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Inhibitory effect of estrogen on Rac1-expression in monocytes

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ABSTRACT

Recruitment of circulating monocytes into the vasculature and release of reactive oxygen species (ROS) promote atherogenesis. Rac1-GTPase is an essential component of the superoxide-producing NADPH-oxidase complex. Estrogens inhibit production of vascular reactive oxygen species.

Angiotensin II as well as overexpression of the constitutively active mutant RacL61 increased ROS production in monocytes. AngII-mediated ROS release was completely inhibited by overexpression of the dominant negative mutant RacN17 or treatment with 17β-estradiol. 17β-Estradiol reduced Rac1-expression concentration- and time-dependently and decreased basal, as well as AngII-induced Rac1 activity. The effects of 17β-estradiol were receptor-mediated. *In vivo*, down-regulation of Rac1 by 17β-estradiol was observed in human mononuclear cells of women with elevated 17β-estradiol levels after controlled ovarian hyperstimulation.

In summary, the data show that down-regulation of Rac1-GTPase contributes to the inhibition of angiotensin II-mediated superoxide release by 17β-estradiol in monocytes.

Introduction

Monocyte recruitment from the circulation into the vessel wall is crucial for atherosclerotic lesion formation and progression. After endothelial dysfunction induced by factors including LDL, hypertension, or diabetes, monocytes attach to the endothelium and migrate into the subendothelial space where they take up lipid, become foam cells and cause early lesion development [1,2]. Monocytes induce lipid peroxidation via the generation of reactive oxygen species (ROS) [3,4]. These modified lipids can induce the expression of adhesion molecules and mediators of inflammation in macrophages and vascular wall cells [5]. Monocyte-derived ROS impair endothelial function and accelerate the progression of atherosclerotic lesions by promoting lipid oxidation, the expression of proinflammatory genes, and by oxidative inactivation of endothelial nitric oxide [6,7]. Therefore, detailed understanding of the regulation and signal transduction of ROS production in monocytes is important.

The NADPH-oxidase complex is regarded as the most important source of the primordial oxygen radical, superoxide, in the vessel wall [8] and in monocytes [9]. Rac1 belongs to the 21 kDa Rho-GTPase family that binds to and hydrolyzes guanosine triphosphate (GTP). Rho proteins have been shown to be central regulators of the actin cytoskeleton. They function as transducers between mechanical forces, cell morphology, and gene regulation. In its active GTP-bound state, Rac1 regulates cell shape, adhesion, movement, endocytosis, secretion, and growth [10,11]. Activation of Rac1-GTPase is necessary for the release of superoxide in the vessel wall [12–14], because Rac1 plays a pivotal role in the assembly and activation of the NADPH oxydase system [12,15]. Inhibition of Rac1 activity has been shown to inhibit oxygen radical release in vascular smooth muscle cells (VSMC) and endothelial cells as well as in phagocytes [12,13,15]. In addition to vascular superoxide production, activation of Rac1 signalling contributes to cellular hypertrophy in cardiac myocytes [12,16]. Furthermore cardiac specific overexpression of constitutively activated Rac1 leads to atrial fibrillation in mice via activation of the superoxide-producing NADPH-oxidase [17].

Despite the importance of Rac1-GTPase for vascular ROS release, the regulation of Rac1 in the cardiovascular system, especially in monocytes, is only partially understood. In VSMC estrogen prevents Rac1-mediated superoxide release [18]. It is thought that the delayed prevalence of cardiovascular disease in women is based on atheroprotective effects of estrogens which are potentially mediated directly through binding to vascular estrogen receptors [19–23]. Although the antioxidative properties of estrogens are among the most prominent vasoprotective functions of sex steroids, the underlying molecular mechanisms are only partially known [13,21,23]. We therefore hypothesized that 17β-estradiol (E2) may regulate Rac1-GTPase expression and activity and, thereby, inhibit the release of ROS from monocytes.

Methods

Purification of human monocytes and cell culture. Human monocytes were isolated from healthy volunteers by Ficoll–Hypaque

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density gradient centrifugation and bead-purification using a negative selection strategy according to the manufacturer's instructions (Miltenyi; Biotec). Monocytes were routinely >92% pure, as determined by FACS analysis staining for CD14. Ethical approval of the Ärztekammer des Saarlandes (No.131/00).

Human monocytic THP1 cells were obtained from American Type Culture Collection and cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, and 0.05 mM beta-mercaptoethanol. To assess cellular fatty acid content, THP1 cells were stained with nile blue for 20 min at room temperature.

Human mononuclear cells after ovarian hyperstimulation. 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 IE subcutaneously daily, Gonal-F; Serono) was given to induce follicular growing. Gonadotropin-releasing hormone analogue injection was continued up to and including the day of ovulation induction (day 10–12). Thirty milliliter 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. Ethical approval of the Ärztekammer des Saarlandes (No. 131/00).

Transfection. THP1 cells were harvested and resuspended in electroporation medium (Optimem 1, Invitrogen) at a concentration of 5×10^7 cells per ml. The following constructs were transfected: insertless vector (pcDNA3) as control, pRK5-myc-Rac1-L61 (constitutively active Rac1 mutant), and pRK5-myc-Rac1-N17 (dominant-negative Rac1 mutant) [24]. Twenty microgram of plasmid DNA and 200 μl of cell suspension were placed in a 0.4-cm cuvette, mixed, and incubated for 30 min on ice. After incubation at 37 °C for 30 s, the cuvette was pulsed with 300 V and 500 micro μF (Electro Cell Manipulator, BioRad). After incubation for 30 min cells were cultured for 48 h and treated with angiotensin II, E2, and vehicle. The transfection efficiency was 40–45% as determined by transfection of green fluorescent protein.

Measurement of ROS. Monocyte superoxide release was analysed by L-012 chemiluminescence assays [25], following transfection (Rac1L61 or Rac1N17) or treatment with AngII, 17-estradiol, ICI or vehicle control. L-012 is a luminol derivative with a high sensitivity for ROS, which does not exert redox cycling by itself. Cell cultures were serum-starved for 24 h and treated as indicated, resuspended in 200 μl RPMI-1640, and equilibrated for 10 min at 37 °C. Reactions were stopped by addition of 10 ml cold 1× PBS before centrifugation (5 min, 400 *g*, 4 °C) and addition of 0.5 mM of L-012. Reactions were equilibrated for 15 min in the dark and serial readings were taken at 1-min intervals using a scintillation counter (Berthold Lumat LB 9501, Bad Wildbad, Germany).

Western blotting. Immunoblotting was performed using Rac1 monoclonal antibody (Santa Cruz Biotechnology Inc., 1:250 dilution). β-Actin was used to control for equal protein loading (Santa Cruz Actin H-196 polyclonal antibody, 1:250 dilution) [17,18].

Rac1 GST-PAK pull down assay. Cells were treated as indicated and washed with ice-cold phosphate-buffered saline, incubated 5 min on ice in lysis buffer (50 mmol/l Tris–HCl, pH 7.4, 2 mmol/l $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 100 mmol/l NaCl, 1 mmol/l 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 10 μl of agarose labelled PAK-1 fusion protein (Upstate, USA) or 20 μl of agarose labelled Rhotekin fusion protein (Upstate, USA) at 4 °C for 60 min. Beads were washed 3 times with MLB buffer, eluted in Laemmli buffer (60 mmol/l Tris [pH 6.8], 2% sodium dodecylsulfate, 10% glycerin, 0.1% bromphenol blue) and analyzed for bound Rac1 content by Western blotting.

Real-time RT-PCR. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems). For human Rac1 the primers were 5′-GTA AAA CCT GCC TGC TCA TC and 5′-GCT TCG TCA AAC ACT GTC TTG. For 18 S the primers were 5′-TTG ATT AAG TCC CTG CCC TTT GT and 5′-CGA TCC GAG GGC CTA ACTA. For quantification, Rac1 mRNA expression was normalized to the expressed housekeeping gene 18 S.

Data analysis. Band intensities were analyzed by densitometry. Mean, standard error of the mean (SEM), one way ANOVA and Newman–Keuls post hoc test for multiple comparison and unpaired Student's *t* tests for single comparison were performed by Sigma Stat Software 3.5. Differences were considered significant at $p < 0.05$.

Results

Inhibition of Rac1-dependent ROS release in THP1 cells by estrogen

To test the effect of Rac1 and E2 on ROS release, THP1 cells were treated with AngII, 100 nM for 2 h. L-012 chemiluminescence assays showed an up-regulation of superoxide production to 297 ± 38% ($p < 0.05$), which was prevented by pretreatment with E2 (100 nM; 16 h; $p < 0.05$; Fig. 1A). Similarly, overexpression of the dominant-negative RacN17 reduced angiotensin-mediated ROS production ($p < 0.05$ vs. Ang; Fig. 1B). Transfection with the constitutively active mutant RacL61 increased ROS release by approximately 3-fold (283 ± 58%; $p < 0.05$ vs. vector). Pre-treatment with the Rac1-specific small molecule inhibitor NSC 23766, which prevents Rac1 activation by inhibition of the Rac-specific guanine nucleotide exchange factors (GEFs) TrioN and Tiam1 without affecting Cdc42 or RhoA activation, reduced AngII induced superoxide production in THP1 cells (Fig. 1C). These data show that E2 inhibits angiotensin II-stimulated free radical release from THP1 cells and that Rac1 activity is both necessary and sufficient for ROS production.

Down-regulation of Rac1-expression by estrogen

Western analysis demonstrated time-dependent down-regulation of Rac1-expression by E2 in THP1 cells, 100 nM with a maximum reduction of 78 ± 10% after 48 h ($p < 0.05$ after 12 h; Fig. 1D). Similarly, treatment with E2 (0.01–1 μM) for 24 h concentration dependently reduced Rac1 protein levels to 87 ± 20%, 48 ± 12%, and 36 ± 20% of control, respectively ($p < 0.05$ for E2 > 100 nM) (Fig. 1E).

Down-regulation of Rac1 activity by estrogen

Rac1 activity was assessed using GST-PAK crib domain pull down assays. AngII (1 μM, 3 h) up-regulated Rac1 activity to 185 ± 21% (Fig. 1F), which was inhibited by pretreatment with 1 μM E2 for 16 h. E2 alone down-regulated basal Rac1 GTP-binding activity to 52 ± 18% of control ($p < 0.05$). The experiments suggest that Rac1 is involved in the E2-induced decrease of oxidative stress.

Down-regulation of Rac1 is mediated by estrogen receptor

To study whether the effects of E2 on Rac1 were receptor-mediated, THP1 cells were treated with AngII (100 nM, 2 h) and/or E2

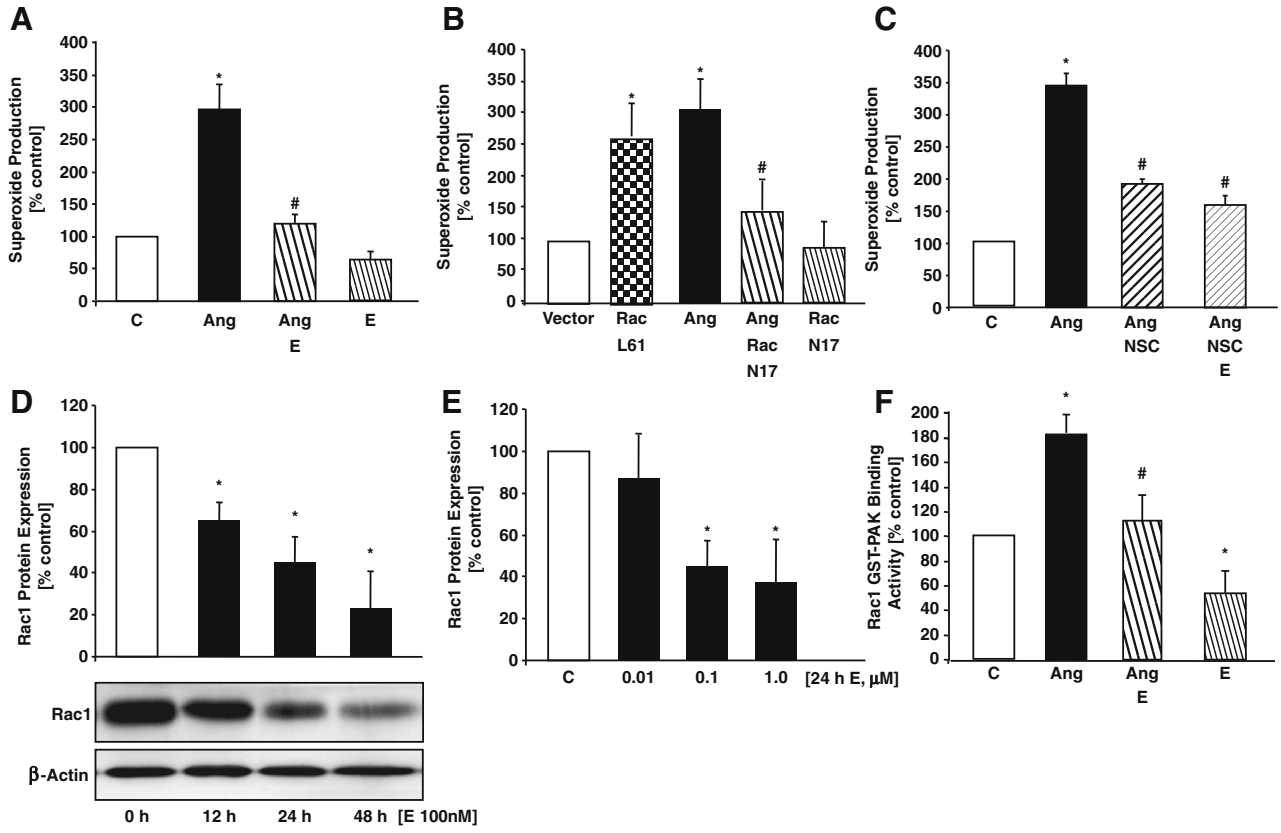

Fig. 1. (A) Free oxygen radical production by angiotensin II (Ang, 100 nM, 2 h) alone and after preincubation with 17β-estradiol (E; 100nM; 16 h) in THP1 cells detected by L-012 chemiluminescence assay. (B) ROS production in THP1 cells transfected with dominant negative RacN17 (N17), constitutively active RacL61 (L61), and empty pcDNA3 vector in the presence and absence of angiotensin II (100 nM; 2 h). (C) Free oxygen radical production by angiotensin II (Ang, 100 nM, 2 h) alone and after preincubation with Rac 1 small molecule inhibitor NSC 23766 (NSC; 100 nM; 21 h) in THP1 cells detected by L-012 chemiluminescence assay; $n = 3–5$, $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. Ang. (D) Representative Western blot and quantification of the time-dependent effects of 17β-estradiol (E; 100 nM) on Rac1 with corresponding β-actin expression in THP1 cells. (E) Concentration-dependent effects of 17β-estradiol after 24 h. (F) Effects of 17β-estradiol (1 μM; 16 h) on angiotensin II (Ang; 1 μM; 3 h) induced Rac1 activity detected by Rac1 GST-PAK pull down assay; $n = 5$, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. Ang.

(100 nM, 16 h) in the presence of ICI 182.780, 1 μM. E2 prevented the AngII-induced up-regulation of superoxide release as previously observed. Co-treatment with ICI showed complete inhibition of E2-induced down-regulation of superoxide production, suggesting receptor-mediated signalling (Fig. 2A). Western blot analysis showed complete inhibition of E2-induced down-regulation of Rac1-GTPase on protein level by co-treatment with ICI (Fig. 2B). E2 prevented AngII induced up-regulation of Rac1 protein expression (AngII 207 ± 23% of control vs. AngII + E2 93 ± 7% of control), whereas ICI abolished these effects (AngII + E2 + ICI 169 ± 8% of control; Fig. 2C), showing that the effect of estrogen receptor inhibition is present in activated and in resting cells. Nile blue staining confirmed that lipid uptake in THP1 cells was increased in the presence of AngII (100 nM; 2 h), which was potently prevented in the presence of E2 (Fig. 2D) [26,27].

Inhibition of superoxide production and Rac1-GTPase in human monocytes by estrogen

In order test the effect of 17β-estradiol on superoxide production and Rac1-expression in human monocytes, isolated human monocytes from healthy volunteers were incubated with AngII (100 nM, 2 h) and E2 (100 nM; 16 h). L-012 chemiluminescence assay showed a 3-fold up-regulation of superoxide production (307 ± 61%, $p < 0.05$), which was prevented by pre-treatment with E2 (100 nM; 16 h; $p < 0.05$). E2 alone had no significant effect on basal ROS production (Fig. 3A). Western analysis demonstrated that Rac1 protein expression was markedly increased after stimulation with AngII (201 ± 20%; $p < 0.01$), pre-treatment with E2 completely prevented the AngII induced effects ($p < 0.05$ vs. Ang), (Fig. 3B).

Inhibition of Rac1-expression by estrogen in vivo

To investigate a possible regulation of Rac1 by E2 *in vivo*, mononuclear cells were collected from women before and during controlled ovarian hyperstimulation prior to *in vitro* fertilization, leading to significant increase of 17β-estradiol blood levels (Fig. 3C). Real-time PCR showed down-regulation of Rac1 mRNA levels to 51 ± 36% in the presence of elevated estrogen levels ($p < 0.05$) (Fig. 3D).

Discussion

This study shows that 17β-estradiol inhibits the expression and activity of Rac1-GTPase in monocytes leading to inhibition of free radical production. The down-regulation of Rac1 by E2 was confirmed *in vivo* in mononuclear cells of women with elevated E2 levels after controlled ovarian hyperstimulation. Atherogenesis is driven by inflammation, lipid accumulation, cell death and fibrosis [2]. The blood monocytes play a crucial role during all stages of atherogenesis [1,2]. Importantly, ROS from monocytes induce peroxidation of LDL [3] and several lines of evidence support a

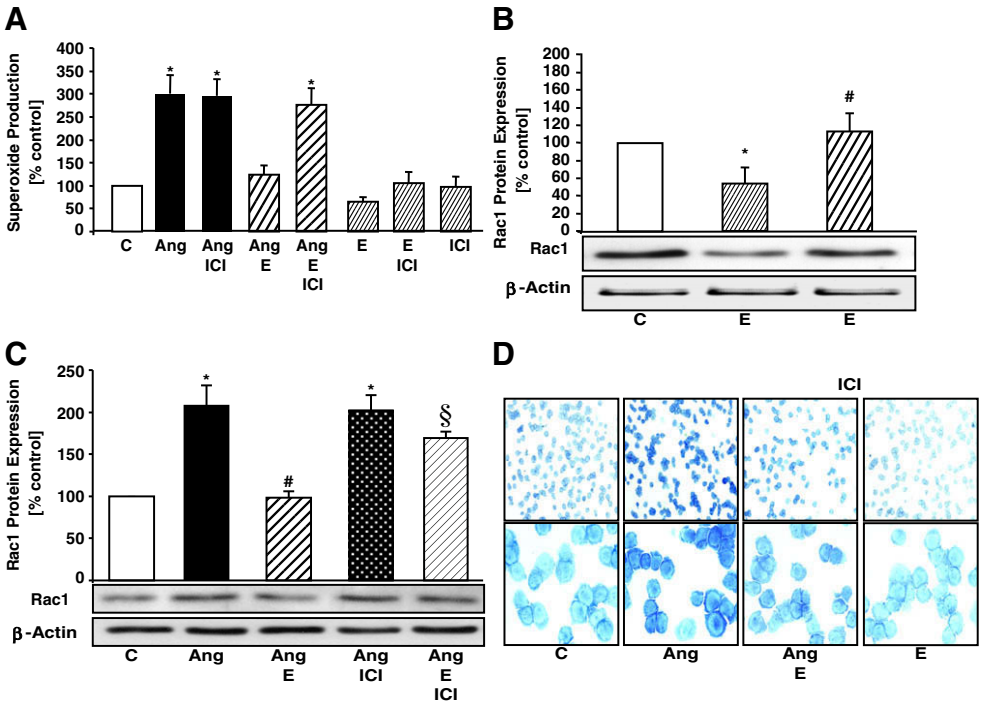

Fig. 2. (A) Effects of 17β-estradiol (E; 1 μM; 16 h) in the presence of ICI 182.780 (1 μM, 16 h) on free oxygen radical production induced by angiotensin II (Ang; 100 nM; 2 h). (B) Representative Western blot and quantification of Rac1 with corresponding β-actin expression after stimulation with 17β-estradiol (E; 100 nM; 16 h) in the presence of ICI 182.780 (1 μM, 16 h) in THP1 cells; $n = 5$, $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. E. (C) Representative Western blot and quantification of the effect of 17β-estradiol (E; 100 nM; 16 h) on Rac1 protein expression related to β-actin after stimulation with angiotensin II (Ang, 100 nM; 2 h) in the presence of ICI 182.780 (1 μM, 16 h) in THP1 cells; $n = 3$, $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. Ang and $^{\S}p < 0.05$ vs. Ang + E. (D) Representative nil blue staining of the effect of 17β-estradiol (E; 100 nM; 16 h) on angiotensin II (Ang, 100 nM; 2 h) induced lipid uptake in 10- and 40-fold magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

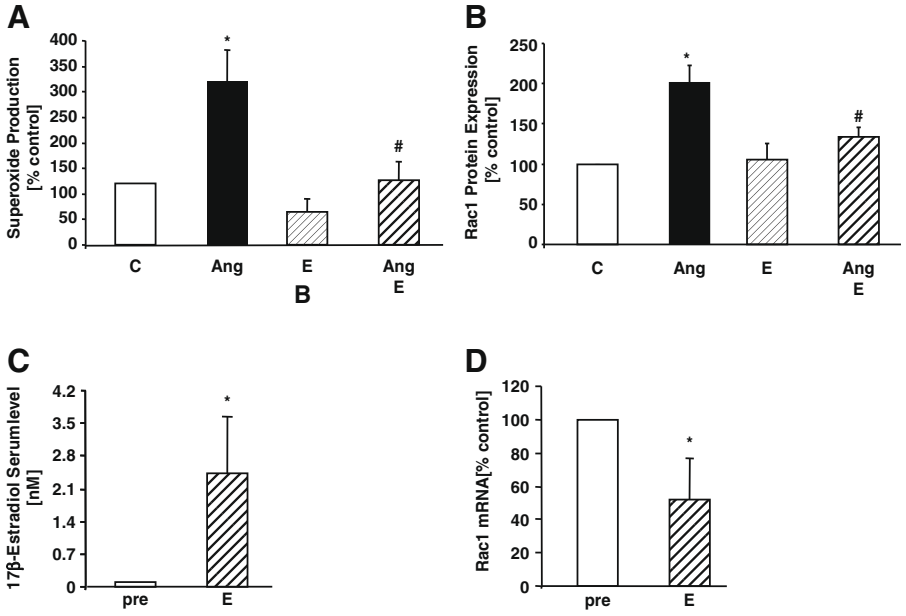

Fig. 3. (A) Free oxygen radical production by angiotensin II (Ang, 100 nM, 2 h) alone and after preincubation with 17β-estradiol (E; 100 nM; 16 h) in human monocytes detected by L-012 chemiluminescence assay. (B) Quantification of Rac1 protein expression in human monocytes stimulated by angiotensin II (Ang 100 nM; 2 h) alone and after preincubation with 17β-estradiol (E; 100 nM; 16 h); $n = 5$, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. Ang. (C) 17β-estradiol serum levels and (D), corresponding Rac1 mRNA expression determined by real-time PCR of mononuclear cells in women undergoing ovarian hyperstimulation; $n = 6$, $^{*}p < 0.05$.

proatherogenic role for oxidized LDL (Ox-LDL) [3,4,28,29]. The primary source of superoxide production in the monocytes is the NADPH-oxidase [9,30,31]. Rac1-GTPase is important for the activation of the NADPH-oxidase [12,15,31,32]. Here we show using overexpression of dominant-negative and active Rac1 mutants, that Rac1 activity is both necessary for ROS production in monocytes and sufficient for ROS release. Treatment with estrogen effectively and completely inhibited this angiotensin II-mediated ROS

release. Angiotensin II increased lipid uptake in monocytes which was prevented in the presence of E2 [26,27]. Western analyses demonstrated that estrogen concentration and time-dependently down-regulated Rac1 protein expression, both alone and in the presence of angiotensin II. Similarly, estrogen inhibited basal and stimulated Rac1 activity. Down-regulation of Rac1-expression by estrogen was completely blocked in the presence of the nonselective estrogen receptor antagonist ICI 182.780, demonstrating a receptor-mediated event. The regulation of Rac1 by estrogen was observed in the monocyte cell line THP1 as well as in freshly isolated human monocytes.

Rac1 signalling is required for fimbriae stimulated monocyte adhesion to human endothelial cells and transmigration across human endothelial cell monolayers [33]. Rac1 contributes to ICAM-1/b2- and VCAM-1/b1-integrin-dependent monocyte adhesion, an effect that is inhibited by estrogen [34]. In addition, Rho-GTPases act as modulators of estrogen receptor (ER) transcriptional activation [35]. We speculate that the observed regulation of Rac1 by estrogen in monocytes is important beyond the regulation of the NADPH-oxidase, however, this has to be addressed in future studies.

Any cell culture study provides only limited evidence to predict effective concentrations in humans. This limitation was the reason why mononuclear cells of young women with elevated estrogen levels undergoing controlled ovarian hyperstimulation prior to *in vitro* fertilization were studied that provided evidence for the concept of the regulation of monocyte Rac1 by E2 *in vivo*. The serum concentrations down-regulating monocytic Rac1 in the women were below the concentrations used in tissue culture suggesting an *in vivo* relevance of our observations.

Similar to our observations in monocytes, treatment with 17β-estradiol prevents superoxide production in vascular smooth muscle cells through receptor-mediated inhibition of Rac1 gene transcription [18]. Rac1-GTPase is involved in the control of oxygen radical release outside the vascular wall in several cell types, including fibroblasts, and cardiac myocytes [15,36]. Based on our data and the literature, we propose that the interaction of the steroid hormone estrogen with the small G protein Rac1 may represent an anti-oxidative defence mechanism that is not limited to monocytes but is important for several cardiovascular cell types.

In summary, these data suggest that Rac1-GTPase gene transcription and activity in monocytes are regulated by estrogen, which may represent a new and potentially relevant molecular mechanism contributing to the cardiovascular effects of estrogens.

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