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Reduction of oxidative stress and AT1 receptor expression by the selective oestrogen receptor modulator idoxifene

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1 The beneficial vasoprotective effects of oestrogens are hampered by their side effects on secondary sexual organs. Selective oestrogen receptor modulators (SERM) such as idoxifene may exert beneficial vascular effects without influencing cancerogenesis in breast or uterus.

2 In order to investigate vascular effects of selective oestrogen receptor modulators, we examined the impact of idoxifene on production of reactive oxygen species as well as AT1 receptor expression in vascular smooth muscle cells (VSMC).

3 Idoxifene caused a concentration- and time-dependent down-regulation of AT1 receptor mRNA expression, as assessed by Northern analysis. The maximal effect was reached with 10 μmol l^{-1} idoxifene after a 4 h incubation period ($33 \pm 7\%$ of control levels). Western blots showed a similar down-regulation of AT1 receptor protein to $36 \pm 11\%$ of control levels.

4 Confocal laserscanning microscopy using the redox sensitive marker 2′,7′-dichlorofluorescein (DCF) and measurement of NAD(P)H oxidase activity in cell homogenates revealed that idoxifene effectively blunted the angiotensin II-induced production of reactive oxygen species.

5 In order to investigate the signal transduction involved in SERM-induced modulation of AT1 receptor expression, VSMC were preincubation with PD98059, genistein, wortmannin, or N^{ω}-Nitro-L-arginine. The results suggested that idoxifene caused AT1 receptor down-regulation through nitric oxide-dependent pathways.

6 In conclusion, idoxifene reduces angiotensin II-evoked oxidative stress in VSMC. This could in part be explained by idoxifene-induced down-regulation of AT1 receptor expression. These results demonstrate that the selective oestrogen receptor modulator idoxifene may exert beneficial vascular effects which could be useful for therapeutic regimen in postmenopausal women at risk for cardiovascular diseases.

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Abbreviations: H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein-diacetate; DMEM, Dulbecco's modified Eagle's medium; DPI, Di-phenylene iodonium; DRB, 5,6-dichlorobenzimidazole; PBS, Phosphate-buffered saline; SERM, selective oestrogen receptor modulator; VSMC, Vascular smooth muscle cells

Introduction

Oestrogen deficiency after menopause is associated with an increased incidence of cardiovascular disease (Wenger *et al*., 1993; Colditz *et al*., 1987). Oestrogen replacement therapy potentially prevents the onset of cardiac events in post-menopausal women (Hong *et al*., 1992; Heckbert *et al*., 1997; Nabulsi *et al*., 1993). Besides the putatively beneficial effects of oestrogens on the cardiovascular system (Liao *et al*., 1995) and osteogenesis (Christiansen, 1996; Lindsay, 1993), oestrogens are thought to facilitate the development of uterus and breast malignancies (Nachtigall & Nachtigall, 1992; Harlap, 1992). In order to overcome these adverse events, selective oestrogen receptor modulators (SERM) have been developed and examined with respect to their effects on various tissues (Mitlak & Cohen, 1999; Weryha *et al*., 1999). These drugs act in the breast and uterus as oestrogen receptor antagonists, omitting the harmful effects of oestrogens, but preserve, presumably, the beneficial effects of oestrogens in bones and the cardiovascular system. Raloxifene, the most prominent member of the SERM family, has been shown to improve endothelial function and to induce endothelial- as well as endothelial-independent vasodilation *via* nitric oxide release and calcium channel blockade (Figtree *et al*., 1999). However, little is known about the mechanisms of action of SERM in vascular smooth muscle cells. Numerous SERMs (Mitlak & Cohen, 1999; Weryha *et al*., 1999) have been developed, and it is probable that these drugs represent distinct chemical compounds which could ultimately lead to differential effects on function and morphology of vascular cells. Therefore, it is imperative to characterize each SERM with regard to its cellular effects.

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Release of reactive oxygen species (Harrison, 1997; Li *et al*., 1997; Laursen *et al*., 1997) and AT1 receptor activation (Rajagopalan *et al*., 1996) are important events in cardiovascular diseases such as hypertension and atherosclerosis. AT1 receptor activation is a major source of free radical production in the vessel wall (Rajagopalan *et al*., 1996) and especially in vascular smooth muscle cells (VSMC) (Griendling *et al*., 1994b). Oestrogens have been reported to down-regulate AT1 receptor expression *in vitro* as well as *in vivo* (Nickenig *et al*., 1998) which could at least in part explain the antioxidative properties of oestrogens.

The present study was conducted to evaluate the effects of the novel SERM idoxifene (Mitlak & Cohen, 1999) on key events in VSMC, namely AT1 receptor regulation and production of reactive oxygen species. The discovery of beneficial effects of SERM on vascular cells would enable novel therapeutic strategies in postmenopausal women and would be of relevance for the treatment of atherosclerosis as well as hypertension in females, and putatively, in men.

Methods

Cell culture

VSMC were isolated from rat thoracic aorta (strain, female Sprague Dawley, 6–10-weeks-old, Charles River 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 Eagles medium (DMEM) without phenol, supplemented with 100 units ml^{-1} of penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 1% non-essential amino acids (100×), and 10% foetal bovine serum (free of steroid hormones, S-15-M, c.c. Pro GmbH, Neustadt/W., Germany). Experiments were performed with cells from passages 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, scraped and processed according to the manufacturer's protocol in order to obtain total cellular RNA. Ten-microgram aliquots were electrophoresed, transferred on Hybond N® membranes in 20×SSC (3 M sodium chloride, 300 mmol l^{-1} sodium citrate), and 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 $\mu g\ ml^{-1}$ denatured salmon sperm DNA (Sigma Aldrich Chemicals) 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.

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 mol l^{-1} NaCl, 0.01 mol^{-1} Tris-HCl, pH 7.6, 0.001 mol l^{-1} EDTA, pH 8.0, 1 $\mu g\ ml^{-1}$ aprotinin, 100 $\mu g\ ml^{-1}$ phenyl-methyl-sulphonyl-fluoride (PMSF)). Equal volumes of 2×SDS gel loading buffer (100 mmol l^{-1} Tris-HCl, pH 6.8, 200 mmol l^{-1} dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) were added and samples were heated to 95°C for 10 min. The samples were then sonicated and centrifuged at 10,000 × *g* for 10 min at room temperature. Twenty-five μg of protein of the supernatant were run through a 10% polyacrylamide gel. Western blotting of proteins was performed in a semi-dry blotting chamber (Pharmacia Biotech/Uppsala, Sweden) and incubated in 5% non fat dry milk in PBS for 1 h and 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 : 50,000 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).

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species production was measured by 2′,7′-dichlorofluorescein (DCF) fluorescence using confocal laserscanning microscopy techniques. Dishes of subconfluent cells were washed and incubated in the dark for 30 min in the presence of 10 $\mu mol\ l^{-1}$ 2′,7′-dichloro-dihydrofluorescein-diacetate (H_2DCF-DA, Molecular Probes, Eugene, OR, U.S.A.). 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 H_2DCF (excitation, 488 nm; emission longpass LP515-nm filter set). 512×512 pixel images were collected by single rapid scans, and identical parameters, such as contrast and brightness, for all samples. In four 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.

NAD(P)H oxidase activity

NADH or NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence assays in a 50 mmol l^{-1} phosphate buffer (buffer A), pH 7.0, containing 1 mmol l^{-1} EGTA, protease inhibitors (Complete®, Boehringer Mannheim, Germany), 150 mmol l^{-1} sucrose, 5 $\mu mol\ l^{-1}$ lucigenin, and either 100 $\mu mol\ l^{-1}$ NADH or 100 $\mu mol\ l^{-1}$ NADPH as substrate (Griendling *et al*., 1994b). Cell cultures were treated as indicated, washed twice with ice-cold phosphate buffered saline, pH 7.4, and scraped from the dishes. After a step of low spin centrifugation, the pellet was resuspended in ice-cold buffer A, lacking lucigenin and substrate. Cells were then mechanically lysed by using a glass/Teflon potter on ice. The total protein concentration was determined using a Bradford assay (BioRad) and adjusted to 1 mg ml^{-1}. One hundred μl aliquots of the protein sample were measured over 10 min in quadruplicates

using NADH or NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501) in 1 min intervals.

Statistical analysis

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

Results

VSMC were grown to confluence. Serum was withdrawn for 24 h before initiation of experiments. Control experiments with vehicle-treated cells showed that AT1 receptor expression remained unaltered during the incubation period (data not shown).

Cells were incubated with 10 μmol l^{-1} idoxifene for 0–16 h and with various concentrations of idoxifene for 4 h followed by RNA isolation and quantification of AT1 receptor mRNA expression by Northern blot analysis. Figure 1A shows a representative experiment revealing the concentration-dependency of idoxifene-induced AT1 receptor mRNA regulation. Figure 1B shows the densitometric analysis of eight separate experiments. The maximal effect was achieved after a 4 h incubation period. Concentration-dependency of idoxifene-induced down-regulation of AT1 receptor mRNA expression was tested after a 4 h incubation (Figure 1C). The maximal effect was reached with 10 μmol l^{-1} idoxifene (33 ± 7% of control levels). Figure 1D includes control experiments with increasing concentrations of raloxifene and 17β-Oestradiol which also down-regulate AT1 receptor mRNA expression.

In order to investigate whether AT1 receptor mRNA down-regulation was accompanied by a similar down-regulation of AT1 receptor protein, VSMC were incubated with 10 μmol l^{-1} idoxifene for 12 h, proteins were isolated and Western blots were performed. Figure 2 shows down-regulation of AT1 receptor protein to 36 ± 11% of control levels in response to incubation with idoxifene for 12 h.

In another set of experiments, we sought to characterize the signal transduction pathways participating in idoxifene-induced AT1 receptor regulation. VSMC were preincubated for 30 min with 1 μmol l^{-1} PD98059, genistein, wortmannin, or N^{ω}-Nitro-L-arginine (10 μmol l^{-1}) before 10 μmol l^{-1} idoxifene or vehicle were added to the culture medium for additional 4 h. RNA was isolated and AT1 receptor mRNA was quantified by Northern blot analysis. Figure 3 shows that neither p42/44 MAP kinase activation, tyrosin phosphoryla-

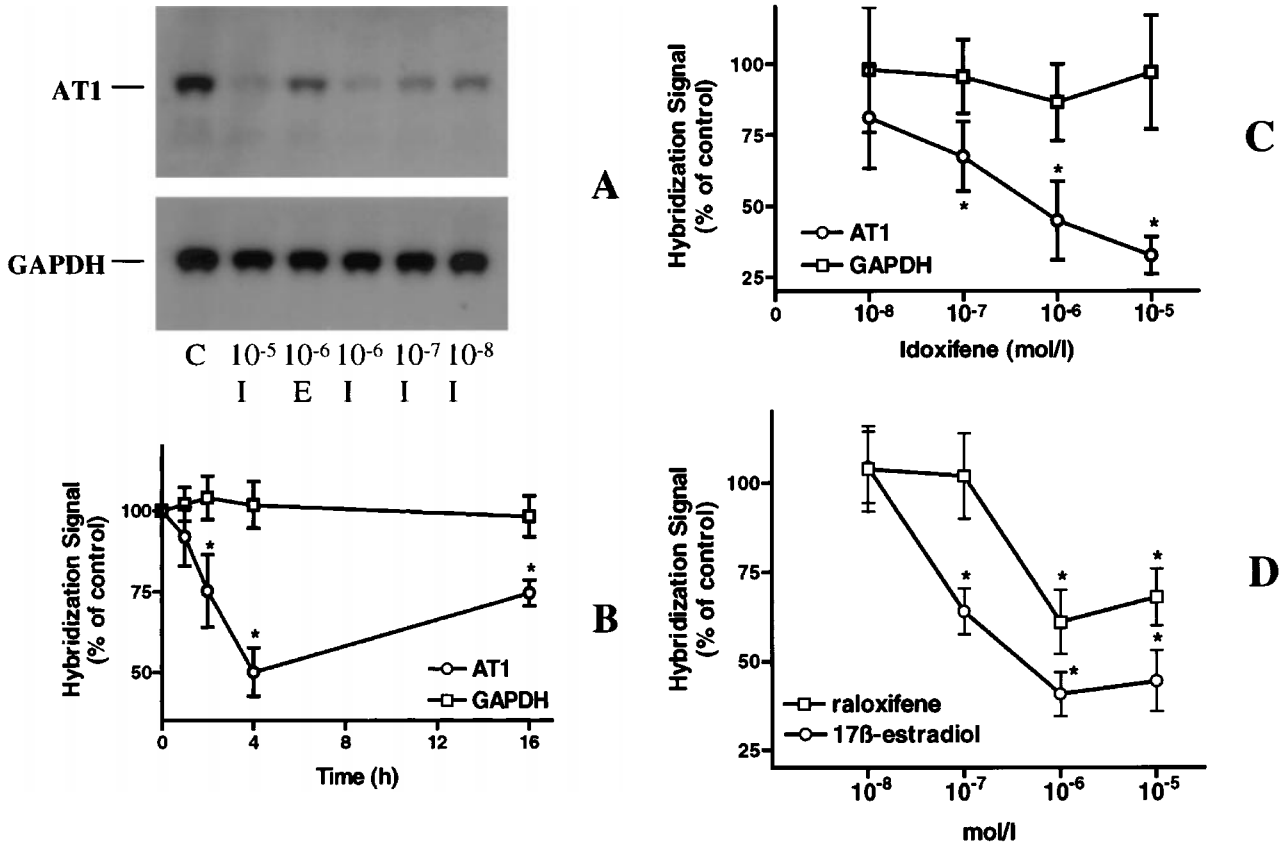

Figure 1 Effect of idoxifene on AT1 receptor mRNA expression. (A) Representative autoradiography of a Northern hybridization of a AT1 receptor cDNA probe to RNA isolated from VSMC stimulated for 4 h with increasing concentrations of idoxifene (I) and 1 μmol l^{-1} 17β-Estradiol (E). Also displayed are the GAPDH signals detected from the same membrane. (B) Densitometric analysis showing the time dependency of the effect of 10 μmol l^{-1} idoxifene on the expression of the AT1 receptor mRNA in VSMC. Mean ± s.e. $n=8$, $^*P<0.05$. (C) Effect of idoxifene at various concentrations on the expression of the AT1 receptor mRNA in VSMC. Densitometric analysis. Mean ± s.e. $n=3$, $^*P<0.05$. (D) Effect of increasing concentrations of 17β-Estradiol and raloxifene on AT1 receptor mRNA expression. Densitometric analysis. Mean ± s.e. $n=3$, $^*P<0.05$.

tion nor PI-3-kinase were involved in idoxifene-induced AT1 receptor down-regulation. In contrast, idoxifene caused AT1 receptor down-regulation through nitric oxide-dependent pathways. The used inhibitors itself had no effect on AT1 receptor mRNA expression. GAPDH and 18S RNA levels were not changed (data not shown). Increase of the concentrations of PD98059, genistein, and wortmannin to 10 and 50 μmol l^{-1} did not enhance the inhibitory properties on the idoxifene effect. Another NO-inhibitor, L-NAME also inhibited the idoxifene-induced AT1 receptor mRNA down-regulation (data not shown).

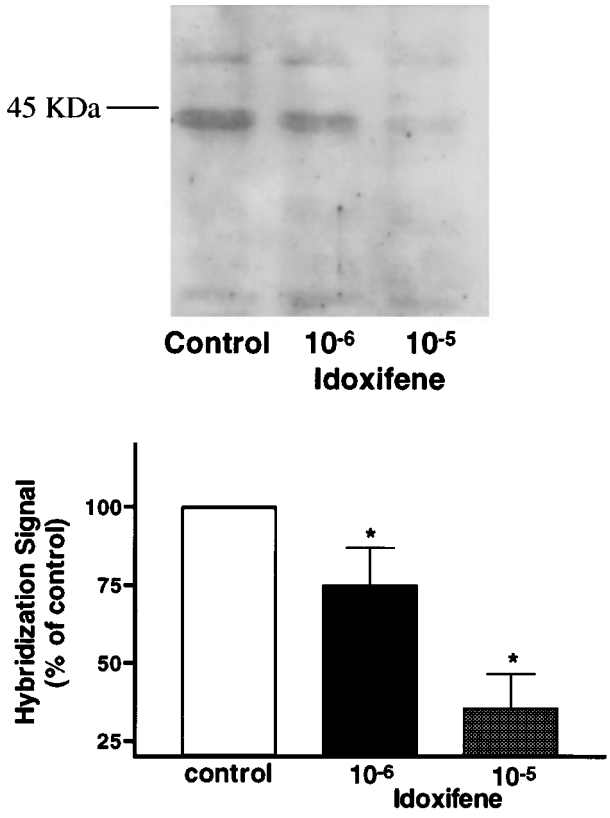

Figure 2 Effect of idoxifene on AT1 receptor protein expression. Western blot of proteins isolated from VSMC stimulated for 12 h with different concentrations of idoxifene. Representative autoradiogram and densitometric analysis. mean ± s.e. $n=3$, $^{*}P<0.05$.

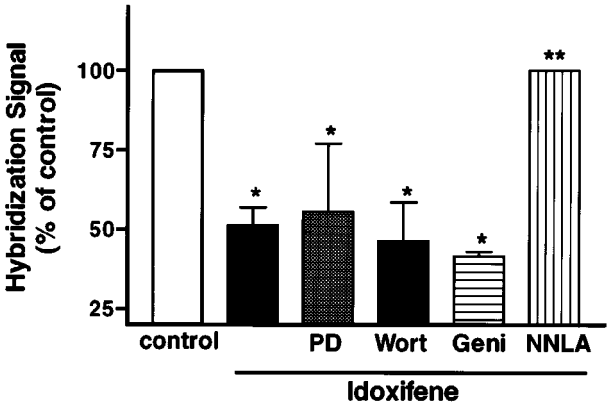

Figure 3 Signal transduction pathways involved in AT1 receptor regulation induced by idoxifene. VSMC were co-incubated with idoxifene (4 h) with the following compounds. PD = PD98059 (1 μmol l^{-1}), Geni = Genistein (1 μmol l^{-1}), Wort = wortmannin (1 μmol l^{-1}), or NNLA = N^{ω}-Nitro-L-arginine (10 μmol l^{-1}). AT1 receptor mRNA was assessed after isolation of total RNA and Northern blot. The densitometric analysis of the Northern blots quantifying AT1 receptor mRNA is depicted. mean ± s.e. $n=3$, $^{*}P<0.05$ Idoxifene, PD, Wort or Geni versus control, $^{**}P<0.05$ NNLA *versus* Idoxifene, PD, Wort or Geni.

Production of reactive oxygen species is another important event in the pathogenesis of vascular diseases. Therefore, experiments were conducted to define the influence of idoxifene on intracellular production of free radicals. VSMC were preincubated with either vehicle or 10 μmol^{-1} idoxifene for 12 h before 1 μmol l^{-1} angiotensin II was added to the cell culture medium. After 3 h, confocal laserscanning using DCF fluorescence revealed that angiotensin II enhanced the production of reactive oxygen species, which was completely inhibited after preincubation with idoxifene. Basal production of free radicals was slightly reduced by idoxifene (Figure 4). Raloxifene, a SERM which also down-regulates AT1 receptor expression, decreased comparably the angiotensin II-evoked oxidative stress.

Angiotensin II releases free radicals predominately through activation of NAD(P)H oxidase. Consequently, NAD(P)H oxidase activity was assessed by lucigenin chemiluminescence in cell homogenates derived from VSMC preincubated with either vehicle or 10 μmol l^{-1} idoxifene followed by a 4 h incubation with 1 μmol l^{-1} angiotensin II. Figure 5 shows that NAD(P)H oxidase activity was enhanced by angiotensin II-stimulation. This was abolished by preincubation with idoxifene.

Discussion

AT1 receptor activation leads to vasoconstriction, sodium and water retention, sympathetic activation, cell growth, and release of free radicals (Griendling *et al*., 1994a). Modulation of AT1 receptor expression substantially influences the magnitude of these cellular effects which play a central role in the pathogenesis of hypertension and atherosclerosis (Rajagopalan *et al*., 1996). Indeed, AT1 receptor activation is one of the most prominent sources of free radical release in the vessel wall that is induced through activation of the NADPH oxidase system (Rajagopalan *et al*., 1996; Griendling *et al*., 1994b).

We have recently shown that oestrogens down-regulate AT1 receptor expression *in vitro* and *in vivo* (Nickenig *et al*., 1998). Based on these findings, the hypothesis was raised that oestrogen-induced AT1 receptor down-regulation could at least in part explain the atheroprotective effects of these reproductive steroids.

Interestingly, the question of whether oestrogens indeed exert vasoprotective effects is subject of intense discussions. In contrast to early observational studies, the HERS trial showed no effect of oestrogens on cardiovascular events in women with manifest coronary heart disease (Hulley *et al*., 1998). Besides others, the missing success of hormone replacement therapy was attributed in part to the concomitant application of medroxyprogesterone. The latter is necessary to overcome the side effects of oestrogens on uterus and breasts. Therefore, the concept of selective

oestrogen receptor modulators (SERM) which act as oestrogen antagonist in uterus and breast but which function as oestrogen agonist in other tissues such as the vasculature seems increasingly attractive. Since cardiovascular diseases are the most frequent death cause in postmenopausal women, the vascular effects of SERM are of special interest. One of those SERM, raloxifene, is able to improve endothelial function and to induce endothelial- as well as endothelial-independent vasodilation *via* nitric oxide release and calcium channel blockade (Figtree *et al*., 1999). Furthermore, raloxifene reduces cardiovascular risk factors such as lipoprotein levels in postmenopausal women (Delmas *et al*., 1997). Little is known about the underlying cellular mechanisms of actions or about the features of other SERM with respect to vascular tissue.

Several SERM have been developed which apparently differ in binding and activation characteristics on oestrogen receptors. Idoxifene has been shown to beneficially influence bone loss and lipid levels in ovarectomized rats without negative side effects on the uterine tissue (Nuttall *et al*., 1998). Therefore, we tested this SERM with respect to AT1 receptor regulation and release of free radicals from vascular smooth muscle cells. The herein presented data imply a potent inhibition of AT1 receptor expression and reactive oxygen species production. Since AT1 receptor activation by angiotensin II leads to the production of reactive oxygen species, it may be concluded that the idoxifene-induced AT1 receptor down-regulation is at least in part involved in the resulting decrease of free radical release. In analogy to 17β-estradiol, this effect is mediated through a nitric-oxide dependent pathway.

These findings may have important implications. AT1 receptor regulation and especially the production of reactive oxygen species are thought to be involved in the initiation as well as progression of vascular damage (Darley-Usmar *et al*., 1997; Laursen *et al*., 1997). Endothelial dysfunction, a prerequisite of atherosclerosis (Vogel, 1997), that is caused by increased availability of reactive oxygen species, may be improved by idoxifene. Furthermore, the incidence of hypertension increases steeply following menopause. There is accumulating evidence that AT1 receptor activation and oxidative stress is crucial for the pathogenesis of hypertension (Harrison, 1997). Therefore, it may be speculated that SERM such as idoxifene could beneficially influence the development

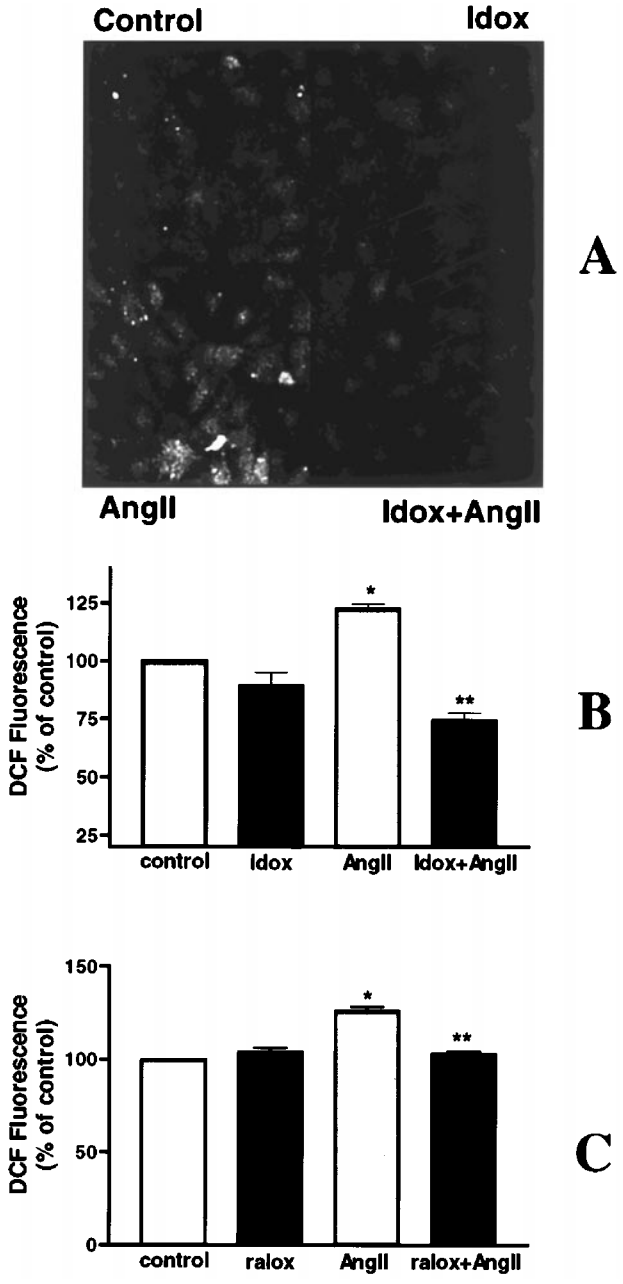

Figure 4 Effect of idoxifene on angiotensin II-induced intracellular production of reactive oxygen species in VSMC. (A) VSMC were preincubated for 12 h with 10 μmol l^{-1} idoxifene (Idox) or vehicle (control), followed by a 3 h incubation with 1 μmol l^{-1} angiotensin II (AngII). Free radical production is visualized through DCF fluorescence. (B) Quantitative analysis of signal intensities are shown as mean $\pm$ s.e., four different experiments with five groups of 25 randomly selected cells each, $^{*}P<0.05$ AngII *versus* control, $^{**}P<0.05$ AngII *versus* AngII + idoxifene. (C) VSMC were pre-incubated for 12 h with 10 μmol l^{-1} raloxifene (ralox) or vehicle (control), followed by a 3 h incubation with 1 μmol l^{-1} angiotensin II (AngII). Quantitative analysis of signal intensities are shown as mean $\pm$ s.e. of four different experiments with five groups of 25 randomly selected cells each, $^{*}P<0.05$ AngII *versus* control, $^{**}P<0.05$ AngII *versus* AngII + raloxifene.

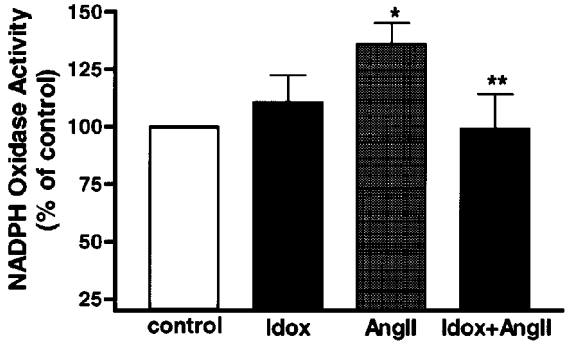

Figure 5 Effect of idoxifene on NAD(P)H oxidase activity in VSMC. VSMC were preincubated for 12 h with 10 μmol l^{-1} idoxifene (Idox) or vehicle (Control), followed by a 4 h incubation with 1 μmol l^{-1} angiotensin II (AngII). Superoxide release of cell homogenates was measured *via* lucigenin enhanced chemiluminescence. mean $\pm$ s.e. $n=3$, $^{*}P<0.05$ AngII *versus* control, $^{**}P<0.05$ AngII *versus* AngII + idoxifene.

of hypertension and atherosclerosis in postmenopausal women by reduction of AT1 receptor expression and decrease of oxidative stress without the oestrogenic effects on secondary sexual organs. The discovery of the mechanisms of vasoprotection by SERM set the stage for clinical trials investigating the prevention of atherosclerosis and its complications by individual compounds.

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