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Atorvastatin limits the pro-inflammatory response of rat aortic smooth muscle cells to thrombin

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Abstract

Thrombin, a serine protease, plays an important role in the progression of atherosclerosis. How atorvastatin could limit the pro-inflammatory response to thrombin was studied in cultured rat aortic smooth muscle cells. The variations in expression of interleukin-6, heme oxygenase-1, p^{22phox} and Mox-1 mRNAs were evaluated by reverse transcriptase–polymerase chain reaction (RT-PCR). Interleukin-6 release was determined using the B9 cell assay. Nuclear factor-kappa B (NF-κB) translocation was analysed by electrophoretic mobility shift assay (EMSA) and RhoA protein translocation by Western blot. Thrombin activated interleukin-6 secretion and mRNA expression in smooth muscle cells in a dose-dependent manner. The greatest effect on mRNA expression was obtained after 1 h of stimulation. Preincubation (72 h) of the cells with various concentrations of atorvastatin prevented this effect. Simultaneous addition of mevalonate overcame this statin effect. Thrombin was without effect on p^{22phox} and heme oxygenase-1 mRNA expression but, after 3 h of stimulation, induced a two-fold increase in that of Mox-1. Preincubation with atorvastatin dose-dependently downregulated this Mox-1 mRNA expression. In addition, thrombin induced NF-κB translocation and membrane translocation of RhoA in smooth muscle cells which were both prevented by pre-treatment of the cells by atorvastatin. These data demonstrate the ability of atorvastatin to prevent the induction by thrombin of a pro-inflammatory phenotype in smooth muscle cells.

Keywords: Atorvastatin; α-Thrombin, human; Interleukin-6; NF-κB (nuclear factor-κB); RhoA

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an endoplasmic reticulum protein, catalyses the reductive deacylation of HMG-CoA to mevalonate (Brown and Goldstein, 1986; Hampton et al., 1996; Istvan and Deisenhofer, 2000). This rate-limiting step in cholesterol biosynthesis can be strongly inhibited by a class of lipid-lowering compounds known as the HMG-CoA reductase inhibitors or statins (Davignon et al., 1992; Stern et al., 2000). Statins are extensively used in medical practice because they are effective in the prevention of cardiovascular events (Tonkin et al., 2000). It appears likely that the beneficial actions of statins involve mechanisms both dependent and independent of their cholesterol-lowering effect (Guijarro and Egido, 1997; Massy et al., 1996; Vaughan et al., 1996). They not only improve the lipid profile but also have direct vascular actions (Vaughan et al., 1996). Recent in vitro studies demonstrated that statins, in addition to their lipid lowering effects, have anti-inflammatory properties and thus may regulate important molecules in vascular biology (Bustos et al., 1998; Vaughan et al., 1996). Because thrombin plays an important role in the progression of atherosclerosis (Harker et al., 1995), we explored whether atorvastatin is able to modulate the pro-inflammatory response to thrombin in rat aortic smooth muscle cells. To see whether atorvastatin can directly interfere with the intracellular regulatory signaling pathway, we investigated the potential preventive effect of atorvastatin on thrombin-induced cytokine production (interleukin-6) and activation of the transcription nuclear factor-kappa B (NF-κB). As several studies have shown that thrombin mediates most of its responses through activation of the G protein-coupled receptor (protease-activated receptor-1, PAR1), this study was conducted specifically to determine whether atorvastatin may have direct lipid-inde-

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pendent effects on cell functions by altering the level of prenylated protein RhoA. In addition, we examined the effect of atorvastatin on the mRNA expressions of p^{22phox} and Mox-1, because recent studies have demonstrated an association between clinical risk factors for atherosclerosis and NAD(P)H oxidase enzymatic activity (Azumi et al., 1999; Guzik et al., 2000).

2. Materials and methods

2.1. Reagents and chemicals

Atorvastatin–calcium was a gift from Pfizer, France. Recombinant human interleukin-6, L-glutamine, non-essential amino acids, sodium pyruvate solution, collagenase type I and moloney-murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase) were purchased from Life Technologies. Dulbecco's modified Eagle's media (DMEM), RPMI 1640 and fetal calf serum were obtained from BioMedia, France. Mevalonate, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), protease inhibitors, bovine serum albumin, trypsin-EDTA and penicillin–streptomycin–amphotericin solutions were from Sigma, France. Mouse monoclonal antibody anti-RhoA and NF-κB consensus oligonucleotide were purchased from Santa Cruz Biotechnology. Promoter-specific transcription factor (SP-1) consensus oligonucleotide was supplied by Promega, and HEPES by Biowittaker. The chemical reagents were obtained from Merck.

2.2. Cell culture

Rat aortic smooth muscle cells were isolated as previously described (Battle et al., 1994). Briefly, male Wistar rats (Iffa Credo, France), weighing 250–280 g, were killed by an overdose of pentobarbital (Sanofi) and the thoracic aortas were aseptically excised, rinsed and placed in phosphate-buffered saline (PBS). Adhering fat and connective tissue were removed by blunt dissection. The adventitia was mechanically dissociated from the media. The media was sliced into 1–2-mm rings, and placed in freshly prepared enzyme solution containing collagenase (367 U/ml), and elastase (4.4 U/ml) (Worthington Biochemical) for 45–60 min at 37 °C. The cell suspension was centrifuged, resuspended in DMEM supplemented with 10% fetal calf serum, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, plated out in 25-cm^2 plastic culture flasks (Corning, France), and kept at 37 °C in a humidified incubator of 95% air/5% CO_2. Smooth muscle cells were characterized by using an antibody raised against α-actin (Gabbiani et al., 1981). Cells between passages 3 and 4 in the exponential phase of growth were used for all of the following experiments. After confluence, the smooth muscle cells were treated with or without atorvastatin and mevalonate for the indicated time and dose in 10% fetal calf serum-DMEM. After treatment with atorvastatin, cells were washed with PBS, incubated for the indicated time in serum-free medium, and subsequently stimulated with human-α-thrombin, purified as previously described (Bezeaud et al., 1985) at different concentrations for different periods of time as indicated in results. Unstimulated cells were considered as controls.

2.3. Reverse transcription–polymerase chain reaction (RT-PCR) analysis

The interleukin-6, p^{22phox}, Mox-1 and heme oxygenase-1 mRNA expressions were assessed in smooth muscle cells by comparative RT-PCR. For these experiments, the cells were cultured on six-well culture plates.

2.3.1. Reverse transcription

Extraction of total RNA from smooth muscle cells was performed with Trizol reagent according to the manufacturer's instructions (Life Technologies). Prior to RT reaction, 1 μg of total RNA was primed with 1 μg of oligo $d(T)_{12-18}$ (Pharmacia Biotech) and heated at 65 °C for 10 min; the tubes were then chilled on ice for 1 min. RT was performed at 37 °C for 60 min using 200 U of M-MLV reverse transcriptase, 20 U RNase inhibitor, 1 × RT buffer (75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl pH 8.3), 10 mM dithiothreitol and 0.5 mM of each dNTP (Sigma).

2.3.2. Polymerase chain reaction

PCR was performed on 3 μl of RT product using 1 × Taq-buffer (1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris–HCl pH 8.3), 1.25 U Taq DNA polymerase (Life Technologies), 0.05 mM dNTP (Pharmacia), 10 pmol of sense and antisense primers and 4×10^5 cpm of a ^{33}P-radiolabelled mix of both primers. The primers were designed on the basis of published rat cDNA sequences and are reported in Table 1. The

Table 1
Primer sequences derived from rat cDNAs, used in the RT-PCR assays

Gene	Sense	Antisense	Annealing temperature (°C)	Amplification size (bp)
IL-6	5′-CTC TGA ATG ACT CTG GCT TTG-3′	5′-CTT GGG ACT GAT GTT GTT GAC-3′	63	389
Mox-1	5′-TCC CTT TAC TCT GAC CTC TG-3′	5′-CAT AAG AAA ACC CCC ACC AC-3′	60	578
p^{22phox}	5′-GGA GTG CTC ATC TGT CTG CTG-3′	5′-GTT GGT AGG TGG CTG CTT GAT-3′	65	306
HO-1	5′-CAG GCA TAT ACC CGC TAC CT-3′	5′-TCT GTC ACC CTG TGC TTG AC-3′	60	209
GAPDH	5′-GTG AAG GTC GGA GTC AAC G-3′	5′-GGT GAA GAC GCC AGT ACT CTC-3′	55	302

interleukin-6, p^{22phox}, Mox-1 and heme oxygenase-1 mRNA expressions were calculated by normalizing to that of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.4. Interleukin-6 bioassay

B9 cells (interleukin-6 murine hybridoma cells) were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES buffer, 2.5 mM sodium pyruvate, (× 5) non-essential amino acids, 10% fetal calf serum and 5 ng/ml of recombinant human interleukin-6. The interleukin-6 content was assayed in smooth muscle cell supernatants by monitoring their ability to induce the proliferation of B9 cells using standardized procedures described by different authors (Aarden et al., 1985, 1987; Rees et al., 1999). After careful washing with PBS, the B9 cells were seeded in a 96-well microtiter plate (100 000 cell/well) and incubated with 10% fetal calf serum-RPMI at 37 °C for 72 h in humidified incubator 5% CO_2. Proliferation of the B9 cells was quantified by the addition of 0.5 mg/ml of MTT for 4 h at 37 °C. Finally, formazan crystals were dissolved by adding an equal volume of isopropanol/HCl. Absorbance values at 540 nm were measured using a micro-plate reader. Each test was run in triplicate. Interleukin-6 concentration was determined by comparison with recombinant human interleukin-6 proliferation rate assay used as a standard.

2.5. Preparation of nuclear extracts and electrophoretic mobility shift assay

2.5.1. Preparation of nuclear fraction

After appropriate treatments, the smooth muscle cells were harvested in 1 ml of ice-cold PBS and centrifuged for 1 min at 2000 rpm, 4 °C. The cell pellet was lysed in 50 μl of hypotonic buffer (buffer A) containing 10 mM HEPES pH 7.9, 10 mM KCl, 300 mM sucrose, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.4% protease inhibitor cocktail and containing 0.5% nonidet P-40 for 5 min on ice, and the samples were vortexed vigorously for 15 s, before centrifuging at 14 000 rpm for 5 s at 4 °C. The cell pellet was washed a second time in 100 μl of buffer A. Then, the lysates were centrifuged at 14 000 rpm for 5 s at 4 °C and the pellets were resuspended in 100 μl of hypertonic buffer (buffer B) composed of 20 mM HEPES pH 7.9, 1 mM KCl, 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail and 20% (v/v) glycerol. They were mixed vigorously by vortexing for 15 s, sonicated for 10 s and centrifuged at 14 000 rpm for 5 s at 4 °C. The nuclear extracts were stored at − 80 °C until used. Protein concentrations of the nuclear extracts were determined (Biorad, protein assay, Bradford).

2.5.2. Electrophoretic mobility shift assay (EMSA)

Binding of transcription factor NF-κB was examined using the consensus oligonucleotide of NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). The oligonucleotide was end-labeled with (^{33}P)γATP (Amersham Pharmacia Biotech.) using T4 polynucleotide kinase (Promega). The labeled probe was purified using a ProbeQuant G-50 microcolumn (Amersham Pharmacia Biotech., 3000 rpm, 1 min) and recovered in Tris–EDTA buffer pH 8.0. Binding reactions included 20 μg of nuclear extracts in incubation buffer containing 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 2.5 mM dithiothreitol, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.25 mg/ml poly(dI-dC)poly(dI-dC) and 20% glycerol. After 10 min, the labeled oligonucleotide (4×10^5 cpm) was added and the mixture was incubated for 20 min at room temperature in a final volume of 10 μl. When indicated, controls for specific or nonspecific competitions were performed using unlabeled NF-κB or SP-1 oligonucleotide (5′-ATT CGA TCG GGG CGG GGC GAG C-3′) and added 20 min before the incubation with the labeled oligonucleotide. Immediately after binding, the nucleoprotein-oligonucleotide complexes were separated from unbound oligonucleotide by electrophoresis on a non-denaturing 4% acrylamide gel at 100 V for 4 h using 0.5 × Tris–borate–EDTA buffer (0.5 × TBE). The gel was then dried and exposed overnight at − 80 °C to autoradiographic film (Kodak Film). Bands were finally scanned and signals were densitometrically analysed.

2.6. Extraction of cytosolic and membrane proteins-bound RhoA

Confluent rat aortic smooth muscle cells were preincubated for 72 h with various concentrations of atorvastatin as indicated in the Results section. One hour before lysis, the cell culture medium was replaced by free-serum DMEM. Cells were then stimulated with thrombin for 30 min, washed twice with PBS and lysed in 200 μl/10 cm^2 ice-cold hypotonic lysis buffer containing 5 mM Tris–HCl pH 7.0, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM $MgCl_2$, 2 mM dithiothreitol and freshly added protease inhibitors and incubated for 30 min on ice. Cells were scraped off and sucked back and forth five times through a 25-gauge needle. Membrane and cytosolic fractions were separated by centrifugation at 100 000 × g for 1 h. The membrane pellet was dissolved in an equal volume of lysis buffer + 1% triton X-100.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

For the RhoA Western blot, aliquots of the cytosolic and membrane fractions, containing 10–60 μg of protein, respectively, were heated for 5 min at 100 °C under reducing conditions (2-β-mercaptoethanol). The protein samples were electrophoresed in a 4% SDS-PAGE stacking gel for

10 min at 100 V and then in a 12.5% SDS-PAGE running gel for 50 min at 200 V. The proteins were then transferred to a nitrocellulose membrane (Amersham, Life Sciences) at 300 mA for 1 h. After blotting, the membranes were stained with ponceau red (Sigma) to assess the efficiency of transfer. The residual binding capacity of the membranes was blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TTBS) for 1 h and then incubated with mouse monoclonal anti-RhoA antibody (1:250 dilution) for 1 h at room temperature. The membranes were washed in TTBS for 15 min four times and then incubated with the second antibody against mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:2500 in 1% bovine serum albumin-TTBS for 1 h. The membranes were washed again in TTBS for 15 min four times, incubated in enhanced chemiluminescence reagents (ECL) (Perkin Elmer, Life Sciences) and the autoradiography was performed at room temperature. The films were scanned and the appropriate exposures were quantified by densitometry.

2.8. Statistical analysis

Data are expressed as means ± S.E.M. of three independent experiments, each performed in triplicate. For the comparison between a treatment group and control, one-way analysis of variance (ANOVA) was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Thrombin induces cytokine mRNA expression

The initial experiments were performed to examine the effect of increasing doses of thrombin on interleukin-6 mRNA expression in rat aortic smooth muscle cells using RT-PCR methodology (Fig. 1A). Smooth muscle cells were incubated with various concentrations of thrombin for 1 h. Unstimulated cells not exposed to thrombin demonstrated constitutive expression of interleukin-6 mRNA (Fig. 1A). After 1 h of exposure to 50 ng/ml of thrombin, the level of interleukin-6 mRNA was significantly increased compared with control conditions. This effect was further enhanced, in a dose-related manner, up to 500 ng/ml. Thus, treatment with thrombin (50, 100 and 500 ng/ml) dose-dependently upregulated interleukin-6 mRNA. Among the doses tested, 100 ng/ml was chosen to study the kinetics of the induction of interleukin-6 mRNA and used in all the following experiments. Fig. 1B shows the time course of the effect of 100 ng/ml thrombin on interleukin-6 mRNA expression. After 1 h of stimulation, we consistently observed that 100 ng/ml of thrombin induced a four-fold increase in interleukin-6 mRNA expression as compared to control untreated cells. This effect was attenuated in a time-dependent manner (Fig. 1B).

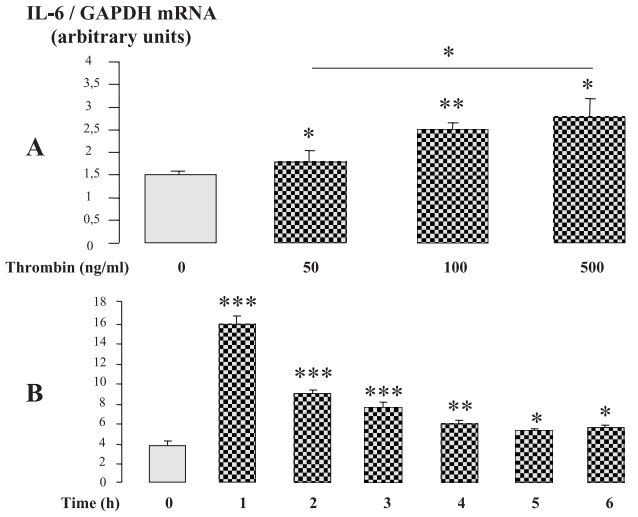

Fig. 1. Dose- (A) and time-dependent (B) effects of thrombin on interleukin-6 mRNA expression. Smooth muscle cells were stimulated with 50, 100 and 500 ng/ml thrombin for 1 h (A). Thrombin induces a dose-dependent increase in interleukin-6 mRNA expression as shown by RT-PCR using the GAPDH housekeeping gene for normalization (A). A dose of 100 ng/ml of thrombin was chosen to study the kinetics of the induction of interleukin-6 mRNA. At this dose, thrombin induced a four-fold increase in interleukin-6 mRNA expression as compared to control unstimulated cells after 1 h of stimulation (B). Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.2. Downregulation of thrombin-induced cytokine mRNA expression by atorvastatin is overcame by mevalonate

The effect of atorvastatin on thrombin-induced interleukin-6 mRNA expression was assessed after 1 h of stimulation by 100 ng/ml thrombin on smooth muscle cells-impregnated with atorvastatin for 72 h (Fig. 2). Preincubation with atorvastatin dose-dependently prevented thrombin-induced interleukin-6 mRNA expression. As shown in Fig. 2, a marked effect was obtained with 0.4 μg/ml atorvastatin, which reduced by 50% the stimulatory effect of thrombin. Atorvastatin alone had no effect (Fig. 2). The preventive effect of atorvastatin (0.4 μg/ml) was not observed when mevalonate (500 μM) was added to statin during preincubation (Fig. 3). Mevalonate alone had no effect (data not shown).

3.3. Thrombin induces cytokine release

We assayed interleukin-6 in smooth muscle cell supernatants to gauge the ability of thrombin to induce cytokine production. The protein level in the supernatant was measured at different times using B9 interleukin-6-dependent cells. A small amount of interleukin-6 was detected in the supernatant of control smooth muscle cells not exposed to thrombin at 1, 2, 4 and 6 h (Fig. 4A). Thrombin increased the level of interleukin-6 released in a time-dependent manner. The peak of interleukin-6 production was observed

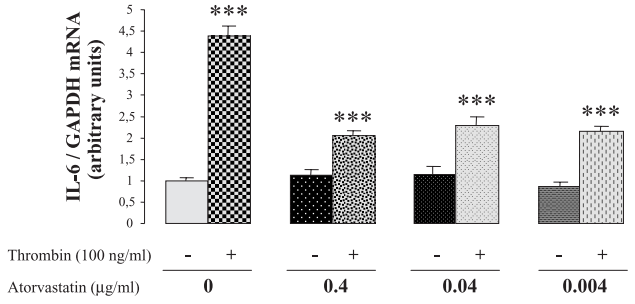

Fig. 2. Atorvastatin (0.4, 0.04 and 0.004 µg/ml) limits thrombin-induced interleukin-6 mRNA expression. Thrombin alone induces a significant increase in interleukin-6 mRNA expression. The effect of atorvastatin on thrombin-induced interleukin-6 mRNA expression was assessed after 1 h of stimulation by thrombin (100 ng/ml) on smooth muscle cells impregnated with atorvastatin for 72 h. 0.4 µg/ml atorvastatin reduced by 50% the effect of thrombin on interleukin-6 mRNA expression, whereas atorvastatin alone had no effect. Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. ***$P<0.001$.

at 6 h. As shown in Fig. 4A, data obtained from this experiment clearly showed that thrombin induced significant levels of interleukin-6 when compared with control unstimulated cells ($P<0.01$). At 24 h, interleukin-6 production in thrombin-stimulated cells was not different from that in unstimulated cells (data not shown).

3.4. Atorvastatin downregulates thrombin-induced cytokine release

In this experiment, we investigated whether atorvastatin could alter the levels of interleukin-6 released under thrombin-stimulation. The effect of atorvastatin on thrombin-induced interleukin-6 release was assessed after 1 h of stimulation by thrombin (100 ng/ml) on smooth muscle cells impregnated with atorvastatin for 72 h (Fig. 4B). Atorvastatin significantly reduced the effect of thrombin on interleukin-6 levels in a concentration-dependent manner (Fig. 4B). The decrease in interleukin-6 production was significant ($P<0.001$) at all the doses of atorvastatin tested. As shown

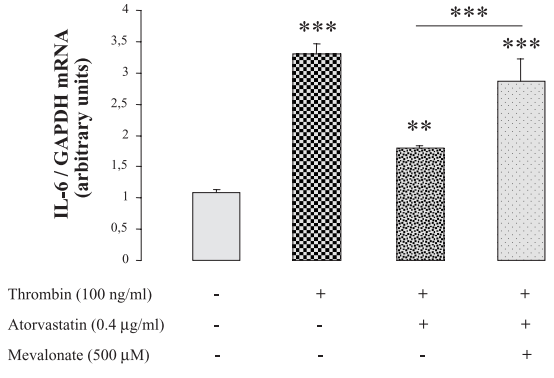

Fig. 3. The effect of atorvastatin (0.4 µg/ml, 72 h) on interleukin-6 mRNA expression was overcome by addition of mevalonate (500 µM, 72 h). Mevalonate alone had no effect (data not shown). Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. **$P<0.01$; ***$P<0.001$.

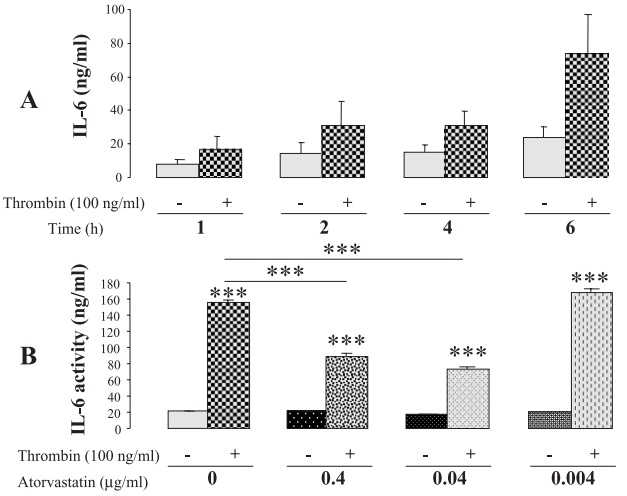

Fig. 4. Time effect of thrombin on interleukin-6 secretion (A). Interleukin-6 secreted by smooth muscle cells after stimulation by thrombin for different times was assessed in the supernatant, after being transferred to B9 interleukin-6-dependent cells. Thrombin induced a time-dependent increase in interleukin-6 secretion and the maximal effect was obtained after 6 h of stimulation. Atorvastatin (0.4, and 0.04 µg/ml) limited thrombin-induced interleukin-6 secretion (B). Thrombin alone induced a significant increase in interleukin-6 secretion. The effect of atorvastatin on thrombin-induced interleukin-6 secretion was assessed after 1 h of stimulation by thrombin (100 ng/ml) on smooth muscle cells impregnated with atorvastatin for 72 h. Atorvastatin (0.4 µg/ml)-impregnated cells secreted 50% less interleukin-6 in response to thrombin than cells without prior incubation with atorvastatin, whereas atorvastatin alone had no effect. Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. ***$P<0.001$.

in Fig. 4B, cells pretreated with 0.4 µg/ml atorvastatin secreted 50% less interleukin-6 in response to thrombin than cells without prior incubation with atorvastatin.

3.5. Effect of thrombin on NAD(P)H oxidase expression

To measure NAD(P)H oxidase expression in smooth muscle cells, the essential subunit of the enzyme, p^{22phox} was quantified at the mRNA level via semiquantitative RT-PCR methodology. Thrombin stimulation by 0 to 500 ng/ml for 1 h (Fig. 5A) and by 100 ng/ml for 1 to 6 h (Fig. 5B) did not induce any significant change in p^{22phox} mRNA expression.

Fig. 6A shows the effect of increasing concentrations of thrombin on Mox-1 mRNA expression. Smooth muscle cells were incubated with thrombin at different doses for 3 h. After stimulation with 100 ng/ml of thrombin, we observed that levels of Mox-1 mRNA were significantly increased ($P<0.001$) compared with unstimulated cells (Fig. 6A).

Fig. 6B demonstrates the time course of the effect of thrombin on Mox-1 mRNA expression. After 3 h of stimulation, thrombin (100 ng/ml) induced a two-fold increase in Mox-1 mRNA expression as compared to control untreated cells ($P<0.001$) (Fig. 6B). This increase in Mox-1 mRNA expression was even greater after 6 h of treatment.

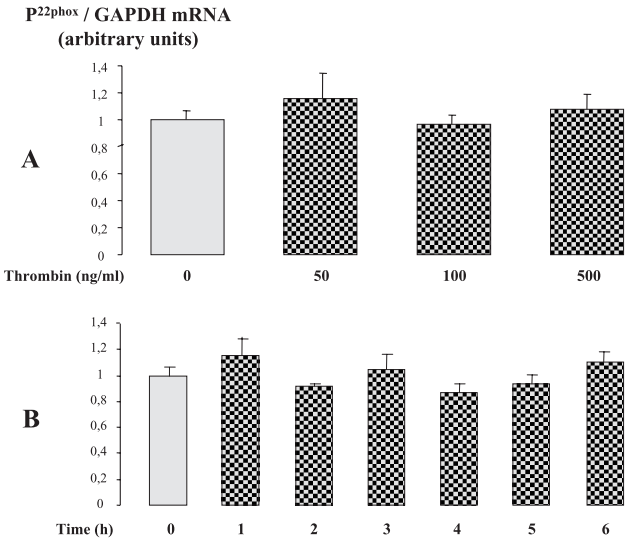

Fig. 5. Dose (A) and time (B) effects of thrombin on p^{22phox} mRNA expression. Rat aortic smooth muscle cells were stimulated with 50, 100 and 500 ng/ml thrombin for 1 h (A) and 100 ng/ml thrombin for 1–6 h (B). Thrombin stimulation did not induce any change in p^{22phox} mRNA expression. Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M.

3.6. Atorvastatin limits thrombin-induced Mox-1 mRNA expression

The effect of atorvastatin on thrombin-induced Mox-1 mRNA expression was assessed after 3 h of stimulation by thrombin (100 ng/ml) on smooth muscle cells impregnated with atorvastatin for 72 h (Fig. 7). RT-PCR analysis revealed

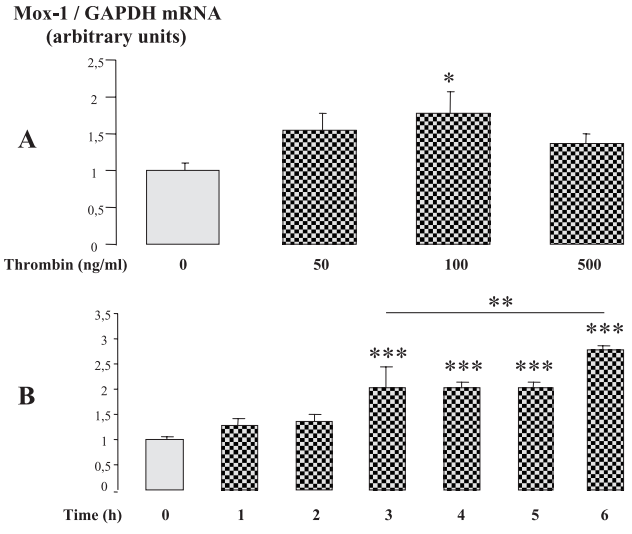

Fig. 6. Dose- and time-dependent effects of thrombin on Mox-1 mRNA expression. Rat aortic smooth muscle cells were stimulated with 50, 100 and 500 ng/ml thrombin for 3 h (A). 100 ng/ml thrombin stimulation increased significantly Mox-1 mRNA expression in smooth muscle cells (A). This dose induced a two-fold increase in Mox-1 mRNA expression after 3 h of stimulation as compared to control unstimulated cells and an even greater increase after 6 h of treatment (B). Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

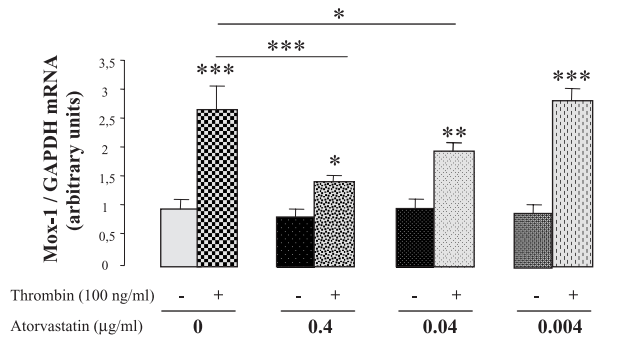

Fig. 7. Atorvastatin (0.4, 0.04 and 0.004 µg/ml) limits thrombin-induced Mox-1 mRNA expression. Thrombin alone induced a significant increase in Mox-1 mRNA expression. The effect of atorvastatin on 100 ng/ml thrombin-induced Mox-1 mRNA expression was assessed after 3 h on smooth muscle cells impregnated with atorvastatin for 72 h. 0.4 µg/ml atorvastatin-impregnation of cells reduced by 40% the effect of thrombin on Mox-1 mRNA expression, whereas atorvastatin alone had no effect. Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

that treatment with atorvastatin dose-dependently down-regulated Mox-1 mRNA expression (Fig. 7). Indeed, as shown in Fig. 7, atorvastatin (0.4 µg/ml) reduced by 40% the effect of thrombin on Mox-1 mRNA expression ($P<0.001$), whereas atorvastatin alone did not induce any effect as compared to control untreated cells.

3.7. Dose and time effects of thrombin on heme oxygenase-1 mRNA expression

Thrombin stimulation for 1 h (0–500 ng/ml) did not induce any significant change in heme oxygenase-1 mRNA expression (Fig. 8A). However, a significant decrease in

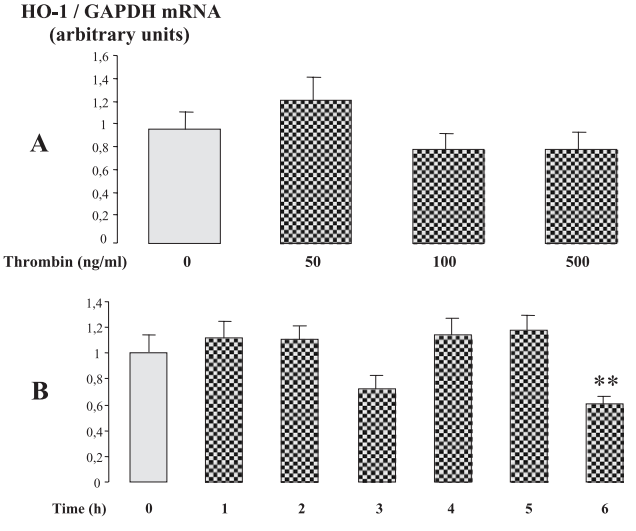

Fig. 8. Dose (A) and time (B) effects of thrombin on heme oxygenase-1 mRNA expression. Thrombin stimulation (0–500 ng/ml, 1 h) induced no significant change in heme oxygenase-1 mRNA expression (A), but thrombin (100 ng/ml) induced a significant decrease in heme oxygenase-1 mRNA expression after 6 h of treatment (B). Unstimulated cells were considered as controls. Results are expressed as means ± S.E.M. **$P<0.01$.

heme oxygenase-1 mRNA expression was observed after 6 h of exposure to thrombin (100 ng/ml) (Fig. 8B).

3.8. Atorvastatin limits thrombin-induced NF-κB activation

We next investigated the transcription factor NF-κB, which is an important regulator of interleukin-6 gene expression. To determine whether thrombin stimulates changes in NF-κB DNA-binding activity, rat aortic smooth muscle cells were stimulated with various doses of thrombin and nuclear extracts were assayed for NF-κB activity using a consensus NF-κB oligonucleotide (Fig. 9). Unstimulated cells possess a basal NF-κB binding activity (Fig. 9, lane 1). EMSA demonstrated enhanced binding activity using an NF-κB motif as a labeled probe and extracts from smooth muscle cells exposed to thrombin stimulation. When nuclear extracts from thrombin-stimulated cells were used, DNA binding protein (Fig. 9, lanes 2 and 3) was increased compared with control unstimulated cells (Fig. 9, lane 1). Active NF-κB was present at 100 ng/ml of thrombin-stimulation, whereas 250 ng/ml induced more enhanced activity than that observed by the lower dose used. Pretreatment of smooth muscle cells with 0.4 μg/ml of atorvastatin resulted in a decrease in thrombin-induced NF-κB activation (Fig. 9, lane 4 vs. lane 2). No effect was observed on NF-κB activation with lower doses of atorvastatin (Fig. 9, lanes 5 and 6 vs. lane 2). An excess of unlabeled NF-κB consensus oligonucleotide completely blocked the appearance of the thrombin-induced binding complex (Fig. 9, lane 8) and thus confirmed the specificity of the shifted autoradiographic bands. When an excess of unlabeled SP1 oligonucleotide was added as a nonspecific competitor, the band associated with active NF-κB did not fade (Fig. 9, lane 7). These results indicate that thrombin-induced activation of NF-κB was decreased under atorvastatin treatment.

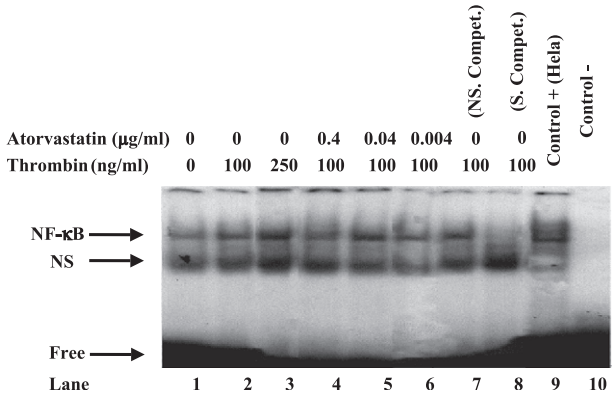

Fig. 9. Effect of atorvastatin on NF-κB activation. The effect of atorvastatin on 100 ng/ml thrombin-induced NF-κB activation was assessed after 1 h on smooth muscle cells impregnated with atorvastatin for 72 h. The EMSA demonstrates that 0.4 μg/ml atorvastatin limits thrombin-induced NF-κB activation (lane 4 vs. lane 2). No effect was observed in cells treated with 0.04 and 0.004 μg/ml atorvastatin combined with thrombin on NF-κB activation as compared to cells treated with atorvastatin alone (lanes 5 and 6 vs. lane 2). 250 ng/ml thrombin stimulation induced a greater increase in NF-κB activation than 100 ng/ml (lane 3 vs. lane 2).

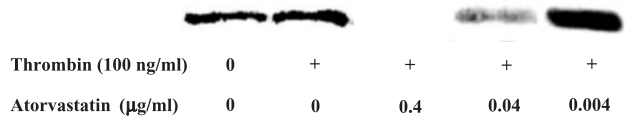

Fig. 10. Atorvastatin prevents RhoA membrane translocation. The Western blot shows that 0.4 μg/ml of atorvastatin prevents thrombin-induced RhoA membrane translocation. No effect was observed on RhoA membrane translocation with lower doses of atorvastatin.

3.9. Atorvastatin prevents RhoA membrane translocation

RhoA was predominately cytoplasmic in unstimulated cells, although a small fraction was present in the membrane. After thrombin treatment, membrane RhoA slightly increased (Fig. 10). However, cytoplasmic RhoA did not change (data not shown) indicating that although a statistically significant change in membrane RhoA was detected, the fraction of RhoA translocated into the membrane represented only a minor part of the total cytoplasmic reservoir. As shown in Fig. 10, treatment of smooth muscle cells with the lipophilic statin, atorvastatin (0.4 μg/ml), prevents this thrombin-induced RhoA membrane translocation. Treatment of cells with lipophilic atorvastatin was associated with a decrease in the quantity of RhoA in the membrane and its appearance in the cytolasmic fraction in a dose-dependent manner and was completely inhibited at the dose of 0.4 μg/ml (Fig. 10). More importantly, the relative steady-state changes in RhoA observed in the membrane compartment after thrombin stimulation exactly paralleled the changes in NF-κB binding.

4. Discussion

α-Thrombin, through activation of the G protein-coupled receptor PAR1 (Coughlin, 1994, 1999; Dery et al., 1998; Strukova, 2001), plays an important role in inflammatory processes as demonstrated by its ability to elicit a variety of cellular responses (Carney et al., 1992; Glusa et al., 1996; Grand et al., 1996; Henrikson et al., 1999), such as the release of several inflammatory cytokines including interleukin-1β, interleukin-6, interleukin-8, etc. by different cell types (Johnson et al., 1998; Naldini et al., 2002). In order to investigate new strategies to modify the pathophysiology of atherosclerosis, now considered as a kind of chronic inflammatory process, we have tested whether HMG-CoA reductase inhibitors, in particular atorvastatin, could regulate the expression of interleukin-6 in rat aortic smooth muscle cells stimulated by thrombin and have explored whether the transcription factor, NF-κB, is involved in this regulation. We also investigated a possible change in levels of prenylated small G-protein RhoA during exposure to thrombin in presence and absence of atorvastatin.

Our results suggest that smooth muscle cells may represent a significant source of interleukin-6 in the inflammatory

response and that cytokine production is an important biological consequence of thrombin activation. Interleukin-6 is secreted by a wide range of cell types (Kohase et al., 1987; Loppnow and Libby, 1990), is linked to a number of disorders (Matsumori et al., 1994; Miyao et al., 1993; Yamauchi-Takihara et al., 1995) and plays critical pathophysiological roles in cardiovascular diseases, such as atherosclerosis (Baggio et al., 1998). Furthermore, interleukin-6 mRNA or protein has been detected immunohistochemically and semi-quantitatively in both experimental animal and human atherosclerotic lesions (Ikeda et al., 1992; Seino et al., 1994; Sukovich et al., 1998). Thus, a decrease in interleukin-6 expression and release in smooth muscle cells when they are exposed to atorvastatin provides evidence for a vasculoprotective effect of statins. Our observation that, the preventive effect of statin was overcome by mevalonate, demonstrated the specificity of the HMG-CoA reductase inhibition.

Over the last decade, a considerable progression in vascular biology research has resulted from the better understanding of the vascular cell inflammatory responses that are mostly mediated through the inhibitor-kappa B (IκB)/nuclear factor-kappa B system (Janssen-Heininger et al., 2000; Valen et al., 2001). In unstimulated cells, inactive NF-κB is sequestered in the cytoplasm as a heterodimer complexed with IκB proteins, which prevents it from entering the nucleus (Chen et al., 1999). When the cells are stimulated, NF-κB is released, translocated to the nucleus and initiates transcription of several pro-inflammatory genes (Thurberg and Collins, 1998; Valen et al., 2001). Our results obtained using EMSA demonstrate that interleukin-6 gene expression is induced by thrombin which apparently increases the DNA-binding activity of NF-κB in smooth muscle cells. We also demonstrated that atorvastatin downregulates interleukin-6 expression at the transcriptional level and that this effect may be mediated, at least in part, via the redox-sensitive transcription factor NF-κB. Our results are similar to those previously reported in several cell types (Ortego et al., 1999).

Various studies have demonstrated that Rho activators include essentially G protein-coupled receptor ligands, growth factors, integrins and cytokines (Sah et al., 2000). A key step in the activation of Rho is the attachment of geranylgeraniol, an isoprenoid intermediate of the cholesterol biosynthesis pathway. This post-translational lipid modification is necessary for the translocation of inactive Rho from the cytosol to the membrane (Laufs and Liao, 2000; Mackay and Hall, 1998; Sah et al., 2000). Therefore, atorvastatin that blocks geranylgeraniol synthesis or geranylgeranyl transferase inhibitors, which prevent the attachment of geranylgeraniol to Rho, both inhibit Rho membrane translocation and activity. Like other members of the Ras superfamily, Rho proteins act as molecular switches to control cellular processes by cycling between active, GTP-bound and inactive, GDP-bound states (Seabra, 1998). Our results provide direct evidence that thrombin induces activation of RhoA in smooth muscle cells and that atorvastatin prevents this activation. Because Rho mediates the activation of the pro-inflammatory transcription factor NF-κB, we suggest that treatment with atorvastatin decreases interleukin-6 gene expression and activity, at least in part, by inhibition of RhoA translocation. Thus, we conclude that atorvastatin, probably via the small G protein RhoA, may regulate pro-inflammatory effects induced by thrombin. Our present findings are consistent with a role of Rho GTPase in mediating cardiovascular disease (Laufs and Liao, 2000) and inhibition of Rho may account for some other of the cholesterol-independent pleiotropic effects of statins (Chong et al., 2001). However, we cannot exclude that this vasculoprotective effect of atorvastatin may be mediated by other mechanisms that have not been explored in the present study and additional studies are needed to help us to understand the exact mechanisms of Rho activation and regulation of cellular functions under physiological and pathophysiological conditions.

In this study, we have also explored the effect of thrombin and atorvastatin on heme oxygenase-1, p^{22phox} and Mox-1 mRNA expressions. Our results indicate that heme oxygenase-1 and p^{22phox} are not modified by exposure to thrombin. In contrast, our data demonstrate that atorvastatin prevents thrombin-induced Mox-1 mRNA expression. However, the present study was not focused on this question and further investigations are required to evaluate the possible antioxidant effect of atorvastatin. Our findings support the idea that the beneficial effects of statins on clinical events involve nonlipid (i.e. serum cholesterol level-independent) mechanisms (Egashira et al., 2000; Sacks et al., 1996; Shepherd et al., 1995), including anti-inflammatory and antioxidant effects (Chen et al., 2002; Gaddam et al., 2002; Lefer, 2002; Wassmann et al., 2002). These effects have been identified in several experimental settings and may be crucial in the treatment of atherosclerotic plaque evolution.

In conclusion, thrombin induces a pro-inflammatory phenotype in vascular smooth muscle cells, which was prevented by atorvastatin impregnation. Our results strongly suggest that atorvastatin, via the inhibition of NF-κB and cytokine gene expression (interleukin-6), could reduce inflammation within the atherosclerotic lesion. These pleiotropic intracellular effects of statins are probably, at least in part, mediated by the inhibition of RhoA isoprenylation. The decrease in Mox-1 gene expression under atorvastatin treatment suggests that this statin may exert cellular antioxidant effects in cultured vascular smooth muscle cells.

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