

**THE EFFECTS OF PSYCHOLOGICAL AND OXIDATIVE STRESS ON THE
TEMPORAL PROFILE OF STRESS PROTEINS AND ANTIOXIDANT STATUS
DURING ATHEROGENESIS IN JAPANESE QUAIL**

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ABSTRACT

This thesis reports the results of four experimental studies which were designed to examine the effects of psychological stress on the interrelationships of heat shock proteins (HSP) and endogenous antioxidants during atherogenesis in an avian model, the atherosclerosis-susceptible (SUS) Japanese quail.

The first study examined the effect of psychological stress in quail fed a cholesterol-supplemented diet for four weeks. As expected, cholesterol-supplemented birds showed significant increases in plaque scores compared to birds fed a non-cholesterol-supplemented diet. The heterophil/lymphocyte ratio was increased in birds exposed to stress and in cholesterol-supplemented birds. Stress or cholesterol feeding alone increased HSP 70 in aortic and heart tissues, whereas lower HSP 70 levels were detected in cholesterol fed birds exposed to stress compared to non-stressed cholesterol fed birds. The stress protocol lowered aortic catalase and heart superoxide dismutase levels, and had little effect on enzymes of the glutathione (GSH) redox cycle. A functional decrease in the antioxidant capacity of the heart as measured by thiobarbituric-acid reactive substances (TBARS) was detected following stress alone or cholesterol supplementation.

In the next longer-term study, I examined the effects of temporal stress during atherogenesis. There were no differences in plaque score between early stressed, recently stressed and non-stressed cholesterol-supplemented birds. Moreover, cholesterol-fed birds exposed to early stress had lower plasma cholesterol levels than cholesterol-fed birds exposed to recent stress or no stress, suggesting that stress may have preconditioned the birds during cholesterol feeding. In general, stressed cholesterol-fed birds had lower HSP 70 and heme

oxygenase (HO-1) levels in their aortic and heart tissue compared to non-stressed cholesterol-fed birds. Furthermore, cholesterol-supplementation alone also increased heart and aortic ferritin and catalytic iron levels, and increased generation of TBARS which is indicative of increased oxidative stress.

In the third study, aortic endothelial cells from random bred wild-type strain (WILD) and SUS and atherosclerosis-resistant (RES) Japanese quail were isolated and characterized. GSH levels were higher in the SUS cells and lowest in RES cells, and glutathione reductase was higher in WILD cells than SUS and SUS cells. Subconfluent monolayers of RES cells had higher HO activity compared with SUS cells whereas HO activity levels were similar between strains at confluence. Catalytic iron levels were higher in SUS cells than WILD and RES cells.

In the final study of this thesis, aortic endothelial cells were exposed to *in-vitro* oxidative challenge to further investigate the role of HO and glutathione in the RES and SUS strains. Overall, the RES cells exhibited higher HO activity and HO-1 protein and lower LDH release and T BARS levels compared with cells isolated from the SUS strain.

In summary, while stress factors imposed did not alter the aortic plaque scores, some associations between and/or cholesterol feeding related to the development of atherosclerosis have been established. These studies provide evidence that some genetic factors in atherosclerosis are manifested at the level of endothelial cells, given the distinct differences in HO and antioxidant components from RES and SUS cells. Therefore, these studies could further characterize the association and possibly the interrelationship of psychological stress to oxidant-induced injury and the subsequent development of atherosclerosis.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AEC	Aortic endothelial cell
ANOVA	Analysis of variance
CAD	Coronary artery disease
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetic acid
GRd	Glutathione reductase
GPx	Glutathione peroxidase
GSH	Glutathione, reduced form
GSSH	Glutathione disulfide
hr	Hour
H ₂ O ₂	Hydrogen peroxide
Hb	Hemoglobin
H/L	Heterophil to lymphocyte ratio
HO	Heme oxygenase
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock protein
LDL	Low density lipoprotein
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
min	Minute
mM	Millimolar
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nmole	Nanomole
OxLDL	Oxidized low density lipoprotein
pmol	Picomoles
RBC	Red blood cell
RES	Atherosclerosis-resistant
ROS	Reactive oxygen species
sec	Second
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SUS	Atherosclerosis-susceptible
TBARS	Thiobarbituric acid reactive substances
t-BHP	Tert-butylhydroperoxide
TCA	Trichloroacetic acid
WBC	White blood cell
WILD	Random-bred

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CHAPTER 1

LITERATURE REVIEW

CARDIOVASCULAR DISEASE

Despite a declining rate of cardiovascular disease (CVD), it is still the major cause of death, disability and illness in Canada (1-3). A considerable body of experimental data has accumulated suggesting that oxidative processes, possibly involving reactive oxygen-derived species (ROS), play a crucial role in the pathogenesis of CVD and atherosclerosis (4). The oxidative transformation of plasma low-density lipoproteins (LDL) markedly increases their atherogenicity (5), and increased levels of oxidized LDL are believed to play an important role in the generation and progression of atherosclerotic plaques (6). According to the oxidative-modification hypothesis, LDL initially accumulates in the extracellular subendothelial space of arteries and, through the action of endothelial cells, is mildly oxidized to minimally modified LDL (mmLDL) (7). Here, mmLDL induces local vascular cells to produce monocyte chemoattractant protein 1 (MCP-1) and macrophage colony-stimulating factor (MCS-F), which stimulates monocyte recruitment and differentiation to macrophages in vessel wall (8). The accumulation of monocytes and macrophages into the sub-endothelial space enriches the microenvironment in ROS through conversion of trapped mmLDL into highly oxidized LDL (recognized by the scavenger and/or oxidized LDL receptors) (7). Scavenger receptors are expressed on endothelial cells (9), macrophages (10), fibroblasts and smooth muscle cells (11). They participate in the recognition and clearance of apoptotic and damaged cells, and by virtue of the presence of oxidatively modified phospholipids on their surface, oxidized LDLs bind to scavenger receptors, are internalized and delivered to lysosomes, where the protein and

cholesterol esters are degraded before free cholesterol is returned to cytoplasm, where it is re-esterified (12). Unlike conventional LDL receptors, scavenger receptors are not down-regulated by high cholesterol levels, so that massive cholesterol accumulation can occur. This can set the stage for focal necrosis within the lesion, and can cause expansion of the lesion. As the lesion enlarges the artery can no longer compensate for the encroachment in the lumen, and at some point flow is impaired (7).

However, the early stages of CVD are not reflected by overt symptoms, and prevention of risk factors is the best defense. Traditional risk factors such as smoking, cholesterol, blood pressure, and glucose intolerance can explain about 21 to 42 % of population variance in CVD (13). The remaining variance can be explained by genetic, psychosocial and other unknown factors. Extensive evidence from prospective and cross-sectional epidemiological studies in post-myocardial infarction patients, and animal studies has demonstrated an association between psychological stress and cardiovascular morbidity and mortality (14-16). Examples of such stressors include social isolation (17), depression (18) and hostility (19), and prolonged periods of negative life events. The key factor is chronicity. This thesis investigated the hypotheses that psychological stress alone or in combination with a cholesterol supplemented diet may exacerbate atherosclerosis.

ANTIOXIDANTS

Dysfunction of endothelial cells induced by oxidized LDL is thought to be an early step in atherogenesis (20). The action of antioxidants to inhibit oxidative modification of lipoproteins has been intensively investigated (21-22). Several clinical studies have reported a correlation of LDL oxidation susceptibility and severity of atherosclerosis (23-24). However, two studies in

cholesterol-fed rabbits reported a dissociation between antiatherogenic actions of antioxidant interventions and their effects on susceptibility of plasma LDL to oxidation (25-26). Thus, vitamin E produced a modest protection of LDL but had no effect on lesion formation, B-carotene had no effect on LDL oxidation but markedly inhibited lesion development and low doses of probucol protected LDL against LDL oxidation *ex vivo* but did not inhibit atherogenesis. However, the interpretation of animal antioxidant studies is not entirely straightforward. Many of these studies have a lack of standardization as control diets amongst studies are not comparable. In particular, the vitamin E content of “standard” laboratory chows can vary considerably and may have major implications on experimental findings (27, 28). Thus, the antioxidant composition of experimental diets should be considered during formulation to control variability.

A number of clinical studies have been conducted to explore the relation between antioxidant status and atherosclerosis. Descriptive studies are useful examining characteristics of a population and its associated disease rates but lack the ability to control for potentially confounding factors such as differences in diet, genetics and environmental factors. For example, four descriptive studies have shown an inverse association between fresh fruit and vegetable consumption in cardiovascular disease (29-32). However, whether this is due to the dietary intake of antioxidants or the replacement by fresh fruits and vegetables of potentially harmful dietary components (e.g. saturated fats) cannot be determined (33).

Case control studies consist of data gathered retrospectively on dietary or lifestyle exposures of interest as well as data on a variety of potentially confounding variables although selection of cases and controls may introduce bias and unknown variables may have a significant impact on results that go unexamined. Thus, one study each to support an

association between vitamin E (34), vitamin C (35), and tissue beta carotene levels (36) in cases with cardiovascular disease compared to controls, suggest that natural antioxidants may reduce the risk of cardiovascular disease.

Prospective studies offer an advantage of measuring exposure of variables prior to the development of disease minimizing the impact of selection bias and the effects that the disease may have on exposure variables such as dietary habits. Although the Health Professionals Follow-up Study supported an inverse association between between the development of coronary artery disease and the intake of vitamin E and beta carotene (37), other prospective cohort studies have only provided support for vitamin E (38-39).

Four large randomized clinical trials have now been completed which examined the effect of antioxidant supplementation on cardiovascular events. However, a combined total of more than 27,000 patients failed to show a significant benefit with vitamin E taken as a dietary supplement for the prevention of coronary artery disease (40-43). Although these randomized clinical trials did not find evidence that antioxidants block the oxidative modification of LDL, the results suggest that an oxidative stress/antioxidant imbalance is involved in the cellular damage by oxidized LDL (44). Thus, endogenous antioxidants (45) are speculated to be important in preventing the development of atherogenesis, and more potent and powerful antioxidants which reduce vascular events may ultimately prove therapeutically beneficial.

HEAT SHOCK PROTEINS

The body has built-in mechanisms for coping with short-term, high demand situations. One such mechanism is the expression of a family of stress proteins, namely heat shock proteins (HSPs). One of the most extensively studied of these is HSP70. These molecular chaperones

are upregulated by a variety of stressors including psychological stress (46-47). However, continued multiple demands may overload the system's response capacity, creating an imbalance that can lead to a breakdown in regulatory processes (48).

It is well established that oxidative stress is associated with expression of HSPs and the development of atherosclerosis (49-50). Berberian *et al.* (51) first reported the elevated expression of HSP70 in human and rabbit arteries and its distribution in relation to necrosis and lipid accumulation, as well as vascular smooth muscle cells and macrophages, in human atherosclerotic plaques. HSP70 was mainly concentrated in the central portions of more thickened atheromas around sites of necrosis and lipid accumulation (52) In contrast, patches of smooth muscle cells were observed in the most complex plaques but without consistent association with necrosis or increased HSP70 (53). The results indicated that elevated HSPs in plaque cells, particularly macrophages, were more stressed within the core of the atheroma, especially in association with necrosis. Thus, HSP70 is expressed in high concentrations in human atherosclerotic lesions (51) and oxidized LDL can induce HSP70 in human endothelial cells *in vitro* (54). Normocholesterolemic rabbits immunized with HSP65 (which is analogous to human HSP60) developed severe atherosclerotic plaques (55) and a strong correlation between serum anti-HSP antibody titers and severity of carotid atherosclerosis has been demonstrated clinically (56). The first stages of atherogenesis may be inflammatory in nature and an autoimmune reaction to HSP60 expressed by endothelial cells might be involved (57). Kleindienst *et al.* (58) proposed that shear force may lead to the expression of HSP60 by intimal cells, followed by recruitment of HSP-sensitized T cells, leading to an inflammatory response that would initiate the process of atherogenesis. In particular, HSP60 was detected on endothelium, smooth muscle cells, and/or mononuclear cells of all carotid and aortic specimens

(58). Whereas vessels of smaller diameter, serving as reference specimens for the normal intima without atherosclerotic lesions and mononuclear infiltration, showed no detectable expression of this HSP (58). HSP47 acting as a chaperone for procollagen has also been found to be associated with atherosclerosis (59). Strong focal expression was evident in atherosclerotic, but not normal arteries and was prevalent in the collagenous regions. All cells expressing type I procollagen also expressed HSP47 (60). Furthermore, heat shock and oxLDL stimulated the expression of HSP47 mRNA by smooth muscle cells. These findings identify HSP47 as a novel constituent of human coronary atheroma, and selective upregulation by stress raises the possibility that HSP47 may be a determinant of plaque stability (60).

There are also data suggesting that the elaboration of these proteins can have a protective effect. Hearts from rabbits exposed to thermal stress exhibited markedly increased functional recovery following low flow ischemia and subsequent reperfusion (61). Similar myocardial protection against ischemia was found in rats subjected to mild heat shock (62). It has been suggested that stress-induced induction of HSPs could be associated with an increase in tissue antioxidant capacity, although definite evidence is presently lacking (63-64). This thesis explored the relationship between HSP elaboration and atherogenesis in the absence and presence of psychological stress.

HEME OXYGENASE

It is becoming clear that other oxidant-induced gene products may also play vital roles in the adaptive and/or protective response of the vasculature to oxidative stress. One such stress-induced protein is heme oxygenase (HO). Heme oxygenase is the rate-limiting enzyme in heme degradation (65). HO was discovered in the course of the inquiry into the capacity of the

reticuloendothelial system to degrade aged hemoglobin (66), and for much of the time HO was studied within the confines of heme metabolism. However, HO-1, an inducible form of HO, can be readily and often markedly upregulated by diverse factors including heme, oxidized LDL, cytokines and growth factors in the vessel wall and inflammatory cells (65). In addition, these inducing stimuli are associated with various cardiovascular disorders, and accumulating evidence suggests that HO-1 induction confers protection against the injury associated with these conditions (67-69).

The heme prosthetic group is ubiquitously distributed within cells, and is present in proteins that carry or store oxygen, mitochondrial and microsomal cytochromes, and numerous other proteins. Disengaged from its linkage with such proteins—as when cells are injured—heme provides a readily transmissible pro-oxidant species that can result in destabilization of cellular organelles. A microsomal enzyme, heme oxygenase (HO) decomposes heme into equimolar amounts of free iron, biliverdin, and carbon monoxide (CO) (70-71) (Fig. 1.1). This reaction requires oxygen and occurs in association with NADPH ferrihemoprotein reductase (NADPH-cytochrome P-450 reductase), which supplies electrons to reduce heme iron or to activate molecular oxygen (72). In avian species, the primary end-product of heme catabolism is biliverdin, whereas in mammals biliverdin is subsequently reduced to bilirubin by bilirubin reductase (65). Since biliverdin and bilirubin are excreted into bile, these compounds are also called bile pigments. Therefore, one of the major functions of HO is heme degradation; however, recent studies have shown that HO is also important for protection against various oxidative stresses as well (73-75).

Three isoforms of HO, which are the products of separate genes, have been described in mammals (76-78). HO-1 is widely distributed in tissues and is highly inducible in virtually all

cell types (79). HO-2 is constitutively present but unresponsive to any of the inducers of HO-1 (76). Histidine residues at H25 for HO-1 and H45 for HO-2 are essential for heme degradation activity (80-81). The third isoform, HO-3, is nearly devoid of catalytic activity and may function chiefly as a heme-sensing or heme-binding protein, or both (78).

HO-1 induction by oxidative stress

HO-1 is an inducible form of the enzyme that is transcriptionally upregulated by numerous chemical or physical stressors, including heme (82), transition metals (83), sodium arsenite (84), hydrogen peroxide (85), heat shock (86), UV radiation (87), thiol scavengers (88), hypoxia (89) and oxidized lipoproteins (67). A common feature of many of these stressors is their ability to alter cellular redox state. HO-1 is also transcriptionally activated through several regulatory mechanisms. Studies of the promoter region of HO-1 have revealed transcriptionally responsive elements, including activator protein I (AP-1), activator protein II (AP-2), NF-kB, interleukin 6 responsive elements, and an antioxidant response element (90-92). In another study, HO-1 which was induced by balloon injury, reduced the risk for restenosis in the femoropopliteal segment after angioplasty in association with inhibition of vascular smooth muscle cell proliferation (93). In another study, the extension of the length of polymorphic (GT) repeats in the HO-1 gene promoter has been reported in patients with chronic pulmonary emphysema (94), and suggests that decreased HO-1 inducibility (e.g., by ROS) may have compromised protection of the lung.

The potential roles of HO reaction products

Biliverdin and bilirubin

Biliverdin and bilirubin form bile pigments and have been considered to be waste products of heme degradation. These products can be toxic at high concentrations, especially in neonates (95). However, it has been suggested that bilirubin may be an antioxidant of physiological importance, since bilirubin suppressed the oxidation of chemically-induced peroxy radicals to a greater extent than alpha-tocopherol, which affords powerful protection against lipid peroxidation (96). Furthermore, potential anti-atherogenic properties of bilirubin may include inhibitory actions against oxidized LDL and scavenging oxygen radicals arising from phospholipids, triglycerides and cholesterol esters (97). The mechanism by which bilirubin reacts with ROS is not completely understood, although its hydrophobic tetrapyrrole structure has been reported to inhibit the activation of superoxide-producing NADPH oxidase (98). Bilirubin has been shown to have an inhibitory effect on protein phosphorylation (99) and protein kinase C activity (100), which both can lead to the inactivation of various proatherogenic factors, such as lipid oxidation (101), immune reactions (102) and inflammatory processes (103).

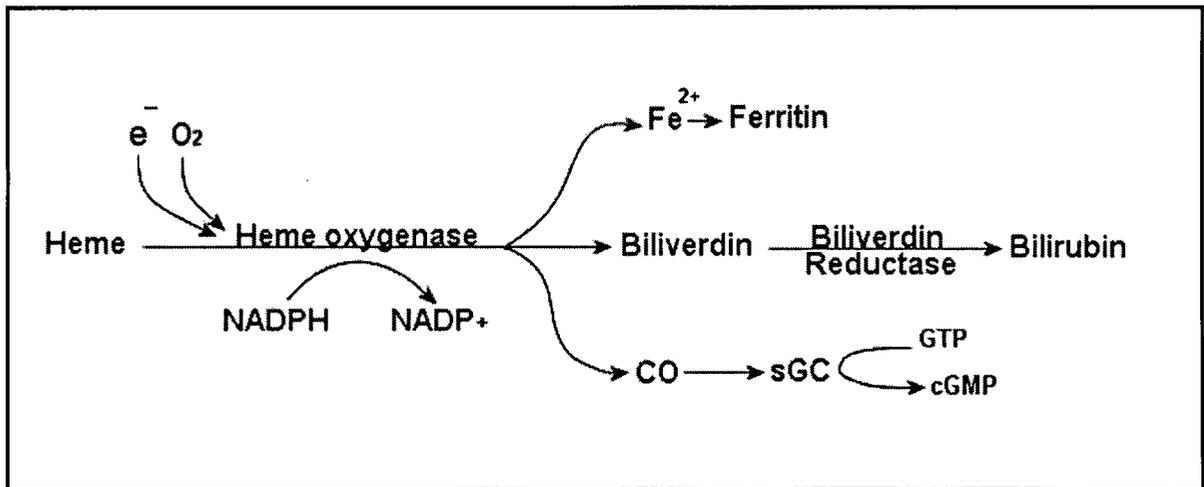


Figure 1.1. Heme degradation pathway in mammals. Heme is degraded by two enzymes. The first reaction is catalyzed by heme oxygenase and requires electrons from NADPH cytochrome P450 reductase and molecular oxygen to yield ferric iron (Fe^{2+}), carbon monoxide (CO) and biliverdin. In the second reaction, biliverdin is degraded to bilirubin by biliverdin reductase.

Carbon monoxide

Carbon monoxide (CO) is another product of the HO reaction, and the similarities between nitric oxide (NO) and CO have suggested that CO may have a physiological role (104). Earlier studies suggested that CO might bind to and activate guanylate cyclase (GC) and enhance intracellular cGMP levels, as has been previously demonstrated for NO (65, 105). However, the physiological relevance of CO as a vasodilator is controversial. NO is a potent activator of GC and increases cGMP production *in-vitro* about 130-fold, whereas the increase in cGMP with CO is only about 4.4-fold (106-107). Nevertheless, since CO is more chemically stable than NO, and there appears to be no enzymatic pathway catalyzing CO degradation *in vivo*, the biological availabilities of NO and CO may be significantly different (65). The biological effects of CO on the formation and actions of cGMP need to be further explored.

Exogenous CO was reported to relax blood vessels *in vivo* (107), and HO activity, as measured by CO production, has been detected in arteries of rodents and humans *in-vitro* (108). Some reports have suggested that CO may act as a physiological regulator of vascular tone through cGMP-mediated responses. Thus, CO has been reported to maintain rat vascular tone in small and medium arteries (106) and the aorta (109). Other reports have suggested CO may play a role in blood pressure regulation in acute hypertension (110). On another note, CO has been shown in mice to mediate anti-inflammatory effects through a pathway involving the mitogen-activated protein kinases (111). Thus, the actions of CO via cGMP-dependant and/or non-cGMP-dependant pathways may explain a number of the potential actions of this HO reaction product.

Ferritin and iron

Ferritin sequesters intracellular free iron, thereby decreasing its stimulatory effect on the formation of damaging ROS such as the hydroxyl radical and the superoxide radical (Fig. 1.2)

(112). HO-1

induction resulting in an increase of catalytic free iron release has been reported to upregulate ferritin (113). Furthermore, the enhancement of intracellular ferritin protein through HO-1 has been reported to decrease the *in-vitro* cytotoxic effects of hemin and hydrogen peroxide in vascular endothelial cells (114). Cytoprotective properties of ferritin against ultraviolet irradiation-induced damage have also been demonstrated (115). Additionally, increased ferritin mRNA has been shown in a rat model of brain ischemia/reperfusion injury (101), in pericardial fluid of patients with coronary artery disease (102) and in human atherosclerotic aortas with abdominal aneurysm

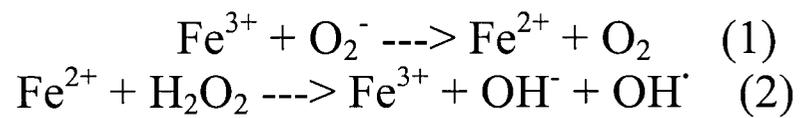


Figure 1.2. The Haber-Weiss reaction can be broken down into two chemical reactions. The first reaction is catalyzed by traces of transition metal ions (1). A superoxide molecule reacts with Fe^{3+} salt to form Fe^{2+} salt and ground state oxygen. The second reaction is known as the Fenton Reaction (2). In this reaction Fe^{2+} salt reacts with hydrogen peroxide to form Fe^{3+} salt, the hydroxyl radical and alcohol.

(118). Thus, the cytoprotective response of ferritin production following HO-1 induction may act to limit intracellular free iron. Indeed, studies in our laboratory have demonstrated increased catalytic iron in cultured aortic endothelial cells from a strain of atherosclerosis-susceptible Japanese quail as compared with an atherosclerosis-resistant strain (Chapter 4). Following exposure to oxidative challenge, the levels of HO-1 expression increased, and ferritin protein was up-regulated in the presence of increased catalytic iron, resulting in a greater cytoprotection of atherosclerosis-resistant cells compared with the atherosclerosis-susceptible strain (Chapter 5).

Genetic models of HO deficiency

Knock-out mice

Recent studies by Poss and Tonegawa (73,119) in HO-1 knockout mice have highlighted the important metabolic and cytoprotective roles of this gene. Mice lacking the HO-1 gene were anemic, unable to modulate iron body stores properly, were more sensitive to hepatic injury, and developed chronic inflammatory disease (119). These findings demonstrated that HO-1 deficient mice develop both serum iron deficiency and pathological iron-loading, and suggest that HO-1 is crucial for the expulsion of iron from tissue stores. Intracellular iron accumulation through reduced mobilization in HO-1 knockout fibroblasts has been shown to be responsible for apoptotic cell death (120). Furthermore, HO-1 knockout mice are very susceptible to lipopolysaccharide and hepatic necrosis (73).

Human HO-1 deficiency

The first case of human HO-1 deficiency has been reported by Yachie *et al.* in 1999 (121). This individual had thrombocytosis, heme in the serum, elevated ferritin and low serum bilirubin in association with severe hemolytic anemia. The patient was diagnosed as HO-1 deficient by gene analysis, and died at the age of 6 (122). Compared with HO-1 knockout mice, the human HO-1 deficiency case seemed to more severely involve the endothelial cells and the reticuloendothelial system, resulting in enlargement of liver and kidney with iron deposition, a defective spleen, intravascular hemolysis and disseminated intravascular coagulation. The patient also had high serum levels of free heme accompanied by elevated serum thrombomodulin and von Willebrand factor, erythrocyte fragmentation, and hyperlipidemia associated with fatty streaks and fibrous plaques. The high concentration of thrombomodulin and von Willebrand factor associated with prominent atherosclerotic changes in this patient indicate that the endothelium sustained injury (121-122). Recent experiments investigating LDL isolated from the patient's plasma revealed a substantial oxidation of hemoglobin in plasma to methemoglobin (108), which may have contributed to formation of oxidized LDL and subsequent observed damage to the endothelium (121-122).

The HO gene

Recent studies have explored the strategy of administering the HO gene with a vector as a means of conferring long-term transgenic expression and tissue protection (124-126).

Recombinant adeno-associated virus (rAAV) is a suitable vector that is nonpathogenic; it elicits an attenuated host inflammatory response and provides long-term expression of therapeutic genes by virtue of its capacity for integration into the host genome. Overexpression and underexpression of human HO can be modulated on a long-term basis in endothelial cells

by introduction of the HO-1 gene with a retroviral vector (124). The finding that overexpression of HO-1 gene in the rat carotid artery reduced neointimal and medial wall area, decreased neointimal thickness and inhibited medial wall DNA synthesis after balloon injury further suggest that HO-1 gene delivery has potential therapeutic applicability (125). An *in vivo* study evaluating this strategy for protection against ischemia-induced injury by Melo *et al.* (126) used rAAV for intramyocardial delivery of the HO-1 gene into normal rat hearts 8 weeks before acute coronary artery ligation, and showed that the long-term, overexpression of HO-1 gene conferred 75% reduction in left ventricular myocardial infarct size, and may be due to the reduction in oxidative stress and apoptotic and inflammatory activities. Finally, adenoviral-mediated gene transfer of HO-1 into rat aortic smooth muscle cells reduced the iron deposition and decreased plaque formation in apo-E deficient mice (127). The results of these preliminary studies raise the possibility that HO-1 may provide a novel therapeutic approach in the treatment of certain vascular diseases.

HO and atherosclerosis

Several lines of evidence suggest a possible physiological and pathophysiological role for HO in the vasculature, as an important protective pathway inhibiting lipid peroxidation prior to and/or during atherosclerosis.

HO-1 is induced by oxidized LDL

As noted earlier, numerous studies have shown that oxidized LDL plays a pivotal role in the development of atherosclerosis. Ishikawa and colleagues (128) have shown that HO-1 is powerfully induced in vascular endothelial cells or smooth muscle cells *in vitro* by oxidized

LDL, whereas HO-1 was not expressed when these cells were exposed to native LDL. Furthermore, other studies have reported HO-1 induction in renal tubular cells and macrophages exposed to oxidized LDL (69,68). Additional reports have examined the components of oxidized LDL responsible for HO-1 induction. HO-1 was induced by arachidonic acid-containing phospholipid (128) and linoleyl hydroperoxide (129), but not lysophosphatidylcholine (67).

Increased HO-1 expression, relative to the native vasculature, has been demonstrated in atherosclerotic lesions (67), apo-E knockout and LDL-receptor deficient knockout mice (130), and Watanabe heritable hyperlipidemic (WHHL) rabbits (131). HO-1 was expressed in vascular endothelial cells and macrophages in the early stages of atherosclerotic lesion formation, and in foam cells and smooth muscle cells residing in the necrotic core of advanced lesions. In all of these atherosclerotic lesions, HO-1 was found to be co-localized with oxidized phospholipids using specific antibodies to HO-1 and LDL. These observations strongly suggest that HO-1 was induced by oxidized phospholipids *in vivo*, and HO-1 induction following oxidant stress is generally thought to counteract rather than mediate deleterious downstream events of ROS formation in the vasculature.

Anti-atherogenic properties of HO-1

Atherosclerosis is a multifactorial disease in which a number of cytokines and growth factors are expressed in the vasculature. The expression of HO-1 appears throughout the entire period of atherosclerotic lesion development (67), suggesting the possible involvement of this enzyme in atherogenesis. In this regard, a report examining the effect of HO-1 expression on monocyte transmigration into the vessel wall, an essential event for fatty streak formation, demonstrated

that HO-1 induction significantly inhibited monocyte transmigration induced by oxidized LDL, whereas HO inhibition had the opposite effect (128). Furthermore, the inhibitory effect of HO-1 on monocyte transmigration could be mediated through the actions of biliverdin and bilirubin, because these compounds can directly inhibit monocyte transmigration (132). Since these compounds have been demonstrated to scavenge ROS and inhibit lipid peroxidation (106-107, 133), they may reduce the inflammatory response to oxidized lipids in the vessel wall. This has been suggested as a possible cause for the inhibitory effects of HO-1 on leukocyte adhesion mediated by the action of bilirubin (132).

There is evidence suggesting that HO influences the development of atherosclerotic lesions in animal models. In LDL receptor knockout mice, HO-1 is abundantly expressed in atherosclerotic lesions following dietary cholesterol feeding (134). In this mouse model, HO overexpression resulted in a reduction of plaque formation, whereas HO inhibition by Sn-protoporphyrin IX promoted lesion development. In the WHHL rabbit model, inhibition of HO-1 by Sn-protoporphyrin IX increased plasma and tissue lipid peroxide levels, and increased atherosclerotic plaque formation (131). However, the fact that the antioxidants vitamin E and probucol exhibited a reduction and increase in atherosclerosis in mouse (135) and rabbit (136) models, respectively, but HO inhibition resulted in increased atherosclerotic lesion formation in both species, suggests a possible role for HO in modulating atherosclerotic lesion formation.

The inhibitory effects of HO on lipid peroxidation are believed to play a crucial role in the putative protective effect of HO against atherosclerosis. Plasma peroxide levels have been shown to be elevated in atherosclerosis to an extent that closely parallels the severity of the disease. Evidence of decreased plasma lipid hydroperoxide levels was found in LDL-receptor mice and WHHL rabbits treated with an HO-1 inducer, whereas increased peroxide levels were

observed with a HO inhibitor (134,131). In the case of WHHL rabbits, HO inhibition resulted in enhanced formation of lipid peroxides in liver and aortic tissue. In the context of the latter studies, this raises the possibility that HO reaction products, such as biliverdin and bilirubin, may reduce the formation of plasma and tissue lipid peroxidation products (131). An interplay between HO and NO pathways may be another mechanism by which HO influences atherosclerosis (136). Experimental studies investigating the effect of HO modulation on NO pathway by measuring plasma NO (nitrite and nitrate) levels found that HO expression was inversely correlated with plasma NO levels, which were significantly decreased after feeding a cholesterol-supplemented diet (134). There is also considerable evidence that NO induction leads to an increase in HO-1 expression (138-139). It has been suggested that the inhibitory action of CO, another product of HO reaction, on vascular smooth muscle cells (140) and platelet aggregation (141) may also provide protection against development of atherosclerosis. Additionally, studies have shown that CO and bilirubin are causative mediators in the antiatherogenic action of NO and its second messenger cGMP (142-143).

Conclusions

Accumulating evidence suggests HO-1 contributes to the balance of prooxidant and antioxidant factors in atherosclerosis. While some studies provide evidence for HO-1 expression during plaque formation as an adaptive response, numerous gene transfer experiments provide evidence for a preventative role for HO-1 overexpression before injury occurs. With regard to the possible relevance of HO-1 in atherosclerosis, it should be noted that HO-1 is induced by most of the established risk factors for the condition, such as oxidized lipoproteins (67), hypertension (116) and hypoxia (89) in vessel wall cells and circulating macrophages, whereas

inhibition of HO-1 results in attenuation of their deteriorative effects. It may, therefore, be speculated that interventions aimed at modulating levels of HO in the vessel wall could provide a novel target for future gene therapies to treat or prevent atherosclerotic diseases. On the other hand, in considering the possible limitations of therapies aimed at inducing HO, there is the concern of a reduction of heme and heme proteins, the substrate for HO, as these proteins function as important prosthetic groups for various crucial enzymes such as cytochrome P450 (140,142) and NO synthase (141-142). It remains to be determined if gene therapy involving HO can be confined to a particular tissue (e.g. endothelium) or result in a more generalized induction to prevent further complications of vascular disorders. Clearly, more information on the elucidation of the precise nature and role of early transductional signaling pathways and transcriptional events activated in HO gene therapy, and understanding the downstream effects responsible for HO antioxidant activity are necessary to support the protective role of HO during atherogenesis, whether it be causal and/or permissive. Additionally, complex interactions in the genome association of HO gene therapy as well as epidemiological studies following such gene therapy will hopefully be the focus of research in the coming years.

THE ATHEROSCLEROSIS-RESISTANT AND –SUSCEPTIBLE JAPANESE QUAIL

A selectively bred strain of Japanese quail have been used in research examining atherosclerosis-related alterations in endogenous antioxidants (146). Japanese quail have numerous advantages, including their small size, ease of maintenance, their short life-span of ~2 years which telescopes the disease process, the rapidity of plaque development and the similarities of the “complex” vascular lesions (focal hemorrhage, calcification, fibrosis) to

those in humans (146-147). Other similarities to the human disorder include the greater susceptibility of males than females to diet-induced atherosclerosis (148) and the implication of viral infection as a possible initiator of lesion formation in both cases (149). Although the major fraction of cholesterol in quail plasma is in the high density lipoprotein (HDL) rather than the low density lipoprotein (LDL) fraction, as in the case in humans, cholesterol feeding in the quail has been shown to cause a large and preferential increase in LDL cholesterol (150-151). Previous studies in our laboratory have shown that atherosclerosis-susceptible (SUS) and -resistant (RES) quail are biochemically distinct in terms of antioxidant profiles, and are consistent with the "oxidation hypothesis" of atherogenesis (152). Furthermore, we have shown that the marked difference in susceptibility of males *versus* females to diet-induced atherosclerosis is unlikely to be due to differences in their respective antioxidant profiles (148). In a study assessing the influence of both genetic and dietary factors in determining the severity of plaque formation in relation to antioxidant enzyme alterations, we observed a significant negative correlation between plasma glutathione peroxidase (GPx) activity and the development of fatty streaks/mild plaques in RES and SUS quail (153). In RES and SUS quail fed a cholesterol-supplemented diet, we found a strong positive genetic correlation of aortic GPx with plaque score, as well as strong negative correlations of aortic glutathione reductase (GRd) with plaque score and aortic cholesterol content suggesting that the role of antioxidant components in the early and late stages of lesion development differs (153). These findings were further analyzed in a recent time-course study of changes in antioxidant components of plasma, red blood cells, heart and aorta in relation to plaque development (154-155). The complex pattern of alterations in endogenous antioxidant components observed in these experiments suggests that the patterns of antioxidant alterations associated with lesion

development is time-dependent. The RES and SUS strains of Japanese quail were used in this thesis to examine the stress induced and atherosclerosis-related alterations in HSPs and endogenous antioxidants.

The overall purpose of this thesis is to examine various factors involved in the link between psychological and oxidative stress, HSPs and antioxidant components in Japanese quail susceptible or resistant to the development of atherosclerosis. Subsequently, I will focus on oxidative stress and the interaction of these components in the aortic endothelial cells of the same two strains of quail.

CHAPTER 2

THE EFFECT OF PSYCHOLOGICAL STRESS ON AORTIC PLAQUE DEVELOPMENT IN THE ATHEROSCLEROSIS-SUSCEPTIBLE (SUS) JAPANESE QUAIL.

PREFACE

This investigation was supported by research grants to D.V. Godin and K.M. Cheng from the British Columbia HealthCare and Research Foundation. A manuscript reporting the studies in this Chapter will be submitted to Molecular and Cellular Biochemistry and will be co-authored with C.N. Nichols, M.E. Garnett, D.V. Godin, and K.M. Cheng. C.R. Nichols contributed to project planning, animal care and designing the stress protocol. M.E. Garnett contributed to technical assistance with the aortic antioxidant assays.

INTRODUCTION

Psychological stress may potentiate diseases via perturbations of the body's neuroendocrine response, such as affecting the hypothalamic-pituitary-adrenocortical (HPA) and sympathetic-adrenomedullary systems (SNS). Chronic stress, for instance, can adversely affect cardiovascular function, which in turn augments risk for diseases of the cardiovascular system (156-157). The influence of chronic stress in the workplace was found to be associated with an increased risk of coronary artery disease (CAD) in some human studies (158-159) but not in others (160-161). Finnish men with exaggerated blood pressure responses exhibited an increased incidence and severity of atherosclerosis (162). It was also found that men with

stress-induced blood pressure elevation and high job demands experienced the greatest atherosclerotic progression (163). Chronic stress may also result in vascular endothelial abnormalities, which can range from subtle dysfunction to overt injury and necrosis (164). Although chronic stress may induce vascular endothelial dysfunction associated with the early stages of atherosclerosis, the precise mechanisms by which this occurs are currently not clear. Udelsman *et al.* (165-166) has shown that chronic restraint stress activates the SNS, which in turn increases heat shock protein 70 (HSP70) mRNA and protein expression in the aorta. HSPs are inducible to increase cellular defenses by a variety of physical stressors, such as mechanical stress (167), infections and disease (168) and exposure to oxygen radicals or cytokines (169-170). While the foregoing observations suggest that HSP's are involved in the pathogenesis of atherosclerosis, and are consistent with the permissive effect of stress on its development, there are data suggesting that the elaboration of these proteins can have a protective effect. Thus, HSP70 induced by heat shock can protect smooth muscle cells against the adverse effects of serum deprivation (52). The exogenous addition of HSP70 to cultured aortic cells from cynomolgous monkeys also increases their survival following exposure to prolonged stress *in vitro* (171). Finally, hearts exposed to thermal stress exhibited markedly increased functional recovery following low flow ischemia and subsequent reperfusion. It has also been suggested that stress-induced increases in HSPs could be associated with an increase in tissue antioxidant capacity (63).

Japanese quail have provided a useful experimental model to investigate antioxidant alterations in atherosclerosis (152-153). Japanese quail develop aortic plaques exhibiting structural features such as intimal thickening, foam cells, focal hemorrhage, calcification, fibrosis and collagen deposition similar to those in the human disorder (146-147), and is

considered a good model for studying atherosclerosis. Our laboratory has also developed a model of chronic stress in Japanese quail (172), which should allow an experimental examination of the influence of chronic stress on the process of atherogenesis.

In the present study, we used a selectively bred strain of atherosclerosis-susceptible Japanese quail (SUS) to test the hypothesis that psychological stress will increase the severity of atherosclerosis. We also measured HSP70 and several endogenous antioxidants in the heart and aortic tissues in the hope of finding some insights into the mechanisms by which stress exacerbates atherosclerosis and the relationship between alterations in antioxidant status and HSP elaboration.

MATERIALS AND METHODS

Animal model

The SUS quail strain was originally acquired from North Carolina State University in 1989. Since then, the quail have undergone further selection at the University of British Columbia Quail Genetic Resource Centre (QGRC) for susceptibility to atherosclerotic plaque formation induced by dietary cholesterol (1% w/w) supplementation (153). The history of the development of the original strain of SUS quail by Shih *et al.* has been described in reference (146).

Experimental Diets

From the time of hatching to eight weeks of age, all birds were fed a commercial turkey starter diet, which is wheat-based and contains 26 % protein. During the experiment, birds were fed

either a synthetic diet prepared by the feed mill at the Agriculture and Agri-Food Canada Research Station at Agassiz, British Columbia according to the National Research Council standards recommended for quail (the “control diet”), or the same synthetic diet supplemented with cholesterol 0.25% w/w (the “cholesterol diet”) (Table 2.1).

Experimental Design

One hundred and twenty males of the SUS strain were used. At 8 weeks of age, the SUS quail were randomly allocated to one of four experimental groups: 1) 4 wks on control diet with no stress; 2) 4 weeks on control diet and stressed (see below for stress protocol); 3) 4 weeks on cholesterol diet with no stress; 4) 4 weeks on cholesterol diet and stressed. The stress groups were exposed to stress five episodes per week for four weeks. Because of the time required to perform the numerous biochemical analyses on blood and aortic tissue, only 24 birds (6 from each experimental group) were processed per day. Thus, the birds were hatched in daily batches so that ages at initial exposure to control or atherogenic diet and at sacrifice were standardized. Each batch of 24 birds was treated as a replication. Thus, a total of 5 replications were performed.

Stress protocol

The stress protocol involved exposure, for a 1-h period once daily, to one of three different stressors between 0700 and 1200h. To minimize habituation, the sequence of stressors was randomized for the treatment period. The stressors used were as follows: a) restraint and music - each bird was restrained by taping both wings to the body, and the bird was then

Table 2.1 Nutritional compositions of control and cholesterol diets fed to atherosclerosis-susceptible Japanese quail

INGREDIENT	CONTROL DIET	CHOLESTEROL DIET
Soy protein flour (50% protein) ¹	34%	34%
Corn starch ⁵	40%	39%
Limestone ²	5%	5%
Mineral premix ³	0.5%	0.5%
Monofos ¹	3%	3%
Sucrose ⁵	2%	2%
Alphacel ³	7%	7%
Vitamin premix ³	0.5%	0.5%
D-L methionine ⁴	0.4%	0.4%
Choline chloride ¹	0.38%	0.38%
Tallow ⁶	5%	5%
Vegetable oil ⁵	3%	3%
Cholesterol ³	0%	0.25%
Cholic acid ³	0%	0.25%

Note: Both the cholesterol and the cholic acid were dissolved in tallow, by warming the tallow and melting the fat to incorporate the sterols.

¹ Van Waters & Rogers, Abbotsford, BC, Canada

² BDH Chemicals, Toronto, ON, Canada

³ ICN Biochemicals Inc., Cleveland, OH, USA

⁴ United States Biochemical Co., Cleveland, OH, USA

⁵ Neptune Food Services, Richmond, BC, Canada

⁶ Cargill Foods, High River, AB, Canada

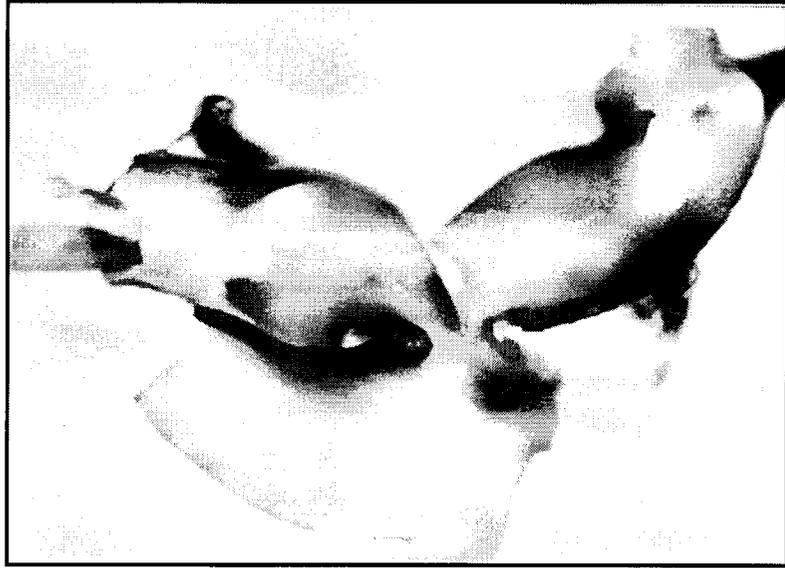
placed in a white plastic carrying case and exposed to loud (Rock and Roll) music (83db, range =73.2 to 95.3db) (Micro-5 Noise Dosimeter; Quest Electronics, WC) for 60 min before being released and returned to their home cages; b) restraint - the birds were restrained by taping both wings to the body, and then they were placed in individual cardboard boxes (44 x 44 x 33 cm) with the lid closed and were left in the dark for 60 min before being returned to their respective home cages via the carrying case; c) irritation - the birds were placed in a white plastic carrying case and a piece of velcro tape was taped loosely around the neck of each bird for 60 min, after which time the tape was removed and the birds were returned to their home cages. The stress protocol employed in this study has been shown to increase HSP70 expression in the myocardium; under these conditions, tolerance does not appear to develop over the experimental period in question (172).

Determination of aortic plaque score

Birds were sacrificed by decapitation and trunk blood was collected for biochemical analysis and heterophil/lymphocyte (H/L ratio) differential counts, which provide a qualitative measure of stress (see next section). Each bird was cut open to expose the aorta. The aortic tree was dissected out immediately proximal to the brachiocephalic arteries and the descending aorta to the iliac branching, opened longitudinally, and examined under a dissecting microscope for gross abnormalities. A score was assigned independently by two investigators (KH and CRN) blinded to the dietary status of the bird using the following scale: 0, normal (Figure 2.1 A); 1, fewer than 10 visible plaques; 2, more than 10 visible plaques and less than 50% area covered; 3, plaques covering greater than 50% area of the

Figure 2.1 A representative aortic tree of an atherosclerosis-susceptible (SUS) Japanese quail. From right to left: Branch 1 (left brachiocephalic artery); Branch 2 (right brachiocephalic artery); Branch 3 (desending aorta). A score (0-4) was assigned according to the dietary status of the bird. (A) Normal aorta with no visable plaques assigned a score of 0; (B) Severe atherosclerosis with large fatty plaques assigned a score of 4.

A.



B.



exposed arterial wall; 4, severe atherosclerosis, large fatty plaques (Figure 2.1 B) (137). The means of the two scores of the independent observers were used for analysis. The left, the right brachiocephalic arteries and the descending aorta were scored separately. Immediately after scoring was completed, the aortic tissue was rinsed in 50 mM Tris 0.1 mM EDTA, pH 7.6 buffer, frozen in liquid nitrogen, and stored at -70°C for later biochemical analyses.

White blood cell differential counts

The H/L ratio has been used extensively as a reliable indicator of physiological and social stress (173-176). One hundred white blood cells (WBCs) were counted from a differential blood smear stained with Wrights Diff-Quik (Dade Behring, Mississauga, Canada) and H/L ratio determined as described by Gross and Siegel (173).

Sample preparation

While some standard analyses (e.g. plasma cholesterol, plaque scoring) were performed on all birds, other analyses required the pooling of material from all three birds comprising each treatment because of limitations in the amount of tissue required (e.g. the hsp analyses on heart and aorta) or the requirement for fresh tissue (forced peroxidation studies).

Trunk blood was collected with a heparinized syringe and centrifuged for 5 min at 1000g at 4°C . A blood smear was made for later white blood cell differential examination. Plasma was collected and stored at -70°C for cholesterol, triglyceride and antioxidant enzyme analyses. Red blood cells (RBCs) were washed twice with isotonic saline (1000 x g, 4°C) and a 0.5 ml

aliquot of packed RBCs collected and stored at -70°C for hemoglobin assay and antioxidant enzyme analyses.

The blotted aorta (weighing approximately 60mg) was minced and subjected to Polytron homogenization (2 x 15 sec bursts at 50% max. speed) at 4°C in 1.2 mls of homogenization buffer (100mM Tris-HCl, pH 7.5) containing concentrated protease inhibitor cocktail [5.7 mM Phenylmethylsulfonylfluoride (PMSF), 15mM EDTA, 100uM Pepstatin, 50uM Leupeptin, 3uM Aprotinin]. For Western blotting, a 400 μl aliquot of aortic homogenate was centrifuged at 17,000 x g for 1.5 h at 4°C and the supernatant fraction was equally divided. The first aliquot was mixed with an equal volume of electrophoresis sample buffer [β -2-mercaptoethanol, glycerol, SDS, Tris [hydroxymethyl] aminomethane-HCl (Tris-HCl), pH 6.8, bromophenol blue], boiled for 5 min and stored at -70°C for later sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The second aliquot was stored at -70°C for total protein determination (177).

Heart tissue (weighing approximately 200 mg) was mixed with 2.5 mls homogenization buffer containing concentrated protease inhibitor cocktail and subjected to Polytron homogenization (2 x 15 sec bursts at 50% max. speed) at 4°C . A 400 μl aliquot of heart was centrifuged at 17,000g for 1.5 h at 4°C and equal amounts (650 μl) of heart supernatant were mixed with electrophoresis sample buffer for SDS-PAGE or stored for total protein determination.

Plasma cholesterol and triglyceride levels

Plasma cholesterol (178-179) and triglycerides (180-181) were quantified spectrophotometrically at 500 nm as the amount of quinoneimine dye formation using

diagnostic kits from Sigma (St. Louis, MO). The absorbance of this dye is proportional to the concentration of cholesterol or triglycerides in the sample.

Aortic cholesterol and triglyceride levels

Aortic cholesterol (178-179) and triglyceride (180-181) levels using aortic homogenates were quantified using diagnostic kits from Sigma (Missouri, USA).

HSP70

Equal amounts (25 µg) of aortic (n=40) or heart tissue (n=80) protein were electrophoresed on a 12% SDS-polyacrylamide gel and then transblotted onto nitrocellulose membranes (0.2 µm pore size) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Richmond, CA). The blots were blocked in TBS-T (Tris-buffered Saline, pH 7.5, 0.05% Tween-20) containing 2% skim milk at room temperature for 1 hr. After two washes in TBS-T for five min intervals, blots were incubated with a mouse monoclonal HSP70 (SPA-810, StressGen Biotechnologies, Victoria, B.C.) for 1 hour at room temperature in TBS-T containing 2% skim milk. For each gel, a purified HSP70 protein standard (SPP-755, StressGen Biotechnologies, Victoria, Canada) served as positive control. After three, 5 min washes in TBS-T blots were incubated with a 1/3000 dilution of alkaline phosphatase-conjugated goat-anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA). After 1 hr at room temperature, blots were washed twice for 5 min intervals in TBS-T, twice for 5 min in TBS, and the colour was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as a substrate (Sigma, Missouri, USA). Densitometry analysis of blots was performed using Sigma Scan Pro (Sigma, St. Louis, MO),

and the results were expressed as a ratio of sample antibodies to purified controls (i.e. sample HSP70/HSP70 standard). This allowed for the semi-quantitative comparison of various samples across different Western blots.

Antioxidants

Glutathione reductase (GRd) activity was determined in plasma (n=120) and heart (n=80) tissue supernatants (182) as the rate of disappearance of NADPH as previously described (152). Limited amounts of tissue available precluded measuring GRd activity in aortic tissue in this experiment. Absorbance at 340 nm was recorded over 5 min to monitor the conversion of NADPH to NADP.

Glutathione peroxidase (GPx) was determined in plasma (n=120) and aortic (n=40) and heart (N=80) supernatants by an indirect coupled assay procedure (182-183) as previously described (152). The enzyme activity for heart and aortic tissues was expressed as nmoles of NADPH oxidized to NADP per minute per mg of wet tissue using an extinction coefficient for NADPH at 340 nm of 6.2×10^6 M/cm. Glutathione peroxidase activity in plasma aliquots was expressed as nmoles NADPH/min/mg Hb.

Superoxide dismutase (SOD) activity was determined in aortic (n=40) and heart (n=80) supernatants using nitroblue tetrazolium (NBT) and expressed as units of SOD formed per mg protein (184). One unit of SOD activity is defined as the amount of enzyme activity that causes 50% inhibition of NBT reduction. The rate of inhibition of NBT reduction by superoxide generated by photoreduction of riboflavin was determined by measuring the absorbance at 560 nm.

Catalase (CAT) activity was measured in aortic tissue (n=40) by the method of Aebi (185). An aliquot (1.5 mls) of aortic tissue supernatant was mixed with 18 μ l of ethanol (95% ethanol/H₂O, 1:1 (v/v)) and incubated for 30 min at 4 °C. Then, 0.1 ml of cold Triton X-100 solution (10% v/v) in (50 mM Tris-0.01 mM EDTA, pH 7.6) was added. Immediately prior to assay, 500 μ l of this mixture was diluted to 10 ml with 50 mM phosphate buffer (pH 7.0). In a 3 ml cuvette, 2.0 ml of this diluted solution was added and the reaction initiated by adding 1.0 ml of freshly prepared 30 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0). After mixing, the rate of decomposition of H₂O₂ was determined from the absorbance change between 15 and 30 sec at 240 nm. Catalase enzyme activity was expressed as K/mg wet tissue, where K is the first order rate constant.

Correction of antioxidant components for blood contamination

Since RBCs contain significant amounts of activity of the enzymes assayed herein, it was necessary to correct tissue cytosolic enzyme activity values for any contribution due to the presence of contaminating RBCs. Hemoglobin (Hb) content was measured in RBC hemolysate and tissue cytosolic fractions by the method of Drabkin and Austin (186). Before assaying for Hb, RBC hemolysate was diluted 1:10 with double distilled H₂O. A 1.0 ml aliquot of 2.4 mM KCN and 1.0 ml aliquot of 1.8 mM K₃Fe(CN)₆ was added to 0.5ml of diluted RBC samples or 1.0 ml of heart tissue homogenate and incubated at room temperature for 30 min. The absorbance was read at 540nm. Hemoglobin concentration was calculated from a standard curve. The RBC activities were expressed on a mg per Hb basis and tissue cytosolic fractions were corrected based on the level of Hb.

Thiobarbituric acid reactive substances

The susceptibility of heart tissue to oxidative damage was quantified in terms of formation of thiobarbituric acid-reactive substances (TBARS) following exposure to increased concentrations of t-BHP *in vitro* (187). Briefly, 0.5 g of heart tissue (n=40) was homogenized in 1.2 ml of homogenizing buffer (50 mM Tris 0.1 mM EDTA, pH 7.6) by Polytron homogenization (2 x 15sec bursts at 50% max. speed) at 4⁰C, and cytosolic fractions were prepared by centrifugation (12,000 x g, 4⁰C, 15 min). Trichloroacetic acid (TCA) (12% w/v) was added to an equal volume of supernatant, combined with thiobarbituric acid solution (0.5%) and boiled for 15 min. The formation of TBARS was determined spectrophotometrically at 532 nm, as described by Tappel and Zalkin (188). Limitation in quantities of tissue available precluded TBARS measurements in aortic tissue.

Statistical analysis of data

Least squares analyses of variance and co-variance were carried out using the JMP (SAS Institute, North Carolina) program.

(1) Plaque score, plasma parameters (cholesterol, triglycerides, GRd, GPx, and SOD):

Plaque scores were square-root transformed before the analyses. ANOVA was first carried out with the model:

$$Y_{ijkl} = \mu + R_i + D_j + S_k + RD_{ij} + RS_{ik} + DS_{jk} + RDS_{ijk} + E_{ijkl}$$

where Y_{ijk} is the parameter measured of the l th individual on the j th diet, exposed to the k th stress treatment in the i th replication. R_i is one of the five replications. D_j is determined by whether the individual was on the cholesterol diet or the control diet. S_k reflects whether the

individual has been exposed to the stress protocol or not. RD_{ij} , RS_{ik} , and DS_{jk} , are the two-way interactions, and RDS_{ijk} is the 3-way interaction term. E_{ijkl} is the error term. In addition, ANOCOVA was carried out on plaque score with the model:

$$Y_{ijkl} = \mu + R_i + D_j + S_k + RD_{ij} + RS_{ik} + DS_{jk} + RDS_{ijk} + A_l + E_{ijkl}$$

where we regressed the plaque score on each of the aortic parameters (A_l) measured on the same individual: aortic cholesterol, triglycerides, GPx, SOD, and CAT. Because these aortic parameters were not measured in all the experimental birds, the sample size for these analyses was reduced to either 80 or 40.

(2) H/L ratio, lymphocyte count, heterophil count, aortic parameters (GPx, SOD, CAT, HSP70, cholesterol, triglycerides) and heart parameters (GRd, GPx, SOD, HSP70 and TBARS):

ANOCOVA was carried out on these parameters with the model:

$$Y_{ijkl} = \mu + R_i + D_j + S_k + RD_{ij} + RS_{ik} + DS_{jk} + RDS_{ijk} + B_l + E_{ijkl}$$

where we regressed these parameters on plasma cholesterol or plasma triglycerides levels (B_l). Aortic and heart parameters were also regressed on H/L ratio.

RESULTS

Heterophil to Lymphocyte ratio

Stress significantly increased the H/L ratio, indicating that the stress protocol was effective (Fig. 2.2). Unexpectedly, dietary cholesterol also increased H/L ratio. Furthermore, the effect of stress and dietary cholesterol was additive in that individuals that were on the cholesterol diet and stressed had the highest H/L ratio. Additionally, the H/L ratio regressed significantly

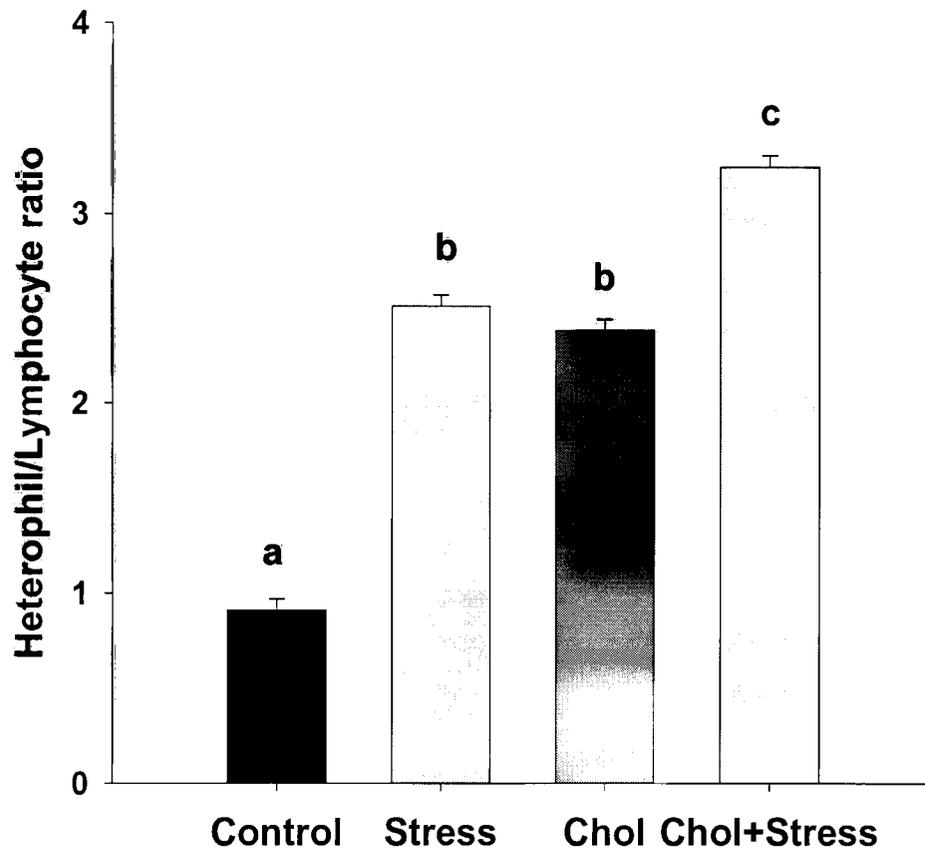


Figure 2.2 Heterophil to Lymphocyte ratio in atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.02$) by analysis of variance.

($P < 0.02$, $r = 0.63$) on plasma cholesterol level such that individuals with high plasma cholesterol levels also had high H/L ratios.

When the components of the H/L ratio, the lymphocyte count and the heterophil count, were examined, it was found that both stress and cholesterol feeding significantly ($P < 0.0001$) lowered lymphocyte counts and increased heterophil counts. Only lymphocyte count regressed significantly ($P < 0.02$, $r = -0.61$) on plasma cholesterol level. Individuals with high plasma cholesterol levels had low lymphocyte counts.

Aortic plaque scores

Plaque scores in all three branches of the aortic tree were significantly higher in birds fed the cholesterol diet. No plaques were found in birds fed the control diet. While stress had no additional effect on plaque score in the right brachiocephalic artery or the descending aorta, the plaque score of the left brachiocephalic artery (branch 1) was significantly higher in stressed birds than in non-stressed birds (Fig. 2.3). Plaque scores in replication 1 were significantly lower than all the later replications, which did not differ from one another. Replication effect was not significant in any of the other parameters measured.

Tissue and plasma lipid profiles

Both plasma cholesterol (Fig. 2.4) and triglyceride (Fig. 2.5) levels were higher in cholesterol-fed birds compared to birds fed a control diet. Unexpectedly, cholesterol and triglyceride levels in the aortic tissue of birds fed the cholesterol diet were significantly lower ($P < 0.02$) than those of the

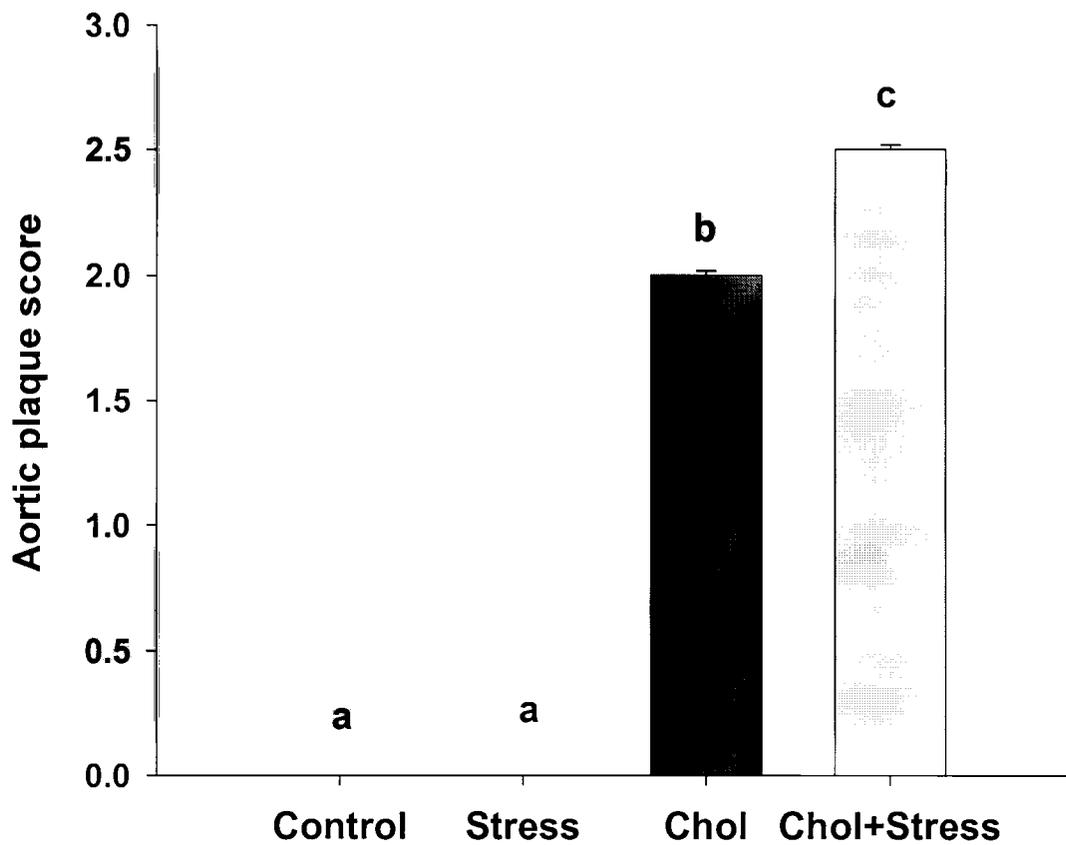


Figure 2.3 Aortic plaque score of the left brachiocephalic artery of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

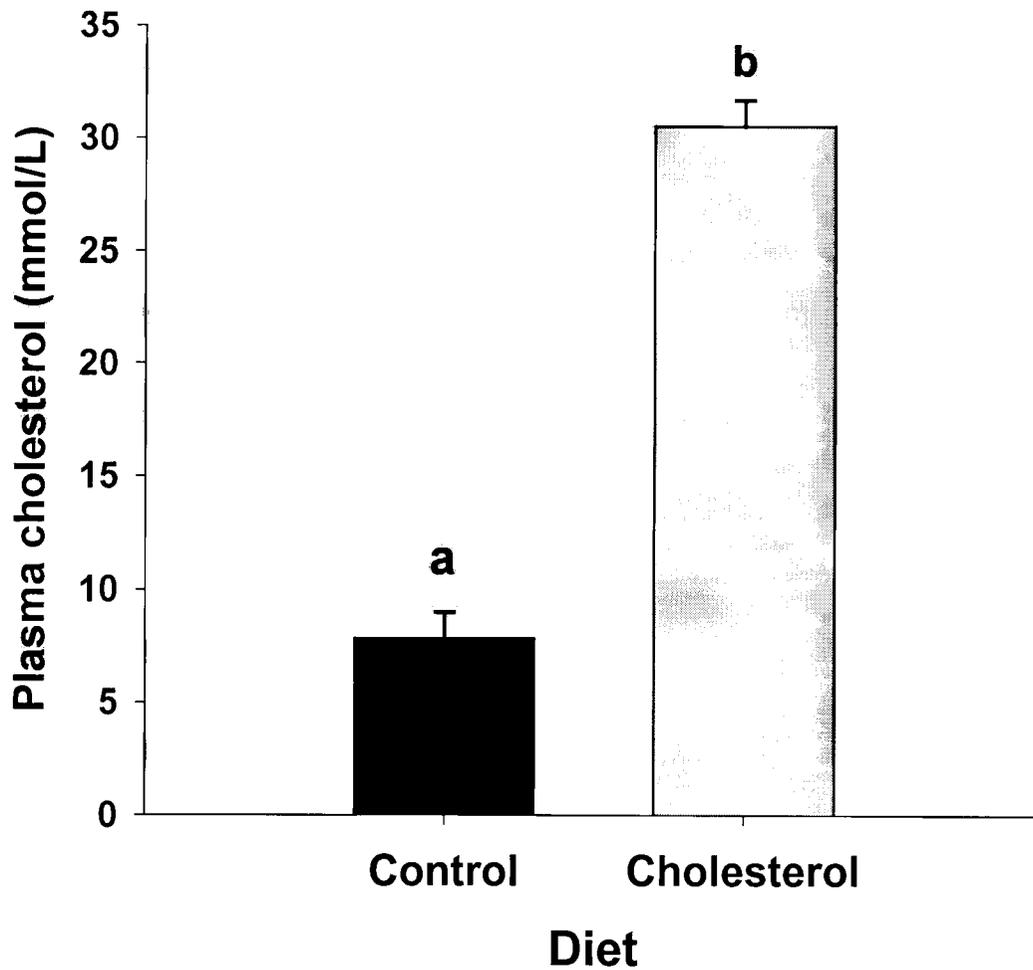


Figure 2.4 Plasma cholesterol levels in atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.0001$) by analysis of variance.

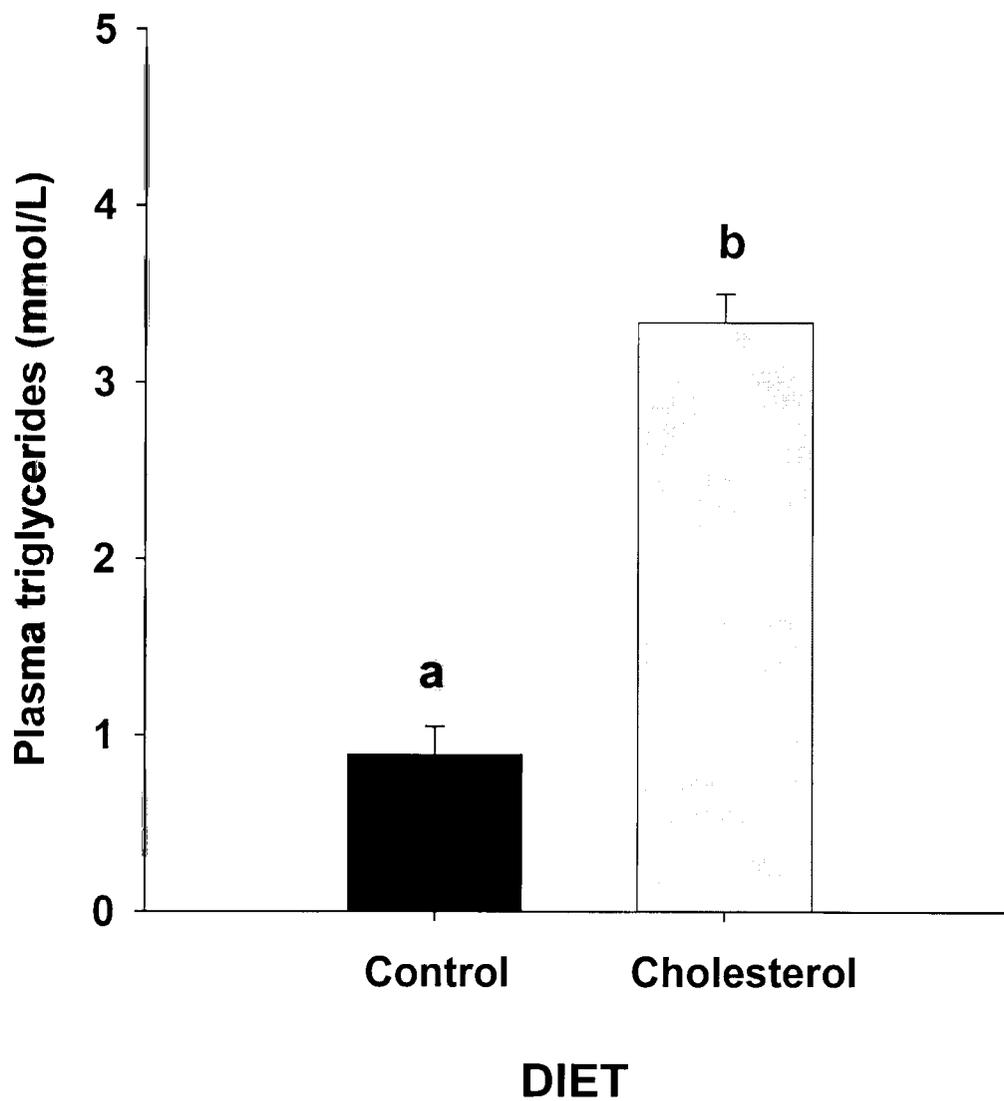


Figure 2.5 Plasma triglyceride levels in atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets. Values in each treatment group are means \pm SE for 20 individual birds. Columns under different letters were significantly different ($P < 0.0001$) by analysis of variance.

controls (data not shown). Stress did not have a significant effect on plasma or aortic cholesterol and triglyceride levels of either the cholesterol fed or control birds.

HSP70

Birds fed a control diet and not stressed had the lowest levels of aortic HSP70 (Fig. 2.6 A,B). Birds which were fed the control diet and stressed had significantly higher levels of aortic HSP70. Cholesterol feeding resulted in even higher aortic HSP70 levels. However, HSP70 levels of cholesterol-fed birds that were stressed were significantly lower than those of non-stressed cholesterol-fed birds. The pattern of heart HSP70 expression was similar to that of aortic HSP70 expression. (Fig. 2.7 A,B).

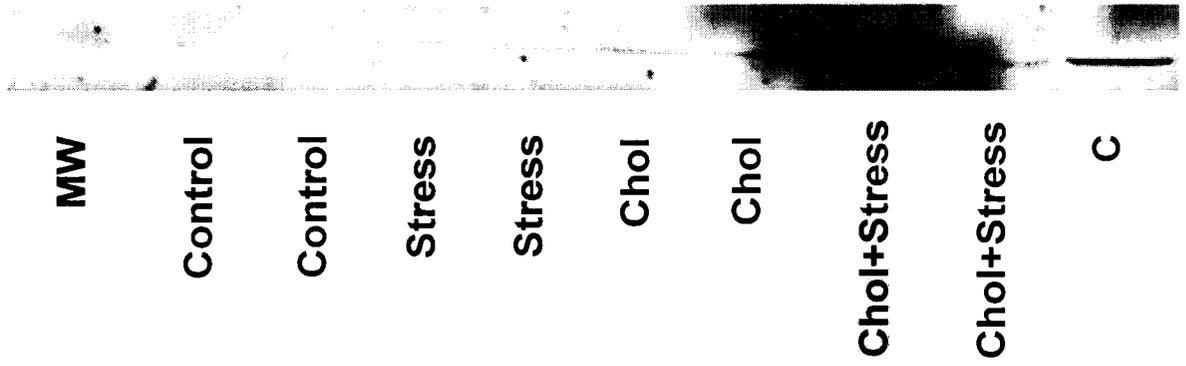
Antioxidant components

Birds on the cholesterol diet had significantly higher GRD levels in the plasma (Fig. 2.8) and the heart tissue (Fig. 2.9) than birds on the control diet. There was a tendency ($P < 0.06$) for stressed birds on the cholesterol diet to have lower plasma GRD levels than non-stressed birds on the same diet.

Cholesterol feeding and stress produced no detectable alterations in aortic GPx activities. Cholesterol-fed birds had significantly higher plasma GPx activities (Fig. 2.10) but lower heart GPx activities (Fig. 2.11) than birds on the control diet. Birds on the control diet that were stressed tended ($P < 0.06$) to have lower heart GPx activities than non-stressed individuals on the same diet.

Figure 2.6. Aortic HSP70 in atherosclerosis-susceptible (SUS) Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. (A) Representative Western blot analysis for HSP70 protein content. MW= molecular weight; C= purified HSP70. Two examples from each treatment are shown. (B) HSP70 immunoreactive protein content in SUS birds in *A* quantified by densitometry. Mean signal intensity in homogenates was normalized to the mean signal in purified HSP70. Means \pm SE for n=10 individual birds. Columns under different letters were significantly different ($P<0.0001$) by analysis of variance.

A.



B.

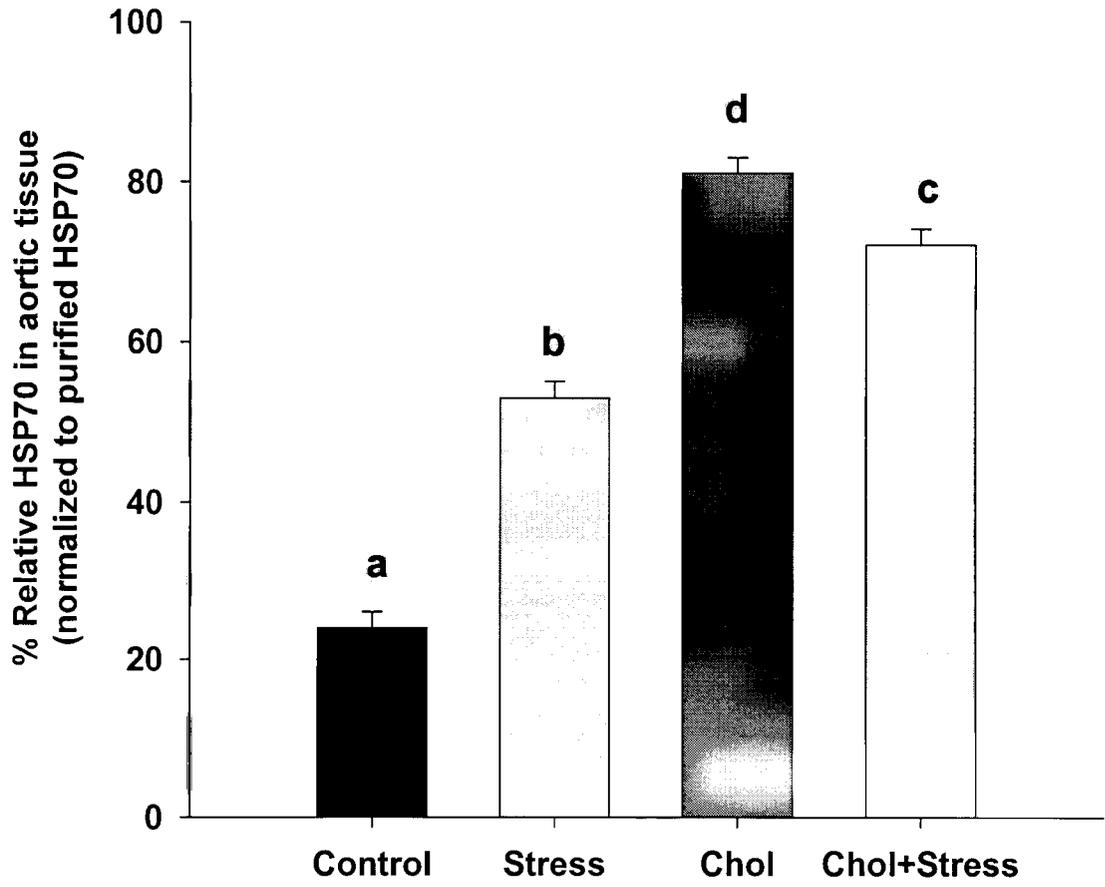
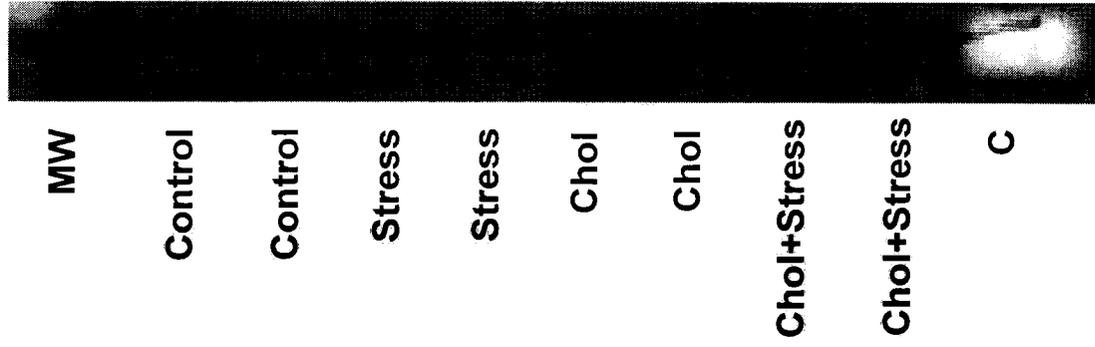
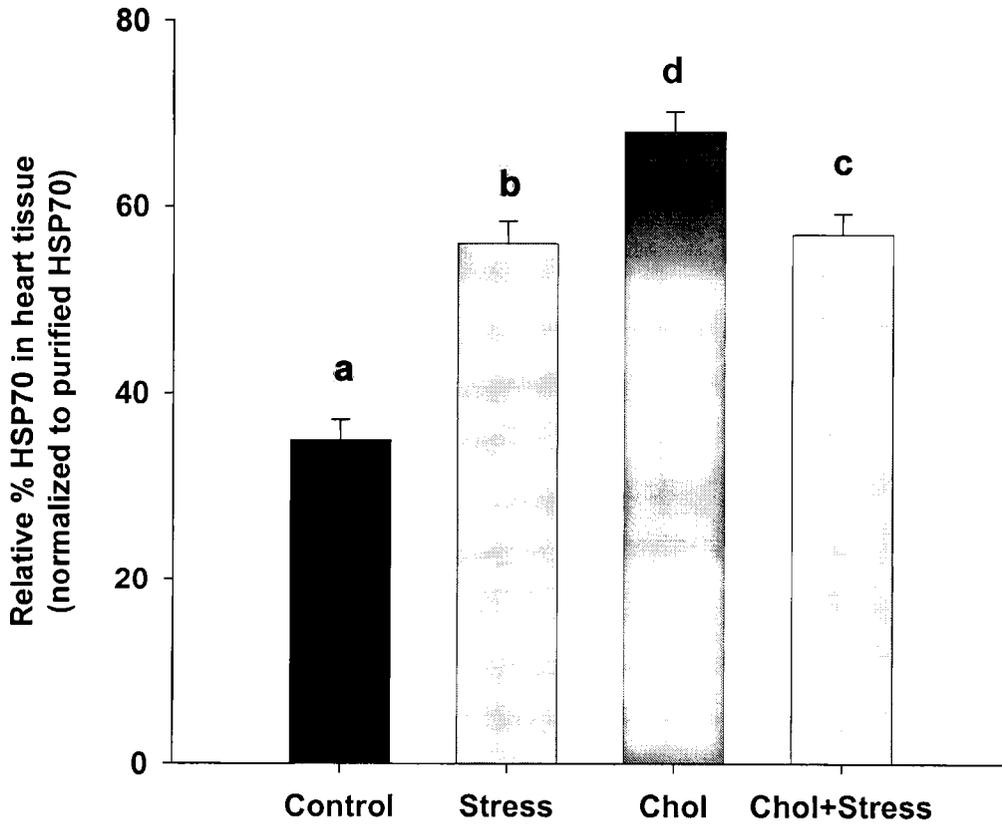


Figure 2.7 Heart HSP70 in atherosclerosis-susceptible (SUS) Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. (A) Representative Western blot analysis for HSP70 protein content. MW= molecular weight; C= purified HSP70. Two examples from each treatment are shown. (B) HSP70 immunoreactive protein content in SUS birds in *A* quantified by densitometry. Mean signal intensity in homogenates was normalized to the mean signal in purified HSP70. Means \pm SE for n=10 individual birds. Columns under different letters were significantly different ($P < 0.0001$) by analysis of variance.

A.



B.



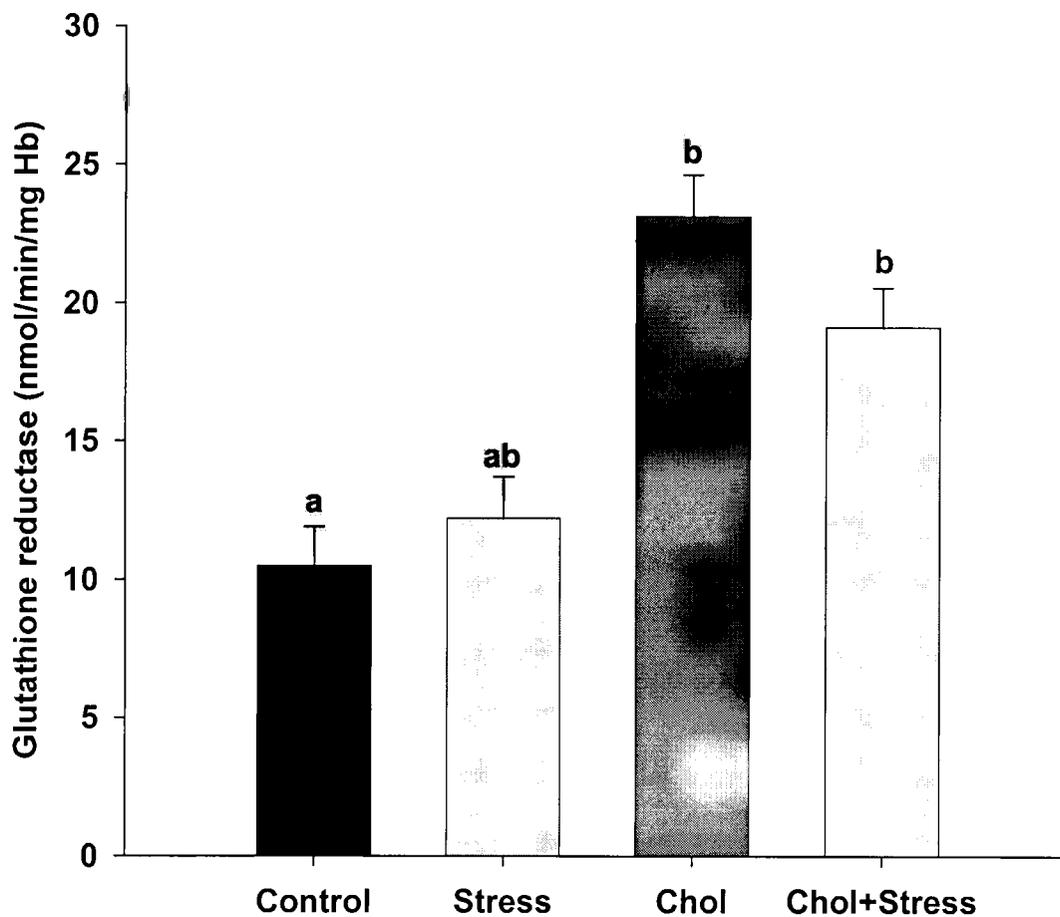


Figure 2.8 Glutathione reductase activity in plasma of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 20 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

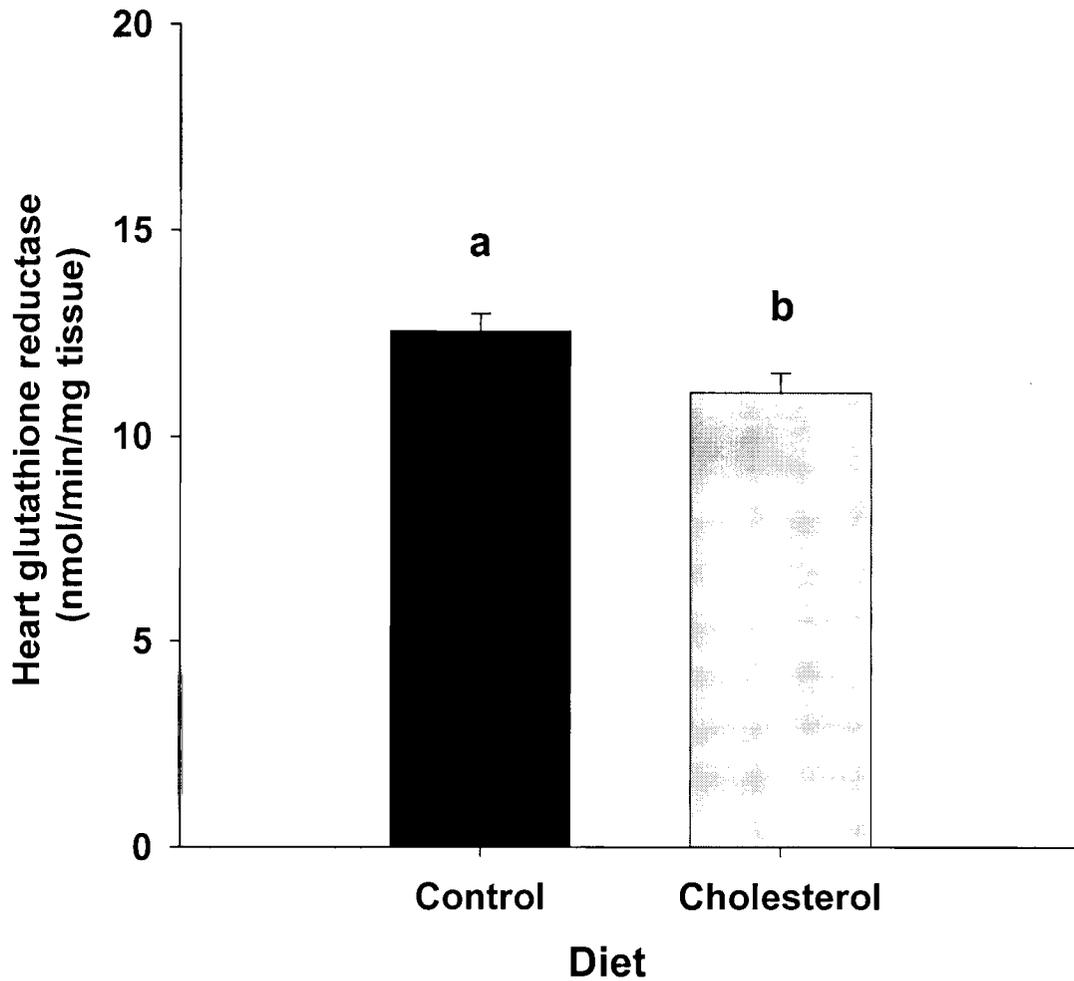


Figure 2.9 Glutathione reductase activity in heart tissue of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets. Values in each treatment group are means \pm SE for 20 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

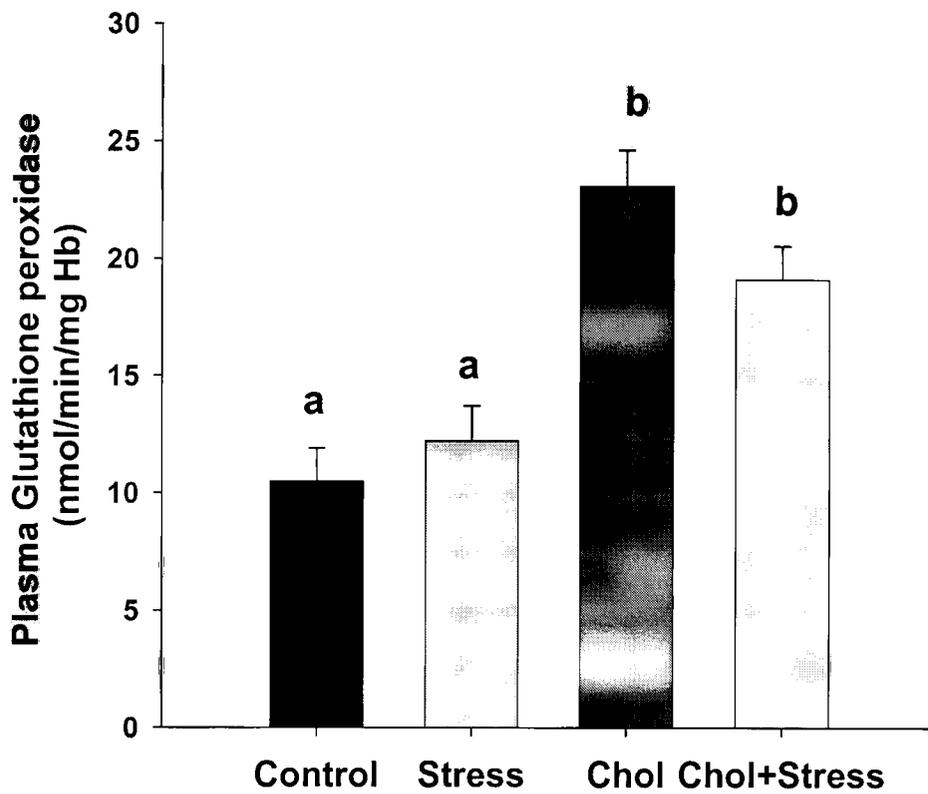


Figure 2.10 Glutathione peroxidase activity in plasma of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

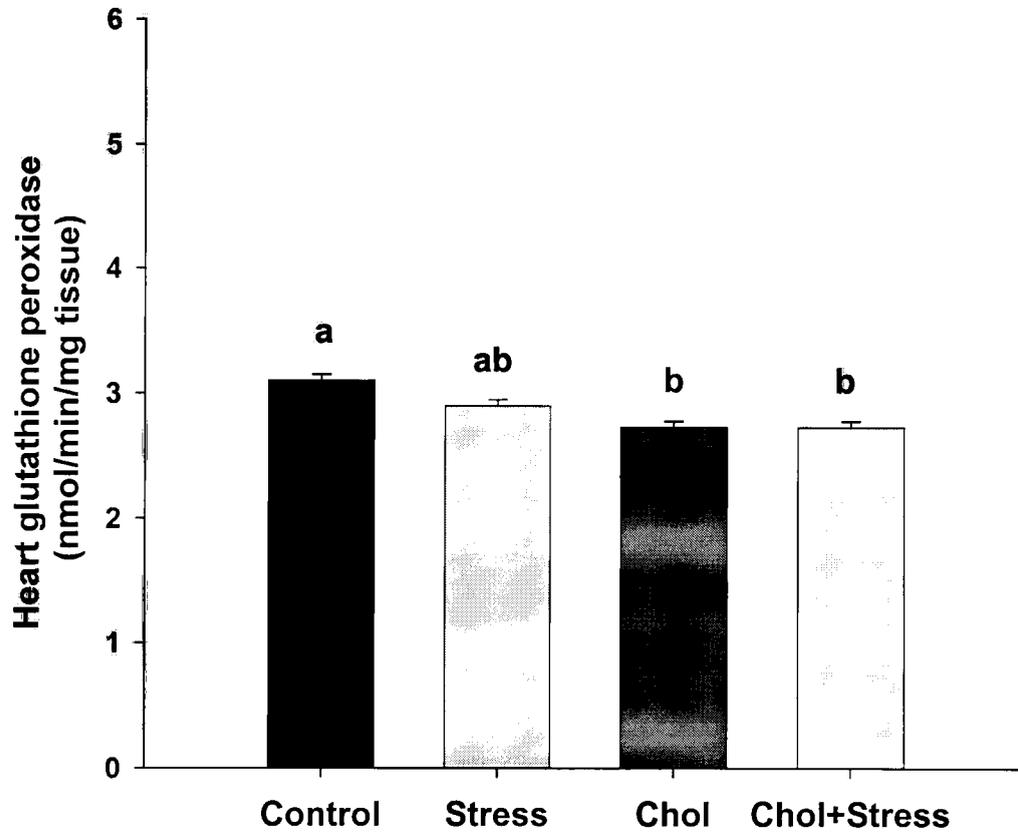


Figure 2.11 Glutathione peroxidase activity in heart tissue of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

Cholesterol feeding resulted in significantly lower ($P < 0.05$) mean SOD activities (24.1 ± 1.25 Units/mg protein) in the aortic tissue compared to birds fed the control diet (29.8 ± 1.16 Units/mg protein). There was also a weak but significant correlation ($P < 0.03$, $r = 0.28$) between aortic SOD activity and plaque score; individuals with high aortic plaque scores also had high aortic SOD activity. Stress did not have a significant effect on aortic SOD activity. Both stress and dietary cholesterol had significant effects on heart SOD activity (Fig. 2.12). Cholesterol-fed birds had lower heart SOD activities compared to birds fed the control diet. Birds that were stressed, regardless of diet, had lower SOD activities compared to non-stressed birds.

Cholesterol feeding alone produced no detectable alterations in aortic catalase activity. Birds exposed to stress, regardless of diet, had significantly lower ($P < 0.05$) mean catalase activity (0.0009 ± 0.006 K/mg wet wt) compared with non-stressed birds (0.0114 ± 0.007 K/mg wet wt).

Thiobarbituric acid reactive substances

Both cholesterol feeding and stress significantly ($P < 0.05$) elevated TBARS levels in heart tissue (Fig. 2.13) but their effects were not additive.

DISCUSSION

The present experiment investigated the effects of stress on atherosclerotic plaque development. We have confirmed that our stress protocol did elicit a stress response. Both the H/L ratio and heart TBARS levels were higher in birds exposed to the stress protocol.

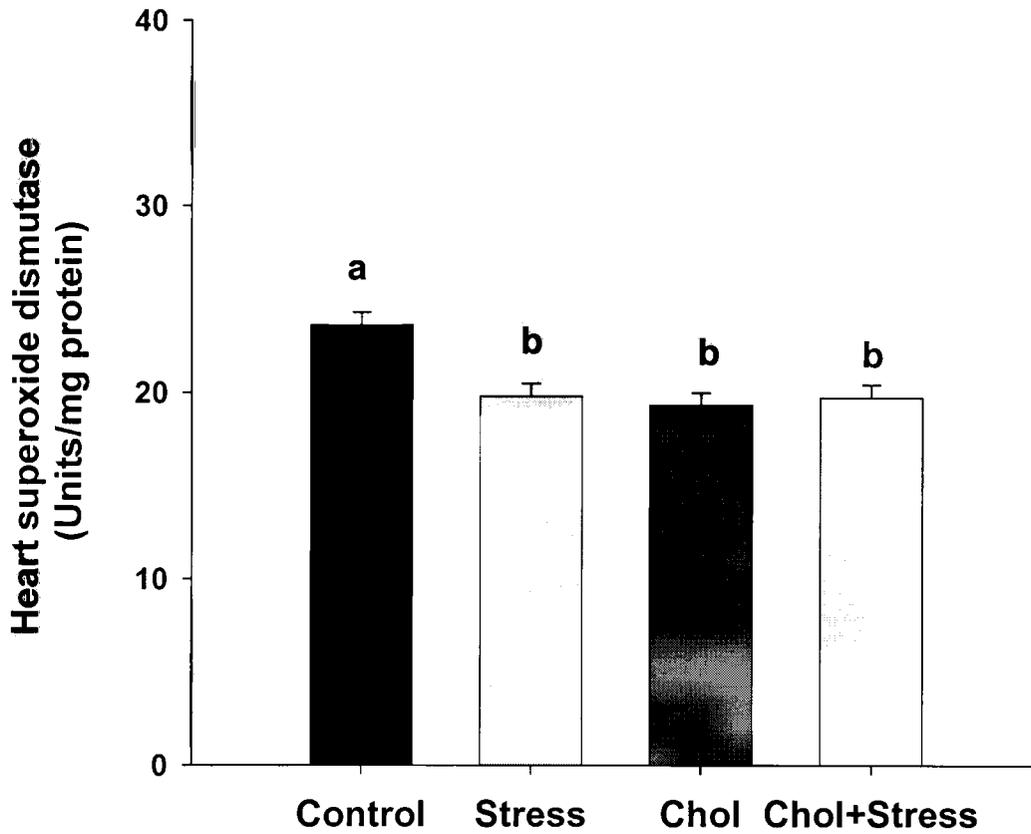


Figure 2.12 Superoxide dismutase in heart tissue of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

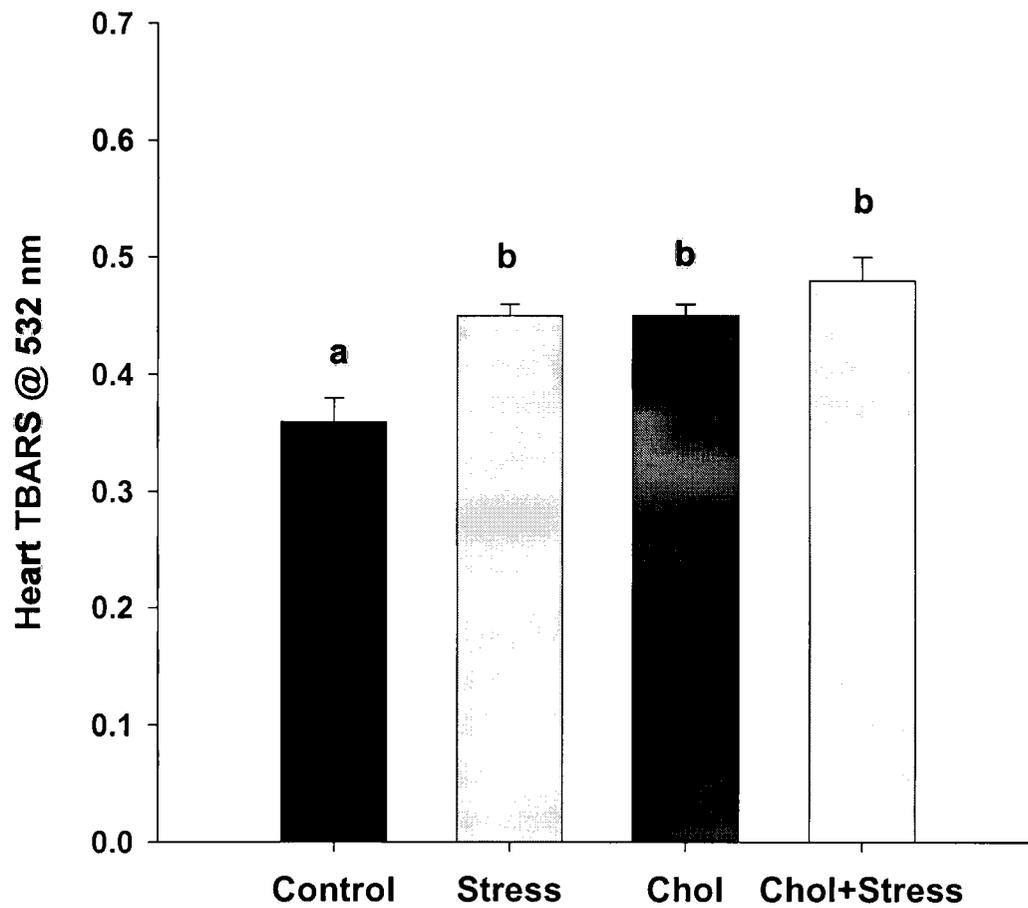


Figure 2.13 Thiobarbituric acid reactive substances (TBARS) in heart tissue exposed to 0.5 mM t-BHP of atherosclerosis-susceptible Japanese quail on control or cholesterol-supplemented diets and exposed to either 4 wks of stress or no stress. Values in each treatment group are means \pm SE for 10 individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance.

Our data also indicated that a high cholesterol diet elicited these same indices of stress in the experimental birds, independent of whether or not the birds were exposed to the stress protocol. While there is much evidence from both clinical and experimental studies indicating that stress can be a contributing factor to the development of atherogenesis (189-195), our hypothesis that psychological stress would increase the severity of atherosclerosis was only marginally supported by our plaque score data. Only the left brachiocephalic artery had a significantly higher plaque score in birds subjected to our stress protocol. In our previous study (154), plaque scores in the two brachiocephalic arteries increased rapidly, whereas plaque score in the descending aorta increased slowly in the early phase of atherogenesis. It seems possible that the brachiocephalic arteries may be subjected to greater stress than the descending aorta. The robust nature of our aortic plaque scoring method may also have been unable to detect small differences in early atherosclerosis, such as fatty streak formation or endothelial dysfunction (196) in the stress treatment groups. However, a previous study using scanning electron microscopy validated the dissecting microscope protocol used in the present experiment (197).

On the other hand, our stress protocol did cause biochemical changes associated with atherogenesis. Both aortic and heart HSP70 levels were lower in cholesterol-fed birds that were stressed. HSP70 is expressed in high concentrations in human atherosclerotic lesions (51) and oxidized LDL can induce HSP70 in cultured human endothelial cells (54). Some reports have suggested that an autoimmune reaction to HSP60 in the early inflammatory stages of atherosclerosis might be involved (55,198), whereas others have proposed that shear force may lead to the expression of HSPs by intimal cells, followed by recruitment of HSP-sensitized T

cells, leading to an inflammatory response that would initiate the process of atherogenesis (58). However, the majority of data suggest that the elaboration of these proteins has a protective effect (16-17, 61-62). Our stress protocol caused a higher level of HSP70 in birds fed the control diet. Cholesterol feeding also increased levels of HSP70 in non-stressed birds. However, HSP70 in stressed cholesterol-fed birds was significantly lower than that of non-stressed birds fed the same diet. These results suggest that the increased HSP70 level was a protective response, but stress may have compromised the protective response towards a high cholesterol diet.

It has been suggested that stress-induced higher levels of HSPs could be associated with an increase in antioxidant capacity (63-64). Heat-shocked *E. coli* increased the levels of HSP70 and the biosynthesis of SOD (64), and Chinese hamster ovarian carcinoma cells had increased SOD activity after exposure to hyperthermia (199). However, we have found no evidence of higher levels of HSPs associated with an increase in antioxidant capacity in the early phase of atherogenesis. Our cholesterol-fed birds generally had lower tissue antioxidant enzyme levels than those in birds fed the control diet, and this supported previously published findings of decreased antioxidant enzyme activities in heart and aortic tissue in humans with CAD (200-201) and in animal models of atherosclerosis (67, 152). In the present study, a positive correlation was found between aortic SOD activities and aortic plaque. However, Godin and coworkers (152) reported that increased plasma SOD activities was common in both RES and SUS strains of quail, and thus aortic SOD activities may not be associated with deposition of aortic plaque in the SUS strain. In a previous study, SUS quail fed the same diet supplemented with 0.5% cholesterol did not exhibit alterations in endogenous antioxidant status (197). However, the fact that lower tissue antioxidant enzymes were observed in the cholesterol-

supplemented birds suggests that at the lower dietary cholesterol content (i.e. 0.25%) used in the present study, the tissue antioxidant status may be able to modulate the development of atherosclerosis. Moreover, the additive protective effect of a high cholesterol intake could conceivably result in a stabilizing effect of membrane phospholipids against oxidation as well as cholesterol acting as an antioxidant to spare membrane fatty acids (202). Our stress protocol, which increased levels of tissue HSP70, significantly lowered aortic catalase and heart SOD levels. Although the combination of stress and cholesterol feeding did not further decrease antioxidant enzymes measured, the results show that the levels of GRd and GPx, and SOD activities of stressed birds fed a cholesterol diet were compromised. Furthermore, our results of increased TBARS in heart tissue following cholesterol feeding support a decrease in the antioxidant capacity of the myocardium and suggest that cholesterol and/or stress may affect the functional antioxidant status of the heart under these conditions.

Our previous studies have reported rapid aortic plaque development in SUS quail fed a 0.5% cholesterol supplemented diet (152,154). Other animal studies have also demonstrated that increased plasma cholesterol level is a reliable method for the induction of atherogenesis (203-204). However, lower levels of dietary cholesterol have been suggested for investigation of the temporal pattern of atherogenesis (205), in that excessively high levels of cholesterol in experimental studies may not accurately reflect the clinical situation – where atherosclerosis develops even with moderate elevations in plasma cholesterol. Therefore, our present study used only a 0.25% cholesterol supplement. As a result, after 4 weeks on the experimental diet, birds had approximately two-thirds the plasma cholesterol and triglycerides levels, and lower plaque scores compared to our previous studies. The progress of atherogenesis quantitated by plaque score may be slower in our present study. This may be the reason why we did not find

significant increases in aortic cholesterol and triglycerides as we did in our previous work. It is therefore imperative to carry out a longer term study to examine the effects of chronic and acute stress during the full course of atherogenesis.

CONCLUSIONS

The studies conducted in this chapter suggest that psychological stress may in part, increase the severity of aortic plaque formation. Furthermore, these experiments characterized some of the basic metabolic differences in plasma lipids, aortic plaque score, HSP70 and endogenous antioxidants between control and cholesterol-supplemented SUS Japanese quail exposed to the stress protocol.

CHAPTER 3

THE INTERRELATIONSHIPS OF PSYCHOLOGICAL AND OXIDATIVE STRESS IN RELATION TO ATHEROGENESIS IN THE ATHEROSCLEROSIS-SUSCEPTIBLE (SUS) JAPANESE QUAIL

PREFACE

This investigation was supported by research grants to D.V. Godin and K.M. Cheng from the British Columbia HealthCare and Research Foundation. A manuscript reporting the studies in this Chapter will be submitted for publication to Molecular and Cellular Biochemistry and will be coauthored with C.R. Nichols, M.E. Garnett, D.V. Godin, and K.M. Cheng. C.R. Nichols contributed to project planning, animal care and designing the stress protocol. M.E. Garnett provided technical assistance with the tissue antioxidant assays.

INTRODUCTION

There is widespread opinion that psychological stress can favor the development and influence the course of coronary artery disease (CAD) (206-208). One important implication of research on psychological risk factors for coronary disease is that interventions targeting these factors could reduce morbidity and mortality (209-211). In understanding the possible influence of psychological stress on CAD, it is useful to make the distinction between chronic and acute risk factors (211). Chronic risk factors – which can be either biological or behavioural – are longstanding and exert their influence over an extended period of time (212). Chronic

psychological stress results in autonomic nervous system activation mediating heightened cardiac responsiveness (213), impairing endothelium-dependent vasomotor responses of the coronary arteries (214) and producing dysfunction of the endothelium (215). An acute risk factor is a transient pathophysiologic change that results from exposure to short-term psychological factors that can trigger clinical events such as increases in heart rate, in the release of catecholamines and corticosteroids, in cardiac output and blood pressure (216). In individuals with atherosclerosis, such physiological changes may increase vulnerability to clinical cardiac events. In a recent report, psychological factors such as mental stress, psychosocial strain, and psychiatric disorders in patients with atherosclerosis were linked to impaired anticoagulant function and the promotion of a hypercoagulable state (217). Other studies have demonstrated that mental stress may result in asymptomatic transient myocardial ischaemia which is not explained by traditional risk factors (218-219). Although chronic and acute psychological stress are hypothesized to increase the risk of clinical cardiac events, it is important to note that because of the complex pathophysiology of coronary disease, psychological stress variables may relate to different stages of the disease process (220-223).

Heat shock proteins (HSPs) may be a biochemical link between stress and ATH. These proteins are highly conserved from bacteria to man (224), and are inducible by a variety of physical stressors, such as mechanical stress (225), infections and disease (226) and exposure to oxygen radicals (227-228). HSP70, the major HSP, has been found during the development of the primary atherosclerotic lesion and in advanced necrotic lesions (51,171,227).

Furthermore, *in-vitro* studies have shown that oxidized low density lipoprotein (LDL), a major component of the atherosclerotic plaque, can induce HSP70 and HSP32 or heme oxygenase (HO) expression. The foregoing observations suggest that HSPs may be involved in

atherogenesis. However, the elaboration of these proteins can also increase aortic cell survival (51,227). Indeed, the addition of exogenous HSP72/73 to cultured aortic cells from cynomolgous monkeys increased their survival following exposure to prolonged heat stress *in vitro* (52). The gene transfer of inducible HO (HO-1) into aortic smooth muscle cells from apo-E deficient mice reduced atherosclerotic plaque formation (127). In low-density lipoprotein (LDL) receptor knockout mice, gene transfer of HO-1 into aortic tissue, and subsequent increased expression of HO-1, resulted in a reduction of plaque formation, whereas HO inhibition using Sn-protoporphyrin IX promoted lesion development (131). Therefore, increases in HSP expression can protect against stress, which has been implicated in the pathogenesis of atherosclerosis.

Animal model studies are of value for investigating the interaction of CAD risk factors, such as acute and chronic psychological stress, HSP expression and antioxidant/oxidant balance. In Chapter 2, it was found that chronic stress increased aortic plaque score in the left brachiocephalic artery. Furthermore, chronic stress increased the heterophil/lymphocyte (H/L) ratio in non-cholesterol-supplemented birds and further increased H/L ratio in the stressed, cholesterol-supplemented treatment group. As well, chronic stress increased HSP70 levels in heart and aortic tissues, while the combination of chronic stress and cholesterol decreased HSP70 levels in these tissues. The stress protocol lowered aortic catalase and heart superoxide dismutase (SOD) levels while, increased thiobarbituric acid-reactive substances (TBARS) levels were detected in heart tissue. Although Chapter 2 examined some of the basic metabolic differences between control and cholesterol-supplemented SUS quail exposed to a 'continuous' stress protocol, the study design did not allow for a temporal investigation of the effects of stress. Therefore, this study was undertaken to investigate the time-course of interrelationships

among stress applied early and measured later and stress applied immediately before sampling, HSP expression and antioxidant status all in relation to atherogenesis, since previous studies have suggested that plaque formation may be biochemically distinct in early and late phases of atherogenesis, at least in terms of changes in antioxidant components (154).

MATERIALS AND METHODS

Animals

The atherosclerosis-susceptible (SUS) Japanese quail strain was acquired by the University of British Columbia Quail Genetic Resource Centre (QGRC) from North Carolina State University in 1989, and has since undergone further selection for susceptibility to atherosclerotic plaque formation induced by dietary cholesterol (1% w/w) (153). The history of this selection process has been described by Shih *et al.* (146).

Experimental Diets

From the time of hatching to eight weeks of age, SUS quail were fed a commercial diet which is 2/3 wheat and 1/3 corn-based, containing 26 % protein. During each experiment, quail were housed in community cages with one treatment group per cage, and were fed experimental diets. These were either a synthetic diet prepared by the feed mill at the Agriculture and Agri-Food Canada Research Station at Agassiz, British Columbia, according to the NRC standard

recommended for quail (the “control diet”) or the same synthetic diet supplemented with cholesterol 0.25% w/w (the “cholesterol diet”) (Table 2.1).

Experimental procedures

One hundred and seventy 8-week old SUS quail were assigned to one of 17 treatment groups (n = 10 per group; Table 3.1). The birds were fed a control diet or the cholesterol diet. In addition, birds were kept in their home cages or assigned to “early” or “recent” stress treatment groups, in which early stress protocols were done Monday-Friday and late stress protocols were done Wednesday-Sunday. Animals in all stress groups were exposed to the stress protocol, as described in Chapter 2, for one hour, five days a week for 4 weeks. “Early” stress treatment groups were exposed to the stress protocol for 4 weeks and were then allowed to recover for 4 or 8 weeks. “Recent” stress groups were exposed to the stress protocol for 4 weeks immediately before being sacrificed. Measurements were taken in groups of birds on cholesterol or control diets for 0, 4, 8 and 12 weeks, respectively. These time points were chosen to encompass both early and late phases of atherosclerotic plaque development.

After exposure to their experimental protocol, quail were sacrificed by decapitation and trunk blood was collected into chilled heparinized tubes and plasma separated by low-speed centrifugation (1000 x g, 5 min, 4⁰C). Aliquots of plasma were collected for analysis of plasma lipids and a blood smear was made for differential blood counting and determination

Table 3.1 Distribution of atherosclerosis-susceptible quail (n=10) into 17 treatment groups.

Age	Control Diet /No Stress	Cholesterol Diet /No Stress	Control Diet / Stress		Cholesterol Diet / Stress	
			(early stress)	(recent stress)	(early stress)	(recent stress)
8 wks	Group 1*					
12 wks	Group 2	Group 3	Group 4		Group 5	
16 wks	Group 6	Group 7	Group 8	Group 9	Group 10	Group 11
20 wks	Group 12	Group 13	Group 14	Group 15	Group 16	Group 17

- *Group 1: 8 weeks old; not fed experimental diets
- Group 2: 12 weeks old; no stress; fed control diet for 4 wks
- Group 3: 12 weeks old; no stress; fed cholesterol diet for 4 wks
- Group 4: 12 weeks old; stressed at 8-12 wks of age; fed control diet for 4 wks
- Group 5: 12 weeks old; stressed at 8-12 wks of age; fed cholesterol diet for 4 wks
- Group 6: 16 weeks old; no stress; fed control diet for 8 wks
- Group 7: 16 weeks old; no stress; fed cholesterol diet for 8 wks
- Group 8: 16 weeks old; stressed at 8-12 wks of age; fed control diet for 8 wks
- Group 9: 16 weeks old; stressed at 12-16 wks of age; fed control diet for 8 wks
- Group 10: 16 weeks old; stressed at 8-12 wks of age; fed cholesterol diet for 8 wks
- Group 11: 16 weeks old; stressed at 12-16 wks of age; fed cholesterol diet for 8 wks
- Group 12: 20 weeks old; no stress; fed control diet for 12 wks
- Group 13: 20 weeks old; no stress; fed cholesterol diet for 12 wks
- Group 14: 20 weeks old; stressed at 8-12 weeks of age; fed control diet for 12 wks
- Group 15: 20 weeks old; stressed at 12-16 wks of age; fed control diet for 12 wks
- Group 16: 20 weeks old; stressed at 8-12 weeks of age; fed cholesterol diet for 12 wks
- Group 17: 20 weeks old; stressed at 12-16 weeks of age; fed cholesterol diet for 12 wks

of H/L ratio, as previously described in Chapter 2. The aortic tree was dissected out, immediately proximal to the brachiocephalic arteries, and the aorta to the iliac branching; the vessels were then opened longitudinally, examined under a dissecting microscope (10-30X) for gross abnormalities and assigned a plaque score of 0-4 as previously described (152).

Plasma cholesterol and triglycerides

Plasma cholesterol (178-179) and triglycerides (180-181) were quantified spectrophotometrically at 500 nm as the amount of quinoneimine dye formation using diagnostic kits from Sigma (St. Louis, MO).

Thiobarbituric acid-reactive substances (TBARS)

Myocardial susceptibility to oxidative stress was quantified in terms of the formation of thiobarbituric acid-reactive substances (TBARS) following exposure to increasing concentrations of t-BHP *in vitro* (187). Briefly, 0.5 g of heart tissue was homogenized in 1.2 ml of homogenizing buffer (50 mM Tris 0.1 mM EDTA, pH 7.6) using a Polytron homogenizer (2 x 15sec bursts at 50% max. speed) at 4⁰C, and cytosolic fractions were prepared by centrifugation (12,000 x g, 4⁰C, 15 min). Trichloroacetic acid (12% w/v) was added to an equal volume of supernatant, combined with thiobarbituric acid solution (0.5%) and boiled for 15 min. The formation of TBARS was determined spectrophotometrically at 532 nm, as described by Tappel and Zalkin (229).

SDS-PAGE and Western blotting

Heart and aortic tissue proteins were electrophoresed on a 12% SDS-polyacrylamide gel, transblotted onto nitrocellulose membranes (0.2 μm pore size), and probed with monoclonal and polyclonal antibodies to HSP70 and HO-1, respectively (Chapter 2). Monoclonal antibodies to HSP70 and polyclonal antibodies to HO-1 were purchased from StressGen (Victoria, BC, Canada). Purified HSP70 and HO-1 proteins were included on each blot to allow for the semi-quantitative comparison between Western Blots.

Heme oxygenase enzyme activity

Heme oxygenase enzyme activity was measured in heart and aortic supernatants by bilirubin generation as previously described (230). Bovine liver (Grand Mason Slaughter House, Surrey, Canada) served as the source of partially purified bilirubin reductase (230). A reaction mixture containing 1 mg/ml of heart or aortic cytosolic protein, 25 μM hemin, 1.0 mg/ml biliverdin reductase, 2 mM glucose 6-phosphate, 1 U glucose 6-phosphate dehydrogenase, 0.25 M sucrose, 20mM Tris, pH 7.4 was preincubated in a final volume of 950 μL at 37⁰C for 10 min in the dark. The reaction was initiated by adding 50 μL of 20 mM NADPH and subsequently scanned with a spectrophotometer (Perkin Elmer, Connecticut, USA). The amount of bilirubin formed was determined from the difference in optical density between 462 and 530 nm (extinction coefficient, 40 $\text{nm}^{-1} \text{cm}^{-1}$ for bilirubin). The reaction mixture without the NADPH-generating system served as a blank. HO enzyme activity was expressed as picomoles of bilirubin formed/mg protein/h.

Ferritin assay

Heart and aortic tissues were assayed for ferritin protein by an enzyme-linked immunosorbent assay (ELISA) method (231). Heart and aortic supernatants were diluted in a coating solution (50 mM sodium carbonate-bicarbonate buffer, pH 9.6), and 96-well plates were coated with 50 μ L of supernatant-coating solution and incubated overnight in a humidified chamber at 4⁰C. Plates were washed three times with PBS-T (10mM PBS, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with blocking buffer (5% BSA/PBS) for 2 h in a humidified chamber at room temperature. Plates were washed three times with PBS-T, and were incubated at room temperature for 1 hr with rabbit anti-rat ferritin antibody (50ul/well, 1:1000; a gift from R.S. Eisenstein, Univ. of Wisconsin), washed four times with PBS-T, and subsequently incubated for 1 h with goat anti-rabbit IgG horse radish peroxidase conjugate (50 μ L/well, 1:5000). Color was developed for 10 min using 3',3',5',5'-tetramethylbenzidine (0.01mg/ml, 10 μ L) and the reaction was stopped by adding 50 μ l 1 M H₂SO₄ to each well. Absorbance was measured at 450nm using a microplate reader (BioTek instruments, Vermont). Purified ferritin from rat liver was used as standard (Sigma). The results were expressed as nanograms ferritin/mg protein.

Catalytic iron

Iron available to participate in free radical reactions was measured using the bleomycin assay (232). The DNA-bleomycin complex combines with the Fe (III) iron in the sample and is

reduced by ascorbic acid, generating a ferryl species which results in degradation of DNA. One product formed during deoxyribose degradation is malondialdehyde (MDA), when reacts with TBA gives a pink chromogen and can follow DNA degradation by bleomycin spectrophotometrically.

All buffers and reagents, except bleomycin, were treated with Chelex-100 (Sigma) to remove extraneous iron. Previously collected heart and aortic supernatants (10 μ l; diluted 1:1 [vol:vol] with double distilled H₂O) were incubated with 50 μ L of MgCl₂ (50 mM), 400 μ L salmon sperm DNA (500 μ g/ml), 10 μ L bleomycin (1.5 U/ml), 50 μ L of Tris (1.0 M, pH 7.4), and 25 μ L of ascorbate (7.5 mM, pH 7.4) for 1 hr at 37⁰C. The reaction was stopped by the addition of 50 μ L of EDTA (100 mM). Thiobarbituric acid (TBA) (0.3% [wt/vol] in 50 mM NaOH) and 1.5 N HCl were added and the mixture was incubated in a water bath at 80⁰C for 20 min. The sample was vortexed, centrifuged at 12,000 x g for 5 min and the absorbance of the supernatant was read spectrophotometrically at 532 nm. Iron content was calculated according to a standard curve and results reported in μ g/mg protein.

Immunohistochemistry

Aortic tissue was mounted on brass pegs using Tissue Tek II O.C.T. compound (Miles Laboratories, Elkhart, IN) and serial sections, 6 μ m thick, were cut on cryostat at -20⁰C. Sections were mounted on poly-l-lysine-coated slides and fixed in 10% neutral buffered formalin for 5 minutes. The slides were then washed in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1% BSA and 1% goat serum at room temperature for 30 minutes.

For detection of HO-1, cryostat sections (6 μm) were incubated overnight at 4⁰C with a 1:25 polyclonal rabbit anti-HO-1 antibody in a humidified chamber. After incubation, the slides were rinsed twice for 5 min in PBS and further incubated with a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody for 30 minutes at 37⁰C, washed twice in PBS, and counterstained with Evans Blue. The slides were mounted in glycerin and viewed with a fluorescent microscope (Zeiss) fitted with a 100-W mercury (Zeiss) lamp.

For detection of ferritin, cryostat sections (6 μm) were pretreated with 3% H₂O₂ in methanol for 10 minutes at room temperature to exhaust endogenous peroxidase activities. The slides were then incubated with 1:50 dilution of rabbit anti-rat ferritin polyclonal antibody (a gift from R.S. Eisenstein, Univ. of Wisconsin) for 2 hr at room temperature. After incubation, the slides were washed three times in PBS and further incubated with a 1:100 dilution of goat anti-rabbit horseradish peroxidase-conjugated antibody. After three washes in PBS, color was developed in 0.1% 3,3'-diaminobenzidine in 0.003% H₂O₂. Negative controls were performed by omission of the rabbit anti-rat ferritin antibody.

Iron Histochemistry

Iron deposits in atherosclerotic lesions were assessed by Perl's Prussian Blue reaction with 3,3'-diaminobenzidine (DAB) intensification (233). Aortic cryostat sections (6 μm) were incubated in equal quantities of 2% potassium ferrocyanide and 2% HCl for 20 min and rinsed in running tap water for 3 min. Next, sections were incubated with 0.5 % DAB in 0.1M PBS (pH 7.4) for 20 min, followed by 15 min in the same medium containing 0.005% H₂O₂. The reaction was stopped by rinsing in deionized H₂O for 30 min. Negative control slides were

carried through the DAB intensification without preincubation with Perl's solution. No positive staining was detected in the negative control sections.

Antioxidant components

Glutathione reductase activity was determined in plasma and heart supernatants as the rate of disappearance of NADPH at 340 nm (182). Heart supernatants were assayed for SOD activity using nitroblue tetrazolium (NBT) and expressed as units of SOD formed per mg protein (184). Hemoglobin (Hb) content was measured in red blood cell (RBC) hemolysate and tissue cytosolic fractions to correct tissue cytosolic enzyme activity values for any contribution due to the presence of contaminating RBCs (184).

Statistical analysis

Least squares analyses of variance (ANOVA) and co-variance (ANOCOVA) were carried out on parameters measured at each time point using the JMP (SAS Institute, North Carolina) program.

(1) Plaque score, plasma parameters (cholesterol, triglycerides, and GRd):

Plaque scores were square-root transformed before the analyses. ANOVA was first carried out with the model:

$$Y_{ijk} = \mu + D_i + S_j + DS_{ij} + E_{ijk}$$

where Y_{ijk} is the parameter measured of the k th individual on the i th diet, exposed to the j th stress treatment. D_i is determined by whether the individual was on the cholesterol diet or the

control diet. S_j reflects whether the individual has been exposed to early stress, stress immediately prior to sampling, or no stress. DS_{ij} is the interaction between diet and stress. E_{ijkl} is the error term. In addition, ANOCOVA was carried out on plaque score with the model:

$$Y_{ijkl} = \mu + D_i + S_j + DS_{ij} + A_l + E_{ijkl}$$

where we regressed the plaque score on each of the aortic parameters (A_l) measured on the same individual: aortic cholesterol, triglycerides, HSP70, HO, HO-1, iron, and ferritin.

(2) H/L ratio, lymphocyte count, heterophil count, aortic parameters (HO, HO-1, iron, ferritin, HSP70, cholesterol, triglycerides) and heart parameters (GRd, SOD, HSP70, HO, HO-1, iron, ferritin and TBARS):

ANOCOVA was carried out on these parameters with the model:

$$Y_{ijkl} = \mu + D_i + S_j + DS_{ij} + B_l + E_{ijkl}$$

where we regressed these parameters on plasma cholesterol or plasma triglycerides levels (B_l). Aortic and heart parameters were also regressed on H/L ratio.

RESULTS

A table highlighting the significant stress and/or diet interactions has been included in Table 3.2. Birds were exposed to early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress treatment groups, and fed a control or cholesterol-supplemented diet.

Table 3.2 Summary of significant stress and/or diet interactions in atherosclerosis-susceptible Japanese quail

Parameter Measured	Stress	Cholesterol	Stress/Cholesterol
Plasma triglyceride	↓ (after 8 wks recovery)	↑ (4, 8, 12 wks)	-
Plasma cholesterol	↑	↑ (4, 8, 12 wks)	↓ (after 4,8 wks recovery)
Plasma GRd	-	↑ (4, 8, 12 wks)	-
H/L ratio	↑ (immediately-4,8,12 wks)	↑ (4,8, 12 wks)	↑ (immediately 4,8,12 wks) ↓ (after 8 wks recovery)
Plaque score	-	↑ (all branches)	-
Heart HSP70	↑ (immediately-4,8,12 wks)	↑ (4, 8, 12 wks)	↓ (after 8 wks recovery) ↓ (immediately- 4wk)
Aortic HSP70	↑ (after 8 wks recovery) ↑ (immediately-4,8,12 wk) ↑ (after 4 wks recovery)	↑ (4, 8, 12 wks)	↓ (immediately-4 wk)
Heart HO-1	↑ (immediately 8,12 wks)	↑ (4, 8, 12 wk)	↑ (immediately-4,8,12 wks) ↓ (after 4, 8 wks recovery)
Aortic HO-1	↑ (immediately 4,8,12 wks)	↑ (4, 8, 12 wk)	↑ (immediately-8 12 wk) ↓ (immediately 4 wk)
Aortic HO activity	-	↑ (4, 8, 12 wk)	↓ (after 4 wks recovery)
Heart HO activity	-	↑ (4, 8, 12 wk)	-
Aortic Ferritin	-	↑ (4, 8, 12 wk)	↓ (immediately- 4wk)
Heart Ferritin	-	↑ (4, 8, 12 wk)	↓ (after 4,8 wks recovery)
Aortic catalytic iron	-	↑ (4, 8, 12 wk)	-
Heart catalytic iron	-	↑ (4, 8, 12 wk)	-
Plasma GRd	-	↑ (4, 8, 12 wk)	-
Heart GRd	-	↑ (12 wk)	-
Heart SOD	-	↑ (12 wk)	-
Heart TBARS	↑ (immediately 4,8,12 wks)	↑ (4, 8, 12 wk)	↑ (immediately 4,8,12 wks)

Heterophil/Lymphocyte ratio

H/L ratios were significantly increased in stressed birds if the blood samples were taken immediately after the completion of the stress protocol (Table 3.3). However, if the blood samples were taken 4 or more weeks after the stress protocol, H/L ratios were similar to those of unstressed birds. The H/L ratio tended ($P < 0.06$) to be higher in older stressed birds than in younger ones.

Similar to results obtained in Chapter 2, H/L ratio was also significantly increased in birds fed the cholesterol diet as compared with birds fed the control diet. Stress and dietary cholesterol have an additive effect on H/L ratio, so that stressed cholesterol-fed birds had higher H/L ratios than birds that were either stressed or on the cholesterol diet alone. Birds on the cholesterol diet for 8 or 12 weeks also had a higher H/L ratio than birds on the cholesterol diet for only 4 weeks. However, we could not separate that out from the age effect. Early stress may have had a pre-conditioning effect on cholesterol-fed birds because the H/L ratios of birds on the cholesterol diet for 12 weeks but that were stressed only during the first 4 weeks were significantly lower than those of unstressed birds on the cholesterol diet for the same length of time.

Plasma cholesterol

Dietary cholesterol supplementation produced a marked elevation in plasma cholesterol levels in all treatment groups (Table 3.4). Stress did not significantly affect plasma cholesterol levels at 4 weeks. However, there was a significant diet x stress interaction after 8 and 12

Table 3.3 Heterophil/Lymphocyte ratio of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. Values are the numerical counts of 100 cells from differential blood smear and expressed as the least squared mean \pm SE for 10 birds per treatment group.

4 weeks	Chol Diet	Control diet
Recent stress	1.91 \pm 0.02 a	1.69 \pm 0.02 b
No stress	1.68 \pm 0.02 b	0.82 \pm 0.02 c

8 weeks	Chol Diet	Control diet
Recent stress	2.16 \pm 0.06 a	1.83 \pm 0.06 b
Early stress	1.66 \pm 0.06 b	0.74 \pm 0.06 c
No stress	1.76 \pm 0.06 b	0.83 \pm 0.06 c

12 weeks	Chol Diet	Control diet
Recent stress	2.22 \pm 0.1 a	1.86 \pm 0.1 bc
Early stress	1.71 \pm 0.1 c	0.74 \pm 0.1 d
No stress	1.92 \pm 0.1 b	0.80 \pm 0.1 d

Table 3.4 Plasma cholesterol levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8 or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. Values are the least squared means \pm SE for 10 birds per treatment group and expressed in mmol/L units.

4 weeks	Chol Diet	Control diet
Recent stress	31.62 \pm 2.04 b	4.79 \pm 2.04 a
No stress	33.12 \pm 2.04 b	7.08 \pm 2.04 a

8 weeks	Chol Diet	Control diet
Recent stress	32.90 \pm 0.59 a	7.09 \pm 0.59 c
Early stress	24.54 \pm 0.59 b	5.90 \pm 0.59 c
No stress	31.86 \pm 0.59 a	4.61 \pm 0.59 d

12 weeks	Chol Diet	Control diet
Recent stress	31.52 \pm 0.94 a	7.25 \pm 0.94 c
Early stress	24.54 \pm 0.94 b	5.58 \pm 0.94 d
No stress	33.39 \pm 0.94 a	4.99 \pm 0.94 d

weeks of cholesterol feeding ($P < 0.05$, and $P < 0.009$, respectively). Birds on the cholesterol diet exposed to early stress (during the first 4 weeks) had significantly lower plasma cholesterol levels at 8 and 12 weeks compared to birds that had not received stress or birds exposed to stress immediately prior to sampling. On the other hand, for birds on the control diet, stress (both early and recent) for the 8 week sampling, and recent stress for the 12 week sampling periods, significantly increased plasma cholesterol levels compared to birds without exposure to stress.

Plasma triglycerides

Dietary cholesterol supplementation produced a marked elevation in plasma triglyceride levels in all treatment groups (Table 3.5). The effect of stress was only significant during the last time period (i.e. at 12 weeks). Birds exposed to early stress (stressed during the first 4 weeks and allowed to recover for 8 wks) had significantly lower ($P < 0.05$) mean plasma triglyceride levels at 12 week time period (1.83 ± 0.37 mmol/L) compared to birds that had not experienced any stress (3.02 ± 0.37 mmol/L) or birds exposed to recent stress (2.50 ± 0.37 mmol/L), regardless of whether the birds were on the control or the cholesterol diet.

Aortic plaque score

The aortic plaque scores (measured at 4, 8 and 12 weeks) in all three vessels were significantly increased by cholesterol feeding (Table 3.6). However, stress did not have any significant effect on atherosclerotic plaque scores measured in any one of the three vessels of

Table 3.5 Plasma triglyceride levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4 (n=20 birds per treatment), 8 (n=30 per treatment) or 12 (n=30 per treatment) weeks. Values are the least squared means \pm SE for 10 birds per treatment group and expressed in mmol/L units.

	Chol diet	Control diet
4 weeks	3.48 \pm 0.39 b	1.10 \pm 0.39 a
8 weeks	3.10 \pm 0.39 b	0.99 \pm 0.39 a
12 weeks	3.88 \pm 0.39 b	0.88 \pm 0.39 a

Table 3.6 Aortic plaque score in left brachiocephalic artery (i.e. branch 1), right brachiocephalic artery (i.e. branch 2), and descending aorta (i.e. branch 3) of atherosclerosis-susceptible Japanese quail on cholesterol supplemented diets for 4, 8, or 12 weeks. Since the stress protocol did not have a significant effect on aortic plaque scores, the data shown was pooled with no stress treatment groups. Birds on control diet did not develop atherosclerotic plaques. Values are the least squared means for 30 (4 weeks time point) and 50 birds (8 and 12 week time points).

	Branch 1	Branch 2	Branch 3
4 weeks	1.61 a	2.46 b	0.47 c
8 weeks	3.64 a	3.64 a	3.46 a
12 weeks	3.69 a	3.8 a	3.24 a

the aortic tree (two brachiocephalic arteries and the descending aorta). Significant regression was found for some biochemical parameters and aortic plaque scores. Specifically, plaque score in the left brachiocephalic artery of birds fed cholesterol for 4 wks significantly regressed on aortic HO activity ($r = - 0.40$; $P < 0.003$). As well, the plaque score for the descending aorta regressed significantly on aortic HO activity ($r = - 0.32$; $P < 0.048$) after 4 wks of cholesterol feeding and regressed significantly on plasma TG ($r = - 0.30$; $P < 0.02$) after 8 wks of cholesterol feeding.

Thiobarbituric acid-reactive substances

Both dietary cholesterol and stress had significant effects on heart TBARS formation (Table 3.7). Heart TBARS were significantly higher in stressed birds if the tissue was sampled immediately after the completion of the stress protocol. However, if the tissue sample was taken 4 weeks or more after the stress protocol (early stressed birds), TBARS values were similar to those of unstressed birds. The results were similar in birds fed the control or cholesterol diet (Table 3.7).

HSP70

Aortic HSP70

Table 3.7 Thiobarbituric acid reactive substances (TBARS) in hearts of atherosclerosis-susceptible Japanese quail on A) control or cholesterol supplemented diets for 4, 8, or 12 weeks; or B) exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocols, or no stress. The data for Part A included stress and no stress treatment groups. Results (absorbance @ 532 nm) are expressed least squared mean for 20 birds per treatment group.

A.

Weeks on diet	Chol Diet	Control diet
4 wks on diet	0.41 ± 0.04 a	0.32 ± 0.03 b
8 wks on diet	0.38 ± 0.03 a	0.34 ± 0.03 b
12 wks on diet	0.38 ± 0.02 a	0.34 ± 0.02 b

B.

Treatment group	Stress	No Stress
4 wks stress	0.41 ± 0.04 a	0.33 ± 0.04 b
8 wks -early stress	0.35 ± 0.01 b	0.35 ± 0.01 b
8 wks -recent stress	0.38 ± 0.01 a	
12 wks-early stress	0.36 ± 0.01 b	0.35 ± 0.01 b
12 wks-recent stress	0.38 ± 0.01 a	

For birds on the control diet, aortic HSP70 was significantly increased in recent stress birds (stress immediately prior to sampling) (Fig. 3.1). After 8 weeks on the control diet, birds that were stressed early (during the first 4 weeks) had HSP70 levels similar to those of unstressed birds. However, after 12 weeks on the control diet, birds that were stressed early (during the first 4 weeks) had HSP70 levels that were significantly higher than those in unstressed birds but lower than recent stressed birds (Fig. 3.1).

HSP70 was also significantly increased in birds fed the cholesterol diet. However, stress and dietary cholesterol did not have an additive effect on aortic HSP70 levels (Fig. 3.1). After 4 weeks on the cholesterol diet, aortic HSP70 levels were significantly lower in recent stressed birds compared with non-stressed birds on the same diet. After 8 and 12 weeks, neither recent nor early stress had any effect on aortic HSP70 levels in birds fed the cholesterol-supplemented diet.

Heart HSP70

For birds on the control diet, heart HSP70 was significantly increased in the recent stressed birds (stressed immediately prior to sampling) (Fig. 3.2). However, after 8 or 12 weeks on the control diet, birds that were stressed early (during the first 4 weeks) had HSP70 levels similar to those of the unstressed birds.

Heart HSP70 was also significantly increased in birds fed the cholesterol diet (Fig. 3.2). Dietary cholesterol supplementation had a greater effect than stress on heart HSP70 levels as

Figure 3.1 Aortic HSP70 levels of atherosclerosis-susceptible (SUS) Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. (A) Representative Western blot analysis for HSP70 protein content (8 wk time point). MW= molecular weight; C-NS= control, no stress; C-RS= control, recent stress; C-ES= control, early stress; CH-NS= cholesterol, no stress; CH-ES=cholesterol, early stress; CH-RS= cholesterol, recent stress; HSP70= purified control. (B) HSP70 immunoreactive protein content in SUS birds in *A* quantified by densitometry. Mean signal intensity in homogenates was normalized to and expressed as a ratio of mean signal in purified HSP70. Values are the least squared mean \pm SE for 10 birds per treatment group.

A.

MW C-NS C-ES C-RS CH-NS CH-ES CH-RS HSP70

B.

4 weeks	Chol Diet	Control diet
Recent stress	0.67 ± 0.04 b	0.64 ± 0.04 b
No stress	0.77 ± 0.04 a	0.32 ± 0.04 c

8 weeks	Chol Diet	Control diet
Recent stress	0.64 ± 0.06 a	0.59 ± 0.05 a
Early stress	0.71 ± 0.04 a	0.41 ± 0.05 b
No stress	0.75 ± 0.06 a	0.38 ± 0.05 b

12 weeks	Chol Diet	Control diet
Recent stress	0.70 ± 0.05 ab	0.67 ± 0.04 b
Early stress	0.83 ± 0.04 a	0.47 ± 0.05 c
No stress	0.75 ± 0.06 ab	0.36 ± 0.05 d

Figure 3.2 Heart HSP70 levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. (A) Representative Western blot analysis for HSP70 protein content (8 wk time point). MW= molecular weight; C-NS= control, no stress; C-RS= control, recent stress; C-ES= control, early stress; CH-NS= cholesterol, no stress; CH-ES=cholesterol, early stress; CH-RS= cholesterol, recent stress; HSP70= purified control. (B) HSP70 immunoreactive protein content in SUS birds in *A* quantified by densitometry. Mean signal intensity in homogenates was normalized to and expressed as a ratio of mean signal in purified HSP70. Values are the least squared mean \pm SE for 10 birds per treatment group.

A.



MW C-NS C-ES C-RS CH-NS CH-ES CH-RS HSP70

B.

4 weeks	Chol Diet	Control diet
Stress	0.61 ± 0.02 b	0.60 ± 0.02 b
No stress	0.68 ± 0.02 a	0.37 ± 0.02 c

8 weeks	Chol Diet	Control diet
Recent stress	0.62 ± 0.02 a	0.53 ± 0.02 c
Early stress	0.57 ± 0.02 b	0.41 ± 0.02 d
No stress	0.62 ± 0.02 a	0.34 ± 0.02 e

12 weeks	Chol Diet	Control diet
Recent stress	0.62 ± 0.03 a	0.53 ± 0.03 c
Early stress	0.57 ± 0.03 b	0.41 ± 0.03 d
No stress	0.62 ± 0.03 a	0.34 ± 0.03 e

non-stressed, cholesterol-fed birds had higher HSP70 levels than stressed birds on the control diet. After 4 weeks on the cholesterol diet, aortic HSP70 levels were significantly ($P < 0.0001$) lower in recent stress birds compared with non-stressed birds on the same diet. After 8 weeks, neither recent nor early stress had any detectable effect on heart HSP70 levels in birds fed the cholesterol diet. After 12 weeks on the cholesterol diet, birds that were stressed early (during the first 4 weeks) had lower heart HSP70 levels than both non-stressed and recently stressed birds on the same diet.

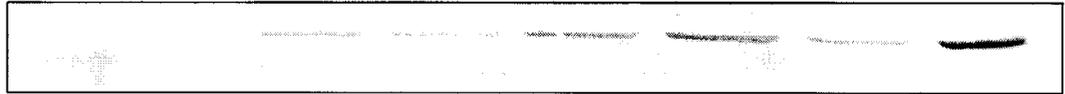
HO-1

Aortic HO-1

Stress had a significant effect on aortic HO-1 levels (Fig. 3.3). Except for the first 4 weeks of cholesterol feeding, aortic HO-1 levels were significantly increased ($P < 0.05$) in recent stress (stress immediately before sampling) birds at 8 (0.7 ± 0.02) and 12 (0.7 ± 0.01) week time points compared with early (8 weeks = 0.57 ± 0.02 ; 12 weeks = 0.65 ± 0.02) or no stress (8 weeks = 0.61 ± 0.02 ; 12 weeks = 0.62 ± 0.02) birds. However, after 8 or 12 weeks on the cholesterol diet, birds that were exposed to early stress (stressed during the first 4 weeks) had HO-1 levels similar to those of the unstressed birds. The results were similar in birds fed the control or cholesterol diet. Aortic HO-1 levels were significantly ($P < 0.0001$) higher in recent stress birds fed the control diet (4 week time point) compared with non-stressed birds on the same diet (Fig 3.3 B). On the other hand, recent stress birds on the cholesterol diet (4 week

Figure 3.3 Aortic HO-1 levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. (A) Representative Western blot analysis for HO-1 protein content (8 wk time point). MW= molecular weight; C-NS= control, no stress; C-RS= control, recent stress; C-ES= control, early stress; CH-NS= cholesterol, no stress; CH-ES= cholesterol, early stress; CH-RS= cholesterol, recent stress; HO-1= purified control. (B) HO-1 immunoreactive protein content in SUS birds exposed to stress or no stress (4 week time point). (C) HO-1 immunoreactive protein content in SUS birds after control or cholesterol supplementation (8 and 12 week time points). Mean signal intensity in homogenates was normalized to and expressed as a ratio of mean signal in purified HSP70. Values are the least squared mean \pm SE for 10 (i.e. B) and 20 (i.e. C) birds per group. (D) Immunohistochemical staining of HO-1 in (1) 0 week, (2) 4 week, and (3) 12 week cholesterol-fed birds. A green fluorescence was indicative of HO-1 staining.

A.



MW C-NS C-ES C-RS CH-NS CH-ES CH-RS HO-1

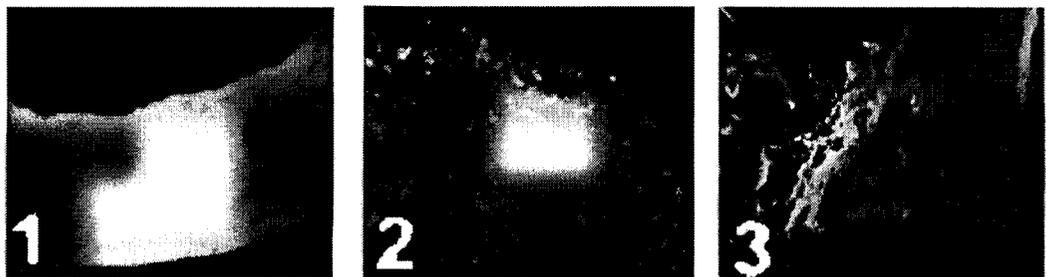
B.

4 weeks	Chol diet	Control diet
Recent Stress	0.78 ± 0.02 b	0.46 ± 0.02 c
No stress	0.84 ± 0.02 a	0.29 ± 0.02 d

C.

	Chol diet	Control diet
8 weeks	0.96 ± 0.03 b	0.3 ± 0.03 c
12 weeks	0.96 ± 0.03 b	0.35 ± 0.03 c

D.



time point) had significantly lower aortic HO-1 levels than non-stressed birds on the same diet (Fig 3.3 B).

Birds on the cholesterol-supplemented diet had approximately 3X higher aortic HO-1 levels compared with birds on the control diet, regardless of whether or not they were stressed (Fig.3.3 C).

Immunohistochemical staining localized aortic HO-1 expression to the intimal layer where staining was greatest in 12>4>0 weeks of cholesterol feeding, respectively (Fig. 3.3 D).

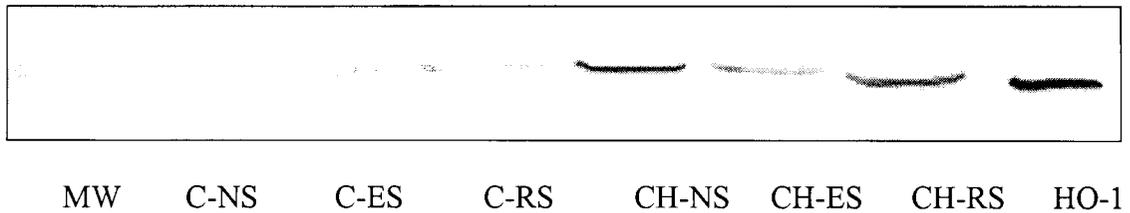
Heart HO-1

Stress had a different effect on heart HO-1 levels in birds on the control diet *versus* those on the cholesterol diet. Except at the first 4 week time point, heart HO-1 levels were significantly ($P<0.0001$) increased in recent stressed birds (stressed immediately prior to sampling) fed a non-cholesterol-supplemented diet (Fig. 3.4). After 8 or 12 weeks on the cholesterol diet, birds that were stressed early (during the first 4 weeks) had HO-1 levels that were similar to those of unstressed birds. Stress did not have any significant effect on HO-1 levels in birds on the control diet during the first 4 weeks.

For birds on the cholesterol diet, heart HO-1 levels (8 and 12 week time point) were significantly ($P<0.0001$) lower in recent stressed birds (stress immediately prior to sampling) compared with non-stressed birds (Fig. 3.4). After 8 or 12 weeks on the cholesterol diet, birds that were stressed early (during the first 4 weeks) had HO-1 levels that were even lower, and were significantly lower ($P<0.05$) than recently stressed or non-stressed birds. In general,

Figure 3.4 Heart HO-1 levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. (A) Representative Western blot analysis for HO-1 protein content (8 wk time point). MW= molecular weight; C-NS= control, no stress; C-RS= control, recent stress; C-ES= control, early stress; CH-NS= cholesterol, no stress; CH-ES=cholesterol, early stress; CH-RS= cholesterol, recent stress; HO-1= purified control. (B) HO-1 immunoreactive protein content in SUS birds in *A* quantified by densitometry. Mean signal intensity in homogenates was normalized to and expressed as a ratio of mean signal in purified HSP70. Values are the least squared mean \pm SE for 10 birds per treatment group.

A.



B.

4 weeks	Chol Diet	Control diet
Recent stress	0.64 ± 0.03 b	0.30 ± 0.02 c
No stress	0.47 ± 0.02 a	0.32 ± 0.03 c

8 weeks	Chol Diet	Control diet
Recent stress	0.75 ± 0.02 b	0.39 ± 0.02 c
Early stress	0.44 ± 0.02 c	0.29 ± 0.02 d
No stress	0.84 ± 0.03 a	0.31 ± 0.02 d

12 weeks	Chol Diet	Control diet
Recent stress	0.71 ± 0.02 b	0.39 ± 0.02 d
Early stress	0.62 ± 0.02 c	0.31 ± 0.02 e
No stress	0.88 ± 0.03 a	0.33 ± 0.02 e

birds on the cholesterol-supplemented diet had significantly higher heart HO-1 levels than birds on the control diet.

After 8 weeks on the cholesterol-supplemented diet, heart HO-1 levels significantly regressed ($r = 0.48$, $p < 0.02$) on plasma triglyceride levels; thus, birds with higher plasma triglyceride levels had higher heart HO-1 levels.

HO enzyme activity

Aortic HO activity

For birds on the control diet, stress had no significant effect on the aortic HO activity. Birds on a cholesterol-supplemented diet had significantly higher aortic HO activity than birds on the control diet (Table 3.8). After 8 weeks on the cholesterol diet, birds that were stressed early (during the first 4 weeks) had significantly lower aortic HO activity than birds that were recently stressed or were not exposed to the stress protocol.

Aortic HO activity regressed significantly ($r = 0.36$; $p < 0.02$) on plasma triglyceride concentration after 4 weeks of cholesterol-supplementation.

Table 3.8 Aortic HO enzyme activity of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. Values are the least squared means \pm SE for 10 birds per treatment group and expressed as pmol of bilirubin formed/mg protein/h.

4 weeks	Chol diet	Control diet
No stress	33.0 \pm 1.40 b	4.0 \pm 1.56 a
Stress	31.0 \pm 1.52 b	4.0 \pm 1.37 a

8 weeks	Chol diet	Control diet
Recent stress	50.96 \pm 1.39 c	5.4 \pm 1.39 a
Early stress	31.2 \pm 1.39 b	5.2 \pm 1.39 a
No stress	49.0 \pm 1.39 c	4.9 \pm 1.39 a

12 weeks	Chol diet	Control diet
Recent stress	43.1 \pm 1.40 b	5.27 \pm 1.40 a
Early stress	46.4 \pm 1.40 b	5.21 \pm 1.40 a
No stress	45.3 \pm 1.40 b	5.09 \pm 1.40 a

Heart HO activity

Stress had no significant effect on heart HO activity. However, birds on a cholesterol-supplemented diet had significantly higher ($P < 0.05$) heart HO activity (control = 4.08 ± 0.60 ; cholesterol 18.26 ± 2.13 pmol of bilirubin formed/mg protein/h) and this was most likely due to the high plasma cholesterol and triglyceride levels (significant regression ($P < 0.0001$) on plasma cholesterol and triglyceride levels after 8 weeks) present in these birds.

Ferritin

Aortic ferritin

For birds on the control diet, stress had no significant effect on aortic ferritin levels. Birds on the cholesterol-supplemented diet had significantly higher aortic ferritin levels than birds on the control diet (Fig. 3.5). During the first 4 weeks (but not 8 or 12 weeks) on the cholesterol-supplemented diet, birds that were stressed had significantly lower aortic ferritin levels than non-stressed birds.

Figure 3.5 (A) Aortic ferritin protein levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. Stress protocol was only significant at 4 week time point and therefore, the data for 8 and 12 week time points was pooled with diet values. Results are the least squared means \pm SE for 20 (4 weeks) or 30 birds (8 and 12 weeks) per group and expressed as ng ferritin per mg protein. (B)

Immunohistochemical staining for ferritin in (1) 0 week, (2) 4 week and (3) 12 week cholesterol-fed birds. A dark brown stain was indicative of positive staining for ferritin.

A.

4 weeks	Chol Diet	Control diet
Recent stress	296.5 ± 21.4 b	116.4 ± 31.2 c
No stress	381.7 ± 23.6 a	109.6 ± 30.3 c

8 weeks	Chol Diet	Control diet
Recent stress	193.4 ± 29.32 b	333.0 ± 29.32 c
Early stress	195.0 ± 28.66 c	249.4 ± 27.80 d
No stress	193.5 ± 29.07 a	312.2 ± 29.31 d

12 weeks	Chol Diet	Control diet
Recent stress	208.5 ± 43.03 b	481.3 ± 46.61 d
Early stress	211.1 ± 43.22 c	513.5 ± 43.07 e
No stress	221.3 ± 43.27 a	524.6 ± 40.42 e

B.



Aortic ferritin regressed significantly ($p < 0.003$) on aortic free iron levels at all time points ($r = 0.38$ at 4 weeks, 0.72 at 8 weeks and 0.74 at 12 weeks). Thus, birds that had high aortic free iron also had high aortic ferritin levels.

Imunohistochemical staining showed large amounts of ferritin protein in the intimal layer of the aorta in 4 and 12 week cholesterol-fed birds (Fig. 3.5b). No ferritin staining was visible in age-matched controls.

Heart ferritin

For birds on the control diet, stress had no significant effect on heart ferritin levels. Birds on the cholesterol-supplemented diet had significantly higher heart ferritin levels than birds on the control diet (Table 3.9). After 8 weeks on the cholesterol diet, birds that were stressed earlier (during the first 4 weeks) had significantly lower heart ferritin levels than birds that were recently stressed (immediately before tissue sampling) or were not exposed to stress protocol. After 12 weeks on the cholesterol diet, birds that were stressed (either recently or earlier) had significantly lower levels of ferritin than non-stressed birds. In addition, 12 week cholesterol-fed birds exposed to the early stress protocol had the lowest ferritin levels.

Catalytic iron

Aortic catalytic iron

Table 3.9 Heart ferritin protein levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 8 or 12 weeks and exposed to either early (stressed for 4 wks and allowed to recover for 4 or 8 wks prior to sampling) or recent (stressed for the 4 wks immediately before sampling) stress protocol, or no stress. Results are the least squared mean \pm SE for 30 birds per treatment group and expressed as ng ferritin per mg protein.

8 weeks	Chol Diet	Control diet
Recent stress	63.7 \pm 2.2 a	24.1 \pm 2.2 c
Early stress	41.6 \pm 2.2 b	23.4 \pm 2.2 c
No stress	66.2 \pm 2.2 a	22.2 \pm 2.2 c

12 weeks	Chol Diet	Control diet
Recent stress	109.3 \pm 2.4 b	23.7 \pm 2.4 d
Early stress	92.8 \pm 2.4 c	23.4 \pm 2.4 d
No stress	124.2 \pm 2.4 a	22.9 \pm 2.4 d

For birds on the control or cholesterol-supplemented diet, stress had no significant effect on aortic catalytic iron. Birds on the cholesterol-supplemented diet had significantly higher aortic catalytic iron levels than birds fed a control diet at all time points (Fig. 3.6).

Iron histochemistry localized dark staining Prussian blue iron to the intimal layer of the aortic tissue (Fig. 3.6 B). Extensive iron staining (dark brown staining) was evident in 12 > 4 weeks of cholesterol-supplementation. No iron (light background staining) was detectable in birds fed a control diet. Interestingly, ferritin and iron co-localized with HO-1 in the intimal layer of the aortic tissue.

Heart catalytic iron

For birds on the control diet, stress had no effect on heart catalytic iron (Table 3.10). Birds on the cholesterol-supplemented diet had significantly higher aortic catalytic iron levels than birds fed a control diet at all time points. After 4 weeks on the cholesterol diet, recent stressed birds had significantly lower ($P < 0.05$) heart catalytic iron levels than non-stressed birds. However, no significant changes were observed at 8 or 12 week time points.

Antioxidant components

Stress had no effect on the antioxidant components measured, namely plasma GRd, heart GRd and SOD, at any of the experimental time points (data not shown). Birds on the cholesterol-supplemented diet had significantly higher plasma GRd activities than birds on the control diet at all time points (Table 3.11).

Figure 3.6 (A) Aortic catalytic iron levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks. Stress had no significant effect on aortic catalytic iron levels and therefore, the data was pooled with diet values. Results are the least squared means \pm SE for 30 birds per treatment group and expressed as μg bleomycin iron per mg protein. (B) Iron staining in aortic tissue using Perls' Prussian Blue reaction with 3,3'-diaminobenzidine intensification (1) 0 week, (2) 4 week and (3) 12 week cholesterol-fed birds. A dark brown colour was indicative of positive staining for iron.

A.

	Chol Diet	Control diet
4 weeks	2.32 ± 1.9 b	0.16 ± 0.2 a
8 weeks	2.31 ± 1.9 b	0.2 ± 0.2 a
12 weeks	4.63 ± 1.9 b	0.2 ± 0.2 a

B.



Table 3.10 Heart catalytic iron levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks. Stress did not have a significant effect on catalytic iron levels, and thus the data were pooled with stress values. Results are the least squared means \pm SE for 60 birds per treatment group and expressed as μg bleomycin iron per mg protein.

	Chol diet	Control diet
4 weeks	0.57 ± 0.031 a	0.14 ± 0.031 b
8 weeks	0.62 ± 0.031 a	0.14 ± 0.031 b
12 weeks	0.70 ± 0.032 a	0.15 ± 0.032 b

Table 3.11 Plasma glutathione reductase (GRd) levels of atherosclerosis-susceptible Japanese quail on control or cholesterol supplemented diets for 4, 8, or 12 weeks. Stress did not have a significant effect on plasma GRd, and thus the data were pooled with stress values. Results are the least squared means \pm SE for 60 birds per treatment group and expressed as nmol NADPH/min/mg protein.

	Chol Diet	Control diet
4 weeks	18.3 \pm 1.28 b	10.4 \pm 1.28 a
8 weeks	18.9 \pm 1.44 b	10.1 \pm 1.44 a
12 weeks	19.5 \pm 1.74 b	11.4 \pm 1.74 a

Heart GRd (control = 9.7 ± 0.4 nmol/min/mg tissue, cholesterol = 10.6 ± 0.4 nmol/min/mg tissue; $P < 0.05$) and SOD (control = 23.57 ± 0.92 Units/mg protein, cholesterol = 25.53 ± 0.92 Units/mg protein; $P < 0.05$) activities were only significantly elevated after 12 weeks of cholesterol supplementation.

DISCUSSION

Psychological stress can contribute to atherosclerotic lesion formation by increasing the probability of endothelial cell injury (234), platelet aggregation and thrombus formation (235-236), and the probability of plaque rupture (237). However, these mechanisms interact with many other factors, such as duration of stress (238), genetic predisposition (239) and antioxidant/oxidant balance (240), in determining the overall risk factor profile. Our previous findings suggested that stress might increase plaque formation and alter HSP expression and antioxidant components (Chapter 2). Thus, in the present study, we investigated the time-course of biochemical changes in stressed, cholesterol-fed animals with particular reference to the relationship between HSP expression and antioxidant alterations using the SUS strain of Japanese quail.

Although stress has been recognized as a risk factor for atherosclerosis (211), the effects of stress on the time-dependent molecular events occurring during the course of atherogenesis are poorly understood. Acute mental stress in humans undergoing cardiac catheterization for atherosclerosis produced constriction of large coronary vessels, possibly caused by local failure of endothelium-dependent dilation (241). Cynomolgous monkeys on a high cholesterol diet

exposed to “chronic social stress” showed coronary artery constriction following acetylcholine infusion, whereas socially stable monkeys fed the same diet and given the same dose of acetylcholine by injection did not show evidence of coronary artery constriction (164). It is well understood that elevations in plasma cholesterol levels are associated with increased risk for CAD; moreover, studies in humans have shown that chronic psychological stress increases plasma cholesterol levels (242-244). Therefore, while the latter studies are consistent with chronic stress-induced functional changes of the coronary vasculature, a lack of attention has been given to the temporal patterns of stress during atherogenesis.

In the present study, we have shown that birds on the control diet and exposed to early stress (stress followed by recovery period) had little effect on the biochemical parameters investigated. However, this is the first report where birds on a cholesterol diet and exposed to early stress had significantly lower plasma cholesterol and triglyceride levels than birds not exposed to early stress. Although statistically significant in this study, there is little information in the literature to support a direct effect of stress on plasma lipid levels. In a study examining the effects of oxidative stress in a trauma patient, the authors concluded that the lower plasma lipoprotein levels observed may be caused by hemodilution in the plasma as a result of trauma (244). Furthermore, hypocholesterolemia has been considered a consequence of increased cholesterol utilization in new cell synthesis after trauma (245), and other studies have suggested that cholesterol is a reliable and inexpensive inflammatory marker in critically ill patients (246). Thus, although significantly lower plasma lipid levels were measured in early stressed birds fed a cholesterol-supplemented diet, future studies are necessary to determine the physiological relevance of these results.

Birds on the cholesterol diet and exposed to early stress also had significantly lower heart HSP70, HO-1 and ferritin, and aortic HO levels compared with non-stressed birds on the same diet. A considerable body of evidence indicates that brief periods of 'stress' prior to induction of various forms of tissue injury can result in preconditioning and attenuation of subsequent damage (247-250). Thus, if increased ferritin (251) and HSP70 (252) levels are indicative of a tissues response to injury, then lower levels may support a pre-conditioning effect following early stress. In the present study, this may be an indication that the birds exposed to early psychological stress (stress followed by recovery period) were less "stressed" by the cholesterol-supplementation later. However, further studies are necessary to support the claim that early psychological stress may precondition tissues to the effect of cholesterol supplementation, and to suggest that some forms of stress may actually be beneficial (253). For example, social support which has been evaluated as positive may buffer the individual from experiencing stress (254). Furthermore, others have shown that individuals who successfully cope with demanding situations may experience cardiovascular growth and regeneration following surgical stress (255). Thus, the prospect of stress, or that successfully coping with stress may improve health, is an intriguing possibility, and future research should include measures which allow for the detection of these positive outcomes.

We observed a greater number of alterations in the biochemical parameters measured in birds immediately after stress when compared to early stressed birds. The H/L ratio was higher during recent stress alone, cholesterol-supplementation alone and the combination of recent stress and cholesterol feeding and confirmed our earlier findings in Chapter 2. Similar findings were reported by Gray *et al.* (256) where the H/L ratio was increased following short- and/or long-term infusion of adrenocorticotrophic hormone and hydrocortisone. Gross and Siegel (173)

suggested the H/L ratio may be short-lived if the stressor was removed. These findings support our results of an increased H/L ratio in recent but not early stressed birds. In the present study, we have shown that birds fed the control diet and exposed to recent stress not only exhibited increased plasma cholesterol levels but also showed increased heart TBARS production and heart and aortic HSP70 and HO-1 levels compared with non-stressed birds on the same diet. The decreased heart tissue antioxidant capacity (as measured by TBARS levels) supports the induction of oxidative stress in tissues by recent stress. In the present study, recent stress rendered the heart tissue more susceptible to oxidative challenge, but if the stress stimulus was removed after short term exposure (as in early stress), its effect may be beneficial because of its induction of endogenous antioxidants (257) and HSPs (252). These results also support the idea that recent psychological stress has dual effects: acutely, recent stress can result in increased susceptibility of some tissues to oxidative stress and, perhaps as a consequence, induce antioxidant systems that minimize the subsequent effects of oxidants. The increased levels of heart and aortic HSP70 and HO-1 may have provided protection during recent stress. Studies have shown that an increase in HSP70 levels can provide protection from a variety of stressors including restraint stress in rat gastric mucosal cells (258), myocardial ischemia in rat hearts (259) and human endothelial cells exposed to oxidized LDL (260). Isosaki and Nakashima (261) have reported that chronic psychological stress increased HSP70 expression in gastric tissue of rats, and the increased HSP70 levels following recent stress in the present study supports our previously reported findings (172). The latter two studies (261,172) indicate that both physiological (i.e. H/L ratio) and cellular (i.e. HSPs) stress responses are increased following recent stress.

Some biochemical changes in cholesterol-fed birds decreased when these birds were exposed to recent stress. Although aortic plaque scores were unchanged, lower aortic and heart HSP70 levels, aortic ferritin and heart HO-1 levels were observed when compared with cholesterol-fed, non-stressed birds, which may suggest that the cholesterol-supplemented birds exposed to recent stress had lower physiological defenses. Moreover, the decreased antioxidant capacity in the heart, as measured by increased TBARS levels, does not coincide with lower heart HSP70 and HO-1 levels. As well, the initial response of increased heart HO-1 during early stages of atherogenesis and the decrease in heart HO-1 levels at later time points may indicate that the combination of recent stress and cholesterol supplementation resulted in the waning of HSP70 and heart HO-1 levels during the later stages of atherogenesis.

Tissue iron levels may be a good indicator of tissue ferritin content (248) and tissue iron levels are correlated to serum ferritin levels (263). Studies have suggested that elevation in serum ferritin levels is associated with psychological stress (264-265), and tissue ferritin has been correlated to an increase in HO-1 levels (266). Others have reported that the 'stress' of exercise in healthy humans correlated with decreased serum ferritin levels (267), whereas another study reported a positive correlation between hyperlipidemia and increased plasma ferritin levels in patients with hyperlipidemia (268). We found an inverse relationship between aortic HO-1 and ferritin levels in cholesterol-supplemented birds exposed to recent stress suggesting that in these birds ferritin levels decreased, possibly independently of HO-1 expression. Eisenstein *et al.* (269-270) reported that HO plays an essential role in the induction of ferritin synthesis by heme, although chelatable iron can regulate ferritin synthesis independently of heme and possibly HO.

Cholesterol-supplementation in non-stressed birds increased nearly all biochemical parameters analyzed in this study compared to stressed birds fed a cholesterol-supplemented diet. Similar to Chapter 2, the 0.25% cholesterol-supplemented diet rapidly increased the aortic plaque score. HSP70 has been detected in early and advanced lesions of human atherosclerosis (51) and HO-1 has been colocalized with iron to the intimal layer of the aorta (67). In this thesis, we localized HO-1, ferritin and iron to the intimal layer of the aorta and provide evidence of oxidative stress at the endothelial cell layer. It is felt that future experiments which focus on the effects of oxidative stress in SUS aortic endothelial cells might yield important information about their physiological function during atherogenesis. The increased levels of RBC and plasma TBARS, plasma GRd, heart GRd and SOD activities following cholesterol-supplementation have been reported previously in SUS birds in our laboratory (152,154,187), aortic tissue in a rabbit model (271) and plasma in humans (272), and are consistent with the proposed oxidative etiology of atherosclerosis (273).

The results obtained in Chapter 2 suggested that psychological stress might be a contributing factor influencing the severity of atherosclerotic plaque formation; however, we did not observe this correlation in the present study. Nevertheless, early and recent stress in combination with cholesterol supplementation did result in alterations of HSP70, HO, ferritin and iron levels in heart and aortic tissue. However, while the mechanism by which psychological stress leads to increased susceptibility to oxidative stress and damage is not well understood, the present study suggests this association may be, at least in part, manifested through HO-1 levels. Further studies to investigate the effect of stress on HSPs and antioxidant components at the cellular level would seem warranted by the results reported herein.

CONCLUSIONS

The studies conducted in this chapter detected no differences in aortic plaque score between early stressed, recent stressed and non-stressed cholesterol-fed birds. However, cholesterol feeding did result in alteration of lipid peroxides (as indicated by increased TBARS), and subsequently increased oxidative stress to heart and aortic tissues as determined by increased levels of HSP70 and HO-1.

CHAPTER 4

HEME OXYGENASE AND ANTIOXIDANT STATUS IN CULTURED AORTIC ENDOTHELIAL CELLS ISOLATED FROM ATHEROSCLEROSIS-SUSCEPTIBLE AND – RESISTANT JAPANESE QUAIL

PREFACE

A manuscript based on the studies in this Chapter has been accepted for publication in *Molecular and Cellular Biochemistry* and was coauthored with D.V. Godin, J. Kurtu and K.M. Cheng. We thank Jamal Kurtu (Quail Genetic Stock Centre, UBC) for his contribution to project planning and animal care and Jenny Hart (Pulmonary Research Centre, St. Pauls. Hospital, Vancouver, B.C) for her technical assistance with the cell cultures.

INTRODUCTION

Abnormalities in endothelial cells (ECs), which line the inside of all blood vessels, have been associated with pathological conditions such as ischemia/reperfusion injury, thrombosis and atherosclerosis (274-276). Vascular ECs are highly susceptible to oxidant injury (277). One possible source of oxidative damage to the arterial wall is oxidized LDL. (276). Such oxidative modification may be atherogenic because many lines of evidence suggest that LDL, an important risk factor for atherosclerosis, must be oxidatively modified to trigger the disease (278). Oxidized LDL (OxLDL) has been shown to stimulate chemotactic recruitment of

monocytes into the artery wall (279) and to promote intracellular cholesterol accumulation and macrophage foam cell formation (280).

Recent experimental studies have shown that antioxidants suppress the progression of atherosclerosis and endothelial dysfunction (281-282). Heme oxygenase (HO), an enzyme essential for heme degradation, has been shown to have such antioxidant properties via the production of bile pigments, carbon monoxide and ferritin induction (283). The expression of HO-1, an inducible isozyme of HO, is increased by a variety of oxidative stresses (284-285) and thus has been considered a “stress protein” that protects cells from oxidative stress (286). Furthermore, several lines of evidence support the role of HO as an intrinsic protective pathway in the vascular wall (67,131,287).

Antioxidant enzymes may inhibit LDL oxidation and reduce the development of atherosclerotic lesions in animal models (288). Enzymes of the glutathione redox cycle (i.e. glutathione peroxidase (GPx) and glutathione reductase (GRd)) are crucial determinants of endothelial sensitivity to oxidative damage including that produced by oxidized LDL (289). Furthermore, the lipid hydroperoxide scavenging activity of GPx (290) suggests a possible modulatory role for this antioxidant during atherogenesis. Arterial smooth muscle cells can oxidize LDL by a superoxide- dependent process, suggesting a role for superoxide dismutase (SOD). Finally, treatment with vitamin C or vitamin E improved endothelium-dependent vasodilation in hypercholesterolemic patients (281,291).

Selected strains of Japanese quail differ strikingly in susceptibility to atherosclerosis (153), and we have previously observed that a high cholesterol diet is associated with differences in antioxidant enzyme components, which closely parallel the severity of atherosclerotic plaque formation in atherosclerosis-susceptible (SUS) and –resistant (RES) quail (152).

In the present study, we developed a method to culture and characterize aortic ECs (AECs) from random bred wild-type (WILD), and selectively bred RES and SUS quail. We examined differences in HO expression between the strains of quail to investigate whether HO is involved in cellular resistance to oxidative stress. Finally, we investigated whether the marked differences in susceptibility to atherosclerosis in these three strains are associated with corresponding differences in antioxidant components. Our results indicate that AECs from RES and SUS quail differ in their HO-catalyzed enzymatic reaction and antioxidant components, and suggest that RES and SUS AECs are a good model for studying oxidant/antioxidant interactions as they relate to the process of atherogenesis.

METHODS

Cell Culture

The SUS and RES strains of Japanese quail (146) used in this study have been maintained by the University of British Columbia Quail Genetic Resource Centre (QGRC) and have undergone divergent selection for susceptibility and resistance, respectively, to atherosclerotic plaque formation when challenged with a diet supplemented with 0.5% cholesterol. Before selection, 8% of the random bred foundation population suffered atherosclerosis when challenged with a high cholesterol diet (146). After divergent selection for 4 generations, 80% of SUS quail but only 4% of RES quail developed atherosclerosis when challenged (146). Additionally, a WILD strain was also used for comparison with the atherosclerosis strains.

Published cell culture techniques for growth of porcine (292), bovine (293) and human AECs (294) were adapted for quail AEC culture. All birds were fed a standard 26% protein

quail diet. Quail were killed by cervical dislocation and cut open to expose the aorta. The aortic tree (the brachiocephalic arteries to their bifurcations and the aorta to the iliac branching) was dissected out and transferred into a sterile beaker containing Dulbecco's phosphate buffered saline (DPBS), streptomycin (500 µg/ml) and penicillin 500 (units/ml). Endothelial cells were isolated from the aorta by digestion for 20 min with collagenase (Worthington I, 0.75mg/ml) in 10 ml of DPBS with antibiotics and sterilized by filtration. In order to remove the endothelial cells from the vessel wall, the intimal surface was gently washed 10X with Eagle's minimum essential medium (EMEM, Sigma, St. Louis, Mo.) supplemented with 30 % fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (1.25 µg/ml). The cells were incubated in humidified air containing 5% CO₂ and 2 ml medium was replaced every 48hr. The cells were cultured at 39⁰ C, which is a compromise between the normal body temperature (42⁰C) of quail and the temperature (37⁰C) of the medium and reagents usually used for the culture of mammalian cells (149).

Cell Morphology and Growth

Endothelial cells grew in a monolayer and were examined daily for growth and morphology. Cells were trypsinized, split in a 1:3 ratio and passaged when they reached confluence, which was usually achieved at 6-7 days. Cells from duplicate dishes were quantified by direct counting of viable cells using trypan blue, and exhibited the typical 'cobblestone' morphology of confluent cultures of AECs (295).

Cell Characterization

Quail AECs were identified by their ability to take up acetylated LDL (Dil-Ac-LDL) (296) and by probing with QH1 (297), an antibody to quail AECs and 1E12 (298), an antibody to smooth muscle cells (SMCs). Cells were grown on gelatin-coated coverslips for cell characterization. For acetylated LDL uptake experiments, the cells were incubated with 10 $\mu\text{g/ml}$ Dil-Ac-LDL at 39 $^{\circ}\text{C}$ in EMEM for 4 h. The media were then removed and the cells were washed once with probe-free medium for 10 min, rinsed with DPBS, and then fixed with 10% neutral buffered formalin (NBF) for 5 min. The Dil-Ac-LDL was visualized by epifluorescence microscopy.

For QH1 and 1E12 probing, cells were fixed in 10% NBF for 10 min. After washing twice with DPBS, the coverslips were incubated with either QH1 (1:100) or 1E12 (1:50) antibodies for 1 h at room temperature. After extensive washing, the cells were incubated with goat anti-mouse IgG coupled to fluorescein isothiocyanate (1:50) overnight at 4 $^{\circ}\text{C}$. The cells were washed with DPBS and examined with a Zeiss photomicroscope equipped with u.v. epifluorescence.

Preparation of cells

Cells were used between passages 3-7 for biochemical assays and incubated overnight in DMEM containing 1% FBS. The cells from each bird were washed 2X with 0.1 M PBS, pH 7.4, ice-cold PBS-EDTA, 1mM pH 8.0, containing 10X protease inhibitor cocktail (5.7 mM PMSF, 15mM EDTA, 100uM Pepstatin, 50uM Leupeptin, 3uM Aprotinin) and cells were detached on ice with a rubber cell-scraper. Cells were centrifuged at 1,000g for 5 min at 4 $^{\circ}\text{C}$ and the cell pellet resuspended in 20 mM Tris-HCl, pH 7.4 containing 10X protease inhibitor cocktail. Cells were sonicated on ice 2X, 15s at 20W using a tabletop sonicator (Branson, Danbury, CT). Homogenates were centrifuged at 4 $^{\circ}\text{C}$ at 12,000g for 30 min. The supernatant was stored at -70 $^{\circ}\text{C}$ and used for biochemical assays.

Protein Determination

Supernatants were analyzed for protein content by the method of Bradford (177) and absorbance read at 595 nm.

Total HO activity

Heme oxygenase enzyme activity was measured in supernatants by bilirubin generation as previously described (230). Bovine liver served as the source of partially purified bilirubin reductase (230). A reaction mixture containing 1 mg/ml of aortic endothelial cell protein, 25 μ M hemin, 1.0 mg/ml biliverdin reductase, 2 mM glucose 6-phosphate, 1 U glucose 6-phosphate dehydrogenase, 0.25 M sucrose and 20mM Tris, pH 7.4 in a final volume of 950 μ L was preincubated at 37⁰C for 10 min in the dark. The reaction was initiated by adding 50 μ L of 20 mM NADPH and subsequently scanned with a spectrophotometer (Perkin Elmer, Connecticut, USA). The amount of bilirubin formed was determined from the difference in optical density between 462 and 530 nm (extinction coefficient, 40 nm⁻¹ cm⁻¹ for bilirubin). The reaction mixture without the NADPH generating system served as a blank. HO enzyme activity was expressed as picomoles of bilirubin formed/mg protein/h.

SDS-PAGE and Western Blot analysis

Equal amounts (10 μ g) of AEC supernatant protein were electrophoresed on a 12% SDS-polyacrylamide gel and then transblotted onto nitrocellulose membranes (0.2 μ m pore size) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Richmond, CA). The blots were

blocked in TBS-T (Tris-buffered saline, pH 7.5, 0.05% Tween-20) containing 2% skim milk at room temperature for 1 h. After two washes with TBS-T for five min intervals, blots were incubated with a rabbit polyclonal anti-HO-1 (SPA-895, StressGen Biotechnologies, Victoria, Canada) for 1 h at room temperature in TBS-T containing 2% skim milk. After three, 5 min washes with TBS-T, blots were incubated with a 1/3000 dilution of alkaline phosphatase-conjugated goat-anti-rabbit IgG (Bio-Rad). After 1 hr at room temperature, blots were washed twice for 5 min intervals with TBS-T, twice for 5 min with TBS, and the colour was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (NBT) as a substrate (Sigma).

To control for slight variations in protein loading between samples, HO-1 controls were included on each blot. Briefly, AEC were incubated in DMEM with 1% FBS and 50 μ M CoCl₂ for 24 h, allowed to recover for 12 h and then collected for Western blot analysis as previously described. Densitometry analysis of blots was performed using Sigma Scan Pro software (Jandel Scientific, San Rafael, CA). The sample HO-1 –to- HO-1 control signal ratio was expressed as relative percentage of HO-1.

Immunohistochemical detection of HO-1

Quail AECs were grown on gelatin-coated glass slides, fixed in methanol for 5 min at -20° C and washed in TBS-T. Cells were incubated overnight at 4° C with 1:25 polyclonal rabbit anti-HO-1 antibody in a humidified chamber. After incubation, slides were washed four times in PBS-T, subsequently incubated with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antibody for 2 h at 37° C, and again washed four times. Slides were mounted in *p*-phenylenediamine and viewed with Zeiss fluorescent microscope.

Ferritin assay

Quail AECs were assayed for ferritin content by an enzyme-linked immunosorbent assay (ELISA) method (231). Antigen solution was prepared by dilution of cell supernatants to 30mg protein/L in coating solution (50 mM sodium carbonate-bicarbonate buffer, pH 9.6). Wells of plates were coated with 50 μ L of antigen solution and incubated overnight in a humidified chamber at 4⁰C. Plates were washed three times with PBS-T (10mM PBS, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with blocking buffer (5% BSA/PBS) for 2 h in a humidified chamber at room temperature. After washing three times with PBS-T, the plates were incubated at room temperature for 1 hr with rabbit anti-rat ferritin antibody (50ul/well, 1/1000; gift from R.S. Eisenstein, Univ. Wisconsin), washed four times with PBS-T, and subsequently incubated for 1 h with goat anti-rabbit IgG horse radish peroxidase conjugate (50 μ L/well, 1/5000). Color was developed for 10 min using 3',3',5',5'-tetramethylbenzidine (0.01mg/ml) and the reaction was stopped by adding 50 μ l 1 M H₂SO₄ to each well. Absorbance was measured at 450nm using a microplate reader (BioTek instruments, Vermont, USA). Purified ferritin from rat liver was used as standard (Sigma). The results were expressed as nanograms ferritin/mg protein.

Bleomycin-chelatable iron

Iron available to participate in free radical reactions was measured using the bleomycin assay (232). All buffers and reagents, except bleomycin, were treated with Chelex-100 (Sigma) to remove extraneous iron. Previously collected AEC supernatants (10 μ l; diluted 1:1 [vol:vol])

with double distilled H₂O) were incubated with 50 µL of MgCl₂ (50 mM), 400 µL salmon sperm DNA (500 µg/ml), 10 µL bleomycin (1.5 U/ml), 50 µL of Tris (1.0 M, pH 7.4), and 25 µL of ascorbate (7.5 mM, pH 7.4) for 1 hr at 37⁰C. The reaction was stopped by the addition of 50 µL of EDTA (100 mM). Thiobarbituric acid (TBA) (0.3% [wt/vol] in 50 mM NaOH) and 1.5 N HCl were added and the mixture was incubated in a water bath at 80⁰C for 20 min. The sample was vortexed, centrifuged at 12,000 x g for 5 min and the absorbance of the supernatant was read spectrophotometrically at 532 nm. Iron content was calculated according to a standard curve and results reported in µg/mg protein

Antioxidant components

Glutathione content

Determination of total glutathione (GSH) was measured according to Moron *et al.* (299). Cell sonicates were precipitated by adding 500 µl of an ice-cold solution of 4% (w/v) TCA and 2 mM EDTA and incubated at 4⁰C for 15 min, after which cell lysates were collected and centrifuged at 12,000g, 4⁰C, for 5 min. The absorbance of the supernatant (200 µL) was measured at 412 nm using 5,5' -dithiobis-(2 nitro-benzoic acid) (Sigma) in 0.1 M phosphate buffer, pH 8.0. Intracellular GSH content was calculated from a standard curve using GSH (reduced form, Sigma) as standard, and the results were expressed as nmoles per mg of cellular protein.

Glutathione reductase

Glutathione reductase (GRd) activity was determined in AECs as the rate of disappearance of NADPH (148) and expressed as nmoles of NADPH oxidized to NADP per minute per mg cell protein. Absorbance at 340 nm was recorded over 5 min to monitor the conversion of NADPH to NADP.

Glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined by a modification (148) of an indirect coupled assay procedure originally outlined by Paglia and Valentine (183) and expressed on the basis of nmoles NADPH oxidized to NADP per minute per mg protein using an extinction coefficient for NADPH at 340 nm of 6.2×10^6 M/cm.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured using NBT and expressed as units of SOD formed per mg protein (148). One unit of SOD activity is defined as the amount of enzyme activity that causes 50% inhibition of NBT reduction. The rate of inhibition of NBT reduction by superoxide generated by photoreduction of riboflavin was determined by measuring the absorbance at 560 nm.

RESULTS

Growth characteristics

Quail AECs were isolated from WILD (n = 3) and RES (n = 10) and SUS (n = 10) birds. Almost all (90-95%) of the growing cells were viable as assessed by trypan blue exclusion. The AECs rapidly attached to the culture dishes as patches of tightly packed cells and within 6 hours, the cells had started dispersing and spreading (Fig. 4.1 A-C, insets). Within 2-3 days, the cells had formed incomplete monolayers of essentially uniform- sized, polygonal-shaped cells (30-50 μm) usually with a centrally located oval nucleus surrounded by granules and a thin peripheral cytoplasm with distinct borders (Fig. 4.1 A-C). After 6-7 days, the cells had grown into single confluent monolayers of tightly packed polygonal cells (Fig. 4.1 D-F). No differences were observable in cell morphology or growth characteristics in AEC isolated from the three strains.

Endothelial cell characterization

Uptake of acetylated LDL using Dil-Ac-LDL was used to verify that the cultured cells were endothelial and not vascular smooth muscle cells. The primary cultures of AECs probed for Dil-Ac-LDL uptake were brightly stained (Fig 4.2 A-C). The fluorescence was localized to the cell membrane and cytoplasm. Endothelial cells from each strain of quail exhibited similar staining patterns (i.e. shape, granular contents, intensity) for Dil-Ac-LDL with low background fluorescence.

Quail AECs were also probed with QH1 and 1E12 antibodies, which identify ECs and SMCs, respectively. Immunofluorescence staining using the QH1 antibody (Fig. 4.2 D-F) showed large amounts of fluorescence within the nucleus and cytoplasm of the three strains.

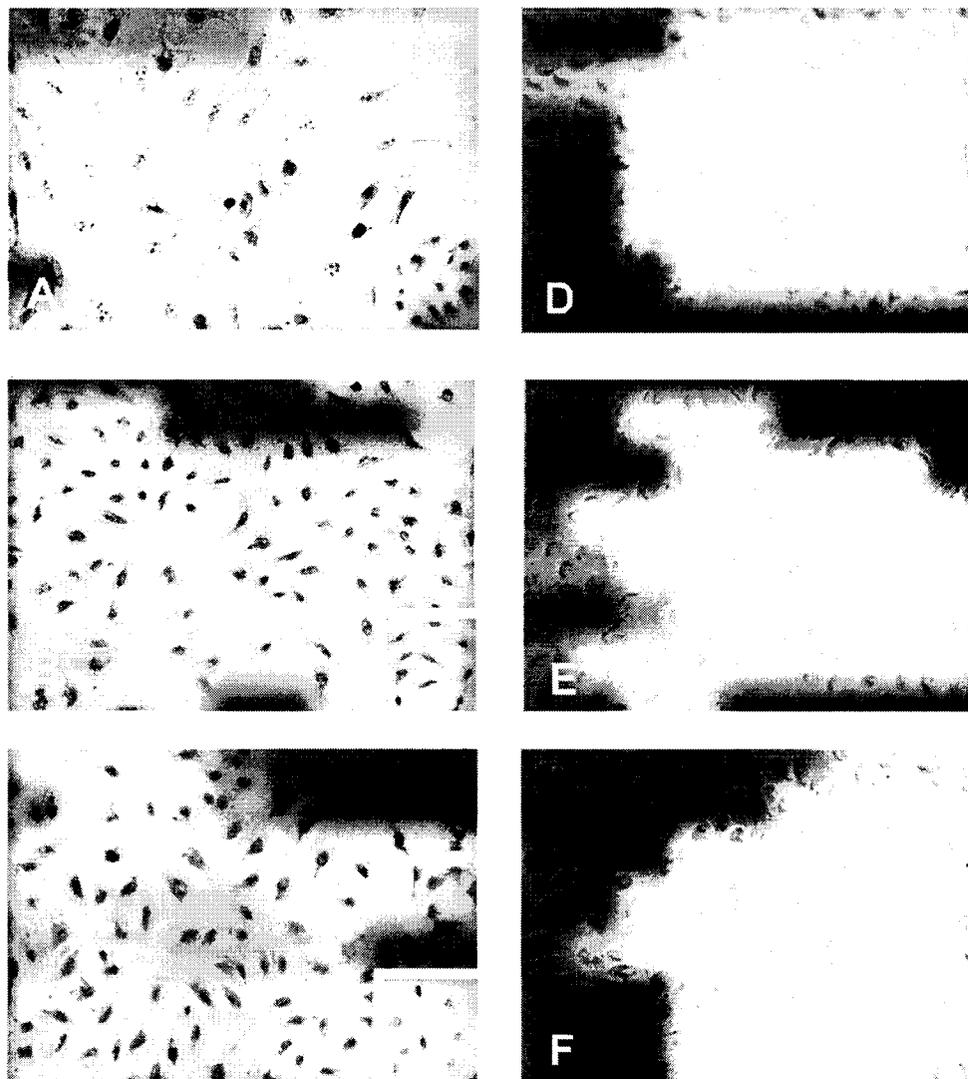


Figure 4.1 Photomicrographs of aortic endothelial cells (AECs). Growing edge of RES (A), SUS (B) and WILD (C) endothelial cell culture, 3 days (x 400), hematoxylin stained; AEC patch attached to culture dish 6 hours after seeding (inset). Phase-contrast confluent monolayer of primary ECs from RES (D), SUS (E) and WILD (F) birds, 7 days (x 400). Note the characteristic cobblestone appearance of the EC culture.

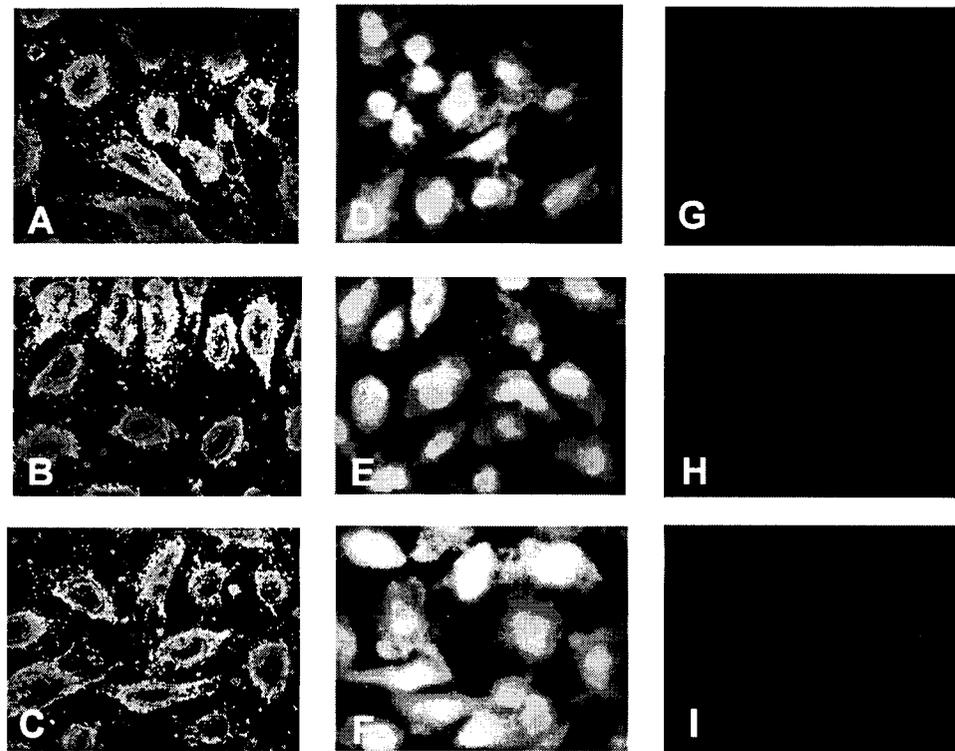


Figure 4.2. Characterization of aortic endothelial cells (AECs). Photomicrographs of atherosclerosis-resistant (RES) (top row), atherosclerosis-susceptible (SUS) (middle row), and random bred (WILD) (bottom row) Japanese quail AECs labeled with Dil-Ac-LDL (A, B, C), and QH1 (D, E, F) and 1E12 (G, H, I) antibodies (x 750).

There was no cross-reactivity in AEC cultures to the 1E12 antibody (Fig. 4.2 G-I), indicative of a relatively pure AEC culture with little or no contamination by SMC.

Heme oxygenase in WILD, RES, and SUS AECs

All biochemical parameters were measured in confluent cells. In addition, HO enzyme activity was also measured in subconfluent cells for comparison. At confluence, AECs from all three strains had similar levels of HO enzyme activity (data not shown). However, in subconfluent (~40%) cells, HO activity was significantly ($P < 0.001$; Least Squared Analysis of Variance) higher in RES cells (56.4 ± 3.66 pmol bilirubin/mg protein/min) than in SUS cells (21.2 ± 4.05) (Fig. 4.3). WILD AEC's were not examined at subconfluence.

Levels of immunoreactive cellular HO-1 protein were significantly ($P < 0.001$) higher in RES cells (10.2 ± 0.65 %) compared with SUS (2.33 ± 2.32 %) and WILD (3.86 ± 3.9 %) AECs (Fig. 4.4 B). Similarly, immunohistochemical detection of HO-1 protein revealed that RES cells had higher levels of HO-1 immunoreactive protein signal than SUS and WILD cells (Fig. 4.4 C). The distribution of the signal for HO-1 was uniform within each strain and was confined to the cytoplasm.

Ferritin protein was not significantly different between AECs from the three strains (data not shown), but catalytic iron levels in SUS cells (15.87 ± 1.95 $\mu\text{g}/\text{mg}$ protein) tended to be higher ($P < 0.053$) than RES cells (9.07 ± 1.77) (Fig. 4.5).

Antioxidant components

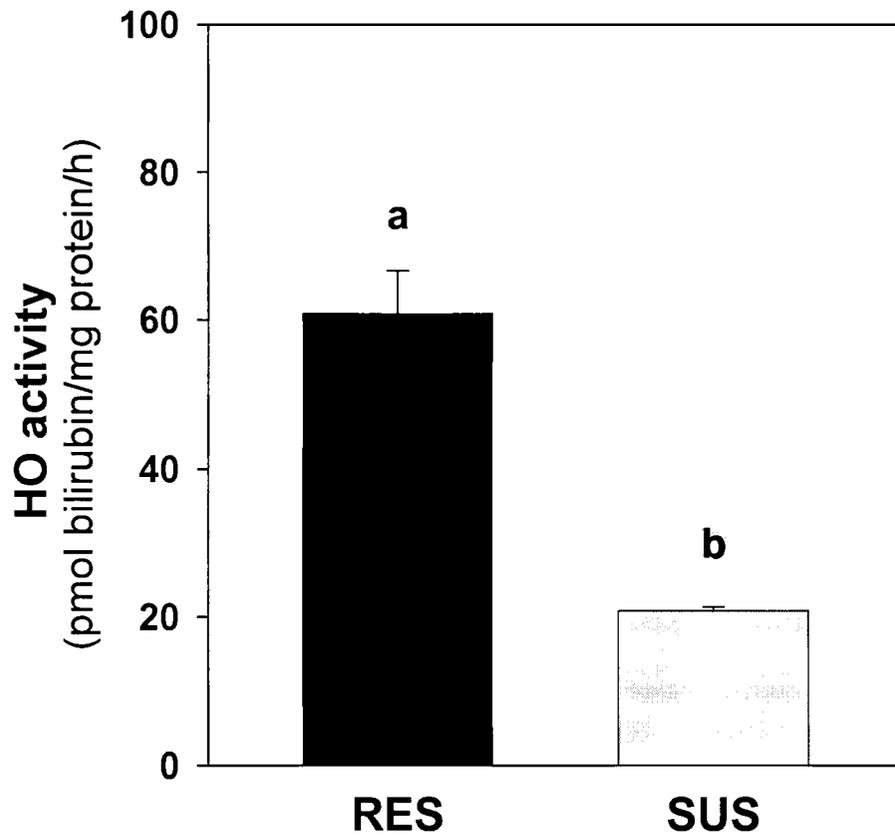
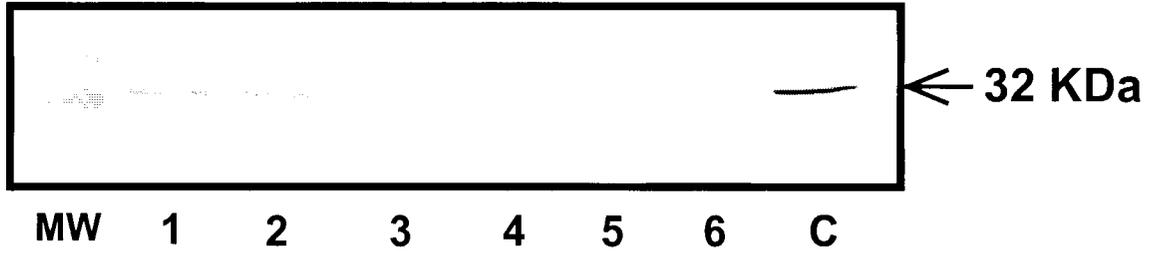


Figure 4.3. HO activity in RES and SUS aortic endothelial cells at subconfluence (40%).

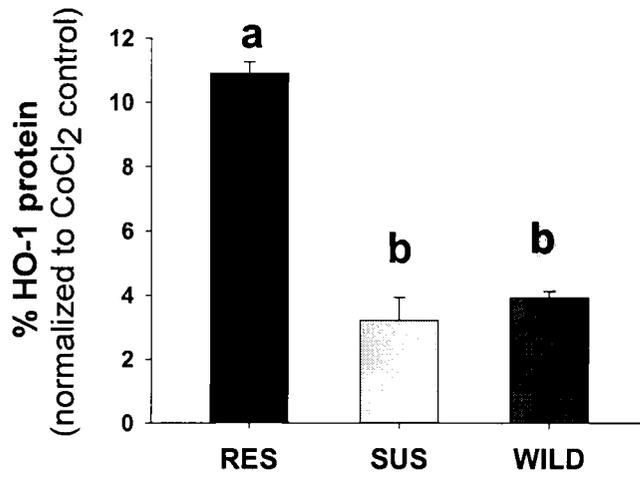
Values are expressed as pmol of bilirubin formed per mg protein per hr, and are means \pm SE of 10 individual birds. Data points under the same letter were not significantly different. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

Figure 4.4 HO-1 expression in RES, SUS and WILD aortic endothelial cells (AECs). (A) Representative Western blot analysis of HO-1-immunoreactive protein content in RES, SUS and WILD AECs. MW= molecular weight; C= CoCl₂ control. Two examples for each strain are shown. (B) HO-1-immunoreactive protein content in RES, SUS and WILD cells in *A* quantified by densitometry. Mean signal intensity in ECs was normalized to mean signal in CoCl₂-treated cells. Values are means ± SE for n=10 (RES, SUS) and n=3 (WILD) individual birds. * P<0.001 vs. SUS and WILD cells. (C) Immunohistochemical detection of HO-1 protein in RES, SUS and WILD cells (representative of 3 experiments).

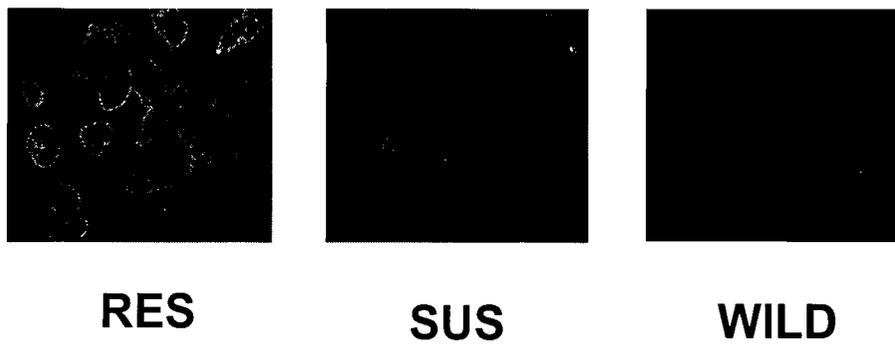
A



B



C



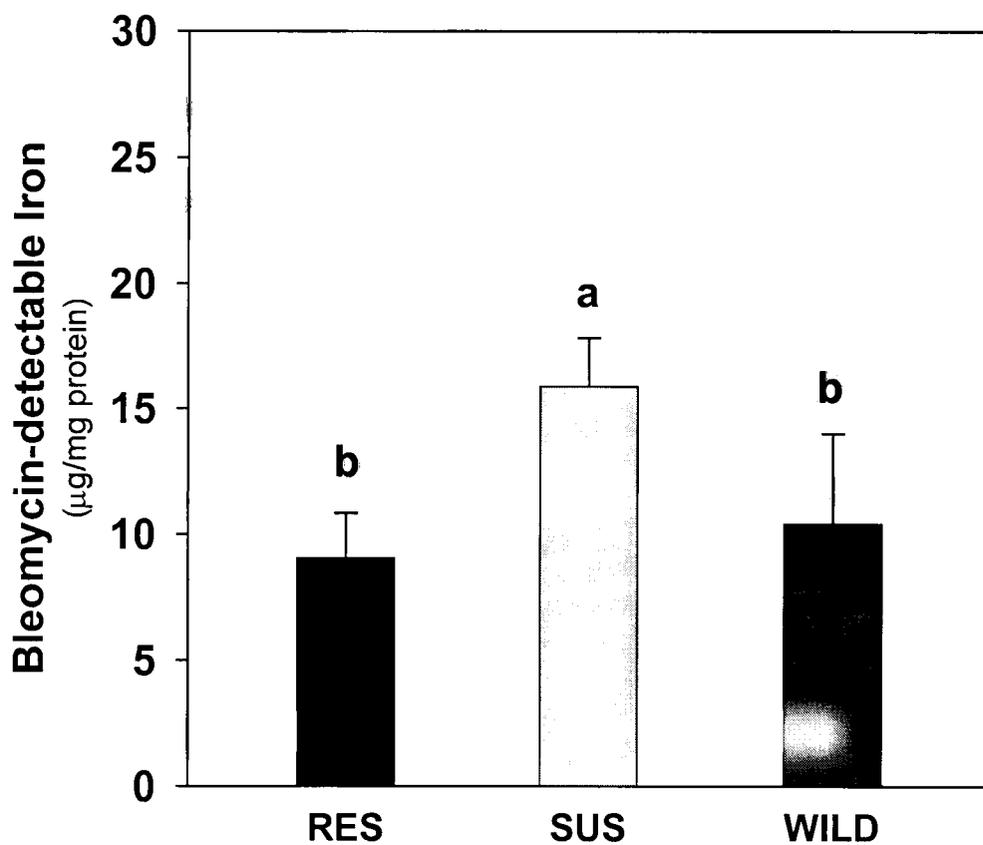


Figure 4.5. Catalytic iron content in RES, SUS and WILD aortic endothelial cells (AECs). Catalytic iron was measured using the bleomycin assay. Values are means \pm SE of 10 (RES, SUS) and 3 (WILD) individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance

Only 5 SUS, 5 RES, and 3 WILD birds were used for measuring antioxidant components. The SUS cells had a significantly ($P < 0.0024$) higher GSH content (43.3 ± 1.89 nmol/mg protein) compared with RES cells (32.78 ± 1.71) (Fig. 4.6 A). WILD cells had GSH content (36.7 ± 3.51) intermediate between SUS and RES. However, SUS cells had a significantly ($P < 0.0001$) lower GRd activity (6.83 ± 0.05 nmol/mg protein) compared with RES (7.77 ± 0.06) and WILD (7.63 ± 0.05) cells (Fig. 4.6 B). SOD activity was lower ($P < 0.0001$) in the WILD strain (10.9 ± 0.08 Units/mg protein) compared with the RES (11.7 ± 0.08) and SUS (11.77 ± 0.08) strains (Fig. 4.6 C). The activities of GPx were not significantly different between the three strains (data not shown).

DISCUSSION

Damage to AECs is important in a number of pathological conditions including atherosclerosis, and oxygen free radicals are known to be major contributors to this injury process. In the present study, we have studied cultured AECs from strains of Japanese quail selected for susceptibility or resistance to dietary-induced atherosclerosis that would be a good model for investigation of AEC alterations in atherosclerosis. This is to our knowledge the first reported culture of AECs from Japanese quail.

Our cultured cells were confirmed as AECs, as all cells were positive for the expression of QH1, a specific endothelial cell antibody (297). Moreover, all of the cells rapidly internalized Dil-Ac-LDL, a characteristic property of ECs (296). Finally, negative 1E12 staining confirmed that the cultures were not contaminated by SMC (298). Multiple independent isolates from

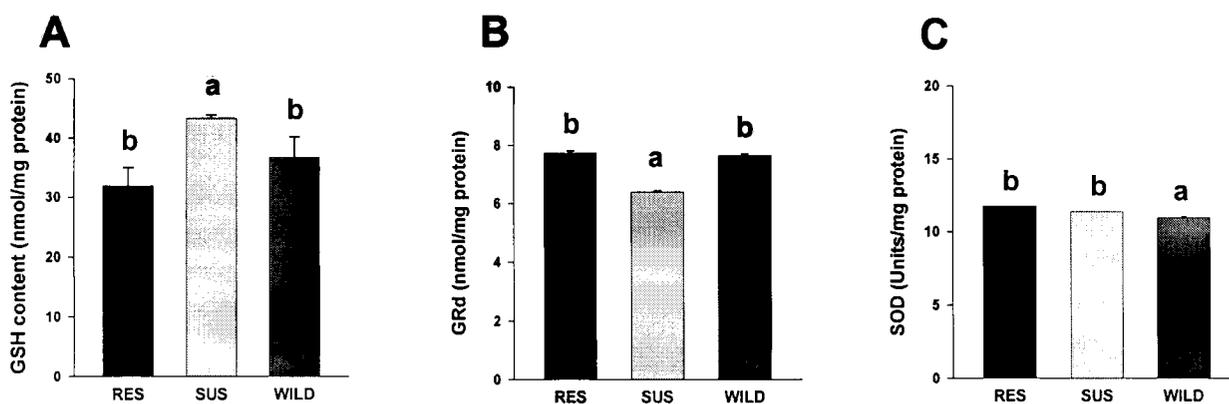


Figure 4.6. Antioxidant components in RES, SUS and WILD aortic endothelial cells. (A) Glutathione (GSH) content, and (B) Glutathione reductase (GRd) and (C) Superoxide dismutase (SOD) enzyme activities. Values are means \pm SE of 5 (RES, SUS) and 3 (WILD) individual birds. Columns under different letters were significantly different ($P < 0.05$) by analysis of variance

strains of WILD, RES and SUS birds by our present methods gave reproducible results for the expression of endothelium-specific markers.

It has been suggested that HO may play an important role in protecting the endothelium against oxidative injury (287). Heme oxygenase has been shown to be inducible by various oxidant stresses and this property may be important in developing resistance to oxidative stress. It has been reported that over- or under-expression of HO-1 alters resistance to oxidative stress (285,300). While subconfluent ECs from the RES strain had greater activity of HO compared with ECs from SUS strains, there were no significant differences in HO activity among the three strains at confluence. Moreover, at confluence, HO-1 protein levels were higher in RES cells compared with WILD and SUS cells. Although HO-1 mRNA was not measured in our cells, it has been reported that HO enzyme activity may be a more accurate measure of HO (301-302). Differences in other enzyme activities and protein synthesis between post-confluent and subconfluent cells have previously been reported (303-305). One possible explanation is that there is a modified enzyme in RES cells that is less stable and loses activity more readily. Such a postulated "modified" enzyme could arise during translation or occur as a post-translational modification event. Differences in cellular growth and proliferation can be excluded as an explanation for differences in HO activity and HO-1 protein levels, as with other gene products (304), because we detected no such differences in cell growth or protein levels during culture.

Our findings of higher levels of bleomycin-detectable "free" iron in SUS cells compared with WILD and RES cells raise the possibility that catalytic iron may be an important determinant in the susceptibility of quail AEC to oxidative stress. Free (i.e. non-chelated) iron

is known to be a powerful catalyst of oxidative reactions (45), and has been implicated in atherogenesis. Thus, iron accumulation has been shown to occur at the onset of lesion formation in a rabbit model of atherosclerosis (305), and serum iron levels have been implicated as an independent risk factor for coronary artery disease (306). The induction of HO is often coupled to an increase in ferritin (283), and it has been suggested that ferritin induction is important in decreasing oxidant damage to cells, possibly through sequestration of catalytic iron. However, differences in ferritin protein levels were not observed in AECs from the three strains, despite higher catalytic iron levels in AECs from SUS strain. These findings raise the possibility that under normal conditions, higher levels of catalytically active iron may predict susceptibility to oxidation, and during oxidative stress, higher levels of catalytically active iron may instigate the oxidation of LDL in SUS birds, and OxLDL in turn may inflict endothelium damage.

Interspecies and inter-strain differences in susceptibility to dietary atherosclerosis are known to exist (307), and the elucidation of molecular determinants responsible for such variations may provide clues concerning the underlying pathological processes. A substantial body of evidence shows that oxidative processes are potent inducers of antioxidant components (289). Studies on cultured ECs have provided evidence that the glutathione redox cycle is a crucial antioxidant defense mechanism during oxidative stress (308-309). Furthermore, the observed increase in GPx and SOD activities in the arterial wall during atherogenesis suggests a possible role for these antioxidants in arterial wall protection (309). Diet-induced alterations in plasma lipid levels may be associated with decreases in the activity of cellular antioxidants (152) and oxidative stress may also lead to inactivation of antioxidant components (310). Finally, lower

catalase and glutathione reductase levels in ECs compared to renal epithelial cells may increase their susceptibility to oxidative stress (311).

Previously, we observed that challenge with an atherogenic diet induced distinct differences in aortic and plasma antioxidant components in SUS quail *versus* RES quail (152). In SUS quail, we found a negative correlation between severity of atherosclerotic lesions and aortic SOD activities, and a positive correlation between plasma SOD and plasma cholesterol and triglycerides. In addition, we found lower aortic GRd in SUS birds compared to RES birds even without exposing the birds to a high cholesterol diet. In the present study, we explored the status of antioxidant systems in cultured AECs from WILD, RES and SUS strains and found lower GRd in SUS cells compared to RES and WILD strains. Sasaki *et al.* (312) suggested that GRd levels are very important in determining the antioxidant capacity of ECs. In addition, *tert*-butyl hydroperoxide (t-BHP) is mostly removed by GPx in fibroblasts (313), yet GRd was rate-limiting at high t-BHP concentrations. Our finding that AECs from SUS strains had lower GRd levels than RES AECs suggests a possible role for this antioxidant component in determining the differences in susceptibility to atherosclerosis between these two strains. We also found that SUS cells had a higher GSH content compared with WILD and RES cells. The higher levels of GSH found in SUS AECs may suggest compensatory increases in certain enzymatic antioxidants. Such a compensatory effect involving GSH has been suggested as a possible cause for its increased concentration in red blood cells of patients with severe familial hypercholesterolaemia (314). Thus, it is clear that vascular endothelial cells are sensitive to the toxicity of reactive oxygen species (ROS), and a number of investigations indicated that endothelial cells are primarily dependant on the GSH redox system for the protection from ROS toxicity (308-309); it may be speculated, therefore, that differences in the glutathione

redox cycle in AECs from the SUS strain result in a greater susceptibility to oxidative stress compared with RES AECs.

CONCLUSIONS

In conclusion, the differences in atherosclerosis susceptibility between selected strains of RES and SUS quail may be due, at least in part, to differences in endothelial HO activities and antioxidant components, and this provides evidence for genetic factors in atherosclerosis that act at the level of ECs. However, differences in antioxidant enzyme activities do not necessarily predict susceptibility to oxidation, especially if there are changes in other antioxidants, either enzymatic or non-enzymatic, that were not examined. In order to further investigate the nature of genetic differences in atherosclerosis, we extended our analyses of RES and SUS AECs to measurements of susceptibility to *in-vitro* oxidative challenge as a functional measure of antioxidant capacity.

CHAPTER 5

EFFECTS OF OXIDANT-INDUCED INJURY ON HEME OXYGENASE AND GLUTATHIONE IN CULTURED AORTIC ENDOTHELIAL CELLS FROM ATHEROSCLEROSIS-SUSCEPTIBLE AND –RESISTANT JAPANESE QUAIL.

PREFACE

A manuscript based on the work described in this Chapter has been accepted for publication in *Molecular and Cellular Biochemistry* and was coauthored with D.V. Godin, J. Kurtu and K.M. Cheng. We thank Jamal Kurtu (Quail Genetic Stock Centre, UBC) for his contribution to project planning and animal care and Jenny Hart (Pulmonary Research Centre, St. Pauls. Hospital, Vancouver, B.C) for her technical assistance with the cell cultures.

INTRODUCTION

The induction of heme oxygenase (HO) is a cellular response to oxidative stress (281). The HO-1 isoenzyme is ubiquitously expressed and is inducible by a variety of oxidant stresses (315). Oxidation releases heme from hemoglobin and other heme proteins. Heme has been shown to promote the formation of reactive oxygen species (ROS) (316), which can lead to cellular injury. HO catabolizes heme to catalytically active iron, carbon monoxide and bilirubin. Although, catalytic iron has been shown to promote the formation of ROS *in vitro* (317), co-induction of ferritin with HO-1 has been demonstrated *in vitro* (301) and *in vivo* (219), suggesting that this may be linked to the sequestration of catalytically active iron (320).

The activation of oxidative-sensitive mechanisms in the arterial wall and increased oxidation of low density lipoprotein (LDL) through free radical-mediated tissue injury may be an important factor in the early formation of atherosclerotic lesions. HO-1 may protect against oxidized lipids such as those produced by oxidative stress (128). HO-1 is present in mice (320) and human (67) atherosclerotic lesions, and oxidized LDL increases HO-1 expression in vascular endothelial cells (67). Inhibition of oxidative modification of lipoproteins by antioxidant interventions has been intensively investigated (321-323). Bilirubin, an end-product of the HO pathway, appears to act as an antioxidant during surgical stress in humans (324-325), it protects low-density lipoprotein (LDL) against peroxy radical-induced oxidation *in vitro* (326), and lower serum bilirubin has been associated with increased risk of ischemic heart disease *in vivo* (101). The glutathione redox cycle (152,187,327-328) has also been shown to play an important role as an endogenous antioxidant defense mechanism in cultured endothelial cells exposed to oxidative stress (329,Chapter 4).

Aging increases the risk and severity of atherosclerosis (330), and may be associated with decreased antioxidant capacity (331). Older animals are more susceptible to atherosclerotic lesion development after consuming an atherogenic diet compared with young animals (332).

We have successfully cultured aortic endothelial cells (AECs) from atherosclerosis-susceptible (SUS) and atherosclerosis-resistant (RES) Japanese quail (Chapter 4). We found that AECs from the SUS strain had lower levels of HO-1 protein and glutathione reductase, and higher levels of catalytic iron compared with RES cells, suggesting that differences in susceptibility to atherosclerosis may be, in part, due to genetic differences in HO and related antioxidant components of AECs. However, these differences in profiles of the endogenous antioxidants examined are not necessarily directly predictive of differences in cell

susceptibility to oxidative stress. We therefore studied the effects of an *in-vitro* oxidative challenge and examined the involvement of HO components and antioxidants in influencing susceptibility to oxidative stress-associated responses in AECs from RES and SUS strains. At the same time, we also investigated the expression of the major 70-kDa heat shock protein (HSP70) (333). The results obtained suggest a differential genetic expression of HO induction and its reaction products in AECs from SUS and RES strains, and demonstrated the influence of the HO system on the susceptibility of AECs to oxidative stress.

MATERIALS AND METHODS

Cell lines and culture conditions

Endothelial cells were derived from five individual atherosclerosis-susceptible and -resistant quail aortas as previously described (Chapter 4). Briefly, cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 15% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml), and amphotericin B (1.25 µg/ml) and maintained at 39⁰ C in a 95% room-air-5% CO₂ humidified chamber. ECs were characterized by the uptake of Dil-Ac-LDL (296), and by probing with EC (297) and smooth muscle cell antibodies (298).

Exposure to oxidative stress

Cells from passages 3-6 were used. The cells were incubated in DMEM containing 1% FBS for 24 h before use in experiments. In a pilot study, cells were exposed to different concentrations of tert-butylhydroperoxide (t-BHP) (0.1-0.5 mM) to determine the concentration of peroxide

necessary to elicit a reliable measurement of oxidative stress as determined by an increase in HO enzyme activity. T-BHP was added to Dulbecco's phosphate-buffered saline (DPBS), and cells from each bird were treated with t-BHP (at a concentration of 0.25 mM) in DPBS for 1 h. Following treatment, the cells were washed by centrifugation at 500 g for 5 min, returned to fresh DPBS medium minus t-BHP, and incubated for the various time periods indicated. Cells were harvested as previously described (Chapter 4), and sonicated on ice 2X, 15s at 20W using a tabletop sonicator (Branson, Danbury, CT). Homogenates were centrifuged at 4°C at 12,000g for 30 min. The supernatants were stored at -70 °C and used for biochemical assays as described below.

Heme oxygenase activity

Heme oxygenase activity was measured in cell supernatants by bilirubin generation as described by Ryter *et al.* (230). Bovine liver served as the source of partially purified bilirubin reductase. A reaction mixture containing 1 mg/ml of cell protein, 25 µM hemin, 1.0 mg/ml biliverdin reductase, 2 mM glucose 6-phosphate, 1 U glucose 6-phosphate dehydrogenase, 0.25 M sucrose and 20mM Tris, pH 7.4 in a final volume of 950 µL was preincubated at 37°C for 10 min in the dark. The reaction was initiated by adding 50 µL of 20 mM NADPH and the resulting mixture subsequently scanned with a spectrophotometer (Perkin Elmer, Connecticut, USA). The amount of bilirubin formed was determined as the difference in optical density between 462 and 530 nm (extinction coefficient, 40 nm⁻¹ cm⁻¹ for bilirubin). The reaction mixture without the NADPH generating system served as a blank. HO activity was expressed as picomoles of bilirubin formed per mg protein per h.

Cell protein content

Cellular protein content was measured by the method of Bradford (177) and absorbance read at 595 nm.

Western blot analysis for HO-1 and HSP70

For detection of HO-1 and HSP70 immunoreactive protein, 25 µg aliquots of supernatants were electrophoresed on a 12% SDS-polyacrylamide gel and then transblotted onto nitrocellulose membranes (0.2 µm pore size) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Richmond, CA). The blots were blocked in TBS-T (Tris-buffered saline, pH 7.5, 0.05% Tween-20) containing 2% skim milk at 4 °C overnight, washed 2X in TBS-T for five min intervals, and incubated with either a 1:500 polyclonal rabbit anti-rat HO-1 (StressGen Biotechnologies, Victoria, Canada) or 1:500 polyclonal rabbit anti-human HSP70 (StressGen) for 2 h at room temperature in TBS-T containing 2% skim milk. After three 5 min washes in TBS-T, blots were incubated for 1 hr with a 1:3000 dilution of alkaline phosphatase-conjugated goat-anti-rabbit IgG (Bio-Rad). Blots were washed twice for 5 min intervals in TBS-T, twice for 5 min in TBS, and the antigen-antibody signal was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (NBT) as a substrate (Sigma, St. Louis, MO). Densitometry analysis of blots was performed using Sigma Scan Pro (Jandel Scientific, San Rafael, CA).

HO-1 and HSP70 positive controls

For the semi-quantitative comparison of various samples across different Western blots, HO-1 and HSP70 immunoreactive protein was induced in a small number of AECs. Cells were incubated for 24 h in DMEM with 1% FBS and 50 μ M CoCl₂ to allow for induction of HO-1 immunoreactive protein. Cells were heat-shocked for 30 min at 45 °C in DMEM supplemented with 10% FBS, and incubated at 39 °C for 8 h to allow for induction of HSP70 immunoreactive protein. Cells were collected for immunoblotting, as described earlier, and included on each treatment blot for HO-1 and/or HSP70 analysis. The results were expressed as % of CoCl₂ or heat-shocked AECs.

ELISA for ferritin

Cells were assayed for ferritin content by an enzyme-linked immunosorbent assay (ELISA) method (231). Antigen solution was prepared by dilution of cell supernatants in coating solution (50 mM sodium carbonate-bicarbonate buffer, pH 9.6). Wells of plates were coated with 50 μ L of antigen solution and incubated overnight in a humidified chamber at 4°C. Plates were washed three times with PBS-T (10mM PBS, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with blocking buffer (5% BSA/PBS) for 2 h in a humidified chamber at room temperature. After washing three times with PBS-T, the plates were incubated at room temperature for 1 hr with rabbit anti-rat ferritin antibody (50ul/well, 1:1000; gift from R.S. Eisenstein, Univ. of Wisconsin), washed four times with PBS-T, and subsequently incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (50 μ L/well, 1:5000). Color was developed for 10 min using 3',3',5',5'-tetramethylbenzidine (0.01mg/ml, 10 μ L,) and the reaction was stopped by adding 50 μ l 1 M H₂SO₄ to each well. Absorbance was measured at 450nm using a microplate reader (BioTek instruments, Vermont, USA). Purified ferritin from

rat liver was used as standard (Sigma). The results were expressed as nanograms ferritin/mg protein.

Immunohistochemical detection of HO-1, HSP70 and Ferritin protein

Cells were grown on gelatin-coated glass slides, fixed in methanol for 5 min at -20°C and washed in PBS-T. Cells were incubated overnight at 4°C with 1:25 polyclonal rabbit anti-HO-1 antibody, 1:50 polyclonal rabbit anti-human HSP70 antibody, or 1:25 polyclonal rabbit anti-rat ferritin antibody in a humidified chamber. For detection of HO-1, slides were washed four times in PBS-T and further incubated with fluorescein isothiocyanate (FITC)- conjugated goat anti-rabbit antibody (1:25) for 2 h at 37°C , and again washed four times. The slides were then mounted in phenylene diamine and viewed with a fluorescent Zeiss microscope equipped with a 100-W mercury lamp. For detection of HSP70, slides were washed four times in PBS-T and further incubated with goat anti-rabbit IgG HRP conjugate for 2 h at 37°C . After three washes in PBS-T, color was developed using 3,3'-diamino-benzidine tetrahydrochloride (Sigma) for the localization of peroxidase activity. For detection of ferritin, slides were further incubated with goat anti-rabbit IgG alkaline phosphatase conjugate for 2 h at 37°C . After three washes in PBS-T, color was developed using Fast Red TR/Naphthol AS-MX (Sigma). Slides stained for ferritin protein were counterstained with hematoxylin. The slides for HSP70 and ferritin were viewed with a Zeiss photomicroscope.

Bleomycin assay

All buffers and reagents, except bleomycin, were treated with Chelex-100 (Sigma) to remove extraneous iron. Cell supernatants (10 μ l; diluted 1:1 [vol:vol] with double distilled H₂O) were incubated with 50 μ L of MgCl₂ (50 mM), 400 μ L salmon sperm DNA (500 μ g/ml), 10 μ L bleomycin (1.5 U/ml), 50 μ L of Tris (1.0 M, pH 7.4), and 25 μ L of ascorbate (7.5 mM, pH 7.4) for 1 hr at 37⁰C and the reaction was stopped by the addition of 50 μ L of 100 mM EDTA. Thiobarbituric acid (TBA) (0.3% [wt/vol] in 50 mM NaOH) and 1.5 N HCl were added and the mixture was incubated at 80⁰C for 20 min. The sample was vortexed, centrifuged at 12,000 x g for 5 min and the absorbance of the supernatant measured spectrophotometrically at 532 nm. Iron content was calculated according to a standard curve and results reported in μ g/mg protein

Glutathione content

We analyzed the effects of t-BHP-induced oxidative stress on glutathione content (299). Briefly, the cells exposed to t-BHP were precipitated by adding 500 μ l of an ice-cold solution of 4% (w/v) TCA and 2 mM EDTA and incubated at 4⁰C for 15 min, after which cell lysates were collected and centrifuged at 12,000g, 4⁰ C, for 5 min. The absorbance of the supernatant (200 μ l) was measured at 412 nm using 5,5' -dithiobis-(2 nitro-benzoic acid) (Sigma) in 0.1 M phosphate buffer, pH 8.0. Intracellular GSH content was calculated from standard curve using GSH (reduced form, Sigma) as a standard, and the results were expressed as nmoles per mg of cellular protein.

Thiobarbituric acid-reactive substances

The extent of lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substances (TBARS). Trichloroacetic acid (12% w/v) was added to an equal volume of supernatant, combined with thiobarbituric acid solution (0.5%) and boiled for 15 min. The formation of TBARS was determined spectrophotometrically at 532 nm, as described by Tappel and Zalkin (229).

LDH

Cell injury was measured by release of lactate dehydrogenase (LDH) according to Nachlas *et al.* (334). Briefly, the supernatant from t-BHP-treated cells was collected from each well and stored at 4 °C. Cell monolayers were treated with 2% Triton X-100 for 30 min at 25 °C to lyse the cell membranes, and then lysate was collected. LDH activity was measured in both the supernatant and cell lysate fractions, and is based on the conversion of tetrazolium salt into a red formazan product. The absorbance was determined at 492 nm. The LDH released from cells was expressed as a percent of the total LDH content (released by Triton X-100 treatment) and determined using the formula: Percent release = LDH activity in supernatant/(LDH activity in supernatant + LDH activity in cell lysate).

Statistical analysis

Least squares analyses of variance were carried out using the JMP (SAS Institute, North Carolina) program.

The ANOVA model was:

$$Y_{ijk} = \mu + S_i + A_j + SA_{ij} + E_{ijk}$$

where Y_{ijk} is the parameter measurement of the k th individual of the i th strain of the j th age group. S_i is whether the AECs came from RES or SUS. A_j is whether the AECs came from young or old birds. SA_{ij} is the interaction between strain and age. E_{ijk} is the error term. Percentage data (HO-1, HSP70, and LDH) were Arc-sine transformed before analysis.

RESULTS

HO enzyme activity

Time-dependant effects were measured after exposure of AECs to 0.25 mM t-BHP for 1 h (Fig. 5.1). There were no significant differences in response between the young and the old birds at any of the time points. The increase in HO activity for SUS AECs began within 2 h of exposure to t-BHP and peaked after 12 h, with a maximal 7.6 fold increase at 12 h. While showing a similar temporal response profile, t-BHP caused a significantly ($P < 0.001$) greater response in RES cells at all time points. RES AECs had a maximal increase in HO activity at 12 h of approximately 10-fold.

HO-1 expression

RES AECs had significantly ($P < 0.002$) higher HO-1 protein levels than SUS cells (Fig. 5.2), even prior to the t-BHP challenge (Fig. 5.2 A,B). Both RES and SUS cells had increased HO-1 protein levels, which peaked at 10 h following the oxidative stress. During this phase, RES AECs had significantly higher HO-1 levels than SUS cells at all time points. At the 10 h time

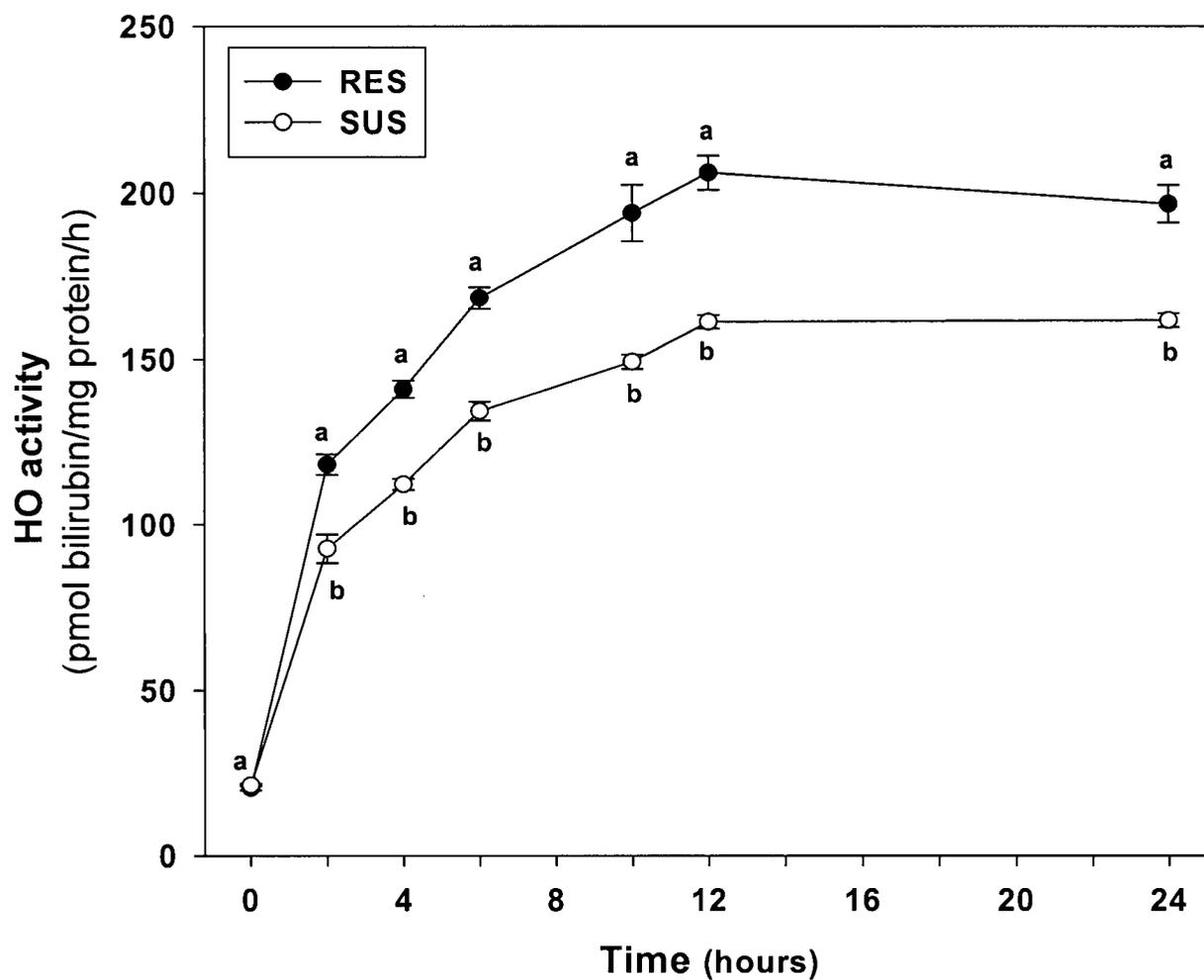
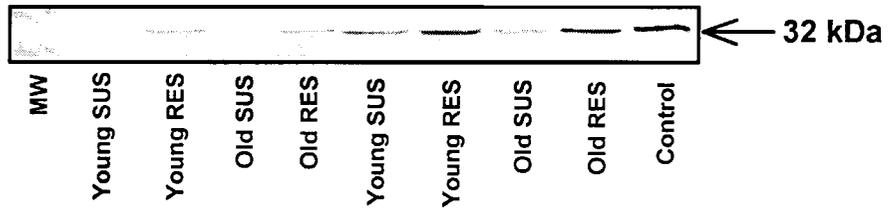


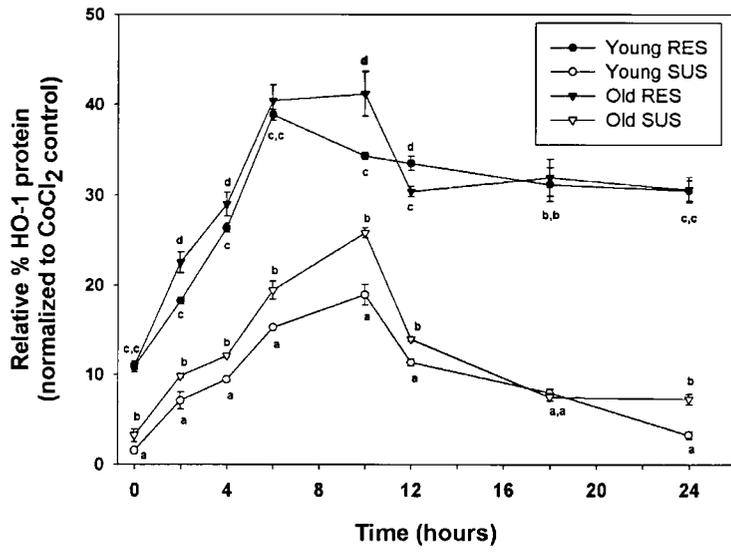
Figure 5.1. Total HO activity in aortic endothelial cells of RES and SUS Japanese quail before and after t-BHP exposure. Values are expressed as pmol of bilirubin formed per mg protein. Each data point represents the mean \pm SE of ten different individuals. Data points under different letters were significantly different ($P < 0.05$) by analysis of variance.

Figure 5.2. (A) Representative of Western blot analyses for HO-immunoreactive protein content in AECs of RES and SUS quail prior to and following oxidative challenge (10 h time-point); MW= molecular weight marker; HO-1=control derived from cobalt chloride-treated AECs. (B) HO-1 immunoreactive protein content in RES and SUS cells prior to and following oxidative challenge. Densitometric values for each sample were normalized to the values for the cobalt chloride-treated cells in each membrane to allow for comparison between membranes. Each data point represents the mean \pm SE of five different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance. (C) Immunohistochemical localization of HO-1 protein in RES and SUS cells (1) young SUS, preexposure; (2) young RES, preexposure; (3) old SUS, preexposure; (4) old RES, preexposure; (5) young SUS, post t-BHP exposure (10 h); (6) young RES, post t-BHP exposure (10 h); (7) old SUS, post t-BHP exposure (10 h); (8) old RES, post-exposure (10 h). Cells were assayed for immunoreactive protein as described in Methods, and were examined with a Zeiss fluorescent microscope at 400 \times .

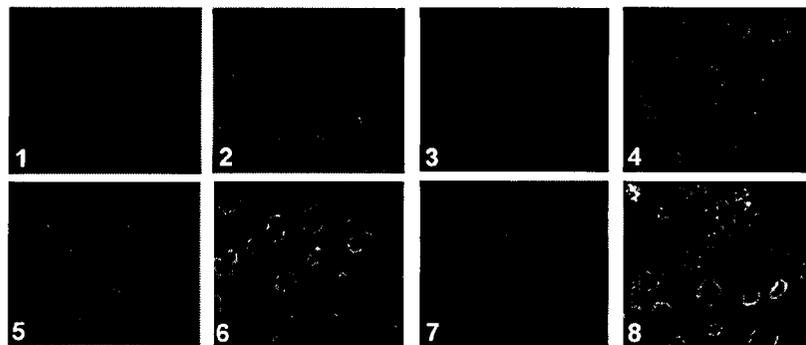
A.



B.



C.



point, there was also a significant age effect in that old AECs had significantly ($P<0.01$) higher HO-1 levels than cells from young birds. Although HO-1 levels started to decrease after 10 h for both strains, RES AECs maintained a significantly higher HO-1 level than SUS cells during this decreasing phase (Fig. 5.2 B).

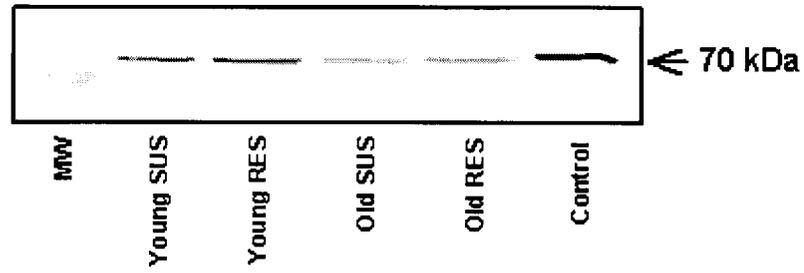
There were no visible differences in the localization of HO-1 protein in t-BHP-treated cells compared with controls (Fig. 5.2 C). Immunohistochemical analysis of control cells showed little cytoplasmic staining with anti-HO-1 in SUS cells, whereas greater staining was visible in RES cells, regardless of age. Following the oxidative stress, SUS cells had increased cytoplasmic staining, predominately around the cell membrane, whereas the RES strain showed an overall stronger response than controls and oxidant-exposed SUS cells.

HSP70

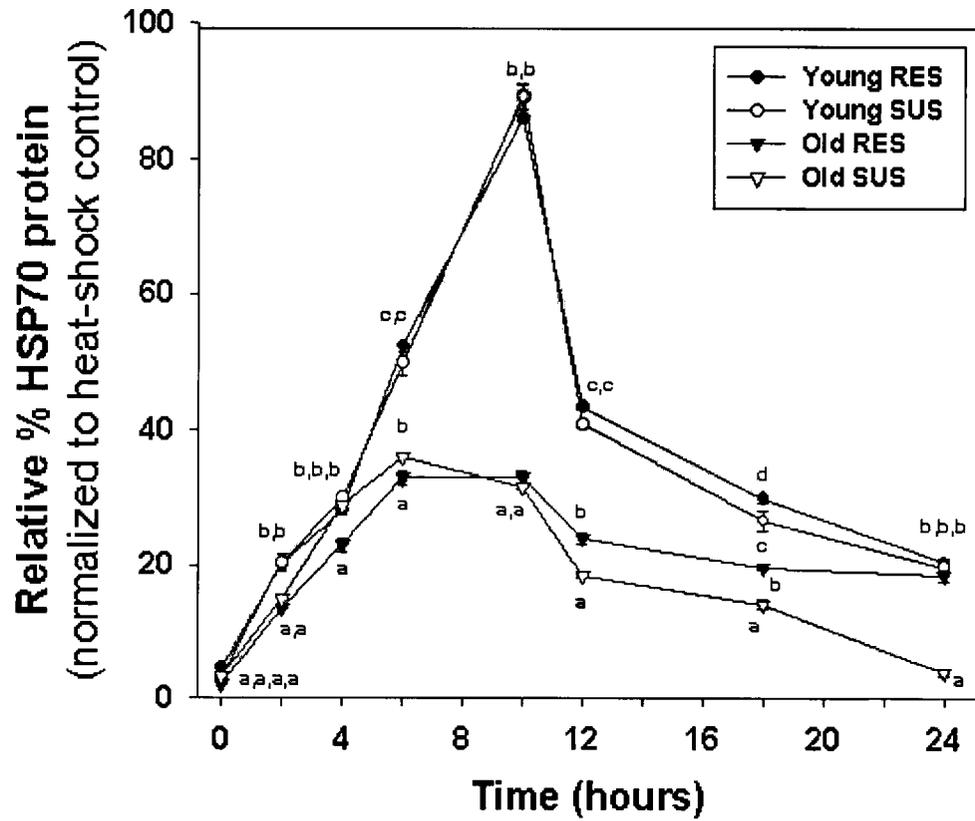
HSP70 was not detectable in cells not subjected to the oxidative challenge. Following exposure to t-BHP, AECs from young birds had significantly ($P<0.001$) higher HSP70 levels than those obtained from old birds at all time points except 4 h (Fig. 5.3 A,B). The greatest difference occurred at 10 h, when HSP70 levels in young AECs were 2.7 fold higher than in old cells (Fig. 5.3 B). Both old SUS and old RES AECs showed similar HSP70 profiles from the time of exposure to 10h, although there were some significant differences at some time points. After the peak at 10 h, HSP70 levels in old SUS cells decreased significantly ($P<0.003$) more rapidly than those in old RES cells. By 24h, old SUS AECs had significantly lower HSP70 level than the other 3 treatment groups (Fig. 5.3 B).

Figure 5.3. (A) Representative of Western blot analyses for HSP70 immunoreactive protein content in aortic endothelial cells of young and old, and RES and SUS quail following oxidative challenge (10 h time-point). MW=molecular weight marker, HSP70=control derived from heat-shocked cells. (B) HSP70 immunoreactive protein content in RES and SUS cells prior to and following oxidative challenge. Densitometric values for each sample were normalized to the values of heat-shocked cells in each membrane to allow for comparison between membranes. Each data point represents the mean \pm SE of five different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

A.



B.



Immunohistochemical analysis of RES and SUS cells showed diffuse nuclear staining with anti-HSP70. Despite comparable HSP70 distributions in AECs from both strains, staining intensity was visibly greater in young AECs compared to old cells.

Ferritin

Ferritin protein in RES AECs increased significantly ($P < 0.0001$) more rapidly than those in SUS cells following t-BHP exposure (Fig. 5.4). Ferritin protein levels were maximal at 12 h in RES AECs but it took 24 h for SUS to reach the same maximal level. There were no significant age differences. It should be noted that the increase in ferritin protein appeared later than that of bleomycin-detectable “free” iron (see below).

Catalytic iron

SUS AECs had significantly ($P < 0.03$) higher levels of catalytic iron than RES cells prior to oxidative stress (Fig. 5.5). However, this difference became non-significant at 2h and 4 h following t-BHP exposure, although catalytic iron increased in both strains. Thereafter, catalytic iron levels in RES AECs decreased to pre-exposure levels by 10 h, whereas levels in SUS AECs remained elevated until 18 h. This difference between the two strains was significant ($P < 0.0001$). There was no significant age difference.

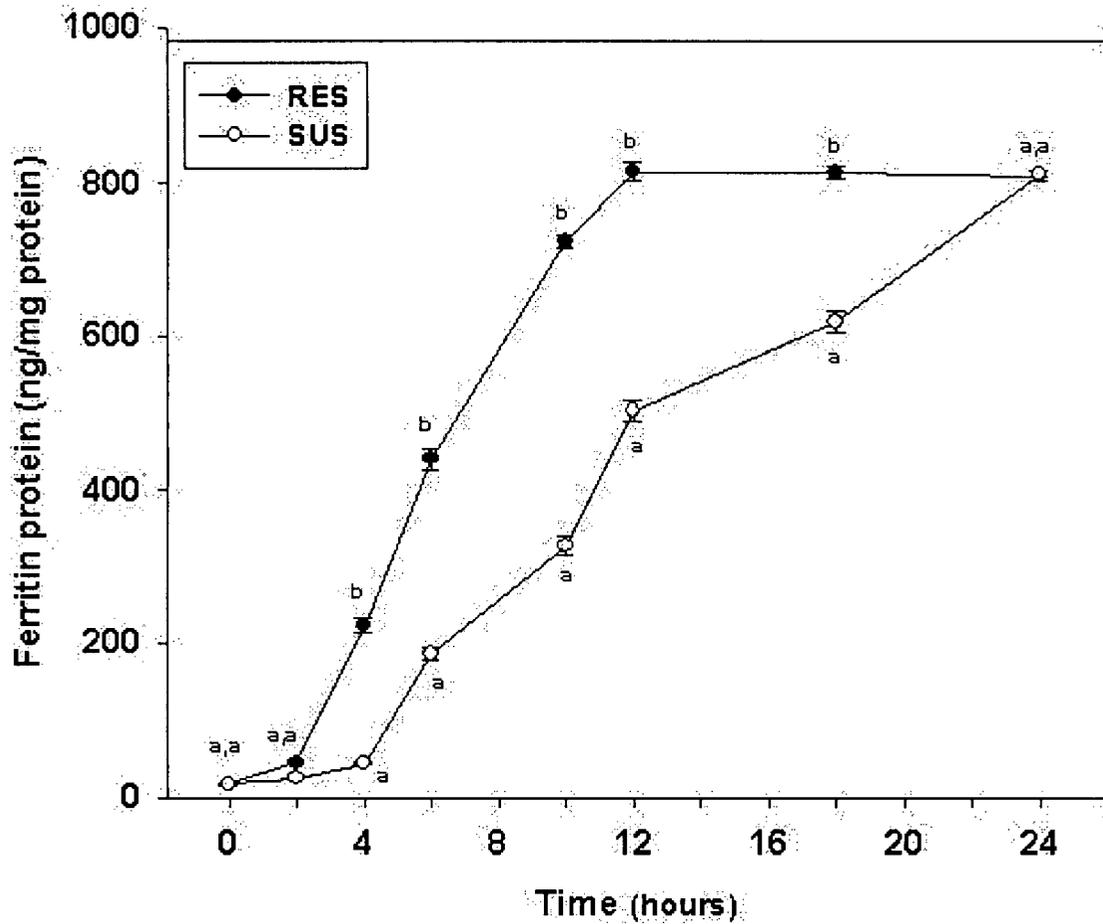


Figure 5.4. Ferritin protein content in RES and SUS aortic endothelial cells at pre- and post-t-BHP exposure. Each data point represents the mean \pm SE of ten different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

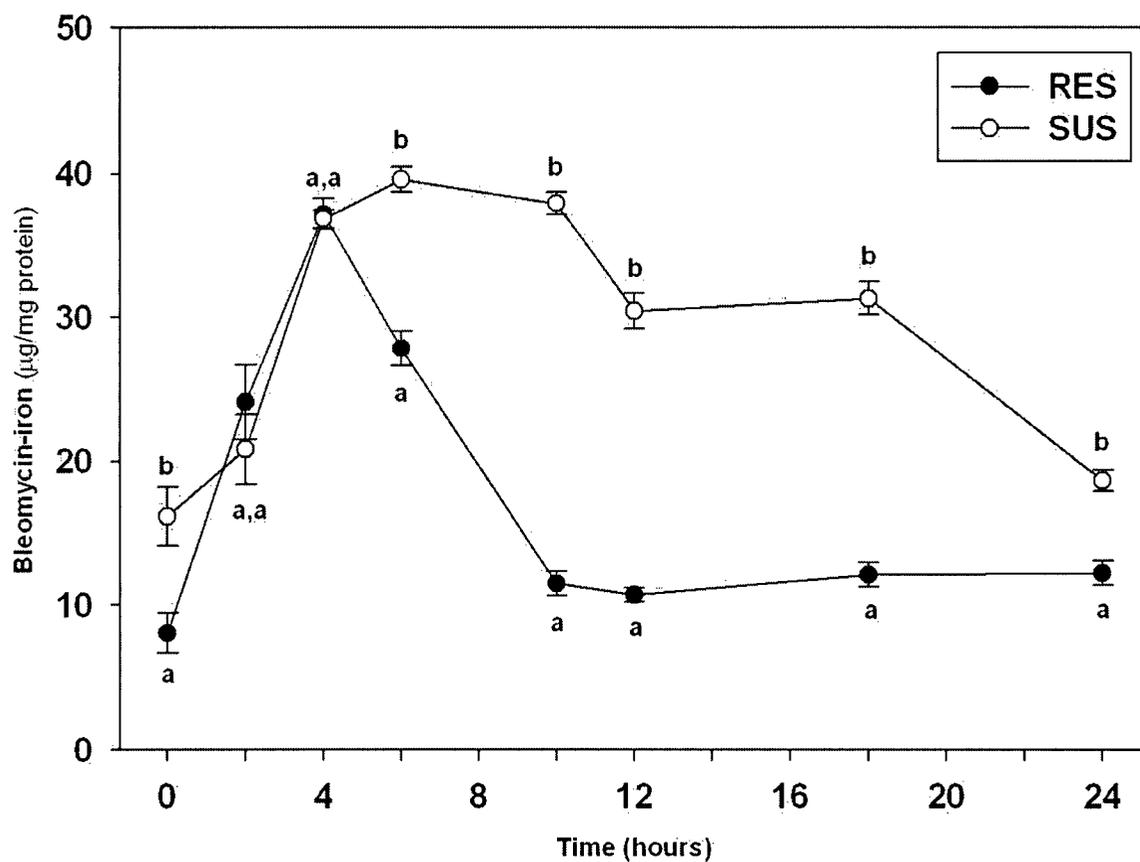


Figure 5.5. Catalytic iron as determined by bleomycin assay in RES and SUS aortic endothelial cells prior to and following oxidative challenge. Each data point represents the mean \pm SE of ten different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

Glutathione

There were significant differences in GSH content between SUS and RES AECs and also between young and old AECs prior to the t-BHP exposure (Fig. 5.6). SUS AECs had significantly ($P < 0.0001$) higher GSH contents than RES, and old AECs had significantly ($P < 0.01$) higher GSH content than young cells. However, during oxidative stress, GSH content in SUS AECs (both young and old) dropped significantly ($P < 0.0001$) more rapidly than those in RES cells. By the end of t-BHP exposure (1 h), GSH content in SUS AECs was already significantly ($P < 0.0001$) lower than that in RES cells (Fig. 5.6). In the next 24 hours, GSH levels in AECs of both strains remained fairly stable and began to recover after 24 h. The age effect was no longer significant.

Measures of lipid peroxidative injury

Damage to cell membranes (e.g. as produced by oxidative injury) causes cytoplasmic LDH release (320). Seven hours following exposure to oxidative stress, SUS AECs began to show a significantly ($P < 0.0006$) higher rate of LDH release as compared to RES AECs (Fig. 5.7). Based on the LDH assay, oxidative challenge was significantly more damaging to SUS AECs than RES cells.

Exposing cells to t-BHP (and other oxidants) increases lipid peroxidation with concomitant formation of TBARS (Fig. 5.8). Although the effect was small, old AECs (RES and SUS) had

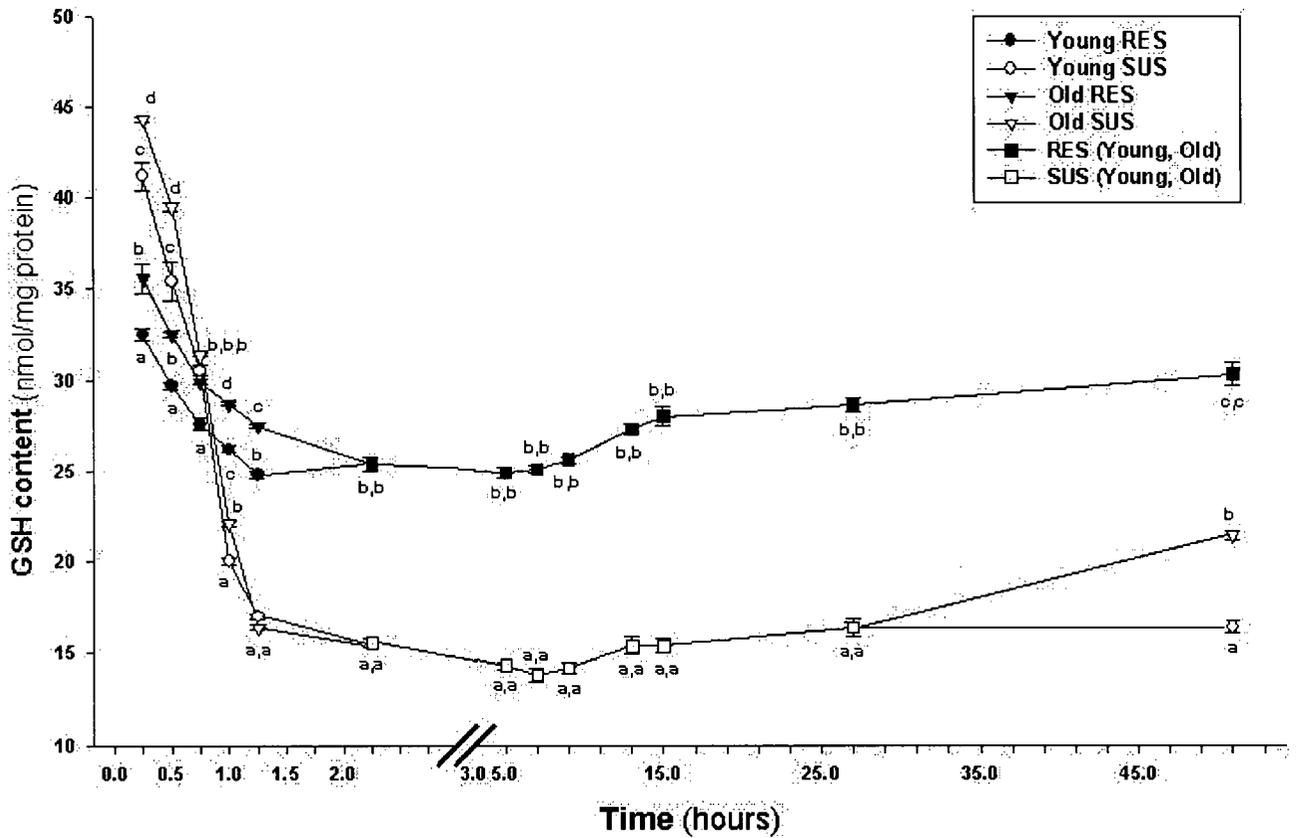


Figure 5.6. Glutathione content in RES and SUS aortic endothelial cells prior to and following oxidative challenge. Each data point represents the mean \pm SE of five different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

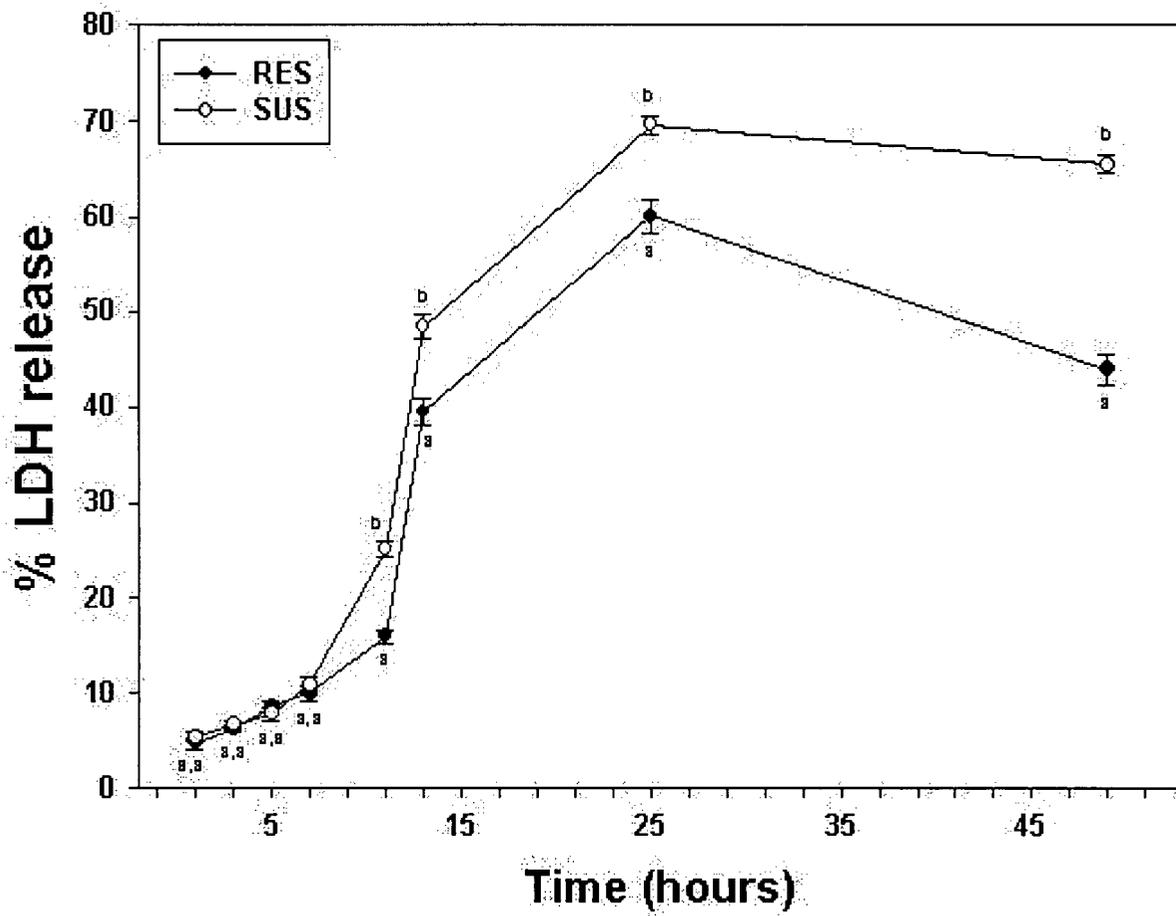


Figure 5.7. Time-course of lactate dehydrogenase release in RES and SUS aortic endothelial cells following oxidative challenge. Each data point represents the mean \pm SE of ten different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

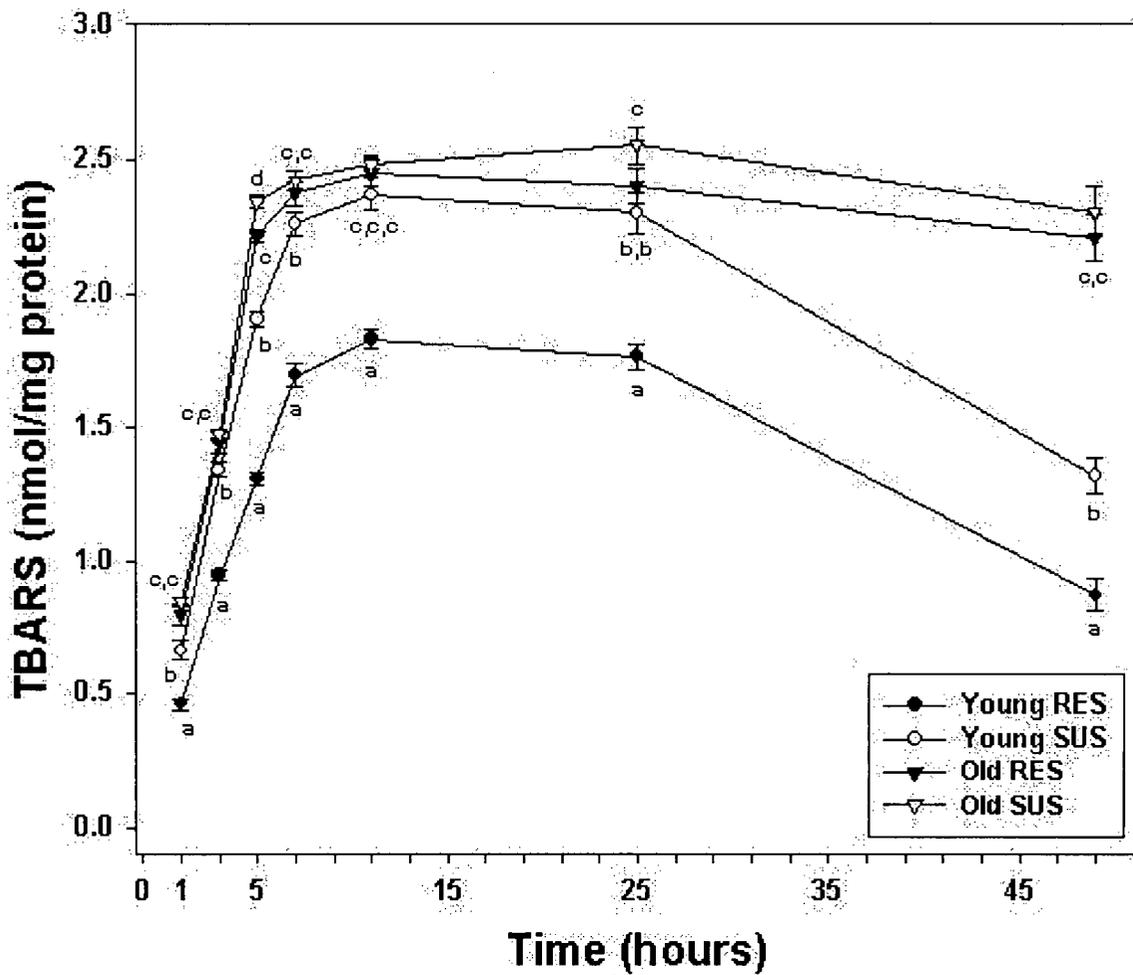


Figure 5.8. Thiobarbituric acid reactive substances in RES (n=5) and SUS (n=5) cells following oxidative challenge. Each data point represents the mean \pm SE of ten different individuals. Data points under different letters were significantly different ($P \leq 0.05$) by analysis of variance.

significantly ($P < 0.0002$) greater TBARS than young AECs prior to t-BHP exposure. Following t-BHP exposure, lipid peroxidation increased in all cells. However, there was a significant ($P < 0.001$) age by strain interaction at all time points examined. From 1 to 24 h after exposure, young RES AECs had significantly lower TBARS levels than old RES and both young and old SUS AECs. There was no significant difference in TBARS formation in old RES, or young or old SUS AECs. At 48 h after oxidative challenge, young RES and SUS AECs showed TBARS levels that were significantly lower than old RES and SUS AECs.

DISCUSSION

The SUS and RES strains of Japanese quail (146) have undergone divergent selection for susceptibility and resistance, respectively, to atherosclerotic plaque formation. When challenged with a diet supplemented with 0.5% cholesterol, more than 80% of SUS quail develop atherosclerosis but only approximately 5% of RES quail do so under the same conditions. Shih *et al.* (146) hypothesized that the reason RES quail were more resistant was because they metabolized and excreted cholesterol faster than SUS quail. Previously, we observed that a cholesterol-supplemented diet induced changes in aortic and plasma antioxidant components which differed between SUS and RES quail (152). In addition, we found that aortic GRd activities in quail fed a control diet were lower in SUS birds compared to RES birds. This led us to a more detailed examination of antioxidant components of AECs of these quail. These experiments confirmed that GRd activity was higher in cultured RES AECs than SUS cells (Chapter 4), and also showed that GSH levels were higher in SUS AECs than

RES cells. HO-1 protein was higher in RES AECs than SUS cells, but catalytic iron levels were higher in SUS AECs than RES cells.

In the present study, we challenged the cultured AECs with a pulse t-BHP oxidative stress. Previous studies have indicated that free radical-mediated lipid peroxidation occurs early after t-BHP exposure (335), and HO-1 levels are a very sensitive marker of this injurious process (336). Treatment with t-BHP causes lipid peroxidation due to free radical formation (337), and accumulation of oxidized glutathione (338). AECs exposed to t-BHP exhibited decreased cell membrane integrity and increased lipid peroxidation, as evidenced by increased LDH release and TBARS production. RES AECs were more resistant to this type of cell injury and they exhibited significantly lower LDH release and significantly lower TBARS levels than SUS cells. Hamster fibroblasts transfected with rat HO-1 showed lower TBARS production following exposure to hyperoxia, compared with non-transfected cells (339). Transfected cells had higher HO activity and HO-1 protein expression (339). Suttner *et al.* (340) demonstrated that rat fetal lung cells transfected with rat HO-1 and exposed to hypoxic conditions showed lower TBARS levels and LDH release. In the present study, we observed higher HO activity and HO-1 protein expression in RES AECs after oxidative stress compared with SUS cells. Thus, HO-1 induction has been shown to confer protection, while its abrogation has been shown to accelerate cellular injuries in various pathological states, including atherosclerosis (127). Furthermore, the higher levels of basal HO-1 in RES cells prior to oxidative stress (23) would also be consistent with a decreased susceptibility to oxidative stress (113,126,341). Our studies have demonstrated a genetic difference in both basal and induced HO-1 expression. The difference between RES and SUS quail in resistance to atherosclerosis may therefore be related to differences in susceptibility to oxidative stress.

In the reaction of HO, heme, a pro-oxidant, is catabolized to catalytic iron (302) and biliverdin (283). At the same time, ferritin may be co-induced to sequester the catalytic iron (301,318) released from heme catabolism. On another note, studies have shown catalytic iron levels may be associated with oxidative alterations in atherosclerosis (342-344). We have detected significantly higher levels of catalytic iron in SUS AECs even before the t-BHP challenge. While catalytic iron levels in AECs of both strains increased to the same extent 4 hours after the t-BHP challenge, levels of iron in the RES strain subsequently decreased and returned to the pre-exposure level by 10 h. In contrast, iron levels in the SUS cells continued to increase after the 4 h time point and remained at a significantly higher level even after 18 h. The more rapid return of catalytic iron to the pre-exposure levels in RES AECs likely contributed to the decreased susceptibility of these cells to oxidative stress, and this may be related to a faster induction of ferritin. Conversely, the sustained catalytic iron levels in SUS cells associated with lower ferritin levels would result in increased susceptibility to oxidative damage. Indeed, catalytic iron has been reported to directly induce an up-regulation of ferritin (113), and the cytoprotective properties of ferritin through limiting intracellular free iron have been demonstrated (114).

It has been shown in Chinese hamster ovary cells that enhanced HO-1 synthesis is maximal when cellular glutathione depletion is greatest (344). In the present study, oxidative stress resulted in a significantly more rapid and greater GSH depletion in SUS AECs. While GSH returned to the pre-exposure level in RES AECs by 45 h, GSH levels remained depressed in AECs from SUS birds. These results provided further evidence that SUS AECs are more susceptible to oxidative stress than RES cells.

Atherosclerosis is a multifactorial disease with a strong heritable component and a prevalence and severity of aortic lesion formation that increases with age - age itself therefore being an independent risk factor for atherogenesis. One of the most characteristic features of aging is a reduced ability to respond to stress and maintain homeostasis (345). Heat shock proteins are crucial for maintenance of cellular homeostasis during normal cell growth and for survival during and after various cellular stresses (346). We have documented greater HSP70 expression in AECs from younger birds (in both strains) when compared with older quail. This corroborates other observations that have shown that the induction of HSP70 after stress is reduced with age in a variety of cells and tissues from rodents (347-348). The steep increase in HSP70 in young AECs coincided with the sharp increase of LDH release and the peak of TBARS production. Moreover, TBARS levels in old RES AECs also peaked at a significantly higher level than those in young RES cells. The defense mechanisms in cells from older birds were less responsive than those of younger cells (349).

CONCLUSIONS

In conclusion, our studies highlight the interplay of HO and HSP70 induction, lipid peroxidation, GSH depletion and increases in catalytically active iron in oxidant-induced cell injury in cultured AECs. The results could have broad implications in our understanding of the roles of HO in the response of AECs to oxidant injury, such as may be involved in the process of atherogenesis. Alterations in these endogenous protective processes could, in conjunction with defects in other antioxidant defense systems or an increase in oxidative stress, predispose cells and tissues to injury (350). In this regard, it has already been shown that ECs from a strain of mice susceptible to dietary atherosclerosis are less able to upregulate expression of heat

shock proteins relative to their atherosclerosis-resistant counterparts (351). Given the importance of oxidants in human disease, it would seem that further investigation of the interplay of genetic factors and endogenous antioxidant systems in determining susceptibility of particular tissues to injury is warranted.

GENERAL SUMMARY AND CONCLUSIONS

Atherosclerotic vascular disease is a major cause of death and disability in adult men and women living in industrialized societies (3). While the relationship between elevated plasma lipids and coronary artery disease (CAD) has been corroborated by epidemiologic (352) as well as pathologic (50) evidence, an increasing body of evidence indicates significant involvement of other factors, including psychological (354) and oxidative stress (355).

Psychological or emotional stress has long been considered a contributing factor to diseases of the cardiovascular system. The concept of stress is central to linking psychological factors to coronary disease, because stress is known to produce hemodynamic (216), endocrine (356) and/or immunologic (357) changes that might possibly affect the development or progression of atherosclerosis. To the extent that these biological processes are influenced by psychological factors, this lends credibility to the biologic plausibility of psychological variables as potential risk factors (358). Furthermore, animal studies of acute and chronic stress (164,359) as well as human epidemiological (360-361) and clinical (362-363) studies provide considerable evidence for the potentially adverse effects of acute and chronic stress on aspects of CAD pathology. However, there remain persistent doubts about the scientific validity and/or clinical relevance of this evidence because of difficulties and inconsistencies in defining and measuring stress in various studies (364), the multifactorial nature of coronary disease and its onset (363), and negative results in some studies (364-365). An exploration of the cellular and biochemical events induced by stress in the context of atherogenesis might yield valuable insights into the processes underlying atherosclerosis and possibly suggest novel approaches for its

management. Moreover, such investigations might also enhance the body of knowledge concerning the oxidative etiology of atherogenesis (366).

This thesis reports the results of four studies which were designed to examine the effects of psychological stress on the interrelationships of heat shock proteins (HSPs) and endogenous antioxidants during atherogenesis in an avian animal model, the atherosclerosis-susceptible (SUS) Japanese quail. These animals were chosen due to their small size and ease of maintenance, their short life-span, which telescopes the disease process, the rapidity of plaque development and the similarities of the vascular lesions produced to those in humans (147,149). However, the ability of this animal model to reproduce a disease state that is similar to the human situation is also variable, and the interaction of experimental treatment effects with the lipid and lipoprotein metabolism of animals does not mimic that of humans, so that extrapolation of results obtained from animal studies to the human is difficult. Variations in feeding behavior and dietary composition between quail animal model versus that of humans (367), differences in lipoprotein composition (368), and susceptibility to atherosclerosis (137; Chapter 4 and 5) are some examples of species related differences.

Chapter 2 compared the effects of psychological stress on aortic plaque development, plasma lipid concentrations, heat shock proteins (HSPs) and endogenous antioxidants in SUS quail. As expected, the birds fed the cholesterol-enriched diet showed highly significant increases in plaque score relative to birds fed the control (i.e. non-cholesterol- supplemented) diet. However, the anticipated increase in the severity of plaque formation with stress was only observed in one of the three aortic branches examined – namely, the left brachiocephalic artery. It must also be mentioned that, contrary to expectation, none of the cholesterol-fed groups showed any evidence of plaque formation in the descending aortic segment. In this

study, the brachiocephalic arteries may have been subject to greater stress (e.g. shear stress) than the descending aorta (369) and the small differences in early atherosclerosis, such as fatty streak formation, may not have been detected by our robust plaque scoring methodology. The heterophil/lymphocyte (H/L) ratio, a quantitative measure of psychological stress, was increased in birds exposed to the stress protocol and in the cholesterol-supplemented birds, indicating in the former case that physiological stress was induced by the experimental protocol used and that cholesterol feeding simulated changes induced by stress (173). However, elevations in H/L ratio may be due to hemoconcentration accompanying stress (370) or elevations in total cholesterol (371). Stress increased HSP70 levels in heart and aortic tissue in birds fed the control diet in agreement with our previous study (172). Although cholesterol feeding alone also increased HSP70 levels in heart and aortic tissue, lower HSP70 levels were detected in heart and aorta tissue of birds exposed to stress and fed the cholesterol diet compared with cholesterol-supplementation alone. Although increased HSP70 expression has been shown to have a protective effect during atherosclerosis (52,61-62), lower HSP levels following a combination of exposure to stress and cholesterol feeding has not been reported and would suggest that effects of stress on HSP70 expression were reduced by cholesterol supplementation. Some reports have suggested that higher levels of HSPs may be associated with an increase in antioxidant capacity (63-64). However, our stress protocol, which increased levels of HSP70 in heart and aorta significantly, lowered aortic catalase and heart superoxide dismutase (SOD) levels, and had a little effect on heart and plasma glutathione peroxidase (GPx) or plasma glutathione reductase (GRd) activities. Furthermore, the measured increases in *in vitro* peroxide-induced thiobarbituric acid-reactive substances (TBARS) generation following stress alone or cholesterol supplementation alone supports a functional decrease in

the antioxidant capacity of the myocardium following stress or cholesterol feeding. This study characterized some of the basic metabolic differences in plasma lipids, aortic plaque score, HSP70 and endogenous antioxidants between control and cholesterol-fed SUS quail exposed to the stress protocol, and set the stage for a longer-term study to investigate the effects of chronic and acute stress during atherogenesis in Chapter 3.

The temporal interrelationships of aortic plaque score, plasma lipids, HSPs and endogenous antioxidants in SUS quail fed a control or cholesterol-supplemented diet and exposed to stress followed by a recovery period (referred to as “early” stress) or a stress protocol administered immediately before sampling (referred to as “recent” stress) were examined in Chapter 3. Once again, the birds fed the cholesterol-enriched diet showed highly significant increases in plaque score and plasma lipids relative to birds fed the control diet. As in Chapter 2, birds exposed to recent stress had increased H/L ratios indicative of a stress response in these birds. Contrary to our expectations, however, there were no differences in aortic plaque score between early stressed, recently stressed and non-stressed cholesterol-fed birds. Birds fed the cholesterol-supplemented diet and exposed to early stress also had lower plasma cholesterol levels compared with birds on the cholesterol diet alone, suggesting that stress preconditioned the birds during cholesterol feeding. Additionally, birds on the cholesterol diet and exposed to stress, in general, had lower HSP70 and inducible heme oxygenase (HO-1) levels in heart and aortic tissue than cholesterol-fed, unstressed birds. The effects of early stress on heart HO-1 in cholesterol-supplemented birds, in which levels increased initially at the 4 wk time point, but then decreased to lower-levels than cholesterol-fed birds exposed to no stress, were surprising. Our data suggest that HSP expression may vary when stressors such as our stress protocol and cholesterol feeding are compounded. Cholesterol supplementation alone also increased heart

and aortic ferritin and catalytic iron, and was associated with increased susceptibility of heart tissue to an *in vitro* oxidative challenge, as reflected in an increased generation of TBARS. These results suggest that cholesterol feeding resulted in increased oxidative stress to heart and aortic tissues, thereby altering the balance of lipid peroxides *in vivo* which, in turn, could further compromise tissue antioxidants. The observation of increases in HO-1 expression in SUS quail during atherogenesis warrants further investigation to study the consequences of stress at a cellular and molecular level in the quail model. The immunohistochemical analyses in Chapter 3 localized HO-1 and ferritin to endothelial cells of the aorta and may suggest that oxidative stress occurs at this level. Indeed, vascular endothelial cells are vulnerable to oxidative damage (372-373), and this has been implicated in atherogenesis (355).

Therefore, in Chapter 4, a technique is described which was used to establish an novel aortic endothelial cell culture from atherosclerosis-resistant (RES) and SUS quail to study HO expression. Aortic endothelial cell (AEC) cultures were established using published protocols, and the resulting cultured cells were identified and characterized as endothelial cells using an endothelial cell-specific antibody (297), the rapid internalization of Dil-Ac-LDL (296), and the negative staining with a smooth muscle-selective antibody (298). Confluent monolayers of cells derived from the resistant lines had higher HO-1 protein and GRd activity and lower catalytic iron and GSH levels when compared with SUS cells. The higher HO may play an important role in conferring protection against oxidative stress to endothelium in the RES strain. Furthermore, catalytic iron may be an important determinant of the susceptibility of quail endothelial cells to oxidative stress. It is conceivable that the lower GRd in AECs from the SUS strain could result in a greater susceptibility to oxidative stress compared with cells from the RES line. Thus, Chapter 4 characterized an *in vitro* endothelial cell culture model of

RES and SUS quail and indicated that further investigation of the nature of genetic differences in atherosclerosis at the cellular level would seem warranted.

The final section of this thesis examined the effects of an *in vitro* oxidative challenge (a functional measure of antioxidant status) to investigate the role of HO and the glutathione redox cycle in RES and SUS AECs. After exposure to oxidative stress *in vitro*, RES cells exhibited higher HO activity and HO-1 protein compared with SUS cells. While catalytic iron increased in both strains, the levels decreased sooner in RES as compared with SUS cells. Furthermore, ferritin levels were significantly higher in RES cells compared with SUS AECs. Finally, LDH release (used as a measure of cell injury) and TBARS were lower, and depletion of GSH was slower in RES cells. These results suggest that differences in atherosclerosis susceptibility between RES and SUS strains may be due, at least in part, to increases in endothelial HO and differences in antioxidant components. It was noteworthy that the results in the present study provide evidence for genetic factors in atherosclerosis that are manifested at the level of endothelial cells which have long been suggested to play a crucial role in the initiation of atherogenesis.

In summary, the results of this thesis indicate that underlying psychological factors related to chronic stress and cholesterol feeding are not equivalently linked to aortic plaque formation in SUS quail. While some associations between psychological stress and/or cholesterol feeding could be related to the development of atherosclerosis (e.g. plasma lipids, H/L ratio), aortic plaque scores were not altered by combination of stress and dietary cholesterol treatment. In contrast, effects of dietary cholesterol alone were associated with the development of aortic plaque, and the effects of stress alone increased physiological and molecular manifestations of stress, notably the induction of HSPs. It has been suggested that

psychological stress may elicit varying effects on the cardiovascular system that may predispose the blood vessel wall to future oxidative injury (374). The LiVicordia Study suggested that psychological and oxidative stress constitute a common set of risk factors and may help explain the higher risk for CAD in Swedish men (47). Further investigations of oxidative stress at the cellular level found distinct differences in HO and antioxidant components in cultured endothelial cells from RES and SUS strains both under basal conditions and in the presence of an *in vitro* oxidative challenge. It is possible that the rapid development of aortic tissue lesions seen in the SUS quail is associated with a greater susceptibility to psychological and/or oxidative damage, and may support a genetic linkage of HO-1 with oxidative stress. Coronary artery disease patients with type 2 diabetes exhibited lower transcriptional activity of HO-1 and had significantly higher leukocyte TBARS levels compared to diabetic patients without CAD (375). It has been postulated that the lower expression level of HO-1 may act synergistically with other risk factors to increase the vulnerability of vasculature to oxidative injury and thus, incidence of CAD in diabetes (376-379). Future directions suggested by the results reported herein include: 1) examining the temporal association in the pre-atherosclerotic aorta (i.e. fatty streak formation) of HSP induction and antioxidant alterations during early lesion formation and/or exposure to psychological stress; 2) examination of HO and antioxidant profiles in other cell types *in vitro* (e.g. smooth muscle cells) in order to further determine the susceptibility of RES and SUS quail to atherosclerosis given that smooth muscle cells proliferate and migrate within the arterial wall during atherogenesis. These studies could conceivably further characterize the association and possibly the relationship of psychological stress to oxidant injury and the subsequent development of atherosclerosis.

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