# The Expression, Regulation and Effects of Inducible Nitric Oxide Synthase in Hibernating Myocardium

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# THESIS SUMMARY

Hibernating myocardium involves the downregulation of regional ventricular contraction, leading to cardiac failure, in response to severe coronary stenoses with recovery of function following revascularisation. This results in improved survival. Experiments described in this thesis address the potential role of inducible nitric oxide synthase (iNOS) in hibernating myocardium. Specifically it was sought to establish a cellular model of hibernating myocardium and investigate the expression, regulation and effects of iNOS in this model. Experiments were performed using primary cultures of neonatal rat ventricular myocytes.

***A cellular model of hibernating myocardium***

Previous models of hibernating myocardium have used whole animals, mostly pigs, with chronic low coronary flow induced by a clamp around the left anterior descending coronary artery. Given that one of the defining features of hibernating myocardium is a reversible downregulation in contractile work in response to low coronary flow, the intrinsic synchronous beating of neonatal rat ventricular myocytes in culture makes this an appropriate choice of model.

A cellular model of hibernating myocardium was established using neonatal rat ventricular myocytes, harvested from neonatal rats aged 1-3 days, subjected to prolonged hypoxia (1% oxygen for 48 hours). In response to hypoxia, it was shown that neonatal rat ventricular myocyte cultures significantly reduced their beating rate (but not the degree of contraction). However this recovered on reoxygenation or with the addition of the inotrope, dobutamine.

Experiments were designed to determine the biochemical characteristics of this cellular model of hibernating myocardium. As described for whole animal models, lactate was mildly increased and cellular ATP content was decreased in response to hypoxia.

***Expression of iNOS in a cellular model of hibernating myocardium***

No evidence of iNOS expression was detected in the cellular model of hibernating myocardium although iNOS expression was consistently demonstrated in response to the addition of Interleukin 1β (IL-1β) to the culture medium. The effects of hypoxia on iNOS expression were further investigated using cultures harvested from neonatal rats aged 7 days. In these cultures there was consistent iNOS expression in response to prolonged hypoxia and in response to the addition of IL-1β, but not in the normoxic controls.

***Regulation of iNOS expression in response to hypoxia***

The role of transcription factors Nuclear Factor kappa B (NFκB) and Hypoxia Inducible Factor 1 (HIF 1) in the expression of iNOS in response to hypoxia were investigated using electromobility shift assays. Nuclear protein binding to both the iNOS NFκB binding site and the iNOS Hypoxia Responsive Element were demonstrated in response to prolonged hypoxia in neonatal rat ventricular myocyte cultures harvested from neonatal rats aged 7 days, suggesting a role for both transcription factors under these hypoxic conditions. However there was no significant upregulation of HIF 1α, compared to normoxic controls, detected on Northern analysis.

Expression of cytokines was investigated by Northern analysis or RT PCR. No IL1β expression was detected, indicating that it has no role in the regulation of iNOS expression under these hypoxic conditions. Expression of Interleukin 6 (IL-6) and Tumour Necrosis Factor α (TNF α) was variable, suggesting a possible contribution of these cytokines to the regulation of iNOS induction under hypoxic conditions.

***The effects of iNOS expression in response to hypoxia***

The downregulation of contractile work in this cellular model of hibernating myocardium, as evidenced by a reduction in intrinsic beating rate in response to hypoxia, was independent of iNOS activity as no iNOS expression was demonstrated in this model. The effects of iNOS expression under conditions of chronic hypoxia were therefore investigated using neonatal rat ventricular myocyte cultures harvested

from neonatal rats aged 7 days. Increased apoptosis was demonstrated in hypoxic cardiac myocytes when compared to normoxic controls. The role of iNOS in hypoxia induced apoptosis was investigated by the addition of an iNOS inhibitor to the culture medium of cells subjected to hypoxic conditions. Results were confirmed using a non specific NOS inhibitor. iNOS inhibition resulted in a significant reduction in apoptosis of hypoxic myocytes indicating that the mechanism of hypoxia induced apoptosis is in part mediated by iNOS.

However, a number of flasks within each experiment showed absolutely no effect of iNOS inhibition suggesting that other pathways to apoptosis may be activated in certain flasks which may have a lower threshold for commitment to cell death. This is supported by the variable TNF $\alpha$ expression demonstrated in both hypoxic and normoxic cultures. In those cultures expressing TNF $\alpha$ there may be direct stimulation of the DR1 receptor, therefore activating the death receptor pathway to apoptosis.

***Conclusions***

The results described suggest that iNOS has no role in the protective downregulation of contractile function in hibernating myocardium. In fact the results implicate iNOS expression as a cause of continuous myocyte loss through apoptosis and may be responsible in part for the deterioration from reversible to irreversible cardiac dysfunction with an associated poorer prognosis in patients with hibernating myocardium.

# DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, to be available for loan and photocopying.

Anke Warner

May 2002

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Coronary Artery Disease and Left Ventricular Dysfunction

### 1.1.1 Coronary Artery Disease

Myocardial ischaemia may be considered in terms of oxygen supply not meeting myocardial demand. Ferrari described myocardial ischaemia as the oxygen supply being inadequate for the rate of mitochondrial oxidation required for myocardial contractile work (Ferrari R 1996). The consequences therefore are mitochondrial impairment and a change in myocardial metabolism and function. Ischaemia that lasts temporarily usually gives rise to the clinical spectrum of angina pectoris through to myocardial infarction.

Reduction in oxygen supply most commonly results from an impairment to coronary blood flow caused through narrowing of the coronary artery lumen by atherosclerotic plaques, or "coronary artery disease". Atherosclerosis is a progressive disease which begins as flat, lipid laden lesions which contain macrophages and smooth muscle cells. Initially these are described as "fatty streaks" (Ross R 1976; Ross R 1976; Stary HC 1984) and they generally begin to appear in the second or third decades. Over time the lesions progress through proliferation of intimal smooth muscle cells, accumulation of macrophages and T lymphocytes, formation of connective tissue matrix and accumulation of lipids. Progression of the atherosclerotic lesions is accelerated in the presence of cardiac risk factors such as hyperlipidaemia, diabetes mellitus, hypertension, smoking and a family history, a risk factor independent of the

others (Higgins M 2000). Eventually the lesions increase in size and begin to impinge on the coronary lumen resulting in reduced myocardial perfusion. This may manifest itself in the clinical syndrome of angina pectoris in middle age or late adulthood.

***Epidemiology***

Ischaemic heart disease is still the leading cause of death worldwide and is expected to remain so in the future. The prevalence of ischaemic heart disease seems to be similar in most Caucasian populations (Cleland JGF 1996) and the overall prevalence in the United States of America has been reported as 3.1% (Rutherford JD 1992). However the prevalence among people over the age of 70 is markedly higher at 30% (Lernfelt B 1990). In fact the lifetime risk of developing ischaemic heart disease is one in two for men and one in three for women (Lloyd-Jones D 1999).

The ischaemic heart disease "event rate", defined as fatal or non fatal myocardial infarction, is variable according to the study population. The event rates in the MONICA study varied from 80 to 800 per 100000 people per year and the case fatality from presentations at 28 days, including prehospital deaths, was approximately 50% (Chambless L 1997). Mortality rate from coronary heart disease has fallen between 1980 and 1990 (Tunstall-Pedoe H 1999) and 70% of the fall has been in people with coronary artery disease, largely by improved treatment and secondary prevention measures (Hunink M 1997). Nevertheless it accounts for 400 000 US deaths per year and 15% ($80 billion) of the US annual health budget. In

Australia in 1998, coronary artery disease accounted for 22% of all deaths (Australian Bureau of Statistics 2001) and for $A894 million of health system resources (National Heart Foundation of Australia 2001).

***Clinical Syndromes***

Acute coronary syndromes, including myocardial infarction and unstable angina, arise from rupture of a vulnerable atherosclerotic plaque in the coronary artery (Zamorano J 1994). Plaque rupture causes exposure of the subintimal collagen which activates platelets (MacIsaac AI 1993). These aggregate and a thrombus forms at the site of the plaque rupture and results in a significant reduction of the luminal diameter. This may explain the stepwise progression of stenotic lesions. If the occlusion caused by the thrombus is severe enough, anginal symptoms will become more prominent. Less exertion may be required to result in the usual anginal pain or even rest ischaemia may ensue. Pain may arise suddenly at rest and may be, but not necessarily so, more severe than previously experienced but there is no irreversible damage to the myocardium. This is referred to as unstable angina. At the extreme end of the clinical spectrum is total occlusion of the coronary artery which, if prolonged, may lead to myocardial necrosis and myocardial infarction. Chest pain at rest which is prolonged is a cardinal feature and may be accompanied by dyspnoea, sweatiness, nausea and in extreme cases hypotension and cardiogenic shock.

Healing of a myocardial infarct involves fibrosis and scar formation and due to the loss of functioning myocytes, left ventricular contractile function is permanently

impaired. The extent of dysfunction depends on the size of the infarct and so extent of myocyte loss. Thus, a large infarct could result in later presentation with cardiac failure due to deterioration in left ventricular function. Alternatively plaque rupture with resultant progression of an atherosclerotic stenosis, whether involving an infarct or not, can represent with chronic stable angina.

Chronic stable angina is generally myocardial ischaemia associated with increased oxygen demand ie physical exertion or effort. A fixed stenosis in the coronary artery may cause no impairment of flow (and therefore myocardial perfusion) at rest but it may result in lack of the required increase in perfusion during activity. Indeed patients may be able to very precisely anticipate what degree of exertion will result in angina symptoms (their threshold for angina). Angina symptoms may be graded by a system proposed by the Canadian Cardiovascular Society in 1972 (Campeau L 1976). On the other hand the threshold for angina in a given patient may be quite variable (Maseri A 1990) due to a component of transiently reduced oxygen supply such as by vasoconstriction or spasm of the coronary artery (Hillis L 1978). Such patients typically have good days, where more activity can be performed before symptoms develop, and bad days when only minimal exertion results in discomfort. Angina may then be precipitated particularly in cold weather or associated with emotional upset.

***Treatment***

If myocardial ischaemia is to be viewed as an imbalance between myocardial oxygen supply and demand, pharmacological treatment may then be directed at improving supply (with resulting increased perfusion or reduced coronary vascular resistance) or reducing demand (by reducing contractility or heart rate).

The organic nitrates act as nitric oxide donors and as such act as vasodilators both in the epicardial arteries and arterioles, causing increased perfusion, but they also induce venous dilation which results in decreased coronary vascular resistance. Their most profound effect is in the large coronary vessels at the sites of stenotic lesions (Horowitz JD 1987). Thus they improve myocardial oxygen supply but through reduced preload and afterload they also reduce myocardial work and so reduce myocardial demand.

Beta adrenoreceptor antagonists reduce myocardial contractility, reduce heart rate and also reduce blood pressure which in turn reduces afterload. All of these mechanisms act to reduce myocardial oxygen demand (Rutherford JD 1992). They may be particularly useful in sympathetically modulated stress, whether physical such as exertion, or emotional upset.

L-type calcium channel blockers such as diltiazem and verapamil interfere with entry of calcium into the cells. This results in negative ionotropy but also in coronary and peripheral vasodilatation and they can also reduce heart rate. Therefore these calcium

channel blockers reduce demand by reducing afterload and reducing contractility and also increase coronary perfusion (Rutherford JD 1992). Not all calcium channel blockers act in the same way. Nifedipine, an example of the dihydropyridines, acts mainly at the level of the vessels to cause vasodilatation but without the myocardial effects. This can result in a reflex tachycardia which can increase myocardial demand and worsen angina (Egstrup K 1993; Minami J 1998). Nevertheless nifedipine can be successfully combined with a beta blocker in order to avoid such deleterious effects (Toal CB 1999).

Perhexiline can be a very useful adjunct to medical treatment of chronic stable angina (Cole PL 1990) but severe adverse effects such as liver and peripheral nerve disturbances (Bouche P 1979; Lewis D 1979) require regular monitoring of blood levels (Horowitz JD 1986).

Should pharmaceutical treatment of angina be ineffective or not tolerated, revascularisation may be appropriate. Percutaneous transluminal coronary angioplasty (PTCA) has emerged as an effective treatment of particularly concentric subtotal stenoses in proximal coronary arteries. Eccentric, calcified or less accessible lesions as well as totally occluded arteries have a lower rate of successful dilatation. Post procedure luminal diameter may be improved with insertion of a stent. The risks associated with PTCA include myocardial infarction and the need for emergency coronary artery bypass surgery in 0.6% of cases and death in 0.3% (Pell JP 2001). Although successful PTCA may allow significant reduction in medication

requirements, the risk of restenosis is 30% (Leimgruber PP 1986) in the first six months post procedure. This is due to intimal hyperplasia with smooth muscle cell proliferation (Nobuyoshi M 1991). Repeat PTCA may then be necessary. The rate of restenosis with coronary stent insertion (Fischman L 1994; Serruys PW 1994) is less than that with PTCA alone. However, the treatment options for in-stent restenosis are somewhat limited. Coronary atherectomy appears to have no advantage over routine PTCA (Elliott JM 1995; Baim DS 1998) but may have specific indications such as treatment of in-stent restenosis. More recently endovascular beta radiation, delivered through stent coated with a beta emitter such as $^{32}P$, has been investigated as a mechanism for reducing the rate of restenosis due to intimal hyperplasia (King SB 1998) however the high incidence of edge restenosis (Albiero R 2000; Wardeh AJ 2001) makes the clinical role of this modality uncertain at this stage.

Coronary artery bypass surgery was introduced in the 1960's (Favaloro RG 1969) and uses a piece of saphenous vein or internal mammary (or more recently also radial) artery to provide a conduit around the stenotic lesion. Vein grafts have a 50-70% incidence of significant atheromatous stenosis by 3-5 years while internal mammary artery grafts have a 90% patency rate at 10 years post bypass (Loop FD 1986). The in hospital mortality rate for bypass surgery is around 1-3% although this rises for elderly patients with the in hospital mortality for octogenarians being 5.6% (Alexander KP 1997).

Initially randomised clinical trials were performed to assess the safety of the technique and it was found that there was no significant difference in mortality between patients treated medically and those undergoing CABG (Murphy ML 1977; European Coronary Surgery Study Group 1979) and symptom improvement was significantly better. Later studies began to suggest that an improvement in survival with surgery was possible (Loeb HS 1979). It was certainly clear that CABG resulted in better symptom control than medical treatment (European Coronary Surgery Study Group 1982; Coronary Artery Surgery Study 1983). However, it appears that the survival benefit of CABG is confined to certain subgroups; those with left main coronary artery disease (Takaro T 1982), significant disease associated with left ventricular dysfunction (Detre K 1981) and possibly triple vessel with a positive exercise test or proximal Left Anterior Descending Coronary Artery (LAD) disease as part of two vessel disease (European Coronary Surgery Study Group 1982). There is no survival benefit from CABG in patients with mild angina or in those with no symptoms following myocardial infarction (Coronary Artery Surgery Study 1983; Coronary Artery Surgery Study 1984).

The treatment of acute coronary syndromes involves the use of many of the antianginal agents discussed previously. In addition, therapy is directed at prevention or dissolution of the thrombus formed at the site of plaque rupture. In the setting of myocardial infarction, thrombolytic agents including, recombinant tissue plasminogen activator (rtPA) and streptokinase, are infused intravenously to reperfuse the threatened myocardium by lysing the occluding thrombus. The advent

of thrombolysis has resulted in significant reductions in mortality (GISSI 1986; ISIS 2 Collaborative Group 1988) from myocardial infarction through early reperfusion and limitation of infarct size (Sheehan FH 1987).

Aspirin and heparin have an important role to play in the adjuvant management treatment of myocardial infarction and have been shown to reduce in hospital cardiac events in patients with unstable angina (Theroux P 1988). Aspirin is also important in the secondary prevention of myocardial infarction and improves survival in stable and unstable angina (Lewis HD, Davis JW et al. 1983). Another antiplatelet agent, clopidogrel, has also been shown to reduce the risk of cardiovascular death, myocardial infarction and stroke in patients with acute coronary syndromes (CAPRIE steering committee 1996; Mitka M 2001). Several trials have confirmed the role of platelet glycoprotein IIb/IIIa antagonists in reducing the risk of myocardial infarction and death in patients with unstable angina (The PARAGON Investigators 1998; The Platelet Receptor Inhibition in Ischaemic Syndrome Management (PRISM) Study Investigators 1998; The Platelet Receptor Inhibition in Ischaemic Syndrome Management in Patients Limited by Unstable signs and Symptoms (PRISM-PLUS) Study Investigators 1998; The PURSUIT Trial Investigators 1998).

***Prognosis***

The average annual mortality in unselected patients with chronic stable angina is 4%, the in hospital mortality for unstable angina is 3-5% for medically treated patients and 7-8% in the first year. The mortality rate for non fatal myocardial infarction is 8-10%

in the first two weeks (Hilton TC 1991). Following myocardial infarction, the presence of left ventricular dysfunction , with an ejection fraction less than 40%, is associated with increased one year mortality compared to those with normal left ventricular function (Mukharji J 1984). Electrical instability (frequent ventricular ectopic beats or ventricular tachycardia) is a risk factor for sudden cardiac death. Ongoing ischaemia is also a risk factor for poorer outcome and the more risk factors, the worse the prognosis (Multicentre post-infarction research group 1983). For example those with three risk factors carry greater than 5 times the cardiac mortality risk than those with only one risk factor. Similar prognostic factors apply to patients with unstable angina.

### 1.1.2 Left Ventricular Dysfunction

Cardiac failure may be defined as the pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood and/or to do so only from an abnormally elevated filling pressure at a rate commensurate with the requirements of the metabolising tissues (Braunwald E 1992). It can be caused by an abnormality in systolic function, ie a defect in expulsion of blood, or by an abnormality in diastolic function which leads to a defect in ventricular filling. The former is the more classic cardiac failure (also known as "congestive cardiac failure") in which myocardial contractile weakness is the underlying mechanism. Nevertheless in many, if not most cases, diastolic and systolic failure

coexist. Cardiac failure due to coronary artery disease, the most common cause of cardiac failure (Sutton GC 1990; Mair FS 1996), is an example of combined systolic and diastolic failure. Myocyte loss through the necrosis of myocardial infarction as well as transient contractile impairment through acute ischaemia are responsible for the systolic component. Acute ischaemia also causes reduced ventricular compliance and therefore the impaired ventricular filling of diastolic failure.

***Epidemiology***

Cardiac failure is emerging as one of the most significant diseases of the new century. During the 1980's , the age adjusted incidence of cardiac failure was 0.07% for men and 0.05% for women (Ho KK 1993) resulting in 400 000 new cases in the United States every year. The prevalence of cardiac failure is approximately 2% (Schoken DD 1992; Ho KK 1993) and this suggests that approximately 2 million Americans are affected. The prevalence rises with age and it is estimated that 10% of people over 80 years of age have cardiac failure (Kannel WB 1991).

Reported mortality rates for cardiac failure vary widely depending on the population studied. Overall the 5 year mortality rate, from time of diagnosis, has been estimated to be 60-80%. (Kannel WB 1991; Ho KK 1993; Dargie HJ 1996) but for severe cardiac failure the annual mortality is as high as 50%. The mortality rate for men is higher than that for women (Burns RB 1997).

Not only is cardiac failure a significant cause of mortality, it is the cause of significant morbidity and therefore hospital admissions in developed nations. In fact it is the leading cause of hospitalisations in older adults, with over 800 000 hospitalisations in the United States in 1993 having cardiac failure as the principle diagnosis (Rich MW 1997). The number of hospitalisations for cardiac failure doubled between 1973 and 1986 (Ghali JK 1990) and continued to rise between the years 1986 and 1993 (Croft JB 1997).

Cardiac failure is also associated with a high incidence of early rehospitalisation. Ten years ago it was estimated that among patients admitted to hospital with cardiac failure, 47% are rehospitalised within 90 days, mostly for recurrent heart failure (Vinson JM 1990). Again this trend persists with a more recent study showing that 25% of patients admitted with cardiac failure were readmitted within 6 weeks, one third by six months and one half by one year (Burns RB 1997). This study also found that cardiac failure leads to a substantial loss of independence for older individuals.

In summary, cardiac failure is common, lethal and causes significant morbidity and loss of independence. It is not surprising then, that $10 billion is spent annually on treatment of cardiac failure in the United States (Rich MW 1997).

***Clinical Presentation***

Breathlessness is the cardinal symptom of heart failure. Initially this occurs on exertion as the failing myocardium cannot increase cardiac output to meet the

increased demands of exertion. Patients find they can no longer do tasks they could previously do with ease. Gradually less exertion is required before breathlessness occurs until minimal exertion causes severe symptoms.

Orthopnoea is breathlessness on lying flat which is relieved by elevating the head. Lying down causes less pooling of blood in the lower extremities and so more venous return into the thoracic cavity. The failing left ventricle cannot cope with the extra blood volume delivered to it and this is experienced as breathlessness. This pulmonary congestion on lying down may also cause a dry non productive cough. An increase in the number of pillows required to be able to breathe comfortably at night is a common indication of deterioration in heart failure symptoms.

Orthopnoea occurs within a minute or two of lying down. Patients find they must sit up, often in front of an open window, to get relief. They may prefer to sleep in an upright position, for example in a recliner chair. Paroxysmal nocturnal dyspnoea on the other hand awakens the patient suddenly at night with shortness of breath and a feeling of suffocation. Unlike orthopnoea, which is relieved quickly on sitting up, paroxysms may last up to 30 minutes or more. The mechanism for these episodes is not clear (Braunwald E 1992).

As cardiac failure becomes more severe, patients may experience a chronic fatigue and weakness. Shortness of breath may occur at rest and pulmonary oedema, a transudation of fluid into the alveoli, results in crackles on auscultation of the chest.

Right heart failure may develop consequent upon left ventricular failure and signs of venous congestion develop. This may manifest itself as peripheral oedema, commonly ankle swelling, and hepatic congestion leading to a dull right upper quadrant or epigastric ache.

***Functional Classification***

The most commonly used classification of patients with cardiac failure was developed by the New York Heart Association and is based on symptoms and the effort required to provoke them. The system allows comparisons between groups of patients as well as the status of a single patient at different times. There are four functional classes described as below :

Class I No symptoms with ordinary activity. No particular limitation of activity.

Class II No symptoms at rest, but dyspnoea or fatigue with moderate or ordinary activity.

Class III Mild (less than ordinary ) activity causes symptoms but comfortable at rest.

Class IV Symptoms are present even at rest. Any activity causes worsening of symptoms.

***Treatment of chronic congestive cardiac failure***

The haemodynamic, hormonal and neurohumoral consequences of congestive heart failure on the kidney lead to salt and water retention and expansion of the

extracellular volume. This may be adaptive in the short term to maintain cardiac output by allowing the left ventricle to operate higher on the Frank-Starling curve (Braunwald E 1992). Higher circulatory volume results in stretching of the left ventricular myocardium and this results in improved left ventricular performance. However increasing chamber dilatation (the "descending limb" of the Frank-Starling curve) and other pathophysiological mechanisms of "remodelling" cause progressive deterioration in cardiac performance. Diuretics serve to reduce extracellular volume and so reduce left ventricular preload. Frusemide, a loop diuretic, one of the most commonly used diuretics in heart failure, also causes venodilation and so further reduces preload. Few clinical trials have been performed to assess the impact of diuretics in heart failure but recently a survival benefit has been shown with spironolactone (RALES study group 1996) when given in addition to both loop diuretics and angiotensin converting enzyme (ACE) inhibitors. Studies have suggested that spironolactone has additional actions to diuresis such as improving endothelial dysfunction, increasing nitric oxide bioactivity, and inhibiting vascular conversion of Angiotensin I to Angiotensin II in patients with heart failure (Farquharson CA 2000), providing other mechanisms for its therapeutic effect in heart failure.

Digitalis glycosides, of which digoxin is the best known, have been used in the treatment of heart failure for over two hundred years. Digoxin binds to the sodium-potassium ATPase pump and has positive ionotropic effects that are independent of catecholamines. No clinical trial to date has proven a survival benefit of digoxin in

the treatment of cardiac failure (Digitalis Investigation Group 1997) but studies have shown improvement in left ventricular ejection fraction (Captopril Digoxin Multicentre Research Group 1988) and symptoms (Uretsky BF 1993) with deterioration upon its withdrawal.

The ACE inhibitors have been shown to improve survival in patients with both mild and severe heart failure (CONSENSUS Trial Group 1987; Newman TJ 1988; SOLVD Investigators 1991). In addition to haemodynamic effects (due to ACE inhibition depressing the circulating levels of Angiotensin II, at least in the short term), ACE inhibitors also inhibit breakdown, and so increase the levels of bradykinin. However, ACE inhibitors do not completely suppress angiotensin II levels in heart failure (Jorde UP 2000) and so interest has been generated in the use of angiotensin II receptor antagonists, but at present their incremental benefit in heart failure remains unproven (Carson PE 2000).

The high levels of noradrenaline associated with severe heart failure (see below) has lead to interest in the use of beta adrenergic blockers in cardiac failure. Early small studies suggested some survival benefit (Svedberg K 1979). More recently, large multicentre clinical trials have been conducted and it has become clear that beta blockers, both conventional ones such as metoprolol and those with additional alpha-1 blocking effects such as carvedilol, not only improve symptoms and exercise tolerance but also improve survival (Bristow MR 1996; Packer M 1996; MERIT-HF Study Group 1999; MERIT-HF Study Group 2000).

In resynchronisation therapy with biventricular pacing, both ventricles are stimulated nearly simultaneously, thus overcoming discoordinated ventricular contraction resulting from intraventricular conduction disturbances. This results in improved haemodynamic parameters (Aurrichio A 1999). Several small studies suggest a benefit in terms of symptomatic improvement but the effects on long term morbidity and mortality are not yet clear (Remme WJ 2001).

Transplantation remains the last resort for treatment of cardiac failure. However, many patients with cardiac failure, particularly those with coronary artery disease as the basis of their cardiac failure, have a number of contraindications to cardiac transplantation. These may include age, concomitant vascular disease or diabetes mellitus. In patients with coronary artery disease, aggressive efforts to treat ischaemia, even with coronary artery bypass surgery, may slow deterioration of cardiac function.

***Prognosis***

Overall the one year and five year survival rates for heart failure after the onset of diagnosis are 75% and 35% respectively (Senni M 1998). A number of factors have been associated with high mortality in patients with cardiac failure. These prognostic factors can be categorised as clinical, haemodynamic, biochemical and electrophysiological. However, prognostic factors predictive of a poor outcome ie highest mortality, are coronary artery disease as the aetiology of cardiac failure; poor

functional class (according to NYHA classification); low left ventricular ejection fraction; high noradrenaline levels and high Atrial Natriuretic Peptide (ANP) levels. For example, those patients able to exercise 12 minutes on a modified protocol had a 55% one year survival rate, whereas those who could only manage 3 minutes had a 15% one year survival (Cleland J 1987). Those patients with a left ventricular ejection fraction between 31-40% had a mortality rate of approximately 7% whereas those with an ejection fraction below 20% had a mortality rate of approximately 27% over the 16 month average study period (Gradman A 1989). Patients with a high plasma noradrenaline level had a 70% one year mortality whereas those with a low noradrenaline level had a one year mortality of approximately 20% (Cohn JF 1984). A high serum ANP indicated a 60% one year survival, whereas a low level indicated approximately an 80% one year survival (Gottlieb SS 1989). More recently other prognostic factors indicating poor outcome have been reported including brain natriuretic peptide (Tsutamoto T 1997), and raised endothelin levels (Pousset F 1997). However the factors that have consistently emerged through multivariate analysis are left ventricular ejection fraction, NYHA functional class and plasma noradrenaline levels (Cohn JN 1988; Madsen BK 1995).

## 1.2 Hibernating myocardium

### 1.2.1 Definition

The discovery that coronary artery bypass grafting can improve survival in patients with triple vessel disease and left ventricular dysfunction together with the importance of left ventricular contractile function as a prognostic indicator in cardiac disease lead to interest in the effects of revascularisation on regional and global contractile function.

It is known that during an episode of severe ischaemia there is regional reduction in contractility involving the ischaemic myocardium but sparing the normally perfused myocardium (Theroux P 1974). This dysfunction may persist for some time after the episode of ischaemia has resolved and perfusion is restored. In some circumstances this dysfunction may persist for a few days (Braunwald E 1983). This persistence of left ventricular dysfunction after reperfusion is termed stunning.

During the early 1980's it was noted by Rahimtoola (Rahimtoola 1985) that in some patients, revascularisation by CABG resulted in improvement in regional wall motion and even global function. Segments, supplied by severely stenosed arteries, that appeared hypokinetic or even akinetic preoperatively, and therefore often assumed to be infarcted or irreversibly damaged, recovered contractility. It was assumed that

these segments were chronically ischaemic although not infarcted and therefore function was reduced. The term hibernating myocardium was coined (Braunwald E 1986) to describe the condition and the distinction was drawn between it (chronic hypoperfusion with correspondingly reduced function and recovery of contractile function after revascularisation) and stunning in which perfusion had been restored but function was persistently but temporarily depressed. It was also found that in some patients with severe coronary disease and reduced myocardial function, there was a transient improvement after an extrasystolic beat (Popio K 1977). Horn et al also reported transient improvement in myocardial function with catecholamine infusion (Horn HR 1974). This transient improvement in response to ionotropic stimulation is the basis of dobutamine stimulated echocardiographic detection of hibernating myocardium.

It was then postulated that hibernating myocardium has a downregulated metabolism, without actually being ischaemic, so that it is an adaptive reduction in function in order to reduce oxygen requirements in conditions of chronically reduced supply (Ferrari R 1996; Hochachka PW 1996). All "unnecessary" cell functions are temporarily reduced to allow the minimum functions required to maintain cell viability. Then when perfusion is restored such as through revascularisation, there is slow recovery of all other cell functions.

Indeed the debate about whether hibernating myocardium is ischaemic or not continues to this day. The view that the myocardium is not ischaemic but a

downregulation of metabolism stems from the biochemical findings in animal models. However, new evidence arising from positron emission tomography (PET) techniques provides some challenges to this view. Schaeffer et al (Schaeffer W 2000) found that contrary to previous definitions of hibernating myocardium, myocardial blood flow as measured by PET using $O^{15}$ water, was not significantly reduced in patients with hibernating myocardium. It has been postulated that hibernation may be repetitive stunning due to repeated ischaemic episodes such that, despite restoration of flow, impairment of function remains chronic. On the other hand Gerber et al (Gerber BL 1998) suggest that $O^{15}$ water yield higher absolute values for myocardial perfusion than does $N^{13}$ ammonia. Some also postulate that repetitive stunning may eventually result in reduced myocardial flow which is secondary to the chronically reduced function (Canty JM 2000).

However, Di Carli et al (Di Carli MF 2000) studied balloon occlusion of the left anterior descending coronary artery in dogs and found that although repeated coronary occlusion resulted in dysfunction that lasted up to a week, the changes in myocardial metabolism were different from those reported in hibernating myocardium.

An interesting perspective is provided by work from Shivalkar et al (Shivalkar B 1999) in which dogs were subjected to severe chronic coronary occlusions by surgical banding of the three coronary arteries. This model was maintained for four weeks. They initially observed early episodic myocardial dysfunction and normal resting

flow, but reduced coronary flow reserve. The dysfunction became persistent and was associated with subendocardial hypoperfusion. Response to ionotropic stimulation was preserved.

It is of course possible that hibernating myocardium represents a combination of repetitive stunning leading to low flow with a downregulated state (with or without superimposed ischaemic episodes) and it typically occurs in the setting of recent or previous myocardial infarction.

### 1.2.2 Prevalence of hibernating myocardium

The prevalence of hibernating myocardium is difficult to estimate, as few prospective studies exist. Bonow (Bonow RO 1996) estimated on the basis of previous reports that 25-40% of patients with chronic coronary artery disease and global left ventricular dysfunction have the potential for significant improvement in left ventricular ejection fraction following revascularisation. These estimates are however subject to selection bias, as they reflect only those patients referred for viability studies who undergo revascularisation.

Al-Mohammad (Al-Mohammad A 1998) et al report a prospective study from a series of consecutive patients undergoing coronary angiography. Of a total of 301 patients, 36 had coronary artery disease and a left ventricular ejection fraction less than or

equal to 30%. 27 of these patients underwent PET imaging before revascularisation. The characteristics of the imaged compared to non imaged patients were reported as similar but the non imaged group had more patients with Class IV (more severe) angina symptoms. 52% of those patients imaged preoperatively (or 39% of all patients with coronary artery disease and severe left ventricular impairment) had evidence of hibernating myocardium on PET imaging. This was defined as the PET characteristics (perfusion-metabolism mismatch) of hibernating myocardium involving 20% or more of the left ventricle. This threshold was chosen on the basis of previous studies as a proportion likely to result in significant improvement in global left ventricular function post revascularisation. The authors therefore concluded that the prevalence of hibernating myocardium in patients with coronary artery disease and ejection fractions less than or equal to 30% was between 39% and 52%. Comparison with recovery of function was not performed as not all patients went on to have a revascularisation procedure.

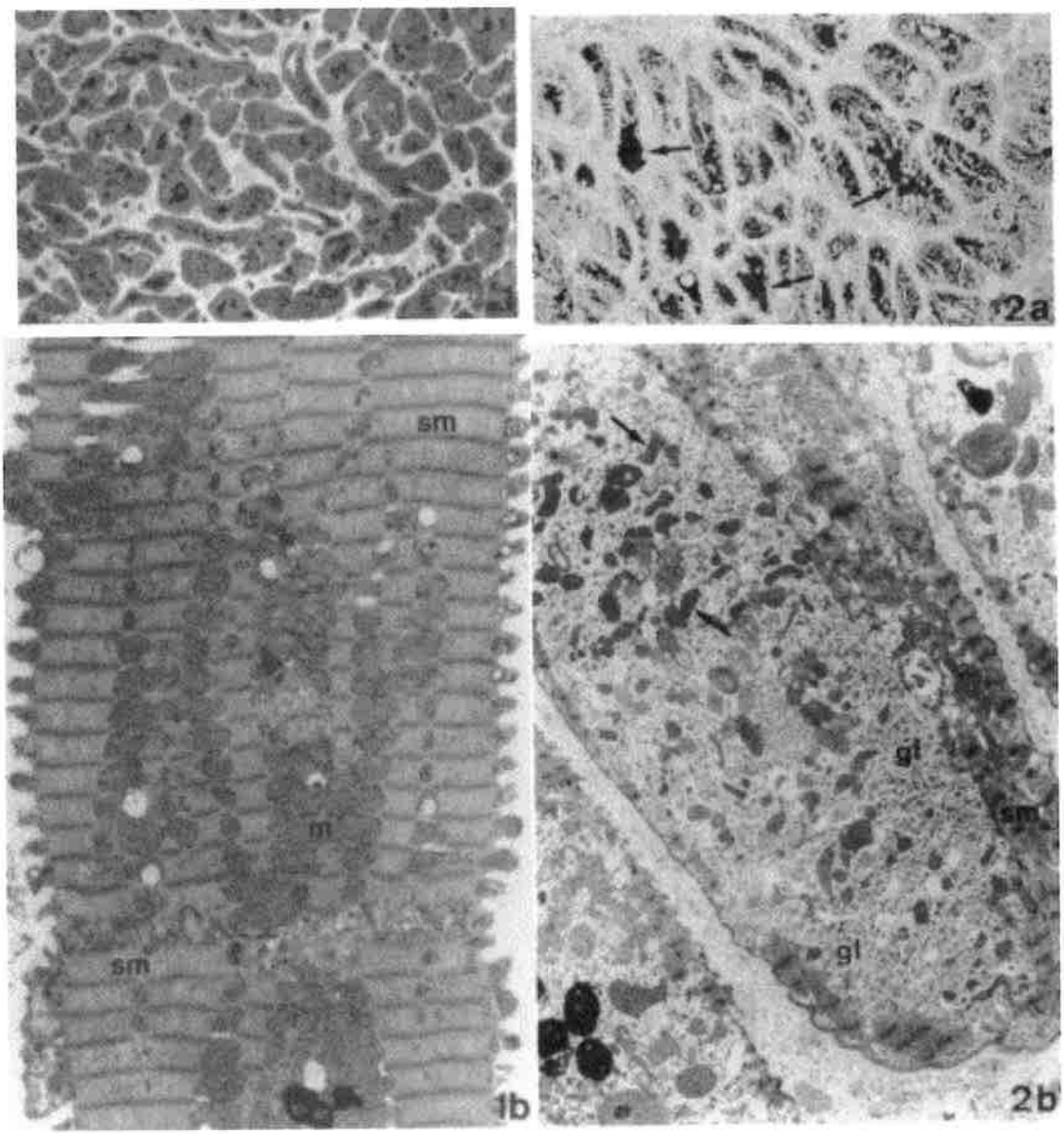
### 1.2.3 Histology

Histological changes in hibernating myocardium have been studied using biopsies from human subjects undergoing revascularisation procedures. Maes et al (Maes A 1994) prospectively identified patients with hibernating myocardium using PET. Biopsies were obtained at bypass surgery and histology was compared to the PET findings and recovery of wall motion abnormality after revascularisation. The

biopsies of hibernating myocardium showed significantly less fibrosis than segments that did not recover function following revascularisation. There was also replacement of sarcomeres with increased glycogen (Figure 1.1).

Elsaesser et al (Elsasser A 1997) also studied patients undergoing coronary artery bypass surgery and also performed PET scanning prior to surgery. They found reduced contractile material, reduced myosin and actin protein and mRNA expression, altered small, dark mitochondria on electron microscopy and fibrosis. The severity of degenerative change and fibrosis correlated inversely with the extent of recovery of contractile function after revascularisation.

Animal models have been used to study hibernating myocardium. Pig models seem to be most commonly reported. Chen et al (Chen C 1996) created a partial occlusion in the LAD of pigs with reduction of resting flow to 60% of baseline. The condition was maintained for 24 hours with eventual recovery of function. Histological findings were similar to those reported in biopsies from patients. Others have continued this pig model of hibernating myocardium for a few weeks (Shen YT 1996) with ongoing ionotropic reserve and recovery of function following revascularisation and yet other groups report a pig model of hibernating myocardium studied for up to 30 days (St Louis JD 2000). Once again histological findings were similar to those found in human subjects. Such pig models have most recently been maintained for three to four months (Fallavollita JA, Perry BJ et al. 1997) and even up to six months (Hughes GC 1999).




A B

**Figure 1.1** Light microscopy (above) and electron microscopy (below) of normal (A) and hibernating (B) myocardium. From Maes et al (Maes A 1994). Hibernating myocardium is characterised by loss of myofibrils (sm), increased glycogen (gl and PAS positive - black arrows - staining on light microscopy), shrunken mitochondria (arrows on electron microscopy).

### 1.2.4 Biochemical Characteristics

***Cardiac metabolism at rest***

The high energy requirements of contracting myocardium is met by ATP production predominantly through free fatty acid metabolism. However post prandially, with increased insulin levels, there is enhanced glucose uptake and reduced fatty acid concentrations due to inhibition of lipolysis. Under these circumstances carbohydrates are the predominant source of myocardial energy requirements. The pathways for fatty acid and carbohydrate metabolism are tightly coupled allowing rapid shifts between energy sources as availability alters.

The oxygen requirements to produce ATP by fatty acid metabolism differs from that of carbohydrate metabolism. Using carbohydrate metabolism, 12 molecules of oxygen are required in the metabolism of one molecule of glucose, to produce 38 molecules of ATP (Grynberg A 1996). One molecule of palmitate on the other hand requires 46 oxygen molecules to produce 130 molecules of ATP. Thus fatty acid metabolism is efficient in terms of amount of ATP produced but inefficient in terms of oxygen consumed. Under normal physiological conditions, without limitation of oxygen supply, fatty acid metabolism is therefore the preferred source of ATP.

***Cardiac metabolism during ischaemia***

During ischaemia there is limitation of oxygen supply and so a switch to carbohydrate metabolism would allow increased production of ATP for the available

oxygen. The degree to which this occurs depends on the degree of ischaemia. With moderate reduction in coronary flow there is increased glucose uptake into myocytes through plasma membrane glucose transporter molecules, GLUT 1 and GLUT 4 (Liedtke AJ 1981; Stanley WC 1997; Young LH 1997). Thus there is reduced coronary flow with an increase in glucose uptake in the myocardium.

Lactate is formed through glycolysis and accumulates in ischaemic tissue as a shift from net lactate uptake to lactate production occurs. If perfusion ceases altogether there is total dependence on anaerobic metabolism and utilisation of glycogen stores within the myocardium. Without perfusion there is no washout of lactate and so intracellular pH decreases until glycolysis is inhibited.

***Cardiac metabolism in hibernating myocardium***

Hibernating myocardium is also thought to be a condition of low coronary flow and limited oxygen supply. There is also increased glucose uptake which forms the basis of detection by PET. There are however some differences in biochemistry which seem to distinguish it from ischaemia.

Using the pig model of hibernating myocardium, Schulz et al (Schulz R 1992) studied the metabolism of hibernating myocardium and the consequences of ionotropic stimulation. The LAD was cannulated and flow was adjusted to reduced regional contractile wall motion by 50%. The corresponding vein was cannulated and sampled for lactate and oxygen content. Transmural biopsies were taken from the LAD

territory for measurement of tissue ATP, creatine phosphate (CP) and glycogen content. Regional myocardial function was reduced after 5 minutes of reduced coronary flow and after 15 minutes, lactate levels were increased, CP was reduced and ATP content in the tissue was reduced compared to controls. After 40 minutes, however, CP levels began to rise again and lactate production began to decrease. Myocardial ATP levels remained reduced compared to controls. Prolonging the low flow conditions to 85 minutes resulted in CP levels which were no longer significantly different from baseline. Dobutamine infusion caused improvement of regional wall motion (contractile function) but with worsening of the metabolic markers of ischaemia: lactate levels increased again and the CP levels decreased.

Through practical necessity the study described used only a short term model of hibernating myocardium. The clinical relevance of these findings may be questioned when it is known that hibernating myocardium is a condition of weeks to months. Indolfi et al (Indolfi C 1996) identified 11 patients with hibernating myocardium in the LAD territory by dobutamine echocardiography (to show inotropic reserve) and studied them at the time of PTCA at rest, during dobutamine infusion and then again three weeks after PTCA. They found that at rest the hypoperfused but viable myocardium had no net lactate production (in fact there was lactate extraction) in the resting state supporting the notion that an adaptation exists in hibernating myocardium. Following dobutamine infusion the arterial-venous lactate difference reduced or reversed indicating a trend towards production. These findings support the results obtained in animal models described above.

### 1.2.5 Treatment

***Medical or Surgical Treatment***

It follows from the definition of hibernating myocardium and from the CASS (Coronary Artery Surgery Study 1983) study observations, that the treatment of choice for hibernating myocardium is revascularisation. Initially this was believed to entail coronary artery bypass surgery but with the advent of PTCA, there is an alternative particularly in single vessel disease. Recent prospective studies have been reported, which support this strategy of revascularisation (CABG or PTCA) rather than medical therapy. Di Carli et al (Di Carli MF 1998) studied 93 consecutive patients with severe coronary artery disease and low ejection fractions and found that those patients with hibernating myocardium on preoperative imaging who underwent coronary artery bypass surgery had an improved four year survival compared with those who were treated medically (75% compared with 30%). It must be noted however that these patients were not randomly assigned to either surgical or medical treatment.

Furthermore, there is evidence to suggest that patients without viable myocardium sent for revascularisation procedures, particularly coronary artery bypass surgery, have a worse prognosis than if treated medically. Haas et al retrospectively evaluated 76 patients with severe coronary artery disease and left ventricular dysfunction, suitable for coronary artery bypass surgery. Thirty five patients were referred for surgery on clinical criteria and 41 patients were assessed by PET and sent for surgery

on the basis of detection of viable myocardium (34 patients). In these patients selected on the basis of PET scanning, there was a 0% mortality rate during hospitalisation and a one year survival rate of 97%. In the group selected for surgery on clinical presentation, without viability assessment by PET, there was an in hospital mortality of 11.4% ($p = 0.04$) and a one year survival rate of 79% ($p = 0.05$).

Allman et al have performed a meta analysis of 24 studies of viability assessment, including 3088 patients, using $^{201}$Thallium, PET or dobutamine echocardiography. In patients with viability detected on any modality revascularisation, compared with medical treatment, was associated with a 79.6% reduction in annual mortality (16% compared with 3.2%, $p < 0.0001$). Patients without viable myocardium had a trend towards higher mortality with revascularisation (7.7%) compared with those treated medically (6.2%) but this did not reach statistical significance.

***Timing of Revascularisation***

The focus in hibernating myocardium has been on recognition and the improved survival with revascularisation. However, there has been little attention given to the timing of revascularisation. It cannot be necessarily assumed that the downregulation and preserved viability remains so indefinitely. If the systolic dysfunction does eventually become irreversible it is important to determine within what time frame revascularisation must be carried out before all the survival benefit is lost through irreversible degeneration of the myocardium. Although few studies have addressed this, Schwarz et al (Schwarz ER 1998) reported on 32 patients awaiting bypass

surgery with hibernating myocardium identified preoperatively by PET. Recovery of function post operatively was compared to duration of time before surgery and to structural degeneration seen on transmural biopsy taken at the time of surgery. It was found that recovery of function was inversely correlated with the duration of time before surgery and also correlated inversely with degenerative changes identified on histology. The authors concluded that early revascularisation should be attempted to rescue myocardium at risk and improve the outcome postoperatively.

It is therefore clear that patients with hibernating myocardium will significantly benefit from revascularisation in terms of improved survival. It is also clear that those patients with regional left ventricular dysfunction due to infarcted myocardium, rather than hibernating or viable myocardium, will have no improvement in prognosis post revascularisation and in these patients the procedure may well carry more risk than benefit. This highlights the importance of preoperative detection of hibernating or viable myocardium and is reflected in the different approaches to methods of detection available and being investigated .

## 1.3 Detection of Hibernation Myocardium

### 1.3.1 Recovery Post Revascularisation

This is by definition the gold standard for identifying hibernating myocardium. It is, however, necessarily a retrospective diagnosis. Given the improved prognosis with revascularisation in patients with hibernating myocardium and the potentially worse outcome as a result of revascularisation in patients with reduced cardiac function and no evidence of reversible ischaemia or hibernation, it is important to be able to preoperatively identify those patients who stand to benefit from these procedures. This would target the revascularisation procedures (mostly coronary artery bypass surgery) to those who would benefit and avoid unnecessary or potentially dangerous operations.

It would be even more advantageous if these methods of detecting hibernating myocardium were noninvasive. Post extrasystolic potentiation of segmental cardiac function during left ventriculography (Popio K 1977) was one of the first methods of prospectively identifying myocardial segments that appeared infarcted (ie were hypokinetic) but were viable. However, its application is limited to those that happen to have an extrasystole during the ventriculogram and the regions of myocardium

studied are those limited to the one view taken of the ventriculogram. Therefore sensitivity is likely to be suboptimal.

A number of imaging techniques have been applied to the problem of identifying viable myocardium prospectively. Evaluation and validation of these techniques is hampered by relatively small studies with different protocols and the inherent problem of referral bias. Nevertheless, PET has become the "gold standard" noninvasive imaging modality in recent years.

### 1.3.2 Positron Emission Tomography

The PET method most extensively validated for the detection of viable but hibernating myocardium involves the near-simultaneous assessment of myocardial flow and myocardial glucose uptake. A number of tracers have been used to estimate myocardial flow including $^{15}O$ labelled water and $^{13}N$ labelled ammonia (Schelbert HR 1979; Bergman SR 1989; Iida H 1989). Both these tracers have short physical half lives (2 and 10 minutes respectively) which allow repeat measuring or immediate progression to metabolic imaging. $^{18}F$ fluorodeoxyglucose (FDG) is the most frequently used tracer for the metabolic assessment of myocardial viability. FDG is a glucose analog which is transported into the myocardial cell by the same carrier as glucose. It is then phosphorylated to FDG-6-phosphate by the enzyme hexokinase

but does not proceed further down the glucose-pyruvate pathway. Instead it accumulates in the cell and reflects myocardial glucose uptake.

Hibernating myocardium is characterised by reduced flow, whether as a primary or secondary event, and enhanced glucose uptake compared with normal myocardium. This is known as a flow-metabolism mismatch and has been found to be highly predictive for viable myocardium which recovered function after revascularisation (Tillisch J 1986; Tamaki N 1989; vom Dahl J 1994). Other patterns observed include normal flow with normal metabolism (ie normal myocardium) and reduced flow with reduced glucose uptake which indicate myocardial infarction and which is not likely to recover function post revascularisation (see Figure 1.2). Unfortunately, despite being considered the most reliable noninvasive method of detecting hibernating myocardium, PET remains an expensive imaging modality with limited availability.

### 1.3.3 Dobutamine Echocardiography

Dobutamine echocardiography relies on the ionotropic reserve of hibernating myocardium to detect prospectively which dysfunctional myocardial segments will recover after revascularisation. Echocardiographic assessment of regional function and myocardial thickening is made before and during low dose (eg less than 10 μg/kg/minute) dobutamine infusion. An incremental protocol starting at 5

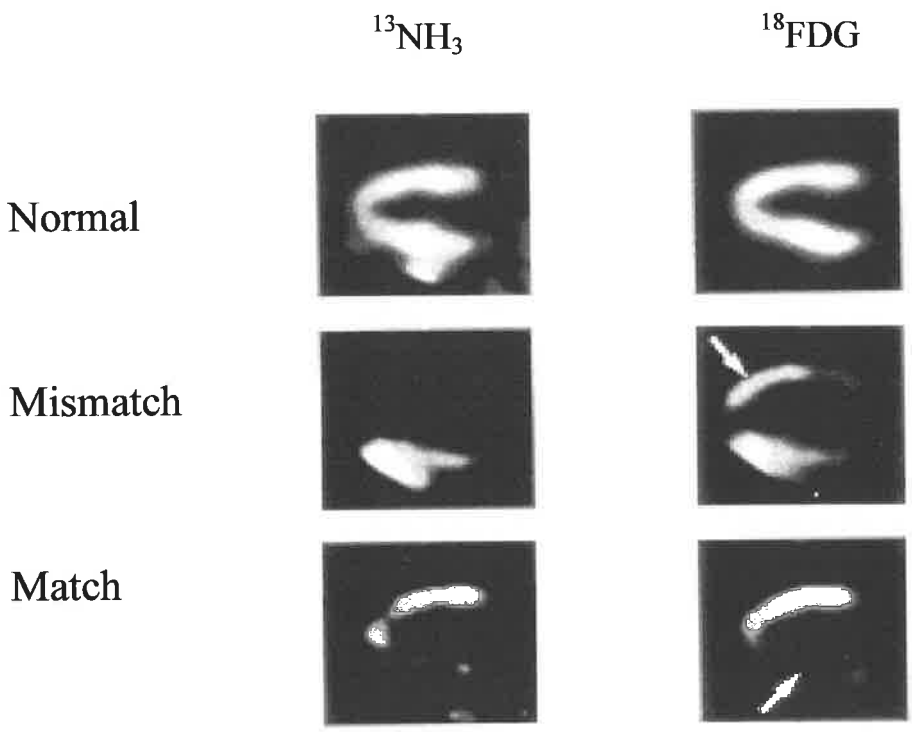



**Figure 1.2** Patterns seen on PET myocardial imaging for detection of hibernating myocardium. From Camici et al (Camici P 1998). For details of interpretation see text.

μg/kg/minute and increasing in step wise fashion to 40 μg/kg/minute is the optimal protocol (Pierard LA 1997). Often a biphasic response is observed with improvement in regional function at low doses of dobutamine and further deterioration at high doses (due to ischaemia) and this is thought to have the highest predictive value for detection of viability and recovery of function (Afridi I 1995).

Comparison of dobutamine echocardiography, PET and thallium-201 imaging with histological findings suggest relatively better sensitivity with PET and thallium-201 compared to dobutamine echocardiography, but possibly improved specificity for the detection of viable myocardium with dobutamine echocardiography compared to thallium-201 (Baumgartner H 1998; Pagano D 1998). When compared with recovery of function post revascularisation dobutamine echocardiography had a sensitivity of 79% and a specificity of 92% (Perrone-Filardi P 1996).

The addition of nitrates may improve detection of functional improvement at low doses of dobutamine but does not seem to prevent the ischaemia induced by higher doses (Ma L 1997). In fact, the observation of a biphasic response is important for the distinction between stunned and hibernating myocardium. In the former, coronary flow has been restored and improvement in function can be expected to be sustained at the higher doses of dobutamine whereas in hibernating myocardium a severe perfusion impairment remains and so at higher doses ischaemia is induced with corresponding worsening of function (Pierard LA 1997).

### 1.3.4 Single Photon Emission Computed Tomography

#### *$^{201}$ Thallium*

$^{201}$Thallium ($^{201}$Tl) is taken up into the myocardial cells in the same way as potassium; by the sodium-potassium ATPase pump (Weich H 1977). Thus, both perfusion and viability are required for uptake. In ischaemic or hypoperfused myocardium, uptake is delayed when compared to normal myocardium and this results in the defect seen on early $^{201}$Tl imaging. On delayed imaging, usually 4 hours later (but with severe ischaemia it may be up to 24 hours later (Yang LD 1990)), activity washes out of normal myocardium but is still accumulating in or washing out significantly more slowly of ischaemic myocytes than normal myocardium and so the defect seen on the earlier images seems to "fill in". This is the basis of $^{201}$Tl redistribution, indicating reversible ischaemia on stress $^{201}$Tl imaging used for the detection of coronary artery disease. In contrast, in an area of previous myocardial infarction there is no redistribution because of lack of viable myocytes.

Nevertheless it was found that a number of these fixed, "non viable" segments did fill in with a reinjection of a small dose of $^{201}$Tl and 87% of these recovered function after angioplasty to the vessel supplying these segments (Dilsizian V 1990; Tamaki N 1991). Intuitively, the stress test associated with these protocols may result in an underestimation of viability due to induction of ischaemia in already compromised segments and so some (Perrone-Filardi P 1996; Gunning MG 1998) have studied the use of rest $^{201}$Tl injections followed by early, 4 hour, with or without 24 hour delayed

imaging to optimise redistribution. However the evidence for an incremental value is inconclusive. Overall, in comparison with recovery of function post revascularisation, the sensitivity of $^{201}$Tl imaging for detection of viable (hibernating) myocardium has been estimated at 89% with a specificity of 50-80% (Ohtani H 1990; Marzullo P 1993).

### *$^{99m}$Technetium ($^{99m}$Tc) labelled perfusion agents*

$^{99m}$Tc labelled perfusion agents, such as $^{99m}$Tc sestamibi and $^{99m}$Tc tetrofosmin, have become very widely used in cardiac nuclear medicine practice because of their favourable imaging characteristics and the lower radiation doses in comparison with $^{201}$Tl. The lack of redistribution, one of the features that make them attractive for the routine detection of ischaemia and in acute studies (Hilton TC 1994; Heller GV 1998), is possibly disadvantageous for imaging of hibernating myocardium. Thus the reduced uptake of these tracers after a rest injection reflects the reduced myocardial perfusion at rest which characterises this condition. Indeed it has been reported that in moderate to severe fixed sestamibi defects, 13-60% of segments were viable by PET scanning (Altehoefer C 1994; Sawada SG 1994). See Figure 1.3.

It has been proposed that interventions capable of increasing coronary flow during tracer uptake could improve the detection of hibernating myocardium with sestamibi or tetrofosmin. Short term nitrate administration significantly increases coronary flow (Cohn PF 1977) and it may be that administration of nitrates could improve tracer

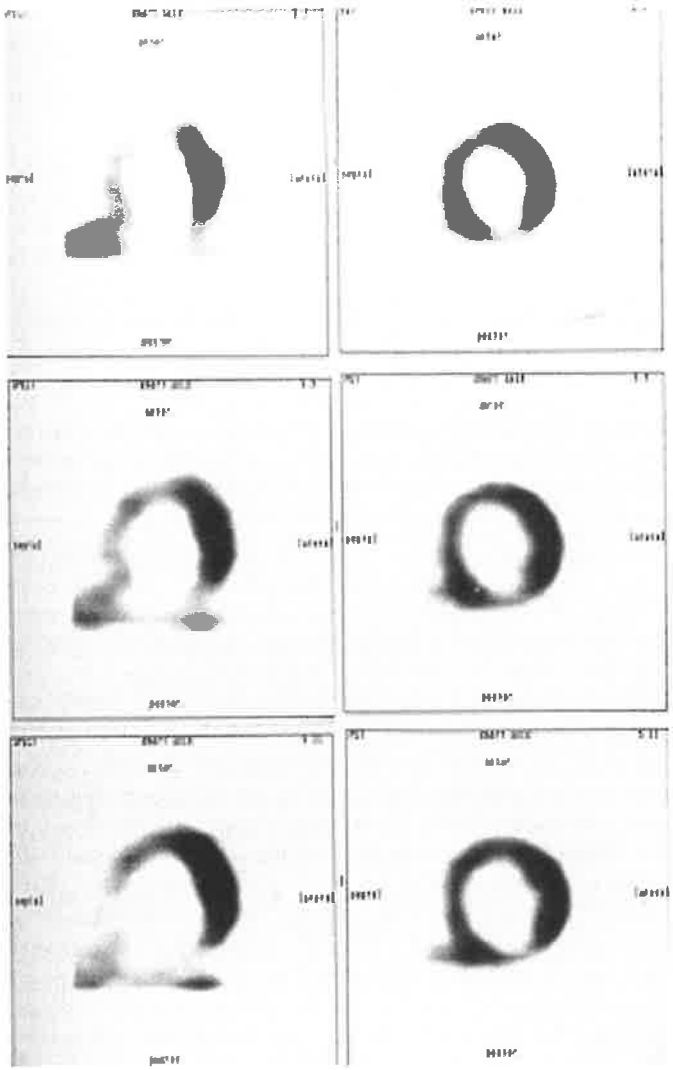


**Figure 1.3** Myocardial PET imaging. Short axis views are shown for rest $^{99m}$Tc sestamibi perfusion images (left column) and $^{18}$FDG metabolism images (right column). Sestamibi images show reduced activity in the anteroseptal and inferior walls with preserved glucose uptake. Thus the myocardium is viable, but hibernating. From Althoefer et al (Altehoefer C 1994).

deliver to the affected myocardium. Some studies have reported a reduction in rest sestamibi defect size with administration of nitrates and promising results in prediction of post revascularisation recovery of regional function (Bisi G 1994; Li ST 1996).

Quantitation of sestamibi or tetrofosmin uptake in myocardial segments may improve stratification of myocardial segments into those that are likely to show recovery of function and those which are not. Activity (counts detected) within segments is compared to peak myocardial activity and is expressed as a percentage. Regional activity has been compared to recovery of function and to histological appearances and amount of fibrosis. Dakik et al (Dakik HA 1997) found that 55% threshold of $^{99m}$Tc sestamibi activity had positive and negative predictive values of 79% and 100% respectively. The extent of fibrosis correlated inversely with sestamibi uptake. Maes et al (Maes AF 1997) in a similar study also found an inverse linear relationship between percent fibrosis and sestamibi uptake and reported an optimal uptake threshold of 50%.

Gating the sestamibi acquisition and acquiring the images during low dose dobutamine infusion allows combination of the information from quantification of sestamibi uptake with improvement in wall motion due to ionotropic reserve. Comparison with dobutamine echocardiography and recovery of function post revascularisation by Lishmanov et al (Lishmanov YU 2000) suggested higher sensitivity than dobutamine echo but marginally lower specificity while Lopez et al

(Yoshinaga K 2001) reported a sensitivity of 76% and a specificity of 100% when compared with PET.

### *$^{18}$FDG SPECT*

While $^{18}$FDG is thought to be the most appropriate tracer for detecting hibernating myocardium, its use is limited by availability of PET cameras and cyclotrons for its production. The short half life makes it difficult to be transported reliably to places remote from the cyclotron. The expense of establishing and maintaining a PET centre and cyclotron makes it unlikely that all population centres will have this technology in the near future. Therefore the adaptation of $^{18}$FDG to SPECT camera technology has seemed an attractive alternative. Bax et al (Bax JJ 1997) estimated the sensitivity and specificity of $^{18}$FDG SPECT as 85% and 75% respectively using recovery of function post revascularisation as the comparative standard. Comparison of $^{18}$FDG SPECT with $^{201}$Tl studies suggest similar accuracy when compared to PET. Other studies have generally found 80-90% concordance between imaging modalities (Table 1.1).

### *$^{123}$I BMIPP*

$^{123}$Iodine labelled fatty acids such as 15-p-[123I]iodophenyl-3-(R,S)-methyl-pentadecanoic acid (BMIPP) have also been proposed as radiopharmaceuticals useful in the detection of hibernating myocardium. Reduced fatty acid uptake in ischaemic tissues has been reported and so the net effect may be reduced activity in hibernating

| Author | No. of patients | Comparative Standards | Results |
|---|---|---|---|
| Burt et al (1995) | 20 | Rest-redistribution Tl-201 | F-18 FDG uptake in 23% of fixed Tl-201 defects |
| | | F-18 FDG PET | 91% concordance |
| Bax et al (1996) | 20 | F-18 FDG PET | 77% concordance |
| | | F-18 FDG PET | 76% concordance |
| Bax et al (1996) | 17 | Functional recovery | 62% PPV, 94% NPV |
| Bax et al (1997) | 55 (10 overlap with above) | Functional recovery | 64% PPV, 91% NPV |
| Bax et al (1997) | 22 (EF<30%) | EF increase > 5% | 100% sensitivity, 80% specificity |
| Chen et al (1997) | 18 | F-18 FDG PET | 90% concordance |
| Srinivassan et al (1998) | 28 | F-18 FDG PET | 94% concordance |

**Table 1.1** Studies of 18-FDG SPECT in detection of hibernating myocardium. From Udelson (Udelson JE 1998). PPV represents positive predictive value; NPV, negative predictive value; EF, ejection fraction.

myocardium (Opie EH 1997). A mismatch, with BMIPP uptake less than perfusion, has been investigated by others (Dobbeleir AA 1999). The role of BMIPP in the detection of hibernating myocardium is still unclear.

***Hypoxia markers***

The hypoxia avid radiopharmaceuticals are taken up in areas of chronically hypoxic or ischaemic myocardium. They are therefore seen as a "hot spot" rather than as a defect. Nitroimidazoles were first developed as radiosensitising agents for use with radiotherapy of tumours. They are taken up in hypoxic cells but are not retained by necrotic cells and so were proposed as a possible marker of hypoxic but viable tissue (Chapman J 1981).

The nitroimidazoles are thought to passively diffuse across the cell membrane where they are reduced with formation of a $R\text{-}NO_2^{\cdot -}$ radical. This occurs in the presence or absence of oxygen. In normoxic conditions, the radical interacts with oxygen, yielding superoxide and non charged nitroimidazole which can then diffuse back across the cell membrane. In the absence of oxygen, the nitroimidazole is further reduced and metabolites (nitroso compounds and hydroxylamines) become less permeable and are retained in the cell, binding to intracellular macromolecules (Sinusas A 1999). Fluorine-18-fluoromisonidazole, a positron emitting tracer, has been investigated in the detection of hibernating myocardium (Martin GV 1992) in a canine model and correlated favourably with both histological findings and with recovery of function after revascularisation. Tc-99m labelled nitroimidazoles have

also been investigated in animal or isolated heart models with similar promising results.

## 1.4 Inducible Nitric Oxide Synthase

### 1.4.1 Introduction

In the 1970's it was discovered that nitric oxide, a relatively simple inorganic gas, could activate guanylate cyclase and so elevate the tissue levels of cyclic guanosine monophosphate (cGMP) (Arnold WP 1977). Gruetter et al (Gruetter CA 1979) found that the activation of guanylate cyclase by nitric oxide resulted in vasodilatation of bovine coronary arteries. Simultaneously, it was discovered by Furchgott et al (Furchgott RF 1980) in rabbit aortas that release of a substance by the endothelium was responsible for acetylcholine induced vasodilatation. For the want of a better label this substance was then referred to as Endothelium Derived Relaxing Factor or EDRF. During the 1980's it was discovered that EDRF caused vasodilatation through activation of guanylate cyclase and increased levels of cGMP (Griffith TM 1985; Mulsch A 1987). The similarity between the actions of nitric oxide and EDRF were not lost on Furchgott and colleagues (Martin W 1985) and in the late 1980's it was realised that EDRF and nitric oxide were one and the same (Palmer RM 1987).

Nitric oxide is synthesised in vivo through the actions of a group of enzymes known as nitric oxide synthases which belong to the cytochrome P450 family of enzymes. Three isoforms of nitric oxide synthase have been identified and they share between 50% and 60% amino acid homology. All three enzymes contain flavin adenine dinucleotide and tetrahydrobiopterin as cofactors. Nitric oxide synthases catalyse the conversion of the amino acid L-arginine to L-citrulline in the presence of oxygen and NADPH and in the process release nitric oxide (NO). Where L-arginine is not available as a substrate, they may function as oxidases and release superoxide and hydrogen peroxide (Stamler JS 1996).

The two 'constitutive' isoforms of nitric oxide synthase were named after the tissue in which they were originally found. Neuronal (nNOS) nitric oxide synthase is also known as NOS1 and is expressed in neurons and epithelial cells and possibly in cardiac myocytes (Harrison RW 2000). Endothelial (eNOS) nitric oxide synthase is also known as NOS3 and was originally described in endothelial cells (Lamas S 1992; Nishida K 1992; Sessa WC 1992) but has since been demonstrated in endocardial cells, kidney epithelial cells, hippocampal pyramidal neurons, skeletal myocytes and cardiac myocytes. Both nNOS and eNOS function at physiological concentrations of calcium which regulates binding of calmodulin, allowing the electron transfer necessary for facilitating conversion of L-arginine to NO and L-citrulline. They have been referred to as "calcium sensitive" isoforms of NOS.

Inducible nitric oxide synthase (iNOS), on the other hand tightly binds calmodulin even at low calcium concentrations and is termed "calcium insensitive". iNOS was first described in macrophages (Lyons CR 1992; Xie QW 1992) but has since been described in a large variety of cell types such as endocardial cells, endothelial cells, vascular smooth muscle cells, fibroblasts, mesangial cells among others. iNOS is also expressed in cardiac myocytes (Shindo T 1995). In cardiac myocytes iNOS seems to localise to the perinuclear space (Buchwalow IB 1997), Golgi complex, mitochondria, plasma membrane and along the contractile fibres.

iNOS is induced in conditions involving inflammation and is thought to be the mechanism of severe hypotension in septic shock (Brady ASB 1992). It seems to have a role in the immune response to certain pathogens and evidence from iNOS knockout mice suggest this may be protective (MacMicking JP 1995; Wei X-Q 1995).

### 1.4.2 Regulation of iNOS Expression

***Induction of iNOS by cytokines***

Inducible nitric oxide synthase expression in the myocardium was originally observed in response to lipopolysaccharide and cytokines (Xie QW 1992). Schultz et al (Schulz R 1992) demonstrated iNOS expression in ventricular myocardium from rats pretreated with endotoxin as well as in isolated adult rat ventricular myocytes in

response to IL-1β and TNF α. These findings have been confirmed by others (Tsujino M 1994; Ungureanu-Longois D 1995; Song W 2000) however Kan et al have found that cardiac myocytes in culture did not express iNOS in response to TNF α alone (Kan H 1999) although the addition of TNF α did enhance iNOS expression in response to IL-1β. In addition to IL-1β and TNF α induction, iNOS is expressed in response to IL-6 (Kinugawa K 1997) although others have found no iNOS expression in response to IL-6 (Shindo T 1995). Expression induced by IL-1β is enhanced by the addition of Interferon gamma (IFN γ) (Balligrand J 1994). In other cell types, such as macrophages (Diaz-Guerra MJ 1999), iNOS may be expressed in response to IFN γ alone. While some studies have found that IFN γ alone also results in iNOS expression in cardiac myocytes (Balligrand J 1994; Luss H 1995), others maintain that addition of another cytokine is required (La Pointe M 1996).The effects of cytokines and lipopolysaccharide on iNOS expression can be diminished by Transforming Growth Factor beta (TGF β) (Roberts AB 1992) and by dexamethasone and osteopontin (Singh K 1995; Kunz D 1996).

Cytokine induction of iNOS expression involves a complex sequence of intracellular signal transduction pathways (Figure 1.5). Of these the best understood is that resulting from IL-1β activation. Singh et al (Singh K 1995) provide evidence that activation of the extracellular signal related (ERK1 and ERK2) kinase members of the Mitogen Activated Protein Kinases (MAPK) is essential for induction of iNOS in response to IL-1β. They also showed that Protein Kinase C (PKC) was involved in

the IL-1β cell signalling pathways as inhibition of PKC resulted in reduced iNOS expression. The involvement of PKC has been confirmed in other studies (La Pointe M 1996). The ERK1/ERK2 kinases then cause increased iNOS expression by activation of the activation protein 1 (AP-1) DNA-protein binding site (Kristof AS 2001).

IFN γ on the other hand, through the Janus family of kinases (JAK1 and JAK2), seems to activate STAT (signal transducer and activator of transcription) 1 alpha which translocates to the nucleus and binds to the IFN γ activated site (GAS) (Kelly RA 1996; Coccia EM 2000). However these are only two of several postulated signal transduction cascades (La Pointe MC 1999; Rao 2000) resulting in binding of different transcription factors to the cis-regulatory elements of the iNOS gene 5' flanking region .

***The iNOS promoter region***

The iNOS promoter regions of the rat, mouse and human iNOS genes have been cloned and analysed. All or most of the mouse iNOS cis-regulatory elements required for transcription control are found in a 1.6 kb 5' flanking region to the promoter (Chu SC 1998). They include Nuclear Factor kappa B (NFκB) binding sites, IFN γ response sites, Nuclear Factor–IL6 response elements, a GAS, and an IFN γ stimulated response element – all of which are involved in conferring iNOS responsiveness to IL-1β and IFN γ.

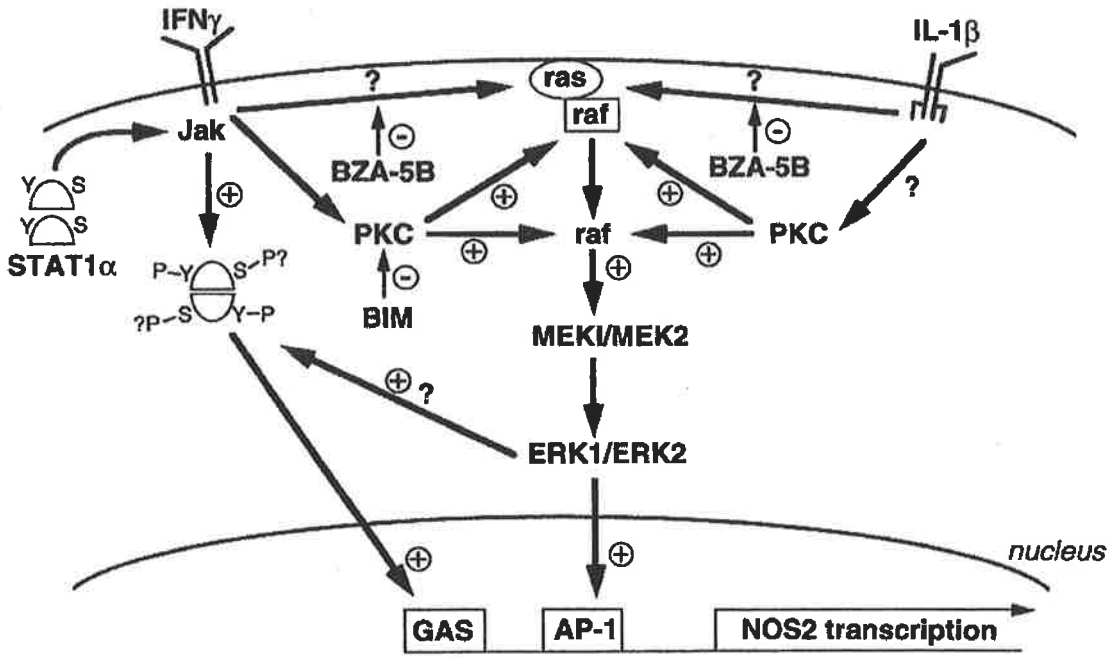



**Figure 1.5** Some of the signal transduction pathways involved in cytokine induced iNOS expression in cardiac myocytes. From Kelly et al (Kelly RA 1996). MEK indicates MAP or ERK kinase kinase; ras and raf are GTPases involved in signal transduction associated with tyrosine kinase receptors; BIM indicates bisindolylmaleimide and inhibitor of diacylglycerol activated PKC; BZA-5B is an inhibitor of farnesyl transferase.

The rat iNOS gene with its promoter region has also been cloned and characterised (Keinanen R 1999). The gene spans 36 kb and comprises 27 exons and 26 introns. The 2.6 kb 5' flanking regulatory region of the rat iNOS gene contains a number of cis-acting regulatory sites : two NFκB binding sites, four AP-1 sites, two interferon stimulated response sites, two GAS, an Octamer (Oct) sequence, two Tumour Necrosis Factor response elements (TNF RE), three Interleukin-6 response sites and a CAAT enhancer binding site (see Figure 1.6). A hypoxia responsive element (HRE) and a cAMP responsive element (CRE) were also identified in the rat promoter region.

The human iNOS gene is located on Chromosome 17 and is approximately 37 kb in size with 26 exons and 25 introns (Chartrain NA 1994). The 5' flanking regulatory sequences occupy a region up to 16 kb in length (de Vera ME 1996) and additional regulatory sequences are found in the 3' flanking region, which may also be important in maximal iNOS activity (Nunokawa Y 1997). Response elements reported in the human iNOS promoter region include AP-1, NFκB, interferon gamma regulatory factor (IRF) binding sites, GAS, NF-IL6, TNF RE, CAAT box, CRE, HRE.

In all three species NFκB (Taylor BS 1998) and AP-1 sites mediate cytokine (TNF α and IL-1 beta) and lipopolysaccharide (Diaz-Guerra MJM 1996) induced iNOS expression. The transcription factor binding the specific TNF responsive elements remain unclear. IFN γ results in activation of the GAS by STAT 1. IFN γ also induces

Interferon Regulatory Factor 1 binding to the IRF binding site which enhances iNOS expression. The octamer sites are activated by Octamer binding proteins (Oct 1 and 2) from the POU family (containing both a homeodomain and a second DNA binding domain and named after the first four factors discovered in this group: Pit-1, Oct-1/2, Unc-86) of transcription factors (Gay RD 1998) and appear to be important in IL-6 induction of iNOS (Sawada T 1997).

The CAAT site is an enhancer and results in increased promoter activity in response to another stimulus. The cyclic AMP responsive site also acts as an enhancer rather than being the sole stimulus for iNOS expression (Ikeda U 1996) but seems to do so by stabilisation of the iNOS mRNA (Oddis CV 1995) resulting in increased protein production per mRNA copy.

In the human iNOS promoter, the specific binding sites, for example NFκB, number up to ten times that found in the mouse iNOS 5' regulatory region (Chu SC 1998) or more. Multiple sites and multiple types of response elements allow a complex interaction in the regulation of iNOS expression. Involvement of many sites results in an amplification cascade, whereas other circumstances, involving activation of fewer sites or of inhibitory sites may result in expression to a lesser degree. Presumably, the more complex the 5' regulatory region, the more "fine tuning" there is in terms of expression in response to different conditions. Chu et al (Chu SC 1998) using A549 cells, a human alveolar epithelium like carcinoma line, reported that the combination

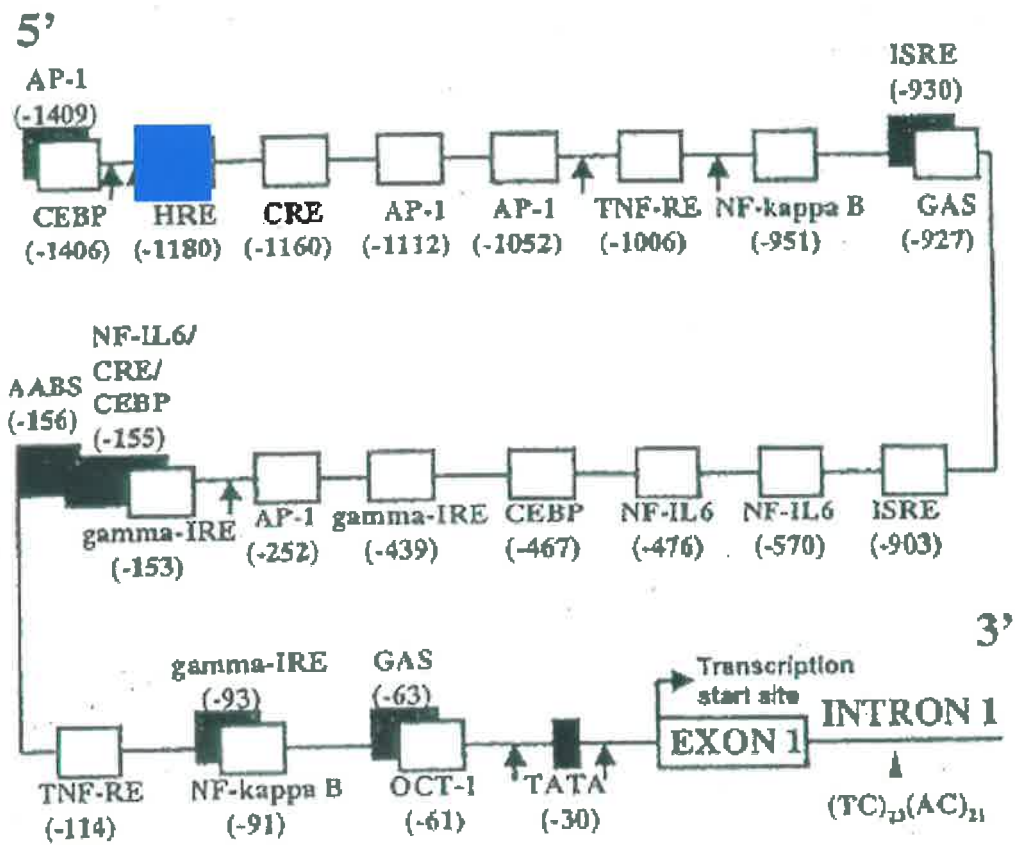



**Figure 1.6** The rat iNOS promoter region, featuring a number of cis-regulating elements. From Keinanen et al (Keinanen R 1999).

of TNF α, IL-1β and IFN γ results in maximal stimulation of the human iNOS promoter. The combination of IL-1β and IFN γ resulted in stimulation that was 41% of maximal and the combination of TNF αand IFN γ resulted in minimal (1.5%) stimulation.

***The Hypoxia Responsive Element***

Hypoxia has recently become recognised as an independent stimulus to induction of iNOS. Initial reports found variable iNOS expression in different tissues in response to hypoxia. Archer et al (Archer SL 1995) studied iNOS expression in rat mesangial cells and the effect of graded hypoxia. Cultured rat mesangial cells were treated with lipopolysaccharide and exposed to different grades of hypoxia ranging from 0% oxygen up to 21% oxygen and found no difference in the level of iNOS transcription at the different oxygen levels. However, the effect of hypoxia alone was not tested.

Kacimi et al (Kacimi R 1997) studied the effect of hypoxia in neonatal rat ventricular myocytes. The cultures were exposed to 1% oxygen and compared with normoxic controls. Nitrite content, as an indicator of nitric oxide production, was measured in the medium. It was found that hypoxia alone had no effect on nitrite release and attenuated IL-1β induced release of nitrite. These findings were confirmed when iNOS mRNA levels were measured; hypoxia resulted in no iNOS mRNA induction, but attenuated the level of iNOS mRNA production in response to IL-1β.

In contrast, Wang et al (Wang D 1999) reported that neonatal rat ventricular myocyte cultures exposed to 5% oxygen did produce iNOS mRNA, but no protein was detected on Western blotting. Also, the levels of mRNA were significantly less than that produced in response to IL-1β. The combination of hypoxia and IL-1β appeared to be synergistic. The authors concluded that hypoxia is a weak stimulus for iNOS induction in cardiac myocytes.

As stated previously (Keinanen R 1999) the iNOS promoter region contains a hypoxia responsive element. Using reporter gene constructs and a transfected murine macrophage cell line, Melillo et al (Melillo G 1995) found that activation of the iNOS hypoxia responsive element was inducible by hypoxia but not by IFN γ or by LPS.

Semenza et al (Semenza G 1997) reported that the transcription factor binding to the hypoxia responsive elements of the hypoxia responsive genes (such as erythropoiten, heme oxygenase 1 and vascular endothelial growth factor) is the Hypoxia Inducible Factor 1 (HIF 1). These authors showed that HIF 1 consists of two subunits as a heterodimer. The HIF 1α subunit is unique to HIF 1, but the HIF 1β subunit is the aryl hydrocarbon receptor nuclear translocation (ARNT) protein and may form other heterodimers. HIF 1α mRNA may be induced by hypoxia but primarily, regulation is mediated by degradation. The HIF 1α subunit is continually synthesised and degraded through the ubiquitin-proteasome system under normoxic conditions. This was shown by Salceda et al (Salceda S 1997) using proteasome inhibitors and has been confirmed by others (Sutter CH 2000). Thus hypoxia confers HIF 1α stability

and allows heterodimer formation and DNA binding activity. Huang et al (Huang LE 1996) confirmed that activation of HIF 1 is dependent upon stabilisation of HIF 1α under hypoxic conditions and went on to report that this is mediated by an oxygen dependent degradation domain (Huang LE 1998) consisting of approximately 200 amino acid residues in the centre of the HIF 1α subunit. Deletion of this domain confers stability of the subunit even under normoxic conditions and when fused to a stable protein, confers oxygen dependent instability.

Taking these studies into account, it seems logical that iNOS, with its hypoxia responsive element, would be induced in cardiac myocytes under hypoxic conditions. Indeed, most recently, Jung et al (Jung F 2000) demonstrated iNOS mRNA in myocardial tissue from rats exposed to hypoxia for three weeks. The authors also demonstrated that neonatal rat ventricular myocytes in vitro expressed iNOS in response to hypoxia. Using electromobility shift assays, they confirmed that this involved DNA binding of HIF 1 to the hypoxia responsive element of the iNOS promoter region. Specific binding was demonstrated by elimination of HIF 1 binding by single base mutation of the HRE.

***The Antioxidant Responsive Element***

The antioxidant responsive element is a cis regulatory element, which is thought to activate genes encoding proteins involved in the protection of eukaryotic cells against oxidative stress. The antioxidant responsive element was characterised in the early 1990's in the 5' flanking region of the glutathione S transferase Ya subunit gene

(Rushmore T 1991). It has also been identified in the 5' flanking region of the NADP(H) quinone reductase gene (Favreau LV 1991; Jaiswal AK 1991). The antioxidant responsive element is activated in response to an increase in planar aromatic compounds and phenolic antioxidants as well as hydrogen peroxide but the specific DNA binding protein has not been identified. The binding site resembles two adjacent AP-1 like sites (Friling RS 1992).

Perhaps not surprisingly, antioxidant responsive element binding activity is increased under hypoxic conditions. This was shown by Waleh et al (Waleh NS 1998) who transfected HepG2 and Hepa cells with plasmid constructs containing the human antioxidant responsive element and the CAT reporter gene and exposed these cultures to 6 or 24 hours of hypoxia.

The iNOS promoter also contains an antioxidant responsive element. Kuo et al (Kuo PC 2000), using rat hepatocytes, report an antioxidant responsive element in the rat iNOS promoter at base pair –1347 and confirmed binding activity in conditions of oxidative stress, resulting in augmented protein expression.

### 1.4.3 Role of iNOS in Myocardial Pathology

iNOS expression and/or activity has been reported in various myocardial conditions, both in animal models or human diseases. The growing number of such studies imply

a significant role for iNOS in the myocardium, but the nature of this role or whether it is adaptive or deleterious remains controversial.

***iNOS in Myocardial Infarction***

Few, if any reports exist on the expression of iNOS in human myocardium during or after myocardial infarction, however Akiyama et al (Akiyama K 1998) report that products of nitric oxide (nitrites and nitrates) are increased in coronary sinus blood from patients with myocardial infarction compared to controls.

A number of animal models of myocardial infarction (Akiyama K 1997; Yamamoto T 1999; Takimoto Y 2000) have shown iNOS expression and activity in infarcted myocardium, compared with remote regions. Wang et al (Wang D 1999), using an LAD ligation model of myocardial infarction in rats, found iNOS expression by Northern blot in the infarct zone. In these areas iNOS was expressed by cardiac myocytes. Furthermore, pretreatment of rats with a specific iNOS inhibitor, S methyl isothiourea (SMT), resulted in reduced infarct size, suggesting a deleterious effect of iNOS in myocardial infarction.

***iNOS in ischaemic preconditioning***

Ischaemic preconditioning is the phenomenon whereby a "mild" ischaemic stress enhances tolerance of the myocardium to subsequent more severe ischaemic events. The initial early phase of preconditioning ("classical preconditioning") lasts approximately 2-3 hours after the first ischaemic episode. This is followed

approximately 24 hours later by a second more prolonged "window of protection" which may last 72 hours (Ferrari R 1999; Edwards RJ 2000). iNOS expression has been demonstrated during the second window of preconditioning and furthermore, addition of iNOS inhibitors has been shown to reduce the protective effects of the second window of preconditioning (Takano H 1998; Guo Y 1999; Imagawa J 1999). Thus, under these circumstances of "mild" ischaemia short of infarction, iNOS appears to have a protective effect.

***iNOS in Cardiac Failure***

iNOS expression has also been documented in congestive cardiac failure. Haywood et al (Haywood GA 1996) were able to study endocardial biopsies from 51 patients with cardiac failure. They used RT PCR to detect iNOS mRNA and immunohistochemistry to detect iNOS protein and found expression in 71% of cardiac failure patients, but in none of the control hearts. By immunohistochemistry they were able to show that iNOS was expressed by the cardiac myocytes themselves. iNOS expression occurred in all forms of heart failure, whether dilated cardiomyopathy, ischaemic heart disease or valvular heart disease. These results have also been confirmed by other groups (Fukuchi M 1998). The fact that iNOS is expressed in heart failure of all causes suggests that it may be a result of, rather than related to, its cause. Nevertheless, the role of iNOS in heart failure has not yet been determined.

***iNOS in septic shock***

Shock and multiple organ failure are complications of severe bacterial, and viral, infections. The profound hypotension associated with this syndrome has been thought to be due to generalised vasodilatation, however there is evidence for a myocardial contractile dysfunction (Ellrodt AG 1985; Reilly JM 1989) which may contribute to the clinical presentation but may also lead to fatal outcomes (Vincent JL 1992). Animal models of septic shock have demonstrated iNOS expression (Wu CC 2000) and specifically in the heart (ter Steege JC 2000). It has been suggested that the myocardial dysfunction associated with septic shock may be due to the myocardial effects of iNOS (Ungureanu-Longois D 1995).

### 1.4.4 Expression of iNOS in Hibernating Myocardium

Two preliminary reports have studied the expression of iNOS in hibernating myocardium in human subjects. Baker et al (Baker CSR 1999) took left ventricular biopsies from seven patients with hibernating myocardium identified preoperatively by PET and confirmed postoperatively by recovery of regional myocardial function. They compared these to biopsies taken from normally contracting myocardium from patients with coronary artery disease and from unused donor hearts and from post mortem samples. iNOS was found in the biopsies from hibernating myocardium but not in normal controls. These results confirm those of Depre et al (Depre CF 1997) who found iNOS expression by immunohistochemistry in hibernating myocardium

from patients undergoing coronary artery bypass surgery and also in myocardium from irreversibly dysfunctional segments but not in segments of normally contracting myocardium.

More recently, Kalra et al (Kalra DK 2002), prospectively studied thirteen patients with left ventricular dysfunction due to ischaemic heart disease scheduled to undergo CABG. They underwent preoperative dobutamine echocardiography and intraoperative myocardial biopsy. Left ventricular function was reassessed three months after revascularisation. Those segments of myocardium found to be "hibernating" showed increased expression of both iNOS and TNF α compared to normal myocardium. Segments with irreversible dysfunction (infarcted) showed highest levels of expression of both iNOS and TNF α.

## 1.5 Nitric Oxide and Myocardium

### 1.5.1 NO and negative inotropy

As discussed above, circumstantial evidence suggests that NO produced by iNOS may have a role in depression of myocardial contractile function in septic shock. It has been demonstrated in a canine model that cytokines cause sustained myocardial dysfunction through production of nitric oxide by iNOS (Oyama J 1998). Early

reports that iNOS expression resulted in reduced positive ionotropy in paced isolated adult rat cardiac myocytes in response to isoprenaline (Balligand JL 1993) also served to stimulate interest in the myocardial depressive effects of nitric oxide.

Mohan et al (Mohan P 1996) examined the effect of nitric oxide donors on isolated papillary muscle function and found a biphasic response with enhanced contractile function at low concentrations but a negative ionotropic effect at high concentrations. These effects were reproduced by 8-bromo-cGMP and so the authors concluded this was a cGMP mediated effect. Others (Weiss HR 1997; Sandirasegarane L 1999) have found a similar negative ionotropic response in response to nitric oxide but found it was cGMP and cAMP independent.

Thus, the mechanism of negative ionotropy caused by nitric oxide in cardiac myocytes is yet to be elucidated. Given the importance of the mitochondrial function to myocardial contractility, attention has also been focussed on the effects of nitric oxide in the mitochondria.

### 1.5.2 NO and mitochondrial function

***NO and the respiratory chain***

Borutaite and Brown (Borutaite V 1996) studied rat heart mitochondria and measured oxygen consumption (changing oxygen content, as an indicator of mitochondrial

respiration) and nitric oxide concentration in the medium with oxygen and nitric oxide electrodes respectively. NO was added to the medium in graded , but sub-micromolar, concentrations in normoxic, hypoxic (52-65 μM oxygen) and high oxygen (126-168 μM oxygen) conditions. NO was found to inhibit mitochondrial respiration in a dose dependent manner and this was rapidly and completely reversible. The effect was greater in hypoxic conditions. Through the addition of respiratory inhibitors or omission of substrates, the authors concluded that the effect was through the interaction of nitric oxide with (possibly competition with oxygen for) cytochrome oxidase (complex IV) and also to some degree with NADH-ubiquinone-1 reductase (complex I). Others have reported similar results (Lizasoain I 1996) or have reported greater effect on different complexes within the respiratory chain (Cassina A 1996) but confirmed the enhanced effect under hypoxic conditions.

Bolanos et al (Bolanos J 1994) studied the effect of nitric oxide produced by iNOS on the mitochondrial respiratory chain in cultured astrocytes. Prolonged incubation for 36 hours produced a 56% reduction in cytochrome oxidase activity but no effect on complex I. There was an increase in glycolysis and lactate formation.

***Effect of peroxynitrite***

Xie et al (Xie Y 1996), using isolated bovine cardiac muscle, confirmed the findings discussed above that nitric oxide inhibits mitochondrial function reversibly, but that the combination of SNAP and pyrogallol (a superoxide releasing agent) resulted in only slowly or partially reversible inhibition of mitochondrial function after washout.

The effect of both SNAP and pyrogallol was inhibited by 100 μM uric acid, a peroxynitrite scavenger. The authors therefore concluded that nitric oxide reversibly inhibits mitochondrial function but peroxynitrite does so less reversibly, or perhaps irreversibly depending on the amount of peroxynitrite generated. The presence of superoxide as a byproduct of mitochondrial respiratory transport in combination with a large quantity of nitric oxide allows the formation of increased amounts of peroxynitrite.

***Relation to myocardial function***

Tatsumi et al (Tatsumi T 2000) used spontaneously beating neonatal rat cardiac myocytes to demonstrate that the nitric oxide release from cytokine induced iNOS resulted in reduced levels of ATP despite normoxic conditions. The activities of the mitochondrial transport chain complexes were also impaired and contractility was reduced. These effects were reproduced by the addition of sodium nitroprusside, a NO donor, but not by 8-bromo-cGMP, indicating a cGMP independent mechanism. It is therefore possible that the negative ionotropic effect of nitric oxide is due to its inhibitory effect on the mitochondrial electron transport chain, particularly its inhibition of cytochrome oxidase.

***Cell death***

Geng et al (Geng Y 1992) used cultured rat vascular smooth muscle cells to study the effects of cytokine induced nitric oxide and authentic nitric oxide gas on the mitochondria. They found that at levels of nitric oxide that reversibly inhibited

mitochondrial respiration, there was no loss of cell viability. These results were confirmed using addition of authentic NO gas, rather than iNOS production of nitric oxide. Once again these effects were not reproduced by addition of 8-bromo-cGMP. However at high doses of cytokines, there was significant loss of cell viability.

## 1.6 Apoptosis

### 1.6.1 Definition

The term "apoptosis" was coined in the early 1970's by Kerr et al (Kerr JF 1972) to describe a distinct form of cell death, different from necrosis. Apoptosis may be thought of as a deliberate mechanism that counters cell proliferation, a programmed cell death. It has a role in cell differentiation, organ development, cell maturation and immunological function. However, deregulated apoptosis may form the basis of atrophic diseases, neurodegenerative conditions and organ failure. On the other hand, inefficient apoptosis could lead to overproliferation, for example that found in neoplasia, autoimmunity and congenital malformations. In contrast to necrosis, apoptosis can be said to involve "suicide" mechanisms and is an active, energy dependent process.

The morphological changes in apoptosis include chromatin condensation and margination which lend a half moon or crescent shape to the nucleus (Majno G 1995). The mitochondria undergo subtle morphological changes early in the process, cytoskeletal alterations can be detected and there is membrane budding. The nucleus begins to fragment and within the nucleus DNA is cleaved into small fragments. This forms the basis of detection of apoptosis by TUNEL (TdT dUTP nick end labelling) or DNA laddering. The cytoplasm condenses and the cell is packaged into several apoptotic bodies which are eventually phagocytosed, preventing an inflammatory response. In contrast, necrosis is characterised by cellular swelling, membrane rupture with release of intracellular contents into the extracellular space and resulting inflammation. Resolution finally occurs through fibrosis.

One of the mechanisms of interaction between cell remnants and phagocytic cells seems to be between phosphatidylserine presented on the surface of apoptotic cells and the phosphatidylserine receptors on the surface of the phagocytic cells. Phosphotidylserine is usually found on the inner leaflet of the plasma membrane and is actively transported from the outer to the inner leaflet by the enzyme aminophospholipid translocase, but in apoptosis phosphatidylserine is exposed on the outer leaflet. This exposure of phosphatidylserine appears to be dissociated from the nuclear events of apoptosis and is not dependent on the inactivation of aminophospholipid translocase but appears to be a non specific reversal of phospholipid position in relation to the plasma membrane (Bratton DL 1997). Phosphatidylserine binds the coagulant protein Annexin V and cells presenting

phosphatidylserine on the outer surface plasma membrane may be detected using labelled Annexin V (Blankenberg FG 1999). This seems to detect apoptosis at an earlier stage than detecting nuclear events (Stuart MC 1998; Rucker-Martin C 1999) with TUNEL, DNA ladders and Hoechst staining (which detects nuclear chromatin condensation).

### 1.6.2 Mechanisms of apoptosis

***Initiation of apoptosis***

One of the best characterised mechanisms for the initiation of apoptosis is the "death receptor" (DR) pathway. Extracellular death signal proteins bind to these death receptors on the cell surface. A number of such receptors have been identified and include Fas, TNF receptor 1, DR3, DR4 and DR5. Their respective ligands are Fas ligand (FasL), TNF α, Apo-3L and TRAIL (TNF-related apoptosis inducing ligand) (Haunstetter A 1998). Fas is the best known death receptor ligand and studies of Fas deficient mice have provided insight into its role and the clinical implications of deregulation of apoptosis. Mice carrying a spontaneous mutation in the Fas ligand exhibit a phenotype of lymphoproliferative and autoimmune disease (Watanabe-Fukunaga R 1992). A human genetic disease, Canale-Smith syndrome, involving a mutation of the Fas gene has also been described (Drappa J 1996) and is also characterised by lymphoproliferation, autoimmune haemolytic disease, thrombocytopenia and a tendency to neoplasia.

The death receptors contain a cytoplasmic domain containing approximately 80 amino acids which are essential for the transmission of proapoptotic signals. This domain has been designated the "death domain". Activation of the death receptors results in signal transduction that involves a unique set of proteins. After ligand binding, the death receptors form complexes through protein-protein interactions of their death domains. This recruits intracellular death domain proteins. For example the Fas receptor and DR4 interact with FADD, the Fas associated death domain protein. FADD is known to directly interact with caspase 8. A summary of events leading to apoptosis is presented in Figure 1.7.

***Caspases as the executioners of apoptosis***

Apoptotic cell death occurs in two phases; first, the commitment to cell death and second, the execution phase which is characterised by the morphological cell changes described above. The caspases are a group of cysteine proteases which have an essential role in the execution phase and have been termed the final executioners of apoptosis (Cohen GM 1997). Activated caspases cleave important intracellular proteins, such as nuclear proteins, signal transduction proteins and cytoskeletal proteins which are essential for cell survival.

The caspases are synthesised as inactive proenzymes which are activated by cleavage at specific sites into a large and a small subunit which must recombine to form the active enzyme. All activating cleavages occur at an aspartate residue. At present 10

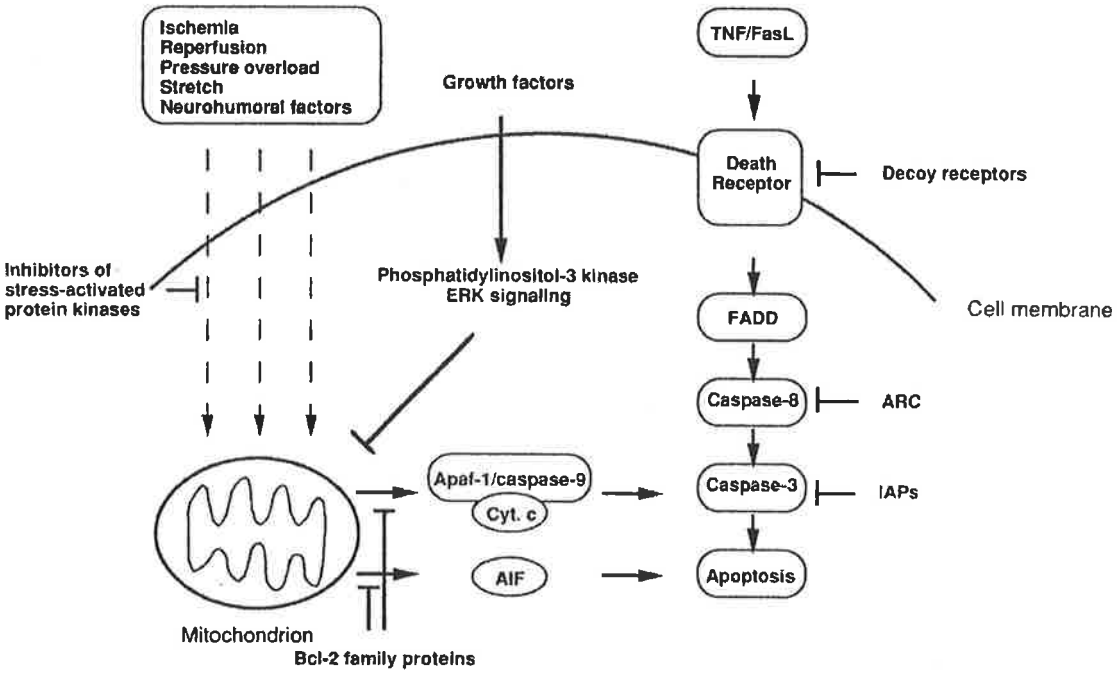



**Figure 1.7** Mechanisms of induction of apoptosis. From Haunstetter et al (Haunstetter A 2000). Cyt c represents cytochrome c; AIF, apoptosis inducing factor; IAPs, inhibitors of apoptosis; ARC, apoptosis repressor with a caspase recruitment domain.

caspases have been described but it is not clear whether they act as a sequential cascade or in parallel with redundant pathways. The caspase proenzymes have a prodomain and an enzymic domain which make up the subunits. The prodomains appear to be important for protein-protein interactions with the death domain proteins, such as FADD. Some caspases, such as caspase 2 and 8, have long prodomains and appear to have a significant regulatory role by interacting with the death domain proteins. It is also thought that "upstream" caspases may activate "downstream" caspases with amplification of activation (Cohen GM 1997).

The caspases with short prodomains seem to exert most of the activity that leads to the lethal proteolytic breakdown of cellular target proteins (Haunstetter A 1998). Caspase 3 is one of these key proteases in the caspase cascade and is responsible partially or fully for the proteolytic cleavage of many important cellular proteins such as poly ADP-ribose polymerase (PARP). Caspase 3 recognises a specific oligopeptide sequence for cleavage, DEVD (aspartate-glutamate-valine-aspartate).

DNA fragmentation requires the prior cleavage of a cytoplasmic inhibitor of the apoptosis specific endonuclease (caspase activated DNase) which then translocates to the nucleus and degrades genomic DNA (Enari M 1998).

***The mitochondria in apoptosis***

It has become apparent that a mechanism of cell commitment to apoptosis exists, that is independent of the death receptors. The mitochondria play a much more important role in the initiation of apoptosis than was first imagined.

Cytochrome c is a haemoprotein containing haem c as a prosthetic group which is bound to the outer surface of the inner mitochondrial membrane. Its function has been thought to be to transport electrons from cytochrome $c_1$ to cytochrome oxidase in the mitochondrial respiratory chain. More recently it has been found to have a role in apoptosis, in fact in the activation of caspase 3 (Liu X 1996). Cytochrome c is released from the mitochondria into the cytoplasm and initiates apoptosis (Kluck RM 1997). It has been found that interaction of apoptosis activating factor 1 (Apaf 1) and cytochrome c and dATP results in conversion of pro-caspase 9 to activated caspase 9 which in turn activates caspase 3 (Li P 1997; Zou H 1997). The mechanism of cytochrome c release from the mitochondria has not been completely elucidated.

A fall in the mitochondrial membrane potential occurs early in the apoptotic process and seems to mark the point of no return in the cell's commitment to apoptosis (Mignotte B 1998). This fall in the transmembrane potential is caused by the opening of pores, a process known as permeability transition. These pores are permeable to compounds up to 1.5 kDa in weight and allow equilibrium of ions and respiratory substrate, leading to the fall in membrane potential and also the arrest of ATP synthesis (Bernardi P 1992) but do not directly account for the release of cytochrome

c from the mitochondria. Permeability transition can be inhibited by N-methylvalyl-4-cyclosporin A and bongkrekic acid resulting in inhibition of apoptosis.

Reactive oxygen species are also important mediators of apoptosis. Exogenous sources of hydrogen peroxide for example, can result in either necrosis or apoptosis depending on the dose (Guenal I 1997). However reactive oxygen species derived from the mitochondria themselves seem to be involved in cell death signalling (Mignotte B 1998). The ubiquinone site in Complex III is the major site of mitochondrial production of reactive oxygen species as this site catalyses the conversion of molecular oxygen to superoxide anion. Disruption of the electron transport chain distal to this site results in accumulation of reactive oxygen species, but the mechanism by which these mediate apoptosis is not clear. Kowaltowski et al (Kowaltowski AJ 1996) suggest that opening of the permeability transition pores is dependent on reactive oxygen species generated by the mitochondria.

Fontaine et al may provide some insight into these mechanisms in their study of rat skeletal muscle mitochondria (Fontaine E 1998). They demonstrated that permeability transition pore opening is related to electron flow through complex I of the mitochondrial electron transport chain. Increased electron flow to complex I resulted in increased sensitivity of permeability transition pore opening to calcium concentrations, ie pore opening occurs at lower calcium concentrations.

***Regulation of apoptosis***

A number of regulatory proteins affect the sequence of events in apoptosis at different levels. Bcl-2 was first discovered as a translocation (t14:18) to the immunoglobulin heavy chain locus in B cell lymphomas and in acute lymphoblastic leukaemia. It seemed to promote tumour cell survival. Since then it has become clear that Bcl-2 is one of a family of proteins, members of which both promote and reduce cell survival. The pro-apoptotic factors in this family include Bax, Bak and Bad, while the anti-apoptotic factors include Bcl-2 and Bcl-xl.

Bcl-2 and other anti-apoptotic factors reside in the outer mitochondrial membrane and act, at least in part, by inhibiting release of cytochrome c from the outer mitochondrial membrane into the cytosol (Kluck RM 1997). In fact both Bax and Bcl-2 can act as membrane channels and it is possible that Bax channels allow release of cytochrome c and Bcl-2 may act to bind cytochrome c tightly preventing its release (Mignotte B 1998; Skulachev VP 1998). Bax and Bcl-2 may also have opposite effects on permeability transition and possibly the consequences of mitochondrial reactive oxygen species.

### 1.6.3 Nitric Oxide, iNOS and apoptosis

As discussed previously, nitric oxide in high doses can be cytotoxic. The mechanism by which this occurs appears to be predominantly apoptosis. Pinsky et al (Pinsky DJ

1999) studied the effects of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) on adult rat ventricular myocytes in primary culture. Apoptosis was detected by DNA ladders and TUNEL. The addition of SNAP resulted in apoptosis of cardiac myocytes in a dose dependent manner.

A previous study by the same group (Pinsky D 1995) suggested that the endogenous source of nitric oxide which generates the concentrations required for cell death is iNOS. Nitric oxide produced by macrophages caused cell death of adjacent cardiac myocytes. Ing et al (Ing DJ 1999) found that cytokines induced apoptosis in neonatal rat ventricular myocytes and that this effect was abolished by a nitric oxide synthase inhibitor. Others have repeated these results using specific iNOS inhibitors (Arstall MA 1999) and have suggested that the mechanism is via production of peroxynitrite as the addition of a peroxynitrite scavenger attenuated the apoptosis induced by iNOS.

Hortelano et al (Hortelano S 1997) provided an interesting insight into nitric oxide induced apoptosis. Using cultured thymocytes, they found that the addition of nitric oxide donors induced apoptosis which could be prevented by the addition of inhibitors of mitochondrial permeability transition. Cell death was preceded by a reduction in mitochondrial transmembrane potential (mitochondrial permeability transition) and exposure of phosphotidylserine residues on the plasma membrane. Isolated mouse liver cell mitochondria also underwent swelling indicative of

permeability transition in response to three different nitric oxide donors (S-nitroso-N-acetylpenicillamine, 1,3-morpholinosydnomine and S-nitroso-glutathione).

Brookes et al (Brookes PS 2000) exposed isolated rat liver mitochondria to nitric oxide donors (NONOates) and measured permeability transition (through mitochondrial swelling) and cytochrome c release. They found that nanomolar concentrations of nitric oxide inhibited permeability transition but at higher concentrations permeability transition was accelerated. However, Borutaite et al (Borutaite V 2000) found that addition of nitrosothiols as nitric oxide donors could open the permeability transition pores in isolated mitochondria, but NONOate nitric oxide donors could not. This suggests that the transnitrosylation associated with nitrosothiol nitric oxide donors is important for permeability transition rather than nitric oxide alone.

Packer and Murphy (Packer MA 1995) are perhaps able to reconcile some of the conflicting results. They found that exposure of isolated mitochondria to both nitric oxide and superoxide together caused permeability transition but neither alone did so. This suggests that nitric oxide interacts with superoxide to form peroxynitrite in the mitochondria and the peroxynitrite causes permeability transition.

### 1.6.4 Hypoxia and apoptosis

Cell death in myocardial infarction has always been ascribed to myocyte necrosis and has been detected by measurement of CK release into the plasma. Indeed this has been the most frequently used test for the diagnosis of myocardial infarction (Pasternak RC 1992) particularly in the absence of diagnostic ECG changes. More recently apoptosis has also been reported in myocardial infarction.

Saraste et al (Saraste A 1997) studied myocardial samples of eight patients who died of AMI and had patent infarct-related arteries at autopsy. Apoptotic cardiomyocytes were observed particularly in the border zones of histologically infarcted myocardium, whereas very few apoptotic cells were present in the remote noninfarcted myocardium. This study provides evidence that in addition to overt necrosis, myocytes also undergo apoptosis during ischaemia-reperfusion injury.

Olivetti et al (Olivetti G 1996) also studied myocardial samples obtained from the region adjacent to and remote from infarction in patients who died within 10 days. The analysis included 20 infarcted and ten control hearts. Apoptotic nuclei were observed in all 20 infarcted hearts in both the regions bordering on and distant from the necrotic myocardium. However, the number of apoptotic nuclei was greater in the peri-infarct region than in that remote from infarction.

Both these studies detected apoptosis in myocardial infarction some days after the clinical event (ie after death). Hofstra et al (Hofstra L 2000) studied in-vivo cell death in the hearts of patients with acute myocardial infarction using imaging with $^{99m}$Tc labelled annexin-V. In the patients with acute myocardial infarction, increased uptake of $^{99m}$Tc labelled annexin-V was seen throughout the infarct zone. No increased uptake was seen in the heart outside the infarct area. Thus, when studied earlier in the clinical course, apoptosis is a feature of the entire infarct zone rather than just the borderzones.

Apoptosis is now well documented in myocardial infarction but it has also been studied in models of hypoxia – reoxygenation. Holly et al (Holly TA 1999) used a rabbit model of myocardial infarction to show that caspases are activated during ischaemia and they found apoptotic nuclei in the infarct region. They found that caspase inhibition (with the "broad spectrum" caspase inhibitor, acetyl-Tyr-Val-Ala-Asp chloromethylketone) reduced the infarct size by 31% and therefore concluded that a significant proportion of cell death in myocardial infarction occurs by apoptotic mechanisms.

The specific trigger mechanisms of apoptosis in ischaemia or infarction are being studied by a number of groups. It is at present unclear whether the important stimulus is the hypoxia, the ischaemia or the reperfusion/reoxygenation. Webster et al (Webster K 1999) used neonatal rat ventricular myocytes cultured in 1% oxygen for 24 hours to study the effects of hypoxia alone on apoptosis. Apoptosis occurred only

with a decrease in pH in the media in the hypoxic environment. Reoxygenation of hypoxic myocytes also induced apoptosis. They concluded that acidosis or reoxygenation was required for apoptosis and that chronic hypoxia alone was not a strong stimulus for apoptosis.

Kang et al (Kang P 2000) studied adult rat ventricular myocytes cultured in 0% oxygen for 6 hours followed by 18 hours of reoxygenation. Apoptosis was detected by annexin V staining. They found that during hypoxia/reoxygenation cell death occurred predominantly by apoptosis. However, during prolonged (up to 24 hours) hypoxia or anoxia, nonapoptotic cell death predominated.

Other studies have also shown induction of apoptosis in myocytes cultured in anoxic (0% oxygen) conditions (Long X 1997; Malhotra R 1999; de Moissac D 2000) but the role of hypoxia (conditions of low oxygen availability, rather than extremely low) is still unclear.

### 1.6.5 Apoptosis in hibernating myocardium

Few human studies documenting the extent of apoptosis in hibernating myocardium exist. However preliminary reports (Angelini A 1998) have found apoptosis in biopsy specimens from patients with hibernating myocardium undergoing coronary artery bypass surgery.

A number of whole animal studies have been performed. Lim et al (Lim H 1999) used a pig model of hibernating myocardium with induced left anterior descending coronary artery stenosis causing reduced coronary flow. This model was maintained for up to three months. Sham operated pigs were used as controls. Histological changes consistent with previous reports in hibernating myocardium were found. Necrosis was not identified but the proportion of apoptotic nuclei was increased seven fold in the hibernating myocardium compared to myocardium in the sham operated group.

Chen et al (Chen C 1997) also used a pig model of hibernating myocardium and maintained it for up to four weeks. Using TUNEL and DNA laddering they found apoptosis in all hibernating segments with little evidence of infarction. Apoptosis was found not only at 24 hours but to a similar degree at four weeks. From this they concluded that apoptosis is an ongoing process in hibernating myocardium and is responsible for continual myocyte loss.

## 1.7 Potential Role of iNOS in Hibernating Myocardium

Current literature is unclear about whether iNOS is consistently expressed in hibernating myocardium as only two preliminary reports (Depre CF 1997; Baker

CSR 1999) exist in human subjects. Whether or not it has any great role to play in the pathophysiology of the hibernation process has not been investigated.

Nitric oxide can have both beneficial and detrimental effects on cardiac myocytes. Through the reversible effects of nitric oxide on mitochondrial function, iNOS may have a role in the downregulation of myocardial function in hibernating myocardium until coronary flow can be restored. On the other hand nitric oxide is known to result in cell death through apoptosis. This would result in irreversible myocyte loss and subsequent irreversible loss of function and a worsening prognosis.

## 1.8 Scope of the Current Study

The aims of the current study are to:

- Establish a cellular model of hibernating myocardium.
- Investigate the expression of iNOS in this model.
- Determine the factors regulating iNOS expression in this model.
- Test the hypothesis that iNOS is responsible for the downregulation in myocardial function in hibernation.
- Determine whether apoptosis is a feature of hibernation and the possible role of iNOS.

Chapter 2 outlines the methods used throughout the study, sources of the methods and any adaptations that have been made specific to the current study.

Chapter 3 describes the establishment of a cellular model of hibernating myocardium using neonatal rat ventricular myocytes. The characteristics of the model are described and compared with the characteristics reported in studies of animal models of hibernating myocardium and of human hibernating myocardium. Myocyte contraction, ionotropic reserve and biochemical characteristics are examined.

Chapter 4 examines the expression of iNOS in this cellular model of hibernating myocardium. The presence or absence of iNOS expression is documented using RT PCR following extraction of total RNA from cultures. Findings are compared to cytokine induction of iNOS, as a positive control, and to normoxic cultures.

Chapter 5 presents the findings of experiments to determine the regulation of iNOS in conditions of chronic oxygen deprivation. The expression of HIF-1$\alpha$ is studied and compared to baseline expression in normoxic controls. Activation of the hypoxia responsive element in the iNOS promoter is examined using electromobility shift assays. Activation of the NF$\kappa$B binding site is also examined and possible mechanisms of activation of this site are investigated by determining expression of cytokines under hypoxic conditions compared to normoxic controls.

Chapter 6 investigates the role of iNOS in chronic hypoxia. The effect on expression of apoptotic proteins Bax and Bcl-2 are determined by Northern analysis. The level of apoptosis in experimental conditions is determined by both caspase 3 activity and annexin V staining and compared with normoxic cultures.

Chapter 7 discusses the results of the current study in the context of the literature and the implication of these results for the understanding of the pathophysiology of hibernating myocardium. The possible clinical implications of these results are also discussed.

# CHAPTER TWO

# METHODS

## 2.1 Isolation of neonatal rat ventricular myocytes

The method for isolation of neonatal rat ventricular myocytes is adapted from that described previously (Springhorn JP 1989). The collagenase concentration was chosen to achieve the maximum viability and yield of cells.

Hearts were extracted from Day 1 to Day 3 neonatal Sprague-Dawley rats. Ten rat pup hearts were harvested for each culture. Each pup was decapitated and a midline cut was made down the sternum. The heart was exposed and the ventricles were cut off and put into calcium- and magnesium-free Hanks Balanced Salt Solution (Life Technologies, Ca, USA) in a sterile petri dish. They were washed briefly and transferred to another sterile petri dish containing calcium and magnesium free Hanks Balanced Salt Solution. The hearts were then cut into small pieces and transferred to a 50 ml sterile polypropylene conical tube containing 50 mg Trypsin (1:250, Trypsin/EDTA solution, Life Technologies) in 50 ml calcium and magnesium free Hanks Balanced Salt Solution. The heart pieces were then gently rotary mixed in the conical tube overnight at 4 ° C.

On the following morning, 20 ml Dubecco's Modified Eagle Medium (DMEM, Life Technologies) containing 10% fetal calf serum (Life Technologies,) and 1% penicillin/streptomycin (Life Technologies) was then added to the trypsin solution, containing the heart pieces, to inactivate the trypsin. The solution was then warmed to 37 ° C and transferred to a sterile 100 ml glass bottle. The solution was discarded and

replaced with 5 ml collagenase solution : 5 mg Type II collagenase (392u/mg, Worthington, NJ, USA) in 25 ml Hanks Balanced Salt Solution. The bottle was shaken in a waterbath at 37 ° C for 1 $^{1}/_{2}$ minutes. The collagenase solution was discarded and replaced with 10 ml of fresh collagenase solution. The bottle was shaken at 37 ° C for 10 minutes. The heart pieces then underwent trituration using a 10 ml sterile pipette and the solution containing cells was removed and transferred to a sterile 50 ml propylene conical tube and kept on ice. 10 ml fresh collagenase solution was added to the remaining pieces and the procedure repeated. The two digests were then pooled.

The cells were centrifuged at 100 g (4K15, Sigma, Germany) for 5 minutes at 4 ° C. The supernatant was discarded and the cells were resuspended in 20 ml low glucose DMEM with 10% fetal calf serum and 1 % penicillin/streptomycin. They were plated into a 75 $cm^2$ culture flask and incubated at 37 ° C for 2 hours. This was done to achieve a purer culture of myocytes due to the differential attachment rate of myocytes and nonmyocytes (ie fibroblasts), the latter attaching more quickly. After preplating, the media containing unattached cells was transferred to another sterile 50 ml polypropylene conical tube. Viable cells were counted using Trypan Blue exclusion in a haemocytometer. Cells were then plated into 25 $cm^2$ culture flasks at a density of 5 x $10^6$ per flask.

The media was changed after 24 hours and the cells were grown to confluence and until spontaneous synchronous beating occurred.

## 2.2 Hypoxic experimental conditions

The optimum hypoxic experimental conditions are probably in a hypoxic chamber which allows manipulation of the culture flasks as necessary during the period of the experiment. However such equipment was not available for this study.

Instead, to allow exposure to hypoxic conditions, primary cultures of neonatal rat ventricular myocytes were grown in 25 $cm^2$ culture flasks and a silicone stopper was inserted in the opening. Two 18G needles were pierced through the silicone cork. Silicone tubing was attached to the needles to allow transfer of gas into and out of the flask.

A hypoxic gas mix cylinder (1% oxygen, 5% $CO_2$ , balance nitrogen ; Linde Gas, NSW, Australia), with an oxygen content identical to that used in previous studies of hypoxia in neonatal rat ventricular myocytes (Wang D 1999; Jung F 2000), was used with a length of silicone tubing leading from the regulator to a plastic pipette which was positioned in the door seal of the incubator. This allowed the gas to be transferred to the culture flasks. Further silicone tubing led from the end of this pipette which was inside the incubator to a conical flask half full of sterile distilled water. The gas was bubbled through the water to humidify it before delivery to the cell cultures. Silicone tubing then lead from the water flask to the branching chain arrangement of tubing which lead to each culture flask (Figure 2.1). Care was taken

to make the lengths of tubing to each flask equal. The hypoxic gas was thus distributed as evenly as possible to each culture flask.

To avoid leaking of hypoxic gas into the incubator and affecting normoxic cultures, silicone tubing collected gas from each culture flask and returned it to a central length of tubing by a "reverse" branching chain arrangement and then via a plastic pipette through the door seal out of the incubator.

The flow rate of the hypoxic gas was controlled with a low flow regulator (Med Vet Medical) such as may be used in a clinical setting, allowing flows as little as 60 ml/minute. The flow from the outlet tubing was checked against that flowing through the water flask by visual assessment, to make sure there was no significant leakage from the system, by holding the end of the outlet tubing under water and comparing bubbling rates. Cells were then incubated under these hypoxic conditions for various times (ranging from 18 to 48 hours), depending on the experiment performed. Similarly, the glucose content of the media varied with the experiment performed. These details are outlined in the chapters covering the specific experiments.

To check whether the cells were becoming hypoxic or not, the oxygen content in the gas mix from the outlet tubing was compared to that in the cylinder using an oxygen sensor (Teledyne Brown Engineering, Sensor Technologies, Ca, USA). The oxygen content of the gas from the outlet tube was 1%, equal to that from the hypoxic gas mix cylinder. This shows that there was no air leak into the system resulting in

increased oxygen to the cells. Ideally media would have been sampled and the oxygen content measured directly, however the act of sampling would necessitate interruption of the hypoxic system and would invalidate the results of that sampling. For this reason, a hypoxic chamber is a more suitable arrangement for experimental manipulations.

## 2.3 Lactate assay

Lactate concentration in media were measured from normoxic and hypoxic cultures of neonatal rat ventricular myocytes. L-lactic acid is converted by lactate dehydrogenase to pyruvate with concomitant reduction of Nicotinamide Adenine Dinucleotide (NAD) to reduced NAD (NADH). The NADH can then be estimated by reduction of iodonitrotetrazolium (INT) producing a colour which may be measured colorimetrically at 510nm. This forms the basis of the assay as previously described (Buttery JE 1985).

Before the start of the hypoxic period, the media was removed from each culture flask. The cells were then washed twice with fresh media and excess carefully removed. Exactly 3 ml of media was then added to each culture flask and cells were incubated in either normoxic or hypoxic conditions. Consistent volumes of media

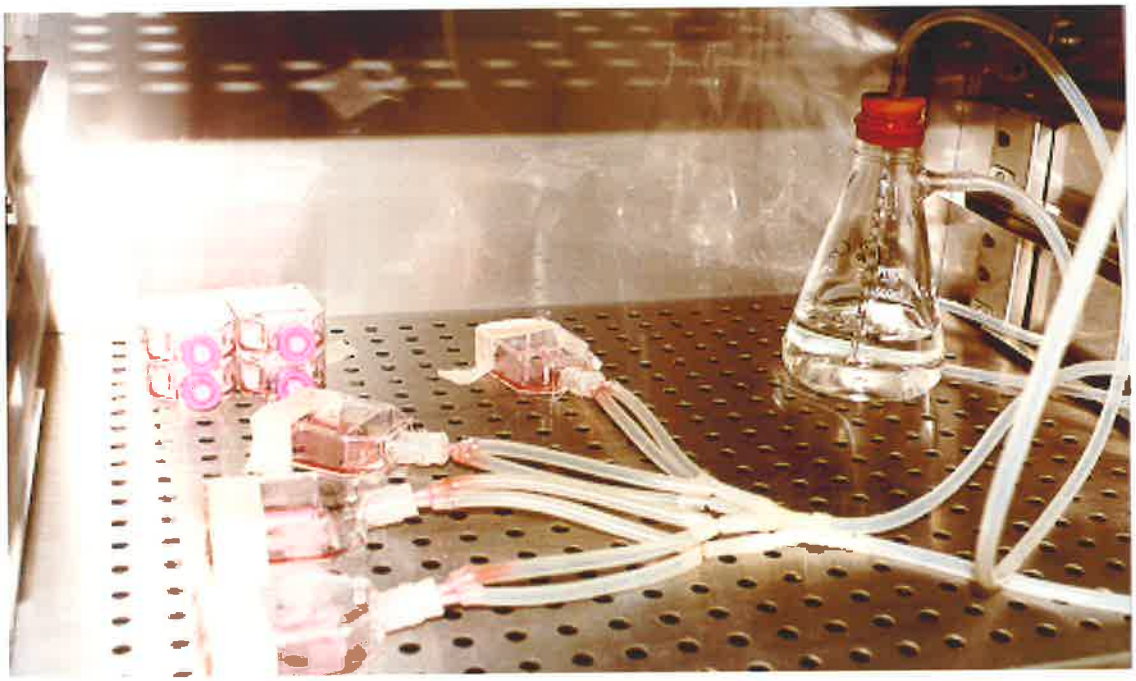


**Figure 2.1 a** Hypoxic gas, which had been passed through sterile Milli Q water to humidify it, was passed into each hypoxic culture flask by a system of silicone tubing in a branching arrangement to ensure that the distance from the outlet tube to each flask was equal. Gas from each flask was then collected from the outlet tube which lead to a collecting tube and then out of the incubator so that the whole incubator was not exposed to the hypoxic gas mix.

**Figure 2.1 b** Gas from the hypoxic gas cylinder was passed into the incubator through a sterile disposable pipette, which was placed through the door seal of the incubator. Silicone tubing piped the gas from the gas cylinder to the pipette and then from the pipette to the branching arrangement of tubes to individual flasks. The outlet gas was collected and similarly passed via a pipette through the door seal and out of the incubator.

were necessary for comparison of lactate production between flasks. Following the experimental period, media was removed and stored at -20 ° C until assayed.

L-lactate standards were made from serial dilutions of a 5 mM standard. To each 25 µl sample (media) or standard was added the following in a glass test tube: 500 µl AMP buffer (0.65 M 2-amino-2-methylpropan-1-ol, pH 9.0), 25 µl BSA solution (70g/l in normal saline) and 100 µl colour reagent. Colour reagent was made by dissolving 80 mg INT (Sigma, Mo, USA) in 20 ml distilled water, centrifuging and decanting the supernatant into 200 mg NAD (Boehringer Mannheim, Germany) and 5 mg phenazine methosulphate (Sigma). For each sample or standard a blank was also made.

10 µl L-lactate dehydrogenase (Boehringer Mannheim, >50 U/mg, 5 mg/ml in 3.2 M ammonium sulphate) was added to each sample or standard and 10 µl 3.2 M ammonium sulphate to each blank at timed intervals (10 seconds). Each tube was vortexed and placed in a 37 ° C waterbath. At 10 minutes 3 ml 0.1M HCl was added to each tube to stop the reaction, the tubes were vortexed and the absorbance at 510 nm was measured. Sample concentrations were calculated from the standard curve.

## 2.4 Adenosine Triphosphate (ATP) assay

Luciferin and the enzyme luciferase are responsible for the light produced by firefly tails but the presence of ATP is required for the reaction to proceed (McElroy WD 1947). With the addition of luciferin and luciferase to a sample containing ATP, the concentration of ATP can be measured from the light emission (peak wavelength emission reported at 560 nm) detected in a luminometer (Sigma 1994). The rapid rise and decay (half life = 1 minute) of the light flash requires consistent timing of the light measurement to obtain reproducible results (Sigma 1994).

ATP was measured in the cellular extract of normoxic and hypoxic cultures of neonatal rat ventricular myocytes. The medium was carefully removed and the cells were washed twice with phosphate buffered saline (PBS). 300 µl 6% perchloric acid was added to each flask to lyse the cells. Cells were then scraped off with a cell scraper and transferred into a microfuge tube and vortexed briefly. The cells were centrifuged for 3 minutes at 15000g at 4 ° C (4K15, Sigma, Germany) to remove cell debris which may interfere with the reaction. The supernatant was collected and neutralised with 5M $K_2CO_3$.

ATP standards were made by dissolving powdered ATP (Sigma) in distilled water and making serial dilutions. A 20 mg/ml solution of luciferase-luciferin (Sigma) was made with distilled water. 100 µl of this solution was added to a glass tube and placed

in the luminometer and emission was recorded. 20 µl of sample or standard was added and after 20 seconds light emission was again recorded. The background emission was subtracted from the sample/standard emission to obtain a corrected value. Sample ATP concentrations were calculated from the standard curve. The total amount of ATP for each plate was expressed in pmol.

## 2.5 Extraction of cellular protein for caspase 3 activity assay

Cellular protein was used to assay caspase 3 activity. After removal of media, cells were washed twice with sterile PBS. This was removed by pipetting and 175 µl of cell lysis buffer (Caspace Assay System, Promega, Wi, USA) was added. Cells were scrapped with a cell scraper and transferred into a microfuge tube. The tubes were then incubated at -80 ° C for 20 minutes. The cells were then thawed and incubated on ice a further 15 minutes before centrifuging at 15000 g for 20 minutes at 4 ° C. The supernatant was collected and stored at -80 ° C until use.

The protein assay (Bio-Rad, Ca, USA) of Bradford (Bradford MM 1976) was used to measure the protein concentration in the cellular extract. Standards were prepared from bovine serum albumin. 5 µl of cellular extract was diluted in 45 µl distilled water. 5 ml reagent (Coumassie blue G-250 dye, BioRad) were added to each sample

or standard and vortexed. Absorbance was read at 595 nm. Sample concentrations were calculated from the standard curve.

## 2.6 Extraction of total RNA

Total RNA was extracted using Trizol LS reagent (Life Technologies) according to manufacturer's instructions. The media was removed from each culture plate and 750 µl Trizol LS reagent was added. The plate was incubated at room temperature for 5 minutes. The cells were then scraped with a cell scraper and transferred to a sterile microfuge tube. 200 µl chloroform was added to each tube and the tube shaken vigorously for 15 seconds. The tubes were then incubated at room temperature for 15 minutes and then centrifuged at 12000 g for 15 minutes at 4 °C. The upper aqueous layer was carefully removed from each tube and transferred to another tube. 500 µl isopropyl alcohol was added and the tube incubated at room temperature for 10 minutes before centrifugation at 12000 g for 10 minutes at 4 °C. The supernatant was discarded and 1 ml ice cold 75% ethanol was added to wash the pellet. Following centrifugation at 7500 g for 5 minutes at 4 °C, the supernatant was discarded and the RNA pellet was allowed to air dry.

The pellet was redissolved in sterile Milli Q water and incubated at 60 °C for 10 minutes to better dissolve the RNA. Absorbance was measured at 260 nm to

determine the concentration. Samples were stored at -80 °C until use. Yield per culture plate varied from 20 to 50 µg depending on plating, age in culture and experimental conditions.

## 2.7 Reverse Transcription Polymerase Chain Reaction (RT PCR)

### 2.7.1 Reverse transcription

2 µg total RNA was incubated with 1 µg Random Hexamer Primers (Geneworks, SA, Australia) and water to 15 µl for 5 minutes at 70 °C to anneal primer and template. A "master mix" for the reverse transcription (RT) step was made using the following for each sample: 5 µl 5x RT buffer , 0.62 µl RNasin 1 µl M-MLV Reverse Transcriptase (200 U/µl) (all from Promega) and 2.13 µl nuclease free water. 10 µl of this mix was added to each primer/template tube. The tubes were incubated for one hour at 37 °C and then for 5 minutes at 94 °C to inactivate the reverse transcriptase.

### 2.7.2 Polymerase chain reaction

A second "master mix" for the polymerase chain reaction (PCR) was made for the number of reactions required. A separate master mix was made for each different primer set. The mix included the following for each reaction: 2.5 µl 5 µM forward primer, 2.5 µl 5 µM reverse primer, 1 µl 10 mM dNTP mix (Geneworks), 0.2 µl of 5 units/µl *Taq* DNA Polymerase (Promega), 3 µl 25 mM $MgCl_2$, 5 µl 10x *Taq* buffer (Promega), nuclease free water.

An aliquot of the mix was added to each reaction tube and cDNA was added, giving a final reaction volume of 50 µl. PCR was performed in a thermocycler (Minicycler, MJ Research, Fl, USA) with an initial 2 minute denaturing step at 94 °C, then 35 cycles of 94 °C for 30 seconds, an annealing temperature of 60 °C for 45 seconds and 72 °C for one minute with a final extension of 72 °C for 6 minutes. The program was altered to an annealing temperature of 52 °C for the IL-1β primers. The PCR product was detected by 1.5% agarose gel electrophoresis and ethidium bromide staining.

## 2.8 Primers and probes

### 2.8.1 Primers

Primers were designed using the program Oligo 4.0 – S (Molecular Biology Insights, Cascade, USA). Primers were designed to be 20-40 bases in length and result in amplification of a product between 400 and 700 base pairs in length. The minimum melting temperature of primers was 52 °C with a maximum of 65 °C. The minimum GC content was 38% and the maximum was 60%. Primers were chosen to avoid primer dimer formation and hairpin loops.

The primer sequences were chosen from known gene cDNA sequences retrieved from GenBank from the National Center for Biotechnology Information (USA) at www.ncbi.nlm.nih.gov . Once designed, the primers sequences were entered into BLAST (www.ncbi.nlm.nih.gov/BLAST/) to check for specificity.

Primers used for amplification of a PCR product intended as a template for the production of RNA probes by transcription were designed with the addition of either the SP6 or T7 promoter at the 5' end of the reverse primer, resulting in an approximately 40 base primer. The product was intended to be approximately 400-500 bases in length for optimum probe length.

***Inducible nitric oxide synthase***

iNOS forward primer:

5' – AAG TTT CTC TTC AGA GTC AAA TCC – 3'

iNOS reverse primer:

5' – TCT TTG AAG GAG CCA TAA TAC TG – 3'

These primers yielded a PCR product 445 bp in length.

***18S***

The 18S primers were taken from Spencer et al (Spencer WE 1999). The T7 promoter (highlighted in bold type) was added to the reverse primer for making RNA probes by transcription. These primers yielded a PCR product 497 bp in length.

18S forward primer:

5' – GGA CCA GAG CGA AAG CAT TTG CC – 3'

18S reverse primer:

5' – **TAA TAC GAC TCA CTA TAG GG**T CAA TCT CGG GTG GCT GAA CGC – 3'

***Hypoxia Inducible Factor 1α***

The SP6 promoter was added to the reverse primer for making RNA probes by transcription. These primers yielded a PCR product 476 bp in length.

HIF 1α forward primer:

5' – GTG GTA TTA TTC AGC ACG ACT TG – 3'

HIF 1α reverse primer:

5' – **ATT TAG GTG ACA CTA TAG AAA** CTC ACT GGG ACT GTT AGG C – 3'

***Bax***

The reverse primer contains the SP6 promoter. The primers were used to make RNA probes for Northern analysis.

Bax forward primer:

5' – GGA TGC ATC CAC CAA GAA GC – 3'

Bax reverse primer:

5' – **ATT TAG GTG ACA CTA TAG GA**G GAC TCC AGC CAC AAA GA – 3'

***Bcl-2***

The reverse primer contains the SP6 promoter. The primers were used to make RNA probes for Northern analysis.

Bcl – 2 forward primer:

5' – CCC CTG GCA TCT TCT CCT TC – 3'

Bcl – 2 reverse primer:

5' – **ATT TAG GTG ACA CTA TAG A**TC CCA GCC TCC GTT ATC CT – 3'

***Tumour necrosis factor α***

TNF α forward primer:

5' – CCA GAA CTC CAG GCG GTG TC – 3'

TNF α reverse primer:

5' –CGG CTG ACG GTG TGG GTG AG – 3'

These primers yielded a PCR product 487 bp in length.

***Interleukin 1 β***

IL-1β forward primer:

5' – ATG GCA ACT GTC CCT GAA CTC AAC T – 3'

IL-1β reverse primer:

5' –CAG GAC AGG TAT AGA TTC AAC CCC TT – 3'

These primers yielded a PCR product 560 bp in length.

***Interleukin 6***

The IL-6 primers used were from those sequences published by Ono et al (Ono K 1998). The reverse primer contains the SP6 promoter. The primers were used to make RNA probes for Northern analysis.

IL-6 forward primer:

5' – CCA GTT GCC TTC TTG GGA CTG ATG – 3'

IL-6 reverse primer:

5' – **ATT TAG GTG ACA CTA TAG AAA** TTT TCT GAC CAC AGT GAG GAA TG – 3'

### 2.8.2 Probes

The probe sequences were chosen to span an intervening intron to avoid amplification of genomic DNA. RNA probes were used for Northern hybridization because of the increased sensitivity compared to DNA probes (Krumlauf R 1994). PCR products, produced with the above primers, were used as templates for transcription of RNA probes. These PCR products were sent for sequencing (Sequencing Facility, Flinders Medical Centre, SA, Australia) and the sequence was then analysed in GenBank using BLAST (as above) to check the sequence of the probe against the published

sequence for the mRNA of interest. The specificity of the probe was also checked in this way. The sequences for the probes used are listed below.

***Inducible nitric oxide synthase***

AAGTTTCTCTTCAGAGTCAAATCCTACCAAGGTGACCTGAAAGAGGAAAA
GGACATTAACAACAACGTGGAGAAAACCCCAGGTGCTATTCCCAGCCCA
ACAACACAGGATGACCCTAAGAGTCACAAGCATCAAAATGGTTTCCCCCA
GTTCCTCACTGGGACTGCACAGAATGTTCCAGAATCCCTGGACAAGCTGC
ATGTGACTCCATCGACCCGCCCACAGCACGTGAGGATCAAAAACTGGGGC
AATGGAGAGATTTTTCACGACACCCTTCACCACAAGGCCACCTCGGATAT
CTCTCGCAAGTCCAAATTATGCATGGGGTCCATCATGAACTCCAAGAGTT
TGACCAGAGGACCCAGAGACAAGCACACCCCAGTGGAGGAGCTTCTGCC
TCAAGCCATTGAATTCATTAACCAGTATTATGG CTCCTTCAAA GA

***Hypoxia Inducible Factor 1α***

GTGGTATTATTCAGCACGACTTGATTTTCTCCCTTCAACAAACAGAATCTG
TCCTCAAACCAGTTGAATCTTCAGATATGAAAATGACCCAGCTGTTCACT
AAAGTGGAATCTGAGGACACGAGCTGCCTCTTCGACAAGCTTAAGAAAG
AGCCCGATGCCCTGACTCTGCTAGCTCCAGCGGCTGGGGACACGATCATA
TCACTGGACTTCGGCAGCGATGACACGGAAACTGAAGACCAACAACTTG
AAGATGTCCCGTTGTACAATGATGTAATGTTCCCCTCTTCTAATGAGAAAT
TAAATATAAATCTGGCAATGTCTCCATTACCTGCCTCTGAAACTCCAAAG

CCACTTCGAAGTAGTGCTGATCCTGCACTGAACCAAGAGGTTGCATTGAA

GTTAGAGTCAAGCCCAGAGTCACTGGGACTTTCTTTTACCATGCCCCAGA

TTCAAGATCAGCCAGCAAGTCCTTCT

***18S***

GGACCAGAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGA

AAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCATAAA

CGATGCCGACTGGCGATGCGGCGGCGTTATTCCCATGACCCGCCGGGCAG

CTTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAAG

CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGGCCTGC

GGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACGGACA

GGATTGACAGATTGATAGCTCTTTCTCGATTCCGTGGGTGGTGGTGCATG

GCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAACG

AGACTCTGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGTCCC

CCAACTTCTTAGAGGGACAAGTGGCGTTCAGCCACCCGAGATTGAGC

***Bax***

GGATGCATCCACCAAGCAGCTGAGCGAGTGTCTCCGGCGAATTGGAGATG

AACTGGACAATAATATGGAGCTGCAGAGGATGATTGCTGACGTGGACAC

GGACTCCCCCCGAGAGGTCTTCTTCCGGGTGGCAGCTGACATGTTTGCTG

ATGGCAACTTCAACTGGGGCCGCGTGGTTGCCCTCTTCTACTTTGCTAGCA

AACTGGTGCTCAAGGCCCTGTGCACTAAAGTGCCCGAGCTGATCAGAACC

ATCATGGGCTGGACACTGGACTTCCTCCGTGAGCGGCTGCTTGTCTGGAT

CCAAGACCAGGGTGGCTGGGAAGGCCTCCTCTCCTACTTCGGGACCCCCA

CATGGCAGACAGTGACCATCTTTGTGGCTGGAGTCCTC

***Bcl - 2***

CCCCTGGCATCTTCTCCTTCCAGCCTGAGAGCAACCGAACGCCCGCTGTG

CACCGAGACACGGCTGCCAGGACGTCGCCTCTACGGCCCCTTGTCGCCAA

CGCTGGGCCTGCGCTCAGCCCTGTGCCACCTGTGGTCCACCTGACCCTCCG

CCGGGCTGGGGATGACTTCTCTCGTCGCTACCGTCGCGACTTTGCAGAGA

TGTCCAGTCAGCTGCACCTGACGCCCTTCACCGCGAGGGGACGCTTTGCC

ACGGTGGTGGAGGAACTCTTCAGGGATGGGGTGAACTGGGGGAGGATTG

TGGCCTTCTTTGAGTTCGGTGGGGTCATGTGTGTGGGGAGCGTCAACAGG

GAGATGTCACCCCTGGTGGACAACATCGCTCTGTGGATGACTGAGTACCT

GAACCGGCATCTGCACACCTGGATCCAGGATAACGGAGGCTGGGAT

***Interleukin 6***

CCAGTTGCCTTCTTGGGACTGATGTTGTTGACAGCCACTGCCTTCCCTACT

TCACAAGTCCGGAGAGGAGACTTCACAGAGGATACCACCCACAACAGAC

CAGTATATACCACTTCACAAGTCGGAGGCTTAATTACATATGTTCTCAGG

GAGATCTTGGAAATGAGAAAAGAGTTGTGCAATGGCAATTCTGATTGTAT

GAACAGCGATGATGCACTGTCAGAAAACAATCTGAAACTTCCAGAAATA

CAAAGAAATGATGGATGCTTCCAAACTGGATATAACCAGGAAATTTGCCT
ATTGAAAATCTGCTCTGGTCTTCTGGAGTTCCGTTTCTACCTGGAGTTTGT
GAAGAACAACTTACAAGATAACAAGAAAGACAAAGCCAGAGTCATTCAG
AGCAATACTGAAACCCTAGTTCATATCTTCAAACAAGAGATAAAAGACTC
ATATAAAATAGTCCTTCCTACCCCAACTTCCAATGCTCTCCTAATGGAGAA
GTTAGAGTCACAGAAGGAGTGGCTAAGGACCAAGACCATCCAACTCATCT
TGAAAGCACTTGAAGAATTTCTAAAGGTCACTATGAGGTCTACTCGGCAA
ACCTAGTGTGCTATGCCTAAGCATATCAGTTTGTGGACATTCCTCACTGTG
GTCAGAAAAT

***Labelling of RNA probes***

The digoxigenin labelling system is based on the steroid hapten digoxigenin, which is derived from the digitalis plant (Figure 2.2). If digoxigenin is coupled as a hapten to a suitable carrier molecule, high affinity antibodies can be easily generated, for example in sheep (Roche Diagnostics Corporation 2000). Since digoxin occurs exclusively in digitalis plants there are fewer endogenous background problems with these antibodies as in the case of other haptens, such as biotin. Digoxigenin can be coupled to nucleotides like dUTP or UTP and incorporated into nucleic acids using polymerases like Klenow polymerase, Taq polymerase, or RNA polymerases. The probes thus generated can be used in standard blotting and hybridization procedures and detected with anti-digoxigenin conjugates. The most frequently used conjugates

are alkaline phosphatase labelled antibodies. If phosphatase labelled antibodies are employed, hybridized probes can be detected either by chemiluminescence or a colour reaction.

Digoxigenin labelled probes were chosen because of the increased sensitivity compared with $P^{32}$ labelled probes. Whereas 10-20 µg total RNA is recommended per sample with $P^{32}$ labelled probes (Sambrook J 1989), with digoxigenin labelled probes, 1 µg total RNA is recommended per lane on the gel and specific mRNA can be detected in as little as 0.1-0.2 µg of total RNA (see instructions for DIG Northern Starter Kit, Roche, Basel, Switzerland).

There are obvious advantages in using nonradioactive probes in terms of ease of use and disposal, the need for radiation licenses, specially allocated equipment, rooms and storage. Furthermore, digoxigenin labelled probes can also be stored at –80° C and reused. The hybridization solution containing probe can also be stored and reused. Finally the exposure times are significantly shorter than for radioactive probes.

Digoxigenin labelled RNA probes were made by transcription using the DIG RNA labelling kit (Roche) according to manufacturer's instructions. A PCR product containing the SP6 or T7 promoter, preferably gel purified, was used as a template. The following reaction was set up in a 200 µl PCR tube: 4 µl PCR product, 2µl

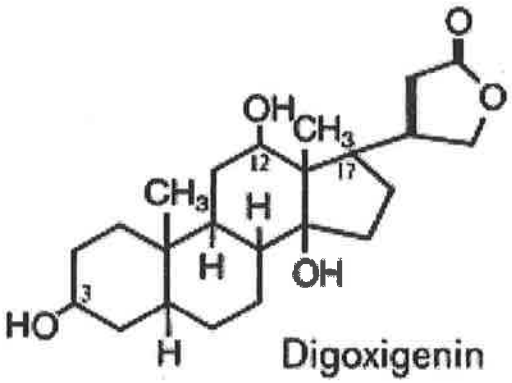



**Figure 2.2** The molecular structure of digoxigenin (Roche Diagnostics Corporation 2000).

nucleotide labelling mix (containing digoxigenin labelled UTP), 2 µl 10 x transcription buffer, 1 µl RNase inhibitor, 2 µl SP6 RNA polymerase (20 U/µl) and 9 µl nuclease free water.

The reaction mix was incubated overnight at 37 °C. 2 µl RNase free DNase was added to digest the template and the reaction mix was incubated at 37° C for a further 15 minutes. 1 µl 0.2 M EDTA pH 8.0 was then added to stop the reaction and the probe was stored at -80° C until use.

## 2.9 Northern hybridization

***RNA gel electrophoresis***

1 g agarose (Progen, Queensland, Australia) was added to 79 ml RNase and DNase free sterile water in a sterile conical flask. The flask was microwaved to dissolve and melt the agarose and then allowed to cool before adding 5 ml 20 x Northern Running Buffer. 20 x Northern Running Buffer was made by dissolving 41.9 g MOPS, 6.8 g Na Acetate, 1.86 g Na EDTA in sterile RNase/DNase free water to a volume of 500 ml, pH 7.0. The solution was autoclaved and stored at 4 °C.

16 ml deionised formaldehyde was added to the agarose and mixed thoroughly and the gel was poured in a fume hood and allowed to set.

1 μg total RNA was used per lane. 3-5 volumes of RNA loading buffer (Sigma) was added to each RNA sample. The samples were denatured at 65 °C for 15 minutes, then cooled on ice and spun briefly in a microfuge. The samples were then loaded onto the gel and electrophoresed at 55 V with 1 x Northern Running Buffer (as above).

***RNA marker***

A marker lane was also run using 1 μg RNA marker (Millennium Markers, Ambion, Tx, USA). To visualise the marker after transfer, the marker lane was cut off the membrane and stained with 0.03% methylene blue, 0.3 M Na acetate pH 5.2 (Wilkinson M 1990) for 3 minutes then rinsed with sterile nuclease free water several times. The marker lane was then scanned (HP Scanjet 6100 C/T, Ca, USA) and the file saved for comparison with the scanned Northern blot.

***Transfer***

Following electrophoresis, the gel was washed three times with sterile nuclease free water and then equilibrated for 30 minutes in transfer buffer, 20 x SSC (175.3 g NaCl, 88.2 g sodium citrate in 1L water, pH7.0, autoclaved). The transfer was prepared in a large plastic "lunch box" with an upside down gel tray inside. 4 wicks

of Whatman 3MM paper were soaked with 20 x SSC and placed on the gel tray. The gel was placed on top of these and a positively charged nylon membrane (Roche) was placed, with the corner cut for orientation.

A piece of 3MM paper soaked in 20 x SSC was placed on the membrane and on this was placed 6 dry sheets of 3MM paper. Interleaved paper towels, cut to the size of the gel, were placed on top in a stack to approximately 1 cm above the upper edge of the lunch box and the lid was firmly closed. Transfer was performed overnight and the membrane was UV crosslinked (Stratalinker UV crosslinker, Stratagene, Tx, USA).

***Hybridization***

The membrane was placed on a gauze square and both were wetted with sterile nuclease free water. The gauze containing the membrane was rolled up and placed into a hybridization bottle and unrolled so that the membrane lay against the inside of the bottle, RNA side inward facing. 5 ml DIG Easy Hyb solution (Roche), prewarmed to 68 °C was added and the membrane underwent prehybridization at 68 °C (Hybaid Stack 'n' Shake hybridization oven, Middlesex, UK) for 30 minutes with gentle rolling.

The RNA probe was denatured at 100 °C, put on ice and then added to 5 ml DIG Easy Hyb solution, which had been prewarmed to 68 °C. The prehybridization

solution was then replaced with the hybridization solution containing the probe and hybridized overnight at 68 °C.

The membrane was washed twice for five minutes with 2 x SSC, 0.1% sodium dodecyl sulphate (SDS) at room temperature and then twice for 15 minutes at 68 °C with 0.5 x SSC, 0.1% SDS.

***Chemiluminescent detection***

The membrane was washed in washing buffer (DIG Wash and Block Buffer kit, Roche) briefly and then incubated in blocking solution (DIG Wash and Block Buffer kit, Roche) for 30 minutes at room temperature with agitation.

The membrane was then incubated in DIG antibody solution, containing 2 µl Anti-digoxigenin-AP (0.75 U/µl, Roche) in 20 ml blocking solution for 30 minutes at room temperature with agitation. It was then washed twice for 15 minutes in washing buffer for 15 minutes at room temperature with agitation and then equilibrated in detection buffer for 5 minutes at room temperature (DIG Wash and Block Buffer kit, Roche).

8 µl CSPD$^R$ (25 mM, Roche) was added to 792 µl detection buffer. The membrane was placed in a plastic bag and heat sealed on three sides. The CSPD solution was added to the bag and air bubbles were removed. The bag was sealed and incubated at

room temperature for 5 minutes. The membrane was then transferred to another bag, which was sealed and incubated at 37 ° C for 15 minutes. The bag was then placed in a film cassette (RNA side up) and taken to the darkroom where a flim was placed over it. The film was exposed for an appropriate time (approximately 15 minutes for 18S probes and up to 2 hours for other probes) and then developed manually using developer and fixer solutions (Sigma).

## 2.10 Quantitation of Northern Blots

Following the chemiluminescent detection of mRNA bands, the film was scanned (HP Scanjet C/T 6100) and the image was analysed using 1D Image Analysis software (Kodak, NY, USA). The nett intensity (mean intensity minus the background intensity) of the band of interest was expressed as a percentage of that of 18S for the same lane on the same blot.

Normalisation to 18S bands allows comparison between lanes to allow for different loading between lanes. However, there is necessarily a significant variation in band density when different experiments are compared. The labelling efficiency can vary each time, probes which are reused will not have the same strength as on the previous occasion. It is not always practical to save up RNA samples and run them on the

same gel, especially when samples may be collected months or even years apart. This may introduce the potential problem of RNA degradation.

## 2.11 Electromobility Shift Assay

The electromobility shift assay ("gel shift assay") is used to detect sequence specific DNA-protein binding. Gel electrophoresis allows separation of free DNA from that bound to a protein. Binding to a protein causes retardation of migration of the DNA through the gel.

Nuclear protein extract is incubated with a known and labelled sequence of DNA from the cis-regulating sequences in the promoter region of the gene of interest. If there is a transcription factor present in the extract, which binds to the regulatory element under investigation, this binding will cause retardation of migration of the known labelled DNA sequence through the gel. This suggests that activation of the regulatory element is present under the experimental conditions studied.

### 2.11.1 Nuclear protein extract

***Solutions***

Lysis buffer contained 10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40$^R$ (Sigma) a non ionic detergent, and 1 mM dithiothreitol (DTT) added fresh.

"Buffer D" contained 50 mM Tris-HCl pH 7.5, 10% sucrose, 0.5 M KCl, 5 mM $MgCl_2$ , 0.1 mM EDTA, 20% glycerol, 1 mM DTT (added fresh)

***Nuclear protein extraction***

Neonatal rat ventricular myocytes grown in monolayer were washed twice with ice cold PBS. 500 µl lysis buffer was added and the cells were collected with a sterile cell scraper and transferred into a microfuge tube. The cells were vortexed, then lysed on ice for 15 minutes.

The cells were then centrifuged at 800 g for ten minutes at 4 °C to collect the nuclei. The supernatant was discarded and the nuclei pellet was washed with 500 µl lysis buffer without NP-40.

The nuclei were resuspended gently in 500 µl "buffer D" using a pipette. Nuclear protein was extracted by rotating samples at 4 °C for 90 minutes. The samples were

then centrifuged at 12 000 g for 30 minutes at 4 ° C. The supernatant was transferred to a new microfuge tube and centrifuged at 12 000 g for 60 minutes at 4 ° C. The supernatant was collected and the protein concentration was measured using the method of Bradford, described (see 2.5).

### 2.11.2 DIG 3' end labelling of double stranded oligonucleotide with terminal transferase

***Oligonucleotide sequence***

The design of the rat iNOS hypoxia responsive element oligonucleotide probe sequence was based on the characterisation of the rat iNOS gene by Keinanen et al (Keinanen R 1999) who reported a hypoxia responsive element at position –1180. It contains the CGTG sequence thought to be the core sequence for Hypoxia Inducible Factor 1α binding (Semenza G 1997). The probe was designed to be 34 base pairs long with a short sequence either side of the core sequence for the hypoxia responsive element. The specificity of the oligonucleotide sequence for the hypoxia responsive element in the iNOS promoter region was verified by search of the BLAST database (see section 2.8.1).

Rat iNOS hypoxia responsive element:

5' – TGA ACT TAG GTC CTT GTA **CGT G**CA AGG CAA GCA C – 3'

The rat iNOS NFκB binding site sequence was also based on the findings of Keinanen et al (Keinanen R 1999) and again the sequence was compared to the BLAST database for confirmation of specificity.

Rat iNOS NFκB binding site:

5' – ACT TGC ACA CCC TAC T**GG GGA CTC TCC** CTT TGG GAA CAG – 3'

***DIG labelling procedure***

Labelling was performed using the DIG Gel Shift Assay Kit (Roche) according to manufacturer's instructions. Firstly the two single stranded oligonucleotides were annealed to form a double stranded oligonucleotide by incubating them in an equimolar ratio in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 8.0) for 10 minutes at 95 °C. The reaction was allowed to cool slowly to room temperature and then diluted with TEN buffer to 3-4 pmol/µl.

The following were mixed on ice: 4 µl 5 x labelling buffer, 4 µl $CoCl_2$, 1µl double stranded oligonucleotide, 1 µl DIG-11-ddUTP solution, 1 µl terminal transferase and sterile Milli Q water to 20 µl.

The reaction was incubated for 15 minutes at 37 °C. The tube was then placed on ice and 2 µl 4 M LiCl and 60 µl chilled ethanol were added. The tube was incubated for

30 minutes at -70 ° C and then centrifuged at 12000 rpm in a microfuge for 15 minutes at 4 ° C. The pellet was washed three times with 500 µl chilled 70% ethanol and then air dried. The pellet was redissolved in TEN buffer.

### 2.11.3 DNA binding reaction

The following reactions were set up: labelled oligonucleotide without nuclear protein extract (negative control), labelled oligonucleotide with nuclear protein extract, labelled oligonucleotide with nuclear protein extract and the addition of excess unlabelled oligonucleotide (to test for nonspecific binding). If the DNA-protein complex is specific, the addition of unlabelled competitor will decrease the intensity of the band. In the presence of unlabelled non competitor, the band will remain.

The reactions were set up using the DIG Gel Shift Assay Kit (Roche). The final reaction volume was 20 µl, comprising 4µl binding buffer, 1 µl Poly d(I-C), 1 µl Poly lysine, 2 µl DIG labelled oligonucleotide (rat iNOS hypoxia responsive element), 4 µl unlabelled oligonucleotide, 5-10 µg nuclear protein extract (except the first reaction) and sterile Milli Q water to a final reaction volume of 20 µl. The samples were incubated at room temperature for 45 minutes.

### 2.11.4 Polyacrylamide gel electrophoresis

A vertical gel apparatus (Biorad Mini PROTEAN II) was assembled using siliconised glass plates. The gel was made using 1 ml 10 x TBE, 575 µl 87% glycerol (Promega) and 14.725 ml sterile Milli Q water in a small beaker with a magnetic stirrer. To this mixture was added 740 µl 2% bisacrylamide and 2.96 ml 40% acrylamide (both from ICN, Ca, USA). 10 µl TEMED (ICN) and 10% ammonium persulphate (freshly made) were added and the solution mixed well. The gel was poured and allowed to set at room temperature. The gel was pre-electrophoresed at 55 V for 1-2 hours at 4 °C using 0.25 x TBE as running buffer. 5µl clear loading buffer (DIG Gel Shift Kit, Roche) was added to each sample from the above binding reactions. Samples were loaded onto the gel and run at 55 V at 4 °C. A lane was loaded with loading buffer containing bromophenol blue to track migration through the gel.

After electrophoresis, the apparatus was disassembled and the gel was lifted with a piece of Whatman 3MM paper cut to size. This was then soaked from underneath with 0.25 x TBE. A presoaked positively charged nylon membrane (Roche) was placed on top of the gel with a corner cut for orientation. The membrane was covered with another sheet of Whatman 3MM paper soaked in 0.25 x TBE. This "sandwich" was placed between two fibre layers presoaked in 0.25 x TBE and the entire "sandwich" was placed in electroblotting apparatus (Mini Trans-Blot Electrophoretic Transfer Cell, Biorad). Transfer was performed at 4 °C for 30 minutes at 300 mA according to manufacturer's instructions.

The membrane was then removed from the electroblotting apparatus and UV crosslinked (Stratalinker, Stratagene). The membrane was rinsed and chemiluminescent detection proceeded as described in section 2.9.

## 2.12 Caspase 3 Activity

Caspase 3 activity, reflecting apoptosis, was assayed using the CaspACE Colorimetric Assay System (Promega). Caspase 3 specifically cleaves at the C-terminal end of the protein sequence Aspartate-Glutamine-Valine-Aspartate (DEVD). The colorimetric substrate provided in the CaspACE Assay System is DEVD-pNA. p-nitroaniline (pNA) is released from the substrate upon caspase 3 cleavage of DEVD. Free pNA produces a yellow colour which may be monitored by absorbance at 405 nm. The amount of yellow colour released upon cleavage is proportional to the caspase 3 activity.

Cellular protein extracts were obtained from primary cultures of neonatal rat ventricular myocytes as described above in section 2.5. The following reaction mix was made for each sample: 32 µl Caspase Assay Buffer (Promega), 2 µl dimethyl sulfoxide (DMSO), 10 µl 100mM DTT, 54 µl cellular protein extract (~1.6 µg/µl), 2 µl DEVD-pNA substrate. The reaction mix was briefly vortexed and transferred to a

96 well plate. A blank, using water instead of cellular protein extract, was also made. The plate was incubated at 37 °C for 4 hours.

Using the 10 mM pNA solution provided (Promega), standard stock solutions were made of the following concentrations : 10 μM, 100 μM and 1 mM. From these the following standards were made for the standard curve: 0, 1, 2, 5, 10, 50 and 100 pmol/μl (μM). The standards were added to the 96 well plate and the absorbance was measured at 405 nm. The absorbances for the samples were corrected for the blank and the concentration was calculated from the standard curve. The amount of pNA liberated over the reaction time was adjusted for the amount of cellular protein extract added to give the amount of pNA liberated per μg cellular protein extract (specific caspase 3 activity).

## 2.13 Annexin V staining for detection of apoptosis

During the early stages of apoptosis, phosphatidylserine is translocated from the inner leaflet of the bilipid plasma membrane layer to the outer leaflet, thus being exposed on the cell surface of apoptotic cells. This serves as a marker for phagocytosis by macrophages (see discussion in Chapter 1). The protein Annexin V has a high affinity for phosphatidylserine and apoptotic cells may be detected by binding to labelled Annexin V, for example biotin labelled Annexin V.

Cells were grown in 9 $cm^2$ slide flasks (Nunc, NY, USA) and after exposure to experimental conditions, were briefly washed twice in sterile PBS. They were incubated with 100 µl Annexin V-Biotin working solution (Roche) for 15 minutes at room temperature. They were then washed twice with HEPES buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and air dried. The cells were then fixed with methanol/ethanol 1:1 for 90 seconds and once again air dried. A blocking step was then performed by incubating the cells with 0.3% hydrogen peroxide for 30 minutes at room temperature to block intracellular peroxidases. 500 µl strepavidin-peroxidase (Roche) working solution was added and the cells were incubated for one hour at room temperature. The cells, adherent to the slides, were then rinsed with HEPES buffer and DAB (diaminobenzidine) substrate solution (Roche) was added. The cells were incubated for 15 minutes at room temperature before a further rinse with HEPES buffer.

The cells were then analysed by light microscopy. Apoptotic cells are stained brown.

## 2.14 Statistical analysis

Results for continuous variables were expressed as mean ± standard error of the mean unless stated otherwise. Comparison of two means was performed using unpaired t test (unequal variances). Differences across groups were analysed using one way

ANOVA followed by Tukey's test to analyse differences between pairs of groups. Significance was considered established with a p value $\leq 0.05$.

# CHAPTER THREE

# A CELLULAR MODEL OF HIBERNATING MYOCARDIUM

## 3.1 Models of hibernating myocardium

One of the main difficulties in studying hibernating myocardium is the lack of readily available myocardial tissue for study. Myocardial biopsy is rarely clinically indicated and the amount of tissue provided by such a procedure is small, for example 10-40 mg (Schwarz E 1996). Biopsy is best done at the time of surgery and may then be restricted to the anterior wall, because of the relative difficulty in accessing other territories. Follow up biopsies would rarely be possible and so generally only cross-sectional studies are reported.

Animal models have usually involved the use of either pigs or dogs (Mertes H 1995; Fallavollita JA 1997; St Louis JD 2000). A stenosis is created in one of the coronary arteries, usually the left anterior descending, by banding or application of a ligature at surgery. The wound is closed and the animal allowed to recover. This model may be kept for a few weeks or even up to a six months. This type of model is expensive, not only because of the initial cost of the animal, but also the housing and maintenance required during the experimental period. A certain expertise in surgery is required to reproducibly induce the same low flow conditions without infarction and with full recovery after surgery. During the learning period a certain wastage of animals may be anticipated.

A cellular model may overcome some of these problems. Primary cell cultures are easier to work with than whole animals and provide enough sample numbers within experimental and control groups for statistical analysis. The turnover is quicker, again allowing more experiments to be done or more experimental conditions to be studied. Few surgical skills are required and the model is less expensive to maintain. The validity of using a cellular model must be tested by comparison to documented characteristics of hibernating myocardium.

## 3.2 Primary cultures of neonatal rat ventricular myocytes

Primary cultures of neonatal rat cardiac myocytes have become widely used in cardiological research. Extraction of cells and maintenance of culture is a relatively simple procedure and the cultures can be used for up to two weeks (Morwinski R 1986). The most attractive feature of these cultures for the establishment of a cellular model of hibernating myocardium is that the myocytes beat spontaneously in culture. Beating cardiac myocytes in culture have been shown to have a fourfold increase in oxygen consumption compared with non cardiac cells or quiescent cell lines (Yamada T 1985). Spontaneously beating neonatal rat cardiac myocytes in culture are therefore performing something analogous to myocardial contraction and consequently, any changes in contractility due, for example to altered oxygen availability, are intrinsic adaptations to these conditions.

In contrast, adult rat cardiac myocytes do not beat spontaneously but can be made to do so by pacing. This is a disadvantage in a model of hibernating myocardium as any adaptation to hypoxia is affected by the continuously imposed contractility (driven by the pacing), theoretically not unlike that done by hibernating myocardium in response to dobutamine infusion. As discussed in Chapter 1, this appears to result in a more ischaemic profile rather than "hibernation".

Neonatal rat cardiac myocytes may, in some respects, have a different phenotype to adult rat cells and so it may be argued that using these cultures as a model for hibernating myocardium may show adaptations which are not representative of adult myocardium. However such differences between adult and neonatal cells have not been well characterised apart from obvious histological appearances. In fact some authors report a high degree of electrophysiological and morphological differentiation in neonatal rat cardiac myocyte cultures (Rohr S 1991). It is perhaps equally or more important to characterise the features of these cultures in chronic hypoxia and compare them to those reported in hibernating myocardium.

### 3.2.1 Spontaneous beating in primary cultures of neonatal cardiac myocytes

After plating, neonatal rat ventricular myocytes grow to confluence when the characteristic synchronous beating can be observed. Single cells exhibit highly

irregular beating and different cells often beat at different rates. When cells are cultured in pairs, partial synchrony may be observed before a steady state is reached and the cells beat in unison (Jongsma HJ 1987). In 83% of cases the final beating rate lay between the baseline rates of the individual cells. Once the cell monolayer has grown to confluence, the whole plate beats synchronously and regularly and the rates are faster than for small groups of cells (Jongsma HJ 1983). These spontaneous beating rates are due to components intrinsic to the tissue (Kucera JP 2000) and vary between different plates from the same harvest, but may relate to many influences including possibly characteristics of the mother rat (Bonner HW 1978) from whose offspring the cells are harvested.

### 3.2.2 Effect of plating density on neonatal rat ventricular myocytes cultures

Millart and Seraydarian (Millart H 1986) investigated the effects of plating density on neonatal rat ventricular myocytes. Cells were plated at high density (1878 ± 96 cells/mm$^2$ ) or low density (748 ± 125 cells/mm$^2$). An accurate cell count could only be made at 24 hours after plating as a proportion of cells die between plating and 24 hours in culture. It was estimated that the proportion of cells seeded that actually attach is 51% for low density plating and 37% for high density plating. The percentage of myocytes was not significantly different at 24 and 48 hours in culture but thereafter, the percentage of myocytes was significantly higher in the high density plates compared to the low density plates (Table 3.1).

| | *Percent myocytes* | | | | *Beating rates (beats/min)* | | |
|---|---|---|---|---|---|---|---|
| Time | 24 hr | 48 hr | 96 hr | 192 hr | 48 hr | 96 hr | 192 hr |
| LDM | 85.7±4 | 81.4±6 | 48.7±5.4 | 33.4±6 | 97±22 | 116±16 | 101±9 |
| HDM | 86.5±4.7 | 77.1±6.3 | 59±7.3 | 51.7±9.8 | 160±30 | 172±33 | 103±20 |
| p | NS | NS | p<0.05 | P<0.01 | p<0.01 | p<0.01 | NS |

**Table 3.1** Percentage of myocytes in culture and beating rates (mean ± SEM) with time in culture for low density plating (LDM) and high density plating (HDM). From Millart and Seraydarian (Millart H 1986).

Following plating, beating rates were significantly faster in the high density plates compared to the low density plates and this persisted at 48 hours (160±30 vs 97±22 bpm, $p < 0.01$) and at 96 hours (172±33 vs 116±16 bpm, $p < 0.01$). In both cases, beating rates tended to become faster over the first 96 hours (although this was not statistically significant) and then decreased.

### 3.2.3 Effect of fibroblasts on neonatal rat ventricular myocyte cultures

From the study described above it seems that non myocytes, largely fibroblasts, can transmit action potentials between aggregates of myocytes. Myocytes generally do not proliferate and confluence is achieved by myocyte hypertrophy and nonmyocyte proliferation. The greatest contribution to the latter is made by fibroblasts. Thus, fibroblasts contribute to the development of synchronous beating in neonatal rat ventricular myocyte cultures.

Although myocytes make up most of the myocardial mass, they comprise only about 30% of the cells in the heart (Zak R 1973). The relative number of myocytes in neonatal rat hearts is approximately 60% (Sasaki R 1968). It is therefore reasonable to speculate that nonmyocytes, the greatest proportion of which are fibroblasts, have a significant role in myocyte cell biology.

Schroedl and Hartzell (Schroedl NA 1983), in a study of the effect of fibroblasts in neonatal rat ventricular myocyte cultures, report that mixed cultures utilised glucose more rapidly than enriched cultures of either myocytes or fibroblasts. The percentage of fibroblasts in each culture was not reported. The mixed cultures also reduced glucose utilisation in response to insulin withdrawal, whereas enriched cultures did not respond in the expected manner to insulin deprivation. Palmitate utilisation was similar in all three culture types. The authors concluded that the mixed cultures may serve as a better model of cardiac metabolism than the enriched cultures.

Suzuki et al (Suzuki T 1997) investigated the effect of fibroblast conditioned serum free medium on serum starved myocytes and found that the serum starved myocytes did not beat but were stimulated to do so by the fibroblast conditioned medium. These findings support the concept that fibroblasts are an essential part of myocardial physiology and a culture which is intended to reflect the myocardial cellular milieu may be best represented by a mixed culture of myocytes and nonmyocytes.

To achieve a purer culture of myocytes, a mitotic inhibitor, for example bromodeoxyuridine, can be used to limit the proliferation of fibroblasts in neonatal rat ventricular myocyte cultures. However adverse effects on the myocytes in culture have been reported (Lokuta A 1994) including depressed spontaneous beating rate and glycogen levels. Thus none was used in the cellular model described in this thesis.

### 3.2.4 Effect of age in culture

Millart and Seraydarian (Millart H 1986) have reported that until 96 hours after plating the spontaneous beating rate remains approximately stable and at 192 hours tends to decrease and continues to do so thereafter.

In the cellular model described in this thesis, the effect of age in culture over the experimental period required for a cellular model of hibernating myocardium was investigated. Neonatal rat ventricular myocyte cultures were incubated in normoxic conditions with 5% $CO_2$. Cells were grown to confluence (reached at 36-48 hours) and culture media replenished. An example of neonatal rat ventricular myocyte culture at baseline, before commencement of experimental conditions, is shown in Figure 3.1. Beating rates were measured by counting the number of beats per 15 seconds and multiplying by 4. This procedure was performed in three random sections of the culture plate to ensure homogeneity. This method is similar to that previously described by Suzuki et al (Suzuki T 1997).

A baseline beating rate was recorded in four flasks after confluence was achieved. Rates were then recorded at 24, 48 and 72 hours following media change. The baseline beating rates were 135 ± 7 beats per minute (bpm) (mean ± SEM, n=4). After 48 hours there was a significant decrease in spontaneous beating rate (90 ± 7)

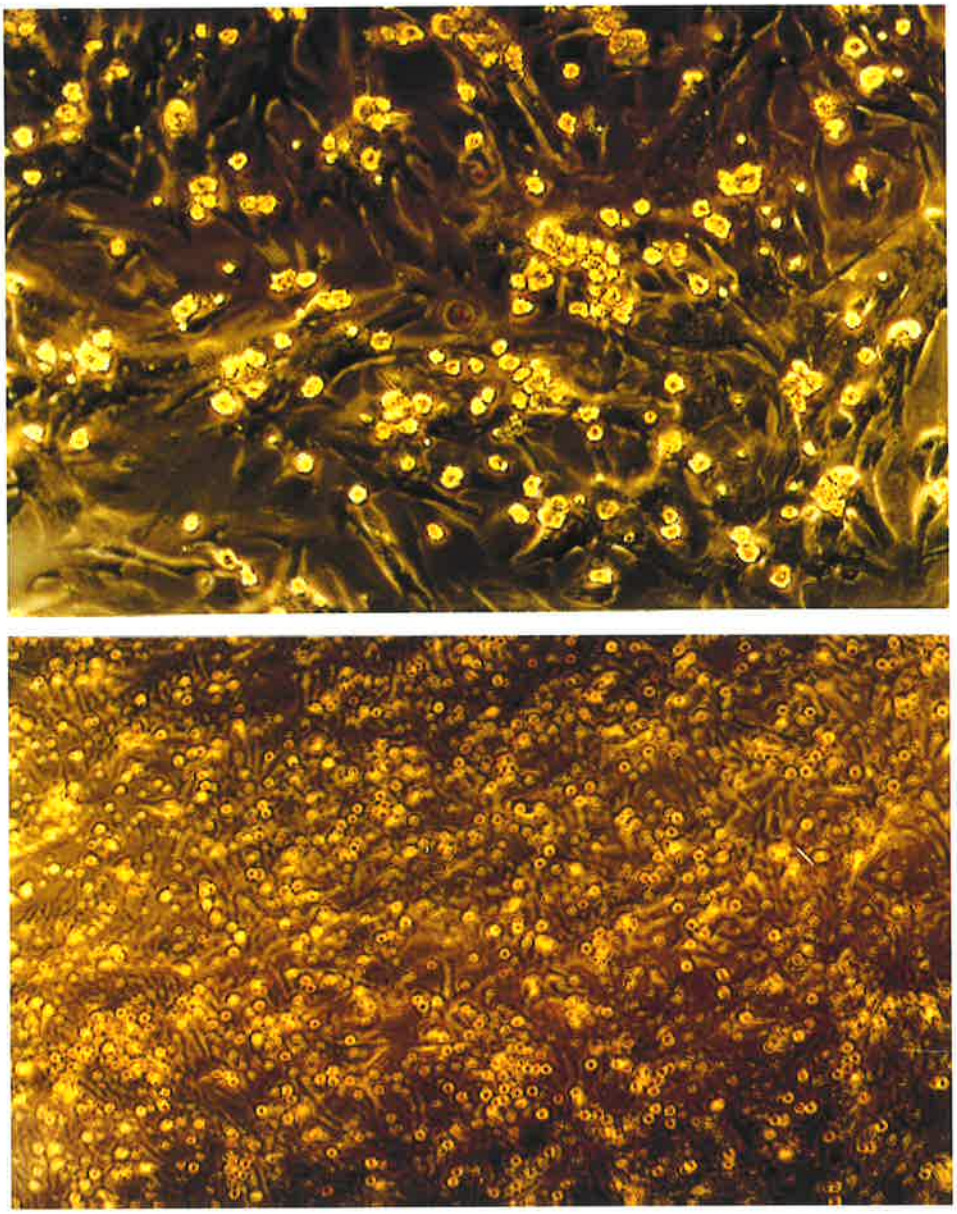


**Figure 3.1** Confluent primary culture of neonatal rat ventricular myocytes before commencement of hypoxic experiment. Top panel shows high magnification (40x), bottom panel shows low magnification (10x).

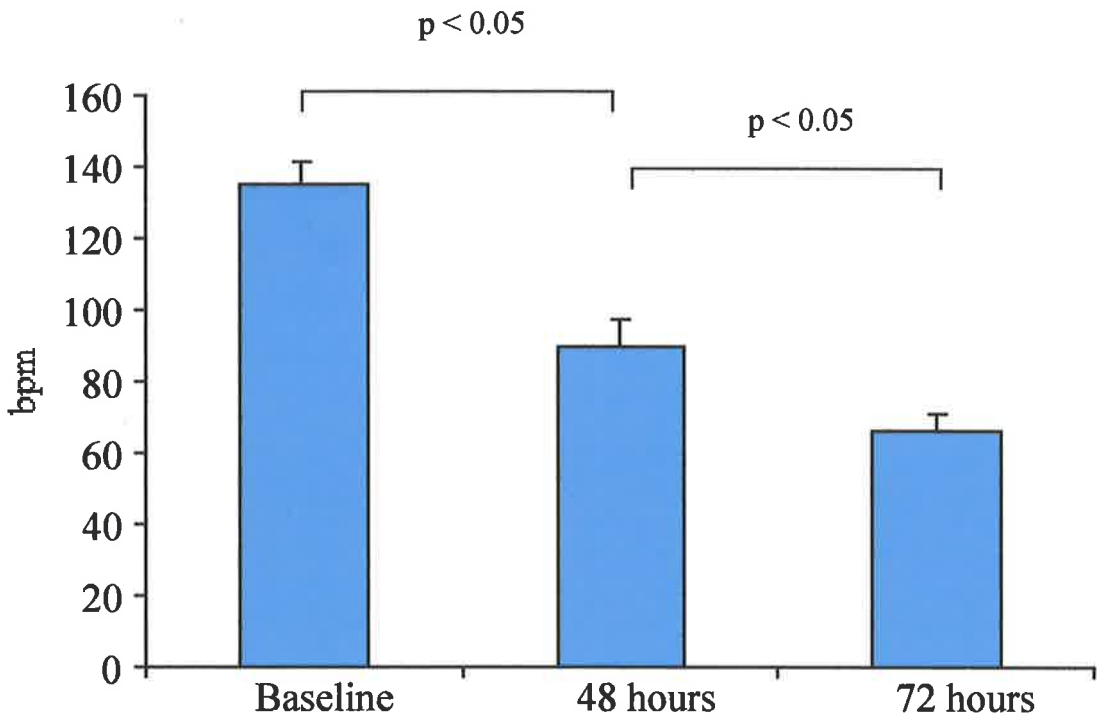



**Figure 3.2** Effect of age in culture on spontaneous beating rate, given as mean ± SEM. n = 4. $p < 0.05$ by ANOVA ($F = 30.4215$). $p < 0.05$ (Tukeys post hoc test).

which decreased further over the next 24 hours (66 ± 5, $p < 0.05$, ANOVA $F=30.4215$, Tukeys post hoc test). This experiment was repeated three times. The results of a representative experiment are shown in Figure 3.2.

## 3.3 A cellular model of hibernating myocardium

***Hypoxia and ischaemia***

Hypoxia is a state of reduced oxygen concentration in tissue, whereas anoxia is the absence of oxygen in tissues. Ischaemia on the other hand may be defined as a condition of oxygen deprivation accompanied by the inadequate removal of metabolites consequent upon reduced perfusion or flow. As discussed in Chapter 1 this is associated with increased lactate production and therefore increased tissue lactate levels.

***Design of a cellular model***

As discussed in Chapter 1, hibernating myocardium is thought to be characterised by reduced oxygen supply but without the biochemical characteristics of ischaemia such as lactate production. There is also reduced substrate supply because of reduced coronary flow but increased glucose extraction.

These factors have been borne in mind in the design of a cellular model of hibernating myocardium. Hypoxia was applied in the form of a gas mixture of 1% oxygen, 5% $CO_2$, with the balance being nitrogen. To simulate a reduced substrate supply, the media was changed from low glucose DMEM to no glucose DMEM at the onset of the experiment. Given the effect of lack of serum on spontaneous beating as discussed above, the experimental medium contained 10% fetal calf serum to optimise beating rates. Culture plates were incubated under hypoxic conditions for 48 hours, to simulate some chronicity while still maintaining spontaneous beating to simulate myocardial contraction, and this was followed by a further 24 hours reoxygenation. No inhibitor of mitosis was added to the media to suppress fibroblast proliferation because of the reported effects on spontaneous beating as discussed above. In view of the fact that the myocardium in vivo is a mix of myocytes and nonmyocytes and because of the described important interactions of fibroblasts and myocytes in culture, a mixed culture (without suppression of fibroblast mitosis) was thought to better represent the in vivo situation. Cells were plated at high density (approximately 5 x $10^6$ cells per 25 $cm^2$ culture plate) with, presumably, variations due to differential cell attachment across experiments, which were unavoidable.

### 3.3.1 Effect of chronic hypoxia on spontaneous beating

Hibernating myocardium adapts to the chronically reduced oxygen supply and coronary flow by reducing the myocardial work done. The aim of the following experiments was to determine whether neonatal rat ventricular myocytes, like hibernating myocardium, reduced their contractility in response to hypoxia and whether this was reversed on reoxygenation as an analogy to hibernating myocardium recovering function on revascularisation.

***Effect of hypoxia on beating rate***

Within the experiment there were five normoxic and five hypoxic flasks. Beating rates were measured at baseline, after 48 hours of hypoxia and then again after 24 hours of reoxygenation. A significant difference was found by two factor ANOVA ($p < 0.01$, $F = 11.7$ for time; $p < 0.05$, $F = 5.2$ for experimental condition). At baseline there was no significant difference in beating rates between normoxic ($161 \pm 4$ bpm) and hypoxic ($168 \pm 4$ bpm, before onset of hypoxia) flasks. At 48 hours, the beating rates in hypoxic flasks ($98 \pm 9$ bpm) were significantly decreased compared to the normoxic controls ($174 \pm 3$ bpm, $p < 0.01$, Tukey's multiple comparison test). Following 24 hours of reoxygenation, the beating rates in the hypoxic flasks ($170 \pm 5$ bpm) had returned to the levels of the control flasks ($153 \pm 15$ bpm) with no statistically significant difference between normoxic and hypoxic flasks after 24

hours reoxygenation. The experiment was performed three times to confirm reproducibility of results.

For comparison across experiments, the rates for each plate were normalised to the baseline rate due to the variability of baseline beating rates between experiments. Data from three separate experiments were pooled and analysed. Once again, a significant difference was found by two factor ANOVA ($p < 0.0001$, $F = 15.7$ for time; $p < 0.0001$, $F = 17.6$ for experimental condition against time). At 48 hours, the mean beating rate of normoxic cultures relative to baseline was 87% ± 6.6. The mean beating rate of the hypoxic cultures relative to baseline was 58% ± 2.9. Thus, overall, there was a decrease in beating rate due to hypoxia which was statistically significant ($p < 0.01$, Tukey's multiple comparison test).

At 72 hours, which corresponded to 24 hours reoxygenation, the beating rate of hypoxic cultures had increased to levels greater than the normoxic controls. The mean beating rate, relative to baseline, of normoxic cultures was 78 % ± 6.3 and that of the hypoxic cultures was 97% ± 5.5. However, this difference did not reach statistical significance. These findings are summarised in Figure 3.3.

***Effect of hypoxia on contraction***

To measure the degree of shortening ie the distance moved by a myocyte edge during spontaneous beating, specialised equipment and edge detection software is required.

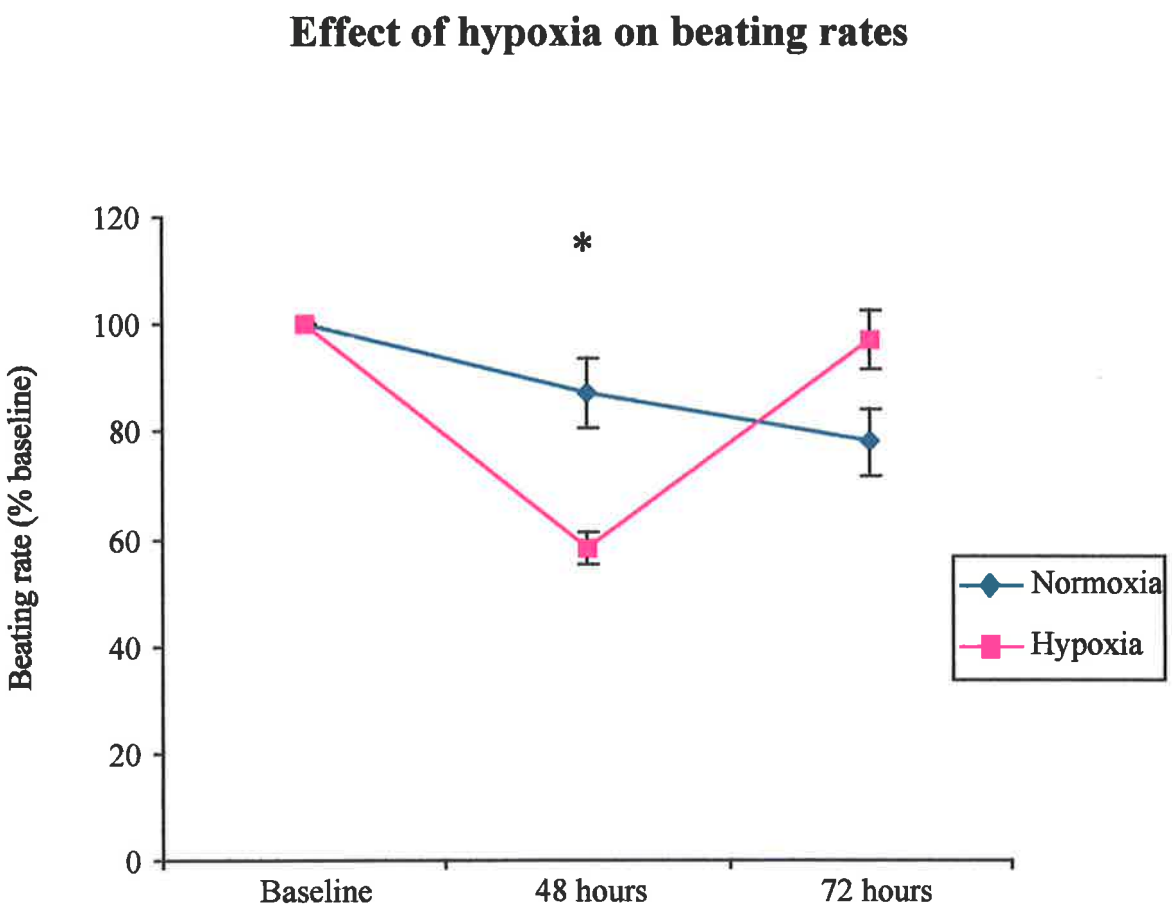
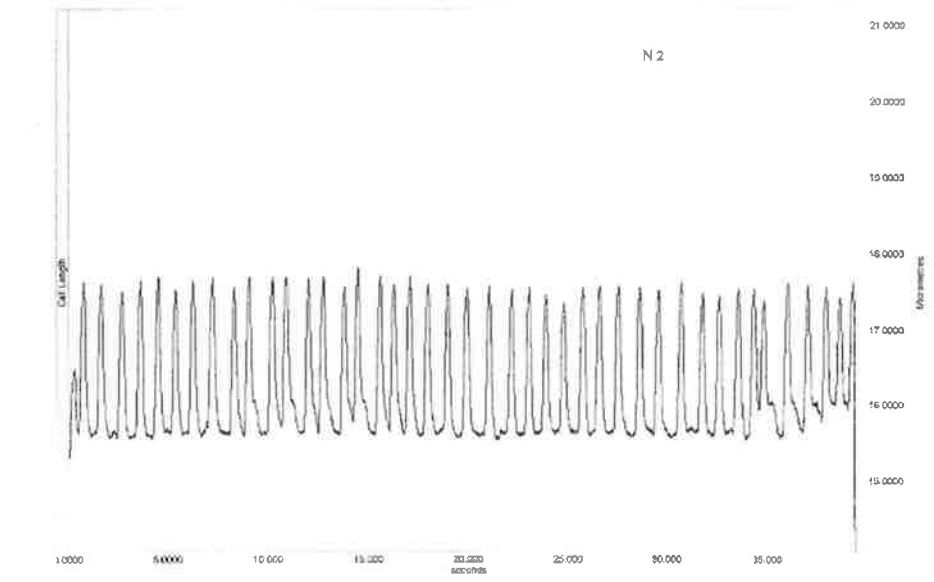



**Figure 3.3** The effect of hypoxia on spontaneous beating rate. Results show pooled data from three experiments each containing five normoxic and five hypoxic flasks. The difference between beating rates from normoxic compared with hypoxic flasks at 48 hours was significant (* indicates $p < 0.01$). The difference between normoxic and hypoxic flasks at 72 hours was not significant, indicating recovery of beating rate after reoxygenation.

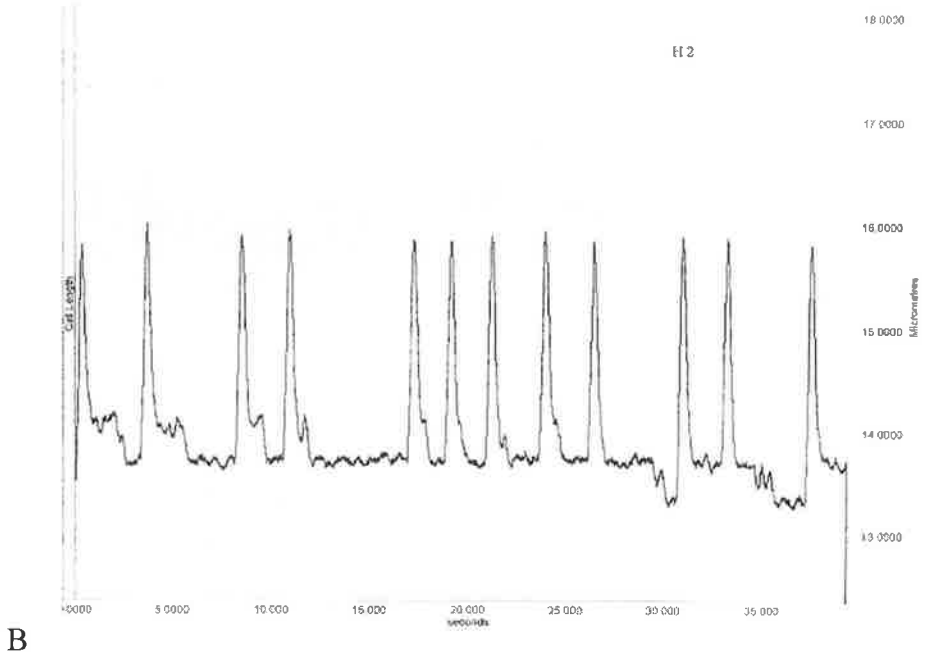
This equipment was not available in our laboratory. A preliminary experiment to assess the effect of hypoxia on contraction in neonatal rat ventricular myocytes was performed with the permission and cooperation of Dr Ted McMurchie of CSIRO, Division of Human Nutrition, Adelaide.

For determination of contraction, a culture plate was observed through a phase contrast microscope which was connected to a video recorder. Edge detection for individual myocytes was performed using the Video Dimension Analyser System (SDR Clinical Technology). Observations were made from three randomly chosen areas on a culture plate. The mean length of contraction for each observation (myocyte "leading edge" chosen for that region) over 30 seconds was calculated by the program. The mean of the three regions in the plate was calculated from these.

The degree of shortening at baseline in the normoxic culture was not significantly different from that of the hypoxic culture at baseline. After 48 hours, the mean shortening in the hypoxic culture (2115 ± 140 nm) was significantly greater than in the normoxic culture (1358 ±165 nm, $p < 0.05$). However the beating rate in the hypoxic culture (17 ± 7 bpm) had quite dramatically decreased compared with the normoxic cells (65 ± 2 bpm, $p < 0.05$). Therefore the "rate-shortening" product was significantly reduced in the hypoxic cells (33559 ± 12650) compared to the normoxic cells (88863 ± 11272, $p < 0.05$). To extrapolate, the contractile "work" done by the myocardial cells in hypoxic culture is reduced compared with those in normoxic




A




B

**Figure 3.4** The effect of hypoxia on degree of shortening. Shown are examples of contraction traces from normoxic (A) and hypoxic (B) culture. The x axis represents time (seconds) and the y axis distance in μm (both to 3 decimal places). For details see section 3.3.1.

culture. Examples of contraction traces from normoxic and hypoxic cells are shown in Figure 3.4.

That the degree of shortening in normoxic cells decreases over time is not surprising. After plating, cells grow to confluence by hypertrophy, largely myocyte hypertrophy, and by cell division. It is the nonmyocytes, especially fibroblasts, that proliferate. Even when confluence is reached, it does not mean that there is no more room for cell growth (Millart H 1986). The initial plating density does not seem to affect myocyte hypertrophy however growth (both by hypertrophy and crowding by fibroblast proliferation) in culture may well mechanically inhibit isotonic contraction. On the basis of these results it was decided to use spontaneous beating rate as the indicator of work done by the neonatal rat ventricular myocytes in culture.

### 3.3.2 Effect of dobutamine on spontaneous beating

One of the hallmarks of hibernating myocardium is inotropic reserve as evidenced by post ectopic potentiation of left ventricular function and by improvement in regional and global function in response to dobutamine. The aim of these experiments was to determine whether neonatal rat ventricular myocytes in hypoxic culture increased their rate of spontaneous beating in response to dobutamine.

Spontaneous beating rates were recorded at 48 hours after hypoxia. Dobutamine was then added at a concentration of 5 ng per ml of media. This concentration was chosen on the basis of preliminary dose-response experiments. Onset of effect was generally within 30 seconds of the addition of dobutamine. The addition of dobutamine necessitated the removal of the culture flask from the hypoxic environment. To minimise effects of reoxygenation, the dobutamine was added quickly (within 10 seconds) and the flask was sealed and beating rates counted within a minute.

The addition of dobutamine caused an increase in spontaneous beating rate in the hypoxic cultures (103 ± 12 bpm) relative to the 48 hour beating rate (61 ± 10, $p < 0.05$).

The experiment was performed three times to confirm consistency of results. Pooling of data across three experiments was done by normalising beating rates to the baseline beating rate for each plate. Beating rates in the hypoxic flasks increased from 47 ± 4% to 75± 6%. The increase in beating rates for the data pooled from four experiments was statistically significant ($p < 0.001$). The results of the pooled data are summarised in Figure 3.5.

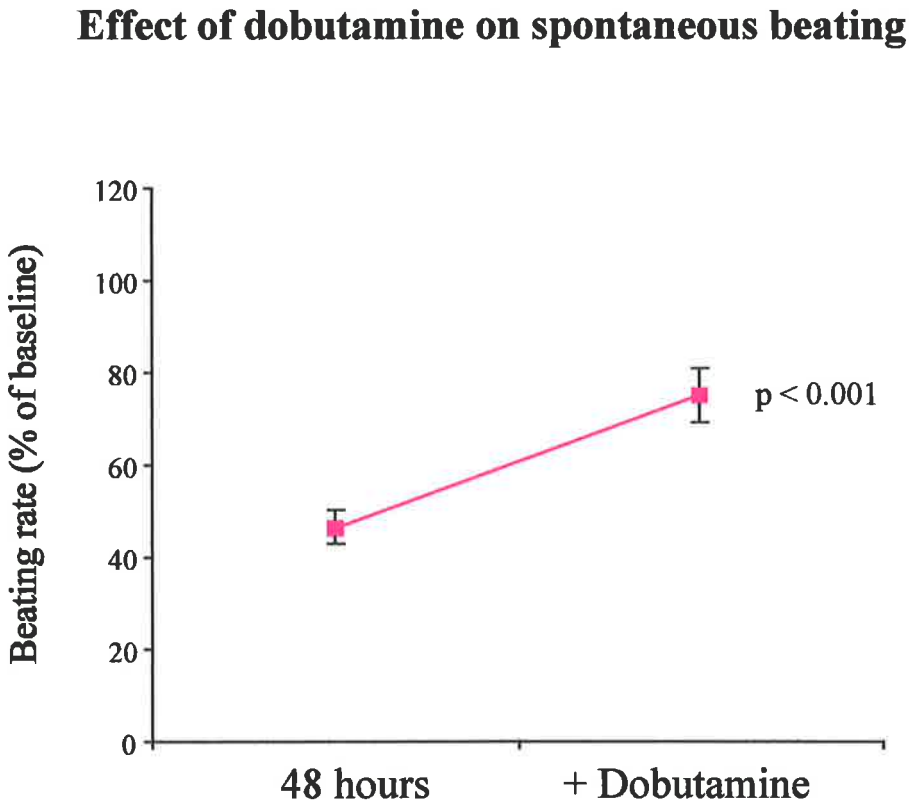



**Figure 3.5** The effect of dobutamine on spontaneous beating rate in cultures of neonatal rat ventricular myocytes after 48 hours of hypoxia. The pooled data from three separate experiments is presented. Addition of dobutamine (5 ng/ml media) resulted in a significant increase in beating rates in hypoxic cultures ($p < 0.001$).

### 3.3.3 The effect of chronic hypoxia on lactate production

Primary cultures of neonatal (Day 1-3 Sprague-Dawley) rat ventricular myocytes were incubated under hypoxic conditions as described in Chapter 2 for 48 hours. The media was collected and the lactate concentration in the media was determined as described in Chapter 2. The number of flasks in the normoxic or hypoxic groups varied between 5 and 8 for each experiment. The experiment was performed 3 times to show reproducibility.

In all experiments the difference in lactate levels in media between normoxic cultures and hypoxic cultures was not statistically significant (unpaired t test). Results from a representative experiment (but that with the greatest number of plates in each condition – normoxic or hypoxic) is shown in Figure 3.6. Lactate concentration in the media of normoxic controls was 2.1 ± 0.2 mM and the concentration in the media of hypoxic flasks was 2.6 ± 0.2 mM (n=8).

To determine if these neonatal rat ventricular myocytes could increase lactate production in response to more severe hypoxia, the experiment was repeated under anoxic (0% oxygen, as described in Chapter 2) conditions. Under these conditions, there was a significant increase in lactate levels in the anoxic cultures (5.7 ± 0.6 mM) compared to the normoxic controls (3.0 ± 0.3 mM, $p = 0.004$). These results are illustrated in Figure 3.7.

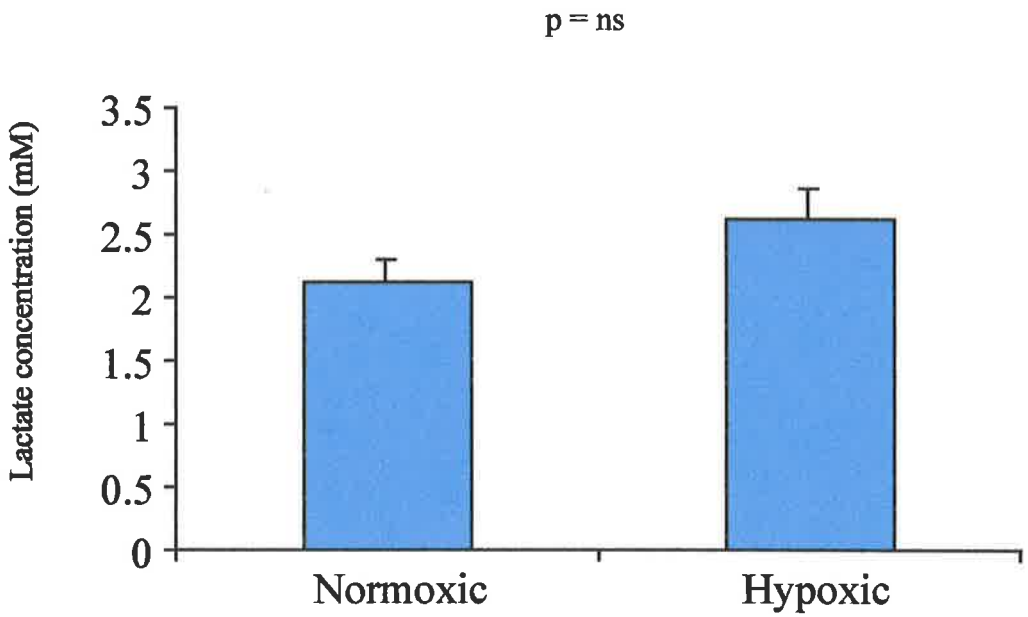



**Figure 3.6** Comparison of lactate concentrations in media, in hypoxic cultures compared with normoxic cultures. Lactate concentrations are expressed as mean ± SEM. There were eight culture flasks in each experimental condition (normoxic or hypoxic). The lactate concentrations in hypoxic cultures were not significantly different from normoxic controls ($p = 0.11$, unpaired t test).

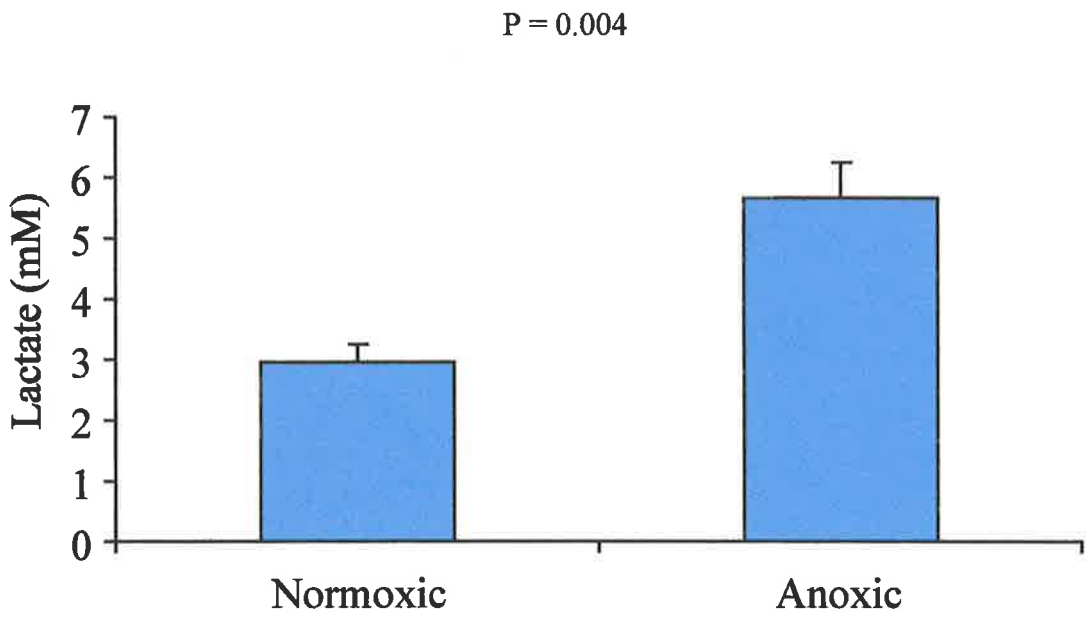



**Figure 3.7** Comparison of lactate concentrations in the media of anoxic (0% oxygen) cultures compared with the media of normoxic cultures. Lactate concentrations are expressed as mean ± SEM. There were six culture plates in each experimental condition (normoxic or anoxic). The lactate concentrations in anoxic cultures were significantly increased compared to normoxic controls ($p = 0.004$, unpaired t test).

### 3.3.4 The effect of chronic hypoxia on ATP production

Primary cultures of neonatal (Day 1-3 Sprague-Dawley) rat ventricular myocytes were incubated under hypoxic conditions as described in Chapter 2 for 48 hours. Cellular extracts were obtained and the ATP content was determined as described in Chapter 2. The ATP levels for hypoxic cells (8442 ± 923 pmol, n = 4) were compared to that of normoxic controls (3517 ± 417 pmol, n = 4). The difference was statistically significant with $p < 0.01$. The data from this experiment is shown in Figure 3.8. The experiment was again repeated twice to demonstrate reproducibility of results. ATP levels were consistently and significantly ($p<0.05$) lower in the hypoxic cultures when compared to normoxic cultures.

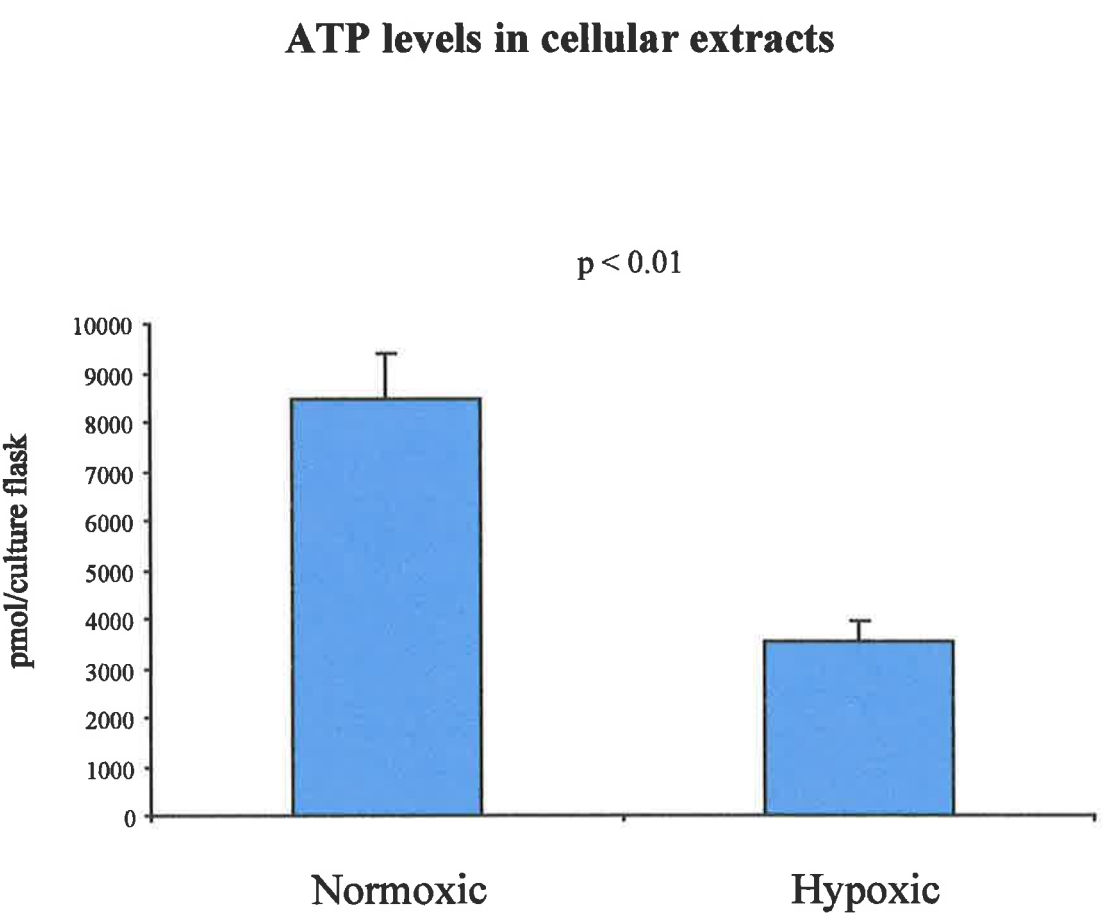



**Figure 3.8** The effect of chronic hypoxia on cellular ATP. Cell extracts from cultures incubated under hypoxic conditions for 48 hours were analysed for ATP content and compared to normoxic controls. There was a significant reduction in intracellular ATP in hypoxic cells ($p<0.01$). Experiment is representative of three performed.

## 3.5 Discussion

In this cellular model of hibernating myocardium, cultures from 1-3 day old neonatal rats, exhibiting maximum beating rate after confluence, were used. These were mixed cultures of myocytes and nonmyocytes (largely fibroblasts) as seen in Figure 3.1. However, such a mixed culture is more representative of myocardium in vivo as up to 40% of neonatal rat myocardium is comprised of non myocyte cells such as fibroblasts (Sasaki R 1968) with an even higher number in adult myocardium (Zak R 1973).

As discussed in section 3.2.3, an enriched myocyte culture may not best reflect cardiac metabolism under conditions of substrate deprivation or hypoxia. This is an important consideration when developing a cellular model of hibernating myocardium. No inhibitor of fibroblast proliferation was therefore added as it may have further altered the cellular responses to hypoxia.

The day of use, once confluence was reached, was chosen to be at maximum intrinsic beating rate. A high plating density was also chosen to optimise intrinsic beating rate (Millart H 1986). One of the most important features of this cellular model of hibernating myocardium is the spontaneous myocardial cell contraction. Without

significant contraction there can be no mechanism for a downregulation of contraction in response to limitation of oxygen supply with subsequent recovery on reoxygenation and this of course is the defining feature of hibernating myocardium.

The neonatal rat ventricular myocytes showed significantly reduced beating rates in response to hypoxia compared to normoxic controls. There was recovery of beating rates after 24 hours reoxygenation. This is consistent with the reduced contractile work seen in human hibernating myocardium and recovery of function after revascularisation. The degree of shortening was also investigated in preliminary experiments using edge detection software, however no change in degree of shortening was found in hypoxic flasks compared to baseline levels, rather there was a decrease in beating rate. Therefore, as beating rate was the parameter most affected, further experiments analysed this alone.

The hypoxic cells retained "contractile reserve" in that an increased beating rate was observed in hypoxic cells with the addition of dobutamine. Increased contractility in response to dobutamine or other inotropic stimuli is also a defining feature of hibernating myocardium. Although the hypoxic conditions were disrupted by the addition of dobutamine, the almost instantaneous effect showed that this disruption was irrelevant to the response observed as reoxygenation alone required several hours for recovery of beating rate.

A feature of hibernating myocardium described in animal models is the adaptation to reduced coronary flow resulting in a reduction in lactate production after an initial increase associated with ischaemia (Schulz R 1992; Chen C 1996). In human hibernating myocardium, no net lactate production was demonstrated as evidenced by a negative arterial-venous difference in blood lactate levels (Indolfi C 1996). In the cellular model of hibernating myocardium described, there was no significant increase in lactate levels in the medium from cells incubated under hypoxic conditions for 48 hours compared to normoxic controls.

On the other hand, incubation under more severe hypoxic conditions (0% oxygen for 48 hours) did result in a significant increase in lactate production. This suggests that in the cellular model described, lactate production is minimised. Again, multiple sampling is not possible in the absence of a hypoxic chamber, as the act of sampling the media results in reoxygenation for the duration of sampling. This may then be more consistent with repeated stunning rather than hibernation.

ATP levels in the cellular extracts of neonatal ventricular myocytes exposed to prolonged hypoxia in this cellular model of hibernating myocardium are decreased compared to normoxic controls. This is consistent with findings in animal models (Schulz R 1992; Chen C 1996) which report reduced ATP levels throughout the period of reduced coronary flow compared to baseline.

None of the animal or human studies of lactate production or ATP levels control for the number of cells present or involved in production of these parameters, clearly for logistical reasons. However, given that under hypoxic conditions cellular loss is not only possible but to be anticipated (Schwarz E 1996), the overall level of production by fewer cells may not give a true indication of cellular adaptation. Nevertheless, for comparison with the existing descriptions of metabolic changes in hibernating myocardium, no such adjustment for number of cells present was made in the current cellular model of hibernating myocardium. This could readily be done by normalising lactate and ATP levels to cellular protein content.

Necrosis in hibernating myocardium, whether in human subjects or experimental animal models, has been assessed by the histological presence of patchy fibrosis (Maes A 1994; Chen C 1996; Elsasser A 1997). No method for detection of necrosis was routinely used in this model. Preliminary samples of media from both hypoxic and normoxic cultures were analysed for creatine kinase (CK) levels (Dimension RXL, Dade Behring) and CK in both normoxic and hypoxic flasks were undetectable. That the lactate levels were not significantly higher in the hypoxic flasks compared to the normoxic controls and that there was recovery of spontaneous beating on reoxygenation, suggested that there was no significant necrosis in this model.

In conclusion, this cellular model of hibernating myocardium is consistent with the clinical definition of hibernating myocardium, as it is characterised by spontaneous contraction which is downregulated in response to oxygen and substrate deprivation

but which recovers on reoxygenation or administration of an inotrope (demonstrating inotropic reserve). Biochemical features such as cellular ATP content and lactate production are also consistent with what has been previously described in animal models.

The advantages of a cellular model include ease of use, a shorter time frame for completion of experiments and enough flasks within each culture to do replicates or apply different experimental conditions (positive and negative controls etc).

# CHAPTER FOUR

# EXPRESSION OF iNOS IN CHRONIC HYPOXIA

## 4.1 Introduction

As discussed in Chapter 1, the effect of hypoxia on iNOS expression in various tissues has been studied with variable results. For example, hypoxia has been shown to increase iNOS expression in rat cultured glial cells (Kawase M 1996), pulmonary endothelial cells (Palmer LA 1998) and in cultured rat hepatocytes (Vargiu C 2000) (although other studies disagree (Inoue T 2000)), but not in rat glomerular mesangial cells (Archer SL 1995) or rat smooth muscle cells (Hong Y 2000). Results reported for cardiac myocytes are also somewhat variable.

***Studies in whole rats***

Rouet-Benzineb et al (Rouet-Benzineb P 1999) studied the expression of iNOS in myocardial tissue from rats exposed to hypoxia for periods up to 21 days and found increased expression of iNOS protein in cardiac myocytes from both right and left ventricles from hypoxic rats compared to normoxic rats. Expression of iNOS was increased threefold in the right ventricle and twofold in the left.

***Studies in primary cultures of neonatal rat ventricular myocytes***

Three studies to date describe the effects of hypoxia on iNOS expression in primary cultures of neonatal rat ventricular myocytes. Kacimi et al (Kacimi R 1997) studied primary cultures of cardiac myocytes from Day 1 neonatal Sprague-Dawley rats. The

cultures were incubated in a hypoxic chamber with a gas mixture of 1% oxygen, 1% $CO_2$, and 98% nitrogen for 48 hours. Expression of iNOS was detected by RT PCR and compared to that in normoxic cultures. The authors report that hypoxia for 48 hours resulted in no production of iNOS mRNA and hypoxia in fact attenuated the expression of iNOS induced by IL-1β.

Wang et al (Wang D 1999) used primary cultures of ventricular myocytes from Day 1-2 Sprague-Dawley rats to investigate the effects of hypoxia on iNOS expression. The cultures were incubated with a gas mix containing 1% oxygen, resulting in a partial pressure of 40 mmHg in the media, for 24 hours. iNOS expression was detected by Northern analysis and also by Western blot. No iNOS expression was detected in normoxic controls. There was significant expression of iNOS in response to IL-1β, used as a positive control. There was a relatively weak induction of iNOS expression at 24 hours in response to hypoxia alone, compared with induction by IL-1β, but enhanced induction of iNOS expression in response to the combination of hypoxia and IL-1β. This contrasts with the findings reported above.

Finally, Jung et al (Jung F 2000) also used primary cultures of cardiac myocytes from Day 1-2 Sprague-Dawley rats and incubated them in hypoxic conditions comprising 1-2% oxygen, 5% $CO_2$, balance nitrogen for up to 36 hours. iNOS expression was demonstrated by detection of iNOS protein with Western blot. Again, there was weakly induced iNOS expression in response to hypoxia alone which was increased with the addition of IL-1β. The authors, in the same article, also report iNOS

expression in rat cardiac myocytes extracted from rats exposed to hypoxia for 12 hours.

## 4.2 Effect of hypoxia on iNOS expression in myocyte cultures from Day 1-3 neonatal rats

### 4.2.1 Methods

To investigate the expression of iNOS in a cellular model of hibernating myocardium, total RNA was extracted from primary cultures of ventricular myocytes, from Day 1-3 neonatal Sprague-Dawley rats. The cultures were exposed to hypoxic conditions, as described in Chapters 2 and 3, for 48 hours. The plates were immediately taken off hypoxia and media decanted. 750 µl Trizol LS solution was added within 10 seconds of the plate being removed from hypoxic conditions to avoid an ischaemia-reperfusion setting. Total RNA yield was typically between 20-40 µg per plate with an OD 260/280 reading of 1.6 or greater. Expression of iNOS was detected by RT PCR, as described in Chapter 2, and compared to normoxic controls and cultures treated with LPS at 10µg per ml media or IL-1β at 10 ng per ml media.

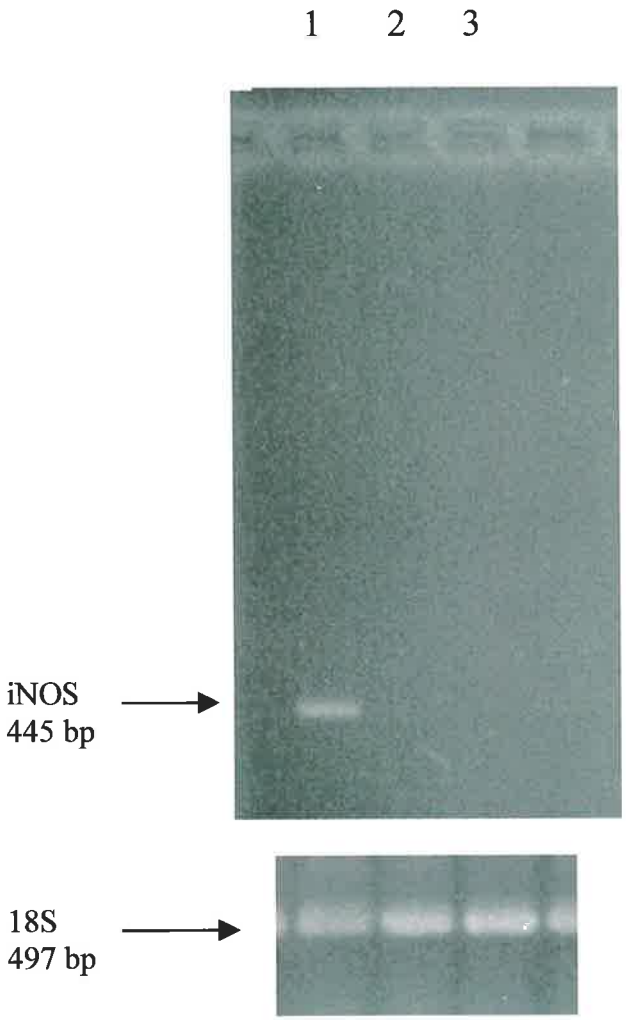



**Figure 4.1** Expression of iNOS in primary cultures of ventricular myocytes harvested from Day 1-3 neonatal Sprague-Dawley rats as detected by RT PCR. Lane 1: positive control, in this case IL-1β induced iNOS expression. Lane 2: normoxic controls and Lane 3: cultures incubated in hypoxic conditions for 48 hours. iNOS expression is detected in response to IL-1β and LPS (not shown), but not to hypoxia. The lower panel represents 18S PCR from same cDNA.

### 4.2.2 Results

iNOS expression was consistently demonstrated in response to the addition of either LPS or IL-1β. No iNOS expression was detected in the normoxic cultures (Figure 4.1). There was also no detectable iNOS expressions in cultures exposed to hypoxia. The experiments were repeated six times. On no occasion was iNOS detected in cultures exposed to hypoxia. The experiment was also repeated with a 5% $CO_2$/ balance $N_2$ gas mix and once again no iNOS expression was demonstrated without the addition of LPS or IL-1β.

## 4.3 Effect of hypoxia on iNOS expression in myocyte cultures from Day 7 neonatal rats

The mammalian fetus lives at reduced partial pressures of oxygen compared to its post natal existence. These fetal conditions have been likened to living at an altitude of 8000 m and require long term adaptations to relative hypoxia (Singer D 1999). Some of these physiological adaptations include improved oxygen transport, reduced metabolic rate and increased glycogen stores, but in more specific ways neonatal hypoxia tolerance is poorly understood. Nevertheless, it may be speculated that such mechanisms may be persistently operating in the cardiac myocytes of Day 1-3 neonatal rats in hypoxic culture. This may be why iNOS expression was not

demonstrated in the cultures, described above, when incubated under hypoxic conditions.

To determine if age of the rats from which the cardiac myocytes are harvested has an effect on iNOS expression under hypoxic conditions, the same set of experiments were performed in primary cultures of ventricular myocytes harvested from Day 7 Sprague-Dawley rats. Day 7 rats were chosen because they still had neonatal features, such as ongoing dependence on their mother (ie not weaned, and eyes had not yet opened), but there were significant differences from the Day 1-3 pups, such as significantly larger size and the early development of fur.

The same hypoxic conditions, were used as described in Chapter 2. The cultures were again incubated for 48 hours under these hypoxic conditions before extraction of RNA and analysis by RT PCR. The ability of these cultures to produce iNOS was again shown by expression in response to the addition of IL-1β at a concentration of 10 ng/ml culture medium.

The addition of IL-1β consistently resulted in iNOS expression both at 5ng or 10 ng per ml of medium. The normoxic cultures did not express iNOS by RT PCR (Figure 4.2). The cultures incubated under hypoxic conditions, in contrast to the cultures from Day 1-3 neonatal rats, did consistently show iNOS expression in response to hypoxia, without the addition of IL-1β. To confirm the consistency of these results, and to gain

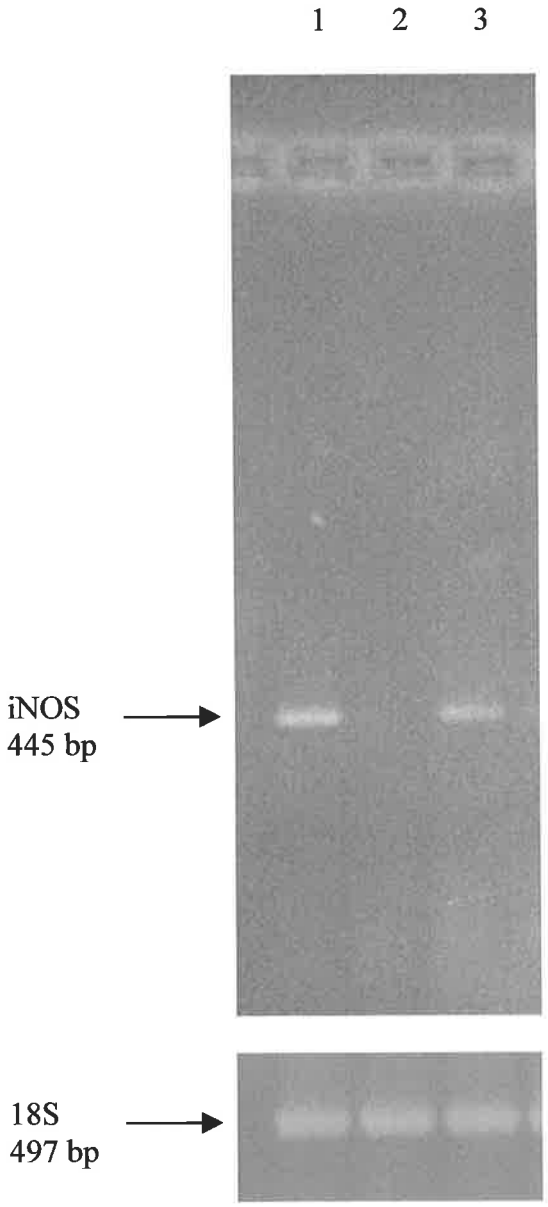



**Figure 4.2** Detection of iNOS expression by RT PCR in primary cultures of ventricular myocytes harvested from neonatal Sprague-Dawley rats aged 7 days. Lane 1 represents cultures incubated with IL-1β, Lane 2 represents normoxic cultures, Lane 3 represents cultures incubated in hypoxia for 48 hours. Lower panel represents PCR with 18S primers from same cDNA.

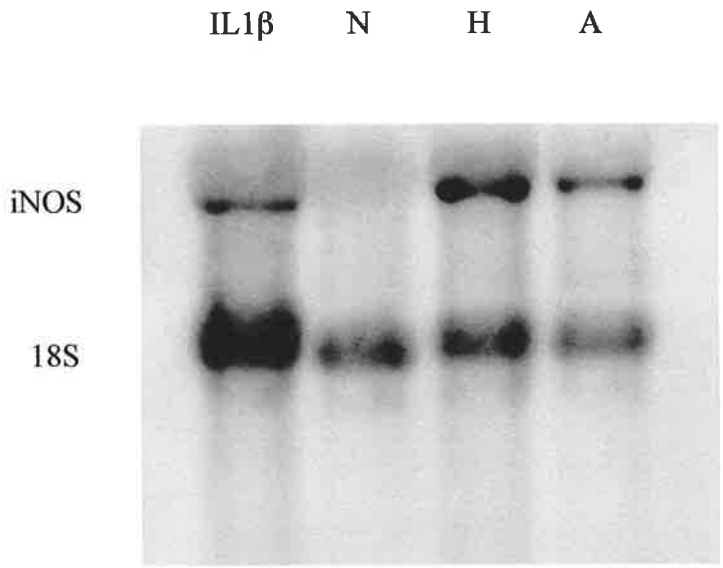



**Figure 4.3** Northern analysis of iNOS expression in response to hypoxia in cardiac myocyte cultures derived from rats aged 7 days. Lane 1 represents IL1β treated cells, lane 2 represents normoxic controls (N), lane 3 represents cells incubated in 1% oxygen (H) and lane 4 represents cells incubated in 0% oxygen (A).

RNA for analysis of the expression of other genes, the experiment was repeated more than ten times.

Further similar experiments were performed to determine whether iNOS expression was detectable after 24 hours of hypoxia and also after overnight incubation (18 hours) under hypoxic conditions. These experiments yielded identical results to those at 48 hours with consistent iNOS expression in hypoxic cultures but none detected in normoxic controls.

Total RNA from flasks incubated under hypoxic conditions for 48 hours was also analysed using Northern hybridization, as described in Chapter 2. This was done to confirm congruence between results obtained from RT PCR and from Northern analysis. The results were similar to those obtained from RT PCR in that no iNOS expression was detected in normoxic controls but iNOS expression was demonstrated in the hypoxic flasks. The results from a representative experiment are shown in Figure 4.3.

The expression of iNOS in cultures incubated in a gas mixture containing no oxygen ("anoxic conditions" – 0% oxygen, 5% $CO_2$, 95% $N_2$) was also investigated using similar experimental procedures. Again, iNOS expression was consistently demonstrated under these conditions (Figure 4.4). The degree of iNOS induction appeared more variable - sometimes more than in hypoxic conditions, sometimes less as assessed by the intensity of the bands on an agarose gel.

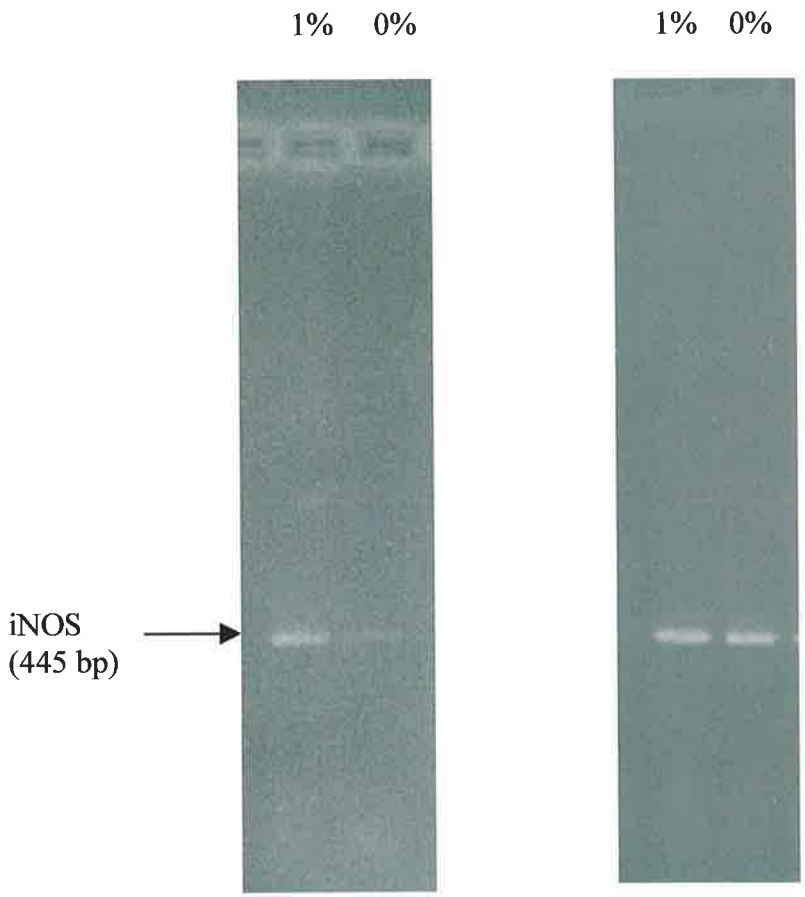



**Figure 4.4** Comparison of iNOS expression in cultures of ventricular myocytes harvested from day 7 neonatal rats, by RT PCR, after incubation with a gas mixture containing 0% oxygen compared with that after incubation with a gas mixture containing 1% oxygen. Results of two experiments are shown. In both panels lane 1 represents 1% oxygen, lane 2 represents 0% oxygen conditions.

## 4.4 Effect of hypoxia on iNOS expression in myocyte cultures from Day 5 and Day 4 neonatal rats

From the results described in the previous section it would appear that the onset of the ability (or requirement) to express iNOS in response to hypoxic conditions occurs between the age of 3 days and 7 days for this strain of rat. To further investigate this, experiments were performed in cultures from Day 5 and then also Day 4 neonatal Sprague-Dawley rats.

Three experiments were performed with cultures from both Day 5 and Day 4 neonatal rats. Experimental conditions were the same as those for the Day 1-3 and the Day 7 cultures.

Once again there was consistent expression of iNOS in response to IL-1β in both Day 4 and Day 5 cultures. No iNOS expression was detected in normoxic control cultures in cells harvested from Day 4 or Day 5 neonatal rats. The expression of iNOS in response to hypoxia (1% oxygen) was variable, not just in terms of relative amounts but in terms of presence or absence of iNOS induction. Results from representative experiments in ventricular myocytes harvested from Day 4 and Day 5 rats are shown in Figures 4.5 and 4.6 respectively.

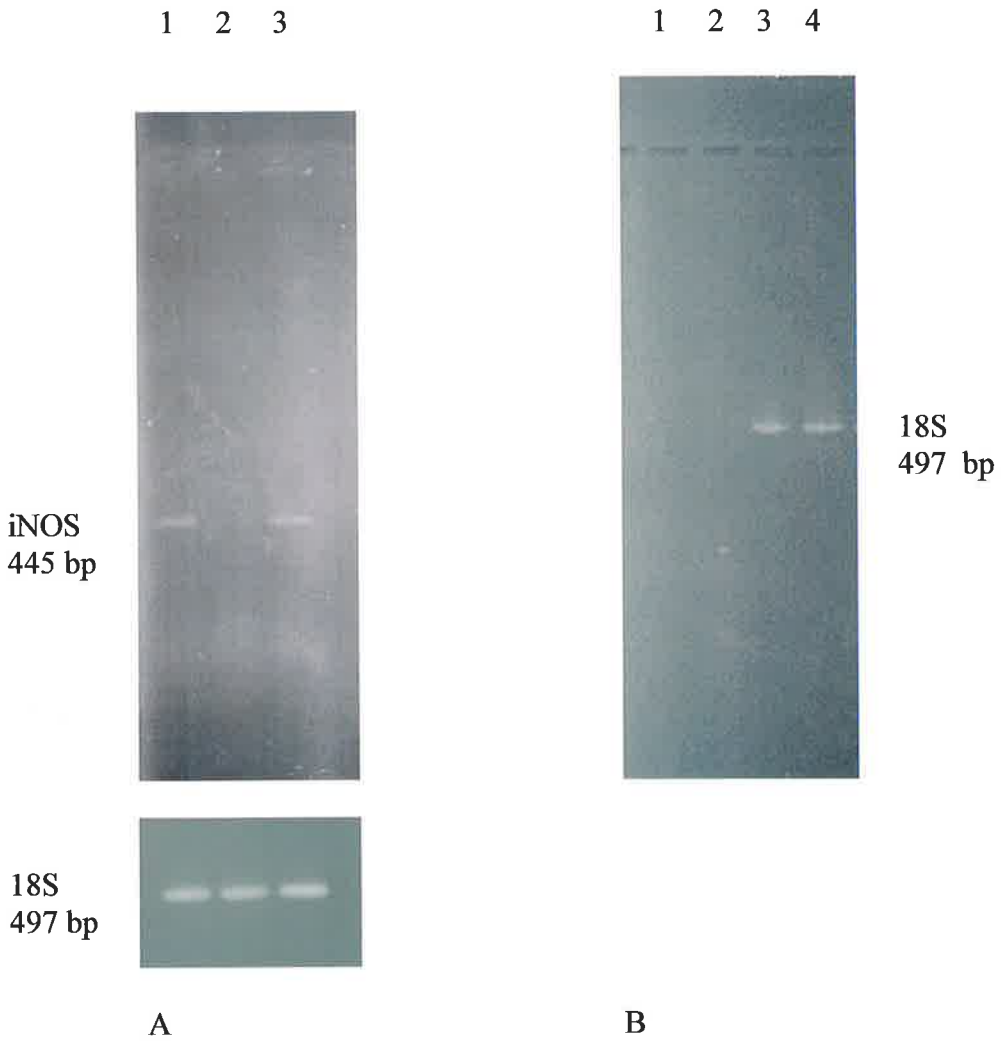



**Figure 4.5** iNOS expression detected by RT PCR in primary cultures of ventricular myocytes harvested from Day 4 neonatal Sprague-Dawley rats. Panels A and B depict separate experiments. **A** shows culture incubated with IL-1β in Lane 1, normoxic culture in Lane 2, hypoxic culture in Lane 3. The lower panel shows PCR with 18S primers using same cDNA. **B** shows no iNOS expression in either the normoxic culture (Lane 1) nor the hypoxic culture (Lane 2). 18S expression is shown in the normoxic culture (Lane 3) and in the hypoxic culture (Lane 4).

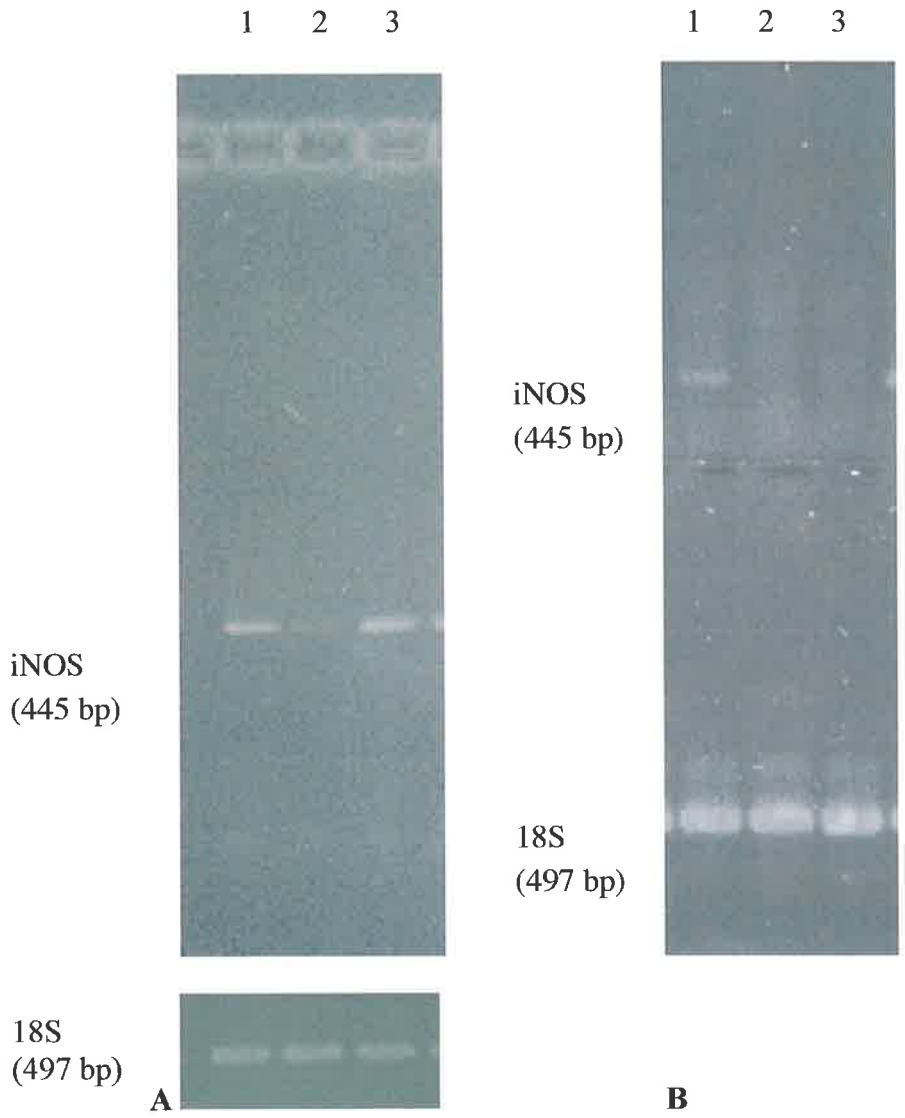



**Figure 4.6** iNOS expression detected by RT PCR in primary cultures of ventricular myocytes harvested from Day 5 neonatal Sprague-Dawley rats. Panels A and B depict separate experiments. **A** shows culture incubated with IL-1β (10 ng/ml media) in Lane 1, normoxic culture in Lane 2, hypoxic culture in Lane 3, with 18S PCR from same cDNA in lower panel. **B** shows iNOS expression in the top half of the gel and 18S expression in the bottom half of the gel. Again culture incubated with IL-1β is in Lane 1, normoxic culture in Lane 2, hypoxic culture in Lane 3.

## 4.5 Expression of HIF 1α in cultures from Day 1-3 rats

As these cultures are able to express iNOS in response to IL-1β, but not in response to hypoxia, other mechanisms of iNOS induction, ie HIF 1α expression, were investigated. RNA samples extracted from the Day 1-3 cardiac myocyte cultures incubated under hypoxic conditions were used to detect the presence of Hypoxia Inducible Factor 1α subunit by RT PCR. This was compared to expression in normoxic controls.

HIF 1α expression was consistently detected in both normoxic and hypoxic cultures. There was no apparent difference on visual inspection of agarose gels, but expression was not quantified. Results of a representative experiment are shown in Figure 4.7.

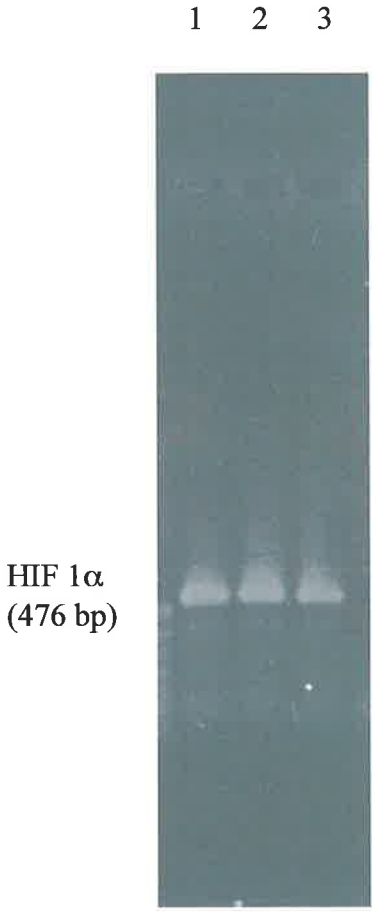



**Figure 4.7** Expression of HIF 1α, by RT PCR, in cultures of ventricular myocytes harvested from Day 1-3 neonatal rats. Lane 1: culture incubated with IL-1β, Lane 2: normoxic culture, Lane 3: hypoxic culture.

## 4.6 Discussion

These results firstly show that cultured ventricular myocytes harvested from neonatal Sprague-Dawley rats are capable of expressing iNOS, as detected by RT PCR, in response to stimuli such as IL-1β and to LPS. They do so consistently in cultures, harvested from rats aged 1-3 days and those aged 7 days, in response to the same doses of cytokines.

Although it might be expected from the rat iNOS promoter region (as discussed in Chapter 1) that these cells would express iNOS in response to hypoxia, the ventricular myocyte cultures harvested from Day 1-3 consistently showed no iNOS expression under hypoxic conditions without the addition of cytokines or LPS. This is consistent with the results reported by Kacimi et al (Kacimi R 1997). On the other hand, cultures of ventricular myocytes harvested from Day 7 neonatal rats consistently *did* show iNOS expression in response to hypoxia without requiring the addition of cytokines or LPS. Cultures of ventricular myocytes harvested from Day 4 and Day 5 neonatal rats showed variable iNOS expression in response to hypoxia.

The fact that cardiac myocytes harvested from Day 1-3 neonatal rats are able to express iNOS in response to cytokines but do not do so in response to hypoxia, may suggest a residual fetal adaptation to hypoxic conditions which is then lost with increasing post natal age. However these fetal adaptations, and particularly the

specific mechanisms, are poorly understood and therefore it is difficult to confirm this.

Alternatively, it may be that in cardiac myocytes from Day 1-3 rats, the mechanism of iNOS induction under hypoxic conditions has not matured. Given that these cultures are able to express iNOS in response to cytokines, these results may also suggest that the mechanisms of iNOS induction under hypoxic conditions are cytokine independent.

To investigate this possibility, HIF 1α expression was analysed. Expression of HIF 1α was ubiquitous in both hypoxic and normoxic cultures. Although these results do not confirm that HIF 1α is functional under these circumstances, it does suggest that potential mechanisms for iNOS induction other than those involving LPS or IL-1β may be present in these cultures. It follows then that these mechanisms therefore may not be required because of other adaptations to hypoxia.

Cultures of ventricular myocytes harvested from Day 7 neonatal rats consistently expressed iNOS in response to hypoxia. On the other hand cultures of ventricular myocytes harvested from Day 4 or Day 5 rats did not. This suggests that onset of consistent iNOS induction under hypoxic conditions occurs at about Day 7 or even Day 6 (not tested). Given that some Day 4 cultures did express iNOS in response to hypoxia and some Day 5 cultures did not, there seems to be a significant degree of variability in onset of consistent iNOS induction under hypoxic conditions even in

rats from the same source (University of Adelaide animal house). A similar variation in onset of glucokinase induction in response to all-trans retinoic acid has been previously reported in hepatocytes harvested from 15 to 17 day old neonatal rats (Cabrera-Valladares G 2001).

This may relate to gradual transition from some adaptations of fetal physiology to a postnatal physiology, particularly in response to such stimuli as hypoxia, occurring at different ages in different environments; or to genetic influences.

The results presented here suggest that iNOS has no role in the downregulation of contractile function seen in the cellular model of hibernating myocardium presented in Chapter 3, as it is not expressed in this model. The reduction in spontaneous beating by ventricular myocytes from Day 1-3 neonatal rats in culture in response to hypoxia and the subsequent recovery of function after reoxygenation is independent of iNOS activity.

It is therefore possible that the role of iNOS in hibernating myocardium of patients with severe coronary artery disease has little to do with the downregulation of metabolism and function seen in these myocardial regions. Although a direct extrapolation from neonatal cells from rats grown in culture to mature myocardium in the intact human being is not possible, these results raise the possibility of alternative roles for iNOS in hibernating myocardium.

# CHAPTER FIVE

# REGULATION OF iNOS EXPRESSION IN CHRONIC HYPOXIA

## 5.1 Introduction

Mechanisms regulating iNOS expression have yet to be completely elucidated. Cytokine induction is at present the best characterised pathway for regulation of iNOS expression. As discussed previously in Chapter 1, the addition of various cytokines alone and/or in combination can induce iNOS expression. Cytokines are produced in response to inflammatory stimuli such as sepsis (for example lipopolysaccharide or endotoxin) or myocardial ischaemia and infarction.

Cytokine production is usually attributed to infiltrating immune cells such as macrophages (Herskowitz A 1995) or nonmyocyte cardiac cells such as fibroblasts (Yue P 1998; Heim A 2000). However, it has recently become more apparent that cytokines can also be expressed by cardiac myocytes as well as fibroblasts. Kapadia et al (Kapadia S 1995) examined TNF α expression in isolated feline hearts after exposure to endotoxin and found by immunohistochemistry that TNF α expression localised to endothelial cells, smooth muscle cells and cardiac myocytes. TNF α expression by cardiac myocytes was further confirmed by in situ hybridization, with TNF α mRNA detected in cardiac myocytes.

TNF α levels have previously been reported to be significantly increased in the hearts of patients with cardiomyopathy, whether idiopathic or ischaemic (Torre-Amione G 1996), however others (Satoh M 1997; Satoh M 1999; Sigusch HH 2000), using immunohistochemistry, have now shown localisation of TNF α to cardiac myocytes.

Myocardial IL-1β expression is less well documented, although IL-1β mRNA has been detected in myocardial tissue in response to the addition of lipopolysaccharide (Kadokami T 2001) and to *Trypanosoma cruzi* infection (Chandrasekar B 1998). However, Ono et al, investigating cytokine expression after myocardial infarction in rat hearts, found by immunohistochemistry that IL-1β expression in the myocardium was confined to infiltrating leukocytes or endothelial cells and vascular smooth muscle cells (Ono K 1998).

Il-6 is expressed in cardiac myocytes both in patients with congestive cardiac failure (Eiken HG 2001) and in primary cell culture (Gwechenberger M 1999; Craig R 2000). IL-6 expression in cardiac myocytes has also been demonstrated in response to hypoxia. Yamauchi-Takihara et al (Yamauchi-Takihara K 1995) reported low levels of IL-6 expression in normoxic neonatal rat ventricular myocyte cultures, but a significant induction in response to hypoxia (0% oxygen, 95% $N_2$ and 5% $CO_2$ ). The experiment was repeated in non myocyte cultures and no induction of IL-6 transcription, above baseline levels, was observed in response to hypoxia. There is also evidence to suggest that hypoxic induction of IL-6 expression is mediated by NFκB (Matsui H 1999).

iNOS expression has been demonstrated in response to LPS and to various cytokines as discussed in Chapter 1. Briefly, in primary cultures of cardiac myocytes, iNOS may be variably expressed in response to TNF α, IL-1β, IL-6 and IFN γ. Expression in response to IL-1β and TNF α is also enhanced by IFN γ. The mechanism of iNOS

induction under conditions of chronic hypoxia without myocardial infarction, and the role of cytokines under these circumstances, remain unclear.

***Transcription factors involved in iNOS regulation under hypoxic conditions***

The transcription factor NFκB has an important role in gene induction in response to IL-1β throughout development into adulthood (Norman DA 1998). NFκB is involved in the induction of iNOS in cardiac myocytes by IL-1β (Kinugawa K 1997) and by TNF α (Kan H 1999). Interestingly, however, NFκB also plays a significant role in the upregulation of IL-1β and TNF α themselves (Chandrasekar B 1998) as well as IL-6. Guha et al (Guha M 2000) studied the effect of chronically high glucose levels on cultured monoblastoid cells and found that chronically high glucose levels resulted in TNF α induction which was mediated by NFκB DNA binding.

Taylor et al (Taylor CT 1999), in human intestinal epithelial cells, found that hypoxia causes increased NFkB activation and also increased TNF α expression. However, epithelial intercellular adhesion molecule (ICAM 1) expression was not increased despite the presence of a NFκB binding site in the ICAM 1 promoter. Thus hypoxia may result in upregulation of some but not all NFκB inducible genes. The authors provide evidence that diminished cAMP responsive element binding may allow enhanced NFκB binding to the TNF α promoter region in their model. Therefore under hypoxic conditions, the interactions between cis-regulatory sequences and their activation by different transcription factors may result in variable induction of

different genes. As discussed in Chapter 1, the iNOS promoter region contains multiple NFκB binding sites. Thus, whether as cause or effect of TNF α expression in hypoxia, NFκB activation may have an important role in the expression of iNOS in cardiac myocytes in hypoxic conditions.

Other transcription factors may also play a role under hypoxic conditions. As discussed in Chapter 1, the iNOS promoter region also contains a hypoxia responsive element which binds the Hypoxia Inducible Factor 1. Although experiments in cultures from day 1-3 neonatal rat ventricular myocytes described in Chapter 4, demonstrated iNOS expression in response to cytokines and LPS (presumably cytokine mediated) but not to hypoxia, HIF 1α was expressed. iNOS expression in response to hypoxia was demonstrated in cultures of ventricular myocytes from day 7 neonatal rats. HIF 1α expression and the potential role of HIF 1 binding to the hypoxia responsive element are therefore also of interest in these cultures.

## 5.2 Expression of cytokines in chronic hypoxia

The expression of TNF α, IL-1β and IL-6 in neonatal rat ventricular myocytes, incubated under hypoxic conditions for 48 hours, was investigated using RT PCR and/or Northern analysis as described in Chapter 2. Northern analysis was used, when expression was very evident on RT PCR, for comparison of expression levels between experiments. Cultures were of ventricular myocytes harvested from day 7

rats and in each case been shown to express iNOS under hypoxic conditions (see Chapter 4) but not under normoxic conditions.

Variable TNF α expression was detected in both normoxic and hypoxic cultures. Using the PCR protocol described in Chapter 2 with an annealing temperature of 60°C, only faint bands were seen but expression was variable in both normoxic flasks and in hypoxic flasks. The experiment was performed 7 times (ie seven separate cell harvests). Representative results are shown in Figure 5.1. PCR was therefore repeated with an annealing temperature of 55 °C to increase sensitivity. Results again showed highly variable TNF α expression, ranging from no expression in either normoxic or hypoxic flasks to expression in either or both. To ensure specific amplification with the reduced annealing temperature, the 487 bp band from two separate experiments (cultures) was then excised, the product extracted and sequenced. The sequence was compared to known sequences from GenBank at the National Center for Biotechnology Information (USA) by entering the sequence into BLAST (www.ncbi.nlm.nih.gov/BLAST/) to confirm the identity and specificity of the PCR product. Thus TNF α expression was confirmed.

In contrast, no expression of IL-1β was detected in either normoxic or hypoxic cultures of neonatal rat ventricular myocytes. This experiment was performed four times.

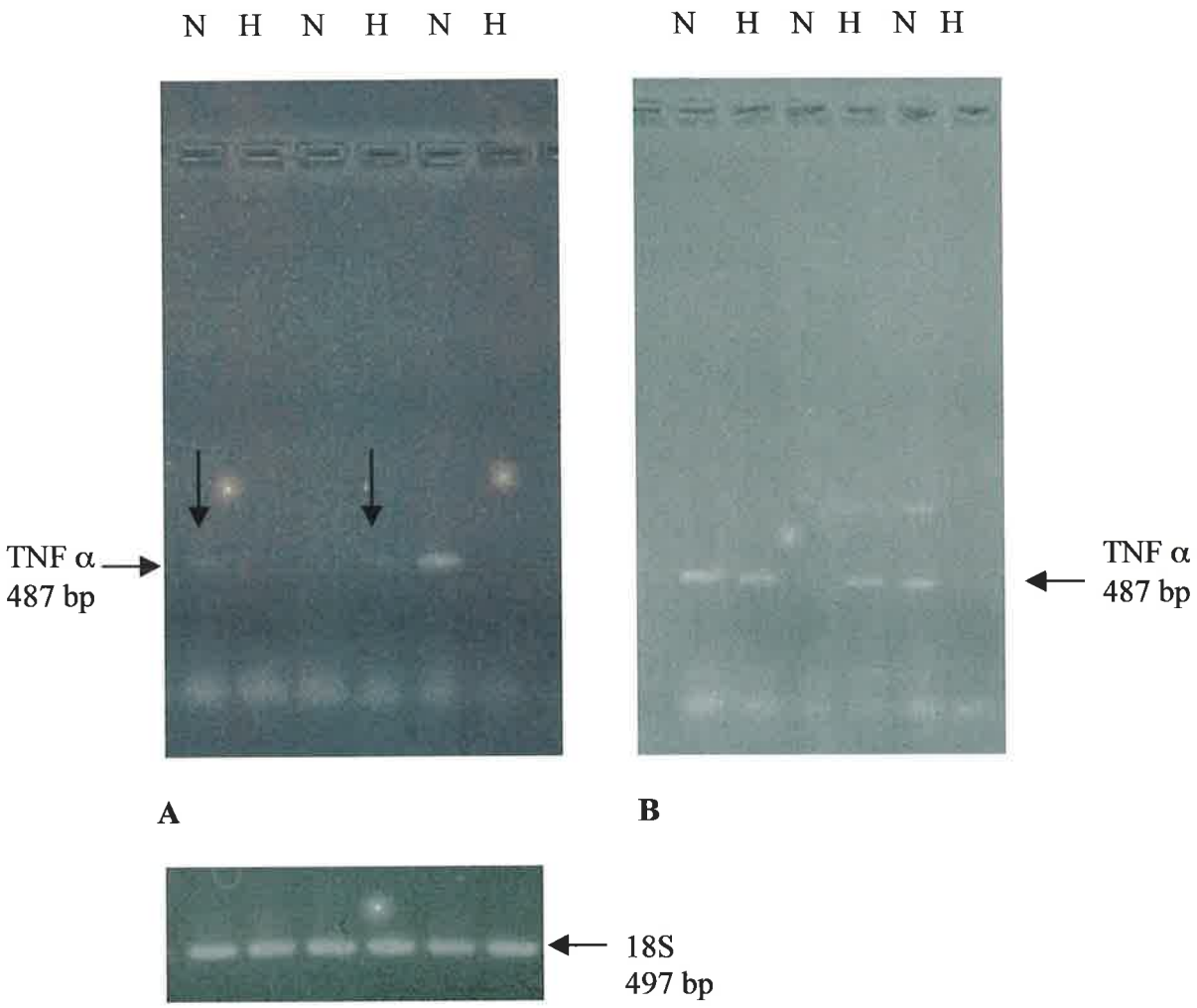



**Figure 5.1** TNF α expression in cardiac myocytes exposed to 1% oxygen for 48 hours (H) compared to expression in normoxic controls (N). The results shown represent three separate experiments where TNF α expression was detected. Panel A shows PCR performed with an annealing temperature of 60°C with 18S shown below, B shows the same PCR with an annealing temperature of 55°C. There is variable expression of TNF α under both normoxic and hypoxic conditions.

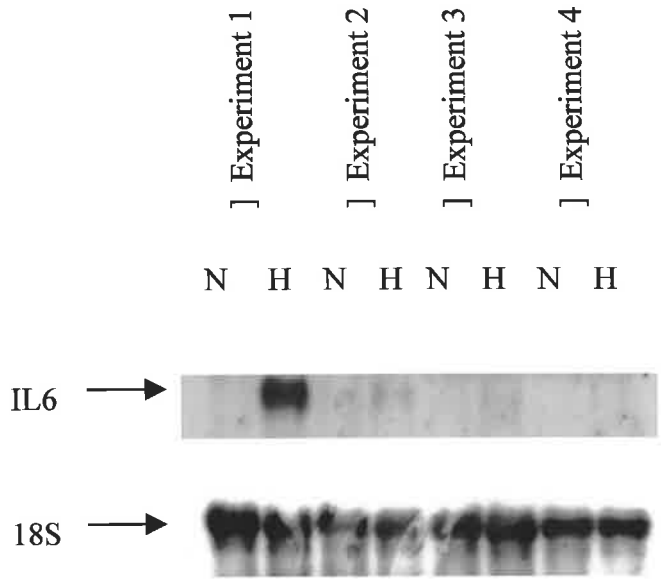



**Figure 5.2** IL6 expression in cardiac myocytes exposed to 1% oxygen for 48 hours (H) compared to expression in normoxic controls (N). The upper Northern blot shows IL6 expression, the lower blot shows 18S. No IL6 is seen in the normoxic controls. IL6 is variably induced in the hypoxic flasks with strong induction demonstrated in Experiment 1 and much less (very mild induction) in Experiments 2 and 3. No IL6 induction is seen in Experiment 4.

Il-6 expression was also investigated, by Northern analysis, and no IL-6 expression was detected in the normoxic controls. IL-6 expression under hypoxic conditions was variable ranging from strong induction in one experiment to none at all in another. In the remaining two experiments there was very weak induction of IL-6 expression detected on Northern analysis. The results of these experiments are shown in Figure 5.2.

## 5.3 Transcription factors binding to the iNOS promoter region in chronic hypoxia

### 5.3.1 Methods

Binding of transcription factors to cis regulatory elements in the iNOS promoter region was detected by electromobility shift assay as described in Chapter 2. Approximately 5 x $10^7$ neonatal rat ventricular myocytes, harvested from day 7 neonatal rats, were cultured in a $T_{75}$ culture plate and incubated under hypoxic conditions for 48 hours to obtain nuclear protein extract. This large number of cells was required in this experiment to obtain a nuclear protein extract of sufficient concentration (1-2 µg/µl) to use in a 20 µl binding reaction volume.

Given that the HIF 1 is constitutively expressed and is continually present in the cytoplasm, albeit constantly degraded unless under hypoxic conditions, it is important to obtain a nuclear protein extract rather than a whole cell protein extract. The centrifugation step following cell lysis was therefore performed at 800 g (Dent CL 1993).

Similarly, in the study of NFκB-DNA binding, NFκB is constitutively expressed in the cytoplasm. It is bound by Inhibitory Factor κB (IFκB), resulting in inhibition of nuclear translocation. Activation of NFκB is caused by phosphorylation and ubiquitination of IFκB (Finco TS 1994; McKinsey TA 1997) with subsequent separation from NFκB and further degradation of IFκB (Henkel T 1993).

### 5.3.2 Hypoxia Responsive Element binding in hypoxia

The experiment was performed four times to demonstrate consistency of the results. Under conditions of prolonged hypoxia (48 hours), nuclear protein binding to the hypoxia responsive element of the iNOS promoter region was consistently observed. This is shown in Figure 5.3 by the band indicating retardation of migration of the labelled DNA through the gel. The band was eliminated with the addition of molar excess of unlabelled hypoxia responsive element DNA sequence, indicating specific binding.

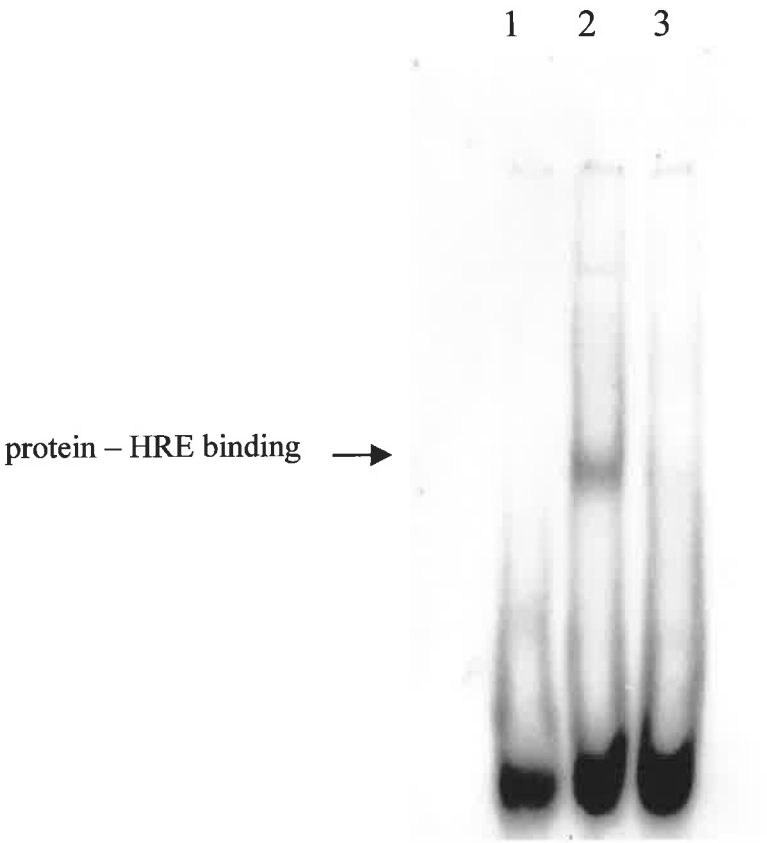



**Figure 5.3** Electromobility shift assay demonstrating binding of nuclear protein extract from neonatal rat ventricular myocytes incubated under hypoxic conditions to the labelled sequence from the hypoxia responsive element (HRE) in the iNOS promoter region. Lane 1 represents labelled oligonucleotide without nuclear extract; Lane 2 addition of nuclear extract to the labelled HRE; Lane 3 represents the nuclear extract, labelled HRE and the addition of molar excess of unlabelled HRE sequence.

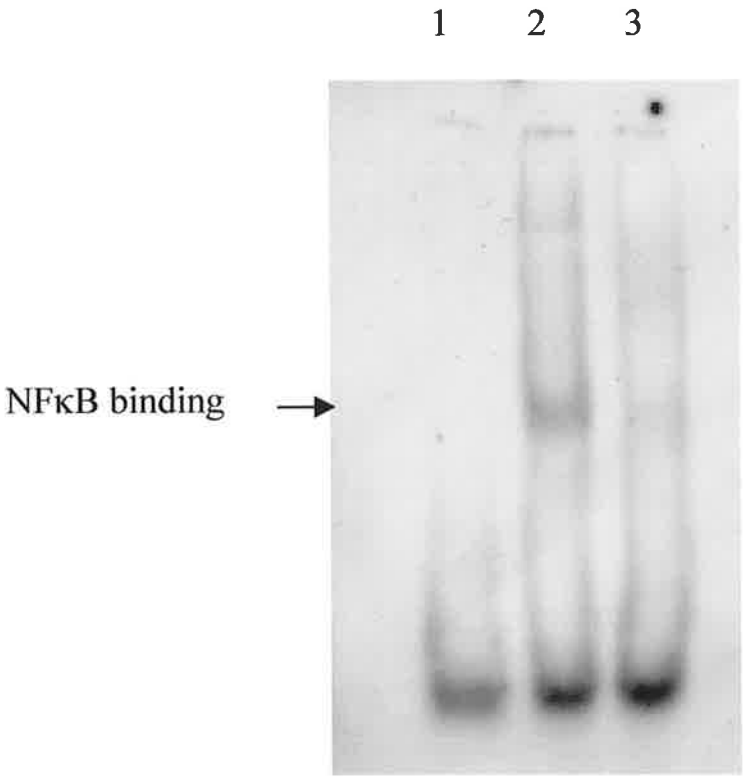



**Figure 5.4** Electromobility shift assay demonstrating binding of nuclear protein extract from same hypoxic cultures to the labelled sequence from the NFκB binding site in the iNOS promoter region. Lane 1 represents labelled NFκB binding site without nuclear extract; Lane 2 addition of nuclear extract to the labelled NFκB binding site; Lane 3 represents the nuclear extract, labelled NFκB binding site and the addition of molar excess of unlabelled NFκB binding site sequence. Once again the band in Lane 2 indicates DNA-protein binding.

### 5.3.3 Binding to the NFκB site under hypoxic conditions

As with Hypoxia Responsive Element binding, demonstration of nuclear NFκB binding to the iNOS promoter region under hypoxic conditions was performed four times to demonstrate consistency of results. Binding to the NFκB site was detected by electromobility shift assay, with elimination of the band by addition of molar excess of unlabelled NFκB binding site sequence. Results of a representative experiment are shown in Figure 5.4.

## 5.4 Expression of Hypoxia Inducible Factor 1 in Chronic Hypoxia

RNA was extracted from normoxic and hypoxic cultures of neonatal rat ventricular myocytes harvested from day 7 neonatal Sprague-Dawley rats as described in Chapter 2. Because HIF 1α is constitutively expressed (Gradin K 1996) (and this was confirmed on initial PCR experiments), expression under hypoxic conditions was compared to that in normoxic controls using Northern analysis as described in Chapter 2. Expression of HIF 1α was normalised to that of 18S as a "housekeeping gene".

There was a significant variation in the baseline levels of HIF 1α mRNA expressed in normoxic control cultures. A significant variation in expression as a proportion of

18S is introduced when experiments analysed on different blots are being compared. The density of the gene of interest compared to the 18S band will depend on relative labelling efficiencies of the probes. This may vary with each transcription reaction and certainly would vary from hybridization to hybridization whether probes are being reused or not. Nevertheless, when comparing expression of HIF 1α in normoxic cultures analysed on the same blot, the variation in baseline HIF 1α expression would appear to be real (see Figure 5.5).

The effect of these "technical" variations on the comparison between normoxic and hypoxic cultures may be taken into account by performing a paired analysis of normoxic and hypoxic cultures. Normoxic and hypoxic flasks from the same ventricular myocyte harvest were analysed on the same Northern blot and paired for the purposes of statistical analysis.

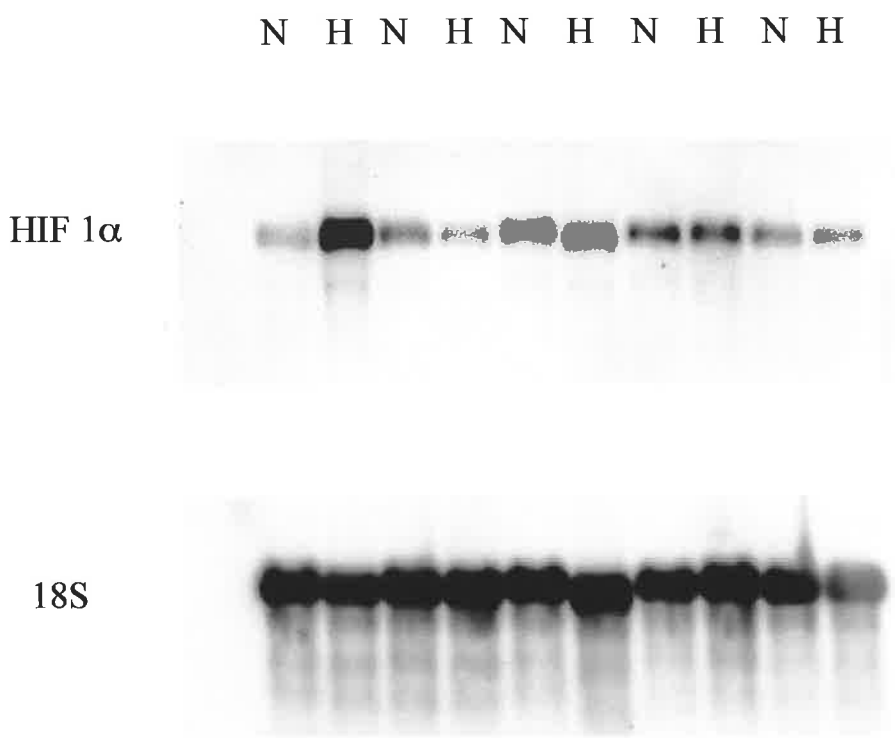



**Figure 5.5** Northern analysis of HIF 1α expression from five experiments, all analysed on the same gel. H represents hypoxic cultures, N represents normoxic controls. 18S expression from the same experiments and same blot is shown in the lower panel. There is a wide variation of baseline HIF 1α expression in the normoxic control cultures.

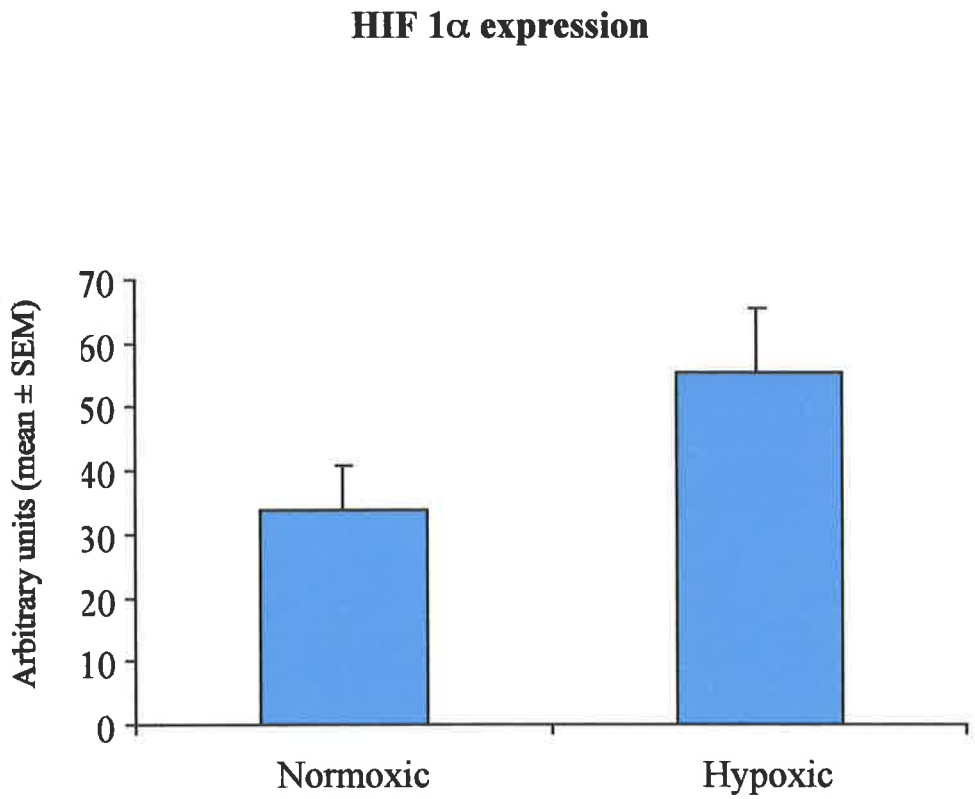



**Figure 5.6** Expression of HIF 1α, normalised to that of 18S, for normoxic and hypoxic cultures (p = ns).

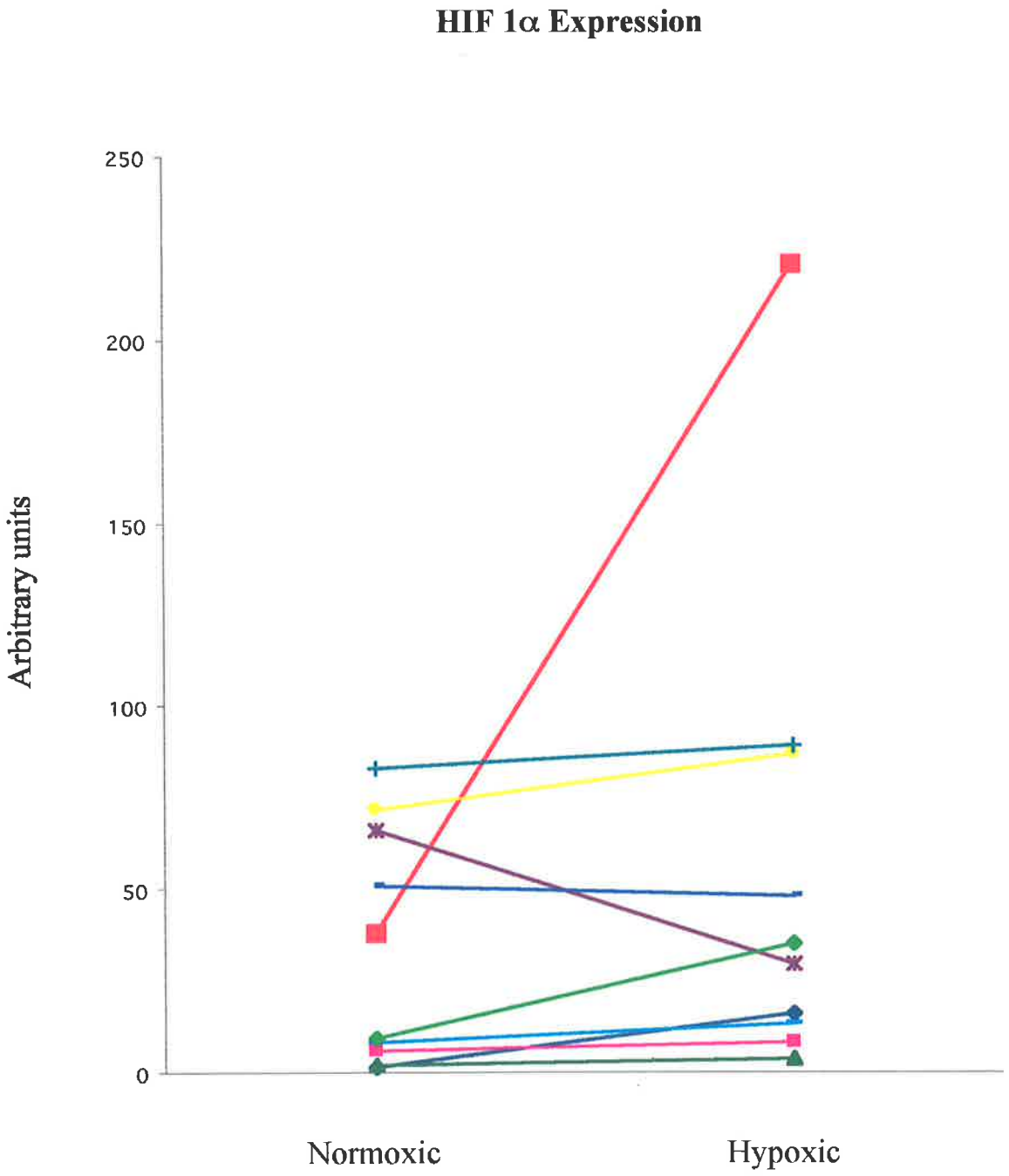



**Figure 5.7** HIF 1α expression (normalised to 18s expression) in normoxic cultures compared to hypoxic cultures. Pairs of cultures from the same harvest of neonatal rat ventricular myocytes are indicated by the lines.

The experiment was performed ten times and no significant difference was found between expression of HIF 1α in normoxic conditions (34 ± 10 arbitrary units) compared to hypoxic conditions (55 ± 21 arbitrary units, p = 0.279). The levels of HIF 1α expression for normoxic and hypoxic cultures are summarised in Figure 5.6. In eight out of ten experiments the level of expression in hypoxic conditions was higher than in normoxic controls, but in two of the experiments the levels were lower (see Figure 5.7).

## 5.5 Discussion

There was highly variable expression of TNF α in both normoxic and hypoxic cultures. The fact that TNF α was expressed in normoxic as well as hypoxic cultures, but iNOS was not expressed in the normoxic cultures suggests that under these experimental conditions, TNF α expression is not a sufficient stimulus for iNOS expression. However, some studies suggest that chronic TNF α expression results in formation of p50/p50 homodimers of NFκB, which translocate but are transcriptionally inactive, with consequently fewer inflammatory effects (Haudek SB 2001). Hypoxia may therefore be the acute stimulus, in some cultures, for the formation of p65/p50 heterodimers NFκB, which are transcriptionally active, with consequently increased iNOS induction.

The variable induction of IL-6 under hypoxic conditions suggests that it may contribute to the levels of iNOS expression by cardiac myocytes under hypoxic conditions. As discussed in Chapter 1, iNOS has been shown to be expressed in response to IL-6 (Kinugawa K 1997). Given that IL-6 was found to be variably induced by hypoxia in the experiments described and that IL-6 expression may variably result in iNOS induction, it is possible that IL-6 may have a role to play in iNOS expression under the hypoxic conditions studied in the present experiments. However, given that iNOS is consistently expressed under these conditions, it is unlikely to be the primary mechanism of iNOS induction.

The addition of IL-1β does consistently result in iNOS expression both in cultures harvested from 1-3 day and 7 day neonatal rats, therefore IL-1β is sufficient for iNOS induction. Theoretically, the mechanism of iNOS induction under hypoxic conditions may be a hypoxia induced increase in IL-1β. However no induction of IL-1β was demonstrated, suggesting that IL-1β has no role in iNOS induction of cardiac myocytes under hypoxic conditions.

NFκB binding was demonstrated consistently in hypoxic myocytes expressing iNOS, consistent with a role for cytokine induction resulting in iNOS expression. Given the variable expression of TNF α under both normoxic and hypoxic conditions, this is unlikely to be the sole reason for NFκB activation and translocation to the nucleus. Other mechanisms may result in direct activation of NFκB under hypoxic conditions. Chandel et al (Chandel NS 2000) report NFκB activation by mitochondrial reactive

oxygen species under hypoxic conditions (1.5% oxygen) in a murine macrophage line, which is independent of LPS activation of NFκB. On the other hand Koong et al (Koong AC 1994) suggest that tyrosine phosphorylation of IFκB under hypoxic conditions results in dissociation of IFκB from NFκB and degradation of IFκB, therefore resulting in NFκB activation.

The results of electromobility shift assays also implicate the involvement of binding to the iNOS HRE in the regulation of iNOS expression in response to prolonged or chronic hypoxia. This suggests involvement of the Hypoxia Inducible Factor 1. Therefore expression of HIF 1α was investigated by Northern analysis. However no significant or consistent upregulation of HIF 1α expression could be demonstrated. This may be attributed to the largely post translational regulation of HIF 1 with reduced ubiquitination and clearance under hypoxic conditions (Huang LE 1996; Salceda S 1997; Sutter CH 2000).

The variation in baseline HIF 1α expression is an interesting finding. While a wide variation in expression of constitutively expressed genes is not unheard of (Lown KS 1994; Le Cluyse E 2000), the baseline variation of HIF 1α expression has not previously been documented. The present thesis contains a statistical analysis of 10 experiments and shows a wide variation in expression in normoxic control cultures, ie baseline conditions. Visual inspection of previous reports of HIF 1α expression (Jung F 2000) also suggest a wide variation in expression in response to hypoxia, however the finding was not specifically discussed.

From the results of experiments outlined in this chapter, it can be concluded that both HRE and NFkB binding play an important role in the regulation of iNOS expression under hypoxic conditions. While the mechanisms of iNOS induction by HRE binding may be more direct, by stabilisation of HIF under hypoxic conditions, NFκB may in part be activated through TNF α expression. Results also suggest that IL-6 is not primarily responsible for iNOS expression under these circumstances, but may have a variable role in enhancing its expression.

# CHAPTER SIX

# EFFECTS OF iNOS EXPRESSION IN CHRONIC HYPOXIA

## 6.1 Introduction

***The role of iNOS in ischaemic preconditioning***

iNOS has been shown to have a role in the delayed phase or "second window" of preconditioning in animal models (Takano H 1998; Guo Y 1999; Imagawa J 1999; Zhao T 2000; Zhao TC 2001). This suggests a protective role for iNOS in ischaemia or conditions of reduced oxygen supply. Given the negative ionotropic effects of nitric oxide may in part be mediated by the reversible effects of nitric oxide in the inhibition of mitochondrial respiration, it is possible that production of nitric oxide by iNOS in hibernating myocardium may contribute to the reversible and potentially protective downregulation of contractile function seen in this condition. Therefore the effects of iNOS on cardiac myocytes under conditions of prolonged hypoxia were investigated.

***iNOS and apoptosis***

iNOS is also known to have deleterious effects on cardiac myocytes such as induction of apoptosis (Ing DJ 1999). The apoptosis of cardiac myocytes in response to cytokines can be inhibited by the addition of an iNOS inhibitor, indicating the important role of iNOS in mediating this process (Arstall MA 1999).

***Hypoxia and the induction of apoptosis***

The role of hypoxia in the induction of apoptosis has been controversial. It is clear that apoptotic cell death plays a significant role in myocyte loss through myocardial

infarction. Previously it was thought that necrosis was responsible for the most if not all myocyte death under these circumstances. However more recently, it has become apparent that apoptosis accounts for a significant proportion of myocyte loss, perhaps up to 90% (Kajstura J 1996).

Myocardial infarction is a process that normally involves a period of severe ischaemia followed by reperfusion. Ironically reperfusion has emerged as a major source of myocyte injury. One study of isolated chick cardiac myocytes, exposed to 1 hour of simulated ischaemia (hypoxia, hypercarbic acidosis, hyperkalaemia and substrate deprivation) ± 3 hours of simulated reperfusion, found that 90% of cell death occurred in the reperfusion phase (Van den Hoek TL 1996). Other studies however estimate that reperfusion accounts for 15% of myocardial cell death (Farb A 1993).

The role of hypoxia, as compared to ischaemia, in the absence of reoxygenation (or simulated reperfusion) in activation of myocyte cell death by apoptosis is less clear. Webster et al (Webster K 1999) investigated the effect of 1% oxygen on apoptosis in cultures of neonatal rat cardiac myocytes and found apoptosis occurred on reoxygenation or with a low pH in the media, but not with hypoxia alone. Others (Long X 1997; Malhotra R 1999; de Moissac D 2000) have found that extremely low oxygen levels (a gas mix containing 0% oxygen) does result in apoptosis of cultured cardiac myocytes. Jung et al (Jung F 2001), however, have demonstrated apoptosis of

cardiac myocytes in response to 1% oxygen without reoxygenation. The mechanism of induction of apoptosis under hypoxic conditions also remains unclear.

## 6.2 The role of iNOS in the downregulation of contraction in chronic hypoxia

Cultures of neonatal rat ventricular myocytes, harvested from Day 1-3 neonates, incubated under conditions of prolonged hypoxia display many of the features of hibernating myocardium as described in Chapter 3. However, as discussed in Chapter 4, these cultures demonstrate no iNOS expression in response to the hypoxic conditions used. It would therefore seem that iNOS has no role in the downregulation of contractile work demonstrated in this cellular model of hibernating myocardium. The reduction in beating rate under prolonged hypoxic conditions, along with the response to dobutamine and the recovery of beating rate following reoxygenation are all independent of iNOS expression in this model.

However, iNOS expression has been documented in hibernating myocardium from human subjects as well as whole animal models, as discussed in Chapter 1. Therefore, other effects of iNOS in hypoxia were investigated using cultures of neonatal rat ventricular myocytes derived from Day 7 neonates, which consistently express iNOS in response to hypoxia as demonstrated in Chapter 4.

## 6.3 Apoptosis in Chronic Hypoxia

### 6.3.1 Detection of apoptosis in cardiac myocytes

Apoptosis of cardiac myocytes was detected using a caspase 3 activity assay as described in Chapter 2. This method was used to provide quantitative data amenable to the statistical analysis of differences between apoptosis in normoxic cultures and hypoxic cultures. The data was analysed with ANOVA followed by Tukeys (post hoc) test to detect the differences between two groups of data.

Annexin V staining was also used to qualitatively detect apoptosis of cardiac myocytes in culture and to confirm findings using a different method of detection.

### 6.3.2 Apoptosis in normoxic cells in culture

The effect of age in culture was investigated to determine apoptosis in normoxic control cultures before proceeding to experiments with hypoxic cultures. Once cultures had grown to confluence, media were changed and cells were maintained in culture under normoxic conditions (5% $CO_2$, 95% air) for 18 (n=5), 24 (n=4) or 48 hours (n=4). Cellular protein was then extracted and assayed for caspase 3 activity.

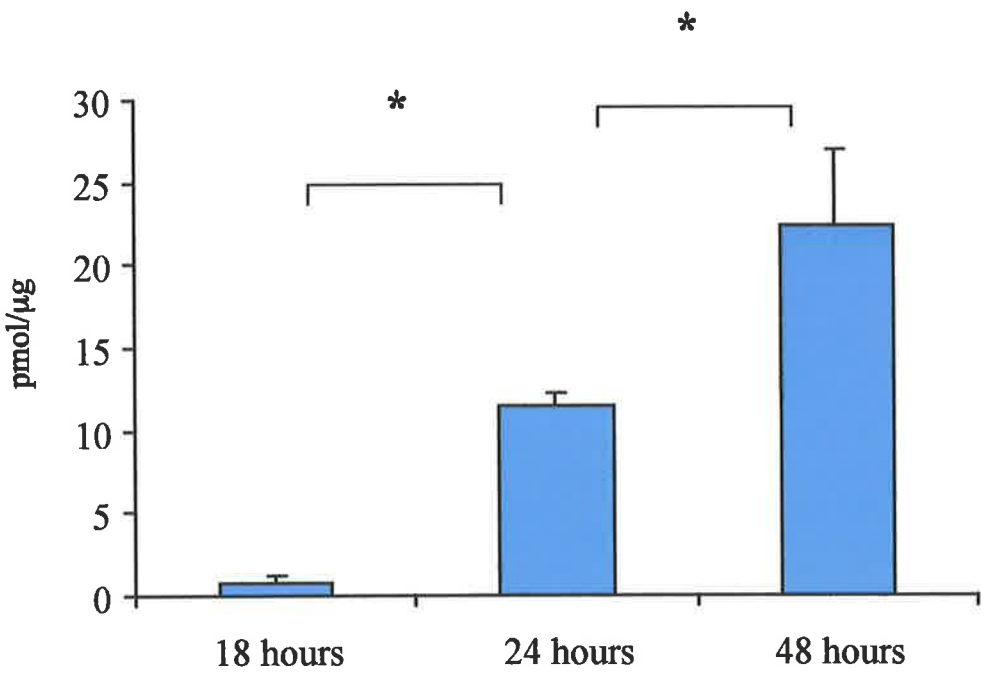



**Figure 6.1** Effect of age in culture on caspase 3 activity in normoxic cells. Increasing age in culture results in increased apoptosis of cardiac myocytes. Caspase 3 activity is expressed as pmol pNA released per µg cellular protein as described in Chapter 2. There was a significant difference between groups ($p < 0.05$, ANOVA, $f = 20.39$). * indicates significant differences between two groups ($p < 0.05$, Tukey's test).

After 18 hours there was minimal caspase 3 activity detectable in normoxic cells (0.7 ± 0.6 pmol pNA released/μg cellular protein). After 24 hours, there was a significant increase in caspase 3 activity (to 11.4 ± 0.8 pmol/μg), compared to the 18 hour cultures ($p<0.05$). After 48 hours there was a further increase in caspase 3 activity (to 22.4 ± 4.5 pmol/μg), compared to the 24 hour cultures ($p<0.05$) (see Figure 6.1). Thus increasing age in culture results in apoptosis of normoxic cells. On this basis it was decided that hypoxic experiments would be performed with 18 hours of hypoxic incubation.

### 6.3.3 The effect of hypoxia on apoptosis of cardiac myocytes

Caspase 3 activity in normoxic cultures was compared with that in cultures incubated under hypoxic conditions for 18 hours, as described in Chapter 2. There was significantly increased caspase 3 activity in the hypoxic cultures (10.4 ± 0.9 pmol/μg) compared with the normoxic cultures (1.0 ± 1.0 pmol/μg, $p < 0.01$ ANOVA, Tukey's post hoc test, see Figure 6.3a). This experiment was repeated twice. The experiments were also performed with cultures incubated for 24 and 48 hours (Figure 6.2). In both of the experimental conditions there was increased caspase 3 activity in the hypoxic cultures compared to the normoxic cultures (25.1 ± 1.5 vs 12.7 ± 0.8 pmol/μg $p<0.05$ for 24 hour experiment; 27.1 ± 3.7 vs 5.7 ± 0.8 $p<0.05$ for 48 hours). It is therefore concluded that hypoxia results in apoptosis of cardiac myocytes in culture. These results support the findings of Jung et al (Jung F 2001).

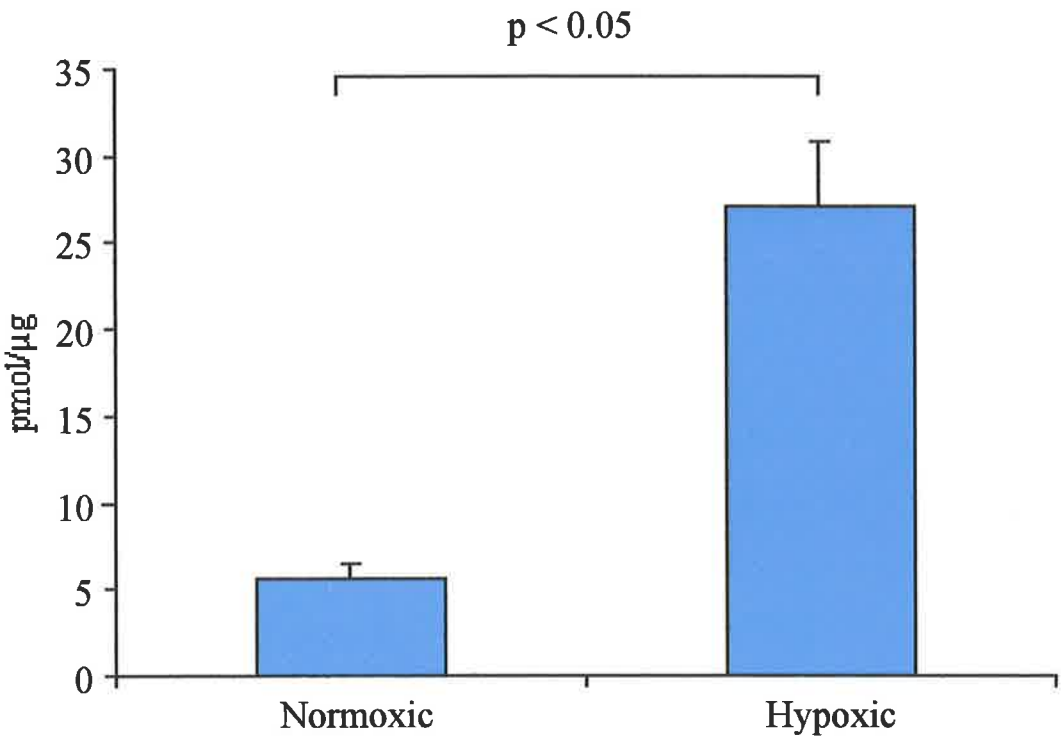



**Figure 6.2** The effect of 48 hours of hypoxia on caspase 3 activity. There is significantly increased caspase 3 activity in hypoxic cultures compared to normoxic cultures ($p < 0.05$, unpaired t test).

## 6.4 Role of iNOS in hypoxia induced apoptosis

To determine whether iNOS has a role in the hypoxia induced apoptosis of cardiac myocytes in culture, hypoxic cultures were incubated with 100 μM S-methyl isothiourea (SMT). SMT is a competitive inhibitor of iNOS at the L-arginine binding site (Southan GJ 1995) and is a more potent inhibitor of iNOS than aminoguanidine in cardiac myocytes in vitro (Wang D 1999) and is a relatively selective inhibitor of iNOS activity over eNOS (Szabo C 1994). For the current study, SMT was used in a concentration of 100 μM in media as described by Wang et al (Wang D 1999).

Caspase 3 activity in hypoxic cells incubated with SMT was compared to those without SMT and to normoxic controls. Results were confirmed with Annexin V staining. Data was analysed by one way ANOVA followed by Tukey's post hoc test.

Results (with n = 3 in each group) show that there was increased caspase 3 activity in hypoxic flasks (10.4 ± 0.9 pmol/μg cellular protein) compared to normoxic controls (1.0 ± 1.0 pmol/μg, $p<0.01$) but no difference in activity in the hypoxic flasks treated with 100 μM SMT (6.9 ± 6.9 pmol/μg) compared to the hypoxic group ($p <$ ns) (Figure 6.3a). This experiment was repeated with similar results (normoxic 0.3 ± 0.3 pmol/μg; hypoxic 11.2 ± 5.0 pmol/μg; hypoxic with SMT 7.6 ± 7.6 pmol/μg).

The third experiment was performed with incubation under hypoxic conditions but using L-NAME (300μM) to inhibit iNOS. This was done to show that the caspase 3 activity in some flasks treated with NOS inhibition is not due to direct effects of SMT on iNOS. This seems unlikely in any case as the result is not consistent within the SMT treated groups, but the concentrations of SMT used were consistent throughout the experiments. In this experiment, there were four flasks in the hypoxia plus L-NAME group instead of three. Once again there was increased caspase 3 activity in the hypoxic group (24.3 ± 5.9 pmol/μg) compared to the normoxic group (0 ± 0 pmol/μg, $p < 0.01$) (see Figure 6.3b). However, in this experiment there was a significant reduction in caspase 3 activity in the hypoxic group treated with L-NAME (4.4 ± 0.9 pmol/μg) compared to the hypoxic group ($p < 0.01$).

On analysis of pooled data from all three experiments, the addition of an iNOS inhibitor resulted in an overall reduction in caspase 3 activity ($p<0.05$), compared with the hypoxic cultures (Figure 6.4). Furthermore, on visual inspection of data from each experiment, iNOS inhibition inhibited caspase 3 activity almost completely in 2 out of 3 flasks. The remaining flasks showed caspase 3 activity equal to or greater than that of the hypoxic cultures. The pooled data is shown in Figure 6.5.

These findings suggest that iNOS is involved in the hypoxia induced apoptosis of cardiac myocytes, but that in some cultures other, iNOS independent, mechanisms of induction of apoptosis are also involved.

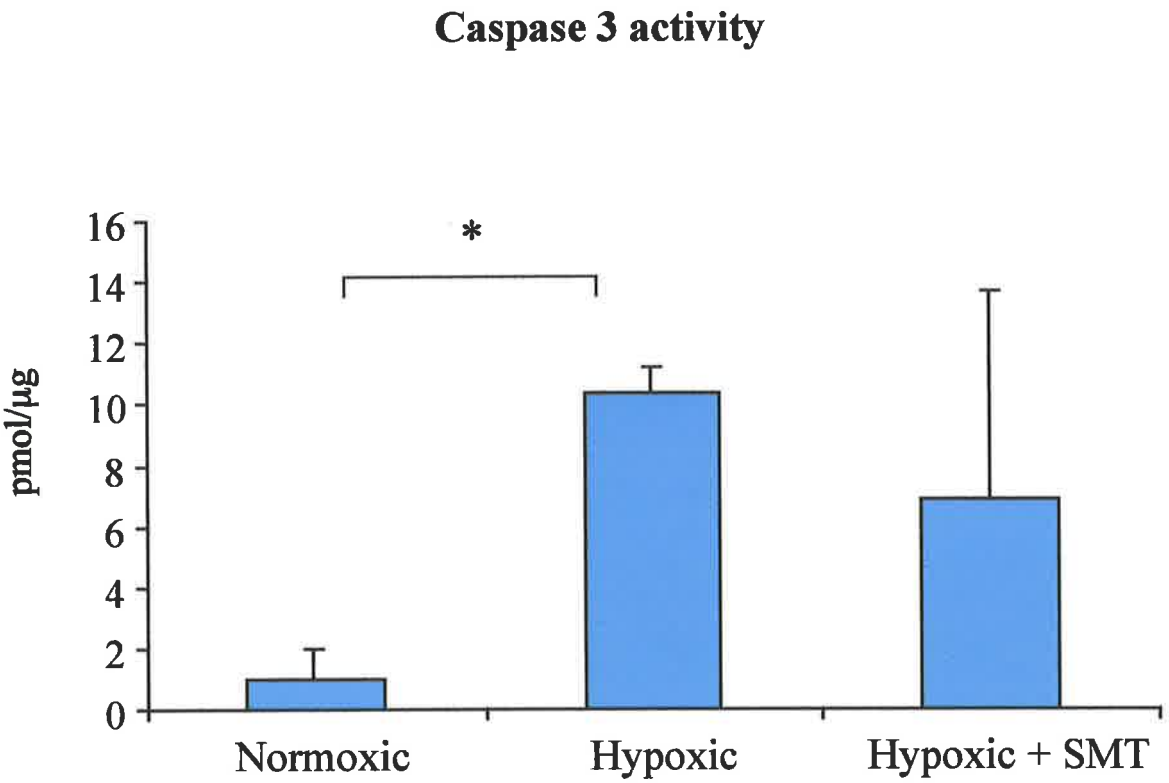



**Figure 6.3a** An example of an experiment to determine the effect of hypoxia (18 hours) on apoptosis of cardiac myocytes and the effect of iNOS inhibition with 100 µM SMT on hypoxia induced apoptosis of cardiac myocytes. Caspase 3 activity is expressed as mean ± SEM (n=3 in each group). * indicates $p < 0.01$. Data were analysed by ANOVA followed by Tukey's post hoc test.

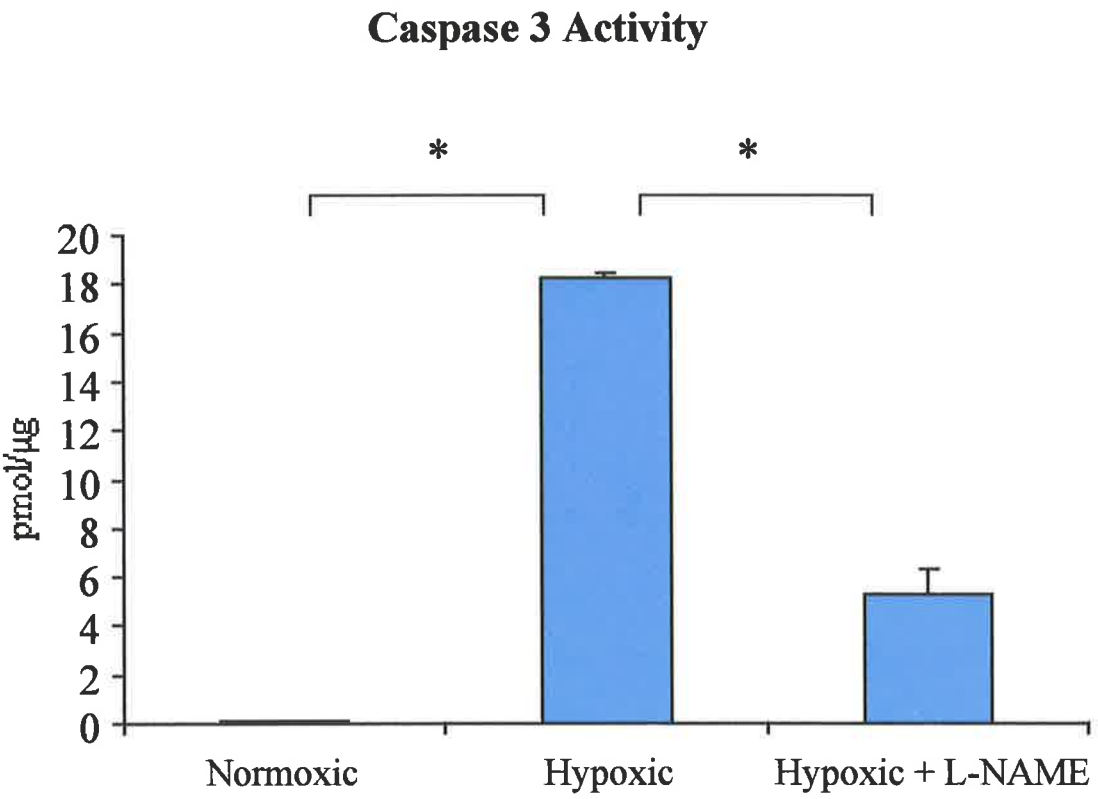



**Figure 6.3b** Results of the third experiment to determine the effect of hypoxia (18 hours) on apoptosis of cardiac myocytes and the effect of iNOS inhibition (with L-NAME). There was significantly increased activity in the hypoxic cultures compared to the normoxic cultures (*$p < 0.001$), as well as a significant difference between hypoxic and hypoxic with L-NAME (*$p < 0.001$).

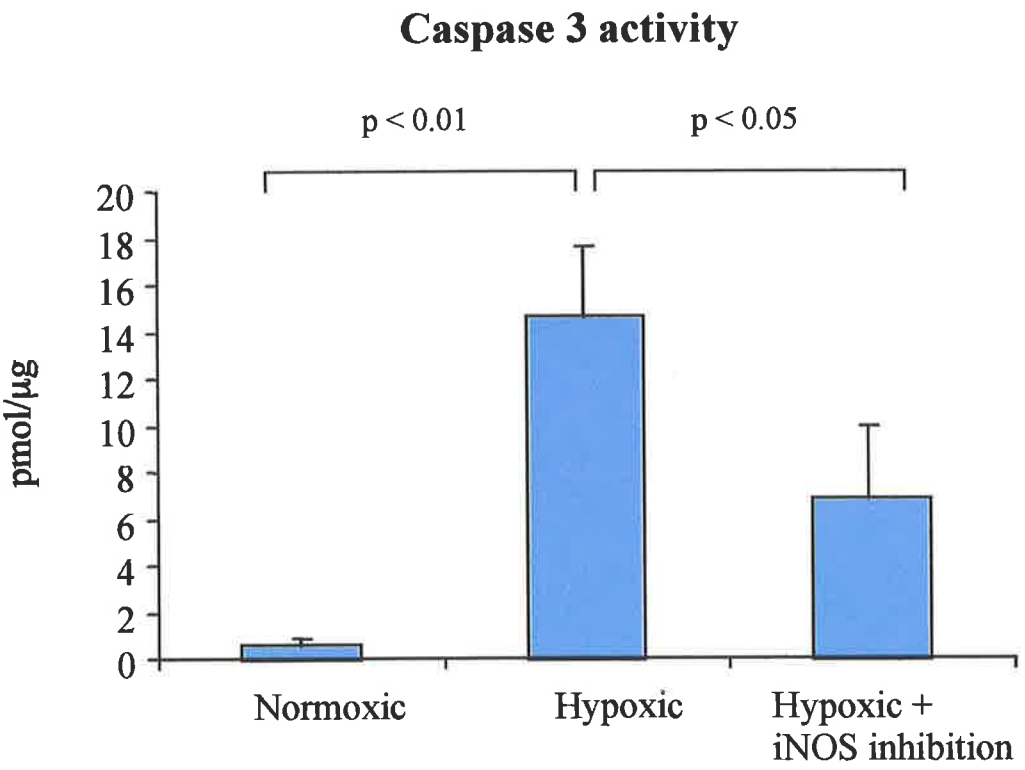



**Figure 6.4** The effect of hypoxia (18 hours) on caspase 3 activity in cardiac myocytes and the role of iNOS in hypoxia induced apoptosis as detected by caspase 3 activity. Data shown is pooled from three separate experiments. Data was analysed by ANOVA with Tukey's post hoc test. There was increased caspase 3 activity in hypoxic flasks compared to normoxic controls ($p < 0.01$) and iNOS inhibition (L-NAME or SMT) resulted in a significant reduction in caspase 3 activity in hypoxic flasks compared to those without iNOS inhibition ($p < 0.05$).

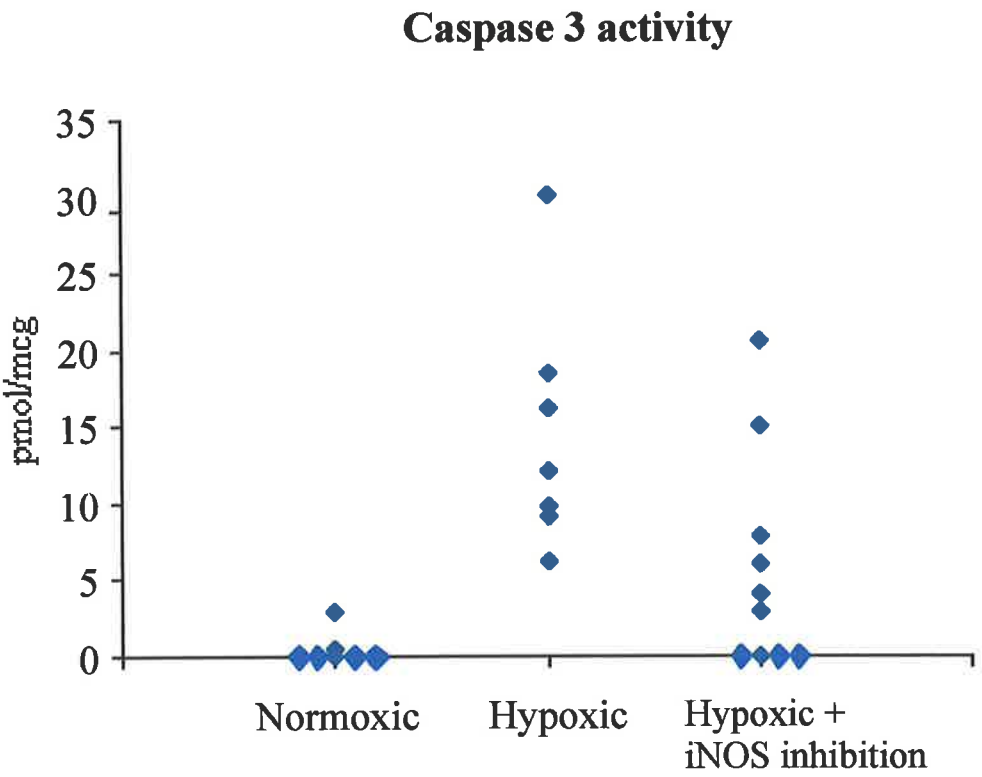



**Figure 6.5** Caspase 3 activity in normoxic controls, hypoxic cultures and hypoxic cultures treated with an iNOS inhibitor. Pooled raw data from three experiments are shown. On visual inspection of data it would seem that a proportion of the hypoxic cultures with iNOS inhibition show virtually no caspase 3 inhibition, while others show caspase 3 activity similar to the normoxic control group.

## 6.5 Confirmation of apoptosis in cardiac myocytes by Annexin V staining

Presentation of phosphatidylserine on the plasma membrane surface of apoptotic cardiac myocytes was detected using Annexin V bound to biotin as described in Chapter 2. Hypoxic cultures were compared to normoxic cultures and to hypoxic cultures treated with 100 μM SMT to assess the effect of iNOS inhibition on hypoxia induced apoptosis. The experiment was repeated twice more.

Annexin V staining of hypoxic cells showed markedly increased apoptosis of cardiac myocytes when compared to normoxic controls. Hypoxia induced apoptosis was inhibited by the addition of SMT, so that the appearances of hypoxic cultures treated with SMT approached those of the normoxic controls. (Figures 6.6, 6.7 and 6.8). These results support the hypothesis that hypoxia induced apoptosis of cardiac myocytes is mediated by iNOS.

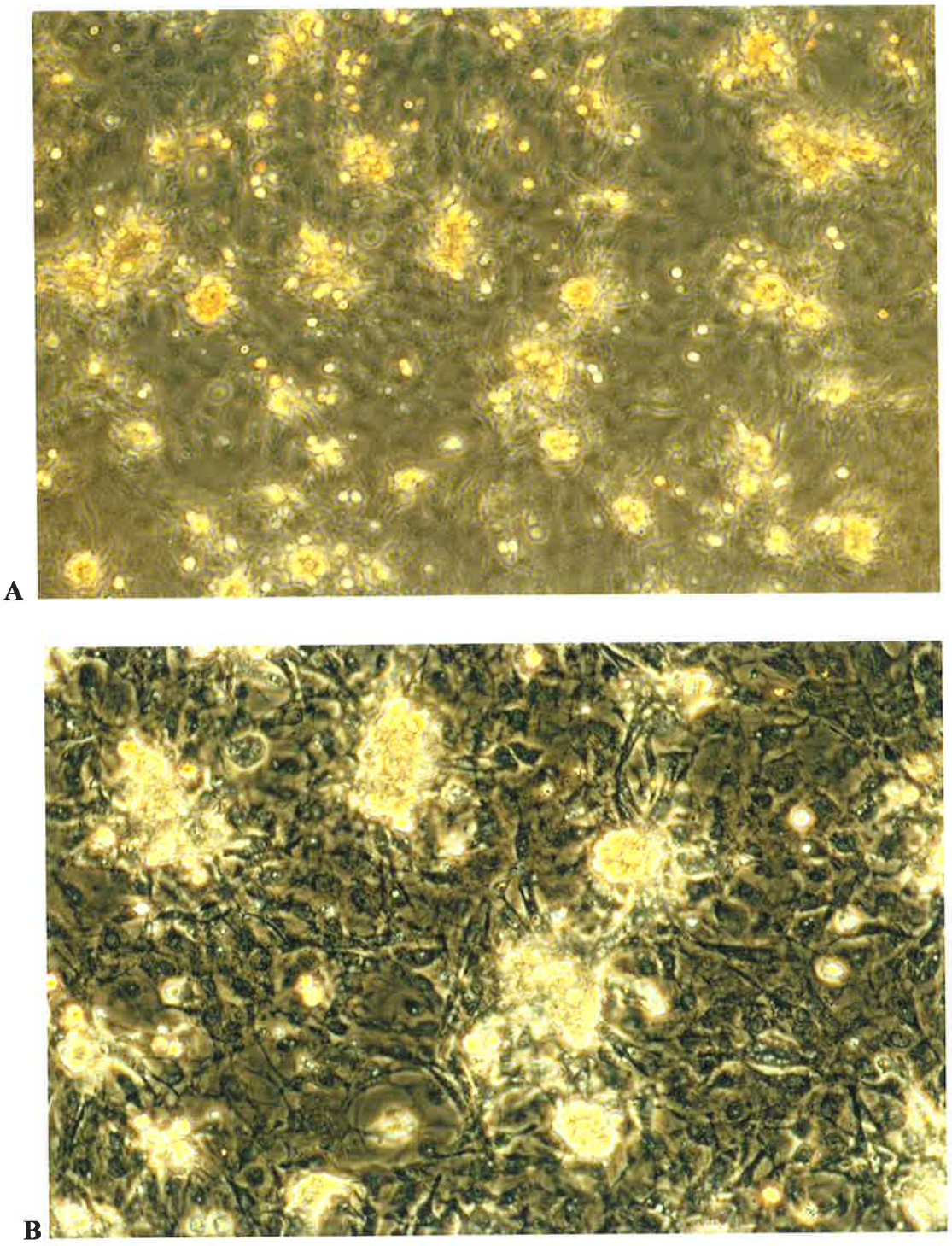



**Figure 6.6** Annexin staining in normoxic cultures. **A** represents low power magnification (10 x), **B** represents high power magnification (20 x). No apoptosis is demonstrated.

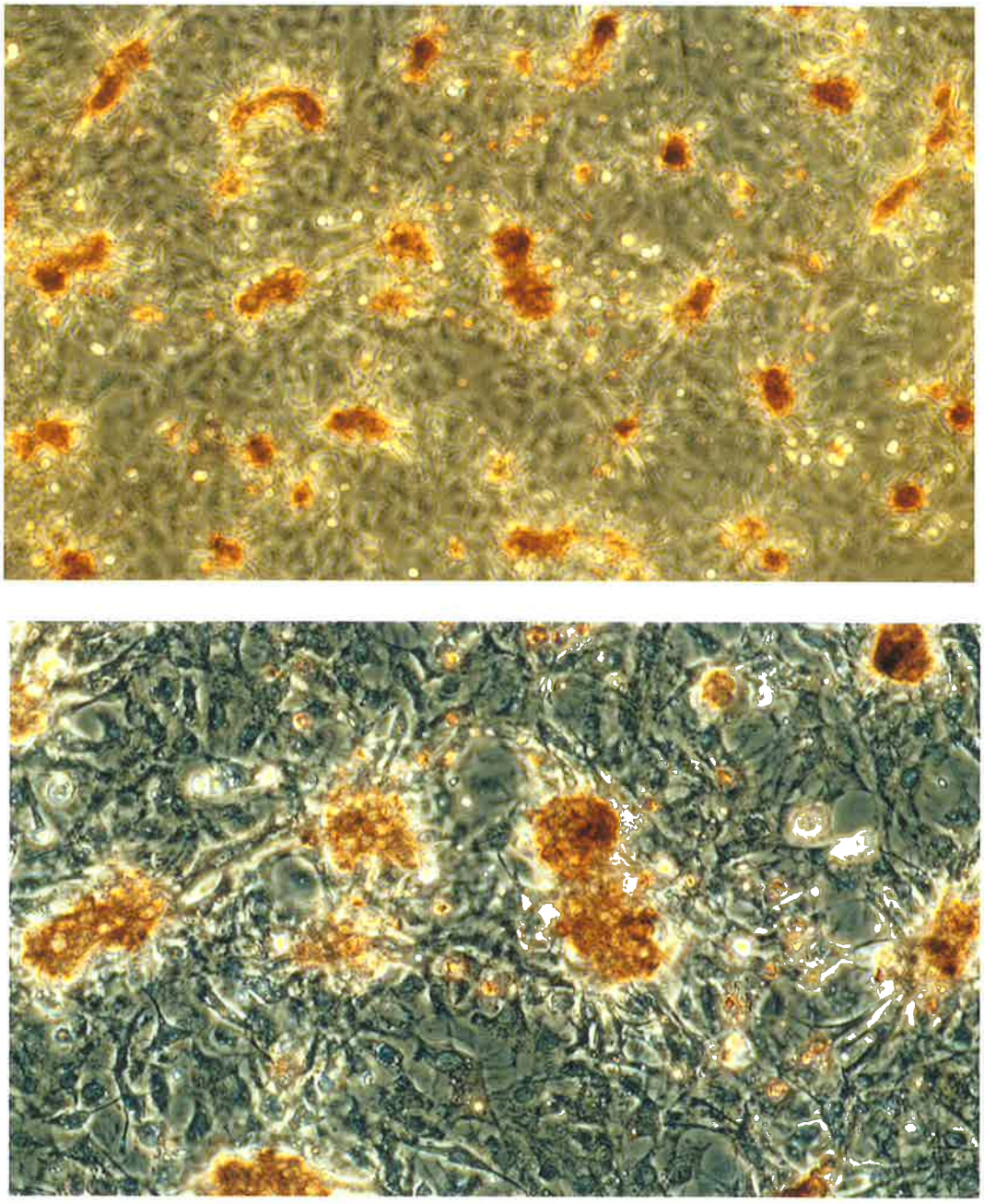


**Figure 6.7** Annexin V staining of hypoxic cultures at low (**A**) and high (**B**) magnification. Brown staining of cells indicates presentation of phosphatidylserine on the surface of the plasma membrane, an early feature of apoptosis.

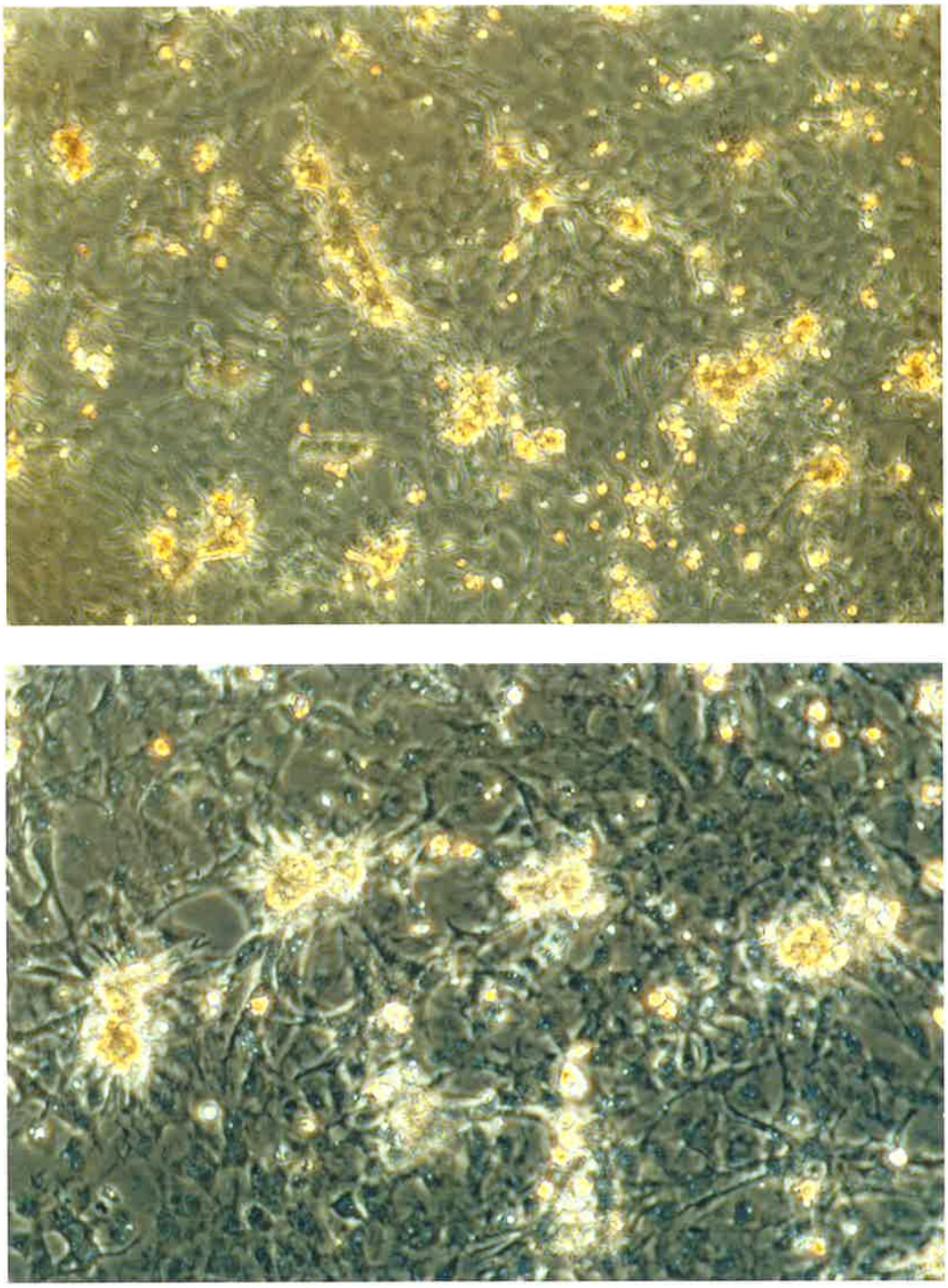



**Figure 6.8** An example of Annexin staining of hypoxic cultures treated with 100μM SMT shown at low (**A**) and high (**B**) magnification.

## 6.6 Expression of apoptotic proteins in chronic hypoxia

### 6.6.1 Expression of Bax by cardiac myocytes under hypoxic conditions

The effect of chronic hypoxia on expression of Bax was investigated by Northern blot as described in Chapter 2. As Bax is constitutively expressed in normoxic cardiac myocyte cultures (Jung F 2001), Northern analysis was used to quantitate Bax expression for statistical analysis. Normalisation to 18S was performed to overcome variation in loading of total RNA onto the gel and paired analysis of normoxic and hypoxic flasks from the same culture was performed to allow for different probe strengths and hybridizations on different membranes done at different times.

Cultures were incubated for 48 hours under hypoxic conditions as discussed in Chapter 2, followed by total RNA extraction before Northern analysis. No significant difference was found between expression of Bax in hypoxic cultures from normoxic controls. The experiment was performed eight times (one pair from each of eight cultures) and expression was analysed by paired t test. A representative experiment is shown in Figure 6.9 and a summary of data from all eight experiments is shown in Figure 6.10.

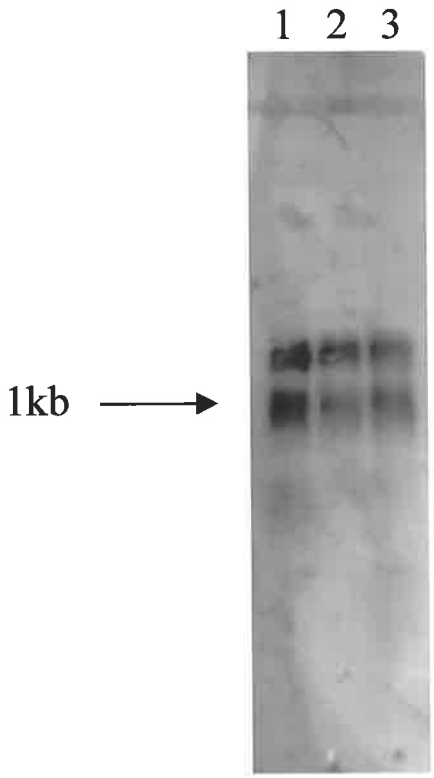



**Figure 6.9** The effect of chronic hypoxia on Bax expression in cardiac myocytes. Expression was analysed by Northern blot and results shown are from a representative experiment. Lane 1 represents IL-1ß treated cells (a positive control for iNOS expression), Lane 2 represents normoxic negative controls and Lane 3 represents hypoxic cultures. The two bands represent splice variants of Bax.

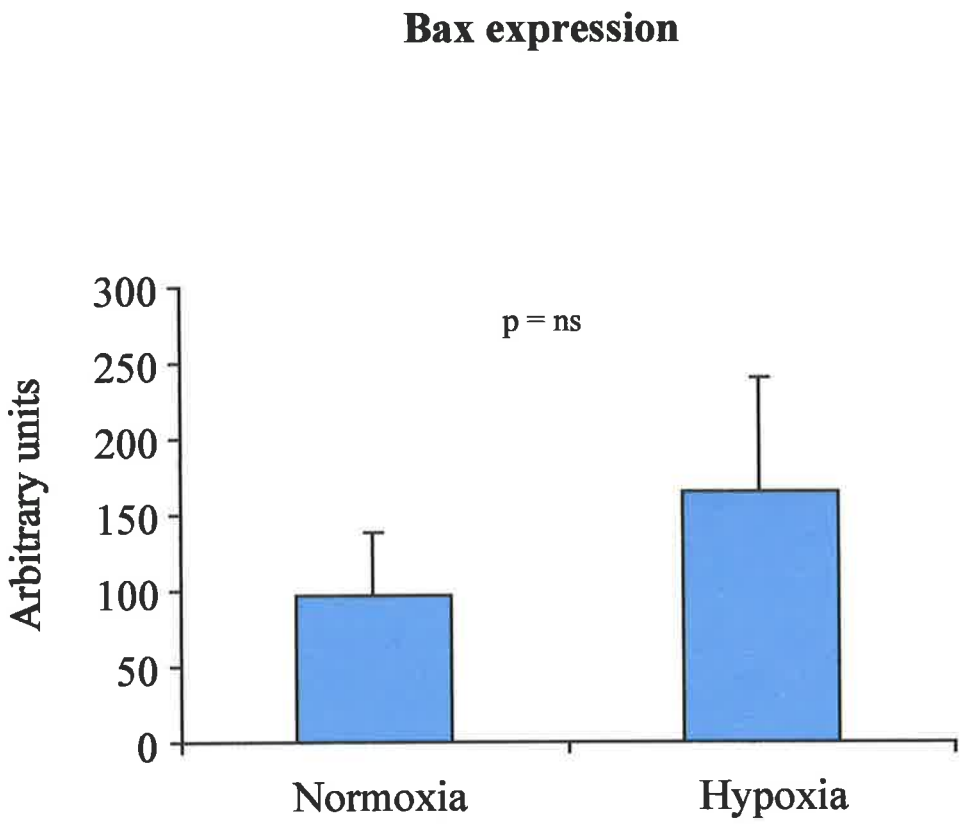



**Figure 6.10** Summary of results of the effect of hypoxia on Bax expression in cardiac myocytes. There was no significant different in Bax expression under hypoxic conditions when compared to normoxic controls. Data represent eight experiments.

Two bands were detected on the Bax Northern blot. The lower band, of 1 kb, represents Bax α (Madison DL 1996), the predominant proapoptotic splice variant of Bax. A number of splice variants have been described (Oltvai ZN 1993) including Bax β, a 1.5 kb transcript (producing a 24 kDa protein), Bax γ which produces a 4.5 kDa protein, Bax δ (Apte SS 1995) which lacks exon 3 compared to Bax α and Bax σ (Schmitt E 2000) which lacks amino acids 159-171 compared to Bax α. Bax ω (Zhou M 1998), which lacks a transmembrane domain, has also been described. Therefore the higher band seen on the current Northern blots probably represents Bax β. Analysis was performed using the 1 kb Bax α band.

### 6.6.2 Expression of Bcl-2 by cardiac myocytes under hypoxic conditions

The effect of hypoxia on expression of Bcl-2 was also investigated, using the same conditions as for Bax, with Northern analysis using probes listed in Chapter 2. The experiment was performed five times. There was no significant change in Bcl-2 expression in hypoxic cultures compared with normoxic controls. The Northern blot results are shown in Figure 6.11 and a summary of data from these experiments is shown in Figure 6.12.

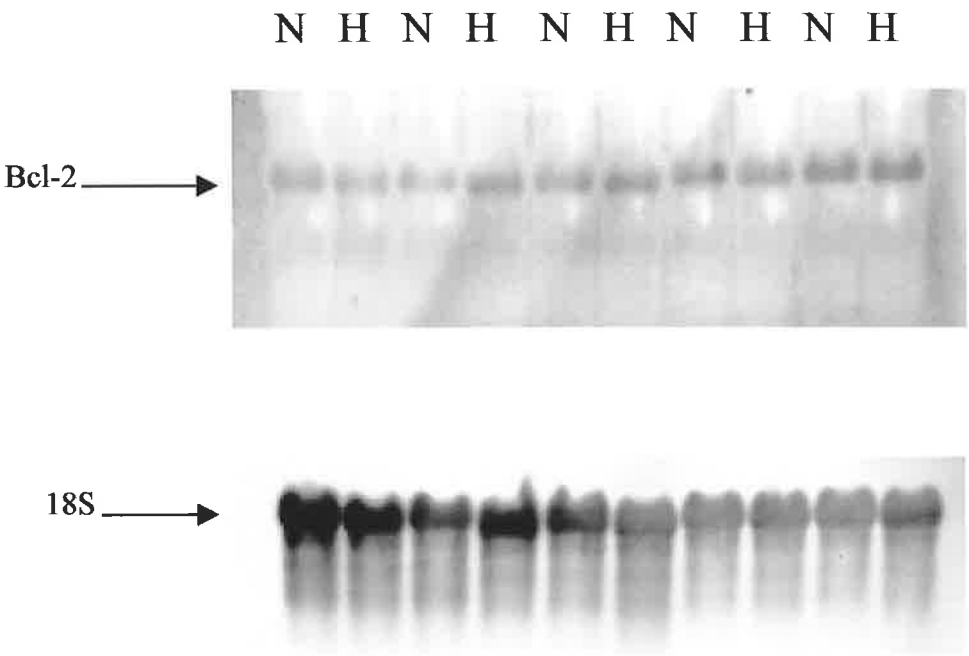



**Figure 6.11** Northern blot of Bcl-2 expression. The bottom panel shows 18S expression for the same membrane. Five experiments (each with a normoxic (N) and hypoxic (H) flask) are shown.

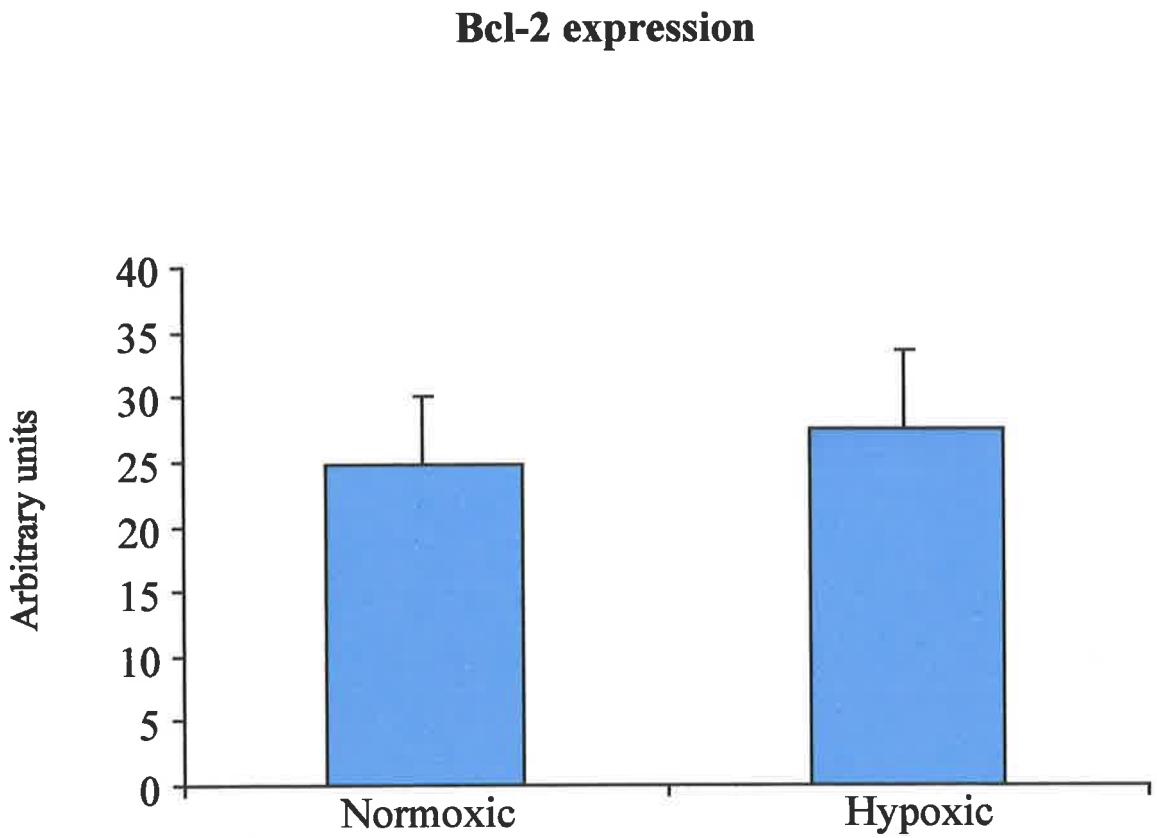



**Figure 6.12** Bcl-2 expression in hypoxic flasks compared with normoxic controls. There was no statistically significant difference ($p = 0.55$, paired t test) in Bcl-2 mRNA levels in hypoxic compared to normoxic cultures. This figure summarises data from five separate experiments (cultures), with one normoxic and one hypoxic flask in each experiment.

## 6.7 Discussion

The results of caspase 3 activity assays described, as well as experiments with Annexin V staining, confirm that hypoxia (using a 1% oxygen gas mix) without reoxygenation does result in apoptosis of cardiac myocytes in culture. These findings are in agreement with those of Jung et al (Jung F 2001). This, however, is in contrast to previous reports (Webster K 1999) where reoxygenation or the presence of low pH in the media (possibly simulating ischaemia) was required for apoptosis to occur. More severe hypoxia (again possibly simulating ischaemia) using a 0% oxygen gas mix (Long X 1997; Malhotra R 1999; de Moissac D 2000), has also been shown to result in apoptosis of cardiac myocytes without a reoxygenation step.

iNOS inhibition resulted in a statistically significant reduction in caspase 3 activity in some (the one using L-NAME as there were more flasks in the L-NAME group) experiments but not in others. This may in part be due to small numbers in each group within each experiment. However, within each experiment there was generally a flask where iNOS inhibition had no effect on caspase 3 activity under hypoxic conditions, while the other flasks had caspase 3 activity levels similar to the normoxic controls (Figure 6.4). Nevertheless, analysis of data pooled from all experiments showing a reduction of caspase 3 activity in the presence of iNOS inhibition compared with hypoxia alone, suggests that the mechanism by which hypoxia

induces apoptosis in cardiac myocytes is in part iNOS mediated. This has not previously been reported.

This "escape" from the effects of iNOS inhibition, where some flasks show no reduction in caspase 3 activity in the presence of iNOS inhibition compared to hypoxic flasks without iNOS inhibition, suggests that in these flasks other triggers to apoptosis, which are independent of iNOS, may be acting. SMT as a direct trigger to apoptosis seems an unlikely explanation as this was added in equal concentrations to all flasks, but did not result in cell death in all flasks. In addition, similar results were obtained using the non specific NOS inhibitor L-NAME. It is possible that in some flasks there is a lower threshold for recruitment of other pathways to apoptosis for example via the death receptor pathways.

This proposed mechanism for the "escape" from iNOS inhibition is supported by the variable expression of TNF α (see Chapter 5) in both hypoxic and normoxic flasks. TNF α may directly activate the DR 1 death receptor, providing an alternate, iNOS independent, pathway to apoptosis. Those flasks which express TNF α in response to hypoxia or those flasks which show further induction of TNF α in response to hypoxia, would be more likely to show activation of the DR 1 receptor with subsequent commitment to apoptotic cell death compared with those flasks that do not express TNF α. This is independent of the iNOS mediated or mitochondrial pathways to apoptosis. Furthermore, activation of death receptor pathways to apoptosis may also enhance recruitment of the mitochondrial pathways through

caspase 8 induced activation of Bid, a proapoptotic protein of the Bcl-2 family, which stimulates mitochondrial cytochrome c release (Li H 1998; Yin XM 2000). Thus, variable expression of TNF α may explain the variable susceptibility of some flasks to iNOS independent triggers of apoptosis.

The experiments performed with Annexin V staining confirm the hypoxia induced apoptosis of cardiac myocytes. There was increased Annexin V binding to the hypoxic cardiomyocytes when compared to normoxic controls. The addition of 100 μM SMT to hypoxic cultures again resulted in less apoptosis of cardiac myocytes than hypoxic cultures without SMT indicating a role for iNOS in the mechanism of hypoxia induced apoptosis of cardiac myocytes. In these experiments however, no "escape" from iNOS inhibition (ie SMT treated cultures, with a degree of apoptosis equivalent to hypoxic cultures) is demonstrated, however for logistical reasons fewer numbers were used in these experiments compared to the caspase 3 activity assay. The lack of Annexin V binding in the SMT group, ie the lack of "escape" from iNOS inhibition, is therefore probably related to the small numbers.

Hypoxia induced apoptosis of neonatal rat cardiac myocytes was not accompanied by significant upregulation of Bax α and nor was it accompanied by downregulation of Bcl-2. While these proteins may be important in the mechanisms (particularly the mitochondrial pathways) of apoptosis, a change in their expression does not seem to account for the increased apoptosis detected under the hypoxic conditions described. If increased numbers were analysed, the increase in Bax expression in hypoxic

compared to normoxic myocytes may become statistically significant, however this requirement for increased numbers supports the notion that upregulation of Bax (or downregulation of Bcl-2) is either not the major mechanism or not the only mechanism for iNOS mediated hypoxia induced apoptosis.

This does not preclude Bax or Bcl-2 activity from contributing significantly to the mechanisms of hypoxia induced apoptosis of cardiac myocytes. Bcl-2 family proteins may be subject to post translational regulation resulting in modification of activity. Phosphorylation of specific Bcl-2 loop sites may result in its inactivation (Haldar S 1997). Ashraf et al (Ashraf QM 2001) in a study of hypoxia in cerebral tissue of newborn pigs report that hypoxia results in increased phosphorylation of Bcl-2, which may prevent its heterodimerisation with Bax, leading to increased proapoptotic Bax activity.

# CHAPTER SEVEN

# DISCUSSION

## 7.1 Validity of The Cellular Model of Hibernating Myocardium

Characterisation of hibernating myocardium is based on the clinical phenomenon of reversible regional myocardial dysfunction in response to chronically low perfusion with subsequent recovery on revascularisation. Inotropic reserve, specifically improvement in contractile function in response to inotropic stimulation whether by dobutamine infusion or post extrasystolic potentiation, is also a characteristic of hibernating myocardium. Characteristic PET appearances and histological features have also been described in human subjects. These are the features with which animal models of hibernating myocardium have been compared, in order to determine the validity of such models.

The cellular model of hibernating myocardium presented in this work conforms to the clinical features characteristic of hibernating myocardium, in that a reduction in contraction (reduction in spontaneous beating rate) is demonstrated in response to chronic hypoxia and substrate deprivation. This is reversible with reoxygenation. In addition, recovery in response to an inotrope can be demonstrated by the addition of dobutamine to the hypoxic cultures.

Hibernating myocardium occurs because of severe coronary artery disease resulting in chronically low perfusion with or without repeated "stunning". It may be argued that a cellular monolayer deprived of oxygen and glucose does not adequately

simulate chronically hypoperfused myocardium in vivo. It is for this reason that an attempt to validate the model by comparison with characteristic features of human hibernating myocardium has been made.

In the cellular model presented, an attempt has been made to preserve the interactions between myocytes and non myocytes through the use of a mixed culture as harvested from the neonatal rat hearts. However, there is no mechanism for infiltration of immune cells which may occur even in hypoperfused myocardium. This allows study of the responses of the myocardial cells (myocytes and nonmyocytes) to hypoxia and substrate deprivation without the confounding influence of immune cells and non myocardial derived growth factors and cytokines.

A more striking difference between the present cellular model of hibernating myocardium and hibernating myocardium described in human subjects is the neonatal derivation of the cells. Not only must the species differences be taken into account, but there may be significant differences in cellular and molecular adaptations to cellular stress, such as hypoxia and glucose deprivation, in neonatal cells compared to adult cells. This again is perhaps incontrovertible, but difficult to document as adult cells in culture demonstrate no spontaneous contraction. It is this feature, the spontaneous intrinsic synchronous beating of neonatal rat ventricular myocytes, which makes them attractive for use in a cellular model of hibernating myocardium. Without this feature, the model would not display the defining characteristics of hibernating myocardium as described above.

Animal models have given valuable insights into possible metabolic and biochemical adaptations in hibernating myocardium. However, these findings have not adequately been investigated in human hibernating myocardium for obvious logistical reasons. Indolfi et al (Indolfi C 1996) investigated biochemical features of hibernating myocardium in human subjects undergoing PTCA. Arterio-coronary sinus lactate differences suggested a net lactate extraction in resting hibernating myocardium with a shift to lactate production after dobutamine infusion. However, Gertz et al (Gertz EW 1981) have demonstrated that a significant amount of lactate can be released by the myocardium when chemical arterial-coronary sinus analysis indicates global myocardial extraction.

Animal models of hibernating myocardium, largely involving LAD clamps maintained in pigs for a few hours, have provided the most information on possible metabolic adaptations in hibernating myocardium (Schulz R 1992; Chen C 1996; Shen YT 1996). However, the short time frame in which the studies were performed raise questions about the relevance of these findings to the clinical condition of hibernating myocardium which can persist for months. Nevertheless, findings point to an initial increase in lactate production with variable decrease in production, or switch to lactate extraction, over time suggesting an adaptation to chronic hypoperfusion. The modest, non significant increase in lactate levels in the media of hypoxic cultures, observed in the cellular model of hibernating myocardium described in this thesis, is therefore consistent with these reports. The decreased

cellular ATP levels demonstrated in the present cellular model of hibernating myocardium is also consistent with the decreased levels of ATP demonstrated in hibernating myocardium in animal models compared to remote, normally perfused myocardium.

Much of the characterisation of human hibernating myocardium has been histological with increased glycogen content and decreased myofibrils described by several authors (Maes A 1994; Elsasser A 1997). The current cellular model of hibernating myocardium was not assessed histologically partly because of the differences in neonatal cells compared with adult cells. Nevertheless, histological adaptations are an important point of comparison for assessment of a model and histology is the method used to determine the lack of significant necrosis (seen as patchy fibrosis) in previously described animal models of hibernating myocardium.

Casey et al (Casey TM 2000) also described a cellular model to investigate the cellular adaptations of cardiac myocytes in response to hypoxia. The authors used neonatal rat cardiac myocytes in cell suspension and reported reduced oxygen consumption in response to hypoxia with preserved intracellular ATP levels and thus suggested an adaptation consistent with hibernation. However the hypoxia was only applied for one hour and the cells were not contracting. Therefore they were doing no spontaneous contractile "work" which would be downregulated in response to the hypoxia and which would subsequently recover on reoxygenation, such that they could be analogous to hibernating myocardium. Again as their model showed no

contraction, no inotropic reserve could be demonstrated. Therefore the cellular model described in this thesis may better reflect the situation in hibernating myocardium.

In summary, the cellular model of hibernating myocardium proposed in this work, is in many ways analogous to human hibernating myocardium, and does demonstrate certain defining features consistent with descriptions of hibernating myocardium in humans and animal models. Specifically, the model does involve spontaneous contraction which is downregulated in response to hypoxia and glucose deprivation and this reduction in contractility is reversible with reoxygenation. The model also demonstrates recovery in response to the addition of dobutamine. The biochemical and metabolic features described are also consistent with those previously reported for animal models. This is the only cellular model of hibernating myocardium reported, which demonstrates these features.

## 7.2 iNOS expression in Chronic Hypoxia

In this study, no iNOS expression was detected in the cellular model of hibernating myocardium. However, cardiac myocyte cultures harvested from rats aged 1-3 days were able to consistently express iNOS in response to the addition of IL-1β to the culture media. This is consistent with the results reported by Kacimi et al (Kacimi R 1997) who demonstrated no iNOS expression in response to hypoxia in cultures of

cardiac myocytes harvested from rats aged 1 day. On the other hand, Wang et al (Wang D 1999) and Jung et al (Jung F 2000) did demonstrate hypoxia induced iNOS expression in cultures harvested from rats aged 1-2 days. In all four studies the species used were Sprague-Dawley rats.

It was for this reason that iNOS expression in response to hypoxia was investigated using primary cultures of cardiac myocytes harvested from rats aged 7 days. These cultures consistently showed iNOS expression in response to hypoxia. Cultures harvested from 5 day old and 4 days old rats were investigated to determine at what age onset of hypoxia induced iNOS expression occurs. Results were variable with some of these cultures demonstrating hypoxia induced iNOS expression, but others not.

The age of onset of iNOS expression in response to hypoxia may therefore be variable, in that some may express iNOS under hypoxic conditions from day one but others may do so later, for example day 4-5 or even day 6-7. This may therefore explain the contrasting results reported previously in the literature (Kacimi R 1997; Wang D 1999; Jung F 2000). As discussed in Chapter 4, similar variation in onset of enzyme induction in response to a specific stimulus has been previously reported. Onset of glucokinase induction in response to all-trans retinoic acid has been reported to occur in hepatocytes harvested from neonatal rats from the same source at 15 to 17 days of age (Cabrera-Valladares G 2001).

Further studies of iNOS expression in cardiac myocyte cultures under conditions of chronic hypoxia were then performed using cultures harvested from rats aged day 7. These studies therefore do not represent a cellular model of hibernating myocardium, as these cultures did not demonstrate consistent or sufficient spontaneous contraction. However, as hibernating myocardium may be thought of as a condition involving tissue hypoxia, study of iNOS expression in these cultures may still give insights into regulation and effects of iNOS expression in hibernating myocardium.

## 7.3 Regulation of iNOS expression under hypoxic conditions

iNOS expression under conditions of chronic hypoxia is, at least in part, regulated by the activation of the HRE in the iNOS promoter region, presumably by binding of Hypoxia Inducible Factor 1. Given that hibernating myocardium is also a condition of prolonged oxygen deprivation due to severe coronary stenoses, activation of the HRE may therefore also play a role in iNOS induction in this condition. Although activation of the HRE occurs through binding of HIF 1, the finding that HIF 1α is not significantly upregulated in conditions of increased HRE binding is not altogether unexpected, as regulation of HIF 1 is largely at a post translational level by stabilisation of HIF 1α under hypoxic conditions. Only modest increases in mRNA levels have been demonstrated in rats exposed to hypoxia (7% oxygen) (Semenza G 1997).

The results of experiments described in Chapter 5 also show that the NFκB binding site in the iNOS promoter region is activated, in addition to that of the HRE, under conditions of prolonged or chronic hypoxia. Similarly, NFκB binding may play an important role in induction of iNOS in human hibernating myocardium. It therefore appears that binding to both HRE and NFκB sites are important in the regulation of iNOS expression under hypoxic conditions. This suggests there are multiple activation pathways or recruitment of stimuli to upregulate iNOS expression.

In order to further elucidate the mechanism of iNOS expression under hypoxic conditions, expression of cytokines were also investigated. Il-1β was not expressed in cardiac myocytes, harvested from day 7 rats, and incubated under hypoxic conditions. However, there was variable induction of both IL-6 and TNF α. The latter may, in part, account for the activation of NFκB and therefore NFκB DNA binding and may contribute to iNOS induction under hypoxic conditions. However, given that expression of TNF α was variable, other pathways to NFκB activation are implicated such as hypoxia induced mitochondrial reactive oxygen species (Chandel NS 2000) as discussed in Chapter 5. Alternatively, a more direct mechanism of NFκB activation may involve tyrosine phosphorylation of IFκB under hypoxic conditions resulting in dissociation of IFκB from NFκB and degradation of IFκB (Koong AC 1994).

Il-6 was also expressed variably by cardiac myocytes under hypoxic conditions and, while not the primary mechanism of iNOS induction, may enhance expression of iNOS in hypoxia.

## 7.4 Effects of iNOS expression under hypoxic conditions

iNOS expression was not detected in the cellular model of hibernating myocardium as described in Chapter 3. Therefore iNOS cannot be associated with the downregulation of contraction seen in this model in response to hypoxia and glucose deprivation. Consequently, iNOS may not have a role in the reversible downregulation of regional contractile function characteristic of human hibernating myocardium. This observation instigated investigations into other effects of iNOS under hypoxic conditions.

The results of experiments described in Chapter 6 demonstrated that hypoxia of cardiac myocytes, without reoxygenation results in apoptosis of these cells. This is in contrast to the findings of Webster et al (Webster K 1999) who found that apoptosis occurred on reoxygenation or with a low pH in the media, but not with hypoxia for 48-72 hours alone. While extremely low oxygen levels (a gas mix containing 0% oxygen) has been previously demonstrated to result in apoptosis of cultured cardiac myocytes (Long X 1997; Malhotra R 1999; de Moissac D 2000), this report confirms

the findings of Jung et al (Jung F 2001) that less severe (1% oxygen) also results in apoptosis of cardiac myocytes in culture.

Furthermore, the addition of an iNOS inhibitor, SMT, resulted in inhibition of hypoxia induced apoptosis of cardiac myocytes in primary culture, suggesting that hypoxia induced apoptosis is mediated, at least in part, by iNOS. This finding has not previously been reported. Some flasks within each experiment, however appeared to show no reduction in apoptosis with iNOS inhibition, ie an "escape" from iNOS inhibition. This suggests other, iNOS independent triggers to apoptosis, are active. Some flasks may therefore have a lower threshold for recruitment of other pathways to apoptosis such as death receptor pathways or generation of reactive oxygen species.

Indeed the demonstration of TNF α induction (reported in Chapter 5) in some, but not all, flasks incubated under hypoxic conditions suggests a mechanism whereby the DR 1 receptor may be activated in some but not all flasks. This would explain why some flasks show significant apoptosis in spite of iNOS inhibition whereas, in other flasks, apoptosis is almost completely inhibited by the addition of an iNOS inhibitor.

No significant up regulation of the pro apoptotic protein Bax nor down regulation of the anti apoptotic protein Bcl-2 were detected under hypoxic conditions. As discussed in Chapter 6, there may be post translational regulatory mechanisms resulting in altered function of either protein which contribute to the mechanisms of hypoxia

induced apoptosis of cardiac myocytes. Therefore Bax and Bcl-2 may play an important role in these experimental conditions, however up- or down regulation respectively may not be the major mechanism. iNOS appears to have no significant effect on Bax or Bcl-2 expression.

## 7.5 The possible role of iNOS in hibernating myocardium

Results from experiments described in this thesis using a cellular model of hibernating myocardium suggest that iNOS has no role in the protective downregulation of contractile function under conditions of chronic hypoperfusion. However, iNOS expression has been demonstrated in human hibernating myocardium (Depre CF 1997; Baker CSR 1999) as discussed in chapter 1. The results of experiments described in rat cardiac myocyte cultures harvested from older neonatal rats suggest that iNOS expression in chronically hypoxic myocardial cells results in myocyte loss by apoptosis. In addition, apoptosis has also been described in human hibernating myocardium.

Therefore, rather than being protective in hibernating myocardium, iNOS expression (by increasing apoptotic myocyte loss) may be responsible for, or contribute to, the change from reversible dysfunction to irreversible myocardial dysfunction in hibernating myocardium, thus resulting in worsening prognosis. iNOS may be a

mechanism whereby hibernating myocardium, when revascularised late, does not recover function.

This mechanism may therefore explain the findings of Kalra et al (Kalra DK 2002) whereby myocardial segments with highest TNF α and iNOS expression showed no recovery of function whereas those with intermediate TNF α and iNOS expression showed improved function post revascularisation.

This has significant clinical implications, particularly for patients with hibernating myocardium awaiting revascularisation procedures or for those in whom such procedures cannot be undertaken. Selective iNOS inhibition as therapy may assist in preserving left ventricular function for longer, although as implicated by the results of experiments using SMT as an iNOS inhibitor, some may be susceptible to other triggers to apoptosis that are iNOS independent. Nevertheless, iNOS inhibition used as therapy may have the potential to result in better recovery of function after revascularisation and improved survival in those who cannot undergo revascularisation procedures.

Clearly further work is required to investigate whether these results in isolated myocardial cells are reflected in the clinical condition of hibernating myocardium and a specific iNOS inhibitor which can be used in a human clinical trial remains to be discovered. However the advantage of targeting such a trigger of apoptosis as iNOS, rather than targeting caspases for example, is that only apoptosis in specific

circumstances is inhibited rather than all apoptosis which may result in malignancy or autoimmune disease. Specific iNOS inhibition applicable to the clinical setting may potentially prevent or limit irreversible myocyte loss in hibernating myocardium before revascularisation and therefore enhance improvement of left ventricular function and therefore prognosis.

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## AMENDMENTS

Chapter 1:

- The following should be included at the end of paragraph 2, page 10:

  The current recommendations on treatment of acute myocardial infarction and unstable angina, along with an evaluation of the evidence for these treatments, are provided on the American Heart Association website (www.americanheart.org).

- The following should be included after the first sentence of paragraph 3, page 10:

  Those patients with unstable angina and a raised Troponin I, indicating myocardial damage, have a relatively increased risk of further acute events and a poorer long term outlook (Ottani F 2000).

  This reference is cited in full:

  Ottani F, Galvani M, Nicolini FA, Ferrini D, Pozzati A, Di Pasquale Jaffe AS (2000). "Elevated cardiac troponin levels predict the risk of adverse outcome in patients with acute coronary syndromes." Am Heart J **140**(6): 917-27

- The following should be inserted before the last sentence of paragraph 3 page 28:

  The increased glucose utilization by hibernating myocardium may not reflect the anaerobic metabolism characteristic of ischaemia, but may reflect an increased expression of the GLUT 1 glucose transporter (Camici P 1997). There is thought to be a decrease in fatty acid metabolism in hibernating myocardium similar to that in ischaemia, as evidenced by a reduced uptake of I-123 BMIPP, a free fatty acid analog (Shimonagata T 1998).

  References in full:

  Camici P, Wijns W, Borgers M, De Silva R, Ferrari R, Knuuti J, Lammertsma AA, Liedtke AJ, Paternostro G, Vatner SF (1997). "Pathophysiological mechanisms of chronic reversible left ventricular dysfunction due to coronary artery disease (Hibernating myocardium)." Circulation **96**(9):3205-14

  Shimonagata T, Nanto S, Kusuoka H, Ohara T, Inoue K, Yamada S, Nishimura Y, Matsubara N, Hori M, Nishimura T, Kubori S (1998). "Metabolic changes in hibernating myocardium after percutaneous transluminal coronary angioplasty and the relation between recovery in left ventricular function and free fatty acid metabolism." Am J Cardiol **82**(5):559-63

- The following should be inserted after the second paragraph, page 29:

  It can be argued that these studies do not take into account the amount of tissue present (eg expression of lactate or ATP per gram of tissue). Also, overall lactate extraction does not exclude local lactate production on a cellular level. Elsasser et al (Elsasser A 2002), using biopsies of human hibernating myocardium, found increased lactate and decreased ATP levels in hibernating myocardium compared with normal myocardium. They expressed levels relative to wet weight and normalised for degree of fibrosis. It is therefore difficult to compare these results to those of previous studies and these results remain to be reproduced by others.

  Reference in full:

  Elsasser A, Muller KD, Skwara W, Bode C, Kubler W, Vogt AM (2002). "Severe energy deprivation of human hibernating myocardium as possible pathomechanism of contractile dysfunction, structural degeneration and cell death." J Am Coll Cardiol **39**(7):1189-98

- Page 50, line 5 should read "an" inhibitor.

Chapter 2 :

- Page 85, line 4 should read:

  The bottle was shaken reasonably vigorously at 37°C for 10 minutes.

- The following should be included at the end of paragraph 2, page 85:

  The yield of cells per isolation was approximately 50-60 million cells.

- The following should be included before the second to last sentence in paragraph 1, page 95:

  The integrity of the RNA was determined by agarose gel electrophoresis.

Chapter 3:

- The following should be included after the first sentence in paragraph 2, page 126:

For example, neonatal cardiac myocytes express T type calcium channels, whereas adult cells do not. Nevertheless, adult cells in prolonged culture dedifferentiate and then do begin to express T channels (Fares N 1996). Carnitine palmitoyltransferase 1, as well as genes associated with cardiac hypertrophy such as beta myosin heavy chain are expressed to a lesser degree in neonatal cells (Xia Y 1996). Neonatal cells have fewer myofilaments and less organisation into sarcomeres (Nag AC 1983). Some studies suggest neonatal cells are more susceptible to oxidative stress than cultures from adult cells (Nag AC 1996).

The references in full:

Fares N, Gomez JP, Potreau D (1996). "T-type calcium current is expressed in dedifferentiated adult ventricular cells in primary culture". C R Acad Sci III **319**(7): 569-76

Xia Y, Buja LM, McMillan JB (1996). "Change in expression of heart carnitine palmitoyltransferase isoforms with electrical stimulation of cultured rat neonatal cardiac myocytes." J Biol Chem **271**(20): 12082-7

Nag AC, Cheng M, Fischman DA, Zak R (1983). "Long term cell culture of adult mammalian cardiac myocyte electron microscopic and immunofluorescent analyses of myofibrillar structure." J Mol Cell Cardiol **15**(5): 301-17

Nag AC, Screepathi P, Lee ML, Reddan JR (1996). "Effect of oxidative insult on young and adult cardiac muscle in vitro." J Mol Cell Cardiol **85**(340): 7-27

- The following should be included between paragraphs 1 and 2, page 141:

  This technique of measuring neonatal cardiac myocyte contraction is not without limitations. It is designed more for adult cells which have an orderly and defined shape. With neonatal cells, given their irregular outline, only one edge can be used (instead of two edges contracting towards each other as seen in paced adult cells). The direction of contraction detected is a vector, as the myofilaments in neonatal cells are aligned in various directions (compared to adult cells). Therefore the measured net contraction may be different in some cells or cell groups compared to others. These difficulties also apply to the situation where adherent beads are used instead of edge detection software.

- The following should be included at the end of the second paragraph, page 142:

  In normoxic cultures, the beating rate rose from 101 ± 9 bpm to 141± 13 bpm ($p < 0.05$).

Chapter 4:

- The following should be included after the second sentence of paragraph 3, page 160:

  The cultures appeared similar in morphology to the cultures isolated from rats aged 1-3 days.

- The following should be included after the first sentence of paragraph 2, page 165:

  These cultures appeared similar in morphology to the cultures isolated from rats aged 1-3 days and from rats aged 7 days.

Chapter 6:

- The following should be included before the last sentence of paragraph 2, page 203:

  It should be noted that this occurred also in the L-NAME experiment. However, because of the increased numbers in the inhibitor group in this experiment, the difference in caspase 3 activity between the hypoxia group and the inhibitor group reached statistical significance.