% FBS or serum-free media did not produce any more DNA degradation than the respective non-drug treated groups. Importantly, all groups showing DNA degradation appear to be due to random digestion, unlike the 200 bp internucleosomal cleavage found in apoptosis that would produce the so-called DNA

ladder. This may be because the cells are undergoing necrosis where DNA degradation is random. Or the cells may be in a late stage of apoptosis where further degradation of 200 bp fragments has occurred.

Bax and Bcl-2 expression was next examined to further clarify the mode of cell death seen in the viablility assay. After performing cycle number trials for both genes of interest (Figure 15), Bax and Bcl-2 primers were duplexed with primers for GAPDH for normalization (Figure 16). When maintained in high serum, the Bax/Bcl-2 ratio was very low in PAC-1 cells (0.564). Bax mRNA increased dramatically when cultures were switched to low serum for 24 hours, but Bcl-2 expression appears stable (Bax/Bcl-2 = 1.882). However, Bcl-2 expression was almost completely lost when cells were maintained in serum-free media (Bax/Bcl-2 = 4.636). When cells were treated with 1 μM DOB in the various high serum the Bax/Bcl-2 ratio (1.095) did increase compared to control. This agrees with the loss in cell viability in this group in the MTS assay. However, in both low and serum-free treatment groups, there was not a further increase in Bax/Bcl-2 (1.010, 1.328 respectively). These data again suggest that while serum-withdrawal does play a role in PAC-1 viability, β_1-AR stimulation does not exacerbate this effect.

Figure 9- PAC-1 cells were transfected with SM-MHC promoter-reporter and treated with increasing doses of PE. The maximal response was seen at 10 μM PE, causing approximately a 6-fold increase in relative luciferase activity.

SM-MHC
Promoter-Reporter Activity

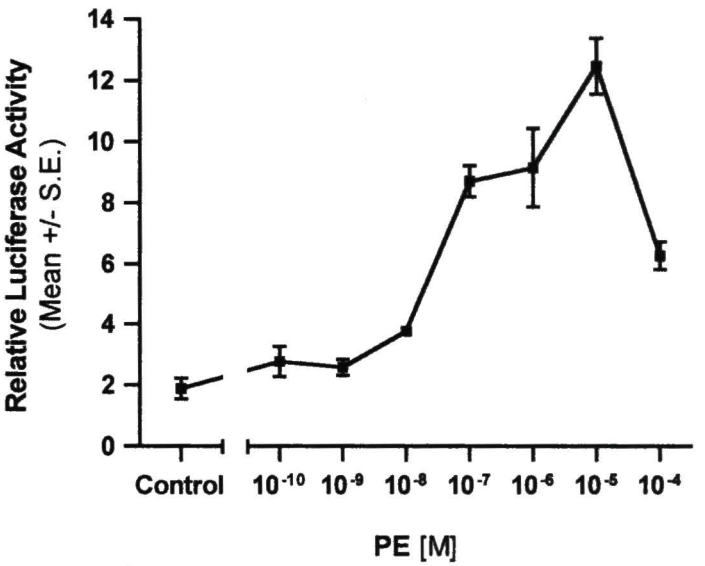


Figure 10- PAC-1 cells were transfected with SM-MHC promoter-reporter then treated with 1 μM PE or 10 μM PZ followed by 1 μM PE. Treatment with the agonist PE significantly increased luciferase activity. Pre-treatment with the antagonist PZ completely blocked this response. (* = $P < 0.01$ compared to untreated control; † = $P < 0.001$ compared to PE alone; n=3.)

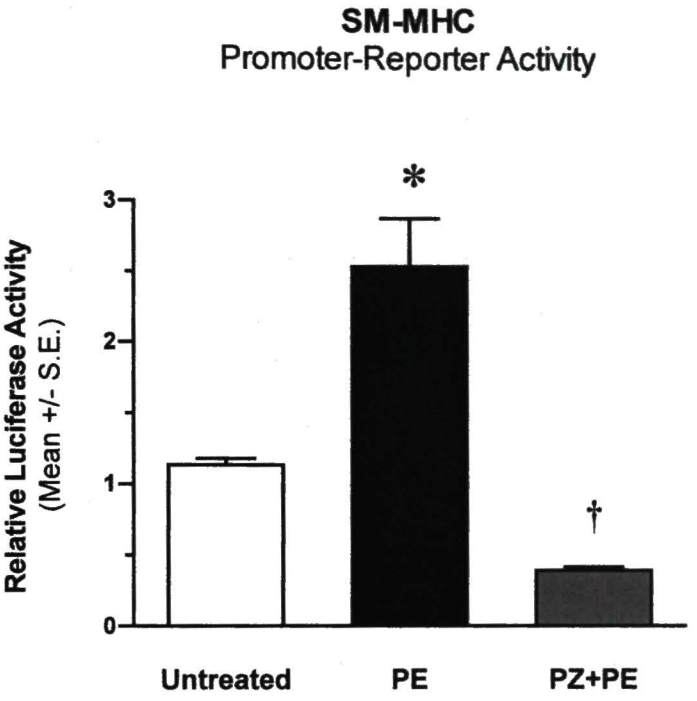
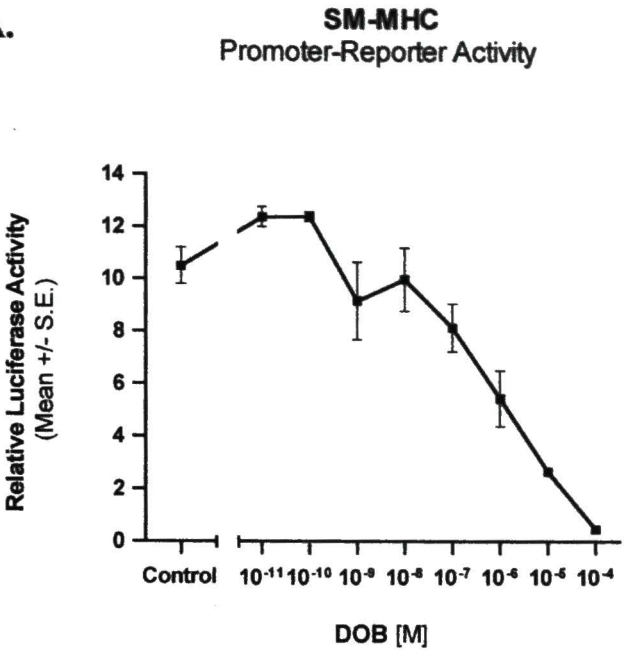

SM-MHC
Promoter-Reporter Activity
Relative Luciferase Activity
(Mean +/- S.E.)
3
2
1
0
*
†
Untreated
PE
PZ+PE

Figure 11- PAC-1 cells were co-transfected with the SM-MHC promoter-reporter (A) as well as the pSV-β-Gal control vector (B) and treated with increasing doses of DOB. Promoter-reporter and β-Gal activity was silenced as drug dosage was increased.

A.


SM-MHC
Promoter-Reporter Activity
Relative Luciferase Activity
(Mean +/- S.E.)
14
12
10
8
6
4
2
0
Control
10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4
DOB [M]

B.

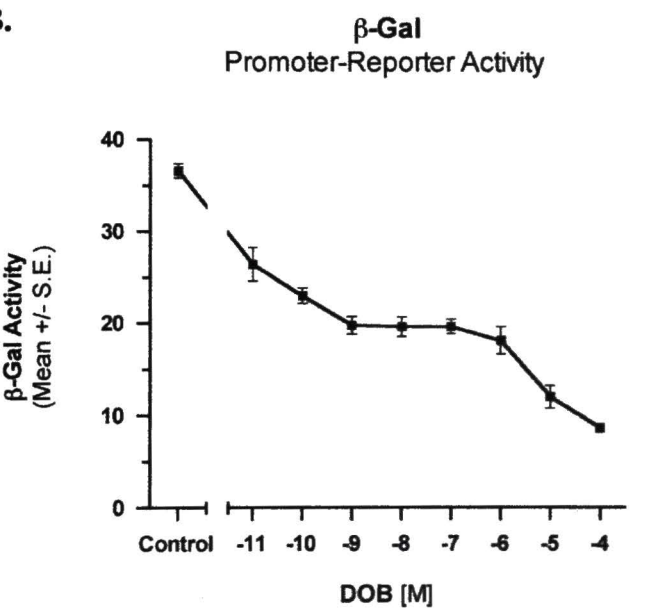
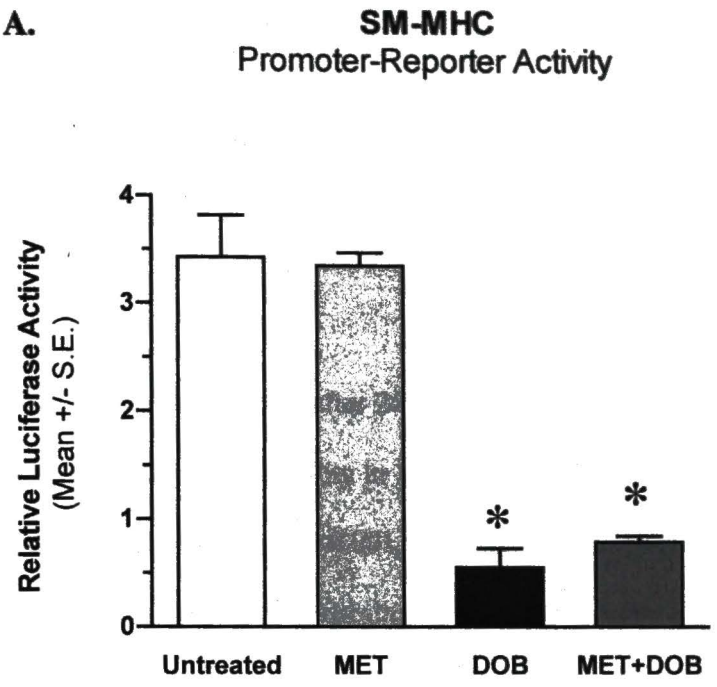

β-Gal
Promoter-Reporter Activity
β-Gal Activity
(Mean +/- S.E.)
40
30
20
10
0
Control
-11 -10 -9 -8 -7 -6 -5 -4
DOB [M]

Figure 12- Treatment of SM-MHC transfected cells with 1 μM DOB caused a 6-fold decrease in promoter-reporter activity. Pre-treatment with 10 μM MET was unable to attenuate this effect (A). This was also true for β-Gal reporter activity, which decreased 1.5-fold in response to DOB (* = $P<0.001$ compared to untreated control; $n=3$.)

A.


SM-MHC
Promoter-Reporter Activity
Relative Luciferase Activity
(Mean +/- S.E.)
4
3
2
1
0
*
*
Untreated
MET
DOB
MET+DOB

B.

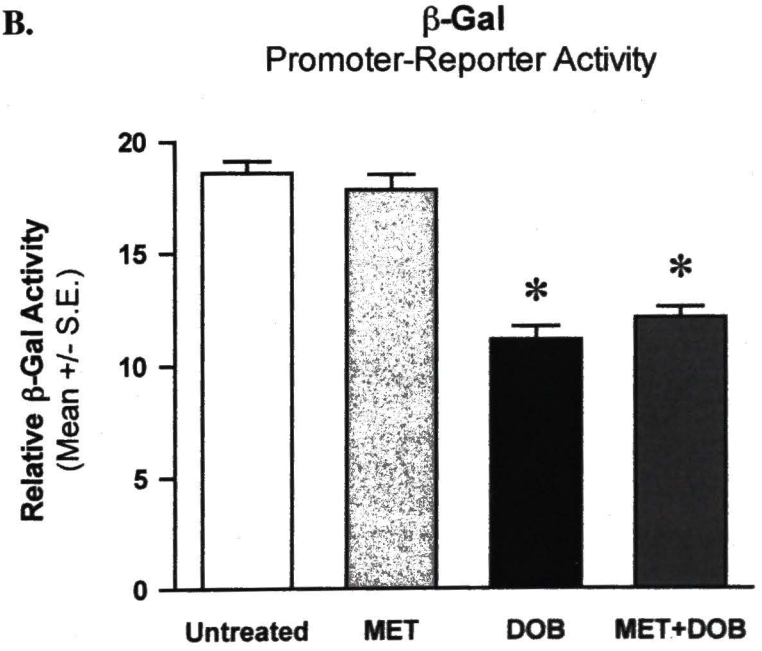

β-Gal
Promoter-Reporter Activity
Relative β-Gal Activity
(Mean +/- S.E.)
20
15
10
5
0
*
*
Untreated
MET
DOB
MET+DOB

Figure 13- MTS viability assay of after 24 hrs treatment with or without 1 μM DOB. DOB treatment reduced cellular viability in 10% FBS supplemented media and in serum-free media compared to control (*=$P<0.001$, †=$P<0.05$ respectively). DOB treatment in 0.4% media did also reduced cellular viability, but not significantly more than control.

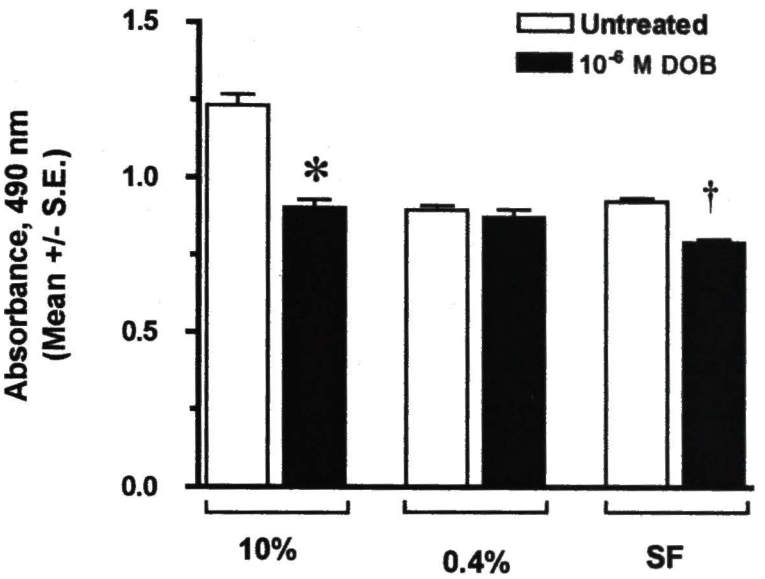

Absorbance, 490 nm
(Mean +/- S.E.)
1.5
1.0
0.5
0.0
Untreated
10-6 M DOB
*
†
10%
0.4%
SF

Figure 14– Fragmented DNA extracted from PAC-1 cells after 24 hrs of treatment do not show the 200 bp laddering pattern characteristic of apoptosis.

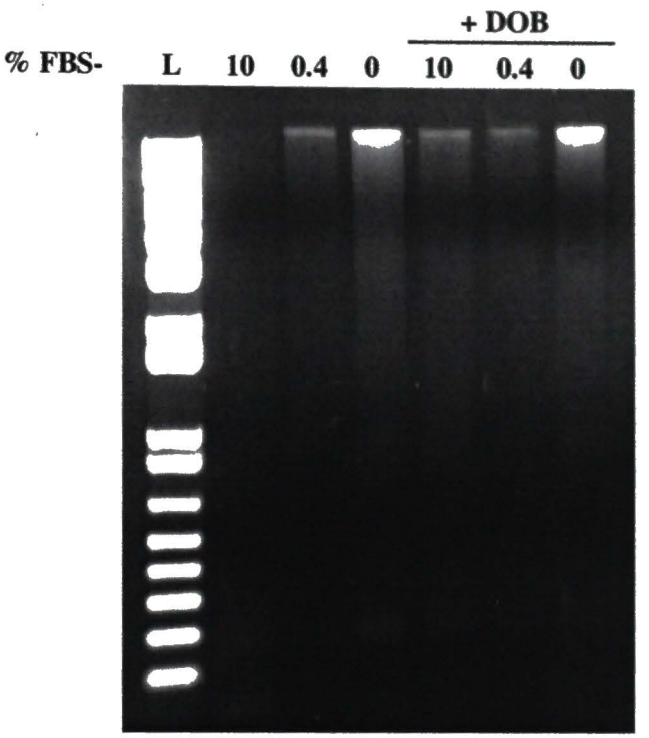

+ DOB
% FBS-
L
10
0.4
0
10
0.4
0

Figure 15- PCR cycle trials shows a maximization of product of both targets at 30 and 40 cycles. 30 cycles was therefore used for duplexing reactions.

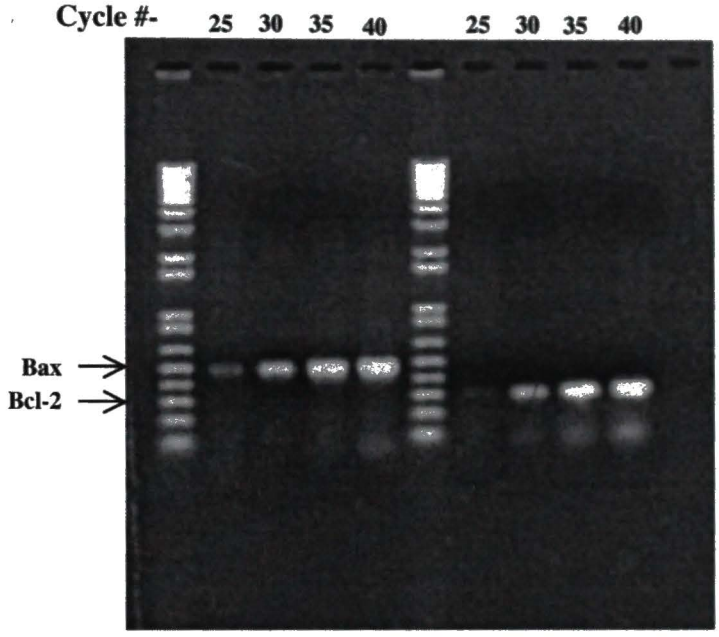
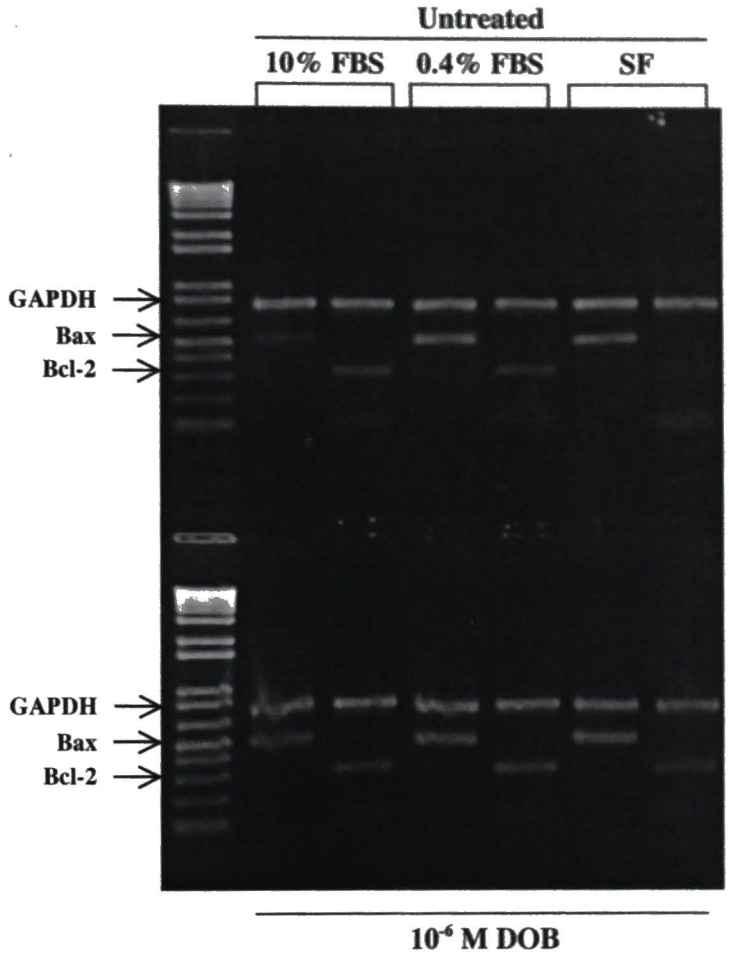

Cycle #-
25 30 35 40
25 30 35 40
Bax
Bcl-2

Figure 16– RT-PCR shows an increase in Bax mRNA in low and no serum, while Bcl-2 expression is almost completely lost when cells are maintained in serum-free media (Bax/Bcl-2 = 0.564, 10% FBS; 1.882, 0.4% FBS; 4.636, SF). DOB treatment with did cause an increase in Bax/Bcl-2 in high serum compared to control, but not in low and serum-free treated groups (Bax/Bcl-2 = 1.095, 10% FBS; 1.010, 0.4% FBS; 1.328, SF).


Untreated
10% FBS
0.4% FBS
SF
GAPDH
Bax
Bcl-2
GAPDH
Bax
Bcl-2
10-6 M DOB

CHAPTER IV

DISCUSSION

In this study, there were disagreements between the promoter-reporter assays and apoptosis assay that have yet to be explained. Some loss of cellular viability occurs when PAC-1 cells are maintained in low or serum-free media. However, when cells were treated with DOB in 0.4% media there was neither a decrease in cell viability nor an increase in Bax/Bcl-2 compared to the untreated low serum group. Since this is the concentration of serum at which the SM-MHC promoter-reporter assays are carried out, one can conclude that loss in promoter-reporter activity in response to DOB is not due to cell death. This does not explain the DOB dose-dependent loss of β-gal activity, nor does it explain the inability of MET to inhibit the effects of DOB.

MET is considered to be specific for the β_1-AR and is highly soluble in water, so the drug should be reaching the receptor. It is possible that DOB could be activating the β_2-AR, although it should be specific at the concentrations used here. However, this is contradicted by other studies in our laboratory with metaproterenol, a specific β_2-AR agonist, which causes an increase in SM-MHC promoter-reporter activity (data not shown).

As for the loss of β-gal reporter activity in response to DOB treatment, this may be explained by the presence of mammalian promoter elements in the pSV-β-Gal vector that are responding to the drug treatment. If this is the case, this vector cannot be used as

a control in our system. This highlights a significant drawback of the promoter-reporter assay system, which is normalization. In hindsight, measurement of SM-MHC protein levels may have been a better index of VSMC hypertrophy in response to adrenergic stimulation. This could be measured by western blot and normalized with GAPDH. The stable expression of GAPDH mRNA in response to DOB treatment as measured by RT-PCR supports the use of this GAPDH as a stable control.

Numerous studies have explored the pathophysiological impact of beta-adrenergic receptor stimulation in the heart. β_1-AR stimulation is associated with increased hypertrophic growth and apoptotic death of cardiomyocytes in transgenic models as well as in primary rat neonate cell culture. Saito *et al.* demonstrated that beta-adrenergic induced apoptosis to be mediated, at least in part, by the calcium-dependent activation of calcineurin[39]. This phosphatase can induce apoptosis by the activation of the pro-apoptotic factor Bad[40]. Calcineurin also plays a role in β-AR induced cardiomyocyte hypertrophy via the dephosphorylation of the NF-AT3[41]. Future studies could determine what role calcineurin plays in β_1-AR mediated VSMC growth and death. For example, β_1-AR stimulation could be carried out in the presence of cyclosporine A or FK506, which inhibit calcineurin activity.

CHAPTER V

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