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Statins Modulate Cyclooxygenase-2 and Microsomal Prostaglandin E Synthase-1 in Human Hepatic Myofibroblasts

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ABSTRACT

Statins have been shown to exert anti-inflammatory and anti-fibrogenic properties in the liver. In the present study, we explored the mechanisms underlying anti-fibrogenic effects of statins in isolated hepatic myofibroblasts and focused on cyclooxyegnase-2, a major anti-proliferative pathway in these cells. We show that simvastatin and fluvastatin inhibit thymidine incorporation in hMF in a dose-dependent manner. Pretreatment of cells with NS398, a COX-2 inhibitor, partially blunted this effect. cAMP levels, essential to the inhibition of hMF proliferation, were increased by statins and inhibited by non-steroidal anti-inflammatory drugs. Since statins modify prenylation of some important proteins in gene expression, we investigated the targets involved using selective inhibitors of prenyltransferases. Inhibition of geranylgeranylation resulted in the induction of COX-2 and mPGES-1. Using gel retardation assays, we further demonstrated that statins potentially activated the NFκB and CRE/E-box binding for COX-2 promoter and the binding of GC-rich regions and GATA for mPGES-1. Together these data demonstrate that statin limit hepatic myofibroblasts proliferation *via* a COX-2 and mPGES-1 dependent pathway. These data suggest that statin-dependent increase of prostaglandin in hMF contributes to its anti-fibrogenic effect. J. Cell. Biochem. 117: 1176–1186, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: STATIN; PROSTAGLANDIN E_2; CYCLOOXYGENASE-2; CELL GOWTH; LIVER; TRANSCRIPTION FACTORS; GERANYLGERANYLATION

Abbreviations: hMF, hepatic myofibroblasts; COX-2, cyclooxygenase; mPGES-1, microsomal prostaglandin E synthase-1; PG, prostaglandin; ET-1, endothelin-1; HS, normal human serum.

Charbel A. Mouawad and May F. Mrad contributed equally to this work.

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Hepatic myofibroblasts (hMF) play a major role in liver fibrogenesis. Upon liver insult, these cells migrate and accumulate at the site of injury. Accumulation of myofibroblasts results from their high mitogenic properties and their enhanced survival capacity, in response to a wide variety of growth factors, cytokines, and lipid mediators produced by the injured liver. The profibrogenic potential of hepatic myofibroblasts relies on their capacity to secrete fibrotic matrix proteins and inhibitors of their degradation [Lotersztajn et al., 2005; Friedman, 2008; Mallat and Lotersztajn, 2013; Schuppan and Kim, 2013].

Cyclooxygenase-2 (COX-2) and microsomal prostaglandin (PG) E_2 synthase-1 (mPGES-1) are responsible for the production of PGE_2 from arachidonic acid [Smith et al., 2011]. PGE_2 plays an important role in many pathophysiological situations including inflammation and fibrosis [Ricciotti and FitzGerald, 2011]. We have previously demonstrated the anti-fibrogenic properties of COX-2-derived PGE_2. Indeed, an increase in PGE_2 in response to various proinflammatory and growth factor factors such as endothelin-1, TNFα, PDGF or sphingosine-1-phosphate results in an increase in cAMP and a reduction of hMF growth, suggesting that factors that induce PGE_2 and COX-2 in hMF may be interesting as antifibrogenic agents [Gallois et al., 1998, 2000; Mallat et al., 1998]. Statins are competitive inhibitors of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase that lower circulating LDL. Beneficial effects unrelated to their cholesterol-lowering capacities were described, including anti-oxidant, anti-inflammatory, and anti-proliferative properties [Shibata et al., 2009; Zhang et al., 2013; Capra and Rovati, 2014]. Mevalonate is the direct product of HMG & CoA reductase and a precursor of isoprenoids, thus important for farnesylation or geranylgeranylation of proteins involved in gene synthesis and cell regulation [Sebti and Hamilton, 1997; Sebti, 2005]. Specific farnesyl or geranylgeranyl transferases are responsible for the transfer of these lipidic moieties to proteins. This post-translational modification is important for the subcellular localization and function of many proteins such as members of the small GTPases families of Ras and Rho [Rikitake and Liao, 2005]. It has been previously shown that isoprenylation plays a role in COX-2 expression in vascular cells and macrophages [Degraeve et al., 2001; Habib et al., 2007; Fukuda et al., 2015]. It has also been shown that statins display anti-fibrogenic properties that may be related to their anti-proliferative effects on hMF [Trebicka et al., 2010; Klein et al., 2012].

We, therefore, investigated whether the anti-fibrogenic effects of statins in hMF may be mediated by COX-2-derived PGE_2 and aimed to elucidate the mechanisms and transcription factors that control PGE_2 formation.

MATERIALS AND METHODS

REAGENTS

Cell culture media and fetal bovine serum (FBS) were from Gibco (Invitrogen Life technologies, NY). L-glutamine, penicillin, streptomycin, sodium pyruvate, and phosphate-buffered saline were from Lonza (Verviers, Belgium). Normal human serum (HS) and *Mycokill* were from PAA (Pasching, Austria). Non-essential amino acids, amphotericin B, monoclonal anti-β actin, bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), and mevalonate were from Sigma–Aldrich (St. Louis, MO). Bradford reagent, chemicals for electrophoresis, and oligonucleotides were from Bio-Rad (Hercules, CA). Simvastatin, fluvastatin, SB203580, FTI-277, GGTI-286, endothelin-1, and ibuprofen were from Merck Millipore (San Diego, CA). Enhanced chemi-luminescense (ECL) and poly (dI/dC) were from General Electric (Piscataway, NJ). Donkey anti-mouse and anti-rabbit antibodies conjugated to peroxidase were from Jackson Immunoresearch Laboratories (West Grove, PA). Rabbit polyclonal antibody against p65-NFκB, p50-NFκB, GATA-6 (H-92), and GATA-4 (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NS-398, cAMP assay kit, and anti-mPGES-1 antibodies were from Cayman chemicals (Ann Arbor, MI). TNFα and EGF were from R&D systems (Minneapolis, MN). [^{3}H-methyl] thymidine (2 mCi/mmol) was from MP Biomedicals (Solon). γ-^{32}P ATP obtained from NEN Life Science Products (Boston, USA). All other chemical reagents were purchased from Amresco (Solon, OH).

CELL CULTURE

Human hMF were obtained by outgrowth from explants of normal liver obtained following surgery of benign or malignant liver tumors. This procedure is in accordance with ethical regulations imposed by the French legislation. Cells were incubated in Dulbecco's modified Eagle's medium containing 10% serum (5% fetal calf serum, 5% pooled human serum), 2 mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% *Mycokill*, 100 U/ml pencillin, 100 μg/ml streptomycin, 0.5 μg/ml amphotericin B, and 5 ng/ml EGF at 37°C, 5% CO_2 in a humid atmosphere. Exhaustive characterization of these cells has already been published [Mallat et al., 1996]. Experiments were performed between passages 4 and 8 without any noticeable difference in results observed with cells obtained from various passages, or from various livers. All experiments and treatments were performed on confluent cells differentiated in serum-free medium over 48 h.

TREATMENT OF CELLS

Treatment of cells was performed in serum- and EGF-free medium. Cells were cultured in 6-well plates for western blot analysis (2×10^5 cell/well) or in 24-well plates for cAMP measurement (5×10^4 cell/well) or for [^{3}H-methyl] thymidine incorporation (5×10^4 cell/well). Cells were cultured for nuclear extraction and RNA isolation (5×10^5cell/100 mm dish). Cells were treated with increasing concentrations of simvastatin or fluvastatin for 48 h alone or in the presence of different concentrations of endothelin-1 (ET-1) added for the last 6 h of incubation. In some experiments, cells were treated with 150 μM mevalonate 30 min prior to the addition of 25 μM simvastatin. To study the implication of p38 MAPK, different concentrations of p38 inhibitor, SB203580, were added for the last 6 h. To study the role of isoprenylation, cells were treated with increasing concentrations of GGTI-286 or FTI-277 for 24 h. For cAMP assay, cells were treated with 25 μM simvastatin or 10 μM fluvastatin for 10 min. For proliferation experiments, cells were treated with human serum (5%) alone or in the presence of increasing concentrations of simvastatin or fluvastatin for 24 h. Cells were treated with 5 or 25 μM simvastatin for 24 h for nuclear extraction and for 6 h for RNA isolation.

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WESTERN BLOTTING FOR COX-2 AND mPGES-1

After treatment, supernatants were collected for PGE_2 analysis. Immunoblot analyses were performed using a monoclonal anti-COX-2-29 antibody [Habib et al., 1993], a rabbit polyclonal antibody against mPGES-1, or a mouse monoclonal anti-β-actin IgG. Secondary antibodies were donkey anti-mouse or anti-rabbit antibodies conjugated to peroxidase. Signals were developed using enhanced chemiluminescence (ECL) kit. Autoradiograms were scanned using Epson 1680 pro scanner and densitometric analysis was performed using Scion NIH software (Scion Corporation Software, Frederick, MD).

MEASUREMENT OF PROSTAGLANDINS AND cAMP

PGE_2 and cAMP were measured using enzyme immunoassay with acetylcholine esterase coupled to PGE_2 or cAMP, respectively, as tracers, as described previously [Pradelles et al., 1985,1989].

PROLIFERATION ASSAY USING [^{3}H]-THYMIDINE

Cells were treated with different concentrations of simvastatin or fluvastatin for 24 h in the presence of 5% pooled human serum. In some experiments, 50 μM of Ibuprofen or 5 μM of NS398 were added 15 min prior to the treatment with statins. One microcurie (uCi) per well of [^{3}H]-thymidine was added for the last 6 h of incubation. Cells were then fixed with 400 μl 10% trichloroacetic acid for 30 min followed by the addition of 400 μl of 0.2 M NaOH.

REAL-TIME PCR

Total RNA was extracted from cell exudates using Tripure reagent (Roche). Total RNA (0.5 μg) was reverse-transcribed with the iScript^{TM} cDNA Synthesis Kit (Biorad). Real-time quantitative PCR was performed using the Master Mix For PCR and the Real-time Quantitative Thermocycler CFX96 using following the manufacturer's protocols (Biorad). Results were calculated based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$) as previously described [Wan et al., 2014]. Primers were as follow: COX-2 F: TGCTGGCAGGGTTGCTGGTGGTA; COX-2 R: GGGCTTCAGCATAAAGCGT TTGCGG; mPGES-1 F: CCTGGTGATGAGCAGCCCGG; mPGES-1 R: GGCAAAGGCCTTCTTCCGCAG; β-actin F: ATGGCCACGGCTGCTTCCAG; β-actin R: CCACAGGACTCCATGCCCAGG.

NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY

Nuclear extracts were prepared as described previously [Habib et al., 2007]. Gel retardation experiments were performed for the binding of NFκB, NF-IL6, and CRE/E-box using the oligonucleotides for human COX-2 NFκB (−223/−214 bp) (sense): 5′-GAGAGTGGGGACTACCCCCTCT-3′, human COX-2 NFκB (−338/−380 bp) (sense): 5′-GGAGAGGGGATTCCCTGCGC-3′, human COX-2 NF-IL6 (−132/−124 bp) (sense): 5′-CCGGGCTTACGCAATTTTTTT-3′, and human COX-2 CRE/E-box (−59/−53 bp) (sense): 5′-TCATTTCGTCACATGGGCTTG-3′, or the binding of GATA and GC box using primers sequences for human mPGES-1 GATA (−644/640 bp) (sense): 5′-ACCCAGATAAACTAG-3′, human mPGES-1 GATA (−293/−289 bp) (sense): 5′-ACTGTAGATAAGGAAA-3′, and human mPGES-1 GC Box (−119/114 and −108/103 bp) (sense): 5′-TGCCGTGGGGCGGGGCGTGGG-3′. Single stranded oligoneculeotides, at a concentration of 100 pmol/μl, were annealed, phosphorylated with [^{32}P]- γ ATP (6,000 Ci/mmol), and incubated with 5 μg of nuclear extracts in reaction buffer as previously described [Habib et al., 2007]. Cold competitor or mutant double strands DNA were added in excess to designated tubes. In supershift experiments, 2 μg of rabbit polyclonal IgG against p65-NFκB, p50-NFκB, GATA-6 (H-92), and GATA-4 (C-20) or unrelated transcription factor antibodies were added 30 min before the addition of the labeled probe. The samples were submitted to electrophoresis on 6% non-denaturing polyacrylamide gel. The gel was dried, and radioactive signals were detected using the Storm PhosphorImager (General Electric).

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 ± S.E.M. Sigmastat® was used as software for statistical analysis and graph drawing. An unpaired *t*-test was used for the comparison of two groups and one way ANOVA followed by the Dunnett test when mentioned. *p*-value of less than 0.05 was considered statistically significant.

RESULTS

STATINS INHIBITION OF HUMAN HEPATIC MYOFIBROBLAST PROLIFERATION IS DEPENDENT ON PROSTAGLANDIN E_2 FORMATION

We first evaluated the effect of two structurally distinct statins on human hMF cell growth. Both simvastatin (5 and 25 μM) and fluvastatin (2 and 10 μM) significantly decreased serum-stimulated [^{3}H]-thymidine incorporation (Fig. 1A). Pretreatment with NS398, a selective COX-2 inhibitor, strongly reduced the inhibitory effect of fluvastatin suggesting a role for COX-2 derived prostaglandin in statin-induced growth inhibition (Fig. 1B). We have previously shown that cAMP mediates PGE_2 induced inhibition of hMF growth [Mallat et al., 1998; Gallois et al., 2000]. Treatment of hMF with 10 μM fluvastatin resulted in a significant increase in cAMP formation, which was blocked by ibuprofen, a non-selective COX-1/COX-2 inhibitor, or with NS398, a selective COX-2 inhibitor (Fig. 1C). Similarly to cAMP, PGE_2 formation in these cells was increased in response to 25 μM simvastatin (Fig. 1D).

SIMVASTATIN AND FLUVASTATIN INCREASE COX-2 AND mPGES-1 EXPRESSION

We next investigated the effect of statins on COX-2 and mPGES-1 expression. Both simvastatin and fluvastatin strongly increased COX-2 compared to untreated cells. Co-treatment of cells with ET-1 significantly synergized the effect of statin (Fig. 2A). Simvastatin also induced mPGES-1 in hMF (Fig. 2B). SB203580, an inhibitor of p38 MAPK, significantly blocked the induction of COX-2 and mPGES-1 by simvastatin suggesting that COX-2 dependent increase of PGE_2 by statins relies, at least, on the p38 MAPK pathway (Fig. 2B). Figure 3C illustrates the densitometry analysis of COX-2 and shows a significant increase in COX-2 in

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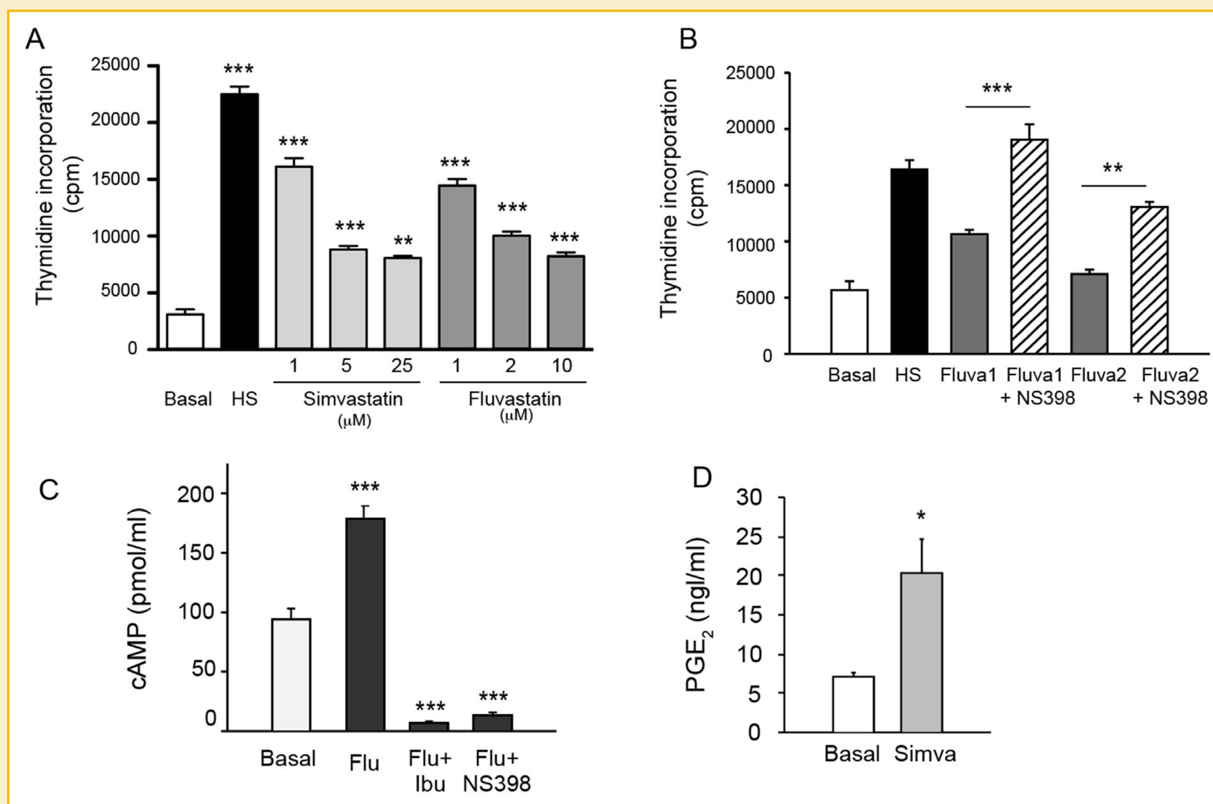

Fig. 1. Effect of statins on DNA synthesis and the release of cAMP and PGE_2 in human hMFs. (A) hMF cells were activated by 5% normal human serum (HS) alone or in the presence of different concentrations of statins for 24 h. One microcurie per well of [^{3}H]-thymidine was added for the last 6 h of incubation. Results are expressed as mean ± SEM of three experiments, $^{***}P < 0.001$ versus HS, one way Anova followed by the Dunnett test (B) To study the role of COX, cells were pretreated with 5 μM NS398 for 15 min prior to the addition of 1 and 2 μM fluvastatin and HS for 24 h. Results are expressed as mean ± SEM of three different experiments, $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired *t*-test. (C) Effect of COX-2 inhibition on statin-induced cAMP release. Cells were treated with 50 μM Ibuprofen or 5 μM NS398 for 15 min prior to incubation with 10 μM fluvastatin for 10 min in the presence of 1mM IBMX. cAMP measurement was assessed according to the manufacture's instruction. Data represent the mean ± SEM of four different experiments, $^{***}P < 0.001$, one way Anova followed by the Dunnett test (D) Effect of statin on PGE_2 release. Cells were treated in serum-free medium with 25 μM simvastatin for 48 h. PGE_2 assay was carried out on supernatants of cells and results are expressed as mean ± SEM of three experiments expressed in ng/ml, $^{*}P < 0.05$, unpaired *t*-test.

response to simvastatin and fluvastatin. We further verified that statins increase the gene expression in response to statins. Real-time PCR was performed and Figure 2D shows that both statins increased the levels of COX-2 and mPGES-1 mRNA.

INHIBITION OF ISOPRENYLTRANSFERASES INCREASES COX-2 AND mPGES-1 EXPRESSION IN hMF

We first assessed whether the effect of mevalonate, the direct product of the HMG-CoA reductase, prevent the statin-dependent induction of COX-2 and mPGES-1. Figure 3A shows a reduction of the expression of these 2 enzymes and the formation of PGE_2 compared to simvastatin-treated cells supporting a role of the mevalonate pathway. Isoprenylation is a post-translational modification of some proteins important for their intracellular localization and activity. Statins, by blocking HMG-CoA reductase affect isoprene level and isoprenylation [Palsuledesai and Distefano, 2015]. We used the geranylgeranyl transferase inhibitor GGTI-286 and the farnesyl transferase inhibitor FTI-277 to investigate the role of prenylation. Pretreatment of cells with 10 μM of GGT-286 resulted in COX-2 and mPGES-1 protein expression (Fig. 3C). A faint band was observed with FTI-277 suggesting that geranylgeranylation and not farnesylation of targets is involved in statin-regulation of COX-2 and mPGES-1 expression. Moreover, Figure 3D shows a strong and statistically significant increase of PGE_2 formation in the presence of GGT-286 and much less with FTI-277.

TRANSCRIPTIONAL REGULATION OF STATIN-DEPENDENT INDUCTION OF COX-2 AND mPGES-1

We further characterized the transcription factors involved in COX-2 and mPGES-1 induction by statins and performed gel retardation assays. Figure 4A illustrates the different potential binding sites for transcription factors located in the human COX-2 proximal promoter. We investigated the two NFκB (−223/−214 bp) and (−338/−380 bp), the CRE/E-box and the NF-IL6 binding sites, in response to simvastatin. As shown in Figure 4B, simvastatin induced protein-DNA complex formation for NFκB (−338/−380 bp) binding sequences compared to untreated cells. TNFα, which has previously shown an increase in NFκB–DNA binding was used as a positive control [Gallois et al., 1998]. The complex was competed with cold NFκB but not CRE/E-box. Supershift analysis using

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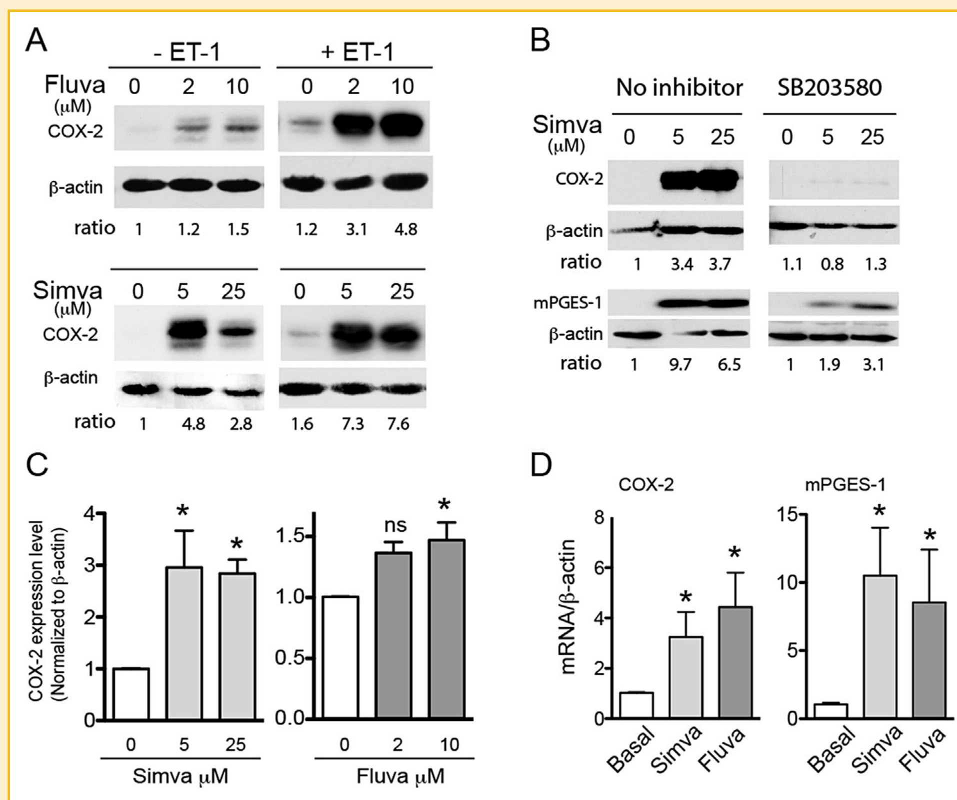

Fig. 2. Effect of statins on the expression of COX-2 and mPGES-1 in hMF. hMF were serum-deprived 48 h prior to incubation with fluvastatin or simvastatin for another 48 h, alone or in the presence of 0.025 μM ET-1 added for the last 6 h of incubation. COX-2 and mPGES-1 expression was investigated in response to simvastatin added for 48 h alone or with 5 μM of SB203580, a p38 MAPK inhibitor. Western blot analyses were carried out using 15 or 30 μg of total cell lysates and selective anti-COX-2 or anti-PGES-1 IgG, respectively. Real-time PCR was carried out as described in the Materials and Methods section. Results are representatives of three different experiments for Western Blot and are the average of three experiments for Q-PCR. (A) Immunoblot of COX-2 in response to simvastatin and fluvastatin, (B) modulation of COX-2 and mPGES-1 by simvastatin and p38 MAP kinase inhibitor, SB203580. (C) Densitometric analysis of COX-2, $^{*}P < 0.05$, versus basal, one way Anova followed by the Dunnett test (D) Q-PCR of COX-2 and m-PGES-1, $^{*}P < 0.05$, versus basal, unpaired *t*-test.

antibodies anti-p50 or p65 NFκB antibodies showed a shift with both antibodies. In addition, simvastatin induced the formation of a DNA-protein complex for CRE/E-box site (Fig. 4C). Double strand CRE/E-box competed with the binding but not cold double strand DNA for NFκB, double mutant CRE/E-box, or single mutant E-box suggesting a role of E-box in statin-dependent effect. No DNA-protein complexes were formed in response to simvastatin for COX-2, NF-IL6 or for COX-2 NFκB (−223/−214 bp) (data not shown).

Analysis of the human promoter of mPGES-1 showed two Barbie boxes (barbituric induced sequence), two GC boxes important in the binding of Egr-1 (early growth response-1), SP1 (specificity protein 1) and two potential GATA binding sites at −293/−289 bp and −644/−640 bp (Fig. 5A). Figure 5B shows that simvastatin induced the formation of a DNA-protein complex for GC box. Finally, there was a DNA–protein complex formation, although faint, in simvastatin-treated cells for GATA (−293/−289 bp) that was strongly potentiated by TNF-α (Fig. 5C). GATA-4 but not GATA-6 antibody showed a supershift of the DNA–protein complex. No DNA–protein complex formation was formed for GATA (−644/−640 bp).

DISCUSSION

Statins were shown in animal models of liver fibrosis and in isolated hepatic stellate cells to inhibit the proliferation and activation of hepatic stellate cells rendering them more quiescent, or inducing their apoptosis [Aprigliano et al., 2008; Trebicka et al., 2010; Klein et al., 2012]. In the present study, we show that two structurally unrelated statins, simvastatin and fluvastatin, inhibit human hepatic myofibroblasts growth *via* a COX-2/mPGES-1 pathway. The role of statin-dependent increase in PGE_2 on hepatic myofibroblasts was investigated. We demonstrated that statins increased PGE_2 and cAMP formation and that statin-dependent proliferation and cAMP generation involve COX-2 activity. Although hepatic myofibroblasts express COX-1, we have shown earlier that in these cells the basal production of PGE_2 is ensured by COX-2, since COX-2 selective inhibitors, SC 58125 or NS398, blocked this synthesis [Gallois et al., 1998; Davaille et al., 2000]. COX-2 derived production of PGE_2 can account for the increase in cAMP and COX-2-dependent inhibition of proliferation. In these cells, we showed that statin also increased the expression of COX-2 and mPGES-1 at a delayed phase. Thus, statins can play a role both

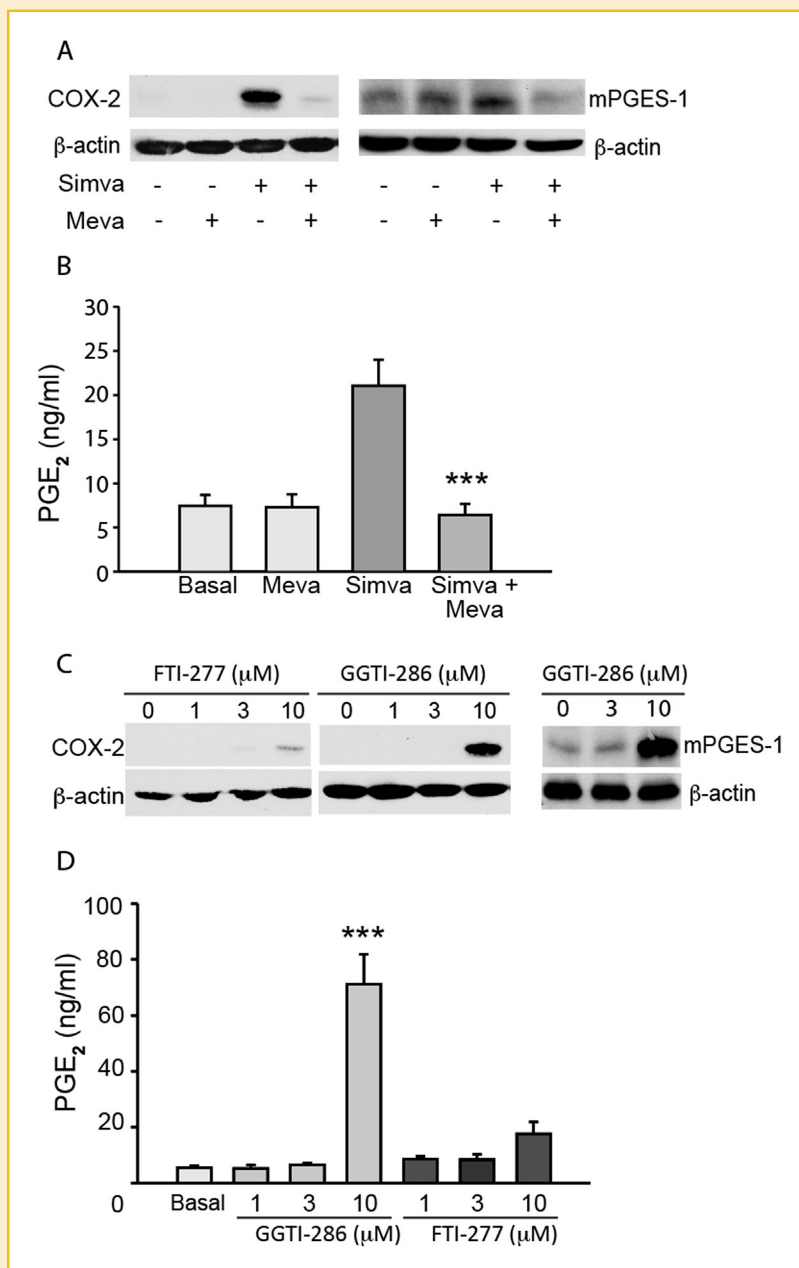

Fig. 3. Molecular regulation of COX-2 and mPGES-1 in hMF: (A) modulation of statin-mediated COX-2 and mPGES-1 expression by mevalonate. Cells were treated with 150 μM mevalonate for 30 min prior to the addition of 25 μM simvastatin for 48 h. The immunoblot is representative of three different experiments. (B) PGE_2 determination. PGE_2 was measured in the supernatant of cells using EIA. Results are expressed as mean ± SEM of 4 experiments. $^{***}P < 0.001$ versus simvastatin alone, unpaired *t*-test. (C) Immunoblots of COX-2 and mPGES-1 in response to increasing concentrations of FTI-277 and GGTI-286 for 24 h (n = 3). (D) Effect of isoprenoids on PGE_2 release: Cells were treated in serum-free medium with increasing concentrations of GGTI-286 and FTI-277 for 24 h. PGE_2 was measured and results are expressed as mean ± SEM of three experiments. $^{***}P < 0.001$ versus basal, unpaired *t*-test.

in early phase of PGE_2 formation and in delayed phase of regulation of these cells associated with a long-lasting increase in COX-2 and mPGES-1 protein and prostaglandin synthesis. Similar regulation of COX-2 pathway has been shown for many stimuli including ET-1 and Sphingosine-1-phosphate in these cells [Gallois et al., 1998; Davaille et al., 2000]. In these cells, the additional regulation of mPGES-1 is in favor of the additional increase of PGE_2 that can play a role in the early phase of proliferation of these cells but also through PGE_2 receptor-dependent gene expression. We cannot exclude in our study the role of PGI_2, which is formed in these cells in response to many stimuli such as ET-1 [Mallat et al., 1996]. Further analysis using EP or IP-receptor antagonists can shed light on the respective roles of these two prostaglandins.

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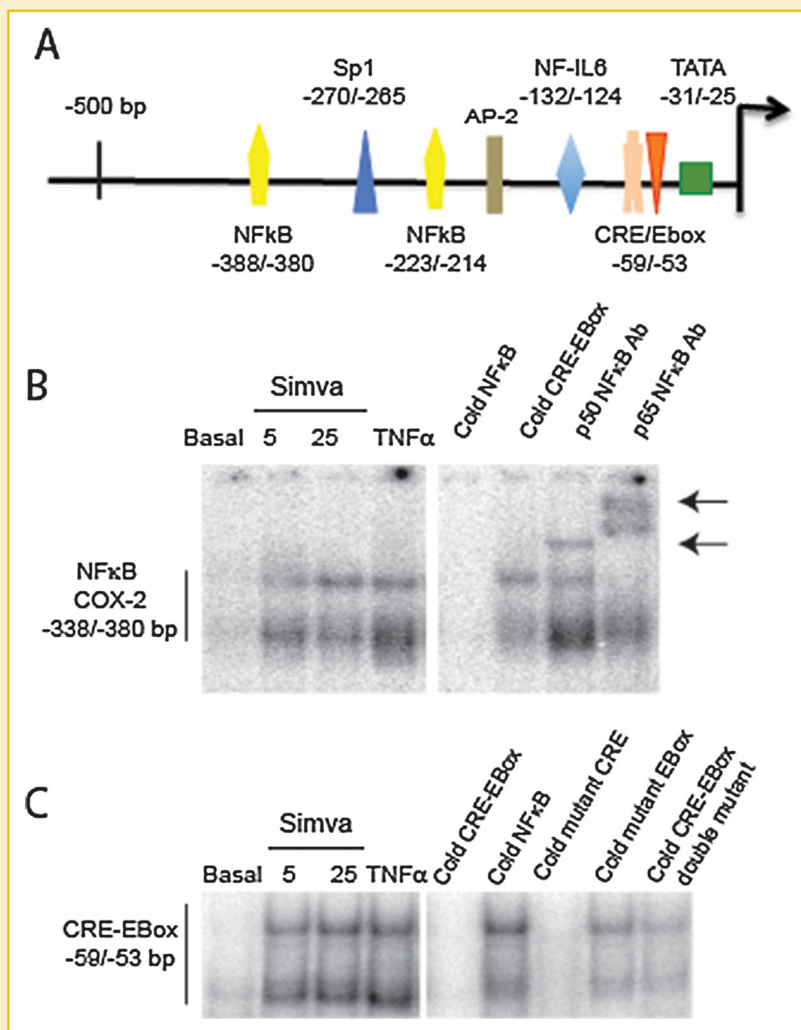

Fig. 4. Electrophoretic mobility of human COX-2 NFκB and CRE/E-box -DNA complexes in response to a 24-h treatment with simvastatin or TNFα: (A) illustration of the sites of regulatory elements in the short region of human COX-2 promoter [Inoue and Tanabe, 1998]. (B) Effect of simvastatin on the in vitro binding of transcription factors to the NFκB cis-acting elements in human COX-2 promoter. Cells were treated with 5 and 25 μM simvastatin for 24 h or 10 ng/ml TNFα for 4 h. Nuclear extracts from these cells were prepared and EMSA was carried out as described in Methods section. In vitro nuclear protein-DNA binding was analyzed using human [^{32}P]-labeled COX-2- NFκB probe. Unlabeled NFκB oligonucleotide was used as a competitor and rabbit polyclonal antibodies specific for p50-NFκB and p65-NFκB were added for the supershift. These controls were performed on the same gel. Free probe and reaction mixtures were electrophoresed on a 6% polyacrylamide, 0.25% TBE gel. Radioactive signals were detected using the Storm PhosphorImager. NFκB-specific and "supershifted" complexes are indicated. (C) Effect of simvastatin on the in vitro binding of transcription factors to the CRE/E-box cis-acting elements in human COX-2 promoter. In vitro nuclear protein-DNA binding was analyzed using human [^{32}P]-labeled COX-2- CRE/E-box probe, chased with and an excess of unlabeled CRE/E-box and mutant CRE oligonucleotides. These controls were performed on the same gel. The figure is representative of two different experiments with similar results.

We furthermore investigated the mechanisms behind COX-2 and mPGES-1 induction by statins. We demonstrate that the upregulation of COX-2 and mPGES-1 expression involves geranylgeranylation, rather than farnesylation and is dependent in part on p38 MAPK. We used selective inhibitors of prenyltransferases to identify the type of prenylation involved. GGTI-286 is described as selective inhibitor of geranylgeranylation whereas and FTI-277, an inhibitor of farnesylation. Our results in hMF are in line with the inhibition of the geranylgeranylation of target proteins rather than farnesylation, although we cannot fully exclude that high concentration of FTI-277 interferes with geranylgeranylation [Delarue et al., 2007].

Members of Rho and Ras small GTPase families are prenylated proteins and are possible targets of statins and are shown to play an important role in vascular proliferation and gene expression [Rikitake and Liao, 2005; Wang and Liao, 2012]. Statin-dependent regulation of COX-2 is variable among different cell types and species. In vascular cells, Sawada and Liao [2014] have shown that only RhoA and its target ROCK1 play a role in statin-dependent inhibition of proliferation. Moreover statins induced eNOS expression in a RhoA-dependent manner and improved vascular functionality [Laufs and Liao, 1998]. We have shown that statins increased COX-2 expression and prostaglandins in part through

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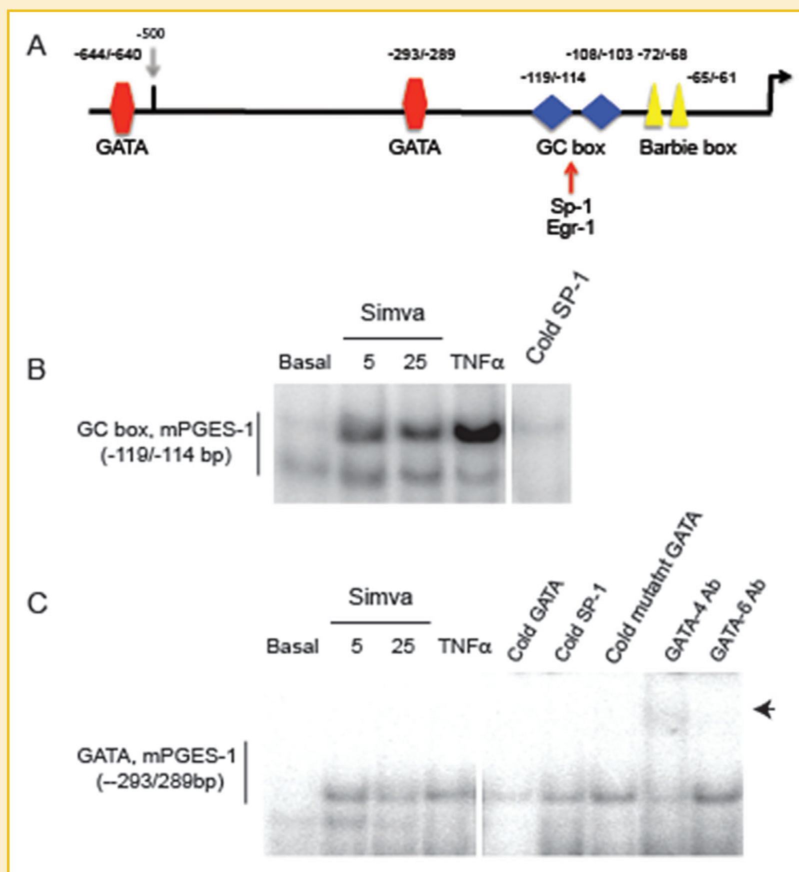

Fig. 5. Electrophoretic mobility of human mPGES-1 GC box and GATA–DNA complexes in response to a 24-h treatment with simvastatin in the presence or absence of TNFα used as a positive control: (A) regulatory elements in the short region of human mPGES-1 promoter adapted with modification from [Forsberg et al., 2000]. (B) Effect of simvastatin on the in vitro binding of transcription factors to the GC box-*cis*-acting elements in human mPGES1-promoter. Cells were treated with 5 and 25 μM simvastatin for 24 h or 2 ng/ml TNFα for 4 h. EMSA was carried as described in Figure 4. In vitro nuclear protein–DNA binding was analyzed using human [^{32}P]-labeled mPGES-1-GC rich probe and an excess of unlabeled GC box-oligonucleotide was the competitor on nuclear extracts of cells. (C) Effect of simvastatin on the in vitro binding of transcription factors to the GATA-cis-acting elements in human mPGES-1 promoter. In vitro nuclear protein–DNA binding was analyzed using human [^{32}P]-labeled mPGES-1- GATA probe, and an excess of unlabeled GATA oligonucleotide was the competitor. Rabbit polyclonal antibodies specific for GATA-4 and GATA-6 were added for the supershift. These controls were performed on the same gel. The figure is representative of two different experiments with similar results.

inhibition of RhoA and ROCK1 in human aortic smooth muscle cells in culture [Degraeve et al., 2001]. In the same cells, fluvastatin and pitavastatin mediated anti-inflammatory and anti-atherosclerotic effects and induced COX-2 expression [Fukuda et al., 2015] and statin increased HDL-dependent induction of COX-2 expression and prostacyclin [Martinez-Gonzalez et al., 2004]. On the contrary, atorvastatin decreased the expression of COX-2 in a model of atherosclerosis in rabbit and in rat vascular smooth muscle cells in culture [Hernandez-Presa et al., 2002] and lowered the expression of EP receptors in atherosclerotic plaques and in macrophages[Gomez-Hernandez et al., 2006].

In endothelial cells and macrophages/monocytes, statins decreased the expression of COX-2. Inhibition of COX-2 expression by statins is described in HUVEC and is associated with anti-angiogenic properties [Massaro et al., 2010]. In monocyte/macrophage, many studies have shown an inhibition of COX-2 and PGE_2 formation by statins in response to LPS or INFγ in human monocytic cells U937 and THP-1 [Habib et al., 2007; Lee et al., 2009] and in vivo in macrophages of atherosclerotic plaques of hypercholesterolemic rabbit treated with atorvastatin [Hernandez-Presa et al., 2002]. The general differences in the regulation of COX-2 expression and prostaglandins formation could be the results of the difference in the activation and levels of expression of targets of statins such as the small GTPase proteins. RhoA, for example, is responsible for blocking COX-2 expression in vascular smooth muscle cells, whereas Rac 2 in monocytic cell line U937 is essential for its expression, resulting in a different and opposite expression of COX-2 in response to statins [Degraeve et al., 2001; Habib et al., 2007; Fukuda et al., 2015]. In human hMF, we show an induction of the prostaglandin synthesis pathway in response to statin similar to the human aortic smooth muscle cells.

p38MAP kinase has also been shown to play a role in COX-2 expression [Barry et al., 1999; Pype et al., 2001; Yamaguchi et al., 2001; Li et al., 2004; Eligini et al., 2009]. We used the largely documented inhibitor of the isoforms α/β of p38 MAP kinase, SB 203580 [Lee et al., 1999], and demonstrated a role p38 MAP kinase in

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statin-dependent induction of COX-2 and mPGES-1, although we cannot exclude a p38 MAP kinase-independent effects of the inhibitor. Yano et al. [2007] demonstrated recently that the RhoA cdc42 are involved in statin-dependent modulation of COX-2 expression in macrophages. One of the possible mechanisms linking statin to p38 MAP kinase is the inhibition of RhoaA, cdc 42, or Rac as described for Raw 264.7 cells.

In the present study, analysis of the transcriptional regulation of COX-2 and mPGES-1 suggests a potential role for CRE/E-box and NFκB nuclear factors in statin-mediated regulation of human COX-2 promoter and for GATA and SP-1 in that of human mPGES-1. The COX-2 promoter has two NFκB binding elements (−214/−223 and −380/−388 bp) in its proximal promoter. Although LPS and PMA were shown to transcriptionally activate the −223/−214 bp promoter region in human monocytes and aortic smooth muscle cells [Inoue and Tanabe, 1998], it is NFκB −388/−380 bp, the second distal NFκB binding site of human COX-2 promoter, that is activated by statin in hMF as shown previously in hMF [Gallois et al., 2000]. Moreover, statins also activated CRE/E-box suggesting a role of cAMP response elements and C/EBPs in statin-dependent activation of COX-2. The contribution of each *cis*-acting promoter element in COX-2 is different among cell types and stimuli. Many transcription factors can bind to CRE. These include ATF-2, c-Jun, c-Fos, and CREB. USF-1/2 binds E-box and activates COX-2 in human gastric epithelial cells in response to PMA and *Helicobacter pylori*. The functional co-activator protein CBP/p300 can also play a role in the activation of the COX-2 promoter activity in response to PMA. Since the binding sites of CRE/E-box are too close, we are not able to distinguish which transcription factor is more likely to be involved, despite the results of the competition with mutant for each binding site.

In the present study, we also analyzed the potential transcription factors involved in mPGES-1 expression. Human mPGES-1 promoter has two GC-rich boxes responsible for the binding of SP-1 and Egr-1 transcription factors, and two GATA binding sites. This promoter region is actively involved in the binding of SP-1or Egr-1 [Ekstrom et al., 2003]. PPARγ inhibits IL-1-dependent induction of mPGES-1 in synovial fibroblasts by interfering with Egr-1 activity and binding [Cheng et al., 2004]. We found a strong GC binding activity in response to statins. Statins were shown to inhibit Egr-1 in vascular smooth muscle cells [Lamon et al., 2009]. GATA are a family of transcription factors characterized by their ability to bind the DNA sequence GATA. The family consists of six members, GATA-1 to 6. GATA-1 to 3 are restricted to the hematopoietic system and GATA-4 to 6 are present in several tissues [Patient and McGhee, 2002]. Statins were shown to activate GATA in vascular cells [Wada et al., 2008; Liu et al., 2009]. We found two potential GATA binding sites in the promoter of mPGES-1. Gel retardation assays showed a specific binding of GATA-4 in response to simvastatin that was enhanced in the presence of TNFα, for the proximal GATA binding site, suggesting a potential role of GATA in statin-dependent regulation of mPGES-1. The direct identification of the transcription factors involved in this activation needs future investigation.

Taken together, we demonstrate that statins inhibit cell growth by increasing the cAMP and the PGE_2 formation in hMF in parallel to an induction of COX-2 and mPGES-1 via the inhibition of geranylgeranylation and the activation of p38 MAPK. NFκB and CRE/E-box binding transcription factors may be important for COX-2 expression. Moreover, the activation of the SP-1 and Egr-1 binding site of mPGES-1 by statins could suggest a role of these transcription factors in the regulation of PGE_2 production. Statin activation of GATA for mPGES-1 transcriptional regulation is novel and implicates the binding of sequences derived from the proximal GATA binding sites.

In conclusion, our results suggest an anti-fibrogenic role of statins via COX-2 and mPGES-1 inhibition and propose the prostaglandin synthesis and signaling as a target for statins in hepatocyte injury and fibrosis. Future investigation on the role of prostaglandin receptors will shed light on the in vivo role of statins and NSAIDS in liver injury and fibrosis.

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