AMERICAN UNIVERSITY OF BEIRUT

MECHANISMS INVOLVED IN STATIN-DEPENDANT EXPRESSION OF HEME OXYGENASE IN SKELETAL CELLS

by
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Last but not least, all gratefulness and respect are to my parents and family members. Part of the effort I have ever given was to make you proud of what you raised. Lara, my sister, you are my idol. I'm sure you'll achieve more than what you dream.

AN ABSTRACT OF THE THESIS OF

Moustafa Abed Al Salam Al Hariri for Master of Science
Major: Biochemistry

Title: Mechanisms involved in Statin-dependant expression of heme oxygenase in skeletal cells.

Introduction: Statins are inhibitors of HMG-CoA reductase and are potent drugs in lowering blood cholesterol. Skeletal muscle cells have been shown to express Heme Oxygenase-1 (HO-1) in response to electrical stress and nitric oxide.

Objectives: Characterize the expression of HO-1 in response to statins in a murine myoblast cell line, C2C12, differentiated into skeletal muscle cells and to assess its role in skeletal cells.

Materials and Methods: Cell Culture, Western Blot, RNA Extraction, RT-PCR, Immuno-Fluorescence, and EMSA.

Results: Statins decreased the expression of MyoD differentiation marker in both differentiated and undifferentiated C2C12 cells. Treatment of cells for 24 hours with different concentrations of simvastatin (5-25 μM) leads to increase expression of HO-1 protein. Addition of spermine NONOate for the last 6 hours resulted in additional increase of HO-1. Involvement of prenylation in the statin-induced upregulation of HO-1 was further assessed using inhibitor of geranylgeranylation (GGTI-286) or farnesylation (FTI-277). GGTI-286 (10 μM) but not FTI-277 induced HO-1 expression. mRNA for HO-1 was also increased in response to simvastatin (25 μM), fluvastatin (10 μM) and GGTI-286 (10 μM) but not in response to FTI-277. Involvement of Activator protein-1 (AP-1), CCAAT/ enhancer binding protein (C/EBP) and Upstream stimulatory factor (USF) transcription factors was assessed by EMSA. C/EBP but not USF was modified in C2C12 after statins treatment. Simvastatin slightly modified AP-1. C2C12 express RhoA and Rac1 and the role of these proteins in HO-1 expression will be investigated. Additional experiments are required to identify the targets of statins in skeletal muscle cells and the role of HO-1 in statin-dependent activation of skeletal muscle cells.

Conclusion: Statins increase HO-1 protein and mRNA levels in skeletal muscle cells. HO-1 expression is suppressed by a geranylgeranylated protein. Inhibition of RhoA/C and ROCK induces HO-1 expression. C/EBP, but not USF, transcription factor is modified due to statins treatment in skeletal muscle cells.

Keywords: Heme Oxygenase-1, prenylation, statins, transcription factor.

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List of Abbreviations

Ang-II: Angiotensin II

AP-1: Activator protein-1

BR: Bilirubin

BV: Bilivirdin

BVR: Biliverdin reductase

C/EBP: CCAAT/ enhancer binding protein

CO: Carbon Monoxide

COX: Cyclo-oxygenase

CRP: C-reactive proteins

DMEM: Dulbecco's modified eagle's medium

DTT: Dithiothreitol

EDTA: Ethylene diamine tetraacetate

eNOS**:** Endothelial Nitric oxide synthase

ERK: Extracellular signal-regulated kinases

FBS: Fetal bovine serum

FDA: Food and drug administration

FITC: Fluorescein isothiocyanate

FPP: Farnesylpyrophosphate

GEF: Guanine nucleotide exchange factor

GGPP: Geranylgeranylpyrophosphate

HDL: High density lipoprotein

HO: Heme oxygenase

I-CAM: Inter-cellular adhesion molecule

IL: Interleukin

INF: Interferon

iNOS: Inducible Nitric oxide synthase

LDL: Low density lipoprotein

LFA: Lymphocyte function-associated antigen

LPS: Lipopolysaccharides

MAPK: Mitogen-activated protein kinases

MHC: Major histocompatibility complex

MMP: Matrix metalloproteases

NF-κB: Nuclear factor-κB

NO: Nitric oxide

NRE: Negative regulatory elements

Ox-LDL: Oxidized LDL

PDGF: Platelet-derived growth factor

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PPAR: Peroxisome proliferator activated receptors

ROCK: Rho-kinase

ROS: Reactive Oxygen Species

SPNO: Spermine NONOate

TAT/C3: Clostridium botulinum C3 exoenzyme

TG: Triglycerides

TGF- β: Transforming growth factor β

TNF-α: Tumor necrosis factor-α

TX: Thromboxane

USF: Upstream stimulatory factor

VLDL: Very-low density lipoprotein

To the spirit of freedom, unity, tolerance, humanity, education, charity, and whole Lebanon, to the soul of Rafik Al Hariri I dedicate this work.

It is not due to my political views, yet it's a simple gratitude to the spirit of the man who made a difference in our society, who gave the civilization a chance to flourish up from the destruction. He's the man that changed the aims of my country's youth and set a role model for the society that is regardless to your financial status you can seek a respective education in prestigious institutes.

Chapter I

Introduction

A. Heme Oxygenases

Heme oxygenases (HO) (EC 1.14.99.3) are microsomal enzymes (1) that catalyze the first and rate-limiting step in the oxidative breakdown of heme to carbon monoxide (CO), biliverdin (BV) that is rapidly reduced to bilirubin (BR) by bilivirdin reductase (BVR), and ferrous iron Fe^{2+} that is sequestered by ferritin (2, 3) (Figure 1). Each of these metabolites has been shown to have beneficial regulatory functions (2) (Figure 2). Heme breakdown requires three moles of molecular oxygen (O_2) as well as reducing equivalents from NADPH cytochrome P-450 (cytochrome c) reductase. Heme molecule, upon binding to the HO apo-protein, serves as both substrate and catalytic cofactor in its own degradation.

Three HO isoforms have been identified and cloned (4). In mammals, 2 active HO isoforms (HO-1, HO-2) with heme catalytic activity have been described (2). HO-1 (previously identified as a 32 kDa heat-shock protein HSP32) is the inducible form (4). Its expression occurs at low levels in most tissues under physiologic conditions, with the exception of the spleen, the site of erythrocyte hemoglobin turnover. In contrast, HO-2 is constitutively expressed under basal conditions in most human tissues, including spleen, liver, kidney, cardiovascular and nervous systems (3), and predominantly expressed in the brain and testes. HO-3 is also a constitutive isoform of HO. It has only been found in rat

tissues, including brain, liver, kidney, and spleen (4), and has a very poor heme degrading capacity and a negligible enzyme activity (5).

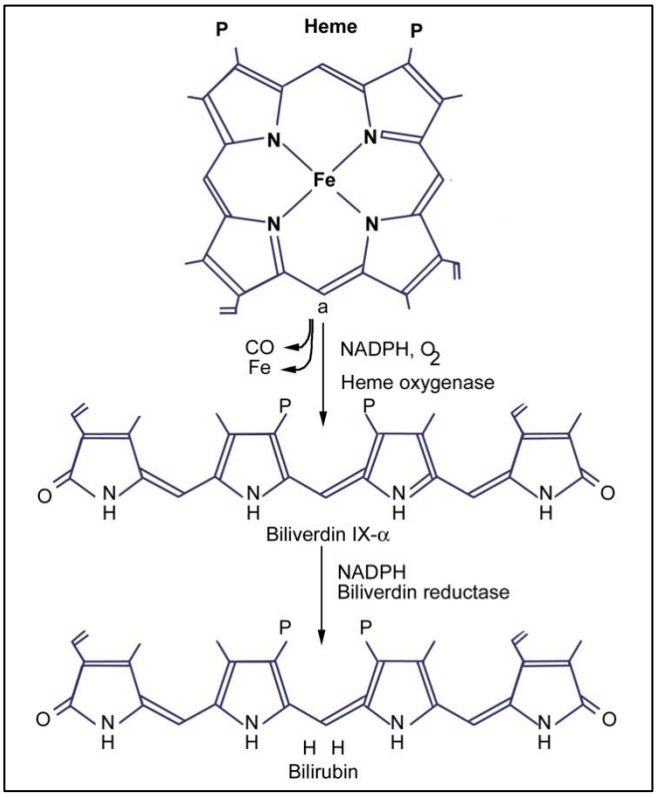

Figure 1 : Heme degradation pathway. Heme is cleaved between *rings A* and *B* by heme oxygenase to yield equimolar quantities of iron (Fe^{3+}), carbon monoxide (CO), and biliverdin (BV). O_2 and NADPH are required for this reaction. BV is then converted to BR by BVR. P: propionyl groups. Adopted from Sikorski et *al.* 2004 (6).

1. Heme Oxygenase Isoforms

HO-1 genes have been cloned and sequenced from different species, including rat, mouse, human, and chicken. Their homology at the amino acid level is around 80%. HO-2 has been cloned from rat, mouse, and human and they share more than 90% protein homology (4). Mouse HO-1gene is localized to chromosome 8 (7) and is consisted of 5 exons and 4 introns spanning about 7 kb. Alam et *al.* have determined the primary structure of the exons and the 5'-flanking region (8).

Mouse HO-1 gene contains a proximal promoter region and two distal enhancers, E1 and E2 located 4 kb and 10 kb, respectively, upstream from the transcription initiation site (8, 9). Alam et al. have described that both of these enhancer regions are required for induction of the mouse HO-1 gene in response to most of the inducers including heme, NO, heavy metals (cadmium), hydrogen peroxide, hyperoxia, LPS, phorbol ester, sodium arsenite, and various electrophiles (10-12).

Mouse HO-1 gene's proximal promoter region contains several sequence elements that are conserved in many species such as mouse and human HO-1 genes. These sequences are consensus binding sites of various transcription factors including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), AP-2, AP-4, specificity protein-1 (SP-1), C/EBP, upstream stimulatory factor (c-Myc:Max/USF), GATA binding factor7 and metal regulatory element (MRE) (8, 10, 13-16).

2. Inducers of HO-1 Expression

HO-1 is induced by wide spectrum of endogenous and exogenous stimuli. Among which are heme and heme derivatives, heat shock, Nitric oxide (NO) and its donors, oxidized lipids, hyperoxia, lipopolysaccharides (LPS), phorbol ester, sodium arsenite, ultraviolet A radiation, quinines, sulfhydryl reagents, heavy metal, hydrogen peroxide, hypoxia, endotoxin, growth factors [platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β)], various electrophiles, curcumin, oxidative stress, exogenous CO, cytokines(IL-1, IL-6, IL-10, TNF-α, INF-γ), shear stress, intensive light, angiotensin II (Ang-II), glucose deprivation, and other injuries (3, 17-21).

HO-1 induction may be mediated by different signaling pathways, including cAMP-dependent mechanisms (22, 23), protein kinase C (PKC) (24), Ca^{2+}-calmodulin dependent protein kinase and the phosphoinositol pathway (25) depending on the cell types and the nature of the stimuli. Mitogen-activated protein kinases (ERK and P38) and tyrosine phosphorylation are also involved in HO-1 induction in some tissues (11, 26, 27).

3. Repressors of HO-1 Expression

Synthetic metalloporphyrins such as cobalt-protoporphyrin- IX (CoPPIX), tin-protoporphyrin (SnPPIX), zinc- or manganese-protoporphyrin-IX (ZnPPIX, MnPPIX), tin and chromium mesoporphyrins (SnMP, CrMP), and iron or zinc deuteroporphyrin-2,4-*bis*-glycol (FeDPBG, ZnDPBG), can act as competitive inhibitors of HO activity *in vitro* (3).

The reduced expression of HO-1 may help to preserve intracellular heme as an important substrate of certain heme proteins, and reduce energy expenditure for heme catabolism. It has been described earlier that hypoxia represses HO-1 expression in many human cells and cell lines whereas as it increases HO-1 expression in rodent cells (28). However, it has been show recently that hypoxia increases the expression of HO-1 in certain types of human cells, such as human retinal pigment epithelium (RPE) cells (29).

HO-1 expression can be reduced by interferon-γ (30) or hypoxia (31) in human glioblastoma, human umbilical vein endothelial cells, coronary artery endothelial cells, astrocytes, and many other human cell lines (32). Bach1, a member of basic leucine-zipper factors, is a heme-regulated transcriptional repressor for the HO-1 gene (33, 34).

4. HO-1 General Biological Functions

Different studies showed that the induction of HO-1 protein synthesis and activity serves as a strong response to oxidative stress that may involve several mechanisms. The overall effect of HO is to lower the cellular concentration of cell toxicants and increase the concentration of cell protectants. Thus, HO activity decreases the cellular levels of the pro-oxidant heme, which catalyzes the decomposition of organic peroxides leading to the generation of alkyl peroxyl radicals that initiate membrane lipid peroxidation (35-37). Moreover, the products of HO's activity (BV/BR, and CO) exert complementary important effects that are described in the following sections.

a. Activities of biliverdin and bilirubin

As mentioned earlier, BV and BR are generated, during heme degradation, by the action of HO and BVR, respectively. These have strong antioxidant properties due to their scavenging capacity to some peroxyl radicals, including superoxide (O_2^-), peroxides (H_2O_2), hydroxides (OH^-), hypochlorous acid (HClO), singlet oxygen (O_2), nitroxides (R_3N^+-O^-), and peroxynitrite ($ONOO^-$) (38-40). Sedlak and Snyder have proposed that the mechanism where BV/BR scavenging effect involves an oxidation of C-10 methylene on BR; thereby converting it to the resonance-stabilized, low-energy, compound BV. The latter is reduced rapidly back to BR by BVR (41, 42). This rapid reduction allows quenching much higher concentrations of oxidants by the reutilization of extremely low tissue BR concentrations, in a manner that is similar to the cellular glutathione reductase system. On the other hand, Stocker has addressed the evidence that BV is not the major product of the reaction between BR and oxyradicals, dioxygen, and singlet oxygen species

(43). Thus, proposing other plausible chemical mechanisms for the regeneration of BR without the requirement of BVR (2, 44-46).

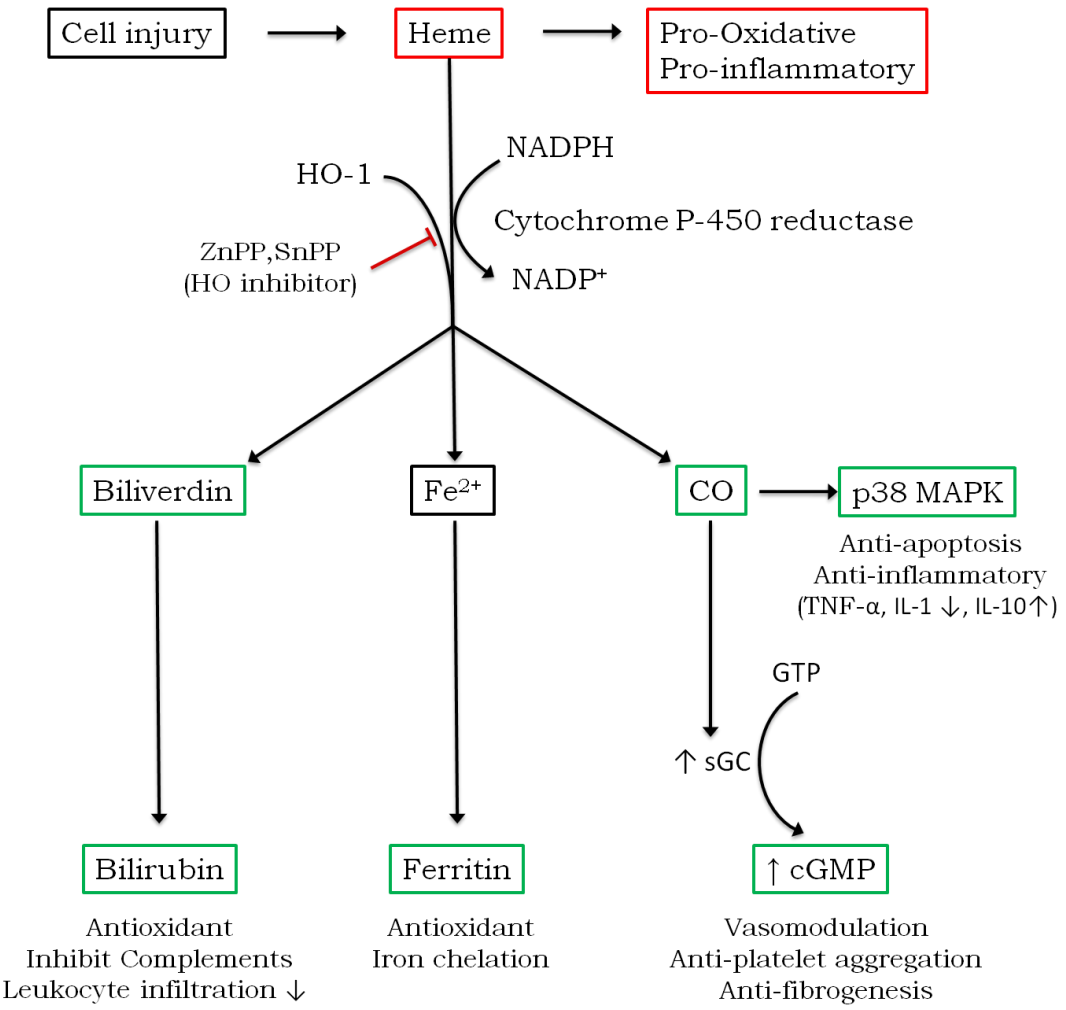

Figure 2: HO enzyme degrades heme to biliverdin, CO, and Fe^{2+}. BV is reduced to BR. BV and BR scavenge reactive oxygen species (ROS), inhibit complement activation and leukocyte infiltration. CO provides anti-inflammatory/anti-apoptotic effects via p38 MAPK activation, and modulates the vascular tone. This leads to diminished platelet aggregation and depressed fibrinogenesis. Fe^{2+} is sequestered by ferritin that prevents cell injury via its anti-oxidant and iron chelation. Modified from Tsuchihashi et *al.* (45)

The direct or indirect effects of BR on cellular oxidants have far-reaching implications in the context of oxidative stress-mediated diseases. Sedlak and Snyder have addressed the involvement of low serum BR concentrations with several pathologic conditions, including ischemic heart disease (47), congestive heart failure (48), peripheral

vascular disease (49), respiratory distress, circulatory failure, sepsis, aspiration, and asphyxia (50).

b. Activities of CO

In biologic systems, CO is generated through two sources that are either heme-dependent or independent. The heme-dependent source corresponds for the major (~80%) generated CO (51). Many studies have shown that CO exerts beneficial effects over these systems by activating, directly or indirectly, a number of intracellular signaling pathways that have anti-aggregant, anti-inflammatory, anti-proliferative, anti-apoptotic, and antithrombotic effects (Fig. 2). These effects occur through one or a combination of the following mechanisms: direct activation of soluble guanylyl cyclase (sGC) leading to an increase in the synthesis of cyclic guanosine monophosphate (cGMP) (52, 53), stimulation of calcium-dependent potassium channels (K^+ Ca) (54), and modulation of different MAPKs that initiate a cascade of transcription regulatory mechanisms designed at protection against oxidative stress (4, 55-57). Beside the cytoprotection effects of CO, it may alter other cellular targets via an interaction with a heme moiety resulting in inhibition including hemoglobin, nitric oxide synthase (NOS), prostaglandin endoperoxide synthase (3), myoglobin, catalase, pyrrolases, cytochrome *c* oxidase, peroxidases, respiratory burst oxidase, cytochrome P450, and tryptophan dioxygenase (4).

c. Role of iron

Although iron is essential in various cellular processes, such as heme synthesis, DNA replication, mitochondrial function, and oxygen sensing, its cytoprotective effects are

less obvious than biliverdin/bilirubin and CO. Iron potentially poses a toxic insult to cells by catalyzing free radical-generating reactions. It promotes the production of Reactive Oxygen Species (ROS) via lipid peroxidation chain reactions, nitric oxide-dependent nitrosylation of thiols, and the Haber–Weiss reaction (58). However, the iron released by HO activity also increases the expression of ferritin, which stores free iron and protect the cell from oxidative stress (59). In addition, HO-1 can repress the expression of a highly active ferrous iron-ATPase transporter involved in iron efflux from cells (60). The ATPase pump removes intracellular iron from the cell protecting it from apoptosis (61).

B. Statins

Increase in blood cholesterol is a risk factor for cardiovascular disease. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) redctase inhibitors (statins) block the regulatory and rate-limiting step of the cholesterol biosynthesis pathway (mevalonate pathway) and reduce blood cholesterol (62). Mevalonate is a precursor in cholesterol synthesis as well as in the synthesis of isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Figure 3). Statins-mediated inhibition of hepatic cholesterol biosynthesis results in a decrease in the hepatic intracellular stores of cholesterol. On the other hand to compensate for this depletion, low density lipoprotein cholesterol (LDL) receptor's expression is increased on the surface of liver cells leading to higher extraction of blood circulating LDL. Some statins (rosuvastain and simvastatin) exert a dual effect over the levels of circulating lipoproteins; they decrease plasma LDL level with a slight (5-10 %) increase in high density lipoprotein cholesterol (HDL) level (63-65). Chronic inhibition of hepatic cholesterol biosynthesis leads to a decrease in

plasma triglycerides (TG) levels due to a lower circulating very-low density lipoprotein (VLDL) levels (66).

1. Origin of statin

Statins are derivatives of hexahydronaphtalne lactones (Figure 4). They are divided into two groups,

i. The natural statins with their semisynthetic derivatives

ii. Statins of synthetic origin

The first statin, mevastatin, was isolated in Japan from a strain of *Penicillium citrinum* and was originally referred as ML-236B and later compactin (67). In 1980, Merck & Co. isolated lovastatin (mevinolin, MK803); the first commercially available statin (FDA approved in 1987), from the strain *Aspergillus terreus* (68). Later different pharmaceutical companies designed and produced other compound-such as pravastatin, simvastatin, fluvastatin, atorvastatin, rosuvastatin and pitavastatin-that showed better efficiency.

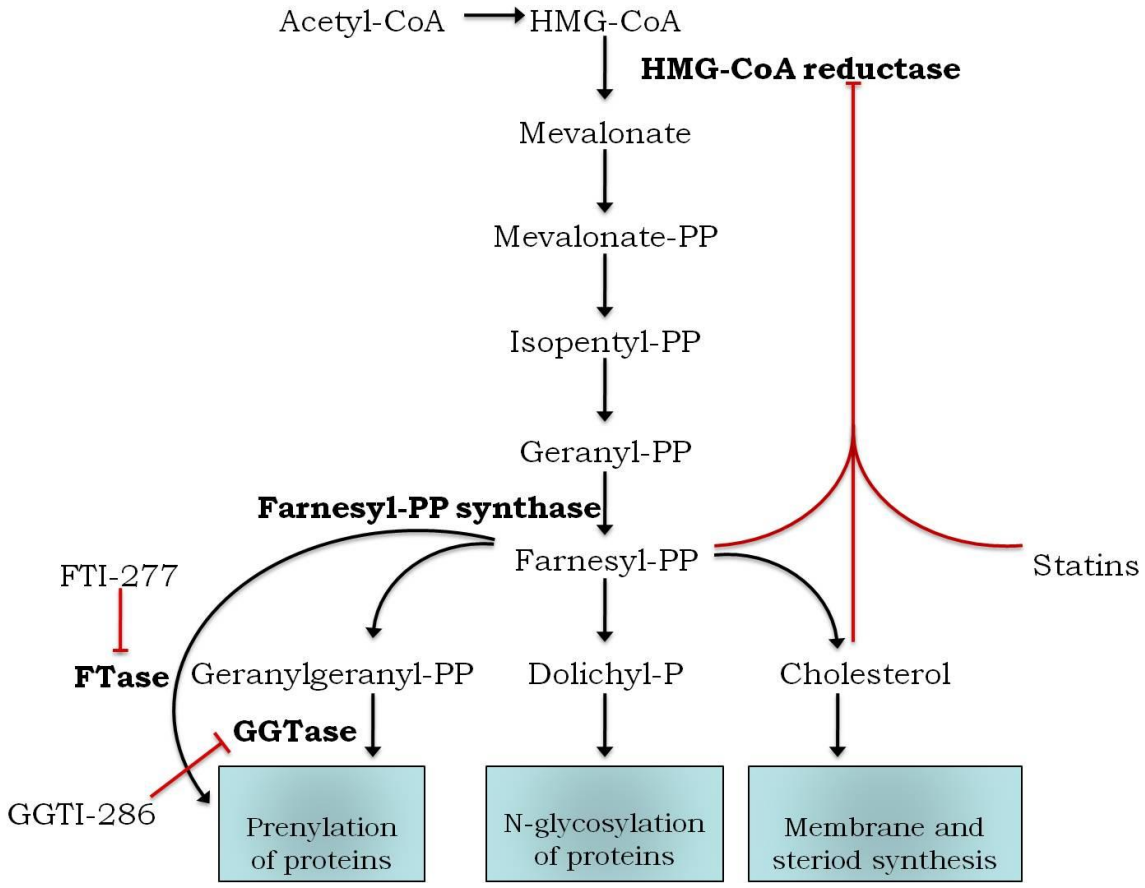

Figure 3 : The mevalonate pathway. 3-hydroxy 3-methylglutaryl-CoA (HMG-CoA) is converted to mevalonate by HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. Mevalonate is then converted to isopentenyl pyrophosphate (isopentenyl-PP; the 5-carbon basic isoprene unit), which is subsequently converted to farnesyl pyrophosphate (farnesyl-PP; a 15-carbon isoprenoid) through a series of enzymatic reactions. After addition of isopentenyl-PP, farnesyl-PP can be converted to geranylgeranyl pyrophosphate (geranylgernayl-PP; a 20-carbon isoprenoid), or alternatively farnesyl-PP can be converted to cholesterol or dolichyl phosphate (dolichyl-P), which is used for N-glycosylation of many proteins including G-protein coupled receptors or growth factor receptors. HMG-CoA reductase is the target of the cholesterol lowering statins. Importantly, in normal cells, cholesterol and isoprenoid products suppress HMG-CoA reductase via post-translational downregulation.

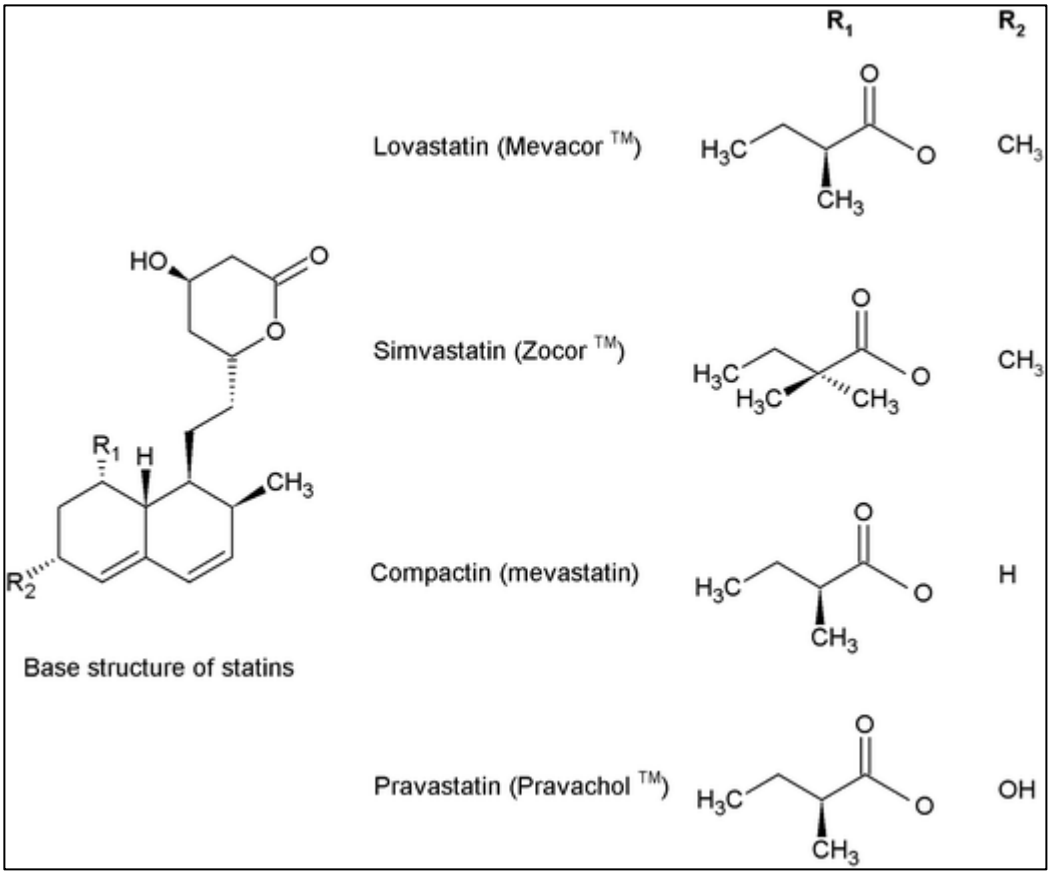

Figure 4: Chemical structures of statins and their derivatives.

2. Pleiotropic effects of statins

Many clinical, epidemiological and pathological studies showed that statins, aside to their cholesterol-lowering effects, reduced cardiovascular events, coronary events (69), stroke, the development of peripheral vascular disease (41), the prevalence of Parkinson's disease and Alzheimer's disease (64, 70), and prevented the rupture of vulnerable atherosclerotic plaque (71). These multiple pleiotropic effects of statins are related to the statin-inhibition of isoprenoids (FPP and GGPP) synthesis that are important lipid attachments for the isoprenylation of a number of proteins including small G proteins such

as Ras, Rho, and Rab (72). Recently, it has been indicated that statins could directly influence cellular processes (proliferation/apoptosis), inflammatory chemokines, and the cellular signaling mediated by these G proteins (73). This diversity of effects, summarized in Table 1, occurs through different mechanisms that shall be discussed in the following sections.

Table 1: Effects of statins.

Lipid-lowering effects
• Decrease LDL • Increase HDL
Pleiotropic effects
• Decrease inflammation • Promote anti-coagulation • Improve thrombogenic profile • Inhibit atherosclerosis • Decrease C- Reactive Proteins (CRP)

a. Anti-inflammation

Statins display an anti-inflammatory immunomodulator activity that is beneficial in atherosclerosis and coronary events, which have important inflammatory components. Statins inhibit adhesion molecules expression [Lymphocyte function-associated antigen-1 (LFA-1) and Inter-Cellular Adhesion Molecule 1 (I-CAM-1)] and the inducible expression of Major histocompatibility complex II (MHC II) molecules on leukocytes, and the expression of chemokine receptors on Th-1 cells. So affecting leukocyte activation,

leukocyte migration to inflammatory sites, and immunologic cytotoxicity (74). Statins reduced serum levels of C-reactive proteins (a serum marker of inflammation) and inflammatory cytokines (IL-6) (69, 75, 76). In addition, statins block the production of several inflammatory cytokines, like Tumor Necrosis Factor-α (TNF-α) and IFN-γ by macrophages (77). It has been shown that prenylation of Rho and Ras proteins have a major implication in inflammation process, through downregulation of endothelial Nitric Oxide synthase (eNOS) expression or activation of NF-κB, respectively. Statins are able to block the effect on Rho and Ras proteins (by decreasing the levels of isoprenoids) and thereby increasing eNOS expression and diminishing the activity of NF-κB (78). Statins might decrease the activity of AP-1 that has an important role in endothelial inflammatory responses, regulating genes responsible for Matrix Metalloproteases (MMPs), cytokines, chemokines, adhesion molecules, inducible nitric oxide synthase (iNOS) and Fas ligand (79, 80). Yano et *al.*(81) showed that statins induce cyclo-oxygenase 2 (COX-2) expression via the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, due to the inhibition of the small G proteins Rho and Cdc42. This activation of COX-2 results in production of 15d-PGJ2, a ligand of peroxisome proliferator activated receptors PPARγ and PPARα (82). PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. PPARγ and PPARα regulate metabolic pathways involved in glucose and lipid homeostasis with clinical application of synthetic PPARα (fibrates) and PPARγ (thiazolidinediones) ligands respectively in the treatment of dyslipidemia and type 2

diabetes. Moreover, PPARs also exert anti-inflammatory actions in atherosclerotic lesion cells, such as macrophages (83).

b. Plaque stabilization

Advanced atherosclerotic plaques are characterized by a core of extracellular lipid droplets, cellular debris and foam cells, surrounded by a fibrous cap of smooth muscle cells and a collagen-rich matrix. T cells, macrophages and mast cells infiltrate the lesion and are abundant in the shoulder regions, which possess active inflammatory properties (84). Macrophages are described to play a role in plaque instability through the production of pro-inflammatory cytokines and proteolytic enzymes (MMPs) weakening the fibrous cap rendering it more vulnerable to rupture. This rupture leads to thrombus formation at such lesions (85-87).

As mentioned earlier, statins modulate several functions of inflammatory cells, present in the plaque, including the adhesion to the endothelium, trans-migration into tissue, the secretion of pro-inflammatory cytokines and the production of free radicals (69, 75, 76, 88). Williams et al. noted that statins significantly reduced plaque content of macrophages and neovascularization (89). Whereas, Sillesen has demonstrated that statins stopped plaque progression, and participated in plaque regression, of intima-media thickness or also reduction of plaque volume (90).

c. Improved thrombogenic profile

Atherosclerosis is often accompanied with increased thrombotic potential that could be explained, in part, by the increases in the production of thromboxane (TX) A_2,

player in platelet aggregation and vasoconstrictor, and by the alterations in platelet membrane (91). Statins exert anti-thrombotic effects by dual mechanism; first, by inhibiting the production of TXA_2, and second, by up-regulating the expression of the COX-2 enzyme, consequently increasing the synthesis of the anti-aggregant, vasodilating agent prostacyclin (PGI_2) (69, 92).

d. Anti-oxidant

Oxidation of LDL by ROS is a central step in the atherosclerotic lesions pathogenesis. Oxidized LDL (Ox-LDL) is thought to attract monocytes into the vessel wall to get activated and lead to the formation of fatty streak, the earliest stages of atheroma, and lesion expansion (93). Moreover, Ox-LDL has important roles in the conversion of foam cells (from macrophages) and in the proliferation of smooth muscle cells. Furthermore, Ox-LDL causes disruptions of vascular tone impairing the normal relaxation of blood vessel (94, 95). This mechanism might involve intervention with NO production derived from endothelium (69, 96).

Different studies have demonstrated an effect of statins mainly fluvastatin on the lowering of the circulating Ox-LDL human. Inami et *al.* have observed a 47.5% reduction in Ox-LDL level due to fluvastatin treatment (97). Tits et *al.* have demonstrated decreased amount of dienes formed during *in vitro* LDL oxidation due simvastatin treatment (98). Evidence for these anti-oxidant effects has only been demonstrated with specific statins and may be unique to those drugs.(99)

C. HO-1 and statins

Since the first evidence of HO-1 induction by statins in rat vascular blood vessels that proposed a model of vascular protection (100), there has been focus over the properties of statins. Recently Ali et *al.* showed that HO-1 induction at different levels such as *HMOX1* promoter activity, mRNA levels, and enzyme activity (101). Chen et *al.* have demonstrated that this induction could be mediated by impeding prenylation of small G proteins, for example Ras protein in particular (102).

D. Preliminary studies

In previous work of Dr. Habib laboratory, it has been demonstrated that HO-1 is induced by NO and NSAIDs in Raw 264.7 mouse macrophages and NIH3T3 mouse fibroblasts using polyclonal antibodies raised against HO-1 and HO-2 selective peptides (103).

In collaboration with Dr. Marie-Jose Alcaraz (Faculty of Pharmacy, University of Valencia, Spain), it was shown in an experimental model of inflammation that the increased expression of HO-1 results in beneficial anti-inflammatory effects that was accompanied by a decrease in Leukotriene B_4 formation and IL-1β and TNFα secretion.

Furthermore, recent studies of the same group showed that statins induced HO-1 expression in both Raw 267.4 cell lines, primary peritoneal macrophages and NIH 3T3 cell lines and that transcription factors such as C/EBP and AP-1 were activated by statins in all cell lines whereas USF transcription factor was also activated in NIH 3T3 cells.

C2C12 cells are murine myoblasts which upon differentiation cells transform from myoblast into myotubes. They represent an *in vitro* model to assess rhabdomyolysis or myotoxicity. It has been previously shown in the laboratory that statins induced expression of HO-1 in these cells involved the inhibition of one or more geranylgeranylated proteins.

These results comprised the preliminary experiments that provoked us to study the mechanism involved in statin-dependent expression of heme oxygenase-1in skeletal cells.

E. Aim

The aims of this study were to characterize the molecular mechanisms behind the statin-dependent induction of HO-1 in skeletal muscle cells and to investigate its cytoprotective role in heme turnover in the skeletal muscle. Our objectives were to:

- Characterize and evaluate the HO-1 expression at the protein and mRNA levels in C2C12 cells in response to statins
- Investigate the role of the small G proteins in the statin-dependent effect
- Analyze the involvement of specific transcription factors by gel shift analyses, like AP-1, C/EBP, and USF
- Evaluate the impact of HO-1 inhibition on cell function
- Study the effect of statins treatment over the differentiation of skeletal cells

Chapter II

Materials and Methods

A. Materials

Cell culture media (DMEM), penicillin, streptomycin, L-glutamine, and trypsin were from Lonza (Basel, Switzerland). Heat-inactivated Fetal Bovine Serum (FBS) was from Invitrogen (Paisley, UK). Simvastatin, fluvastatin, FTI-277, GGTI-286, Y-27632, and NSC23766 were from EMD-Calbiochem (San Diego, CA, USA). Spermine NONOate (SPNO) was from Cayman Chemical Co (Ann Arbar, MI). Bovine serum albumin (BSA), Hemin Chloride, Triton X-100, Formamide, ethidium bromide, Hoechst H 33258, avidin-fluorescein isothiocyanate(FITC), Dithiothreitol (DTT), and mouse monoclonal anti α-actin IgG were from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), hepes, sodium chloride, Tween 20, and MOPS were from Amresco (Solon, OH, USA). Tris, acrylamide, bis-acrylamide, agarose, sodium dodecyl sulfate (SDS), TEMED, glycine, and Bradford reagent were from BioRad (Hercules, CA, USA). Polyclonal anti-mouse IgG and polyclonal anti-rabbit IgG coupled to horse radish peroxidase were from Jackson ImmunoResearch Laboratories (PA, USA). Tripure, and Poly dI/dC were from Roche (Basel, Switzerland). [^{32}P]-γ-ATP pure 1000 Ci/mmol was from NEN-PerkinElmer (Wellesley, MA, USA). RT-PCR kit was from Finnzyme (Keilaranta, Finland). PCR and EMSA primers were from TIB Molbiol (Berlin, Germany). Formaldehyde and ethylene diamine tetraacetate (EDTA) were obtained from Fisher Scientific (London, UK). Biotinylated (polyclonal anti-mouse IgG and polyclonal anti-rabbit IgG) antibodies and

enhanced chemiluminesence (ECL) reagent were from Amersham General electric (Little Chalfont Buckinghamshire, UK). Autoradiograph films were obtained from AGFA (Mortsel, Belgium). Rabbit polyclonal antibodies anti C/EBP α, C/EBP β, and C/EBP δ and anti USF1 and USF2 were from Santa Cruz Biotechnology (CA, USA). Chloroform and Isopropanol were obtained from UniChem (Mumbai, India). Spin-X centrifuge tube filter was from Corning (NY, USA).

B. Methods

1. Cell culture

Murine myoblasts cell line, C2C12 CRL-1772 ™ (ATCC, VA, USA) was kindly provided by Dr G. Nemer. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM Hepes. For their differentiation into myotubes, confluent cells were incubated with DMEM contatining 0.5% fetal bovine serum for 3 days. Cells were incubated at 37ºC and 5% CO2 in a humid atmosphere.

2. Treatment of cells

For treatment, cells were incubated in medium containing 0.5% fetal bovine serum in absence or presence of different concentrations of simvastatin or fluvastatin, GGTI-286, a geranylgeranyl transferase inhibitor, FTI-277, a farnesyl transferase inhibitor mevalonic acid, TAT/C3 exoenzyme, a Rho A/C inhibitor, NSC23766, a Rac Inhibitor, Y-27632, a Rho Kinase Inhibitor, hemin chloride, or SPNO .

3. Western blot analysis

After incubation, cells ($1.5x10^5$/well, 12- well plates) were washed with PBS containing Ca^{2+}/Mg^{2+} and lysed on ice in 200 µl of lysis buffer (20 mM Tris/HCl pH 7.5 containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.33 mM Benzamidine). Cell lysates were centrifuged at 15000 g at 4 ºC. Total protein concentration was determined using Bradford assay (Bio-Rad) with BSA as standard.

a. SDS-PAGE

10-30 µg of total cell proteins were heated to 95 °C for 5 minutes in Lammeli buffer solution (0.1 mM Tris pH 6.8, 10% glycerol, 0.01% bromophenol blue dye, 5% beta-mercaptoethanol, and 2% SDS) and submitted to SDS-PAGE using 12% polyacrylamide gel (29:1 acrylamide:bis). Migration was allowed to proceed in 1X migration buffer (final concentrations 0.05 M Tris, 0.384 M glycine, and 2% SDS). The gels were then transferred to a supported nitrocellulose membrane (Bio-Rad, porosity 0.45 µm) using a semi-dry transfer apparatus. Membranes were stained with Ponceau red.

b. Western blot analysis

Membranes were incubated overnight in tris buffer saline tween (TBST) (25 mM Tris pH 7.4, 0.25 M NaCl, and 0.1% Tween-20) -5% non-fat dry milk, at 4°C. Immunoblot analysis was performed using polyclonal rabbit anti-mouse HO-1 peptide and donkey anti-rabbit IgG coupled to horse-raddish peroxidases, both at a dilution of 1:2000 in TBST-5% milk, were used. Membranes were stripped off their antibodies by washing two times with stripping buffer pH 2.2 (0.2 M glycine, 0.1% SDS, and 1% Tween-20) for 30 min and

redeveloped with monoclonal mouse anti- α-actin (Sigma Aldrich) at a dilution of 1:10000, and donkey anti-mouse IgG coupled to horse-raddish peroxidase. Protein band signals were developed on Agfa autoradiography films using enhanced chemiluminescence (ECL kit Amersham) according to manufacturer instructions, and scanned using Epson scanner. Bands were quantified by Scion Image.

4. RNA extraction

Total RNA was extracted from differentiated C2C12 cells ($2.5x10^5$ cells/ 60 mm dish) using 500 µl of Tripure (Roche) according to the manufacturer's instructions. Briefly, 100 µl chloroform was added, mixed and the mixture was centrifuged at 12,000 g for 15 min at 4 ºC. 250 µl of isopropanol was added to the collected aqueous phase followed by centrifugation at 12000 g for 10 min at 4 ºC. The RNA pellet was washed with 500 µl of 75% ethanol followed by centrifugation at 12, 000 g for 5 min at 4 ºC and left to dry. The pellet was dissolved in 30 µl of DEPC-treated water, incubated for 5 min at 60 ºC and then quenched. RNA concentration was measured at 260 nm, and 260/280 ratio was determined. Total RNA was checked by electrophoresis of 2 µg of RNA sample with 2X RNA loading dye (10X MOPS, formamide, formaldehyde, ethidium bromide, and loading buffer containing 50% glycerol, 1 mM EDTA, and 0.4% bromophenol blue) on 1% agarose gel in 1X Tris Boric EDTA buffer (TBE) (0.1 M Tris base, 0.1 M boric acid, and 20 mM EDTA pH 8.3) at 80 volts for 30 min.

5. RT-PCR

2 μg of total RNA were reverse transcribed into single-stranded cDNA using the Phusion® RT-PCR kit (Finnzyme), according to manufacturer's instructions. cDNA amplification reaction was done using the Phusion® RT-PCR kit (Finnzyme) according to the manufacturer's instructions. Primer sequences for the genes of interest are shown in table 2:

Table 2: PCR conditions. The primer sequences, annealing temperature and the amplified fragment size of each primer pair are optimized to the following values.

Gene	Direction	Sequence	Annealing	Fragment size
HO-1	Forward	5'-GAATTCAGCATGCCCCAGGATTTG-3'	55	0.7 kb
	Reverse	5'-TCTAGACTAGCTCAATGTTGAGCAGGA-3'		
β-Actin	Forward	5'-GTGACGAGGCCCAGAGCAAGAG-3'	61	1 kb
	Reverse	5'-AGGGGCCGGACTCATCGTACTC-3'		
MyoD	Forward	5'-GCAGGCTCTGCTGCGCGACC-3'	55	0.5 kb
	Reverse	5'-TGCAGTCGATCTCTCAAAGC-3'		
Desmin	Forward	5'- GCGCCAAGCCAAGCAGGAGA-3'	61	0.5 kb
	Reverse	5'- GAGGACGGGGCCAGGACACT-3'		

PCR was performed using the iCycler (BioRad) programmed for a 1 min denaturation at 98 ºC (1 cycle) followed by 25 or 30 cycles for: 30 sec at 98 ºC, 30 sec annealing, 45 sec extension at 72 ºC, and finally one cycle of 10 min extension at 72 ºC. The annealing temperature was determined for each gene and the number of cycles of each gene was optimized in the lab. The optimized conditions are listed in Table 2. Upon gel

electrophoresis (1% agarose gel in 1X TBE buffer), single bands appeared and their sizes were confirmed by the psk-MpI ladder.

6. Nuclear extraction and EMSA

Subconfluent differentiated C2C12 ($1x10^6$ cells/100 mm dish) were treated and washed with 4 ml of PBS containing 2 mM EDTA. After centrifugation at 10,000 g for 30 sec, the pellet was resuspended in 800 µl of Buffer A (10 mM Tris/HCl, pH 8 contatining 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 1 µl/ml Pepstatin) and incubated for 15 min on ice. This was followed by the addition of 50 µl NP40 (10%), shaking for 10 sec, and centrifugation at 16000 g for 30 sec. The pellet was resuspended in 200 µl of Buffer C (20 mM Tris/HCl pH 8 containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 µl/ml Pepstatin) and incubated on a shaker for 20 min at 4ºC. After centrifugation at 15,000 g for 5 min at 4ºC, the pellet was discarded and the supernatant was assayed for protein content using the Bradford Assay (Bio-Rad).

a. <u>Primers annealing</u>

Single stranded oligonucleotides (forward and reverse) diluted concentration of 100 pmol/µl were incubated in 50 µl Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA) at a final concentration of 2 pmol/ µl, and then annealed by mixing 5 µl of each primer with 90 µl of TEN annealing buffer (10 mM Tris/ HCl pH 7.9, 0.5 M EDTA, and 400 mM NaCl) after boiling the samples for 2 min. Samples were left to cool down to room temperature overnight. The annealed primers were stored at -20ºC. Primer sequences of the probes were as follow:

HO-1 CEBP sense 5'- GCTGGATGTTGCAACAGC-AGC -3'

Consensus AP-1 sense 5'- CGCTTGATGACTCAGCCGGAA-3'

HO-1 USF sense 5'- GGCCACCACGTGACCCGC-3'.

b. Oligonucleotides labeling

Annealed primers were labeled with ^{32}P by mixing 1 µl of the oligonucleotide (5 pmol), 1 µl of 10x Polynucleotide Kinase buffer, 0.5 µl of T4 Polynucleotide Kinase, 5 µl of [^{32}P]-γ-ATP (4500 Ci/mmol) in a total volume of 10 µl. After incubation for 1 h at 37ºC, labeled oligonucleotides were migrated on 12% Bis-Acrylamide gel (containing 38:2 acrylamide:bis, water, 1.6% APS, 10x TBE and TEMED) in 1x TBE buffer at 110 volts for 30 min. Purified probes on the gel were cut and incubated overnight in 400 µl TEN buffer at 37ºC with vigorous shaking and transferred to a spin column (Spin-X) and centrifuged for 10 sec at 14,000 g. 1 ml of absolute ethanol was then added, and the samples are incubated for 20 min at -20ºC to precipitate the probe. After centrifugation at 20000 g for 15 min at 4ºC, the obtained pellet was washed with 700 µl of 70% ethanol and centrifuged again at 20000 g at 4ºC for 10 min. The supernatant was decanted, and 50 µl TE 1x were added. The probe was stored at -20ºC, after quantification of its radioactivity using a β counter.

c. Gel preparation and casting

6% polyacrylamide non-denaturing gel (Bis-Acrylamide 29:1, 1.6% APS, 10 x TBE, H2O and Temed) was prepared and poured into the assembled apparatus, then left to

polymerize in half an hour. The gel was pre-run in 0.25x TBE for 30 min at 200 volts before running the samples.

d. Binding reaction

To initiate reaction between the labeled oligos and C2C12 nuclear extract, 5 μg of protein nuclear extract were mixed with 4 μl of binding buffer (20 mM Tris/HCl, pH 7.9, 120 mM KCl, 25 mM MgCl2, 2 mM EDTA, 25% Glycerol, 2 mM DTT), 1 μl of Poly dI-dC (Roche), and water to make a final volume of 20 μl. Cold competitor was added in excess to designated tubes. The radioactive probe was added at the final step and tubes were incubated at room temperature for 20 min. The samples were then loaded in the wells and the gel was run at 200 volts for 2-3 hours. For supershift analysis, anti C/EBP (α, β, or δ) or USF (1 or 2) were added to designated tubes. Briefly, 2 μg of the antibody was added 30 min before the addition of the radioactive probe and the reaction was performed as described above.

e. Gel drying and imaging

The gel was dried for 3 hours at 80ºC under vacuum in a Bio-Rad dryer. The dried gel was exposed overnight to a phosphoimager screen and scanned using the STORM Phosphoimager (General Electrics). The gel was next exposed to and also developed on an X-Ray film (Agfa) after incubation at -80ºC.

7. Immuno-fluorescence

Cells ($1x10^5$ cells/12-well plate) were seeded over gelatin-covered cover-slips. After differentiation and incubation, cells were fixed to the slips with 4%

paraformaldehyde for 20 min, then permeabilized with 0.2% Triton X-100 for 30 min. Cover slips were then blocked by 0.5 ml PBS- 0.5% BSA for 30 min. Cells were incubated with polyclonal rabbit anti-mouse HO-1 IgG solution (0.5 ml, 1/250) for 1 hour. Secondary Biotinylated goat anti-rabbit IgG solution (0.2 ml, 1/250) was added for 1 hour. Next, avidin-FITC (Fluorescence-Avidin DCS vector A-2011) (0.4 ml, 1/1000) was added for 1 hour in dark. Finally, Hoechst H 33258 solution (0.3 ml, 1/20) was added to each sample for 10 min in dark. Each incubation step was followed by 4 washing steps with PBS-sodium azide (NaN_3) for 5 min each. The control sample was prepared without the incubation with polyclonal rabbit anti-mouse HO-1 IgG.

8. Statistical analysis

Sigmastat® and Sigmaplot® were used for statistical analysis and graph design Results of protein was expressed as the mean ± standard error mean (SEM) from at least 3 independent experiments. For comparison between multiple groups, statistical significance was tested by one way ANOVA. An unpaired t-test was used for the comparison of 2 groups, and a P value of less than 0.05 was considered statistically significant.

Chapter III

Results

A. Effect of statins on HO-1 expression at the protein and mRNA levels

Different molecules were described to induce HO-1 in many cell types. This modulation is complex and involves transcriptional and post-transcriptional mechanisms. In a previous study conducted in our lab, by Attar et *al*. (104), it has been shown that spermine NONOate (SPNO)-donor of NO-induces HO-1 expression in a dose-dependent manner in C2C12, murine myoblast, cell line at 6 hours (Figure 5). As shown in figure 5 HO-1 was 34, 66 and 117 % induced after treatment by 0.02, 0.1 and 0.5 µM SPNO, respectively.

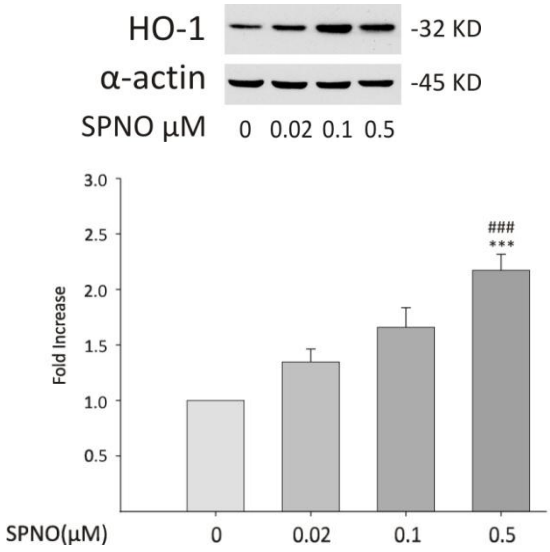

Figure 5: NO induces HO-1 protein expression in C2C12. Differentiated C2C12 were incubated for 6 hours in the absence or presence of 0.02, 0.1 or 0.5 µM SPNO. Cells were lysed and western blot analysis was carried out using 20 µg of total cell lysates and a selective anti-HO-1 IgG or anti-α-actin IgG. Enhanced chemiluminescence was used to develop signals. Densitometric analysis-shown to the bottom- is the ratios of HO-1 to α-actin of the western blots. Data are mean ± s.e.m. of 7 separate experiments with similar results. ***$p<0.001$ versus control, ###$p<0.001$ versus NO 0.02 µM.

1. Effect of statins on HO-1 expression

We first repeated the tests on the effect of statins on HO-1 expression using both simvastatin and fluvastatin in C2C12 cell line in the absence or presence of SPNO.

Treatment of differentiated cells with 5 or 25 μM of simvastatin in the absence or presence of 0.02 or 0.1 μM NO is represented in the western bolt and densitometric graph (Figure 6). Simvastatin alone at 5 μM increased expression of HO-1 (24 %). Similarly, at 25 μM simvastatin induced HO-1 expression (50 %). SPNO augmented the induction of HO-1. This was apparent when low concentrations of both simvastatin and SPNO were used.

Similarly, incubation of differentiated C2C12 cells with 5 or 10 μM of fluvastatin in the absence or presence of 0.02, 0.1 or 0.5 μM NO is represented in the western blot and densitometric graph (Figure 7). Neither fluvastatin 5 μM nor 10 μM alone induced the expression of HO-1. Higher concentrations were toxic for the cells. Co-treating cells with SPNO lead to induction of HO-1 that reached more than 2 fold increase with NO 0.5 μM.

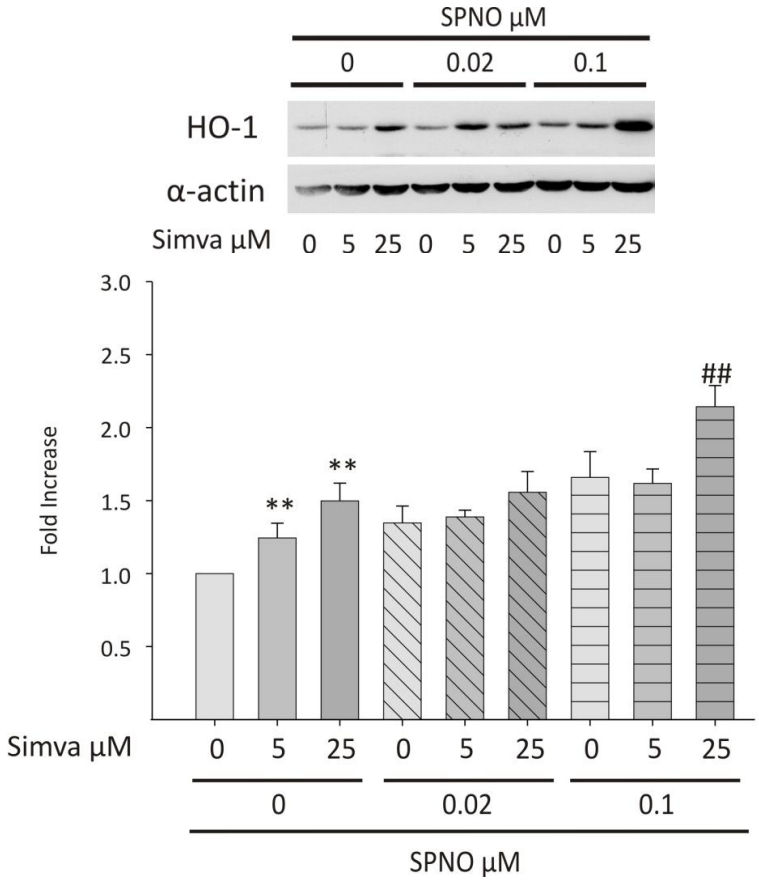

Figure 6: Simvastatin induces HO-1 protein expression in C2C12. Differentiated C2C12 were incubated for 24 hours with 5 or 25 µM simvastatin in the absence or presence of 0.02, 0.1 or 0.5 µM SPNO for the last 6 hours. Western blot analysis was performed as described in the legend for figure 5. Data are mean ± s.e.m. of 7 separate experiments with similar results. **p<0.005 versus control, ##p<0.005 versus NO 0.1 µM.

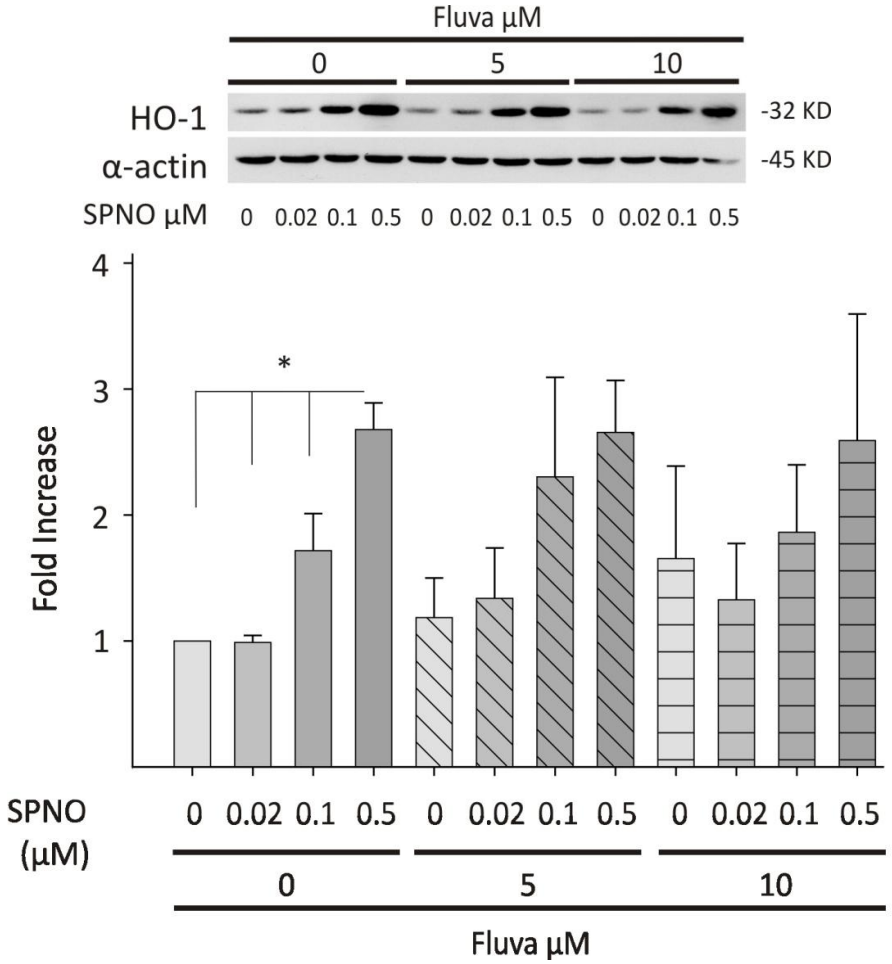

Figure 7: Fluvastatin failed to induce HO-1 protein expression in C2C12. Differentiated C2C12 were incubated for 24 hours with 5 or 10 µM simvastatin in the absence or presence of 0.02, 0.1 or 0.5 µM SPNO for the last 6 hours. Western blot analysis was performed as described in the legend for figure 5. Data are mean ± s.e.m. of 3 separate experiments with similar results. *$p<0.05$ versus control.

As mentioned earlier, statins are potent inhibitors of HMG-CoA reductase, which catalyze the rate limiting step in the biosynthesis of cholesterol and other lipid-based molecules (FPP and GGPP) (Figure 3). To verify that statins induce HO-1 through the inhibition of mevalonate pathway, cells were incubated with mevalonic acid to overcome the inhibition effect of statins. Differentiated C2C12 cells were pretreated with mevalonic acid 30 minutes prior to the addition of statins. Figure 8 shows that mevalonate reduced the induction of HO-1 by simvastatin. Basal expression of HO-1 was also modified.

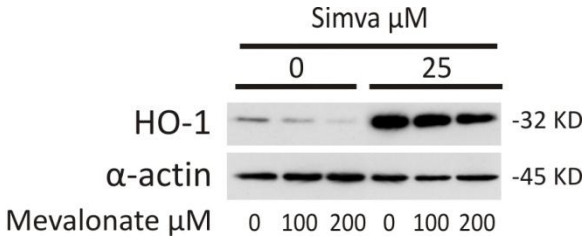

Figure 8: Mevalonic acid abolishes Simva-induced HO-1 expression. Differentiated C2C12 cells were incubated with 100 or 200 µM mevalonic acid 30 min prior to the addition of 25 µM of simvastatin to be then incubated for 24 hrs. Western blot analysis was performed as described in the legend for figure 5. Data illustrate one representative experiment out of 3 separate.

2. HO-1 cellular localization

HO-1 is well known as a microsomal protein. We checked whether there's any localization change after statin induced HO-1 by Immunocytochemistry. C2C12 cells were seeded, differentiated, and treated. HO-1 was detected using selective anti HO-1 antibody. As shown in figure 9, HO-1 was strongly induced by both simvastatin and hemin, with a uniform distribution. In our conditions, fluvastatin showed minimal induction of HO-1.

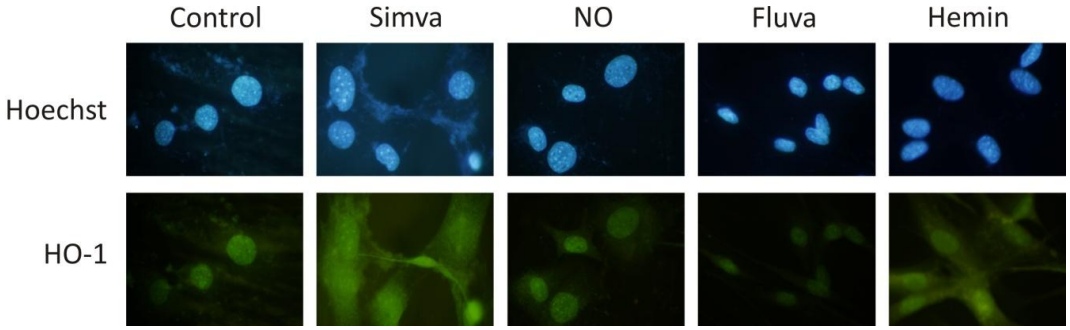

Figure 9: Cellular localization of HO-1 protein assessed by Immuno-Fluorescence. Differentiated C2C12 cells were incubated with 0.5 µM of SPNO and 30 µM Hemin (6 hrs), 25 µM simvastatin, and 10 µM fluvastatin (24 hrs). Cells were subjected to incubations with polyclonal rabbit anti-mouse HO-1 IgG, Biotinylated anti rabbit antibodies, avidin-FITC and Hoechst according to the protocol. Microscopic magnification is 40x. Results represent 2 experiments.

3. Modulation of HO-1 gene expression

We examined the effect of different HO-1 inducers and statins on HO-1 mRNA levels using reverse transcriptase- RT-PCR. As shown in figure 10 simvastatin (Simva), fluvastatin (Fluva) and hemin were able to induce HO-1 gene expression in the absence or presence of NO.

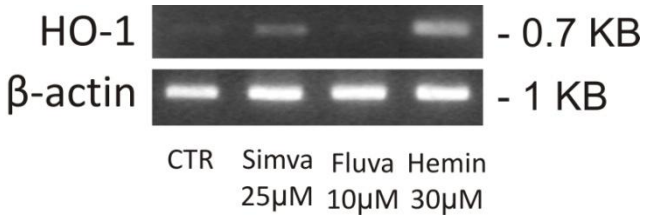

Figure 10: Effect of simvastatin, fluvastatin, or Hemin on the mRNA level of HO-1 as assessed by RT-PCR. Differentiated C2C12 in 60 mm DISHES were treated for 24 hours with simvastatin (25 μM), or fluvastatin (10 μM) or for 6 hours with Hemin (30 μM). Total RNA were extracted using Tripure reagent (Roche). Reverse transcriptase reaction was performed using 2 μg of total RNA and PCR was performed using selective HO-1 or β-actin primers. Results represent 5 experiments.

The effect of prenylated proteins over the HO-1 gene expression was assessed by incubating differentiated C2C12 cells with 10 μM GGTI-286. As shown in figure 11, GGTI-286 increased the relative levels of HO-1 mRNA as compared to the control and simvastatin incubated cells.

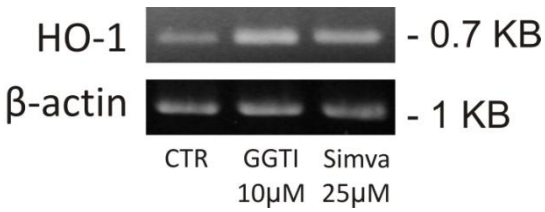

Figure 11: Effect of GGTI on the mRNA level of HO-1 as assessed by RT-PCR. C2C12 in 60 mm were treated for 24 hours with either GGTI or simvastatin. RNA extraction and RT-PCR was performed as describes in legend of figure 11. Results represent 4 experiments.

4. Effect of prenyltransferase inhibitors on HO-1

To further assess the involvement of any prenylated proteins in the statin-dependent regulation of HO-1 expression, we tested the effect of two inhibitors of protein prenylation, FTI-277 and GGTI-286, inhibitors of farnesyl and geranylgeranyl transferases, respectively. As shown in figure 12, treatment of C2C12 cells with 10 μM of GGTI-286 for 24 hours induced significantly HO-1 expression (2 fold increase) whereas no significant increase was obtained with FTI-266 incubation.

HO-1 induction by GGTI-286 was further increased when cells were treated with 0.1 μM SPNO for the last 6 hrs (Figure 13). This additional effect on HO-1 expression was clear at 3 and 10 mM of GGTI-286 and was similar to the effect of effect.

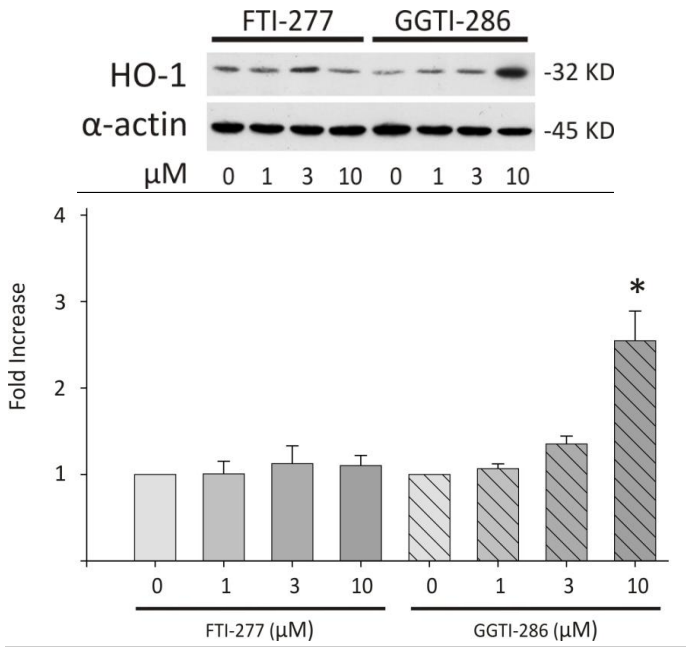

Figure 12: HO-1 protein expression in response to farnesyl transferase inhibitor (FTI-277) and geranylgeranyl transferase inhibitor (GGTI-286). Differentiated C2C12 cells were incubated with 1, 3, or 10 μM of FTI-277 or GGTI-286 for 24 hours. Western blot analysis was performed as described in the legend for figure 5. Data are mean ± s.e.m. of 6 separate experiments with similar results. *$p<0.005$ of GGTI-286 (10 μM) versus control, $p<0.05$ of both GGTI-286 (10 μM) versus GGTI-286 (1 μM) and versus GGTI-286 (1 μM).

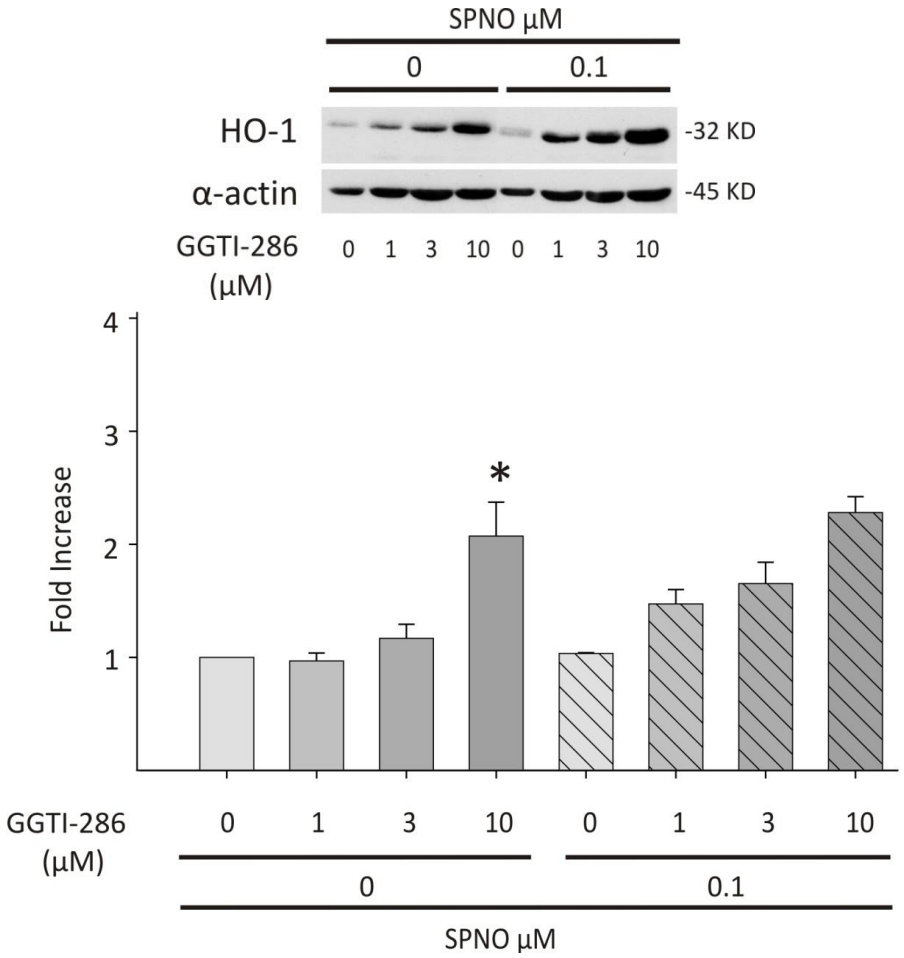

Figure 13: NO augmented the induction of HO-1 protein expression by GGTI-286. Differentiated C2C12 cells were incubated with 1, 3, or 10 μM GGTI-286 for 24 hours in the absence or presence of 0.1μM SPNO for the last 6 hours. Western blot analysis was performed as described in the legend for figure 5. Data are mean ± s.e.m. of 3 separate experiments with similar results. *$p<0.005$ of GGTI-286 (10 μM) versus control, $p<0.05$ of both GGTI-286 (10 μM) versus GGTI-286 (1 μM) and versus GGTI-286 (1 μM).

B. Role of small G-proteins in HO-1 expression

Small G-proteins are known to play an important role in cellular signaling. These proteins require post-translational modifications including prenylation to be active and exert their functions. Prenylation is responsible for their translocation to plasma membrane. Since HO-1 was induced in the presence of GGTI-286, we investigated the role of some geranylgeranylated proteins in the expression of HO-1. It was reported that many

Rho GTP-bound proteins require geranylgeranylation and are important in gene expression. Thus we checked the role of two small G proteins, RhoA/C and Rac, in HO-1 expression.

1. Role of RhoA/C protein

To assess the involvement of RhoA/C in the regulation of HO-1 expression, we first used; Y-27632 (ROCK inhibitor) and C3 exoenzyme, TAT/C3 (RhoA/C inhibitor). ROCK (Rho-kinase) is a serine/threonine-specific protein kinase which is activated by GTP-bound RhoA(105). TAT/C3 is a toxin from Clostridium botulinum that is described to selectively block Rho A/C by ribosylation (106).

a. <u>ROCK inhibitor</u>

Treating differentiated C2C12 cells with 10 μM Y-27632 showed induction in HO-1 protein expression at 3 hours (Figure 14).

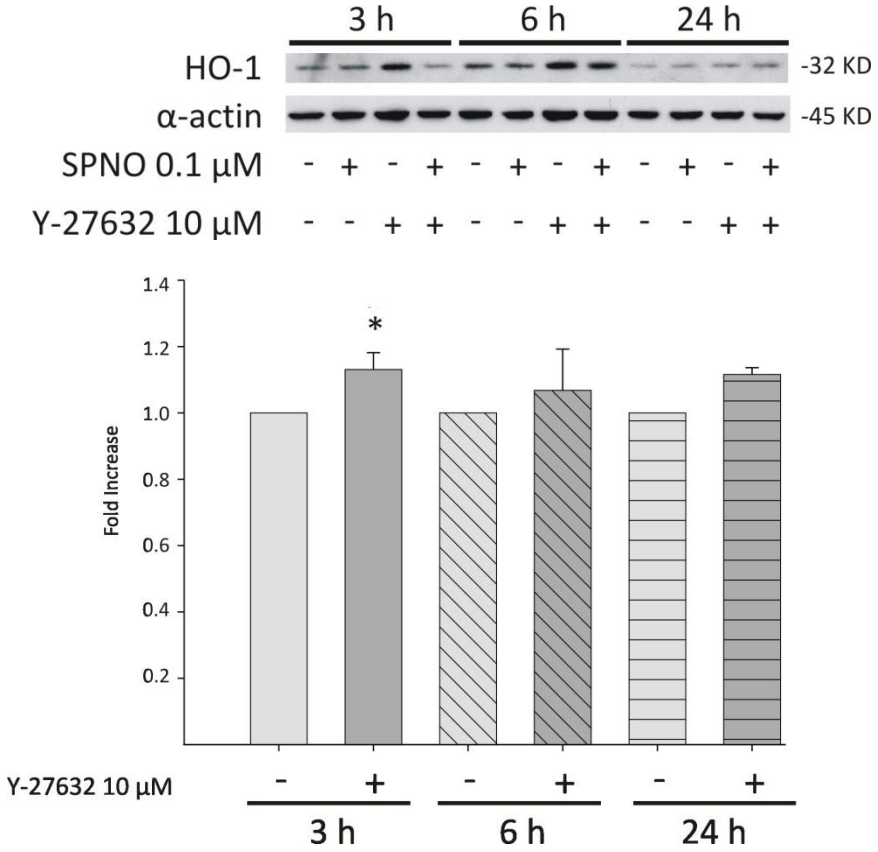

Figure 14: Effect of RhoA/C over HO-1 expression assessed by Y-27632. Differentiated C2C12 cells were incubated with 10 µM Y-27632 for 3, 6 or 24 hours in absence or presence of 0.1 µM of SPNO. Western blot analysis was performed as described in the legend for figure 5. Data are mean ± s.e.m. of 4 separate experiments with similar results. *$p<0.05$ versus control.

b. <u>RhoA/C inhibitor</u>

Treating differentiated C2C12 cells with 20 µg/ml TAT/C3 for different incubation points showed an induction in HO-1 protein expression at 3 and 6 hours (Figure 15).

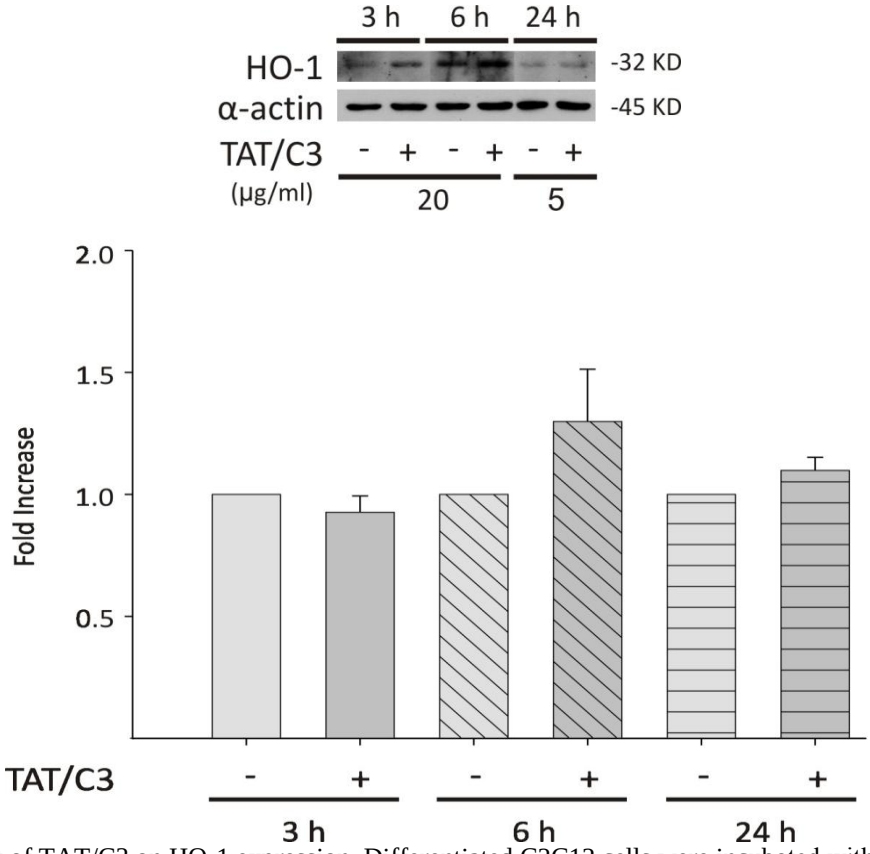

Figure 15: Effect of TAT/C3 on HO-1 expression. Differentiated C2C12 cells were incubated with 5 μg/ml (for 24 hours) or 20 μg/ml (for 3 or 6 hours) of TAT/C3 exoenzyme. Western blot analysis was performed as described in the legend for figure 5. Autoradiogram is representative of 6 separate experiments with similar results.

2. Role of Rac protein

To assess the role of Rac protein in the regulation of HO-1 expression, we used NSC23766, an inhibitor of Rac1 and Rac2. Differentiated C2C12 cells were incubated with 50 μM of NSC23766 for 24 hours in absence or presence of 0.1 μM SPNO added for the last 6 hours. NSC23766 was able to significantly elevate the level of HO-1 expression (Figure 16). Co-treatment with SPNO lead to higher HO-1 expression compared to NSC23766 alone.

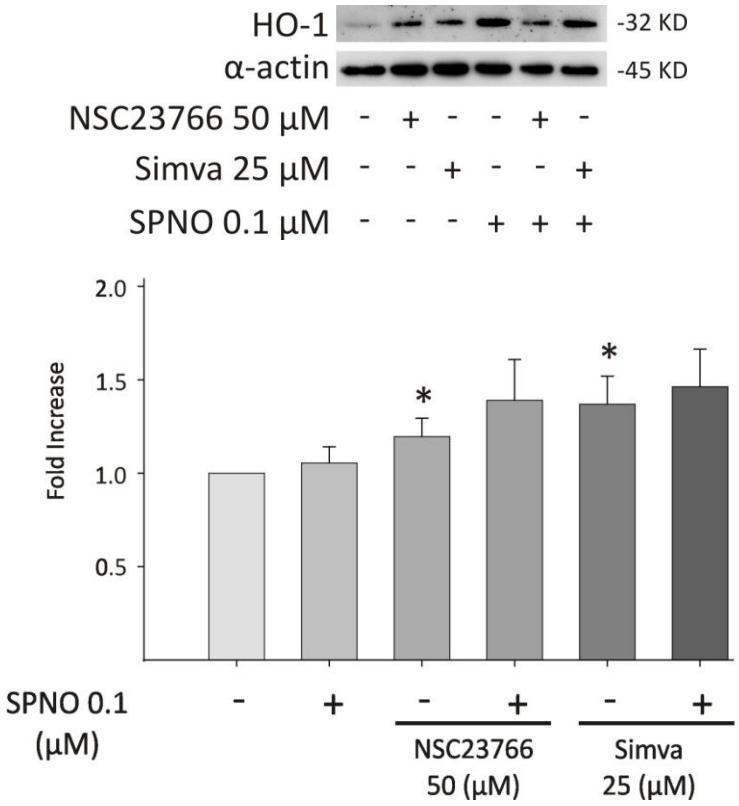

Figure 16: Effect of Rac over HO-1 expression assessed by NSC23766. Differentiated C2C12 cells were incubated with 50 µM NSC23766 or 25 µM simvastatin for 24 hours in absence or presence of 0.1 µM SPNO. Western blot analysis was performed as described in the legend for figure 5. Autoradiogram is representative of 6 separate experiments with similar results. *p<0.05 versus control.

C. Gel retardation of HO-1 DNA complexes by statins

To further study the regulation of HO-1 expression in C2C12 cells, we checked the activation of transcription factors in response to stains through their binding to special DNA sequences that are present in the HO-1 gene promoter. We checked for C/EBP (CCAAT/ enhancer binding protein), USF (Upstream Stimulatory Factor) and AP-1(Activator Protein-1) binding in response to statins. DNA sequences containing C/EBP or USF binding sites were prepared based on sequences from proximal region of murine HO-1 promoter, and DNA sequence containing AP-1 binding sites was prepared based on

consensus AP-1 region (Santa Cruz). Simvastatin was able to promote complex formation of C/EBPβ but not USF 1 or 2, whereas; fluvastatin showed no effect. Moreover, simvastatin showed slight AP-1 dependent complex formation (Figs. 17, 18 and 19). Supershift analyses using specific antibodies that identify different isoforms of C/EBP and USF were used and showed that C/EBP β or both USF 1 and 2 are the major transcription factors to interact with HO-1 promoter region.

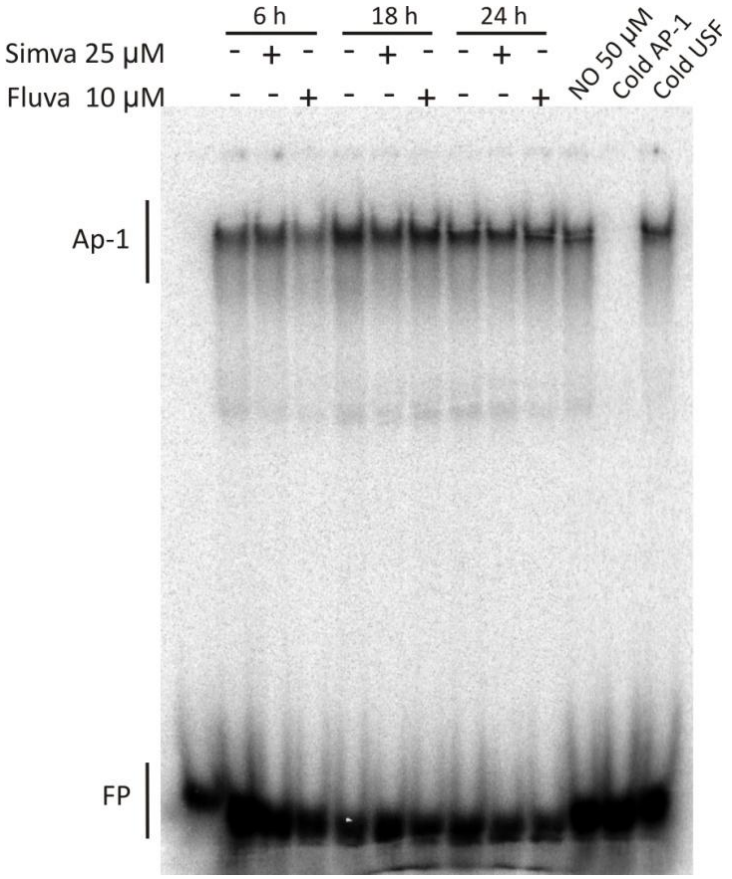

Figure 17: Electrophoretic mobility of consensus AP-1 DNA complexes in response to treatment with 25 μM simvastatin or 10 μM fluvastatin. *In vitro* nuclear protein-DNA binding was analyzed using mouse [^{32}P]-labeled consensus AP-1 probes. An excess of unlabeled AP-1DNA strand was used as competitor. Free probe and reaction mixtures were electrophoresed in a 6% polyacrylamide gel, then dried and exposed to phosphoscreen. The result is representative of 2 experiments.

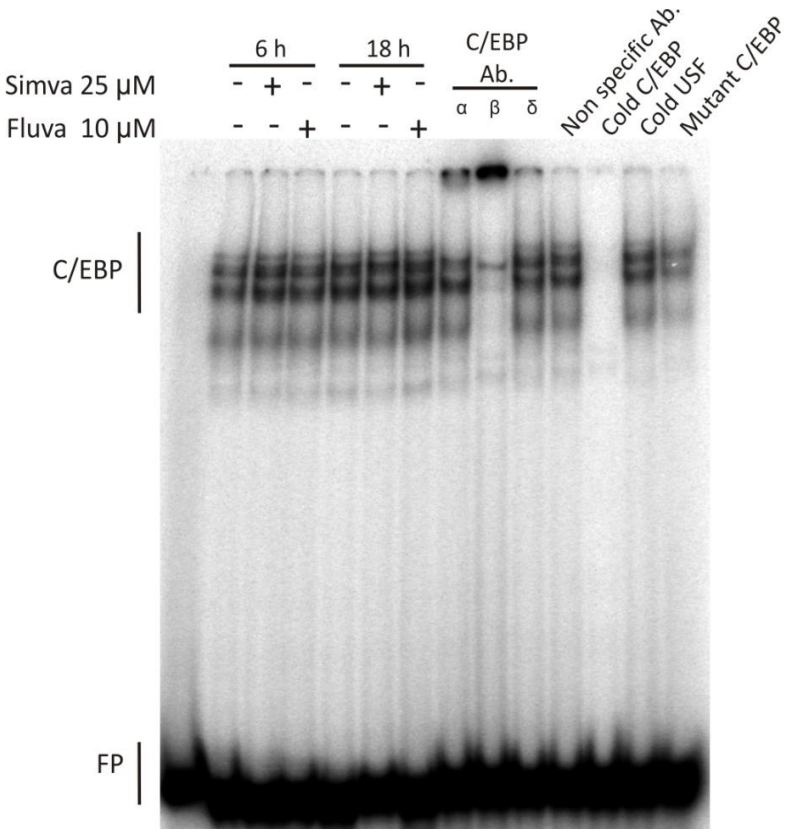

Figure 18: Electrophoretic mobility of HO-1 C/EBP DNA complexes in response to treatment with 25 µM simvastatin or 10 µM fluvastatin**.** *In vitro* nuclear protein-DNA binding was analyzed using mouse [^{32}P]-labeled HO-1 C/EBP probes. An excess of unlabeled C/EBP DNA strand was used as competitor. For supershift analysis, polyclonal rabbit anti-mouse IgGs specific for C/EBP (α, β, or δ) were added to nuclear protein, treated with simvastatin 18 hours, 30 min prior to the addition of the radioactive probe. Free probe and reaction mixtures were electrophoresed in a 6% polyacrylamide gel, then dried and exposed to phosphoscreen. The result is representative of 3 experiments.

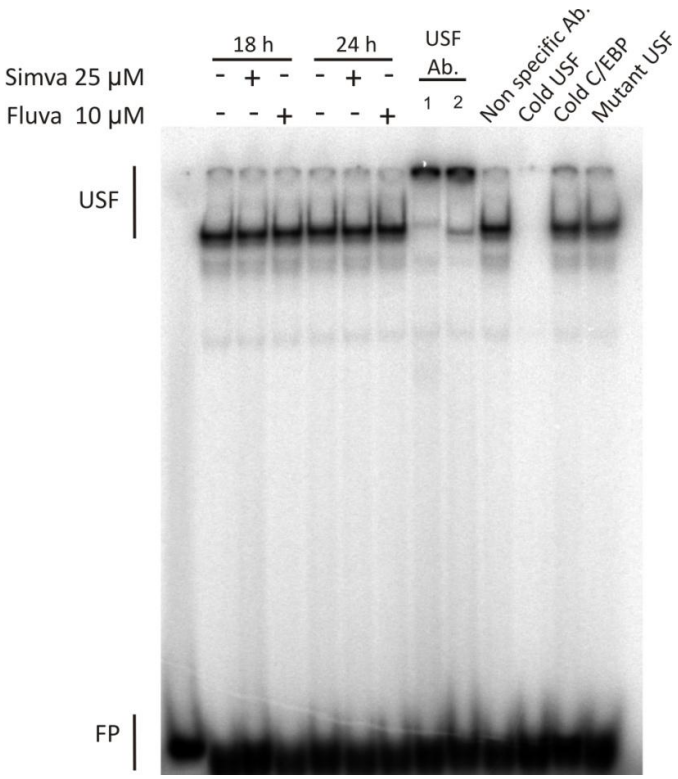

Figure 19: Electrophoretic mobility of HO-1 USF DNA complexes in response to treatment with 25 µM simvastatin or 10 µM fluvastatin**.** *In vitro* nuclear protein-DNA binding was analyzed using mouse [^{32}P]-labeled HO-1 USF probes. An excess of unlabeled USF DNA strand was used as competitor. For supershift analysis, polyclonal rabbit anti-mouse IgGs specific for USF (1 or 2) were added to nuclear protein, treated with simvastatin 18 hrs, 30 min prior to the addition of the radioactive probe. Free probe and reaction mixtures were electrophoresed in a 6% polyacrylamide gel, then dried and exposed to phosphoscreen. The result is representative of 4 experiments.

D. Effect of statins over C2C12 Differentiation

There are specific genes that express during C2C12 differentiation from myocytes to myotubes, and are used as differentiation markers. Among those, the Myogenic Transcription Factor MyoD and the muscle-specific filament proteins desmin are studied to assess C2C12 cell differentiation. We investigated the effect of statins on differentiation of C2C12 cell. Differentiated or undifferentiated cells were incubated for 24 hrs with 25 μM simvastatin or 10 μM fluvastatin. RT-PCR was performed to amplify differentiation markers genes MyoD and desmin as well as HO-1 and normalized to β-actin. Figure 20 shows that in undifferentiated C2C12 cells and due to the incubation by statins, the expression of MyoD was less compared to the control sample. On the other hand, fully-differentiated C2C12 cells showed no difference in the expression of MyoD in treated and control.

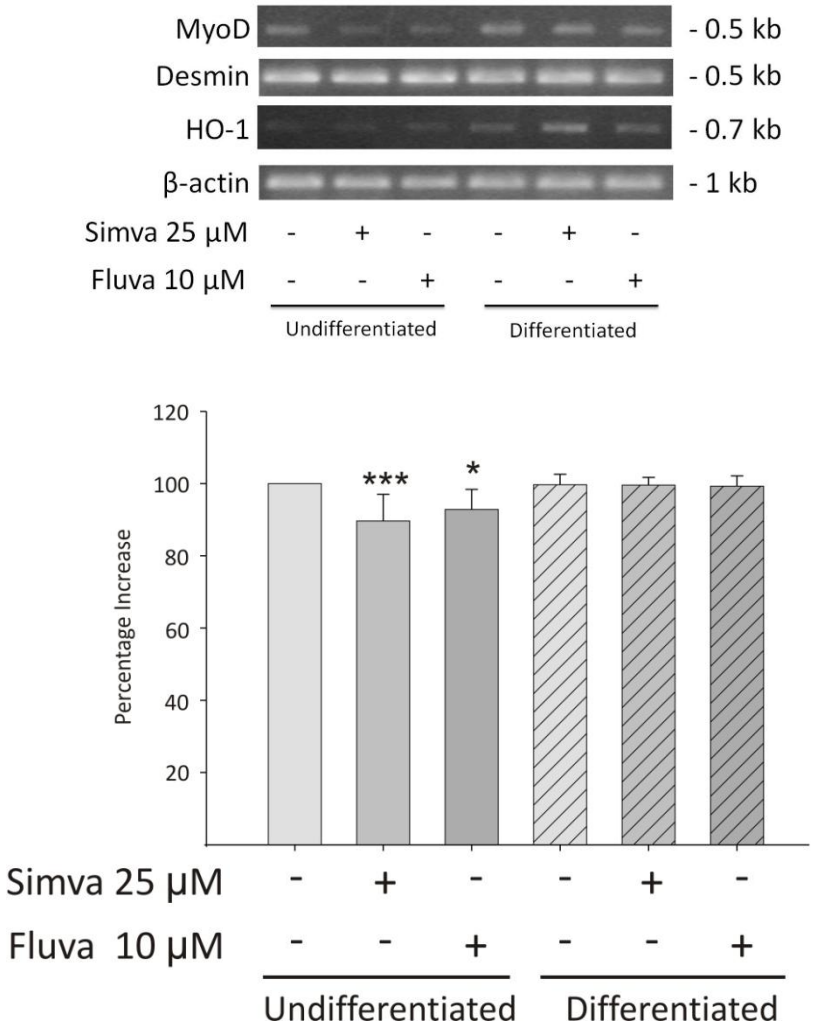

Figure 20: Effect of simvastatin or fluvastatin on the mRNA level of differentiation markers as assessed by RT-PCR. Differentiated or undifferentiated C2C12 cells in 60 mm dishes were treated for 24 hours with fluvastatin (10 µM) or simvastatin (25 µM). RNA extraction and RT-PCR was performed as describes in legend of figure 12. Results represent 3 experiments. Densitometric analysis-shown to the bottom- is the ratio of MyoD to β-actin of the PCR. Data are mean ± s.e.m. of 3 separate experiments with similar results. ***$p<0.001$ versus control, *$p<0.05$ versus control.

Chapter IV

Discussion

Statins are potent cholesterol lowering drugs. They are inhibitors of HMG-CoA reductase enzyme, a key and rate limiting enzyme in the cholesterol biosynthesis (mevalonate) pathway (Figure 3) (62). Aside to their cholesterol lowering effects, statins showed additional beneficial effects related to the inhibition of isoprenoids synthesis (FPP and GGPP). Isoprenoids are important lipid moieties involved in post-translational modification of proteins. HO-1 is largely described as cytoprotective in most cells (69). We addressed the effect of statins on HO-1 expression in skeletal cells. For this purpose we used differentiated skeletal cells, C2C12, and assessed the effect of statins on HO-1 expression and the mechanisms underlying this regulation.

In a previous study done in our lab, Attar et *al.* (104) have optimized the conditions by which HO-1 is induced in C2C12 cell line. They further showed that simvastatin induces HO-1 protein expression, which was augmented by the NO. We have pursued this study and confirmed the induction of HO-1 by simvastatin rather than fluvastatin. This was confirmed at the protein level, both by western blot analyses and immunofluoresence detection of HO-1 in the cells.

The induction of HO-1 by simvastatin, which shows *in vivo* a higher risk of rhabdomyolysis than fluvastatin, might play a role in the reduction of hemolysis. Future studies on the effect of statins on the viability of cells in the absence of HO-1 activity will be addressed.

In some previous studies the induction of HO-1 was shown to be dependent on the statins-inhibition of mevalonate pathway. Pretreatment of cells with mevalonate prior to the addition of simvastatin prevented HO-1 induction confirming that the effect of statins involved mevalonate synthesis. This suggested that statins, by their inhibition of mevalonate pathway, decrease the cellular production of isoprenoids, so abolishing the repressor effect of the isoprenylated protein from HO-1. However, there are some examples where statins exert their function independent to mevalonate pathway. Fujii et *al.* showed that simvastatin inhibits lymphocyte function-associated molecules 1 (LFA-1) through a mevalonate independent pathway (107). Moreover, Grosser et *al.* showed that in human umbilical vein endothelial cells (ECV 304) simvastatin induced HO-1 expression independent of mevalonate pathway (108).

Lee et *al.* (100) were the first to demonstrate that simvastatin induces HO-1 expression in *in vitro* study in aortic vascular smooth muscle cells. In macrophages, both simvastatin and fluvastatin induce HO-1 at the protein and mRNA level (109).

To further analyze we checked the isoprenylation type involved in this modification. Two isoprenylation inhibitors were used; FTI-277 and GGTI-286, inhibitors of Farnesyl transferase and geranylgeranyl transferase, respectively. Our results show that GGTI-286, but not FTI-277, significantly induced HO-1 protein expression. The induction of HO-1 was observed at the mRNA level of GGTI-286 treated cells. These observations suggest that geranylgeranylated proteins inhibits basal HO-1 expression in the cells and that the suppression of these proteins by either statins or specific inhibitors such as GGTI-286 results in the induction of the protein. Since members of the small G protein family of

Rho are geranylgrenaylated and were shown to be involved in gene expression, we analyzed the role of Rho A/C and Rac in HO-1 expression in differenmtiated C2C12 cells. We used selective blockers of RhoA/C and Rac.

NSC23766 is a specific inhibitor of Rac1 GDP/GTP exchange stimulated by GEF (Guanine nucleotide exchange factor). It showed a significant induction of HO-1 expression after 24 hours treatment (Figure 15). Taking these observations together lead us to conclude that RhoA/C through its associated protein (ROCK) and Rac 1 protein repress the expression of HO-1 in resting conditions, and statins could overcome this repression by decreasing the levels of GGPP.

Our results show that both RhoA/C and Rac are involved in the regulation of HO-1 expression. TAT/C3 exoenzyme that is a selective inhibitor of geranylgeranylated Rho A/C by ribosylation (106) and NSC23766 is a specific inhibitor of Rac1 GDP/GTP exchange stimulated by GEF (Guanine nucleotide exchange factor) induced HO-1 expression ROCK (Rho Kinase) is a Rho-associated coiled-coil forming protein with serine/threonine kinase activity. It is activated by RhoA/C to phosphorylate many proteins and control a broad range of cellular functions, such as smooth muscle contraction and proliferation, angiogenesis and gene expression (110, 111). Y-27632, specific inhibitor of ROCK, showed a significant induction of HO-1 expression in differentiated C2C12 cells. (Figure 13).

Taking these observations together lead us to conclude that RhoA/C through its associated protein (ROCK) and Rac 1 protein repress the expression of HO-1 in resting conditions, and statins could overcome this repression by decreasing the levels of GGPP.

In an attempt to analyze transcriptional regulation of HO-1 by statins in C2C12 cells, we analyzed the activation of some of the transcription factors by statins in C2C12 cells. We investigated the role of three transcription factors AP-1, C/EBP and USF in statin-dependent induction of HO-1. Gel retardation analyses using sequences derived HO-1 promoter showed a major role of C/EBPα and β but not USF or AP-1. Studies have shown a role of AP-1 and USF in HO-1 induction. In NIH3T3 cells, it has been shown that statins activate C/EBP and USF-1 in a mevalonate dependent manner whereas only C/EBP was activated in macrophages (14).

Recently, Kozakowska et *al.* (unpublished data) investigated the effect of HO-1 over the differentiation of C2C12 cells. We investigated the effect of statins, due to their HO-1 induction, over the differentiation of C2C12 cell by exploring the expression of two differentiation markers in C2C12, MyoD and desmin. In our conditions we noticed that statins decrease the expression of MyoD in undifferentiated C2C12 cells. On the other hand, there was no evident modification in the level of MyoD expression in the fully differentiated C2C12 cells. This could be an index to the cytotoxicity after statins treatment. No modification was observed over the expression level of desmin gene.

In summary, we inspected the mechanism of HO-1 induction due to statin in C2C12 cells. Our results propose that simvastatin releases the repression of RhoA/C and/or

Rac 1 proteins, or other geranylgeranylated proteins, from HO-1. This release leads to the induction of HO-1 that involves a strong transcriptional activity of AP-1 and C/EBP β but not USF.

As any study, there were many limitations for this study. It's an *in vitro* model by using a myoblast cell line C2C12. The differentiation of these myoblast into myotubes contributed to an additional limitation, due to the fact that there would be variability by the differentiation efficiency of the cells. Differentiated cells didn't tolerate a long period incubation with the treatments, especially treatment by statins. Semi-quantitative PCR technique was also a limitation since it took a lot of optimization among the primers used. Finally, we faced many problems in the band quantification software that we used, and it was the only available software in our lab.

In future experiments, we recommend to assess the implication of transcription by the use of dominant negative vectors for specific transcription factors that are optimized in our lab, and the effect of statin over HO-1 enzyme activity in C2C12. Finally, we propose to establish an *in vivo* model to compare the effect of statins over mouse skeletal muscles.

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