CHAPTER FOUR: DISCUSSION

The HMG-CoA reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis. These agents slow the progression and foster the regression of atherosclerosis, resulting in an improvement of cardiovascular outcomes in humans with elevated or normal serum cholesterol levels [102, 103]. Evidence is steadily increasing for the beneficial, lipid-independent or "pleiotropic" effects of statins in cardiovascular diseases [59, 70, 103]. This study demonstrates for the first time that atorvastatin treatment exerts beneficial cardiovascular effects in STZ-diabetic rats, including improvement of endothelial function and attenuation of vascular inflammation and reduction of oxidative excess via ERK and NF-κB pathway. As the chosen atorvastatin dose was too low to alter the lipid profile, these changes were independent of its lipid-lowering property.

4.1 Improvement of atorvastatin on endothelial dysfunction independent of cholesterol-lowering effect

Endothelial-derived factors normally play an important role in maintaining vascular homeostasis by regulating vasomotion, inflammation, thrombosis, and smooth muscle proliferation [41]. In contrast, endothelial dysfunction, as represented by the reduced ability of blood vessels to relax in response to endothelium-dependent relaxants, has been implicated in the pathogenesis and clinical course of major cardiovascular disease [41, 104]. There is an overwhelming evidence demonstrating the development of endothelial dysfunction in animal models of diabetes [2-6, 99] as well as in human blood vessels from diabetic patients [7, 8].

Statins are inhibitors of the cholesterol synthesis and very effective agents to lower cholesterol levels. Atorvastatin is one of the most powerful statins [105]. Clinical trials have shown the benefit of statin therapy in the reduction of cardiovascular morbidity and mortality [106]. The primary mechanism for beneficial effects is an effective lowering of serum LDL cholesterol. However, growing evidence indicates that some mechanisms are incompletely explained by lipid lowering [107]. For example, the onset of effects of statins on vasomotor function has been observed to be much more rapid than the effects

of lipid lowering [52]. Tsunekawa *et al*. found an improved endothelial function within three days due to statin treatment without affecting lipid levels [108]. Therefore, we studied whether endothelial dysfunction was modulated by atorvastatin due to pleiotropic effects. We examined the function of a skeletal muscle vascular bed *in vivo* in a rat model of diabetes mellitus. The anaesthetized autoperfused rat allows to measure the perfusion pressure in the hindlimb [109], thus, application of KHS-induced, flow-mediated vasodilatatory responses could be observed as decrease of hindlimb perfusion pressure. The method is valid to analyze endothelial dysfunction in the resistance vasculature [110]. The presence of endothelial dysfunction in the experimental diabetes model used in the present work, STZ-induced diabetes, has been demonstrated in several studies [4, 6, 99]. A report by Dai *et al*. [111] in STZ-diabetic rats provided evidence that the degree of endothelial impairment increased with duration of diabetes in that model. In agreement with these previous works, we observed considerable reduced endothelium-dependent vasodilatator function in STZ-diabetic rats 48 days after induction of diabetes mellitus.

Our study shows that atorvastatin treatment significantly leads an increased flow-mediated endothelium-dependent vasodilatatory response in diabetic rats. Since this effect was not associated with SNP-induced, endothelium-independent vasodilation, it is conceivable that an amelioration of endothelial dysfunction is the mediator of this action. Previous studies have demonstrated that statins attenuate the development of endothelial dysfunction in patients with coronary artery disease and in postmenopausal women [50, 112]. Regarding the effects of statin treatment in diabetes mellitus, the available data are conflicting, with some studies studies suggesting no effect of statins on endothelial dysfunction [113, 114]. However, two recent studies have reported an improvement of endothelial function in type 2 diabetes [115, 116]. The first study using simvastatin had a target LDL cholesterol lower than 80 mg/dl, whereas the second study, following a similar protocol, used atorvastatin. Both studies included subjects with diabetic dyslipidemia. Atorvastatin has been shown to improve the flow-mediated vasodilatation in type 1 diabetes [117]. A common characteristic of all mentioned studies is that their protocols intend to lower LDL cholesterol levels. Although in the last few years, experimental and clinical studies have emphasized that the cardiovascular protective effects of HMG-CoA reductase inhibitors are also involving direct pleiotropic effects on

the vascular wall, the importance of those pleiotropic effects in relation to lipid lowering remained to be clarified. The improvement in endothelium-dependent vasodilation with atorvastatin in diabetes was not associated with basal total or LDL cholesterol levels or the change in these lipid levels as a result of treatment. Our study demonstrates for the first time in a model of diabetic endothelial dysfunction that atorvastatin is able to normalize endothelium-dependent vasodilatation without affecting the diabetic dyslipidemia rather than lowering serum LDL cholesterol. This implies that in our model pleiotropic effects are responsible for the most part of the beneficial effects of atorvastatin.

4.2 Anti-oxidative effect of atorvastatin in diabetic rats

Multiple mechanisms contribute to endothelial cell dysfunction in diabetes mellitus. Loss of endothelial NO bioavailability and increased oxidative stress are largely considered key mechanisms. The bioavailability of NO reflects a balance between its production via NOS and its degradation, particularly by oxygen-derived free radicals [118]. Actually, NO has a half-life of only a few seconds *in vivo*. Therefore, its biological activity is determined decisively by oxygen-derived free radicals such as superoxide. Many of the metabolic derangements known to occur in diabetes, including hyperglycemia, excess of free fatty acid liberation, and insulin resistance, mediate abnormalities in endothelial function by affecting the synthesis or degradation of NO [119]. It should be emphasized that some endothelium-impaired function disorders may be associated with a decrease in NO bioavailability despite increased NO synthesis. It is reported that the expression of eNOS and its mRNA appears to be increased in endothelial cells incubated in high glucose [118]. However, in the same work authors have also reported a threefold elevation in superoxide production, which is probably more relevant in the pathomechanism of hyperglycemia-induced endothelial dysfunction. Thus, the mechanisms underlying endothelial dysfunction are likely to be multifactorial but seem to be, at least in part, secondary to increased NO degradation caused by activation of superoxide-producing enzymes, such as the xanthine oxidase [35] and a NAD(P)H oxidase[19].

With respect to oxygen-derived free radicals and oxidants, strong evidence has accumulated that the generation of ROS (oxidative stress) plays an important role in the etiology of diabetic complications [18]. This hypothesis is supported by evidence that many biochemical pathways strictly associated with hyperglycemia (glucose auto-oxidation, polyol pathway, prostanoid synthesis, protein glycation) can increase the production of free radicals. Furthermore, exposure of endothelial cells to high glucose leads to augmented production of superoxide anion, which may quench nitric oxide, thereby reducing the efficacy of a potent endothelium-derived vasodilator system that participates in the general homeostasis of the vasculature. Nevertheless, in the presence of high O_2^-, the bioactivity of NO is not only diminished but also transformed into peroxynitrite ($ONOO^-$), a toxic oxidant molecule with proatherogenic effects [120]. In further support of the consequential injurious role of oxidative stress, many of the adverse effects of high glucose on endothelial functions, such as reduced endothelial-dependent relaxation and delayed cell replication, are reversed by antioxidants [121, 122].

Possible sources of oxidative excess in vasculature are NAD(P)H oxidase [19, 34], xanthine oxidase [35], uncoupled NO synthase [37], and the mitochondria [36]. Studies within the past several years have revealed that NAD(P)H oxidase family members are major sources of reactive oxygen species that appear to play a pivotal role in the progression of vascular disease. There is increasing evidence for superoxide generation by NAD(P)H oxidases contribute to the development of vascular (particularly endothelial) dysfunction [18]. Pro-oxidant processes, such as the uncoupling of eNOS and increases in NAD(P)H oxidase protein and activity have been reported in diabetes [71], as confirmed in this study.

There are conflicting reports in which eNOS expression was shown to increase [5, 123] or decrease [124, 125] in the diabetic rat model. This discrepancy may be explained by the severity of diabetes and the degree of hyperglycemia induced. Enhanced oxidative stress and eNOS expression in the aorta have been demonstrated in the early stage in diabetic rats [5]. Since constitutively expressed eNOS produces low concentrations of NO, which is necessary for good endothelial function and integrity, the contradiction of increased eNOS and impaired endothelial dysfunction may be due to the loss of NO

bioavailability. High glucose increases eNOS protein expression, but decreases NO release finally, and the inhibitory effect of high glucose on NO production is restored by the addition of SOD [126]. In certain disease conditions, NO production is not altered or even increased, but its bioavailability is reduced because of O_2^- generation within the endothelium. Loss of endothelial NO bioavailability caused by its enhanced biodegradation in reaction with O_2^-, yielding peroxynitrite ($ONOO^-$), is the key feature of such diverse vascular disease states as hypertension, diabetes, atherosclerosis, heart failure, and cigarette smoking. In addition, eNOS produces not only NO, but may also be a source of marked amounts of O_2^- even in intact endothelial cells. eNOS can uncouple and generate superoxide anions particularly when the availability of its co-factor tetrahydrobiopterin (BH_4) is limited [37], such as in the process of diabetes. Oxidative excess will result in a reduction of tetrahydrobiopterin and an increase in dihydrobiopterin (BH_2). When this occurs, formation of the active dimer of eNOS with oxygenase activity and production of NO is curtailed (uncoupling of eNOS). The reductase function of eNOS is activated and more ROS are formed. So NOS goes from its oxygenase function, producing NO, to its reductase function, producing ROS, with the consequent exaggeration of oxidative excess and a deleterious effect on endothelial and vascular function. Of note, the extent of eNOS uncoupling is dependent on another significant enzymatic source of O_2^- in the endothelial cells, namely NAD(P)H oxidase.

In the present study, we tested the hypothesis that atorvastatin ameliorates endothelium-dependent vascular relaxtion via its antioxidant property in diabetes. Animal and human investigation have demonstrated that statin therapy reduces oxidative stress [70, 104]. Chen *et al* showed that lovastatin reduced the maximal rate of oxidation induced by leukocytes isolated from rabbits and totally blocked the initiation of LDL in higher amounts, while it had no significant effect on plasma cholesterol levels *in vivo* [127]. Also, simvastatin showed a dose-dependent antioxidant effect on LDL and HDL oxidation [128]. These findings suggest a possible role of HMG-CoA reductase inhibitors as anti-oxidative agents, especially regarding the short-term effects that are not dependent on the mevalonate pathway. It appears that there are several mechanisms whereby statins may reduce oxidative stress. Statins have been shown to reduce NAD(P)H-dependent superoxide formation by a monocyte-derived cell line in culture [129]. NAD(P)H

oxidases have been suggested as one of the most important sources of superoxide in human coronary arteries and their activities are increased in patients with diabetes mellitus [71]. Experiments with human endothelial cells have shown that statin treatment can inhibit oxLDL-induced NAD(P)H oxidase expression and superoxide anion formation [130]. Statins also have other antioxidative properties in addition to their effects on NAD(P)H oxidases. An extra beneficial effect reported with statins is that they can potentiate the synthesis of BH_4, which may prevent the uncoupling of eNOS and shift the balance away from NOS-generated superoxide production to the generation of NO [131]. Our study showed that diabetic rats had increased NAD(P)H activity and expression of eNOS compared with healthy controls, and these were diminished by atorvastatin therapy, which implicates that the reduction of diabetic oxidative excess such as NAD(P)H and eNOS by atorvastatin contributes to the beneficial effect of atorvastatin on endothelial dysfunction in STZ-diabetic experimental rats.

4.3 Anti-inflammatory effect of atorvastatin in a diabetic rat model

An additional mechanism by which diabetes chronically impairs endothelial function independently of oxidative stress includes the increase in inflammation [132]. Inflammatory processes represent a hallmark of atherosclerosis and involve the release of inflammatory cytokines, the activation of their distinct signaling cascades, and the recruitment and accumulation of leukocytes via cell adhesion molecules [133]. Cell adhesion molecules mediate firm attachment of circulating leukocytes to the vascular endothelium, a critical, early step in the induction of inflammatory states [134]. ICAM-1 (CD54) and VCAM-1 (CD106), two important markers of endothelial dysfunction, are largely responsible for recruiting immune cells to atherosclerotic lesions and for activating lymphocytes in lesions [135]. Proinflammatory cytokines, including interferon-γ, tumor necrosis factor-α, and interleukin (IL)-1β can increase T cell localization to inflammatory sites through induction of ICAM-1 and VCAM-1 [136, 137]. Moreover, the accumulation of inflammatory cells can further increase the levels of oxidative stress, since they are a rich source of superoxide radicals and are involved in changes of matrix hemostasis.

Statins have been reported to have anti-inflammatory properties in animal models and cell culture studies [50, 51, 75, 86]. Statins can suppress the proinflammatory mediators such as TNF-α, IFN-γ, and iNOS [76, 138]. High glucose levels have been shown to increase the expression of ICAM-1 and VCAM both *in vitro* [139] and *in vivo* [140]. Atorvastatin has been shown to reduce levels of circulating soluble ICAM-1 and VCAM-1 [141-143]. The underlying mechanism of increased expression of endothelial cell adhesion molecules in response to glucose is correlated with the loss of NO production [140] and activation of NF-kB by increased oxidative stress [139]. In this study, we also examined whether atorvastatin can protect against the inflammatory endothelial events mediated by diabetic conditions, namely enhanced cytokines: TNF-α and IL-1β, and increased proinflammtory vascular adhesive molecules: ICAM-1 and VCAM-1 in skeletal muscles. As exspected, we found that enhanced expression of TNF-α and IL-1β in STZ-diabetic rats, which may account for overproduction of ICAM-1 and VCAM-1 in skeletal quandriceps. Atorvastatin treatment attenuated STZ-mediated inflammatory markers, and the effect of inflammation reduction was independent of decreases in lipids and lipoproteins. The improved endothelium-dependent vasodilatation after statin treatment correlated with reduced inflammatory markers in this study, implying that the vascular protective effect of atorvastatin on STZ-diabetic experimental rats partly seems to be mediated by the anti-inflammation capability of atorvastatin.

4.4 ERK1/2, and NF-κB signaling pathways implicated in the improvement of endothelial dysfunction by atorvastatin

Despite extensive research on molecular mechanisms of statins, little is known about the interactions of these drugs with intracellular signaling transduction and transcription factors. All the contributing factors in endothelial dysfunction such as growth factors, angiotensin II, oxidative stress, elevated low-density lipoprotein, and cytokines, have been shown to activate mitogen-activated protein kinases (MAPK) signaling in a variety of cell types. The MAPKs constitute a family of serine/threonine kinases that mediate the transduction of external stimuli from the cell surface to the nucleus, usually resulting in the phosphorylation and subsequent activation of transcriptional factors, producing altered gene transcription [144]. Three MAPK families that differ in their substrate

specificity and responses to stress have been identified in vertebrates: c-Jun aminoterminal kinase (JNK) or stress-activated protein kinase (SAPK), extracellular-regulating kinase (ERK), and p38 MAPK [145]. These protein kinases share 60~70% identity, but differ in the sequence and size of their activation loop, and are activated by different stimuli. MAPKs are proline-directed serine and threonine protein kinases that are activated by dual specificity kinases by phosphorylation of threonine and tyrosine in a Thr-Xaa-Tyr motif in a loop near the active site [146]. Activated MAPKs play a key role in activating transcription factors and down stream kinases, leading to the induction of immediate-early gene expression, and subsequent changes in other cellular processes [147]. The magnitude and duration of the stress, as well as the cell type involved, are important factors in determining which pathways are activated, and the particular outcome reflects the balance between these pathways [148]. Among them, the ERK signaling module is a vital mediator of a number of cellular fates including growth, proliferation and survival. At least five different ERK proteins have been identified in mammalian cells, ERK1 to 5 [149]. The more highly studied and abundantly expressed ERK family members, ERK1 and ERK2, also referred to as p42/p44 MAP kinases, are directly regulated by two MAPK kinases, MEK1 and MEK2. ERK1/2 proteins are directly phosphorylated by MEK1/2 at both a threonine and adjacent tyrosine residue within a dual specificity motif (Thr-Glu-Tyr).

NF-κB is one of the key regulators of transcription of a variety of genes involved in immune and inflammatory responses. The most abundant form of the transcription factor is a heterodimer that contains a p50 and p65 subunit, the latter comprising a powerful transcriptional activation domain. NF-κB can be activated by a variety of stresses such as oxidants, xenobiotics, viruses and pro-inflammtory cytokines [150]. In uninduced cells, NF-κB is kept inactive in the cytoplasm through binding of an inhibitory protein IκB. After stimulation by a variety of inducers, such as TNF-α, IL-1β or lipopolysaccharide (LPS), the IκB protein becomes phosphorylated, ubiquitylated and degraded by the 26S proteasome. Release of IκB unmasks the nuclear localization signal and causes translocation of NF-κB to the nucleus, allowing activation of transcription [151]. The induction of NF-κB is thought to be critical in the regulation of inflammatory responses [151]. Target proteins include adhesion molecules such as selectins, ICAM-1 and

VCAM-1 [152] and various pro-inflammmatory cytokines such as IL-2 [153], IL-6 [154], TNF-α [155] and iNOS [156].

Many studies have reported that oxidative stress is important in the activation of NF-κB and in the elicitation of inflammatory cytokines. Moreover, numerous studies have demonstrated that activation of NF-κB can often be prevented by antioxidants, and have led to the prevailing theory that NF-κB is an oxidant-sensitive transcription factor [157]. MEK kinase regulates the activation of NF-κB by activating IκB kinase [158]. ERK signaling pathways have been implicated in NF-κB activation induced by mechanical deformation or oxidative stress through phosphorylation of IκB in rat osteosarcoma ROS 17/2.8 cells and HeLa cells [159, 160]. Overexpression of ERK1/2 can activate NF-κB [161]. The activation of ERK is directly linked to the increased TGF-β1 production and the nuclear translocation of NF-κB, because PD98059, a specific inhibitor of MAPK/ERK kinase [162], prevented these responses [163]. Recently, NF-κB has also been reported to be downstream of ERK signaling participating in H_2O_2-induced suppression of osteoblast differentiation [164].

Because activation of NF-κB is involved to regulate the transcription of many genes, including cytokines, chemokines, adhesion molecules, and growth factors, it is speculated that NF-κB activation is involved in endothelial dysfunction [152], initiates and propagates the immune/inflammatory response and amplifies free radical production, which leads to further development of diabetes. If NF-κB activation is a critical step in the diabetogenic process, selective modulation of NF-κB activation or of its downstream effect could lead to the attenuation of the disease outcomes. In this study, we examined the effects of atorvastatin on the activation of ERK1/2 and NF-κB signal transduction pathway in quadriceps of diabetic rats. The interactions of atorvastatins on vascular cell signaling and gene expression may explain vascular protective effects not directly related to cholesterol lowering. Atorvastatin has been shown to reduce activation of transcription factor NF-κB in cultured vascular smooth muscle and mononuclear cells [165] as well as in atherosclerotic lesions in the rabbit [166]. Janssen-Heininger *et al*. [167] showed that cytokines and oxidants cooperate in the activation of transcription factors through distinct pathways, and suggest that anti-inflammatory and antioxidative therapies may be required

in concert to prevent the activation of NF-κB-regulated genes important in the development of diabetes. We found that atorvastatin inhibited the expression of the transcription factor NF-κB, possibly due to the inhibition of ERK phosphorylation, which is correlated with the modulation of endothelial function, inflammation and oxidative stress by atorvastatin. The inhibitory effects of atorvastatin on NF-κB expression and ERK1/2 in this study is associated with their ability to scavenge oxygen-free radicals and regulate inflammatory reaction [168]. However, previous studies also showed conflicting results regarding the interaction of statins and NF-κB signaling. Decreased NF-κB activity in mesangial cells has been detected on treatment with lovastatin [94] whereas simvastatin has been found to increase NF-κB activation in endothelial cells [169]. The disparity may relate with the duration and severity of stress and the cell type. Our investigation, namely that atorvastatin inhibits STZ-induced NF-κB expression and ERK1/2 activation independently of lipid-lowering, clearly supports the notion that HMG-CoA reductase inhibitors curtail NF-κB signaling and regulate downstream transcription.

Statins have been shown to affect intracellular pathways in several cell types. For example, Simvastatin 10 μmol/L decreased MAPK activity in human coronary artery endothelial cells [170]; pravastatin and lovastatin suppressed the increment of MAPK activity in activated mesangial cells [171], and cerivastatin almost completely prevented Ang II-induced ERK phosphorylation in rat aortic vascular smooth muscle cells [172]. Evidence shows that statin also can inhibit IFN-γ-induced expression of ICAM-1 in vascular endothelial and smooth muscle cells by inhibiting the ERK 1/2 signaling pathway [173]. As ERK may be involved in the pathway of NF-κB, we found in this study that ERK1/2 was activated under diabetic conditions, whereas atorvastatin reduced the phosphoylation of ERK1/2, which at least may partly explain the decreased expression of NF-κB. Considering the pluripotency of ERK signaling, other ERK-regulated transcription factors may also be involved in this process in addition to NF-κB.