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Simvastatin-dependent up-regulation of heme oxygenase-1 via mRNA stabilization in human endothelial cells☆

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ABSTRACT

Heme oxygenase (HO)-1, which is the inducible isoform of the rate-limiting enzyme of heme degradation, has potent antioxidant and anti-inflammatory effects and is an emerging therapeutic target for the treatment of cardiovascular disease. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also termed statins, induce HO-1 gene expression in endothelial cells, but the underlying regulatory mechanisms are not well studied. To further investigate the statin-specific HO-1 regulation, we examined HO-1 gene expression by simvastatin in cell cultures of human endothelial cells. Simvastatin-dependent HO-1 gene activation was significantly reduced by pharmacological inhibition of the p38 MAPK and phosphotidylinositol-3-kinase (PI3K)/Akt pathways. Although HO-1 is considered to be primarily regulated at the transcriptional level, simvastatin induced activity of a human HO-1 promoter gene construct only to a minor extent. By contrast, studies with actinomycin D indicated that the half-life of HO-1 mRNA was significantly prolonged in the presence of simvastatin suggesting a post-transcriptional mode of HO-1 regulation. The increased HO-1 mRNA stability by this compound was blocked by inhibition of PI3K/Akt, but not by that of p38 MAPK. In conclusion, statin-dependent up-regulation of endothelial HO-1 is mainly regulated by stabilization of HO-1 mRNA via a PI3K/Akt-dependent signaling pathway.
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1. Introduction

Heme oxygenase (HO)-1, which catalyzes the enzymatic degradation of heme (Tenhunen et al., 1968), produces carbon monoxide (CO), iron and biliverdin, all of which have important physiological functions (Maines, 1997; Ryter et al., 2006; Gozzelino et al., 2010). HO-1 is up-regulated by multiple oxidative stress stimuli and provides antioxidant cytoprotection (Applegate et al., 1991). More recently, HO-1 has also been shown to exhibit potent immunomodulatory and anti-inflammatory effects, that have initially been described in HO-1$^{-/-}$ mice (Poss and Tonegawa, 1997) and have essentially been confirmed in a human case of genetic HO-1 deficiency (Yachie et al., 1999). Accumulating evidence indicates that targeted overexpression of HO-1, either by gene therapy or by pharmacological induction, has beneficial effects in various models of inflammation (Immenschuh and Ramadori, 2000; Wagener et al., 2003; Abraham et al., 2007). More specifically, HO-1 has been suggested to be a therapeutic target for the treatment of cardiovascular disease such as atherosclerosis (Ishikawa et al., 2001; Immenschuh and Schroder, 2006; Stocker and Perrella, 2006).

Inhibitors of 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, also termed statins, markedly up-regulate HO-1 gene expression in a cell- and species-specific manner (Grosser et al., 2004a,b; Lee et al., 2004; Chen et al., 2006; Hsu et al., 2006). Statins are beneficial in the primary and secondary prevention of coronary heart disease (Maron et al., 2000). Their therapeutic effects have not only been ascribed to the cholesterol-reducing properties, but also to cholesterol-independent pleiotropic effects such as anti-inflammatory modulation of the endothelium (Liao and Laufs, 2005; Heeba et al., 2009). Specific regulation of endothelial gene expression has been implicated in the protective effects of statins and HO-1 could be an important target gene of statins (Greenwood and Mason, 2007; Ali et al., 2010). This assumption is supported by recent findings indicating that genetic HO-1 deficiency causes major phenotypical alterations of the endothelium (True et al., 2007) and that the beneficial effects of endothelial HO-1 induction overlap with those of statins (Soares et al., 2002,

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2004). Remarkably, statins regulate gene expression via distinct transcriptional and post-transcriptional mechanisms (Laufs and Liao, 1998; Masuya et al., 1998). To further investigate the regulatory mechanisms and signaling pathways of statin-dependent HO-1 induction, we examined the effects of simvastatin on HO-1 gene expression in human endothelial cells.

In the current study, it is reported that the simvastatin-dependent induction of endothelial HO-1 gene expression is mainly regulated by post-transcriptional stabilization of HO-1 mRNA via activation of the phosphotidylinositol-3-kinase (PI3K)/Akt survival pathway.

2. Materials and methods

2.1. Materials

Cell culture media and fetal bovine serum were obtained from Gibco (Eggenstein, Germany) and Lonza (Walkersville, MD), polyvinylidene difluoride membranes were from Millipore (Bedford, MA). Simvastatin lactone was provided by Merck, Sharp and Dohme (Munich, Germany) and was prepared as described previously (Grosser et al., 2004a,b). All other chemicals were purchased from Sigma and Roche Applied Science, unless otherwise indicated.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) cells were obtained from Lonza (Walkersville, MD) and the human endothelial cell line ECV304 was from the European Collection of Cell Cultures. HUVEC were grown in EGM media as recommended by the manufacturer. ECV304 cells were grown in M199 media as described previously (Grosser et al., 2004a,b). Cell cultures were grown in 150-mm dishes under air/CO_2 (19:1) at 100% humidity and were incubated with control media or simvastatin at the indicated concentrations for 24 h. Specific pharmacological inhibitors of p38 MAPK (SB202190 and SB203580), ERK (PD098059) and PI3K (LY294002) were purchased from Calbiochem and were added to the culture medium 30 min prior to treatment with simvastatin. For measurements of mRNA stability, cells were incubated with simvastatin and after an incubation time of 6 h, cells were subsequently treated with actinomycin D (AD) at a concentration of 1 μg/ml alone or in combination with SB203580 or LY294002, as indicated.

2.3. RNA isolation and Northern blot analysis and hybridization

Total RNA isolation was performed with the peqGOLD TriFast kit for RNA isolation from PEQLAB Biotechnologie (Erlangen, Germany) according to the manufacturer's instructions. Northern blot analysis was essentially performed as described previously (Grosser et al., 2004a,b). The probe for hybridization was the *Eco*RI restriction fragment of the human HO-1 cDNA (clone2/10) that was kindly provided by Dr. Rex Tyrrell (School of Pharmacy and Pharmacology, University of Bath, UK) (Keyse and Tyrrell, 1989). The HO-1 cDNA probe was ^{32}P-labeled with the random prime DNA-labeling kit from Roche Applied Science. Equal loading was assessed by a second hybridization with a β-actin cDNA probe (Grosser et al., 2004a,b). Quantification of HO-1 mRNA content was performed with computer-assisted videodensitometry.

2.4. Western blot analysis

Cells were washed with 0.9% NaCl and thereafter lysis was performed as described (Grosser et al., 2004a,b). The lysate was centrifuged for 5 min at 13,000 × *g* at 4 °C and the protein concentration in the supernatant was determined by the BCA™ protein assay kit (Pierce, Rockford, IL). 50 μg of total protein was separated on a 12% SDS-polyacrylamide gel and was electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline containing 5% skim-milk or 5% BSA, 50 mM Tris/HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20 for 1 h at room temperature. The primary antibodies against HO-1 (Stressgen, Victoria, BC, Canada), β-actin and glyceraldehyde-3-dehydrogenase (GAPDH) (Hytest, Turku, Finland) were used at 1:1000 dilutions. Primary antibodies against phosphorylated and total ERK1/2 and JNK were from Cell Signaling, and primary antibodies against phosphorylated p38 and total p38 MAPK were from Biomol. Primary antibodies were applied at the concentrations recommended by the manufacturer. The secondary anti-rabbit peroxidase-conjugated antibody was obtained from Alexis (Grünberg, Germany). The ECL chemiluminescent detection system (GE Healthcare, Germany) was used for detection according to the manufacturer's instructions. Equal loading was assessed with antibodies against β-actin or GAPDH.

2.5. Plasmid constructs, transfection and luciferase assay

The luciferase reporter gene constructs pHO4.9luc was obtained from Dr. Norbert Leitinger (University of Virginia, Charlottesville, VA) (Kronke et al., 2003). After growth for 24 h, transfection of plasmid DNA into ECV304 cells was performed by using FuGENE (Roche Applied Science), as described previously (Wijayanti et al., 2004). Cells were transfected with 1 μg of the reporter plasmid. Transfection efficiency was controlled using 0.1 μg of *Renilla* luciferase expression vector pRL-SV40 (Promega, Germany), as described previously (Grosser et al., 2004a,b). Cells were lysed with luciferase lysis reagent (Promega, Germany), and luciferase activity was determined with a commercial Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Cells were treated for 24 h with simvastatin or other reagents, as indicated. Relative light units of *Firefly* luciferase activity were normalized with *Renilla* luciferase activity.

3. Results

3.1. Statin-dependent induction of HO-1 gene expression in human endothelial cells

Statins have previously been shown to induce HO-1 gene expression *in vivo* and in cell culture (Grosser et al., 2004a,b; Lee et al., 2004; Chen et al., 2006; Hsu et al., 2006). To further investigate the mechanisms that regulate the statin-dependent induction of HO-1 gene expression in the endothelium, we extended our previous studies on the statin-dependent HO-1 stimulation in human endothelial cells (Grosser et al., 2004a,b). In accordance with earlier findings, treatment of primary HUVEC with simvastatin leads to a marked up-regulation of endogenous HO-1 gene expression. This regulatory pattern was similar to that observed in cell cultures of the human endothelial cell line ECV304 (Fig. 1). In addition, HO-1 gene expression was also induced in HUVEC and ECV304 cells by lovastatin, which is another widely used statin (data not shown).

3.2. Statin-dependent induction of HO-1 gene expression is mediated via activation of p38 MAPK and PI3K/Akt in human endothelial cells

Statins have previously been shown to have different effects on the activation of MAPKs and the PI3K/Akt survival pathway in various cell cultures. Whereas Lee and colleagues have initially

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