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Modulation of COX-2 Expression by Statins in Human Aortic Smooth Muscle Cells

INVOLVEMENT OF GERANYLGERANYLATED PROTEINS*

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Cyclooxygenase (COX)-2 and COX-1 play an important role in prostacyclin production in vessels and participate in maintaining vascular homeostasis. Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which is crucial in cholesterol biosynthesis. Recently, cholesterol-independent effects of statins have been described. In this study, we evaluated the effect of two inhibitors of HMG CoA reductase, mevastatin and lovastatin, on the production of prostacyclin and the expression of COX in human aortic smooth muscle cells. Treatment of cells with 25 μM mevastatin or lovastatin resulted in the induction of COX-2 and increase in prostacyclin production. Mevalonate, the direct metabolite of HMG CoA reductase, and geranylgeranyl-pyrophosphate reversed this effect. GGTI-286, a selective inhibitor of geranylgeranyltransferases, increased COX-2 expression and prostacyclin formation, thus indicating the involvement of geranylgeranylated proteins in the down-regulation of COX-2. Furthermore, *Clostridium difficile* toxin B, an inhibitor of the Rho GTP-binding protein family, the Rho selective inhibitor C3 transferase, and Y-27632, a selective inhibitor of the Rho-associated kinases, targets of Rho A, increased COX-2 expression whereas the activator of the Rho GTPase, the cytotoxic necrotizing factor 1, blocked interlukin-1α-dependent COX-2 induction. These results demonstrate that statins up-regulate COX-2 expression and subsequent prostacyclin formation in human aortic smooth muscle cells in part through inhibition of Rho.

The competitive inhibitors of 3-hydroxymethylglutaryl coenzyme A (HMG CoA)[1] reductase, also called statins, inhibit the rate-limiting step in the synthesis of cholesterol by blocking the conversion of HMG CoA to mevalonate (1). In this way statins are clinically useful for primary and secondary prevention of atherosclerosis (2, 3). However, some of their beneficial effects in therapy seem unrelated to the decrease in low density lipoprotein-cholesterol.

By modulating the initial part of the cholesterol synthesis pathway, statins decrease the level of numerous important intermediate compounds including isoprenoids that contain geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). Isoprenoids are lipid attachments involved in posttranslational modification of some proteins such as the γ subunit of the heterotrimeric G proteins, the small G proteins Ras, and Ras-like proteins such as Rho, Rap, Rab ,or Ral (4, 5). Statins can thus modulate various biological or physiological mechanisms.

Cyclooxygenases are involved in the metabolism of arachidonic acid to prostaglandins (PGs) and thromboxane (TX) A_2 (6). In vascular biology, the two major products of COX are TXA_2, which is mainly formed by the constitutive form of COX, COX-1 in activated platelets, and prostacyclin or PGI_2, which is mainly produced in vascular cells by COX-1 and the inducible form of COX, COX-2 (7, 8). TXA_2 participates in platelet aggregation and vascular contraction, whereas PGI_2 acts as an anti-aggregant for platelets and a vasodilator. PGI_2 plays an important role in vascular physiology as illustrated by the therapeutic effect of stable analogs of PGI_2 such as iloprost (9). Platelets from patients suffering from hypercholesterolemia are characterized by hypersensitivity to various aggregating agents. Notarbartolo *et al.* (10) have shown that simvastatin decreased platelet aggregation in hypercholesterolemic subjects and supported a decrease in the thromboxane platelet production, although the underlying mechanism of the statin effect on platelet function remains unclear.

In this study, we demonstrated in human aortic smooth muscle cells (hASMC) that two different statins, mevastatin and lovastatin, increased COX-2 expression and PGI_2 formation. We further demonstrated using selective inhibitors of geranylgeranyltransferases and modulators of Rho GTPases that geranylgeranylated proteins such as Rho seem to be responsible for COX-2 down-regulation, which is prevented by statins.

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[1] The abbreviations used are: HMG CoA, 3-hydroxymethylglutaryl coenzyme A; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; PG, prostaglandin; TX, thromboxane; COX, cyclooxygenase; PGI_2, prostacyclin; hASMC, human aortic smooth muscle cells; IL, interleukin; HIV, human immunodeficiency virus; MOPS, 4-morpholinepropanesulfonic acid; CNF, cytotoxic necrotizing factor; NOS, nitric oxide synthase.

EXPERIMENTAL PROCEDURES

Materials—hASMC and the corresponding culture media (SmGM, SmBM) were from Clonetics (Biowhittaker Europe, Verviers, Belgium). Mevastatin, lovastatin, mevalonate, squalene, farnesyl-pyrophosphate, geranylgeranyl-pyrophosphate, arachidonic acid, and actinomycin D were from Sigma-Aldrich. Statins in lactone form were dissolved in 0.1 M NaOH to generate the active form and the pH adjusted to 7.4 by adding 0.1 M HCl as described previously (11, 12). Prenyltransferase inhibitors (farnesyltransferase inhibitor FTI-277 and geranylgeranyltransferases inhibitor GGTI-286) were from Calbiochem (La Jolla, CA). Recombinant human IL-1α was from R&D (Minneapolis, MN). The selective inhibitor of Rho-associated kinase, Y-27632, was from TOCRIS Cookson Ltd (Bristol, UK). Electrophoresis reagents were from Euromedex (Souffelweyersheim, France), nitrocellulose membrane (Hybond-C extra) and enhanced chemiluminescence (ECL) from Amersham Pharmacia Biotech (Les Ulis, France). Donkey anti-mouse IgGs conjugated to peroxidase were from Jackson Immuno-Research laboratories (West Grove, PA). HECAMEG® was from Vegatec (Villejuif, France). Trizol and Albumax® were from Life Technologies Inc. *Escherichia coli* cytotoxic necrotizing factor 1 was prepared as described previously (13), and *Clostridium difficile* toxin B was a kind gift of Dr. Ingo Just (Hannover University, Hannover, Germany). *Clostridium botulinum* C3 transferase was used as a fusion protein with the HIV TAT protein transduction domain that allows for rapid entry into cells as described by Sebbagh *et al.* (14).

Cell Culture and Incubation—hASMCs were grown in SmGM culture medium supplemented with 5% fetal bovine serum, 5 mg/l insulin, 2 μg/l fibroblast growth factor, 10 μg/l human recombinant epidermal growth factor, 50 mg/l gentamicin, and 50 μg/l amphotericin B according to Clonetics. hASMCs were used at passage 9. Cells were subcultured in 12-well plates or 60-mm dishes and cultured until subconfluence was reached. The medium was then replaced by a serum-free culture medium containing 0.5% Albumax® for 48 h prior to the addition of statins or the other reagents. For statins, cells were further co-incubated in the same medium with or without the different isoprenoids in the absence or presence of IL-1α for 48 h. Shorter incubation periods were used for GGTI-286, FTI-277, and toxins as indicated. The concentration of ethanol or Me_2SO did not exceed 0.3% and did not alter COX expression. The absence of cellular toxicity by statins, isoprenyltransferase inhibitors, and toxins was evaluated by neutral red assay (15).

Prostacyclin Assay—After stimulation, supernatants were collected to measure the stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, using an enzyme immunoassay with acetylcholinesterase-labeled 6-keto-$PGF_{1\alpha}$ as tracer (16).

Assessment of Cell Apoptosis—Cells were cultured for 48 h in the absence or presence of 25 μM each statin as described above. Culture medium was carefully removed and replaced by 500 μl of a 5 μg/ml solution of Hoechst 33342 in phosphate buffer saline. Dishes were incubated at 37 °C for 30 min in the dark, and cells were overlaid with a coverslip and immediately examined under fluorescence microscopy (14).

Western Blot Analysis—After incubation, hASMCs were washed twice in phosphate-buffered saline, lysed in 200 μl of lysis buffer (20 mM Tris/HCl, pH 7.5, 20 mM HECAMEG®, 1 mM EDTA, and 1 mM benzamidine). Protein content was determined by a microbicinchoninic acid assay (Pierce) with bovine serum albumin as standard. Western blot analysis was performed as described previously (17). Briefly, 10 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Immunoblotting was performed as described previously using one monoclonal antibody COX-229 and two specific monoclonal antibodies (COX-110 and COX-111) for COX-2 and COX-1 analysis, respectively (17, 18). Membranes were further incubated with a donkey anti-mouse IgG conjugated to peroxidase. Excess antibody was washed and positive bands were revealed by ECL chemiluminescence reagents according to the manufacturer's instructions. Autoradiograms were scanned using an Arcus II Agfa scanner, and densitometric analysis was performed using Sigma Gel® software (SPSS, Chicago, IL).

RNA Extraction and Northern Blot Analysis—hASMCs in 60-mm culture dishes were incubated as described above and washed once with phosphate-buffered saline. For the mRNA stability experiments, cells were incubated for 24 h with IL-1α in the presence or absence of mevastatin. 5 μM actinomycin D was then added for various periods of time. Total RNA were extracted with Trizol® according to the manufacturer's instruction. Northern blot analysis was performed as described previously (19). 10 μg of total RNA was fractionated on a formaldehyde/MOPS/EDTA/1% agarose gel and stained with ethidium bromide. RNA was transferred to nitrocellulose membrane and cross-linked by UV irradiation (Stratalinker® UV cross-linker, Stratagene). The cDNA probes used were a 2.1 kilobases of human COX-2 cDNA fragment (20) and β-actin cDNA fragment (CLONTECH Laboratories Inc, Palo Alto, CA). cDNA was labeled using a Ready to Go kit (Amersham Pharmacia Biotech) and [α-^{32}P]dCTP (PerkinElmer Life Sciences). Membranes were first prehybridized for 4 h and then hybridized overnight at 42 °C with the COX-2 probe (10^6 cpm/ml) in 50 mM Tris/HCl buffer, pH 7, containing 50% formamide, 10× Derhardt's solution, 1 M NaCl, 1% SDS, 5% dextran sulfate, 0.1% pyrophosphate, and 100 μg/ml salmon sperm DNA. Membranes were washed twice for 10 min with 2× SSC/0.1 SDS at room temperature, twice in 1× SSC/0.1 SDS at 60 °C, and once in 0.1× SSC/0.1 SDS at 60 °C. For β-actin detection, membranes were hybridized with 0.5 10^6 cpm/ml. Signals were quantified using a Fuji bioimaging analyzer (Fuji, Tokyo, Japan), and the ratio of COX-2/β-actin was determined.

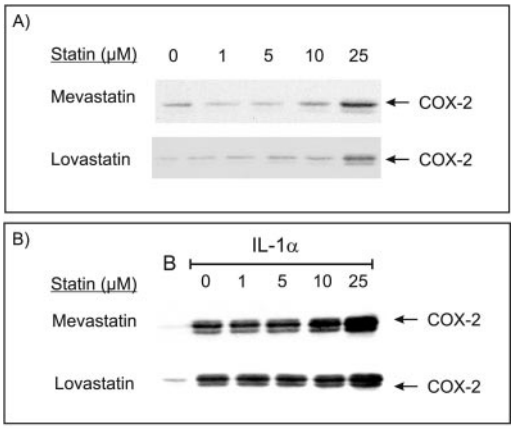

FIG. 1. **Induction of COX-2 by mevastatin and lovastatin in the absence or presence of IL-1α.** *Panel A*, hASMC cells were incubated for 48 h in the absence or presence of two different statins at increasing concentrations (1, 5, 10, or 25 μM). *Panel B*, the same protocol as in *panel A* except that the cells were incubated with 0.5 ng/ml IL-1α. Western blot analysis was performed as described under "Experimental Procedures." Each blot is representative of five different experiments except for 10 μM where two experiments were performed.

Statistics—Results are shown as average mean ± S.E. of n different experiments. Data were analyzed by Student's paired t test. A p value <0.05 was accepted as significant.

RESULTS

Effects of Statins on Prostacyclin Release and COX-2 Expression—Exposure of human aortic smooth muscle cells to 25 μM mevastatin or lovastatin for 48 h led to a statistically significant increase in prostacyclin production compared with basal conditions. Cells secreted 43.2 ± 6.8 and 46.2 ± 6.7 ng/ml of 6-keto-$PGF_{1\alpha}$ ($n = 9$) when incubated with 25 μM mevastatin and lovastatin, respectively, compared with 35.6 ± 5.4 in untreated cells ($p < 0.01$). An increase in prostacyclin production was also observed in the presence of 0.5 ng/ml IL-1α (405 ± 61 and 412 ± 59 ng/ml of 6-keto-$PGF_{1\alpha}$, $n = 9$ for 25 μM mevastatin and lovastatin, respectively, compared with 299 ± 43, $n = 9$, for IL-1α alone, $p < 0.01$).

Western blot analysis of these cells using a selective antibody for COX-2 showed an increase in COX-2 expression in cells treated with 25 μM mevastatin or lovastatin compared with untreated cells (Fig. 1*A*). Under these conditions, neither lovastatin nor mevastatin induced apoptosis, as assessed by examination of Hoechst 33342-stained cells (data not shown). Treatment of the cells with increasing concentrations of statins along with 0.5 ng/ml IL-1α caused an increased induction in COX-2 expression at 25 μM statins as detected at the protein level (Fig. 1*B*). Under these conditions, no modification of COX-1 expression by statins alone or in the presence of IL-1α was observed (Fig. 2).

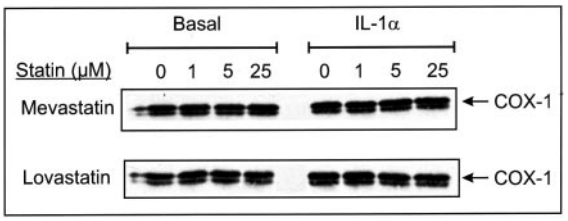

FIG. 2. **Absence of modulation of COX-1 expression by statins.** hASMC were treated in the absence or presence of 0.5 ng/ml IL-1α and 1, 5, or 25 μM statins. *Basal* corresponds to untreated cells. Western blot analysis of COX-1 expression was performed as described under "Experimental Procedures." Note that human COX-1 migrates as a protein doublet. Each blot is representative of two separate experiments.

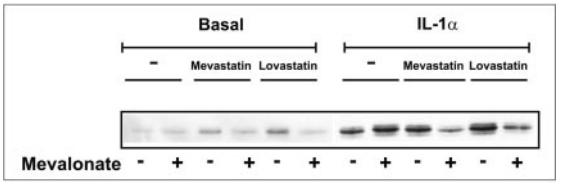

FIG. 3. **Mevalonate reversed COX-2 induction by statins.** hASMC were co-incubated for 48 h in the absence or presence of 100 μM mevalonate, 25 μM statins, and 0.5 ng/ml IL-1α. Western blot analysis of COX-2 was performed as described under "Experimental Procedures." Results are representative of four separate experiments.

Effect of Mevalonate on Statin-induced COX-2—To determine the mechanism of COX-2 protein induction by statins, cells were first co-incubated with mevastatin or lovastatin in the presence of different compounds of the cholesterol biosynthesis pathway. We tested the effect of mevalonate, the direct HMG CoA reductase metabolite, to check whether the effect of statins is due to direct inhibition of this enzyme. We incubated cells with 25 μM mevastatin or lovastatin together with 100 μM L-mevalonate. Induction of COX-2 by statins, both in the absence or presence of IL-1α, was reversed by L-mevalonate (Fig. 3). Mevalonate alone did not modulate in a statistically significant manner the IL-1α-dependent COX-2 induction (18% increase in cells treated by IL-1α + mevalonate compared with IL-1α alone, unpaired *t* test, $n = 4$). Up-regulation of COX-2 by mevastatin or lovastatin was not modified after treatment with 10 μM squalene, the late metabolite in the cholesterol synthesis pathway (data not shown), suggesting that regulation of cellular cholesterol level is not involved in this effect.

Involvement of Isoprenoids in the Regulation of COX-2—The implication of the isoprenoid compounds in the modulation of COX-2 expression both under basal conditions or after incubation with IL-1α was further confirmed by testing the importance of the isoprenoids intermediates, FPP and GGPP. As shown in Fig. 4, co-treatment of cells with 10 μM GGPP completely reversed the induction of COX-2 by mevastatin or lovastatin in the presence or absence of IL-1α. In contrast, 10 μM FPP did not significantly modify the effect of statins or of IL-1α (Fig. 4; 7% increase in cells treated by IL-1α + FPP compared with IL-1α alone, unpaired *t* test, $n = 3$). These findings suggested to us that geranylgeranylated proteins negatively regulate COX-2 expression. To test this hypothesis further, we used GGTI-286, a recently described selective inhibitor of geranylgeranyltransferase (21). Induction of COX-2 by GGTI-286 alone was detected at 10 μM after 24-hour of incubation. In the presence of IL-1α, the increase in COX-2 expression was clear at 5 and 10 μM (Fig. 5). 10 μM GGTI-286 also increased PGI_2 production in a statistically significant manner (50.6 $\pm$ 10.9 compared with 12.3 $\pm$ 2.8 ng/ml of 6-keto-$PGF_{1\alpha}$ for untreated cells, $n = 4$, $p < 0.03$). We further used a selective inhibitor of farnesyltransferases, FTI-277, to check whether farnesylation was involved in COX-2 expression (22). Since it has been reported that FTI-277 had low IC_{50} for inhibiting farnesyltransferases (IC_{50} = 20 nM compared with the IC_{50} for GGTI-286 = 3 μM), we first checked the effect of low concentrations of FTI-277 (0.3–3 μM) and showed no modification of COX-2 expression nor prostacyclin formation. In separate experiments, we verified as well that further treatment of cells with 10 μM FTI-277 did not alter COX-2 expression (Fig. 5).

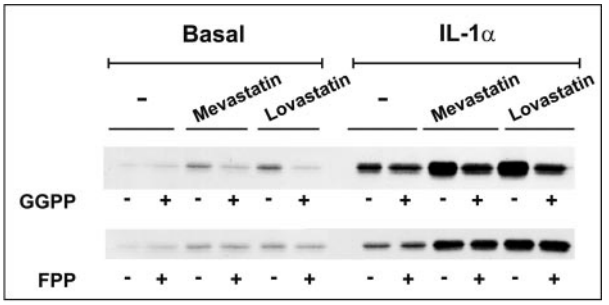

FIG. 4. **Effect of GGPP or FPP on the COX-2 induction by statins in the absence or presence of IL-1α.** hASMC were treated for 48 h in the absence or presence of 10 μM GGPP or FPP, 25 μM statins, and 0.5 ng/ml IL-1α. Western blot analysis of COX-2 expression was performed as described under "Experimental Procedures." Results are representative of three different experiments.

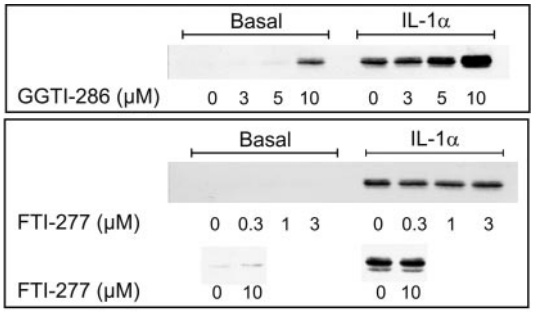

FIG. 5. **Effect of inhibitors of geranylgeranyltransferases (GGTI-286) and farnesyltransferases (FTI-277) on COX-2 expression.** hASMC were treated for 24 h in the absence or presence of 3, 5, and 10 μM GGTI-286 or 0.3, 1, 3 μM FTI-277. Results are representative of three different experiments. In two separate experiments, cells were treated with 10 μM FTI-277. Western blot analysis of COX-2 expression was performed as described under "Experimental Procedures."

Treatment of Cells with Mevastatin or GGTI-286 Increases COX-2 mRNA Level—We next performed Northern blot analysis of COX-2 mRNA to demonstrate that modulation of COX-2 protein by statins or GGTI-286 was also obtained at the RNA level. Northern blot analysis was carried out with 25 μM mevastatin, the concentration required to induce COX-2 protein. Cells were incubated in 60-mm plates for 24 and 36 h with IL-1α in the absence or presence of mevastatin. Incubation of cells with mevastatin in the presence of IL-1α resulted in a 3.2- and 5.8-fold increase in COX-2 mRNA level at 24- and 36-h incubation times, respectively, compared with cells treated with IL-1α alone (Fig. 6*A*).

We next compared the stability of the COX-2 mRNA of IL-1α-treated cells in the presence or absence of mevastatin. Cells were treated with 0.5 ng/ml of IL-1α in the presence or absence of 25 μM mevastatin for 24 h. 5 μM actinomycin D was then added to block transcription, and total RNA was extracted after an incubation period of 0.5, 1, 2, 4, or 8 h. Fig. 6*B* reveals little difference in the mRNA stability of COX-2 IL-1α and IL-1α + mevastatin-treated cells with half-lives of 4.7 and 5.5 h, respectively.

We finally tested the effect of GGTI-286 on COX-2 mRNA. Cells were incubated with 10 μM GGTI-286 for 8, 12, and 18 h. Fig. 7 shows an increase in COX-2 mRNA levels by GGTI-286

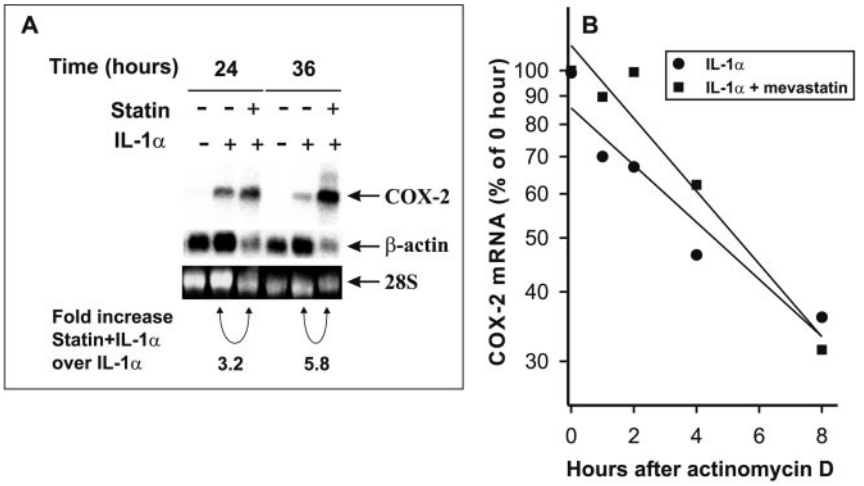

FIG. 6. **Northern blot analysis of COX-2 mRNA and its half-life in the presence of statins.** *Panel A*, hASMC cells were treated for 24 or 36 h in the absence or presence of 25 μM mevastatin and 0.5 ng/ml IL-1α. Northern blot analysis of COX-2 mRNA was performed as described under "Experimental Procedures." The blot was stripped and reprobed for β-actin. The ratio of COX-2 to β-actin was determined. Results are expressed as fold increase of IL-1α + mevastatin over IL-1α alone. Total mRNA loadings were checked by ethidium bromide staining of the gels, and ribosomal 28 S are reported. Differences in β-actin corresponded to difference in RNA loading. Results are representative of three different experiments. *Panel B*, time-course of COX-2 mRNA decay. hASMC were treated for 24 h in the presence of 0.5 ng/ml IL-1α with or without 25 μM mevastatin. After addition of 5 μM actinomycin D, total RNA was extracted after 0.5, 1, 2, 4, and 8 h. Northern blot analysis was performed as in *panel A*. The ratio of COX-2 to β-actin was determined, and time 0 was taken as 100%. Results are representative of two different experiments.

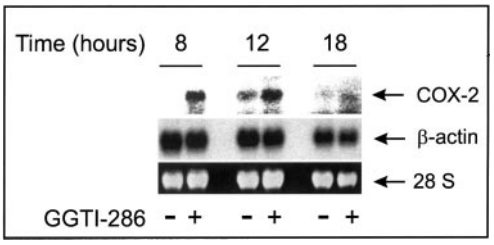

FIG. 7. **Time-course of COX-2 mRNA formation by GGTI-286.** hASMC cells were treated with 10 μM GGTI-286. Northern blot analysis of COX-2 mRNA was performed as described under "Experimental Procedures." Total mRNA loadings were checked by ethidium bromide staining of the gels, and ribosomal 28 S are reported. Results are representative of three different experiments.

at 8 and 12 h followed by a decrease at 18 h.

Effect of Modulators of Rho GTPases on Prostacyclin Formation and COX-2 Expression—The Rho family of GTP-binding proteins contains many geranylgeranylated proteins that play an important role in cell adhesion, actin dynamics, or regulation of gene transcription and includes Rho, Rac, and Cdc42 proteins (23, 24). To determine whether the inhibition of these proteins mediates the effects observed by statins, we incubated the cells in the presence or absence of IL-1α or *C. difficile* toxin B (toxin B), an inhibitor of the different Rho GTPases (25). Treatment of hASMC for 6 h with 2 nM of toxin B induced COX-2 as shown in Fig. 8*A*. Toxin B also increased COX-2 mRNA (Fig. 8*B*). In parallel, we tested the effect of *E. coli* cytotoxic necrotizing factor 1 (CNF1), a toxin reported to activate Rho GTPase proteins by preventing Rho GTP hydrolysis (26, 27). Treatment of cells with 30 nM CNF1 inhibited induction of COX-2 by IL-1α (Fig. 8*C*). Both toxins modulated PGI_2. Toxin B increased PGI_2, whereas CNF1 inhibited IL-1α-dependent formation (Table I).

We further tested the effect of *C. botulinum* C3 transferase, a selective inhibitor of Rho A and C proteins, on COX-2 expression and prostacyclin formation. We used a fusion protein with the TAT protein of HIV with the *C. botulinum* C3 transferase to allow rapid introduction of the protein into cells (14). Treatment of the cells with 20 μg/ml of TAT-C3 transferase resulted in an induction of COX-2 with a maximal expression at 6 h (Fig. 9*A*). In the same samples, PGI_2 was statistically increased

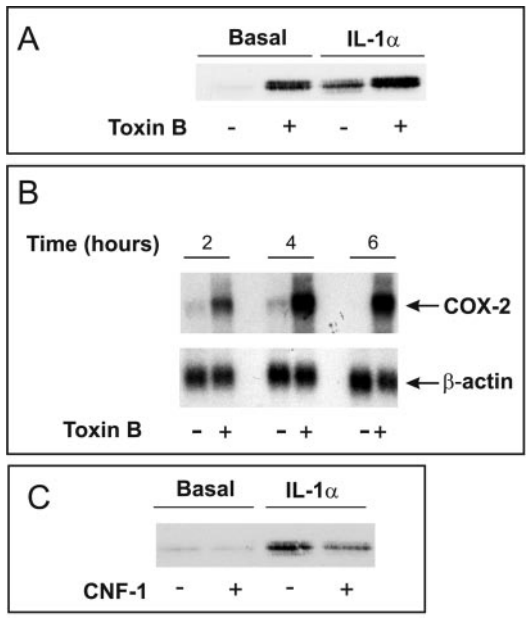

FIG. 8. **Effect of *C. difficile* Toxin B or CNF1 on COX-2 expression in hASMC.** *Panel A*, Western blot analysis of COX-2 after incubation of cells for 6 h in the presence or absence of 0.5 ng/ml IL-1α with or without 2 nM Toxin B. *Panel B*, time-course of COX-2 mRNA formation by 2 nM of Toxin B. *Panel C*, Western blot analysis of COX-2 after incubation of cells for 6 h in the presence or absence of 0.5 ng/ml IL-1α with or without 30 nM CNF1. Western and Northern blot analyses were performed as described under "Experimental Procedures." Results are representative of three experiments for the Western blots of COX-2 and two experiments for the Northern blots.

(Table I). Finally, since the serine/threonine kinases ROCK are among the identified targets of Rho, we tested the effect of a selective inhibitor of the ROCK I and II, Y-27632, on COX-2 expression (28). Cells were incubated with 10 μM Y-27632 for 3, 6, and 24 h. This treatment resulted in the increase in COX-2 expression at 6 and 24 h as shown in Fig. 9*B*.

DISCUSSION

We have shown for the first time that inhibitors of HMG CoA reductase, lovastatin and mevastatin, increase the expression

TABLE I
Prostacyclin formation in response to inhibitors or activators of Rho GTPases

Cells were incubated in the absence or presence of 0.5 ng/ml IL-1α with or without Toxin B or CNF1 for 6 hours. In a different set of experiments, cells were incubated in the presence or absence of TAT-C3 transferase for 6 hours. Each treatment group was compared with its control.

Treatment	IL-1α[a]	
	−	+
	ng/ml	
No treatment	10.1 ± 1 (n = 4)	38 ± 2.2 (n = 4)
Toxin B (2 nM)	31.4 ± 4.6[b] (n = 4)	44.3 ± 4.5 (n = 4)
CNF1 (30 nM)	5.3 ± 0.8 (n = 3)	20.7 ± 1.3[c] (n = 4)
No treatment	7.4 ± 1 (n = 3)	
TAT-C3 transferase (20 μg/ml)	13.1 ± 1.6[d] (n = 3)	

[a] Values indicate the amount of 6-keto-$PGF_{1\alpha}$.
[b] $p < 0.02$ versus cells with no treatment.
[c] $p < 0.01$ versus IL-1α-stimulated with no treatment.
[d] $p < 0.05$, versus cells with no treatment.

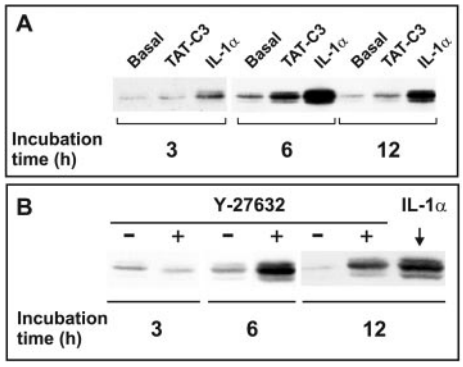

FIG. 9. **Effect of TAT-C3 transferase and Y-27632 on COX-2 expression.** hASMC were incubated for 3, 6, and 12 h in the absence or presence of 0.5 ng/ml IL-1α or 20 μg/ml TAT-C3 transferase (*Panel A*) or for 3, 6, and 24 h in the absence or presence of 0.5 ng/ml IL-1α or 10 μM Y-27632. Western blot analysis of COX-2 was performed as described. Results are representative of two to three different experiments for *panel A* and two different experiments for *panel B*.

of COX-2 in human aortic smooth muscle cells. We confirmed the implication of the mevalonate pathway and isoprenoids in the negative modulation of the expression of COX-2 by demonstrating that the direct metabolite of HMG CoA reductase, mevalonate and the isoprenoid, GGPP, reversed the induction of COX-2 by statins and that GGTI-286, the inhibitor of geranylgeranyltransferases, induced COX-2 as for statins. Our results suggest that geranylgeranylated proteins are involved in the down-regulation of COX-2. Although COX-2 expression has been reported to implicate activation of farnesylated proteins such as Ha-Ras or Ki-Ras, farnesylation does not seem important in the present study (29–32). On the other hand, squalene, the precursor of cholesterol, did not modify COX expression, suggesting that regulation of cellular cholesterol level is not involved in this effect. Our results are different from those reported by Inoue *et al.* (33) who showed that some statins reduce the level of inflammatory elements in human umbilical vein endothelial cells such as IL-1, IL-6, and COX-2. Falke *et al.* had also demonstrated a moderate inhibition of the release of prostacyclin in human umbilical vein endothelial cells and bovine aortic smooth muscle cells in culture (34). The divergence in the regulation of COX-2 expression might be cell type- and species-dependent or also related to the class of statins (33). The participation of vascular COX-2 in inflammation is still ambiguous. In our study, the increase in the expression of COX-2 might reflect either an adverse pro-inflammatory role of statins on vasculature or a positive effect if COX-2 expression in vessels were considered as beneficial in participating for instance in physiological functions or anti-inflammatory processes. Although COX-2 expression is detected in atherosclerotic plaque where it is distributed in the intima and media (35) and urinary prostacyclin derivatives increased in patients with atherosclerotic plaques (36), the consequence of endothelial and smooth muscle cell increase in COX-2 expression is still a matter of debate. An increase in urinary prostacyclin derivatives has been shown recently in two murine models of atherosclerosis, ApoE-deficient mice and low-density lipoprotein receptor-deficient mice on a high fat diet (37, 38). Selective inhibition of COX-2 failed to decrease the extent of atherosclerosis in these models suggesting that COX-2, although expressed in the atherosclerotic lesions, does not participate in its progression (38). Little is known about the roles of PGI_2 and PGE_2 on vessels. PGI_2 and PGE_2 inhibit vascular proliferation and cell-cell interaction (39, 40). In normal volunteers, COX-2 contributes to the formation of PGI_2 *in vivo* since selective inhibitors of COX-2 decreased its systemic formation (8). Laminar shear forces have also been demonstrated to increase COX-2 expression in cultured endothelial cells (41). As suggested recently by FitzGerald and Patrono, PGI_2 may be part of a homeostatic defense mechanism limiting the consequences of platelet activation *in vivo* (42). Vascular PGE_2 has also been reported to inhibit the expression of adhesion molecules such as ICAM-1 (43), although this prostaglandin is considered to have pro-inflammatory effects. This suggest that COX-2-dependent release of PGE_2 could have a regulatory role in limiting inflammatory responses and consequently have a protective role in cardiovascular disease (44). Thus, statin therapy might increase the vasodilator, anti-thrombotic, or anti-inflammatory properties of the vascular wall by increasing PGI_2 and PGE_2 in a COX-2-dependent manner. Whether this regulation occurs *in vivo* following statin administration and is similarly important in all vascular beds remains to be seen.

Our results are comparable with those described recently on the modulation of expression of nitric oxide synthases by statins and geranylgeranylated proteins. Laufs and Liao (45), using GGPP, reported that treatment of human endothelial cells with mevastatin increased NOS-III expression as a result of inhibition of geranylgeranylation. Finder *et al.* (46) also showed that mevastatin and GGTI-298, another GGTI similar to GGTI-286, increased NOS-II expression in rat pulmonary artery smooth muscle cells. Rho GTPases are geranylgeranylated proteins important in cell migration, contraction, cell shape, adhesion, and gene expression (23, 24). It has been shown that one of these proteins, Rho, is linked to the activation, contraction, or proliferation of vascular cells (47). Moreover, Rho (A or C) controls the expression of different proteins in vessels including NOS-II (48), NOS-III (45), TGFβ (49), and pre-pro-endothelin-1 (ET-1) expression (50, 51). In our system, *C. difficile* toxin B and CNF1, selective inhibitor and activator of all Rho GTPases, respectively, affected COX-2 expression either at the basal level or after activation by IL-1α. This suggests that Rho GTPases participate in COX-2 regulation. The further demonstration that both *C. botulinum* C3 transferase and Y-27632, the selective inhibitors of Rho and ROCK, respectively, induced COX-2 expression stressed the role of these proteins in the negative regulation of COX-2 expression and PGI_2 formation and that these geranylgeranylated small G proteins are one of the targets of statins.

Since it has been described that induction of COX-2 in some cells could participate in the apoptosis process, we tested whether statins induced apoptosis in HASMC. Although it has been described that some statins could induce apoptosis or sensitize hASMC to death receptor-induced apoptosis (52, 53),

careful examination of Hoechst 33342-stained cells showed no evidence of cell death under our conditions. Observation under phase contrast, however, evidenced some morphological changes with a slight increase in rounded cells that appear to become less adherent.

The effect of statins on COX-2 expression was also noted in the presence of IL-1α, a cytokine important in the atherosclerotic vascular wall and largely described to activate COX-2 (17, 20, 54). We treated cells with IL-1α first to verify the normal induction of COX-2 in hASMC as reported previously (55, 56) and to further check whether statins could modify COX-2 induction. We showed that these two statins increased COX-2 expression in the presence of IL-1α.

The mechanism by which inhibition of Rho increases COX-2 expression is not clear. Rac1 has been shown to down-regulate Rho activation (57). Signaling through IL-1 receptors implicates activation of Rac, which in turn is involved in the activation p38 MAP kinases and NF-κB, both of which are important in the regulation of COX-2 expression (23, 58, 59). The balance between these two GTPases might be important in determining gene expression, *i.e.* COX-2. Up-regulation of COX-2 is also induced by protein kinase A activation in response to prostaglandins for example in different cell types including macrophages, vascular cells, and hepatic stellate cells (60–62). In view of the results presented here, it may be of interest that protein kinase A is known to phosphorylate and inactivate Rho A (63) and induce COX-2 (61, 62). Further investigation is required to understand whether or not these mechanisms are involved in the regulation of COX-2 expression by statins and if they are similar in untreated and IL-1α-stimulated cells

In the present study, statin did not modify the half-life of the mRNA of COX-2 in IL-1α-activated cells indicating that transcriptional regulation is essentially implicated in the induction of COX-2 by statins. Our results are similar to those reported for NOS-II where regulation at the transcriptional level has been demonstrated in response to Toxin B, C3 transferases (48), and Y-27632 (64) but different from those indicating that statins and Rho GTPase inhibitors could increase the stability of the NOS-III mRNA (45, 46). Recently, Slice *et al.* have demonstrated in NIH 3T3 cells that $G\alpha_{13}$ is able to increase COX-2 promoter activity through activation of Rho A (65, 66). These data contrast with ours, which show an inhibition of COX-2 induction by Rho and Rho-associated kinases. This difference in the regulation of COX-2 might be cell-type and species dependent.

We obtained increase in prostacyclin synthesis and COX-2 expression at high concentrations of lovastatin or mevastatin corresponding to 20–100 times the therapeutic doses. It seems that these concentrations are essential for the inhibition of geranylgeranylation of proteins, *i.e.* Rho GTPases. Previous studies have reported modification of protein expression using high concentrations of lovastatin or mevastatin in different cultured cells (45, 46, 67). It is noteworthy to mention that some of these reported effects, *i.e.* the modulation of NOS-II and NOS-III expression, were further demonstrated *in vivo* in mice or rats treated with statins or the Rho kinase inhibitor Y-27632 (68, 69). Although we are not sure that the up-regulation of COX-2 by statins we described *in vitro* will occur *in vivo* at lower concentration of statins, it remains interesting to test whether statins or direct inhibitors of Rho or the Rho kinases such as Y-27632 can modulate *in vivo* the expression of COX-2.

Clinical trials of statin therapy showed an improvement in cardiovascular end points, which is incompletely explained by low-density lipoprotein cholesterol modifications. Cholesterol-independent mechanisms have been suggested to explain the beneficial effect of statins beyond their effects on low density lipoprotein cholesterol (70). Statins have also been reported to reduce stroke incidence (71). Therefore, statins could be responsible for a large favorable effect on endothelial function, plaque architecture and stability, cellular adhesion, migration and proliferation, thrombosis, and inflammation. The many *in vitro* and *in vivo* data support a role of Rho in vascular function and gene expression. Rho might be one of the potential targets of statins. Additional studies are required to understand how Rho is activated and how it regulates cellular functions under physiological conditions (72, 73).

In summary, by preventing geranylgeranylation of some proteins including Rho, statins increase the expression of COX-2 in human vascular smooth muscle cells. Inhibition of Rho activity in vessels may be important to restore vascular function and could account for the cholesterol-unrelated effects of statins.

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