0026-895X/01/5903-646–654$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:646–654, 2001

Vol. 59, No. 3
220/887818
Printed in U.S.A.

Inhibition of Geranylgeranylation Reduces Angiotensin II-Mediated Free Radical Production in Vascular Smooth Muscle Cells: Involvement of Angiotensin AT1 Receptor Expression and Rac1 GTPase

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Received April 16, 2000; accepted November 16, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) may exert pleiotropic effects on vascular cells independent of lowering plasma cholesterol. To elucidate the molecular mechanisms involved in these effects, we investigated the impact of statins on production of reactive oxygen species (ROS) in rat aortic vascular smooth muscle cells (VSMC). Exposure of VSMC to angiotensin II caused production of ROS via angiotensin AT1 receptor activation. Pretreatment with atorvastatin inhibited angiotensin II-induced ROS production. Atorvastatin decreased AT1 receptor mRNA levels in a time- and concentration-dependent manner and consistently reduced AT1 receptor density. L-Mevalonate but not hydroxy-cholesterol reversed the inhibitory effect of atorvastatin on AT1 receptor transcript levels. Inhibition of geranylgeranyl-transferase but not of farnesyl-transferase mimicked the effect of atorvastatin on AT1 receptor gene expression. Atorvastatin did not decrease AT1 receptor gene transcription but did reduce the half-life of the AT1 receptor mRNA. AT1 receptor activation by angiotensin II increased the expression of the GTPase rac1, enhanced rac1 GTP-binding activity, and increased the geranylgeranyl-dependent translocation of rac1 to the cell membrane. In contrast, statins inhibited rac1 activity and membrane translocation. Consequently, specific inhibition of rac1 with *Clostridium sordellii* lethal toxin blocked angiotensin II-induced production of free radicals. Finally, treatment of rats with atorvastatin caused down-regulation of aortic AT1 receptor mRNA expression and reduced aortic superoxide production in vivo. Cholesterol-independent down-regulation of AT1 receptor gene expression and inhibition of rac1, leading to decreased ROS production, demonstrates a novel regulatory mechanism of statins that may contribute to the beneficial effects of these drugs beyond lowering of plasma cholesterol.

Hypercholesterolemia is a major risk factor for the development of atherosclerosis, and lowering of low-density lipoprotein cholesterol levels significantly reduces the mortality of cardiovascular diseases (Yusuf et al., 1988; Farmer and Gotto, Jr, 1996). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors—"statins"—lower plasma cholesterol concentrations by blocking the conversion of HMG-CoA to mevalonate and thus inhibiting cholesterol biosynthesis (Goldstein and Brown, 1990; Levine et al., 1995). Recent large clinical trials demonstrated that the therapeutic use of statins results in a decreased incidence of ischemic stroke and myocardial infarction and in a reduction of mortality in hypercholesterolemic subjects (Anonymous, 1994; Shepherd et al., 1995). The effects of statins have been mainly attributed to their cholesterol-lowering properties, but there is growing evidence that some beneficial effects of these agents may be independent of plasma cholesterol levels (Vaughan et al., 1996; Anonymous, 1998). However, the mechanisms by which statins may exert beneficial effects independent of lipid-lowering have not been completely identified.

The release of reactive oxygen species (ROS) is thought to be involved in the pathogenesis of atherosclerosis by, for

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), the Bundesministerium für Bildung, Forschung und Technologie (BMBF), the Köln Fortune Program/Faculty of Medicine, University of Cologne, and a Gödecke research grant.

S.W. and U.L. contributed equally to this study.

ABBREVIATIONS: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; statins, HMG-CoA reductase inhibitors; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells; AT1 receptor, angiotensin II type 1 receptor; DRB, 5,6-dichlorobenzimidazole; GGTI, geranylgeranyl-transferase inhibitor; FTI, farnesyl-transferase inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DCF, 2′,7′-dichlorofluorescein; n.s., not significant; LT, *Clostridium sordellii* lethal toxin; SHR, spontaneously hypertensive rats.

example, enhancing proliferation of vascular smooth muscle cells (VSMC) and inducing apoptosis of vascular cells (Darley-Usmar et al., 1997; Laursen et al., 1997; Li et al., 1997). The majority of ROS occurring in the vascular wall seems to originate from membrane bound NAD(P)H oxidase. The latter is an enzymatic system composed of several subunits, including p22phox, gp91phox/mox1, p40phox, p47phox, and p67phox (Griendling and Ushio-Fukai, 1998; Babior, 1999; Suh et al., 1999). Especially in vascular cells, little is known about the detailed molecular mechanisms involved in ROS generation via NAD(P)H oxidase. Studies on phagocytes suggest that the small GTP-binding protein rac1 plays a pivotal role in the activation and assembly of the NAD(P)H oxidase (Dusi et al., 1995; Rinckel et al., 1999).

In vascular cells, activation of the angiotensin AT1 receptor by angiotensin II is one of the most prominent mechanisms of ROS production in vitro as well as in vivo (Griendling et al., 1994; Rajagopalan et al., 1996). The expression level of the AT1 receptor is subject to regulation and influences through up- or down-regulation the activity of the renin-angiotensin system (Griendling et al., 1993; Timmermans et al., 1993). The AT1 receptor is regulated by various pathophysiologically important agents and hormones, such as lipoproteins and estrogen in vitro as well as in vivo, suggesting the important role of this receptor in the development of atherosclerosis and cardiovascular diseases (Nickenig and Murphy, 1994, 1996; Nickenig et al., 1997, 1998a,b).

To elucidate the plasma cholesterol-independent molecular mechanisms involved in the beneficial effects of statins in cardiovascular diseases, we investigated the interference of HMG-CoA reductase inhibitors with angiotensin II-induced free radical release and tried to gain insight into mechanisms underlying this potential antioxidative effect of statins.

Experimental Procedures

Materials. Angiotensin II, 5,6-dichlorobenzimidazole (DRB), L-mevalonate, 25-hydroxy-cholesterol, lucigenin, *Taq* DNA polymerase, nucleotides, salts, and other chemicals were purchased from Sigma Chemical (Deisenhofen, Germany). GGTI-286 and FTI-276 were obtained from Calbiochem (Bad Soden, Germany). Moloney murine leukemia virus reverse transcriptase was purchased from Life Technologies (Eggenstein, Germany). [^{32}P]dCTP, [^{32}P]UTP, ^{125}I-angiotensin II, and Hybond N-nylon membranes were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany). [^{35}S]GTPγS (1250 Ci/mmol) was supplied by New England Nuclear (Boston, MA). 2′,7′-Dichlorodihydrofluorescein diacetate was purchased from Molecular Probes (Eugene, OR). Antibiotics, calf serum, and cell culture medium were obtained from Life Technologies (Eggenstein, Germany). RNA-clean was purchased from AGS (Heidelberg, Germany). Atorvastatin was a gift of Gödecke-Parke-Davis (Freiburg, Germany). Losartan, simvastatin, and lovastatin were a gift of Merck-Sharp-Dohme (Haar, Germany). Simvastatin, lovastatin, and mevalonate were chemically activated by alkaline hydrolysis, as described previously (Laufs et al., 1997). *Clostridium sordellii* lethal toxin was kindly provided by K. Aktories (University of Freiburg, Freiburg, Germany).

Cell Culture. VSMC were isolated from rat thoracic aorta (strain, male Sprague-Dawley, 6–10 weeks old, Charles River Wega GmbH, Sulzfeld, Germany) by enzymatic dispersion and cultured over several passages. Cells were grown in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium, supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids (100×), and 10% fetal calf serum. Experiments were performed with cells from passage 5 to 11.

Animals. Male, 18-week-old, spontaneously hypertensive rats (SHR) (Hoechst Marion Roussel, Frankfurt/Main, Germany) were put on a standard chow or on a standard chow supplemented with atorvastatin at a dose of 50 mg/kg body weight/day for 30 days. After treatment, the rats were killed by decapitation and the aortas were carefully excised. Animal experiments were performed in accordance with the German animal protection law.

mRNA Isolation, Northern Blot Analysis, and Polymerase Chain Reactions. Total cellular RNA from VSMC was isolated with RNA-clean according to the manufacturer's protocol. Approximately 1×10^6 VSMC were needed to obtain 10 μg of total RNA. Aliquots (10 μg) were electrophoresed through 1.2% agarose/0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. After capillary transfer on Hybond N-membranes, the RNA was UV-cross-linked. Northern blots were prehybridized for 2 h at 42°C and hybridized for 15 h at 42°C with a random-primed, [^{32}P]dCTP-labeled, rat AT1 receptor cDNA probe. The rat AT1 receptor cDNA probe was a 824-base-pair fragment generated from an AT1 receptor cDNA template by the polymerase chain reaction using the primer pair 5′-GTCATGATCCCTACCCTCTACAGC-3′ and 5′-CCGTAGAACAGAGGGTTCAGGCAG-3′ and *Taq* DNA polymerase. Blots were exposed to film, and autoradiographic signals and band intensities of 18S ribosomal RNA were quantified by laser densitometry.

After excision, rat aortas were quickly frozen in liquid nitrogen and homogenized with a motorized homogenizer. Total cellular RNA was isolated with RNA-clean, and 1-μg aliquots were electrophoresed as described above. The original AT1 receptor cDNA (Ca18b; Murphy et al.) was digested with *Msc*I and self-ligated. The resulting plasmid lacking the region from base 446 to 734 was linearized by digestion with *Sac*I, and the deletion mutated AT1 receptor cDNA was in vitro transcribed with the Megascript-Kit (Ambion, Austin, TX) following the manufacturer's instructions. One microgram of the isolated total RNA and 10 pg of the AT1 receptor mutant mRNA were mixed and reverse transcribed using random primers and Moloney murine leukemia virus reverse transcriptase for 60 min at 42°C and 10 min at 75°C. The single-stranded cDNA was amplified by polymerase chain reaction using *Taq* DNA polymerase. Twenty-eight cycles were performed under the following conditions: 30 s, 94°C; 45 s, 55°C; 45 s, 72°C. The sequences for AT1 receptor sense and antisense primers were 5′-ACC-CTC-TAC-AGC-ATC-ATC-TTT-GTG-GTG-GGG-3′ and 5′-GGG-AGC-GTC-GAA-TTC-CGA-GAC-TCA-TAA-TGA-3′, respectively. The same cDNA samples were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification (22 cycles) to confirm that equal amounts of RNA were reverse transcribed. The primers employed were 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′ and 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′. PCR amplification gave 479 bp, 191 bp, and 452 bp of fragments originated from AT1 receptor mRNA, mutated AT1 receptor mRNA, and GAPDH mRNA, respectively. Equal amounts of RT-PCR products were loaded on 1.5% agarose gels, and absorbance of ethidium bromide-stained DNA bands were quantified.

Radioligand Binding Assays. VSMC were collected, homogenized, and resuspended in an incubation buffer containing 50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 10 mM $MgCl_2$, and 0.1% bovine serum albumin. AT1 receptor density and affinity were investigated in saturation experiments using increasing amounts of ^{125}I-angiotensin II as radiolabeled ligand (0.25–8 nM). Losartan (10 μM) was used to determine nonspecific binding. Experiments were carried out at 24°C for 60 min. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters, and radioactivity was determined in a gamma counter (Beckman, Munich, Germany). All experiments were performed in duplicate. Maximal density (B_{max}) and affinity (K_d) of binding sites were obtained by nonlinear regression analysis.

Nuclear Run-On Assays. VSMC were collected and washed. After lysis for 10 min on ice, nuclei were isolated by centrifugation through 0.6 M sucrose. The nuclei (~3–5 × 10^8/reaction) were used

to carry out the transcription in a reaction mixture containing 40% glycerol; 50 mM Tris/HCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 0.5 mM levels of CTP, GTP, and ATP; and 0.2 to 0.3 μM [^{32}P]UTP (>3000 μCi/mmol) at 30°C for 30 min. Reactions were terminated by addition of RNA-clean, and the radioactive RNA was isolated and purified. Approximately 5×10^6 to 1×10^7 cpm of the [^{32}P]UTP-labeled RNA were dissolved in hybridization solution (100 mM TES, pH 7.4, 0.3 M NaCl, 100 μg/ml *Escherichia coli* tRNA). Plasmids (5 μg) containing cDNAs for the AT1 receptor (insert from pCa18b subcloned in pKS+ Bluescript; Stratagene, Heidelberg, Germany) or GAPDH (rat GAPDH in pIBI30; International Biotechnology, New Haven, CT), and a plasmid (KS+ Bluescript) without insert were linearized, denatured, and applied to nylon membranes using a dot blot apparatus. These membranes were prehybridized for 2 h at 42°C in 100 mM TES, 0.3 M NaCl, 100 μg/ml *E. coli* tRNA, and 5 × Denhardt's solution and were then hybridized at 42°C for 15 h. Membranes were exposed to film, and autoradiographic signals were quantified by laser densitometry. The relative intensity of the AT1 receptor signal was determined as the ratio of AT1 receptor to GAPDH intensity.

Measurement of Reactive Oxygen Species. Intracellular reactive oxygen species production in VSMC was measured by 2′,7′-dichlorofluorescein (DCF) fluorescence using confocal laser-scanning microscopy techniques. Dishes of subconfluent cells were washed and incubated in the dark for 30 min in the presence of 10 mM 2′,7′-dichlorodihydrofluorescein diacetate. Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Jena, Germany), equipped with a 25×, numerical aperture 0.8, oil-immersion objective (Plan-Neofluar, Carl Zeiss) and Zeiss LSM 410 confocal attachment, and reactive oxygen species generation was detected as a result of the oxidation of dichlorodihydrofluorescein (excitation, 488 nm; emission long-pass LP515-nm filter set). Images (512- × 512-pixel) were collected by single rapid scans and identical parameters, such as contrast and brightness, for all samples. Five groups of 25 cells for each sample were randomly selected from the image and fluorescent intensity was taken. The relative fluorescence intensity is an average of values of all experiments.

For measurement of superoxide release in intact vessel segments, aortas were excised carefully and placed in chilled, modified Krebs-HEPES buffer [pH 7.4; 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM $CaCl_2$, 1.20 mM $MgSO_4$, 20.0 mM NaHEPES, 1.03 mM K_2HPO_4, 25.0 mM $NaHCO_3$, 11.1 mM D-(+)Glucose]. Connective tissue was removed, and aortas were cut into 5-mm segments. The aortic rings were placed in Krebs-HEPES buffer aerated with 95% O_2 and 5% CO_2 and were incubated for 30 min at 37°C. Then the samples were transferred into scintillation vials containing 2 ml of Krebs-HEPES buffer with 5 μM lucigenin. Chemiluminescence was assessed over 10 min in a scintillation counter (Berthold Lumat LB 9501) in 1-min intervals. Background signals were subtracted. The vessel segments were then dried, and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue.

Western Blotting. Total cell lysates and membrane and cytosolic proteins were isolated and separated on SDS-polyacrylamide gel electrophoresis, as described previously (Laufs and Liao, 1998). Immunoblotting was performed using a rac1 monoclonal antibody (sc-217, 1:250 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunodetection was accomplished using a goat anti-rabbit secondary antibody (1:4000 dilution), and the enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Autoradiography was performed at 24°C, and the appropriate exposures were quantified by densitometry.

Assay for rac1 GTP-Binding Activity. The rac1 GTP-binding activity was determined by immunoprecipitation of [^{35}S]GTPγS-labeled rac1. Briefly, membrane and cytosolic proteins were isolated, and proteins (20 μg) from control and treated VSMC were incubated for 30 min at 37°C in a buffer containing 20 nM [^{35}S]GTPγS, 2 μM GTP, 5 mM $MgCl_2$, 0.1 mM EGTA, 50 mM NaCl, 4 mM creatinine phosphate, 5 U phosphocreatinine kinase, 0.1 mM ATP, 1 mM dithiothreitol, 100 μg/ml leupeptin, 50 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The assay was terminated with excess unlabeled GTPγS (100 μM). Samples were then resuspended in 100 μl of immunoprecipitation buffer containing 1% Triton-X, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The rac1 antiserum was added to the mixture at a final dilution of 1:75. The samples were allowed to incubate for 4 h with gentle mixing. The antibody–G-protein complexes were then incubated with 50 μl of protein A-Sepharose (1 mg/ml, Santa Cruz Biotechnology Inc.) for 2 h, and the immunoprecipitate was collected by centrifugation at 12,000*g* for 10 min. Preliminary studies using Western analysis of the supernatant indicated that rac1 was completely immunoprecipitated under these conditions. The pellets were washed four times in a buffer containing 50 mM HEPES, pH 7.4, 100 μM NaF, 50 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, and 0.1% SDS. The final pellet containing the immunoprecipitated [^{35}S]GTPγS-labeled rac1 proteins was counted in a liquid scintillation counter (LS 1800; Beckman, Munich, Germany). Nonspecific activity was determined in the presence of excess unlabeled GTPγS (100 μM).

Statistical Analysis. Data are presented as means ± S.E.M. obtained in at least three separate experiments. Statistical analysis was performed using analysis of variance and Mann-Whitney-U test. $p < 0.05$ indicates statistical significance.

Results

Statins Reduce the Production of Reactive Oxygen Species. To evaluate the effect of statins on intracellular production of reactive oxygen species, VSMC were preincubated for various time points with vehicle, 10 μM atorvastatin, 200 μM L-mevalonate, or both, followed by a 3-h incubation with 1 μM angiotensin II. Preliminary studies with various incubation periods of angiotensin II indicated that ROS production was increased after 30 min and was within the maximum after 3 h (data not shown). ROS production was quantified by DCF fluorescence laser-microscopy. A representative microscopic scan is shown in Fig. 1A, and data analysis of three separate experiments is illustrated in Fig. 1B. Incubation with atorvastatin alone for 12 h had no significant effect on basal ROS production (108 ± 9% of control; n.s. versus control), whereas stimulation with angiotensin II led to a marked increase of ROS production (201 ± 7% of control; $p < 0.05$ versus control). Preincubation with atorvastatin (12 h) significantly reduced angiotensin II-induced ROS production to 92 ± 13% of control ($p < 0.05$ versus angiotensin II). L-Mevalonate did not affect basal ROS production (106 ± 17% of control; n.s. versus control) but reversed completely the effect of atorvastatin on angiotensin II-induced ROS production (197 ± 35% of control; $p < 0.05$ versus control; n.s. versus angiotensin II). Measurements at various time points revealed that statins exerted no effect on the basal release of free radicals. Moreover, inhibition of angiotensin II-induced ROS production by atorvastatin required long-term preincubations (12–24 h), whereas short-term exposition for 1 to 4 h showed no effect (data not shown). Angiotensin II-mediated ROS production was completely prevented by preincubation with the AT1 receptor antagonist losartan or diphenylene iodonium, an inhibitor of flavoprotein containing oxidoreductases such as NAD(P)H oxidase (data not shown).

Statins Down-Regulate AT1 Receptor Gene Expression. To assess whether atorvastatin caused reduction of

angiotensin II-induced ROS production via modulation of AT1 receptor gene expression, we examined the effect of atorvastatin on AT1 receptor mRNA expression by Northern blot experiments. Cells were treated with 10 μM atorvastatin or vehicle, and total cellular RNA was extracted at the time points indicated in Fig. 2A. Figure 2A illustrates representative autoradiographic results revealing a time-dependent reduction of the transcript level. Densitometric data of three separate experiments show the atorvastatin-induced down-regulation of AT1 receptor mRNA hybridization signal compared with 18S ribosomal RNA band intensities, relative to vehicle-treated control levels at 0 h (100%). The effect was maximal after 4 h incubation with 10 μM atorvastatin (55 $\pm$ 5% of control; $p < 0.05$ versus control). Repetitive addition of atorvastatin after 4, 8, and 12 h caused a more profound decrease of the AT1 receptor mRNA for up to 24 h (35 $\pm$ 6% of control after 24 h; $p < 0.05$ versus control; data not shown). Figure 2B demonstrates the concentration-dependent effect of atorvastatin on AT1 receptor mRNA expression. VSMC were incubated with vehicle or 0.1 nM to 100 μM atorvastatin. AT1 receptor down-regulation was significant with 1 and 10 μM atorvastatin ($p < 0.05$ versus control) and reached a maximum at 100 μM atorvastatin (15 $\pm$ 4%; $p < 0.05$ versus control). In a set of control experiments cells were serum-deprived for 24 h, and RNA was subsequently isolated after incubation with vehicle. AT1 receptor mRNA was not significantly altered during the time course of the assay, suggesting that the AT1 receptor mRNA expression level remained

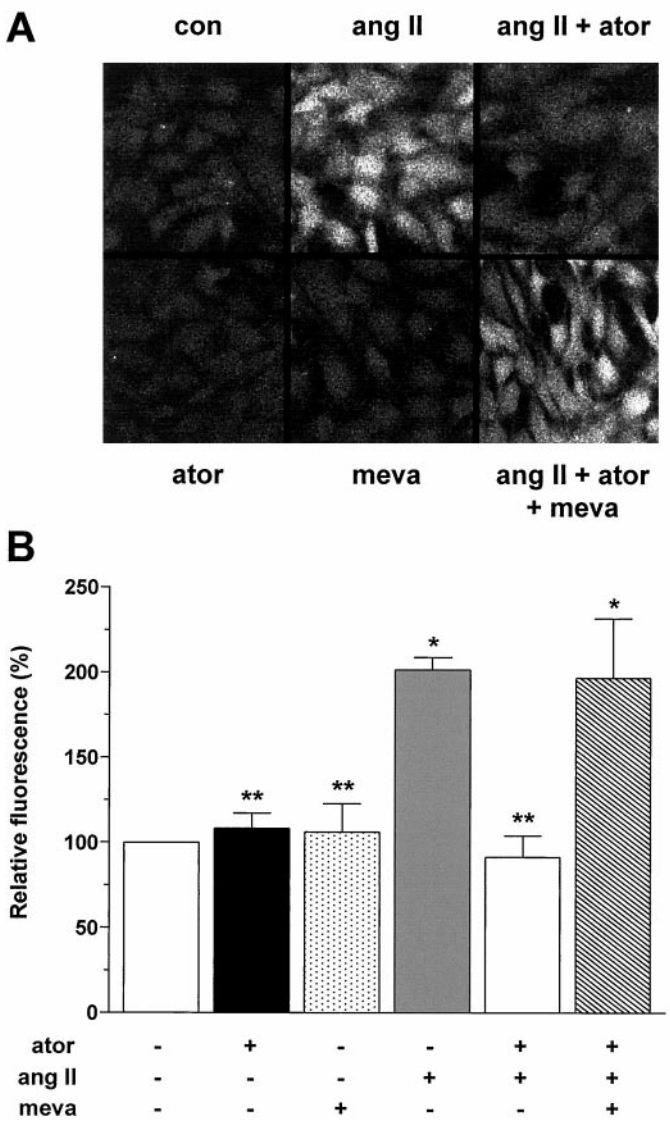

Fig. 1. Effect of HMG-CoA reductase inhibition on angiotensin II-induced intracellular production of reactive oxygen species in VSMC. A, representative microscopic scan. VSMC were preincubated for 12 h with vehicle (con), 10 μM atorvastatin (ator), 200 μM L-mevalonate (meva), or both, followed by a 3-h incubation with 1 μM angiotensin II (ang II). Free radical production is visualized through DCF fluorescence. B, quantification of free radical production measured by DCF fluorescence laser-microscopy. Data analysis of three separate experiments expressed as relative fluorescence (mean $\pm$ S.E.M.). *$p < 0.05$ versus control; **$p < 0.05$ versus angiotensin II.

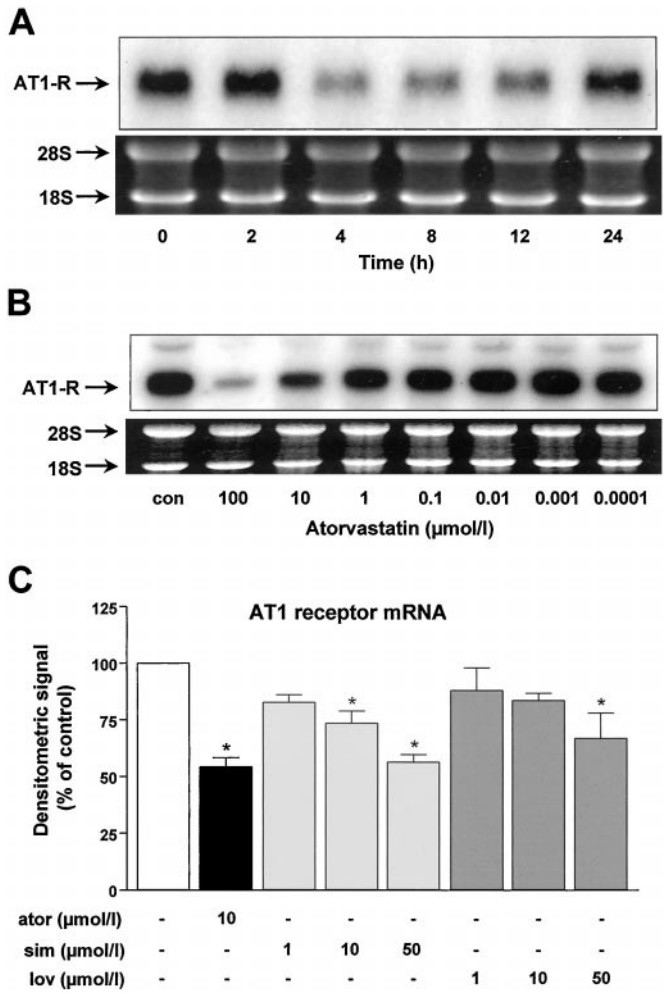

Fig. 2. Effect of HMG-CoA reductase inhibition on AT1 receptor mRNA expression. A, VSMC were exposed to 10 μM atorvastatin. Hybridization of an AT1 receptor cDNA probe to Northern blots of 10 μg of total RNA/lane extracted from VSMC at the indicated time points. Representative autoradiogram and corresponding ethidium bromide-stained ribosomal RNA. B, Northern blot analysis of 10 μg total RNA/lane extracted from VSMC after 4 h incubation with 0.1 nM to 100 μM atorvastatin or vehicle. Autoradiogram showing the AT1 receptor mRNA levels and corresponding ribosomal RNA. C, VSMC were exposed for 4 h to vehicle; 10 μM atorvastatin (ator); 1, 10, or 50 μM simvastatin (sim); or lovastatin (lov), and subsequently Northern hybridizations of 10 μg of total RNA/lane were carried out. Densitometric data are from three separate experiments. Each column represents the relative hybridization signal (mean $\pm$ S.E.M.) normalized to the treatment with vehicle (100%). *$p <$ 0.05 versus control.

stable for up to 24 h after withdrawal of serum and incubation with vehicle (data not shown).

To evaluate whether the reduced level of AT1 receptor mRNA was translated to a reduction of AT1 receptor expression, ^{125}I-angiotensin II radioligand binding assays were performed. After a 24-h treatment of VSMC with either 10 μM atorvastatin or vehicle, AT1 receptor binding sites were measured in a membrane saturation binding assay. AT1 receptor density was significantly decreased in atorvastatin-treated VSMC (B_{max}, 823 $\pm$ 136 fmol/mg of protein; $p < 0.05$ versus control) compared with vehicle-treated cells (B_{max}, 1424 $\pm$ 274 fmol/mg of protein). Treatment with atorvastatin did not change the affinity for the radioligand [K_d = 4.0 (1.2/6.7) nM versus K_d = 5.5 (−1.2/12.2) nM for vehicle-treated cells; n.s.]. Additional competition binding experiments, in which ^{125}I-angiotensin II (0.05 nM) was competed by increasing concentrations of losartan or atorvastatin (0.1 nM-10 μM), revealed that atorvastatin does not specifically bind to the AT1 receptor (data not shown).

To compare the effects of different HMG-CoA reductase inhibitors on AT1 receptor transcript levels, VSMC were incubated for 4 h with vehicle; 10 μM atorvastatin; 1, 10, or 50 μM simvastatin or lovastatin. Figure 2C shows that simvastatin and lovastatin also caused a significant, concentration-dependent reduction of the AT1 receptor mRNA levels, with a maximum of 67 $\pm$ 11% of control ($p < 0.05$ versus control) for lovastatin, and 56 $\pm$ 3% of control ($p < 0.05$ versus control) for simvastatin, comparable with the effect of atorvastatin (54 $\pm$ 4% of control; $p < 0.05$ versus control). In all experimental set-ups, 18S ribosomal RNA was not altered (data not shown).

Statins Decrease AT1 Receptor mRNA Stability. To assess the effect of atorvastatin on AT1 receptor gene transcription rate, we measured the de novo synthesis of AT1 receptor mRNA. VSMC were treated for 4 h with either 10 μM atorvastatin or vehicle, and nuclear run-on assays were performed. Figure 3A shows a representative autoradiogram of radiolabeled, de novo synthesized mRNA to AT1 receptor, GAPDH, and plasmid DNA. Incubation with atorvastatin had no effect on the rate of de novo synthesis of AT1 receptor mRNA (95 $\pm$ 16% of control; n.s.). To investigate whether atorvastatin caused its effect by influencing AT1 receptor mRNA stability, cells were incubated for 2 h with either 10 μM atorvastatin or vehicle before transcription was blocked with 50 μg/ml DRB. Total RNA was then extracted at the indicated time points, and Northern hybridizations were performed. Figure 3B illustrates that atorvastatin caused a destabilization of the AT1 receptor mRNA with a decrease of the mRNA half-life from 6 h to approximately 2.5 h. GAPDH mRNA remained stable during the experimental period (data not shown).

Statin-Induced Down-Regulation of AT1 Receptor Gene Expression Is Mediated by Impaired Geranylgeranylation. To assess whether the atorvastatin-induced down-regulation of AT1 receptor mRNA expression was caused by specific inhibition of HMG-CoA reductase, VSMC were treated for 4 h with either L-mevalonate (200 μM) or 25-hydroxycholesterol (5 μg/ml) in addition to 10 μM atorvastatin or vehicle. AT1 receptor mRNA was assessed by Northern blotting (Fig. 4). Mevalonate completely prevented the inhibitory effect of atorvastatin on AT1 receptor mRNA, whereas addition of hydroxycholesterol did not reverse the atorvastatin-induced reduction of AT1 receptor transcript levels. Furthermore, cells were incubated for 4 h with the specific farnesyl-transferase inhibitor FTI-276 (10 nM) or geranylgeranyl-transferase inhibitor GGTI-286 (50 μM). GGTI, but not FTI, caused a marked down-regulation of the AT1 receptor mRNA. In all experimental set-ups, 18S ribosomal RNA was not altered (data not shown).

Statins Block the Angiotensin II-Mediated Activation of rac1. We have previously shown that statins inhibit the posttranslational isoprenylation of small GTP-binding proteins in vascular smooth muscle cells (Laufs et al., 1999). Rac1 GTPase is presumably important for NAD(P)H oxidase activation and free radical release. Rac1 is posttranslationally geranylgeranylated. Therefore, the effect of angiotensin II and statins on the expression of rac1 was assessed in total cell lysates and in membrane fractions. Rac1 was present in

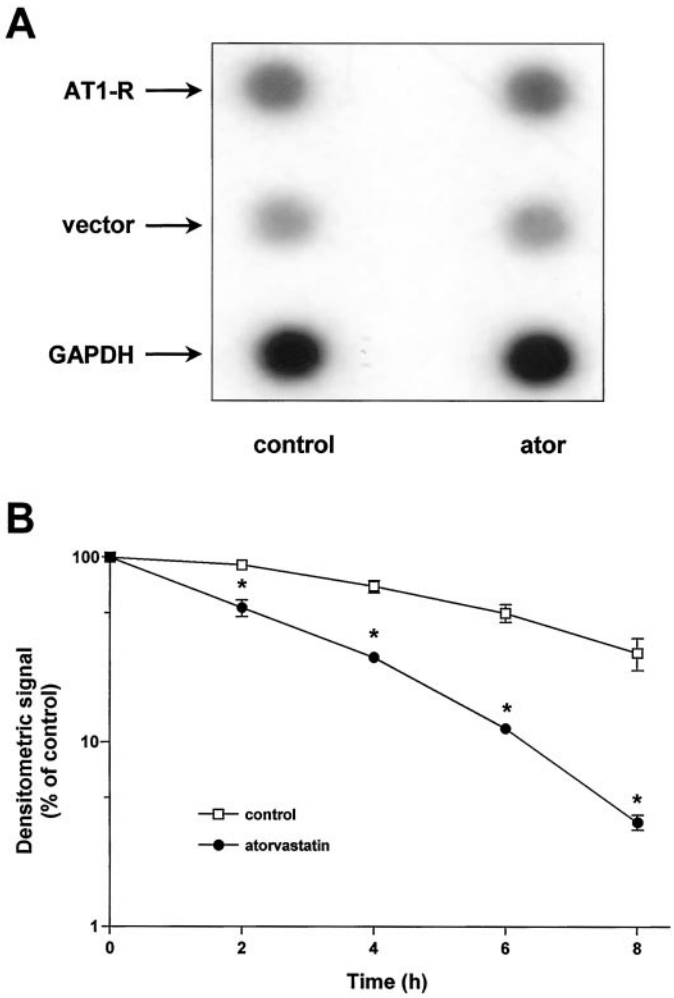

Fig. 3. Effect of HMG-CoA reductase inhibition on AT1 receptor gene transcription rate and mRNA stability. A, VSMC were challenged with either vehicle or 10 μM atorvastatin for 4 h, and nuclei were isolated and used for nuclear run-on assays. Radioactive de novo synthesized mRNA was hybridized to AT1 receptor (AT1-R), plasmid (vector), or GAPDH (GAPDH) cDNA. Representative autoradiogram. B, VSMC were pretreated with either vehicle (□) or 10 μM atorvastatin (●) for 2 h before cells were exposed to 50 μg/ml DRB. Total RNA was isolated at the indicated time points, and Northern hybridizations were performed with an AT1 receptor cDNA probe. Each point represents the relative hybridization signal of four separate experiments (mean $\pm$ S.E.M.) normalized to the mRNA level obtained from cells pretreated for 2 h before the addition of DRB to the culture medium. *$p < 0.05$ versus control.

VSMC under basal culture conditions. Treatment with angiotensin II (1 μM, 3 h) induced rac1 expression in total cells and increased the translocation of rac1 to the cellular membrane (Fig. 5A). Preincubation of VSMC with atorvastatin (10 μM, 12 h) decreased the membrane translocation of rac1 under basal conditions and completely prevented the angiotensin II-mediated increase of rac1 expression in the cell membrane. Expression of rac1 in the cytosol was increased by atorvastatin.

To determine whether angiotensin II and atorvastatin affect rac1 activity (i.e., GTP-bound state), we immunoprecipitated [^{35}S]GTPγS-labeled rac1 from the membrane of VSMC. Under basal conditions, VSMC had a membrane-associated rac1 activity of 2.1 $\pm$ 0.1 fmol/mg/min. Treatment with angiotensin II (1 μM, 3 h) increased rac1 GTP-binding activity by 2-fold (4.0 $\pm$ 0.6 fmol/mg/min; $p < 0.05$). Atorvastatin (10 μM, 12 h) not only completely inhibited angiotensin II-induced rac1 GTP binding, but reduced basal rac1 membrane-associated activity by half (0.9 $\pm$ 0.2 and 0.8 $\pm$ 0.7 fmol/mg/min, respectively; $p < 0.05$) (Fig. 5B).

Inhibition of rac1 Reduces Angiotensin II-Induced Free Radical Release. The effect of rac1 on angiotensin II-mediated release of free radicals was measured by DCF fluorescence laser-microscopy. *Clostridium sordellii* lethal toxin (LT) is a glucosyltransferase that specifically inhibits rac1 GTPase independent of isoprenylation (Aktories, 1997). VSMC were pretreated for 12 h with either vehicle or LT (200 ng/ml) before angiotensin II (1 μM) was added for additional 3 h. Figure 6 (representative microscopic scan and data analysis of three separate experiments) shows that angiotensin II-induced ROS production was significantly reduced by rac1 inhibition with LT. In contrast, LT had no effect on the basal release of free radicals.

Statin Treatment Causes Down-Regulation of Aortic AT1 Receptor mRNA Expression and Reduction of Vascular Free Radical Production in Vivo. To investigate whether statins are capable of modulating vascular AT1 receptor gene expression in vivo, we assessed AT1 receptor mRNA concentrations by means of quantitative RT-PCR in RNA isolated from aortic segments of SHR treated with atorvastatin and from control animals. Figure 7A demonstrates the densitometric analysis of these experiments (n = 5 per group) revealing that aortic AT1 receptor mRNA expression was significantly down-regulated to 45 $\pm$ 12% in rats treated with atorvastatin ($p < 0.05$ versus control).

In addition, vascular production of ROS was measured

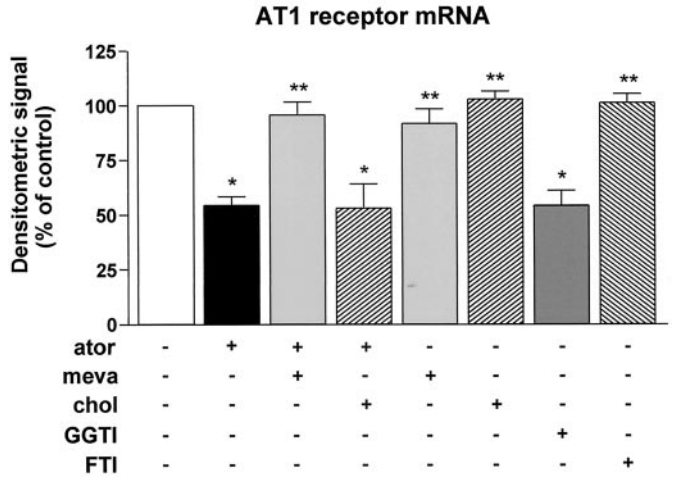

Fig. 4. Effect of isoprenoids on HMG-CoA reductase inhibition-induced down-regulation of AT1 receptor mRNA expression. VSMC were treated for 4 h with either L-mevalonate (200 μM; meva) or 25-hydroxycholesterol (5 μg/ml; chol) in addition to 10 μM atorvastatin (ator) or vehicle. In addition, cells were treated with geranylgeranyl-transferase inhibitor (50 μM; GGTI-286) or farnesyl-transferase inhibitor (10 nM; FTI-276). Subsequently, Northern blots of 10 μg of total RNA/lane were performed. Each column represents the relative hybridization signal (mean $\pm$ S.E.M.) normalized to the treatment with vehicle (100%) of three separate experiments. *$p < 0.05$ versus control; **$p < 0.05$ versus atorvastatin.

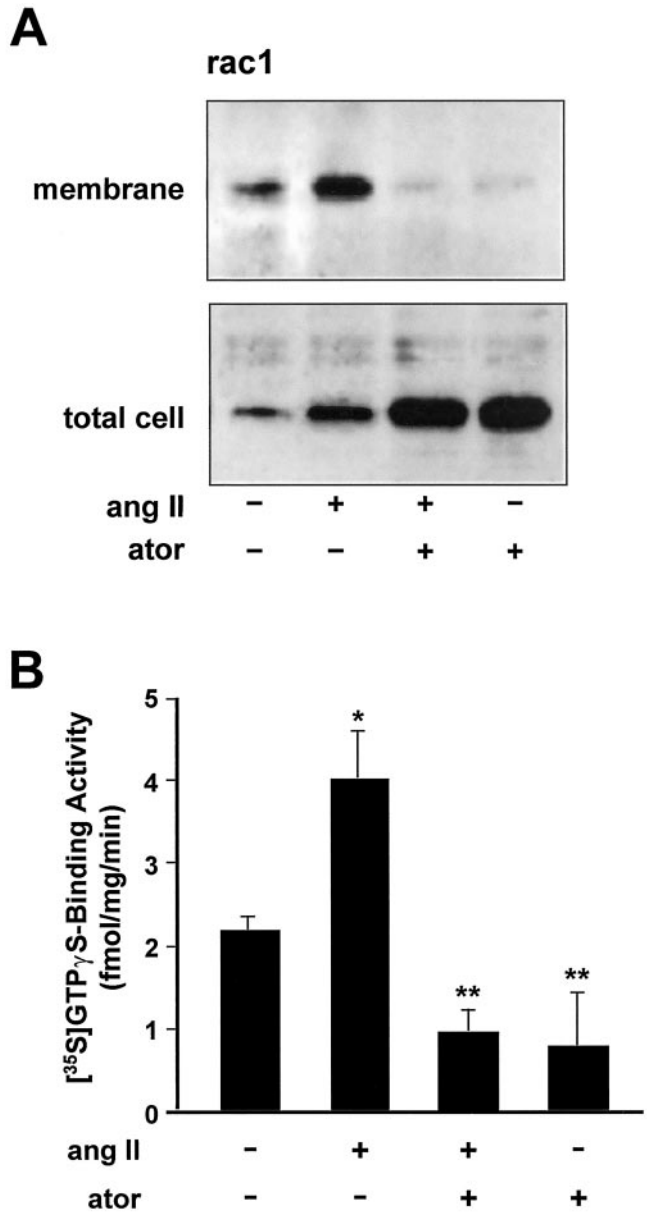

Fig. 5. Effect of HMG-CoA reductase inhibition and angiotensin II on rac1 expression and activity. A, representative immunoblot (30 μg of protein/lane) showing the effects of atorvastatin (10 μM, 12 h; ator) in the presence and absence of angiotensin II (1 μM, 3 h; ang II) on rac1 protein expression in total cell lysates and cell membranes of VSMC. B, [^{35}S]GTPγS-binding assays showing the effect of angiotensin II (1 μM, 3 h; ang II) and atorvastatin (10 μM, 12 h; ator) on the membrane-associated rac1 GTP-binding activity. Data analysis of three separate experiments (mean $\pm$ S.E.M.). *$p < 0.05$ versus control; **$p < 0.05$ versus angiotensin II.

with lucigenin chemiluminescence assays in intact isolated aortic segments of control and statin-treated SHR. Figure 7B illustrates that treatment with atorvastatin caused a significant decrease of superoxide production in the vessel wall to 59 ± 6% of control levels ($n = 5$ per group; $p < 0.05$ versus control).

Discussion

Inhibition of the HMG-CoA reductase leads to decreased angiotensin II-induced release of free radicals from vascular smooth muscle cells. This effect is mediated by two important mechanisms. First, statins down-regulate AT1 receptor gene expression. The molecular mechanism is the destabilization of the AT1 receptor mRNA mediated by impaired geranylgeranylation. Furthermore, we show that angiotensin II activates the small GTP-binding protein rac1. Statins inhibit the activation of rac1 by angiotensin II by inhibiting the geranylgeranyl-dependent translocation of rac1 from the cytosol to the cell membrane. Finally, direct inhibition of rac1 reduces free radical release induced by angiotensin II. Consistently, HMG-CoA reductase inhibition causes down-regulation of vascular AT1 receptor mRNA expression and reduction of vascular ROS production in vivo.

Beside their cholesterol-lowering properties (Levine et al., 1995), statins exert direct effects on VSMC, such as down-regulation of monocyte chemoattractant protein-1 (Bustos et al., 1998), induction of apoptosis (Guijarro et al., 1998), and inhibition of cell cycle progression (Laufs et al., 1999). In

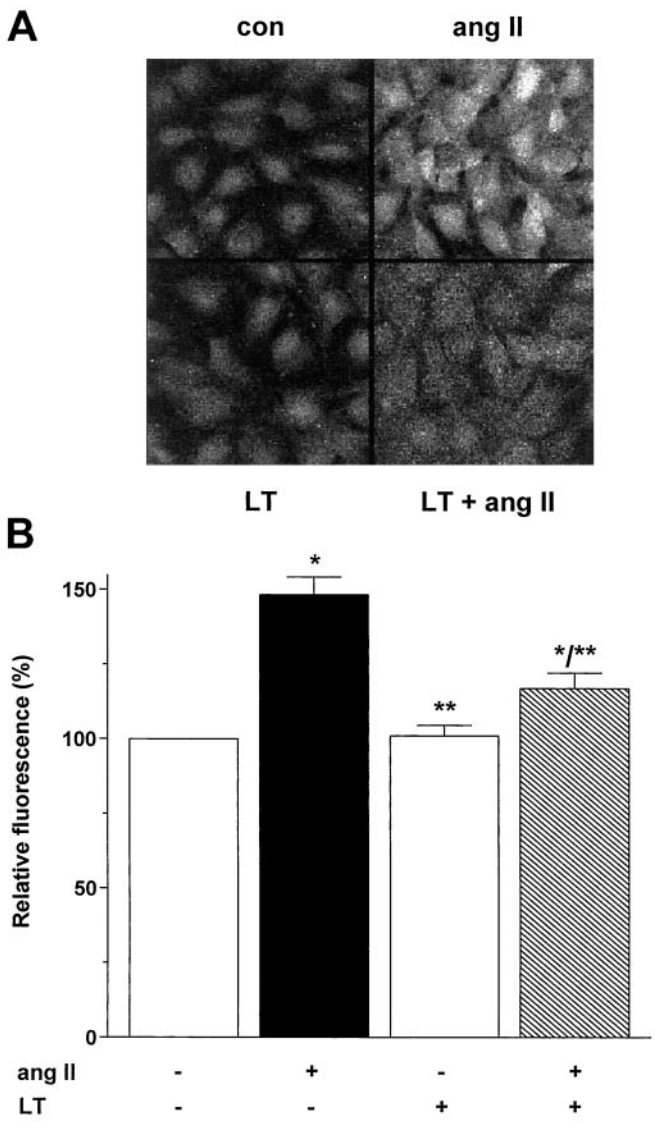

Fig. 6. Effect of rac1 inhibition on angiotensin II-induced intracellular production of reactive oxygen species. A, representative microscopic scan. VSMC were preincubated for 12 h with either vehicle (con) or *Clostridium sordellii* lethal toxin (200 ng/ml; LT), followed by a 3-h incubation with angiotensin II (1 μM; ang II). B, quantification of free radical production measured by DCF fluorescence laser-microscopy. Data analysis of three separate experiments expressed as relative fluorescence (mean ± S.E.M.). *$p < 0.05$ versus control; **$p < 0.05$ versus angiotensin II.

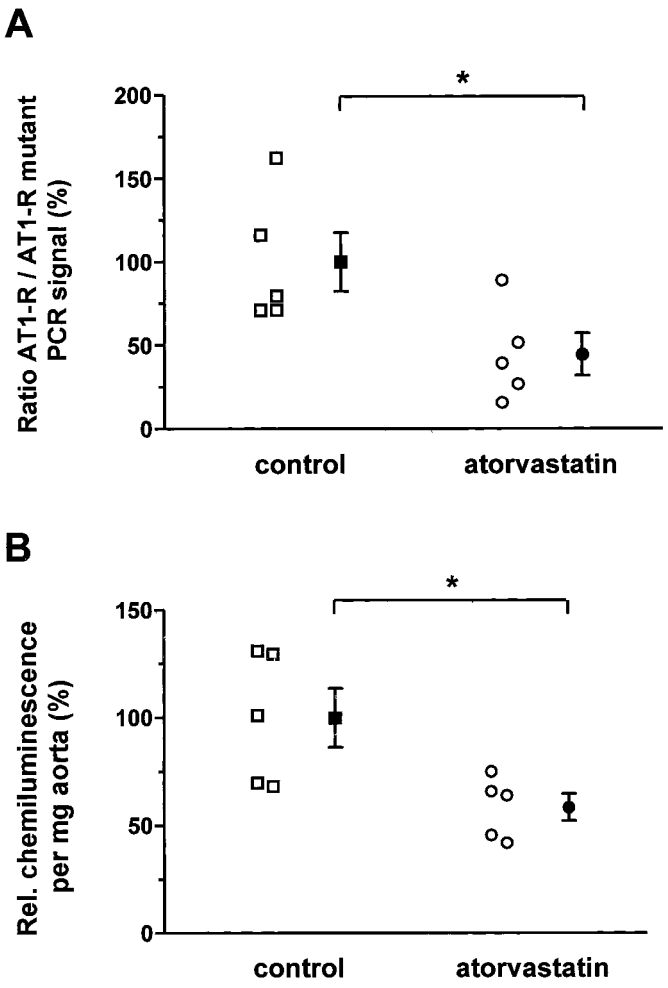

Fig. 7. Effect of HMG-CoA reductase inhibition on aortic AT1 receptor mRNA expression and free radical production in vivo. Male, spontaneously hypertensive rats (SHR) were put on standard chow (control) or on standard chow plus atorvastatin (50 mg/kg/day) for 30 days. A, aortic segments were isolated and homogenized. AT1 receptor mRNA expression was assessed by quantitative RT-PCR. Densitometric analysis of the ratio of AT1 receptor wild-type and AT1 receptor mutant PCR signal. Aortas of control animals (squares) were compared with atorvastatin-treated SHR (circles). Open symbols, individual data; closed symbols, mean ± S.E.M. ($n = 5$ per group). *$p < 0.05$ versus control. B, superoxide production in intact isolated aortic segments of control (squares) and statin-treated SHR (circles) was measured with lucigenin chemiluminescence assays. Aortic rings (5 mm) were transferred to scintillation vials containing Krebs-HEPES buffer with 5 μM lucigenin, and chemiluminescence was recorded over 10 min. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue. Open symbols, individual data; closed symbols, mean ± S.E.M. ($n = 5$ per group). *$p <$ 0.05 versus control.

endothelial cells, treatment with statins leads to a cholesterol-independent decrease of endothelin-1 synthesis and to an up-regulation of the expression of endothelial nitric-oxide synthase, contributing to an improved endothelial function (Endres et al., 1998; Hernandez-Perera et al., 1998; Laufs et al., 1998). However, the pathogenesis of endothelial dysfunction and atherosclerosis is closely connected to reactive oxygen species, which are involved in proliferation, contraction, and apoptosis of vascular cells (Darley-Usmar et al., 1997; Harrison, 1997; Laursen et al., 1997; Li et al., 1997; Yang et al., 1998). It was demonstrated that angiotensin II is a predominant factor leading to an increased production of free radicals in VSMC by activation of the NAD(P)H oxidase via stimulation of the AT1 receptor (Griendling et al., 1994; Rajagopalan et al., 1996). Our findings demonstrate that statins lead to a decreased angiotensin II-induced intracellular production of free radicals. In addition to the above-mentioned interactions of statins with nitric oxide production and other vasoactive factors, these findings reveal another potent pleiotropic effect of statins. The antioxidative properties of HMG-CoA reductase inhibitors are based on at least two different mechanisms both generated through reduced isoprenylation rather than through decreased cholesterol synthesis.

First, statin-mediated reduction of AT1 receptor gene expression may impair angiotensin II effects on intracellular signaling, which ultimately causes a diminished activation of the NAD(P)H oxidase. Decreased ROS production as well as AT1 receptor down-regulation after statin treatment is reversed with L-mevalonate, indicating that these effects are mediated through HMG-CoA reductase inhibition. Mevalonate alone had no effect on AT1 receptor regulation and basal ROS production. This indicates that basal cellular isoprenoid concentrations are sufficient for required prenylations of downstream targets, so that an increase of isoprenoid intermediates after adding mevalonate exerts no additional effect. However, after inhibition of the HMG-CoA reductase by statins, isoprenoid depletion leads to the described effects on AT1 receptor regulation and ROS production, which can then be reversed by addition of mevalonate. Geranylgeranylation but not farnesylation or decreased cholesterol synthesis is involved in the statin-induced down-regulation of AT1 receptor expression. The decrement in mRNA levels is not mediated through a decrease in AT1 receptor gene transcription rate. It is well established that post-transcriptional modulation is the predominant mechanism for the regulation of AT1 receptor gene expression (Nickenig and Murphy, 1994, 1996; Nickenig et al., 1997, 1998b). Consistently, statins lead to a decreased AT1 receptor mRNA stability. The second messenger systems involved in these events are less clear. Decreased mevalonate synthesis caused by HMG-CoA reductase inhibition leads to changes in isoprenoid metabolism (Goldstein and Brown, 1990). Isoprenoid intermediates are important for the post-translational modification and function of numerous proteins, such as the family of small GTP-binding proteins (Laufs and Liao, 1998). It was shown, for example, that up-regulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors is mediated by an increase in mRNA stability via blocking of the geranylgeranylation of rho GTPase (Laufs and Liao, 1998). If AT1 receptor regulation by statins involves similar geranylgeranyl-dependent pathways is currently unknown.

Second, direct effects of statins on ROS-generating enzymes independent of AT1 receptor regulation may also account for reduced radical production. The NAD(P)H oxidase is a multicomponent enzyme complex. Superoxide is produced upon assembly of the cytosolic proteins rac1, p67phox, p47phox, and p40phox with the transmembrane proteins gp91phox/mox1 and p22phox (Griendling and Ushio-Fukai, 1998; Babior, 1999; Suh et al., 1999). The GTPase rac1 seems to be crucial for the agonist-mediated activation of the enzyme. This requires translocation of rac1 from the cytosol to the cell membrane, as demonstrated in neutrophils (Dusi et al., 1995; Rinckel et al., 1999). Assembly and composition of the NAD(P)H oxidase may vary dependent on the cell type. Most of the knowledge concerning the NAD(P)H oxidase system has been recruited from investigations in phagocytes, and it is not at all clear whether superoxide production via NAD(P)H oxidase is comparable in vascular cells. Indeed, little is known about mechanisms of superoxide production by the NAD(P)H oxidase in vascular cells. In this respect, the role of rac1 in the process of radical generation in vascular cells has not yet been elucidated. Our data suggest that rac1 translocation from the cytosol to the membrane is a prerequisite for angiotensin II-induced ROS production, which may lead to the assembly of the NAD(P)H oxidase subunits within the cell membrane. Moreover, this process is inhibited by statins resulting in a reduced angiotensin II-driven ROS release. This is mediated through diminished geranylgeranylation of the small GTPase rac1, which is required for its translocation to the cell membrane. Consequently, specific inhibition of rac1 reduces angiotensin II-induced ROS production, suggesting the importance of rac1 in angiotensin II-caused NAD(P)H oxidase activation. These results demonstrate detailed mechanisms of superoxide production by the NAD(P)H oxidase in vascular cells and are in agreement with previous findings from polymorphonuclear cells (Bockoch and Prossnitz, 1992; Diebold et al., 1994; Day et al., 1997). Interestingly, rac1 expression in total cell homogenates increases after statin treatment, maybe because of a negative feedback regulation elicited by decreased membrane-bound rac1. In our experimental settings, statins and LT did not alter basal production of free radicals. This finding and the reversal by mevalonate exclude nonspecific effects of statins or LT on DCF fluorescence. According to these DCF fluorescence data, atorvastatin does not exert direct radical scavenging properties. However, no direct measurements of nitric oxide, peroxynitrite, hydroxyl radical, hydrogen peroxide, or superoxide were undertaken, so that direct antioxidant properties of the statin cannot be fully excluded. Moreover, the effect of statins on other enzymes influencing the amount of ROS released in VSMC, such as xanthin oxidase or superoxide dismutases, was not investigated.

Finally, our data indicate that the observed in vitro effects of HMG-CoA reductase inhibition are also relevant in vivo, because statin treatment caused decreased aortic AT1 receptor expression and reduced aortic ROS production in rats.

Beside their lipid-lowering properties in hypercholesterolemic subjects, statins exert additional beneficial therapeutic effects (Vaughan et al., 1996). Subgroup analyses of clinical trials suggested that there may be therapeutic benefits of treatment with statins that are independent of plasma cholesterol levels (Anonymous, 1998; O'Driscoll et al., 1997). Statins seem to influence various aspects potentially in-

volved in the pathogenesis of chronic cardiovascular diseases. The presented results provide a novel regulatory mechanism of HMG-CoA reductase inhibitors modulating vascular function through antioxidative properties by rac1 inhibition and AT1 receptor down-regulation, which may help us understand the complexity of interactions of these drugs with the vessel wall.

Acknowledgments

We thank K. Aktories for kindly providing LT.

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