

Role of Nitric Oxide and CCAAT/Enhancer-Binding Protein Transcription Factor in Statin-Dependent Induction of Heme Oxygenase-1 in Mouse Macrophages

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Abstract

The effect of statins on heme oxygenase-1 (HO-1) was compared in 2 murine cell lines, RAW 264.7 and J774A.1 cell lines, and in primary peritoneal macrophages of BALB/c or C57BL/6 mice. The role of endogenous nitric oxide and the type of transcription factors involved were explored. Simvastatin and fluvastatin induced HO-1. Pretreatment of cells with *l*-NMMA or 1400 W, two different nitric oxide synthase inhibitors, partially blocked statin-dependent induction of HO-1 in RAW 264.7 and J774A.1 but not in primary peritoneal macrophages. Induction of HO-1 by statins was dependent on p-38 MAP kinase activation in all types of macrophages. In RAW 264.7 cells, both statins increased the activity of reporter genes linked to the proximal 1.3 kbp promoter of HO-1 (EC_{50} of $1.4 \pm 0.3 \mu\text{M}$ for simvastatin and $0.6 \pm 0.03 \mu\text{M}$ for fluvastatin). This effect was significantly blocked by 1400 W ($80 \pm 5.2\%$ inhibition, $p < 0.02$) and mevalonate, the direct metabolite of HMGCoA reductase. Gel retardation experiments implicated C/EBP β , AP-1 but not USF, for both RAW 264.7 and primary peritoneal macrophages of C57BL/6 mice. Collectively we showed a differential role of endogenous nitric oxide between macrophage cell lines and primary macrophages and an effect of statins in the protection against inflammation by increasing HO-1 expression.

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Introduction

Statins are lipid-lowering agents that act as competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [1]. Studies have shown beneficial and protective effects of statins in reducing mortality and morbidity beyond their capacity to lower cholesterol. These pleiotropic effects vary from improvement of endothelial function to anti-thrombotic and anti-inflammatory properties [2]. In macrophages, it has been shown that statins decrease the production of proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF) α [3,4] and monocyte chemoattractant protein MCP-1 [5], expression of adhesion molecule, tissue factor [6] as well as cyclooxygenase-2 and prostaglandin synthesis [7]. Some of these beneficial effects, such as the improvement of endothelial function and anti-oxidant capacities were demonstrated *in vivo* [8]. Heme oxygenase-1 (HO-1) is the inducible isoform of heme oxygenase, a microsomal enzyme responsible for the oxidative degradation of heme. The resulting end products contribute to the anti-oxidant, anti-inflammatory and anti-apoptotic actions of HO [9,10]. Many inducers have been reported for HO-1 including, pro and anti-inflammatory cytokines [11], lipopolysaccharide (LPS) [12] and nitric oxide (NO) [13]. HO-1 has been shown to be a downstream

effector of IL-10 [14] and to play a role in the resolution of inflammation [15]. Stimulation of HO-1 expression by a large number of stimuli is regulated mainly at the transcriptional level which is controlled by many regulatory elements located in the proximal and distal sites of the HO-1 promoter gene 5' flanking region [16,17]. Controversial roles of endogenous NO in HO-1 expression in RAW 264.7 cells were described [18,19,20]. However the intracellular pathways involved in this regulation in primary cells were not investigated. We have recently reported a role of C/EBP β and δ and USF-1 and -2 in statin-dependent induction of HO-1 in NIH 3T3 fibroblasts [21]. Since macrophages are important players in inflammation and since statins were shown to have anti-inflammatory effects, we aimed to study the regulation of HO-1 and the mechanisms underlying this regulation in different types of macrophages, primary cells and murine cell lines, mainly by investigating the role of endogenous NO and the transcription factors involved.

Experimental Procedures

Materials

Cell culture media were from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) and lipofectamine 2000[®] were obtained from

Gibco (Invitrogen, New York, USA). Penicillin and streptomycin were purchased from Cambrex (Rockland, USA). Chemicals for electrophoresis and Bradford reagent were from Bio-Rad (Hercules, USA). Simvastatin, fluvastatin, FTI-277, GGTI 286, *l*-NMMA, 1400 W and SB203580 were from Calbiochem (San Diego, USA). Monoclonal anti- β actin, lipopolysaccharide 0111:B4, bovine serum albumin (BSA), *N*-(1-Naphthyl)- ethylenediamine dihydrochloride 98%, sulphanilamide and sodium nitrite were from Sigma Aldrich (St. Louis, USA). Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were from MP Biomedicals (Costa Mesa, CA, USA). Spermine NONOate (SPNO) was from Cayman chemicals (Ann Arbor, USA). Enhanced chemiluminescence (ECL) was from Amersham (General Electric Healthcare Life Sciences, USA) and Roche (Mannheim, Germany). Fugene 6[®] was from Roche. Modified Thioglycollate Medium Brewer was obtained from Becton Dickinson (Sparks, USA). Donkey anti-mouse or anti-rabbit antibodies conjugated to peroxidase were from Jackson Immuno-research Laboratories (Pennsylvania, USA). Rabbit polyclonal antibodies against C/EBP α (14AA), C/EBP β (C-19) and C/EBP δ (C-22) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). C3 exoenzyme and GST-cytonecrotic factor -1 were kind gifts of Dr. Jacques Bertoglio (INSERM U 749, Institut Gustave Roussy, Villejuif, France). DNA oligonucleotides were obtained from TIB Molbiol (Berlin, Germany). ELISA assays for murine IL-6 and TNF α were from eBioscience (San Diego, CA). Sn-protoporphyrin was from Frontier Scientific Inc. (Logan, UT, USA). HO-1 promoter regions (1.3 kbp and 15 kbp-luciferase constructs) were developed as previously described [17,22]. All other chemical reagents were purchased from Amresco (Solon, USA).

Cells

RAW 264.7 and J774A.1 cells were obtained from ATCC (Manassas, VA, USA). Elicited mouse peritoneal macrophages (eMPM) were harvested from BALB/c or C57BL/6 mice. Approval for use of animals was obtained from the Institutional Animal Care and Use Committee of the American University of Beirut (permit # 06-10-026). Mice were injected i.p. with 1 ml of sterile 3.85% thioglycollate broth for 48 to 72 hours and the peritoneal cavity was washed with 10 ml of RPMI 1640 culture medium containing 2% FBS. Macrophages were allowed to adhere for 2 hours and washed twice before treatment.

Cell Culture

Murine RAW 264.7 and J774A.1 cells were grown in DMEM culture medium supplemented with 20 mM HEPES buffer (pH 7), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, at 37°C in 10% CO₂. For treatment, cells were cultured in 12-well plates for western blot analysis (1 \times 10⁶ cell/well) or transfection experiments (0.5 \times 10⁶ cell/well), and in 60 mm dishes for electrophoretic mobility shift assay. Subconfluent cells were treated in serum-free culture medium alone or in the presence of simvastatin or fluvastatin for 24 hours and various concentrations of LPS. In some experiments, NO synthase inhibitors, *l*-NMMA (1 mM) or 1400 W (10 μ M), or p38 MAP kinase inhibitor, SB203580, (10 μ M) were added 30 minutes prior to the addition of statins. The concentration of DMSO did not exceed 0.2%. The cellular toxicity by statins was evaluated by neutral red assay [23] and did not exceed 10%. At the end of treatment, supernatants of cell culture were kept at -70°C until IL-6, TNF α and nitrite were measured.

Western Blot Analysis

At the end of the treatment, cells were washed with phosphate-buffer saline and lysed in 300 μ l of lysis buffer (20 mM Tris/HCl, pH 8, containing 1 mM EDTA, 1% Triton and protease inhibitor cocktail). Protein content was determined using the Bradford Protein Assay with BSA as standard. Immunoblotting was performed as described previously [24] using 20 μ g of total protein. Blotting was performed using specific polyclonal antibodies against HO-1 (1/2000), HO-2 (1/1000) [24], inducible NOS (1/1000) [25] and monoclonal antibody against β -actin (1/2000) (Sigma-Aldrich) and immunoblotting was carried out as previously described [21].

Determination of Nitrite Concentration

Nitrite concentrations were determined according to the Griess method with sodium nitrite used as a standard [26]. The optical density was immediately measured at 540 nm on a 96-well plate reader (Multiskan EX, Thermo Electron). Nitrite concentrations were obtained in μ M.

Measurement of IL-6 and TNF α Concentrations

Mouse IL-6 and TNF α were measured in the supernatants of cell culture according to the manufacturer's instruction (eBioscience, San Diego, CA). Results were expressed in pg/ml.

Electrophoretic Mobility Shift Assay

The sequences of the primers used to prepare the double strand DNA probe were reported earlier and the experiments were performed as previously described [21]. Briefly, 5 μ g of nuclear extract and 5 \times 10⁴ cpm of the desired probe were allowed to bind in 20 mM Tris/HCl, pH 8, containing 120 mM KCl, 25 mM MgCl₂, 2 mM EDTA, 25% Glycerol and 2 mM DTT for 30 min on ice. For supershift experiments, antibodies were incubated with the nuclear extracts for 30 minutes on ice before the addition of the labelled probe. Binding activities were measured by Storm 860[®] phosphorImager.

Reporter Assay

RAW 264.7 cells were seeded at 0.5 \times 10⁵ cells/well in 12-well plates one day before transfection. pHO-1-1.3luc or pHO-1-15luc gene promoter vectors were transiently transfected by FuGene 6[®] (Roche) according to the manufacturer's instructions. Cells were treated 24 hours post-transfection for an additional 24 hours with different concentrations of statins. In some experiments, cells were incubated for 30 minutes with 10 μ M 1400 W prior to the addition of statins. Luciferase activity was evaluated as described in [21]. Results were normalized to the protein concentration. Fold activation was calculated by dividing the corrected values of treated cells with the control value, which was attributed a value of 1.

Data Analysis

Autoradiograms obtained from western blot analyses were scanned using Epson 1680 pro scanner and densitometric analysis was performed using Scion NIH software (Scion Corp., Frederick MD). Results are shown as average \pm S.E.M. Data was analyzed by student's *t*-test (Sigma Stat[®], Systat Software, Inc., San Jose, CA, USA).

Results

Effect of Statins on HO-1 Expression in Murine Macrophages

We first checked that statins induced HO-1 in RAW 264.7 cells. Treatment of murine macrophage cell line RAW 264.7 for 24 hours with different concentrations of simvastatin (1, 5 and 25 μ M) or fluvastatin (0.4, 2 and 10 μ M) increased HO-1 expression (Figure 1A). Induction of HO-1 was slightly detectable at 5 μ M simvastatin and 2 μ M fluvastatin; maximal statistically significant induction was observed with 25 μ M simvastatin and 10 μ M fluvastatin (2.9 ± 0.5 and 2.3 ± 0.3 fold of HO-1 expression of control, mean \pm S.E.M., $n = 4$, $p < 0.005$ and $p < 0.01$, t -test, for simvastatin and fluvastatin, respectively). We next examined the effect of co-treatment of macrophages with statins and lipopolysaccharide (LPS). LPS increased HO-1 expression in a dose-dependent manner (Figure 1). Treatment of cells with simvastatin (Figure 1B) or fluvastatin (Figure 1C) further amplified this expression. Strong additive effects between statins and LPS were evident at low concentrations of LPS, i.e. 4 and 20 ng/ml. We next checked the intermediates of the mevalonate pathway. SpermineNONOate or SPNO, a strong NO donor, was used as a control for testing the effect of isoprenoid derivatives. Induction of HO-1 by simvastatin+SPNO was abrogated after treatment with 10 μ M geranylgeranyl-pyrophosphate but not with farnesyl-pyrophosphate (Figure 2A). Moreover, geranylgeranyltransferase inhibitor alone, GGTI-286 but not farnesyltransferase inhibitor, FTI-277, increased SPNO-induced HO-1 expression in RAW 264.7 cells suggesting a role of geranylgeranylated proteins in modulating HO-1 expression (Figure 2B). Inactivation of Rho A/C by C3- exoenzyme induced expression of HO-1 (Figure 2C) whereas activation of Rho members by cytonecrotic factor (CNF)-1 showed a decrease in HO-1 expression in the presence of statins (Figure 2C).

We next investigated the role of endogenous NO formation in statin-dependent induction of HO-1 in different macrophages. We first analyzed the formation of nitrite, one of the breakdown products of NO, in response to statins. Simvastatin and fluvastatin were able to increase nitrite release in RAW 264.7 cells (10 ± 0.6 μ mol/L for basal untreated cells compared to 20.9 ± 0.7 μ mol/L for simvastatin and 19.1 ± 0.5 μ mol/L for fluvastatin, $n = 23$) and to induce iNOS protein expression with a fold increase over control of 1.5 ± 0.2 for simvastatin and 1.4 ± 0.1 for fluvastatin ($n = 3-4$, $p < 0.05$, t -test) (Figure 3B). We studied in parallel the effect of statins on HO-1 expression in J774A.1 cells, another murine macrophage-like cell line. Similar findings to Raw 264.7 cells were obtained in statin and iNOS induction; statins increased nitrite release (5.2 ± 0.6 μ mol/L for basal untreated cells compared to 8.5 ± 0.3 μ mol/L for simvastatin and 8.2 ± 0.2 μ mol/L for fluvastatin, $n = 13$) (Figure 3A) (1.5 fold induction of control, $n = 2$). To analyze the role of endogenous NO in statin-dependent induction of HO-1, we pretreated RAW 264.7 cells for 30 minutes with *l*-NMMA, a broad inhibitor of NO synthases or 1400 W, a more selective inhibitor of iNOS, prior to the addition of simvastatin. Figure 3C shows a significant reduction in HO-1 expression ($35 \pm 4.7\%$ for 1400 W and $64 \pm 3.2\%$ for *l*-NMMA, ($n = 3$, $p < 0.01$ and $p < 0.001$, t -test, for 1400 W and *l*-NMMA, respectively) (Figure 3C). In these cells, *l*-NMMA and 1400 W blocked significantly nitrite formation in response to LPS ($85 \pm 2\%$ inhibition, $n = 5$ and $60.2 \pm 4.5\%$ inhibition, $n = 4$, respectively). We performed similar experiments in primary murine elicited macrophages. No nitrite was detected by the Griess measurement method in eMPM from BALB/c mice in

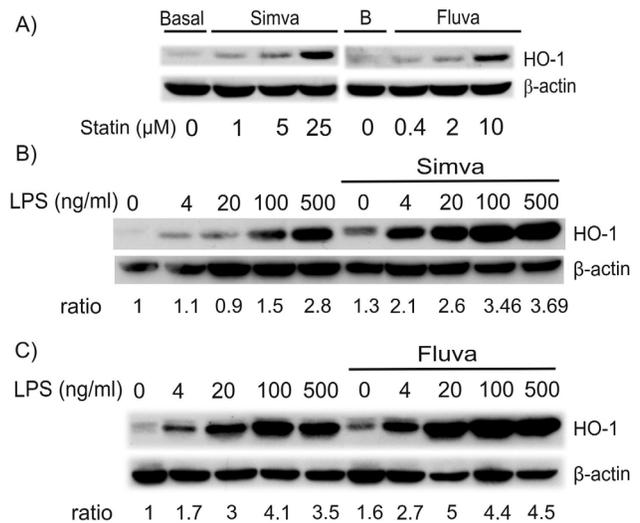


Figure 1. HO-1 induction by statins in RAW 264.7 cells. Cells were incubated for 24 hours with simvastatin or fluvastatin. 20 μ g of total proteins were loaded on a 12% SDS polyacrylamide gel and blotted for HO-1 and then stripped and blotted for β -actin. HO-1 band intensities were measured by densitometry and normalized to the intensity of β -actin bands. Fold increase was calculated as a ratio of the result of stimulated cells to that of the control cells. (A) Dose-response of simvastatin or fluvastatin in RAW 264.7 cells. The western blot is representative of 4 experiments with similar results. B corresponds to basal untreated cells. (B & C) Effect of statins on LPS induced HO-1 expression. RAW 264.7 cells were incubated for 24 hours with increasing concentrations of LPS alone or in the presence of 25 μ M simvastatin (B) or 10 μ M fluvastatin (C). Results are representative of 3 experiments. doi:10.1371/journal.pone.0064092.g001

response to statins or LPS. Treatment of these cells with *l*-NMMA did not modify HO-1 induction in response to simvastatin (Figure 3D). Since macrophages from BALB/c are Th-2 responder cells with a major production of ornithine from *l*-arginine [27], we checked peritoneal macrophages from Th-1 responder mouse strain C57BL/6, which produce NO when activated by LPS. In these cells, treatment with LPS resulted in a significant increase of nitrite ($2-10$ μ M, $n = 3$) compared to undetectable levels in untreated macrophages. Pretreatment with *l*-NMMA did not modify HO-1 induction in response to simvastatin although it blocked significantly nitrite formation ($88 \pm 3.2\%$ inhibition, $n = 3$, of LPS treated cells) (Figure 3D).

Since p38 MAP kinase was shown to play an important role in HO-1 induction in response to different inducers [28] and since statins were shown to modulate p38 MAP kinase, we compared the involvement of p38 MAP kinase activity among the different macrophages. Treatment of RAW 264.7 cells with simvastatin increased p38 MAP kinase with a peak after 1 hour treatment (Figure 4A) whereas 10 μ M SB203580, a p38 MAP kinase inhibitor, decreased significantly simvastatin-dependent induction of HO-1 in all three macrophages models providing evidence for a role of p38 MAP kinase in HO-1 expression in all macrophages primary and cell lines (Figure 4B).

Effect of Statins on Cytokines Release in eMPM

IL-6 and TNF α concentrations were measured in the supernatant of eMPM cells prepared from C57BL/6. Treatment of cells with 100 ng/ml LPS for 24 hours resulted in an increase in cytokines production in cells (11540 ± 3210 pg/ml of IL-6 ($n = 5$) and 84350 pg/ml of TNF α ($n = 2$) compared to undetectable levels in controls). Pretreatment of cells with

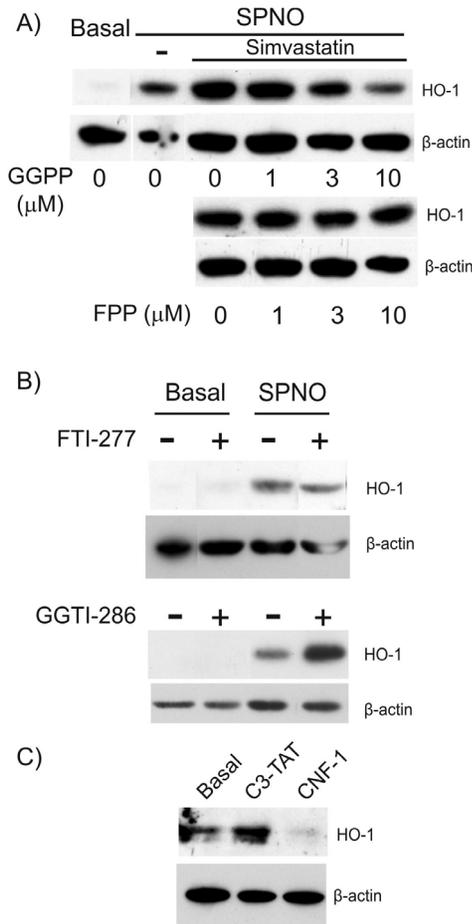


Figure 2. Role of prenylation in simvastatin-dependent induction of HO-1. (A) RAW 264.7 cells were co-treated with 25 μ M simvastatin and increasing concentrations of GGPP and FPP for 24 hours in the presence of 125 μ M SPNO. Western blotting of HO-1 was performed as described in the legend for figure 1. Results are representative of 3 experiments for GGPP and 2 for FPP. (B) RAW 264.7 cells were incubated with 10 μ M of GGTI-286 or FTI-277 for 24 hours. Results are representative of 3 experiments. (C) RAW 264.7 cells were treated with 5 μ g/ml of C3-exoenzyme and 1 μ g/ml of CNF-1 for 24 hours. Results are representative of 3 experiments with similar results.
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25 μ M simvastatin reduced IL-6 production by 40 \pm 6% (n = 5) and TNF α by 50% \pm 3% (n = 3). Sn-protoporphyrin (50 μ M) inhibited LPS-dependent IL-6 by 81 \pm 3% (n = 4) but not TNF α synthesis and reversed the inhibition observed for TNF α +simvastatin but not IL-6+ simvastatin (96% \pm 5%, n = 3).

Transcriptional Regulation of HO-1 Promoter Gene by Statins

Previous studies have shown in RAW 264.7 an ability of statins to induce AP-1 and to modulate NF κ B in macrophages [7,20]. We have previously shown that statins increase HO-1 promoter activity in NIH 3T3 cells [21]. To investigate whether HO-1 induction by statins involved transcriptional regulation in this more physiologically relevant model, we initially studied the effect of statins on HO-1 promoter activity in RAW 264.7 cells. Cells were transfected with expression vectors encoding the murine HO-1 promoter genes of 1.3 kbp and 15 kbp [16]. As shown in Figure 5A left panel, 25 μ M simvastatin increased the

1.3 kbp and the 15 kbp HO-1 promoter luciferase activity by 2.9 \pm 0.3 (n = 6, ***P*<0.001, paired *t*-test) and 3.2 \pm 0.4 (n = 3, **P*<0.01, paired *t*-test), compared to untreated cells, respectively. 10 μ M fluvastatin also increased reporter activity by 3.4 \pm 0.4 (n = 6, ***P*<0.001, paired *t*-test) and 2.4 \pm 0.2 (n = 3, **P*<0.01, paired *t*-test) for 1.3 kbp and the 15 kbp HO-1 promoters, respectively. Pretreatment of cells with 200 μ M mevalonate prior to statins addition resulted in an inhibition of statin-dependent 1.3 kbp HO-1 promoter activity by more than 68 \pm 6.6% (n = 3, *p*<0.01, *t*-test). We further assessed the role of endogenous NO in the promoter activity of HO-1. We inhibited endogenous NO synthesis with 1400 W, the more selective inhibitor of iNOS, despite the strong inhibition of HO-1 protein induction by *l*-NMMA. Figure 5A, right panel, shows that pretreatment of cells with 10 μ M of 1400 W significantly reduced simvastatin-dependent HO-1 promoter activity by 80 \pm 5.2% (n = 3, *p*<0.02, *t*-test). 1400 W alone had no effect on the promoter activity. 1.3 kbp HO-1 promoter activities were increased dose-dependently by both statins with significant increase at concentrations as low as 1 μ M of simvastatin or fluvastatin with an IC₅₀ of 1.4 \pm 0.3 μ M for simvastatin (Figure 5B, left Panel) and 0.6 \pm 0.03 μ M for fluvastatin (Figure 5B, right Panel). We have previously shown that statins activation of HO-1 involved C/EBP β and δ as well as USF-1 and USF-2. To determine the transcription factors involved in the response to statins, we performed gel retardation assays in RAW 264.7 cells and eMPM of C57BL/6. Cells were treated with 25 μ M simvastatin or 10 μ M fluvastatin for 6, 12 or 24 hours and nuclear proteins were subjected to gel retardation assay after incubation with double-stranded (ds) DNA sequences for (HO-1) C/EBP, AP-1 or USF. As shown in figures 6A for RAW 264.7 and 6B for eMPM of C57BL/6, both statins induced protein- DNA complexes for C/EBP binding sequence compared to control at 24 hours. The complex was competed with an excess of cold ds-DNA C/EBP but not with an excess of ds-DNA GATA or USF. Antibodies against C/EBP β but not against C/EBP α or δ were able to cause a supershift in the C/EBP-protein complex suggesting a role for the β isoform in the promoter activation (Figure 6). Significant binding was also obtained in response to statins at 12- and 24- hours treatment and this binding was competed with an excess of cold AP-1 but not with an excess of the double-stranded oligonucleotides containing the GATA or USF recognition sequence (Figure 6C for RAW 264.7 and Figure 6D for macrophages from C57BL/6). In these cells, gel retardation assays for USF showed a strong basal signal of DNA binding in untreated cells which was not modified upon treatment with statins at different periods of time (n = 3, data not shown).

Discussion

In this study, we compared the effect of statins on HO-1 expression in 2 murine macrophages cell lines, RAW 264.7 and J774A.1, and in primary macrophages. Because NO is an important cellular mediator in macrophages induced in response to inflammation and since NO induces HO-1 gene, we investigated its role in statin-dependent induction of HO-1. We showed that NO synthase plays a role in some types of macrophages. We first observed an important additive effect between low concentration of LPS and statins on HO-1 expression for all types of macrophages, cell lines and primary macrophages. The results in RAW 264.7 cells are consistent with the data by Leung et al [18]. Since LPS increased NO in a large majority of cells, we investigated blocking endogenous NO synthase activity on HO-1

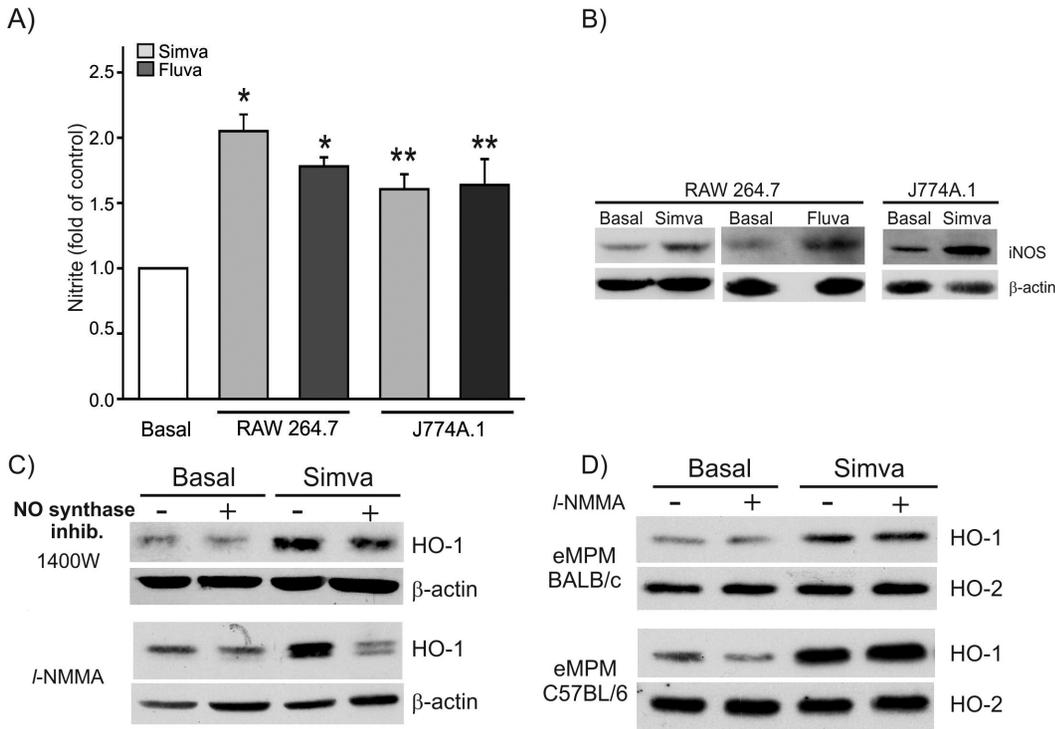


Figure 3. Role of NO and NO activity in statin-dependent induction of HO-1. (A) Nitrite formation in response to statins. RAW 264.7 or J774A.1 cells were incubated for 24 hours with 25 μ M simvastatin or 10 μ M fluvastatin. Nitrite was measured in the supernatant by the Griess method. Results correspond to mean \pm S.E.M of the fold increase of nitrite release in treated RAW 264.7 (n=17, *p<0.001, t-test) or J774A.1 cells (n=10–12, **p<0.02, t-test) compared to untreated basal cells. (B) iNOS induction by statins. 40 μ g of total proteins were loaded on a 7% SDS polyacrylamide gel and blotted for iNOS or β -actin. Results are representative of 2–3 experiments. (C) Effect of 2 NO synthase inhibitors on HO-1 expression in RAW 264.7 cells. Cells were pre-treated for 30 minutes with 10 μ M 1400 W or 1 mM l-NMMA prior to the addition of 25 μ M simvastatin for 24 hours. Results are representative of 3 experiments. (D) Effect of l-NMMA on HO-1 expression in eMPM cells. eMPM were prepared from BALB/c or C57BL/6 mice and treated as mentioned in the previous paragraph. Results are representative of 3 experiments for eMPM from BALB/c and 2 from C57BL/6.

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expression. Our results show that endogenous NO is partially responsible for the statin-dependent upregulation of HO-1 protein in RAW 264.7 and J774A.1 cell lines. We also observed a significant increase in nitrite formation and iNOS expression in response to LPS and the results suggested that NO is a player in the intracellular signal transduction pathways leading to statin-dependent induction of HO-1 in these macrophage cell lines. In RAW 264.7 cells, this result was confirmed by the effect of statins on HO-1 promoter activity. However, in primary peritoneal macrophages, a different role of NO was observed where statin increased HO-1 expression in a NO synthase-independent manner. This was obtained in primary peritoneal macrophages from BALB/c mice that did not produce endogenous NO probably due to a predominant arginase activity in these mice [27], and in macrophages from C57BL/6 mice that have the capacity to produce NO. These observations suggested different mechanisms of regulation of HO-1, which might be due to macrophage subsets and types, the mice models and cell lines [29]. Our results on the role of endogenous NO in statin-dependent HO-1 expression show a different intracellular signaling between macrophages. Although primary macrophages could constitute a better model for murine macrophages than the cell lines RAW 264.7 and J774.1A, a lot of scientists still rely on these cell lines as models of cultured macrophages. Both cell lines were initially prepared from BALB/c mice but reports showed clearly that these

cells maintained their capacity to produce NO in response to LPS [30].

In this study, we also investigated the role of p38 MAP kinases in response to statins. p38 MAP kinases were shown to play a central role in HO-1 and in C/EBP induction by many cell regulators [31,32]. Moreover, statins activate p38 MAP kinase [28]. We showed in the present study that inhibition of p38 MAP kinases decreased HO-1 expression in response to statins in all macrophages whereas intracellular NO is not involved in primary eMPM from BALB/c and C57BL/6 mice. This suggested both common and divergent pathways between the different macrophages for the regulation of HO-1 expression in response to statins with possible interaction.

In the present study, we showed that inhibition of geranylgeranylation and not farnesylation, and more particularly RhoA/C is involved in HO-1 expression by statins unlike Chen et al who showed that both inhibition of farnesylation and geranylgeranylation played a role in the induction of HO-1 by statins [20]. We further demonstrated that RhoA/C is a potential target for statins in the regulation of HO-1 expression. Our results are different from the ones described by Grosser et al. [33] who showed that statin-dependent activation of HO-1 did not involve inhibition of HMG CoA reductase in ECV 304, a human cell line earlier described as an endothelial immortalized cell line and further characterized as a human bladder cancer cell line [34]. The mevalonate-independent

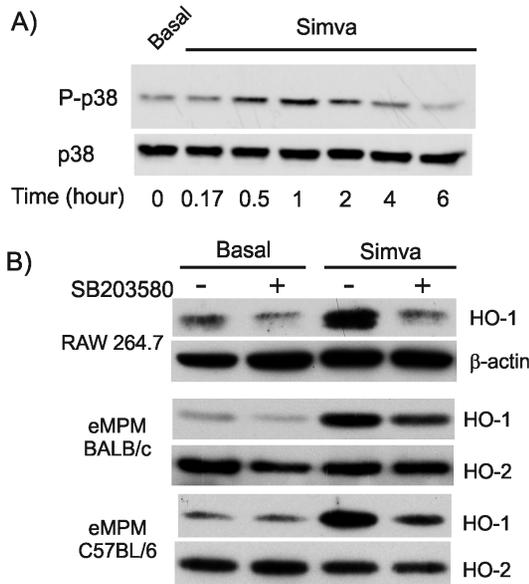


Figure 4. Implication of P38 MAP kinase pathway in statin-dependent induction of HO-1. (A) Phosphorylation of P38 by 25 μ M simvastatin at different times in RAW 264.7 cells. Results are representative of 3 experiments. (B) Effect of p38 MAP kinase inhibitor, SB203580 on HO-1 induction in RAW 264.7 and eMPM. Cells were pretreated for 30 minutes with 10 μ M of SB203580 prior to the addition of 25 μ M of simvastatin for 24 hours. Western blotting of HO-1 was performed as indicated earlier. Results are representative of 3 experiments.
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effect was probably due to the very high concentrations of statins (up to 300 μ M). However, Ali *et al.* have shown that the increase in HO-1 in human primary endothelial cells in response to statins was mevalonate-dependent [35]. The only significant effect of statin that was mevalonate-independent was by Weitz-Schmidt *et al.* who described a unique example where statin inhibits lymphocyte function-associated antigen-1 (LFA-1) interactions with adhesion molecules including ICAM-1 [36,37]. Our results converge with the mevalonate-dependent type of regulation of HO-1.

We also examined the transcriptional regulation of HO-1 by statins. Previous studies have shown an activation of AP-1 transcription factor in response to statins in RAW 264.7 cells [20] and checked the effect of statins on AP-1 promoter using truncated HO-1 promoters in ECV 304 cell line [33]. We have recently reported a role for C/EBP β and δ as well as USF-1 and -2 in statin-dependent induction of HO-1 in NIH 3T3 [21]. In the present study, we compared the role of C/EBP isoforms, AP-1 and USF in this more physiologically relevant system and tested for the first time their activation and demonstrated that only C/EBP β and AP-1 but not C/EBP δ or USF-1/-2 are activated in response to statin. Reporter analysis using 1.3 kbp HO-1 gene proximal promoter accounted for the majority of statin-dependent effect and its activity was strongly reduced in the presence of NO synthase inhibitor and mevalonate. Our results are not supportive of a role of the distal promoter as previously described by Grosser *et al.* in ECV 304 cell line [33]. Thus, regulation of the HO-1 promoter C/EBP in response to statins may differ among the different cell types.

In primary eMPM cells, we showed that simvastatin treatment resulted in reduced IL-6 and TNF α levels. Statin-

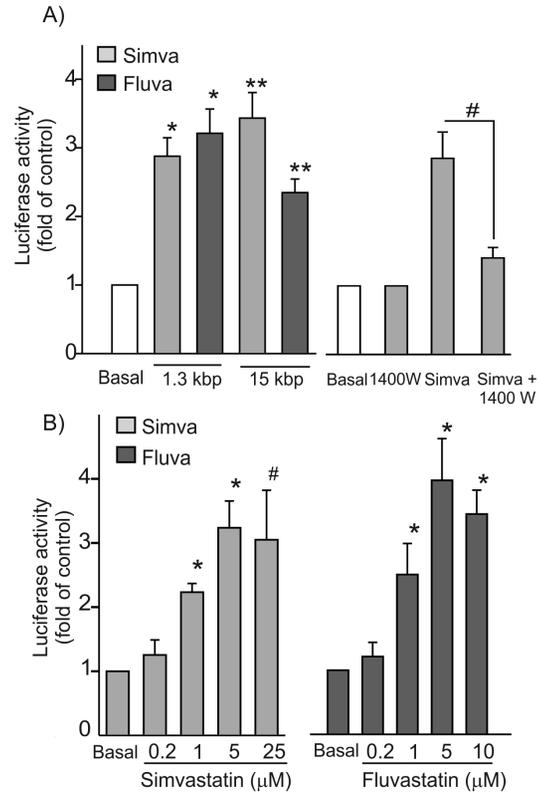


Figure 5. Regulation of HO-1 promoter activity by statins in RAW 264.7 cells. (A, left panel). Cells were transfected with 1.3 or 15 kbp HO-1 Luciferase gene promoter vectors and treated 24 hours later with 25 μ M simvastatin or 10 μ M fluvastatin for an additional 24 hours. Luciferase activity was measured and normalized to total protein and expressed as fold increase compared to untreated (control) cells attributed the value of 1. Results represent mean \pm S.E.M. of 3–6 different experiments. (A, right panel) Effect of 1400 W on the 1.3 kbp HO-1 Luciferase promoter activity by simvastatin in RAW 264.7 cells. Cells transfected with the 1.3 kbp HO-1 Luciferase promoter were incubated 24 hours post-transfection with 10 μ M 1400 W for 30 minutes prior to the treatment with 25 μ M simvastatin for 24 hours. Results represent 3 different experiments. (B) Dose-response of simvastatin and fluvastatin on 1.3 kbp HO-1 Luciferase promoter activity. Cells were transfected as described earlier and treated with different concentrations of statins for 24 hours. Results represent 3 different experiments.
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dependent inhibition of TNF α although modest was restored to normal using Sn-protoporphyrin, a selective inhibitor of HO-1 activity, suggesting an involvement of HO-1 in statin-dependent effect on TNF α formation. In macrophages/monocytes, numerous reports suggest a participation of HO-1 in the resolution of inflammation [15]. HO-1 is expressed in response to many proinflammatory cytokines and in many inflammatory settings [9,10,38]. Future investigation in *in vivo* models of inflammation will help confirm the protective anti-inflammatory role of HO-1 mediated by statins. Statins have been attributed many anti-inflammatory effects as part of their beneficial pleiotropic effects. This included decrease in reactive oxygen species, NADPH oxidase, matrix metalloproteinases, adhesion molecule expression and cytokine formation [39,40,41]. Very few studies addressed the regulation of HO-1 by statins in human macrophages. Recently, Gueler *et al.* described a role of statin-induced HO-1 in protection against kidney failure [42]. HO-1 is induced in atherosclerotic plaques [43] and in dendritic cells [44] and played a role in protection against injury.

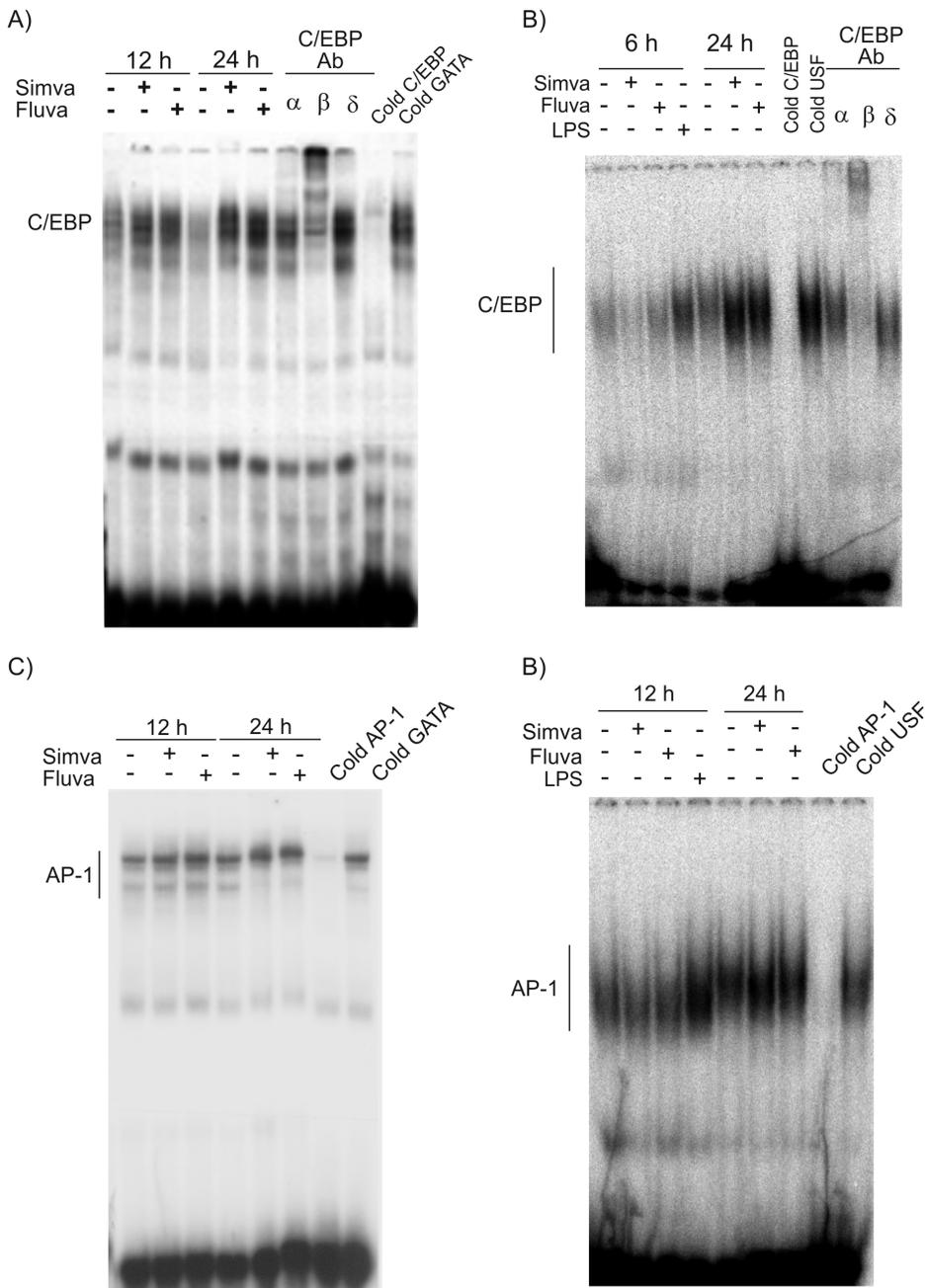


Figure 6. Transcriptional regulation of HO-1 by statins in mouse macrophages. Gel retardation analyses for C/EBP in (A) Raw 264.7 cells or (B) eMPM of C57BL/6 in response to statins. Cells were incubated with 25 μM simvastatin or 10 μM fluvastatin for 6, 12 or 24 hours. Nuclear extracts were prepared and incubated with labeled double stranded DNA C/EBP as described in Materials and Methods. DNA-protein complexes were detected after migration on non denaturing acrylamide. Storm PhosphorImager was used to detect radioactive bands. Competition was done with unlabeled C/EBP, GATA, or USF double-stranded DNA added in excess in 24 hour fluvastatin treated cells. For supershift experiments, nuclear extracts were incubated for 30 min in the absence or presence of 2 μg of C/EBPα, β or δ antibodies prior to the addition of the labeled probe. Results are representative of 3 different experiments with similar results showing a formation of C/EBP-β- protein complex. (C & D) Gel retardation analyses for AP-1-protein complex formation in response to statins at 12 and 24 hours in Raw 264.7 cells and eMPM of C57BL/6, respectively Competition was done with unlabeled AP-1, GATA or USF double-stranded DNA added in excess of 24 hours- fluvastatin treated cells. Results are representative of 3 different experiments with similar results. doi:10.1371/journal.pone.0064092.g006

Although the anti-inflammatory and anti-oxidant effects of statins in our study and others [45] were obtained at higher therapeutic plasma circulating dose, the regulatory effect was specifically targeting inhibition of HMG CoA reductase without toxicity. This was supported by the effect of mevalonate, the

direct product of HMG CoA reductase and the isoprenoid-derivatives, which reversed the induction of HO-1 by statins. Studies conducted *in vitro* and *in vivo* helped despite using high concentration of drugs to understand the mechanistic underlying

the protective effects of statins in patients with high risk of cardiovascular diseases and vascular inflammation [8].

In summary, we have compared the role of NO in statin-dependent induction of HO-1 in different models of macrophages. The mechanisms behind the different role of NO in statin induction of HO-1 will need future investigation. Our data suggest and implicate a strong transcriptional activity which involved C/EBP β and AP-1 but not USF-1/–2 or C/EBP δ in response to statins and support anti-inflammatory effects of statins and HO-1 induction.

References

- Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. *Nature* 343: 425–430.
- Liao JK (2005) Clinical implications for statin pleiotropy. *Curr Opin Lipidol* 16: 624–629.
- de Bont N, Netea MG, Rovers C, Smilde T, Demacker PN, et al. (1998) LPS-induced cytokine production and expression of LPS-receptors by peripheral blood mononuclear cells of patients with familial hypercholesterolemia and the effect of HMG-CoA reductase inhibitors. *Atherosclerosis* 139: 147–152.
- Pahan K, Sheikh FG, Nambodiri AM, Singh I (1997) Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest* 100: 2671–2679.
- Tanimoto A, Murata Y, Wang KY, Tsutsui M, Kohno K, et al. (2008) Monocyte chemoattractant protein-1 expression is enhanced by granulocyte-macrophage colony-stimulating factor via Jak2-Stat5 signaling and inhibited by atorvastatin in human monocytic U937 cells. *J Biol Chem* 283: 4643–4651.
- Tuomisto TT, Lumivuori H, Kansanen E, Hakkinen SK, Turunen MP, et al. (2008) Simvastatin has an anti-inflammatory effect on macrophages via upregulation of an atheroprotective transcription factor, Kruppel-like factor 2. *Cardiovasc Res* 78: 175–184.
- Habib A, Shamsedeen I, Nasrallah MS, Antoun TA, Nemer G, et al. (2007) Modulation of COX-2 expression by statins in human monocytic cells. *Faseb J* 21: 1665–1674.
- Zhou Q, Liao JK (2009) Statins and cardiovascular diseases: from cholesterol lowering to pleiotropy. *Curr Pharm Des* 15: 467–478.
- Ryter SW, Alam J, Choi AM (2006) Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 86: 583–650.
- Wu L, Wang R (2005) Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* 57: 585–630.
- Mitani K, Fujita H, Fukuda Y, Kappas A, Sassa S (1993) The role of inorganic metals and metalloporphyrins in the induction of haem oxygenase and heat-shock protein 70 in human hepatoma cells. *Biochem J* 290 (Pt 3): 819–825.
- Lutton JD, da Silva JL, Moqattash S, Brown AC, Levere RD, et al. (1992) Differential induction of heme oxygenase in the hepatocarcinoma cell line (Hep3B) by environmental agents. *J Cell Biochem* 49: 259–265.
- Polte T, Abate A, Dennery PA, Schroder H (2000) Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. *Arterioscler Thromb Vasc Biol* 20: 1209–1215.
- Lee TS, Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 8: 240–246.
- Alcaraz MJ, Fernandez P, Guillen MI (2003) Anti-inflammatory actions of the heme oxygenase-1 pathway. *Curr Pharm Des* 9: 2541–2551.
- Alam J (1994) Multiple elements within the 5' distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. *J Biol Chem* 269: 25049–25056.
- Alam J, Den Z (1992) Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J Biol Chem* 267: 21894–21900.
- Leung PO, Wang SH, Lu SH, Chou WH, Shiau CY, et al. (2011) Simvastatin inhibits pro-inflammatory mediators through induction of heme oxygenase-1 expression in lipopolysaccharide-stimulated RAW264.7 macrophages. *Toxicol Lett* 207: 159–166.
- Chen YC, Shen SC, Lee WR, Lin HY, Ko CH, et al. (2002) Nitric oxide and prostaglandin E2 participate in lipopolysaccharide/interferon-gamma-induced heme oxygenase 1 and prevent RAW264.7 macrophages from UV-irradiation-induced cell death. *J Cell Biochem* 86: 331–339.
- Chen JC, Huang KC, Lin WW (2006) HMG-CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways. *Cell Signal* 18: 32–39.
- Mrad MF, Mouawad CA, Al-Hariri M, Eid AA, Alam J, et al. (2012) Statins modulate transcriptional activity of heme-oxygenase-1 promoter in NIH 3T3 Cells. *J Cell Biochem* 113: 3466–3475.
- Alam J, Cai J, Smith A (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5' sequences are required for induction by heme or heavy metals. *J Biol Chem* 269: 1001–1009.
- Zhang SZ, Lipsky MM, Trump BF, Hsu IC (1990) Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell Biol Toxicol* 6: 219–234.
- Alcaraz MJ, Habib A, Creminon C, Vicente AM, Lebreton M, et al. (2001) Heme oxygenase-1 induction by nitric oxide in RAW 264.7 macrophages is upregulated by a cyclo-oxygenase-2 inhibitor(1). *Biochim Biophys Acta* 1526: 13–16.
- Habib A, Bernard C, Lebreton M, Creminon C, Esposito B, et al. (1997) Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J Immunol* 158: 3845–3851.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, et al. (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126: 131–138.
- Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 164: 6166–6173.
- Dunoyer-Geindre S, Dimitrova Y, Fish RJ, Satta N, Reber G, et al. (2005) Fluvastatin increases the expression of adhesion molecules, monocyte chemoattractant protein-1 and tissue factor in HUVEC stimulated by patient IgG fractions containing antiphospholipid antibodies. *Thromb Haemost* 93: 339–345.
- Ricardo SD, van Goor H, Eddy AA (2008) Macrophage diversity in renal injury and repair. *J Clin Invest* 118: 3522–3530.
- Wang WW, Jenkinson CP, Griscavage JM, Kern RM, Arabolos NS, et al. (1995) Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem Biophys Res Commun* 210: 1009–1016.
- Alam J, Cook JL (2007) How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Respir Cell Mol Biol* 36: 166–174.
- Chio CC, Chang YH, Hsu YW, Chi KH, Lin WW (2004) PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutylryl cAMP. *Cell Signal* 16: 565–575.
- Grosser N, Hemmerle A, Berndt G, Erdmann K, Hinkelmann U, et al. (2004) The antioxidant defense protein heme oxygenase 1 is a novel target for statins in endothelial cells. *Free Radic Biol Med* 37: 2064–2071.
- MacLeod RA, Dirks WG, Matsuo Y, Kaufmann M, Milch H, et al. (1999) Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *Int J Cancer* 83: 555–563.
- Ali F, Hamdulay SS, Kinderlerer AR, Boyle JJ, Lidington EA, et al. (2007) Statin-mediated cytoprotection of human vascular endothelial cells: a role for Kruppel-like factor 2-dependent induction of heme oxygenase-1. *J Thromb Haemost* 5: 2537–2546.
- Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, et al. (2001) Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med* 7: 687–692.
- Weitz-Schmidt G (2003) Lymphocyte function-associated antigen-1 blockade by statins: molecular basis and biological relevance. *Endothelium* 10: 43–47.
- Lee TS, Chang CC, Zhu Y, Shyy JY (2004) Simvastatin induces heme oxygenase-1: a novel mechanism of vessel protection. *Circulation* 110: 1296–1302.
- Liao JK (2005) Effects of statins on 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition beyond low-density lipoprotein cholesterol. *Am J Cardiol* 96: 24F–33F.
- Liao JK, Laufs U (2005) Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* 45: 89–118.
- Forrester JS, Libby P (2007) The inflammation hypothesis and its potential relevance to statin therapy. *Am J Cardiol* 99: 732–738.
- Gueler F, Park JK, Rong S, Kirsch T, Lindschau C, et al. (2007) Statins attenuate ischemia-reperfusion injury by inducing heme oxygenase-1 in infiltrating macrophages. *Am J Pathol* 170: 1192–1199.
- Cheng C, Noordeloos AM, Jeny V, Soares MP, Moll F, et al. (2009) Heme oxygenase 1 determines atherosclerotic lesion progression into a vulnerable plaque. *Circulation* 119: 3017–3027.
- Chauveau C, Remy S, Royer PJ, Hill M, Tanguy-Royer S, et al. (2005) Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory cytokine but conserves IL-10 expression. *Blood* 106: 1694–1702.
- Laufs U, Endres M, Stagliano N, Amin-Hanjani S, Chui DS, et al. (2000) Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest* 106: 15–24.

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Author Contributions

Conceived and designed the experiments: CAM MFM JA AH. Performed the experiments: CAM MFM MAH HS. Analyzed the data: CAM MFM EH JA AH. Contributed reagents/materials/analysis tools: EH JA AH. Wrote the paper: CAM MFM AH.