Alterations in Non-Enzymatic Antioxidants in Ischemic-Reperfused Isolated Rat Myocardium: Effects of Schisandrin B and Diabetes Mellitus

by

Ho-Yan YIU, B.Sc. (Hons.)

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in Partial Fulfillment of the Requirements for the Degree of

Master of Philosophy in Biochemistry

Hong Kong August 2000
Department of Biochemistry
The Hong Kong University of Science and Technology

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The Hong Kong University of Science and Technology

August 2000
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<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>AGES</td>
<td>advanced glycosylation end products</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CCl₄</td>
<td>carbon tetrachloride</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<tr>
<td>CPR</td>
<td>cardiopulmonary resuscitation</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>FS</td>
<td><em>Fructus Schisandrae</em></td>
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<td>G-6-PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<td>GPX</td>
<td>selenium glutathione peroxidase</td>
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<td>GRD</td>
<td>reduced glutathione</td>
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<td>GS⁺</td>
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<td>glutathione S-transferases</td>
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<td>ischemic heart disease</td>
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<tr>
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<td>-------------</td>
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<td>NADPH</td>
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<td>non-insulin-dependent diabetes mellitus</td>
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<td>O₂⁻*</td>
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<td>reactive oxygen species</td>
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<tr>
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<td>streptozotocin</td>
</tr>
<tr>
<td>VC</td>
<td>ascorbic acid</td>
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<tr>
<td>VE</td>
<td>α-tocopherol (Vitamin E)</td>
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Abstract

Isolated rat hearts were subjected to increasing periods of ischemia and reperfusion for examining the changes in the extent of myocardial IR injury and the level of non-enzymatic antioxidants (VC, VE and GSH). Hearts subjected to prolonged period (40 min) of ischemia followed by reperfusion were inflicted with sustainable tissue damage, which was indicated by the increased extent of LDH leakage measured in the coronary effluent.

Hearts subjected to IR challenge resulted in a marked decrease in tissue VC level, but myocardial VE and GSH level remained relatively unchanged, except under a more severe IR condition. These results suggest that myocardial VC appeared to be first line of antioxidant defense against IR-induced oxidative stress, with myocardial VE served as the ultimate antioxidant defense.

Pretreatment with Sch B and α-lipoic acid (LA) produced the cardioprotective effect on IR-induced injury to various degrees. Although both Sch B and LA pretreatment could suppress the IR-induced LDH leakage, the effect of LA seemed to be more potent. However, while Sch B pretreatment improved the extent of contractile force recovery of the ischemic-reperfused myocardium, no effect was produced by LA pretreatment. The cardioprotection afforded by Sch B pretreatment was associated with increases in myocardial VC and VE levels, with the effect on VE being more prominent. In contrast, the cardioprotective action of LA pretreatment was found to be associated with elevations in myocardial VC and VE level, but the effect on tissue VC level appeared to be much more dramatic.

Hearts isolated from short-term (2-week) diabetic rats were more resistant to IR injury induced by ischemia followed by 20-min of reperfusion. However, this protective effect observable in diabetic hearts diminished or disappeared with the prolongation in reperfusion time from 20 to 40 min, which was corroborated by the decreased in contractile force recovery.

Differential changes in non-enzymatic antioxidant levels were observed in the diabetic myocardium upon IR challenges. The exposure to oxidative stress arising from the diabetic state may play an important role in priming the myocardium in responding to
oxidative challenge. However, prolongation in ischemic and reperfusion period could overcome this preconditioned effect, indicated by the drastic increase in LDH leakage and decrease in contractile force recovery.

The diabetic-associated changes in hearts subjected to IR challenge were partially reversed by insulin treatment, but not Sch B. The inability of Sch B pretreatment to protect against IR injury in diabetic hearts suggests the involvement of a common antioxidant mechanism in the cardioprotection afforded by Sch B and diabetic state. Sch B pretreatment could slightly protect against IR injury in insulin-treated diabetic hearts, and the result suggests that Sch B pretreatment may produce beneficial effect on myocardial non-enzymatic antioxidants in insulin-treated diabetic rats under oxidative stress condition.
1.1 **Oxygen-derived Free Radicals**

Any atom or molecule that contains one or more unpaired electrons can be classified as free radical (Halliwell and Gutteridge, 1989; Halliwell, 1994). The unpaired electrons are able to alter chemical reactivity, usually making the radical more reactive than the corresponding atom or molecule.

Oxygen is the terminal oxidant, which is essential for respiration and other oxidation reactions in aerobic organisms. Currently, oxygen constitutes about 21% of the atmosphere. During the reduction of molecular oxygen, reactive oxygen species (ROS) may be formed, including the oxygen-centered free radicals (Anderson, 1996). These reactive species are implicated in many diseases including atherosclerosis (Witzum, 1994), respiratory tract disorders (Cross *et al.*, 1994), neurodegenerative diseases (Jenner, 1994), inflammatory bowel disease (Grisham, 1994), cancer (Ames, 1989; Cerutti and Trump, 1991; Cerutti, 1994; Beckman and Ames, 1997) and also in ageing (Ames, 1988 and 1989; Beckman and Ames, 1998).
1.1.1 Oxygen metabolism and its related sources of oxy-radicals

In living tissues, ionizing radiation can split water to generate the hydroxyl radical \( ^\cdot \text{OH} \), which is extremely reactive at the site of formation. Many enzymatic and autoxidation reactions within the body produce superoxide anion radical (\( \text{O}_2^\cdot \)), via the addition of an electron to molecular oxygen (Reaction 1). In the case of phagocytic cells such as neutrophils, monocytes, macrophages and eosinophils, superoxide is generated as part of their response to foreign organisms or particles. In chronic inflammation, this normal protective mechanism may become damaging (Grisham, 1994). Superoxide is poorly reactive but can take part in further reactions leading to the formation of more reactive species. For example, two superoxide radicals can combine to form hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (Reaction 2), which can react with reduced metal ions (e.g. \( \text{Fe}^{2+} \) and \( \text{Cu}^+ \)) to form \( \text{OH}^- \) and the highly damaging \( ^\cdot \text{OH} \) (Reaction 3). Superoxide may contribute further by reducing the metal ions, which have become oxidized during the reaction.

Reaction 1: \( \text{O}_2 + e^- \rightarrow \text{O}_2^\cdot \)

Reaction 2: \( 2\text{O}_2^\cdot + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \)

Reaction 3: \( \text{H}_2\text{O}_2 \rightarrow ^\cdot \text{OH} + \text{OH}^- \) \hspace{1cm} (Fenton reaction)

Another physiological free radical is nitric oxide (\( ^\cdot \text{NO} \)), which is also produced by phagocytes as well as the vascular endothelium as a relaxing factor (Moncada and Higgs, 1993). Superoxide can react with nitric oxide to produce peroxynitrite (\( \text{O}_2^\cdot + ^\cdot \text{NO} \rightarrow \))
ONO$^*$), which decomposes into toxic products including $^*$OH, nitrogen dioxide and nitronium ion (NO$_2^*$).

Hydroxyl radical is a highly reactive three-electron reduction product of molecular oxygen, which has an extremely short half-life and a very limited diffusion capacity. It binds and oxidizes DNA, lipids and proteins, and it reacts with structures from its close neighborhood mainly by hydrogen abstraction from the unsaturated bonds (Kehler, 1989; Gate et al., 1999). For instance, production of $^*$OH close to DNA can lead to modification of purines or pyrimidines or to strand breakage (Mello Filho et al., 1984) whereas production of $^*$OH close to an enzyme might cause enzyme inactivation (Gee et al., 1985). Furthermore, reaction of $^*$OH with a biological molecule will produce another reactive radical; such radical diffuses away from the site of formation and attacks other biomolecules. The best example of the deleterious effect of such “secondary” radicals in vivo is the ability of $^*$OH to initiate lipid peroxidation by abstracting hydrogen atoms from unsaturated bonds of lipid molecules to form carbon-centered and peroxyl radicals.

1.2 Defense Mechanism Against Free Radicals

Defense mechanisms have evolved within the body to reduce the levels of reactive oxygen species (ROS) and limit the damage they produce. This includes the enzymatic and non-enzymatic systems, which convert oxidants into non-toxic molecules, thus protecting the organism from the deleterious effects of oxidative stress.
1.2.1 Protection by enzymes

Superoxide dismutase (SOD) converts the superoxide anion $\text{O}_2^{-*}$ into a less toxic product, namely $\text{H}_2\text{O}_2$ and $\text{O}_2$. Two forms of SOD exist, a manganese containing SOD (MnSOD, present in mitochondria) and a copper-zinc dependent SOD (CuZn SOD) present in the cytosol (Halliwell and Gutteridge, 1992). These enzymes are the first line of enzymatic antioxidant defense against oxidative stress. On the other hand, catalase (CAT) is the second enzyme that acts in cellular detoxification and is involved in converting $\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}$ and $\text{O}_2$.

In $\text{H}_2\text{O}_2$ detoxification, the selenium-dependent glutathione peroxidase (GPX) converts $\text{H}_2\text{O}_2$ into water via the oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG). GPX exists also in an insoluble form associated with the membrane (phospholipid hydroperoxide glutathione peroxidase), which acts on lipid hydroperoxide (Ursini et al., 1986). There also exists a second enzyme that involved in the metabolism of glutathione, namely glutathione reductase (GRD). GRD is a flavoprotein which permits the conversion of GSSG to GSH at the expense of the oxidation of NADPH to NADP$^+$. This reaction is essential for the availability of GSH in vivo. Deprivation of trace elements, such as Cu, Mn, Zn, Se, or vitamins such as riboflavin, can lead to the inactivation of antioxidant enzymes and the subsequent oxidative stress associated diseases (Sipowicz et al., 1997).
1.2.2 Protection by GSH-dependent non-enzymatic system

Beside antioxidant enzymes, there is an array of small molecules in the biological system that can quench free radicals directly or act as co-substrates for the antioxidant enzymes. GSH, ascorbic acid (VC), α-tocopherol (VE) and α-lipoic acid (LA) are some well-known examples.

GSH is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, which represents the major non-protein thiol in the body. This molecule is found in large quantities (millimolar concentrations) in organs such as the kidney, the liver, the lungs and the intestines which are frequently exposed to exogenously or endogenously generated toxins. In contrast, very little amount of GSH is found in body fluids (micromolar concentrations) (DeLeve and Kaploowitz, 1991). In the cell, GSH plays a role in protein synthesis, amino acid transport, DNA synthesis, and more generally, in cellular detoxification. GSH is involved in the conversion of \( \text{H}_2\text{O}_2 \) to water, and in the reduction of lipid hydroperoxides. GSH may react directly with oxy-radicals such as \( \text{O}_2^\bullet \), \( \bullet \text{OH} \) and \( \text{RO}^\bullet \) by a radical transfer process, yielding the thyl radical, GS•, which can eventually form GSSG. On the other hand, GSH can be conjugated to xenobiotics via glutathione S-transferases (GST), a reaction which increases the hydrophilic properties of xenobiotics, thereby favoring their elimination. Since GSH enters poorly into the cells (except in epithelial cells), intracellular GSH is derived mainly from synthesis (Denke and Fanburg, 1991), and the main source of plasma GSH is from the liver (Kuplowitz et al., 1985). GSH is
synthesized from L-glutamine, L-cysteine, and glycine via two enzymes: \( \gamma \)-glutamylcysteine synthase and glutathione synthase.

The free radical scavenging activity of GSH is augmented by its interaction with VC and VE. GSH, VC and VE interact synergistically in antioxidant defense (Casini et al., 1990; Meister, 1994). VC protects against lipid peroxidation in the plasma (Frei et al., 1989) and in the endoplasmic reticulum by a vitamin E dependent pathway (Wefers and Sies, 1988). VC also prevents endothelial dysfunction in chronic heart disease by inhibiting NO degradation (Hornig et al., 1998), and it also neutralizes oxidant molecules produced during macrophage activation in the presence of tobacco smoke (Anderson et al., 1988).

VE is the major lipophilic antioxidant that reduces free radicals such as lipoperoxides or oxygen radicals (Frei and Ames, 1998). The oxidized VE can be reduced by glutathione (Packer et al., 1979) or VC (Bode et al., 1990). Diet supplementation with VE is associated with an inhibition of the oxidation of low-density lipoprotein (LDL) (Reaven et al., 1995), a reduction in the risk of atherosclerosis (Nyssoner et al., 1994) and coronary heart disease (Stampfer et al., 1993). It protects against endothelium injury (Boogaerts et al., 1984; Hennig et al., 1989) and myocardial membrane injury (Janero and Burghardt, 1989).

VE is located in the cell membrane and is very effective in breaking the chain reactions of lipid peroxidation. Both GSH and VC are located in the cytosol and thus serve as the first line of defense against ROS. VC can spare GSH while GSH can regenerate VC.
Scheme 1. The regeneration and recycling pathways of Lipoic Acid (LA) and the major cell antioxidants - GSH, VC and VE
from its oxidized form. VC and GSH are unable to inhibit lipid peroxidation by trapping free radical directly in the lipid bi-layer. However, both VC and GSH can help to regenerate VE from its oxidized form. The putative regeneration and recycling pathway of GSH, VC and VE is shown in Scheme 1.

1.2.3 Protection by sequestration of metal ions

An overabundance of free metal ions, particularly ferrous and cuprous ions, can catalyze the formation of hydroxyl radical via the Fenton reactions. Hence the biological system is equipped with specific metal chelators that can keep the presence of these free metal ions in check. Transferrin, an iron-binding protein, is present in such a quantity that the amount of reactive iron available for catalyzing the generation of *OH from O₂⁻ and/or H₂O₂ is usually negligible (Aruoma and Halliwell, 1989). On the other hand, most of the copper ions are bound by ceruloplasmin and albumin. In addition to the binding of copper ions, ceruloplasmin can catalyze the conversion of ferrous to ferric ion, which is unreactive towards H₂O₂ (Blake et al., 1987).

1.3 Myocardial Ischemia-Reperfusion (IR) Injury – A Consequence of Myocardial Revascularization

Recently, it has become apparent that reperfusion, the restoration of blood flow after a period of ischemia, can place ischemic organs at risk of further cellular necrosis and thereby limit the recovery of function. While the concept of lethal reperfusion injury to
parenchymal cells is not universally accepted, it is widely recognized that the microvasculature, particularly the endothelial cells lining of microscopic blood vessels, is very vulnerable to the deleterious consequences of ischemia and reperfusion (IR). Indeed, IR-induced microvascular dysfunction has been described in most organs and it is recognized as a potentially serious problem that is encountered during a variety of standard medical and surgical procedures, such as thrombolytic therapy, organ transplantation, coronary angioplasty and cardiopulmonary bypass (Grace, 1994). In addition, similar consequences also resulted from a period of global ischemia in patients undergoing cardiac transplantation or open-heart surgery (Burton, 1988); the reintroduction of electrolytes, oxygen and cellular elements to the previously ischemic myocardium initiates lethal biochemical changes. In this respect, reperfusion, although essential for cell survival, carries with it the risk of inducing irreversible cell damage or cell death. Reperfusion of the previously ischemic myocardium may indeed be a “double-edged sword”. “Ischemia-reperfusion injury” is a term refers to this paradoxical phenomenon.

The central role of the readmission of oxygen in the genesis of reperfusion injury has led to the suggestion that oxygen-derived free radicals and the consequent oxidative stress may contribute to reperfusion injury. Over the past 10 years, direct observation of free radical production during ischemia and reperfusion has been achieved by electron spin resonance (ESR) spectroscopy measurement using spin-trapping techniques (Tosaki et al., 1990; Blasig et al., 1994) in isolated heart (Arroyo et al., 1987; Maupoil and Rochette, 1988; Shandelya et al., 1993) and open-chest heart models (Bolli et al., 1989; Li et al.,
1993). These studies showed an increase in oxygen free radical production during the ischemic period, but even more so during the first few minutes of reperfusion when a sudden burst of free radical production occurred. It has been proposed that the oxidation of cellular constituents by oxygen radicals and other toxic oxygen metabolites represents a major form of tissue injury that may occur in reperfused heart (Hess and Manson, 1984; Becker and Abrosio, 1987). Moreover, the involvement of free radicals has been indirectly shown by the protective effect of antioxidant enzymes as well as free radical scavengers (Das and Engelman, 1990). In addition, the involvement of free radical-mediated damage has been supported by the presence of lipid peroxidation product in the reperfused myocardium (Rao et al., 1983).

1.3.1 Influence of risk factors for cardiovascular diseases on reperfusion-induced microvascular dysfunction

It has been strongly suggested by recent studies that risk factors such as hypercholesterolemia, hypertension, and diabetes enhance the vulnerability of the microvasculature to the injurious effects produced by IR.

Elevated levels of plasma cholesterol appear to exacerbate the dysfunctional responses elicited by IR in all segments of the microvasculature. Endothelium-dependent relaxation of arterioles is impaired in even mildly hypercholesterolemic experimental animals and humans (Harrison, 1997; Steinberg et al. 1997). The capillary filtration response to IR is also exaggerated during hypercholesterolemia (Harris and Granger, 1996).
Experimentally induced diabetes is also associated with a significant enhancement in the recruitment of rolling, adherent and emigrating leukocytes, more albumin leakage and an accelerated formation of oxidants in venules exposed to IR (Panes et al., 1996; Salas et al., 1996; Salas et al., 1999). Unlike the responses observed in hypercholesterolemic animals wherein the exaggerated albumin extravasation from postischemic venules is tightly linked to the accompanying enhancement of leukocyte-endothelial cell adhesion, a leukocyte-independent mechanism appears to mediate the IR-induced endothelial barrier dysfunction in diabetes.

Relatively little is known about the influence of hypertension on the responses of the microvasculature to IR. Although a number of studies suggest that hypertension is associated with altered inflammatory responses, it remains unclear whether chronic arterial hypertension enhances or attenuates the responses elicited by inflammatory stimuli, such as IR (Granger, 1999).

1.3.2 Involvement of oxygen-derived free radicals in the pathogenesis of myocardial IR injury

The reperfusion of the ischemic myocardium, depending on the severity and duration of the preceding blood flow deprivation, may be a potential source of accelerated and amplified myocardial injury (Hearse, 1977; Braunwald and Kloner, 1985; Hearse and Bolli, 1992). In this context, it has been suggested that oxygen radical-mediated tissue
damage may play a critical role (McCord, 1985; Flaherty and Weisfeldt, 1988). Previous studies have shown that post-ischemic reperfusion is associated with a burst of free radical production and increased accumulation of lipid peroxide. It is possible that myocardial ischemia and reperfusion can alter the defense mechanisms against oxygen radicals, and the main cellular antioxidant became more susceptible to oxidative damage. Therefore, the balance between free radical products and endogenous antioxidants could be critical in the progression of tissue damage.

1.3.3 Free radical-related mechanism of myocardial IR injury

A huge body of experimental evidence has suggested the involvement of ROS in development of myocardial IR injury (Bernier et al., 1986; Bolli, 1988; Mergner et al., 1991). As regards the sources of ROS in ischemic-reperfused myocardium, a number of possibilities have been proposed. However, their relative contribution to myocardial IR injury is still unclear. It is likely that the development of myocardial IR injury involves a multiplicity of free radical related events.

1.3.3.1 Xanthine oxidase

Given the widespread endothelial localization of xanthine oxidase and the capacity of this enzyme to generate \( O_2^- \) and \( H_2O_2 \), much attention has been given to xanthine oxidase as a contributor to IR-induced distant organ injury. Plasma xanthine oxidase activity increases dramatically following aortic occlusion-reperfusion, as well as after IR to
hepatic or intestinal IR. This increase is associated with pulmonary, hepatic and myocardial injury. The fact that inhibition of xanthine oxidase blunts the pulmonary and liver dysfunction suggests that xanthine oxidase or its products may contribute to reperfusion-induced distant organ injury.

It has been known for some time that ischemic tissues accumulate hypoxanthine, a substrate for xanthine oxidase, from the catabolic breakdown of ATP due to oxygen starvation. Xanthine dehydrogenase is likewise converted to the oxygen-reducing xanthine oxidase during ishemia. That is to say that during the period of low blood flow, a new form of enzyme, xanthine oxidase, appears. Concomitantly, one of the two necessary substrates (hypoxanthine) for that enzyme appears. The second substrate, molecular oxygen, is supplied when the tissue is reperfused. At that point, all substrate components necessary for the reaction to proceed are present, and a burst of tissue-damaging superoxide production occurs (Scheme 2).

1.3.3.2 Leukocytes

Neutrophils are known to produce $O_2^{-*}$ and $H_2O_2$ and to secrete myeloperoxidase, an enzyme that catalyzes the formation of hypochlorous acid from $H_2O_2$ and chloride ions. Activated neutrophils also produce potent proteases capable of degrading virtually all components of the endothelial basement membrane as well as junctional proteins that maintain endothelial barrier function. Leukocytes exposed to ischemic tissue may re-enter the systemic circulation in an activated state upon reperfusion. These activated
Scheme 2. Proposed mechanism for superoxide production during reperfusion of ischemic tissues
neutrophils have been implicated as mediators of IR-induced distant organ injury (Carden et al., 1993 and 1998; Xiao et al., 1997).

1.3.3.3 Arachidonic acid metabolism

Arachidonic acid is an essential fatty acid which is metabolized to diverse biologically active products. The arachidonic acid pathway constitutes one of the main mechanisms for the production of pain and inflammation, as well as controlling homeostatic function.

The majority of the known mediators of inflammation arise from the membrane-bound arachidonic acid. Their intracellular release by activation of phospholipases facilitates their enzymatic metabolism into a host of pharmacologically potent products.

The synthesis of eicosanoids, including leukotrienes, thromboxanes and prostaglandins involves oxygen free radical-mediated processes (Mason et al., 1980). The biosynthesis of the eicosanoid-derived inflammatory mediators which, according to the present pharmacological knowledge, are of importance for the understanding of the pathologic alterations on a molecular level, is initiated by the two enzymes: cyclooxygenase and 5-lipoxygenase (Loschen and Ebeling, 1991). Cyclooxygenase, also known as prostaglandin H synthetase, catalyzes the first step in the synthesis of prostaglandins and other eicosanoids by inserting molecular oxygen into the arachidonic acid molecule. As a result, two endoperoxides, PGG₂ and PGH₂, are formed. A trace amount of hydroperoxide is required to react with the heme (Fe³⁺) at the active site of the enzyme to
form a peroxyl radical that can abstract a hydrogen atom from the arachidonic acid molecule. It has been shown that the addition of GSH and GPX to remove traces of lipid peroxides in the reaction mixture could decrease the rate of PGG₂ formation (Capdevila et al., 1995). However, an excessive presence of lipid peroxides can inactivate cyclooxygenase, and this represents an important feedback control of prostaglandin synthesis. Besides, it has been reported that cyclooxygenase can metabolize certain xenobiotics to more toxic intermediate, which may react with oxygen to form reactive oxygen radicals (Smith et al., 1991).

1.3.3.4 Catecholamine oxidation

Catecholamines are neurotransmitters that have long been known to play vital roles in many adult physiological processes, from heart stimulation to motor control. The autoxidation of catecholamines has been proposed to be a source of oxygen radicals leading to damage in myocardial IR injury (Singal et al., 1982). During ischemia, catecholamines are over-secreted in the ischemic myocardium, which is innervated by sympathetic nerve endings (Newman et al., 1971). These catecholamines are degraded spontaneously by autoxidation and result in excessive production of electrons (Hess and Manson, 1984). Molecular oxygen presented to the myocardium during reperfusion acts as an electron acceptor, resulting in the formation of ROS (Scheme 3). In this regard, autoxidation of 6-hydroxydopamine has been largely attributed to its cytotoxicity in catecholaminergic neurons (Heikkila and Cabbit, 1981).
Scheme 3. Autoxidation of catecholamine and the subsequent generation of reactive oxygen species

\[ \text{O}_2^\cdot + \text{H}_2\text{O}_2 + \cdot\text{OH} \]

Reactive Oxygen Species
1.3.3.5 Mitochondrial electron transport chain

Mitochondria have long been considered as one of the major sources of partially reduced form of oxygen within the reperfused myocyte. The main function of mitochondria is energy production, which is mediated by the electron transport process occurring in the inner membrane of the mitochondrion. Molecular oxygen undergoes tetravalent reduction to water in the cytochrome oxidase complex located at the terminal part of the mitochondrial electron transport chain. Under normal circumstances, oxygen free radicals are not released from the complex because they are firmly bound. However, during myocardial ischemia, an increase in reducing equivalents in the electron transport chain favors leakage of energetic electrons from the electron transport chain. This will cause univalent reduction of molecular oxygen during reperfusion, which in turn results in \( H_2O_2 \) and \( O_2^- \) production.

Ubisemiquionone and ubiquinol are the main sources of mitochondrial \( O_2^- \), whereas amine oxidase in the outer mitochondrial membrane produces \( H_2O_2 \). The release of \( H_2O_2 \) by NADH cyanide-independent oxidase in cardiac mitochondria has been demonstrated (Vandeplassche et al., 1989).

1.4 Myocardial Consequences Caused by Free Radicals

Many experimental data have shown that reperfusion can result in transient or permanent myocardial injury (i.e. reperfusion injury), which is likely mediated by oxygen free
radicals (Goldhaber and Weiss, 1992; Ambroso and Chiarello, 1991; Ferrari et al., 1992; Luchesi, 1990). Oxygen free radicals cause peroxidation of membrane lipids, resulting in structural and functional changes. These mechanisms can explain some manifestations of the reperfusion injury such as myocardial stunning and reperfusion arrhythmias (Luchesi, 1990; Ambroso and Chiarello, 1991; Ferrari et al., 1992). Structural damages observed in the myocardium include swollen mitochondria with marked ultrastructural change (Duncan, 1988), and the development of vacuoles and hyper-contrasted myofibrils (Burton, 1988). In addition to the undesirable functional changes such as myocardial stunning and reperfusion arrhythmias, activated neutrophil infiltration and vasoconstriction could also be observed. In addition, accumulation of calcium, inactivation of essential enzymes, membrane pumps (Wang et al., 1988), ion exchangers (Reeves et al., 1986) and ion channels (Kaneko et al., 1989) were the observable biochemical changes in the myocardium upon IR-induced oxidative challenge.

### 1.4.1 Lipid peroxidation

Lipid peroxidation is one of the major forms of free radical-mediated tissue damage in the myocardium. It occurs in polyunsaturated fatty acids (LH) located in cell membrane and can be initiated by a hydroxyl radical, *OH, which captures a hydrogen atom from a methylene carbon in the polyalkyl chain of the fatty acid molecule.

Initiation

\[
\text{LH} + \cdot\text{OH} \rightarrow \text{L'} + \text{H}_2\text{O}
\]
Propagation

\[ \text{LOO}^* + \text{LH} \rightarrow \text{LOOH} + \text{L}^* \]

Under aerobic conditions, a fatty acid molecule with an unpaired electron undergoes a molecular rearrangement by reaction with \( \text{O}_2 \) to generate a peroxyl radical (LOO*). This product is highly reactive and can combine with other peroxyl radicals to alter membrane proteins. These radicals can also abstract hydrogen atoms from the adjacent fatty acid molecules to form a lipid hydroperoxide, with resultant formation of lipid free radical and the subsequent propagation of lipid peroxidation. Thus, the peroxidation of unsaturated fatty acids can induce the conversion of several fatty acid side-chains into lipid hydroperoxides, which in turn leads to the propagation of a reaction chain (Scheme 4). The end products of lipid peroxidation (e.g. reactive carbonyl species) can inhibit protein synthesis (Fraga et al., 1989), alter vascular permeability (Messent et al., 1997), inflammatory response and chemotactic activity of leukocytes (Rossi et al., 1991) and react with nitrogenated bases of DNA (Park and Floyd, 1992).

1.4.1.1 Consequences of lipid peroxidation

Lipid peroxidation can alter the fluidity of cell membrane, cause damage to membrane structure and polymerize membrane components (Niki et al., 1991). These types of membrane alterations are capable of impairing the ability of cell membrane to maintain
Fatty acid with 3 double bonds

\[ \text{Hydrogen abstraction} \]

\[-H^*\]

Molecular rearrangement

Conjugated diene with UV absorbance at 234 nm

\[ \text{O}_2 \text{ uptake} \]

Peroxy radical: abstracts \( H^* \) from another fatty acid causing an autocatalytic chain reaction

\[ \text{Lipid hydroperoxide} \]

\[ \text{Cyclic peroxide} \]

\[ \text{Cyclic endoperoxide} \]

Fragmentation to aldehydes (including malondialdehyde) & polymerization products

Scheme 4. Lipid peroxidation pathway
its ionic gradients, resulting in the accumulation of cytosolic calcium (Steenbergen et al., 1987). The rise in cytosolic Ca$^{2+}$ may be mediated through the activation of Na$^+$-Ca$^{2+}$ exchange, an event secondary to a rise of intracellular Na$^+$ due to free radical-mediated inactivated of sodium/potassium adenosine triphosphatase (Na$^+$-K$^+$ ATPase) activity (Murphy et al., 1988). Overloading of intracellular Ca$^{2+}$ can activate the production of free radicals from many sources, including the respiratory burst of leukocytes, arachidonic acid release and the subsequent release of ROS, the conversion of xanthine dehydrogenase to xanthine oxidase (Bondy and LeBel, 1993) and the activation of nitric oxide synthase (Dawson, 1995). In addition, high levels of intracellular calcium taken up by mitochondria can stimulate mitochondria-derived free radical production.

On the other hand, the reaction of free radicals with protein molecules results in the formation of protein radicals, which may cause further cellular damage in the myocardium. For example, hydroxyl radical may inactivate membrane transport protein, as a result, normal ion transport in cellular membrane may be severely affected, leading to altered contractility. An overview of the myocardial consequences caused by ROS is summarized in Scheme 5.

1.4.2 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) catalyzes the following reaction:

Pyruvate + NADH $\longrightarrow$ Lactate + NAD$^+$ + H$^+$
Scheme 5. An overview of the myocardial consequences caused by reactive oxygen species

- Constriction of coronary vasculature
- Inhibited Endothelial-derived relaxing factor
- Free radicals $R^*$
- DNA, protein
- Reactive Oxygen Species $O_2^\cdot$, $OH^\cdot$, $^{1}O_2$
- Inhibited $Na^+\cdot-K^+\cdot$ ATPase
- Increased cytosolic $Na^+$
- Activated $Na^+\cdot-Ca^{2+}$ exchange
- Arrhythmias
- Activation of contractile elements "Hypercontraction"
- Activation of calcium-dependent protein kinase & phospholipase
- Mitochondrial accumulation of calcium
- Decreased ATP generation
LDH is one of the house keeping enzymes present in cardiomyocytes. Under anaerobic condition, LDH can reduce pyruvate to lactate, generating NAD⁺. Upon myocardial tissue injury, LDH will be leaked out from the myocardium; therefore, it can serve as a biochemical index of cardiac injury.

1.4.3 Malondialdehyde (MDA)

MDA, a highly reactive dialdehyde, is produced as one of the end products of peroxidation. It can react with the free amino-group of proteins, phospholipids or nucleic acids, to produce inter and intra-molecular 1-amino-3-iminopropene bridges and structural modifications of biological molecules. In several pathologies such as diabetes (Sato et al. 1979), hyperlipemia (Wsterbauer et al. 1990), atherosclerosis (Plachta et al. 1992), apoplexy (Kawamoto et al. 1986), and liver diseases (Rouach et al. 1997), it has been shown that the extent of lipid peroxidation increased significantly. In addition, the fatty acid moieties of cholesterol ester located in plasma low-density lipoproteins (LDL) can also be oxidized during oxidative stress. Oxidized LDL is considered to be the key event involved in the development of atherosclerosis (Galle et al. 1995).

1.4.4 Contractile force recovery

Upon the myocardial damage induced by IR, the contractile performance will be deteriorated. Myocardial stunning, a term referring to myocardial contractile dysfunction that follows a period of coronary occlusion even after flow has been restored during
reperfusion, represents the combined results of ischemia and reperfusion injury. Stunning represents a flow-contraction mismatch that is typically seen in the clinical setting of thrombolytic therapy for acute myocardial infarction. Stunning has also been described in the recovery phase after exercise-induced ischemia, in the post-cardiac operation period, in the setting of unstable angina and following coronary angioplasty. The proposed mechanisms for the observed wall motion abnormalities include abnormal energy utilization by myofibrils, production of cytotoxic oxygen free radicals, altered calcium flux and accumulation of neutrophils in the previously ischemic tissue.

1.5 Clinical Implications of Myocardial IR Injury – Coronary Heart Disease (CHD)

Coronary Heart Disease (CHD, International Classification of Diseases 410 – 414, 9th Revision) or Ischemic Heart Disease (IHD) is nowadays the predominant cause of mortality and disability in most developed countries, and is rapidly emerging as a serious health problem in many developing nations also. The entire population has become more aware of the seriousness of heart diseases and coronary heart problems. Cardio-pulmonary resuscitation (CPR) training, one of the first aid measure for heart attack, is offered in schools, places of business, church and community functions, and people seems to recognize that prevention of coronary heart disease is a crucial considerations for the maintenance of good health.
The heart needs a constant supply of oxygen and nutrients carried by the blood in the coronary arteries. The damage or malfunction of heart following the narrowing or blockage of the coronary arteries is defined as CHD. The inadequate supply of blood to heart would cause chest pain known as angina (pain usually felt in the chest or sometimes in the left arm and shoulder).

When the extent of coronary blockage was exaggerated, it would result in further damage of myocardial tissue called myocardial infarction (heart attack). The part of the heart that does not receive oxygen may be permanently damaged and become dead (National Institutes of Health, 1993).

1.5.1 Conventional therapy of CHD

A variety of drugs are available for improving the blood flow through coronary arteries. An anticoagulant is a drug that delays the clotting (coagulation) of blood. When a clot plugs a blood vessel and an anticoagulant is given, the drug tends to prevent new clots from forming or the existing clot from enlarging. Anticoagulants are also given to certain people at risk for forming blood clots, such as those with artificial heart valves or having atrial fibrillation. However, anticoagulant drugs only have little effect on ameliorating the progress of the disease because it does not dissolve an existing blood clot.

Angina pectoris can be treated with drugs that affect the supply of blood to the heart muscle or the heart's demand for oxygen. Examples of drugs affecting blood supply are
coronary vasodilators, which cause blood vessels to relax. Nitroglycerin is the coronary vasodilator most often used. It relaxes the veins (reducing the amount of blood that returns to the heart and easing the heart's workload) and the coronary arteries (increasing the heart's blood supply). Other drugs such as the beta-blockers, calcium channel blockers and peripheral vasodilator, can also be used to reduce the heart’s workload. Unfortunately, the therapeutic effect of these drugs is far from satisfactory since the damage to coronary arteries as well as myocardial tissue has already been done. They are only helpful in providing symptomatic relief that are of no curative value.

If drug treatment fails to relieve the symptom, or if examination shows severe narrowing of coronary arteries, such high-risk situations should be treated by surgical treatment. Coronary artery bypass graft operation is done to reroute or "bypass" blood around clogged arteries and improve the supply of blood and oxygen to the heart. In some patients, alternative surgical treatment such as balloon angioplasty, laser angioplasty, stents or atherectomy (plaque removal) is used. Balloon angioplasty is a procedure used for dilating (widening) narrowed arteries. A catheter is inserted with a deflated balloon at its tip into the narrowed part of the artery. Then the balloon is inflated to compress the plaque and to enlarge the inner diameter of the blood vessel so blood can flow through more easily. Then the balloon is deflated and the catheter is removed. It is a less traumatic and less expensive alternative to bypass surgery for some patients with coronary artery disease.

1.6 Diabetes Mellitus
Diabetes mellitus (diabetes) is a multifactorial disease associated with a high risk for vascular complications. It is characterized by symptoms such as excessive thirst, weight loss in the mid of increase food intake, excessive urination, elevated blood glucose level and the excretion of glucose in urine.

1.6.1 Classification and etiology of diabetes mellitus

There are three main types of diabetes: insulin-dependent diabetes mellitus (IDDM or Type 1), non-insulin-dependent diabetes mellitus (NIDDM or Type 2), and gestation diabetes. IDDM usually develops at any time from infancy to the mid-to late 30s, although it can occur later in life. The pancreas does not produce any insulin at all. This may be due to a combination of hereditary and environmental factors, but also it is thought that it may be caused by damage to the pancreas by virus or other infection. In NIDDM, which usually occurs in those who are over 40s, is often linked with being overweight. The body still produces insulin, but either insufficient amount is produced or the body cannot use the insulin properly to control the glucose increase in the blood after a meal. This may be due to a combination of factors including being overweight. Like IDDM, NIDDM can be seen to occur in family members, which suggests the involvement of genetic component in the pathogenesis of the disease.

1.6.2 Diabetic complications
Several studies have demonstrated that diabetic patients have an increased risk of suffering from coronary heart disease (Hahn et al., 1990), stroke (Abott et al., 1987), pancreatic cancer (Gullo et al., 1994), kidney failure (nephropathy) (Luke, 1992), cataract/blindness associated with retinopathy (Merimee, 1990) and foot ulceration associated with neuropathy (Kozak and Rowbotham, 1984). Late complications of diabetes will develop in the majority of diabetic patients.

Both experimental and clinical studies suggested that cardiovascular complications represent by far the most common and devastating clinical manifestation as well as the major cause of hospital admissions for diabetic patients. The major incidence of cardiovascular disease is not fully explained by hyperglycemia or by an association with other known microvascular risk factors. Epidemiologic studies have recorded a three-fold increase in the relative risk of myocardial infarction compared with matched non-diabetic populations (Jacobs et al., 1991). The consequences of diabetic microangiopathy and macroangiopathy represent the principal cause of mortality and disability in patients with diabetes mellitus. In microangiopathy, the thickening of capillary basement membranes results in the increase in vascular permeability, which can contribute to the development of diabetic retinopathy and nephropathy. Macroangiopathy mainly consists of atheromatous development in large blood vessels, which is morphologically similar to the non-diabetic atheroma. Macrovascular lesions can in turn lead to hypertension and coronary heart disease.
Although the pathogenetic mechanism and age distribution differ between Type I and Type II diabetes, atherosclerosis develops in both types of diabetic populations at an accelerated rate. Arterial hypertension, which is present in up to 60% of patients with Type II diabetes as well as different forms of dyslipidemia, may amplify or further accelerate the vascular disease that affects most diabetic patients. This also explains the higher prevalence of large-vessel lesions in this population.

1.6.3 Pathogenesis of diabetes mellitus

The pathogenesis of the functional defects in diabetes is not fully understood, but it is likely to be multi-factorial with genetically determined susceptibility. Nevertheless, there are a few potentially unifying mechanisms early in the evolution of the disease that merit consideration.

Non-enzymatic glycosylation of “Maillard reaction” was first described by Louis Mallard (1912) who observed the browning of amino acids during the heating with reducing sugars. In this reaction, the Schiff bases formed between protein and glucose molecules are eventually forming the highly cross-linked advanced glycosylation end products (AGEs) through a complex series of irreversible rearrangements (Brownlee et al., 1988). The cross linking of proteins can cause the thickening of glomerular basement membrane and coronary artery, leading to hypertension, atherosclerosis (Brownlee et al., 1988) and nephropathy (Makita et al., 1991). Similar events may occur in the development of retinopathy (Merimee, 1990) and neuropathy (Monnier et al., 1986).
The sorbitol pathway consists of two enzymes, aldose reductase and sorbitol dehydrogenase, which sequentially, catalyze the conversion of glucose to sorbitol and then to fructose, with the utilization of NADPH and NAD⁺ in the respective reaction (Tomlinson et al., 1994). Under physiological conditions, glucose is metabolized through the glycolytic pathway and the flux through the sorbitol pathway is low and the level of sorbitol measurable in most tissues is negligible (Holcomb et al., 1974). However, under hyperglycemia, the highly saturated hexokinase causes a significant flux of glucose through the sorbitol pathway catalyzed by the low-affinity aldose reductase (Tomlinson et al., 1994). The accumulation of sorbitol causes the redox imbalance in cells, and the study done by Tilton et al. (1995) showed that vascular dysfunction (in ocular tissues, peripheral nerve and aorta) and electrophysiological dysfunction (in peripheral nerve) induced by diabetes are more causally linked to the increased sorbitol dehydrogenase-catalyzed oxidation of sorbitol to fructose.

The glyoxalase system catalyzes the conversion of methylglyoxal (a 2-oxoaldehyde) to D-lactate through the intermediary of S-D-lactoylglutathione. The involvement of this system in diabetic complications has been proposed by Thornalley and co-workers (1988). It was observed that the blood concentrations of methylglyoxal, S-D-lactoylglutathione and D-lactate were increased in IDDM and NIDDM patients. In addition, IDDM patients with retinopathy had a significantly higher activity of glyoxalase I and a significantly lower activity of glyoxalase II in red blood cells than those in patients without retinopathy (Thornalley et al., 1989).
Although being described in separate entities, the aforementioned mechanisms involved in the development of diabetic complications are not totally unrelated to each other. While it is no doubt that hyperglycemia is the primary cause for all the diabetes related metabolic changes, oxidative stress, which will be discussed in the later session, seems to be a common factor associated with all these diabetes-related alterations in cellular metabolism.

1.6.4 Streptozotocin-induced diabetes mellitus

Streptozotocin (STZ) is a mixture of α- and β-stereoisomers, which occurs as pale yellow crystals or platelets. It is an antibiotic originally derived from the soil microorganism Streptomyces achromogenes. It has been investigated for use in diabetes, since it has specific toxic action on pancreatic β-cells. When administered by intraperitoneal injection, streptozotocin increased the incidences of kidney and pancreatic islet cell tumors in rats of both sexes, liver tumors in female rats, and peritoneal sarcomas in male rats. The animal will subsequently become unable to produce adequate amounts of insulin and IDDM is thus induced. The symptoms of streptozotocin-induced IDDM rats include weight loss, increase water intake and urine production, and glucosuria.

1.7 Rationale for the Study

1.7.1 Traditional Chinese herbs – sources of antioxidants
It has become apparent that reperfusion, the restoration of blood flow after a period of ischemia, can place ischemic organs at risk of further cellular necrosis and thereby limit the recovery of function. It is widely recognized that the microvasculature, particularly the endothelial cells lining microscopic blood vessels, is very vulnerable to the deleterious consequences of ischemia and reperfusion. Many investigations have shown that free radical-mediated process is involved in the pathogenesis of myocardial IR injury. Although the conventional dietary supplements such as VC or VE possess antioxidant activities and could help to lower the extent of IR-induced myocardial damage, they could only act as free radical scavengers, which are quickly consumed under oxidative stress. In addition, the prophylactic effects of these conventional antioxidants are limited by their pro-oxidant action when administered at high dose (Bowry \textit{et al.}, 1992). Under these unfavorable circumstances, other antioxidants, which are able to display a new mode of action other than free radical scavenging, should be of pharmacological interest to explore.

The practice of traditional Chinese medicine emphasizes on the prevention of diseases. Many Chinese herbs have long been used for delaying the onset of senility and safeguarding health. Scientific investigations have shown that most of these “tonifying” herbs possess antioxidant activity. Given that traditional Chinese herbs work by maintaining and/or restoring the balance of body functions in preventing diseases, antioxidants isolated from these Chinese herbs may produce antioxidant action through a mechanism more than that of a free radical scavenger. Instead, they may restore or
enhance the endogenous antioxidant defense system to counteract the consequences caused by free radical induced oxidative stress.

1.7.2 Schisandrin B – an active Chinese herb-derived antioxidative agent

Fructus Schisandrae (FS), the fruit of Schisandra chinensis (Turcz.) Baill., has been used as tonic and astringent in traditional Chinese medicine for centuries. A recent study has demonstrated that FS was the antioxidant component herb in a Chinese formula “Sheng Mai San” used for the treatment of CHD (Ko et al., 1995). Schisandrin B (Sch B, see Fig. 2.2a), a dibenzo[a,c]cyclooctadiene lignoid derivative isolated from FS, has early been shown to produce free radical scavenging actions (Zhao et al., 1990) and inhibit lipid peroxidation (Mak et al., 1996) under in vitro conditions, as well as protect against carbon tetrachloride (CCl₄)-induced hepatotoxicity (Ip et al., 1995). The ensemble of these results suggests that Sch B is an active antioxidative agent.

Regarding the myocardial effect, previous studies in our laboratory have demonstrated that Sch B pretreatment could produce cardioprotection on post-ischemic reperfused heart (10-min ischemia followed by 15-min reperfusion) in rats. The myocardial protection was found to associate with an enhancement in myocardial glutathione antioxidant status, as indicated by significant reductions in both the extent of IR-induced GSH depletion and inhibition of GPX and GRD activities (Li et al., 1996; Yim et al., 1999). However, the effect of Sch B on the myocardial non-enzymatic antioxidants, namely VC and VE, has yet to be determined. Given that non-enzymatic antioxidant level is crucial in
determining the myocardial tissue susceptibility to oxidative challenge, it is of interest to investigate the relative change in myocardial GSH, VC and VE under the condition of IR-induced oxidative stress. Results from previous studies have demonstrated the cardioprotective action of Sch B upon a 10-min ischemic challenge followed by 15-min of reperfusion. However, as to whether the protective effect of Sch B could extend to conditions under prolonged period of ischemia followed by reperfusion remains to be explored.

Myocardial complications, including myocardial infarction and heart failure resulting from diabetes have been described in various clinical and experimental settings. In recent years, the diabetes syndrome has been associated with enhanced lipid peroxidation, which can contribute to tissue damage under chronic conditions (Nourooz-Zadeh et al., 1997). It has been found that generalized increase in tissue oxidative stress existed in STZ-induced diabetic rats was well reflected in the suppression of glutathione antioxidant system (Mak et al., 1996). Given the efficacy of Sch B in protecting against myocardial IR injury, it would be of therapeutic interest to examine whether Sch B could protect the heart from IR injury under diabetic status.

1.7.3 Alpha-lipoic acid (LA) – A powerful metabolic antioxidant

LA (see Fig. 2.2b) is a substance found in food and manufactured in small quantities by the body. In 1988, it was discovered that LA was a powerful metabolic antioxidant. When taken with a comprehensive antioxidant regime, LA replenishes VC and GSH and
helps to recycle VE. As an added benefit, LA promotes cellular and muscular energy and functions as a co-enzyme in the metabolism of sugars.

As an antioxidant, LA is unique because it is both fat- and water-soluble, it can enter all area of the cell affected by free radicals. Because of this property, it can enter all areas of the cell affected by free radicals. LA also helps to convert pyruvate into acetyl co-enzyme A, one of the main fuels driving the Krebs Cycle, which provides the body with its energy. As a result, the body’s rate of metabolism of fats and carbohydrates can be improved.

1.7.4 Rat model of myocardial IR injury

Given the involvement of free radical-mediated process in pathogenesis of myocardial IR injury and CHD (Ji et al., 1991; Gey, 1993; Kaul et al., 1993), the search of pharmacological agents from natural herbs that can enhance myocardial antioxidant defense has been an area of intense research (Hearse, 1991; Jayakumari et al., 1992; Rice and Diplock, 1993). In the present study, a model of myocardial IR injury using Langendorff isolated-perfused rat heart system was adopted to simulate the conditions of myocardial infarction. The effect of antioxidant pretreatment in the protection against myocardial IR injury was investigated.
1.7.5 Objective of the study

Given the involvement of free radical-mediated processes in the pathogenesis of myocardial IR injury, the primary objective of this thesis work is to examine the change in non-enzymatic antioxidant status of the myocardium under oxidative stress conditions induced by IR. The effect of Sch B pretreatment, which has previously been shown to protect against myocardial IR injury in the isolated rat heart model, was examined in an effort to explore the biochemical mechanism involved in the cardioprotection in relation to the interplay among non-enzymatic antioxidants. In view of the change in myocardial antioxidant status under diabetic conditions, the extent of IR injury induced in hearts isolated from diabetic rats was examined, and the effect of Sch B pretreatment was also investigated. In the drug pretreatment studies, the effect of α-lipoic acid (LA) pretreatment was also investigated for comparison.
Chapter 2

MATERIALS AND METHODS

2.1 Herbal Extraction

2.1.1 Isolation of Schisandrin B from Fructus Schisandrae

*Fructus Schisandrae* (FS), the fruit of *Schisandra chinensis* (Turcz.) Baill., was purchased from an authenticated source in Hong Kong. It was dried by heating in an oven at 60°C overnight. The dried FS was then ground into powder and extracted three times with petroleum ether (w/v, 1:2). The pooled extract was dried under reduced pressure at 30°C to obtain an FS extract at a yield of 13% (w/w).

FS extract (500 g) was applied onto a preparative silica gel column (10 cm x 40 cm) equilibrated with hexane. Eluates were monitored by silica gel TLC (hexane/acetone (v/v, 65:35), detected by UV 254 with standard Schisandrin B (Sch B) as reference. Stepwise elution was started with 3 L of hexane and followed by 12 L of hexane/acetone (95:5). Fractions containing Sch B were then eluted out by 12 L of hexane/acetone (85:15). The Sch B fractions were then pooled and concentrated under reduced pressure at 30°C. After standing overnight at room temperature, Sch B was obtained in crystal form (racemic mixture) at a yield of 0.2% (w/w) with respect to crude FS extract. Recrystallized Sch B was assessed for purity (>95%) by HPLC method (Fig. 2.1) and then used for the experiments. The chemical structure of Sch B (Fig. 2.2a) was confirmed by comparing
Fig. 2.1  Elution profile of Sch B (racemic mixture)
Fig. 2.2  Chemical structure of (a) Sch B and (b) α-Lipoic acid
the TLC and spectral characteristics (\(^{1}H\)- and \(^{13}C\)-NMR and mass spectra) with an authentic standard obtained from the Institute of Materia Medica, Chinese Academy of Medical Science, Beijing.

2.2 Sch B and α-Lipoic Acid Pretreatments

2.2.1 Animal care

Adult female Sprague Dawley rats weighing 220 - 250 g were used in the study examining the effect of Sch B and α-lipoic acid (LA) (Fig.2.2b) pretreatments on myocardial ischemia-reperfusion (IR) injury. All animals were housed in air/humidity-controlled room, with 12 hours dark-light cycle at approximately 22°C and allowed food and water ad libitum in the Animal Care Facilities of the Hong Kong University of Science and Technology throughout the experiment.

2.2.2 Drug pretreatment protocols

Sch B and LA were dissolved/suspended in olive oil (vehicle) at a dose of 1.2 mmol/kg. All animals were pretreated intragastrically with the respective drug preparation at the indicated daily dose for 3 consecutive days. Control animals were given the appropriate vehicle instead (i.e. olive oil, 10 ml/kg). Animals were randomly assigned to 3 main groups: (1) Control, olive oil treated; (2) Sch B treated; (3) LA treated. The animals
within each main group were further divided into 6 groups subjected to various periods of ischemia and reperfusion. The experimental design was described later in Section 2.4.3.

All drug pretreatments were performed between 0930 and 1130 hour. Twenty-four hours after the last dosing, control or drug-pretreated rats were anesthetized with diethylether, the heart was then quickly excised and subjected to IR challenge.

2.3 Short-Term (2-week) Diabetes Mellitus

2.3.1 Induction of diabetes and animal care

Diabetes was induced in female Sprague Dawley rats of 8 – 10 week old by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 45 mg/kg (prepared in ice-cold 0.1 M citrate buffer, pH 4.5). Non-diabetic animals were injected with the buffer only (1 ml/kg body weight). Diabetic state was confirmed by the presence of glucosuria as determined by using glucose test strips (Glukotest, Boehringer Mannheim UK Ltd.) on the second day. All animals were housed in air/humidity-controlled room, with 12 hours dark-light cycle, and allowed food and water ad libitum throughout the experiment. Non-diabetic and diabetic rats were assigned to various IR time-course as described later in Section 2.4.3. There were 5 animals in each time-course for both non-diabetic and diabetic groups.
2.3.2 Insulin and Sch B pretreatments on short-term diabetic animals

The short-term diabetic rats were randomly assigned to the following treatment groups of 5 animals in each:

(1) Dia, diabetic control;
(2) Dia + Ins, diabetic with insulin pretreatment;
(3) Dia + Sch B, diabetic with Sch B pretreatment;
(4) Dia + Ins + Sch B, diabetic with both insulin and Sch B pretreatments.

The day of STZ injection was assigned as day 0. Insulin treatment began on day 3 and continued until the night before sacrifice (day 13). Animals with insulin pretreatment received twice daily intramuscular injections of insulin (2U Actrapid MC + 2U Monotard MC at 1100 hour and 3U Actrapid MC + 3U Monotard MC at 2100 hour). Sch B pretreatment commenced on day 11 for 3 consecutive days and was orally given to the animal at a daily dose of 1.2 mmol/kg. Diabetic animal in control group was orally given with olive oil (10 ml/kg) alone on day 11 for 3 consecutive days. The hearts of the animals were excised and subjected to various time courses of ischemia and reperfusion on day 14.

2.4 Rat Model of Myocardial Ischemia-Reperfusion (IR) Injury

2.4.1 Preparation of perfusion buffer
The perfusion buffer used in this study was a modified Krebs-Henseleit bicarbonate solution (pH 7.4) containing (in mM) 120 NaCl, 25.4 NaHCO₃, 4.8 KCl, 1.2 KH₂PO₄, 0.86 MgSO₄, 1.25 CaCl₂ and 11 glucose. Perfusion buffer was freshly prepared on the day of experiment and was adjusted to pH 7.4. The buffer was then filtered through a 0.45 mm filter (acetatePlus, supported Plain, 47 mm, 100/RK, Micron Separation Inc.) before use. The normoxic perfusion buffer was gassed with 95% O₂-5% CO₂ gas mixture (analytical grade, Hong Kong Oxygen Co.).

2.4.2 Preparation of isolated and perfused rat heart

Twenty-four hours after the last dosing, control or drug pretreated Sprague-Dawley rats were anesthetized with diethylether. Hearts were then excised and immediately chilled in ice-cold and heparinized (50 unit/ml) 0.9% saline. The arrested heart was cannulated via the aortic root in a warm and moisturized chamber of the non-recirculating Langendorff perfusion apparatus (Fig. 2.3). The heart was retrogradely perfused at a constant perfusion pressure regulated by a peristaltic pump. The heart and perfusion buffer were maintained at 37°C via a constant temperature water-jacketing system. The apex of the heart was attached via a metal hook to an unextendable thread connected to a force displacement transducer (Grass FT03). Isometric contractions of the heart were recorded on a polygraph (Grass Model 7-8P), with a resting tension of 1.5 g. The isolated perfused rat hearts were allowed a 30-min of normoxic perfusion for equilibration before the start of ischemic challenge. All hearts used in the IR experiment had an initial coronary flow
Fig. 2.3  Schematic representation of the non-recirculating Langendorff perfused rat heart model
rate ranging from 6 to 11 ml/min and an initial heart rate between 160 to 200 beats/min during the equilibration period.

2.4.3 Experimental protocol for IR challenge

After an initial 30-min of perfusion for equilibration, the isolated heart prepared from non-diabetic or diabetic animals with or without drug pretreatment was subjected to a 20- or 40-min period of “no-flow” normothermic global ischemia followed by a 20- or 40-min period of reperfusion. The non-ischemic (i.e. non-IR) hearts were subjected to either a 60- or 80-min perfusion after equilibration, without subjected to ischemia. The schematic diagram showing the time course of non-IR perfusion and IR protocols was shown in Fig. 2.4. The isometric contractions of the heart were continuously recorded during the whole experiment. Coronary effluent (1-min fraction) sample was collected every 10 min during the 30-min equilibration period, as well as every minute during the 20- or 40-min reperfusion for the IR hearts. The samples collected were kept on ice until assay for lactate dehydrogenase (LDH) activity.

2.5 Assessment for Myocardial IR Injury

To assess the IR-induced myocardial injury, the extent of LDH leakage from the myocardium and the contractile force recovery during reperfusion period were measured, which served as biochemical and functional index, respectively, for the ischemic and reperfused myocardium.
Non-IR Hearts:

**Group 1**
60 min perfusion

| 30-min equilibration | 40-min perfusion | 20-min perfusion |

**Group 2**
80 min perfusion

| 30-min equilibration | 40-min perfusion | 40-min perfusion |

IR Hearts:

**Group 3**
20-I, 20-R

| 30-min equilibration | 20-min ischemia | 20-min reperfusion |

**Group 4**
20-I, 40-R

| 30-min equilibration | 20-min ischemia | 40-min reperfusion |

**Group 5**
40-I, 20-R

| 30-min equilibration | 40-min ischemia | 20-min reperfusion |

**Group 6**
40-I, 40-R

| 30-min equilibration | 40-min ischemia | 40-min reperfusion |

Fig. 2.4 Experimental protocols

After an initial 30-min of perfusion for equilibration, the isolated heart prepared from control or drug pretreated animals was either subjected to a 20- or 40-min period of “no-flow” normothermic global ischemia followed by an additional 20- or 40-min period of reperfusion. The non-ischemic (i.e. non-IR) hearts were subjected to either a 60-min perfusion (control for Group 3, 4, 5), with the coronary effluent being collected on the last 20-min perfusion, or 80-min perfusion (control for Group 6), with the coronary effluent being collected on the last 40-min perfusion, after equilibration, without subjected to ischemia.
2.5.1 IR-induced lactate dehydrogenase (LDH) leakage

The LDH activity of the coronary effluent was assayed by adding 500 µl of the sample into a mixture of 50 µl 20 mM pyruvate and 350 µl 0.1 M phosphate buffer (pH 8.0). The reaction was initiated by addition of 50 µl of NADH (3 mM)) solution and absorbance changes of the reaction mixture in a final volume 1 ml were monitored spectrophotometrically at 340 nm for 2 min at 25°C, using Perkin-Elmer model Lambda 2 spectrophotometer. Enzyme activity was estimated using an extinction coefficient for reduced form of NADH at 340 nm of 6.22 x 10³ M⁻¹cm⁻¹ and expressed as U/L of coronary effluent. One unit (U) represents the activity of enzyme that can catalyze the oxidation of 1 µmol NADH per min. The extent of myocardial damage was estimated by computing the area under the curve (AUC) of the graph plotting the percent of LDH activity (%) (with respect to the mean pre-ischemic value measured during the equilibration period) against the reperfusion time (1 to 20 or 40min) (Fig. 2.5).

The extent of IR-induced myocardial damage would be quantified by calculating the difference in the extent of LDH leakage between the IR and non-IR perfused hearts, i.e. their difference in AUC of the graph plotting the percent of LDH activity (%) against the reperfusion time (min) (Fig. 2.5). The extent of LDH leakage (AUC) of the non-IR heart (640 ± 33, n=5) would serve as a basal value under the present experimental conditions; the IR-induced LDH leakage could be obtained by subtracting the basal value from that obtained from the IR hearts.
Fig. 2.5  Measurement of ischemia-reperfusion (IR)-induced LDH leakage in isolated perfused rat hearts

The extent of myocardial damage was estimated by computing the area under the curve and was expressed as arbitrary unit (AU). The extent of LDH leakage (%) (with respect to the mean preischemic value measured during the equilibration period) of the non-IR heart served as a basal value. The extent of myocardial damage induced by IR can be quantified by calculating the difference in AUC (shaded area) between the IR and non-IR curves. Each data point is the mean, with n=5.
Fig. 2.6  Estimation of contractile force recovery

Contractile Force Recovery (%) = \frac{H_{rep}}{H_{equ}} \times 100
2.5.2 Contractile force recovery

The extent of contractile force recovery was estimated by comparing the contractile force recorded at 20\textsuperscript{th} or 40\textsuperscript{th} min after reperfusion with that at 30\textsuperscript{th} min of equilibration. The ratio of reperfused/equilibrated value was multiplied by 100\% and the contractile force recovery was expressed as a percentage. Figure 2.6 illustrates a recording of contractile force and the estimation of contractile force recovery.

2.6 Myocardial Non-enzymatic Antioxidants Status

At the end of IR experiment, heart tissue was rapidly homogenized. The myocardial tissue homogenate was used for measuring the tissue level of non-enzymatic antioxidants, namely reduced glutathione (GSH), ascorbic acid (VC) and \(\alpha\)-tocophenol (VE) levels, as well as malanodialdehyde (MDA).

2.6.1 Preparation of myocardial tissue homogenate

After IR challenge, the entire myocardium removed from the Langendorff perfusion apparatus was immediately placed in ice-cold homogenizing buffer with 50 mM Tris[hydroxymethyl]-amino methane (Tris)-0.1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.6. The myocardium was then homogenized in homogenizing buffer (1:10, w/v) using two 10-second bursts of an Ika Ultra Turrax T25 homogenizer at 13,500 rpm. A 10-second interval was allowed between the bursts in order to avoid overheating.
2.7 Biochemical Analysis

2.7.1 Myocardial MDA level

Myocardial MDA level was measured by the HPLC method modified from Young and Trible (1991). The thiobarbituric acid reaction was carried out by mixing 250 µl of 10% homogenate, 250 µl 1.22 M phosphoric acid and 500 µl 0.5% thiobarbituric acid. The reaction mixture was incubated in a boiling water bath for 1 hour in sealed glass tubes and then cooled to room temperature. An aliquot (400 µl) of sample or standard was mixed with 80 µl NaOH and 720 µl HPLC grade methanol immediately before HPLC analysis. After centrifugation, the supernatant which contained the MDA-thiobarbituric acid adduct was chromatographed on a µ-Bondapak C-18 column (200 mm x 3.9 mm L.D., Waters) using a mobile phase of 50% methanol and 50% 25 mM phosphate buffer at pH 6.5. The separation was monitored by a Waters 474 Scanning Fluorescence detector set at excitation and emission wavelengths of 532 nm and 553 nm, respectively or by a Waters 996 Photodiode Array Detector, with the eluted peaks being obtained at 245 nm. Tissue MDA content, expressed as nmol/g tissue, was estimated from a calibration curve using acid-hydrolyzed 1,1,3,3-tetramethoxypropane as standard.

2.7.2 Myocardial GSH level

Myocardial GSH level was measured by an HPLC method modified from Reed et al. (1980). An aliquot of 500 µl myocardial tissue homogenate was mixed with an equal
volume of 10% (v/v) perchloric acid (PCA). After centrifugation at 632 g for 10 min using a Beckman GS 6R centrifuge, 500 μl of the supernatant was mixed with 50 μl iodoacetic acid (IAA, 2%, w/v) and 450 μl of 2 M KOH-2.4 M KHCO₃ solution. The sample was kept in dark at room temperature for an hour before the addition of 1 ml FDNB (1% in absolute ethanol). The derivatized sample was stored at 4°C before analysis.

HPLC using μ-Bondapak C-18 (200 mm x 3.9 mm I.D., Waters) column was employed to analyze GSH, in which the FDNB derivative of GSH was eluted at a characteristic elution time. Solvent A consisted of methanol (HPLC grade, Fisher)-water (4:1, v/v). Solvent B was obtained by dissolving 108.8 g sodium acetate trihydrate with 210 ml Milli-Q water, 150 ml glacial acetic acid and 640 ml methanol. The column was equilibrated with 25% solvent B at a flow rate of 1 ml/min, and 20 μl sample was applied with an isocratic elution of 25% solvent B for 10 minutes. Then the percentage of solvent B was increased to 95% in the next 15 minutes. After maintaining at 95% for 10 minutes, the percentage of solvent was decreased back to 25% in 1 minute. The separation was monitored by a Water 996 Photodiode Array Detector and the eluted peaks were obtained at 355 nm. GSH standard (Sigma, USA) were eluted at 25 min (Fig. 2.7). GSH content in the sample can be estimated by measuring the peak area using standard calibration curve and expressed as nmol/mg tissue.
Fig. 2.7 Elution profile of 1-fluoro-2, 4-dinitrobenzene derivatives of thiols
2.7.3 Myocardial VC level

Myocardial VC level was determined by HPLC according to the method of Liau et al. (1993) with minor modifications. Briefly, 200 µl of myocardial homogenate was deproteinized by incubating with 200 µl 10% perchloric acid for 30 min on ice. The deproteinized sample was mixed with 400 µl mobile phase (20 mM ammonium dihydrogenphosphate, 0.15% (w/v) meta-phosphoric acid, pH 2.95), and the mixture was centrifuged at 3,000 g for 10 min. An aliquot (20 µl) of the supernatants was injected as described above. The sample was analyzed by a µ-Bondapak C-18 column (200 mm x 3.9 mm I.D., Waters) using the mobile phase maintained at a flow rate of 1 ml/min at room temperature. The separation was monitored by Waters 996 Photodiode Array Detector, with the eluted peaks being obtained at 245 nm. Myocardial VC level was estimated from a standard calibration curve and expressed as µg/g wet tissue.

2.7.4 Myocardial VE level

Myocardial VE level was determined by HPLC method as described by Sadrzadeh et al. (1994) with minor modifications. Briefly, a 200 µl aliquot of myocardial homogenate (10%, w/v) was mixed with 200 µl of 1% VC in methanol and was then extracted with 600 µl of hexane. The extract was dried under a stream of nitrogen. The dried sample was re-dissolved in methanol and the sample solution was injected as described in the previous section. The sample was analyzed by a µ-Bondapak C-18 column (200 mm x 3.9 mm I.D., Waters) using methanol as the mobile phase maintained at a flow rate of 1
ml/min at room temperature. A Waters 474 Scanning Fluorescence detector set at excitation and emission wavelengths of 292 nm and 330 nm, respectively, was used to monitor the eluant. Myocardial VE level was estimated from a standard calibration curve and expressed as μg/g wet tissue.

In the HPLC analyses described above, all chromatographic data were recorded and analyzed by computer software (Millennium 2010 Chromatography Manager, Waters).

2.8 Statistical Analysis

For the statistical analysis, data were expressed as arithmetic mean and standard error of the mean (SEM), unless otherwise specified. The data were analyzed by one-way analysis of variance (one-way ANOVA) to test for any significant difference among the groups. Duncan's test was used for identifying the two groups with significantly difference from each other (p < 0.05).
Chapter 3

RESULTS

3.1 Ischemia-Reperfusion Injury in Isolated and Langendorff-Perfused Rat Hearts

3.1.1 Changes in the extent of LDH leakage and contractile force recovery upon increasing periods of ischemia and reperfusion

In this study, isolated and Langendorff perfused rat hearts were subjected to increasing periods of ischemia and reperfusion, the myocardial damage caused by ischemia-reperfusion (IR) was assessed by measuring the extent of lactate dehydrogenase (LDH) leakage and contractile force recovery. As shown in Fig. 3.1a, an abrupt increase in LDH leakage was observed in isolated hearts subjected to 20-min of ischemia followed by 20- or 40-min of reperfusion. The LDH activity measurable in the coronary effluent was found to be maximal at the first minute after reperfusion, it then declined gradually and reached a steady level as the reperfusion continued, with the value being slightly higher than that of the perfused (i.e. non-IR) control 20 min after the reperfusion. However, when the ischemic period was prolonged to 40 min, the extent of LDH leakage, as shown in Fig. 3.1b, was also found to increase dramatically at the first minute of reperfusion, which then declined for the next 1 to 2 min of reperfusion. But the LDH activity increased gradually again and reached a steady level, with the value being much higher than that of the non-IR control 20 min after the reperfusion.
**Fig. 3.1** Extent of LDH leakage upon increasing periods of ischemia and reperfusion

After an initial 30-min of perfusion for equilibration, the isolated rat heart was subjected to a (a) 20- or (b) 40-min period of "no-flow" normothermic global ischemia followed by 20- or 40-min reperfusion. The non-ischemic control (i.e. non-IR) hearts were perfused for 60 or 80 min after equilibration, without subject to ischemia.
For the non-IR control hearts, which were either perfused for 60 or 80 min following the equilibration period, showed a low and stable LDH activity comparable to that of the equilibration period.

The extent of LDH leakage was quantified by computing the area under the curve of the graph plotting the percent of LDH activity with respect to the mean pre-ischemic value measured during the equilibration period (see Section 2.3.2). It was found that the extent of LDH leakage of IR-hearts for various ischemic and reperfusion periods was generally higher than that of non-IR control hearts (Fig. 3.2). The extent of LDH leakage of non-IR hearts served as a base-line value under their respective experimental conditions (either a 20- or 40-min of reperfusion). The IR-induced LDH leakage was obtained by subtracting the base-line value from that of the respective IR hearts (value shown in the parentheses in Fig. 3.2). As shown in Fig. 3.2, the increase in perfusion time of non-IR control hearts caused a one-fold increase in the extent of LDH leakage. After a 20-min of ischemia followed by 20-min of reperfusion, the extent of LDH leakage was significantly higher (2.5-fold) than that of the non-IR control. No significant increase in the extent of LDH leakage was observed when the reperfusion period was prolonged to 40 min. However, after a 40-min period of ischemia followed by 20-min of reperfusion, the extent of LDH leakage was significantly increased by 9-fold, when compared with the non-IR (60-min perf.) control. When the reperfusion time following 40-min of ischemia was prolonged to 40 min, the extent of LDH leakage was increased by 7-fold, when the compared with the non-IR (80-min perf.) control. The increase in ischemic time from 20 to 40 min caused significant increases in the extent of LDH leakage following either 20- or 40- min of reperfusion.
Fig. 3.2 The extent of LDH leakage from control (perf) and ischemic-reperfused (IR) isolated rat hearts

The extent of myocardial damage was estimated by computing the area under the curve (AUC) of the graph plotting the percent of LDH activity (%) (with respect to the mean pre-ischemic value measured during the equilibration period) against the reperfusion time (1 to 20 or 40 min). The non-ischemic control (i.e. non-IR) hearts were subjected to 60- or 80-min perfusion (perf) after equilibration without ischemia. The number in parenthesis represents the extent of IR-induced LDH leakage with the base-line value (AUC of the respective non-IR group) being subtracted. Each bar represents the mean ± SEM, with n=5.

* significantly different from the 60-min perf group
† significantly different from the 80-min perf group
*# significantly different from the 20-I, 20-R group
†# significantly different from the 40-I, 20-R group
*# significantly different from the 20-I, 40-R group
While the increase in perfusion time (60 to 80 min) for non-IR hearts did not change the contractile force recovery, a 20-min period of reperfusion following 20-min of ischemia resulted in an incomplete recovery (83%) of contractile force in ischemic-reperfused hearts (see Fig. 3.3). But a 40-min of reperfusion was able to make an almost complete recovery (97%). However, after 40-min period of ischemia, contractile force recovery measured at 20-min of reperfusion was only 55% and no further improvement was found when the reperfusion time was extended to 40 min. The increase in ischemic time resulted in significant decreases in contractile force recovery following 20- or 40-min of reperfusion.

3.1.2 Changes in tissue MDA level in ischemic and reperfused rat hearts

Increase in perfusion time from 60 to 80 min did not cause any significant change in myocardial malondialdehyde (MDA) level (Fig. 3.4). On the other hand, while IR did not produce any significant changes in myocardial MDA level, the increase in reperfusion time from 20 to 40 min caused a slight but insignificant increase. The increase in ischemic time did not produce any significant change in myocardial MDA level following 20- or 40-min of reperfusion.

3.1.3 Changes in tissue VC, VE and GSH levels in ischemic and reperfused rat hearts

Regarding the tissue ascorbic acid (VC), α-tocopherol (VE) and reduced glutathione (GSH) levels in ischemic and reperfused rat myocardium, the changes in the level of
Fig. 3.3 Changes in contractile force recovery in ischemic and reperfused rat hearts

The contractile force can serve as a functional parameter for the isolated and perfused heart. The recovery of contractile force was estimated by comparing the contractile force recorded at 20th or 40th min after reperfusion with that at 30-min of equilibration. The ratio of reperfused/equilibrated values was multiplied by 100%, and the contractile force recovery was expressed as a percentage. Each bar represents the mean ± SEM, with n=5.

* significantly different from the 60-min perf group
‡ significantly different from the 80-min perf group
* significantly different from the 20-I, 20-R group
a significantly different from the 20-I, 40-R group
Fig. 3.4 Changes in tissue MDA level in ischemic and reperfused rat hearts

The extent of myocardial oxidative damage was assessed by measuring the tissue MDA level. Each bar represents the mean ± SEM, with n=5.
these non-enzymatic antioxidants with respect to various IR conditions were shown in Fig. 3.5 - 3.7.

The results showed that while the increase in perfusion time in non-IR hearts did not alter tissue VC level, there was a significant depletion in tissue VC level for IR-hearts subjected to various conditions of IR (Fig. 3.5). While the 40-min (but not 20-min) period of reperfusion following 20-min of ischemia did not cause further reduction in VC level, it resulted in significant decrease in VC level in hearts subjected to a 40-min period of ischemia, when compared with that of the 20-min reperfusion. The increase in ischemic time (from 20 to 40 min) caused a significant decrease in VC level following 40-min of reperfusion, but not in the case of 20-min reperfusion. Consequently, the reduction (50%) in VC level was most significant in IR-hearts subjected to 40-min of ischemia followed by 40 min of reperfusion.

The increase in perfusion time in non-IR control hearts did not change the tissue VE level (Fig. 3.6). A 20- or 40-min period of ischemia followed by 20-min of reperfusion did not change the tissue VE level, when compared with the non-IR control (Fig. 3.6). On the other hand, myocardial VE level was significantly (17%) or substantially (15%, but not statistically significant) reduced, respectively, after 20- or 40-min of ischemia followed by 40-min of reperfusion (Fig. 3.6). There was, however, no significant difference in tissue VE level between hearts subjected to 20-min and 40-min of ischemia followed by 20- or 40-min of reperfusion.
Fig. 3.5  Changes in tissue VC level in ischemic and reperfused rat hearts

Data was expressed as mean ± SEM, with n=5.

* significantly different from the 60-min perf group

‡ significantly different from the 80-min perf group

† significantly different from the 40-I, 20-R group

a significantly different from the 20-I, 40-R group
Fig 3.6  Changes in tissue VE level in ischemic and reperfused rat hearts

Date was expressed as mean ± SEM, with n=5.

* significantly different from the 60-min perf group

# significantly different from the 20-I, 20-R group
Fig. 3.7  Changes in tissue GSH level in ischemic and reperfused rat hearts

Data was expressed as mean ± SEM, with n=5.

† significantly different from the 80-min perf group

‡ significantly different from the 40-I, 20-R group

₃ significantly different from the 20-I, 40-R group
As shown in Fig. 3.7, the increase in perfusion time did not produce any significant change in tissue GSH level in non-IR control hearts, while the 20-min of reperfusion following 20- or 40-min of ischemia slightly increased the myocardial GSH level, a 40-min reperfusion following a 40-min of ischemia caused a significant decrease (16%) in GSH level, when compared with that of the respective non-IR control (i.e. 80-min perf.). The increase in ischemic time (from 20 to 40 min) caused a significant decreased in GSH level only in heart subjected to 40-min of reperfusion.

### 3.2 Schisandrin B and Lipoic Acid Pretreatment

Previous studies in Dr. Robert Ko’s laboratory have demonstrated that isolated hearts prepared from Sch B pretreated rats afforded a dose-dependent protection against tissue damage induced by 10-min of ischemia followed by 15-min of reperfusion. The myocardial protection was associated with an enhancement of myocardial enzymatic antioxidants, namely glutathione reductase, glutathione peroxidase and glutathione transferases activities. The cardioprotective action produced by Sch B pretreatment against IR challenge was found to be optimal in hearts isolated from rats following a 3-day pretreatment with Sch B at a daily dose of 1.2 mmol/kg (Yim et al., 1999). The same regimen of Sch B pretreatment was used in the present study, which focused on examining the changes in the level of non-enzymatic antioxidants, namely VC, VE and GSH, in hearts subjected to increasing periods of ischemia and reperfusion.

In addition, α-lipoic acid (LA), an essential cofactor in mitochondrial dehydrogenases found to possess antioxidant properties, was also investigated as positive control.
3.2.1 Effect of Sch B and LA pretreatment on IR-induced LDH leakage and contractile force recovery in isolated rat hearts

As shown in Fig. 3.8, while isolated hearts prepared from rats pretreated with Sch B at a daily dose of 1.2 mmol/kg for 3 days did not show any changes in the extent of LDH leakage with the increase in perfusion time, varying extents of protection against myocardial damage induced by various IR conditions were observed. When hearts isolated from Sch B pretreated animals were reperfused for 20 min following a 20-min period of ischemia, the extent of LDH leakage was significantly reduced by 49%, when compared with the unpretreated IR control. However, the Sch B pretreated hearts were subjected to a 40-min period of reperfusion following 20-min of ischemia, only a small protection against IR injury was observed, as indicated by the slight and insignificant reduction in the extent of LDH leakage. Sch B pretreatment also protected the heart against the IR injury induced by 40-min of ischemia followed by 20-min of reperfusion, but did not produce any protective effect when the reperfusion time was increased to 40 min.

LA pretreatment at a daily dose of 1.2 mmol/kg for 3 days did not affect the extent of LDH leakage in non-IR hearts. While LA pretreatment did not cause significant protection against IR injury induced by 20-min of ischemia followed by 20-min of reperfusion, it was able to protect against myocardial damage induced by 20- or 40-min of ischemia followed by 40-min of reperfusion. A more potent protective action was produced in LA pretreatment, as compared with the Sch B pretreated, in hearts subjected to 40-min of ischemia followed by 20-min of reperfusion, with the exent of

69
Fig 3.8  Effect of Sch B and LA pretreatment on IR-induced LDH leakage in ischemic and reperfused rat hearts

The number in parenthesis represents the extent of IR-induced damage of the IR group with the base-line value (AUC of the respective non-IR (perf) group) being subtracted. Each bar represents the mean ± SEM, with n=5.

* significantly different from the control 60-min perf group

† significantly different from the control 80-min perf group

# significantly different from the control 20-I, 20-R group

‡ significantly different from the control 20-I, 40-R group

†† significantly different from the control 40-I, 20-R group

* significantly different from the control 40-I, 40-R group

† a significantly different from the Sch B 40-I, 20-R group

70
LDH leakage being reduced by 67%, when compared with the untreated IR control (Fig. 3.8).

As regards the contractile force recovery, Sch B pretreatment did not produce any effect in non-IR hearts as well as hearts subjected to 20-min of ischemia followed by 20- or 40-min of reperfusion (Fig. 3.9). When subjected to 40-min of ischemia, the extent of contractile force recovery of Sch B pretreated hearts was significantly enhanced (25%) following the 40-min of reperfusion, when compared with the non-pretreated control.

Hearts pretreated with LA did not show any detectable changes in contractile force recovery, with or without the IR challenge, when compared with the untreated controls (Fig. 3.9).

3.2.2 Effect of Sch B and LA pretreatment on tissue MDA level in the ischemic-reperfused hearts

Both Sch B and LA pretreatment significantly decreased the tissue MDA level in non-IR perfused hearts.

While Sch B pretreatment did not produce any effect on the tissue MDA level in hearts subject to either 20- or 40-min of ischemia followed by 20-min of reperfusion, it significantly reduced (24 and 16%, respectively) tissue MDA level in ischemic hearts following 40-min of reperfusion (Fig. 3.10), when compared with the untreated IR controls.
Fig. 3.9 Effect of Sch B and LA pretreatment on the contractile force recovery in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

* significantly different from the control 60-min perf group

* significantly different from the control 80-min perf group

* significantly different from the control 20-I, 20-R group

* significantly different from the control 20-I, 40-R group

* significantly different from the control 40-I, 40-R group
Fig. 3.10  Effect of Sch B and LA pretreatment on tissue MDA level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

*significantly different from the respective unpretreated control group
In contrast, LA pretreatment significantly reduced the tissue MDA level (14%) in hearts subjected to 40-min of ischemia followed by 20-min of reperfusion, when compared with the untreated IR controls. Reperfusing LA pretreated hearts for 40 min following 20- or 40-min of ischemia did not show any detectable change in tissue MDA level.

3.2.3 Effect of Sch B and LA pretreatment on tissue non-enzymatic antioxidant levels in the ischemic-reperfused hearts

3.2.3.1 Tissue VC level

While Sch B pretreatment did not alter tissue VC level in non-IR perfused hearts, it significantly increased the tissue VC level by 19% in hearts subjected to 20-min of ischemia followed by 20-min of reperfusion, when compared with the untreated IR control (Fig. 3.11). However, when the reperfusion time was extended to 40 min, no detectable difference in tissue VC level were observed when compared to the untreated IR control. Sch B pretreatment did not produce any effect on tissue VC level in hearts subjected to 40-min of ischemia followed by 20-min of reperfusion. But as the reperfusion time was increased to 40-min, tissue VC level of Sch B pretreated hearts was increased by 20%, when compared with the untreated control.

LA pretreated hearts showed a slight (8%) but significant decrease in tissue VC level after 80-min perfusion, when compared with the untreated control (Fig. 3.11). In contrast, LA pretreated hearts showed significantly higher level of tissue VC under all IR conditions when compared with the respective untreated control. The effect
Fig. 3.11  Effect of Sch B and LA pretreatment on tissue VC level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

- significantly different from the control 60-min perf group
- significantly different from the control 80-min perf group
- significantly different from the control 20-I, 20-R group
† significantly different from the control 20-I, 40-R group
✛ significantly different from the control 40-I, 20-R group
* significantly different from the control 40-I, 40-R group
produced by LA pretreatment on tissue VC level was more potent than that of Sch B, with a maximal enhancement of 73% following 40-min of ischemia and a subsequent 40-min of reperfusion, when compared with the untreated control.

3.2.3.2 Tissue VE level

Both Sch B and LA pretreatment did not alter tissue VE level in non-IR perfused hearts.

Sch B pretreatment did not produce any changes in tissue VE level in hearts subjected to either 20- or 40-min of ischemia followed by 20-min of reperfusion (Fig. 3.12). However, when the reperfusion period was prolonged to 40 min, the Sch B pretreated hearts showed a significantly higher level of tissue VE following the IR challenge, with the values being 61 and 49%, respectively, higher than the untreated control after 20- and 40-min of ischemia followed by reperfusion.

LA pretreatment significantly increased (47%) tissue VE level in hearts subjected to 40-min of ischemia followed by 40-min of reperfusion, when compared with the untreated control. No detectable changes in tissue VE level were observed in LA pretreated hearts subjected to 20- or 40-min of ischemia followed by 20-min of reperfusion or 20-min of ischemia followed by 40-min of reperfusion.
Fig. 3.12  Effect of Sch B and LA pretreatment on tissue VE level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

* significantly different from the control 60-min perf group

# significantly different from the control 20-I, 20-R group

† significantly different from the control 20-I, 40-R group

* significantly different from the control 40-I, 40-R group
3.2.3.3 Tissue GSH level

Both Sch B and LA pretreatment slightly but significantly reduced tissue GSH level in 80-min perfused hearts, when compared with the untreated control (Fig. 3.13). There were no significant changes detectable in tissue GSH level in Sch B pretreated hearts under various IR conditions, when compared with the respective untreated control (Fig. 3.13).

On the other hand, LA pretreated hearts subjected to 20-min ischemia followed by 40-min of reperfusion or 40-min of ischemia followed by 20-min of reperfusion showed a significantly lower level of tissue GSH when compared with the respective untreated control.

3.3 Ischemia-Reperfusion Injury in Isolated Hearts from STZ-Induced Short-Term Diabetic Rat

3.3.1 Changes in the extent of LDH leakage and contractile force recovery in diabetic hearts subjected to increasing periods of ischemia and reperfusion

While the diabetic state did not alter the extent of LDH leakage in perfused (non-IR) hearts, it significantly reduced the extent of LDH leakage by 41 and 65%, respectively, after 20- and 40-min ischemia followed by 20-min of reperfusion (see Fig. 3.14). However, the extent of LDH leakage in diabetic heart became significantly greater
Fig. 3.13  Effect of Sch B and LA pretreatment on tissue GSH level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

* significantly different from the control 80-min perf group

† significantly different from the control 20-I, 40-R group

‡ significantly different from the control 40-I, 20-R group
**Fig. 3.14 Effect of diabetes on IR-induced LDH leakage in ischemic and reperfused rat hearts**

The number in parenthesis represents the extent of IR-induced damage of the IR group with the base-line value (AUC of the respective non-IR (perf) group) being subtracted. Each bar represents the mean ± SEM, with n=5.

- **x** significantly different from the non-dia control 60-min perf group
- **γ** significantly different from the non-dia control 80-min perf group
- **#** significantly different from the non-dia control 20-I, 20-R group
- **†** significantly different from the non-dia control 20-I, 40-R group
- **‡** significantly different from the non-dia control 40-I, 20-R group
than that of the non-diabetic heart by 11% following 40-min of ischemia and a subsequent 40-min of reperfusion.

The diabetic state did not alter the extent of contractile force recovery in perfused hearts as well as hearts subjected to 20- or 40-min of ischemia followed by 20-min of reperfusion (Fig. 3.15). However, when subjected to 20- or 40-min of ischemia followed by 40-min of reperfusion, significant decreases (14 and 51%, respectively) in the extent of contractile force recovery were observed in diabetic hearts, when compared with the non-diabetic control.

3.3.2 Changes in tissue MDA level in diabetic hearts subjected to increasing periods of ischemia and reperfusion

The increase in perfusion time from 60 to 80 min in diabetic hearts caused a significant decrease (20%) in tissue MDA level, when compared with the non-diabetic control (Fig. 3.16). Diabetic hearts subjected to 20-min of ischemia followed by 20-or 40-min of reperfusion showed a decrease (33 and 18%, respectively) in tissue MDA level. No significant changes in tissue MDA level were detected in hearts subjected to 40-min of ischemia followed by 20- or 40-min of reperfusion. Tissue MDA level in diabetic hearts was significantly lower (30 and 33%, respectively) than that of the non-diabetic control following 20-min of ischemia and a subsequent 20- and 40-min reperfusion. However, the diabetic state did not produce any change in tissue MDA level in hearts subjected to 40-min of ischemia followed by 20- or 40-min of reperfusion, when compared with the respective non-diabetic control.
Fig. 3.15  Effect of diabetes on the contractile force recovery in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

* significantly different from the non-dia control 60-min perf group

† significantly different from the non-dia control 80-min perf group

‡ significantly different from the non-dia control 20-I, 20-R group

§ significantly different from the non-dia control 20-I, 40-R group

* significantly different from the non-dia control 40-I, 40-R group
Fig. 3.16  Effect of diabetes on tissue MDA level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

\(^\gamma\) significantly different from the non-dia control 80-min perf group
\(^\#\) significantly different from the non-dia control 20-I, 20-R group
\(^\dagger\) significantly different from the non-dia control 20-I, 40-R group
\(^a\) significantly different from the dia 60-min perf group
3.3.3 Changes in tissue VC, VE and GSH levels in diabetic heart subjected to increasing periods of ischemia and reperfusion

3.3.3.1 Tissue VC level

Hearts isolated from diabetic animals showed a significantly higher (22%) tissue VC level after 60-min perfusion, when compared with the non-diabetic control (Fig. 3.17). Myocardial VC level was significantly depleted under all IR conditions in diabetic hearts. After 20-min of ischemia followed by 20-min of reperfusion, no significant difference was observed in tissue VC level between diabetic and non-diabetic hearts. However, VC level in diabetic heart became significantly higher (45%) than that of the non-diabetic control when the reperfusion period was extended to 40 min. After 40-min of ischemia followed by 20- and 40-min of reperfusion, tissue VC levels in diabetic hearts were observed to be 33 and 39%, respectively, higher than that of the non-diabetic control.

3.3.3.2 Tissue VE level

The diabetic state did not alter tissue VE level in perfused hearts (Fig. 3.18). While diabetic hearts subjected to 20-min of ischemia followed by 20-min of reperfusion showed a significant increase (45%) in tissue VE level when compared with the diabetic non-IR control, a 40-min of ischemia followed by 20-min of reperfusion also caused an increase (31%, but not statistically significant) in myocardial VE level. When the diabetic hearts were subjected to 20- and 40-min of ischemia followed by 20-min of reperfusion, tissue VE level was significantly higher (42 and 69%, respectively) than that of the
Fig. 3.17  Effect of diabetes on tissue VC level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

* significantly different from the non-dia control 60-min perf group
† significantly different from the non-dia control 20-I, 40-R group
‡ significantly different from the non-dia control 40-I, 20-R group
* significantly different from the non-dia control 40-I, 40-R group
a significantly different from the dia 60-min perf group
b significantly different from the dia 80-min perf group
c significantly different from the dia 20-I, 20-R group
e significantly different from the dia 40-I, 40-R group
Fig. 3.18  Effect of diabetes on tissue VE level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

x significantly different from the non-dia control 60-min perf group

a significantly different from the non-dia control 20-I, 20-R group

† significantly different from the non-dia control 40-I, 20-R group

a significantly different from the dia 60-min perf group
respective non-diabetic control (Fig. 3.18). Following 20- or 40-min of ischemia and a subsequent 40-min of reperfusion, tissue VE level in diabetic hearts did not differ significantly with that of the respective non-diabetic control.

3.3.3.3 Tissue GSH level

The diabetic state did not alter tissue GSH level in perfused hearts (Fig. 3.19). While diabetic hearts subjected to 20-min of ischemia followed by 20-min of reperfusion showed a significant increase (13%) in tissue GSH level, a 40-min of ischemia followed by 40-min of reperfusion significantly reduced myocardial GSH level by 13%, when compared with the diabetic non-IR control. When the diabetic hearts were subjected to 20-min of ischemia followed by 20-min of reperfusion, tissue GSH level was significantly higher (17%) than that of the non-diabetic control. However, tissue GSH level in diabetic hearts became significantly lower (18%) than that of the non-diabetic control when the reperfusion period was extended to 40 min (Fig. 3.19). No significant differences in tissue GSH level were observed between diabetic and non-diabetic hearts subjected to 40-min of ischemia followed by 20- and 40-min of reperfusion.

3.4 Effects of Insulin and Sch B Pretreatment on IR Injury in STZ-Induced Short-term Diabetic Hearts

In this part of study, isolated hearts were subjected to a 20-min period of ischemia followed by 20-min of reperfusion. Diabetic animals were pretreated with either or
Fig. 3.19  Effect of diabetes on tissue GSH level in ischemic and reperfused rat hearts

Each bar represents the mean ± SEM, with n=5.

\( ^\gamma \) significantly different from the non-dia control 80-min perf group

\( ^a \) significantly different from the non-dia control 20-I, 20-R group

\( ^\dagger \) significantly different from the non-dia control 20-I, 40-R group

\( ^\ddagger \) significantly different from the non-dia control 40-I, 20-R group

\( ^a \) significantly different from the dia 60-min perf group

\( ^b \) significantly different from the dia 80-min perf group

\( ^c \) significantly different from the dia 20-I, 20-R group
both insulin and Sch B, prior to the subjection of the isolated hearts to IR challenge. Diabetic animals received twice-daily intramuscular injections of insulin treatment, which began on day 3 (with the day of STZ injection assigned as day 0) and continued until the night before the IR challenge (day 13). Sch B pretreatment (1.2 mmol/kg, suspended in olive oil) was orally given on day 10 for 3 consecutive days.

3.4.1 Effect of insulin and Sch B pretreatment on IR-induced LDH leakage and contractile force recovery

Following a 20-min of ischemia and a subsequent 20-min of reperfusion, the extent of LDH leakage in diabetic hearts was shown to be significantly lower than that of the non-diabetic control by 44% (see Fig. 3.14). Diabetic heart pretreated with insulin resulted in a significant increase in the extent of LDH leakage by 1-fold, when compared with the diabetic control. However, no significant changes in the extent of LDH leakage were found in diabetic hearts pretreated either with Sch B per se or both insulin and Sch B, when compared with the respective unpretreated control (Table 3.1).

As shown in Table 3.1, insulin treatment did not change the extent of contractile force recovery in the ischemic-reperfused diabetic hearts. A slight but significant decrease (11%) in the extent of contractile force recovery was observed in Sch B pretreated diabetic hearts, when compared with the unpretreated control. Similarly, Sch B pretreatment also significantly decreased (16%) the extent of contractile force recovery in insulin-treated diabetic hearts, when compared with the unpretreated control.
Table 3.1  Effect of insulin and Sch B pretreatment on the extent of LDH leakage, contractile force recovery and tissue MDA level in ischemic and reperfused diabetic rat hearts

<table>
<thead>
<tr>
<th></th>
<th>LDH (AUC - AUC_{pre})</th>
<th>% Contractile force recovery</th>
<th>MDA (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dia control</strong></td>
<td>570 ± 28</td>
<td>89 ± 1</td>
<td>28.0 ± 1.0</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dia + Ins</strong></td>
<td>1283 ± 68 †</td>
<td>85 ± 2</td>
<td>36.2 ± 1.2 †</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dia + Sch B</strong></td>
<td>863 ± 66</td>
<td>79 ± 1 †</td>
<td>38.3 ± 2.1 †</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dia + Ins + Sch B</strong></td>
<td>900 ± 92</td>
<td>71 ± 1 ††#</td>
<td>19.7 ± 1.3 ††#</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolated hearts were subjected to a 20-min period of ischemia followed by 20-min of reperfusion. Diabetic animals were pretreated with either or both insulin and Sch B prior to the IR challenge. Insulin treatment was begun on day 3 (with the day of STZ injection assigned as day 0) until the night before the IR challenge (day 13). Sch B pretreatment (1.2 mmol/kg, suspended in olive oil) was orally given on day 10 for 3 consecutive days. Each value represents the mean ± SEM, with the number of animals indicated in the parentheses.

† significantly different from the dia group
‡ significantly different from the dia + Ins group
# significantly different from the dia + Sch B group
3.4.2 Effect of insulin and Sch B pretreatment on tissue MDA level in ischemic-reperfused diabetic hearts

Insulin treatment significantly increased (29%) tissue MDA level in the ischemic-reperfused diabetic hearts (Table 3.1). Sch B pretreatment also caused a similar increase (37%) in tissue MDA level, when compared with the untreated control. On the other hand, the combined pretreatment with Sch B and insulin caused a significant decrease (30%) in tissue MDA level in the ischemic-reperfused hearts, when compared with the untreated control.

3.4.3 Effect of insulin and Sch B pretreatment on tissue VC, VE and GSH levels in ischemic-reperfused diabetic rat hearts

As shown in Table 3.2, insulin pretreatment resulted in a significant increase (17%) in tissue VC level in the ischemic-reperfused diabetic hearts, when compared with the untreated control. While Sch B pretreatment per se did not cause any significant change in tissue VC level, diabetic hearts obtained from animals pretreated with both insulin and Sch B resulted in a further increase (32%) in tissue VC level, when compared with the untreated control.

Tissue VE level in diabetic and ischemic-reperfused hearts was significantly decreased by 33 and 13%, respectively, by insulin and Sch B pretreatment (Table 3.2). A slight but insignificant decrease in tissue VE level was observed after IR in diabetic hearts pretreated with both insulin and Sch B, when compared with the untreated control.
<table>
<thead>
<tr>
<th></th>
<th>VC (μg/g tissue)</th>
<th>VE (μg/g tissue)</th>
<th>GSH (nmol/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dia control</strong> (n = 5)</td>
<td>40.6 ± 1.6</td>
<td>32.0 ± 1.5</td>
<td>1.075 ± 0.015</td>
</tr>
<tr>
<td><strong>Dia + Ins</strong> (n = 7)</td>
<td>47.4 ± 0.5 †</td>
<td>21.6 ± 1.2 †</td>
<td>1.058 ± 0.008</td>
</tr>
<tr>
<td><strong>Dia + Sch B</strong> (n = 6)</td>
<td>42.7 ± 0.7</td>
<td>27.7 ± 0.9 ‡</td>
<td>1.072 ± 0.028</td>
</tr>
<tr>
<td><strong>Dia + Ins + Sch B</strong> (n = 5)</td>
<td>53.4 ± 1.3 †† #</td>
<td>29.6 ± 1.5 #</td>
<td>1.053 ± 0.023</td>
</tr>
</tbody>
</table>

Isolated hearts were subjected to a 20-min period of ischemia followed by 20-min of reperfusion. Diabetic animals were pretreated with either or both insulin and Sch B prior to the IR challenge. Insulin treatment was begun on day 3 (with the day of STZ injection assigned as day 0) until the night before the IR challenge (day 13). Sch B pretreatment (1.2 mmol/kg, suspended in olive oil) was orally given on day 10 for 3 consecutive days. Each value represents the mean ± SEM, with the number of animals indicated in the parentheses.

† significantly different from the dia group
‡ significantly different from the dia + Ins group
# significantly different from the dia + Sch B group
However, when compared with the insulin-treated diabetic control, tissue VE level was increased by 37% in insulin and Sch B pretreated ischemic-reperfused diabetic heart.

As shown in Table 3.2, there were no detectable changes in tissue GSH level in diabetic and ischemic-reperfused hearts pretreated with either insulin and Sch B, or both.
Chapter 4

DISCUSSIONS

Previous study in Dr. Ko’s laboratory has shown that hearts isolated from rat pretreated with Schisandrin B (Sch B), a dibenzocyclooctadiene derivative isolated from the fruit of Schisandria Chinensis, showed a dose-dependent protection against ischemia-reperfusion (IR) injury in rats, with an optimum dosage being 1.2 mmol/kg/day for 3 days (Yim and Ko, 1999a). When subjected to 10-min ischemia followed by 15-min reperfusion, the myocardial protection produced by Sch B pretreatment was found to be associated with an enhancement in myocardial glutathione antioxidant status, as indicated by significant reductions in both the extent of IR-induced myocardial reduced glutathione (GSH) depletion and inhibition of glutathione peroxidase (GPX) and glutathione reductase (GRD) activities. In the present study, in order to further investigate the underlying mechanism involved in the protection of Sch B pretreatment against myocardial IR injury, isolated rat hearts were subjected to increasing periods of ischemia and reperfusion and the changes in the extent of myocardial IR injury and myocardial non-enzymatic antioxidants, namely ascorbic acid (VC), α-tocopherol (VE) and GSH, were examined in control and Sch B pretreated animals. The effects of a thiol-enhancing antioxidant, lipoic acid (LA), were also examined for comparison.

Experimental and clinical studies have shown that diabetic state can increase oxidative stress in tissues including that of the myocardium (Feuvray, 1996). The extent of myocardial IR injury and the associated alteration in tissue non-enzymatic antioxidants in
hearts isolated from streptozotocin (STZ)-induced short-term (2-week) diabetic rats were investigated, and the effects of insulin and Sch B treatment on IR injury induced in diabetic hearts were also examined.

4.1 Myocardial Injury Induced by Increasing Periods of Ischemia and Reperfusion

It is now well established that reperfusion of the previously ischemic myocardium can cause tissue injury (Alanen et al., 1980; Forman et al., 1990; Takemura et al., 1992; Kaul et al., 1993). In the present study, reperfusion following a 20-min period of isothermic global ischemia resulted in an abrupt increase in lactate dehydrogenase (LDH) leakage in isolated rat hearts during the first minute of reperfusion (Fig. 3.2). The LDH leakage, indicative of tissue damage, subsided with increasing reperfusion time, with the value approaching the control level and being level off 20 minutes after the commencement of reperfusion. In contrast, reperfusion following a 40-min period of ischemia caused a biphasic change in LDH leakage, with the value being much higher than the control level up to 40 minutes of reperfusion (Fig. 3.1b). The increase in ischemic period from 20 to 40 minutes resulted in a greater degree of myocardial damage upon reperfusion, as assessed by the extent of LDH leakage (Fig. 3.2). While the prolongation of reperfusion time up to 40 min did not increase the extent of LDH leakage in hearts subjected to 20-min of ischemia, it exaggerated the LDH leakage by 66% in hearts subjected to 40-min of ischemia (Fig. 3.2). The changes in the extent of LDH leakage under various IR conditions were paralleled by alterations in the extent of contractile force recovery
measured in the ischemic-reperfused hearts at the end of the reperfusion period. While the increased period of ischemia reduced the extent of contractile force recovery upon reperfusion, the prolonged period of reperfusion improved the contractile force recovery in hearts following 20-min of ischemia (Fig. 3.3). However, when subjected to 40-min of ischemia, the prolongation of reperfusion period did not produce any enhancing effect on the extent of contractile force recovery in ischemic-reperfused hearts. Under the present experimental conditions, the results indicated that isolated hearts subjected to a 40-min period of ischemia were inflicted with sustainable tissue damage, which was not recovered up to 40 minutes of reperfusion.

With regard to the pathogenesis of myocardial IR injury, a key event elicited by reperfusion is the re-introduction of molecular oxygen, which leads to the subsequent formation of oxygen-derived free radicals (OFR). Over the past years, experimental findings from several laboratories have unequivocally demonstrated the increase in OFR formation in the myocardium during post-ischemic reperfusion (Limm et al., 1993; Baker et al., 1988; Maupoil and Rochette, 1988). While OFR have been implicated in the pathogenesis of tissue injury in the ischemic-reperfused hearts (Bolli, 1991; Flitter, 1993; Jeroudi et al., 1994; Kukreja and Hess, 1994), they can cause oxidation of lipid molecules in cellular and subcellular membranes as well as oxidative damage in biomolecules such as proteins and nucleic acids, leading to the disruption of structural and functional integrity (Noronha-Dutra and Steen, 1982; Esterbauer et al., 1991; Ambrosio et al., 1991; Manciet and Copeland, 1992; Nagy et al., 1996). The observation of LDH leakage from the ischemic-reperfused myocardium may possibly be a result of IR-induced injury in
cellular membranes of cardiomyocytes caused by lipid peroxidation. Under normal conditions, the LDH activity in the coronary effluent of isolated perfused rat hearts should be very low due to the intracellular localization of LDH. However, when the structural integrity of cardiomyocyte membrane is destructed by the IR challenge, LDH will leak out from cardiomyocytes, resulting in the increase in LDH activity in the coronary effluent. The extent of LDH leakage is therefore conveniently used as a biochemical marker of IR injury in isolated and perfused hearts.

In addition, the OFR-induced membrane damage can cause dysfunction of sacroplasmic reticulum and the enhanced calcium influx across the sacrolemma of cardiomyocytes, leading to the excitation-contraction uncoupling of cardiac muscle and intracellular calcium overload (Kaneko et al., 1989). The measurement of impairment in contractile force in ischemic-reperfused heart serves as a functional marker for assessing the extent of IR injury. The finding of the extent of contractile force recovery in reperfused heart being primarily dependent on the duration of the preceding ischemia is consistent with the reports by Manche et al. (1995) and Haramaki et al. (1998). In the present study, a longer ischemic period (i.e. 40 min) resulted in an incomplete recovery of contractile force upon reperfusion up to 40 minutes. On the contrary, reperfusing of hearts subjected to a 20-min period of ischemia for 40 minutes resulted in an almost complete recovery of contractile force (Fig. 3.3).

The level of malondialdehyde (MDA), one of the end-products of lipid peroxidation, was measured in myocardial tissue as an indirect index of oxidative tissue damage. However,
no detectable changes in myocardial MDA level were found under all IR conditions examined in the present study. Neither the extent of LDH leakage nor the extent of contractile force recovery correlated with the level of tissue MDA in ischemic-reperfused hearts. It has been reported that when isolated-perfused hearts exposed to exogenously generated free radicals or subjected to 60-min period of ischemia, the increase in myocardial MDA level was only found in hearts having been exposed to the most extreme oxidative challenge, i.e., higher concentration of free radical or longer duration of ischemia followed by reperfusion (Ballagi-Pordany et al., 1991). The finding of undetectable changes in tissue MDA level of ischemic-reperfused hearts may be due to the relatively short ischemic periods adopted in the present study. As a result, the limited extent of lipid peroxidation reaction triggered by OFR arising from the reperfusion of the ischemic myocardium can be effectively quenched by myocardial antioxidant mechanism. The formation of lipid peroxidation end-products (i.e. MDA) is therefore undetectable.

4.2 IR-Induced Changes in Myocardial Non-enzymatic Antioxidants

The cellular antioxidant system, which is composed of both enzymatic and non-enzymatic antioxidants, plays an important role in the protection against OFR induced oxidative tissue damage (Porreca et al., 1994). The enzymatic antioxidants consist of antioxidant enzymes including catalase, superoxide dismutase, GPX and GRD, whereas the non-enzymatic antioxidants are mainly composed of VC, VE and GSH. Since the interplay among these three non-enzymatic antioxidants in scavenging OFR plays a crucial role in antioxidant defense (Wells and Xu, 1994; Tritto et al., 1998; Gate et al.,
1999; Venditti et al., 1999), in the present study, the dynamic changes in tissue level of these antioxidants were examined in the myocardium subjected to various IR conditions. Among the three studied antioxidants, namely VC, VE and GSH, VC level was depleted by 34% following 20-min of ischemia and 20-min of reperfusion, the VE and GSH levels remained relatively unchanged (Fig. 3.5–3.7). The increased ischemic period (40-min) did not further deplete (VC) or alter (VE, GSH) myocardial antioxidant levels, but the prolonged reperfusion time (40-min) caused decreases in either VE level following 20- and 40-min of ischemia or VC and GSH levels following 40-min of ischemia. Under the experimental condition of 40-min ischemia and 40-min reperfusion in which the ischemic-reperfused myocardium sustained the highest degree of IR injury in terms of LDH leakage and contractile force recovery, the myocardial VC and GSH levels were significantly depleted by 52 and 16%, respectively. However, the depletion of myocardial VE level by 15% did not attain statistical significance when compared with the non-IR (i.e. 80-min perf.) control.

Non-enzymatic antioxidants (VC, VE, GSH) may work synergistically in cellular antioxidant defense (Wells and Xu, 1994; Haramaki et al., 1998). VC and GSH are water-soluble antioxidants that serve as the first line of defense in combating OFR (Haramaki et al., 1995; Tritto et al., 1998). In this regard, a marked depletion of myocardial VC was observed after IR challenge (Fig. 3.5). However, myocardial GSH level was only moderately depleted even under a more severe IR condition. It has been reported that GSH can facilitate the regeneration of VC form its oxidized form (Wells and Xu, 1994; Haramaki et al., 1995; Tritto et al., 1998). Under the present experimental
conditions, the burst of OFR formation induced by IR could effectively be scavenged by myocardial VC and the relatively small extent of VC depletion may not trigger the GSH-mediated regeneration of VC (Gate et al., 1999). As a result, the myocardial GSH level remained relatively unchanged until when the heart was subjected to 40-min of ischemia followed by 40-min of reperfusion. The further decrease in myocardial VC level was accompanied by a significant depletion in myocardial GSH that may be utilized for the regeneration of VC. It has been reported that leakage of tissue VC (Pietri et al., 1990; Sharm et al., 1994) and GSH (Lesnfsky et al., 1989; Rigobello and Bindoli, 1993) into the coronary effluent occurred during the post-ischemic reperfusion of the myocardium. The observation of only VC, but not GSH, depletion was not consistent with the postulation that the decrease in VC level was caused by leakage from damaged cellular membranes of the ischemic-reperfused myocardium. However, VC and GSH may preferentially be transported across cellular membranes in their oxidized forms into the coronary effluent, thereby resulting in the depletion of these antioxidants (Haramaki et al., 1999).

VE is a lipophilic antioxidant responsible for terminating the lipid peroxidation reactions occurring in cell membranes (Campo et al., 1998; Venditti et al., 1999). Unlike VC, myocardial VE level remained relatively unchanged under various IR conditions except in the case of 20-min of ischemia followed by 40-min of reperfusion (Fig. 3.6). While the mechanism involved in the protracted reperfusion time course associated depletion of tissue VE in ischemic hearts remained to be elucidated, the relatively stability of myocardial VE against IR-induced depletion may be related to the finding that VC, even
at a low concentration in the myocardium, could recycle VE (Packer et al., 1979) and that GSH prevented the loss of VE under oxidative stress conditions in vitro (Leedle and Aust, 1990). The observation that more than 50% of myocardial VC and GSH being remained following 40-min of ischemia and 40-min of reperfusion supports the possible redox cycling of VE by VC or GSH. In addition to myocardial tissue (Serbinova et al., 1992; Venditti et al., 1999), the redox cycling of VE has been demonstrated in several in vitro systems, including human low density lipoproteins (Kagan et al., 1992), mitochondrial and microsomal membranes (Kagan et al., 1990a and 1990b) and human erythrocyte membranes (Constantinescu et al., 1993). The ability of the ischemic-reperfused myocardium to maintain tissue VE level is consistent with the finding of undetectable changed in myocardial MDA level under all IR conditions examined in the present study (Fig. 3.4).

4.3 Myocardial Protection against IR Injury by Schisandrin B and Lipoic Acid Pretreatment

Previous studies have demonstrated the cardioprotection afforded by Sch B pretreatment against IR injury induced by 10-min of ischemia followed by 15-min of reperfusion in isolated rat hearts (Yim and Ko, 1999a and 1999b). Using the same treatment regimen in the present study, Sch B and LA were found to protect against myocardial IR injury induced by longer periods of ischemia (20–40-min) and reperfusion (20–40-min), to varying degrees, as assessed by measuring the extents of LDH leakage and contractile force recovery. Despite the observation that Sch B pretreatment produced a better
cardioprotective effect in terms of the reduction in LDH leakage under the condition of 20-min ischemia followed by 20-min of reperfusion, LA pretreatment produced a more sustainable protective action, particularly under conditions of protracted time course of ischemia and reperfusion (Fig. 3.8). LA, but not Sch B, pretreatment could significantly reduce the extent of LDH leakage in hearts subjected to 40-min of ischemia followed by 40-min of reperfusion. As regards the extent of contractile force recovery, the Sch B pretreated and ischemic-reperfused hearts consistently outperformed those of the LA pretreated hearts, with the extent of recovery being increased by 25% when compared with the untreated or LA pretreated hearts following 40-min of ischemia and 40-min of reperfusion (Fig. 3.9). The beneficial effect of Sch B pretreatment on the IR-induced impairment in contractility suggests the possible action of Sch B in restoring the calcium homeostasis in ischemic-reperfused cardiomyocytes.

Under the condition of oxygenated perfusion, the Sch B and LA pretreated hearts had a significantly lower level of tissue MDA (Fig. 3.10). As similar to the case of untreated hearts, there were no detectable changes in myocardial MDA level upon various IR challenges in either Sch B or LA pretreated hearts when compared with the non-IR control. However, tissue MDA level is, in general, lower in the drug pretreated hearts, when compared with the untreated control, even after IR challenges. The result suggests that Sch B and LA pretreatment may suppress the lipid peroxidation reactions in myocardial tissue, possibly through the enhancement of myocardial antioxidant status. In this regard, the significant decreases in tissue MDA level in Sch B pretreated hearts subjected to 20- or 40-min of ischemia followed by 40-min of
reperfusion were found to be associated with significant elevations in myocardial VE level (Fig. 3.10 and 3.12). However, the significant increases in tissue VC level in LA pretreated and ischemic-reperfused hearts were not paralleled by any apparent decrease in myocardial MDA level, except in the case of 40-min ischemia followed by 20-min reperfusion (Fig. 3.10 and 3.11).

While tissue VC level was depleted by IR challenges to varying extents in Sch B pretreated hearts, it remained more or less the same as the pre-ischemic value in LA pretreated hearts (Fig. 3.11). As a result, all LA pretreated and ischemic-reperfused hearts had myocardial VC levels significantly higher than those of the untreated control. The higher level of tissue VC level in LA pretreated and ischemic-reperfused hearts were associated with a smaller extent of LDH leakage, an indication of myocardial protection against IR injury (Fig. 3.8 and 3.11). The elevation in tissue VC level was only observed in Sch B pretreated hearts subjected to 20-min of ischemia followed by 20-min of reperfusion, when compared with the untreated control (Fig. 3.11). Although the increased period of post-ischemic reperfusion tended to decrease tissue VE level in control hearts, it significantly increased tissue VE level in Sch B pretreated hearts (Fig. 3.12). But the LA pretreatment did not produce any apparent effect in this regard. Following 40-min of ischemia and 40-min of reperfusion, both Sch B and LA pretreated hearts possessed a significantly higher (21 and 26%, respectively) level of tissue VE (Fig. 3.12). However, only the LA pretreatment could reduce the extent of IR-induced LDH leakage (Fig. 3.8). Instead, the Sch B pretreated hearts showed a better contractile force recovery 40 minutes after post-ischemic reperfusion (Fig. 3.9). Both Sch B and LA
pretreatment decreased myocardial GSH level in non-IR (i.e. 80-min perf.) hearts, but in contrast to the unpretreated control, the IR challenge (40-min of ischemia followed by 40-min of reperfusion) did not deplete the tissue GSH in Sch B and LA pretreated hearts (Fig. 3.13). The relatively stability of myocardial GSH upon IR-induced depletion was accompanied by protection against IR injury in Sch B and LA pretreated hearts, as assessed by the extent of contractile force recovery and LDH leakage, respectively. Interestingly, while myocardial GSH levels of control and Sch B pretreated hearts was maintained at similar levels under various IR conditions, LA pretreatment tended to result in a lower myocardial GSH level. The latter may be related to the possible effect of the thiol-containing LA on the feedback mechanism involved in the regulation of cellular GSH level.

4.4 Comparison Between Cardioprotective Effect of Sch B and LA Against IR Injury

The ensemble of results indicates that both Sch B and LA pretreatment could suppress the IR-induced leakage of LDH from myocardial tissue, with the effect of LA being more potent. On the other hand, while Sch B pretreatment could improve the extent of contractile force recovery of the ischemic-reperfused myocardium, LA pretreatment did not seem to produce any effect. Under the present experimental conditions, the cardioprotection afforded by Sch B pretreatment was associated with increases in myocardial VC and VE levels, with the effect on VE being more prominent. The cardioprotective action of LA pretreatment was also found to be associated with
elevations in myocardial VC and VE levels, but the effect on tissue VC level appeared to be much more dramatic. Given the involvement of OFR in the pathogenesis of myocardial IR injury (Alanen et al., 1980; Forman et al., 1990; Takemura et al., 1992; Kaul et al., 1993), the increased levels of non-enzymatic antioxidants, VC and VE, can scavenge the over-produced OFR, thereby protecting the myocardium against oxidative tissue damage and functional impairment. Using isolated-perfused rat hearts, previous studies have shown that the cardioprotection afforded by Sch B pretreatment against IR injury with a shorter reperfusion time course (15-min) was associated with increases in myocardial GSH level and activities of GPX and GRD (Yim and Ko, 1999a). In the present study, a time-dependent increase in myocardial VE level was observed in post-ischemic myocardium when a protracted reperfusion time course (20 to 40 min) was adopted. However, no detectable change in myocardial GSH level was observed. Since GSH can increase tissue VE level by enhancing the redox recycling of VE via the direct reduction of α-tocopheroxyl radical (Chan et al., 1991), the ability of Sch B pretreatment to increase myocardial VE level under oxidative stress condition induced by IR may therefore be an event secondary to the enhanced myocardial glutathione redox status. The possible regeneration of VE at the expense of GSH may result in the undetectable change in tissue GSH level in Sch B pretreated and ischemic-reperfused myocardium.

LA is a thiol-containing antioxidant both in vitro and in vivo (Packer et al., 1995). LA treatment was found to be protective against experimentally induced IR injury in the brain (Cao and Phillis, 1995; Panigraphi et al., 1996; Wolz and Kneiglsttein, 1996) and the heart (Serbinova et al., 1992; Schouheit et al., 1995), as well as experimentally
induced diabetic neuropathy (Naganatsu et al., 1995; Packer et al., 1997; Cameron et al., 1998), all of which the pathogenesis of tissue damage involves the increase in tissue oxidative stress. While the molecular mechanism involved in the enhancement of myocardial VC level by LA pretreatment in ischemic-reperfused myocardium remains to be determined, it is possible that LA can increase tissue VC level by facilitating the regeneration of VC via a thiol-mediated reductive reaction (Wells et al., 1994). In this regard, it has been reported that GSH can facilitate the regeneration of VC from its oxidized form (Wells et al., 1990). VC, in addition to being OFR scavenger, can regenerate VE via reductive action (Gate et al., 1999). The increased tissue VE level in LA pretreated and ischemic-reperfused hearts (Fig. 3.2), which probably results from the enhancement of tissue VC redox status, may at least in part contribute to the protection against IR-induced oxidative tissue damage.

In summary, the cardioprotection afforded by Sch B and LA pretreatment against IR injury may at least in part be mediated by facilitating the interplay among myocardial non-enzymatic antioxidants. The resultant enhancement in tissue antioxidant status in the drug pretreated hearts is reflected by the lower tissue MDA level under non-IR condition as well as the protection against IR injury. While the mechanisms underlying the differential ability between Sch B and LA in suppressing the extent of LDH leakage and improving contractile force recovery in ischemic-reperfused myocardium remain to be elucidated, they are likely related to pharmacological actions other than antioxidant activity of the drugs.
4.5 Differential Response of Diabetic Heart to IR Challenge

The influence of short-term diabetes on the extent of myocardial IR injury and the IR-induced alterations in the myocardial non-enzymatic antioxidant levels were examined. When diabetic hearts were subjected to 20-min of ischemia followed by 20-min of reperfusion, the extent of LDH leakage was significantly reduced by 72% when compared with the non-diabetic heart (Fig. 3.14). The difference in the extent of LDH leakage between diabetic and non-diabetic hearts became more apparent under the condition of 40-min ischemia followed by 20-min of reperfusion. However, the protective effect observable in diabetic hearts diminished or disappeared with the increase in reperfusion time from 20 to 40 min. This was corroborated by the observation of poorer contractile force recovery in diabetic and ischemic hearts subjected to 40-min period of reperfusion when compared with the non-diabetic control (Fig. 3.15). The protection against IR injury, as assessed by the extent of LDH leakage, in diabetic hearts subjected to 20-min of ischemia followed by 20-min of reperfusion was associated with a significant decrease (33%) in myocardial MDA level when compared with the non-diabetic control (Fig. 3.16). However, no detectable change was observed when the hearts were subjected to 40-min of ischemia followed by 20-min of reperfusion, even though there was a significant reduction in the extent of LDH leakage. The decreased extent of IR-induced LDH leakage in diabetic hearts may be related to the decrease in susceptibility of cardiomyocyte membranes to lipid peroxidation. The post-ischemic reperfusion associated oxidative stress can lead to the oxidation of membrane lipids and the subsequent leakage of LDH from the damaged cellular membrane of cardiomyocytes.
The observation of decrease in MDA level (Fig. 3.16) and increase in tissue VE level (Fig. 3.18) in ischemic-reperfused diabetic hearts supports this postulation. In addition, the possible alteration in membrane lipid composition in diabetic tissues may be ascribable to the decreased membrane sensitivity to oxidation (Verges, 1999). As reperfusion proceeded following a prolonged period (40-min) of ischemia, the resistance of membrane lipids to oxidation would be overwhelmed by the prevailing OFR-mediated reactions (Romaschin et al., 1987), eventually leading to the increased extent of LDH leakage (Fig. 3.14).

The susceptibility of diabetic hearts to IR injury seemed to be dependent on the reperfusion time course, in which the prolonged reperfusion period revealed a greater extent of IR injury in diabetic hearts as assessed by the extent of contractile force recovery. However, a shorter period of post-ischemic reperfusion showed a smaller extent of LDH leakage in diabetic hearts under the present experimental conditions. Results obtained from other studies regarding the susceptibility of diabetic hearts to IR injury have been inconsistent. While diabetic hearts were found to be more sensitive than the non-diabetic counterpart to anoxic or ischemic injury (Regan et al., 1974 and 1981; Fein et al., 1980), it has also been reported that diabetic hearts showed a greater resistance to IR injury regardless of the abnormalities in cardiac function (Tani and Neely, 1988).

While whether or not the diabetic heart is more susceptible to IR injury remains to be determined, the finding of differential response of diabetic hearts to IR challenge, as
reported by the present study, may be related to the diabetes associated metabolic changes in the myocardium. In this regard, the increase in reliance on fatty acids as energy substrate is believed to be an important contributing factor to the development of biochemical changes in diabetic hearts (Lopaschuk, 1989). The accumulation of fatty acids in myocardial tissue could inhibit the overall glucose utilization (Nicholl et al., 1991). This postulation is strengthened by the observation that pharmacological interventions aimed at overcoming the inhibition of glucose oxidation by fatty acids in acute diabetic rats was found to be beneficial in ameliorating contractile failure caused by mild myocardial ischemia (Hekimian and Feuvray, 1986; Lopaschuk and Spafford, 1989).

Under the present experimental conditions, the inability of diabetic hearts to utilize glucose for energy metabolism during post-ischemic reperfusion may at least in part contribute to the marked decrease in the extent of contractile force recovery. In addition, the increased level of acyl derivatives of long-chain fatty acids in diabetic hearts may facilitate their incorporation into cellular membranes, leading to functional perturbation on the membrane proteins (Knabb et al., 1986). It has been reported that microsomes of sarcoplasmic reticulum prepared from diabetic rat hearts showed a depressed ATP-dependent calcium transport activity (Lopaschuk et al., 1983). Results obtained from a recent study indicated that long-chain acyl carnitine could elevate intracellular calcium concentration in a dose-dependent manner, in which the increase in calcium concentration induced by acyl carnitine was dependent on both calcium influx from the extracellular space and calcium release from the sarcoplasmic reticulum stores (Netticadan et al., 1999). In this regard, the IR-induced calcium overload may be aggravated in diabetic hearts, leading to the exaggeration of contractile force impairment.
Despite the higher tissue level of VC in non-IR and IR diabetic hearts than the respective non-diabetic control, IR caused depletion of myocardial VC in both diabetic and non-diabetic hearts (Fig. 3.17). Prolonged reperfusion of post-ischemic (20-min of ischemia) diabetic hearts, as in contrast to the non-diabetic control, could increase myocardial VC level. On the other hand, while myocardial VE level in diabetic hearts was similar to that of non-diabetic control under non-IR condition, reperfusion of the post-ischemic (20 or 40 min of ischemia) diabetic hearts for 20 min caused a significant increase in myocardial VE level (Fig. 3.18). However, the prolonged reperfusion (40-min), as also observed in the case of non-diabetic control, decreased myocardial VE level, with values not being significantly different from the non-diabetic control. Myocardial GSH level in diabetic hearts did not differ from that of the non-diabetic control (Fig. 3.19). Instead of showing slight and insignificant changes in tissue GSH level upon IR challenge as in the case of non-diabetic hearts, post-ischemic reperfusion of diabetic hearts for 20 min following a 20-min period of ischemia increased myocardial GSH level, with the value being significantly higher than those of the diabetic non-IR and non-diabetic IR control. Prolongation of reperfusion time, however, caused depletion in myocardial GSH level, with the value being significantly lower than that of the non-diabetic IR (20-I, 40-R) control.

Results obtained from the present study indicated the differential changes in myocardial non-enzymatic antioxidant levels upon IR challenges in control and diabetic hearts. The myocardial protection against IR injury in diabetic hearts, as assessed by the extent of
LDH leakage, was associated with increases in tissue VE and GSH/VC levels. Given that non-enzymatic antioxidants play an important role in cellular antioxidant defense (Wells and Xu, 1994; Haramaki et al., 1998), the enhanced tissue antioxidant status under oxidative stress condition in hearts obtained from short-term (2-week) diabetic rats may contribute to the protection against IR-induced injury, as indicated by the decrease in the extent of LDH leakage. It has been reported that metabolic changes occurring during the early phase of diabetes may "chemically" precondition the myocardium, rendering it more resistant to IR injury (Tosaki et al., 1996). This is consistent with the observation of enhanced antioxidant response in diabetic hearts upon relatively mild IR challenge in the present study. While the mechanism involved in the "chemical" preconditioning of diabetic heart remains unclear, it is possible that the exposure to oxidative stress arising from the diabetic state may play an important role in priming the myocardium in responding to oxidative challenge such as IR. However, prolonged exposure to metabolic changes, as in the case of chronic diabetes, could lead to the development of heart failure (Baynes and Thorpe, 1999). By the same token, exposure of diabetic (pre-conditioned) hearts to severe oxidative stress condition, as in the case of 40-min ischemia followed by 40-min of reperfusion, could overcome the preconditioning effect, leading to drastic increase in LDH leakage and impairment in contractile force.

4.6 Effect of Insulin and Sch B Pretreatment on IR Injury in Diabetic Hearts
Under the IR condition of 20-min of ischemia followed by 20-min of reperfusion, the extent of IR-induced LDH leakage and the level of myocardial MDA in diabetic hearts were significantly lower than the non-diabetic control (Fig. 3.14 and Fig. 3.16). Insulin treatment partially reversed the diabetes-associated changes in hearts subjected to IR challenge, with the extent of LDH leakage and myocardial MDA level being significantly increased when compared with the untreated diabetic control. The result indicated that the change in susceptibility of diabetic hearts to IR injury was mainly caused by the diabetic state. The increased susceptibility of insulin-treated diabetic hearts to IR injury was associated with a marked decrease in myocardial VE level and a slight but significant increase in myocardial VC level (Table 3.2). The negative correlation between VE level and sensitivity to IR injury of diabetic hearts supports the important role of myocardial VE in the antioxidant defense against IR-induced oxidative stress. This is also consistent with the finding in non-diabetic hearts that myocardial VE seemed to be the ultimate antioxidant defense against IR-induced oxidative stress.

Unlike the case of non-diabetic hearts, Sch B pretreatment could not protect against IR injury induced in diabetic hearts, as assessed by the extent of LDH leakage. Instead, the extent of contractile force recovery was significantly reduced in the Sch B pretreated and ischemic-reperfused diabetic hearts (Table 3.1). As similar to the case of insulin treatment, Sch B pretreatment could also partially reverse the diabetes associated decrease in myocardial MDA level and increase in myocardial VE level, but to a lesser extent than that of insulin treatment. The inability of Sch B pretreatment to protect against IR injury in diabetic hearts suggests the involvement of a common antioxidant
mechanism in the cardioprotection afforded by Sch B and the diabetic state. This may possibly be related to the enhancement of interplay among non-enzymatic antioxidants in combating OFR.

Sch B pretreatment could also reduce the extent of IR-induced LDH leakage in insulin-treated diabetic hearts although the extent of reduction did not reach statistical significance when compared with the insulin-treated diabetic control (Table 3.1). However, the extent of contractile force recovery in Sch B pretreated hearts was found to be significantly smaller than that of the insulin-treated diabetic control. Under the condition of diabetes with insulin treatment, the myocardial protection afforded by Sch B pretreatment was associated with a marked decrease in myocardial MDA level as well as significant increases in myocardial VE and VC levels, when compared with the insulin-treated diabetic control (Table 3.1 and 3.2). The result suggests that Sch B pretreatment may produce beneficial effect on myocardial non-enzymatic antioxidants in insulin-treated diabetic rats under oxidative stress condition. The striking difference between control and diabetic hearts with regard to the ability of Sch B pretreatment in modulating the post-ischemic contractile force recovery remains to be further investigated.

4.7 Conclusions

Isolated rat hearts were subjected to increasing periods of ischemia and reperfusion for examining the changes in the extent of myocardial IR injury and the level of myocardial non-enzymatic antioxidants (VC, VE and GSH). Sustainable tissue damage was inflicted
in hearts subjected to prolonged period (40 min) of ischemia followed by reperfusion, as indicated by the increased extent of LDH leakage and impairment in contractile function.

While myocardial VC level was markedly depleted by IR challenge, myocardial VE and GSH levels remained relatively unchanged except under a more severe IR condition in which slight decreases both antioxidants were observed. Under IR condition, myocardial VC appeared to be the first line of defense against oxidative stress, whereas myocardial VE served as the ultimate antioxidant defense.

Sch B and LA pretreatment were found to protect against myocardial IR injury induced by increasing periods of ischemia and reperfusion to vary degrees. Both Sch B and LA pretreatment could suppress the IR-induced LDH leakage, with the effect of LA being more potent. While Sch B pretreatment could improve the extent of contractile force recovery of the ischemic-reperfused myocardium, LA pretreatment did not seem to produce any effect.

Under the present experimental conditions, the cardioprotection afforded by Sch B pretreatment was associated with increases in myocardial VC and VE levels, with the effect on VE being more prominent. On the other hand, the cardioprotective action of LA pretreatment was found to be associated with elevations in myocardial VC and VE levels, but the effect on tissue VC level appeared to be much more dramatic.
Diabetic hearts were more resistant to IR injury induced by ischemia (20-40 min) followed by 20-min of reperfusion, as assessed by the extent of LDH leakage. However, the protective effect observable in diabetic hearts diminished or disappeared with the increase in reperfusion time from 20 to 40 min. This was corroborated by the observation of poorer contractile force recovery in diabetic and ischemic hearts subjected to 40-min of reperfusion.

Differential changes in myocardial non-enzymatic antioxidant levels upon IR challenges were observed in control and diabetic hearts. Notably, while myocardial VE level in diabetic hearts was similar to that of non-diabetic control under non-IR condition, reperfusion of post-ischemic diabetic hearts for 20 min caused a significant increase in myocardial VE level. Instead of showing slight and insignificant changes in tissue GSH level upon IR challenge as in the case of non-diabetic hearts, post-ischemic reperfusion of diabetic hearts for 20 min following a 20-min period of ischemia increased myocardial GSH level. It is possible that the exposure to oxidative stress arising from the diabetic state may play an important role in priming the myocardium in responding to oxidative challenge. However, exposure of diabetic hearts to severe oxidative stress condition, as in the case of 40-min of ischemia followed by 40-min of reperfusion, could overcome the preconditioning effect, leading to drastic increase in LDH leakage and impairment in contractile force.

Insulin treatment partially reversed the diabetes-associated changes in hearts subjected to IR challenge. The increased susceptibility of insulin-treated diabetic hearts to IR injury
was associated with a marked decrease in myocardial VE level and a slight but insignificant increase in myocardial VC level. Sch B pretreatment could not protect against IR injury induced in diabetic hearts, as assessed by the extent of LDH leakage. Instead, the extent of contractile force recovery was significantly reduced in the Sch B pretreated and ischemic-reperfused diabetic hearts. The inability of Sch B pretreatment to protect against IR injury in diabetic hearts suggests the involvement of a common antioxidant mechanism in the cardioprotection afforded by Sch B and the diabetic state.

Sch B pretreatment could slightly protect against IR injury in insulin-treated diabetic hearts. The myocardial protection afforded by Sch B pretreatment was associated with a marked decrease in myocardial MDA level as well as significant increases in myocardial VE and VC levels. The result suggests that Sch B pretreatment may produce beneficial effect on myocardial non-enzymatic antioxidants in insulin-treated diabetic rats under oxidative stress condition.
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