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Negative feedback regulation of reactive oxygen species on AT1 receptor gene expression

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1 Free radicals as well as the AT1 receptor are involved in the pathogenesis of cardiovascular disease. Both the intracellular mechanisms of AT1 receptor regulation and the effect of free radicals on AT1 receptor expression are currently unknown. This study investigates the role of free radicals in the modulation of AT1 receptor expression and in the angiotensin II-induced AT1 receptor regulation.

2 AT1 receptor mRNA was assessed by Northern blotting and AT1 receptor density by radioligand binding assays, respectively, in vascular smooth muscle cells (VSMC). Free radical release was measured by confocal laser scanning microscopy. AT1 receptor mRNA transcription rate was determined by nuclear run-on assays and AT1 receptor mRNA half-life was measured under transcriptional blockade.

3 Angiotensin II caused a time-dependent decrease of AT1 receptor mRNA expression in rat VSMC in culture ($30 \pm 6\%$ at 4 h with 100 nM angiotensin II). This was followed by a consistent decrease in AT1 receptor density. Angiotensin II caused release of reactive oxygen species in VSMC which was abolished by preincubation with 100 μM diphenylene iodonium (DPI). DPI inhibited partially the down-regulating effect of angiotensin II on the AT1 receptor. Incubation of VSMC with either hydrogen peroxide or xanthine/xanthine oxidase caused a dose-dependent decrease in AT1 receptor mRNA expression which was not mediated by a decreased rate of transcription but rather through destabilization of AT1 receptor mRNA. Experiments which included preincubation of VSMC with various intracellular inhibitors suggested that free radicals caused AT1 receptor downregulation through activation of p38-MAP kinase and intracellular release of calcium. However, angiotensin II-induced AT1 receptor expression was not inhibited by blockade of p38-MAP kinase activation or intracellular calcium release.

4 Free radicals may at least in part mediate angiotensin II-induced AT1 receptor regulation through direct post-transcriptional effects on AT1 receptor mRNA expression which involves intracellular release of calcium and activation of p38-MAP kinase. These findings may help to clarify the intracellular mechanisms involved in AT1 receptor regulation and reveal a novel biological feature for reactive oxygen species.

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Abbreviations: DCF-DA, 12,7dichlorodihydrofluorescein-diacetate; DMEM, Dulbecco's modified Eagles medium; DPI, Diphenylene iodonium; DRB, 5,6-dichlorobenzimidazole; PBS, Phosphate-buffered saline; VSMC, Vascular smooth muscle cells

Introduction

Reactive oxygen species are thought to be involved in the pathogenesis of cardiovascular diseases such as hypertension and atherosclerosis. The free radicals are released from various types of cells residing either in the vessel wall or circulating in the blood. In this respect, endothelial as well as vascular smooth muscle cells are known to be potent sources of these reactive oxygen species (Darley-Usmar *et al*., 1997; Rajagopalan *et al*., 1996; Laursen *et al*., 1997). It was recently shown that these molecules participate in proliferation of vascular smooth muscle cells, promote the development of hypertension, and influence apoptosis of vascular cells (Rajagopalan *et al*., 1996; Laursen *et al*., 1997; Li *et al*., 1997; Wolin, 1996; Bretschneider *et al*., 1997). These effects may be related to either oxidative scavenging of nitric oxide or to direct cellular effects of free radicals (Darley-Usmar *et al*., 1997). Despite recent scientific efforts, the molecular mechanisms of free radical-induced cellular events remain poorly understood. It is only in part known how reactive oxygen species such as hydrogen peroxide and O_2^- influence the expression of genes such as the AT1 receptor which are important for the development of cardiovascular diseases (Wolin, 1996).

The angiotensin AT1 receptor mediates angiotensin II-driven effects such as vasoconstriction and cell growth suggesting a significant role in the pathogenesis of hypertension and atherosclerosis (Caponi *et al*., 1981; Peach, 1997). The expression of the AT1 receptor, which is subject to various regulative influences, balances the efficacy of the entire renin angiotensin system. Numerous agonists, such as low-density lipoprotein, growth factors, estrogen, insulin, and angiotensin II, have been identified that cause modulation of AT1 receptor expression (Peach, 1997; Schiffrin *et al*., 1984; Lassegue *et al*., 1995; Nickenig & Murphy, 1994; 1996; Nickenig *et al*., 1997, 1998a,b). Nevertheless, the detailed intracellular mechanisms

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involved in AT1 receptor expression are less clear. Therefore, this study aimed to characterize the second messengers used by angiotensin II, the most prominent modulator of AT1 receptor gene expression. We investigated the role of reactive oxygen species in this setting of homologous AT1 receptor regulation and gained to clarify whether free radicals exert direct positive or negative feedback effects on AT1 receptor gene expression in vascular smooth muscle cells.

Methods

Materials

Angiotensin peptides, salts, 5,6-dichlorobenzimidazole (DRB), hydrogen peroxide, xanthin/xanthin oxidase, and other chemicals were purchased from Sigma Chemical (Deisenhofen, Germany). [^{32}P]-dCTP, Hybond N-nylon membranes and [^{125}I]-angiotensin II were obtained from Amersham (Braunschweig, Germany). Antibiotics, serum and cell culture medium were purchased from Gibco BRL (Eggenstein, Germany). RNA-clean was purchased from AGS (Heidelberg, Germany).

Methods

Cell culture VSMC were isolated from rat thoracic aorta (strain, female Wistar Kyoto, 6–10 weeks old, Charles River Wega GmbH, Sulzfeld, Germany) by enzymatic dispersion as described previously (Chamley *et al*., 1979) and cultured over several passages according to Ross 1970). Cells were grown in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagles medium (DMEM) supplemented with 100 ml^{-1} of penicillin, 100 μg ml^{-1} streptomycin, 1% nonessential amino acids (100×) and 10% foetal bovine serum. Experiments were performed with cells from passage 5–15.

mRNA isolation and Northern analysis

After the indicated treatments, culture medium was aspirated and the cells were lysed with 1 ml RNA-clean (AGS, Heidelberg, Germany), scraped and processed according to the manufacturer's protocol in order to obtain total cellular RNA. Ten-microgram aliquots 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 in 20× SSC (3 M sodium chloride, 300 mM sodium citrate) the RNA was cross-linked to the membranes using a Stratalinker 1800 (Stratagene, Heidelberg, Germany). Northern blots were prehybridized for 2 h at 42°C in a buffer containing 50% deionized formamide, 0.5% SDS, 6× SSC, 10 μg ml^{-1} denatured salmon sperm DNA (Sigma Chemicals, Deisenhofen, Germany) and 5× Denhardt's solution and were then hybridized for 15 h at 42°C with a random-primed, [^{32}P]-dCTP-labelled, rat AT1 receptor cDNA probe, in the same buffer but without Denhardt's solution. 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′-GTCATGATCCCTACCC-TCTACAGC-3′ and 5′-CCGTAGAACAGAGGGTTCAGG-CAG-3′ and Taq polymerase.

Radioligand binding assays

Experimental cells were washed three times with phosphate-buffered saline (PBS). Cells were collected and after a brief centrifugation the pellet was resuspended in 1 ml ice-cold 50 mM Tris-HCl, pH 8.0 and homogenized by repeated trituration through a 22-gauge needle. The membranes were pelleted by centrifugation at 12,000 r.p.m. for 10 min at 4°C. Homogenization and centrifugation was repeated twice. The final pellet was resuspended in an incubation buffer in the absence of DTT (50 mM Tris/HCl, 50 mM NaH_2PO_4, 10 mM $MgCl_2$, 0.2% bovine serum albumin). Angiotensin II receptor density and affinity were investigated in saturation experiments using increasing amounts of [^{125}I]-angiotensin II as radiolabelled ligand (0.125–2 nM). Ten μM of Dup753 was used to determine nonspecific binding. The assay was performed in a total volume of 250 μl incubation buffer. The incubation was carried out at 24°C for 60 min. These conditions allowed a complete equilibration of the receptor with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatmann GF/C filters (Whatman, Clifton, New Jersey); the filters were washed immediately three times with 5 ml of ice-cold incubation buffer and radioactivity was determined in a gamma-counter. All experiments were performed in triplicate. The maximal density (B_{max}) and apparent affinity (K_d) of binding sites were obtained by non-linear regression analysis.

Measurement of free radical release

These experiments were based on modifications of previously described methods (Bass *et al*., 1983; Ohba *et al*., 1994). Intracellular ROS production was measured by 2,7dichlorofluorescein (DCF) fluorescence using confocal laserscanning microscopy techniques. Dishes of sub-confluent cells were washed in buffer E1 (containing NaCl 135 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM $MgCl_2$ 1 mM, glucose 10 mM, pH 7.5) and incubated in the dark for 30 min in the same buffer containing 10 mM 2,7dichlorodihydrofluorescein-diacetate (DCF-DA, Molecular Probes, Eugene, OR, U.S.A.). DCF-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivate DCFH and thereby trapped within the cell. In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent DCF. Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope, equipped with a ×20 objective and Zeiss LSM 410 confocal attachment, and reactive oxygen species generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission 515–540 nm). The images were collected by single rapid scans, and identical parameters, such as contrast and brightness, for all samples. In three separate experiments, five groups of 25 cells each were randomly selected from the image and fluorescent intensity was taken. The relative fluorescence intensity are average values of all experiments and each value reflects measurements performed on a minimum of 100 cells for each sample.

Calcium imaging and confocal laser scanning microscopy

$[Ca^{2+}]_i$ was monitored using the fluorescent dye fluo-3, AM (Molecular Probes, Eugene, OR, U.S.A.). Cells grown on coverslips were loaded for 60 min in IMDM cell culture medium with 10 μM fluo-3, AM, dissolved in dimethyl sulphoxide (final concentration 0.1%) and pluronic™ F-127 (Molecular Probes) which facilitates the solubilization of fluo-3, AM (final concentration <0.025%). After loading, the coverslips were rinsed three times in E1-buffer. The experiments were performed at 37°C. Fluorescence data were recorded using an inverted confocal laser-scanning microscope (LSM 410; Zeiss, Jena, Germany) equipped with a 25×

objective, numerical aperture 0.80 (Plan-Neofluar, Zeiss). Fluorescence was excited by the 488 nm line of an argon-ion laser. Emission was recorded using a LP 515 nm filter set. Processing of images (512×512 pixels, 8 bit) was carried out by the Time-software facilities of the confocal setup. Full-frame images were aquired and stored automatically at 4 s intervals to a 16-megabyte video memory of the confocal setup. The minimum, maximum, mean, standard deviation, and integrated sum of the pixel values in a region of interest (selected using an overlay mask) were written to a data file and routinely exported for further analysis to the commercially available Sigma Plot (Jandel Scientific, Erkrath, Germany) graphic software. Because fluo-3 does not permit the use of ratio measurements, data are presented in arbitrary units as percentage of fluorescence variation (F/F_0) with respect to the resting level F_0 which was set to 100%.

Nuclear run-on assays

After a 24 h incubation of VSMC with hydrogen peroxide or vehicle, cells were dispersed with trypsin, and washed with 150 mM potassium chloride, 4 mM magnesium acetate, 10 mM Tris-HCl, pH 7.4. After centrifugation in a Beckman GS-GR tabletop centrifuge, using a GH 3.8 rotor (1200 r.p.m., 5 min, 4°C), the cell pellet was resuspended in 2 ml of the same buffer containing 0.5% Nonidet P-40 (Sigma). After lysis for 10 min on ice, the nuclei were isolated by centrifugation (2000 r.p.m., for 5 min, at 4°C) through 4 ml of 0.6 M sucrose, with the same equipment. The supernatants were carefully removed and the nuclear pellet was resuspended in a buffer containing 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$ and 0.1 mM EDTA. These were stored at $-80°$ until used for assays. Nuclei ($\cong 5-20 \times 10^8$ nuclei/reaction) were used to carry out the transcription, in a reaction mixture containing 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM levels of CTP, GTP and ATP, and 0.2–0.3 μM [^{32}P]-UTP (>300 μCi mmol^{-1}), at 30°C for 30 min. Reactions were terminated by addition of 800 μl of RNA-clean, and the radioactive RNA was isolated and purified by collection of the eluate from a Bio-Rad P-30 spin column. Approximately $5 \times 10^6 - 1 \times 10^7$ c.p.m. of the [^{32}P]-UTP-labelled RNA were dissolved in hybridization solution (100 mM TES, pH 7.4, 0.3 M NaCl, 100 μg ml^{-1} *Escherichia coli* tRNA). Plasmids (5 μg) containing cDNAs for the AT1 receptor (a *Hind*III-*Not*I cDNA insert from pCa18b subcloned in pKS+ -Bluescript) or GAPDH (rat GAPDH in pIBI30; International Biotechnology, New Haven, CT, U.S.A.) 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^{-1} *E.coli* tRNA, 5 × Denhards solution, and were hybridized at 42°C for 16 h. Membranes were washed for 10 min at room temperature in 2 × SSC and for 15 min at 50°C in 2 × SSC/0.1% SDS. The filters were exposed to film for 12–48 h, and autoradiographic signals were quantified by laser densitometry.

Western blotting

VSMC samples were washed in phosphate-buffered saline (PBS) at 0°C and homogenized in 5 volumes of ice cold suspension buffer (0.1 M NaCl, 0.01 M Tris-Cl, pH 7.6, 0.001 M EDTA, pH 8.0, 1 μg ml^{-1} aprotinin, 100 μg ml^{-1} phenyl-methyl-sulphonyl-fluoride (PMSF)). Aliquots were taken for quantification of total protein (BioRad DC Protein Assay). Equal volumes of 2 × SDS gel loading buffer (100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) were added and samples were heated in a boiling water bath for 10 min. The samples were then sonicated in a chilled water bath for 5 min. Centrifugation at 10,000 × *g* for 10 min at room temperature. Twenty five ug of protein of the supernatant were run through a 10% polyacrylmide gel. Western blotting of proteins was performed in a semi-dry blotting chamber (Pharmacia Biotech/Uppsala, Sweden) using 1 × Transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol. The membrane was stained with Ponceau red to verify appropriate protein transfer and equal loading for each lane. It was then incubated in 5% non fat dry milk in PBS for 1 h, washed with PBS-Tween (0.1%). The first antibody (AT1 (N-10): sc 1173, rabbit polyclonal IgG, Santa Cruz) was diluted 1 : 500 and incubated with the membrane for 1 h at room temperature. The second-horse radish peroxidase- (HRP) labelled antibody (anti-rabbit, Sigma) was diluted to 1 : 5000 and incubated with the membrane for 1 h at room temperature. Following the last washing step, ECL detection was carried out following the manufacturer's instructions (Amersham). Exposure to film was performed at room temperature for 2–20 min.

Statistical analysis

Data are presented as means ± standard error. Statistical analysis was performed using the ANOVA test.

Results

Cells were grown to confluency and serum was removed from the culture medium 24 h before initiation of experimental treatments to obviate its effects. In order to confirm previous findings (Lassegue *et al*., 1995; Nickenig & Murphy, 1996), VSMC were incubated for 0–24 h with either vehicle or 100 nM angiotensin II. Total RNA was isolated at the indicated time points and AT1 receptor as well as GAPDH mRNA expression was quantified using Northern Blot analysis. Figure 1 illustrates densitometric data from five separate experiments. Following incubation with vehicle, the AT1 receptor mRNA remained stable over the time course of the experiment (Figure 1A). Angiotensin II caused a time-dependent decrease of AT1 receptor mRNA with a maximum of 30 ± 6% in comparison to control levels 4 h after addition of angiotensin II to the culture medium (Figure 1B). GAPDH mRNA levels were unchanged during stimulation with vehicle and increased slightly upon challenge of VSMC with angiotensin II (Figure 1A,B).

Radioligand binding assays showed that angiotensin II-induced down-regulation of AT1 receptor mRNA led to the expected decrease of AT1 receptor density. Stimulation of VSMC with 100 nM angiotensin II for 12 h caused a decrease in the AT1 receptor density from B_{max} 600 ± 20 fmol mg^{-1} protein under basal conditions to 160 ± 10 fmol mg^{-1} protein after stimulation with angiotensin II. AT1 receptor affinity was not significantly altered (K_d 0.24 (0.1–0.4) versus 0.19 (0.04–0.43) nM).

The following experiments were conducted to examine whether angiotensin II causes release of free radicals in this experimental set-up in which angiotensin II-induced AT1 receptor down-regulation was encountered. Therefore, VSMC were incubated for various periods of time with 100 nM angiotensin II and free radical release was assessed with confocal laser scanning microscopy using H_2-DCFH-DA (2,7-Dihydrodichlorofluorescein-diazetat). The maximal effect was

evident between 2 and 8 h. Figure 2A shows a representative microscopic scan comparing vehicle (A) and angiotensin II (B)-pretreated cells (3 h incubation). Angiotensin II led to a significant release of reactive oxygen species in these cultured VSMC. Additionally, the effect of 100 μM diphenylene iodonium (DPI), an inhibitor of flavoprotein containing oxidoreducates such as NADH/NADPH oxidase on free radical release was tested under co-incubation with either vehicle (C) or angiotensin II (D). Whereas DPI had no significant effect on basal levels, the angiotensin II-induced production of free reactive oxygen species was completely inhibited. Figure 2B shows the summarized evaluation of separate experiments. Angiotensin II led to an $180 \pm 41\%$ increase in free radical release compared to control levels, DPI alone reduced these levels to $82 \pm 20\%$, and DPI inhibited angiotensin II-induced free radical release to $84 \pm 21\%$.

In order to explore the angiotensin II-induced production of reactive oxygen species on the modulation of AT1 receptor mRNA expression, VSMC were incubated with vehicle, vehicle and 100 μM DPI, 100 nM angiotensin II, or 100 nM angiotensin II and 100 μM DPI. RNA was isolated after a 4 h incubation and AT1 receptor as well as GAPDH levels were assessed by Northern blots. The densitometric analysis of three separate experiments revealed that the angiotensin II-caused AT1 receptor mRNA down-regulation was significantly inhibited by DPI ($44 \pm 11\%$ versus $79 \pm 6.5\%$) (Figure 3). These data suggested that release of free radicals *via* activation of NADH/NADPH oxidase is involved in angiotensin II-caused AT1 receptor down-regulation.

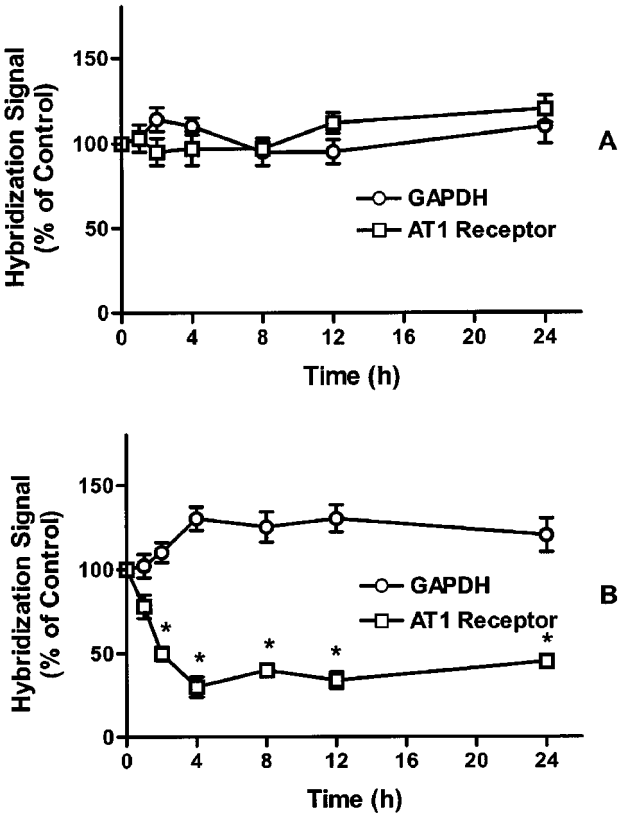


Figure 1 Quantification of Northern hybridization signal intensity. Time course of the AT1 receptor as well as GAPDH mRNA in the presence of either vehicle (A) or 100 nM Angiotensin II (B). Northern hybridizations were performed as described in Experimental Procedures. Each point represents the relative hybridization signal normalized to the 0 h treatment with vehicle (100%) from five separate experiments $\pm$ s.e. $^*P < 0.05$.

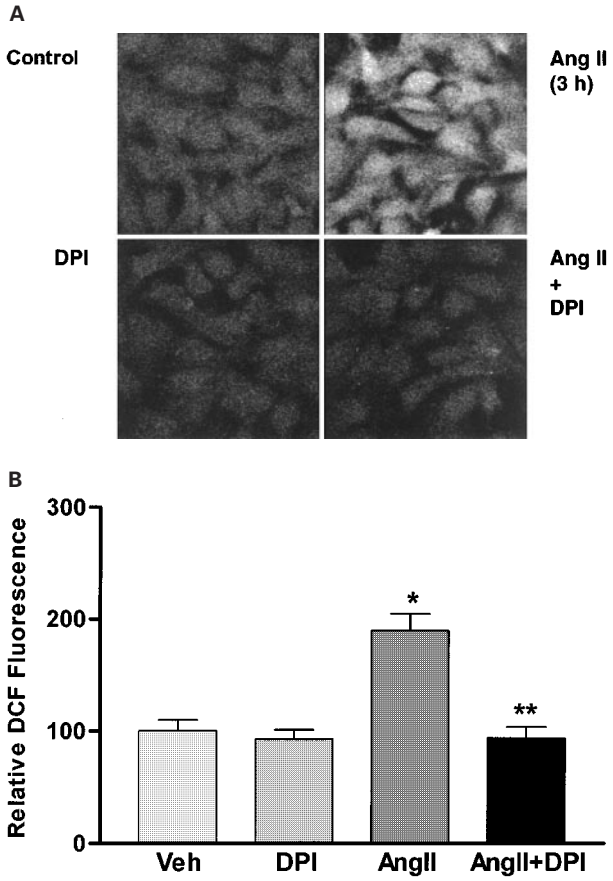


Figure 2 Effect of angiotensin II on free radical release in VSMC. (A) Representative microscopic laserscan of VSMC incubated for 3 h with vehicle (A), 100 μM DPI (B), 100 nM angiotensin II, or DPI and angiotensin II (D). Free radical release is visualized through DCF fluorescence. (B) Quantification of angiotensin II-induced release of free radicals. VSMC were incubated for 3 h with vehicle (control), 100 μM DPI, 100 nM angiotensin II (AngII), or DPI and angiotensin II (D). Free radical release is visualized through DCF fluorescence. means $\pm$ s.e. $^*P < 0.05$.

Consequently, VSMC were stimulated with hydrogen peroxide or xanthine/xanthine oxidase, to examine whether free radicals exert direct effects on AT1 receptor mRNA expression. Figure 4 demonstrates densitometric data from three separate experiments in which cells were stimulated for 24 h with 0–1.6 μU ml^{-1} xanthine oxidase (0.1 mM purin) or 0–100 μM hydrogen peroxide (+0, 0.1, 1, or 10 μM Fe^{3+}-NTA, respectively) before AT1 receptor and GAPDH mRNA was assessed by Northern analysis. Hydrogen peroxide as well as xanthine oxidase caused a dose dependent down regulation of AT1 receptor mRNA (maximal $10 \pm 2.9\%$ or $60 \pm 13.5\%$ of control levels, respectively). Also shown is a representative Northern blot which reveals the time-dependency. AT1 receptor mRNA expression was down-regulated after a 4 h incubation with 100 μM hydrogen peroxide (+10 μM Fe^{3+}-NTA). The maximal effect was reached after 24 h. The selectivity of this effect was also tested. Hydrogen peroxide at a concentration of 10 μM had no effect on the serotonin 5 HT2 receptor mRNA or endothelin-1 mRNA expression. One hundred μM hydrogen peroxide down-regulated the endothelin-1 mRNA to $61 \pm 9\%$ and the 5HT2 mRNA to $80 \pm 6\%$ of control levels. (data not shown).

ROS led also to down-regulation of AT1 receptor protein expression. Figure 5 shows the analysis of Western Blot experiments of proteins isolated from VSMC incubated for

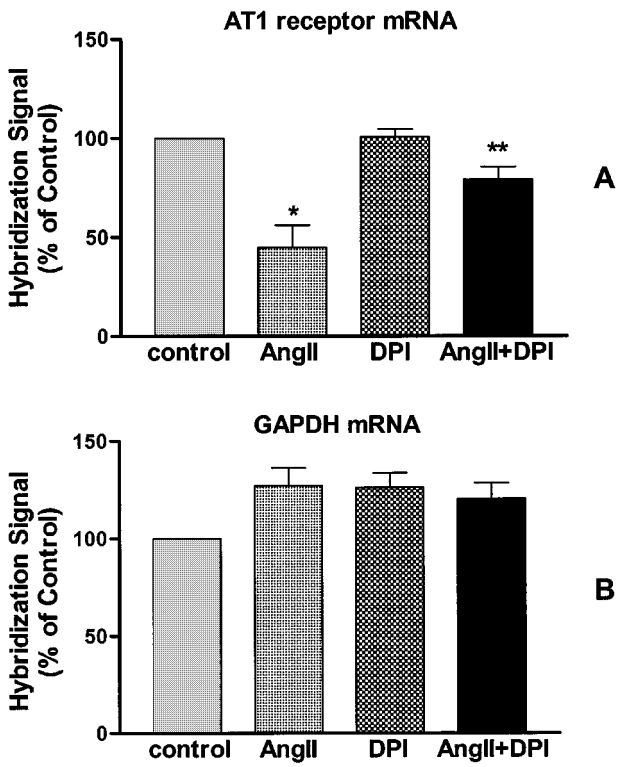


Figure 3 Effect of DPI on angiotensin II-induced AT1 receptor down-regulation. VSMC were incubated for 4 h with vehicle (control), 100 nM angiotensin II (AngII), 100 μM DPI, or angiotensin II and DPI. AT1 receptor and GAPDH mRNA were quantified by Northern Blots. Densitometric analysis of three separate experiments $\pm$s.e. $^*P<0.05$ vehicle versus angiotensin II, $^{**}P<0.05$ angiotensin II versus DPI + angiotensin II.

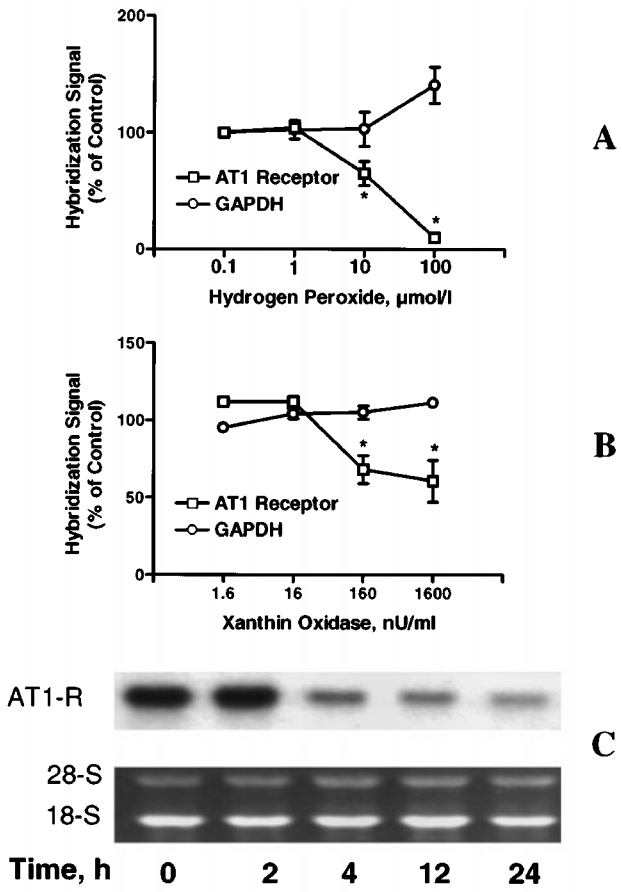


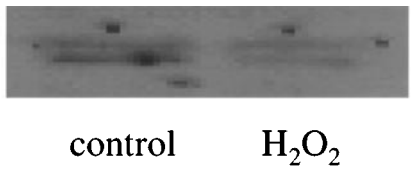
Figure 4 Effect of xanthine oxidase and hydrogen peroxide on AT1 Receptor mRNA. VSMC were incubated for 24 h with vehicle, 0.1–100 μM hydrogen peroxide (A) or 0–1.6 μU ml^{-1} xanthine oxidase (B). AT1 receptor and GAPDH mRNA were quantified by Northern Blots. Densitometric analysis of three separate experiments. mean $\pm$ s.e. $^*P<0.05$ VSMC were incubated for 0–24 h with 0.1–100 μM hydrogen peroxide (C). Representative Northern Blot showing AT1 receptor and 18S and 28S mRNA expression.

24 h with vehicle of 100 μM hydrogen peroxide. The AT1 receptor protein was down-regulated to 30.4 $\pm$ 3.8% of control levels.

Nuclear run-on assays assessed the effect of hydrogen peroxide on AT1 receptor mRNA transcription rate. VSMC were incubated for 4 h with either 0.1 mM hydrogen peroxide (+10 μM Fe^{3+}-NTA) or vehicle. Nuclei were isolated and *de novo* mRNA synthesis was measured. Figure 6 shows that hydrogen peroxide exerted no significant effect on AT1 receptor mRNA transcription rate. As a positive control, cells were treated for 4 h with 20 ng/ml EGF (epidermal growth factor) leading to a significant decrease of AT1 receptor transcription rate.

VSMC were preincubated with either vehicle or 0.1 mM hydrogen peroxide for 12 h before 50 μg ml^{-1} DRB (5,6-dichlorobenzimidazole) were added to the culture medium in order to inhibit RNA polymerase II-mediated gene transcription. AT1 receptor mRNA levels were measured 0–6 h after addition of DRB to the cell culture with Northern analysis. This enabled the measurement of AT1 receptor mRNA decay during the experimental period. Figure 7 displays the AT1 receptor mRNA decay following incubation with vehicle or hydrogen peroxide. Whereas the half-life of the AT1 receptor mRNA was calculated as approx. 5.5 h under control conditions, hydrogen peroxide caused a decrease of AT1 receptor mRNA half-life to 2 h suggesting that ROS induce destabilization of the AT1 receptor mRNA.

Further studies investigated the signalling pathways involved in ROS-induced AT1 receptor down-regulation. Cells were incubated with 100 μM hydrogen peroxide (+10 μM Fe^{3+}-NTA) in the presence of either 1 μM PD98059, 1 μM



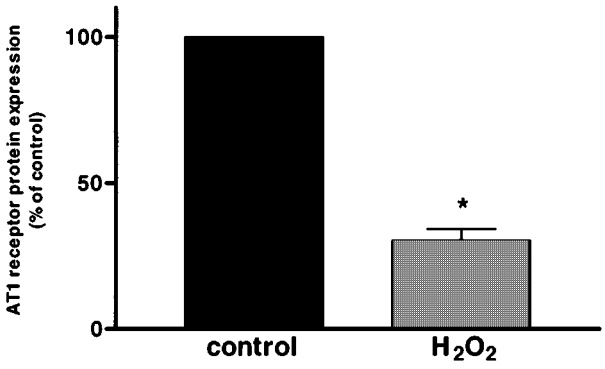


Figure 5 Effect of hydrogen peroxide on AT1 receptor protein expression. VSMC were incubated for 24 h with 100 μM hydrogen peroxide. Proteins were isolated and Western blots were performed to quantify AT1 receptor protein. Representative Western blot and densitometric analysis. mean $\pm$ s.e. $^*P<0.05$.

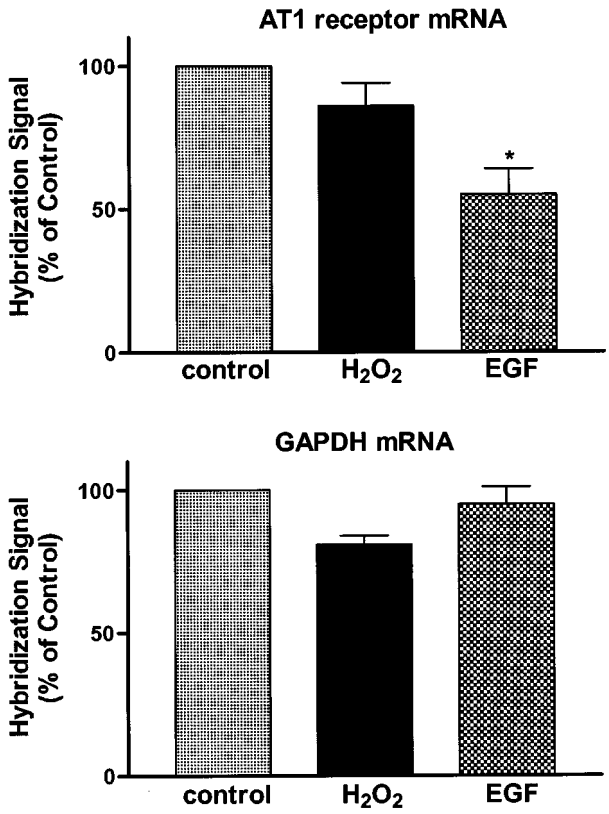


Figure 6 Effect of hydrogen peroxide on *de novo* mRNA synthesis in VSMC. VSMC were incubated with vehicle, 100 μM hydrogen peroxide, or 20 ng ml^{-1} EGF. Nuclei were isolated and nuclear run-on assays were performed. Autoradiograms were quantified by laser densitometry. Each point represents three separate experiments $\pm$ s.e. $^*P<0.05$.

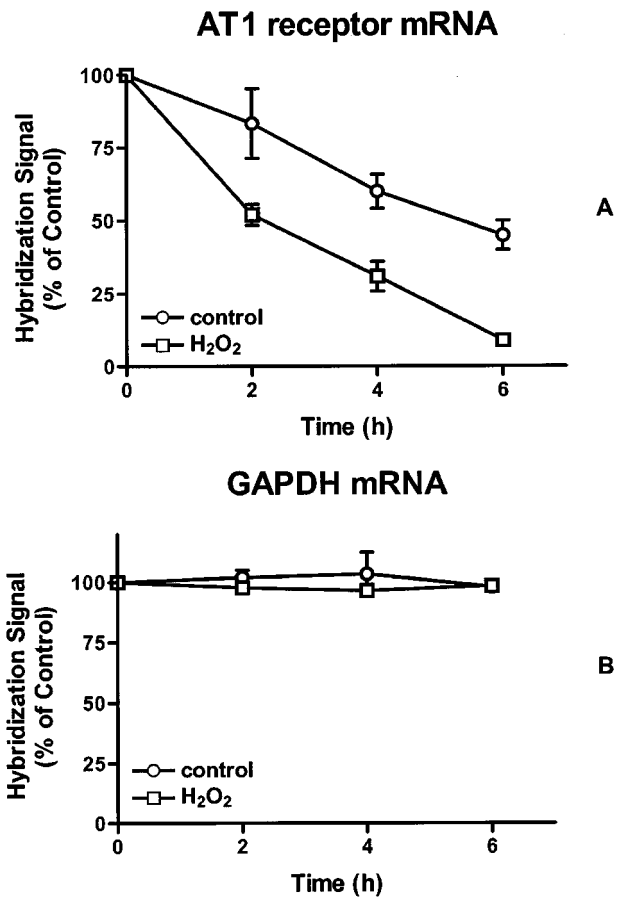


Figure 7 Effect of hydrogen peroxide on AT1 receptor mRNA stability. VSMC were incubated for 12 h with either 100 μM hydrogen peroxide or vehicle before 50 μg ml^{-1} DRB was added. Total RNA was isolated at the indicated time points and AT1 receptor mRNA was quantified by Northern analysis. Each point represents data from five separate experiments $\pm$ s.e. $^*P<0.05$.

SB203580, 1 μM herbimycin or 20 μM Bis-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (MAPTAM). PD98059 inhibits activation of p42/44 MAP kinase, SB203580 the activation of p38-MAP kinase, herbimycin inhibits tyrosine phosphorylation and MAPTAM acts as intracellular calcium chelator. Figure 8 reveals that only inhibition of p38-MAP kinase with SB203580 and incubation with MAPTAM prevented the ROS-induced AT1 receptor down-regulation. GAPDH mRNA levels remained unchanged. Experiments with increasing concentrations of up to 100 mmol/l of SB203580, PD98059, or MAPTAM showed similar results (data not shown). Furthermore, VSMC were incubated for 4 h with 1 μM angiotensin II in the presence or absence of 20 μM MAPTAM, 1 μM SB203580, or 1 μM PD98059. Angiotensin II-induced AT1 receptor down-regulation (40 ± 6% of control levels) was inhibited neither by SB203580 (43 ± 4%), by PD98059 (43 ± 10%) nor by MAPTAM (38 ± 11%).

Finally, experiments assessed the influence of hydrogen peroxide on the cytosolic release of calcium. Figure 9 shows an representative calcium transient upon stimulation with 100 μM hydrogen peroxide ($+10$ μM Fe^{3+}-NTA) and an summarizing graph demonstrating that increasing concentrations of hydrogen peroxide caused an increase in cytosolic calcium in VSMC supporting the notion that calcium signalling may play an important role in ROS-induced AT1 receptor regulation. Additional experiments confirmed that angiotensin II also caused an enhanced release of intracellular calcium. Preincubation with DPI did not inhibit this angiotensin II effect (data not shown).

Discussion

Angiotensin II causes AT1 receptor down regulation through mechanisms which involve the release of free radicals. Free radicals down-regulate AT1 receptor gene expression in part *via* activation of p38-MAP kinase and release of intracellular calcium through destabilization of AT1 receptor mRNA.

The AT1 receptor and its regulation play a pivotal role in blood pressure, fluid and electrolyte homeostasis and are involved in the pathogenesis of atherosclerosis and hypertension (Griendling *et al*., 1993). The AT1 receptor is regulated by steroids, growth factors, low-density lipoprotein, estrogen, insulin and angiotensin II (Peach, 1997; Schiffrin *et al*., 1984; Lassegue *et al*., 1995; Nickenig & Murphy, 1994; 1996; Nickenig *et al*., 1997; 1998a,b). The latter causes AT1 receptor down-regulation which establishes a negative feedback regulation on the AT1 receptor level within the RAS. The mechanisms involved in AT1 receptor regulation are only partially understood. Currently, it is believed that transcriptional and especially posttranscriptional events lead to modulation of AT1 receptor gene expression (Nickenig & Murphy, 1996). In this context, it has been shown that inducible (de)-stabilization of AT1 receptor mRNA is one decisive component of AT1 receptor regulation. The second messenger systems involved in these events are less clear. AT1 receptor up-regulation by insulin is dependent on tyrosine

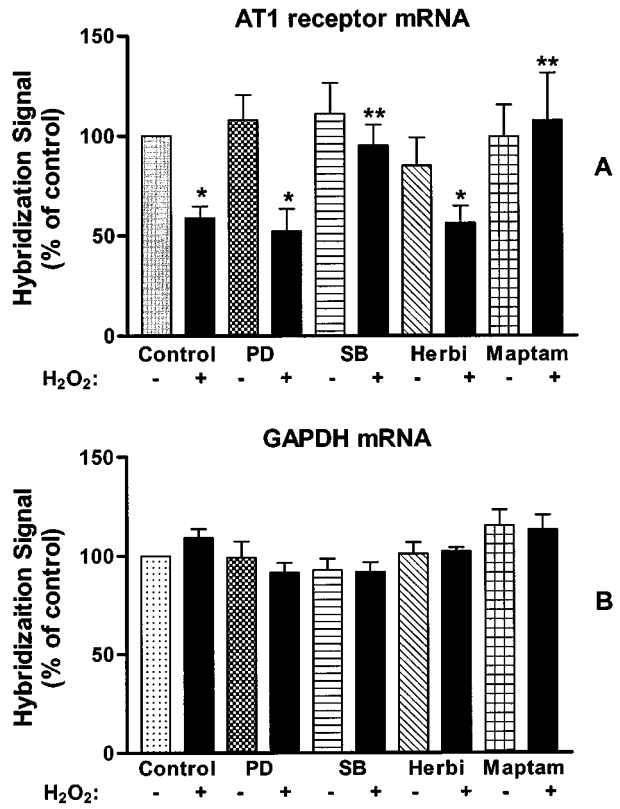


Figure 8 Second messenger involved in hydrogen peroxide-induced AT1 receptor mRNA down-regulation. Cells were incubated for 24 h with 100 μM hydrogen peroxide in the presence of either 1 μM PD98059 (PD), 1 μM SB203580 (SB), 1 μM herbimycin (Herbi) or 20 μM Bis-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (Maptam). Inhibitors were added 30 min prior to hydrogen peroxide. AT1 receptor and GAPDH mRNA was assessed by Northern analysis. Each point represents data from five separate experiments $\pm$ s.e. $^*P<0.05$ control versus H_2O_2. $^{**}P<0.05$ H_2O_2 versus H_2O_2 + MAPTAM or SB.

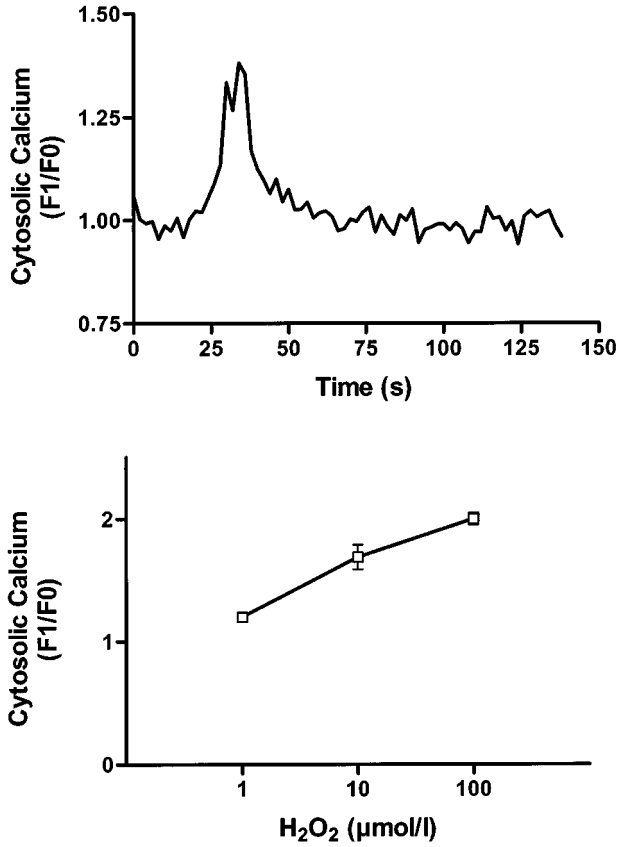


Figure 9 Intracellular calcium release in vascular smooth muscle cells. Confluent cells were stimulated with 1–100 μM hydrogen peroxide before the intracellular calcium concentration was monitored with confocal microscopy. Representative graph generated from cells stimulated with 100 μM hydrogen peroxide (A) and summarized data (B) of stimulation with increasing concentrations of hydrogen peroxide.

phosphorylation and potentially on p42/44-MAP kinase activation (Nickenig *et al*., 1998b).

The intracellular signalling pathways participating in angiotensin II-caused AT1 receptor down-regulation are still unknown, although it has been speculated that calcium, protein kinase C as well as cyclic AMP may play a role (Lassegue *et al*., 1995; Wang *et al*., 1997). One of the most prominent features of AT1 receptor regulation is the release of reactive oxygen species (Griendling *et al*., 1994). These free radicals have been implicated in the pathogenesis of cardiovascular disease due to recent reports which have shown the relevance of reactive oxygen species in general cellular events such as proliferation, contraction and apoptosis (Dariey-Usmar *et al*., 1997; Bretschneider *et al*., 1997; Yang *et al*., 1998). Current efforts concentrate on exploration of direct effects of free radicals on distinct cellular functions. Accordingly, it was demonstrated that free radicals influence calcium release in myocardial cells, stimulate insulin-like and vascular endothelial growth factor production, cooperate with p53, suppress endothelin production, induce expression of adhesion molecules and plasminogen activator inhibitor 1 and 2, and modulate cation channels (Kawakami & Okabe, 1998; Delafontaine & Ku, 1997; Ruef *et al*., 1997; Johnson *et al*., 1996; Saito *et al*., 1998; Bowie *et al*., 1996; Hsieh *et al*., 1998; Chiu *et al*., 1997; Koliwad *et al*., 1996; Okada *et al*., 1998). Interestingly, it was also reported that free radicals cause cyclic AMP release in murine vascular smooth muscle cells (Tan *et al*., 1995), a fact that could connect the above mentioned role of cyclic AMP and free radicals in the regulation of AT1 receptor expression. It is well known that the transcription factor NF-κB is activated *via* oxidative stress (Bretschneider *et al*., 1997; Bowie *et al*., 1996). One could speculate that this transcription factor participates in the demonstrated AT1 receptor regulation. On the other hand, AT1 receptor expression is predominately modulated through post-transcriptional and not transcriptional pathways, and it has not been demonstrated so far that NF-κB acts as mRNA binding protein which influences AT1 receptor mRNA stability.

More recent data have shown that angiotensin II causes phosphorylation of p38 and p42/44 MAP kinase *via* intracellular release of free radicals in VSMC (Ushio-Fukai *et al*., 1998). However, exogenously applied hydrogen peroxide activated exclusively p38-MAP kinase and not p42/44 MAP kinase. Both pathways are obviously involved in the mitogenic activity of free radicals and angiotensin II in VSMC. This is in good agreement with the herein presented data, since activation of p38-MAP kinase is apparently also one prerequisite of ROS-induced AT1 receptor regulation, confirming the importance of MAP kinase for direct cellular effects of free radicals.

In addition, hydrogen peroxide-induced AT1 receptor downregulation is dependent on the intracellular release of calcium. In this context, it has been shown that intracellular calcium is involved in modulation of AT1 receptor down-regulation (Lassegue *et al*., 1995). Moreover, it has been

demonstrated that free radicals lead to a transient increase of cytosolic calcium in cells originated from a prostate tumour (Sauer *et al*., 1997). Therefore, intracellular calcium handling seems to be a substantial part of ROS-exerted activation of intracellular signalling. The present study combines the above mentioned data with respect to the AT1 receptor regulation. Hydrogen peroxide leads to AT1 receptor down-regulation *via* release of intracellular calcium in VSMC.

Nevertheless, many cellular events leading from free radical release to modulation of gene expression still remain obscure and require further investigations. The presented findings identify free radicals as one possible mediator of angiotensin II-induced AT1 receptor regulation, display the variety of biological effects exerted by this reactive oxygen species, and establish another mechanism involved in AT1 receptor regulation. In any event, numerous other signal pathways are also influenced by reactive oxygen species and angiotensin II such as C-Jun-N-terminal kinase, JAK/Stats, akt/protein kinase B, Fyn and JAK2 (Ushio-Fukai *et al*., 1999; Abe & Berk, 1999). The presented data cannot exclude that these and other pathways are also involved in the free radical-induced AT1 receptor activation. The data on angiotensin II-induced AT1 receptor regulation suggest that other pathways than reactive oxygen species are additionally required in these regulative pathways. Cyclic AMP could be another candidate which mediates the angiotensin II-driven AT1 receptor down-regulation in VSMC (Wang *et al*., 1997).

The radical-mediated negative feedback regulation of the AT1 receptor may be interpreted as a cellular self-protecting mechanism which helps to avoid that VSMC enter a potentially self-destructing fate, starting off with AT1 receptor activation and finishing with an overwhelming and putatively lethal release of reactive oxygen species. Free radicals decisively influence the G-protein coupled AT1 receptor upstream of post-transcriptional mechanisms. It is tempting to speculate that this could represent a general mechanism underlying regulation of G-protein coupled receptors in cardiovascular cells which could be of significance for diseases such as hypertension, atherosclerosis and heart failure.

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