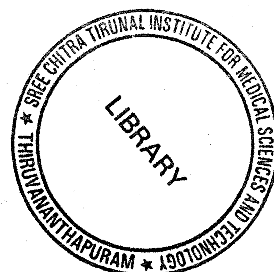
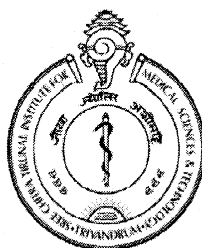
RESPONSE OF CARDIAC FIBROBLASTS TO HYPOXIA: A PROFILE OF DELAYED CELL CYCLE PROGRESSION AND AUGMENTED PRODUCTION OF MODULATORS OF INFLAMMATION

SAPNA SUBRAYAN

PhD Thesis – AUGUST 2007





SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695 011, INDIA

RESPONSE OF CARDIAC FIBROBLASTS TO HYPOXIA: A PROFILE OF DELAYED CELL CYCLE PROGRESSION AND AUGMENTED PRODUCTION OF MODULATORS OF INFLAMMATION

A thesis presented

by

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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695 011

CERTIFICATE

I, **Sapna S**, hereby certify that I had personally carried out the work depicted in the thesis entitled **"Response of cardiac fibroblasts to hypoxia: a profile of delayed cell cycle progression and augmented production of modulators of inflammation"** under the direct supervision of **Dr. K Shivakumar**, Scientist F, Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help was sought and is acknowledged.

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This is to certify that **Ms. Sapna S**, in the Division of Cellular & Molecular Cardiology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled **"Response of cardiac fibroblasts to hypoxia: a profile of delayed cell cycle progression and augmented production of modulators of inflammation"** was carried out under my direct supervision.

Date: 4/8/2007

Dr. K Shivakumar (Guide)

The thesis entitled

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submitted by

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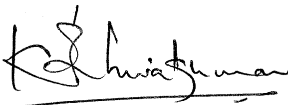

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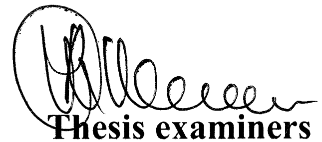

Thesis examiners

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ABBREVIATIONS

ALP	Alkaline phosphatase
Ang II	Angiotensin II
AP-1	Activating protein-1
APC/C	Anaphase promoting complex
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia-related
bFGF	Basic fibroblast growth factor
BIM	Bisindolylmaleimide
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CREB	cAMP response element-binding
DAB	3, 3' - diamino benzidine
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylene diamine tetraacetic acid
ERK1/2	Extracellular signal regulated kinase 1/2
ET-1	Endothelin-1
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

HEPES	N-[2-hydroxyethyl] piperazine-n'-[2-ethanesulfonic acid]
HFCM	Hypoxic fibroblast-conditioned medium
HIF	Hypoxia inducible factor
HRP	Horse radish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-jun N-terminal kinase
LDH	Lactate dehydrogenase
M199	Medium 199
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MEFs	Mouse embryo fibroblasts
MMP	Matrix metalloproteinase
MOPS	(3-[N-morpholino] propane sulfonic acid)
MPF	Mitosis promoting factor
NAC	N-acetyl cysteine
NAD	Nicotinamide adenine dinucleotide
NFCM	Normoxic fibroblast-conditioned medium
NFκB	Nuclear factor kappa B
NO	Nitric oxide
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidine dithiocarbamate
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
POPOP	1,4-bis[5-phenyl-2-oxazolyl]-benzene

PPO	2,5-diphenyloxazole
RB	Retinoblastoma protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
sICAM-1	Soluble ICAM-1
SMCs	Smooth muscle cells
SOD	Superoxide dismutase
SP	Substance P
TCA	Trichloroacetic acid
TEMED	N, N, N', N' - tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumore Necrosis factor-α
VCAM-1	Vascular cell adhesion molecule - 1
VEGF	Vascular endothelial growth factor

SYNOPSIS

Fibroblasts are the most abundant cell type in the heart, representing about two-thirds of the total myocardial cell population and more than 90% of the non-myocytes. They are the only source of fibrillar type I and type III collagens in the heart, and are a major source of matrix metalloproteinases and tissue inhibitors of metalloproteinases that are involved in matrix turnover. Fibrillar collagens form major components of the cardiac ECM, which contributes to the functioning of the heart as a syncytium by providing a protein scaffold that supports myocytes, tethers myocyte bundles and transmits the force of contraction across the myocardial muscle mass. Thus, fibroblasts play an important role in regulating cardiac function in the normal adult and during development. In response to injury, they contribute to the initiation and progression of remodeling and repair through phenotypic transformation into myofibroblasts that are capable of multiple functional responses, such as proliferation, ECM metabolism, and production of autocrine and paracrine mediators that influence cardiomyocyte growth and ECM homeostasis.

Unlike cardiomyocytes that have limited proliferative potential, cardiac fibroblasts are undifferentiated cells that remain quiescent in the normal heart but retain the capacity to proliferate throughout adult life and do so in response to a variety of mitogenic stimuli. Many pathologic states of the heart are associated with fibroblast hyperplasia. In fact, fibroblast hyperplasia is central to the role played by these cells in the regulation of multiple aspects of cardiac function in the context of myocardial injury.

In the diseased myocardium, however, a multitude of factors can act as positive and negative modulators of fibroblast proliferation and the balance between positive and negative influences determines the functional response of these cells in a given situation. The most prevalent and hence the most widely studied cardiovascular diseases are associated with hypoxia. Hypoxia is a major factor influencing the extent of cell injury

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and response in the injured myocardium. Cellular responses to hypoxia have been studied extensively using cell lines and in the setting of pathologic states, including tumor hypoxia and pulmonary hypertension.

The effects of hypoxia on cell proliferation depend upon several factors, including the cell type, degree of hypoxia, the extent of cell differentiation and the tissue or organ of origin, making generalizations with respect to mechanisms difficult. Therefore, although a general scheme has evolved for control of cell cycle progression in proliferating cells, involving the coordinated actions of cyclin-CDKs and several cell cycle regulatory proteins, the precise mechanisms that regulate cell cycle progression in cardiac fibroblasts exposed to hypoxia warrants scrutiny. Further, cardiac fibroblasts are relatively resistant to apoptosis in response to a variety of pro-apoptotic stimuli, including hypoxia, and the molecular pathways mediating apoptosis and cell cycle progression are coordinately regulated. Thus, it is possible that the relative resistance of cardiac fibroblasts to hypoxic injury (apoptosis) is related to the regulation of cell cycle progression in these cells. The postulation provides a compelling rationale for investigating the regulation of cell cycle progression in cardiac fibroblasts under hypoxia.

Intrinsic difficulties in evaluating the modulation of cardiac cell function by hypoxia *in vivo* include the existence of a heterogeneous cell population in the heart, and the inability to produce a uniform hypoxic insult. Against this backdrop, the objectives of the study were to set up and characterize an *in vitro* cell culture model of hypoxia to evaluate the effects of hypoxia on the cardiac fibroblast cell cycle. An additional objective was to examine the production of modulators of inflammation and collagen by hypoxic cardiac fibroblasts.

Isolation and culture of adult rat cardiac fibroblasts

Cardiac fibroblasts were isolated from the ventricular tissue of young adult Sprague Dawley (2-3 months) rats by a series of enzymatic digestions using collagenase, trypsin, DNase and pancreatin. Selective enrichment of cardiac fibroblasts in cultures was achieved by pre-plating the isolated cells for 150 minutes. The fibroblastic nature of the cells in culture was ascertained morphologically and by immunocytochemistry. Cells in passage 2 or 3 were used for the experiments.

Characterization of the *in vitro* hypoxia model

GasPak anaerobic systems with anaerobic/aerobic GasPak envelopes from Becton Dickinson, USA, were used to generate hypoxic or normoxic environments. The pO_2 of the hypoxic medium to which the cells were exposed was about 3% as against 15% of the normoxic medium. Intracellular lactate levels were undetectable at 24 hours of hypoxia and there was no change in the pH of the medium (~7.5) during this interval. Loss of viability was assessed by flow cytometric analysis of the sub G_0/G_1 population, which represents the percentage of damaged cells. About 2% of the cells were found to be non-viable in hypoxic and normoxic cultures. Syto13/PI staining and reversibility of hypoxia-induced changes upon re-oxygenation confirmed that cell viability was not affected by 48 hours of hypoxic incubation.

1. Hypoxia delays cell cycle progression in cardiac fibroblasts

The effects of hypoxia on basal and serum-induced fibroblast proliferation were assessed in terms of [^{3}H]-thymidine incorporation into DNA over 24 hours, and population doubling time (T_d). The results indicated that -

- Hypoxia significantly decreases basal and serum (10%)-induced DNA synthesis in adult rat cardiac fibroblasts
- Hypoxia significantly increases the population doubling time from 27.7 hours in normoxic cultures to 54.14 hours in hypoxic cultures
- The effect of hypoxia was reversible, as re-oxygenation restored population doubling time and the rate of DNA synthesis

Molecular basis of hypoxia-induced decrease in cell proliferation

a) Identification of hypoxia-sensitive cell cycle checkpoint in cardiac fibroblasts by flow cytometry

Cells exposed to 48 hours of hypoxia/normoxia were trypsinized, fixed in 70% ethanol and stained with propidium iodide in the presence of RNase. Flow cytometric analysis of the stained cells was performed at an excitation wavelength of 488 nm and emission wavelength of 620 nm. The cell cycle phase distribution profile showed that hypoxia induces accumulation of cells at G_1, with a corresponding decrease in the S-phase cell population, suggesting that the G_1/S checkpoint in the cardiac fibroblast cell cycle is hypoxia-sensitive.

b) Identification of signaling pathways mediating the hypoxia effect

Cells were subjected to hypoxia in the presence of specific inhibitors of superoxide anion (SOD, 200U/ml), p42/44 MAPK (PD 098059, 10μM) and p38 MAPK (SB 203580, 10μM), and [^{3}H]-thymidine incorporation into DNA was determined. SOD had no effect on the hypoxic inhibition of DNA synthesis, but it decreased fibroblast DNA synthesis under normoxic conditions. p42/44 MAPK was found to act as positive modulator and p38 MAPK as negative modulator of cardiac fibroblast proliferation.

c) Western blot analysis of checkpoint-related cell cycle regulatory protein(s)

Deceleration of the cell cycle at G_1/S suggested that hypoxia may alter the expression of p27, the CDK inhibitor known to regulate cyclin E/CDK2 activity and therefore progression of cells from G_1 to S phase. Western blot analysis demonstrated p27 induction in cells exposed to hypoxia for 24 hours, suggesting the involvement of this cell cycle regulatory protein in the anti-proliferative effect of hypoxia.

d) p38 MAPK may mediate hypoxia-induced p27 expression

Exposure of cells to hypoxia in the presence of SB203580 was found to attenuate hypoxia-induced p27 expression, indicating that p38 MAPK may mediate hypoxia-induced p27 expression. However, in the absence of data documenting changes in MAPK activity in hypoxic cells, it cannot be concluded that p38 MAPK and p42/44 MAPK are causally linked to the hypoxia-induced decrease in proliferation.

e) Soluble factors from hypoxic cardiac fibroblasts inhibit fibroblast proliferation

To ascertain if soluble factors from hypoxic cardiac fibroblasts would exert autocrine effects on fibroblast proliferation, basal levels of DNA synthesis over 24 hours were assessed in cardiac fibroblasts exposed to hypoxic fibroblast-conditioned medium (HFCM). HFCM caused a 46% decrease in [^{3}H]-thymidine incorporation in cardiac fibroblasts suggesting that oxygen deprivation may not only exert direct effects on the cell cycle but also trigger the release of factors from these cells that can amplify the effects of hypoxia.

The observation that HFCM affects cardiac fibroblast DNA synthesis suggested that these cells may be a source of autocrine/paracrine factors whose expression may be altered under hypoxic conditions. Consistent with the postulation, a marked increase (4.7 fold) in the levels of TNF-α was observed in culture supernatants of hypoxic cardiac fibroblasts. As TNF-α is known to influence proliferation in a variety of cell types,

experiments were performed to ascertain if it may contribute to the observed effects of HFCM on fibroblast DNA synthesis. Basal levels of DNA synthesis in synchronous sub-confluent cultures of normal cardiac fibroblasts were found to be significantly reduced by incubation with TNF-α at concentrations similar to that in HFCM (200pg/ml).

2. Hypoxia augments production of modulators of inflammation by fibroblasts

In addition to TNF-α, the present study also determined the levels of IL-1β, IL-4, IL-6 and soluble ICAM-1 (sICAM-1), which are important modulators of inflammation, in cardiac fibroblast culture supernatants under normal and hypoxic conditions. Supernatants from normoxic and hypoxic cultures had detectable levels of all these factors, except IL-1β. Besides TNF-α, hypoxia caused a marked increase in the production of sICAM-1 but not of IL-4 and IL-6.

a) Hypoxia augments sICAM-1 production in cardiac fibroblasts by p42/44 MAPK- and PKC-dependent mechanisms

sICAM-1, released from its cell surface isoform by proteolytic cleavage, is believed to be a negative modulator of inflammation that attenuates cell-cell interactions by competitively inhibiting the action of the pro-inflammatory cell surface ICAM-1. A marked increase in sICAM-1 production was observed under hypoxic conditions, which was not associated with changes in cell surface ICAM-1 transcript levels, as revealed by RT-PCR, implying post-transcriptional and/or post-translational mechanisms. Experiments involving pharmacological inhibitors showed that the effect of hypoxia on sICAM-1 production is mediated by p42/44 MAPK and PKC pathways.

b) Antioxidants increase sICAM-1 production by cardiac fibroblasts

Attempts to ascertain if the hypoxia-induced increase in sICAM-1 is mediated by reactive oxygen species led to the serendipitous finding that the antioxidant N-acetyl cysteine (NAC) causes a three-fold increase in sICAM-1, a response elicited by its antioxidant property as confirmed by additional experiments involving another antioxidant, pyrrolidine dithiocarbamate (PDTC). The effect of NAC was mediated by post-transcriptional and/or post-translational mechanisms independent of p38 MAPK, p42/44 MAPK and PKC signaling.

3. Cardiac fibroblast collagen synthesis

The effect of hypoxia on collagen synthesis in confluent cultures of adult and neonatal rat cardiac fibroblasts was evaluated in terms of [^{3}H]-proline incorporated into collagen, determined following collagenase digestion. The results suggested that hypoxia *per se* had no effect on collagen synthesis in adult and neonatal rat cardiac fibroblasts.

Significance of the findings

The three principal findings of this study are:

1) Hypoxia retards G_1/S transition in cardiac fibroblasts

As cycling cells are more vulnerable to apoptotic cell death, and stalling cells in G_0/G_1 provides additional time to repair any damage to DNA or cellular proteins that may occur from ambient stress, the finding that hypoxia causes delayed cell cycle progression at the G_1/S transition in cardiac fibroblasts may represent a mechanism integral to cell survival under hypoxic conditions to ensure their role in post-infarct repair and remodeling.

2) Cardiac fibroblasts are an intra-cardiac source of pro-inflammatory mediators whose expression may be modulated by hypoxia and redox status

That fibroblasts are an important intra-cardiac source of TNF-α and sICAM-1 whose levels are augmented by hypoxia suggests a mechanism by which these cells, as the most abundant cell type in the heart, may modulate local inflammatory responses within the ischemic myocardium. Further, given that sICAM-1 may be a negative modulator of inflammation, the increase in sICAM-1 production induced by NAC and PDTC suggests a mechanism by which these clinically used antioxidants may exert anti-inflammatory effects within the myocardium.

3) Hypoxic fibroblast-derived factors reduce normoxic cardiac fibroblast proliferation suggesting that these factors may exert autocrine/paracrine effects on cardiac cells

The finding that hypoxic fibroblast-conditioned medium reduces cardiac fibroblast proliferation suggests that soluble factors produced by these cells under hypoxia may exert autocrine/paracrine effects on co-resident cardiac cells and may contribute to the response elicited by the primary stimulus, hypoxia.

Together, the study provides insights into how hypoxia may modulate multiple aspects of cardiac fibroblast function in ischemic states of the heart.

I have already published three articles, and two more are about to be communicated. The ones published are in the American Journal of Physiology Heart and Circulatory Physiology (2006), Molecular and Cellular Biochemistry (2007) and Cell Biology International (2007).

I. INTRODUCTION

I.1. CARDIAC INTERSTITIUM – A MAJOR DETERMINANT OF CARDIAC FUNCTION

The myocardium consists of two distinct but related compartments, parenchyma and stroma. Parenchyma, the contractile element of the heart, is composed of terminally differentiated cardiomyocytes that have lost their replicative capacity at or soon after birth. Cardiac myocytes represent one-third of the myocardial cell population and are highly specialized cells responsible for the pump function of the heart. The stromal compartment, or the interstitium, is a dynamic metabolic entity composed of a scaffold of structural proteins and non-myocytes, including fibroblasts, ECs and vascular SMCs [101, 185]. The interstitial protein scaffold or the ECM is an elaborate network of interconnected proteins, including the fibrillar collagens I (~80%) and III (10%), smaller amounts of collagens IV, V, VI, elastin and fibronectin, interacting with integrins and focal adhesion kinases at the cell/matrix junction [20]. ECM provides a structural framework to support the myocytes, prevent myocyte slippage, and provide a structural and mechanical continuum for the functioning of the heart as a syncytium. Maintenance of the quantitative relationship between the parenchymal and stromal compartments is vital for the structural and functional integrity of the myocardium [23, 131, 183].

In many disease states of the heart, a disproportionate accumulation of ECM proteins, especially fibrillar collagens, leads to compromised cardiac function [38, 39, 182]. In cardiac pathologies characterized by myocyte injury and loss, a cascade of processes is initiated that includes enhanced degradation of the collagenous matrix facilitating slippage and removal of dead myocytes followed by 'reparative/replacement fibrosis' through enhanced deposition of fibrillar collagens at the site of injury [33]. Further, hemodynamic changes within the myocardium, as in hypertensive cardiomyopathy, lead to enhanced synthesis and disproportionate accumulation of

fibrillar collagen even in the absence of myocyte loss, a process referred to as 'reactive fibrosis' [11, 184]. Such alterations in the quantity and composition of the interstitial protein scaffold due to fibrosis are primarily physiological and adaptive, aimed at restoring normal myocardial function, but may eventually lead to distorted tissue structure, abnormal myocardial stiffness and electrical discontinuity between surviving myocytes [41]. In recognition of the centrality of the interstitium in overall cardiac health, the term "interstitial heart disease" has been coined to indicate disease states that predominantly involve the extracellular space [182, 186].

The contribution of the interstitium to cardiac function and its role in the initiation and progression of cardiac dysfunction has aroused considerable interest in cardiac fibroblasts, the cells primarily responsible for ECM turnover.

I.2. CARDIAC FIBROBLASTS

Fibroblasts represent the majority of cardiac interstitial cells (>90%) and about two-thirds of the myocardial cell population. In the heart, they are the only source of fibrillar collagens types I and III, the predominant proteins of the interstitial protein scaffold [11, 184]. They are also a major source of MMPs and TIMPs, key enzymes involved in ECM protein turnover [54, 185]. Thus, cardiac fibroblasts contribute to the maintenance of ECM homeostasis, and hence to the structural and functional integrity of the myocardium. Further, increasing evidence over the years supports the view that cardiac fibroblasts are an intracardiac source of growth factors, cytokines and immunomodulatory factors with autocrine/paracrine functions [12, 25, 108, 199]. Whereas cardiac fibroblasts are primarily responsible for the maintenance of ECM homeostasis in the normal myocardium, in the diseased heart, they contribute to tissue repair and remodeling.

I.3. CARDIAC FIBROBLASTS IN PATHOLOGIC STATES OF THE HEART

Cardiac fibroblasts play a cardinal role in the wound healing response following myocardial injury associated with conditions such as myocardial infarction, hemodynamic overload, and myocarditis [33, 39, 82]. In pathologic states of the heart or in response to injury, they undergo transformation into an activated phenotype, the myofibroblast, which exhibits increased rate of proliferation, chemotactic migration to the site of injury, altered ECM metabolism, and coordinated temporal elaboration of factors that can modulate fibrotic and inflammatory responses [25, 54, 55, 108]. These varied responses of fibroblasts are important determinants of cardiac function post-injury, especially as these are the most abundant cell type in the heart.

Several factors prevalent in the myocardium can influence cardiac fibroblast function in a setting of injury [19, 27, 116, 138]. A common metabolic stress associated with many disease states of the heart is hypoxia.

I.4. HYPOXIA

Hypoxia results from oxygen supply insufficient to meet metabolic requirements, and is a major factor influencing the extent of cell injury and response in many cardiac disorders including myocardial ischemia and infarction. It is a powerful regulator of gene expression affecting multiple aspects of cell function including proliferation [50, 51], energy metabolism [84,187], expression of various proteins such as cytoskeletal proteins, ECM proteins [15, 34], proteins associated with cell survival [21] and factors modulating growth and inflammation [66, 122, 159, 192].

Cellular responses to hypoxia have been studied extensively using cell lines [22, 62, 63, 71] and in pathologic states, including tumor hypoxia [72, 78, 157] and pulmonary

hypertension [44, 45, 53, 197]. The responses elicited by hypoxia are found to vary depending on the cell type, degree and duration of hypoxia, the extent of cell differentiation, and the tissue, organ and species of origin [62]. For example, hypoxia inhibits cell cycle progression in MEFs [63, 71] but induces proliferation in pulmonary artery adventitial fibroblasts [44, 45, 168], pulmonary artery SMCs [197] and airway SMCs [40]. Further, in contrast to the enhanced proliferation observed in hypoxic porcine aortic ECs [160], proliferation in bovine aortic and bovine pulmonary artery ECs is inhibited by hypoxia [177]. The influence of hypoxia on the different functional capabilities of cardiac fibroblasts in a setting of injury has received relatively less attention despite the relative abundance of these cells in the heart.

I.5. BROAD OBJECTIVES OF THE STUDY

This study focused mainly on the regulation of cardiac fibroblast proliferation by hypoxia. An additional objective was to examine the effects of hypoxia on the production of modulators of inflammation and collagen by these cells. These distinct aspects of cardiac fibroblast function are important determinants of the outcome of myocardial response to injury in ischemic states.

I.5.1. Hypoxic modulation of cardiac fibroblast proliferation

Cardiac fibroblasts are undifferentiated cells that remain quiescent in the normal heart but, unlike cardiomyocytes, retain the capacity to proliferate throughout adult life and do so in response to several mitogenic stimuli [96, 144]. Fibroblast hyperplasia is a feature common to many pathologic states of the heart and is central to the role played by these cells in cardiac remodeling and reparation. In the diseased heart, a multitude of factors can act as positive or negative modulators of cell cycle progression and the

balance between these opposing influences will ultimately determine the functional response of cardiac fibroblasts in a given situation. However, barring a few reports [96, 144] on cell cycle arrest in cardiac fibroblasts induced by IL-1β, a cytokine associated with inflammatory states of the heart, little information is available on the effects of various pathogenic stimuli, such as hypoxia, on the kinetics of the cardiac fibroblast cell cycle and the molecular events mediating cell cycle entry and exit.

In this regard, it is noteworthy that a general mechanistic scheme has evolved in cycling cells for the initiation, progression and completion of the cell cycle, or the exit from the cell cycle [57, 70, 79]. The transition between states of quiescence and proliferation is determined by a cascade of tightly orchestrated events coordinately regulated by changes in the expression and/or activity of an array of proteins, kinases and transcription factors [77, 91, 132, 190]. However, within such a general scheme, there are several levels at which pro- and anti-mitogenic stimuli can exert control over the cell cycle.

Hypoxia has been reported to inhibit DNA synthesis in human [3] and rat cardiac fibroblasts [73, 200]. However, in these studies, the decrease in DNA synthesis was not correlated with changes in cell proliferation and population doubling time. Moreover, the experiments were carried out in confluent cardiac fibroblast cultures under serum-free conditions when cell proliferation cannot occur due to cell cycle arrest resulting from contact inhibition and serum withdrawal. Thus, modulation of the cardiac fibroblast cell cycle by hypoxia and the underlying molecular mechanisms remain largely unexplored and warrant further examination.

Another rationale for examining hypoxic-modulation of the cardiac fibroblast cell cycle is that these cells are relatively resistant to apoptosis in response to a variety of pro-apoptotic stimuli, including hypoxia, simulated ischemia, and staurosporine, [120] and

that the molecular pathways mediating apoptosis and cell cycle progression are coordinately regulated [1, 57, 93, 113], raising the possibility that the resistance of cardiac fibroblasts may be related to the regulation of cell cycle progression in these cells.

I.5.2. Hypoxic modulation of growth factor and cytokine expression in cardiac fibroblasts

Despite the recognition of the role of cardiac fibroblasts as an intra-cardiac source of growth factors and cytokines that can modify the cytokine milieu of the myocardium and exert autocrine/paracrine effects [12, 25, 82, 108, 198, 199], hypoxic modulation of the production of these factors by cardiac fibroblasts has not been addressed. In contrast, hypoxia is reported to induce, in a variety of cell types, including cardiomyocytes [90, 195], vascular SMCs [6, 172] and ECs [9, 123, 202], the production of factors that modulate proliferation, growth and inflammation.

In this context, it is pertinent to note that intrinsic difficulties in evaluating the effects of hypoxia on cardiac fibroblasts *in vivo* include the existence of a heterogeneous cell population in the heart, confounding influences from other factors prevailing in the myocardium and the inability to produce a uniform hypoxic insult.

Against this backdrop, the specific objectives of this study were to

- set up and characterize an *in vitro* cell culture model of hypoxia
- evaluate the effects of hypoxia on
  - the cardiac fibroblast cell cycle
  - production of modulators of inflammation: TNF-α, IL-1 β, IL-4, IL-6, and sICAM-1
  - collagen synthesis, and
- delineate mechanisms underlying these cellular responses to hypoxia

I.6. RESULTS AT A GLANCE

The three principal findings of this study and their significance can be summarized as follows:

I.6.1. Hypoxia delays cardiac fibroblast cell cycle progression at G_1/S transition, which is reversed upon re-oxygenation

Cycling cells are reportedly more vulnerable to apoptotic cell death, and arresting the cell cycle at G_1/S may prevent cell damage by providing additional time to repair any damage to DNA or cellular proteins that may occur from ambient stress. Hence, the finding that hypoxic cardiac fibroblasts exhibit reversible cell cycle deceleration at the G_1/S transition, causing accumulation of cells in the G_0/G_1 phase of the cell cycle, may represent a mechanism integral to cell survival under hypoxic conditions and is consistent with the role played by these cells in post-infarct repair and remodeling.

I.6.2. Cardiac fibroblasts are an intra-cardiac source of pro-inflammatory mediators whose expression may be modulated by hypoxia and redox status

The observation that cardiac fibroblast-conditioned medium had detectable levels of soluble factors such as IL-4 , IL-6, TNF-α, and sICAM-1 is consistent with a role for cardiac fibroblasts as an intracardiac source of several growth factors and cytokines. Moreover, the finding that hypoxia augments the production of TNF-α and sICAM-1 by fibroblasts suggests a mechanism by which these cells may modulate local inflammatory responses within the ischemic myocardium. Further, given that sICAM-1 may be a negative modulator of inflammation, the increase in sICAM-1 production induced by NAC and PDTC, observed serendipitously in this study, indicates that these clinically used antioxidants may exert anti-inflammatory effects within the myocardium.

I.6.3. Hypoxic fibroblast-derived factors reduce normoxic cardiac fibroblast proliferation, suggesting that these factors may exert autocrine/paracrine effects on cardiac cells

The attenuation of cardiac fibroblast proliferation by HFCM suggests that soluble factors produced by these cells under hypoxia may exert autocrine/paracrine effects on co-resident cells and amplify the response elicited by the primary stimulus, hypoxia.

Together, the study provides insights into how hypoxia may modulate multiple aspects of cardiac fibroblast function in ischemic states of the heart.

II. REVIEW OF LITERATURE

II.1. CARDIAC PARENCHYMA AND STROMA

The myocardium consists of two compartments, the parenchyma and the stroma that supports the parenchyma. The parenchymal compartment consists of highly differentiated cells, the cardiac myocytes. Myocytes account for 33% of the cardiac cell population and occupy about 70-75% of the total volume of the heart. They have a highly specialized structure and express a complex assembly of contractile proteins that enable their contraction and relaxation, which in turn leads to the phasic contractile activity of the heart. Cardiac myocytes, therefore, are the functional units that confer the pump function. Being terminally differentiated, myocytes of the postnatal heart have limited capacity to proliferate but grow by hypertrophy in response to mitogenic stimuli [23]. Hypertrophy refers to growth in terms of cell size and cell protein content and, in the case of myocytes, also includes changes in the contractile proteins in terms of quantity and alignment within the cell. Hypertrophic response, which involves an increase in myocardial mass and wall thickness can be physiologic, as in the case of exercise and normal growth, or can be pathologic as seen in conjunction with chronic hypertension, valvular disease and ischemia/infarction [20, 185].

The stromal component of the heart, representing the cardiac interstitium, provides a substratum in which the myocytes are embedded to facilitate the functioning of the heart as a syncytium [182]. The interstitium is a complex network of interstitial cells, or the non-myocytes, and the acellular compartment composed of the structural proteins of the ECM. Cardiac interstitial cells or non-myocytes are of mesenchymal origin and include resident fibroblasts, capillary ECs and monocytic cells and vascular cells from coronary arteries and veins. The interstitial cells constitute more than two-thirds of the myocardial cell population of which cardiac fibroblasts represent a majority [182].

Cardiac ECM is composed mainly of fibrillar collagens [type I (~80%) and type III collagens (~10%)] along with smaller amounts of other collagens (IV, V, VI), elastin, laminin, proteoglycans, glycosaminoglycans and other proteins [182]. Cardiomyocytes are primarily covered by a basement membrane consisting predominantly of collagen type IV. Fibrillar collagens, the major structural proteins of cardiac ECM, form weaves and struts of collagen fibrils that surround the myocytes and connect myocytes to each other. These loose weaves of collagen fibrils are referred to as endomysial collagen. Several myocytes enclosed in endomysial collagen are surrounded by collagen sheath forming perimysial bundles. The perimysial bundles are in turn ensheathed by a collagenous fascia, the epimysium [25]. Collagen fibers also support the intracoronary arterioles that provide blood supply to the myocardium. The structural protein scaffold of the interstitial compartment ensures adequate support to the myocytes and non-myocytes, prevents myocyte slippage or overstretching of the myocytes, and also prevents rupture of the heart during cardiac systole and diastole [185]. Thus, the interstitium provides a structural, functional and mechanical continuum to ensure transmission of force of contraction from individual myocytes to the cardiac muscle and thereby to the heart as a whole, resulting in the ejection of blood. In addition to the structural proteins, the ECM also accommodates a diverse spectrum of growth factors, proteases and other molecules of physiologic and pathologic significance [25]. The cardiac interstitium, therefore, is a highly differentiated, dynamic metabolic entity which helps maintain the structural and functional integrity of the myocardium [185]. As is the case with any organ, maintenance of the relative proportions of the parenchymal and stromal compartments is critical to sustaining normal cardiac function [23, 25, 183].

Overriding importance has been assigned to the myocyte compartment in normal myocardial function and in the initiation and progression of cardiac dysfunction

associated with several cardiac disorders. However, increasing evidence over the years suggests that it is the continued dysregulation and accumulation of the cardiac interstitium that impairs cardiac function in many instances. In fact, the term "interstitial heart disease" has been coined to indicate disease states that may predominantly involve the extracellular space [182, 186]. A distinctive feature of cardiac ECM dysregulation is the accelerated and aberrant ECM remodeling leading to disproportionate and excessive accumulation of matrix proteins especially fibrillar collagens, a condition referred to as cardiac fibrosis. In the heart, the only source of fibrillar collagens is cardiac fibroblasts, the cell type that is primarily responsible for ECM homeostasis [54].

II.2. CARDIAC FIBROBLASTS

Fibroblasts are the major cell type of the heart numerically, comprising about 90% of the interstitial cells. These undifferentiated cells remain quiescent in the normal heart but retain the capacity to proliferate in response to mitogenic stimuli. Apart from being the only source of fibrillar collagens, cardiac fibroblasts are a major source of MMPs and TIMPs, the key enzymes involved in ECM turnover [11, 54, 184, 185]. Recent findings support the idea that these cells are an intracardiac source of growth factors and cytokines that can affect functions of co-resident cardiac cells by autocrine and paracrine mechanisms [108]. Cardiac fibroblasts also serve as intermediate sensors and amplifiers of stimuli originating from the other cells of the myocardium, including myocytes and infiltrating immune cells [12, 25, 199].

In the normal myocardium, cardiac fibroblasts are primarily responsible for the regulation of ECM metabolism and hence the maintenance of ECM homeostasis. In the diseased myocardium, however, cardiac fibroblasts are activated or phenotypically transformed to myofibroblasts, a cell type of intrinsic importance in the initiation and

progression of myocardial remodeling as part of wound healing and repair post-injury [12]. Several factors prevailing in the diseased myocardium trigger the phenotypic transformation of fibroblasts and also determine the functional capabilities of the resultant myofibroblast phenotype [25]. Central to the role of cardiac fibroblasts in myocardial reparation and remodeling are the functional endpoints acquired by these cells upon activation to myofibroblasts, namely, chemotactic migration, hyperplasia, enhanced matrix production and release of soluble modulators of inflammation within the myocardium [25, 54, 55, 108]. Cardiac fibroblast proliferation and altered matrix turnover are almost always associated with pathologic conditions of the heart, unlike cardiac myocyte hypertrophy [25]. This further underlines the importance of cardiac fibroblasts in the normal and diseased myocardium.

II.2.1. Cardiac fibroblasts – role in myocardial wound healing and repair

In response to myocardial injury, a series of processes occur that are primarily adaptive in nature but can at times progress to a stage where organ function can be partially or largely impaired. These processes constitute the wound healing response of the myocardium [170, 185].

II.2.2. Phases of wound healing

The exudative phase appears within minutes to hours. The characteristics of this phase include vasodilatation, vascular hyperpermeability to macromolecules such as plasma fibrinogen and fibronectin, and increased lymphatic flow. This phase is marked by the recruitment of circulating immune-inflammatory cells to sites of injury and macrophage activation. The initiation and progression of these changes are regulated by the individual or concerted action of specific chemical mediators like histamine, serotonin, bradykinin, and/or prostaglandins.

The inflammatory phase occurs over the course of several days resulting in the formation of granulation tissue. During this phase, cells mediating the wound healing response such as macrophages, ECs and fibroblasts are activated and as a result respond to chemotactic signals by migrating into an extravascular fibrin-fibronectin gel. This constitutes the granulation tissue, and represents a provisional matrix to support the cells involved in tissue repair. The activation of fibroblasts into the myofibroblast phenotype that is capable of increased collagen synthesis and enhanced production of modulators of inflammation occurs in this phase in response to factors like TGF-β released from activated cells including macrophages. Myofibroblasts have prominent nuclei and abundant rough endoplasmic reticulum and Golgi-vesicular transport organelles. These cells also express actin filaments that enable their contractility and motility and chemotactic migration to sites of tissue injury.

In the fibrogenic phase, myofibroblasts within the fibrin-fibronectin gel proliferate and concomitantly synthesize collagen type III in large amounts. The synthesis and deposition of collagen type I follows, restoring the normal type I to type III ratio. Type I collagen is the dominant fibrillar collagen in the normal and diseased heart. Fibrous tissue accumulation has been detected in the injured myocardium as early as the 7th day post-injury and continues to accumulate throughout the fibrogenic phase for the ensuing 4-6 weeks. Such continued accumulation of collagen within the myocardium leads to cardiac fibrosis. Cardiac fibrosis can be "reparative or replacement fibrosis" where the newly synthesized collagen replaces the injured myocytes, as occurs in pathologic conditions such as ischemia and infarction or in the senescent myocardium [33]. Fibroblasts, however, may get activated and cause fibrosis even in the absence of myocyte loss. This is termed "reactive fibrosis" and may occur as a reaction to inflammatory changes in the heart or during changes in hemodynamics resulting in mechanical stress to cardiac cells,

as in a setting of hypertensive heart disease [11, 184]. Prolonged, uncontrolled synthesis and excessive accumulation of fibrillar collagen as part of the wound healing process represents an adaptive process gone awry and is a major factor contributing to myocardial stiffness and compromised cardiac function following ventricular remodeling [41, 147, 182, 186]. The impairment of pump function manifests as ventricular dysfunction initially during systole and at later stages during diastole.

Collagen degradation, another component of tissue repair, is reported to occur prior to collagen accumulation and is mediated by increased collagenolytic activity of MMPs, a major source of these enzymes being fibroblasts. Such collagenolytic activity may facilitate the slippage and removal of damaged myocytes from the site of injury before fibrous tissue accumulation occurs. TIMPs regulate MMP activity.

During the phase of <u>fibrous tissue remodeling,</u> the fibrous tissue formed during the previous phase may undergo further remodeling by way of retraction leading to scar thinning.

The various processes constituting the wound healing mechanisms are regulated by inflammatory cytokines such as IFNγ, IL-1, TNF-α and members of the IL-6 family, and fibrogenic growth factors including Ang II, aldosterone and TGF-β released from the cells of the myocardium and infiltrating inflammatory cells [116]. Although initiation of the wound healing response is dominated by cells such as macrophages derived from the circulation, cardiac fibroblasts play a significant role in regulating the subsequent phases [39]. The functional attributes of activated cardiac fibroblasts are modulated by the aforementioned pro-inflammatory and profibrogenic factors associated with pathologic states of the heart. Mechanical stretch and alterations in ECM have also been shown to affect cardiac fibroblast function significantly [116]. Moreover, cardiac fibroblasts are a source of inflammatory mediators and growth factors, including Ang II, TGF-β, IL-1β,

IL-6, IL-8, ET-1, prostaglandins and NO [19]. These factors in turn can regulate the process of remodeling in addition to affecting the growth and function of co-resident cells by autocrine/paracrine mechanisms.

As is evident from the foregoing discussion, the cardiac fibroblast is a pivotal effector cell in tissue remodeling whose diverse functional capabilities are regulated by several factors prevailing in the injured myocardium [19]. A better understanding of the factors that modify cardiac fibroblast gene expression, growth and functions, and delineation of the underlying mechanisms, is of intrinsic importance in preventing or restricting pathologic remodeling and improving cardiac function in the context of myocardial injury.

A factor known to be associated with many cardiovascular diseases is hypoxia. Over the past several years, a substantial body of work has focused on tissue and cellular responses to hypoxia and the molecular mechanisms underlying such responses that ensure survival of the organism under challenging conditions of oxygen deficit.

II.3. HYPOXIA

Molecular oxygen is essential for the normal development and growth of most multicellular organisms and is required for aerobic metabolism in all mammalian cells. Any decrease in its availability below normal levels can have profound physiological and health consequences [122]. Hence, a complex physiological network has evolved that ensures optimal oxygenation of all cells and maintenance of oxygen homeostasis at the tissue level. This network involving the lungs, erythrocytes, vasculature and the heart facilitates the capture, binding, transport, and delivery of molecular oxygen to the tissues [122]. Hypoxia is a state in which convective or diffusive oxygen transport fails to meet the metabolic oxygen requirements of the tissue. Hypoxia can occur as a result of

decrease in partial pressure of oxygen in the inhaled air or due to failure of one or more of the components of the organ systems involved in oxygen intake, transport and delivery [149]. Depending on the etiology, hypoxia can be classified into four different types.

II.3.1. Types of hypoxia

Hypoxia can result from a reduction in the amount of oxygen passing into the blood due to decreased oxygen pressure in the lungs, reduced gas exchange area, exposure to high altitudes where the pO_2 of atmospheric air is low, or in lung disease [149]. This type of hypoxia is termed hypoxic hypoxia. Anemic or hypemic hypoxia is seen in conjunction with reduced oxygen carrying capacity of the blood. This occurs from a reduction in the amount of blood hemoglobin or a reduced number of red blood cells [141, 149]. Ischemic or stagnant hypoxia results from poor circulation of blood to the tissues, as occurs in the case of ischemia associated with coronary and peripheral artery diseases. Histotoxic hypoxia is seen in association with carbon monoxide and cyanide poisoning and in alcohol abuse where the tissues are unable to utilize oxygen despite normal blood levels [141, 149].

Hypoxia is encountered in a variety of conditions – both physiologic and pathologic. The developing embryo or the fetus may be exposed to hypoxic conditions *in utero* [106, 194]. Further, hypoxia is an extremely common physiological stressor at high altitudes due to oxygen-thin atmosphere [149, 159]. Hypoxia also manifests under clinical conditions such as wound healing, anemia, myocardial infarction, ischemic heart disease, stroke, retinopathy, chronic obstructive pulmonary disease and cancer [141].

II.3.2. Responses to hypoxia

Hypoxia activates highly complex oxygen sensing systems within cells leading to compensatory physiological mechanisms aimed at optimizing oxygen delivery to metabolizing tissues. If the hypoxic episode persists, the physiological changes may culminate in severe pathological consequences at the cell, tissue, organ and the organism level [51, 66, 84, 122, 159, 187, 192].

Oxygen sensing, the initial event leading to changes that constitute the physiologic responses to hypoxia, is mediated at the organism level by several chemoreceptors including the neuroepithelial bodies in the airway, carotid bodies in the arterial circulation and the SMCs of the pulmonary vasculature [66, 122, 191]. Under hypoxia, the oxygen sensors initiate respiratory changes including increased alveolar ventilation and respiratory alkalosis, and cardiovascular system changes such as pulmonary vasoconstriction, reduced myocardial contractility, decreased maximum oxygen consumption, and switch from anaerobic to aerobic mode of metabolism [149]. Pulmonary vascular smooth muscle constriction and dilation of peripheral vessels including the coronary and cerebral vasculature comprise the changes at tissue level aimed at optimizing blood flow to tissues under hypoxic conditions [122]. Another adaptive change at the tissue level is the increased transport of iron to erythroid tissues resulting in increased heme biosynthesis and erythropoeisis, which in turn enhances the oxygen carrying capacity of blood. Further, hypoxia is associated with enhanced angiogenesis which decreases oxygen diffusion distance, and also modulation of local blood flow mediated by alterations in vascular tone [192]. These varied responses help maintain homeostasis with minimum energy expenditure. Though primarily adaptive, some of these changes may exert adverse influences on tissues as in the case of cardiovascular changes such as right ventricular hypertrophy and pulmonary hypertension

that may result in congestive heart failure [149]. The physiologic responses to hypoxia are the result of a multitude of events elicited by hypoxia at the cellular level [122].

II.3.3. Cellular hypoxia

Cells have intrinsic mechanisms that enable them to sense and maintain oxygen homeostasis, the failure of which can lead to cellular dysfunction and/or irreversible cell damage. This raises the question as to what the normal levels of oxygen for tissues or cells are, and what is perceived as hypoxia by these systems. While atmospheric air contains 21% oxygen, arterial pO_2 is only about 14% [156]. Mammalian tissues under normoxic conditions exhibit a wide range of oxygen concentrations (3% to 9%), much lower than the arterial pO2, across a distance of 400μm from a blood supply. Thus, the cells within a tissue are exposed to different concentrations of oxygen depending on the specific localization of the cells and the functional status of the tissue. Heart cells receive an oxygen supply of <10% under conditions of systemic normoxia [156]. Hence, it appears that "normoxia" for cells is an adjustable variable, which is set according to the oxygen levels available under normal conditions of growth. Accordingly, cells perceive a decrease in oxygen concentration as hypoxia when the oxygen concentration is below their normoxic set point and is sub-optimal in comparison with their metabolic requirement [156].

II.3.3.1. Cellular responses to hypoxia

Hypoxia is a powerful regulator of gene expression and can affect multiple aspects of cell function. Hypoxia-induced modifications in cell function are initiated as part of cellular mechanisms of adaptation to hypoxia and are aimed at preventing cell damage and maintaining cell survival under the potentially deleterious hypoxic conditions [192].

Although hypoxia responses are thought to be evolutionarily conserved in all mammalian cells, cellular response to hypoxia vary between cell types depending on the degree of hypoxia, the extent of cell differentiation, the cell type and the tissue/organ/species [51, 62]. These variations in hypoxia response can be explained in terms of the diverse energy requirements, the differences in microenvironments, and the differences in the oxygen concentration that the cells are normally exposed to. Further, tissues and cells also differ in their sensitivity to hypoxia, which forms the basis of their classification as oxyregulators or hypoxia-sensitive and oxyconformers or hypoxia-tolerant systems [21, 83, 84]. Cellular responses to hypoxia range from alterations in the metabolic state of the cell to perturbations in ion exchange and alterations in cell membrane permeability [84, 105, 141, 164]. These varied responses are mediated at the molecular level by alterations in the activity of signaling molecules, gene expression changes of an array of transcription factors, and modification of the target protein function [192].

II.3.3.2. Molecular mechanisms mediating cellular responses to hypoxia

A family of transcription factors called HIFs plays a central role in orchestrating the cellular response to hypoxia. HIFs represent the link between oxygen sensors and effectors at the cellular, local and systemic levels [28, 74, 124, 129, 161, 192]. The lack of O_2 is translated into many adaptive responses, a process that is largely controlled by HIF-1. HIF-1 is a phosphorylation-dependent and redox-sensitive heterodimeric protein composed of two subunits – the variable α subunit HIF-1α or HIF-2α or HIF-3α and the constitutively expressed HIF-1β subunit. The activity and protein abundance of HIF-1β are not affected by hypoxia. HIF-1α constitutes the most prominent member of the 3 α-subunits. Under normoxic conditions, the alpha subunit is continuously degraded so that

the basal levels are below detection limits. HIF-1α degradation is accomplished by a family of enzymes, the prolyl hydroxylases, also referred to as oxygen sensors [192]. Activation of HIF-1 by hypoxia involves several steps including stabilization of the alpha subunit against proteosome-mediated degradation by decreasing prolyl hydroxylase activity and levels. The HIF-1α subunit, activated by hypoxia, binds to the HIF-1β subunit to modulate the expression of genes involved in several physiological processes including angiogenesis, proliferation, cell survival and cell death, erythropoiesis, energy metabolism and oxygen chemoreception [192].

Apart from HIF proteins, a variety of other transcription factors and signaling molecules are involved in eliciting the hypoxia-induced changes. p53 and NFκB are among the transcription factors activated by hypoxia [94, 125, 157, 161, 164]. Hypoxia has been reported to increase levels of the tumor suppressor protein, p53, and its accumulation in the mitochondria [158]. p53 is associated with the activation of several processes that in turn mediate the effects of hypoxia on cells. For example, exposure of neonatal rat cardiac myocytes to prolonged hypoxia results in substantial cell loss by apoptosis and is associated with increased expression of p53 and its trans-activating ability [111]. Hypoxia activates NFκB by tyrosine phosphorylation, inactivation and dissociation of IκB, the inhibitory subunit that binds and inactivates NFκB [94]. Transcription factors such as activating protein-1 (AP-1), CREB, GATA-1, GATA-2 and forkhead protein are also reported to be regulated by hypoxic stress [10, 128, 164]. Many hypoxia-inducible genes have their promoter and/or enhancer elements in their regulatory regions as binding sites for one or more of these hypoxia-responsive transcription factors. For example, most of the hypoxia-sensitive genes encoding cytokines such as VEGF, bFGF, and TNF-α have NFκB binding sites in or near their promoters, and therefore are regulated by NFκB [164]. Hypoxic induction of these transcription factors is followed by

their translocation to the nucleus where they modulate the expression of a wide array of genes encoding proteins with varied cellular functions [192].

Some of the effector molecules modulated by hypoxia include those regulating

- Oxygen transport through erythropoiesis and iron metabolism

  - Erythropoietin, transferrin, transferrin receptor, ceruloplasmin

- Oxygen transport by vascular regulation

  - VEGF, iNOS, heme oxygenase 1, ET-1

- Anaerobic energy production by glucose uptake and glycolysis

  - Glucose transporter 1, phosphofructokinase L, aldolase A, GAPDH, phosphoglycerate kinase 1, enolase 1, lactate dehydrogenase A

- Mitochondrial function – cytochrome oxidase, manganese SOD (MnSOD)

- Cytoskeletal structure and membrane permeability

Several signaling pathways, including cAMP-protein kinase A, Ca^{2+}-calmodulin, p42/44 MAPK, SAPK or p38 MAPK and phosphatidylinositol 3-kinase/Akt, mediate the effects of hypoxia through activation/inactivation of hypoxia-sensitive factors/proteins culminating in a functional phenotype that is characteristic of the response to hypoxia [13, 14, 89, 133, 163, 164]. Hypoxic conditions are also associated with an increase in intracellular oxidant stress, which is postulated to result from increased ROS production within complex III of mitochondria. Oxidants produced under hypoxic conditions may act as second messengers and trigger additional signaling pathways involved in the regulation of transcriptional, translational and post-translational modifications under low-oxygen conditions [32, 52, 56, 105, 160]. Hypoxia-sensitive signaling pathways and factors are

reported to modify the expression of several genes controlling mitochondrial respiration and metabolism, activities of mitochondrial enzymes such as cytochrome oxidase and MnSOD, intercellular interactions, cytoskeletal structure, membrane transport, antioxidant defense mechanisms and cell survival [164].

II.3.3.3. Effects of hypoxia on energy metabolism

Hypoxia initiates a series of metabolic adjustments that help the cells adapt to the decrease in energy efficiency resulting from decreased availability of oxygen. The adaptive metabolic changes elicited by hypoxia in cells can be classified into the 'defense phase' which extends for a limited duration of hypoxia following which the cells activate the 'rescue phase' mechanisms. Whereas the defense phase may be present in both hypoxia-sensitive and hypoxia-tolerant cell types, the rescue phase is characteristic of hypoxia-tolerant systems [84].

During the defense phase, cells exhibit balanced suppression of ATP demand and supply pathways, down-regulation of ion pumping by channel/spike arrest and down-regulation of protein synthesis by translational arrest. For example, cardiac myocytes exhibit a decline in contractility under hypoxic conditions [26], a process by which the cell minimizes energy utilization. The translational arrest and consequent modulation of protein synthesis, induced by hypoxia, is reported to be a reversible response in hypoxia–tolerant but not in hypoxia–sensitive cells [84].

Extended hypoxia leads to activation of rescue phase responses in hypoxia-tolerant cells, involving preferential translation and overproduction of specific proteins such as glycolytic enzymes that confer energy efficiency and survival advantage by initiating significant gene-based metabolic reprogramming [84, 192]. Such metabolic reprogramming enables the cells to maintain reduced activities of energy demand and

energy supply pathways of metabolism. Thus, the most significant and protective feature of the hypoxia response is regulated metabolic depression to a hypo-metabolic state, a response resulting from several metabolic adjustments [84]. In hypoxia-tolerant cells, energy-requiring processes such as protein synthesis, Na^{+}/K^{+}- ATPase, protein degradation, urea biosynthesis and gluconeogenesis, are inhibited under hypoxia resulting in a net decrease in protein turnover by about 10-fold [84, 122]. These cells are also capable of using anaerobic metabolism to sustain reduced rates of energy turnover during hypoxia, thereby conserving the fermentable fuel, preventing deleterious end product (H^{+}) accumulation and extending survival time. Metabolism in hypoxia-sensitive cells like the brain cells, on the other hand, exhibit a high obligatory rate of energy consumption and the forced suppression of metabolism observed in response to hypoxia is, in effect, metabolic failure [84, 122].

Thus, the major metabolic changes associated with hypoxia include a switch from aerobic metabolism (oxidative phosphorylation) to anaerobic metabolism (glycolysis), increase in carbohydrate consumption to compensate for its inefficient utilization under hypoxic conditions, decreased energy usage by shutting down non-essential cell functions, and metabolic depression to a stable hypo-metabolic state consequent upon the coordinated down-regulation of energy supply and demand processes [84].

II.3.3.4. Effect of hypoxia on protein synthesis

In neonatal rat cardiac myocytes, hypoxia has been reported to attenuate RNA levels and protein synthesis, suggesting transcriptional as well as translational arrest [29]. Chronic hypoxia decreases norepinephrine-mediated hypertrophy of neonatal rat cardiac myocytes [107]. In contrast, hypoxia has been shown to induce hypertrophy in pulmonary artery ECs, characterized by increased numbers and size of cell organelles, including

ribosomes, rough endoplasmic reticulum, and Golgi apparatus [53]. These reports support the postulation that the response to hypoxia is cell type-specific.

A plethora of reports on the effects of hypoxia on different cell types suggests that apart from its well-documented effects on cell growth and metabolism, hypoxia also influences the production of factors that can exert autocrine/paracrine actions and determine tissue response.

II.3.3.5. Effect of hypoxia on the production of autocrine/paracrine factors by cells

Hypoxia modulates the expression of immunomodulatory factors, cytokines and growth factors, and adhesion molecules, all of which may modify intercellular interactions in disease states by autocrine/paracrine mechanisms [6, 53, 172, 192].

The autocrine/paracrine effectors whose expression in different cell types is modulated by hypoxia, both under basal conditions and in response to pathogenic factors, include

- growth factors and cytokines regulating **vascular growth and vascular tone** – PDGF (-AA, BB and AB), bFGF, IGF-1, TGF-β, ILs (-1, -6 and -8), endothelium-derived growth factor and tissue factor [53]
- **vasoactive agents** – Ang II, ET-1, bradykinin, NO, PAF, prostacyclin, prostaglandins, thromboxane A2, and leukotrienes [53]
- factors such as PDGF and PAF that regulate **endothelial cell activation and vascular remodeling** [6, 53]
- factors like VEGF, PDGF and bFGF that induce **endothelial cell proliferation,** which in turn may contribute to **angiogenesis** as an adaptive response that augments blood supply to affected tissues [192]
- **immunomodulatory factors** such as TNF-α, PAF, and prostaglandins, which regulate the tissue inflammatory response [30, 53]

- factors like IL-6, which contributes to inflammation by increasing **endothelial cell permeability** [123, 172, 195]
- **adhesion molecules** such as ICAM-1 and VCAM-1 that may modulate intercellular interactions and inflammatory responses [9, 90, 202]

Hypoxic modulation of multiple aspects of inflammation, from activation of inflammatory cells to adhesion and extravasation, can have significant impact on the initiation and progression of inflammatory processes.

Among the multiple effects of hypoxia, regulation of cell cycle progression has been investigated extensively in a variety of cell types [22, 62, 63, 71] and in relation to several pathologic states, including cancer [72, 78, 157].

II.3.3.6. Effects of hypoxia on cell cycle progression and cell survival

A significant change exhibited by most aerobic cells under hypoxic conditions is a reversible inhibition or delay in cell cycle progression, a response that contributes to the reduction in utilization of available residual energy [24, 51, 62, 67]. Hypoxia is found to delay cell cycle progression in a variety of cell types including bovine aortic and pulmonary artery ECs [177], MEFs [62, 63, 67, 71] and splenic B-lymphocytes [67]. The hypoxia-induced inhibition of proliferation in many cell types, including murine embryo fibroblasts, is reported to be associated with changes in the cell cycle regulatory apparatus. The cell cycle may halt completely and irreversibly in hypoxia-sensitive cells leading to death whereas in hypoxia-tolerant cells, the cell cycle arrest can be transient lasting only for the duration of the stress and is resumed when the stress is removed [7, 8].

In certain cell types, however, hypoxia causes hyperplasia as demonstrated in the pulmonary vasculature. Hypoxia induces human pulmonary fibroblast and pulmonary smooth muscle cell proliferation [44, 45], mediated by PDGF and PAF. Hypoxic-

induction of pulmonary artery adventitial fibroblast proliferation is reported to involve multiple pathways including p42/44 MAPK, JNK, p38 MAPK, and PKC [53, 65]. Hypoxia also regulates proliferation of aortic [160] and pulmonary artery ECs [177].

An understanding of the steps involved in cell cycle progression and the proteins that regulate cell cycle is necessary to determine how hypoxia modulates cell cycle activity in various cell types.

II.3.4. The cell cycle

The cell cycle is a highly ordered process by which cells duplicate their genome and transmit identical copies of the initial genetic material to daughter cells. In other words, cell cycle is the process by which cells reproduce and is the basis of growth and development in all living organisms. The mechanism of cell proliferation or cell cycle is regulated by a complex system of cyclins and CDKs, and the regulatory proteins that modulate the activities of these two components [57].

In cycling or proliferating cells, chromosomes undergo recurrent structural changes in a time-dependent cyclic manner based on which the chromosomal cycle is classified into interphase, prophase, metaphase, anaphase and telophase. Prophase, metaphase, anaphase and telophase together constitute mitosis [51]. Interphase is the time between the completion of anaphase of one cycle and the onset of prophase of the very next chromosomal cycle. Unlike mitosis, where changes in the chromosomal DNA are microscopically evident, the interphase is not marked by gross changes but conceals within it many key molecular events that define the cell division cycle. The interphase is further divided into G_1 (Gap 1) phase, S (Synthesis) phase, and G_2 (Gap 2) phase. The G_2 phase is followed by the various phases of mitosis the durations of which vary greatly. The G_1 phase extends for about 10-14 hours in dividing cells whereas the duration of the

S phase is about 3-6 hours and that of the G_2 phase is about 2-4 hours. Mitosis accounts for only a short duration in the entire cell cycle, about one hour [51]. The intracellular changes associated with the various phases of cell cycle are as follows:

G_1 (Gap 1) phase - This constitutes the changes mediating the growth of cells and preparation of chromosomes for replication in the subsequent phase. In this phase, the cells are not metabolically quiescent. The DNA is compactly folded as chromatin and the genes and proteins necessary for DNA synthesis and replication are activated. The proteins that help the cells progress through the S phase are synthesized and accumulated. During the G_1 phase, cells are subject to stimulation by extracellular mitogens and growth factors. The cells proceed through G_1 in response to these stimuli.

S (Synthesis) phase - During this phase, the synthesis of DNA and centrioles occurs. The double helical DNA is replicated into two daughter DNA molecules. Histones and various proteins responsible for the activity of chromosomes bind rapidly to the newly formed DNA molecules. DNA replication produces 'dyads' made up of two identical sister chromatids held together by a ring of proteins called 'cohesins'.

G_2 (Gap 2) phase - This phase is marked by proof reading of replicated DNA and repairing any mismatches and/or mispairing in the newly synthesized DNA. In addition to this, proteins required for mitosis are accumulated and the cell is prepared for mitosis. During this phase, the chromosomes appear as thin threads within the nucleus, attached to microtubules radiating from centrioles.

M (Mitosis or Maturation) phase - Mitosis ensures chromatin condensation, nuclear envelope breakdown, chromatid separation and cytokinesis, resulting in the formation of two daughter cells genetically identical to the parent cell.

G_0 (Gap 0) phase - Non-cycling or non-proliferating cells, exemplified by terminally differentiated myocytes and neuronal cells or cells that have exit the cell cycle to enter a

stage of quiescence, are said to be at G_0. This phase may extend from about 8 hours to years, during which the cells are not committed to progress into the G_1 phase of the cell cycle. G_0 phase cells show G_1-phase DNA content but are metabolically quiescent and exhibit low rates of RNA and protein synthesis and low enzymatic activity. Apparently, G_0 represents a stage when there is active repression of genes needed for mitosis, resulting in the exit of cells from the cell cycle and entry into a state of differentiation or quiescence. Transformed cells are incapable of entry into G_0 and repeat the cell cycle indefinitely, as seen in tumor cells [57].

II.3.4.1. Regulation of the cell cycle

Normal undifferentiated cells at quiescence are dependent on external mitogenic stimuli to move them out of G_0 and through the early part of G_1. For all mammalian cells with proliferative potential, a general scheme has emerged for the control of cell proliferation in response to mitogen stimulation. This scheme involves the cell cycle-specific stimulation of a binary system of cell regulators consisting of a family of regulatory subunits called the cyclins, which bind to (and help to activate) the catalytic subunits, the cyclin-dependent protein kinases (CDKs) that are essential for the progression of cells through the cell cycle checkpoints [51, 57, 87].

Cyclins – Cyclins are the molecular timers for cell cycle progression. Cell cycle control is exerted through signal transduction pathways and regulatory mechanisms that are connected to extracellular signals and involve the expression and assembly of different kinase holoenzymes and their precisely ordered activation. Cyclins are the regulatory subunits or enzyme cofactors and cyclin-dependent kinases (CDKs) are the catalytic subunits within these holoenzymes. Cyclins help determine the sub-cellular localization, substrate specificity, interaction with upstream regulatory proteins, and timing of CDK activation. Cyclins undergo sequential binding to the CDK complexes throughout the cell

cycle to form active complexes that phosphorylate nuclear control proteins that have specific functions during different stages of the cell cycle. Unlike CDK expression, which is reported to be more or less constitutive, cyclin expression is "cyclic," the levels of each cyclin independently increasing or decreasing within the phases of the cell cycle and responsive to proliferative signals. Depending on cell cycle phase-specificity, cyclins are classified into

G_1 cyclins – cyclin D1 (D2, D3)

G_1/S cyclin – cyclin E

S phase cyclin – cyclin A (also termed late G_1/S cyclin due to its presence at later stages of G_1/S transition)

D and E cyclins are sometimes referred to as 'Start' cyclins since they govern the entry into, progression through and the exit from the G_1 phase in response to external mitogenic cues. The expression of G_1 cyclins is induced by growth factors and inhibited by antiproliferative agents. Hence these molecules, especially the D type cyclins that regulate the transition through the initial part of G_1, function as nuclear regulators and, indirectly, as growth factor sensors and ECM protein detectors [57]. In cycling or proliferating cells, cyclin D expression is increased during the G_1 phase and persists till the onset of the S phase. Cyclin E expression is essential for the transition of cells through G_1/S checkpoint of the cell cycle and its levels are increased towards the later stages of G_1, persisting through the G_1/S checkpoint and at the initiation of the S phase. Cyclin A expression is evident during the later stages of G_1/S checkpoint and is present throughout the S phase and during G_2/M checkpoint and M phase. It is responsible for the progression of the cell cycle through the S phase, G_2/M and is active during the M phase. B group cyclins form complexes with specific CDK (CDK1) to form M-phase promoting factor that could accelerate the entry into mitosis in early G_2 cells. The degradation of

cyclins A and B occurs at the completion of metaphase. Cyclin B degradation is necessary for the exit of cells from mitosis [57].

Cyclin-dependent kinases – The cell cycle comprises the oscillation of certain cell cycle proteins, namely the cyclins and their intranuclear protein kinase partners, the cyclin-dependent kinases (CDKs), in a cyclical, phasic fashion. The activation of CDKs, which propels the cell cycle through various stages, is achieved by the association of these kinases with specific cyclins followed by phosphorylation by an activating kinase. In the resultant cyclin-CDK complex, cyclin is the regulatory subunit and CDK is the catalytic partner and these complexes phosphorylate specific protein substrates thereby activating or inhibiting them, which in turn regulate cell cycle progression. CDKs are expressed constitutively and are activated in a cell cycle phase-dependent manner. CDKs govern the checkpoints and the phase transitions within cell cycle [51].

An assemblage of cyclin/CDK complexes orchestrates the advance of the cell through the phases of its growth cycle. The cell cycle can therefore be viewed as a recurring CDK cycle in which specific patterns of cyclin/CDK complexes enable the transition of cells through the phases and checkpoints of the cell cycle. The specific cyclin-CDK combinations for the different phases of cell cycle are as follows:

G_1 cyclins or cyclin D – bind CDK4 (CDK 6)

G_1/S cyclins E and A – bind CDK2

Mitotic cyclins A and B – bind CDK1

The molecular events promoting progression of cells through the cell cycle can be summarized as follows:

The cell moves out of G_0 and through G_1 to the G_1/S boundary in response to growth or mitotic stimuli. These extracellular signals bind to membrane receptors and activate signaling pathways that initiate the translation and increased expression of D cyclins (D1, D2, D3), their protein kinase partners CDK4 and CDK6, marking the initiation of the cell cycle at G_1 [87]. The D cyclins then associate with CDK4 and CDK6 forming complexes and resulting in the phosphorylation and activation of the bound CDKs. The activated CDKs then phosphorylate the retinoblastoma gene product, RB. In non-proliferating cells, RB is in a hypophosphorylated form and is bound to E2F transcription factor thereby inhibiting the transcriptional activity of E2F, which is essential for overcoming the G_1/S transition point and progression to S phase [2]. The phosphorylation of RB by CDK4/6 leads to its dissociation from E2F, allowing this transcription factor to initiate the transcription of various genes such as cyclin E that are required for traversing the restriction point. Later in the cycle, cyclin D targets the hyperphosphorylated RB for dephosphorylation mediated by phosphoprotein phosphatase type I and making it available for binding to and inactivating E2F.

The transcriptional up-regulation by E2F leads to an increase in cyclin E expression with the levels and the associated CDK2 activity peaking in late G_1. Cyclin E binds to and activates CDK2, and functions to initiate the transcription of S phase genes and their products. Increased expression of cyclin A occurs at G_1/S transition and persists through the S phase and G_2 to M. Cyclin A associates with and activates CDK2 and the active complex localizes to nuclear/chromosomal replication foci. A concomitant transcription of S phase genes leads to accumulation of protein products required for chromosomal duplication and progression of DNA synthesis, such as the origin

recognition complex (ORC) and DNA polymerase. Cyclin A/CDK2 activity is present at G_1/S transition and peaks at G_2, suggesting a role for this complex in cell cycle progression at both G_1/S and G_2/M checkpoints. When cells complete the S phase and enter G_2, Mitosis promoting Factor (MPF) is synthesized. Cyclin A/CDK2 activity is essential for the entry of cells into prophase and progression through mitosis and promotes the initiation of chromosome condensation, disassembly of nucleoli, and activation of MPF [51]. The MPF is composed of cyclin B and CDK1 and this holoenzyme activates several proteins that are essential for mitosis. The resultant activities trigger nuclear envelope breakdown, spindle formation and chromatid segregation. On activation and translocation of MPF to the nucleus, cells enter metaphase and undergo changes characteristic of this phase. The progression of cycling cells from metaphase to anaphase requires the activation of Anaphase-promoting complex (APC/C), a multi-subunit ubiquitin ligase which mediates the changes that mark anaphase [57]. The exit of cells from anaphase requires the degradation of cyclin B and inactivation of CDK1 by the proteolytic activity of APC/C. The last phase of mitosis, the telophase, is marked by events mediating cytokinesis leading to the separation of the two daughter cells, complete with a copy of the genetic material and cytoplasmic elements.

Control of cell cycle activity can be exerted during the various stages of the cell cycle and also during cell cycle checkpoints, which are the points of transition from one phase to the next [113].

Cell cycle checkpoints – Cell cycle checkpoints are the transition states within a cell cycle where the cells and the cell cycle events are monitored to ensure the coordinated and error-proof completion of each stage of the cell cycle before the cell moves on to the next phase. At these points, the cells are screened for damage and, accordingly, the cell cycle

regulatory proteins direct the cells towards repair or death, depending on the extent of damage. Thus checkpoints represent a proof-reading mechanism for preventing the replication of damaged or un-repaired genome and, therefore, progression and propagation of mutated or damaged cells [51]. The major cell cycle checkpoints are G_1/S transition, G_2/M transition, Spindle checkpoint and G_0/G_1 transition.

G_1/S transition – this checkpoint appearing at late G_1, is designated 'Start' or 'Restriction point' and is sensitive to the availability of extracellular mitogens. This transition ensures that the enzymes and other proteins required for the S phase have been synthesized and that the cell is ready to replicate DNA. Within this transition is the G_1 DNA damage checkpoint, which senses damage to the DNA, and activates pathways that delay cell cycle progression in G_1 before DNA synthesis [57]. Activation of the DNA damage checkpoint involves the PI3K homologs ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related protein (ATR), which function at both G_1 and G_2. This checkpoint imposes a G_1/S block mediated by activation of proteins such as ATM, ATR, and p53. In the presence of genomic damage, p53 is activated that in turn up-regulates the expression of p21, a CDK inhibitor, to interrupt cycling by inhibiting RB phosphorylation. This provides time for DNA repair. The progression of G_1-phase cells to the S-phase, in the absence of DNA damage, is a critical regulatory step in both normal and neoplastic cell growth [87, 113]. Once a cell transits this checkpoint and enters the S phase, the progression through the rest of the cycle is not dependent on extracellular influences and hence the cell becomes committed to completing DNA synthesis. The progression from the S phase to G_2 lacks checkpoint control.

G_2/M transition – checks whether the DNA has been correctly and fully replicated and that the daughter chromosomes lack any replication error such as mispairing of

nucleotides or incompletely synthesized DNA. This function of G_2/M is mediated by the presence of a DNA replication check point that imposes a cell cycle arrest or delay in cell cycle progression at G_2, before the cell enters the mitotic phase [51]. Unlike G_1 checkpoint, the G_2 checkpoint does not require p53 but functions by inhibiting the activation of CDK1, the protein kinase essential for the transition of cells at this point of cell cycle. The S and M phases of the cell cycle are tightly coupled and the G_2/M transition is important in preventing division of cells containing incompletely replicated DNA. This transition phase involves nuclear envelope dissolution and chromosome condensation.

Spindle check point – ensures that a functional mitotic spindle is formed as a part of karyokinesis. This checkpoint also detects any failure of spindle fiber to attach to kinetochore and arrests the cells at metaphase (hence, also referred to as M checkpoint). Spindle checkpoint senses the improper alignment of the spindle itself and blocks cytokinesis and triggers apoptosis of the cell if the damage is irreparable. The checkpoint, in the event of inappropriate spindle formation, directs the binding of components of the spindle checkpoint pathway to the ubiquitin-conjugating system and inhibits APC/C required for the progression of cells to anaphase [51].

G_0/G_1 transition – a major determinant of cell growth is the efficiency of the cells to move from G_0 to the G_1 phase of the cell cycle across the G_0/G_1 transition. The transition involves the activation of quiescent G_0 cells with unduplicated DNA and the progression of the activated cells into G_1. This switch is mediated by the induction of immediate early gene products such as Fos and Myc in response to mitogenic stimuli. However, apart from triggering immediate early gene expression in cycling cells, mitogens also ensure

that the cells traverse the entire duration of G_1 and the G_1/S transition [87]. Most of the cell cycle checkpoints are linked to the apoptosis machinery, which enables the channeling of cells to apoptosis when cell or DNA damage, and replication error, is irreparable [1, 57, 93, 113].

Cell cycle control is exerted by both positive and negative regulators. The regulators of CDK activity can affect cell cycle progression. These include the inhibitors of CDKs, the accelerators or activators of CDKs and brakes or negative regulators of CDKs [57].

Cyclin-dependent kinase inhibitors – Activation of CDKs by cyclins leads to a wave of phosphorylation driving the cell through the corresponding checkpoint. The CDKs are subject to regulation by another class of proteins, the CDK Inhibitors (CDKIs), designated by their molecular weight. The CDKIs bind to CDKs, cyclins or the cyclin-CDK complexes thereby inhibiting the catalytic activity of CDKs and preventing cell cycle progression [165, 166, 167]. Two families of CDKIs are involved in cell cycle regulation and these can convert the response of cells from growth to differentiation.

The **Cip/Kip family of CDKIs** includes p21 (also known as Cip1 or Waf1), p27 (Kip1) and p57 (Kip2) that function at several points of the cell cycle, targeting the activity of CDKs 4, 6, and 2. These inhibitors are widely implicated in G_1/S arrest induced in various cell types in response to stress conditions, including hypoxia.

The **INK4 family of CDKIs** are constitutively expressed and inhibit CDK4 and the cyclin-CDK complexes containing CDK4 as the catalytic subunit. The two important members of this family are p16 (INK4a) and p19(ARF). p16 specifically inhibits CDK4/6 whereas p19 binds to MDM2 and prevents p53 destruction, thereby causing a p53-

mediated increase in p21 levels, the CDKI capable of inhibiting CDK4 and CDK6. The other members of this family include p14, p15 (INK4B) and p18 (INK4C).

II.3.5. Hypoxia and cell cycle arrest

The oxygen sensitivity of the different phases of the cell cycle and the checkpoints differ in different cell types. Accordingly, hypoxia may delay/arrest cell cycle progression at G_1/S [63] or mid-S phase [71] or metaphase [50]. Hypoxia may exert its inhibitory effect through the regulation of one or more of the cell cycle regulatory proteins such as RB, p53, cyclins (D, E and A), CDKs (2, 4 and 6), and CDKIs (p21, p27). The modulation of expression and/or activity of these proteins under hypoxic conditions has been found to be mediated by several pathways [43, 44] including p38 MAPK, p42/44 MAPK, Jun kinase, tyrosine kinase, and transcription factors such as HIF-1α [28, 67].

II.3.6. Cardiac fibroblasts and hypoxia

As stated earlier, a plethora of factors prevalent in the normal and diseased myocardium have been demonstrated to regulate the various functions of cardiac fibroblasts [19]. However, the effects of hypoxia on these cells have received very little attention, as borne out by the following account.

Hypoxia is reported to enhance collagen type I synthesis in human cardiac fibroblasts but decrease basal levels of total protein synthesis. Further, hypoxia was found to modify the effects of TGF-β, thyroid hormone, Ang II and bFGF on collagen and protein synthesis in these cells [3]. Hypoxia enhanced MMP-2 synthesis in neonatal rat cardiac fibroblasts and augmented the stimulatory effects of ET-1, Ang II and IL-1β on MMP-2 synthesis [16].

Despite the fact that fibroblasts are an intracardiac source of growth factors and cytokines, little is known about the effects of hypoxia on the elaboration of such factors by cardiac fibroblasts.

As stated earlier, the capacity of cardiac fibroblasts to proliferate when stimulated by mitogenic stimuli is central to their role in the normal and injured myocardium. Apart from a single report on G_1/S arrest of the cardiac fibroblast cell cycle by IL-1β, information on the cardiac fibroblast cell cycle is scanty. *Agocha et al (1997)* [3] have reported that hypoxia inhibits DNA synthesis in human cardiac fibroblasts and modifies the response of these cells to several growth factors. Two other reports from the same laboratory [73, 200] examined gender-specific effects of hypoxia on rat cardiac fibroblast DNA synthesis, and the expression of cyclin D1, cyclin B1, NFκB, p53, ERK1/2, Bcl2, HIF-1 and Jun kinase. However, in all these studies, the observed inhibition of DNA synthesis was not corroborated with changes in cell number or proliferation doubling time. Further, these experiments were performed under serum-free conditions on confluent (contact-inhibited) fibroblast cultures that had been synchronized by serum-deprivation; under these conditions, cell proliferation cannot be expected to occur.

It is important to note that cardiac fibroblasts are resistant to apoptosis under hypoxic conditions [111, 120], and that the pathways mediating the cell cycle, cell survival, and cell death are coordinately regulated [93]. Delineation of the molecular mechanisms underlying hypoxic regulation of cell cycle activity may therefore provide useful insights into the pathways that confer hypoxia-resistance or tolerance on cardiac fibroblasts, in contrast to hypoxia-sensitive cells.

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Fine chemicals

M199, DMEM, BSA, Collagenase type IA, Collagenase type VII, Trypsin, DNase, HEPES, FBS, EDTA, glucose, monoclonal anti-vimentin antibody, anti-mouse IgM antibody, anti-human von Willebrand antibody, monoclonal anti-desmin antibody, SIGMA FAST^{TM} (Fast Red TR/Naphthol AS-MX, ALP substrate tablets set), SP, BIM, PD098059, NAC, SOD, TRI reagent, DEPC, SDS, Tris base, agarose and sodium acetate were purchased from Sigma-Aldrich, MO, USA. Glycine buffer, LDH and NAD for lactate assay and immunostaining kit for desmin were purchased from Sigma-Aldrich, MO, USA. SB203580, the p38MAPK inhibitor, and the fine chemicals for cDNA synthesis including RT buffer, RNase inhibitor, random primers, dNTPs and M-MLV Reverse Transcriptase were purchased from Promega Corporation, Madison, WI, USA. The Dual Quantitative PCR kit for rat ICAM-1 was purchased from Maxim Biotech, Inc., Rockville, MD, USA.

III.1.2. Routine Chemicals

Calcium chloride, disodium hydrogen phosphate, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, POPOP, PPO, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, toluene, TCA, chloroform, isopropanol, hydrochloric acid, ethanol and ether were purchased from SISCO Research Laboratories, India.

III.1.3. Radiochemicals

[^{3}H]-thymidine (Sp. radioactivity – 17.2Ci/mmol) was obtained from the Bhabha Atomic Research Center, India. [^{3}H]-proline (Sp. radioactivity – 28Ci/mmol) was procured from Amersham Radiochemicals, UK.

III.1.4. ELISA Kits

Levels of TNF-α, IL-1β, IL-4, IL-6, were determined using the rat cytokine/chemokine LINCO*plex* kit (Linco Research, Missouri, USA). ELISA kit for sICAM-1was purchased from R&D systems, USA.

III.1.5. Cell culture-ware

35mm and 100mm cell culture-treated dishes were purchased from Nunc, Denmark. Cell culture filter ware was procured from Millipore, USA. GasPak environment systems and the anaerobic/aerobic envelopes to generate hypoxic/normoxic conditions were from Becton Dickinson (USA).

III.1.6. Equipments used

ELISA reader (Bio-Tek instruments, USA), 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-flaker (Hoshizaki, Japan), pH meter (Labindia, India), CO_2 incubator (Nuaire, USA), phase-contrast microscope (Nikon, Japan), laminar flow hood (CLAS, India), magnetic stirrer (Schott, Germany), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), electrophoresis unit (Biorad laboratories, USA), Mini Blot (Biorad laboratories, USA), Programmable Thermal Cycler (MJ Research Inc, USA), submarine electrophoresis unit (Bangalore Genei, India), Syngene Bio Imaging (Ingenius, Canada), BD FACScan flow cytometer (Becton Dickinson, USA) and UV-Transilluminator (Bangalore Genei, India).

III.2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III.2.1. Acrylamide 30%

29% (w/v) acrylamide and 1% (w/v) N,N'-methylene bisacrylamide in deionized water

III.2.2. Agarose gel (1%) for electrophoresis of DNA or RNA samples

For DNA - 200mg agarose in 20ml of 0.5X TBE

For RNA - 200mg agarose in 20ml of 1X MOPS buffer

III.2.3. Blocking solution

2.5% (w/v) skim milk in TBST containing 0.1% Tween-20

III.2.4. Cardiac fibroblast growth medium (pH 7.4)

M199 with Earle's salts containing FBS (10%), benzyl penicillin (50U/ml) and gentamycin (0.04mg/ml)

III.2.5. DAB substrate solution

6mg DAB in 10ml Tris (pH 7.6) containing 10μl of 30% H_2O_2

III.2.6. DEPC-treated deionized water

1ml of DEPC in one liter of deionized water, stirred overnight at room temperature and autoclaved

III.2.7. Dissociation medium for fibroblast isolation

The medium consisted of sodium chloride (116.4mM); HEPES (20mM); sodium dihydrogen phosphate (1.15mM); glucose (5.55mM); potassium chloride (5.37mM); magnesium sulfate (0.81mM) and adjusted to pH 7.4. Deoxyribonuclease (5.5μg/ml), pancreatin (0.025mg/ml), BSA (1mg/ml), $CaCl_2$ (1mM), and antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin) were

added to the medium under sterile conditions at the time of isolation. The dissociation medium for the first two digestions had collagenase type IA (1mg/ml) while that for subsequent digestions had trypsin 1:250 (0.75mg/ml).

III.2.8. DNA/RNA gel-loading dye

bromophenol blue (0.25%); xylene cyanol FF (0.25%); EDTA (1mM); glycerol (50%) in DEPC-treated deionized water

III.2.9. Electrode buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS – PAGE)

Tris base (25mM), glycine (192mM), SDS (0.1%) in deionized water

III.2.10. Ethidium bromide (Stock solution)

1mg in 1ml deionized water; 5μl of this stock solution was added to 20ml of 1% agarose gel for DNA/RNA electrophoresis

III.2.11. EDTA (0.5M, pH 8.0)

930mg EDTA in 5 ml DEPC-treated deionized water

III.2.12. Lysis buffer

0.1M sodium hydroxide containing 0.1% SDS

III.2.13. MOPS electrophoresis buffer [10X]

MOPS (0.2M, pH 7.0), sodium acetate (3M, pH 5.0; *Ref. 3. 2. 20*), EDTA (0.5M, pH 8.0; *Ref. 3. 2. 11*) in DEPC-treated deionized water

1X MOPS buffer was prepared from this stock solution by 1:10 dilution.

III.2.14. Phosphate-buffered saline (PBS) (pH 7.4)

Sodium chloride (137mM), potassium chloride (2.7mM), disodium hydrogen phosphate (10.14mM), potassium dihydrogen phosphate (1.76mM)

III.2.15. Propidium iodide staining solution

PI (25μg/ml), RNase (40μg/ml), and nonidet P-40 (0.3%) in PBS

III.2.16. Resolving Gel for SDS–PAGE (12%)

6.0ml 30% acrylamide (*Ref. 3. 2. 1*), 3.8ml 1.5M Tris (pH 8.8), 0.15ml 10% SDS, 0.15ml 10% ammonium persulfate and 6μl TEMED were added to 4.9ml of deionized water

III.2.17. Scintillation cocktail

PPO (0.6%) and POPOP (0.02%) in toluene

III.2.18. SDS gel-loading buffer [1X]

SDS (2% w/v), bromophenol blue (0.03%), β-mercaptoethanol (0.3%), glycerol (10% v/v) in Tris buffer (0.067M, pH 6.8)

III.2.19. Serum-free medium

M199 containing antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin)

III.2.20. Sodium acetate (3M, pH 5-6)

1.23 g sodium acetate in 5 ml DEPC-treated deionized water

III.2.21. Stacking gel for SDS – PAGE (5%)

0.83ml 30% acrylamide (*Ref. 3. 2. 1*), 0.63ml 1M Tris (pH 6.8), 0.05ml 10% SDS, 0.05ml 10% ammonium persulfate and 5μl TEMED were added to 3.4ml of deionized water

III.2.22. Substrate solution for alkaline phosphatase

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

III.2.23. Towbin's buffer (Transfer buffer)

3.027g Tris base, 14.4g glycine, 200ml methanol made up to 1L with deionized water

III.2.24. Tris borate EDTA buffer (TBE) (5X, pH 8.3)

54g Tris base; 27.5g boric acid; 20ml EDTA (0.5M, pH 8.0) made up to 1L with deionized water

III.2.25. Tris-buffered saline (10X, pH 7.6)

24.2g Tris base, 80g sodium chloride in 1L deionized water

III.2.26. Tris-buffered saline with Tween-20 (TBST) [1X]

1X TBS containing 0.1% Tween-20

III.2.27. Tris-CaCl2 buffer (4X, pH 7.4)

Tris (200mM), $CaCl_2$ (200mM)

III.2.28. Trypsin-EDTA solution

0.25mg/ml trypsin and 0.2mg/ml EDTA in PBS (pH 7.4)

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

III.3.1. Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague-Dawley rats following the method of *Kumaran and Shivakumar (2002)* [98] with some modifications.

Rats (2-3 months old) were anaesthetized with ether. Heart was excised and collected in PBS *(Ref. III.2.14)* containing antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin) and amphotericin (2.5μg/ml). The atria were removed and the ventricular tissue was washed in PBS, minced into bits of approximately 1 mm^3 size and subjected to a series of 12 digestions in dissociation medium *(Ref. III.2.7)*. Digestion was aided by gentle shaking of the flask containing tissue bits on an orbital shaker maintained at 37°C. The digests from the first two digestions with collagenase type IA (1mg/ml) were discarded. The supernatants from subsequent digestions in dissociation medium containing trypsin (0.75mg/ml) were mixed with M199 containing 10% FBS *(Ref. III.2.4)* to inactivate trypsin and centrifuged at 1900 rpm for 5 minutes. The cell pellets were pooled, re-suspended in M199 supplemented with 10% FBS, seeded in two 35 mm cell culture dishes and incubated in a humidified CO_2 incubator at 37°C in 95% air-5% CO_2.

Neonatal rat cardiac fibroblasts were isolated from 2-3 day old Sprague Dawley pups. The ventricular tissues from three or four rat pups were used and the procedure for isolation was the same as that for adult rat cardiac fibroblasts, except for the enzyme composition of the dissociation medium used for tissue digestion. The dissociation medium used for all the digestions contained both collagenase (0.3mg/ml) and trypsin (0.3mg/ml), along with DNase and pancreatin.

III.3.1.1. Selective enrichment of cardiac fibroblasts in culture

A pre-plating step ensured removal of contaminating cell types and selective enrichment of cardiac fibroblasts in the cultures. Freshly prepared adult rat cardiac fibroblasts cells were maintained in a CO_2 incubator at 37°C for 150 minutes. At the end of this period, the supernatant containing unattached cells and debris was discarded, the dishes with the adherent fibroblasts were rinsed 3-4 times and incubated with M199 containing 10% FBS. At 24 hours after isolation, the dishes were washed and incubated with M199 containing 10% FBS *(Ref. III.2.4)*. The pre-plating time for neonatal fibroblasts was 90 minutes.

III.3.1.2. Sub-culture of cardiac fibroblasts

At confluence, the culture supernatant was removed, cells were washed with PBS *(Ref. III.2.14)* and trypsinized at 37^0C in trypsin-EDTA solution *(Ref. III.2.28)*. Trypsinization was stopped by addition of M199 containing 10% FBS *(Ref. III.2.4)* and the detached cells were collected immediately by centrifugation at 1900 rpm for 5 minutes. The cell pellet was suspended in M199 containing 10% FBS *(Ref. III.2.4)* and seeded in fresh culture dishes at a split ratio of 1:3.

III.3.2. Characterization of cardiac fibroblasts in culture

Fibroblastic nature of the cells in culture was ascertained by morphology and immunocytochemistry.

III.3.2.1. Analysis of morphology

Sub-confluent and confluent cultures were examined under an inverted phase contrast microscope for morphologic characteristics.

III.3.2.2. Immunocytochemical analysis for vimentin, desmin and von Willebrand factor

Immunocytochemical staining was done as described by *Eghbali et al (1991)* [55]. Cells from passage 2 or 3 grown to 60-70% confluence were washed with PBS *(Ref.III.2.14)* and fixed in 70% ice-cold methanol for 30 minutes. The fixed cells were treated with 3% hydrogen peroxide for 5 minutes, incubated for 10 minutes with 2% BSA in PBS and for 60 minutes with primary antibody diluted in PBS containing 1% BSA. The cells were then incubated for 30 minutes with diluted HRP- or ALP-conjugated secondary antibody. Following PBS wash, the cells were incubated for up to 10 minutes with either the HRP-specific substrate reagent containing 3-amino, 9-ethyl-carbazole (AEC) or the ALP-specific substrate *(Ref.III.2.22)*. After color development, the cells were counterstained with hematoxylin, mounted in glycerol and observed under a microscope.

Monoclonal anti-vimentin, anti-mouse IgM and anti-mouse IgG antibodies were diluted 1:50; anti-human von Willebrand factor was diluted 1:800. Immunostaining for desmin was done using a commercially available kit.

III.3.3. Induction of hypoxia

The GasPak disposable hydrogen + CO_2 generator envelopes containing sodium borohydride and sodium bicarbonate + citric acid tablets and palladium pellets were used to generate hypoxic conditions. Upon addition of deionized water, the sodium borohydride tablet generates hydrogen which, in the presence of palladium catalyst, reduces the chamber oxygen to water, resulting in a chamber pO_2 of about $< 0.1\%$, as indicated by anaerobic indicator strips. To generate normoxic conditions, GasPak disposable CO_2 generator envelopes containing sodium bicarbonate + citric acid tablet were used. The sodium bicarbonate + citric acid tablet in both normoxic and hypoxic

systems release CO_2 upon addition of deionized water to provide a pCO_2 of about 5%, which was checked using a CO_2 indicator. The desired chamber oxygen and CO_2 levels were reached within 50 minutes, at 37°C.

III.3.3.1. Setting up the *in vitro* hypoxia system

Cardiac fibroblast cultures were exposed to hypoxic/normoxic conditions for 24 hours and the pH and pO_2 of the media were determined.

III.3.3.2. Effects of hypoxia on cardiac fibroblasts

III.3.3.2.1 ***Syto 13/ PI staining of hypoxic cardiac fibroblasts***

Synchronized sub-confluent cultures of cardiac fibroblasts were exposed to hypoxia for 24 hours. Cell viability was assessed by double staining of the cell nuclei with two DNA probes - the permeant DNA intercalating green probe, Syto 13 (1µM), and the non-permeant intercalating orange probe, PI (6µg/ml), in PBS *(Ref.III.2.14)*. Staining was carried out for 5 minutes at 37°C and the fluorescence was analyzed.

III.3.3.2.2 ***Determination of intracellular and extracellular lactate levels***

Confluent cultures of cardiac fibroblasts were subjected to 24-48 hours of hypoxia in M199 with 10% FBS *(Ref.III.2.4)*. Lactate levels were assayed as described by *Pallotti et al (2004)* [143]. After determining the cell number in each sample, the cells were lysed and the cell lysates and known volumes of the media were de-proteinized by precipitation with ice-cold 10% TCA and centrifuged. The supernatants were used for spectrophotometric determination of lactate levels at 340 nm. The intracellular and extracellular lactate levels were calculated using the formulae:

Intracellular lactate (nmoles/10^6 cells) =

$$(OD_{340} \times 5.25 \times 10^6) / [6.22 \times \text{Total cell no.}]$$

Extracellular lactate (μmoles/10^6 cells) =

$$\frac{OD_{340} \times \text{Total vol. deproteinized} \times \text{Total medium vol.} \times 10^6}{6.22 \times \text{vol. used in cuvette} \times \text{vol. used for deproteinization} \times \text{total cell no.}}$$

III.3.3.2.3 ***Flow cytometric analysis of cell viability and cell cycle phase distribution***

Sub-confluent cultures of cardiac fibroblasts exposed to normoxia/hypoxia were trypsinized, washed with Ca^{2+}-/ Mg^{2+}-free PBS *(Ref.III.2.14)* and fixed in 70% ethanol in PBS for 1 hour at 4°C. The fixed cells were re-suspended in PBS at a density of 1-2 x 10^6cells/ml and treated with 0.25% pepsin solution for 10 min at 37°C. The pepsin solution was removed by centrifugation, and the cells stained with PI staining solution *(Ref. III.2.15)* at RT for 20 min. Flow cytometric analysis of the stained cells was performed at an excitation wavelength of 488 nm and emission wavelength of 620 nm. Based on DNA content, the cells were sorted into G_0/G_1, S and G_2/M populations. The fraction of the cell population with cell size below that characteristic of the G_0/G_1 population (sub G_0/G_1) was used as a measure of viability loss in response to hypoxia [169].

III.3.3.2.4 ***Measurement of DNA synthesis***

DNA synthesis was measured in terms of [^{3}H]-thymidine incorporation into TCA-insoluble material, as described earlier [98], with some modifications.

Sub-confluent cultures at passage 2 or 3 were synchronized by serum-deprivation for 24 hours and incubated with 10% serum-containing or serum-free M199 with [^{3}H]-thymidine at 2μCi/ml. At 24 hours, the culture supernatant was discarded; cell layer was washed with PBS, lysed in lysis buffer *(Ref. III.2.12)* and precipitated with 10% ice-cold TCA. The TCA precipitates were washed with 5% ice-cold TCA and 70% alcohol and air dried. Radioactivity was determined using a scintillation counter.

III.3.3.2.5 ***Determination of cell number and population doubling time***

Synchronous sub-confluent cardiac fibroblast cultures at passage 2 or 3 were subjected to 24 hours of hypoxia in 10% serum-containing M199 *(Ref. III.2.4)*. Where necessary, hypoxic incubation was followed by re-oxygenation for 24 to 48 hours. Increase in cell number was calculated from the cell numbers at the start and termination of the treatment as follows:

% of increase in cell number = $[(N2 - N1) / N1] \times 100$

The population doubling time in response to various treatments was calculated using the formula, Doubling time, $T_d = 0.693 / k$, where

rate constant, $k = (2.3 \log N2/N1) / \Delta t$

N2 = the number of cells per dish at the end of the treatment

N1 = the number of cells at the onset of the treatment

Δt = the duration of the treatment (in hours)

III.3.3.2.6 ***Western blot analysis of cyclin-dependent kinase inhibitor, p27***

Western blot analysis was carried out as described by *Maniatis et al (1982)* [115]. Briefly, sub-confluent cardiac fibroblast cultures in M199 containing 10% FBS *(Ref. III.2.4)* were exposed to 24 hours of hypoxia/normoxia and lysed in SDS-gel loading buffer (1X) *(Ref. III.2.18)*. The lysates were denatured by incubation in boiling water bath for 10 minutes and cooled on ice. 25μl (2μg/μl) aliquots of samples were electrophoretically fractionated on 10% SDS-PAGE minigels *(Refs. III.2.16. & III.2.21)* and electroblotted onto nitrocellulose membrane. The membrane with the transferred proteins (ascertained by Ponceau S staining) was blocked for 45 minutes with skim milk *(Ref. III.2.3)* and incubated overnight at 4°C with mouse monoclonal anti-p27 antibody prepared at 1:200 dilutions in TBST *(Ref. III.2.26)* containing 5% BSA. The unbound primary antibody was removed by washing with TBST and the membrane was incubated for 1.5 hour with HRP-conjugated anti-mouse secondary antibody diluted 1:800 in TBST containing 5% BSA. After removal of the unbound secondary antibody, the membrane was incubated with DAB *(Ref. III.2.5)*, washed in TBST, dried and scanned.

The membrane was re-probed with anti-β actin antibody. The images were captured on Syngene gel documentation system.

III.3.3.2.7 ***Preparation of conditioned medium***

Synchronous confluent cultures were exposed to hypoxia for 24 hours in serum-free M199 *(Ref. III.2.19)*. Culture supernatants from hypoxic and normoxic cultures were collected under sterile conditions, centrifuged at 3,000rpm for 5 minutes to remove cell debris and used for the experiments.

III.3.3.2.8 ***Determination of levels of cytokines and sICAM-1***

Synchronous confluent cultures of cardiac fibroblasts were exposed to normoxia/hypoxia for 24 hours in serum-free M199 *(Ref. III.2.19)*. The levels of TNF-α,

IL-1β, IL-4 and IL-6, and sICAM-1 in culture supernatants were analyzed by sandwich ELISA using commercially available kits, following the manufacturer's instructions.

III.3.3.2.9 ***Isolation of total RNA***

Total RNA was isolated from confluent cultures of cardiac fibroblasts by an improvement of the single-step method reported by *Chomczynski and Sacchi (1987)* [37] using TRI reagent (5 ml/100 mm culture dish) to lyse the cells. RNA isolated from the lysates, as per instructions provided with the TRI reagent, was dissolved in DEPC-treated water *(Ref. III.2.6)*. The yield of RNA was determined spectrophotometrically and intactness was ascertained by 1% agarose gel electrophoresis *(Ref. III.2.2)* following standard protocol [115].

III.3.3.2.10 ***cDNA synthesis***

10μg total RNA in 10μl of DEPC-treated water and 3.0μl random primers were mixed and heated at 70°C for 5 min. The heated mixture was then snap-cooled on ice. To this were added 6.0μl DEPC-treated water, 6.0μl 5X RT buffer, 2.5μl dNTPs, 0.5μl RNAse inhibitor, and 2.0μl M-MLV reverse transcriptase. The reaction mix was incubated at 37°C for one hour, heated for 5 minutes at 90°C and then snap-cooled on ice. The cDNA preparations were stored at -20°C until use.

III.3.3.2.11 ***Polymerase Chain Reaction***

Dual Quantitative PCR kit for rat ICAM-1, obtained from Maxim Biotech, enabled co-amplification of the target ICAM-1 gene with 18S rRNA in a single reaction tube. The PCR reaction mix contained 35.0μl of PCR master mixture *(ICAM-1 and 18S primers in optimized Dual PCR buffer)*, 0.3μl of Taq DNA polymerase and 2-5μl of sample cDNA or positive control made up to 50μl with DNase–free water. The cDNAs of ICAM-1 and 18S rRNA transcripts in the reaction mix were co-amplified over 35 cycles

at an annealing temperature of 60°C by standard PCR protocol [115]. The PCR-amplified samples were stored at 4°C until analysis on 1% agarose gel *(Ref. III.2.10)*.

III.3.3.2.12 ***Identification of signaling pathways***

Cells were pre-treated for 30-60 min with pathway-specific pharmacologic inhibitors such as those specific for p42/44 MAPK [PD098059, 10μM], p38 MAPK [SB203580, 10μM], PKC [BIM, 1μM], and ROS [SOD (200U/ml), NAC (5mM), PDTC (50μM)], and exposed to various treatments in the presence of the inhibitors.

III.3.3.2.13 ***Measurement of net collagen production***

Collagen synthesis was measured as described by *Diegelmann and Peterkofsky (1972)* [48] with some modifications. Synchronous confluent cultures of cardiac fibroblasts were incubated for 24 hours in DMEM containing [^{3}H]-proline at 1μCi/ml and 50μg/ml ascorbic acid. The cells were lysed in 1% Triton-X 100 containing 5mM N-ethylmaleimide. The cell lysate and culture supernatant were pooled and the mixture divided into two aliquots. One aliquot was digested for 5 hours at 37°C with 30μg/ml collagenase Type VII in Tris-$CaCl_2$ buffer (pH 7.4) *(Ref. III.2.27)*. The proteins in collagenase-treated and -untreated aliquots were precipitated with 10% ice-cold TCA and radioactivity in the TCA-insoluble fraction was determined by scintillation counting. Net collagen synthesis was calculated using the formula:

Collagen (% of total protein) =

[Collagenase-released cpm x 100] / [(Non-collagen cpm x f) + Collagenase-released cpm]

A correction factor of f = 5.4 for non-collagen protein was used to adjust for the relative abundance of proline and hydroxyproline in collagen-containing proteins.

III.4. STATISTICAL ANALYSIS

Following overall comparison by one-way ANOVA, differences between experimental groups were assessed by Student's t-test and significance determined at $p \leq 0.05$. Values were expressed as Mean ± SD.

IV. RESULTS

IV.1. CHARACTERIZATION OF RAT CARDIAC FIBROBLASTS

IV.1.1. Morphological analysis

Cardiac fibroblasts were isolated from adult rat ventricular tissue and grown in culture as described under Methods. Pre-plating for 150 minutes post-isolation ensured selective enrichment of fibroblasts, which constituted >99% of the cells in these cultures. The fibroblastic nature of the cells was ascertained by morphologic analysis and immunocytochemical staining. At 150 minutes after isolation *(Figure 1)*, the cells had a dense nest-like morphology and attained a spindle-like appearance by 24 hours. At confluence, the cultures exhibited a monolayer pattern *(Figure 2)*. Cells at passages 2 and 3 were used for the experiments.

IV.1.2. Immunocytochemical staining of cells in culture

Cells were tested for immunoreactivity with antibodies against the cytoskeletal proteins, vimentin and desmin, and the perinuclear Factor VIII-associated antigen. The cells were positive for vimentin *(Figure 3)* but negative for Factor VIII-related antigen *(Figure 4)* and desmin (data not shown) confirming their fibroblastic nature and ruling out the presence of ECs and SMCs in these cultures.

IV.2. CHARACTERIZATION OF *IN VITRO* HYPOXIA MODEL

The anaerobic GasPak systems from Becton Dickinson, USA *(Figure 5)* were used to set up the hypoxia system. The system characteristics in terms of the pH and pO2 of the medium under hypoxic versus normoxic (control) conditions were assessed. The pH of the hypoxic and normoxic media were comparable at approximately 7.5. pO_2 of the medium under hypoxic culture conditions was ~3%, compared to the normoxic pO_2 of ~15% *(Table 1)*. pCO_2 in both normoxic and hypoxic systems was about 5%.

Figure 1. Photomicrograph of cardiac fibroblasts 150 minutes after isolation (100X)

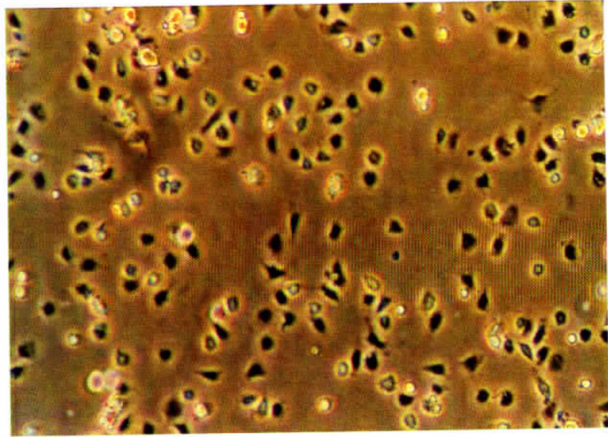


Figure 2. Photomicrograph of cardiac fibroblasts at confluence (100X)

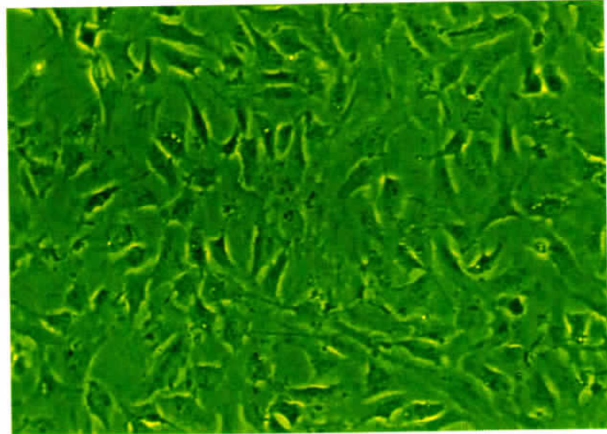
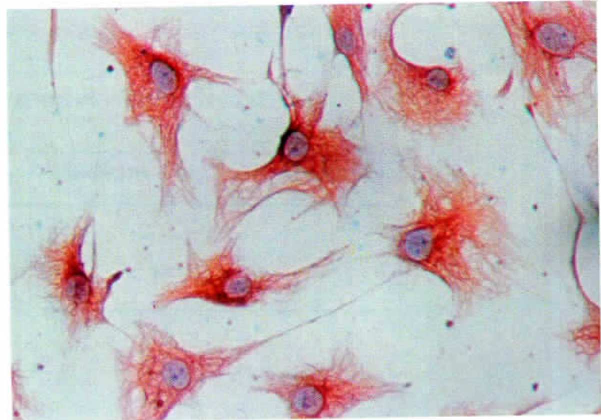


Figure 3. Photomicrograph of vimentin-positive cardiac fibroblasts (200X)

Sub-confluent cultures were methanol-fixed and incubated with anti-vimentin antibody, ALP-conjugated secondary antibody and ALP-specific chromogen. Nuclei were counter-stained using hematoxylin.



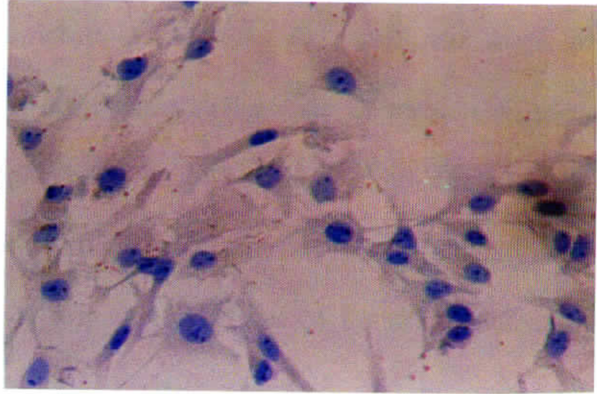


Figure 4. Photomicrograph of Factor VIII-negative cardiac fibroblasts (200X)

Sub-confluent cultures were methanol-fixed, and incubated with anti-Factor VIII antibody, ALP-conjugated secondary antibody and ALP-specific chromogen. Nuclei were counter-stained using hematoxylin.

Figure 5. Setting up *in vitro* cellular hypoxia system

GasPak anaerobic systems containing CO_2 generator envelope or (H_2 + CO_2) generator envelope for normoxic or hypoxic conditions respectively

Table 1. Hypoxia model characteristics

	Chamber pO_2	Medium pO_2	Medium pH
Hypoxia	< 0.1%	~ 3%	7. 5
Normoxia	~ 20%	~ 15%	7. 5

IV.2.1. Measurement of lactate production in cells exposed to hypoxia

A possible metabolic outcome of hypoxia being intracellular lactate accumulation, which may lead to lactic acidosis and compromised viability [69, 188], the levels of intracellular and extracellular lactate were determined in hypoxic cultures. Confluent cultures of cardiac fibroblasts at passage 3 were exposed to 24 and 48 hours of hypoxia in M199 containing 10% FBS. Intracellular and extracellular lactate levels were determined in cell lysates and cell culture supernatants, respectively. After 24 hours of incubation *(Table 2)*, intracellular lactate was undetectable in both normoxic and hypoxic cells whereas extracellular lactate was detectable in both the groups. Extracellular lactate in 24-hour hypoxic cultures was significantly higher than in normoxic cultures. Intracellular lactate was, however, detectable upon prolonged exposure of the cells (48 hours) to hypoxia. Compared to the value at 24 hours of hypoxia, extracellular lactate increased nearly two-fold at 48 hours of hypoxia *(Table 2)*.

Table 2. Lactate production by hypoxic cardiac fibroblasts

	Normoxia (24 hr.)	**Hypoxia (24 hr.)**	**Hypoxia (48 hr.)**
IC lactate (nmol/10^6 cells)	**ND**	**ND**	**30.32 ± 9.4**
EC lactate (μmol/10^6 cells)	**39.76 ± 5.4** ^{a}	**64.03 ± 5.1** ^{b}	**112.15 ± 15. 3** ^{c}

Confluent fibroblast cultures were exposed to hypoxia for 24 or 48 hours in M199 containing 10% FBS. Intracellular (IC) and extracellular (EC) lactate levels were determined in the cell lysates and the culture medium, respectively. Difference between the groups was analyzed by Student's t-test and the significance level set at $p \leq 0.05$.

b *vs.* ***a,*** $p = 0.022$; ***c*** *vs.* ***b,*** $p = 0.025$

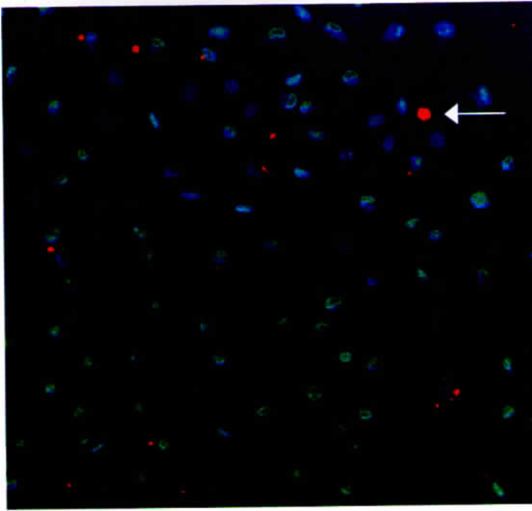
IV.2.2. Syto 13/propidium iodide staining for assessment of membrane damage and compromised viability under hypoxic conditions

Syto 13/PI staining was carried out to detect early changes in membrane integrity and morphological changes that may lead to loss of viability, as shown by red fluorescence due to PI uptake. The hypoxic and normoxic cultures were found to take up the permeant dye, Syto 13, and flouresce green. However, only <1% of the cells took up the non-permeant intercalator, PI *(Figure 6)*, showing that there was no significant loss of viability.

IV.2.3. Analysis of cell viability by flow cytometry

Flow cytometric analysis of the distribution of cells over different phases of the cell cycle provides a sub G_0/G_1 population that represents cells with marked membrane damage and DNA fragmentation. Based on this, loss of viability in cells subjected to 48 hours of hypoxia was evaluated by flow cytometry. Hypoxic and normoxic incubations of cells were carried out in M199 containing 10% FBS and the cells were fixed in 70% ethanol, stained with PI and subjected to flow cytometric analysis of the sub G_0/G_1 population. Loss of viability was meager and comparable in hypoxic and normoxic cultures at 48 hours *(Table 3)*. Non-viable cells accounted for <2.5% of the cell population in control and hypoxic cultures.

Figure 6. Assessment of cell viability by Syto13/PI staining



Photomicrograph of Syto13/PI-stained cardiac fibroblasts at 24 hours of hypoxia. Cells were ashed in PBS and stained with Syto13 and PI. The arrow shows a non-viable cell that has taken up PI and fluoresces red.

Table 3. Flow cytometric analysis of cell viability under hypoxic conditions

	Percentage of cells in sub G_0/G_1 phase
Normoxia (n=6)	1.84 ± 0.11
Hypoxia (n=6)	2.12 ± 0.431

ardiac fibroblasts exposed to 48 hours of hypoxia in M199 containing 10% FBS were rypsinized, fixed in 70% ethanol and stained with PI. The percentage of sub G_0/G_1 cells was ssessed by flow cytometry at an excitation wavelength of 488nm and emission wavelength of 20nm.

IV.3. MODULATION OF CARDIAC FIBROBLAST FUNCTIONS BY HYPOXIA

Cardiac fibroblasts play a major role in modulating myocardial function in the normal and the diseased heart. The functions of fibroblasts are regulated by the myriad of factors prevailing in the myocardium. This study examined the possibility that hypoxia, a factor prevalent in many cardiac pathologies, may modulate three important functional endpoints of cardiac fibroblasts such as proliferation, elaboration of autocrine/paracrine factors and collagen synthesis, which are common in myocardial tissue repair post-injury.

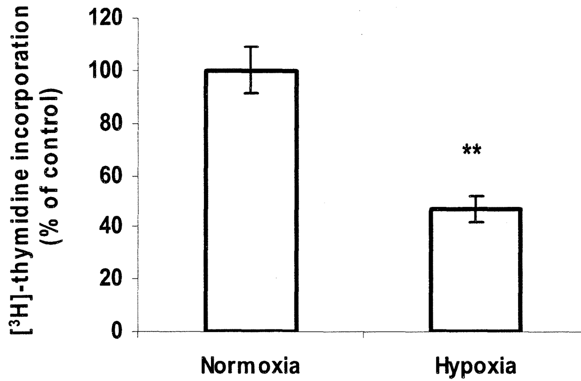
IV.3.1. Effects of hypoxia on fibroblast proliferation

Cardiac fibroblast proliferation is a central component of the role played by these cells in the context of myocardial injury. Further, cardiac fibroblasts are relatively resistant to apoptotic cell death induced by various pro-apoptotic stimuli prevalent in the diseased myocardium, including hypoxia [111, 120]. Since the pathways mediating cell cycle progression and apoptosis are linked, it seemed reasonable to examine the effects of hypoxia on cardiac fibroblast proliferation. Experiments were therefore carried out to assess changes in DNA synthesis and population doubling time in response to hypoxia (T_d).

IV.3.1.1. Hypoxia decreases DNA synthesis in adult rat cardiac fibroblasts

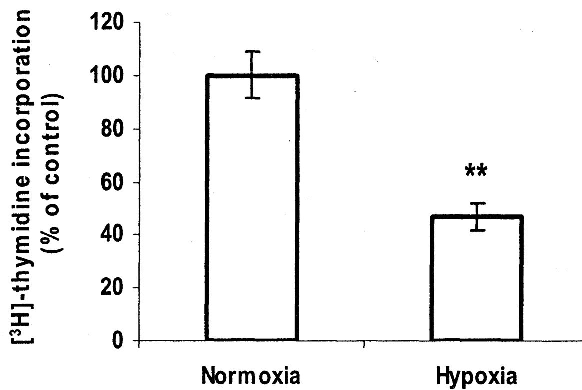
DNA synthesis, measured in terms of [^{3}H]-thymidine incorporation, was found to be significantly reduced in cells exposed to 24 hours of hypoxia in M199 without FBS *(Figure 7)* and in M199 containing 10% FBS *(Figure 8)*. The extent of inhibition of DNA synthesis was comparable under both basal (53%) and serum-stimulated conditions (47%).

Figure 7. Effect of hypoxia on basal levels of DNA synthesis in cardiac fibroblasts



*Synchronized sub-confluent cultures of cardiac fibroblasts were incubated under hypoxic conditions for 24 hours in serum-free M199 containing [^{3}H]-thymidine at 2μCi/ml. DNA synthesis was measured as described under 'Methods'. Mean ± SD of values (cpm/dish) from 5 determinations each in the hypoxic and normoxic groups were compared. The results are presented as percent of control. Significance was determined by Student's t-test and the significance level set at $p \leq 0.05$. **$p < 0.001$*

Figure 8. Effect of hypoxia on serum-induced DNA synthesis



*Synchronized sub-confluent cultures of cardiac fibroblasts were incubated under hypoxic conditions for 24 hours in M199 containing 10% FBS and [^{3}H]-thymidine at 2μCi/ml. DNA synthesis was measured as described under 'Methods'. Mean ± SD of values (cpm/dish) from 5 determinations each in the hypoxic and normoxic groups were compared. The results are presented as percent of control. Significance was determined by Student's t-test and the significance level set at $p \leq 0.05$. **$p < 0.001$*

IV.3.1.2. Hypoxia decreases cardiac fibroblast proliferation

In order to confirm that the decrease in DNA synthesis under hypoxic conditions reflects changes in proliferation, cells exposed to hypoxia were analyzed for cell number and population doubling time (T_d). Asynchronous sub-confluent cultures of cardiac fibroblasts were used for the experiment. Cell number per dish was determined after 24 hours of seeding and cells were incubated under hypoxic (H+FBS) or normoxic (N+FBS) conditions for 24 hours in M199 with 10% FBS. To compare the effect of hypoxia on proliferation with that of serum withdrawal, a third set of cells was exposed to normoxic conditions for 24 hours in M199 without serum (N-FBS). At the end of 24 hours of hypoxia/normoxia, the number of cells in the three groups [(H+FBS), (N+FBS) and (N-FBS) was determined. From the cell number data, population doubling time was calculated as described under 'Methods'. Hypoxia was found to decrease serum-induced proliferation of cardiac fibroblasts *(Figure 9)*. While the population doubling time in cycling cells grown in serum-containing medium (N+FBS) under normoxic conditions was 27.7 hours, the population doubling time of hypoxic cells (H+FBS) was 54.1 hours and that of serum-starved normoxic cells (N-FBS) 192.5 hours *(Figure 9)*.

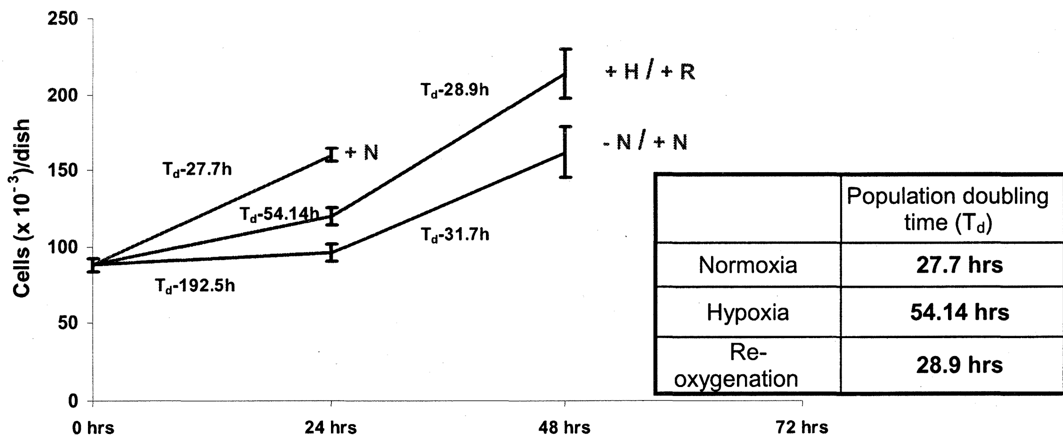
IV.3.1.3. The effects of hypoxia on fibroblast proliferation are reversible

Following 24 hours of hypoxic incubation, cells were re-oxygenated for 24 hours in M199 with 10% FBS. It was found that the effects of hypoxia on cell number and population doubling time are reversed upon re-oxygenation *(Figure 9)*. The population doubling time of 54.1 hours of hypoxic cells (H+FBS) was restored to about 28.9 hours at the end of 24 hours of re-oxygenation.

Similar to the reversal of the hypoxia effect, the effect of serum-deprivation on cell proliferation was reversed by serum supplementation *(Figure 9)*. The population doubling time of 192.5 hours in serum-starved cultures (N-FBS) was restored to 31.7 hours upon replenishing the medium with 10% FBS.

Reversibility of the hypoxic inhibition of proliferation was also assessed by pulse labeling studies. Following 24 hours of hypoxia, the cells were pulse-labeled with [^{3}H]-thymidine over 0-4 hours and 24-28 hours of re-oxygenation. Cell number was determined in these dishes after which the cells were lysed and processed for measurement of DNA synthesis as described under 'Methods'. [^{3}H]-thymidine incorporation at the end of 24 hours of re-oxygenation was two-fold higher than that at the onset of re-oxygenation *(Figure 10)*.

Figure 9. Hypoxia-induced changes in cardiac fibroblast proliferation are reversed upon re-oxygenation



	Population doubling time (T_d)
Normoxia	**27.7 hrs**
Hypoxia	**54.14 hrs**
Re-oxygenation	**28.9 hrs**

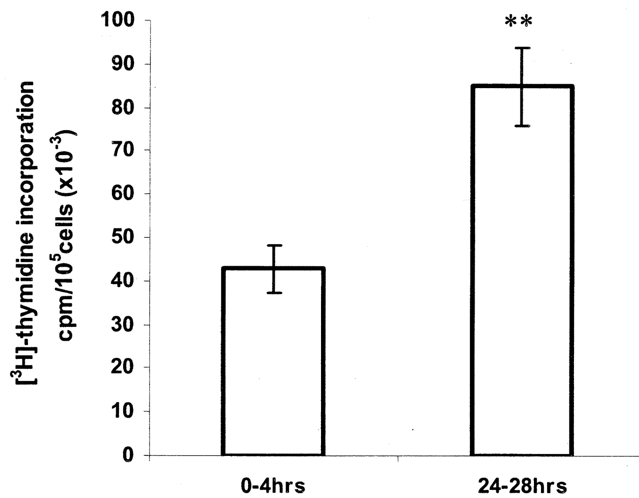
+ N - *cells exposed to normoxia for 24 hours in M199 containing 10% serum*

- N/ + N - *cells exposed to normoxia for 48 hours, first 24 hours in serum-free medium and the next 24 hours in medium containing 10% serum*

+ H/ + R - *cells in M199 with 10% serum exposed to hypoxia for 24 hours followed by re-oxygenation for 24 hours*

Sub-confluent cultures of cardiac fibroblasts were subjected to 24 hours of hypoxia (0-24 hrs) in M199 containing 10% FBS followed by re-oxygenation for 24 hours (24-48 hrs). To compare the effects of hypoxia with serum-starvation, cultures were incubated under normoxic conditions in serum-free M199 for 24 hours followed by 24 hours of incubation in M199 with serum. Changes in cell number and the population doubling time (T_d) of hypoxic cells and serum-starved cells were compared with that of normoxic cardiac fibroblasts grown in M199 containing 10% FBS. Values represent the Mean ± SD of 5 determinations.

Figure 10. Rate of DNA synthesis during re-oxygenation



*Sub-confluent cultures of cardiac fibroblasts were exposed to hypoxia for 24 hours and re-oxygenated for another 28 hours in M199 with 10% FBS. Cultures were pulse-labeled with [^{3}H]-thymidine at 2μCi/ml during 0-4 hours or 24-28 hours of re-oxygenation. Radioactivity was determined as described under 'Methods' and expressed as cpm/10^5 cells. Values represent the Mean ± SD of 5 determinations. Difference between the groups was analyzed by Student's t-test and significance was determined at $p \leq 0.05$. ** $p < 0.001$*

IV.3.2. Elucidation of the molecular basis of inhibition of cardiac fibroblast proliferation by hypoxia

To elucidate the mechanisms underlying hypoxic inhibition of cardiac fibroblast proliferation, subsequent experiments focused on plausible signaling pathways, the hypoxia-sensitive cell cycle checkpoint, changes in the expression of p27 and on the role of soluble factors from hypoxic cardiac fibroblasts.

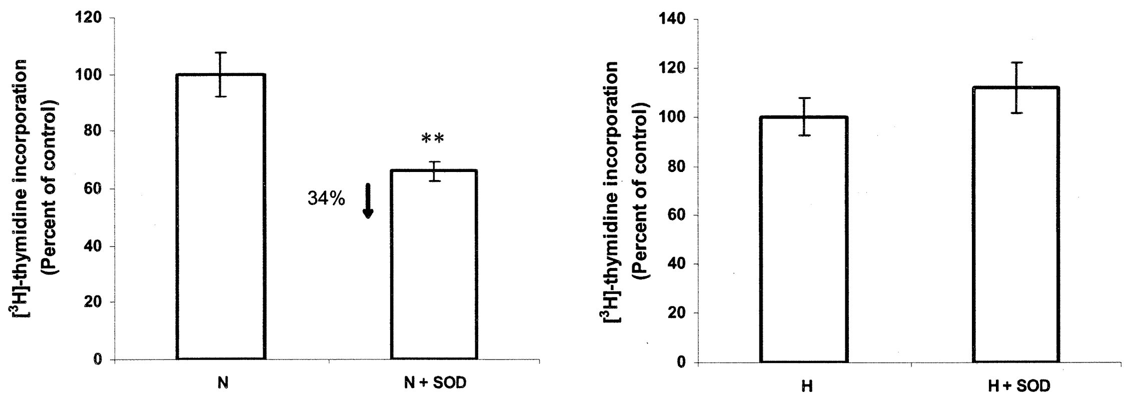
IV.3.2.1. p38 MAPK and p42/44 MAPK are modulators of cardiac fibroblast proliferation

Hypoxia has been shown to regulate proliferation in several cell types through the activation/inactivation of p38 MAPK and p42/44 MAPK [43, 44]. Further, hypoxic conditions are known to be associated with increased production of ROS such as superoxide [105], a potent second messenger known to mediate a wide range of cellular responses, including proliferation [160]. To examine the possibility that these different molecular pathways may mediate the observed attenuation of proliferation by hypoxia, experiments were carried out in the presence of inhibitors specific for superoxide (200U/ml SOD), p42/44 MAPK (10μM PD098059 in 0.1% DMSO), and p38 MAPK (10μM SB203580 in 0.1% DMSO). Synchronized sub-confluent cultures of cardiac fibroblasts at passage 3 were pre-treated for one hour with pathway-specific inhibitors followed by exposure to hypoxia/normoxia for 24 hours in M199 containing the inhibitors and [^{3}H]-thymidine. 0.1% DMSO, the solvent for PD098059 and SB203580, was included in the control groups.

Synchronized sub-confluent cultures of cardiac fibroblasts were pre-treated with SOD and exposed to 24 hours of hypoxia in serum-free M199 containing SOD. Results indicate that SOD did not affect the hypoxic inhibition of basal levels of cardiac fibroblast proliferation, but decreased DNA synthesis in normoxic cardiac fibroblasts by 34% *(Figure 11)*.

The effects of PD098059 and SB203580 on proliferation of synchronized sub-confluent cultures of cardiac fibroblasts under normoxic and hypoxic conditions were analyzed. It was found that p42/44 MAPK inhibition does not modify the effect of hypoxia on cardiac fibroblast proliferation, but reduces normoxic cardiac fibroblast proliferation by 32% *(Figure 12)*. Inhibition of the p38 MAPK pathway increased DNA synthesis by 38% and 69% in hypoxic and normoxic cells, respectively *(Figure 13)*.

Figure 11. SOD decreases DNA synthesis in normoxic but not hypoxic cells



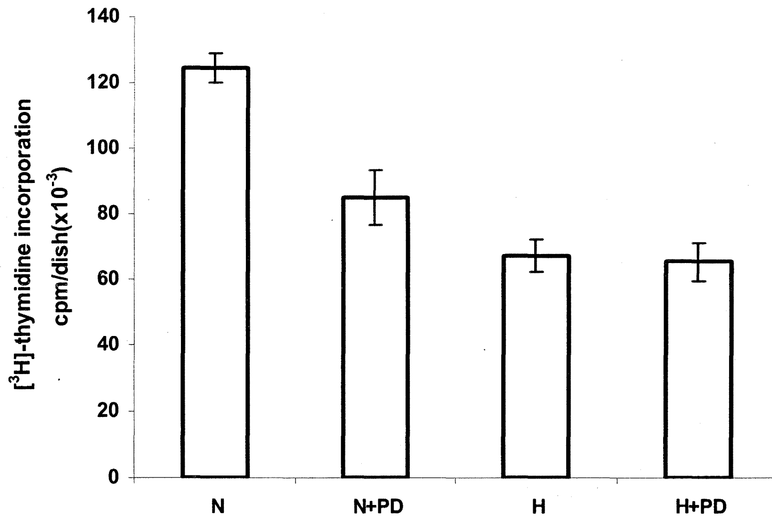
Synchronized sub-confluent cultures were pre-incubated with SOD (200 U/ml) for one hour and exposed to hypoxia for 24 hours in serum-free M199 containing SOD and [^{3}*H]-thymidine at 2μCi/ml. DNA synthesis was measured as described under 'Methods'. Values represent the Mean ± SD of 5 determinations and are presented as percent of the control group, N being the control for N + SOD and H the control for H + SOD. Difference between the groups was analyzed by Student's t-test and significance was determined at* $p \leq 0.05$.

N – Normoxia *N + SOD – Normoxia in the presence of SOD*

H – Hypoxia *H + SOD – Hypoxia in the presence of SOD*

*N + SOD versus N, **p < 0.001* *H +SOD versus H, ns*

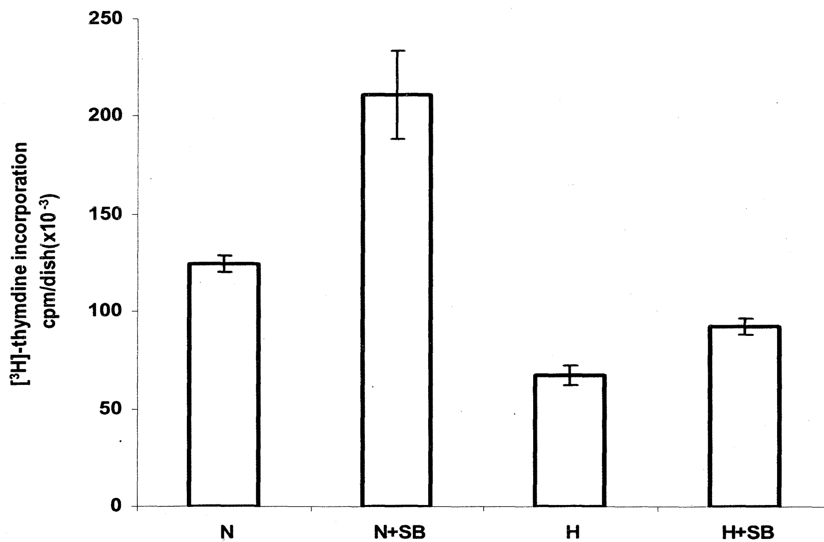
Figure 12. p42/44 MAPK inhibitor decreases DNA synthesis in normoxic but not hypoxic cardiac fibroblasts



Synchronized sub-confluent cultures of cardiac fibroblasts were pre-incubated with PD098059 (10μM) for one hour and exposed to hypoxia for 24 hours in the presence of PD098059 and [^{3}H]-thymidine (2μCi/ml) in M199 with 10% FBS. DNA synthesis was measured as described under 'Methods'. Values represent the Mean ± SD of 5 determinations. The effects of various treatments were evaluated by Student's t-test. $p \leq 0.05$ was considered significant.

H versus N	$p < 0.001$
N+PD versus N	$p < 0.001$
H+PD versus H	*p, ns*

Figure 13. p38 MAPK inhibitor increases DNA synthesis in normoxic and hypoxic cardiac fibroblasts



Synchronous sub-confluent cultures of cardiac fibroblasts were pre-incubated with SB203580 (10μM) for one hour and exposed to hypoxia for 24 hours in the presence of PD098059 and [^{3}H]-thymidine (2μCi/ml) in M199 with 10% FBS. DNA synthesis was measured as described under Methods. Values represent the Mean ± SD of 5 determinations. The effects of various treatments were evaluated by Student's t-test. $p \leq 0.05$ was considered significant.

H versus N, $p < 0.001$;   N+SB versus N, $p = 0.049$;   H+SB versus H, $p < 0.001$

IV.3.2.2. G_1/S transition in the cardiac fibroblast cell cycle is hypoxia-sensitive

The inhibition of proliferation by hypoxia prompted investigations on the hypoxia-sensitive checkpoint(s) in the cardiac fibroblast cell cycle. To identify the hypoxia-sensitive checkpoint, sub-confluent cultures were subjected to 48 hours of hypoxia in M199 with 10% FBS and the cell cycle phase distribution based on cell size and DNA content was analyzed by flow cytometry. The cell cycle profile showed that hypoxia resulted in marked accumulation of cells at the G_0/G_1 phase, with a concomitant decrease in the percentage of cells in the S phase, compared to the normoxic controls *(Table 4)*, suggesting that G_1/S transition is oxygen-sensitive. The percentage of cells in G_2/M phase was also decreased in hypoxic compared to normoxic cultures.

IV.3.2.3. Cyclin-dependent kinase inhibitor, p27, is up-regulated in hypoxic cardiac fibroblasts

In different cell types, hypoxic inhibition of cell cycle progression at G_1/S has been shown to involve the induction of the CDKI, p27, which is known to arrest cells at G_1/S by inhibiting the activation of CDK2 and Cyclin E/CDK2 complexes. To examine the possibility that the observed delay in G_1/S transition in hypoxic cardiac fibroblasts may be associated with alterations in the expression of p27, the levels of this protein in lysates prepared from sub-confluent cultures exposed to 24 hours of hypoxia in M199 with 10% FBS were analyzed by Western blots. The cells were not serum-starved prior to hypoxia in order to avoid cell cycle arrest due to serum deprivation and associated changes in the cell cycle regulatory protein profile. The blots with hypoxic and normoxic samples were probed for p27, and re-probed with antibody against β-actin, the loading control. As shown in *Figure 14*, exposure of cells to 24 hours of hypoxia was found to induce the expression of p27.

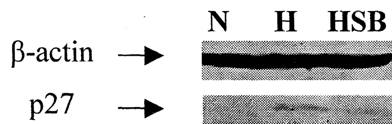
To evaluate the effect of p38 MAPK inhibition on the expression of p27 under hypoxic conditions, cells were exposed to hypoxia with/without SB203580 (10μM) and the levels of p27 were analyzed. *Figure 14* shows that pre-treatment with SB203580 for one hour followed by hypoxic incubation for 48 hours in the presence of the inhibitor attenuated p27 expression. The modest decrease in p27 expression correlates well with the modest increase in DNA synthesis in cells exposed to hypoxia in the presence of SB203580 *(Figure 13)*.

Table 4. G1-S checkpoint in cardiac fibroblast cell cycle is hypoxia-sensitive

	G_0/G_1	S	G_2/M
Normoxia (n=6)	29.89	32.83	37.27
Hypoxia (n=6)	58.96	18.92	22.12

Sub-confluent cultures of cardiac fibroblasts were exposed to 48 hours of hypoxia in M199 with 10% FBS. At the end of the incubation, the cells were trypsinized, washed in ice-cold PBS and fixed in 70% ice-cold ethanol. The fixed cells were stained with PI and used for flow cytometric analysis of cell cycle phase distribution, as described under 'Methods'. Typical values from a representative experiment (one of 6) are presented in the Table as percent distribution in each phase. ***Page 75a provides representative cell cycle histograms of normoxic and hypoxic cardiac fibroblasts.***

Figure 14. Hypoxia modulates Cyclin-dependent kinase inhibitor, p27, by p38 MAPK-mediated mechanisms

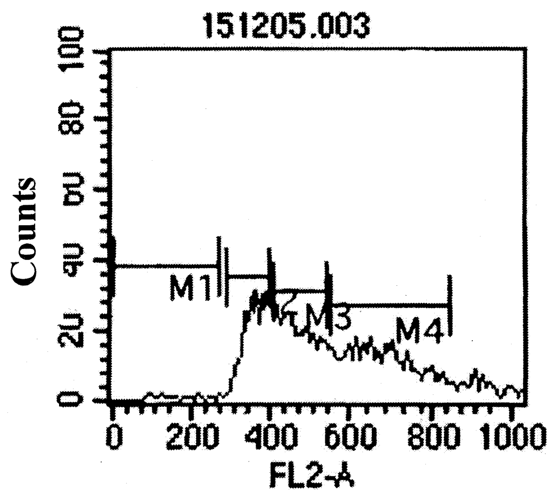


Sub-confluent cultures of cardiac fibroblasts were exposed to hypoxia for 24 hours. Western blot analysis of lysates of hypoxic and normoxic cells was performed using monoclonal anti-p27 antibody, with β-actin as the loading control. A representative picture showing the profile of p27 expression in hypoxic cardiac fibroblasts is shown.

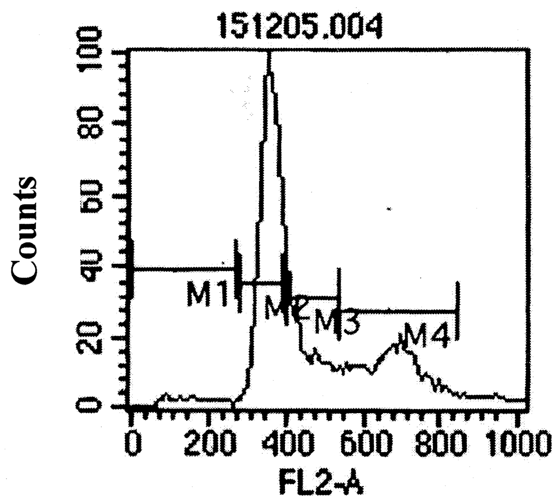
N – cells exposed to normoxia for 24h

H – cells exposed to hypoxia for 24h

HSB – cells exposed to hypoxia for 24h in the presence of the p38 MAPK inhibitor, SB203580 (10μM)



Normoxia



Hypoxia

	Phase Distribution (%)	
	Normoxia	**Hypoxia**
M1- Sub G0/G1	0.95	0.53
M2- G0/G1	29.6	58.6
M3- S	32.5	18.8
M4- G2/M	36.9	21.9

Representative cell cycle histograms of normoxic and hypoxic cardiac fibroblasts, recorded as described in Table 4 legend.

IV.3.2.4. Soluble factors produced by hypoxic cardiac fibroblasts inhibit cardiac fibroblast proliferation

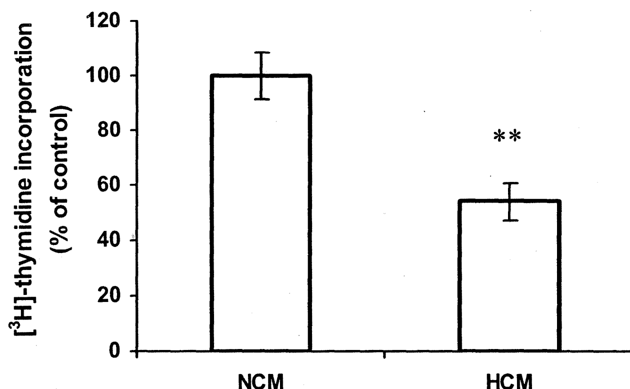
Cardiac fibroblasts are known to be an intra-cardiac source of several growth factors and cytokines that can affect various aspects of cell function, including proliferation. To examine the possibility that hypoxic cardiac fibroblasts may produce soluble factors that can affect DNA synthesis in normoxic cardiac fibroblasts, synchronized sub-confluent fibroblast cultures at passage 3 were incubated for 24 hours with hypoxic- and normoxic-conditioned media containing 2μCi/ml [^{3}H]-thymidine. Serum-free culture supernatants from confluent serum-deprived cardiac fibroblast cultures exposed to hypoxia for 24 hours and their matched normoxic controls were used as the hypoxic fibroblast-conditioned medium (HFCM) and normoxic fibroblast-conditioned medium (NFCM), respectively. HFCM was found to significantly reduce (46%) basal DNA synthesis in normoxic cardiac fibroblasts *(Figure 15)*, showing that soluble factors from hypoxic cardiac fibroblasts can affect DNA synthesis in normoxic cardiac fibroblasts.

IV.3.3. Hypoxia enhances the production of TNF-α and sICAM-1

The effect of HFCM on cardiac fibroblast proliferation *(Figure 15)* suggested that these cells produce factors whose levels are modified by hypoxia. In an attempt to identify these factors, the levels of TNF- α, IL-1β, IL-4, IL-6, and sICAM-1 in hypoxic and normoxic cardiac fibroblast-conditioned media were determined by ELISA. Culture supernatants from normoxic and hypoxic fibroblasts had detectable levels of all these factors, except IL-1β. IL-4 and IL-6 production was comparable in hypoxic and normoxic fibroblasts *(Figure 16)*. However, hypoxia significantly augmented the production of TNF- α *(Figure 17)* and sICAM-1 *(Figure 18)*. The levels of TNF- α in HFCM were

about 4.7 fold higher than in NFCM *(Figure 17)*. Hypoxia increased sICAM-1 production by about 60% over the control *(Figure 18)*.

Figure 15. Hypoxic fibroblast-derived factors decrease basal levels of DNA synthesis in normoxic cardiac fibroblasts

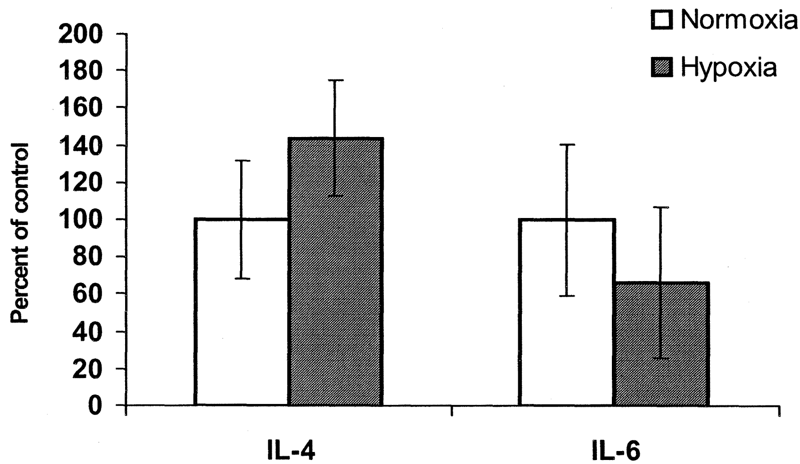


*Synchronized confluent cultures of cardiac fibroblasts were incubated in serum-free M199 for 24 hours under hypoxia and the cell culture supernatants were used for the conditioned medium experiments. Sub-confluent, synchronized fibroblast cultures were incubated for 24 hours with [^{3}H]-thymidine at a concentration of 2μCi/ml in conditioned media from hypoxic/normoxic cultures and DNA synthesis assessed as described under 'Methods'. Values represent the Mean ± SD of 5 determinations. Significance was evaluated by Student's t-test and $p \leq 0.05$ was considered significant. **$p < 0.001$*

NCM – cells exposed to conditioned medium from normoxic cardiac fibroblasts

HCM – cells exposed to conditioned medium from hypoxic cardiac fibroblasts

Figure 16. Production of IL-4 and IL-6 are unaltered under hypoxic conditions

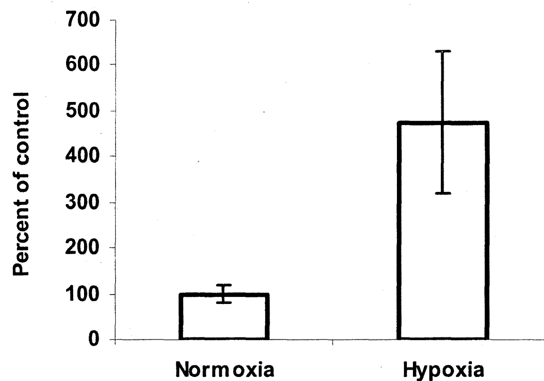


Synchronized confluent cultures of cardiac fibroblasts were incubated in serum-free M199 for 24 hours under hypoxia/normoxia and the cell culture supernatants were used for determining IL-4 and IL-6 levels. Values represent the Mean ± SD of 5 determinations. Significance was evaluated by Student's t-test and $p \leq 0.05$ was considered significant.

Hypoxia – culture supernatants from hypoxic cells

Normoxia – culture supernatants from normoxic cells

Figure 17. Hypoxia enhances production of TNF-α by cardiac fibroblasts

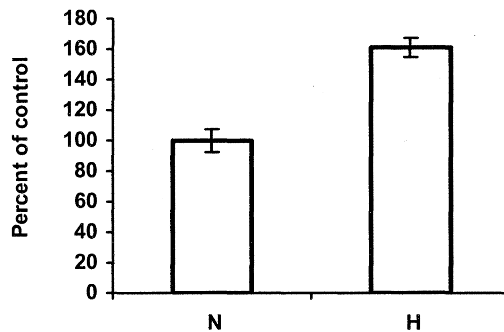


Synchronized confluent cultures of cardiac fibroblasts were incubated in serum-free M199 for 24 hours under hypoxia/normoxia and the cell culture supernatants were used for determining levels of TNF-α. Values represent the Mean ± SD of 5 determinations. Significance was evaluated by Student's t-test and $p \leq 0.05$ was considered significant. Hypoxia versus Normoxia, $p = 0.0017$

Hypoxia – culture supernatants from hypoxic cells

Normoxia – culture supernatants from normoxic cells

Figure 18. Hypoxia enhances sICAM-1 production in cardiac fibroblasts



Synchronized confluent cultures of cardiac fibroblasts were incubated in serum-free M199 for 24 hours under hypoxia/normoxia and sICAM-1 levels in the cell culture supernatants were determined. Values represent the Mean ± SD of 12 determinations. Significance was evaluated by Student's t-test and $p \leq 0.05$ was considered significant. H versus N, $p < 0.001$

H – culture supernatants from hypoxic cells

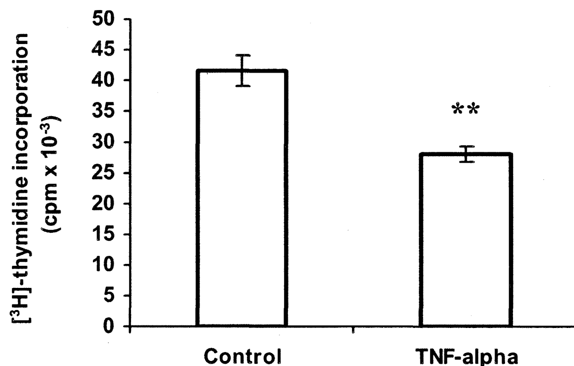
N – culture supernatants from normoxic cells

IV.3.3.1. TNF-α inhibits basal levels of DNA synthesis in cardiac fibroblasts

The observation that hypoxia markedly enhances TNF-α production raised the possibility that TNF-α may contribute to the observed effect of HFCM on cardiac fibroblast proliferation.

Synchronous sub-confluent cultures of cardiac fibroblasts were incubated with [^{3}H]-thymidine in NFCM spiked with recombinant TNF-α at concentrations detected in HFCM (200pg/ml) and DNA synthesis was analyzed as described under 'Methods'. TNF-α was found to reduce basal levels of DNA synthesis in normoxic cardiac fibroblasts by about 33% *(Figure 19)*, which is comparable to the extent of inhibition of DNA synthesis in cells treated with HFCM *per se*.

Figure 19. Effect of TNF-α (200pg/ml) on DNA synthesis in cardiac fibroblasts



*Synchronized sub-confluent cultures of cardiac fibroblasts were incubated for 24 hours in serum-free M199 containing TNF-α (200pg/ml) and [^{3}H]-thymidine (2μCi/ml). DNA synthesis was determined as described under 'Methods'. Values represent Mean ± SD of 5 determinations. Significance was evaluated by Student's t-test and $p \leq 0.05$ was considered significant. **$p < 0.001$*

IV.3.3.2. PKC and p42/44 MAPK pathways mediate the augmented production of sICAM-1 in hypoxic cardiac fibroblasts

sICAM-1 is produced by proteolytic cleavage and release of the soluble ectodomain of cell surface ICAM-1. The pro-inflammatory cell surface ICAM-1 is known to be expressed on cardiac fibroblasts and its proteolytic cleavage is mediated by several pathways including MAPK, PKC and ROS in other cell types. To evaluate the involvement of these molecular mediators in the hypoxia-induced augmentation of sICAM-1 production in cardiac fibroblasts, confluent cultures of cardiac fibroblasts were pre-incubated with PD098059 (10μM), the PKC inhibitor BIM (1μM) or the antioxidant NAC (5mM) for one hour followed by incubation under hypoxic/normoxic conditions in medium containing the inhibitor and 0.1% FBS. At the end of hypoxic incubation, sICAM-1 levels in culture supernatants were measured by ELISA. Both PD098059 and BIM reversed the stimulatory effect of hypoxia on sICAM-1 production *(Figure 20)*,

suggesting the involvement of p42/44 MAPK and PKC in the stimulation of sICAM-1 production in cardiac fibroblasts by hypoxia.

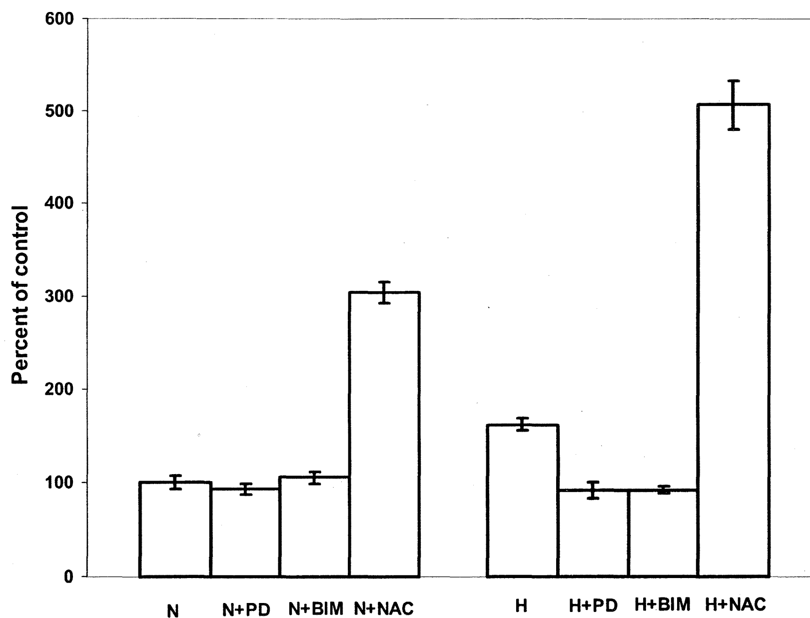
IV.3.3.3. NAC and PDTC augment sICAM-1 production in cardiac fibroblasts

Studies using pharmacologic inhibitors, designed to ascertain the role of different signaling pathways in the hypoxia-induced increase in sICAM-1, led to the serendipitous finding that the antioxidant NAC causes a 3-fold increase in basal and hypoxia-stimulated sICAM-1 production *(Figure 20)*. To ascertain that the effect of NAC is due to its antioxidant property, the effect of another antioxidant, PDTC, on sICAM-1 production was assessed. As shown in *Figure 21*, PDTC (50μM) augmented (65%) the production of sICAM-1 in normoxic cardiac fibroblasts. As sICAM-1 is reported to have an anti-inflammatory role, the findings are consistent with the reported anti-inflammatory effects of these clinically used antioxidants in inflammatory conditions [181].

IV.3.3.4. PKC and p42/44 MAPK pathways are not involved in the NAC-mediated increase in sICAM-1 production by cardiac fibroblasts

In order to delineate the pathways mediating the effect of NAC on cardiac fibroblast sICAM-1 production, cells were pre-treated for one hour with the inhibitors of p42/44 MAPK (PD098059 - 10μM), p38 MAPK (SB203580 - 10μM) and PKC (BIM - 1μM) pathways and then exposed to NAC (5mM) for 24 hours in medium containing the inhibitors and 0.1% FBS. Meager effects of the MAPK inhibitors notwithstanding, the results indicated that the stimulatory effect of NAC on sICAM-1 production involves mechanism(s) independent of p42/44 MAPK, p38 MAPK and PKC signaling cascades *(Figure 21)*.

Figure 20. Pathways mediating the effect of hypoxia on sICAM-1 production by cardiac fibroblasts

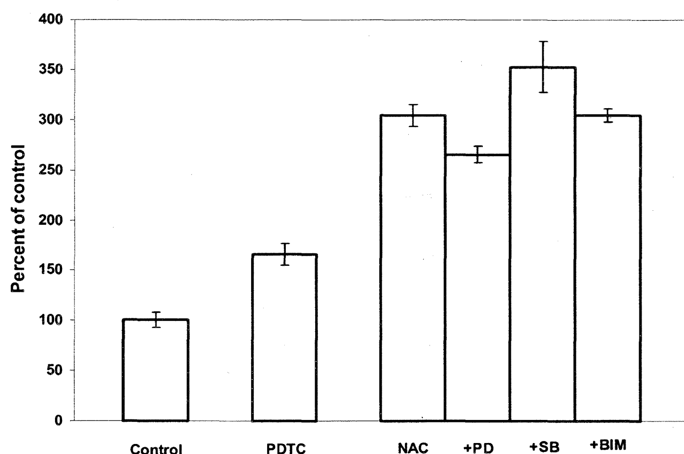


Confluent cultures of cardiac fibroblasts at passage 2-3 were serum-deprived for 24 hours and then exposed to hypoxia for another 24 hours in serum-free M199 with or without PD098059 (10μM), BIM (1μM) or NAC (5mM). sICAM-1 levels in cell culture supernatants were determined by ELISA. Data represent the Mean ± SD of 12 determinations. The effects of various treatments were evaluated by Student's t-test, and $p<0.05$ was considered significant.

H versus N, $p < 0.001$ *N+NAC versus N, $p < 0.001$*

H+PD versus H, $p < 0.001$ *H+BIM versus H, $p < 0.001$* *H+NAC versus H, $p < 0.001$*

Figure 21. Antioxidants NAC and PDTC augment sICAM-1 production by cardiac fibroblasts



Confluent cultures of cardiac fibroblasts were serum-deprived for 24 hours and then exposed to PDTC (50μM) or NAC (5mM) for 24 hours in M199 without FBS. Where indicated, the NAC treatment was carried out in the presence of PD098059 (10μM), BIM (1μM), or SB203580 (10μM). sICAM-1 levels in cell culture supernatants were determined by ELISA. Data represent the Mean ± SD of 12 determinations. The effects of various treatments were evaluated by Student's t-test, and p<0.05 was considered significant.

+ PD – Cells treated with NAC in the presence of PD098059

+ SB – Cells treated with NAC in the presence of SB203580

+ BIM – Cells treated with NAC in the presence of BIM

PDTC versus Control, p < 0.001; NAC versus Control, p < 0.001

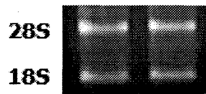
+PD versus NAC, p = 0.003; +SB versus NAC, p = 0.04

IV.3.3.5. Hypoxia and NAC do not up-regulate cell surface ICAM-1 transcription

To examine the possibility that the increase in sICAM-1 in response to hypoxia and NAC involves a transcriptional increase in cell surface ICAM-1 mRNA, confluent cultures of cardiac fibroblasts were exposed to hypoxia or NAC for 24 hours. Total RNA was isolated, the purity ascertained by 1% agarose gel electrophoresis *(Figure 22)* and cDNA synthesized as per the protocol described under Methods. The cDNAs were co-

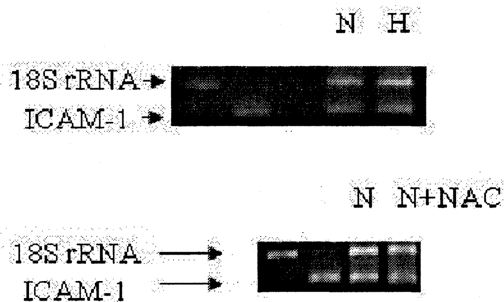
amplified with primers specific for rat ICAM-1 and 18S rRNA. As evident from *Figure 23*, neither hypoxia nor NAC was found to increase cell surface ICAM-1 transcript levels.

Figure 22. Agarose gel electrophoresis of RNA samples



RNA samples were subjected to agarose gel electrophoresis, as described under Methods. The presence of intact 28S and 18S bands was documented using Syngene Bio Imaging system.

Figure 23. RT-PCR analysis of ICAM-1 transcript levels in cells treated with hypoxia or antioxidant NAC

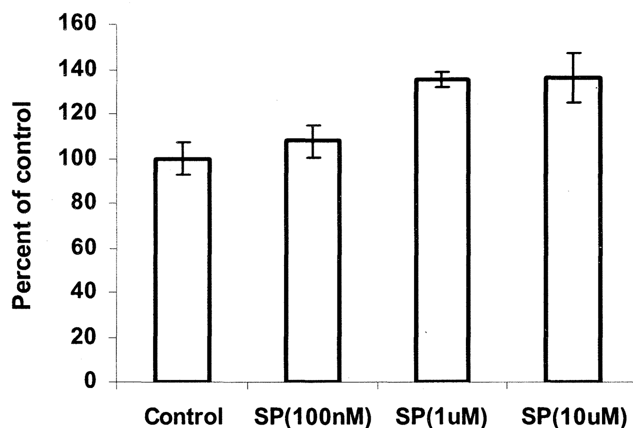


Confluent cultures of cardiac fibroblasts were serum-deprived for 24h and then exposed to hypoxia or treated with 5mM NAC for 24 hours. Total RNA was isolated and cDNA synthesized from 10 μg of RNA by reverse transcription at 37°C for 60 min using random primers. The cDNAs were co-amplified over 35 cycles with primers specific for rat ICAM- 1 and 18S rRNA as the internal control and the PCR products were analyzed by 1% agarose gel electrophoresis followed by densitometric scanning. Upon normalization to the respective 18S rRNA, no significant differences in ICAM-1 mRNA levels were found between control and hypoxia-treated cells in two experiments.

IV.3.3.6. SP increases sICAM-1 production by cardiac fibroblasts through p42/44 MAPK- and PKC-dependent mechanisms

The idea that cardiac fibroblasts are a source of modulators of inflammation, as borne out by this study and others [25], was further tested. SP, a proinflammatory neuropeptide released from the peptidergic innervations of ischemic myocardium, is reported from this laboratory to be mitogenic to adult rat cardiac fibroblasts [98]. Its role as a proinflammatory agent in several cell types [60, 81, 119] prompted experiments to evaluate its effects on sICAM-1 production by cardiac fibroblasts. Confluent cultures of cardiac fibroblasts at passage 3 were incubated with varying concentrations of SP (100nM to 10μM) for 24 hours in M199 supplemented with 0.1% FBS. sICAM-1 levels were determined in culture supernatants by ELISA. It was observed *(Figure 24)* that SP at ≥1μM concentration increases sICAM-1 production in cardiac fibroblasts. Using pathway-specific pharmacologic inhibitors as outlined in the preceding sections, it was found that the inhibition of p42/44 MAPK and PKC reverses the effect of SP on sICAM-1 production *(Figure 25)*. Further, the increase in sICAM-1 was not associated with an increase in ICAM-1 transcript levels *(Figure 26)*.

Figure 24. Effect of varying concentrations of SP on sICAM-1 production by cardiac fibroblasts



Confluent cultures of cardiac fibroblasts were serum-deprived for 24 h and then exposed to SP at the indicated concentrations for 24h. sICAM-1 levels in cell culture supernatants were determined by ELISA. Data, expressed as Mean ± SD of 5 determinations, were analyzed by Student's t-test, and $p \leq 0.05$ *was considered significant.*

SP (100nM) versus Control, ns

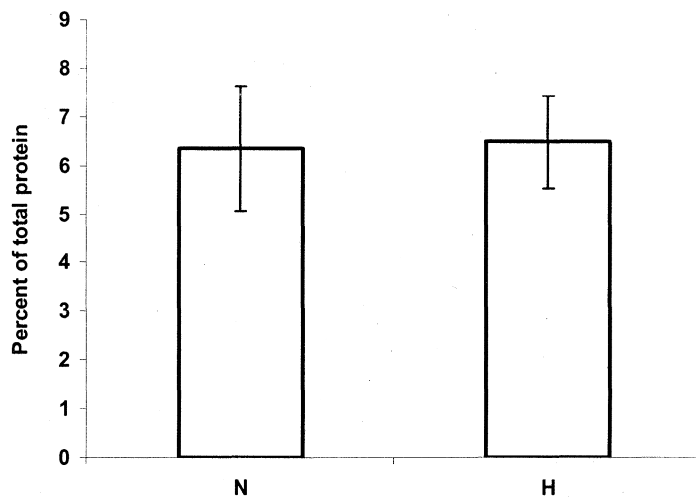
SP (1μM) versus Control, $p < 0.001$

SP (10μM) versus Control, $p < 0.001$

IV.3.4. Hypoxia does not alter collagen synthesis in cardiac fibroblasts

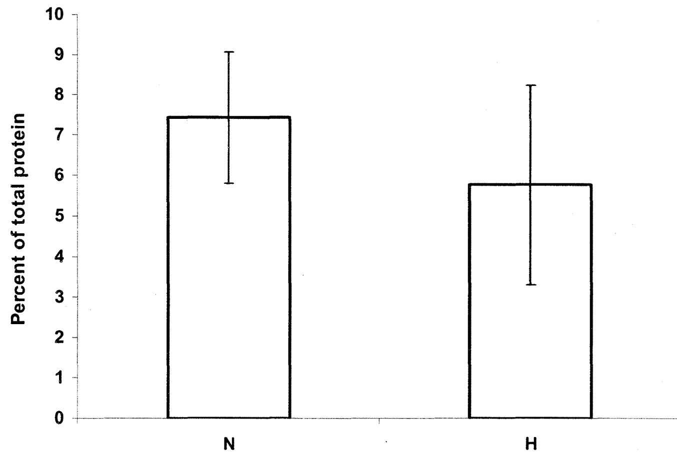
To examine the effects of hypoxia on collagen synthesis, confluent cultures of cardiac fibroblasts were incubated for 24 hours under hypoxic conditions in DMEM (without proline) supplemented with ascorbate and 1μCi/ml [^{3}H]-proline. Collagen synthesis, measured from [^{3}H]-proline incorporated into the collagenase-digestible fraction of TCA-insoluble protein, was found to be unaffected by hypoxia in cardiac fibroblasts from adult *(Figure 27)* and neonatal *(Figure 28)* rat hearts.

Figure 27. Effect of hypoxia on collagen synthesis by adult rat cardiac fibroblasts



Confluent cultures of adult rat cardiac fibroblasts were serum-deprived for 24h and incubated in DMEM containing [^{3}H]-proline at 1μCi/ml for 24h under hypoxic conditions. Collagen synthesis was measured as the collagenase-sensitive fraction of the total proline incorporated. Values represent the Mean ± SD of 5 determinations.

Figure 28. Effect of hypoxia on collagen synthesis by neonatal rat cardiac fibroblasts



Confluent cultures of neonatal rat cardiac fibroblasts were serum-deprived for 24h and incubated in DMEM containing [^{3}H]-proline at 1μCi/ml for 24h under hypoxic conditions. Collagen synthesis was measured as collagenase-sensitive fraction of the total proline incorporated. Values represent the Mean ± SD of 5 determinations and significance was analyzed by Student's t-test with $p \leq 0.05$ set as the level of significance. H versus N, ns.

V. DISCUSSION

Molecular oxygen is essential for normal development, growth and survival of most multicellular organisms, and for aerobic metabolism in mammalian cells. While atmospheric air contains 21% oxygen, the arterial pO2 is about 14%. Mammalian tissues under normoxic conditions exhibit oxygen concentrations of 3% to 9%, which is much lower than the arterial pO2. Heart cells receive an oxygen supply of <10% under conditions of systemic normoxia. The cells within a tissue are exposed to different concentrations of oxygen depending on the specific localization of the cells and the functional status of the tissue. Hence, "normoxia" for cells is an adjustable variable, set according to the oxygen levels available under normal conditions of growth [156]. Accordingly, cells perceive a decrease in oxygen as hypoxia when the oxygen concentration is below their normoxic set point and is insufficient for metabolic requirements. Cells have intrinsic mechanisms that enable them to sense and maintain oxygen homeostasis, the failure of which can lead to cellular dysfunction and/or irreversible cell damage [99, 192]. Oxygen sensing ensures the activation of several adaptive mechanisms that help the cells prevent or attenuate hypoxia-induced cell damage and dysfunction [21, 26, 83, 84].

Cellular responses to hypoxia vary depending on several factors that determine the sensitivity or resistance of cells to hypoxia. Of the multiple aspects of cell function known to be modified by hypoxia, hypoxic modulation of cell cycle progression has been studied extensively using cell lines [22, 62, 63, 71] and in pathologic states such as tumor hypoxia [72, 78, 157] and pulmonary hypertension [44, 45, 53, 197].

Cardiac fibroblasts play an important role in regulating myocardial function by maintaining ECM homeostasis and elaborating autocrine/paracrine effectors. Several pathogenic stimuli are known to modify cardiac fibroblast function [12, 25, 108, 199]. Given that a hyperplastic response is central to the role played by cardiac fibroblasts in

the diseased heart, and that hypoxia is intrinsic to several pathologic states of the heart, modulation of fibroblast proliferation by hypoxia warrants scrutiny.

This work was carried out to examine the modulation of three distinct aspects of cardiac fibroblast function by hypoxia and to delineate the underlying molecular mechanisms. The investigations focused primarily on the regulation of cell cycle progression in hypoxic cardiac fibroblasts. Additionally, the study examined the effects of hypoxia on collagen synthesis and the production of autocrine/paracrine effectors by these cells.

V.1. CHARACTERIZATION OF RAT CARDIAC FIBROBLASTS

The fibroblastic nature of cells isolated from rat ventricles was ascertained by morphology and immunocytochemical staining [4]. The adherent cells had spindle morphology and formed a monolayer at confluence *(Figures 1 & 2)*. Confluent cultures did not exhibit either 'cobblestone' or 'hill and valley' morphology, ruling out the presence of ECs and SMCs respectively, as confirmed by negative immunostaining for factor VIII-related antigen *(Figure 4)* and desmin (data not shown). The cells stained positive for the cytoskeletal protein, vimentin *(Figure 3)*.

V.2. CHARACTERIZATION OF *IN VITRO* HYPOXIA MODEL

Inherent difficulties associated with the evaluation of cardiac fibroblast responses to hypoxia *in vivo* are manifold, including confounding influences from co-resident cells and the diverse array of growth factors/cytokines in the heart, and the inability to induce a uniform hypoxic insult across the tissue. Hence, one of the specific objectives was to set up and characterize an *in vitro* system of hypoxia, to be used for the experiments.

The *in vitro* hypoxia model used for the study provided a hypoxic atmosphere with a pO_2 of <0.1% as against the normoxic pO_2 of ~20%. The pCO_2 in normoxic and hypoxic chambers was ~5%. Medium pH was comparable in both normoxic and hypoxic chambers and was about 7.5, ruling out a change in pH due to hypoxia. The medium pO_2 was found to be ~3% in the hypoxic chambers whereas that in the normoxic medium was about 15%. It is pertinent to note that although the atmospheric pO_2 is 20%, the arterial pO_2 is reported to be about 14% and, under conditions of normoxia, cardiac tissue pO_2 is <10%. In the heart, a 30 to 60% decrease in oxygen levels resulting in a pO_2 of ~ 1 to 3% is considered moderate hypoxia [156]. Hence the hypoxic conditions (medium pO_2 ~3%) used in the present study may be considered moderate.

V.2.1. Measurement of lactate production in cells exposed to hypoxia

A switch from aerobic to anaerobic metabolism is among the adaptive mechanisms initiated in cells under hypoxic conditions [84, 192, 187]. A toxic product of anaerobic metabolism is lactate, the intracellular accumulation of which may result in lactic acidosis, loss of proton gradient across the cell membrane, and consequent cell damage and death [69, 188]. Active transport of lactate ions by the proton-linked monocarboxylate transporter (MCT) to the extracellular space is reported to be the mechanism by which cells adapt to an increase in intracellular lactate [88, 193]. Hypoxia is reported to up-regulate the expression of MCT in many cell types [178]. Continued efflux of lactate and the resultant increase in extracellular lactate concentration may, however, inhibit the monocarboxylate transporter and cause intracellular lactate accumulation [143, 193].

In this study, intracellular and extracellular lactate levels were determined in cardiac fibroblast cultures exposed to 24 and 48 hours of hypoxia. At 24 hrs, intracellular

lactate was undetectable in normoxic and hypoxic cultures but extracellular lactate was detectable in both the groups and was found to be considerably enhanced in hypoxic cultures *(Table 2)*, suggesting increased lactate production by hypoxic fibroblasts and efflux to the extracellular milieu. Upon prolonged exposure to hypoxia (48 hours), extracellular lactate was approximately twice that observed at 24 hours and intracellular lactate was detectable, possibly due to reduced efflux of lactate resulting from increased extracellular lactate.

V.2.2. Analysis of cell viability by Syto 13/PI staining and flow cytometry

Hypoxia is known to cause damage and loss of viability in a variety of cell types including myocytes [111, 114, 173], ECs [118] and neurons [36, 97]. Early changes in membrane integrity and loss of viability in cells exposed to 24 hours of hypoxia were analyzed by Syto 13-PI staining. <1 % of the total cell population was stained with PI, suggesting no significant loss of viability *(Figure 6)*.

Cell death upon prolonged exposure to hypoxia (48 hours) was evaluated by flow cytometry where the damaged cells form the sub G_0/G_1 population characterized by cell size and DNA content less than that of G_0/G_1 cells. The percentage of cells in the sub G_0/G_1 phase was meager and comparable in fibroblast cultures exposed to 48 hours of hypoxic/normoxic incubation *(Table 3)*. The data suggest that despite the increase in intracellular lactate levels upon prolonged exposure to hypoxia (48 hours), cell viability remained unaffected. These findings are consistent with earlier reports which show that, compared to cardiac myocytes [111, 114] and fibroblasts of lung and skin origin [120], cardiac fibroblasts are resistant to death induced by hypoxia and several other pro-apoptotic stimuli.

V.3. MODULATION OF CARDIAC FIBROBLAST FUNCTIONS BY HYPOXIA

V.3.1. Hypoxia inhibits cardiac fibroblast proliferation

Hypoxia is known to act as a modifier of proliferative capacity in various cell types [44, 63, 168, 177]. Incubation of adult rat cardiac fibroblasts under hypoxic conditions for 24 hours was found to inhibit basal and serum-induced DNA synthesis *(Figures 7 & 8)* and the magnitude of inhibition was comparable in the presence and absence of serum. Consistent with the effect on DNA synthesis, cardiac fibroblasts exposed to hypoxia for 24 hours in M199 with 10%FBS showed only a 36% increase in cell number as against the 82% increase in cell number under normoxic conditions *(Figure 9)*. The corresponding population doubling times of hypoxic and normoxic fibroblasts were 54.1 hours and 27.7 hours, respectively. The data on DNA synthesis, cell number and population doubling time confirm that hypoxia inhibits cardiac fibroblast proliferation even in the presence of serum. In comparison with the anti-proliferative effect of hypoxia, serum-withdrawal was found to cause a greater inhibition of cell cycle progression, as the population doubling time increased to 192.5 hours after 24 hours of serum-withdrawal, with no increase in cell number *(Figure 9)*. Thus, while serum-deprivation induces absolute cell cycle arrest, hypoxic stress may only cause a delay and not a complete cessation of cell cycle progression.

The inhibition of cardiac fibroblast proliferation by hypoxia is reversed by re-oxygenation, as the cell number was found to increase by 78% and the corresponding population doubling time was found to be 28.9 hours *(Figure 9)* at 24 hours of re-oxygenation. In another set of experiments, cells were pulse-labeled with $[^3H]$-thymidine for 4 hours at the onset of re-oxygenation when the hypoxia effect would be expected to persist, and at 24 to 28 hours of re-oxygenation when prolonged re-oxygenation may be

expected to reverse the hypoxia effect. Consistent with the data on cell number and population doubling time *(Figure 9)*, rates of DNA synthesis were found to increase nearly two-fold (Figure 10). These data confirm that re-oxygenation restores rates of proliferation to normoxic levels.

Together, the data suggest that hypoxia causes a reversible inhibition of proliferation in cardiac fibroblasts, as in many cell types such as MEFs and fibroblast cell lines [62, 63, 71]. However, hypoxia is also known to enhance proliferation of ECs [160], pulmonary artery SMCs [197] and pulmonary adventitial fibroblasts [44, 45, 168].

V.3.2. Elucidation of the molecular basis of inhibition of cardiac fibroblast proliferation by hypoxia

V.3.2.1. Signaling pathways mediating the anti-proliferative effect of hypoxia

Hypoxia activates multiple signaling pathways [89, 164] including those involving ROS, MAPKs, SAPKs, cAMP-protein kinase (Protein Kinase A), Ca2+-calmodulin, PKC, mTOR and PI3K/Akt, many of which are known to regulate the cell cycle [65, 85, 168]. Hypoxic states are associated with the production of ROS, probably from the mitochondrial electron transport complexes [56]. ROS, including superoxide anion, are known to act as second messengers and have been implicated in mediating multiple aspects of cell function, including cell proliferation [32, 52, 105, 160]. Among hypoxia-activated pathways are the SAPK and MAPK family of enzymes that have a positive or negative influence on cell cycle progression, depending on the cell type and the stimulus [44, 127, 160, 162, 163, 201]. The SAPKs (p38 MAPK and JNK) are generally believed to be activated in response to stress conditions although recent reports suggest that these pathways may also be activated by mitogenic factors. In contrast to the SAPKs, the

MAPKs (p42/44 MAPKs) are strongly activated by mitogenic stimuli, and may only occasionally be activated under conditions of stress [42, 43, 102].

The results presented in *Figure 11* show that, whereas treatment with SOD decreased basal levels of DNA synthesis in normoxic cardiac fibroblasts, the antioxidant enzyme failed to reverse or alter the effect of hypoxia on DNA synthesis. The findings indicate a role for ROS in the proliferation of cardiac fibroblasts under normoxic conditions but not in the hypoxia-induced decrease in proliferation. It is noteworthy, in this regard, that mitochondrial ROS is known to mediate, at least in part, the hypoxia-induced proliferation of ECs [160].

Inhibition of the p42/44 MAPK pathway decreased proliferation (32%) in normoxic cardiac fibroblasts *(Figure 12)*, suggesting that p42/44 MAPK may serve as a positive regulator of cardiac fibroblast proliferation. The finding is consistent with the growth-promoting role of the p42/44 MAPK pathway reported in mitogen-activated cells [49, 95, 155]. Further, p42/44 MAPK is reported to mediate the hypoxia-induced proliferation of porcine aortic ECs [160]. Given the positive influence of p42/44 MAPK on fibroblast proliferation, inactivation of p42/44 MAPK may be a mechanism that decreases proliferation under hypoxic conditions. In fact, the refractoriness of hypoxic cells to p42/44 MAPK inhibition *(Figure 12)* supports the postulation that hypoxia may decrease p42/44 MAPK activity in cardiac fibroblasts leading to diminished proliferation.

In contrast to the effect of p42/44 MAPK inhibition, the inhibition of p38 MAPK *(Figure 13)* led to a marked increase (69%) in proliferation of normoxic cells, showing that p38 MAPK is a negative regulator of cardiac fibroblast proliferation. This finding is consistent with reports that suggest the involvement of p38 MAPK in mediating anti-proliferative effects [31, 49, 103, 148, 154]. Any overriding role of p38 MAPK in the hypoxic inhibition of proliferation would, however, have resulted in comparable levels of

DNA synthesis in normoxic and hypoxic cells treated with the inhibitor. On the contrary, the data show that the difference in DNA synthesis between normoxic and hypoxic cells is maintained regardless of the presence of the inhibitor. It appears therefore that p38 MAPK may not be responsible for the reduction in proliferation under hypoxic conditions. The meager increase in proliferation in hypoxic cells treated with the inhibitor may result from a general proliferative response elicited by p38 MAPK inhibition, as seen in normoxic cells.

The findings *(Figures 12 and 13)* indicate that whereas p38 MAPK may act as negative modulator of cardiac fibroblast proliferation, p42/44 MAPK activation may positively influence proliferation in these cells. Additional experiments examining the activation status of p38 MAPK and p42/44 MAPK in hypoxic fibroblasts are required to ascertain the role played by these pathways in mediating the fibroblast responses to hypoxia.

V.3.2.2. G_1/S checkpoint of cardiac fibroblast cell cycle is hypoxia-sensitive

Hypoxia is known to inhibit cell proliferation by delaying the progression of cells through specific cell cycle transition points referred to as hypoxia-sensitive checkpoints. To identify the hypoxia-sensitive checkpoint in the cardiac fibroblast cell cycle, sub-confluent cultures subjected to hypoxia were analyzed for cell cycle phase distribution by flow cytometry. In order to ensure that the cell cycle profile is modulated by hypoxia *per se* and not by serum-withdrawal, which is known to induce cell cycle arrest [145], the cells were not synchronized by serum-deprivation prior to exposure to hypoxia, and hypoxic incubation was carried out in M199 supplemented with 10% serum. Several reports suggest that during hypoxia, cells at the oxygen-dependent restriction point are stalled immediately from progressing through the cell cycle whereas the cells in other

phases complete the cycle and are arrested at the restriction point in the second cycle [8]. Hence, to avoid the effect of asynchronous distribution of cells across the cell cycle phases, and to ensure accumulation of cells at the hypoxia-sensitive checkpoint, hypoxic incubation was carried out for approximately two cycles (48 hours).

The cell cycle profile of hypoxic fibroblasts showed the accumulation of cells at G_0/G_1 with a concomitant decrease in the percentage of cells in S phase *(Table 4)*, indicating the activation of the G_1/S checkpoint. The finding is consistent with several studies which show that the transition at G_1/S is oxygen-sensitive in many cell types [7, 63, 68, 71].

Although hypoxia caused a marked increase in the percentage of cells in G_0/G_1, a significant fraction (18.92%) of the cells were at the S phase and G_2/M (22.12%), suggesting that hypoxia may cause a deceleration at the G_1/S transition rather than absolute cell cycle arrest. Such a possibility is supported by the data on DNA synthesis *(Figure 8)* and cell number *(Figure 9)*.

Cell cycle deceleration or arrest under hypoxic conditions is known to be an adaptive response that protects cells by minimizing cellular energy expenditure and preventing cell proliferation under the potentially deleterious conditions of oxygen deficit. The initiation and completion of DNA synthesis, and therefore the progression of cells through S phase, are energy-dependent and several reports show that mammalian cells with proliferative potential tend to accumulate in G_0/G_1, the stage preceding DNA synthesis. Additionally, cell cycle arrest at G_1/S may provide protection against DNA damage that may result if DNA synthesis were to occur under conditions such as hypoxia that are unfavorable for proliferation [7]. As molecular mechanisms mediating the cell cycle and apoptosis are linked [93, 113], DNA damage consequent upon cell cycle reentry and/or proliferation of hypoxic cells may trigger apoptosis mediated by certain

cell cycle effectors such as p53 and cyclin A/CDK2 [1, 97, 111, 158]. Interestingly, it has been reported that compared to ECs and lung fibroblasts in which hypoxia elicits a proliferative response [160, 172], cardiac fibroblasts are found to be more resistant to hypoxic cell damage [120]. Cycling cells, therefore, are found to be more vulnerable to the lethal effects of hypoxia.

The reversibility of the functional alterations elicited by hypoxia in various cell types is determined by the degree of resistance or tolerance of cells to hypoxia, with irreversible alterations often being associated with hypoxia-sensitive cells [84]. Apparently, a reversible inhibition of proliferation through delayed cell cycle progression at the G_1/S checkpoint may underlie the reported relative-resistance of cardiac fibroblasts to hypoxia-induced damage [111, 114, 120] , consistent with their role in the ischemic and post-infarct myocardium. It is noteworthy that IL-1β, a cytokine associated with many pathologic states of the heart, is reported to induce G_1/S arrest in cardiac fibroblasts [96].

V.3.2.3. Delayed cell cycle progression under hypoxic conditions may involve up-regulation of p27

An important molecular mechanism implicated in hypoxia-associated G_1 arrest is a decrease in CDK2 activity primarily mediated by p27, the CDKI belonging to the CIP/KIP family of proteins [61, 63, 67].

In the present study, western blot analysis of p27 in asynchronous sub-confluent cultures of cardiac fibroblasts exposed to 24 hours of hypoxia showed that hypoxia induces p27 expression in cardiac fibroblasts *(Figure 14)*. In normoxic fibroblasts, p27 expression was very low or undetectable. In this context, it is pertinent to note that the function of p27 is strongly linked to the progression of cells from G_0 to S and it is shown

to be a likely tumor suppressor gene [46, 180]. The forced expression of p27 is found to result in G_1 arrest in most cell types [174], while ablation of its synthesis delays withdrawal from the cell cycle. Further, p27 is reported to be constitutively expressed in post-mitotic cardiomyocytes [5], and down-regulation of p27 activity in neonatal cardiomyocytes is associated with increase in proliferation [171]. Growth arrest by contact inhibition and serum withdrawal is reported to be mediated at least in part by p27 [150, 151]. The data presented in this study *(Figure 14)* are consistent with previous reports on induction of p27 in other cell types under hypoxic conditions. Studies by *Gardner et al (2001)* [63] suggest that p27 may be a major regulator of cell cycle progression in moderately hypoxic normal MEFs, as p27 null cells failed to arrest under these conditions and hypoxia-induced G_1 arrest in normal cells was overcome by decreasing p27 activity by antisense approaches. Additional evidence of the involvement of p27 is provided by *Goda et al (2003)* [67] who have demonstrated HIF-dependent increase in the expression of p27 during hypoxia-induced cell cycle arrest in MEFs and splenic B-lymphocytes. However, there are also conflicting data on the role played by this cell cycle regulatory protein in the induction of G_1/S arrest by hypoxia. For example, reports by *Green et al (2001)* [71] suggest that p27, expressed in MEFs in response to hypoxia, is required for cell cycle re-entry rather than cell cycle arrest.

Interestingly, inhibition of the p38 MAPK pathway was found to attenuate p27 expression in cardiac fibroblasts, suggesting that it is a regulator of p27 expression in these cells. Further, the decrease in p27 expression in hypoxic cells upon p38 MAPK inhibition was associated with a partial reversal of the decrease in proliferation *(Figure 13)*, pointing to the link between p27 expression and cell proliferation. Thus, the results presented in this study *(Figures 13 & 14)* are consistent with the involvement of p27 in mediating hypoxia-induced cell cycle deceleration in cardiac fibroblasts. Confirmatory

evidence would require experiments involving abrogation of this cell cycle regulatory protein using anti-sense approaches.

V.3.3. Soluble factors produced by hypoxic cardiac fibroblasts inhibit proliferation of cardiac fibroblasts

An emerging concept in relation to cardiac fibroblast function is that these cells are an important source of several factors with autocrine/paracrine effects on cell growth and function. For example, these cells are reported to be a source of a number of factors including VEGF, bFGF, PDGF and TGF-β that are known to modulate endothelial cell proliferation [199]. Myofibroblasts are also known to express VEGF, a factor known to promote angiogenesis [35]. Further, peptide factors produced by fibroblasts are reported to induce cardiomyocyte hypertrophy [76, 109]. The production of growth factors and cytokines by cardiac fibroblasts is regulated by a myriad of pathophysiologic stimuli.

The present study examined the possibility that hypoxia may induce or modulate the production of soluble factors by cardiac fibroblasts that may affect proliferation by autocrine mechanisms and found that conditioned medium from hypoxic fibroblasts decreases basal levels of DNA synthesis in cardiac fibroblasts under normoxic conditions *(Figure 15)*. The finding suggests that in addition to its direct effects on cardiac fibroblast cell cycle progression, oxygen deprivation may trigger the release of soluble factors from these cells that can contribute to, and amplify, the direct effects of hypoxia.

The effects of HFCM on fibroblast proliferation prompted studies on the production of TNF-α, IL-1β, IL-4, IL-6 and sICAM-1 that are associated with cardiovascular pathologies and are important modulators of inflammation [47, 75, 117, 136, 137, 140]. Supernatants from normoxic and hypoxic fibroblast cultures had detectable levels of IL-4, IL-6, TNF-α and sICAM-1. Hypoxia caused a dramatic increase

in TNF-α and sICAM-1 levels *(Figures 16, 17 & 18)*. Though *Long et al (1993)* [110] have reported that hypoxia causes an increase in the transcript levels of IL-1β in rat cardiac fibroblasts, IL-1β protein was undetectable in supernatants from normoxic and hypoxic cultures.

The finding that cardiac fibroblasts are a source of TNF-α is particularly significant because this cytokine has been implicated in the pathogenesis of several cardiovascular diseases, including myocardial infarction, chronic heart failure, atherosclerosis, and viral myocarditis [17, 59, 100, 104, 121]. The striking increase (~5-fold) in the production of TNF-α under hypoxic conditions *(Figure 17)* led to experiments aimed at examining the possibility that this cytokine, at concentrations detected in culture supernatants of hypoxic fibroblasts, may affect cardiac fibroblast proliferation. Cells exposed for 24 hours to NFCM spiked with 200pg/ml recombinant TNF-α showed a decrease in DNA synthesis, compared to cells exposed to NFCM alone. The extent of inhibition by TNF-α was about 32% as against the 46% decrease caused by HFCM, suggesting that TNF-α may contribute, at least in part, to the observed effects of HFCM on fibroblast proliferation. TNF-α, possibly in combination with other fibroblast-derived factors, may therefore modulate the proliferative response of cardiac fibroblasts under hypoxic conditions.

V.3.4. Hypoxia augments sICAM-1 production in cardiac fibroblasts

In addition to TNF-α, cardiac fibroblast culture supernatants were found to have detectable basal levels of sICAM-1 *(Figure 18)*. sICAM-1 is the soluble form of ICAM-1 comprising the extracellular domains of the membrane-bound ICAM-1 and is produced by diverse cell types including ECs, carcinoma cells, keratinocytes, and astrocytes [175]. Several pro-inflammatory factors like TNF-α, IL-1β and LPS are reported to induce

proteolytic cleavage and release of sICAM-1 from cells *in vitro* [64, 80]. Although a pro-inflammatory role has been attributed to sICAM-1 via the production of pro-inflammatory mediators such as TNF-α, IFN-γ, IL-6 and macrophage inflammatory protein-2 [142], it is known to exert a predominantly anti-inflammatory effect by competitively inhibiting the interactions between leukocytes and cell surface ICAM-1 [175, 64]. In fact, it has been shown that signaling inhibitors that block ICAM-1 shedding augment pro-inflammatory events [175]. In contrast, cell surface ICAM-1 is known to augment the inflammatory process by directing leukocyte adhesion and trafficking to sites of inflammation [176]. It is noteworthy that increased circulating levels of sICAM-1 have been reported in cardiovascular pathologies including atherosclerosis, angina, acute myocardial infarction and coronary and peripheral artery disease in which hypoxia is a major factor influencing the extent of injury and inflammation [18, 86, 130, 139].

Cardiac fibroblasts have been shown to express cell surface ICAM-1 constitutively, and in response to pro-inflammatory factors such as TNF-α and IL-1β [90]. *Kacimi et al (1998)* [90] have reported that hypoxia does not affect cell surface ICAM-1 expression in cardiac fibroblasts although hypoxia is found to modulate ICAM-1 expression in diverse cell types [202]. The present study *(Figure 18)* provides evidence that cardiac fibroblasts are a source of sICAM-1 and that hypoxia enhances its release from these cells, which may represent a mechanism by which fibroblasts can regulate the complex inflammatory process in hypoxic states of the myocardium.

V.3.4.1. Molecular mechanisms mediating the effect of hypoxia on sICAM-1 production

Pharmacologic inhibition of p42/44 MAPK and PKC did not alter basal levels of sICAM-1 production but attenuated the stimulatory effect of hypoxia, suggesting a role for these pathways in augmented sICAM-1 release from cardiac fibroblasts under hypoxic conditions *(Figure 20)*. This is consistent with reports that suggest the involvement of MAPK and PKC in the regulation of adhesion molecule expression in response to pro-inflammatory stimuli [175].

RT-PCR profile of ICAM-1 transcript levels *(Figure 23)* revealed that the increase in sICAM-1 production in response to hypoxia is not associated with transcriptional up-regulation of cell surface ICAM-1. The finding is consistent with the report from *Kacimi et al (1998)* [90] which shows that ICAM-1 mRNA levels are unaffected in neonatal rat cardiac fibroblasts over 6-12 hours of hypoxic incubation [90]. Together, the data suggest that hypoxia may post-transcriptionally increase sICAM-1 production by mechanisms involving p42/44 MAPK and PKC.

In addition to the MAPK and PKC pathways, the role of reactive oxygen intermediates in mediating the hypoxic-stimulation of sICAM-1 release was ascertained using the ROS scavenger, NAC. Contrary to expectation, NAC caused a 3-fold increase in both basal and hypoxia-stimulated sICAM-1 levels *(Figure 20)*, a response elicited possibly by its antioxidant property since another antioxidant, PDTC, also enhanced the production of sICAM-1, although to a lesser extent (65%). Experiments involving pharmacologic inhibition of p42/44 MAPK, p38 MAPK and PKC suggested that the stimulatory effect of NAC on sICAM-1 generation was by mechanisms largely independent of these signaling cascades *(Figure 21)*. Further, RT-PCR analysis of ICAM-1 mRNA *(Figure 23)* suggested that NAC induces sICAM-1 release in cardiac fibroblasts by post-transcriptional mechanisms.

NAC and PDTC are clinically important antioxidants used in many disease states [153]. Although two earlier reports indicate that these antioxidants may exert a pro-inflammatory effect by inducing ICAM-1 expression in human umbilical vein ECs [134, 181], NAC and PDTC are generally believed to limit the extent of inflammation and there is growing interest in the mechanisms of their action. Several reports suggest that NAC down-regulates the inflammatory cascade by decreasing membrane ICAM-1 expression [153]. NAC is also known to be an inhibitor of NFκB, a transcription factor involved in the regulation of ICAM-1 expression in cells [90, 139, 175]. The findings presented in this study show that NAC and PDTC may also promote cleavage of ICAM-1, which in turn may lead to a reduction in the levels of cell-surface ICAM-1 and an increase in the concentration of sICAM-1 and consequent competitive inhibition of cell surface ICAM-1. Together, these may represent an additional mechanism by which these antioxidants exert their anti-inflammatory effects.

The findings that hypoxia and NAC modulate sICAM-1 production *(Figure 20)* are in accord with the reported link between redox status and adhesion molecule expression [139] and suggests the involvement of redox balance in sICAM-1 production as well. The observation that hypoxia and antioxidants may regulate the expression of positive and negative immunomodulatory factors in cardiac fibroblasts prompted experiments to ascertain whether SP, another modulator of inflammation, would alter sICAM-1 release from these cells.

V.3.5. SP increases sICAM-1 production by cardiac fibroblasts through p42/44 MAPK- and PKC-dependent mechanisms

SP is a neuropeptide released from capsaicin-sensitive peptidergic innervations within tissues in response to stress and nociceptive stimuli. It is a potent peripheral and

coronary vasodilator [196] and its role in inflammation has been established in several inflammatory conditions, including acute intestinal inflammation, rheumatoid arthritis and pancreatitis [60, 81, 119, 146]. SP is also involved in tissue repair and fibrosis [92] and may enhance inflammatory response by vasodilatation, activation of inflammatory cells and by paracrine mechanisms involving the expression of cytokines and other pro-inflammatory mediators, including cell adhesion molecules [112, 135]. In the myocardium, SP is released from peptidergic innervations [189] under pathologic conditions such as hypoxia and ischemia [126, 179]. However, the possibility that SP may modulate metabolic events or inflammation in the heart under normal and/or pathologic conditions remains largely unclear.

It was reported earlier from this laboratory that SP is mitogenic to cardiac fibroblasts [98]. The finding presented in this study *(Figure 24)* furnishes evidence for another mechanism by which SP may modulate cardiac fibroblast function. SP ($\geq$ 1μM) was found to enhance sICAM-1 production in cardiac fibroblasts *(Figure 24)*, a response mediated by p42/44 MAPK- and PKC-dependent mechanisms as suggested by experiments using pharmacologic inhibitors *(Figure 25)*. SP is reported to enhance the expression of cell surface ICAM-1 in human umbilical vein ECs [135] and dermal microvascular ECs [152], which in turn may exert a pro-inflammatory effect. However, the increase in sICAM-1 release induced by SP in cardiac fibroblasts may indicate that although this peptide is believed to elicit pro-inflammatory responses in different cell types, it may also help balance the inflammatory reaction by enhancing the production of the anti-inflammatory sICAM-1.

As with hypoxia and NAC, the increase in sICAM-1 release induced by SP was not associated with an increase in ICAM-1 transcript levels *(Figure 26)*, suggesting post-transcriptional mechanisms of induction. Further experiments are required to clarify the

precise step(s) at which the post-transcriptional increase in sICAM-1 occurs in response to hypoxia, NAC and SP. It would be interesting to examine the functional significance of enhanced sICAM-1 production by hypoxic cardiac fibroblasts.

Being the most abundant cell type in the heart, even modest increments in the production of modulators of inflammation by cardiac fibroblasts in response to factors like hypoxia and altered redox status that prevail in a setting of ischemia and reperfusion may influence local inflammatory responses within the myocardium. Together, the findings suggest that cardiac fibroblasts may play a role in inflammatory myocardial remodeling in a setting of hypoxic injury.

V.3.6. Hypoxia does not affect collagen synthesis by cardiac fibroblasts

Fibroblasts are the only source of fibrillar collagens in the heart and several factors prevailing in the diseased myocardium are known to modulate collagen turnover in these cells [25]. The present study shows that collagen synthesis in adult rat cardiac fibroblasts is unaffected by hypoxia *per se (Figure 27)*. Collagen synthesis in neonatal rat cardiac fibroblasts *(Figure 28)* is also unaltered under hypoxic conditions suggesting no developmental-stage specific differences in the response. The findings are in disagreement with the report from *Agocha et al (1997)* [3] suggesting that hypoxia increases collagen synthesis in human cardiac fibroblasts. The discrepancy may be due to species difference. Moreover, the study used human ventricular tissue obtained from a recipient of heart transplant who had developed cardiomyopathy secondary to viral myocarditis. Although unaffected segments of the septum were used for obtaining the cells, the pathophysiologic status of the tissue may also explain the discordance.

V.4. SIGNIFICANCE OF THE FINDINGS

1) Hypoxia retards G_1/S transition in cardiac fibroblasts

Hypoxia-tolerant cells are reported to initiate several adaptive mechanisms to survive the hypoxic stress. One of the responses associated with hypoxia adaptation is the inhibition or deceleration of cell cycle progression [51, 62, 67]. As stated earlier, cycling cells are reported to be more vulnerable to the lethal effect of hypoxia, especially in the S phase [7, 8, 58]. In the present study, hypoxia was found to retard G_1/S transition in cardiac fibroblasts and there was no loss of viability in cardiac fibroblasts exposed to hypoxia. Together, these data suggest that induction of reversible cell cycle deceleration in cardiac fibroblasts by hypoxia may represent a mechanism integral to cell survival under the potentially deleterious conditions of hypoxia.

2) Cardiac fibroblasts are a source of immunomodulatory factors whose expression may be regulated by hypoxia and redox status

Consistent with the emerging concept that cardiac fibroblasts are an intra-cardiac source of modulators of inflammation, the present study shows that these cells elaborate factors such as IL-4, IL-6, TNF-α, and sICAM-1. Further, modulation of the expression of these factors by hypoxia and antioxidants suggests that cardiac fibroblasts may modify local inflammatory responses within the myocardium in pathophysiologic states marked by the prevalence of hypoxia and alterations in redox status.

3) Soluble factors from hypoxic cardiac fibroblasts may exert autocrine and paracrine effects within the heart

The attenuation of cardiac fibroblast proliferation by HFCM is consistent with the postulation that soluble factors such as TNF-α produced by these cells under ischemic conditions may exert autocrine/paracrine effects on co-resident cells. In fact, investigations carried out by *Shivakumar et al* in pursuance of this finding show that hypoxic cardiac fibroblast-derived factors exert paracrine effects on cardiomyocytes and compromise their viability (unpublished data).

Together, the findings presented in this study provide insights into how hypoxia may modulate distinct aspects of cardiac fibroblast function that are central to the role played by these cells in the remodeling and reparation of the diseased myocardium.

V.5. LIMITATIONS OF THE STUDY

Although several lines of evidence support a role for p27 in G_1/S arrest, there are reports suggesting that it is the coordinated regulation of the activity of several cell cycle proteins that determines the cell cycle profile. In that sense, inability to evaluate the role of various signaling pathways and cell cycle proteins like RB, cyclin E, cyclin D, cyclin A, and p21 in the hypoxia-induced delay in G_1/S transition is a limitation of this study. Attempts to determine the levels of cyclins and the phosphorylation status of RB were not successful on account of several problems commonly encountered in Western blot analysis, including low concentration of proteins from sub-confluent cultures, low protein transfer and binding, non-specific staining and high background, and inconsistency in the protein profile between samples of the same group.

Nevertheless, subsequent work in the laboratory, undertaken as a sequel to this study, indicates that the expression profile of cyclin A, cyclin E and cyclin D in hypoxic cardiac fibroblasts is consistent with the activation of the G_1/S restriction point. Currently, efforts are underway to examine p42/44 MAPK and p38 MAPK activities under hypoxia.

VI. SUMMARY AND CONCLUSIONS

VI.1. SUMMARY

The findings of the present study can be summarized as follows:

- Hypoxia decreases basal and serum-induced proliferation of adult rat cardiac fibroblasts
- The hypoxic inhibition of proliferation is reversible, as re-oxygenation restores population doubling time and rate of DNA synthesis
- Exposure of cardiac fibroblasts to hypoxia retards G_1/S transition, thereby delaying their entry into the S phase
- Hypoxia-induced deceleration of cell cycle progression is mediated, at least in part, by the CDKI, p27
- p27 expression in cardiac fibroblasts is regulated by p38 MAPK
- p38 MAPK may act as negative modulator and p42/44 MAPK as positive modulator of cardiac fibroblast proliferation; preliminary data support a role for the latter but not the former in the hypoxic inhibition of cardiac fibroblast proliferation
- The decrease in proliferation in hypoxic cells may not be mediated by superoxide anion, as SOD failed to reverse the hypoxia effect
- Hypoxia triggers release of factors from cardiac fibroblasts that can reduce fibroblast proliferation under normoxic conditions
- Hypoxia, SP and the clinically used antioxidants, NAC and PDTC, enhance sICAM-1 production in cardiac fibroblasts
- Whereas hypoxia and SP increase sICAM-1 production in cardiac fibroblasts by p42/44 MAPK- and PKC-dependent mechanisms, the NAC effect is independent of these pathways

- Hypoxia *per se* has no effect on collagen synthesis in neonatal and adult rat cardiac fibroblasts

VI.2. CONCLUSIONS

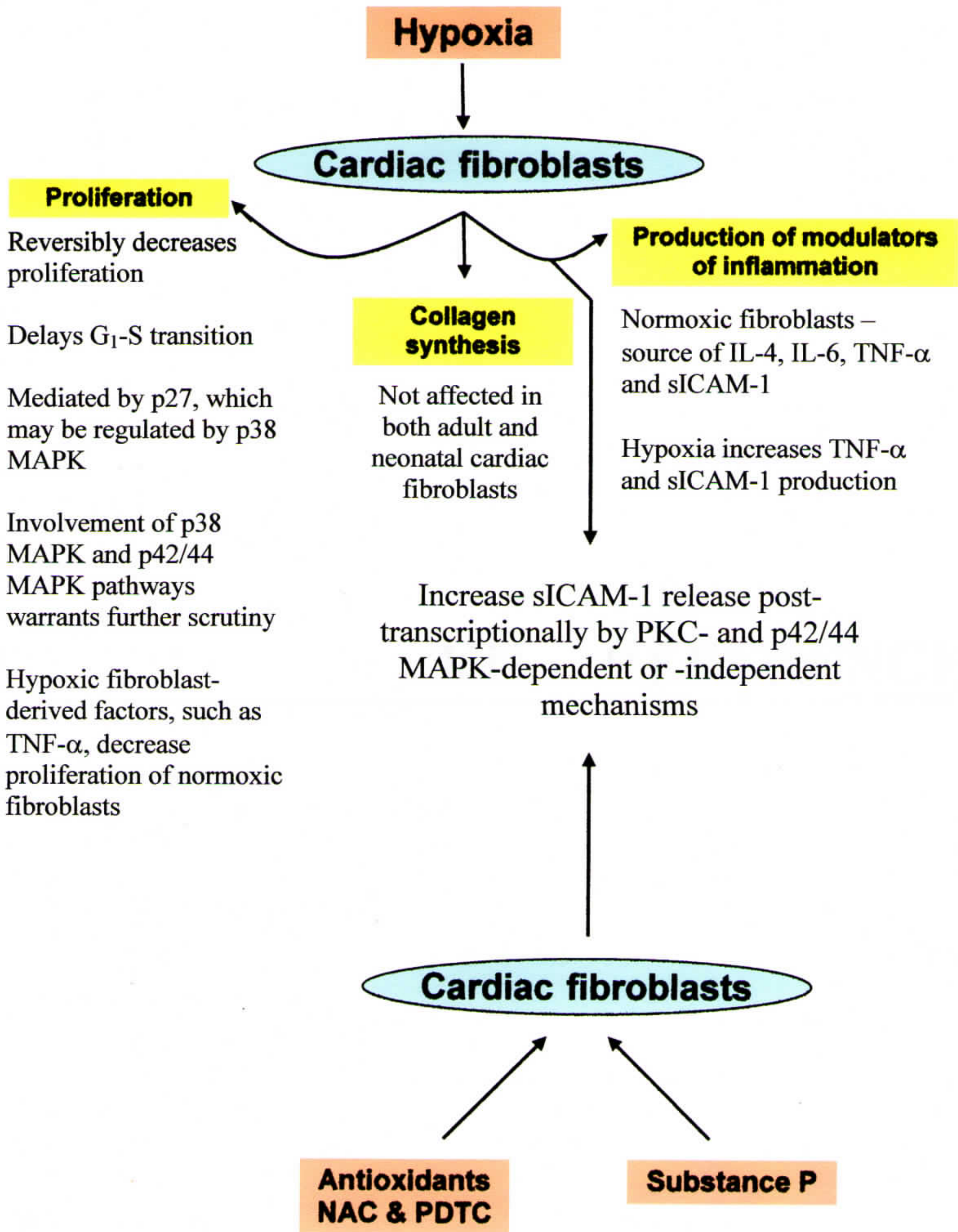
This study suggests that hypoxia may modulate multiple aspects of cardiac fibroblast function. The functional significance of the findings may be manifold because fibroblasts are the most abundant cell type in the heart that play an important role in inflammatory and fibrotic remodeling of the diseased myocardium.

VI.3. FUTURE DIRECTIONS

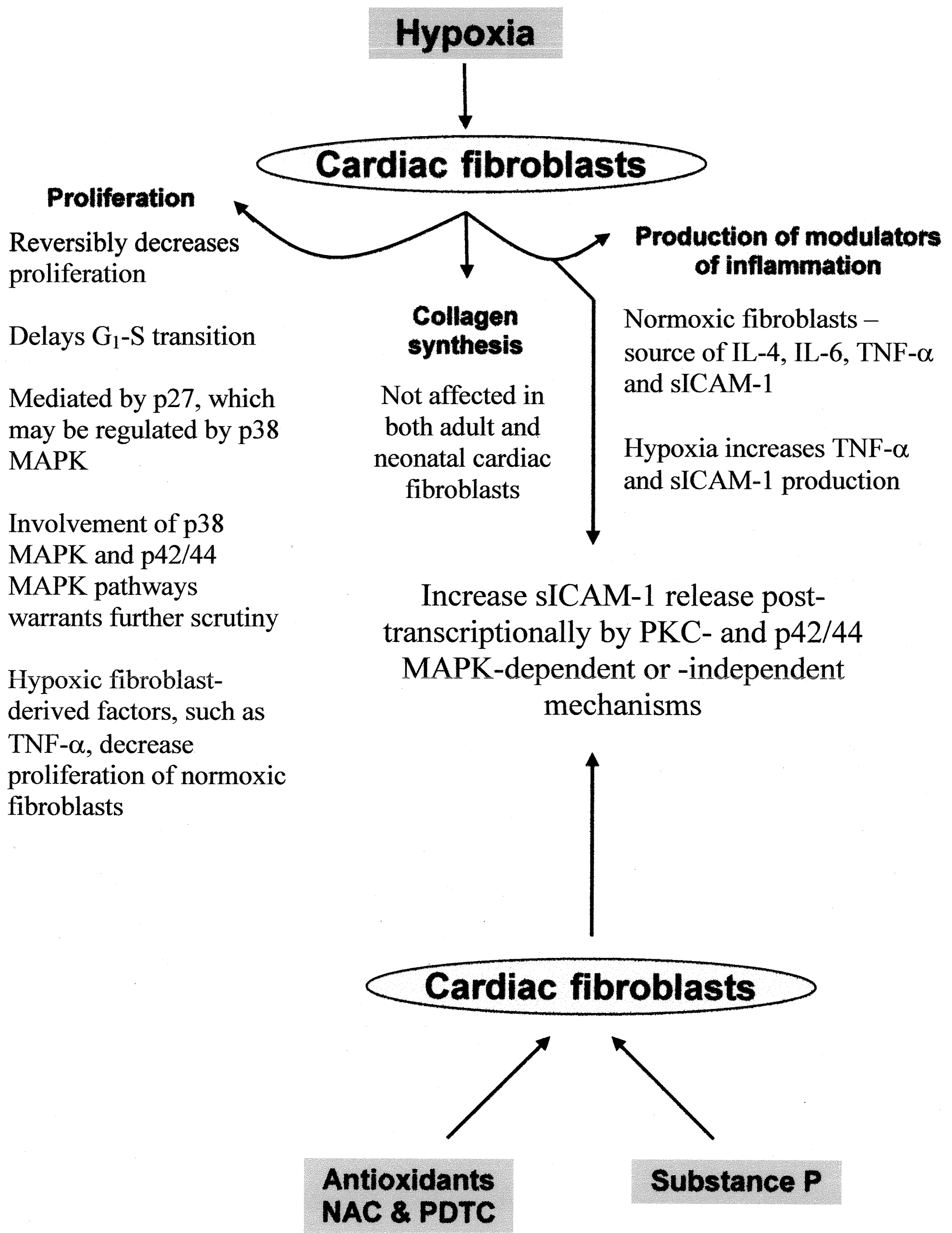
1] To clarify the molecular mechanisms underlying hypoxia-induced inhibition of cardiac fibroblast proliferation, focusing on signaling pathways and molecular events related to G_1/S transition

2] To examine the possibility that cell cycle progression and cell survival are coordinately regulated in cardiac fibroblasts under hypoxia

Cell cycle arrest in response to stress is associated with two functional endpoints i) differentiation as in post-mitotic cells, and ii) survival as in pre-mitotic or undifferentiated cells. On the basis of this study, it can be postulated that cardiac fibroblasts are driven to a survival mode under hypoxia by delaying cell cycle progression at the G_1/S checkpoint. To ascertain if cell cycle progression and cell survival are coordinately regulated in these cells under hypoxia, it would be important to examine i) whether selective abrogation of cell cycle regulatory proteins leads to reversal of cell cycle deceleration and cell death, and ii) whether cell survival-related proteins such as Bcl-2 contribute to cell cycle deceleration in hypoxic cardiac fibroblasts.



Hypoxia
Cardiac fibroblasts
Proliferation
Reversibly decreases proliferation
Delays G1-S transition
Mediated by p27, which may be regulated by p38 MAPK
Involvement of p38 MAPK and p42/44 MAPK pathways warrants further scrutiny
Hypoxic fibroblast-derived factors, such as TNF-α, decrease proliferation of normoxic fibroblasts
Collagen synthesis
Not affected in both adult and neonatal cardiac fibroblasts
Production of modulators of inflammation
Normoxic fibroblasts – source of IL-4, IL-6, TNF-α and sICAM-1
Hypoxia increases TNF-α and sICAM-1 production
Increase sICAM-1 release post-transcriptionally by PKC- and p42/44 MAPK-dependent or -independent mechanisms
Cardiac fibroblasts
Antioxidants NAC & PDTC
Substance P



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VIII. LIST OF PUBLICATIONS

Articles published:

1. *S. Sapna, S. K. Ranjith and K. Shivakumar.* Cardiac fibrogenesis in magnesium deficiency: a role for circulating angiotensin II and aldosterone. American Journal of Physiology Heart & Circulatory Physiology **291**:436-440, 2006.
2. *S Sapna and K Shivakumar.* Substance P enhances sICAM-1 release from adult rat cardiac fibroblasts by a p42/44 MAPK- and PKC-mediated mechanism. Cell Biology International **31**: 856-859, 2007.
3. *S Sapna and K Shivakumar.* Hypoxia and antioxidants enhance soluble ICAM-1 release from cardiac fibroblasts. Molecular and Cellular Biochemistry (2007, article in press)

Manuscripts communicated/to be submitted:

1. Paracrine effects of hypoxic fibroblast-derived factors on the MPT-ROS threshold and viability of adult rat cardiac myocytes (Co-author) - Communicated to Journal of Molecular and Cellular Cardiology.
2. Hypoxia retards cardiac fibroblast cell cycle progression at G_1/S by a p27-mediated mechanism - To be communicated to Journal of Molecular and Cellular Cardiology