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MITOGENIC EFFECT OF SUBSTANCE P ON CARDIAC FIBROBLASTS: A NEURAL PATHWAY OF ACTIVATION OF CARDIAC CONNECTIVE TISSUE GROWTH

submitted by

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CERTIFICATE

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This is to certify that Sri Kumaran C in the Division of Cellular and Molecular Cardiology of this institute has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 011, India. The work relating to his thesis entitled *"Mitogenic Effect of Substance P on Cardiac Fibroblasts: A Neural Pathway of Activation of Cardiac Connective Tissue Growth"* was carried out under my direct supervision.

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K. C.

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The cardiac system represents one of the most exciting challenges to human ingenuity. Critical to our survival, it consists of a tantalizing array of interacting phenomena, from ionic transport, membrane channels and receptors through cellular metabolism, energy production to fiber mechanics, microcirculation, electrical activation to the global, clinically observed, function, which is measured by pressure, volume, coronary flow, heart rate, shape changes and responds to imposed loads and pharmaceutical challenges. It is a complex interdisciplinary system requiring the joint efforts of life sciences, the exact sciences, engineering and technology to understand and control the pathologies involved.

Sideman S and Beyar R, 1993

MITOGENIC EFFECT OF SUBSTANCE P ON CARDIAC FIBROBLASTS: A NEURAL PATHWAY OF ACTIVATION OF CARDIAC CONNECTIVE TISSUE GROWTH

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MITOGENIC EFFECT OF SUBSTANCE P ON CARDIAC FIBROBLASTS: A NEURAL PATHWAY OF ACTIVATION OF CARDIAC CONNECTIVE TISSUE GROWTH

For a long time, defective myocyte function was believed to be solely responsible for cardiac failure and very little attention was focused on the contribution of fibroblasts to the structural and functional integrity of the myocardium although they represent two-thirds of the myocardial cell population. Terminally differentiated myocytes lose their replicative ability soon after birth, but fibroblasts retain their ability to proliferate in response to humoral and mechanical stimuli even in the adult heart. Consequently, cardiac fibroblasts play a central role in wound healing and myocardial remodeling under various pathological conditions. Modulation of cardiac fibroblast function has therefore attracted increasing attention in the past decade. Identification of a renin-angiotensin system (RAS) in cardiac fibroblasts paved the way to extensive studies on the effects of angiotensin II on these cells. The emphasis on RAS and its role in fibrogenesis has, however, nearly precluded investigations on the possible involvement of other growth factors in regulating myocardial fibroblasts.

The discovery that the myocardium is innervated not only by cholinergic and adrenergic nerves but also by peptidergic nerves that synthesize and secrete neuromodulatory peptides, such as substance P (SP), raises the possibility that

these neuropeptides may modulate myocardial metabolism under normal and/or pathological conditions. Substance P, a potent peripheral and coronary vasodilator, is reported to be involved in inflammation, tissue repair and fibrosis. More recently, it has been shown that SP acts as a trophic agent in certain cell types, such as arterial endothelial cells and skin fibroblasts. Although SP is known to be released from nerve endings in the heart under pathological conditions such as hypoxia and ischemia, no attempt has been made to understand its action on cardiac cells. Moreover, as SP immunoreactivity is reported to be marked in the connective tissue of the heart, myocardial fibroblasts may be particularly susceptible to its action. It is surprising therefore that regulation of fibroblast function by SP has not hitherto been investigated.

The present study had three objectives: 1) to set up an *in vitro* model of cardiac fibroblasts from adult rats in culture, 2) to test the hypothesis that SP is a stimulus for cardiac fibroblast proliferation and to delineate the underlying mechanism(s), with focus on the possible involvement of calcium and superoxide anion, and 3) to probe the role of substance P in the activation of cardiac fibroblasts in magnesium deficiency, based on observations that magnesium deficiency produces significantly higher circulating levels of substance P and blockade of the SP receptor attenuates the cardiac lesions of magnesium deficiency.

Isolation of myocardial fibroblasts from adult rats. Cardiac fibroblasts were isolated from adult Sprague-Dawley rat ventricles following repeated digestions of the ventricular tissue with an enzyme cocktail consisting of collagenase, pancreatin and trypsin. The cells were pre-plated for 150 minutes when the fibroblasts adhered to the culture dish leaving other contaminating cell types of the heart in the supernatant. The fibroblastic nature of the cells was confirmed by immunocytochemistry using monoclonal antibodies against vimentin, a fibroblast-specific cytoskeletal protein, desmin and factor VIII.

SP exerts mitogenic action on adult cardiac fibroblasts. Exposure of cells to SP, at 10 μmol/L, for 24 hours increased incorporation of [^{3}H]-thymidine into DNA in these cells significantly. To ascertain whether the increase in DNA synthesis with SP reflects a proliferative response, cell count was performed following treatment with SP. Further, to determine the concentration range over which SP exerts mitogenic effect on adult cardiac fibroblasts *in vitro*, the dose-response was studied. Even a dose of 1 nmol/L SP produced a small but significant increase in cell number. Increasing the concentration of SP from 1 nmol/L to 10 μmol/L caused a progressive increase in cell number. A concentration of 10 μmol/L SP was chosen for further experiments.

SP does not influence total protein synthesis and net collagen production. In order to determine if SP has any effect on total protein synthesis, the incorporation of [^{3}H]-phenylalanine into proteins was measured in confluent cultures of adult cardiac fibroblasts. SP had no effect on total protein synthesis at 20 hours, suggesting that SP does not exert a hypertrophic action on these cells. This was confirmed by a lack of effect of SP on cell protein content per dish.

As cardiac fibroblasts are the main source of collagens, the effect of substance P on net collagen production (collagen deposition) was determined. The cell monolayer and medium were pooled up and used for determination of collagen-associated hydroxyproline content. Results showed that treatment of adult cardiac fibroblasts with 10 μmol/L SP does not alter net collagen production by these cells.

SP enhances superoxide production in cardiac fibroblasts. Consistent with its postulated role in tissue injury and repair, SP has been shown to promote superoxide production in certain cell types. Therefore, experiments were designed to ascertain if SP increases endogenous superoxide production, measured in terms of nitroblue tetrazolium reduction, in cardiac fibroblasts and to examine whether superoxide mediates the mitogenic action of SP on cardiac fibroblasts. SP was found to significantly increase endogenous superoxide generation in confluent

cardiac fibroblasts while in proliferating cells, the increase was even more marked. The effect was not dependent on extracellular calcium.

Superoxide anion mediates the mitogenic action of SP on cardiac fibroblasts. Although SP increases superoxide generation in certain cell types, such an effect has not been shown to be linked to a mitogenic response. In the present study, the anti-oxidants, N-acetyl cysteine and superoxide dismutase, abolished the increase in cell number in response to SP, suggesting that SP-induced cellular hyperplasia may be mediated by reactive oxygen species.

SP enhances calcium uptake by cardiac fibroblasts. SP enhanced ^{45}Ca influx into cardiac fibroblasts by 21% at 30 min and 170% at 60 min. In the untreated cells, there was a linear increase in ^{45}Ca uptake upto 30 min followed by a dip whereas in the SP-treated cells, the dip was not observed so that the difference between control and SP-treated cells was much higher at 60 min.

Calcium is involved in the mitogenic action of SP on cardiac fibroblasts. SP is reported to stimulate hydrolysis of phosphatidyl inositol 4, 5-bisphosphate (PIP_2) with resultant changes in calcium homeostasis in some cell types. It was hypothesized that calcium may be involved in the expression of SP effects on cardiac fibroblasts. When extracellular calcium was blocked by EGTA, the SP-induced increase in [^3H]-thymidine incorporation was abolished. This, in

conjunction with the increase in ^{45}Ca uptake in response to SP, suggested a role for calcium influx in mediating the mitogenic action of SP.

SP acts via the NK-1 receptors on cardiac fibroblasts. Spantide, an NK-1 receptor antagonist, attenuated the increase in cell number in response to SP, showing that SP acts via NK-1 receptors on cardiac fibroblasts. Spantide was also found to inhibit the stimulatory effect of SP on superoxide generation, confirming the involvement of NK-1 receptors in mediating this response.

The data, taken together, suggest that substance P may function, via NK-1 receptors, as an activator of a hyperplastic but not hypertrophic response in adult cardiac fibroblasts and that alterations in redox state and calcium homeostasis may act in concert to mediate its mitogenic action.

Substance P and magnesium deficiency. It has been reported by Weglicki *et al* that circulating substance P levels increase in the first week of magnesium deficiency. The authors hypothesized that SP might be involved in neurogenic inflammation which triggers inflammatory responses in magnesium-deficient animals, with subsequent free radical production and lesion formation.

Serum from rats, magnesium-deficient for 6 days, had a marked stimulatory effect on superoxide production in and proliferation of cardiac fibroblasts in culture. Anti-oxidants, N-acetyl cysteine and superoxide dismutase, abolished the

serum-induced enhanced proliferative response in cardiac fibroblasts suggesting that the increased cellular proliferation is mediated by superoxide anion. Further, serum from magnesium-deficient rats had a significant stimulatory effect on net collagen production in cardiac fibroblasts. The increase in superoxide and proliferation with serum from magnesium-deficient rats but not from magnesium-sufficient rats was partially inhibited by the NK-1 receptor antagonist, spantide. The observations suggested that NK-1 receptor-mediated pathway may, at least partly, activate cardiac fibroblasts and contribute to cardiac fibrosis in magnesium deficiency. A role for other factors in cardiac fibrogenesis associated with magnesium deficiency warrants further examination.

Significance of the study

As stated earlier, factors that regulate fibroblast proliferation in the context of injury are yet to be identified. The work reported here suggests that SP might have important regulatory roles over cardiac fibroblasts. The present investigation revealed for the first time that SP, acting via the neurokinin -1 (NK -1) receptor, is a mitogenic stimulus for cardiac fibroblasts and that concerted increases in superoxide production and calcium influx may contribute to its mitogenic action. Further, the role of calcium in cardiac fibroblast function is a neglected area of research although calcium handling by myocytes has been studied extensively. The

present study suggests that calcium may play a significant role in mediating the actions of SP on cardiac fibroblasts.

The involvement of substance P in the pathogenesis of cardiomyopathic lesions in magnesium deficiency was also investigated. Magnesium deficiency produces cardiac fibrosis but the underlying mechanisms are unclear. A significant increase in the circulating levels of pro-oxidant and mitogenic factors, especially substance P, has been reported in a model of acute magnesium deficiency. Consistent with the hypothesis that these factors may activate cardiac fibroblasts, in the present study, serum from magnesium-deficient rats was found to have a more marked effect on fibroblast proliferation, net collagen production, and superoxide generation in these cells, compared to control. The increased proliferative response to serum from magnesium-deficient rat was mediated by superoxide anion. NK-1 receptors partly mediated the effect, showing the involvement of factors like substance P, that act via these receptors, in magnesium deficiency.

In summary, the findings have uncovered a new vista by pointing to a mechanism of paracrine regulation of cardiac interstitium by peptidergic neurons.

I. INTRODUCTION

I. 1. CELLULAR COMPONENTS OF THE MYOCARDIUM

The myocardium is composed of a myocytic compartment and a nonmyocytic compartment consisting primarily of interstitial tissue. Cardiac myocytes occupy 70-75% of the total volume of the myocardium, but account for only 33% of the cells present. The cells found in the nonmyocytic compartment of ventricular myocardium include mainly endothelial cells, vascular smooth muscle cells, and fibroblasts. These cells together constitute two thirds of the cell population of the heart of which more than 90% are fibroblasts (Brilla *et al*, 1992; Eghbali *et al*, 1992). Terminal differentiation of cardiac myocytes occurs at, or shortly after, birth in mammals while cardiac fibroblasts, residing within the interstitial space of the myocardium and the walls of arteries and veins, remain undifferentiated (Claycomb, 1992; Li *et al*, 1996; Soonpaa *et al*, 1996).

I. 2. CARDIAC INTERSTITIUM AND FIBROBLASTS

The cardiac interstitium, a complex 3-dimensional collagenous extracellular matrix, forms a scaffolding that plays an important role in maintaining the structural and functional integrity of the myocardium and produces mechanical synchronization throughout contraction and relaxation. The interstitium maintains, distributes, and stores mechanical forces during systole and plays a significant role in their redistribution during ventricular relaxation. Any deviation from the

normal extracellular matrix architecture, either due to an excessive deposition or degradation of collagens, may lead to abnormal functioning of the heart (Weber *et al* 1991; Brilla *et al* 1994).

Cardiac fibroblasts, the main cellular component of the interstitium, synthesize fibrillar collagens, types I and III, that are the chief components of the extracellular matrix (Weber *et al*, 1988; Bashey *et al*, 1992). They also produce matrix metalloproteinases (MMPs), enzymes that degrade collagen. Tissue inhibitors of matrix metalloproteinases (TIMPs) are also synthesized by fibroblasts (Weber *et al*, 1995). It is clear, therefore, that fibroblasts play a cardinal role in collagen turnover and myocardial interstitial homeostasis. Thus, contrary to the dogma that prevailed two decades ago, that improper functioning of the myocytic compartment alone is the basis of heart failure, it is now evident that mechanisms involving the myocardial interstitium, and regulation of myocardial fibroblasts, contribute to the development of cardiac failure (Weber *et al* 1991; Brilla *et al* 1994).

I. 3. CARDIAC FIBROBLAST ACTIVATION

Pathophysiological stimuli such as hemodynamic overload, myocardial infarction and myocarditis lead to myocardial remodeling, a cascade of events that includes myocyte hypertrophy and apoptosis, molecular phenotypic transformation

due to re-induction of fetal gene program and activation of cardiac fibroblasts and consequent alterations in the quantity and composition of the extracellular matrix which determines the progress of cardiac dysfunction (Colucci, 1997). During myocyte necrosis, fibroblasts mediate a *replacement fibrosis* that restores structural integrity of the tissue. In addition, alterations in hemodynamics may lead to the activation of cardiac fibroblasts resulting in enhanced collagen synthesis and disproportionate accumulation of fibrillar collagen (Chapman *et al*, 1990; Lee *et al*, 1999). This *reactive fibrosis* occurring in the absence of myocyte loss results in distorted tissue structure, increased tissue stiffness and electrically isolated myocytes. In such conditions, cardiac myocytes increase in size (hypertrophy), whereas cardiac fibroblasts increase in number (hyperplasia) and produce extracellular matrix proteins, such as collagens and fibronectins (Weber *et al*, 1988; Bashey *et al*, 1992). Activation of cardiac fibroblasts plays a central role in processes known to be associated with cardiac remodeling, a physiological process to restore normal cardiac functioning. Nevertheless, abnormal proliferation of cardiac fibroblasts with excessive accumulation of extracellular matrix proteins, as in left ventricular remodeling, leads ultimately to cardiac dysfunction (Weber *et al*, 1991; Brilla *et al*, 1994).

Recognition of the role of cardiac fibroblasts in maintaining extracellular matrix architecture led to the search for regulators of cardiac fibroblast function under normal and pathological conditions.

I. 4. REGULATORS OF CARDIAC FIBROBLAST FUNCTION

With the discovery of the cardiac renin-angiotensin system and receptors for angiotensin II on cardiac fibroblasts (Dostal *et al*, 1992a; Dostal *et al*, 1992b; Dostal and Baker, 1999), extensive investigations on the effects of angiotensin II on fibroblasts were undertaken. Angiotensin II stimulation of cardiac fibroblasts was found to induce hyperplasia and enhance production of matrix proteins such as fibronectins and collagens (Sadoshima and Izumo, 1993; Schorb *et al*, 1993; Sadoshima *et al*, 1995; Dostal *et al*, 1996; Simm and Diez, 1999). Moreover, angiotensin II is known to activate cardiac fibroblasts to produce factors that exert a paracrine effect on cardiac myocytes (Gray *et al*, 1998). The emphasis on the cardiac renin-angiotensin system and its role in fibrogenesis has, however, nearly precluded investigations on the possible involvement of other growth factors in regulating myocardial fibroblasts though a variety of growth factors, including neurotransmitters, hormones, and cytokines, are increased systemically and/or locally in the heart in response to several pathological stimuli. Many of these

agents are known to modulate fibroblast activity, including proliferation, migration, and extracellular matrix synthesis.

I. 5. SUBSTANCE P

Ischemia and hypoxia are known to stimulate cardiac peptidergic nerve endings to release the neuropeptide, substance P, which is suggested to play a role in the resistance of the myocardium to ischemia and reperfusion injury (Milner *et al* 1989; Ustinova *et al*, 1995). Substance P is a potent peripheral and coronary vasodilator (Christie *et al*, 1989; Nakamura *et al*, 1990; Yatani *et al*, 1990). A member of the tachykinin family (Pernow, 1983), substance P exerts its effects by binding to high affinity neurokinin-1 receptors (Nakanishi, 1991). It has been implicated in the regulation of several physiologic and pathophysiologic processes such as pain, smooth muscle contraction, and intestinal secretion (Nakanishi, 1991). Several *in vitro* and *in vivo* studies suggest a role for substance P as a pro-inflammatory agent. It is a chemoattractant for monocytes (Ruff *et al*, 1985; Wiedermann *et al*, 1989) and neutrophils (Wiedermann *et al*, 1989), a stimulator of macrophage phagocytosis (Bar-Shavit *et al*, 1980), and mast cell degranulation (Brain, 1997). Substance P induces the secretion of interleukin-1, interleukin-6, and tumor necrosis factor-α from monocytes and macrophages (Brain, 1997; Castagliuolo *et al*, 1997). *In vivo* studies show that substance P plays a pro-

inflammatory role in acute intestinal inflammation (Castagliuolo *et al*, 1998), pancreatitis (Bhatia *et al*, 1998; Shrikhande *et al*, 2001), and lung disease (Bozic *et al*, 1996). In addition, substance P acts as a growth-promoting factor for various cell types like endothelial cells (Villablanca *et al*, 1994), vascular smooth muscle cells (Nilsson *et al*, 1985; Payan, 1985), synoviocytes (Lotz *et al*, 1987), T-lymphocytes (Payan *et al*, 1983) and skin fibroblasts (Nilsson *et al*, 1985).

Though its presence in the heart is documented, no attempt has been made to elucidate its effects on cardiac cells. Light-microscopic autoradiograms revealed that substance P binding sites in the heart occur within clusters of connective tissue cells or in rarely observed parasympathetic ganglia (Walsh *et al*, 1996). No evidence was found to suggest the presence of substance P receptors on endothelial cells, cardiac muscle fibers or smooth muscle fibers. Substance P binding sites were observed on connective tissue cells in the heart, suggesting that cardiac fibroblasts may be particularly susceptible to its action.

I. 6. SUBSTANCE P IN MAGNESIUM DEFICIENCY

It has been known for a long time that magnesium deficiency produces a cardiomyopathy, characterized by focal necrosis and fibrosis (Bloom, 1988). Several lines of evidence, from this laboratory and elsewhere, support the view that oxidative injury may contribute to the cardiac lesions of magnesium deficiency

(Freedman *et al*, 1990; Weglicki *et al*, 1992a; Kramer *et al*, 1994; Kumar *et al*, 1997; Kumar and Shivakumar, 1997). Further, this laboratory has obtained evidence of significant alterations in rates of collagen turnover and fibroplasia in the heart which point to a wound healing response in the myocardium, possibly following oxidative damage (Kumar *et al*, 1997). Intriguingly, in this model of acute magnesium deficiency, while serum magnesium levels were significantly reduced, cardiac tissue levels of the element remained unaltered, indicating that a fall in serum magnesium levels (hypomagnesemia), without a reduction in tissue magnesium, would suffice to trigger cardiac lesions (Weglicki *et al*, 1996; Kumar *et al*, 1997). The question therefore arises as to what, if not a fall in tissue magnesium levels, causes the reported changes in the heart.

Acute magnesium deficiency is reported to induce an inflammatory response in rodents as evidenced by elevated circulating levels of histamine, interleukin-1, interleukin-6, tumor necrosis factor-α, and endothelin between 11^{th} and 21^{st} day of magnesium deficiency (Weglicki *et al*, 1992b). A neurogenic inflammatory response was observed in this model with elevated plasma and cardiac tissue substance P and calcitonin gene-related peptide during the first week on a magnesium-deficient diet (Weglicki and Phillips, 1992). These findings led to the hypothesis that substance P, whose circulating and cardiac levels increase in

the first week of magnesium deficiency, may trigger the cascade of later inflammatory events in this model (Weglicki *et al*, 1996). This is supported by the observations that substance P is a pro-inflammatory agent that acts as a chemoattractant for monocytes (Ruff *et al*, 1985; Wiedermann *et al*, 1989) and neutrophils (Wiedermann *et al*, 1989) and induces secretion of interleukin-1, interleukin-6 and tumor necrosis factor-α from these cells (Brain, 1997; Castagliuolo *et al*, 1997). Moreover, cardiac lesion formation and the extent of fibrosis in magnesium-deficient rodents was markedly reduced upon administration of substance P receptor blockers (Weglicki *et al*, 1994; Kramer *et al*, 1997). Therefore, a role for humoral factors, especially substance P, in the pathobiology of magnesium deficiency has been postulated (Weglicki *et al*, 1996).

I. 7. THE PRESENT STUDY

Against this backdrop, the objectives of the present study were

1. to ascertain if substance P exerts trophic action on cardiac fibroblasts and to delineate the underlying mechanisms
2. to test the hypothesis that humoral factors, especially substance P, play a role in cardiac fibrogenesis associated with magnesium deficiency, and
3. to establish cultures of cardiac fibroblasts from adult rats that would serve as a model for these investigations.

I. 8. RESULTS AT A GLANCE

I. 8. 1. Substance P is mitogenic to cardiac fibroblasts

Substance P, acting via NK-1 receptors, stimulated cellular hyperplasia over a range of 1 nM to 10 μM. It elicited no change in net collagen production, total protein synthesis or cell protein content but increased ^{45}calcium uptake and superoxide generation. EGTA, N-acetyl cysteine and SOD attenuated the hyperplastic response to substance P. A combination of substance P and EGTA enhanced superoxide generation without an increase in DNA synthesis, showing that an increase in superoxide production does not result in hyperplasia when extracellular calcium is chelated. Together, the data suggested that substance P may activate, via NK-1 receptors, a hyperplastic but not hypertrophic response in adult cardiac fibroblasts and that alterations in redox state and calcium homeostasis may act in concert to mediate its mitogenic action.

I. 8. 2. Humoral factors activate cardiac fibroblasts in magnesium deficiency

Probing the mechanism of cardiac fibrogenesis in magnesium deficiency, the present study furnished evidence that serum from magnesium-deficient rats has a more marked effect than serum from magnesium-sufficient rats on mitogenesis, net collagen production and superoxide generation in cardiac fibroblasts from

young adult rats. The enhanced mitogenic response was abolished by superoxide dismutase and N-acetyl cysteine, showing that it is mediated by superoxide anion. Further, a modest inhibitory effect of the neurokinin-1 receptor antagonist, spantide, suggested that factors acting via neurokinin-1 receptors may partly modulate cardiac fibroblast function in magnesium deficiency. The findings are consistent with the postulation that humoral factors may activate cardiac fibroblasts via a superoxide-mediated mechanism and contribute to the fibrogenic response in magnesium deficiency.

The findings presented in this thesis point, for the first time, to a neural pathway of activation of cardiac fibroblasts.

II. REVIEW OF LITERATURE

II. 1. CARDIAC PARENCHYMA AND STROMA

In an elegant review article on cardiac connective tissue, Weber *et al* (1995) bring out the importance of stroma to organ function. Organs, composed of parenchyma and stroma, maintain a harmonic relationship between the two components. Parenchyma is constituted of highly differentiated cells having very specialized function. Stroma consists of pluripotent, undifferentiated cells and a structural protein network formed largely of fibrillar collagens. The two integrated components respond to various stimuli independently but influence each other to maintain homeostasis.

About 35 years ago, it was realized that the quantitative relationship between parenchyma and stroma in different organs was largely unexplored as an overriding importance had been assigned to parenchyma, in both normal and pathological conditions (Montfort and Perez-Tamayo, 1962). Later it was found that in several pathological conditions fibrillar collagen turnover is indeed dynamic (Laurent, 1987). The prevailing paradigm that heart disease is largely explicable in terms of highly differentiated myocytes and their contractile proteins became untenable. The stromal component of the myocardium, formed mainly of collagens, preserves the architecture of the heart, prevents slippage or overstretching of the myocytes, prevents rupture of the heart, and coordinates

transmittal of mechanical force from the myocytes to the blood. Epimysial collagen covers the entire muscle system. Perimysial collagen is found as the weaves surrounding groups of myocytes, as connecting strands between weaves, and as coiled perimysial fibers that may assist in contraction. Endomysial collagen is found around individual myocytes and as struts between myocytes. The regulation of stroma and, in turn, fibroblast collagen turnover are therefore attracting greater interest (Weber *et al*, 1995).

The heart consists of a myocytic and a nonmyocytic compartment. The myocytic compartment, constituting the parenchyma, is composed of highly differentiated myocytes that express different contractile proteins and occupy 70-75% of the total volume of the heart. They lose the ability to divide soon after birth and further growth of the myocyte compartment occurs through enlargement, or hypertrophy of the individual cells. This hypertrophic process can be physiologic, i.e. resulting from normal growth and exercise, or it can be the pathologic hypertrophy which is associated with chronic hypertension, valvular disease and ischemia/infarction. Stroma includes nonmyocytic cells and a structural protein network formed largely of fibrillar collagens. Two thirds of the cell population of the heart are nonmyocytic cells, mainly the fibroblasts, endothelial cells, and vascular smooth muscle cells. More than 90% of the nonmyocytic compartment is

formed of fibroblasts, pluripotent cells that retain the ability to replicate even in the adulthood. While myocytes respond to pathophysiological stimuli by hypertrophic growth, fibroblasts increase in number (hyperplasia) and synthesize more extracellular matrix proteins (Claycomb *et al*, 1992; Li *et al*, 1996; Soonpaa *et al*, 1996).

II. 2. CARDIAC FIBROBLASTS

Fibroblasts are cells that synthesize fibrillar collagens, types I and III, that together form the major component of the cardiac interstitium (Weber *et al*, 1988; Bashey *et al*, 1992). Moreover, these cells synthesize proteolytic enzymes called matrix metalloproteinases (MMPs) that degrade collagens. They are also responsible for the transcription of tissue inhibitors of matrix metalloproteinase (TIMP) mRNAs whose translation products inhibit the activity of MMPs (Weber *et al*, 1995). Fibroblast activity and function, therefore, determine the extracellular matrix architecture of the heart. During myocardial remodeling, a cascade of events occurs in the myocardium that includes a shift to fetal gene expression, molecular phenotypic transformation, myofibroblast formation, a change in the quantity and composition of collagenous extracellular matrix (Colucci, 1997). Important to matrix and tissue remodeling is the regulation of collagen turnover,

based on the behavior of fibroblasts and fibroblast-like cells, and governed by signals derived locally or from the circulation.

Fibroblasts are activated in response to various pathopysiologic stimuli and consequently, collagen turnover is altered. In the event of myocyte injury, fibroblasts proliferate and synthesize more collagen and this occurs as part of a reparative process termed replacement or reparative fibrosis. Nevertheless, fibroblasts get activated even in the absence of myocyte loss during changes in hemodynamics resulting in mechanical stress to cardiac cells (Chapman *et al*, 1990). This phenomenon, termed reactive fibrosis, also results in excessive collagen production. Both these processes alter the structural composition of the heart, leading to cardiac dysfunction (Weber *et al*, 1991; Brilla *et al*, 1994)) .

II. 3. WOUND HEALING IN THE HEART

A series of reactions that can be divided into several phases occur in the heart in response to different pathophysiological stimuli like myocardial infarction and myocarditis (Weber *et al*, 1995).

II. 3. 1. Exudative phase: This appears within minutes to hours of an injury and is characterized by vasodilation; increased vascular permeability to macromolecules such as plasma fibrinogen and fibronectin and lymphatic flow. Several chemical factors like histamine, serotonin, bradykinin, and/or

prostaglandins may act alone or in combination to initiate and sustain these responses.

II. 3. 2. Inflammatory phase: Following exudative phase, an inflammatory phase occurs in vascularized tissues that over a course of several days eventuates in formation of granulation tissue. This is a prerequisite to subsequent repair when parenchymal regeneration is not possible, as in the case of heart. Inflammatory cells like macrophages, endothelial cells, and fibroblasts develop increased motility and get primed to respond to chemotactic signals and migrate into an extravascular fibrin-fibronectin gel of granulation tissue. Phenotypic transformation of fibroblasts into myofibroblasts occurs in this phase. Myofibroblasts have prominent nuclei and abundant rough endoplasmic reticulum and Golgi vesicular transport organelles. They express actin filaments that facilitate their motility and contractility. Transforming growth factor - β_1 may be involved in this phenotypic transformation of fibroblasts and their enhanced synthesis of collagen (Eghbali *et al*, 1991; Campbell *et al*, 1997).

II. 3. 3. Fibrogenic phase: Myofibroblasts proliferate in the gel as their synthesis of type III fibrillar collagen increases. The synthesis and appearance of type I collagen follows restoring the normal type I to III collagen ratio. In the heart, myofibroblasts persist indefinitely. Type I collagen is the dominant fibrillar

collagen in the normal and diseased heart. Evidence of fibrillar collagen accumulation after myocyte loss is first detectable by microscopy on day 7. Fibrous tissue continues to accumulate over the ensuing 4-6 weeks.

Collagen degradation is also an early component of tissue repair that has received less attention. Matrix metalloproteinases (MMP) reside in the myocardium in latent form. When activated, MMP-1 (interstitial collagenase) degrades fibrillar collagen into characteristic one and three-quarter fragments: MMP-2 and MMP-9 are gelatinases that degrade these smaller fragments. A transient increase in collagenase activity appears in infarcted left ventricle together with a concomitant increase and contribution in collagenolytic activity of gelatinases. An increase in collagenase (MMP-1) mRNA expression does not appear until day 7 and only in the infarcted ventricle, while changes in MMP-1 activity or mRNA expression are not observed at sites remote to the infarct. Tissue inhibitors of matrix metalloproteinases (TIMP) neutralize collagenolytic activity. Fibroblast-like cells, not inflammatory or endothelial cells, are responsible for the transcription of MMP-1 and TIMP mRNAs.

II. 3. 4. Fibrous tissue remodeling: The fibrogenic component of healing substitutes for lost parenchymal cells. Ultimately, fibrous tissue may undergo

retraction and/or resorption. Retraction is expressed as scar thinning and appears 6 weeks after myocardial infarction.

II. 4. ROLE OF FIBROBLASTS IN WOUND HEALING

- ✯ Migration and proliferation
- ✯ Phenotypic transformation of fibroblasts into myofibroblasts
- ✯ Increased MMP and TIMP epxression
- ✯ Enhanced collagen synthesis
- ✯ Fibrogenesis - a substitute for parenchymal loss

II. 5. REGULATION OF CARDIAC FIBROBLAST FUNCTION

It is clear from the foregoing discussion that fibroblasts play a significant role in healing processes as they control collagen turnover, an important event during tissue remodeling (Weber *et al*, 1991; Brilla *et al*, 1994). Greater accumulation or degradation of collagens and alterations in the ratio of different types of collagens lead to structural changes in the heart with consequent organ dysfunction (Colucci, 1997). Fibroblast migration, proliferation, and phenotypic transformation into myofibroblasts are important events in wound healing (Weber *et al*, 1995). A variety of growth factors, including neurotransmitters, hormones, and cytokines, are increased systemically and/or locally in the heart in certain pathological conditions. Many of these agents are known to modulate fibroblast

activity and regulation of cardiac fibroblast function has received increasing attention in the last two decades.

A brief description on the action of different growth factors and cytokines on cardiac fibroblasts follows.

II. 5. 1. Angiotensin II

The discovery of cardiac renin angiotensin system (RAS) has led to numerous investigations on the effects of the components of RAS on cardiac cells (Dostal *et al*, 1992a; Dostal *et al*, 1992b; Kuizinga *et al*, 1998; Dostal and Baker, 1999). Angiotensin II (Ang II) promotes hypertrophy of cardiac myocytes by activation of Ang II type 1 (AT_1) receptor (Miyata and Haneda, 1994). Activation of AT_1 receptors induces transcription of immediate-early genes (*c-fos, c-jun, jun B, Egr-1, and c-myc*) and the re-expression of cardiac fetal gene products such as skeletal α-actin and atrial natriuretic factor (Sadoshima and Izumo, 1993). Ang II-mediated increases in protein synthesis and induction of fetal isogene program in the absence of DNA synthesis result in growth of cultured myocytes resembling that in whole heart models of hypertrophy (Everett *et al*, 1994; Schunkert *et al*, 1995).

The AT_1 receptors responsible for Ang II-stimulated cardiac hypertrophy have been thought to reside on the myocytes (Sadoshima *et al*, 1995; McWhinney

et al, 1997) . However, recent studies raise questions regarding the validity of this assumption. First, both the intact myocardium and isolated cardiac cells maintained in culture exist as co-populations of myocytes and nonmyocyte cells, predominantly fibroblasts (Long, 1996). Abundant Ang II receptors have been identified on neonatal (Villarreal *et al*, 1993) and adult rat cardiac fibroblasts (Crabos *et al*, 1994). Binding of Ang II to the fibroblast AT_1 receptor initiates proliferative growth (Schorb *et al*, 1993) and increases net collagen production (Villarreal *et al*, 1993; Crabos *et al*, 1994). Furthermore, activation of the AT_1 receptor increases transforming growth factor-β_1 (TGF-β_1) mRNA levels and bioactivity in neonatal (Fisher *et al*, 1995) and adult (Lee *et al*, 1995) rat cardiac fibroblast culture models. TGF-β_1 acts as a potent stimulant for myocyte growth and fetal contractile protein gene transcription (Long, 1996), suggesting a potential paracrine mechanism for Ang II-mediated cardiac myocyte hypertrophy. Second, when efforts are made to strictly limit fibroblast contamination of cardiac myocyte preparations, the putative direct effects of Ang II on myocytes are also diminished. Finally, direct receptor identification techniques such as microscopic autoradiography reveal localization of AT_1 receptors predominantly to fibroblasts in isolated cardiac cells maintained in culture under basal conditions (Kim *et al*, 1995). Fibroblast AT_1 receptor binding assessed by autoradiography of tissue

sections of intact myocardium is further increased in pathological settings such as myocardial infarction and co-localizes with enhanced collagen deposition (Sun and Weber, 1994; Lefroy *et al*, 1996; Ju *et al*, 1997). These data support the notion that AT_1 receptors reside primarily on fibroblasts in heart tissue and that effects of Ang II on myocytes occur via paracrine mechanisms mediated by fibroblasts.

In cardiac fibroblasts, AT_1 stimulation was found to stimulate DNA synthesis and cell proliferation and also to increase the synthesis of extracellular matrix proteins (Villarreal *et al*, 1993; Crabos *et al*, 1994; Crawford *et al*, 1994; Brilla *et al*, 1995; Ohkubo *et al*, 1997), which suggests that cardiac fibroblasts contribute to remodeling of the cardiac interstitium under a variety of physiological and pathological conditions. Ang II, acting via AT_1, initiates early biochemical events, including rapid production of diacylglyceraol and inositol 1,4,5-triphosphate by phospholipase C-mediated hydrolysis of inositol phospholipids and activation of protein kinase C [PKC] (Booz *et al*, 1994; Inagami and Kitami, 1994; Berck and Corson, 1997; Matsubara and Inada, 1998). Ang II also induces a rapid increase in expression of the growth-associated nuclear proto-oncogenes and stimulates tyrosine phosphorylation of multiple substrates, including p44 and p42 mitogen-activated protein/extracellular signal-regulated kinases [ERKs] (Schorb *et al*, 1994; Sadoshima *et al*, 1995; Bernstein and

Marrero, 1996; Dostal *et al*, 1996; Eguchi *et al*, 1996; Sadoshima and Izumo, 1996; Zou *et al*, 1996).

In cardiac fibroblasts, angiotensin II-induced synthesis of fibronectin and transforming growth factor-β is regulated through downstream signaling of EGF-R transactivated by Ca^{2+}/calmodulin-dependent tyrosine kinases. Induction of fibronectin mRNA by Ang II was regulated by two different mechanisms, namely, transcriptional control by binding of the *c-fos/c-jun* complex to the activator protein-1 (AP-1) site and post-transcriptional control by autocrine or paracrine effects of TGF-β, which is exerted by increasing the mRNA stability via de novo protein synthesis and which up-regulates fibronectin mRNA (Moriguchi *et al*, 1999).

II. 5. 2. Norepinephrine

Catecholamines have been implicated in hypertrophic changes in the heart as increases in the levels of catecholamines have been detected in hypertensive patients and hypertrophy is induced with catecholamine infusions in experimental animals (Bhambi and Eghbali, 1991). *In vitro* studies have shown that norepinephrine activates α_1-adrenergic receptors on the myocytes to induce hypertrophy and fetal gene expression (Knowlton *et al*, 1993). Norepinephrine is found to increase DNA synthesis and collagen synthesis in cardiac fibroblasts and

this effect was abolished by β-blockers suggesting the involvement of β-adrenergic receptors in mediating these changes in cardiac fibroblasts (Long *et al*, 1993). β-adrenergic stimulation of cardiac fibroblasts has been shown to induce the expression of transforming growth factor-β in these cells (Fisher and Absher, 1995) and this factor is suggested to exert a an autocrine/paracrine effect on cardiac cells. It is possible that, in both myocytes and fibroblasts, norepinephrine acting via α_1-adrenergic or β-adrenergic receptors, respectively, causes the expression of peptide growth factors that can exert autocrine or paracrine effects on growth and gene expression (Long *et al*, 1993; Takahashi *et al*, 1994).

β_2-Adrenergic receptor stimulation is found to increase inducible nitric oxide synthase message stability thereby enhancing the production of nitric oxide by cardiac fibroblasts in response to interleukin-1β. Therefore, cardiac fibroblasts could participate in a paracrine mechanism whereby the direct positive inotropic effect of β_1-adrenergic stimulation of myocytes is opposed by β_2-adrenergic enhancement of nitric oxide production, a negative inotropic event, in fibroblasts (Gustafsson and Brunton, 2000).

II. 5. 3. Tumor necrosis factor-α

Tumor necrosis factor-α has a unique capacity to substantially increase AT_1 mRNA levels in cardiac fibroblasts. TNF-α is a pleiotropic cytokine that plays an

important role in the response to tissue injury and wound healing. Increased amounts of this cytokine have been detected in regions of the infarcted heart where AT_1 up-regulation is known to occur. TNF-α increases the density of functional AT_1 receptors on cultured cardiac fibroblasts which suggests a role for this cytokine in wound healing (Gurantz *et al*, 1999). TNF-α has also been found to decrease collagen synthesis and activate matrix metalloproteinases that degrade collagen. This suggests that TNF-α may contribute to ventricular dilatation and myocardial failure by promoting the remodeling of interstitial collagen (Siwik *et al*, 2000). TNF-α stimulates proliferation and expression of fibronectin in cardiac fibroblasts (Jacobs *et al*, 1999).

II. 5. 4. Transforming growth factor-β

Treatment of cultured cardiac fibroblasts with transforming growth factor - β induces the expression of α-smooth muscle actin, characteristic of the transformation to myofibroblasts (Eghbali *et al*, 1991), and raises atrial natriuretic peptide concentrations in the medium (Cameron *et al*, 2000). Cardiac gene expression of atrial natriuretic peptide and that of brain natriuretic peptide are markedly elevated after myocardial infarction (Cameron *et al*, 2000).

The cells synthesizing ANP were shown by in situ hybridization and immunocytochemistry to be fibroblasts invading the infarct. The appearance of

ANP expression coincides with the transition of these cells to myofibroblast phenotype (Cameron *et al*, 2000). TGF-β induces an increase in collagen production and secretion and enhances the abundance of mRNA levels for collagen type I and III in rat cardiac fibroblasts in culture (Booz and Baker, 1995). Expression of connective tissue growth factor, a cysteine-rich protein, in cardiac fibroblasts is induced by TGF-β. Connective tissue growth factor triggers many of the cellular processes underlying fibrosis, such as cell proliferation, adhesion, migration and the synthesis of extracellular matrix (Chen *et al*, 2000).

Transforming growth factor-β_1 and collagen type I and III mRNA expressions are markedly increased in the infarcted heart and the increase in levels of transforming growth factor-β_1 mRNA precedes increases in mRNA levels for extracellular matrix proteins. Blockade of transforming growth factor-β_1 does not lead to increased expression of extracellular matrix proteins. These data suggest a role for transforming growth factor-β_1 in remodeling in the myocardium by promoting extracellular matrix protein expression (Eghbali, 1992; Lijnen *et al*, 2000; Sun *et al*, 2000).

II. 5. 5. Endothelin-1

After myocardial ischemia, circulating levels of endothelin-1 increase. Endothelin-1 stimulates DNA synthesis and cell proliferation in cultured neonatal

and adult rat cardiac fibroblasts. The mitogenic action of endothelin-1 on cardiac fibroblasts is via activation of protein kinase C and concurrent treatment of cardiac fibroblasts with the pro-inflammatory cytokine interleukin-1β which, like endothelin-1, is released after myocardial ischemia, attenuates the ET-1-induced increases in DNA synthesis and cell number (Piacentini *et al*, 2000).

II. 5. 6. Adenosine

Adenosine is known to exert a cardioprotective effect in myocardial ischemia and reperfusion, and it has been proposed as a mediator of ischemic preconditioning. The transduction pathways involved in the response to adenosine have not been definitely established. Adenosine inhibits cardiac fibroblast proliferation via A(2B) receptors and this is mediated by inhibition of MAP kinase activity. Pharmacologic or molecular biological activation of A(2B) receptors may prevent remodeling associated with hypertension, myocardial infarction, and myocardial reperfusion injury after ischemia (Dubey *et al*, 1998; Kuizinga *et al*, 1998; Dubey *et al*, 2001) .

II. 5. 7. Insulin-like growth factor-1

Fibroblasts are highly resistant to programmed cell death though in the recent past there are several reports that provide evidence of apoptotic cell death in these cells (Brilla *et al*, 1995; Rupp *et al*, 1999). Recently, it has been shown that

osmotic stress induced apoptosis in cardiac fibroblasts and treatment with insulin-like growth factor-1 promoted a dose-dependent increase in cell survival against osmotic cell death in these cells (Mockridge *et al*, 2000). It is suggested that insulin-like growth factor-1 regulates osmotic stress-induced apoptosis via the activation of the PI3-K/Akt pathway (Mockridge *et al*, 2000).

II. 6. SUBSTANCE P

In the early eighties, peptidergic innervation of the heart was discovered. Immunocytochemistry revealed the presence of the neuropeptide substance P, secreted by peptidergic neurons in the heart (Reinecke *et al*, 1980; Weihe *et al*, 1981; Furness *et al*, 1982). In dogs, the neuropeptide, substance P was found to increase coronary blood flow as well as cardiac output (Maxwell, 1968; Losay *et al*, 1977). It was speculated that substance P-immunoreactive nerve fibers may be part of an intrinsic regulatory system for coronary blood flow (Priola, 1980). Substance P is a potent vasodilator that is found to counteract deoxycorticosterone acetate (DOCA) - salt-induced hypertension by modulating both cardiac output and peripheral resistance (Kohlmann *et al*, 1995). Ischemia of the myocardium stimulates cardiac sensory nerve endings to release substance P (Milner *et al*, 1989). It has been shown that substance P plays a role in protection of the isolated heart against ischemic damage. This observation suggests a role of substance P in

the resistance of the myocardium to ischemia and reperfusion injury (Ustinova *et al*, 1995).

Substance P is a tachykinin that is co-localized with calcitonin gene-related peptide (CGRP) in a unique subpopulation of cardiac afferent nerve fibers. These neurons are activated by nociceptive stimuli and exhibit both sensory and motor functions that are mediated by the tachykinins and/or CGRP. Sensory signals (e.g., cardiac pain) are transmitted by peptides released at central processes of these neurons, whereas motor functions are produced by the same peptides released from peripheral nerve processes. The major targets for the tachykinins within the heart are the intrinsic cardiac ganglia and coronary arteries. Intrinsic cardiac ganglia contain cholinergic neurons that innervate the heart and coronary vasculature. Tachykinins can stimulate NK-3 receptors on these neurons to increase their excitability and evoke spontaneous firing of action potentials. This action provides a mechanism whereby tachykinins can indirectly influence cardiac function and coronary tone. Tachykinins also have direct effects on coronary arteries to decrease or increase tone. Stimulation of NK-1 receptors on the endothelium causes vasodilation mediated by nitric oxide. This effect is normally dominant, but NK-2 receptor-mediated vasoconstriction can also occur and is augmented when NK-1 receptors are blocked. It is proposed that these ganglion stimulant and vascular

actions are manifest by endogenous tachykinins during myocardial ischemia. Substance P evokes bradycardia mediated by cholinergic neurons in experiments with isolated guinea pig hearts (Chang *et al*, 2000). Intravenous injection of substance P causes a brief decrease in heart rate and a long-lasting decrease in blood pressure. The negative chronotropic response to substance P is attenuated by muscarinic receptor blockade with atropine and augmented by inhibition of cholinesterases with physostigmine. Therefore, it seems that substance P can evoke bradycardia through stimulation of postganglionic cholinergic neurons (Tompkins *et al*, 1999; Hoover *et al*, 2000).

Substance P is an 11-amino acid peptide member of the tachykinin family (Pernow, 1983). It exerts its effects by binding to its high affinity NK-1 receptor subtype (Nakanishi, 1991). Substance P has been implicated in the regulation of several physiologic and pathophysiologic processes such as pain, smooth muscle contraction, and intestinal secretion (Nakanishi, 1991). A large body of *in vitro* and *in vivo* studies suggests a role for substance P as a pro-inflammatory agent. It is a chemoattractant for monocytes (Ruff *et al*, 1985; Wiedermann *et al*, 1989) and neutrophils (Wiedermann *et al*, 1989), a stimulator of macrophage phagocytosis (Bar-Shavit *et al*, 1980), and mast cell degranulation (Brain, 1997). Substance P induces the secretion of interleukin-1, interleukin-6, and tumor necrosis factor

from monocytes and macrophages (Brain, 1997; Castagliuolo et, 1997). *In vivo* studies show that substance P plays a pro-inflammatory role in acute intestinal inflammation (Castagliuolo *et al*, 1998), pancreatitis (Bhatia *et al*, 1998; Shrikhande *et al*, 2001), and lung disease (Bozic *et al*, 1996).

Substance P has been shown to stimulate superoxide production in synovial cells (Tanabe *et al*, 1996b) and human neutrophils (Tanabe *et al*, 1996a; Sterner-Kock et al, 1999). Tanabe *et al* (1996b) have reported that NK-1 receptor /phospholipase C-linked DAG formation with resulting activation of protein kinase C is the signal transduction pathway for substance P-stimulated oxyradical production in synovial cells. The ability of gastrointestinal tissue to react to tachykinins with liberation of free radicals as part of an inflammatory reaction has also been demonstrated (Lordal *et al*, 1997).

Besides acting as a pro-inflammatory mediator and a neurotransmitter, substance P induces mitogenesis in several cell types including T lymphocytes (Payan *et al*, 1983), skin fibroblasts (Nilsson *et al*, 1985), smooth muscle cells (Nilsson *et al*, 1858; Payan, 1985), and synoviocytes (Lotz *et al*, 1987). In addition, it acts synergistically with growth factors to stimulate skin fibroblast proliferation (Kahler *et al*, 1996) and corneal epithelial cell migration (Nakamura *et al*, 1997). Induction of mitogenesis and migration by substance P suggests a

possible beneficial role for this peptide in tissue healing during chronic inflammation (Katayama and Nishioka, 1997). Indeed, several *in vivo* studies show that substance P and NK-1 receptors are up-regulated during chronic inflammation disorders characterized by diffuse tissue damage (Mantyh *et al*, 1988; Sharkey, 1992). Furthermore, substance P depletion by the neurotoxin capsaicin (Kjartansson *et al*, 1987) or administration of a substance P antagonist (Benrath *et al*, 1995) delays wound healing in experimental animals.

Substance P binding sites have been identified in the nervous system as well as peripheral tissues such as in immune (Ruff *et al*, 1985), epithelial (Pothoulakis *et al*, 1998), and endothelial cells (Mantyh *et al*, 1988). Sequence analysis of the cloned NK-1 receptor has demonstrated that it belongs to the G-protein-coupled receptor (GPCR) family, and displays a typical 7-transmembrane-spanning domain structure (Hershey and Krause, 1990). The NK-1 receptor has been shown to associate with a variety of G_{α} protein isotypes, such as $G_{\alpha 0/11}$, $G_{\alpha i}$, $G_{\alpha s}$ (Nishimura *et al*, 1998; Roush and Kwatra, 1998). Thus, substance P binding to NK-1 receptor and consequent activation of G protein subunits results in the activation of a variety of second messengers, including inositol 3-phosphate kinase resulting in hydrolysis of phosphoinositides, cAMP formation (Otsuka and Yoshioka, 1993), and arachidonic acid release (Kahler *et al*, 1993). These events lead to mobilization of

intracellular calcium and enhancement of protein kinase activity, including protein kinase C (Otsuka and Yoshioka, 1993). For example, it has been reported that, in Chinese hamster ovary cells expressing the substance P receptor clone (CHO-SPR), substance P induces calcium entry through activation of cation channels. Further, it has been suggested that inositol 1,4,5-trisphosphate (IP_3) may regulate both calcium entry and Ca^{++} mobilization from intracellular stores in CHO-SPR cells (Mochizuki-Oda *et al*, 1994). In neutrophils, substance P stimulates the hydrolysis of PIP_2 into diacylglycerol (DAG) and IP_3 with a rise in intracellular calcium (Tanabe *et al*, 1996a). Substance P and related tachykinins have also been shown to evoke calcium signaling in cultured myenteric neurons by the influx of extracellular Ca^{++} through L- and N-type calcium channels. These signals are abolished upon removal of extracellular Ca^{++} or by the addition of calcium channel blockers, lanthanum chloride and nickel chloride (Sarosi *et al*, 1998).

In the heart, light-microscopic autoradiograms revealed that substance P binding sites occurred within clusters of connective tissue cells or in rarely observed parasympathetic ganglia (Walsh *et al*, 1996). No evidence was found to suggest the presence of substance P receptors on endothelial cells, cardiac muscle fibers or smooth muscle fibers. Autoradiograms revealed prominent small foci of intense autoradiographic reactions dispersed intermittently around the periphery of

the great vessels and coronary arteries, among the interstitial connective tissue of the heart, and along the cusps of the cardiac valves (Walsh *et al*, 1996).

II. 7. SUBSTANCE P IN MAGNESIUM DEFICIENCY: A NEW HYPOTHESIS

II. 7. 1. Magnesium deficiency

Acute magnesium-deficiency leads to crystalluria, calcification and degenerative changes in various organs, prominent especially in the heart (Arsenian, 1993). Greenberg *et al* (1936) observed myocardial degeneration with fibrosis and polyblastic infiltration in magnesium-deficient rats. Histologically, a wide-spread inflammatory lesion involving loose mesenchymal tissues in magnesium-deficient rat was noticed. In the heart, the lesion begins as focal acute perivascular inflammation and progresses through stages of necrosis, granulomatous inflammation and fibroblastic proliferation to scar formation. Heggtveit *et al* (1964) have reported an array of myocardial which include focal myocardial necrosis, which enlarges and develops areas of calcification. As deficiency advances, fragmentation, vacuolization and eventual myocyte loss are detected with progressive increase in macrophages, fibroblasts and collagen. Electron microscopic studies revealed mitochondrial swelling and loss of internal fine structure. Mitochondria calcify and coalesce to form mineralized masses within the sarcoplasm of degenerating myocytes. Calcification of large arteries,

including the aorta, was also observed in magnesium-deficient animals. Biochemical alterations like elevated serum and cardiac tissue levels of thiobarbituric acid reactive substances (Kumar *et al*, 1997), impaired anti-oxidant defense in the myocardium (Kumar and Shivakumar, 1997) and generation of ferryl myoglobin radical (Wu *et al*, 1994) are demonstrated in these animals. Further, significant alterations in collagen turnover rates and fibroblast proliferation provided evidence of a wound healing response in the myocardium (Kumar, *et al*, 1997).

Several theories have been proposed to explain the formation of cardiac lesions in magnesium deficiency. Heggtveit *et al* (1964) proposed a calcium overload hypothesis based on reduced activity of magnesium-dependent Na^+-K^+ ATPase detected in the hearts of magnesium-deficient animals. This causes Na^+ accumulation in the myocytes, resulting in the reversal of Na^+-Ca^{++} exchanger and the consequent calcium overload and myocyte damage (Ahmad and Bloom, 1989; Reuter and Seitz, 1968).

Wu *et al* (1994) studied the oxidation states of intracellular myoglobin and cytochrome oxidase aa_3 in isolated perfused rat heart subjected to acute magnesium-deficiency. Within 30 minutes of exposure to low magnesium, more than 80% of the myocardial oxymyoglobin was deoxygenated and increased levels

of reduced cytochrome oxidase aa_3 was observed. Further, it was observed that deoxymyoglobin was converted to ferryl-myoglobin which is proposed to be responsible for the myocardial damage observed in magnesium deficiency. Later, it was shown that the extent of ferryl-myoglobin -mediated cardiac muscle damage can be reduced by concurrent administration of ascorbate to the animals on magnesium-deficient diet (Wu *et al*, 1994).

β-Adrenergic receptor activation in the heart plays an important role in cardiac cell signaling pathways as it mediates an inotropic effect (Feldman, 1991). β-Adrenergic receptors belong to G-protein-coupled receptor family of receptors and their activities are altered during cardiac failure (Feldman *et al*, 1987). Magnesium is crucial in the activation of G-protein coupled receptors as it stimulates nucleotide hydrolysis through high-affinity binding sites on G-proteins and hormone-receptor nucleotide exchange through low-affinity binding sites (Gilman, 1987; Higashijima *et al*, 1987). Magnesium deficiency increases the levels of inhibitory subunit of G-protein (G_i) and exacerbates ischemia-induced muscle damage in the heart. It has been hypothesized that catecholamines that are elevated during magnesium deficiency may play a role in altered G-protein function (Bing *et al*, 1995).

II. 7. 2. Neurogenic inflammation in magnesium deficiency

Probing the molecular pathology of magnesium deficiency, Weglicki *et al* have reported that acute magnesium deficiency produces a pro-inflammatory condition marked by elevations in the circulating levels of pro-oxidant and mitogenic factors (Weglicki *et al*, 1992b). In a rat model, cardiac lesions develop after 3 weeks on the magnesium-deficient diet; significant elevation of several cytokines, IL-1. IL-6 and TNF-α also occurs (Weglicki *et al*, 1992b; Weglicki and Phillips, 1992). It has been found that free radicals play a role in myocardial damage (Kumar *et al*, 1997; Kumar and Shivakumar, 1997). Based on the identification of elevated levels of circulating substance P and calcitonin gene-related peptide, a role for neurogenic peptides in the pathogenesis of the cardiac lesions of magnesium deficiency has been proposed (Weglicki *et al*, 1996). During the second week, circulating histamine, PGE2 and TBAR-materials were elevated and red cell glutathione was reduced, all prior to the elevation of inflammatory cytokines during the third week. When the animals were treated with the substance P receptor blocker, CP-96345, the levels of substance P and CGRP remained elevated; however, increases in histamine, PGE2, TBAR-materials, and the decrease in red cell glutathione were inhibited; also, the development of cardiac lesions was inhibited significantly. These data support a central role for neurogenic

peptides, especially substance P, in the development of cardiomyopathic lesions during magnesium deficiency (Weglicki *et al*, 1994; Kramer *et al*, 1997).

The importance of inflammatory processes in the pathology of magnesium deficiency and the sequence of events leading to the inflammatory response and cardiac tissue repair remains unclear. Though increased DNA and collagen syntheses in magnesium-deficient rats have been reported (Kumar *et al*, 1997), no attempt has been made to identify the factors that activate myocardial fibroblasts during magnesium deficiency although fibroblasts are known to play a central role in the context of tissue injury. It is possible that these humoral factors may modulate cardiac fibroblast function in magnesium deficiency.

III. MATERIALS AND METHODS

III. 1. MATERIALS

III. 1. 1. Fine chemicals

Substance P, spantide, M199, Dulbecco's modified Eagle's medium, bovine serum albumin, nitroblue tetrazolium, collagenase type IA, trypsin, deoxyribonuclease, pancreatin, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), fetal bovine serum, ethylenediamine tetraaceitic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), verapamil, diltiazem, N-acetyl cysteine, insulin-tranferrin-sodium selinite media supplement, penicillin, gentamycin, monoclonal anti-vimentin antibody, anti-mouse IgM (μ-chain specific) antibody, anti-human von Willebrand factor antibody, monoclonal anti-rabbit IgG (γ-chain specific) antibody, monoclonal anti-desmin antibody, anti-mouse IgG (whole molecule) antibody, SIGMA *FAST*™ (Fast Red TR/Napthol AS-MX, Alkaline Phosphatase Substrate Tablets Set), and activated charcoal were purchased from Sigma Chemical Company, USA.

III. 1. 2. Routine chemicals

Calcium chloride, chloramine T, citric acid monohydrate, copper sulfate, *p*-dimethylaminobenzaldehyde, glucose, hydroxyproline, lanthanum chloride heptahydrate, magnesium chloride, magnesium sulfate heptahydrate, potassium chloride, potassium dihydrogen phosphate, potassium hydroxide, 1,4-bis[5-phenyl-

2-oxazolyl]-benzene, 2,5-diphenyloxazole, potassium-sodium tartrate, sodium acetate trihydrate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium dihydrogen phosphate, sodium acetate trihydrate, sodium hydroxide, sodium dodecyl sulfate, Toluene, *n*-butanol, concentrated hydrochloric acid, concentrated sulfuric acid, perchloric acid, ethanol, ether, Folin-Ciocalteau reagent, glacial acetic acid, triton X-100, and trichloroacetic acid were obtained from Sisco Research Laboratories, India.

III. 1. 3. Radiochemicals

[^{3}H]-thymidine (Specific activity 17,000 mCi/mmol), [^{3}H]-phenylalanine (Specific activity 6400 mCi/mmol) and ^{45}calcium chloride (Specific activity 12.35 mCi/g) were obtained from Bhabha Atomic Research Center, India.

III. 1. 4. Equipment used

Liquid scintillation counter (Wallac 1409), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), water-bath (LKB, Sweden), ice-machine (Hoshizaki, Japan), pH meter (Elico, India), CO_2 Incubator (Nuaire, USA), phase contrast microscope (Nikon, Japan), laminar flow hood (CLAS, India), and EASYpure UV/UF compact reagent grade water system (Barnstead, USA).

III. 1. 5. Cell cultureware

35mm, 60mm, and 100mm cell culture dishes, multidishes, and T25 cell culture flasks were purchased from Nunc, USA. Cell culture filterware was from Millipore, USA.

III. 1. 6. Animal feed

Animal feed, based on the nutrient requirements of rats proposed by the American Institute of Nutrition (Anonymous, 1977), was purchased from Zeigler Bros. Inc., USA. Magnesium-sufficient and magnesium-deficient diets contained 0.0515% and 0.0008% magnesium, respectively.

Table 1. Composition of rat feed (g/100g)

Cellulose	50.0 g
Casein lactate	20.0 g
Corn starch	15.0 g
Cellulose	5.0 g
Fat, Corn oil	5.0 g
American Institute of Nutrition vitamin mix	1.0 g
American Institute of Nutrition mineral mix	3.5 g
Dimethionine	0.3 g
Choline bitartrate	0.2 g

III. 2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III. 2. 1. Aldehyde perchloric acid solution

15.0 g p-diaminobenzaldehyde, 62.0 ml of *n*-propanol, 26.0 ml of perchloric acid

III. 2. 2. Alkaline sodium dodecyl sulfate solution

2% (w/v) Na_2CO_3, 1% (w/v) Sodium dodecyl sulfate, 0.04% (w/v) NaOH, 0.16% (w/v) sodium tartrate

III. 2. 3. Chloramine T solution

1.41g chloramine T, 10.0 ml *n*-propanol, 10.0 ml distilled water, 80.0 ml citrate buffer, pH 6.0

III. 2. 4. Citrate buffer, pH 6.0

5.0 g/100 ml citric acid monohydrate, 1.2 ml/100 ml glacial acetic acid, 12.0 g/100 ml sodium acetate trihydrate, 3.4 g/100 ml sodium hydroxide

III. 2. 5. Cupric solution

4% (w/v) $CuSO_4$. $5H_2O$

III. 2. 6. Dissociation medium, pH 7.4

NaCl, 116.4 mM; HEPES, 20 mM; NaH_2PO_4, 1.15 mM; Glucose, 5.55 mM; KCl, 5.37 mM; $MgSO_4.7H_2O$, 0.81 mM

III. 2. 7. Growth medium

Dulbecco's Modified Eagle's Medium (DMEM), M199, and Fetal Bovine Serum (FBS) in the ratio 7:2:1

III. 2. 8. HEPES-buffered physiological salt solution, pH 7.4 (HBPSS)

NaCl 145 mmol/l, KCl 5 mmol/l, $MgCl_2$ 1 mmol/l, $CaCl_2$ 1.2 mmol/l, HEPES 5 mmol/l, Glucose 10 mmol/l

III. 2. 9. Kreb's Ringer phosphate-buffered saline, pH 7.4 (KRPBS)

50 mM HEPES, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 1 mM NaH_2PO_4, 2 mM glucose, 1 mM $CaCl_2$

III. 2. 10. Lysis buffer

0.1 M NaOH, containing 0.1% sodium dodecyl sulfate

III. 2. 11. Magnesium deficient serum-containing medium (MgD)

DMEM, M199, and serum from magnesium-deficient rat in the ratio 7:2:1

III. 2. 12. Magnesium sufficient serum-containing medium (MgS)

DMEM, M199, and serum from magnesium-sufficient rat in the ratio 7:2:1

[Magnesium levels of incubation media for the control (MgS) and test (MgD) groups were comparable.]

III. 2. 13. Nitroblue tetrazolium reduction assay buffer

KRPBS, pH 7.4, containing 1.25 mg/ml nitroblue tetrazolium and 17 mg/ml bovine serum albumin

III. 2. 14. Phosphate-buffered saline, pH 7.4 (PBS)

NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10.14 mM; KH_2PO_4, 1.76 mM

III. 2. 15. Reagent A

100 parts of alkaline sodium dodecyl sulfate solution and 1 part of cupric solution

III. 2. 16. Scintillation cocktail

2 Triton X-100 : 1 Toluene containing 0.4% PPO

III. 2. 17. Serum-free medium

DMEM, and M199 in the ratio 8:2, containing Insulin-Transferrin- Sodium Selenite media supplement (working range of insulin, transferrin, and sodium selenite - 5 μg, 5 μg, 5 ng/ml)

III. 2. 18. Substrate solution

1.0 mg/ml Fast Red TR (4-Chloro-2-methylbenzenediazonium), 0.4 mg/ml Naphthol AS-MX (3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), 0.15 mg/ml levamisol prepared in 0.1 M Tris buffer, pH 7.4

III. 2. 19. Trypsin/EDTA solution

PBS, pH 7.4, containing 0.5 mg/ml trypsin, and 0.2 mg/ml EDTA

III. 3. ISOLATION, CULTURE AND CHARACTERIZATION OF CARDIAC FIBROBLASTS

III. 3. 1. Isolation of cardiac fibroblasts

Cardiac fibroblasts from adult rats were isolated following the method of Eghbali *et al* (1991) with some modifications.

The entire procedure was done in a laminar flow hood in a tissue culture laboratory with all precautions to maintain sterile conditions. Adult Sprague Dawley rats (150 g -200 g body weight) were anesthetized with ether and the thoracic cavity was exposed. The heart was excised and washed free of blood using PBS (*Ref.* III. 2. 14) containing antibiotics (penicillin, 0.12 mg/ml; gentamycin, 0.04 mg/ml). The ventricular tissue was separated and minced into bits of approximately 1 mm^3 size. The tissue bits were subjected to a series of ten digestions, each of 10 minutes duration, in dissociation medium (*Ref.* III. 2. 6) containing collagenase type IA (1.15 mg/ml), trypsin (0.50 mg/ml), pancreatin (0.60 mg/ml), deoxyribonuclease (5.5 μg/ml), penicillin (0.12 mg/ml) and gentamycin (0.04 mg/ml) at 37°C with mild shaking. The supernatants from the fourth to tenth digestions were collected and cells were pelleted at 3000 rpm for 5

minutes. The cells were pooled and resuspended in 4. 0 ml of growth medium (*Ref.* III. 2. 7), with antibiotics, and were equally distributed into two 35 mm dishes and incubated in a humidified CO_2 incubator at 37°C in an atmosphere of 95% air and 5% CO_2 to maintain the pH at 7.4.

III. 3. 1. 1. Selective enrichment of cardiac fibroblasts

Cardiac fibroblasts were separated from other contaminating cell types by a pre-plating method. Soon after isolation, the cells seeded on two 35 mm culture dishes were incubated in a CO_2 incubator at 37°C for 150 minutes. The unattached cells were discarded and the adherent fibroblasts were washed twice with growth medium (*Ref.* III. 2. 7). Fresh growth medium was then supplied to the plates. At 24 hours, fresh growth medium was added, after washing the cells three to four times with growth medium.

III. 3. 1. 2. Sub-culture of cardiac fibroblasts

At confluence, the conditioned medium was removed and the cells were washed twice with PBS (*Ref.* III. 2. 14) and trypsinized at 37°C in trypsin/EDTA solution (*Ref.* III. 2. 19) for 3-5 minutes. Trypsinization was stopped by 10% fetal bovine serum and the detached cells were collected immediately by centrifugation at 3000 rpm for 5 minutes. The pellet was resuspended in growth medium (*Ref.* III. 2. 7). Cells were seeded in fresh culture dishes at a ratio 1:3.

III. 3. 2. Characterization of cardiac fibroblasts

Fibroblastic nature of the cells was assessed by morphological analysis, immunocytochemistry, and growth kinetics.

III. 3. 2. 1. Morphological analysis

The cells were examined using an inverted phase contrast microscope at magnifications of 100X, 200X, and 400X.

III. 3. 2. 2. Immunostaining for vimentin

Cells from passage 3, grown on cover slips to 60-70% confluence, were washed thrice with 0.1 M Tris buffer, pH 7.4, and fixed in 70% ethyl alcohol for 30 minutes. Following this, cells were washed thrice with 0.1 M Tris buffer, pH 7.4. Nonspecific binding was blocked with 2% bovine serum albumin in 0.1 M Tris buffer, pH 7.4, for 10 minutes. 50 μL of primary antibody diluted in 0.1 M Tris buffer, 7.4, was added and incubation continued for 30 minutes. The cells were washed three times with 0.1 M Tris buffer, pH 7.4, and incubated for 30 minutes with 50μL of secondary antibody conjugated with alkaline phosphatase diluted in 0.1 M Tris buffer, pH 7.4. Cells were washed three times with 0.1 M Tris buffer, pH 7.4, and 200 μL of substrate solution (*Ref.* III. 2. 18) was added. After 30-45 minutes of incubation, cells were washed in distilled water and mounted in glycerol. All incubations were at room temperature.

Monoclonal anti-vimentin, anti-mouse IgM and anti-mouse IgG antibodies were diluted 1:50; anti-human von Willebrand factor was diluted 1:200; monoclonal anti-rabbit IgG was diluted 1:100 and monoclonal anti-desmin was diluted 1:20.

III. 3. 2. 3. Growth kinetics of cardiac fibroblasts

Growth kinetics of cardiac fibroblasts was studied following the method of Kovacks and Fleishmajer (1974).

Cells from passage 3 were plated in 4-well plates at a density of 15 x 10^3 cells per well. Cell count was determined using a Neubauer chamber at 0, 24, 48, 72 and 96 hours after seeding. A graph was plotted with time on x-axis and cell number on y-axis. The best fitting curve was constructed by connecting the mean points. A time interval was selected in the middle of the exponential phase where the relationship between time and log cell density is linear. The beginning of the time interval was marked as t_1 and the end as t_2. The time interval was taken as Δt i.e., $t_2 - t_1$. The cell densities (cells/ml) at t_1 and t_2 were taken as N_1 and N_2, respectively. The growth rate constant, k, was calculated using the formula:

$k = [2.3 \log (N_2/N_1)] / \Delta t \text{ hours}^{-1}$.

The doubling time, T_d, was determined using the formula:

$T_d = 0.693/k$ hours

III. 4. ACTION OF SUBSTANCE P ON CARDIAC FIBROBLASTS

III. 4. 1. Measurement of DNA synthesis

DNA synthesis was measured in terms of ^{3}[H]-thymidine incorporation into TCA-insoluble material, as described by Shivakumar *et al* (1992) with some modifications.

Cardiac fibroblasts from passage 3 or 4 were seeded at a density of 50 x 10^3 per 35 mm dish in growth medium (*Ref.* III. 2. 7). The cells were allowed to grow for 24 hours and then serum-deprived for 24 hours. The cells were exposed to 10 µmol/L substance P in serum-free medium (*Ref.* III. 2. 17) for an additional 24 hours. During the last four hours, cells were pulsed with [^{3}H]-thymidine at 2.5 µCi/ml. At 24 hours, medium was discarded and cells were washed thrice with PBS (*Ref.* III. 2. 14) , lysed in lysis buffer (*Ref.* III. 2. 10) and precipitated with an equal volume of 10% ice-cold TCA for 1 hour at 5°C. The precipitate was pelleted at 15,000 rpm for 10 min at 5° C. The pellet was washed with 1.0 ml of 5% ice-cold TCA and then with 1.0 ml of absolute ethanol. Dried pellet was dissolved in 1.0 ml of 0.25 N NaOH. A 300 µl aliquot was added to 10.0 ml of scintillation cocktail (*Ref.* III. 2. 16) and radioactivity was measured by Liquid Scintillation Spectroscopy.

III. 4. 2. Measurement of cell proliferation

To determine whether substance P increases fibroblast proliferation, cells from passage 3 were seeded at a density of 50 x 10^3 per 35 mm dish and allowed to grow for 24 hours in growth medium (*Ref.* III. 2. 7). Following serum-deprivation for 24 hours, the cells were exposed to SP at 1 nmol/L, 100 nmol/L and 10 µmol/L for 24 hours. Cell count was done using a Neubaeur counting chamber.

III. 4. 3. Measurement of total protein synthesis

Protein synthesis was measured as described by Schorb *et al* (1993) Confluent cultures of cardiac fibroblasts, serum-deprived for 24 hours, were incubated in serum-free medium (*Ref.* III. 2. 17) containing 10 µmol/L substance P and [^{3}H]-phenylalanine at 2.5 µCi/ml. Reaction was stopped at 24 hours by removing the conditioned medium and washing the cells three times with PBS (*Ref.* III. 2. 14). The cells were lysed in 1.0 ml of lysis buffer (*Ref.* III. 2. 10) and precipitated with 10% ice-cold TCA for 1 hour at 5°C. The precipitate was pelleted at 15,000 rpm for 10 min at 5°C and the pellet processed for determination of radioactivity.

III. 4. 4. Measurement of total protein content

Cardiac fibroblasts from passage 3 or 4 were grown to confluence and serum-deprived for 24 hours and then exposed to serum-free medium (*Ref.* III. 2.

17) containing 10 μmol/L substance P for 24 hours. The cells were washed thrice with PBS (*Ref.* III. 2. 14), lysed in 1.0 ml of lysis buffer (*Ref.* III. 2. 10) and the proteins were precipitated with an equal volume of 10% ice-cold TCA. Acid-precipitable material was dissolved in 0.25 N NaOH. Total protein estimation was carried out by a modified Lowry assay (Winterbourne, 1993). 0.3 ml aliquot was mixed with 1.0 ml of reagent A (*Ref.* III. 2. 15). The solutions were mixed thoroughly and kept at room temperature for 10 minutes. Then, 0.1 ml of Folin-Ciocalteau phenol reagent, diluted with an equal volume of water, was added while mixing vigorously. Absorbance at 660 nm was measured after 45 minutes of addition of Folin-Ciocalteau phenol reagent.

III. 4. 5. Measurement of net collagen production

Net collagen production (present in cell monolayer and medium) was determined by a hydroxyproline-based assay, as described by Villarreal *et al* (1993).

Confluent cultures of cardiac fibroblasts in 100 mm dishes were serum-deprived for 24 hours and treated with 10μmol/L substance P in serum-free medium (*Ref.* III. 2. 17) for an additional 24 hours. Following this, the conditioned media were collected and the cells lysed in 1.0 ml of lysis buffer (*Ref.* III. 2. 10). The cell lysate and corresponding medium were pooled and precipitated with 10%

ice-cold TCA for 1 hour in ice. Precipitates were pelleted at 15,000 rpm for 10 minutes at 5°C, washed in 5% ice-cold TCA and then with ethanol. Dried pellets were hydrolyzed in 6 N HCl for 16-18 hours at 110°C. Hydrolysates were neutralized with 12 N KOH and decolorized by passing through a column of activated charcoal. The hydrolysates were made up to 5.0 ml. An aliquot of 1.0 ml was taken for the estimation of hydroxyproline.

The sample was oxidized with 1.0 ml of chloramine T solution (*Ref.* III. 2. 3) at room temperature for 30 minutes with occasional shaking. Oxidation was stopped by adding 1.0 ml of aldehyde-perchloric acid solution (*Ref.* III. 2. 1) and incubating at 60°C for 20 minutes. The colored complex produced by the reaction between the oxidation product of hydroxyproline and *p*-diaminobenzaldehyde was measured spectrophotometrically at 558 nm.

III. 4. 6. Measurement of superoxide generation

Superoxide generation was measured in terms of nitroblue tetrazolium reduction, as described by Siwik *et al* (1999) with some modifications.

Confluent or sub-confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours and exposed to substance P at 10 μmol/L in NBT reduction assay buffer (*Ref.* III. 2. 13) for 4 hours. The medium was discarded and the cells were washed in PBS (*Ref.* III. 2. 14) thrice, and lysed with 0.5 ml distilled water.

The lysates were centrifuged at 1000 rpm for 10 minutes. The absorbance of supernatants was measured spectrophotomtrically at 490 nm.

III. 4. 7. Measurement of ^{45}calcium uptake

^{45}Calcium uptake by cardiac fibroblasts was assayed following the method of Jiang *et al* (1997).

Cells grown to 90% confluence were pre-incubated for 10 minutes in 1.0 ml of HBPSS (*Ref.* III. 2. 8) at 37^{0}C. $^{45}Ca^{2+}$ influx measurements were initiated by adding 5 μCi/ml of $^{45}CaCl_2$ and the cells were incubated for 0, 15, 30 and 60 minutes. At the end of each time point, 1.0 ml of ice-cold HBPSS (*Ref.* III. 2. 8), containing 2 mM lanthanum chloride was added and the medium was discarded within 10-15 seconds. The cells were washed in 100 ml of HBPSS (*Ref.* III. 2. 8), containing 1.0 mM lanthanum chloride by immersing the plates in the buffer for 15 seconds. The plates were drained and the cells were solubilized in 0.5 ml of 0.1 N NaOH for determination of radioactivity. "Zero time" assay was carried out by adding radioactive calcium to ice-cold HBPSS (*Ref.* III. 2. 8), containing lanthanum chloride (final concentration 1 mM), and immediately terminating the reaction and processing for determination of radioactivity. The plates were washed as per the standard procedure. The zero time value was subtracted from every uptake measurement.

III. 4. 8. Effect of spantide on substance P-induced increase in cell number

Sub-confluent, serum-deprived cardiac fibroblasts were pre-treated with the neurokinin-1 receptor antagonist, spantide (Nilsson *et al*, 1985; Villablanca *et al* 1994), at 15 μg/ml for 15 minutes and then exposed to 10 μmol/L substance P for 24 hours in serum-free medium (*Ref.* III. 2. 17) in presence of 15 μg/ml spantide. The cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was performed using a Neubaeur counting chamber.

III. 4. 9. Effect of spantide on substance P-induced increase in superoxide generation in cardiac fibroblasts

Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours followed by pre-treatment with spantide at 15 μg/ml for 15 minutes. The cells were then exposed to substance P at 10 μmol/L in NBT reduction assay buffer (*Ref.* III. 2. 13), containing spantide at 15 μg/ml, for 4 hours. NBT reduction was measured as described in III. 4. 6.

III. 4. 10. Effect of EGTA on substance P-induced increase in [^{3}H]-thymidine incorporation

Cardiac fibroblasts were seeded at a density of 50 x 10^3 per 35 mm dish in growth medium (*Ref.* III. 2. 7) and incubated for 24 hours. Following this, cells

were serum-deprived for 24 hours and then treated with 1 mM EGTA for 15 minutes. The cells were exposed to 10 μmol/L substance P in serum-free medium (*Ref.* III. 2. 17) containing 1 mM EGTA for an additional 24 hours. During the last four hours, the cells were pulsed with [^{3}H]-thymidine at 2.5 μCi/ml and processed for measurement of radioactivity as described in III. 4. 1.

III. 4. 11. Effect of superoxide dismutase on substance P-induced increase in cell number and superoxide production

Cells from passage 3 or 4 were seeded in 35 mm dishes at a density of 5 x 10^3 per 35 mm dish and allowed to grow for 24 hours. Following this, the cells were serum-deprived for 24 hours and then exposed to substance P at 10 μmol/L for 24 hours in serum-free medium (*Ref.* III. 2. 17) containing 200 U/ml superoxide dismutase. The cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was done.

Serum-deprived, sub-confluent cultures of fibroblasts were exposed to substance P at 10 μmol/L for 24 hours in serum-free medium containing 200 U/ml of superoxide dismutase. During the final four hours, the cells were transferred to NBT reduction assay buffer containing substance P at 10 μmol/L and superoxide dimutase at 200 U/ml. At 24 hours, NBT reduction was measured as described in III.4.6.

III. 4. 12. Effect of N-acetyl cysteine on substance P-induced increase in cell number

Following serum-deprivation for 24 hours, sub-confluent cells were exposed to 10 μmol/L substance P for 24 hours in serum-free medium (*Ref.* III. 2. 17) containing 5 mM N-acetyl cysteine. The cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was performed.

III. 4. 13. Effect of EGTA on substance P-induced superoxide generation

Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours and then exposed to substance P at 10 μmol/L in NBT reduction assay buffer (*Ref.* III. 2. 13) with 1.0 mM EGTA for 4 hours. NBT reduction was measured as described in III. 4. 6.

III. 4. 14. Effect of calcium-free medium on substance P-induced superoxide generation

Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours. The cells were then exposed to 10 μmol/L substance P in NBT reduction assay buffer (*Ref.* III. 2. 13) without calcium for 4 hours. NBT reduction was measured as described in III. 4. 6.

III. 4. 15. Effect of EGTA, calcium-free incubation, calcium channel blockers on superoxide generation

Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours. The cells were then incubated in nitroblue tetrazolium reduction assay buffer containing 1 mM EGTA, or 10 μM diltiazem or 50 μM verapamil for 4 hours. Another batch of cells was incubated in nitroblue tetrazolium reduction assay buffer without calcium for 4 hours. NBT reduction under these conditions was measured as described in III. 4. 6.

III. 5. DOES SUBSTANCE P REGULATE CARDIAC FIBROBLASTS IN MAGNESIUM DEFICIENCY?

III. 5. 1. Serum from magnesium-deficient rats

Magnesium deficiency was induced in rats as reported earlier from this laboratory (Kumar *et al*, 1997; Shivakumar and Kumar, 1997). Sprague-Dawley rats weighing 65 ± 5 g were randomly divided into two groups and were fed a magnesium-deficient or magnesium-sufficient diet. The rats were housed in wire-bottomed cages and were pair-fed the appropriate diet and de-ionized triple-distilled water was provided *ad libitum*. On the 6^{th} day, the rats were anesthetized with ether and killed. Blood was collected from the abdominal aorta. Serum was

separated by centrifugation, filtered through 0.22 μm membrane and stored at -80°C. Hypomagnesemia was confirmed in these animals.

III. 5. 2. Measurement of DNA synthesis

Cardiac fibroblasts from passage 3 or 4 were seeded at a density of 50 x 10^3 per 35 mm dish in growth medium (*Ref.* III. 2. 7) and allowed to grow for 24 hours. They were transferred to serum-free medium (*Ref.* III. 2. 17) for 24 hours and then were exposed to MgD (*Ref.* III. 2. 11) for an additional 24 hours. During the last four hours, the cells were pulsed with [^{3}H]-thymidine at 2.5 μCi/ml. Cells exposed to MgS (*Ref.* III. 2. 12) served as control. Radioactivity associated with trichloroacetic acid insoluble material was detected as described in III. 4. 1.

III. 5. 3. Measurement of cell proliferation

Cardiac fibroblasts isolated from normal adult rats were seeded at a density of 50 x 10^3 per 35 mm dish and allowed to grow for 24 hours in growth medium (*Ref.* III. 2. 7). The cells were transferred to serum-free medium (*Ref.* III. 2. 17) for 24 hours. Following serum-deprivation, cells were exposed for 24 hours to MgD (*Ref.* III. 2. 11). Cells exposed to MgS (*Ref.* III. 2. 12) served as control. At 24 hours, the cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was performed.

III. 5. 4. Measurement of net collagen content

Confluent cultures of cardiac fibroblasts in 100 mm dish were serum-deprived for 24 hours and exposed to either MgD (*Ref.* III. 2. 11) or MgS (*Ref.* III. 2. 12) and incubated for an additional 24 hours. Following this, net collagen content of the medium and the corresponding cell lysate was measured as described in III. 4. 5.

III. 5. 5. Measurement of superoxide generation

Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours and exposed to MgS (*Ref.* III. 2. 12) or MgD (*Ref.* III. 2. 11) for 24 hours. The cells were then transferred to NBT reduction assay buffer (*Ref.* III. 2. 13) and incubated for 30 minutes. NBT reduction was estimated as described in III. 4. 6.

III. 5. 6. Effect of spantide on magnesium-deficient serum-induced increase in cell number and superoxide generation

Serum-deprived, sub-confluent cells were pre-treated with spantide at 15 μg/ml in growth medium (*Ref.* III. 2. 7) for 15 minutes and then exposed for 24 hours to MgS (*Ref.* III. 2. 12) or MgD (*Ref.* III. 2. 11) with and without 15 μg/ml spantide. At 24 hours, the cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was performed.

Serum-deprived, confluent cultures were pre-treated with spantide at 15 μg/ml for 15 minutes and then exposed for 24 hours to MgS or MgD with or without 15 μg/ml spantide. At 24 hours, cells were transferred to NBT reduction assay buffer (*Ref.* III. 2. 13) and incubated for 30 minutes. NBT reduction was estimated as described in III. 4. 6.

III. 5. 7. Effect of N-acetyl cysteine and superoxide dismutase on magnesium deficient serum-induced increase in cell number

Serum-deprived, sub-confluent cells were exposed for 24 hours to MgS (*Ref.* III. 2. 12) or MgD (*Ref.* III. 2. 11) containing 5 mM N-acetyl cysteine or 200 U/ml superoxide dismutase. At 24 hours, the cells were detached using trypsin/EDTA solution (*Ref.* III. 2. 19) and cell count was done.

III. 6. STATISTICAL ANALYSIS

In experiments with substance P, cells exposed to serum-free medium without substance P served as control. In experiments designed to examine the role of humoral factors in magnesium deficiency, cells exposed to serum from rats on magnesium-sufficient diet served as control. Values were computed on per dish basis and expressed as Mean ± SD. Collagen production in response to substance P was expressed as nmol/dish and [^{3}H]-thymidine incorporation into DNA in response to magnesium-deficient serum and ^{45}calcium uptake were expressed as cpm/dish. Data from all the other experiments were expressed as percentage over control. Student *t* test was used to examine differences between experimental groups. Significance was determined at $p<0.05$.

IV. RESULTS

Substance P is released from peptidergic neurons in the heart in response to ischemia and hypoxia (Milner et al, 1989; Ustinova et al, 1995). However, its action on cardiac cells has not hitherto been studied. More recently, substance P has been postulated to play a role in the pathobiology of magnesium deficiency.

This Section deals with three aspects:

1. ***Setting up an in vitro model of cardiac fibroblasts in culture***
2. ***Investigations to ascertain if substance P elicits a proliferative response in vitro in cardiac fibroblasts and to delineate the underlying mechanism(s)***
3. ***Attempts to elucidate the role of humoral factors, especially substance P, in the activation of cardiac fibroblasts in magnesium deficiency.***

IV. 1. CHARACTERIZATION OF CARDIAC FIBROBLASTS

IV. 1. 1. Morphological analysis

Selective enrichment of cardiac fibroblasts was achieved by a differential attachment procedure. Cardiac fibroblasts attached to cell culture dishes by 150 minutes of incubation at 37°C in a CO_2 incubator, leaving other cell types unattached. As shown in Figure 1, the attached cells had a dense, nest-like morphology. Some attached cells had begun to spread out. Figure 2 shows the morphology of the cells at 24 hours after isolation. At 48 hours, most of the cells had spread out to attain typical spindle-shaped morphology (Figure 3). The cells

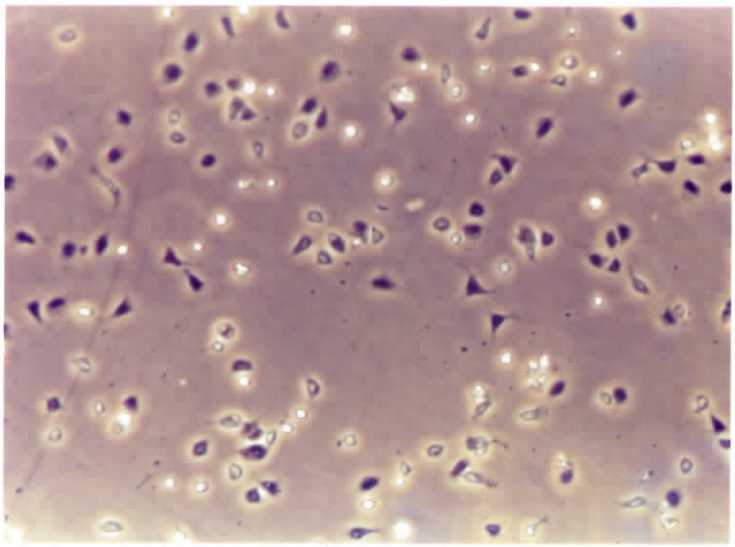


Figure 1. Photomicrograph of cardiac fibroblasts 2 1/2 hours after isolation. Magnification 100X

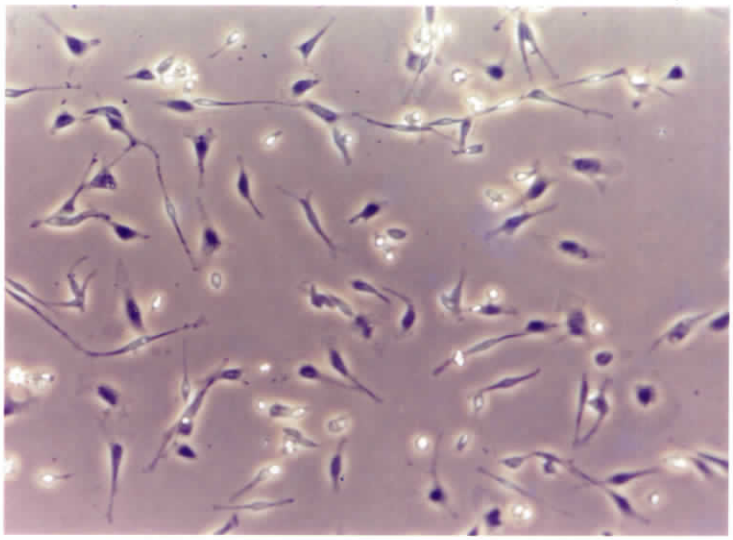


Figure 2. Photomicrograph of cardiac fibroblasts 24 hours after isolation. Magnification 100X

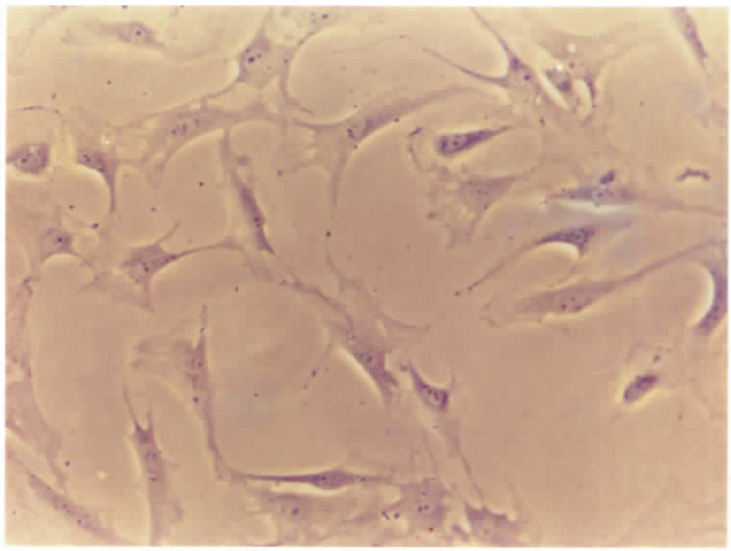


Figure 3. Photomicrograph of cardiac fibroblasts 48 hours after isolation. Magnification 100X

had well-defined nuclei containing several nucleoli. Some cells appeared irregular in shape. Figure 4 shows the cardiac fibroblast monolayer, at confluence.

IV. 1. 2. Immunostaining for vimentin

Cells from passage 3 grown on cover slips were tested for immunoreactivity with antibodies for cytoskeletal and cell surface proteins. The cells did not stain with anti-desmin or anti-factor VIII antibody, indicating the absence of smooth muscle and endothelial cells in these cultures. However, ≥ 99% cells reacted positively, when stained with anti-vimentin antibody, confirming the fibroblastic nature of the cells (Figure 5 & 6).

IV. 1. 3. Growth kinetics

Figure 7 shows the growth curve of myocardial fibroblasts over 4 days following seeding. There was no lag phase following seeding and a rapid proliferative phase was observed between days 2 and 4. The population doubling time was calculated to be about 24 hours.

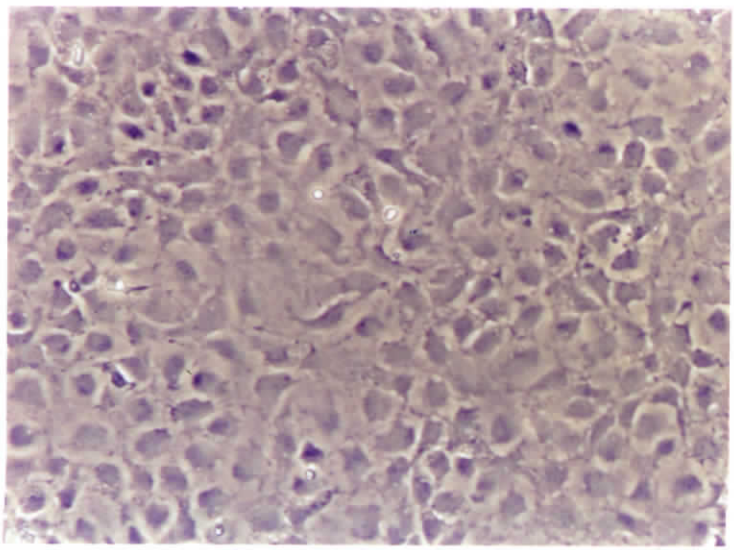


Figure 4. Photomicrograph of cardiac fibroblasts at confluence.
Magnification 100X

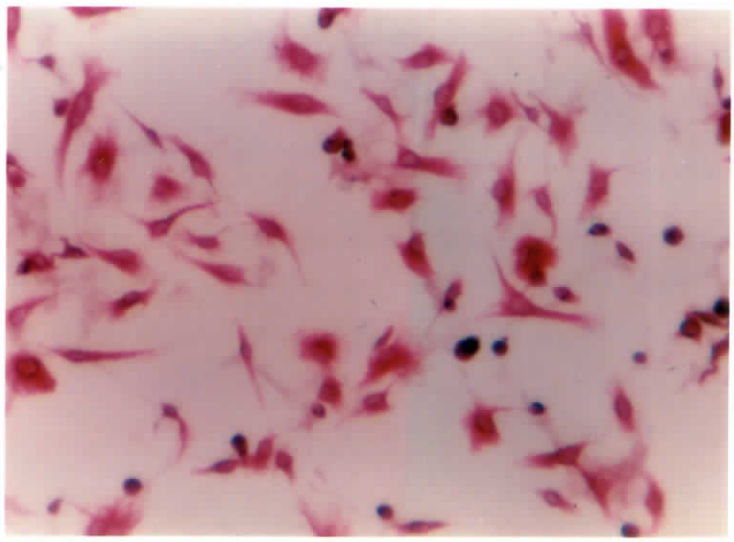


Figure 5. Cardiac fibroblasts stained with anti-vimentin antibodies.
Magnification 100X

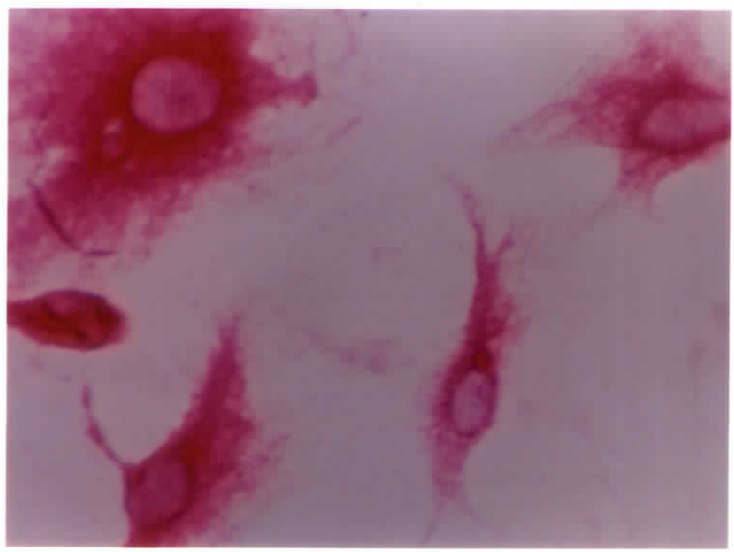
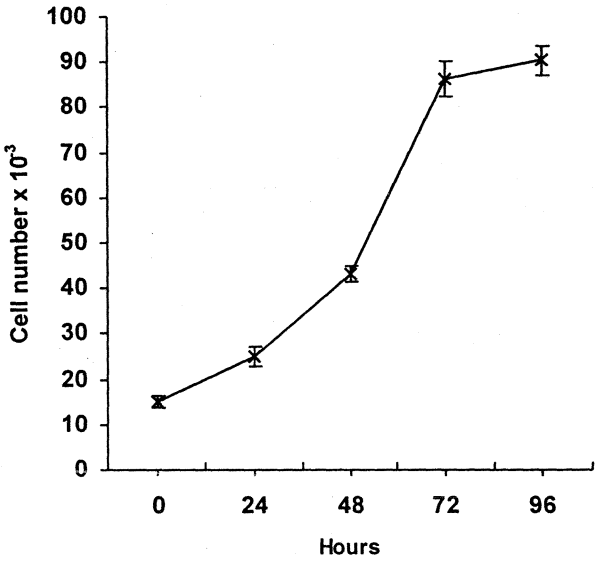


Figure 6. Cardiac fibroblasts stained positive for vimentin.
Magnification 200X

Figure 7. Growth curve of adult myocardial fibroblasts in culture



Normal adult myocardial fibroblasts were seeded at 15 x 10^3 cells per well in a 4-well plate. Cell number was determined at the specified intervals.

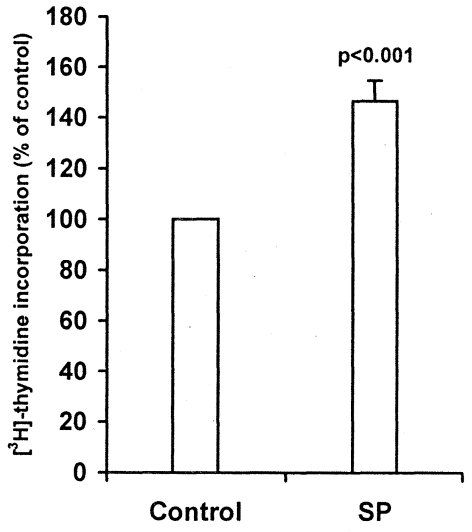
IV. 2. SUBSTANCE P IS MITOGENIC TO CARDIAC FIBROBLASTS

IV. 2. 1. Substance P increases incorporation of [^{3}H]-thymidine into DNA

Substance P is reported to be mitogenic to certain cell types like skin fibroblasts (Nilsson *et al*, 1985), endothelial cells (Villablanca *et al*, 1994) and vascular smooth muscle cells (Nilsson *et al*, 1985; Payan, 1985). To examine whether substance P exerts a growth-promoting action on cardiac fibroblasts,

quiescent cultures of cardiac fibroblasts were exposed to substance P for 24 hours in serum-free medium. Labeling of fibroblast DNA with radioactive thymidine was enhanced by about 46% (Figure 8) in substance P-treated cells ($n=20$, $p<0.001$). Moreover, it was observed that even in the absence of insulin and transferrin in the culture medium, substance P had a stimulatory effect of comparable magnitude on [^{3}H]-thymidine incorporation, indicating that substance P *per se* exerts the effect even in the absence of other growth factors (data not presented).

Figure 8. Effect of substance P on DNA synthesis



Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were treated with SP at 10 μmol/L for 24 hours. [^{3}H]-thymidine was included in the medium at 2.5 μCi/ml during the final 4 hours of incubation with SP. Values were computed on per dish basis and expressed as Mean ± SD of 20 separate determinations and compared with untreated control (=100).

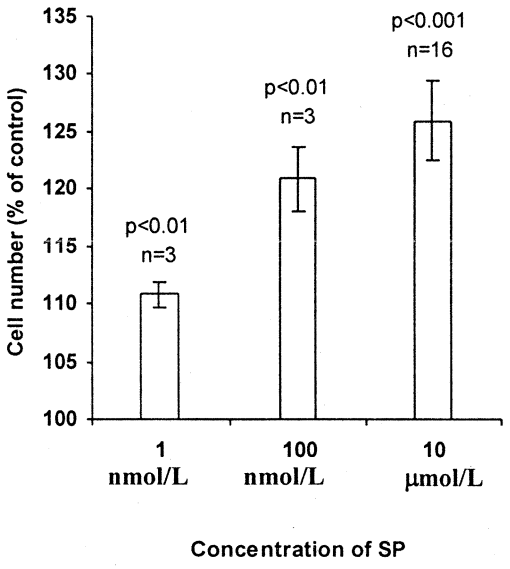
IV. 2. 2. Substance P stimulates cardiac fibroblast proliferation in a dose-dependent manner

To ascertain whether the increased DNA synthesis in cardiac fibroblasts upon exposure to substance P reflects a proliferative response, cell count was performed following treatment with substance P for 24 hours. Further, to determine the concentration range over which substance P exerts mitogenic effect on adult cardiac fibroblasts *in vitro* the dose-response was studied. As shown in Figure 9, even a dose of 1 nmol/L SP produced a small but significant increase in cell number. Increasing the concentration of SP from 1 nmol/L to 10 μmol/L caused a progressive and significant increase in cell number ($n=16$, $p<0.001$) A concentration of 10 μmol/L substance P, at which the effect was maximal, was chosen for further experiments.

IV. 2. 3. A. Substance P does not alter total protein synthesis

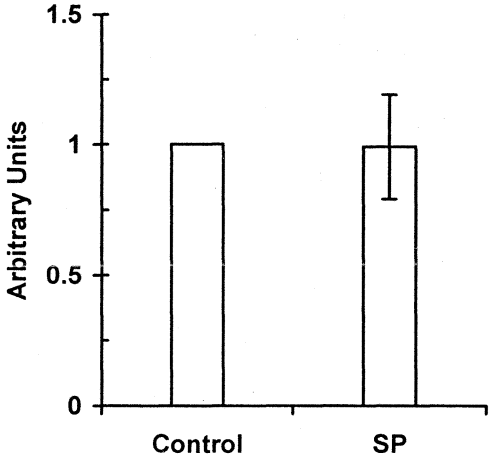
The effect of substance P on total protein synthesis in cultured cardiac fibroblasts was determined in terms of incorporation of [^{3}H]-phenylalanine into acid-precipitable material. Confluent cultures of fibroblasts were serum-deprived for 24 hours and exposed for 20 hours to substance P at 10 μmol/L in serum-free medium containing [^{3}H]-phenylalanine. Figure 10 shows that substance P had no effect on protein synthesis ($n=12$).

Figure 9. Effect of SP on cell number – dose response



Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were treated with SP at the indicated concentrations for 24 hours. Cell count was performed using a Neubauer counting chamber. Values are expressed as Mean ± SD. p values are with respect to control (=100).

Figure 10. Effect of SP on total protein synthesis



Following serum-deprivation, confluent cultures of fibroblasts in 35 mm dishes were exposed to SP at 10 μmol/L and [^{3}H]-phenylalanine at 2.5 μCi/ml for 20 hours. Acid-precipitable radioactivity was measured. Values were computed on per dish basis and expressed, using arbitrary units, as Mean ± SD of 12 separate determinations and compared with untreated control.

B. Substance P does not alter total protein content

Total protein content of confluent cultures of adult cardiac fibroblasts exposed to 10 μmol/L substance P for 24 hours was measured by a modified Lowry assay. As Figure 11 shows, substance P had no effect on total protein content over 24 hours (n=12).

These results showed that substance P does not exert a hypertrophic action on cardiac fibroblasts.

IV. 2. 5. Substance P does not influence net collagen production

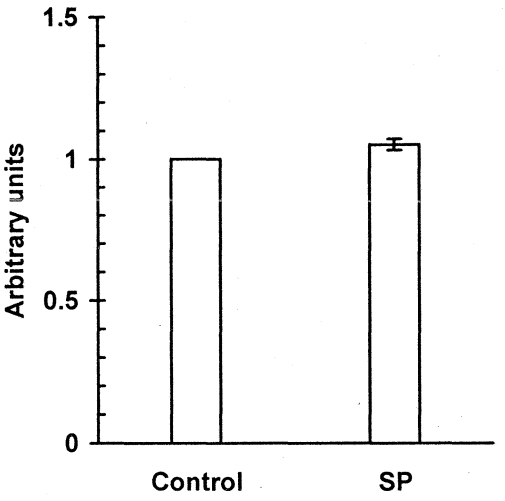
As cardiac fibroblasts are the main source of collagens (Bashey *et al*, 1992), the effect of substance P on net collagen production (collagen deposition) was determined. The cell monolayer and medium were pooled up and used for determination of collagen-associated hydroxyproline content. Results presented in Figure 12 show that treatment of adult cardiac fibroblasts with 10 μmol/L substance P for 24 hours did not alter net collagen production by these cells (n=6).

IV. 2. 6. Substance P acts via neurokinin-1 receptor

Spantide, a neurokinin- 1 receptor antagonist, attenuates substance P-induced proliferative response. Substance P is known to act via the neurokinin-1 (NK-1) receptor in different cell types (Nakanishi, 1991). In the present study,

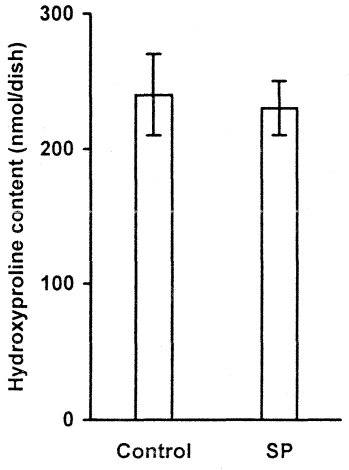
spantide, an NK-1 receptor antagonist (Nilsson *et al*, 1985; Villablanca *et al*, 1994), attenuated the increase in fibroblast proliferation in response to substance P, showing that SP acts via NK-1 receptors ($n=8$, $p<0.001$) (Figure 13).

Figure 11. Effect of SP on total protein content



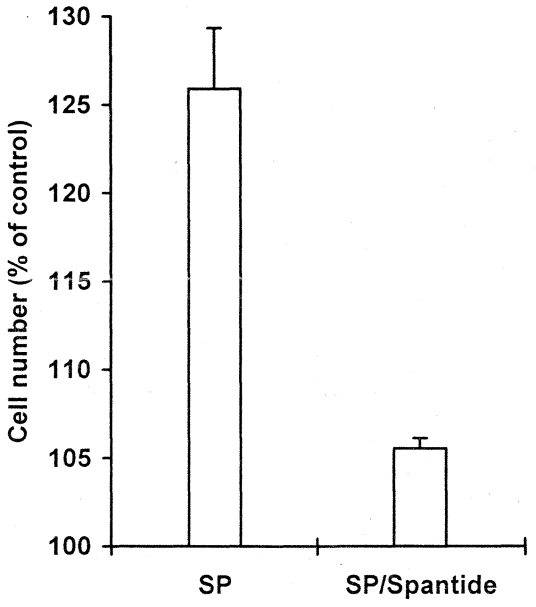
Following serum-deprivation, confluent cultures of fibroblasts in 35 mm dishes were exposed to SP at 10 μmol/L for 24 hours. Protein content was determined by a modified Lowry assay. Values were computed on per dish basis and expressed, using arbitrary units, as Mean ± SD of 12 separate determinations and compared with untreated control.

Figure 12. Effect of SP on net collagen production by cardiac fibroblasts



Confluent cultures in 100 mm dishes were serum-deprived for 24 hours and then exposed to SP at 10 μmol/L for 24 hours. Net collagen production was measured in terms of hydroxyproline content of the monolayer and the medium. Values are Mean ± SD of 6 separate measurements.

Figure 13. Effect of spantide on SP-induced increase in cell number



To assess the effect of spantide on SP-induced fibroblast proliferation, sub-confluent, serum-deprived cells in 35 mm dishes were incubated with 15 μg/ml of spantide in presence of SP for 24 hours, after pre-treatment of the cells with spantide for 15 min (n=8). Values are Mean ± SD. Control (=100) vs SP, $p<0.001$; SP vs SP/Spantide, $p<0.001$

MECHANISM OF ACTION

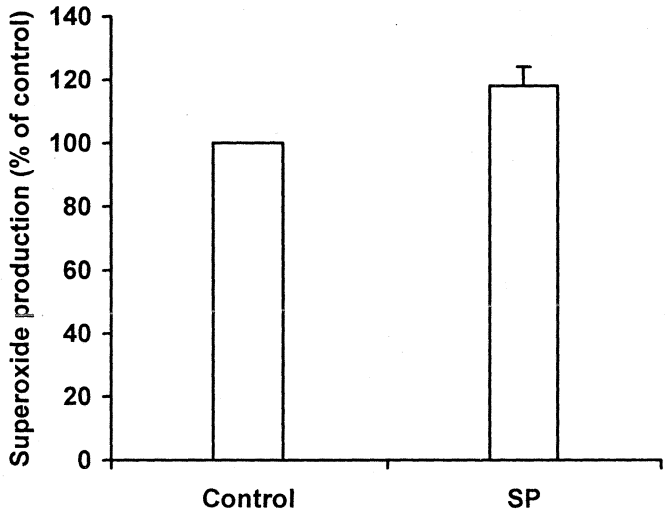
The findings showed that substance P exerts a hyperplastic effect on cardiac fibroblasts via NK-1 receptors. Following this, studies were undertaken to probe the mechanisms underlying the mitogenic effect of substance P on cardiac fibroblasts. Substance P has been shown to modulate calcium homeostasis and superoxide production in certain cell types (Tanabe et al, 1996a; Tanabe et al, 1996b; Sarosi et al, 1998) but these changes have not been linked to its mitogenic effect. Based on these observations, experiments were designed to investigate whether substance P increases superoxide generation and calcium influx in cardiac fibroblasts and if these are related to its mitogenic action. Superoxide generation and calcium uptake in cardiac fibroblasts in response to substance P were measured in terms of nitroblue tetrazolium reduction and ^{45}Ca uptake, respectively.

IV. 2. 7. Substance P enhances superoxide generation

In the present study, substance P was found to enhance superoxide production consistently. In confluent cultures (Figure 14), following exposure to substance P for 4 hours, the increase was found to be about 18% ($n=20$, $p<0.001$). However, in sub-confluent cultures (Figure 15), treatment with substance P for 24

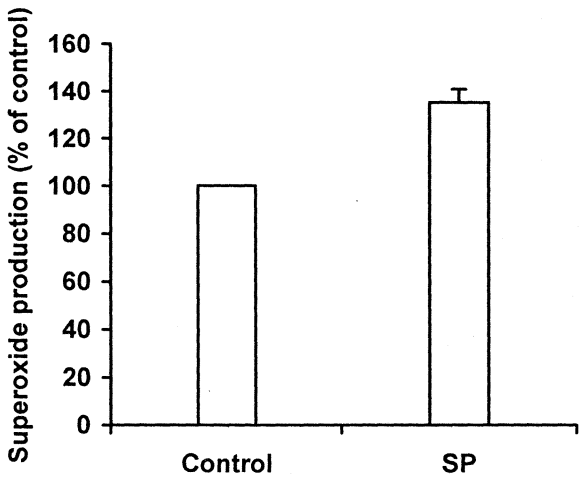
hours resulted in a 35% increase in superoxide generation (n=4, $p<0.001$) (about 2-fold higher, vis a vis Figure 14).

Figure 14. Effect of SP on superoxide production in confluent cultures of fibroblasts



Confluent cultures of cardiac fibroblasts in 35 mm dishes were exposed to SP at 10 μmol/L (n=20) in KRPBS for 4 hours in presence of NBT. Values are Mean ± SD and are compared with control (=100). Control vs SP, p<0.0.001

Figure 15. Effect of SP on superoxide production in sub-confluent cultures of fibroblasts



Sub-confluent cultures in 35 mm dishes were serum-deprived for 24 hours and then exposed to SP at 10 μmol/L (n=4) for 24 hours. During the last 4 hours, the cells were transferred to KRPBS containing NBT to measure superoxide generation. Control vs SP, $p<0.001$

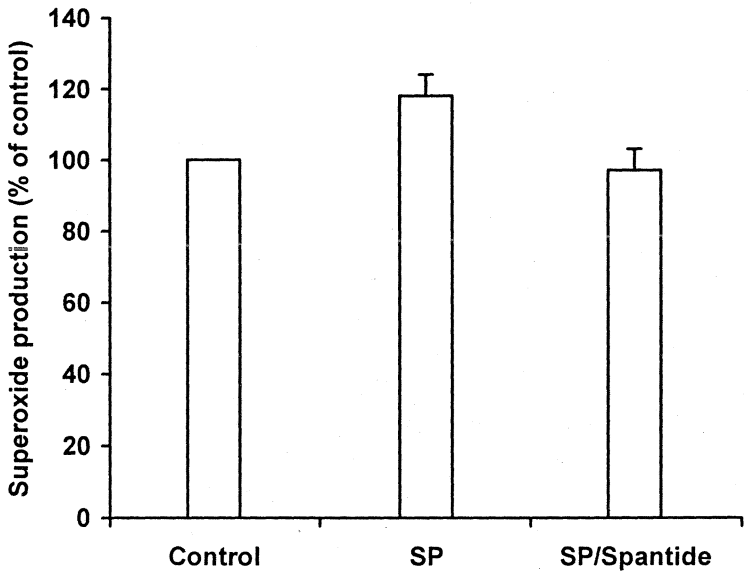
Spantide blocks substance P-stimulated superoxide generation. Spantide was also found to abolish the stimulatory effect of substance P on superoxide generation (Figure 16), confirming involvement of NK-1 receptors in mediating increased superoxide generation ($n=20$, $p<0.001$).

IV. 2. 8. Substance P enhances ^{45}calcium uptake

Figure 17 shows kinetics of ^{45}Ca uptake by cardiac fibroblasts. Substance P enhanced ^{45}Ca influx into cardiac fibroblasts by 21% at 30 min ($n=8$, $p<0.001$) and 170% at 60 min ($n=8$, $p<0.001$). In untreated cells, there was a linear increase in

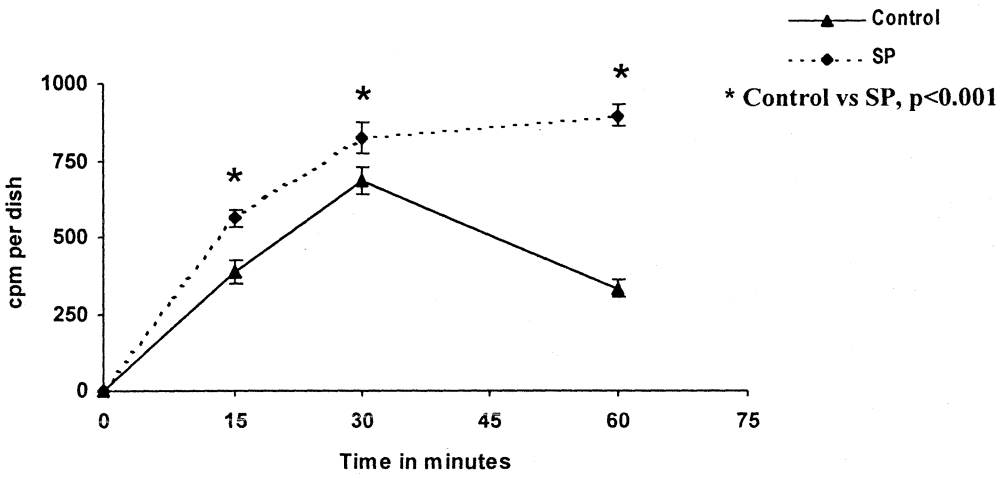
^{45}Ca uptake up to 30 min followed by a dip whereas in substance P-treated cells, the dip was not observed so that the difference between control and substance P-treated cells was much higher at 60 min.

Figure 16. Effect of spantide on SP-enhanced superoxide production



Confluent cultures of cardiac fibroblasts in 35 mm dishes were exposed to SP at 10 μmol/L (n=20) in KRPBS for 4 hours in presence of NBT. To assess the effect of spantide (n=20), cells were incubated with spantide at 15 μg/mL in presence of SP, after pre-treatment of the cells with spantide for 15 min. Values are Mean ± SD. Control vs SP, $p<0.0.001$; SP vs SP/Spantide, $p<0.001$

Figure 17. Effect of SP on ^{45}calcium uptake



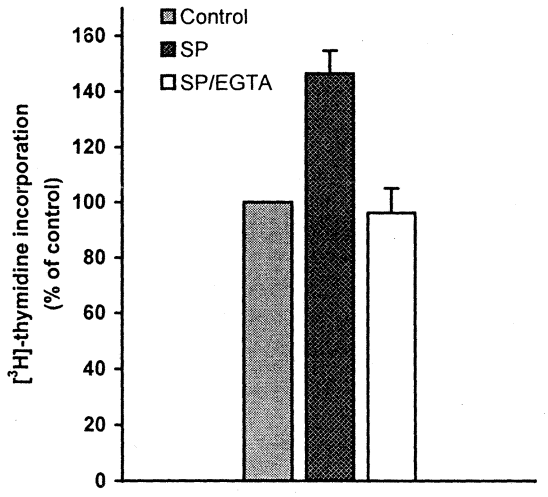
Fibroblasts in 35 mm dishes were exposed to SP at 10 μmol/L for different periods of time as shown. ^{45}Calcium uptake was measured, as described under Methods, in HBPSS following addition of ^{45}calcium at 5 μCi/ml. Values are expressed as Mean ± SD of 8 separate measurements.

IV. 2. 9. Extracellular calcium is required for substance P-triggered mitogenesis

Substance P is reported to stimulate hydrolysis of phosphatidyl inositol 4, 5-bisphosphate (PIP_2) with resultant changes in calcium homeostasis in some cell types. Therefore, it was hypothesized that extracellular calcium may be involved in the expression of substance P effects on cardiac fibroblasts. When extracellular calcium was blocked by EGTA, the substance P-induced increase in [^{3}H]-thymidine

incorporation was abolished (n=20, $p<0.001$) (Figure 18). This, in conjunction with the increase in ^{45}Ca uptake in response to SP (Figure 17), suggested a role for calcium influx in mediating the mitogenic action of substance P.

Figure 18. Effect of EGTA on SP-stimulated DNA synthesis

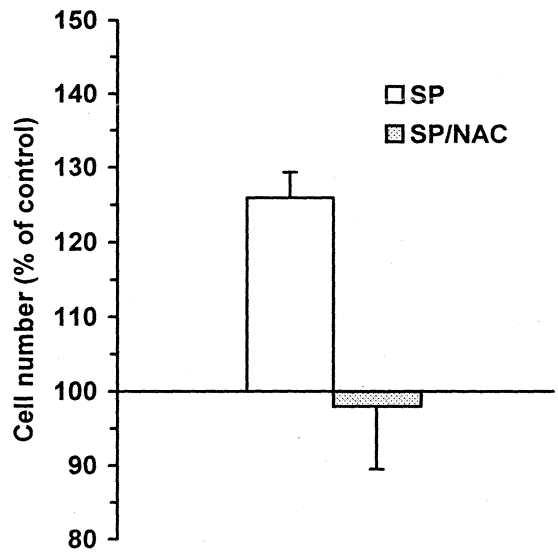


Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were treated with SP at 10 μmol/L for 24 hours. [^{3}H]-thymidine was included in the medium at 2.5 μCi/ml during the final 4 hours of incubation with SP. To assess the effect of EGTA on SP-induced DNA synthesis, cells were incubated with 1 mmol/L EGTA in presence of SP, after pre-treatment of the cells with EGTA for 15 min. Values were computed on per dish basis and expressed as Mean ± SD of 20 separate determinations.
Control vs SP, $p<0.001$; SP vs SP/EGTA, $p<0.001$

IV. 2. 10. Anti-oxidants inhibit substance P-stimulated cellular hyperplasia

The anti-oxidant, N-acetyl cysteine, abolished the increase in cell number in response to SP, suggesting that substance P-induced cellular hyperplasia may be mediated by reactive oxygen species (n=8, $p<0.001$) (Figure 19).

Figure 19. Effect of N-acetyl cysteine on SP-induced cell proliferation

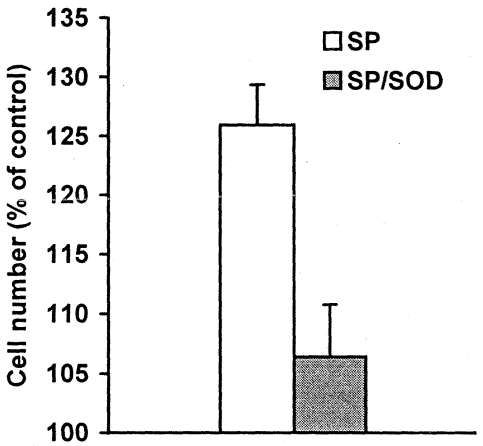


To assess the effect of N-acetyl cysteine, sub-confluent, serum-deprived cultures in 35 mm dishes were treated with 10 μmol/L of SP in presence of 5 mmol/L N-acetyl cysteine (n=8) for 24 hours. Values are Mean ± SD. Control (=100) vs SP, $p<0.001$; SP vs SP/NAC, $p<0.001$

In order to confirm the involvement of free radicals in substance P-mediated mitogenesis in cardiac fibroblasts, cells were exposed to substance P in

the presence of 200 U/ml superoxide dismutase, a free radical scavenger. The inhibitory effect of superoxide dismutase on substance P-induced mitogenesis (Figure 20) confirmed the role of superoxide in the hyperplastic response (n=4, $p<0.001$).

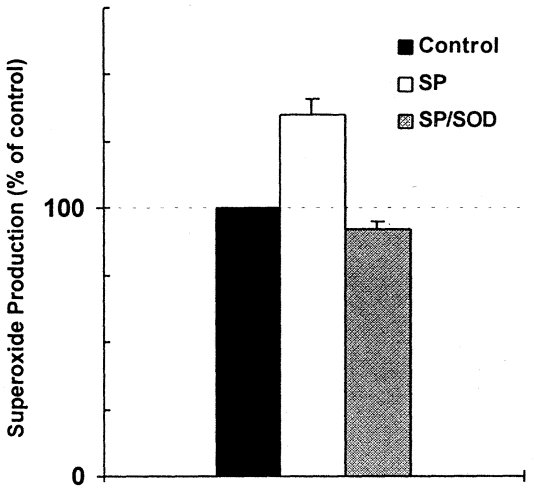
Figure 20. Effect of superoxide dismutase on SP-induced cell proliferation



To assess the effect of superoxide dismutase, sub-confluent, serum-deprived cultures in 35 mm dishes were treated with 10 μmol/L of SP in presence of 200 U/ml SOD (n=4) for 24 hours. Values are Mean ± SD. Control (=100) vs SP $p<0.001$; SP vs SP/SOD, $p<0.001$

In the presence of superoxide dismutase, there was no significant increase in superoxide generation in serum-deprived, sub-confluent cardiac fibroblasts exposed to substance P at 10 μmol/L for 24 hours (Figure 21).

Figure 21. Effect of superoxide dismutase on SP-enhanced superoxide generation in sub-confluent cultures of fibroblasts



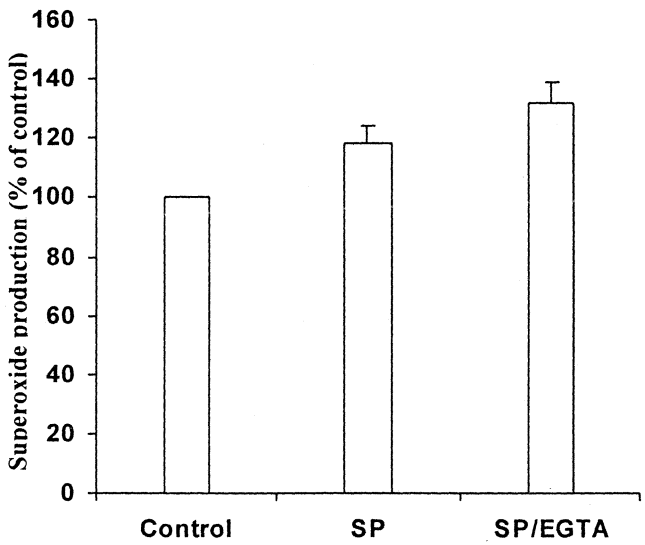
Sub-confluent cultures in 35 mm dishes were serum-deprived for 24 hours and then exposed to SP at 10 μmol/L (n=4) with or without SOD at 200 U/ml (n=4). During the last 4 hours, the cells were transferred to NBT reduction assay buffer to measure superoxide generation. Values are expressed as Mean ± SD. Control vs SP, $p<0.001$; SP vs SP/SOD, $p<0.001$

IV. 2. 11. EGTA and substance P enhance superoxide generation

Since substance P was found to stimulate calcium influx and substance P-induced hyperplasia was dependent on extracellular calcium, experiments were done to investigate the role of extracellular calcium in substance P-stimulated increase in superoxide generation. Cells were exposed to substance P in the presence of EGTA, a calcium chelator, and superoxide generation was measured in

terms of nitroblue tetrazolium reduction. Surprisingly, the effect of a combination of EGTA and substance P was more pronounced than substance P alone (31.5% vs 18%; n=20; $p<0.001$) (Figure 22).

Figure 22. Effect of EGTA on SP-enhanced superoxide production

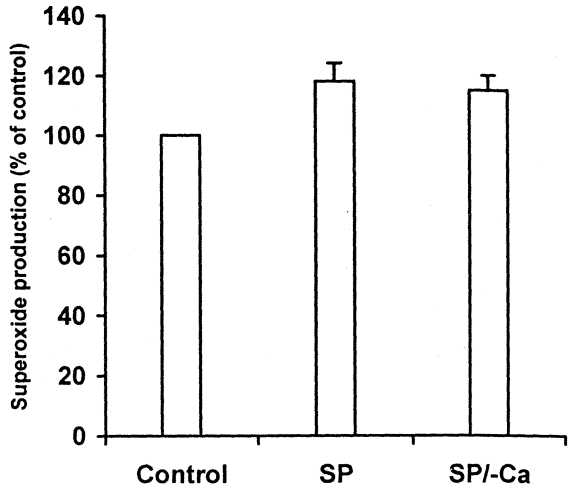


Confluent cultures of cardiac fibroblasts in 35 mm dishes were exposed to SP at 10 µmol/L (n=20) in KRPBS for 4 hours in presence of NBT. To assess the effect of EGTA (n=20), cells were incubated with EGTA at 1 mmol/L in presence of SP, after pre-treatment of the cells with EGTA for 15 min. Values are Mean ± SD.
Control vs SP, $p<0.001$; SP vs SP/EGTA, $p<0.05$

IV. 2. 12. Calcium-free medium and substance P increase superoxide generation

Exposing cardiac fibroblasts to substance P in calcium-free medium increased endogenous superoxide production in these cells (n=4, $p<0.01$) (Figure 23).

Figure 23. Effect of calcium-free incubation on SP-enhanced superoxide production



Confluent cultures of cardiac fibroblasts in 35 mm dishes were exposed to substance P at 10 μmol/L in KRPBS with or without calcium for 4 hours in presence of NBT. Values are expressed as Mean ± SD. Control vs SP, $p<0.001$ (n=20); Control vs SP/(-)Ca^{++}, $p<0.01$ (n=4)

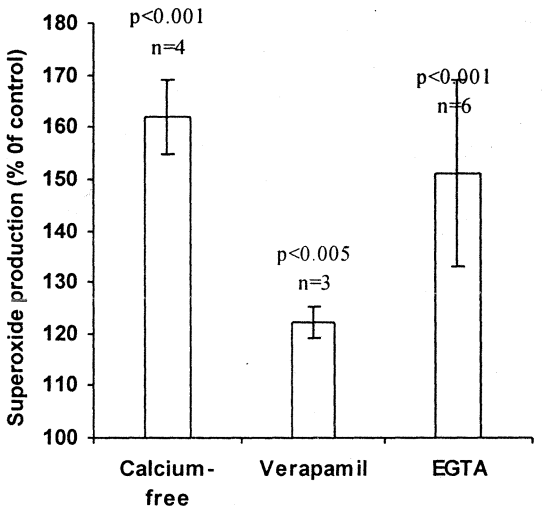
As a combination of substance P and EGTA had a more pronounced effect on superoxide production than substance P alone, experiments were carried out to ascertain the effects of EGTA, calcium-free incubation and calcium channel blockers per se on superoxide generation in cardiac fibroblasts.

IV. 2. 13. EGTA *per se* enhances superoxide generation

Blocking extracellular calcium with EGTA was found to increase the production of superoxide anion in cardiac fibroblasts. Treatment of confluent

cardiac fibroblast cultures with 1 mM EGTA for 4 hours produced about 51% increase in superoxide production (Figure 24).

Figure 24. Superoxide production in cardiac fibroblasts in response to calcium-free incubation, verapamil and EGTA

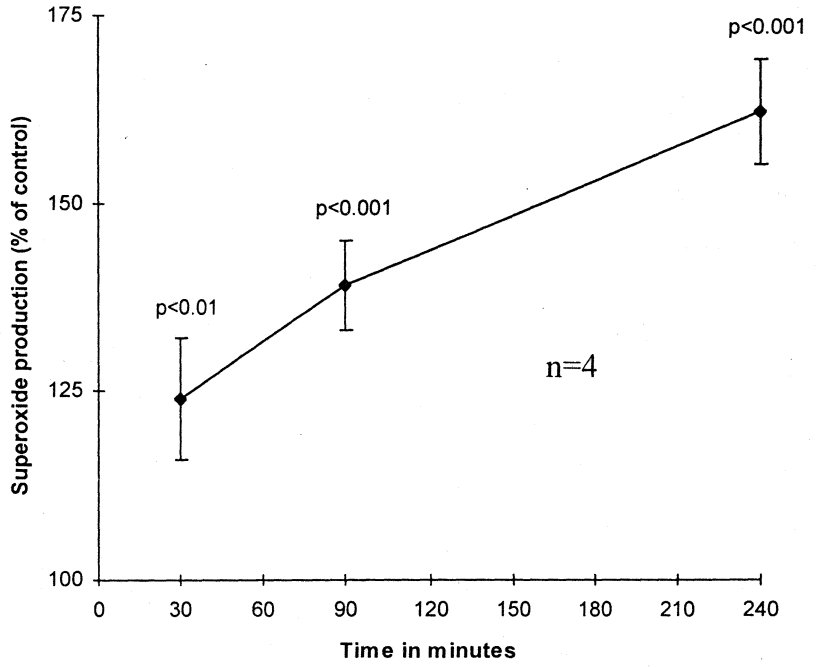


Confluent cultures of cardiac fibroblasts in 35 mm dishes were subjected to the indicated treatments for 4h in KRPBS and NBT assay was performed as described under methods. Results are expressed as percent of control (baseline, 100%). Values are expressed as Mean ± SD.

IV. 2. 14. Calcium-free incubation *per se* enhances superoxide generation

Calcium-free incubation for 4 hours caused a 62% increase in superoxide levels in cardiac fibroblasts (Figure 24). Moreover, exposure to calcium-free conditions had incremental effect with time (Figure 25).

Figure 25. Superoxide production in cardiac fibroblasts upon calcium-free incubation: time-course



Confluent cultures of cardiac fibroblasts in 35 mm dishes were subjected to calcium-free incubation in KRPBS for 30, 90 and 240 minutes and NBT assay was performed as described under "Methods". Values are expressed as Mean ± SD, as percent of control (=100).

IV. 2. 15. Calcium channel blockers enhance superoxide generation in cardiac fibroblasts

Treatment of confluent cultures of cardiac fibroblasts for 4 hours with calcium channel blockers resulted in enhanced superoxide production in these cells. Verapamil, at 50 μM increased superoxide anion production by about 22% in

cardiac fibroblasts (Figure 24). Diltiazem, at 10 μM, elicited a similar response, of comparable magnitude (data not shown).

MECHANISM OF CARDIAC FIBROGENESIS IN MAGNESIUM DEFICIENCY: A ROLE FOR SUBSTANCE P?

Having established that substance P is mitogenic to cardiac fibroblasts, an attempt was made to test the hypothesis that humoral factors, and substance P in particular, may modulate cardiac fibroblast function in magnesium deficiency.

Magnesium deficiency is known to produce a cardiomyopathy, characterized by focal necrosis and fibrosis (Bloom, 1988). The possibility that oxidative injury may contribute to the cardiac lesions of magnesium deficiency is supported by several lines of evidence (Freedman et al, 1990; Dickens et al, 1992; Kramer et al, 1994; Kumar and Shivakumar, 1997). Further, significant alterations in collagen turnover rates and fibroblast proliferation provided evidence of a wound healing response in the myocardium, following possible oxidative damage (Kumar et al, 1997). No attempt has, however, been made to identify the factors that activate myocardial fibroblasts during magnesium deficiency although fibroblasts are known to play a central role in the context of tissue injury (Weber et al, 1991; Brilla et al, 1994). Probing the molecular pathology of magnesium deficiency, several studies show that acute magnesium deficiency produces a pro-inflammatory condition marked by elevations in circulating levels of pro-oxidant and mitogenic factors (Weglicki et al, 1992). It

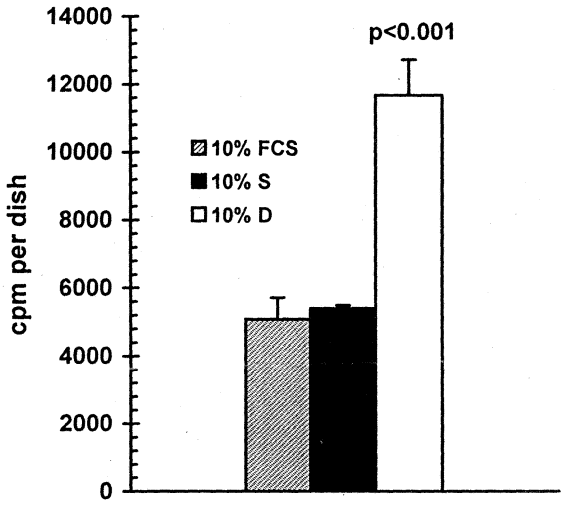
appears that humoral factors may play a role in the cardiovascular consequences of hypomagnesemia. The involvement of humoral factors in the pathogenesis of the cardiomyopathy of magnesium deficiency is particularly relevant as cardiac tissue levels of magnesium remain unaltered even as serum levels decrease in the rodent model of acute magnesium deficiency (Weglicki et al, 1996; Kumar et al, 1997).

IV. 3. HUMORAL FACTORS MODULATE CARDIAC FIBROBLAST FUNCTION IN MAGNESIUM DEFICIENCY

IV. 3. 1. DNA synthesis

Serum from rats on magnesium-deficient diet for six days increased incorporation of [^{3}H]-thymidine into fibroblast DNA by about 116% over control in 24 hours while the increase in [^{3}H]-thymidine incorporation in cells exposed to magnesium-sufficient serum was comparable to that with 10% fetal bovine serum (Figure 26).

Figure 26.Effect of rat sera on DNA synthesis



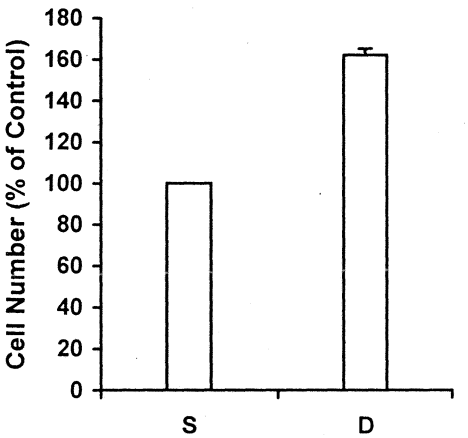
Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were exposed for 24 hours to serum (10%) from magnesium-sufficient (S) or –deficient (D) rats, including [^{3}H]-thymidine at 2.5 μCi/ml in the medium during the final 4 hours of incubation. Values were computed on per dish basis and expressed as Mean ± SD of 4 separate determinations.

IV. 3. 2. Fibroblast proliferation

Proliferative response was studied in quiescent cardiac fibroblasts following exposure to magnesium-deficient serum for 24 hours. Cells exposed to sera from magnesium-sufficient rats served as control. Serum from magnesium-deficient rats

had a more marked mitogenic effect (67% over control) on cardiac fibroblasts (Figure 27).

Figure 27. Effect of rat sera on cell number



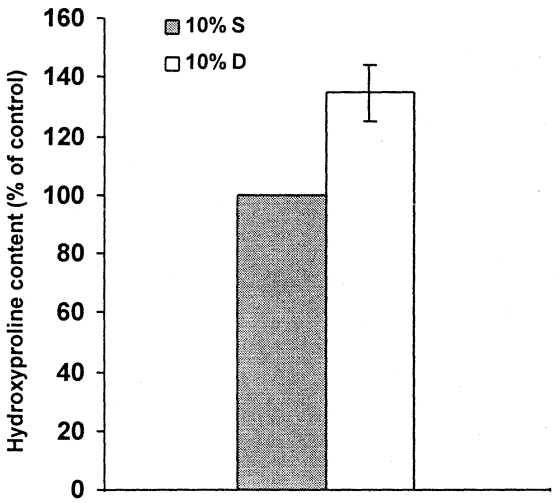
Serum-deprived, confluent cultures of fibroblasts in 35 mm dishes were exposed for 24 hours to serum (10%) from magnesium-sufficient (S) and –deficient (D) rats. Cell count was performed using a Neubauer counting chamber. Values are expressed as Mean ± SD (n=12). S vs D, $p<0.001$

IV. 3. 3. Net collagen production

Serum-deprived, confluent cultures of cardiac fibroblasts were exposed to magnesium-deficient serum for 24 hours. The conditioned medium and the corresponding cell lysates were pooled up and processed for hydroxyproline estimation. Net collagen production was 35% higher in cells exposed to

magnesium-deficient serum (Figure 28) than in cells exposed to magnesium-sufficient serum.

Figure 28. Effect of rat sera on net collagen production

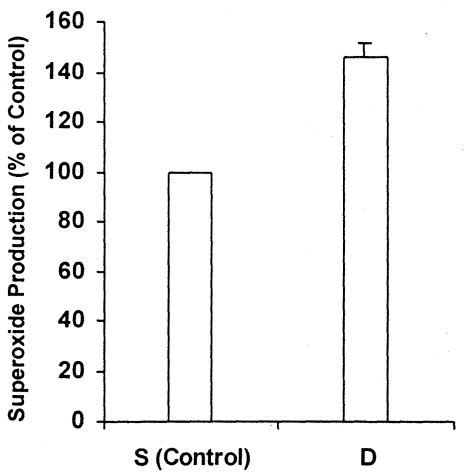


Serum-deprived, confluent cultures of fibroblasts in 100 mm dishes were exposed to serum (10%) from Mg-sufficient and Mg-deficient rats for 24 hours. At 24 hours, the conditioned medium and the corresponding cell lysates were pooled and acid-precipitated. The protein precipitate was hydrolyzed in 6N HCl at 110°C for 16-18 hours and was processed for the estimation of collagen by a hydroxyproline-based assay. S vs D, $p<0.001$

IV. 3. 4. Superoxide generation

Magnesium-deficiency is reported to increase oxidative stress in the myocardium as evidenced by elevated circulating and cardiac levels of thiobarbituric acid reactive substances and decreased anti-oxidant defense (Kumar *et al*, 1997a; Kumar and Shivakumar, 1997). The pro-oxidant nature of magnesium-deficient serum was therefore studied by exposing confluent cultures of cardiac fibroblasts to magnesium-deficient serum and measuring endogenous superoxide production. Following 24 hours of exposure to sera, cells were subjected to NBT reduction assay for 30 minutes. Serum from magnesium-deficient rats was found to increase free radical production in these cells by about 46% over control (Figure 29).

Figure 29. Effect of rat sera on superoxide production

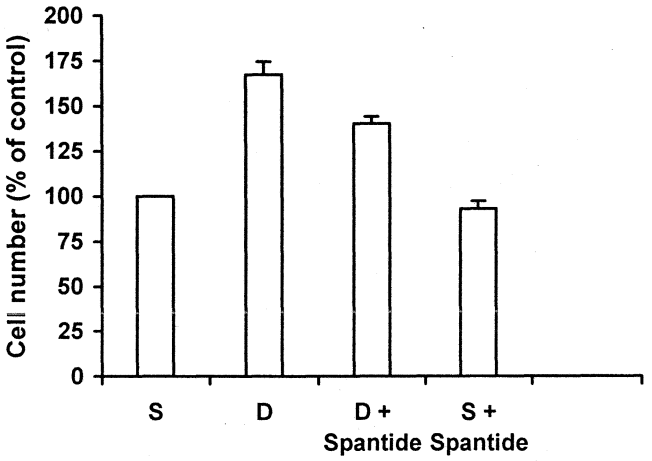


Confluent cultures of fibroblasts in 35 mm dishes were exposed to serum (10%) from Mg-sufficient (S) and Mg-deficient (D) rats for 24 hours. At 24 hours, the cells were incubated in KRPBS for 30 minutes in presence of NBT. Values are Mean ± SD (n =12). S vs D, $p<0.0.001$

IV. 3. 5. Effect of spantide

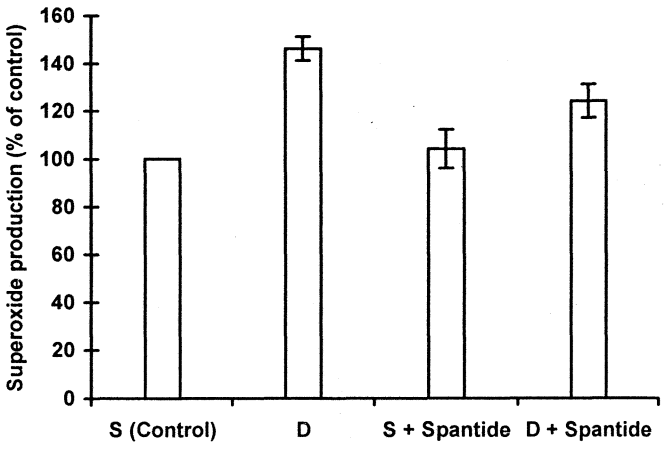
Weglicki *et al* (1994) have found elevated cardiac tissue and circulating levels of the neuropeptide, substance P, and have hypothesized that it triggers an inflammatory reaction leading to cardiac muscle damage (Weglicki *et al*, 1996). To ascertain whether substance P contributes to the effects of magnesium-deficient serum on cardiac fibroblasts, cell proliferation and superoxide production in response to magnesium-deficient serum were studied in presence of spantide (Nilsson *et al*, 1985; Villablanca *et al*, 1994). Spantide was found to reduce the magnesium-deficient serum-induced increases in cell number (Figure 30) and superoxide production by 14% and 15%, respectively (Figure 31). The stimulation of cell proliferation and superoxide generation by serum from magnesium-sufficient rats was not significantly influenced by neurokinin-1 receptor blockade (Figures 30 & 31).

Figure 30. Effect of spantide on serum-induced fibroblast proliferation



Serum-deprived, confluent cultures of fibroblasts in 35 mm dishes were exposed for 24 hours to serum (10%) from magnesium-sufficient (S) and –deficient (D) rats. To assess the effect of spantide on serum-induced increase in cell number, cells were exposed to rat serum with or without spantide at 15 μg/mL. Cell count was performed using a Neubauer counting chamber. Values are expressed as Mean ± SD (n=12). S vs D, $p<0.001$; S vs S/Spantide, ns; D vs D/Spantide, $p<0.0.001$

Figure 31. Effect of spantide on serum-induced superoxide production in cardiac fibroblasts



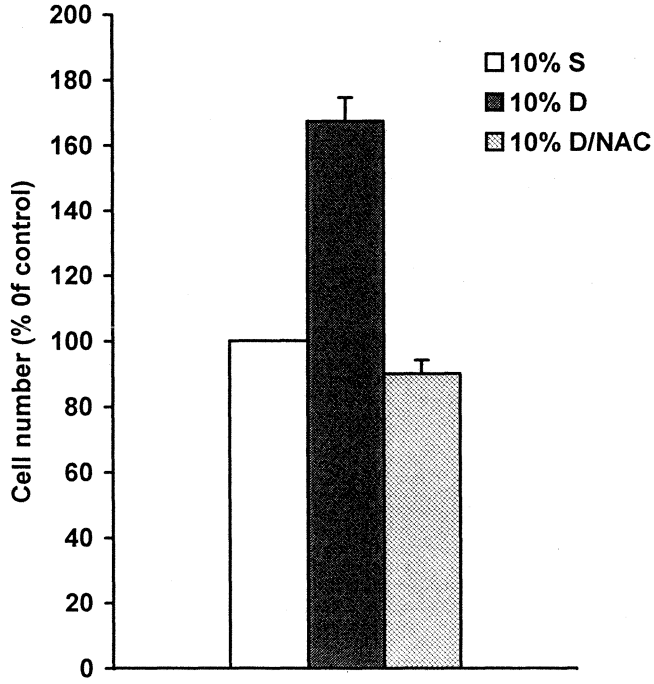
Confluent cultures of fibroblasts in 35 mm dishes were exposed to serum (10%) from Mg-sufficient (S) and Mg-deficient (D) rats for 24 hours with or without spantide at 10 μmol/L. At 24 hours, the cells were incubated in KRPBS for 30 minutes in presence of NBT. Values are Mean ± SD (n =12). S vs D $p<0.001$; S vs S/Spantide, ns; D vs D/Spantide, $p<0.001$

IV. 3. 6. Effect of N-acetyl cysteine and superoxide dismutase

To investigate whether free radicals mediated the more marked proliferative response in cardiac fibroblasts exposed to magnesium-deficient serum, cell proliferation with magnesium-deficient serum was determined in presence of the antioxidants, N-acetyl cysteine and superoxide dismutase. Figure 32 & 33 show that the greater proliferative response was abolished by N-acetyl cysteine and

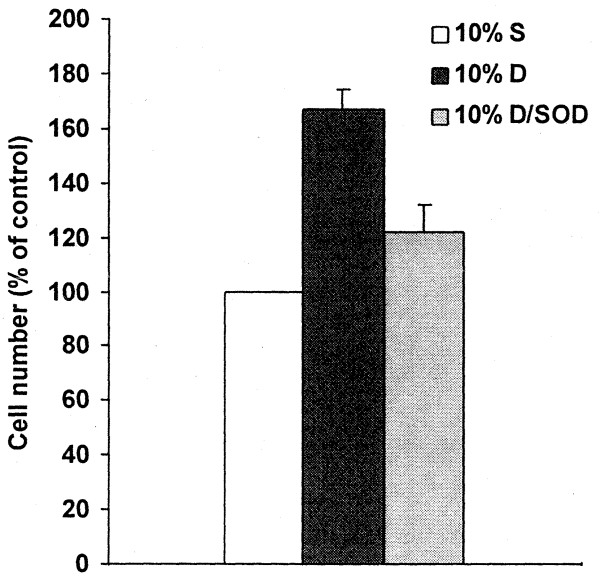
superoxide dismutase, respectively, suggesting that it may be linked to reactive oxygen species.

Figure 32. Effect of N-acetyl cysteine on serum-induced proliferative response



Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were exposed to serum (10%) from Mg-sufficient (S) and Mg-deficient (D) rats for 24 hours with or without N-acetyl cysteine at 5 mM. Cell count was performed using a Neubauer counting chamber. Values are expressed as Mean ± SD. S vs D, $p<0.001$; D vs D/NAC, $p<0.001$

Figure 33. Effect of superoxide dismutase on serum-induced proliferation



Serum-deprived, sub-confluent cultures of fibroblasts in 35 mm dishes were exposed to serum (10%) from Mg-sufficient (S) and Mg-deficient (D) rats for 24 hours with or without superoxide dismutase at 200 U/ml. Cell count was performed using a Neubauer counting chamber. Values are expressed as Mean ± SD. S vs D, $p<0.001$; D vsD/SOD, $p<0.001$

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V. DISCUSSION

Induction of cellular proliferation (Kahler *et al*, 1996) and migration (Nakamura *et al*, 1997) by substance P implies a role for this neuropeptide in the context of tissue injury. As fibroblasts are involved in tissue repair (Weber *et al*, 1988; Bashey *et al*, 1992), the present study tested the postulation that substance P may exert mitogenic action on cardiac fibroblasts and examined the underlying mechanisms. In addition, an attempt was made to delineate the role of neuropeptides like substance P in the context of myocardial damage associated with magnesium deficiency.

V.1. DIRECT EFFECTS OF SUBSTANCE P ON CARDIAC FIBROBLASTS

In the present study, the use of cardiac fibroblasts in culture permitted evaluation of the ability of substance P to exert direct effects on cardiac fibroblasts in the absence of any complicating systemic effects such as increase in cardiac work load, inotropic mechanisms, or tissue metabolism. Further, the study of cardiac fibroblasts in the absence of growth factors following serum-deprivation constituted a more suitable environment for the study of cardiac fibroblast growth responses and was more physiological, because fibroblasts *in vivo* are in growth quiescence and are not normally exposed to serum, except at wound sites.

V.1.1. Characterization of cardiac fibroblasts

Fibroblasts isolated by an enzymatic digestion method from the ventricular tissue showed typical fibroblast morphology on adhesion and growth characteristics. The cells exhibited density-dependent growth and formed a monolayer upon confluence. Growth kinetics revealed absence of a lag phase following seeding and a rapid proliferation phase between days 2 and 4 (Figure 7). The population doubling time was determined to be about 24 hours which is consistent with other reports available in the literature (Bashey *et al*, 1992). Immunocytochemistry targeting the fibroblast-specific cytoskeletal protein, vimentin, showed that ≥ 99% of the cells isolated were fibroblasts (Figures 5 & 6). In addition, they stained negative for desmin and factor VIII-related antigen.

V. 1. 2. Substance P is mitogenic to cardiac fibroblasts

The findings of the present study showed that substance P elicits a hyperplastic response *in vitro* in adult cardiac fibroblasts, as evidenced by an increase in the incorporation of [^{3}H]-thymidine into DNA and cell number (Figures 8 & 9). The magnitude of the hyperplastic response to substance P was comparable to that reported with skin fibroblasts in which about 18% and 38% of [^{3}H]-thymidine-labeled nuclei were observed in response to substance P at 1 nmol/L and 10 μmol/L, respectively (Nilsson *et al*, 1985). The stimulatory action of substance P

was mediated by the NK-1 receptor as spantide attenuated the hyperplastic response to substance P (Figure 13).

The hyperplastic effect of SP on cardiac fibroblasts, though modest, was very consistent and was validated by different observations, such as increase in cell number (Figure 9) and thymidine incorporation (Figure 8), dose response (Figure 9) and the abolition or attenuation of its action by the receptor antagonist (Figure 13), EGTA (Figure 18), N-acetyl cysteine (Figure 19) and SOD (Figure 20).

Substance P did not have any effect on total protein synthesis or protein content per dish in confluent cultures (Figures 10 & 11)). Thus, it appears that substance P elicits a hyperplastic but not a hypertrophic response in adult cardiac fibroblasts. The neuropeptide did not influence net collagen production (Figure 12). As fibroblast proliferation generally precedes enhanced collagen synthesis, it is possible that the lack of an effect of SP on net collagen production in the present study reflects an incomplete response of the cells under *in vitro* conditions. *In vivo*, other factors may act upon fibroblasts, following activation by SP, to increase collagen production. Consistent with our finding, substance P has been shown to enhance proliferation of pulp cells without an increase in the production of matrix proteins, suggesting that it may enhance proliferative activity without influencing functional activity (Trantor *et al*, 1995).

Admittedly, a concentration of substance P, higher than its plasma concentration, was required to elicit a proliferative response in cardiac fibroblasts. The dose response was reported to display a bell-shaped distribution for DNA synthesis in endothelial cells, requiring substance P concentration of 10 μmol/L to 100 μmol/L for a growth-stimulating response (Villablanca *et al*, 1994). In rheumatoid synoviocytes, however, substance P was found to induce mitogenesis at 10 nmol/L to 1 μmol/L (Lotz *et al*, 1987). It appears therefore that the cell type in question and the experimental conditions employed are determinants of the effective concentration of substance P. In the present study, substance P produced a small but significant stimulatory effect on cell number at a concentration as low as 1 nmol/L but the effect was more pronounced at 100 nmol/L and 10 μmol/L (Figure 9). It may be noted that the cells were exposed to substance P in the absence of other serum-derived growth factors. *In vivo*, a lower concentration of substance P may act synergistically with other growth factors to exert more pronounced effects. It is important to note that although the plasma concentration at which substance P can be detected in the blood is several orders of magnitude smaller than the concentrations required to stimulate cardiac fibroblast proliferation in the present study, cardiac fibroblasts *in vivo* may be exposed to higher local concentrations of substance P since three sources of substance P are

locally available to fibroblasts: substance P released from perivascular peripheral afferent nerve endings, circulating substance P, and substance P released from endothelial cells (Linnik et al, 1989; Villablanca *et al*, 1994). Moreover, pathological conditions like ischemia are reported to stimulate cardiac afferent nerve endings to release substance P (Milner *et al*, 1989; Ustinova *et al*, 1995).

V. 2. MECHANISM OF ACTION OF SUBSTANCE P: Calcium and superoxide mediate the mitogenic action of substance P on cardiac fibroblasts

V. 2. 1. Substance P alters calcium homeostasis

Substance P has been shown to modulate calcium homeostasis in many cell types (Tanabe *et al*, 1996a; Tanabe *et al*, 1996b; Sarosi *et al*, 1998). For example, it has been reported that, in Chinese hamster ovary cells expressing the substance P receptor clone (CHO-SPR), substance P induces calcium entry through activation of cation channels. Further, it has been suggested that inositol 1,4,5-trisphosphate (IP_3) may regulate both calcium entry and Ca^{++} mobilization from intracellular stores in CHO-SPR cells (Mochizuki-Oda *et al*, 1994). In neutrophils, substance P stimulates the hydrolysis of PIP_2 into diacylglycerol (DAG) and IP_3 with a rise in intracellular calcium (Tanabe *et al*, 1996a). Substance P and related tachykinins have also been shown to evoke calcium signaling in cultured myenteric neurons by the influx of extracellular Ca^{++} through L- and N-type calcium channels. These

signals are abolished upon removal of extracellular Ca^{++} or by the addition of calcium channel blockers, lanthanum chloride and nickel chloride (Sarosi *et al*, 1998).

In contrast, very little is known about regulation of calcium homeostasis in fibroblasts and the role of calcium in mediating the effects of growth factors on fibroblasts. The results of this study showed that substance P has a stimulatory effect on ^{45}Ca uptake by the cells (Figure 17). This is the first demonstration of agonist-induced enhancement of ^{45}calcium uptake by cardiac fibroblasts. Further, it was found that chelation of extracellular calcium using EGTA completely abolishes the stimulation of fibroblast proliferation by substance P (Figure 18), suggesting that the mitogenic effect of substance P is dependent on calcium influx from the extracellular space. Thus, the data showed that changes in calcium homeostasis may play a role in the stimulation of cardiac fibroblast proliferation by substance P.

V. 2. 2. Substance P enhances superoxide generation

Substance P has also been shown to stimulate superoxide production in synovial cells (Tanabe *et al*, 1996b) and human neutrophils (Tanabe at al, 1996a). Tanabe *et al* (1996b) have reported that NK-1 receptor /phospholipase C-linked DAG formation with resulting activation of protein kinase C is the signal transduction pathway for substance P-stimulated oxyradical production in synovial

cells. The ability of gastrointestinal tissue to react to tachykinins with liberation of free radicals as part of an inflammatory reaction has also been demonstrated (Lordal *et al*, 1997). In the present study, substance P was found to consistently increase superoxide production in cardiac fibroblasts (Figure 15). Exposure of sub-confluent cultures to substance P for 24 hours elicited a more pronounced (about 2-fold higher) effect than exposure of confluent cultures to substance P for 4 hours (Figures 14 & 15). The effect of SOD (Figure 21) further confirmed the stimulatory action of substance P on superoxide generation. Probing a role for extracellular calcium in the enhancement of superoxide production by substance P, fibroblasts were exposed to substance P under calcium-free conditions. It was observed that, while the effect of substance P on superoxide production remained unaffected in a calcium-free medium, a combination of substance P and EGTA had a more pronounced effect on superoxide generation than substance P alone (Figure 22). However, it was found subsequently that EGTA (Figure 24) and calcium-free incubation (Figures 24 & 25) *per se* enhanced superoxide production in adult cardiac fibroblasts (Shivakumar and Kumaran, 2001). Thus, it is not clear from these experiments whether the effect of substance P on superoxide production is dependent on extracellular calcium. It is intriguing that both substance P (Figures 14 & 15) and EGTA (Figure 24) stimulate superoxide generation although the

former enhances calcium uptake and the latter chelates extracellular calcium. The mechanisms underlying the effects of substance P and extracellular calcium on superoxide production may be different and need to be addressed.

Importantly, the inhibitory effect of the antioxidant, N-acetyl cysteine, on the mitogenic action of substance P showed that substance P-induced fibroplasia is mediated by a redox-sensitive mechanism (Figure 19). Further, the effect of superoxide dismutase suggested a role for superoxide anion, at least in part, in mediating the mitogenic action of substance P (Figure 20). This is the first demonstration of the involvement of reactive oxygen species (ROS) in mediating the mitogenic action of substance P and is in agreement with the current thinking that ROS may exert critical signaling functions to regulate cellular growth in response to growth factors (Kunsch and Medford, 1999; Irani, 2000). Superoxide anion-induced spectrum of cellular alterations includes such divergent effects as cell growth and cell death, depending largely on the cell type. This notion is supported by the report that, while apoptosis occurs in cardiomyocytes, cardiac fibroblasts undergo enhanced proliferation in response to superoxide anion (Li P-F *et al*, 1999). Platelet-derived growth factor {PDGF} (Sundaresan *et al*, 1995) and thrombin (Patterson *et al*, 1999) have been shown to stimulate ROS production in smooth muscle cells and suppression of ROS was found to inhibit PDGF- and

thrombin-induced mitogenesis in these cells. Further, angiotensin II elicits a hypertrophic response in smooth muscle cells (SMCs) via the production of both superoxide and H_2O_2 (Ushio-Fukai *et al*, 1998; Zafari *et al*, 1998).

It has been reported that free radical generation and alterations in calcium homeostasis may be related in some cell types. Oxidants have been shown to stimulate calcium signaling by increasing cytosolic calcium concentration (Suzuki *et al*, 1997), suggesting a possible physiological role of ROS and oxidative stress in the regulation of Ca-induced signaling. Increase in intracellular Ca^{++} was detected in response to hydrogen peroxide (H_2O_2) treatment of vascular smooth muscle cells {VSMCs} (Roveri *et al*, 1992) and, in endothelial cells treated with H_2O_2 (Doan *et al*, 1994), there was a transient release of Ca^{++} from intracellular stores. Further, even a link between substance P-induced superoxide production and calcium flux has been suggested. Tanabe *et al* (1996a) have reported that NK-1 receptor/G protein- coupled IP_3 formation with resulting IP_3 - induced transient increase in $[Ca^{++}]_i$ is the main signal transduction pathway for substance P-stimulated $O_2^{\cdot}$ production in human neutrophils although in synovial cells superoxide production was not found to be linked to IP_3-induced calcium mobilization (Tanabe *et al*, 1996b). In the present study, while the mitogenic action of substance P was dependent on superoxide production (Figure 20), a combination of substance P and

EGTA enhanced superoxide generation (Figure 22) but without an increase in [^{3}H]-thymidine incorporation (Figure 18). The observations showed that an increase in superoxide production in response to substance P does not result in increased cell proliferation when extracellular calcium is chelated. It appears therefore that substance P-induced fibroplasia may depend on both calcium- and superoxide-sensitive pathways and that blocking either of these would abolish the proliferative response. Thus, while the literature points to the link between ROS and calcium homeostasis in certain cell types, the results of the present study suggested for the first time that substance P-triggered calcium- and oxidant- signaling pathways may, in addition, be functionally coupled to a hyperplastic response in cardiac fibroblasts.

V. 2. 3. Model of substance P-induced cardiac fibroblast proliferation

Substance P is reported to exert its mitogenic action via its G protein-coupled NK-1 receptor and activation of extracellular signal-regulated kinases {ERKs} (Castagliuolo *et al*, 2000). The substance P antagonist, [d-Arg (1), d-Trp (5,7,9), Leu (11)] substance P, has been shown to inhibit both G protein-coupled receptor (GPCR)-mediated signal transduction and cellular DNA synthesis in Swiss 3T3 cells and the activation of ERK-1 and ERK-2, induced by GPCR agonists (Sinnett-Smith *et al*, 2000). Moreover, it has been shown that activation of ERK-1

and ERK-2 is required for DNA synthesis in cardiac fibroblasts (Pages *et al*, 1993). ERKs represent an important intracellular target of an altered redox environment and, in fact, activation of ERKs by ROS has been implicated in the growth response of smooth muscle cells stimulated with growth factors (Irani, 2000). Thus, a model of substance P-induced mitogenesis in cardiac fibroblasts, mediated by calcium- and redox-sensitive pathways and activation of ERKs, is consistent with the data presented here and recent literature and may provide a basis for future investigations.

In summary, a fibroproliferative response in the heart is important for recovery from injury and subsequent remodeling of the interstitium but factors that regulate fibroblast proliferation in the heart remain largely unidentified. The present study establishes substance P as an 'activator' of a mitogenic response in cardiac fibroblasts and proposes that substance P-induced alterations in redox state and calcium homeostasis may act in concert to mediate its mitogenic action. The findings are novel in that they point to a paracrine mechanism of peptidergic neuronal control of cardiac fibroblast proliferation which would be particularly relevant in conditions like myocardial ischemia that stimulate cardiac sensory nerve endings and promote increased local release of substance P and other neuropeptides. Future investigations should delineate the calcium- and oxidant-

signaling pathways, and any cross-talk between them, that seems to mediate the mitogenic action of substance P on cardiac fibroblasts.

V. 3. SUBSTANCE P AND MYOCARDIAL CHANGES IN MAGNESIUM DEFICIENCY

Investigations in this laboratory and elsewhere have demonstrated that dietary deficiency of magnesium produces reparative cardiac fibrosis following myocardial injury (Bloom, 1988; Kumar *et al*, 1997). This laboratory has reported evidence of alterations in collagen metabolism and fibroblast proliferation in a rodent model of acute magnesium deficiency (Kumar *et al*, 1997). These observations are in line with histological evidence of fibroplasia in the cardiac lesions of magnesium deficiency, reported earlier (Heggtveit *et al*, 1964). The findings point to the involvement of fibroblasts in a wound healing response in the heart in magnesium deficiency but the underlying mechanisms remain unclear.

Interestingly, it has been observed (Weglicki *et al*, 1996; Kumar *et al*, 1997; Shivakumar and Kumar, 1997) that while serum levels of magnesium are significantly reduced (hypomagnesemia), cardiac tissue levels of the element are well-preserved in this model, raising the question as to what, if not a fall in tissue magnesium, causes the changes observed in the heart (Shivakumar, 2001). The importance of immunomodulatory processes in the pathobiology of magnesium deficiency has been recognized. Weglicki *et al* (1992) have reported significant

elevations in the circulating and cardiac tissue levels of neuropeptides (substance P and calcitonin gene-related peptide) in the first week of acute magnesium deficiency and have ascribed a role for these factors in the cardiomyopathic changes in light of the observation that a substance P antagonist reduced the cardiac lesions (Weglicki *et al*, 1994; Kramer *et al*, 1997). Based on these observations, a neurogenic inflammatory trigger and its consequent inflammatory cascade is postulated to initiate the cardiomyopathic lesions of magnesium deficiency (Weglicki *et al*, 1996). However, controversy exists over the role of neuropeptides in the pathology of magnesium deficiency. Brugère *et al* (2000) failed to observe similar elevations in the circulating levels of substance P at day 4 in a rodent model of acute magnesium deficiency though they confirmed the occurrence of an inflammatory response in magnesium-deficient rats as evidenced by the presence of activated macrophages and an elevation of plasma concentration of interleukin-6, a known mediator of acute phase response, as early as day 4 in animals on a magnesium-deficient diet.

The present study was undertaken to test the hypothesis that humoral factors, including substance P, may contribute to the activation of cardiac fibroblasts in magnesium deficiency reported earlier (Kumar *et al*, 1997). The study also attempted to ascertain if such activation is free radical-mediated. The

experimental approach consisted in comparing the effects of sera from magnesium-sufficient and -deficient rats on proliferation, net collagen production (deposition) and superoxide generation in isolated fibroblasts; use of anti-oxidants permitted evaluation of the role of superoxide in the mitogenic response. The possibility that the incubation medium for the test group, with 10% magnesium-deficient serum, has lower levels of magnesium was ruled out by atomic absorption spectrophotometry before these experiments were carried out.

V. 3. 1. Serum from magnesium-deficient rats enhances proliferative response in cardiac fibroblasts via a free radical-mediated pathway

Cardiac fibroblasts exposed to serum from rats on magnesium-deficient diet for six days showed a more pronounced proliferative response, compared to cells exposed to serum from magnesium-sufficient rats (Figures 26 & 27). In addition, net collagen production in cardiac fibroblasts increased significantly on exposure to magnesium-deficient serum (Figure 28). Moreover, the observation that serum from magnesium-deficient rats enhances oxidative stress in cardiac fibroblasts (Figure 29) further supports the postulation that the myocardium undergoes oxidative stress during magnesium deficiency. It is noteworthy that several studies have linked reactive oxygen species to cellular hyperplasia (Irani, 2000). Therefore, an attempt was made to examine whether free radicals mediate the

enhanced proliferative response in these cells. As shown in Figure 32, N-acetyl cysteine significantly reduced the hyperplastic effect of serum from magnesium-deficient rats. This observation emphasized the role of reactive oxygen species in magnesium deficiency. Further, superoxide dismutase also attenuated the enhanced hyperplastic effect of the magnesium-deficient serum suggesting that the greater mitogenic effect of serum from magnesium-deficient animals may be related to superoxide generation (Figure 33).

In the present study, spantide, an NK-1 receptor antagonist, was found to decrease the stimulatory effects of magnesium-deficient but not magnesium-sufficient serum on proliferation (Figure 30) and superoxide production (Figure 31) by about 14% ($p<0.001$) and 15% ($p<0.001$), respectively, suggesting that neuropeptides acting via NK-1 receptors may modulate cardiac fibroblast proliferation, although to a meager extent, in this model. Definitive conclusions regarding a role for neuropeptides would require further experimental evidence. Clearly, involvement of factors other than those acting via NK-1 receptors needs to be examined.

The findings of the study suggested for the first time that in hypomagnesemia, serum factors may stimulate cardiac fibroblast proliferation via a superoxide anion-mediated mechanism and contribute to the fibrogenic response

in the heart, even when cardiac tissue levels of magnesium are well-preserved. The NK-1 receptor-mediated pathway seems to have only a modest role in modulating cardiac fibroblast function in magnesium deficiency. The present study establishes a good model to investigate serum factors that may be involved in the process of wound healing associated with dietary deficiency of magnesium.

Recently, it was postulated that a fall in serum magnesium levels may trigger a temporal sequence of events involving vasoconstriction, hemodynamic alterations and vascular endothelial injury to produce pro-inflammatory, pro-oxidant and pro-fibrogenic effects, resulting in initial perivascular myocardial fibrosis which, in turn, would cause myocardial damage and replacement fibrosis (Shivakumar, 2001). It was also postulated that angiotensin II may be the prime mover of the pathogenetic cascade in magnesium deficiency (Shivakumar, 2001). The possible involvement of angiotensin II is suggested by experimental evidence that it is a potent vasoconstrictor (Ito *et al*, 1995) having marked effects on vascular cells (Touyz *et al*, 1997; Zhang *et al*, 1997), including endothelial cells (Pastore *et al*, 1999). Angiotensin II also has a pro-inflammatory action as it is known to activate endothelial cells and stimulate leukocyte adhesion to these cells (Grafe *et al*, 1997). Further, it is an important regulator of fibroblast function and collagen turnover in the heart (Weber *et al*, 1995). Future studies should therefore

probe the possible role of the renin angiotensin system in the inflammatory cascade and cardiac lesions observed in magnesium deficiency. It is pertinent to point out that while humoral factors may, as evidenced by this study, influence cardiac fibroblast activity in the early stages of magnesium deficiency, growth factors locally generated in the myocardium, including components of the renin-angiotensin system, may also contribute significantly to fibrogenesis as deficiency advances.

V. 4. EXTRACELLULAR CALCIUM AND OXIDATIVE STRESS IN CARDIAC FIBROBLASTS: IS THERE ANY LINK?

As a spin-off from investigations on the involvement of extracellular calcium in substance P-mediated mitogenesis and superoxide generation in cardiac fibroblasts, it was observed that alterations in extracellular calcium may induce oxidative stress in cardiac fibroblasts.

Mitogenic action of substance P was found to be dependent on both extracellular calcium and superoxide anion (Figures 18 & 20). When extracellular calcium was chelated using EGTA, substance P elicited no proliferative response in cardiac fibroblasts but stimulated superoxide generation (Figures 18 & 22). In fact, a combination of substance P and EGTA produced a greater effect on superoxide production than substance P alone in these cells (Figure 22). This led to

the question whether chelation of extracellular calcium *per se* increases oxidative stress in cardiac fibroblasts. Surprisingly, EGTA enhanced free radical production in cardiac fibroblasts (Figure 24) and this observation was later supported by the fact that calcium-free incubation also increased superoxide production in these cells (Figures 24 & 25). Further, treatment of cardiac fibroblasts with L-type calcium channel blockers enhanced oxidative stress (Figure 24). Thus, by using three different experimental strategies - exposure to EGTA, calcium-free conditions and L-type calcium channel blockers - it was found that interventions that may interfere with calcium influx and possibly reduce intracellular calcium resulted in enhanced endogenous superoxide production in cardiac fibroblasts.

Intriguingly, the observation on the effect of calcium channel blockers is not in line with reports on their anti-oxidant property and the postulation that their anti-oxidant activity may have to do with their chemical structure and biophysical interaction with membranes (Mak *et al*, 1992). On the contrary, the data point to a pro-oxidant effect of L-type calcium channel blockers in cardiac fibroblasts. It is not clear if the effect is related to their pharmacological action as calcium antagonists since it is possible that in these quiescent cells, most of the L-type calcium channels may remain closed. However, in the course of investigations on the correlation between intracellular calcium concentration and membrane

potential in monolayers of density-arrested normal rat kidney fibroblasts, Oe-Roos *et al* (1997) observed spontaneous repetitive spike-like increases in $[Ca^{2+}]_i$ synchronously throughout the monolayer which were paralleled by depolarizations of the plasma membrane. Moreover, the calcium spikes disappeared in calcium-free solutions and could be blocked by L-type calcium channel blockers (Oe-Roos *et al*, 1997). Anyway, given the effects of EGTA and calcium-free incubations on these cells, the present study underscores the link between extracellular calcium levels and superoxide generation.

The results are consistent with an earlier observation of a biphasic effect of calcium on NBT reduction (Babizhayev, 1988). Using a model of oxidative stress consisting of liposomes and ufasomes and a superoxide generating system, it was shown that while low calcium concentrations (10^{-6} M to 10^{-5}) enhanced NBT reduction, higher concentrations (5×10^{-4} M) had an inhibitory effect. Further, EGTA was found to abolish the inhibitory effect of higher calcium concentration. It is also noteworthy that Snyder *et al* (1995) have reported that removal of calcium from the culture medium or treatment of hepatocytes with calcium channel blockers potentiates the hepatotoxicity of aluminium maltolate while thapsigargin, which increases cytosolic calcium by inhibiting the sequestration of the ion by the endoplasmic reticulum, attenuates the effect of aluminium maltolate (Snyder *et al*,

1995). The authors postulate that manipulations that would reduce intracellular calcium levels may enhance cellular vulnerability to injury. On the basis of the present data, its is tempting to speculate that augmented superoxide production may represent a mechanism of such increased vulnerability.

The observations may have a bearing on some important issues. Primarily, they show for the first time that withdrawal of extracellular calcium or treatment with calcium channel blockers increases superoxide generation in adult cardiac fibroblasts. In this context, it is to be noted that calcium-free perfusion of the heart is associated with the problem of myocardial calcium overload upon readmission of calcium (Nayler and Grinwald, 1981). Extending this phenomenon, the data suggest that exposure of the heart to unphysiologically low extracellular calcium, as in hypocalcemic cardioplegia, may, in addition, subject the fibroblasts to increased oxidative stress. It is possible, therefore, that inclusion of anti-oxidants in cardioplegic solutions may confer benefits. Second, it is now well-recognized that reactive oxygen species may act as second messengers and exert critical signaling functions (Kunsch and Medford, 1999). Therefore, possible oxyradical-mediated effects of calcium channel blockers on cardiac fibroblasts need to be probed further, especially as these compounds are known to accumulate in biological membranes upon prolonged exposure to therapeutic levels (Mak, *et al*,

1992). Third, given the stimulatory effect of EGTA on superoxide generation, it appears that results of experiments involving the use of calcium chelators should be interpreted with circumspection. Last, the possibility that myocardial fibroblasts may contribute to the cardiac effects of calcium channel blockers and alterations in extracellular calcium concentration presents a new perspective that warrants further examination.

VI. SUMMARY AND CONCLUSIONS

VI. 1. A NEURAL PATHWAY OF ACTIVATION OF CARDIAC FIBROBLASTS

Recognizing the important role played by cardiac fibroblasts in maintaining the structural and functional integrity of the myocardium, this laboratory has been interested in signals that regulate cardiac fibroblasts.

As a prerequisite to identifying factors that modulate cardiac fibroblast function, cultures of adult cardiac fibroblasts were established and characterized. The present study focused on the action of substance P, a neuropeptide, on these cells and the underlying mechanisms.

Substance P, acting via NK-1 receptors, stimulated fibroblast proliferation in a dose-dependent manner without inducing a hypertrophic response. The neuropeptide did not influence total protein synthesis, total protein content or net collagen production. As fibroblast proliferation generally precedes enhanced collagen synthesis, the lack of an effect of substance P on collagen production in the present study may represent an incomplete response of the cells under *in vitro* conditions. Attempts to delineate the mechanism of action of substance P revealed that it increases both calcium uptake by cardiac fibroblasts and endogenous superoxide production in these cells. A concerted action of increased calcium influx and superoxide anion production seemed to mediate the mitogenic effect of substance P on cardiac fibroblasts.

Having established that substance P is mitogenic to cardiac fibroblasts, a role for the neuropeptide in activating cardiac fibroblasts in magnesium deficiency was investigated on the basis of the postulation that substance P may initiate neurogenic inflammation leading to the cardiovascular pathology of magnesium deficiency. The results suggested a modest role for NK-1 receptor-mediated pathways in activating cardiac fibroblasts in magnesium deficiency although serum factors markedly increased fibroblast proliferation and net collagen production in these cells. A free radical-mediated pathway was found to be responsible for the enhanced proliferative effect of serum from magnesium-deficient rats. By stressing the role of humoral factors, the present study suggests a mechanism of initiation of cardiac lesions in response to a fall in serum magnesium levels when the myocardial magnesium level is well-preserved.

Together, the findings presented in this thesis uncover a neural pathway of activation of cardiac fibroblasts.

VI. 2. FUTURE DIRECTIONS

The present study shows that Substance P is mitogenic to cardiac fibroblasts by a mechanism involving increased calcium influx and superoxide anion production. Further, it may play a modest role in magnesium deficiency.

Future studies should probe

1. the calcium- and oxidant-signaling pathways, and any cross-talk between them, that seem to mediate its mitogenic effect
2. the possible role of substance P in inflammatory cardiac diseases
3. the expression of neurokinin receptors on cardiac fibroblasts in magnesium deficiency, and
4. the involovement of the renin-angiotensin system in the pathobiology of magnesium deficiency.

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VIII. LIST OF PUBLICATIONS

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