Down-regulation of Rac-1 GTPase by Estrogen

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Rac1 GTPase is essential for the activation of the NAD(P)H oxidase complex and, thereby, regulates the release of reactive oxygen species (ROS) in the vessel wall. 17β-estradiol (E2) inhibits vascular ROS production. To elucidate the underlying molecular mechanisms we investigated the potential regulation of Rac1 by E2 in vascular smooth muscle cells. Treatment of vascular smooth muscle cells with angiotensin II as well as overexpression of the constitutively active mutant RacL61 increased ROS release as assessed by dichlorofluorescein fluorescence, whereas inhibition of Rac1 by Clostridium sordellii lethal toxin or overexpression of dominant-negative RacN17 inhibited ROS production. Treatment with E2 (100 nm) completely prevented angiotensin II-induced NAD(P)H oxidase activity and ROS production. E2 time and concentration dependently decreased angiotensin II-induced and basal Rac1 mRNA and protein expression as well as Rac1 activity. Down-regulation of Rac1 expression by E2 was mediated by inhibition of gene transcription (nuclear run-on assays), but E2 had no effect on Rac1 mRNA stability. Regulation of Rac1 was mediated by estrogen receptors since co-incubation with ICI 182,780 prevented down-regulation of Rac-1 GTPase by estradiol. E2 time and concentration dependently decreased angiotensin II-induced and basal Rac1 mRNA and protein expression as well as Rac1 activity. Down-regulation of Rac1 expression by E2 was mediated by inhibition of gene transcription (nuclear run-on assays).

Despite the importance of Rac1 GTPase for vascular ROS release, the regulation of Rac1 in the cardiovascular system is only partially understood. It is thought that the reduced prevalence of cardiovascular disease in women is based on atheroprotective effects of estrogens. The latter are potentially mediated directly through binding to vascular estrogen receptors (12–17). Although the antioxidative properties of estrogens are among the most prominent vasoprotective functions of sex steroids, the underlying molecular mechanisms are only partially known. Furthermore, it is not known whether small GTPases are regulated by steroid hormones. We hypothesized that 17β-estradiol may regulate Rac1 GTPase expression and activity and, thereby, inhibit the release of ROS from VSMC.

MATERIALS AND METHODS

Materials—Angiotensin II, l-2-mercaptoethanol and chemicals were purchased from Sigma. [32P]dCTP and Hybond N-nylon membranes were obtained from Amersham Biosciences. [35S]GTPγS was supplied by PerkinElmer Life Sciences. H,D,Fl-Da was purchased from Molecular Probes (Eugene, OR). Antibiotics, calf serum, and cell culture medium were obtained from Invitrogen. RNA-clean was purchased from AGS (Heidelberg, Germany). Clostridium sordellii lethal toxin was kindly provided by K. Aktories (Freiburg, Germany). O. RhoN19 and RacN17 were a kind gift from A. Hall (London, UK) (2).

Cell Culture—VSMC were isolated from female 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% CO2 atmosphere at 37 °C in Dulbecco’s modified Eagles medium without phenol supplemented with 100 units/ml of penicillin, 100 g/ml streptomycin, 1% nonessential amino acids (100 ×), and 10% fetal calf serum (free of steroid hormones, S-15-M, c.c.pro GmbH). Experiments were performed with cells from passage 5–10. Cells were kept in quiescent medium without fetal calf

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vacular smooth muscle cells; DRB, 5,6-dichlorobenzimidazole riboside; DCF, 2',7’-dichlorofluorescein; RT, reverse transcription; GST, glutathione S-transferase; ER, estrogen receptor; SHR, spontaneously hypertensive rats; E, estradiol.
serum 24 h prior to treatment. Cellular viability under all treatment conditions was determined by cell count, morphology, and trypan blue exclusion.

**Animal Treatment**—Female, spontaneously hypertensive rats put on a standard chow and were ovariolectomized or sham-operated (control group) 16 weeks after birth. For treatment, 17β-estradiol pellets (containing 1.7 mg of estradiol each, 60-day release, Innovative Research) were implanted subcutaneously. E2 levels were determined by radioimmunoassay (DPC Biermann, Bad Nauheim, Germany). The thoracic aorta was harvested 5 weeks after surgery. All animal experiments were conducted in accordance to the German animal protecting law.

**Transfection**—Female VSMC were harvested and resuspended in electroporation medium (OptiMEM 1, Invitrogen) at a concentration of 5 × 10^6 cells per ml. Following concentration, cells were transfected using a BD BioLytex transfection kit following the manufacturer’s instructions. Luciferase expression was determined using the luciferase assay system (Prism 7700 Sequence Detection System, PE Biosystems). For polymerase chain reaction (RT-PCR) was performed with the TaqMan Hot-Start II Probe Master (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative fluorescence intensities were obtained from at least three independent experiments with technical triplicates. Differences were analyzed by Student’s t tests and analysis of variance for multiple comparisons. Data are presented as mean ± S.E.M.

**RNA Pull Down Assay**—A glutathione-S-transferase (GST)-PAK-CD fusion protein, containing the central PAK domain region from human PAK1 (22) was used to determine Rac1 activity as described (23). *E. coli* strain transformed with the GST-PAK-CD construct was grown at 37°C to an absorbance of 0.3. The construct was a kind gift of R. C. Roovers and J. G. Collard, The Netherlands Cancer Institute, Amsterdam, The Netherlands. Expression of recombinant protein was induced by addition of 0.1 mmol/liter isopropyl thiogalactoside for 2 h. Cells were harvested, resuspended in lysis buffer (50 mmol/liter Tris-HCl, pH 7.5, 0.5 mmol/liter Na2S2O4, 10% glycerol, 20% sucrose, 2 mmol/liter diethiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin), and then sonicated. Cell lysates were centrifuged at 4°C for 20 min at 45,000 × g, and the supernatant was incubated with glutathione-coupled Sepharose 4B beads (Amersham Biosciences) for 30 min at 4°C. Protein bound to the beads was washed three times in lysis buffer, and the amount of bound fusion protein was estimated using Coomasie-stained SDS gels.

Vascular smooth muscle cells were treated as indicated and washed with ice-cold phosphate-buffered saline, incubated 5 min on ice in lysis buffer (50 mmol/liter Tris-HCl, pH 7.4, 2 mmol/liter MgCl2, 1% Nonidet P-40, 10% glycerol, 100 mmol/liter NaCl, 1 mmol/liter benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin), and then centrifuged for 5 min at 21,000 × g at 4°C. Aliquots were taken from the supernatant to compare protein amounts. Equal amounts of supernatant protein were incubated with the bacterially produced GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads at 4°C for 30 min. The beads and proteins bound to the fusion protein were washed three times in an excess of lysis buffer, eluted in Laemmli sample buffer (60 mmol/liter Tris, pH 6.8, 2% SDS, 10% glycerin, 0.1% bromphenol blue), and then analyzed for bound Rac1 molecules by Western blotting.

**Northern Blotting**—Northern blotting in the presence and absence of 5-8-dichloro-7-hydroxynitazoxide riboside (DRE) (Sigma) using ^32P-labeled, full-length Rac1 cDNA and p22phox cDNA was performed as described previously (19).

**Measurement of Reactive Oxygen Species**—Intracellular reactive oxygen species generation was measured by 2,7′-dichlorofluorescein (DCF) fluorescence using confocal laser scanning microscopy technique. Briefly, dishes of subconfluent cells were washed and incubated in the dark for 30 min in the presence of 10 mmol/liter 2′,7′-dichloro-dihydrofluorescein-diacetate (H2DCF-DA). 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, equipped with a 25×, numerical aperture 0.8, oil-immersion objective (Plan-Neofluar, Carl Zeiss). For detection of intracellular reactive oxygen species, a 500-nm light was used to excite the DCF fluorophore (excitation, 488 nm; emission longpass LP515-nm filter). The cells were detected with a 100× oil objective. Dishes were illuminated with a rectangle of a 5-mm long horizontal cross section of the cell area. The illumination of the entire cell area was excluded. To account for background fluorescence, a separate image was taken of the cell area with the DCF fluorophore but without excitation. The background fluorescence was subtracted from the fluorescence image of the cells. Prolonged illumination was avoided to prevent photobleaching (19).

**Measurement of Rac1 and 18 S mRNA Levels**—Total RNA was isolated and Rac1 and 18 S mRNA were quantitated using real-time RT-PCR. In some experiments, Rac1 mRNA levels were determined by subtracting the amount of Rac1 mRNA standardized to 18 S mRNA prior to transcription from the amounts post transcription. For some experiments, transcription was performed in the presence of 0.2 mmol/liter ^32P-labeled [UTP (>3000 Ci/mmol)]. The transcribed radioactive RNA was hybridized with nylon membranes dotted with linearized pKS + BlueScript, Rac1, and glyceraldehyde-3-phosphate dehydrogenase cDNA, 5 μg of each, as described in detail previously (4, 24). Quantification using dot-blot did not differ from quantifications by real-time RT-PCR.

**Human Mononuclear Cells**—Blood samples of patients from the gynecology outpatient clinic scheduled for planned in vitro fertilization were investigated. Controlled ovarian hyperstimulation following the long-protocol was initiated in all patients with the gonadotropin-releasing hormone analogue triptorelin, 0.1 mg subcutaneously daily, starting in the midluteal phase of the previous cycle until pituitary desensitization was achieved. Then gonadotropin therapy (recombinant follicle-stimulating hormone 150–200 IU subcutaneously daily, Gonal-F, Serono) was given to induce follicular growth. Controlled ovarian hyperstimulation hormone analogue injection was continued up to and including the day of ovulation induction (day 10–12). 30 ml of EDTA plasma were taken before and after 6–10 days of follicle-stimulating hormone treatment. Estradiol levels were evaluated, and mononuclear cells were separated immediately by standard Ficoll gradient centrifugation.

**Data Analysis**—Band intensities were analyzed by densitometry. All values are expressed as mean ± S.E. compared with controls. Paired and unpaired Student’s t tests and analysis of variance for multiple comparisons were employed. Post-hoc comparisons were performed with the Newman-Keuls test. Differences were considered significant at p < 0.05.
Down-regulation of Rac-1 GTPase by Estrogen

RESULTS

Inhibition of Rac1-dependent ROS Release in Vascular Smooth Muscle Cells by E2—To test the effect of E1 and E2 on ROS release, VSMC were treated with angiotensin II, 1 μM for 3 h. DCF fluorescence laser microscopy showed a 2-fold up-regulation of ROS production (208 ± 22%, p < 0.005), which was prevented by pretreatment with E2, 100 nM. Pretreatment with E2 for 6, 12, 24, 36, and 48 h time dependently inhibited angiotensin II-induced ROS release (167 ± 22, 135 ± 17, 99 ± 8, 95 ± 23, and 79 ± 23% of control, respectively) (representative microscopic scan is shown in Fig. 1A, data analysis in B). E2 alone had no significant effect on basal ROS production.

In addition, VSMC intracellular superoxide anion formation in the presence of NADPH and NADH was detected by lucigenin assays as described by Griendling et al. (21). Angiotensin II mediated an up-regulation of both NADH (243 ± 109%) and NADPH oxidase activity (307 ± 126%), which was inhibited after pretreatment with E2 (100 nM, 16 h) (n = 3, * p < 0.05) (Fig. 1C).

Clostridium sordellii lethal toxin inhibits Rac1 activity by specific glycosylation (1). Treatment with lethal toxin (200 ng/ml, 16 h) completely abolished angiotensin II-stimulated oxygen radical release (Fig. 2A). Similarly, overexpression of the dominant-negative RacN17 reduced angiotensin-mediated ROS production (Fig. 2B). Transfection with the constitutively active mutant RacL61 increased ROS release by 2-fold (Fig. 2B). E2 completely reversed angiotensin II-mediated ROS release in cells transfected with empty vector but had no significant effect after transfection with RacL61. These data show that E2 inhibits angiotensin II-stimulated free radical release from VSMC and that Rac1 activity is both necessary and sufficient for ROS production. The experiments suggest that Rac1 is involved in E2-induced decrease of oxidative stress.

Down-regulation of Rac1 Expression by E2—Western analysis demonstrated time-dependent down-regulation of Rac1 expression by E2, 100 nM, by 28 ± 12%, 39 ± 6.7%, 46 ± 6%, 61 ± 7%, and 78 ± 10% after 6, 12, 24, 36, and 48 h, respectively (p < 0.05 after 12 h) (Fig. 3, A and B). Similarly, treatment with E2 (0.01–10 μM) for 24 h concentration dependently reduced Rac1 protein levels to 91 ± 8.4%, 54 ± 6%, 33 ± 4%, and 49 ± 10% of control, respectively (p < 0.05 for E2 ≥ 100 nM) (Fig. 3C). As shown previously, angiotensin II (1 μM, 3 h) increased Rac1 protein expression by 2-fold (4). In the presence of E2, 100 nM, the angiotensin II effect was completely abolished (Fig. 3, D and E). Next, the effects of E2 on Rac1 mRNA levels were studied. Northern blots showed significant concentration-dependent as well as time-dependent down-regulation of Rac1 mRNA levels similar to the effects of E2 on Rac1 protein (Fig. 4, A–C).

In contrast to the down-regulation of Rac1 mRNA, the expression the NAD(P)H oxidase subunits p22phox and nox1 was not significantly altered by E2 (1 μM, 16 h, n = 5) (Fig. 4, D and E).

Down-regulation of Rac1 Activity by E2—Rac1 activity was assessed using GST-PAK Crib domain pull down assays (22, 23). Angiotensin II (1 μM, 3 h) up-regulated Rac1 activity to 175 ± 21% (Fig. 5), which was time dependently inhibited by pretreatment with 100 nM E2 for 6, 12, and 24 h. E2 alone (24 h) down-regulated basal Rac1 GTP-binding activity by 52 ± 18% (p < 0.05).

Inhibition of Rac1 Gene Transcription by E2—To elucidate the mechanism of down-regulation of Rac1 mRNA expression by E2, the rate of Rac1 gene transcription and mRNA stability were studied in the presence and absence of E2 (1 μM, 16 h). Nuclear run on assays showed a reduction of Rac1 transcription to one-third compared with untreated cells (34.5 ± 10%, p < 0.05) (Fig. 6A). In contrast, DRB studies showed no significant alteration of Rac1 mRNA half-life in the presence of estrogen (Fig. 6B). Down-regulation of Rac1 expression by E2 is mediated by inhibition of gene transcription.

Down-regulation of Rac1 Mediated by Estrogen Receptor—To study whether the effects of E2 on Rac1 were receptor-mediated, VSMC were treated with E2 (0.01–1 μM, 16 h) in the presence of ICI 182.780, 1 μM. Co-treatment with ICI showed complete inhibition of E2-induced down-regulation of Rac1 expression, suggesting receptor-mediated signaling (Fig. 7A). To verify the expression of estrogen receptor α (ERα) and β (ERβ) in vascular smooth muscle cells, Western analysis was performed. Both receptor subtypes were expressed abundantly. Treatment with E2 (0.01–10 μM, 16 h) lead to concentration-dependent up-regulation of ERα and ERβ expression (Fig. 7, B and C).

Inhibition of Rac1 Expression by E2 in Vivo—To investigate a possible regulation of Rac1 by E2 in vivo, spontaneously hypertensive rats (SHR) were ovariecotomized and were treated with 17β-estradiol pellets (containing 1.7 mg of estradiol each, 60-day release) for 5 weeks. E2 plasma levels dropped to 1.6 ± 0.5 pg/ml in ovariecotomized SHR compared with 35.7 ± 12 pg/ml in sham-operated rats and to 61 ± 21 pg/ml after estrogen replacement. Aortic superoxide production (20) was up-
regulated (160 ± 27%, p < 0.05) in the ovariectomized animals, which was completely reversed by E2 replacement (Fig. 8A). To test the effects of E2 on Rac1 expression in vivo, real-time PCRs were performed in the aortas of these animals (Fig. 8B). In E2-deficient rats, there was a trend toward up-regulation of Rac1 mRNA (126 ± 33% of control, n = 5, p = non-significant). Treatment of ovariectomized rats with E2 down-regulated vascular Rac1 expression (58 ± 19%, n = 4, p < 0.05). Similarly, aortic protein expression was reduced (42 ± 12%) after treatment with E2 (n = 3, p < 0.05) (Fig. 8C). These data suggest that estrogen regulates vascular Rac1 expression in vivo.

Inhibition of Rac1 Expression in Mononuclear Cells of Women with Elevated E2 Levels—To assess whether the cell culture and animal studies may have significance in humans, mononuclear cells were collected from women before and during controlled ovarian hyperstimulation prior to in vitro fertilization, leading to significant increase of 17β-estradiol levels (Fig. 9A). Real-time PCR showed down-regulation of
Rac1 mRNA levels to 51 ± 36% in the presence of elevated estrogen levels (n = 6, p < 0.05) (Fig. 9B).

**DISCUSSION**

This study shows that 17β-estradiol inhibits the expression and activity of Rac1 GTPase leading to inhibition of free radical production in vascular smooth muscle cells. Similar effects were observed in the vessel wall in vivo. Down-regulation of Rac1 by E2 was not limited to VSMC but was observed in mononuclear cells of women with elevated E2 levels after controlled ovarian hyperstimulation.

An important step in the pathogenesis of endothelial dysfunction and the progression of atherosclerosis is the activation of NAD(P)H oxidase enzyme complex in VSMC by angiotensin II, the primary source of superoxide production in the vessel wall (25). Rac1 GTPase plays a pivotal role during the assembly of the NAD(P)H system (3, 10, 26). Here we show, using overexpression of dominant-negative and active Rac1 mutants, that Rac1 activity is both necessary for ROS production in vascular smooth muscle cells and sufficient for ROS release. In agreement with previous studies (27), E2 effectively and completely...
inhibited angiotensin II-mediated ROS release. More specifically, E2 prevents angiotensin II-mediated NADH and NADPH oxidase activity. But E2 did not significantly reduce ROS after transfection with the active Rac1GTPase. Indeed, Western and Northern analyses demonstrated that E2 concentration and time dependently down-regulated Rac1 protein and mRNA expression, both alone and in the presence of angiotensin II. Similarly, E2 inhibited basal and stimulated Rac1 activity. The molecular mechanism is the inhibition of Rac1 gene transcription, whereas E2 had no significant effect on Rac1 mRNA half-life. The estrogen receptors α and β were abundantly expressed in VSMC and up-regulated by treatment with E2. Down-regulation of Rac1 expression by E2 was completely blocked in the presence of the nonselective estrogen receptor antagonist ICI 182,780, demonstrating a receptor-mediated event.

To test the relevance of these findings in vivo, a well characterized animal model of estrogen deficiency by ovariectomy and E2 replacement therapy was studied (20). In the aortas of ovariectomized SHR significant down-regulation of Rac1 mRNA and protein expression by E2 was observed. Depression of Rac1 by estrogen replacement strongly correlates with reduced vascular oxidative stress. The presented cell culture data assign an essential role to Rac1 in NAD(P)H oxidase-mediated radical release. Thus, it may be suggested that estrogen-induced inhibition of Rac1 reduces production of ROS in vitro as well as in vivo.

To further extend these findings to the human situation, mononuclear cells of young women with elevated estrogen levels undergoing controlled ovarian hyperstimulation prior to in vitro fertilization were studied. Elevation of serum 17β-estradiol correlated with a decrease of Rac1 mRNA expression. These data suggest that estrogen may regulate Rac1 GTPase in humans, but additional studies are needed before conclusions regarding a potential effect of estrogen replacement therapy, especially in combination with progesterone, should be drawn (27).

In the vascular wall, estrogens exert anti-oxidant effects in addition to the inhibition of Rac1 GTPase in VSMC, which are primarily located in the media of the arterial wall. ROS release from the endothelium as well as the adventitia may play an important role in vivo. 3-nitrotyrosine immunoreactivity as well as expression of the NAD(P)H oxidase subunit gp91phox have been shown to increase in the endothelium and adventitia of mice treated with angiotensin II (28, 29). Importantly, recent work by Wagner et al. shows that E2 decreases the function of the NAD(P)H oxidase in endothelial cells, which is mediated by down-regulation gp91phox (30). The expression of its homologues in vascular smooth muscle cells, nox1, was not significantly altered by E2, suggesting a potential dichotomy between endothelial cells and VSMC, which may help to address the cell-specific function of NAD(P)H oxidases in different cell types in further studies. Down-regulation of gp91phox in the endothelium and Rac1 in the media are likely complementary effects in vivo. In addition, estrogen reduces oxidative stress by down-regulation of the AT1 receptor (7, 12, 27). The estrogen-induced reduction of ROS is closely connected to another beneficial action of estrogen on vascular cells, namely the up-regulation of endothelial nitric-oxide synthase activity (12, 30, 31). Therefore, the well established increase of NO bioavailability is caused by increased NO production and decreased superoxide release.

It is thought that the putative vasoprotective effects of estrogens are at least in part mediated via reduction of oxidative stress. Decreased Rac1 expression and activity may resemble a novel and important mechanism by which estrogens interfere with free radical production. In addition, recent evidence suggests an important role for Rac1 GTPase for the control of oxygen radical release outside the vascular wall in several cell types, including leukocytes, fibroblasts, and cardiac myocytes (10, 32). Inhibition of Rac1 by expression of dominant-negative N17rac1 has been shown to protect from hypoxia/reoxygenation-induced cell death in a variety of cell types including vascular smooth muscle cells, fibroblasts, endothelial cells, and ventricular myocytes (33). In cardiomyocytes, Rac1 has been identified as a mediator of hypertrophy (11, 34, 35). Inhibition of Rac1 activity in the heart, e.g. by inhibition of Rac1 isoprenylation using HMG-CoA reductase inhibitors, has been shown to prevent the hypertrophic phenotype as well as cardiac ROS production (36, 37). Interestingly, estrogen has been reported to prevent cardiac hypertrophy by a mechanism yet unknown (16, 38). We speculate that the antihypertrophic effects of estrogen could at least in part be mediated by regulation of Rac1 GTPase.

In summary, Rac1 GTPase gene transcription and activity are regulated by E2, which may be an important molecular mechanism contributing to the cardiovascular effects of estrogens.

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