ELSEVIER

Biochemical Pharmacology 64 (2002) 497–505

Biochemical Pharmacology

Reduction of bFGF-induced smooth muscle cell proliferation and endothelin receptor mRNA expression by mevastatin and atorvastatin

Cang-Bao Xu, Emelie Stenman, Lars Edvinsson*

Department of Medicine, Division of Experimental Vascular Research, University Hospital of Lund, S-22185 Lund, Sweden

Received 12 February 2002; accepted 22 May 2002

Abstract

The anti-atherosclerosis mechanisms of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) occur *via* both cholesterol-dependent and cholesterol-independent mechanisms. The present study used aortic and cerebral vascular smooth muscle cells (SMC) from rat to investigate whether atorvastatin and mevastatin affect basic fibroblast growth factor (bFGF)-induced SMC proliferation and the mRNA expression of endothelin A (ET_A) and endothelin B (ET_B) receptors. Cell proliferation was assessed by MTT and real-time PCR was used to quantify ET_A and ET_B receptor mRNA. bFGF-induced concentration and time dependent SMC proliferation and up-regulation of the mRNA expression of ET_A and ET_B receptors. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors inhibited bFGF-induced proliferation of SMC ($P < 0.01$). In aortic SMC atorvastatin and mevastatin significantly inhibited bFGF-induced mRNA expression of endothelin ET_A and ET_B receptors ($P < 0.05$). Although in cerebral SMC the inhibitory effect of the statins was comparable in size with that seen in aortic SMC, only reached borderline significance ($P = 0.06$) for ET_A receptor mRNA but not for ET_B. The findings suggested a direct effect of statins on the vascular wall beyond their well-known lipid lowering effect in anti-atherosclerosis. Furthermore, the specific antagonists of ET_A and ET_B receptors (FR139317 and BQ788, respectively) significantly inhibited bFGF-induced SMC proliferation ($P < 0.001$). The results suggested that endothelin receptors and the mevalonate pathway were involved in bFGF-induced SMC proliferation.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Basic fibroblast growth factor; Endothelin-receptors; Proliferation; Smooth muscle cells; Statins; Atherosclerosis

1. Introduction

Proliferation of SMC is one of the key elements in the development of atherosclerotic lesions [1]. bFGF and ET-1 derived from vascular intimal endothelial cells have been implied to regulate medial SMC proliferation which regularly is seen in atherosclerosis, restenosis, and vascular replication after stroke; this occurs *via* autocrine and paracrine mechanisms [2,3].

The beneficial effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) in anti-atherosclerosis is mainly considered as due to their ability to reduce cholesterol biosynthesis [4]. Clinic trials of statins have demonstrated an improvement in cardiovascular end-points [5]. However, the improvement in cardiovascular end-points is incompletely explained by the reduction of LDL cholesterol [5,6]. Recent studies have suggested that statins have additional benefits beyond their lipid lowering properties in protection against atherosclerosis [6,7]. Such nonlipid mechanisms may involve: (i) up-regulation of endothelial nitric oxide synthase and down-regulation of inducible nitric oxide synthase [8,9]; (ii) attenuation of inflammatory responses to cytokines [9,10]; (iii) influence on vascular cell migration [11], proliferation [12,13], and adhesion molecule expression [14]; and (iv) reduction of lipoprotein oxidation and amelioration of free radical injury [15,16].

Studies have revealed that statins may directly reduce vascular SMC proliferation [12,13]. Statins inhibit the conversion of HMG-CoA to mevalonate, which is a rate-limiting step of *in vivo* cholesterol biosynthesis [17,18]. However, mevalonic acid is the precursor not only for cholesterol synthesis, but also for many nonsteroidal

* Corresponding author. Tel: +46-46-2220603; fax: +46-46-2220616.
E-mail address: lars.edvinsson@med.lu.se (L. Edvinsson).

Abbreviations: SMC, smooth muscle cells; bFGF, basic fibroblast growth factor; ET-1, endothelin-1; ET_A receptor, endothelin A receptor; ET_B receptor, endothelin B receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MAPK, mitogen-activated protein kinases; LDL, low-density lipoprotein.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0006-2952(02)01189-9

isoprenoid compounds [6]. Hence, statins may have a direct effect on the arterial wall in addition to their lipid lowering properties, which may contribute to the anti-atherosclerotic benefits. However, in contrast to their lipid lowering ability, the direct effect of statins on the arterial wall is less well-studied. Statins reduce the production of isoprenoids and mevalonate when they inhibit cholesterol biosynthesis. Early intermediates of cholesterol synthesis and isoprenylated proteins are necessary for cell proliferation and other important cell functions, and their metabolism needs to be increased during cell activation.

Both bFGF and ET-1 are important mitogenic factors in atherosclerosis and restenosis. They may act *via* autocrine/paracrine mechanisms and are evoked in the process of arterial wall injury, myocardial infarction and stroke. Local bFGF and ET-1 production from arterial mural cells could contribute to the formation of atherosclerosis, restenosis, and SMC replication. Studies have demonstrated that isoprenoids and mevalonate are important mitogenic signaling molecules [12] that act on ras-MAPK [10]. This signal transduction pathway contributes to cell proliferation [19] and to the expression of receptors [19,20]. Therefore, the present study was designed to investigate whether atorvastatin and mevastatin can affect bFGF-induced proliferation and mRNA expression of ET_A and ET_B receptors in vascular SMC.

2. Materials and methods

2.1. Chemicals

bFGF (GibcoBRL), epidermal growth factor (GibcoBRL), atorvastatin (Pfizer), mevastatin (Sigma), collagenase/dispase (Roche), penicillin, and streptomycin (GibcoBRL), FR139317 and BQ788 (Sigma).

Atorvastatin, mevastatin, bFGF, FR139317 and BQ788 were prepared following the instructions from the companies and finally dissolved in phosphate-buffered saline (PBS) (GibcoBRL) containing 0.1% bovine serum albumin (BSA). PBS containing 0.1% BSA treated identically but without the above reagents served as controls.

2.2. Culture of vascular SMC

Male Sprague–Dawley rats (body weight 160 g) were anesthesed by CO_2, the animals rapidly decapitated and the brains removed carefully. The whole brain was gently homogenized in PBS with a glass homogenizer several times. Cerebral vessels were then isolated by 15% dextran density centrifugation at 3500 *g* for 45 min. The cerebral vessels were collected from the bottom of the centrifugation tubes and incubated with collagenase/dispase (1 mg/mL) at 37° for 1 hr. The collagenase/dispase was removed by centrifugation 2000 *g* for 10 min. The segments of cerebral vessel were then explanted into Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) to grow cerebral SMC [21]. The DMEM was supplemented with 10% fetal bovine serum (GibcoBRL, heated inactivated, Batches No. 10108-165), bFGF, 2 ng/mL; EGF, 5 ng/mL; penicillin, 100 U/mL; and streptomycin, 100 μg/mL. Subcultures were obtained by use of 0.25% trypsin–1 mM EDTA (GibcoBRL).

Rat aortic SMC (RASMC) were grown as previously described with a slight modification [22]. Shortly, aortic endothelium was removed by scraping the intimal surface with a surgical blade. The medial layer of the aortic wall was removed and cut into 1 mm × 1 mm segments. The segments were grown in the same medium as mentioned before.

SMC were cytochemically identified with a fluorescent microscope by more than 95% positive reaction of immunofluorescent staining with a monoclonal antibody (mouse IgG) against alpha-smooth muscle actin and a secondary antimouse antibody labeled with FITC (Boehringer Mannheim), and in addition with a typical "hill and valley" growth pattern. Cell viability was checked through all experiments by trypan blue (GibcoBRL) exclusion (>95%). SMC from passage 5 to 15 were used for the experiments.

2.3. Assay of cell proliferation

SMC proliferation was assayed using a cell proliferation kit (MTT) (Boehringer Mannheim) [23]. Briefly, the cells were seeded in a 96-well plate (Falcon) at a density of about 4000 cells in 100 μL of 10% FBS supplied DMEM medium and incubated at 37°, 5% CO_2 for 24 hr. The medium was then changed to serum free (SF) DMEM and incubated for another 24 hr to arrest cell growth. After the 24 hr of SF starvation, atorvastatin (0.5–2 μM), and mevastatin (25–100 μM) were added 4 hr before bFGF [24]. The concentration of atorvastatin is comparable to the plasma concentration used clinically [25]. The stimulus, bFGF, was added into the cultures for 24–48 hr of incubation. Control cultures received the same volume of PBS containing 0.1% BSA. At the last 4 hr of the 24–48 hr incubation, 10 μL of the MTT labeling reagent was added into each well. At the end of 24–48 hr of incubation, 100 μL of the solubilization solution (supplied in the kit) was added into each well, and the plate allowed to stand overnight in humidified incubator at 37°. The dissolved purple formazan was read in an ELISA reader (Titertek® Multiskan). All experiments were performed at least three times in more than triplicate in a 96-well plate. The results are presented as percent of SF.

2.4. Real-time quantitative RT-PCR

The 25-cm^2 flask (Falcon) subconfluent SMC were rinsed with PBS twice and incubated with SF for 24–48 hr. After the 24–48 hr of SF starvation, bFGF 10 ng/mL was added to SMC cultures for a further 1/2, 1, 3, and 6 hr of incubation. In statin experiments, atorvastatin (2 μM) or mevastatin (100 μM) was added 4 hr before bFGF administration. At

the end of the incubation, the SMC cultures were rinsed twice with cold PBS and directly lysed in the TRIzol reagent (GibcoBRL) for extraction of total mRNA. Real-time quantitative reverse transcription (RT)-PCR was used to quantity ET_A and ET_B receptor mRNA in the sample. Identical experiments were repeated four to six times.

2.4.1. Total RNA isolation

After the SMC had been lysed in the TRIzol reagent, total RNA was extracted with chloroform/TRIzol reagent (1:5) and precipitated using isopropyl alcohol [26]. The final pellet was washed with 75% ethanol and dissolved in diethypyrocarbonate-treated nuclease-free water (Promega).

2.4.2. Reverse transcription

Reverse transcription of total RNA to cDNA was carried out using the Gene Amp RT kit (PE Applied Biosystems) in a Perkin-Elmer 2400 PCR machine at 42° for 30 min [27].

2.4.3. Real-time quantitative PCR for quantitating the expression ET_A and ET_B receptor mRNA

The real-time quantitative RT-PCR was performed with the GeneAmp SYBR Green PCR kit (PE Applied Biosystems) in a Perkin-Elmer real-time PCR machine (PE, GeneAmp 5700 sequence detection system) [28]. The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Specific primers for rat ET_A and ET_B receptors were designed as below:

ET_A receptor forward:	5′-ATT GCC CTC AGC GAA CAC-3′
Reverse:	5′-CAA CAA AGC AGA AAG ACG GTC-3′
ET_B receptor forward:	5′-GAT ACG ACA ACT TCC GCT CCA-3′
Reverse:	5′-GTC CAC GAT GAG GAC AAT GAG-3′

The housekeeping gene, elongation factor-1 (EF-1), mRNA continuously expressed to a constant amount in the cells, was compared with the house keeping gene β-actin in a pilot study by real-time PCR (data not shown). EF-1 was used as a reference in this study, but both were equally well constant in the tests. Rat EF-1 primers were designed as below:

EF-1 forward:	5′-GCA AGC CCA TGT GTG TTG AA-3′
Reverse:	5′-TGA TGA CAC CCA CAG CAA CTG-3′

The PCR reaction was performed in a 50 μL volume and started at a temperature of 50° for 2 min, 95° for 10 min, and the following 40 PCR cycles with 95° for 15 s and 60° for 1 min. Dissociation curves were run after the real-time PCR, and no nonspecific amplification was detected in the present study. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by GibcoBRL Custom Primers (Life Technologies, Inc.). The PCR products of ET_A (64 bp), ET_B (86 bp), and EF-1 (96 bp) were visualized with agarose gel electrophoresis. To make a real-time RT-PCR standard curve, cDNA from reverse transcription of total RNA was diluted with the PCR buffer in three sequential log concentrations (1:0, 1:10, and 1:100).

Data were analyzed with the comparative cycle threshold (CT) method. To evaluate the amount of ET_A or ET_B receptor mRNA in a sample, EF-1 mRNA was assessed in the same sample simultaneously. The CT values of EF-1 mRNA was used as a reference to quantity the relative amount of ET_A or ET_B receptor mRNA. The relative amount of mRNA was calculated with the CT values of ET_A or ET_B receptor mRNA in relation to the CT values of EF-1 mRNA in the sample.

2.5. Cell counting

Cells were staining with 0.2% trypan blue and counted in a Burker hemocytometer chamber counter under a light microscope.

2.6. Statistics

Data are expressed as the mean ± SEM. One-way analysis (ANOVA) with Dunnett's post test (control vs. treated groups) or two-way unpaired t test were performed. $P < 0.05$ was considered as significant. Analyses were performed using a Prism 3.0 software package (GraphPad Software, Inc.).

3. Results

3.1. Real-time RT-PCR standard curves

To make real-time RT-PCR standard curves, cDNA from reverse transcription of the total RNA was diluted with the PCR buffer in three sequential log concentrations (1:0, 1:10, and 1:100). ET_A and ET_B receptor, EF-1 mRNA were assessed by real-time RT-PCR described in Section 2. There were clear linearity relationships (correlation 98–99% and slope of 3.249–3.275) between log concentrations of cDNA and the real-time RT-PCR CT values of ET_A and ET_B receptor mRNA and EF-1 mRNA. The PCR products of ET_A (64 bp), ET_B (86 bp), and EF-1 (96 bp) were visualized with agarose gel electrophoresis (data not shown).

3.2. Effect of bFGF on cerebral and aortic SMC proliferation

bFGF-induced time and concentration-dependent increases in proliferation of both aortic (A) and cerebral

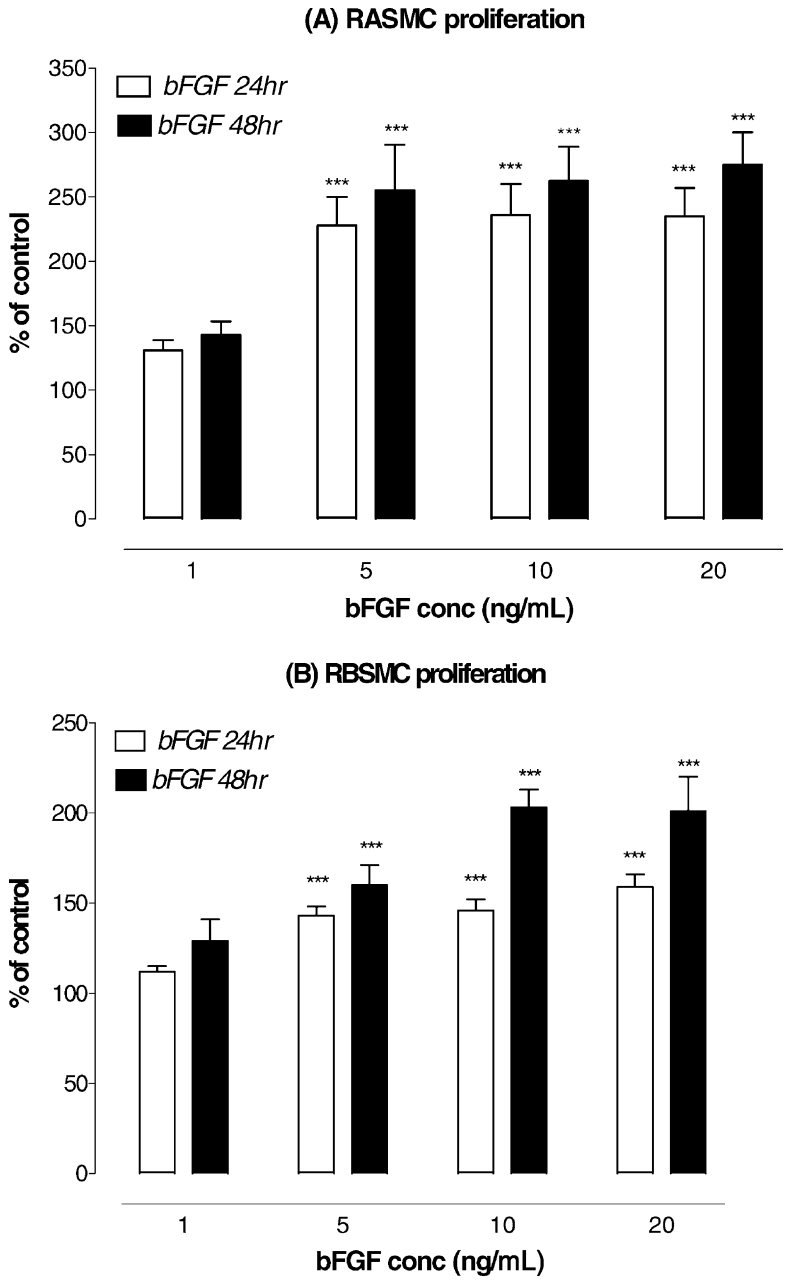

Fig. 1. RASMC and RBSMC were seeded in 96-well plates. After 24 hr SF starvation, bFGF 1, 5, 10, and 20 ng/mL were added into wells and incubated for another 24 and 48 hr. At the end of the last 4 hr of the 24–48 hr incubation, SMC cell proliferation was assessed by using MTT. Control only received the same volume of 0.1% BSA in PBS. Mean data with SEM were derived from 16 wells and presented as percent of control (without bFGF). Statistical analysis was performed by one-way analysis (ANOVA) and Dunnet's post test. $^{***}P < 0.001$.

(B) SMC with a near maximal effect at 10 ng/mL bFGF (Fig. 1). Therefore, in the subsequent studies 10 ng/mL bFGF was used.

3.3. Atorvastatin and mevastatin inhibition of bFGF-induced proliferation of SMC

Statin has two subtypes: type 1 includes mevastatin, simvastain, lovastatin and pravastatin, and type 2 is fully synthetic HMG-CoA reductase inhibitor including atorvastation, fluvastatin, cerivavastatin and rosuvastatin [17]. Mevastatin and atorvastatin were chosen for the present study to represent each type of statins. To test whether statins have a direct effect on SMC proliferation, atorvastatin (0.5–2 μM), and mevastatin (25–100 μM) were added to SMC cultures 4 hr before bFGF. Atorvastatin concentration-dependently inhibited bFGF-induced proliferation (Fig. 2) of aortic (A) and cerebral vascular (B) SMC. However, mevastatin required higher concentrations to reach the same degree of inhibition as atorvastatin on bFGF-induced proliferation (Fig. 2) of aortic (C) and cerebral (D) SMC.

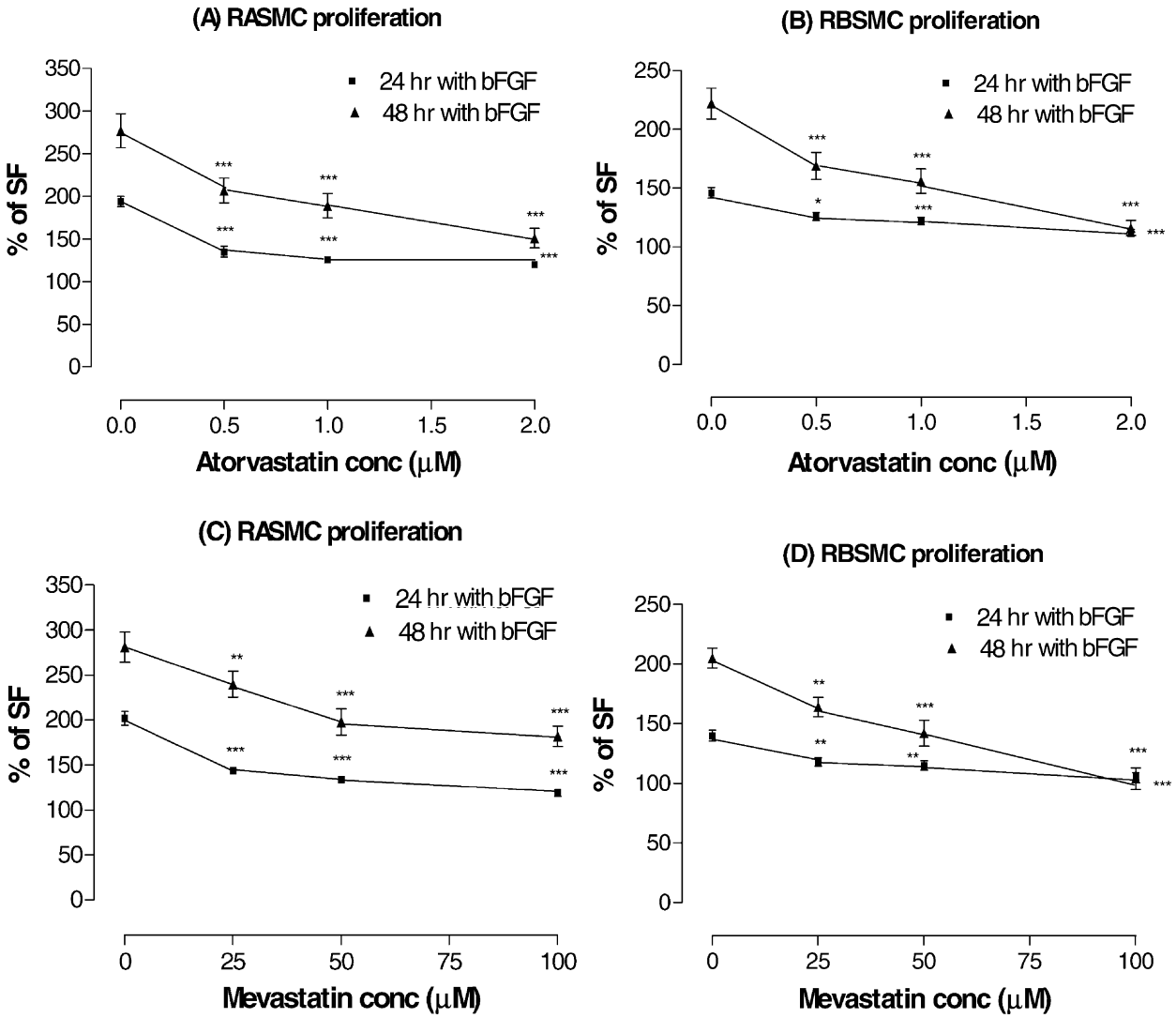

Fig. 2. (A–D): RASMC and RBSMC were seeded in 96-well plates. After 24 hr SF starvation, atorvastatin (0.5–2 μM) or mevastatin (25–100 μM) were added to the SMC cultures 4 hr before bFGF (10 ng/mL) for 24–48 hr incubation. At the end of the last 4 hr of the 24–48 hr incubation, SMC cell proliferation was assessed by using MTT. Mean data with SEM were derived from 12 wells and presented as percent of SF. Statistical analysis was performed by one-way analysis (ANOVA) and Dunnet's post test. Compared with controls (without statins), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.4. Time course of mRNA expression of ET_A and ET_B receptors induced by bFGF

In order to examine whether bFGF may cause an up-regulation of SMC ET_A and ET_B receptors, SMC cultures were stimulated with bFGF (10 ng/mL) for 1/2, 3, and 6 hr and then assessed for the mRNA expression of ET_A and ET_B receptors by real-time quantitative PCR. The time course of bFGF-induced mRNA expression of ET_A and ET_B receptors on SMC were slightly different (Fig. 3). The mRNA expression of ET_A receptors had a peak at 3 hr while the mRNA expression of ET_B receptors reached a maximum already at 1 hr. Although aortic and cerebral SMC had a similar time-response manner to bFGF, the ET_A receptors response of to bFGF was stronger in aortic than in cerebral SMC while bFGF-induced a larger expression of ET_B in cerebral SMC (Fig. 3).

3.5. Effect of statins on bFGF-induced expression of the ET receptor mRNA

To examine the hypothesis that statins could have an inhibitory effect on the mRNA expression of ET_A and ET_B receptors in SMC, atorvastatin (2 μM), and mevastatin (100 μM) were added 4 hr before bFGF. The mRNA expression of ET_A and ET_B receptors was assessed after the addition of bFGF for 1 hr for the ET_B receptor and 3 hr for the ET_A receptor (Fig. 3). Both atorvastatin and mevastatin had a significant inhibitory effect on bFGF-induced the expression of ET_A and ET_B receptor mRNA in aortic SMC ($P < 0.05$) (Fig. 4). In cerebral SMC the inhibitory effect of the statins reached borderline significance ($P = 0.06$) for ET_A receptor mRNA but not for ET_B, although the mean values were comparable with that seen in aortic SMC.

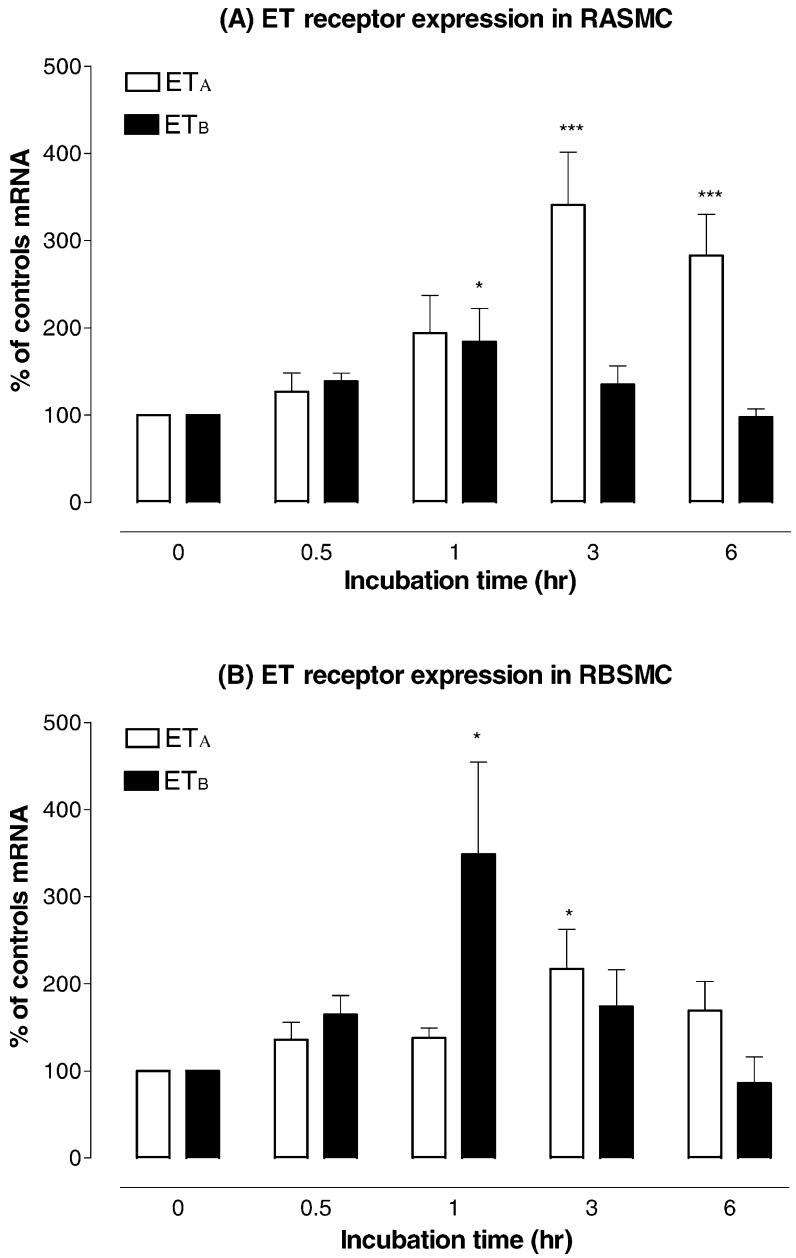

Fig. 3. (A–B): 10 ng/mL bFGF were added to 24 hr SF starved RASMC and RBSMC in 25 cm^2 flasks for 1/2, 1, 3, and 6 hr incubation. At the end of the incubation, the SMC cultures were rinsed twice with cold PBS and directly lysed in the TRIzol reagent for extraction of total mRNA. Real-time RT-PCR was used to quantify ET_A and ET_B receptor mRNA. Identical experiments were repeated four to six times. Mean data with SEM were derived from four to six flasks and presented as percent of controls (without bFGF). Statistical analysis was performed by one-way analysis (ANOVA) and Dunnet's post test. $^{*}P < 0.05$ and $^{***}P < 0.001$.

3.6. Effects of the endothelin receptor antagonists on bFGF-induced proliferation of SMC

Since bFGF-induced up-regulation of ET_A and ET_B receptor mRNA and this effect occurred at the early stage of bFGF-induced SMC proliferation, the up-regulation of endothelin receptors might be associated with the SMC proliferation. To test whether endothelin receptors are required for bFGF-induced SMC proliferation, specific ET_A and ET_B antagonists FR139317 and BQ788 were used in the further study. Thirty minutes before addition of bFGF, FR139317, and BQ788 were used at high concentrations (10 μM) that are known to block ET_A and ET_B receptors [29], respectively. Both antagonists significantly inhibited the bFGF-induced SMC proliferation (Fig. 5). The ET_B antagonist BQ788 had a more pronounced inhibitory effect than FR139317 and overcame more than 50% the bFGF effect. Unexpectedly, bosentan (10 μM), a dual antagonist of both ET_A and ET_B receptors [30], did not have an additional inhibitory effect over that of FR139317 or BQ788 on bFGF-induced SMC proliferation (results not shown).

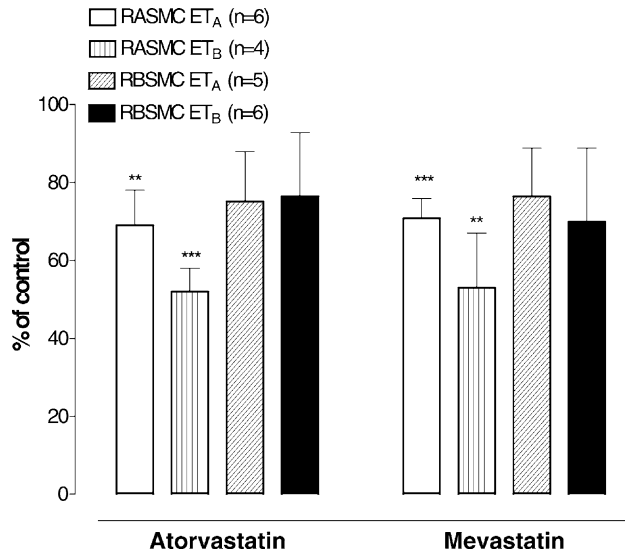

Fig. 4. Atorvastatin (2 μM) and mevastatin (100 μM) were added to 24 hr SF starved-RASMC and RBSMC 4 hr before bFGF (10 ng/mL). ET_A receptor mRNA was quantified by using real-time RT-PCR at 1 hr incubation with bFGF for ET_A and at 3 hr incubation with bFGF for ET_B. Mean data with SEM were derived from four to six flasks and presented as percent of controls (without statins). Identical experiments were repeated four to six times. Statistical analysis was performed by unpaired two-way *t* test. Compared with controls, $^{**}P < 0.01$; $^{***}P < 0.001$. N, number of experiments.

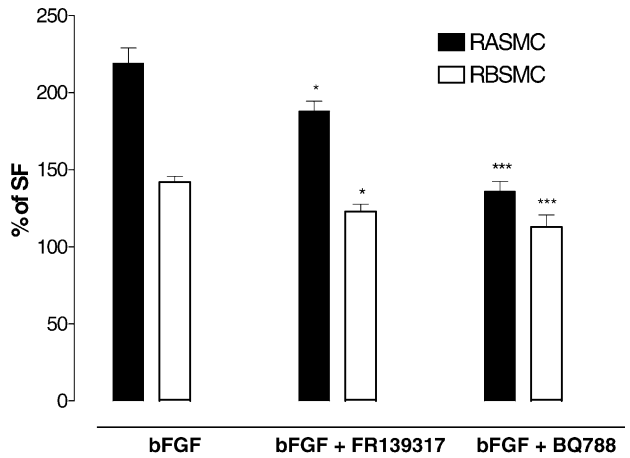

Fig. 5. Twenty four hour SF starved-RASMC and RBSMC in 96-well plates were received 10 μM of FR139317 or BQ788 before 30 min of bFGF (10 ng/mL). The SMC cultures were then incubated for 24 hr. SMC cell proliferation was assessed by MTT at the last 4 hr of the 24 hr incubation. Mean data with SEM were derived from 9 wells and presented as percent of SF. Statistical analysis was performed by unpaired two-way *t* test. Compared with bFGF, $^{*}P < 0.05$; $^{***}P < 0.001$.

4. Discussion

The present study has demonstrated that bFGF induces proliferation and up-regulates the mRNA expression of ET_A and ET_B receptors in cerebral and aortic vascular SMC. Aortic vascular SMC were more sensitive to bFGF-induced mitogenic stimulation than cerebral vascular SMC (RBSMC). Our findings are supported by Diglio *et al.* [21] who observed that RASMC had significantly higher growth rate (2–4-fold) in response to 10% fetal bovine serum stimulation than rat cerebral SMC. The HMG-CoA reductase inhibitors, atorvastatin, and mevastatin, inhibit bFGF-induced proliferation of SMC and the mRNA expression of endothelin ET_A and ET_B receptors. This suggests that the mevalonate pathway and endothelin receptors are involved in bFGF-induced SMC proliferation. In agreement with our findings, studies have demonstrated that statins can inhibit SMC proliferation induced by bFGF [12] and platelet-derived growth factor [12,24]. However, this has not been shown in cerebral SMC. The depletion of intracellular mevalonate and the reduction in the formation of certain prenylated proteins by statins may contribute to the antiproliferative effect [13,24]. A schematic hypothesis of the present study is shown in Fig. 6. It provides a key to follow the results and connections between the intracellular mevanolate pathway and the ET-1 autocrine loop. Both the mevanolate pathway and ET-1 autocrine loop contribute to bFGF-induced vascular SMC proliferation.

The development of an atherosclerostic lesion is a complicated process starting from endothelial dysfunction that leads to migration and proliferation of SMC in the intimal layer [1]. The overweight of SMC proliferation is continuously seen in atherosclerotic lesion from initiation through progression to the ultimate lesion [1,2]. A high blood concentration of LDL-cholesterol is one of the most important ‘‘risk factors’’ for atherosclerosis and this contributes to the endothelial dysfunction. Atorvastatin is currently used in the clinic for the prevention and treatment of atherosclerosis [17,25]. Statins inhibit the conversion of HMG-CoA to mevalonate, which is the rate-limiting step for *in vivo* cholesterol biosynthesis, and, therefore, they prevent the progression of atherosclerosis by lowering LDL-cholesterol. Cholesterol, the major product of the mevalonate pathway, is required for cell membrane formation in proliferating cells. However, it is unlikely that the reduction of cholesterol production can explain the inhibitory effect of statins on cell proliferation because mevalonate, but not cholesterol, abolishes the inhibitory

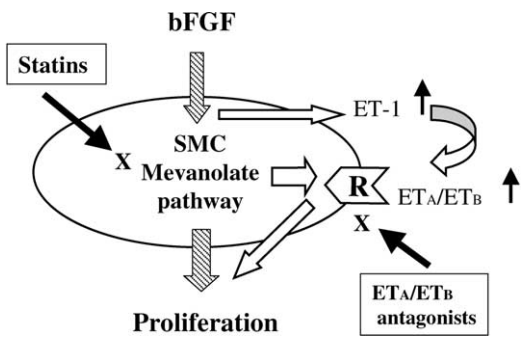

Fig. 6. SMC, smooth muscle cells; bFGF, basic fibroblast growth factor; empty arrows show ET-1 autocrine loop and dashed arrows show mevanolate pathway. Filled arrow with X = blocker.

effect of statins on cell proliferation [13]. Several proteins that are involved in growth factor signal transduction pathways are lipid-modified by isoprenoids derived from mevalonate pathways. Both farnesylation and geranylgeranylation of signal transduction proteins are required in PDGF-induced SMC proliferation [24]. One of possible mechanisms for the inhibitory effect that we observed on cell proliferation is that statins interfer with growth factor signal transduction mechanisms that require prenylated proteins (see Fig. 6).

Basic fibroblast growth factor has been shown to promote proliferation and migration of SMC [31,32]. Extracellular signal-regulated kinase activity induced by balloon catheter injury to the rat carotid artery was inhibited by an anti-bFGF antibody, which further implies a specific role for bFGF in the vascular SMC extracellular signal-regulated kinase MAPK pathway [31]. Statins have been shown to inhibit ras-MAPK activity in aortic SMC and this inhibitory effect may occur *via* inhibition of the isoprenylation of ras, which in turn inhibits the ras-raf-MAPK pathway [13]. On the other hand, statins reduce the production of isoprenoids and mevalonate that are necessary for cell proliferation [13]. Walter *et al.* [33] have demonstrated that statin therapy in man significantly reduces in the development of restenosis after stent implantation.

The present study revealed that both atorvastatin and mevastatin inhibit bFGF-induced SMC proliferation and more interestingly the expressions of ET_A and ET_B receptor mRNA (see Fig. 6). The results suggest a direct effect of the statins on the vascular wall beyond lipid lowing. Since atorvastatin and mevastatin inhibit both bFGF-induced proliferation of SMC and the expression of endothelin receptor mRNA, it seems that the mevalonate pathway is required for both bFGF-induced SMC proliferation and the endothelin receptor expression. This finding makes it also likely that bFGF induces SMC proliferation that is associated with the up-regulation of endothelin receptors. In order to find a link between bFGF-induced SMC proliferation and the expression of endothelin receptors, FR139317 and BQ788 were, therefore, used in the further study. In addition, we could also demonstrate that antagonists of ET_A and ET_B receptors inhibit SMC proliferation induced by bFGF. The ET_B receptor antagonist BQ788 had a more pronounced inhibitory effect on bFGF-induced SMC proliferation. In agreement with our findings, Hahn *et al.* [34] reported that growth factors could induce expression of ET-1 mRNA and secretion of ET-1. However, quiescent SMC did not constitutively express ET-1 mRNA. In addition, growth factors increase ET-1 binding to vascular SMC [35]. Furthermore, ET-1 enhances growth factors-induced vascular SMC proliferation *via* its receptors [36,37]. Thus, the statins inhibit bFGF-induced SMC proliferation, at least, partly *via* inhibition of the expression of endothelin receptors. This together with the inhibitory effect of atorvastatin and mevastatin on bFGF-induced SMC proliferation suggest that the mevalonate pathway and the formation of endothelin receptor are required for bFGF-induced SMC proliferation. Increased endothelin-1 and its receptors were found in human atherosclerotic lesions which suggest a possible role of ET-1 and its receptors in atherogenesis [38]. The increased expression of endothelin receptors in vascular SMC induced by bFGF seen in the present study implies a further stimulatory effect on SMC proliferation after arterial injury and in the pathogenesis of atherosclerosis and restenosis. This ET-1 autocrine loop in SMC may contribute to the pathogenesis of vasospasm and atherosclerosis. The role of endothelin receptors in bFGF-induced SMC proliferation is, however, not completely clear yet. bFGF might induce release of ET-1, and ET-1 may enhance SMC proliferation *via* endothelin receptors. On the other hand, endothelin receptors might also directly cross-talk with bFGF mitogenic signal transduction, since ET_B receptor antagonists could abolish relative large amount (more than 50%) of bFGF-induced SMC proliferation.

Our group has previously demonstrated that endothelin receptors are up-regulated during organ culture [29], and this is further enhanced by cytokines such as TNF-α and IL-1β [39]. The present study has shown that bFGF strongly results in endothelin receptor mRNA expression in SMC and the mevalonate pathway is required in bFGF up-regulation of endothelin receptors. Our findings further suggest that growth factors and cytokines play an important role in the regulation of endothelin receptor expression. The up-regulation of endothelin receptors by bFGF is associated with the bFGF-induced mitogenic effect on SMC as seen in the statin experiments, i.e. both the mitogenic effect and the up-regulation of endothelin receptors are sensitive to statins. This suggests that bFGF-induced proliferation and expression of endothelin receptors occur at least in part *via* a similar signal transduction pathway and that the mevalonate pathway is required for both bFGF-induced SMC proliferation and the expression of endothelin receptors.

Acknowledgments

This study was supported by grants from the Swedish Research Council (project no. 5958). The assistance of Elisabeth Nilsson is gratefully acknowledged.

References

[1] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–9.
[2] Reidy MA. Factors controlling smooth-muscle cell proliferation. Arch Pathol Lab Med 1992;116:1276–80.
[3] Alberts GF, Peifley KA, Johns A, Kleha JF, Winkles JA. Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. J Biol Chem 1994;269:10112–8.

[4] Blumenthal RS. Statins: effective antiatherosclerotic therapy. Am Heart J 2000;139:577–83.
[5] Scandinavian Simvastatin Survival Study Group. Randomized trial of cholesterol lowering in 4444 participants with coronary heart disease: the Scandinavian Simvastatin Survival Study. Lancet 1994;344:1383–9.
[6] Koh KK. Effects of statins on vascular wall: vasomotor function inflammation and plaque stability. Cardiovasc Res 2000;47:648–57.
[7] Vaughan CJ, Delanty N. Neuroprotective properties of statins in cerebral ischemia and stroke. Stroke 1999;30:1969–73.
[8] Mital S, Zhang X, Zhao G, Bernstein RD, Smith CJ, Fulton DL, Sessa WC, Liao JK, Hintze TH. Simvastatin upregulates coronary vascular endothelial nitric oxide production in conscious dogs. Am J Physiol Heart Circ Physiol 2000;279:2649–57.
[9] Pahan K, Sheikh FG, Namboodiri AM, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. J Clin Invest 1997;100:2671–9.
[10] Takata M, Urakaze M, Temaru R, Yamazaki K, Nakamura N, Nobata Y, Kishida M, Sato A, Kobayashi M. Pravastatin suppresses the interleukin-8 production induced by thrombin in human aortic endothelial cells cultured with high glucose by inhibiting the p44/42 mitogen activated protein kinase. Br J Pharmacol 2001;134:753–62.
[11] Vincent L, Chen W, Hong L, Mirshahi F, Mishal Z, Mirshahi-Khorassani T, Vannier JP, Soria J, Soria C. Inhibition of endothelial cell migration by cerivastatin an HMG-CoA reductase inhibitor: contribution to its anti-angiogenic effect. FEBS Lett 2001;495:159–66.
[12] Negre-Aminou P, van Vliet AK, van Erck M, van Thiel GC, van Leeuwen RE, Cohen LH. Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors comparison with other human cell types. Biochim Biophys Acta 1997;1345:259–68.
[13] Raiteri M, Arnaboldi L, McGeady P, Gelb MH, Verri D, Tagliabue C, Quarato P, Ferraboschi P, Santaniello E, Paoletti R, Fumagalli R, Corsini A. Pharmacological control of the mevalonate pathway: effect on arterial smooth muscle cell proliferation. J Pharmacol Exp Ther 1997;281:1144–53.
[14] Weber C, Erl W, Weber KS, Weber PC. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. J Am Coll Cardiol 1997;30:1212–7.
[15] Hussein O, Schlezinger S, Rosenblat M, Keidar S, Aviram M. Reduced susceptibility of low density lipoprotein (LDL) to lipid peroxidation after fluvastatin therapy is associated with the hypocholesterolemic effect of the drug and its binding to the LDL. Atherosclerosis 1997;128: 11–8.
[16] Chen L, Haught WH, Yang B, Saldeen TG, Parathasarathy S, Mehta JL. Preservation of endogenous antioxidant activity and inhibition of lipid peroxidation as common mechanisms of antiatherosclerotic effects of vitamin E lovastatin and amlodipine. J Am Coll Cardiol 1997;30:569–75.
[17] Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science 2001;292:1160–4.
[18] Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol 2001;21:1712–9.
[19] Czuwara-Ladykowska J, Gore EA, Shegogue DA, Smith EA, Trojanowska M. Differential regulation of transforming growth factor-beta receptors type I and II by platelet-derived growth factor in human dermal fibroblasts. Br J Dermatol 2001;145:569–75.
[20] Schmidlin F, Loeffler S, Bertrand C, Landry Y, Gies JP. PLA2 phosphorylation and cyclooxygenase-2 induction through p38 MAP kinase pathway is involved in the IL-1beta-induced bradykinin B2 receptor gene transcription. Naunyn Schmiedebergs Arch Pharmacol 2000;361:247–54.
[21] Diglio CA, Grammas P, Giacomelli F, Wiener J. Rat cerebral microvascular smooth muscle cells in culture. J Cell Physiol 1986;129: 131–41.
[22] Erlinge D, Heilig M, Edvinsson L. Tyrphostin inhibition of ATP-stimulated DNA synthesis cell proliferation and fos-protein expression in vascular smooth muscle cells. Br J Pharmacol 1996;118:1028–34.
[23] Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A, Boyd MR. Comparison of in vitro anticancer drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Natl Cancer Inst 1990;82:1113–8.
[24] Negre-Aminou P, van Erck M, van Leeuwen RE, Collard JG, Cohen LH. Differential effect of simvastatin on various signal transduction intermediates in cultured human smooth muscle cells. Biochem Pharmacol 2001;61:991–8.
[25] Igel M, Sudhop T, von Bergmann K. Metabolism and drug interactions of 3-hydroxy-3-methylglutaryl coenzyme A-reductase inhibitors (statins). Eur J Clin Pharmacol 2001;57:357–64.
[26] Shabaana AK, Venkatasubramani R, Narayan NS, Hoessli DC, Dharmalingam K. Cytokine profiles in paraffin-embedded biopsy samples of lepromatous leprosy patients: semi-quantitative measure of cytokine mRNA using RT-PCR. Int J Lepr Other Mycobact Dis 2001; 69:204–14.
[27] Moller S, Edvinsson L, Adner M. Transcriptional regulated plasticity of vascular contractile endothelin ET(B) receptors after organ culture. Eur J Pharmacol 1997;329:69–77.
[28] Qing X, Svaren J, Keith IM. mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. Am J Physiol Lung Cell Mol Physiol 2001;280:L547–54.
[29] Adner M, Cantera L, Ehlert F, Nilsson L, Edvinsson L. Plasticity of contractile endothelin-B receptors in human arteries after organ culture. Br J Pharmacol 1996;119:1159–66.
[30] Besse S, Tanguy S, Riou B, Boucher F, Bulteau AL, Le Page C, Swynghedauw B, de Leiris J. Coronary and aortic vasoreactivity protection with endothelin receptor antagonist, bosentan, after ischemia and hypoxia in aged rats. Eur J Pharmacol 2001;432:167–75.
[31] Koyama H, Olson NE, Dastvan FF, Reidy MA. Cell replication in the arterial wall: activation of signaling pathway following in vivo injury. Circ Res 1998;82:713–21.
[32] Jackson CL, Reidy MA. Basic fibroblast growth factor: its role in the control of smooth muscle cell migration. Am J Pathol 1993;143: 1024–31.
[33] Walter DH, Schachinger V, Elsner M, Mach S, Auch-Schwelk W, Zeiher AM. Effect of statin therapy on restenosis after coronary stent implantation. Am J Cardiol 2000;85:962–8.
[34] Hahn AW, Resink TJ, Scott-Burden T, Powell J, Dohi Y, Buhler FR. Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function. Cell Regul 1990;1:649–59.
[35] Bonin PD, Leadley Jr RJ, Erickson LA. Growth factor-induced modulation of endothelin-1 binding to human smooth-muscle cells. J Cardiovasc Pharmacol 1993;22:125–7.
[36] Yang Z, Krasnici N, Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ET_A and ET_B receptor blockade. Circulation 1999;100:5–8.
[37] Di Luozzo G, Bhargava J, Powell RJ. Vascular smooth muscle cell effect on endothelial cell endothelin-1 production. J Vasc Surg 2000;31: 781–9.
[38] Iwasa S, Fan J, Shimokama T, Nagata M, Watanabe T. Increased immunoreactivity of endothelin-1 and endothelin B receptor in human atherosclerotic lesions. A possible role in atherogenesis. Atherosclerosis 1999;146:93–100.
[39] Leseth KH, Adner M, Berg HK, White LR, Aasly J, Edvinsson L. Cytokines increase endothelin ET_B receptor contractile activity in rat cerebral artery. Neuroreport 1999;10:2355–9.