EARLY CARDIAC MYOCYTE RESPONSES TO HYPOXIA, ISCHEMIA, AND REOXYGENATION

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RESUMEN: Respuestas Tempranas de Miocitos Cardíacos a Hipoxia, Isquemia y Reoxigenación

La isquemia miocárdica es la causa más prevalente de morbilidad y mortalidad en las poblaciones occidentales. Las múltiples condiciones asociadas a la isquemia comparten la propiedad común de contracción reducida, anormal o perdida debido a una entrega de oxígeno disminuída a la región del miocardio. En todas las condiciones el disbalance entre aporte y demanda de oxígeno puede ser transitorio, relacionado a periodos de ejercicio físico y/o estrés mental. Como consecuencia, los tejidos que experimentan episodios isquémicos repetidos sufrirán daño tanto por la isquemia (hipoxia, ácido láctico) como por la reperfusión subsecuente (estrés oxidativo). La isquemia miocárdica es acompañada comúnmente por hipertrofia que implica crecimiento cardiaco incrementado y expresión genética alterada. Al incrementar el espesor de la pared del ventrículo izquierdo y las distancias de difusión del oxigeno, la hipertrofia exacerba la isquemia y lleva a una patologia progresiva y autoperpetuante.

En estudios con modelos de hipoxia simulada hemos empezado a develar los eventos moleculares que determinan las respuestas de los miocitos cardiacos al estrés redox. Este trabajo identificó una red única de señales intracelulares, iniciadas por situaciones de hipoxia e hiperoxia, que promueven ya sea modificaciones adaptativa/defensivas o muerte celular por apoptosis. Los eventos tempranos de señalización en respuesta a la hipoxia severa incluyen cambios en el metabolismo lípido, en el manejo del calcio, en el pH intracelular y en la actividad de proteina kinasa C. Estos cambios culminan en la inducción de los genes de respuesta inmediata al estrés temprano, incluyendo *c-fos y c-jun*, que codifican información para la transcripción del factor AP1. Los blancos periféricos de las respuestas tempranas incluyen ó-actina, péptido atrial natriurético y genes de enzimas glicolíticas. La hipoxia crónica implica desensibilización de cAMP, protelna kinasa A y apoptosis incrementada. La reoxigenación causó una activación transitoria débil de la proteina kinasa activada por mitógeno, una activación fuerte y sostenida de la cascada de proteina kinasa activada por estrés, inducción de los blancos periféricos incluyendo AP1, supresión de actividad Sp1 y una oleada adicional de apoptosis.

Palabras claves: Insuficiencia cardiaca congestiva, Enfermedad de arterias coronarias, Proteina kinasa, Expresión genética, Angina, Miocardio en hibernación, Segundos mensajeros

RÉSUMÉ: Réponses précoces des myocytes cardiaques à l'hypoxie, l'ischémie et la réoxygénation.

L'ischémie du myocarde est la cause la plus fréquente de morbidité et de mortalité parmi les populations occidentales. Les multiples conditions associées à l'ischémie ont pour propriété commune la contraction réduite, anormale ou annulée, du fait d'un apport réduit d'oxygène à la région du myocarde. Quelles que soient les conditions, le déséquilibre entre l'apport et la demande d'oxygène peut être transitoire, en rapport avec des périodes d'exercice physique et/ou de stress mental. La conséquence de ce déséquilibre est que les tissus subissant des épisodes ischémiques répétés seront endommagés, autant du fait de l'ischémie (hypoxie, acide lactique) que de la reperfusion subséquente (stress oxydatif). L'ischémie du myocarde s'accompagne généralement d'une hypertrophie impliquant une augmentation de la croissance cardiaque et une altération de l'expression génétique. En accroissant l'épaisseur de la paroi du ventricule gauche et les distances de diffusion de l'oxygène, l'hypertrophie exacerbe l'ischémie et conduit à une pathologie progressive et s'auto-perpétuant.

Dans des études faites à partir de modèles d'hypoxie simulée, nous avons commencé à démêler les événements moléculaires qui déterminaient les réponses des myocytes cardiaques au stress réduction-oxydation. Ce travail a permis d'identifier un réseau unique de signaux intracellulaires, déclenchés par des situations d'hypoxie et d'hyperoxie et qui favorisent soit des modifications adaptatives / défensives, soit la mort des cellules par apoptose. Les événements précoces de signalisation en réponse à l'hypoxie sévère comportent des changements dans le métabolisme des lipides, le contrôle du calcium, le pH intracellulaire et l'activité de la protéine kinase C. Ces changements aboutissent à l'induction des gènes de réponse immédiate au stress précoce, comprenant c-*fos* et c-*jun* qui codifient l'information pour la transcription du facteur AP1. Les cibles périphériques des réponses précoces comprennent la α-actine, le peptide atrial natriurétique et les gènes d'enzymes glycolytiques. L'hypoxie chronique implique la désensibilisation du cAMP, de la protéine kinase A et une apoptose augmentée. La réoxygénation provoque une faible activation transitoire de la protéine kinase activée par mitogènes, une activation forte et soutenue de la cascade de protéine kinase activée par stress, l'induction des cibles périphériques incluant l'AP1, la suppression de l'activité de Sp1 et une vague supplémentaire d'apoptose.

Mots-clés : Insuffisance cardiaque congestive, Maladie des artères coronaires, Protéine kinase, Expression génétique, Angine, Myocarde en hibernation, Seconds messagers.

SUMMARY: Myocardial ischemia is the most prevalent cause of morbidity and mortality in Western populations. The multiple ischemia-associated conditions share the common property of reduced, abnormal, or lost contraction due to impaired oxygen delivery to a region of the myocardium. In all conditions the imbalance between oxygen supply and demand may be transitory, related to periods of physical exertion and/or mental stress. As a consequence, tissue experiencing repetitive ischemic episodes will suffer damage both from the ischemia (hypoxia, lactic acid), and from subsequent reperfusion (oxidative stress). Myocardial ischemia is commonly accompanied by hypertrophy involving increased cardiac growth and altered gene expression. By increasing left ventricle wall thickness and oxygen diffusion distances, hypertrophy exacerbates the ischemia and leads to a progressive and self-perpetuating pathology.

In studies using models of simulated ischemia we have begun to unravel the molecular events that determine the responses of cardiac myocytes to redox stress. This work identified a unique network of intracellular signals, initiated by hypoxic and hyperoxic stresses, that promotes either adaptive/defensive modifications, or cell death through apoptosis. Early signaling events in response to severe hypoxia include changes in lipid metabolism, calcium handling, intracellular pH, and protein kinase C activity. These culminate in the induction of immediate-early stress response genes including c-fos and cjun which code for transcription factor APl. Downstream targets for the early responses include skeletal a-actin, atrial natriuretic peptide, and glycolytic enzyme genes. Chronic hypoxia involves down regulation of cAMP, protein kinase A, and enhanced apoptosis. Reoxygenation caused a weak transient activation of mitogen activated protein kinase, strong sustained activation of the stress activated protein kinase cascade, induction of downstream targets including APl, suppression of Spl activity, and an additional wave of apoptosis.

Key Words: Congestive heart failure, Coronary artery disease, Protein kinase, Gene expression, Angina, Hibernating myocardium, Second messengers.

INTRODUCTION

Pathophysiology

Heart disease is the most common cause of death in Western populations (reviewed in (Mangano, 1990)). The clinical syndrome of cardiac failure follows a complex pathway of pathophysiologic interactions that result in progressively deteriorating cardiac pump function causing inadequate perfusion of body tissues and/or congestion. Despite multiple approaches to therapy including polypharmacology and surgery, the prognosis of congestive heart failure (CHF) remains poor. The overall 5- year survival for congestive heart failure is 50%, and patients with New York Association class IV have a dismal 1-year survival rate of less than 50% (Mangano, 1990).

Hypertension and coronary artery disease (CAD) are the most frequent underlying causes of myocardial failure (Mangano, 1990; Swan, 1990; Lamas, 1993; Chobanian, 1992; Birkenhager, 1991). With the general availability of effective antihypertensive therapies, the contribution of CAD has become more prominent (Lamas, 1993). CAD, which involves the progressive narrowing of coronary arteries by the atherosclerotic process, initiates a cascade of responses in the myocardium and vasculature. A simplified scheme of events is depicted in Fig 1. Unfortunately, for many patients the first indication of CAD is sudden complete vessel blockage due to a combination of thrombosis and endovascular disruption, resulting in myocardial infarction (MI) (Swan, 1990; Krayenbuehl and Hess, 1992; Yeung et al., 1992). The stage is set for MI by the progressive narrowing and thrombogenicity of the vascular surfaces during the early phases of CAD. In a larger number of patients, chest pain due to myocardial ischemia is the presenting complaint, as coronary reserve and blood flow are gradually restricted (Carbajal and Deedwania, 1991). Clinical and molecular events at the earliest stages of CAD are poorly understood because asymptomatic patients do not normally seek medical attention, and because until quite recently there were no good animal models of CAD. However, all forms of ischemia cause abnormal contractility and decreased cardiac output; these in turn trigger compensatory mechanisms as described below (Homans et al., 1986; Wilde et al., 1990; Kawai et al., 1990; Bolli et al., 1989; Galinanes et al., 1993; Baker et al., 1988). Ischemia also causes muscle loss through myocyte cell death, either through infarction (which can involve more than 50% of the left ventricle), or gradually, through less well understood mechanisms, in reponse to chronic hypoxia and cycles of ischemia and reperfusion (Gottlieb et al., 1994; Tanaka et al., 1994; Ivey et al., 1995; Williams et al., 1994; Swan, 1990). Because cardiac myocytes do not regenerate, this loss is permanent.

A number of secondary factors contribute to CHF progression and clinical deterioration (for reviews see (Lamas, 1993; Francis and Chu, 1995; Marian and Roberts, 1995; Nadal-Ginard and Mahdavi, 1993; Glennon et al., 1995; Morgan and baker, 1991; lee and lindpainter, 1993; Neyses and Pelzer, 1995; van Bilsen and Chien, 1993; Yamazaki et al., 1994; Schwartz et al., 1995; Scwartz, 1995)). Decreased cardiac output resulting from muscle loss or malfunction causes a drop in systemic blood pressure, triggering a cascade of compensatory mechanisms designed to raise the blood pressure and/or improve cardiac output.

Enhanced production of atrial natriuretic factor (ANF) is one of the earliest features of heart disease (Kovacic-Milivojevic and Gardner, 1992). As the name implies, this peptide promotes diuresis, and its production is probably regulated by baroreceptors in and around the cardiac atria. Ischemia-associated hypoxia stimulates the release of vasoconstrictor peptides including endothelin-l (ET-1) (Wadsworth, 1994; Watanabe et al., 1990; Malek et al., 1993; Bodi et al., 1995). The renin-angiotensin-aldosterone system is activated by reduced cardiac output, resulting in further vasoconstriction with increased renal and other systemic perfusion pressures (lee and lindpainter, 1993; Yamazaki et al., 1994). The result is increased cardiac work load and oxygen demand, further exacerbating the ischemia. Reduced cardiac output activates neurohormonal systems and increases plasma catecholamine levels. These

hormones stimulate cardiac muscle contraction and put even more strain on oxygen requirements.

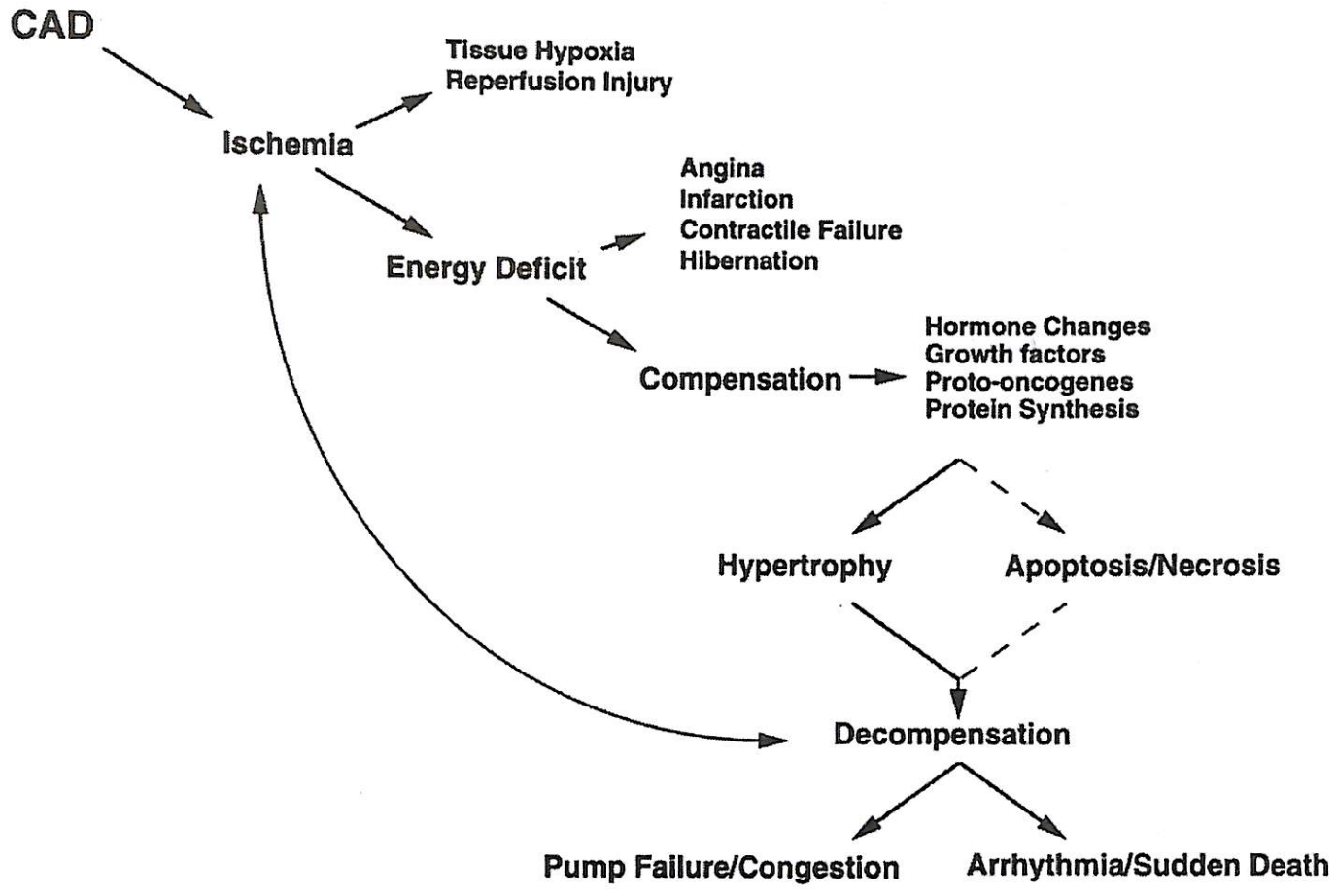

Fig. 1. Stages in the Progression of Ischemic Heart Disease

In addition to perpetuating oxygen deficits, the chronic elevation of catecholamines, ET-1, and AT-II may also play a role in the development of myocardial hypertrophy. Myocardial hypertrophy is a principal adaptive response of the heart to many kinds of stress, and frequently accompanies ischemic heart disease. Hypertrophic cardiomyopathy (HCM) (Marian and Roberts, 1995; Schwartz et al., 1995; Scwartz, 1995) and CAD associated cardiomyopathy may share common initiating signals and developmental pathways (see references listed above for reviews). In both cases, the initiating stimulus appears to be circulatory insufficiency caused by reduced contractility. Although the mechanisms for translating a contractile deficit into a growth response are presently unknown, the end result is a dramatic stimulation of cellular biosynthetic pathways, increased myocyte mass, and extensive thickening of the left ventricular wall. During hypertrophy, certain myofilament genes are selectively activated, new sarcomeres are assembled, and individual cardiac myocytes enlarge and change shape. There are changes in the balance of muscle and non-muscle proteins, changes in ion transport channels, and changes in myocardial vascularity. Hypertrophy is a major component of so-called "myocardial remodeling", which refers to changes in the overall composition, architecture, and functioning of the heart following myocardial infarction and during the progression of congestive heart failure. In recent years, many of the molecular signals and targets of the hypertrophic process have been identified, and these regulatory pathways constitute possible new targets for therapy.

To the degree that adaptive mechanisms are successful, a period of adequate compensation follows in which the combination of neurohumoral support and increased cardiac mass produce an improvement in cardiac output.

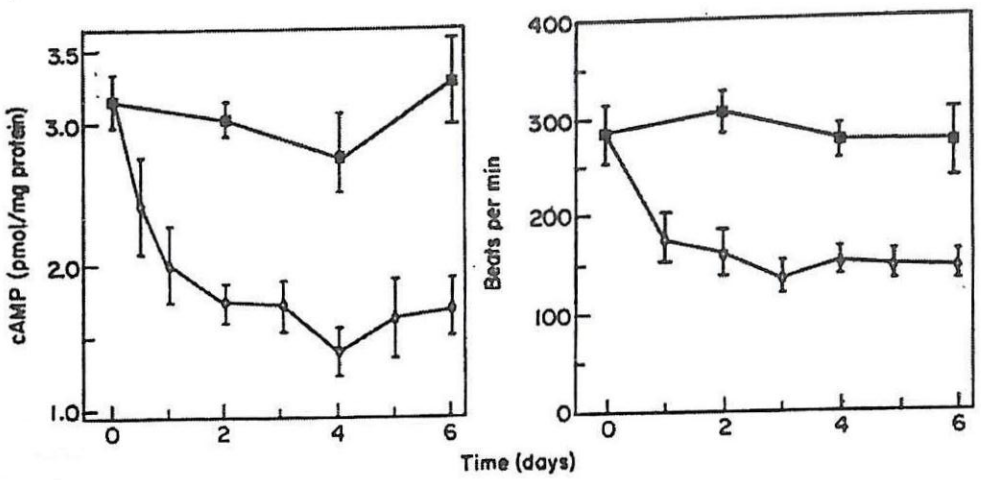

Fig. 2. Changes in cAMP and Contractility of Heart Cells under Chronic Hypoxia.

Heart cell cultures were exposed to atmospheric oxygen, pO_2=160mmHg, or hypoxia, pO_2=48mmHg five to seven days after isolation as described in ref (Webster and Bishopric, 1992). Cyclic AMP measurements are the result of duplicate determinations from six separate experiments. Contraction and motion characteristics were measured using a computerized motion analysis edge detection system as described previously (Webster and Bishopric, 1992). During this period, the remodelling process is accompanied by several membrane-associated changes that may critically affect cardiac function. cAMP production is depressed (Feldman et al., 1987; Neumann et al., 1988; Chen et al., 1991), ,β-adrenoreceptors are down-regulated (Gwathmey et al., 1987; Barnett, 1991), sarcoplasmic reticulum calcium ATPase (SERCA) expression and function are depressed, and sodium-calcium exchanger activity increases (reviewed in (Arai et al., 1994; Wankerl and Schwartz, 1995)). Together, these changes reduce inotropic responsiveness, depress the response to sympathoadrenal stimulation, impair diastole (relaxation), and increase the probability of arrhythmia. The ability of further hypertrophy to strengthen the heart is limited, and eventually the system begins to collapse; the underlying ischemia still persists, blood pressure may remain high, and the hypertrophied muscle itself creates physical and bioenergetic problems. Ultimately, adaptive resources are exhausted. Death may result either from arrhythmia or gradual pump failure, with approximately equal probability.

Pharmacology

Heart failure therapy is targeted at both the heart and the vasculature. Approaches to prevent, treat, or reverse CAD include lifestyle modulations, drug combinations, angioplasty, and surgery. These have been reviewed recently and extensively elsewhere (Gibbons and Dzau, 1996; Swynghedauw and Camm, 1994; Wickelgren, 1996; Wickelgren, 1996; Mcnamara, 1995). Goals of current therapy are to improve function and prevent further damage. Since contractile insufficiency due to muscle damage is a principal causes of death in CHF, effective treatment must not only improve organ function by increasing contractile output and efficiency, but must ultimately stem further myocardial tissue loss.

A number of pharmacological approaches have been used to improve cardiac functions, with varied but limited success. The only inotropic agent widely used for chronic CHF therapy is digitalis. It has been used for at least 2 centuries, and works by inhibiting Na^+/Ca^{2+} exchange, thereby increasing intracellular calcium (reviewed in (Taylor, 1996; Gheorghiade, 1996). Digitalis is mildly inotropic *in situ*, with a very narrow therapeutic index. Recent multiphase trials (DIG) demonstrated the positive impact of digitalis therapy on quality of life and no significant effect on mortality. Angiotensin converting enzyme (ACE) inhibitors and nitrates work at least in part by dilating peripheral blood vessels, reducing blood pressure and relieving cardiac work load (reviewed in (Swynghedauw and Camm, 1994)). Calcium channel antagonists and 5-adrenoreceptor blockers (5-blockers) may help reduce arrhythmias and control blood pressure. Chronic blocker therapy may exert additional beneficial actions, possibly related to the preservation of active adrenoreceptors, although these effects have not been fully explained (Barnett, 1991; Feurstein and Ruffolo, 1996; Poole-Wilson, 1996). Combined therapy using ACE-I,

digoxin and diuretics is standard practice; unfortunately, the overall impact on mortality is minimal (see (Taylor, 1996) for reviews). To date, only ACE inhibitors have been convincingly demonstrated to have beneficial effects on both the quality of life and mortality in congestive heart failure. Calcium channel antagonists have no beneficial effects in CHF, although some agents in this class appear to be safe for use in the treatment of CHF-associated hypertension and angina (Poole-Wilson, 1996). Some promising newer agents with ß-blocking functions may eventually be incorporated into the standard treatment regimens (Taylor, 1996).

Apart from digoxin, success with positive inotropic drugs has been limited. Two approaches to inotropic therapy are relevant in the context of this discussion. The phosphodiesterase-3 inhibitors (PDIs), of which amrinone and milrinone (Sterling Pharmaceuticals) are prototypes, improved contractility and peripheral vascular resistance in short-term clinical trials, but significantly increased mortality during chronic use (Wetzel and Hauel, 1988; Monrad et al., 1986; Cottney et al., 1990; Packer, 1991). These drugs act by selectively repressing cardiac and smooth muscle phosphodiesterase type III activity. The suppression of this enzyme raises cAMP levels, resulting in increased cAMPdependent protein kinase A (PK-A) and myosin light chain kinase (MLC-K) activity . The immediately beneficial consequences of PDIs are thus (1) increased contractility due to PKA-mediated phosphorylation of cardiac calcium channel proteins and increased intracellular calcium, and (2) peripheral vasodilation due to phosphorylation of smooth muscle MLC, which causes smooth muscle relaxation.

The disappointing outcome of clinical trials of these agents, despite their promising short term results, has alerted investigators to the complexity of heart failure pathophysiology, as well as to the dangers inherent in increasing bioenergetic expenditure in this setting. A newer class of inotropic agent works by enhancing the sensitivity of myofilament proteins to available intracellular calcium (Ruegg and Morano, 1989; Keane et al., 1990; Hajjar and Gwathmey, 1991) These drugs include pimobendan (Boehringer Ingleheim), EMD 53998 (E. Merck-Darmstadt), and levosimendan (Orion Pharmaceuticals Inc.) all of which have differing amounts of intrinsic PDI activity in addition to calcium sensitizing properties. Calcium sensitizers may have a unique ability to strengthen the heart in a bioenergetically favorable manner (Lee and Allen, 1990; Ferroni et al., 1990; Lee et al., 1989; Kubo and et al, 1992; Haikala et al., 1992) without significantly increasing intracellular calcium or cAMP. However, their clinical efficacy has yet to be established.

Further progress in heart disease research will require the identification of new molecular targets and may involve gene therapy techniques (reviewed in (Prentice and Webster, 1995)). By analyzing models of heart disease at the cellular and molecular levels, we are beginning to understand the signaling pathways, genes, and proteins that determine the responses of cardiac myocytes and vascular cells to extracellular stresses, including pressure and tension changes, ischemia, reperfusion, and hypertrophy. One unifying feature of the responses to these stimuli is the modulation of the activities of protein kinases. In the remaining part of this communication we will review recent work demonstrating critical roles for three separate protein kinase pathways in the transmission of myocardial stress responses.

Models of Heart Disease

Although there is a wealth of information on the clinical aspects of both acute and chronic ischemia, the molecular control mechanisms and cellular responses to ischemia are poorly understood. This is due in part to the limited availability of animal models of heart failure and the technical and ethical limitations of performing molecular analyses on whole animals and humans. In our molecular approach to analyze signaling pathways and gene expression we have developed cellular models of ischemia using isolated cardiac myocytes. We present here three models that represent different ischemia syndromes (1) a model of chronic myocardial hypoxia (Webster and Bishopric, 1992) with parallels to chronic ischemia (Carbajal and Deedwania, 1991) and hibernating myocardium (Braunwald and Rutherford, 1986; Rahimtoola, 1989) where the key change involves a depression of protein kinase A activity; (2) a model of acute cardiac arrest involving severe hypoxia, glucose depletion, and contractile failure (Webster et al., 1993; Webster et al., 1994; Webster et al., 1993), which is accompanied by increased protein kinase C activity and nuclear accumulation of Fos and Jun family proteins; and (3) a model of ischemia and reperfusion in which myocytes are subjected to cycles of hypoxia and reoxygenation, causing a strong activation of stress activated protein kinases (SAPK/JNKs) (Laderoute et al., 1996; Laderoute and Webster, 1997).

(1) Chronic Model of Myocardial Hypoxia

Contractile insufficiency and malfunction are integral features of myocardial ischemia and end stage heart failure. At least four separate mechanisms have been proposed to account for these: (1) decreased sensitivity of myofilament contractile proteins to calcium resulting from waste metabolite build up, in particular increased acidosis, inorganic phosphate, and adenosine in the ischemic tissue (Allen and Orchard, 1987; Allen et al., 1989; Ruegg and Morano, 1989), (2) failure of the action potential and calcium transient due to depletion of energy reserves (Stern et al., 1988), (3) changes in the turnover rates of high energy phosphates (Bittl et al., 1987; Weiss et al., 1989; Marshall, 1988), and (4) depressed adrenergic responsiveness due to changes in adenine cyclase regulation, receptor activity and regulatory proteins Gs and/or Gi. While each of these factors may regulate contractility within a particular set of conditions, many studies have found poor correlation between loss of contractility and changes in any of these parameters, suggesting that other regulatory mechanisms and oxygen sensors must exist (Matthews et al., 1986; Downing and Chen, 1990; Lodge and Gelband, 1988; Smith et al., 1982).

In our studies, to simulate conditions of chronic low flow hypoxia in vitro, cardiac myocytes were isolated from neonatal rat hearts, cultured by standard procedures (Webster and Bishopric, 1992), and exposed to a PO_2 of approximately 5 mm Hg with 5% CO_2 at 37°C in a self contained environmental chamber (described in (Webster and Bishopric, 1992)). The chamber is equipped with continuously recording microscope, oxygen electrode, and pH electrode. To prevent metabolic buildup or substrate depletion, a high volume of buffered medium was added to the cells initially (12ml per 60mm plate), and replenished with pre-equilibrated, deoxygenated medium every 8-lOh. Under these conditions the lactate concentration never exceeded 3mM, glucose concentration remained > 5 mM, and pH was maintained at 7.0 ± 0.2. Contraction frequency and motion characteristics were determined by periodic microscopic examination of cultures with a 40x phase objective and CCTV camera (Hitachi). Images were stored on videocassette and analyzed by computerized motion edge analysis. Our methods for calcium, gene expression, cAMP, and ATP analyses have been described previously (Webster and Bishopric, 1992). Under these conditions the cardiac myocyte cultures could be maintained for several weeks without visual deterioration or significant leakage of creatine kinase. These conditions are similar to chronic ischemia or hibernating myocardium in that cell viability is preserved in the face of diminished oxygen supply.

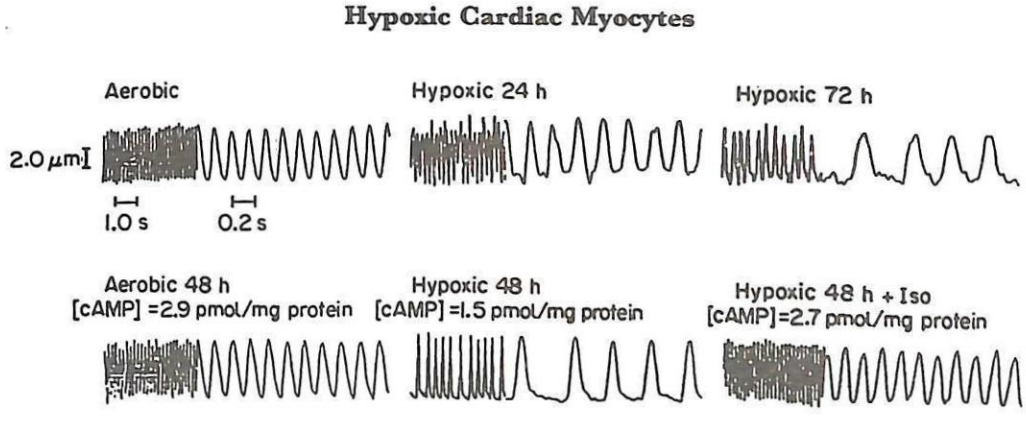

Fig. 3. Motion Characteristics and Effect of Isoproterenol

Analyses were made as described above in Fig 2, traces are typical of myocyte membrane motions at the different time points during hypoxic exposure. Routinely, areas of maximum motion were selected for analysis in both aerobic and hypoxic cultures. Concentrations of cAMP indicated on the Figure were measured in parallel duplicate plates before and after addition of isoproterenol. Note irregular motion in hypoxic cultures.

Under these conditions of simulated low flow ischemia, we monitored and correlated changes in contractility, ATP, cAMP, calcium flux, and gene expression at intervals over a 5 day period. The changes in ATP were small owing to the replacement of phosphorylation by glycolysis; glycolysis increased by 8 fold within hours of exposure to hypoxia, and remained elevated for the duration of the hypoxic period. Changes in the other parameters are shown in Figs 2 -4. The principal effects of chronic hypoxia were (1) a > 50% fall in intramyocyte cAMP (Fig. 2a); (2) a gradual decrease in the beating rate, (Fig. 2b); (3) slower sequestration of calcium during muscle relaxation (diastole) resulting in a longer, irregular shaped transient (Fig. 3); reversibility of these effects by isoproterenol treatment (Fig. 4); (4) changes in the expression of PK-A regulated gene promoters, and (5) induction of glycolytic enzyme genes (Fig 5). All of these parameters appeared to be causally related through depressed PK-A activity. The effects of chronic hypoxia on contractility and calcium transients were rapidly reversed by adding the ~adrenergic agonist, isoproterenol, which activates adenyl cyclase and re-established aerobic cAMP levels. Hypoxic induction of transfected cAMP-dependent promoters was reversed by adding cAMP inducers such as forskolin or phosphodiesterase inhibitors (Fig. 5). These results indicate that in the chronically hypoxic myocyte, cAMP levels drop gradually, with corresponding changes in signal transduction and gene expression. This can be envisioned as part of an adaptive-survival response that preserves energy through PK-A-mediated depression of contractility and induction of anaerobic metabolism. That these effects promote cell survival under hypoxia was indicated by the lethality of phosphodiesterase inhibitors and cAMP agonists in hypoxic but not normoxic myocytes (see ref. (Webster and Bishopric, 1992)). Thus, depressed cAMP may be an important adaptation for cardiac myocytes to survive extended periods of low flow hypoxia, as may be the case in hibernating myocardium (Cohen et al., 1988; Flameng et al., 1981; Spirito and Maron, 1990; Krayenbuehl and Hess, 1992).

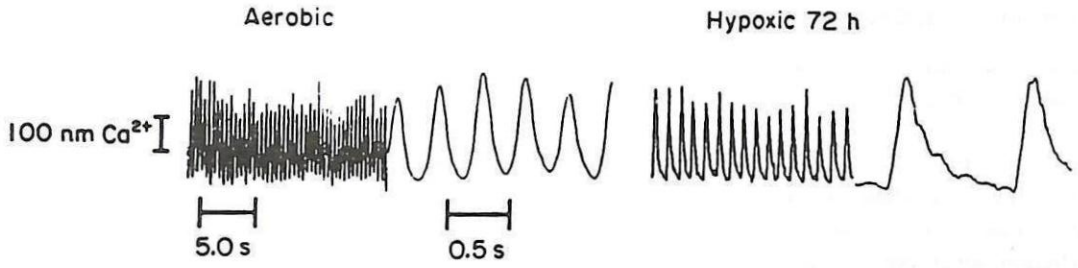

Fig. 4. Hypoxia Mediated Effects on Calcium Flux

Calcium flux was measured using an ACAS 470 Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI) after incorporation of the calcium selective fluorescent dye indo1 (Webster and Bishopric, 1992) and calibration as recommended by the manufacturer. Cells were grown in cover-slip diches (Nunc, Inc., Naperville, Il) and loaded with 20pLg/ml of indo-l for 2h immediately prior to analysis. Indo- 1 loading efficiency was not affected by oxygen conditions. After loading, dishes were sealed in the prevailing environment and transfered to the cytometer stage. The compartment was maintained at 37ºC and flushed continuously with the appropriate gas mixture. Multiple point scans were taken on cells incubated under aerobic or hypoxic atmospheres. Traces show representative point scans with two time scales on a 72h aerobic culture and after 72h under hypoxia.

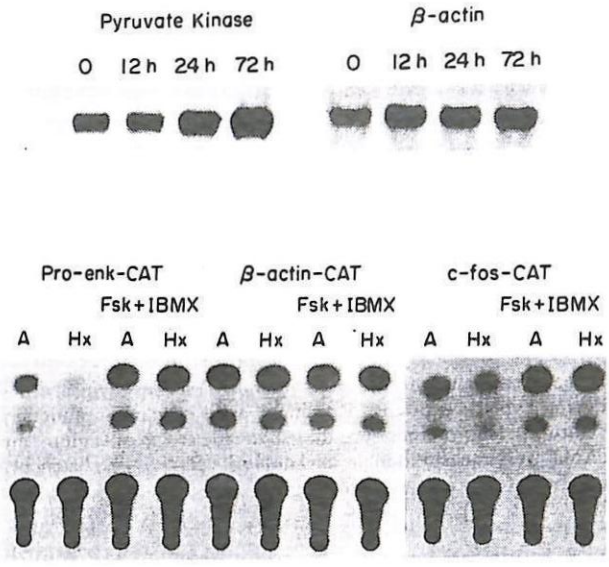

Fig. 5. Effects of Hypoxia on Gene Expression in Isolated Cardiac Myocytes.

Top panel: total RNA was isolated from hypoxic (pO_2=4mm Hg) cultures of cardiac myocytes at the times indicated, blotted and probed as described in reference (Webster and Bishopric, 1992). Bottom panels: transient expression of the transfected promoters linked to the CAT gene was assayed using equal amounts of protein also as described in reference (Webster and Bishopric, 1992).

(2) Acute model of cardiac arrest

Acute, severe ischemia results when a coronary artery or capillary becomes completely occluded. This condition causes rapid contractile failure of the affected tissue and is accompanied by hypoxia, nutrient depletion, and waste metabolite build up, especially inorganic phosphate and lactic acid. Severe ischemia, when prolonged more than a few minutes, results in infarction of the affected region, and protracted global myocardial dysfunction.

To simulate acute ischemia in vitro, spontaneously contracting, confluent cultures of cardiac myocytes were incubated under a PO_2 of <4 mm Hg with a shallow covering of medium (minimal essential medium (GIBCO) with 5% fetal calf serum). The medium contained 3-4 mM glucose, in the low physiological range. As in the chronic hypoxia model (Webster and Bishopric, 1992), glycolysis is induced; a key feature of this model is progressive glucose depletion, as with ischemic tissue *in situ* (Allen and Orchard, 1987). Under these conditions, glucose was consumed at a rate of 2.3 +/- 0.4 llmol/min./10^6cells, about 8 times faster than glucose consumption by parallel aerobic cultures (Webster et al., 1993). There was no significant change in medium pH during the relatively brief period of exposure to hypoxia used in these experiments.

Under these conditions of simulated acute severe ischemia, contractility began to decrease immediately after the cultures became hypoxic, and continued gradually to complete contractile failure at 3 to 4 hours (Fig 6 a&b). Changes in contractility were apparent before glucose was depleted, and before there was any significant change in ATP (see Fig 7). Contractile failure involved a progressive decrease in the amplitude of contraction without significant change in the beating frequency (Fig 5a). These effects on contractility have been described previously in isolated cardiac cells and tissues and in whole hearts subjected to hypoxia or ischemia (Allen and Orchard, 1987; Stern et al., 1988; Matthews et al., 1986; Downing and Chen, 1990). Contractile failure was reversible under the conditions described here; recovery was complete within lh when the cells were reoxygenated within 4h of hypoxia (n=6). Although a number of factors may contribute to the loss in contractility (Stern et al., 1988; Allen and Orchard, 1987), the combination of hypoxia and low glucose was critical because neither condition alone mediated the effect. It seems probable that an "emergency" response is triggered when the cellular energy reserve is threatened by blockade of both oxidative and glycolytic energy pathways. Reducing contraction is an obvious means to conserve energy, and other

cellular and biochemical energy conserving measures are probably implemented at the same time. The receptors and signaling pathways that promote these responses are not known, but the synergy between oxygen and glucose starvation suggests that cross-talk exists between the two stress response pathways.

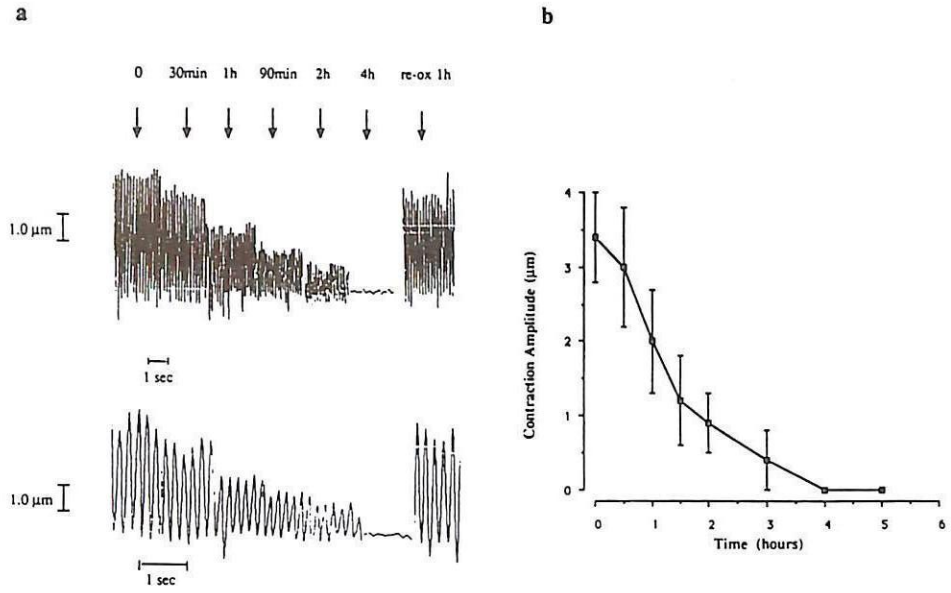

Fig. 6. Loss of contractility of cardiac myocytes during exposure to hypoxia Cardiac myocytes were exposed to hypoxia and low glucose as described in Reference (Webster et al., 1993). (a) shows the contractility changes recorded from a single myocyte and is representative of a typical response. (b) includes measurements from four experiments with four seperate myocyte preparations. A decline in contractility was apparent within the first 30 min of exposure to hypoxia and complete failure occurred within 4 h. There was no apparent decrease in the frequency of contraction until failure.

As one approach to identify possible components of this stress response, we analyzed changes in immediate early gene expression and protein kinase activity during the period of contractile failure. mRNA transcript levels of *c-fos* and *c-jun*, encoding components of the transcription factor APl,were elevated by 5-10 fold within the first hour of hypoxia (Webster et al., 1993). *c-fos* levels were maximal after 2h and began to decrease significantly after 4h, whereas *jun* transcript levels were sustained for at least 6 hours. All transcript levels returned to basal or lower within 12h. Intense Fos and Jun immunoreactivity was seen in the nuclei of cells subjected to hypoxia for 2 - 4 h, comparable to that associated with phorbol ester treatment. Severe, acute hypoxia thus induces rapid accumulation of Fos and Jun proteins in the nuclei of hypoxic cells, coincident with contractile failure

To investigate the signal transduction pathway for this response we tested the effects of selective kinase inhibitors on the hypoxia-mediated induction of fos and jun transcripts (Webster et al., 1993). The induction of both *c-fos* and cjun mRNAs was repressed by the protein kinase A-selective inhibitor KT5720 (Kamiya Biomedical), and essentially eliminated by the protein kinase C inhibitor staurosporine. It is likely that PK-A inhibition had its major effect on basal rather than on hypoxia-induced PKA activity. The potent inhibition of both *c-fos* and *c-jun* inductions by staurosporine suggests that protein kinase activation, including activation of PK-C, plays a role in the hypoxia mediated induction of these proto-oncogenes.

PK-C activation may have a number of effects on the cell in addition to protooncogene induction, including phosphorylation of calcium, potassium, and sodium channels, and phosphorylation of thin-filament regulatory proteins that control calcium sensitivity. Activation of PK-C could thus result in decreased responsiveness to calcium and hence decreased contractility (Gwathmey et al., 1987; Gwathmey and Hajjar, 1990). In our studies, the intracellular resting calcium concentration was 236 nM ± 5 under aerobic conditions, and 275 nM ± 14 following contractile failure under acute hypoxia, but this increase in intracellular calcium was associated with depressed rather than increased

contractility. Contractile failure was also accompanied by enhanced potassium efflux through a glibencamide-insensitive channel, as measured in rhubidium14l-loaded cells (data not shown). These results are consistent with a role for PK-C activation, possibly in combination with decreased intracellular pH, in the development of contractile failure in this model.

Taken together, these results suggest that conditions that simulate severe ischemia are accompanied by contractile failure, activated protein kinase C, strong transient inductions of *fos* and *Jun* transcription, and rapid accumulation of Fos and Jun (AP-1 complex) proteins in the myocyte nuclei. Contractile failure and induction of immediate early genes may be components of a stress response to hypoxia and low glucose. AP-1 has been implicated in many ischemia-associated stress responses (Gunn et al., 1990; Safirstein et al., 1990; Schiaffonati et al., 1990; Brand et al., 1992; Kindy et al., 1991) and probably has numerous target genes under its regulation. We have previously shown that AP-1 regulates the expression of the muscle specific skeletal a-actin gene (Bishopric et al., 1992), and others have demonstrated a similar activation of the atrial natriuretic peptide (ANP) gene (Schiaffonati et al., 1990; Sen et al., 1990) by AP-1. Both skeletal a-actin and ANP genes are activated during myocardial hypertrophy (Parker and Schneider, 1991). Therefore there may be a molecular link between ischemia and hypertrophy through the hypoxia-mediated activation of PKC, induction of AP-1, and the targeting of these to positive regulatory elements in the skeletal a-actin and ANP gene promoters.

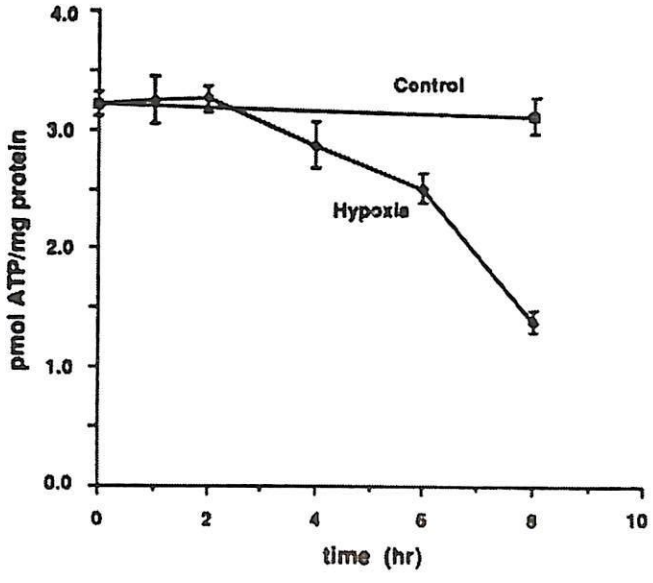

Fig.7. ATP levels during Hypoxia. ATP was measured in duplicate plates at each point as described in Reference (Webster et al., 1993). Bars represent S.E.M. (n=3).

(3) Model of ischemia and reperfusion

Intermittent myocardial ischemia imposes two extremes of redox stress on cardiac tissues. Hypoxic stress occurs during the ischemic phase, as discussed above, and oxidative stress results during reoxygenation, when the ischemic tissue is reperfused. During the ischemic phase, hypoxia triggers metabolic and ionic changes (Allen et al., 1989; Macleaod, 1989; Cascio et al., 1992), changes in cyclic nucleotide levels, protein kinase activities, and immediate early stress genes (described above and in references(Webster et al., 1989; Feldman et al., 1987; Webster et al., 1993; Neumann et al., 1988; Webster and Bishopric, 1992)). In addition, sustained hypoxia is associated with a decline in intracellular redox buffers (Werns and Lucchesi, 1990; Cowan et al., 1993). Restoration of blood flow induces the formation of reactive oxygen intermediates (ROIs) and suboxidative cell injury, exacerbated by the loss of redox buffering capacity. This reperfusion damage, including myocyte death due to both necrosis and apoptosis, can be limited by pre-exposure to antioxidants or to antibodies that block leukocyte adhesion (Gottlieb et al., 1994; Ma et al., 1993). ROIs damage cells directly by oxidizing cellular

components, and indirectly by activating inflammation(Satriano et al., 1993; Ivey et al., 1995; Galinanes et al., 1993). The relative contribution of reperfusion damage to the progression of ischemic heart disease has not been determined. Likewise, the signal transduction pathways for redox stress response in cardiac myocytes have not been defined.

Several recent reports indicate that signal pathways involving the stress- and mitogen-activated protein kinases (JNK/SAPK and MAPK/ERKs) may have important roles in the myocardial response to redox

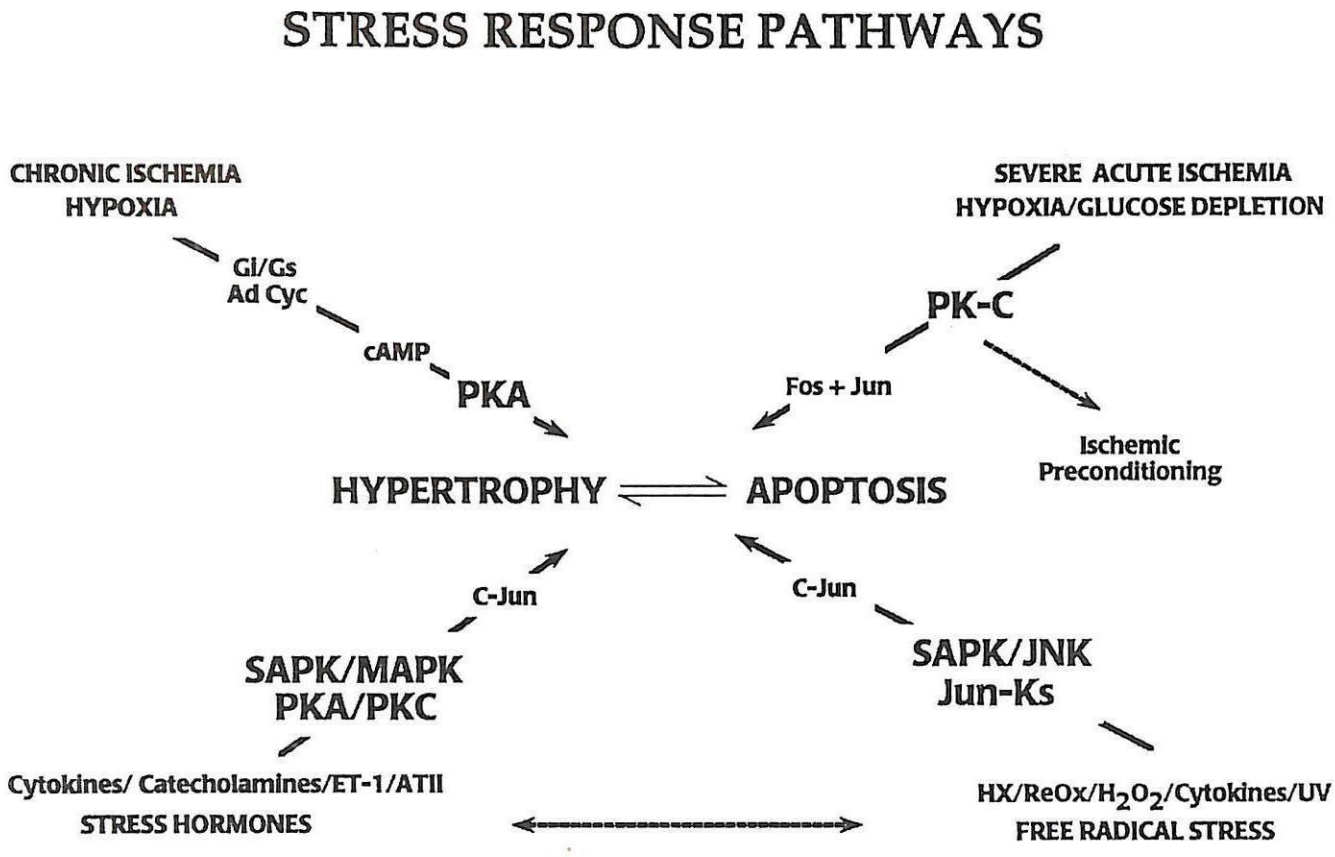

Fig. 8. Summary of Stress Responses and Second Messengers that Contribute to the progression of Myocardial Hypertrophy.

stress and hypertrophy. These pathways consist of multiple serine, threonine and tyrosine kinases that relay growth and stress-related signals from extracellular stimuli to the cell nucleus (reviewed in (Cano and Mahadevan, 1995; Karin, 1995; Sanchez et al., 1994; Kyriakis et al., 1994)). JNK/SAPKs were originally identified as serine/threonine kinases which phosphorylate the arnino terminal transactivation domain of the transcription factor c-Jun. MAPKs/ERKs are defined as mitogen activated protein kinases, or extracellular signal regulated kinases (designated here as MAPKs). Activation of these pathways through plasma membrane or cytoplasmic receptors results in the translocation of a terminal kinase to the cell nucleus. Once in the nucleus, the kinase modifies specific target proteins by phosphorylation, leading to changes in gene expression and other cell functions. Although there is cross-talk between the JNK/SAPK and MAPK pathways, they are activated by different extracellular stimuli, are subject to different regulatory mechanisms, and involve different intermediate kinases and terminal target proteins. Unlike MAPKs, the JNK/SAPKs are characteristically activated by survival-threatening stresses, such as protein synthesis inhibitors, inflammatory cytokines, ultraviolet irradiation, osmolarity changes, sodium arsenite, okadaic acid, muscarinic receptor stimulation, and heat shock (reviewed in (Cano and Mahadevan, 1995; Moriguchi et al., 1995)). A number of stimuli induce both pathways, albeit with different potencies and kinetics, and both pathways can be induced by activated Ras (Westwick et al., 1994). MAPK and JNK/SAPK signaling pathways are

probably involved in coordinating growth and/or repair responses in the nucleus, cytoplasm, and cytoskeleton and may in some instances be involved in the initiation of apoptosis (Bagrodia et al., 1995; Xia et al., 1995; Johnson et al., 1996; Verheij et al., 1996).

The activation of JNK/SAPKs appears to be closely linked with redox events. The pathway is potently activated by treatment of cells with irradiation, H_20_2, and cytokines (Devary et al., 1992; Guyton et al., 1996; Verheij et al., 1996; Moriguchi et al., 1995; Lo. et al., 1996). In an experimental model of ischemia and reperfusion in the kidney the JNK/SAPK pathway was activated in a manner that correlated with ATP depletion (Pombo et al., 1994; Morooka et al., 1995). Modest and transient activations of cardiac myocyte MAPKs have been described in response to hypoxia and reoxygenation in neonatal rat, and release from metabolic inhibition in chick (Seko et al., 1996; Yao et al., 1995), but the initiating signals were not identified. Strong inductions of JNK/SAPK have been reported in ischemia-reperfusion models of perffused rat hearts (Knight and Buxton, 1996; Bogoyevitch et al., 1996) and our laboratory recently reported similar strong (10 -20 fold) inductions of JNK/SAPK in an in vitro model of hypoxia and reoxygenation (Laderoute et al., 1996; Laderoute and Webster, 1997). In these latter studies we demonstrated that the inductions were quenched by adding antioxidants and correlated with depressed intracellular levels of reduced glutathione. Therefore the JNK/SAPK cascade appears to be a component of ischemia and reperfusion at least in these experimental models. It is intriguing that transcription factor c-Jun is a target of the several different kinases that are activated during both acute hypoxia and hypoxia-reoxygenation. It seems likely that these signals form an early initiation event that results in hypertrophy (Webster et al., 1993; Glennon et al., 1995; Laderoute and Webster, 1997).

ABSTRACT

The salient features of this review are summarized in Figure 5, in which we compare the component kinases and protooncogenes of the signaling pathways initiated by redox and sympathetic stimuli. All these stimuli are likely to be involved in ischemic heart disease. Similar or related kinase pathways may be activated by two other hypertrophy mediators, angiotensin II and endothelin 1 (Bogoyevitch et al., 1993; Bodi et al., 1995; Ito et al., 1991; Paul and Ganten, 1992;). Contractility and hypertrophy are two critical parameters that may determine short and long term survival of the cardiac system in the face of ischemic stress. To preserve cell integrity there must be a balance between bioenergetic input and output; if ATP is used more rapidly than it is generated the cell will not survive (Hochachka, 1986; Hochachka et al., 1996a; Hochachka et al., 1996b). Therefore one of the immediate adaptive responses to ischemia is to modify contractility in conformity with the reduced ATP generating capacity. "Hibernating myocardium" may represent a clinical example of this type of contractile adaptation; this term refers to an area of underperfused, non-contracting heart muscle that recovers activity when the oxygen supply is restored. In this situation, reduced contraction may preserve myocyte survival for an extended time period under chronic ischemia. Our model of chronic hypoxia implicates cAMP as a possible mediator of this condition (Webster and Bishopric, 1992). Another example of reversible contractile failure occurs in the acute severe hypoxia model. In this model of low-flow ischemia, contractile failure is associated with sustained ATP levels and prolonged cell survival (Webster et al., 1993). and is reversible on replenishment of oxygen or glucose. The oxygen sensor(s) that initiate contractile failure in this situation not been identified. While reduced contractile activity may be an adequate short term solution to ischemia for the affected myocytes, the resulting depressed cardiac output may be inadequate to sustain necessary functions. Circulatory compromise brings about the secondary activation of neurohormonal systems whose net effects are to(l) increase cardiac output and (2) induce long-term trophic responses in the cardiac muscle.

While many components of the stress response pathways are still unidentified, including -the oxygen sensors, it is clear that protein kinases A, and C, and the JNK/SAPK pathways are important intermediates in the regulation of both contractility and hypertrophy in response to ischemic stress. It has been recognized for some time that sympathoadrenal stimulation through norepinephrine release regulates both contractility and hypertrophy (Bishopric et al., 1989; Bishopric and Kedes, 1991; Bishopric et al., 1992; Neyses and Pelzer, 1995; Glennon et al., 1995). Protein kinase C and A and associated alterations of IP_3 and calcium handling are likely to be directly involved in regulating contractility (Feldman et al., 1987; Gwathmey et al., 1987; Hajjar and Gwathmey, 1991; Gwathmey and Hajjar, 1990; Webster et al., 1993). Contractility is altered directly by changes in the calcium transient, as well as by changes in the phosphorylation state of specific proteins that

modify ion transport and myofiber reactivity (Garvey et al., 1988; Lee and Allen, 1990; Ruegg and Morano, 1989; Hajjar and Gwathmey, 1991; Sen et al., 1990). The latter include phospholambam, L-type calcium entry channel, SR proteins, and troponins (Garvey et al., 1988; Arai et al., 1994; Wankerl and Schwartz, 1995). Activation of AP- 1 by norephinephrine and other factors may be a critical initial event in the coordinated trophic response that results in myocardial hypertrophy. AP- 1 transactivates several hypertrophy-associated genes, including skeletal a-actin (Bishopric et al., 1992). Therefore a simplified schema for the induction of ischemia-associated myocardial hypertrophy contains five steps: (1) onset of a contractile deficit (2) secondary neurohumoral activation (3) activation of intracellular protein kinases A and C by neurohumoral factors, including norepinephrine and angiotensin II (4) induction and nuclear accumulation of immediate-early gene products, including transcription factor AP1 and (5) transcriptional activation of hypertrophy associated genes, including skeletal a-actin and ANP.

Our results demonstrate that hypoxia activates the same protein kinases and protooncogenes as those activated by sympathoadrenal stimulation. From this, it can be proposed that hypoxia induces hypertrophy through these same signal transduction pathways. In the same way, cycles of hypoxia and reperfusion may also induce hypertrophy via redox-activated pathways; in this case the activation of PKC during hypoxia and of JNK/SAPK during reoxygenation may each contribute to the induction of hypertrophy. The hypertrophy that accompanies ischemic cardiomyopathy *in vivo* may thus be the end result of signals from three different redox stress response pathways in addition to those mediated by sympathoadrenergic stimulation.

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