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Pitavastatin suppresses mitogen activated protein kinase-mediated *egr-1* induction in human vascular smooth muscle cells

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Abstract

Statins have been demonstrated to elicit a broad range of cellular events resulting in an attenuation of the inflammatory response and enhanced protection to the components of the vessel wall. The present study was designed to examine the effect of pitavastatin on pathways associated with the proinflammatory gene, early growth response (*Egr*)-1, in human vascular smooth muscle cells. Pretreatment with pitavastatin resulted in a dose-dependent reduction in *Egr*-1 protein and suppressed *Egr*-1 mRNA expression in response to phorbol 12-myristate 13-acetate (PMA). A reduction in *Egr*-1 expression reduced the activation of NGFI-A binding protein (NAB)-2, an *Egr*-1-dependent gene. Furthermore, these events appeared to be dependent on the ability of pitavastatin to attenuate signaling cascades associated with extracellular regulated kinase (ERK) 1/2, but not p38 and c-Jun N-terminal kinase (JNK).

Keywords

Early growth response (*Egr*)-1; statins; mitogen-activated protein kinase (MAPK); smooth muscle cells

1. Introduction

The clinical effectiveness of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) in the reduction of cardiovascular events is understood to involve cellular mechanisms supplementary to their ability to reduce serum low-density lipoprotein (LDL)-cholesterol (Calabro and Yeh, 2005; Davignon, 2004). Furthermore, the capacity of statins to target multiple cellular pathways may be particularly ideal in the treatment of multifactorial diseases such as atherosclerosis. Atherogenesis is characterized by the presence of numerous pro-inflammatory mediators and a self-amplifying molecular crosstalk which supports lesion formation, maturation and potentially plaque rupture (Packard and Libby, 2008). Elucidation of pathways which globally influence these processes and identification of the molecular

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targets of statins are critical to both the enhanced efficacy and safety of these drugs. Although several classes of inducible factors have been implicated in cardiovascular disease, early growth response (Egr)-1 is an immediate-early gene product and a zinc finger transcription factor which soundly fits these criteria, as it is induced early in disease, regulates pathways associated with the initiation and propagation of lesion development, and contributes to molecular events associated with thrombosis (Fahmy and Khachigian, 2007; Harja et al., 2004; Khachigian, 2006; McCaffrey et al., 2000; Mechtcheriakova et al., 1999).

A variety of stimuli relevant to vascular disease such as angiotensin II, platelet-derived growth factor (PDGF), shear stress, acute mechanical injury and chlamydia pneumoniae, transiently induce Egr-1 in vascular smooth muscle cells (Haas et al., 2007; Ling et al., 1999; Rupp et al., 2005; Santiago et al., 1999a; Silverman et al., 1997). Mitogen-activated protein kinases (MAPKs) including extracellular regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) have been linked to Egr-1 induction, however, the cell type and particular stimulus may be critical in dictating the activation identities of these pathways. The role of vascular smooth muscle Egr-1 in atherogenesis is highlighted by experimental and clinical evidence linking this transcription factor to lesion development. LDL-receptor deficient mice fed a high-fat diet exhibit a progressive increase in Egr-1 protein in smooth muscle cells of atherosclerotic lesions, and knockout of the Egr-1 gene on an ApoE-deficient background results in a reduction in lesion size (Harja et al., 2004; McCaffrey et al., 2000). Similarly, fibrous cap tissue in human lesions from patients with obstructive carotid atherosclerosis exhibit a five-fold increase in Egr-1, with the most intense staining located in alpha-smooth muscle-actin-positive cells surrounded by invading macrophages (Du et al., 2000). When activated, Egr-1 has been demonstrated to regulate over 300 genes influencing inflammation and/or atherogenesis including other transcription factors (peroxisome proliferator-activated receptor gamma (PPAR γ), NGFI-A binding protein 1/2 (NAB-1/2)), growth factors and cytokines (insulin-like growth factor-2 (IGF-2), transforming growth factor β (TGF β), vascular endothelial growth factor (VEGF)), cell-cycle regulatory proteins (p57, cyclin D1), extracellular matrix proteins (vascular cell adhesion molecule 1 (VCAM1), fibronectin, collagen) and a variety of signaling molecules (Rad, Notch3, receptor activity-modifying proteins (RAMP1)) (Fu et al., 2003; Khachigian, 2006).

Pitavastatin is a synthetic statin, which competitively inhibits HMG-CoA reductase and lowers serum LDL cholesterol by 40% in hypercholesterolemic patients (Aoki et al., 1997; Saito et al., 2002). We have previously reported that pitavastatin supports antiatherogenic mechanisms such as the suppression of oxLDL uptake and foam cell formation by targeting CD36, provides anti-inflammatory actions in macrophages, and alters arterial homeostasis by favoring fibrinolysis over thrombosis (Han et al., 2004a; Han et al., 2004b; Markle et al., 2003). Here, we investigate the hypothesis that the vasoprotective effects of pitavastatin are mediated, in part, by an attenuation of Egr-1 induction in vascular smooth muscle cells.

2. Materials and methods

2.1 Cell culture and treatment protocols

Human aortic smooth muscle cells (HASMCs; ATCC) from normal aortic tissue were cultured in SmGM-2 medium (Cambrex) supplemented with 5% fetal bovine serum (FBS). Cells were plated and grown to 75% confluency at or before the 7th passage. Prior to treatments, cells were washed with phosphate-buffered saline (PBS) and quiesced for 4 h in media containing 0.5% FBS and 10mM Hepes (pH 7.4). Pitavastatin (0 – 20 μ mol/L) or vehicle (dimethyl sulfoxide; DMSO) was then added directly to wells. After 20h (24h total quiescing time), cells were exposed to phorbol 12-myristate 13-acetate (PMA; 100ng/ml) for 1h unless otherwise noted and cells were harvested as described. Where appropriate, MAPK inhibitors for ERK (U0126;

10 μ mol/L), p38 (SB202190; 30 μ mol/L) and JNK (420119; 20 μ mol/L) were added 1h prior to stimulation with PMA (MAPK inhibitors purchased from Calbiochem).

2.2 Protein and total RNA preparation

After treatment, whole cell lysates or total RNA was prepared for western blotting and real-time PCR, respectively. For whole cell lysates, cells were washed with PBS and scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail (Sigma)). Cells were rotated on an orbital shaker at 4°C for 15 minutes and then spun at 13,000 RPM. The supernatant was collected immediately and protein concentration was determined using the Lowry method. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) as per the manufacturer's protocol.

2.3 Western blot analysis

Total protein from each treatment group was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk/PBST (0.1% Tween) and probed with anti-Egr-1 (1:1000; Cell Signaling), anti-NAB-2 (1:500; Santa Cruz), anti-basal or -phosphorylated ERK/p38/JNK (1:1000; Cell Signaling) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Santa Cruz) overnight at 4°C. Membranes were then washed in PBST. Blots were probed with the species-appropriate horseradish peroxidase-conjugated IgG secondary antibody (1:2500) in 1% milk PBST for 1 hour at room temperature. Membranes were washed and treated with ECL PLUS (GE Healthcare) and exposed to BIOMAX MR Film (Eastern Kodak Co.).

2.4 Real time PCR (qPCR)

For qPCR analysis, 50 ng of total RNA was reverse transcribed with the high capacity cDNA reverse transcription kit (ABI). Primers were designed using IDT software and SYBR Green master mixes were prepared for PCR. Each RT-PCR reaction was performed in triplicate with primers for human Egr-1 and GAPDH, and detected using an ABI 7500 system (Applied Biosystems). Results are presented as the amount of Egr-1 mRNA present relative to the basal, untreated condition and normalized to the level of GAPDH mRNA. Specificity of the PCR primer sets was verified with dissociation curve analysis of the reactions in comparison to no-template and no-RT controls.

2.5 Statistical analysis

Results are presented as the mean $\pm$ standard error (S.E.M.). Data were analyzed by ANOVA followed by the Newman-Keuls post hoc test. The null hypothesis was rejected at $P < 0.05$. Densitometric analysis of western blotting is expressed as protein of interest to GAPDH ratio or fold increase over control after correction for equal loading by GAPDH.

3. Results

Although largely absent under non-stressed conditions, Egr-1 induction has been observed in vascular smooth muscle cells in response to proinflammatory stimuli relevant to atherogenesis (Ling et al., 1999; Santiago et al., 1999b; Silverman et al., 1997; Silverman et al., 1999). Consistent with these findings, exposure of HASMCs to PMA led to a time-dependent induction of Egr-1 protein expression which was detected as early as 30min (Fig. 1A). As Egr-1 induction was maximal at 1h, this time-point was used in subsequent studies. Shown in Figure 1B, pre-treatment of smooth muscle cells with pitavastatin produced a dose-dependent reduction in PMA-induced Egr-1 protein expression. A significant attenuation of Egr-1 induction was observed with as low as 0.1 μ mol/L pitavastatin, a concentration previously

determined to be physiologically relevant and achievable in patients (Tsujimoto et al., 2006). In the absence of PMA, basal expression of Egr-1 was unaltered by pitavastatin (1–10 $\mu\text{mol/L}$) (Fig. 1C). Real-time PCR (qPCR) analysis was subsequently used to examine the expression of Egr-1 transcript in the presence or absence of pitavastatin. Using this approach, PMA was found to produce a dramatic induction of Egr-1 transcript at 1h (Fig. 1D). While pitavastatin had no effect on basal levels of Egr-1 transcript, Egr-1 mRNA induction by PMA was significantly ($P<0.05$) reduced by pitavastatin pretreatment. Collectively, these findings are consistent with an inhibitory influence of pitavastatin on smooth muscle cell Egr-1 activation. Egr-1 has been established as a signaling nexus resulting in the induction of various molecular mediators relevant to atherogenesis. In an effort to associate the observed effects of pitavastatin on Egr-1 protein expression with a functional response, NAB-2 was examined as a model Egr-1-dependent gene. The expression of NAB-2 protein was previously shown to supervene Egr-1 induction and to be maximal 2h post-stimulus (Silverman et al., 1999). Consistent with these reports, NAB-2 protein expression was upregulated 2h following exposure of smooth muscle cells to PMA (Fig. 1E). Importantly, NAB-2 induction was reduced in cells pretreated with pitavastatin (Fig. 1E).

Previous reports have linked stimuli-induced activation of Egr-1 to the mitogen-activated protein kinase (MAPK) signaling pathways, however, statins have been shown to both inhibit and activate these pathways (Hasan and Schafer, 2008; Kamimura et al., 2004; Matsumoto et al., 2004; Tsujimoto et al., 2006; Yamakawa et al., 2003). Under present experimental conditions, PMA-mediated induction of Egr-1 was shown to be partially dependent on both ERK and JNK, but not p38, as inhibitors of these kinases significantly suppressed Egr-1 induction (Fig. 2A). We thus sought out to determine if these signaling pathways play a role in the observed effects of pitavastatin on Egr-1. Specifically, we examined both basal (inactive) and phosphorylated (active) levels of ERK, p38 and JNK. Western blot analysis revealed a robust induction of phosphorylated ERK, and to a lesser extent p38 and JNK in response to PMA, without altering basal levels of these kinases (Fig. 2B). Importantly, pitavastatin suppressed PMA-induced ERK phosphorylation in a dose-dependent manner (Fig. 2B). While a trend towards a reduction in ERK phosphorylation was observed at 0.01 and 0.1 $\mu\text{mol/L}$ pitavastatin, this reached statistical significance at 1 $\mu\text{mol/L}$, at which activation of this kinase was reduced by ~35% (Fig. 2C). While pitavastatin appeared to slightly suppress p38 and JNK phosphorylation, this did not reach statistical significance at any of the concentrations examined (1–20 $\mu\text{mol/L}$) (Fig. 2C and data not shown). Basal levels of MAPKs were unaltered by pitavastatin. These findings implicate multiple signaling pathways involved in PMA-induced smooth muscle cell stimulation, however, only ERK remains as a clear upstream target of pitavastatin-mediated suppression of Egr-1 induction.

4. Discussion

Statins have been recently heralded for therapeutic effects involving a vast number of molecular targets which compliment their capacity to inhibit HMG-CoA reductase and reduce serum cholesterol (Calabro and Yeh, 2005; Davignon, 2004). One explanation for this phenomenon is that statins target transcription factors which globally regulate downstream mediators associated with cardiovascular disease. In this study, we demonstrate a role of pitavastatin in diminishing PMA-mediated induction of Egr-1, a transcription factor that has been linked to inflammatory-based pathologies such as atherosclerosis in human vascular smooth muscle cells (Du et al., 2000; Fahmy and Khachigian, 2007; Harja et al., 2004; Mechtcheriakova et al., 1999).

We provide evidence that the molecular actions of pitavastatin involve a reduction in the capacity of cells to activate ERK, a known modulator of Egr-1 induction (Hasan and Schafer, 2008; Kamimura et al., 2004). PMA was found to promote the activation of all three MAPK

pathways. These findings highlight the concept of redundant signaling mechanisms modulating similar downstream mediators (e.g. transcription factors), similar to that observed in smooth muscle cells exposed to oxidative stress (Hasan and Schafer, 2008; Tsujimoto et al., 2006). Despite this generalized activation to PMA, pitavastatin appears to selectively suppress ERK-mediated induction of Egr-1. Previous studies support a link between pitavastatin and ERK inactivation, however, ERK phosphorylation was only reduced by high concentrations (10–100 $\mu\text{mol/L}$) of pitavastatin and occurred in response to lysophosphatidylcholine to inhibit smooth muscle cell proliferation (Yamakawa et al., 2003). Interestingly, work by others has argued against a role of ERK in response to pitavastatin, demonstrating an enhancement of p38 and JNK phosphorylation in response to this statin (Tsujimoto et al., 2006). Discrepancies amongst signaling networks may be the product of differential MAPK activation depending on the underlying stimulus and cellular conditions. Whether the pathophysiological phenotype of Egr-1 expression is altered with regards to its inherent upstream activation pathways is currently unknown.

That pitavastatin suppressed NAB-2 expression suggests inhibition of Egr-1 produces a functional response in terms of Egr-1-dependent gene activation. Although classically associated with infiltrating cells from the circulation, such as macrophages and T cells, cytokines and growth factors (e.g. IL-1 and VEGF) are secreted from vascular smooth muscle cells in response to Egr-1 activation, and play an important role in determining the extent of inflammation in the vascular wall (Yan et al., 2000; Zhu et al., 2007). Conversely, Egr-1 was shown to downregulate nitric oxide synthase, the enzyme responsible for the generation of the antiproliferative, vasodilatory and antithrombogenic gas nitric oxide (NO) (Fu et al., 2003). The emergence of pharmacological inhibitors of NF κ B activation, another ubiquitous, proinflammatory transcription factor, provides an analogous therapeutic strategy for targeting Egr-1; one in which multiple downstream mediators can be simultaneously targeted by a common upstream factor (Gilmore and Herscovitch, 2006).

The classification of Egr-1 as an early-response gene that is transiently induced following pathological stimuli may put to question the importance of this transcription factor in the chronic setting of atherogenesis. However, cellular events occurring throughout lesion development including endothelial damage, macrophage infiltration, LDL oxidation and accumulation, and smooth muscle cell migration and proliferation, likely occur in a cyclic manner (Lamon and Hajjar, 2008). To this end, molecular mediators may serve to periodically activate Egr-1 in the micro-environment, thereby initiating cascades and cellular events which perpetuate lesion formation on a chronic basis. As such, our findings that Egr-1 induction is attenuated in the presence of pitavastatin raises the potential of this drug to serve as a continuous prophylactic against an oscillating pattern of vascular injury.

Although the present study utilized an *in vitro* cell culture model with human-derived smooth muscle cells, previous work has confirmed the capacity of statins to modulate Egr-1 *in vivo*. For example, simvastatin inhibited the expression of tissue factor in advanced atherosclerotic lesions of apolipoprotein E deficient mice, in an Egr-1-dependent manner (Bea et al., 2003). Importantly, the regulation of smooth muscle cell proliferation, migration, and associated intimal thickening is believed to be a critical component of Egr-1-driven atherogenesis and response to vascular injury (Harja et al., 2004; Khachigian, 2006; McCaffrey et al., 2000; Ohtani et al., 2004; Silverman et al., 1999; Yan et al., 2000). The present study demonstrates that pitavastatin diminishes the induction of Egr-1 protein and mRNA in smooth muscle cells in response to the model agonist, PMA. Furthermore, these effects appear to be upstream of transcriptional activation of Egr-1 synthesis via a reduction in ERK signaling. We interpret the finding that pitavastatin suppresses Egr-1 as an important mechanism by which this drug exerts its anti-inflammatory and vasoprotective actions.

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Figure 1.

A. Representative western blot and densitometric analysis for time-course activation of Egr-1 in human aortic smooth muscle cells by PMA. B. Representative western blot and densitometric analysis of Egr-1 in cells pretreated with increasing concentrations of pitavastatin (0.01–10 $\mu\text{mol/L}$) prior to 1h stimulation with PMA. C. Western blot of basal Egr-1 expression in response to pitavastatin (1–10 $\mu\text{mol/L}$). D. Quantification of Egr-1 mRNA by real-time PCR. E. Representative western blot of NAB-2 expression in response to pitavastatin (1–20 $\mu\text{mol/L}$) prior to 2h stimulation with PMA. All data normalized to GAPDH. * $P < 0.05$ relative to control. † $P < 0.05$ relative to PMA alone.

Figure 2.

A. Representative western blot and densitometric analysis of Egr-1 expression in human aortic smooth muscle cells in response to PMA (1h) ± inhibitors of MAPK pathways. B. Representative western blots of phosphorylated MAPKs pretreated with increasing concentrations of pitavastatin (0.01–10 μmol/L) prior to 1h stimulation with PMA. C. Densitometric analysis of phosphorylated forms of ERK, p-38 and JNK pretreated with 1 μmol/L pitavastatin prior to 1h stimulation with PMA.