Basic research

Atheroprotective effects of 17β-oestradiol are mediated by peroxisome proliferator-activated receptor γ in human coronary artery smooth muscle cells

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Abstract

Introduction: 17β-oestradiol (E2) mediates vasculoprotection in various preclinical and clinical models of atherosclerosis and neointimal hyperplasia. However, the molecular mechanisms underlying these effects are still not fully elucidated. Previous studies have demonstrated the essential role of the peroxisome-proliferator-activated-receptor-γ (PPARγ) in mediating vasculoprotective effects of E2 *in vivo*. The aim of the current study was to investigate whether PPARγ mediates vasculoprotective mechanisms of E2 in human coronary artery smooth muscle cells (HCASMC).
Material and methods: Primary HCASMC were stimulated with E2 (10 nM), the selective oestrogen receptor α (ERα) agonist propylpyrazole triol (PPT) (50 nM) and the selective ERα antagonist methyl-piperidino-pyrazole (MPP) (1 μM), respectively. Changes in PPARγ mRNA, protein expression, and DNA binding affinity were assessed.
Results: E2 significantly increased PPARγ expression in HCASMC (1.95 ±0.41-fold; $n = 5$; $p = 0.0335$). This effect was mimicked by ERα agonist PPT (1.63 ±0.27-fold; $n = 7$; $p = 0.0489$) and was abrogated by co-incubation with ERα antagonist MPP (1.17 ±0.18-fold; $n = 3$; $p_{vs.\ control} > 0.05$). PPARγ-DNA binding activity to PPRE remained unchanged upon stimulation with E2 (0.94 ±0.11-fold; $n = 4$; $p_{vs.\ control} > 0.05$). Pharmacological inhibition of PI3K/Akt by LY294002 abrogated E2-induced expression of PPARγ (0.24 ±0.09-fold; $n = 3$; $p_{vs.\ E2} = 0.0017$).
Conclusions: The present study identifies PPARγ as an important downstream mediator of E2-related atheroprotective effects in HCASMC. PPARγ agonism might be a promising therapeutic strategy to prevent neointimal hyperplasia and consecutive cardiovascular events in postmenopausal women with depleted E2 plasma levels.

Key words: 17β-oestradiol, peroxisome-proliferator-activated-receptor-γ, propylpyrazole triol, oestrogen receptor α, human coronary artery smooth muscle cells.

Introduction

17β-oestradiol (E2) is the most potent naturally occurring representative of steroidal oestrogens in mammals [1]. A vast body of evidence links E2 to vasculoprotection in a wide range of preclinical and clinical models of atherosclerosis and neointimal hyperplasia [2–4]. E2 has been demonstrated to favourably affect vascular biology: E2 mediates vasodi-

lation by an increase in nitric oxide, reduces oxidative stress, and hampers vascular smooth muscle cell proliferation [5–7]. These effects of E2 are mediated by the nuclear oestrogen receptors ERα and ERβ, as well as by the 7 transmembrane G protein-coupled receptor GPR30/GPER, all of which are expressed in vascular smooth muscle cells [8, 9]. Although the lack of E2 has been associated with an increased cardiovascular risk, hormone replacement therapy has failed to demonstrate clinical net benefits in postmenopausal women who are at risk of cardiovascular events [10–12]. From this arises the as yet unmet medical need to identify druggable downstream targets for novel antiatherosclerotic therapeutic strategies.

Peroxisome proliferator-activated receptor γ (PPARγ) is another nuclear receptor that regulates fat and carbohydrate metabolism. Its activation has been shown to reduce vascular inflammation and atherogenesis *in vitro* and *in vivo* [13, 14]. Recently, our group has demonstrated that PPARγ is a downstream target of E2, which mediates the atheroprotective effects of E2 in an atherosclerotic mouse model [15]. In this former study, we demonstrated that PPARγ expression in murine aortas declines when production of E2 is hampered by ovariectomy, whereas subcutaneous application of E2 restores aortic PPARγ levels in ovariectomised mice [15]. In the same study, E2 diminished the atherosclerotic plaque burden of $ApoE^{-/-}$ mice and improved endothelial relaxation capacity. This effect was mimicked by PPARγ agonist pioglitazone and abolished by co-administration of the selective PPARγ antagonist GW9662. While this former study was the first to demonstrate that the atheroprotective effects of E2 depend on the functioning of the transcription factor PPARγ, the underlying molecular mechanisms on a cellular level remain unclear.

In the present study, we aimed to elucidate the molecular pathway that links PPARγ to E2 signalling in human coronary artery smooth muscle cells. Furthermore, our objective was to examine whether atheroprotective signalling of E2 is mediated by PPARγ.

Material and methods

HCASMC cell culture

Primary human coronary artery smooth muscle cells (HCASMC) were purchased from PromoCell (Heidelberg, Germany). Cells were cultivated at 37°C in 5% (v/v) CO_2. All experiments were conducted at 80–90% confluence unless stated otherwise. Drug treatment was carried out in phenol red-free smooth muscle cell basal medium (PromoCell) and its corresponding supplement mix containing foetal calf serum (5% (v/v)), epidermal growth factor (0.5 ng/ml), fibroblast growth factor (2 ng/ml), and insulin (5 µg/ml).

Reagent preparation

E2 was purchased from Sigma-Aldrich (Taufkirchen, Germany) and reconstituted in dimethyl sulfoxide (DMSO, Carl Roth GmbH, Karlsruhe, Germany), generating a 10^{-5} M stock solution. This E2 stock solution was further diluted (1 : 10) with sterile H_2O (Ampuwa, Fresenius, Bad Homburg vor der Höhe, Germany) and with cell culture medium (1 : 100) to a final concentration of 10^{-8} M.

The selective agonist of ERα, propylpyrazole triol (PPT), was purchased from Tocris Bioscience (Bristol, United Kingdom). PPT was reconstituted in DMSO as a 50 µM stock solution. PPT was further diluted in cell culture medium to a final concentration of 50 nM prior to use.

2-Chloro-5-nitro-N-phenylbenzamide (GW9662, Sigma-Aldrich), a selective antagonist of PPARγ, was reconstituted in DMSO and stored as a 10^{-2} M stock solution. The 10^{-6} M GW9662 working solution was obtained by dilution in cell culture medium.

Quantification of PPARγ mRNA levels by qPCR

PPARγ mRNA expression levels of HCASMC were quantified using qPCR. HCASMC were stimulated with E2 (10 nM), with the ERα agonist PPT (50 nM), or with the pathway inhibitors SB203580 (1 µM) (Promega, Madison, USA), LY294002 (5 µM) (Cell Signaling, Danvers, USA), or L-N^G-Nitroarginine (L-NNA) (10 µM) (Tocris) for 4 h to 24 h. DMSO (0.1% (v/v)) served as control. Cells were then washed with PBS and harvested with Trizol® (Ambion life technologies/Thermo Fisher Scientific Inc., Waltham, USA). Chloroform (Merck, Darmstadt, Germany) was added at a ratio of 1 : 5, and the RNA-containing phase was separated by centrifugation (18,000 × g; 4°C; 15 min). RNA was precipitated by addition of isopropanol. Samples containing the precipitated RNA were spun (18,000 × g; 4°C; 15 min), the supernatant was discarded, and the RNA was washed twice with ethanol (75% (v/v)). Finally, the RNA was dried and eluted in RNAse-/DNAse-free H_2O (Gibco/Thermo Fisher Scientific Inc.) at 56°C for 10 min. RNA was reversely transcribed into cDNA using the Omniscript RT kit (Qiagen GmbH, Hilden, Germany). PCR amplification and quantification of cDNA fragments was accomplished using TaqMan® probes and the appropriate master mix (Thermo Fisher Scientific Inc.). Data were generated on a 7500 Fast Real-Time PCR system and analysed using 7500 software v.2.0.6 (both Thermo Fisher Scientific Inc.). TaqMan® probes used in this study are specified in Table I.

Table I. Overview of TaqMan® probes used in this study

TaqMan® Gene Expression Assay ID	Target gene	Vendor	Cat no.
Hs03003631_g1	Eukaryotic 18S rRNA	Thermo Fisher Scientific Inc.	4331182
Hs01115513_m1	PPARG	Thermo Fisher Scientific Inc.	4331182

Quantification of PPARγ protein expression levels by western blot

HCASMC were stimulated with E2, PPT, or DMSO for 24 h. Subsequently, HCASMC were washed with PBS and solubilised with ice-cold RIPA lysis buffer containing: 1 M Tris-HCl: 10 ml; 50% (v/v) nonident P40 substitute: 2 ml; 10% (w/v) deoxycholic acid: 5 ml; 5 M NaCl: 6 ml; 0.5 M EDTA: 0.4 ml; 0.1 M Na3VO4: 2 ml; and 0.5 M NaF: 0.4 ml. Cells were then harvested using a cell scraper (Sarstedt, Nümbrecht, Germany) and sonicated for 5 min. The protein-containing supernatant was separated from the cell debris by centrifugation (18,000 × g; 4°C; 30 min). Proteins were separated by SDS gel electrophoresis as described [16]. Proteins were blotted onto nitrocellulose membranes by western blotting, and protein immunodetection was performed as follows: nitrocellulose membranes were sequentially exposed to blocking reagent (5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline, 0.1% (w/v) Tween 20 (TBST)), primary antibodies (PPARγ (Santa cruz, Heidelberg, Germany), GAPDH (Hytest, Turku, Finland)), and the appropriate HRP-conjugated secondary antibody (Sigma-Aldrich). Bands were visualised using the enhanced chemiluminescence Prime Western Blotting System (Sigma-Aldrich) and quantified by Image J software (National Institute of Health, Bethesda, USA). The PPARγ signal was normalised to GAPDH.

Quantification of nuclear PPARγ protein expression

HCASMC were stimulated with E2 (10 nM) or DMSO (0.1% (v/v)) for 24 h. The nuclear extract was separated from the cytosolic fraction using a nuclear/cytosol fractionation kit (PromoCell) in accordance with the manufacturer's instructions. In brief, cells were harvested with trypsin/EDTA, centrifuged (600 × g, 4°C; 5 min), and the supernatant was discarded. The cytosolic fraction was isolated after the addition of cytosol extraction buffers, which were supplied with the kit. The nuclear fraction was extracted using the provided nuclear extraction buffer following centrifugation (16,000 × g, 4°C; 5 min). PPARγ protein expression levels were quantified by western blotting as described in 2.4.

Measuring PPARγ DNA binding activity in nuclear extracts

HCASMC were stimulated with E2 (10 nM) or DMSO (0.1% (v/v)) for 24 h, and the nuclear fraction was isolated using the nuclear extraction kit by Abcam (Cambridge, United Kingdom). Cells were lysed in hypotonic buffer, and nuclear protein was extracted using nuclear extraction buffers, provided with the kit. Transcription factor binding activity to dsDNA was studied using the PPARγ transcription factor assay kit (Abcam). The assay is based on the enzyme-linked immunosorbent assay (ELISA) principle. Nuclear protein fractions were pipetted onto a 96-well plate that had been coated with dsDNA containing the recognition sequence for PPARγ, PPAR response element (PPRE). Adherent PPARγ was visualised by the appropriate primary and HRP-conjugated secondary antibodies, the latter of which catalysed a colorimetric reaction. Light extinction at 450 nm was quantified using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland).

Quantification of HCASMC proliferation by BrdU staining

HCASMC were grown on cover slips in 24-well plates until 80% confluence was reached. Cells were stimulated with E2 (10 nM), E2 (10 nM) + the selective PPARγ antagonist GW9662 (1 µM), or DMSO (0.1% (v/v)) for 24 h. Meanwhile, replicating cells were labelled with 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) (10 µM). Subsequently, cells were fixed and permeabilised with 4% (w/v) paraformaldehyde (PFA; 4°C; 30 min). Then, DNA was hydrolysed by sequential exposure to 1 M HCl (4°C; 10 min) and 2 M HCl (37°C; 20 min), which was neutralised with 0.1 M sodium borate buffer pH 8.5 for 12 min. Finally, cells were washed with phosphate-buffered saline (PBS) and sequentially exposed to blocking agent (5% (v/v) donkey serum in PBS, room temperature, 1 h), primary anti-BrdU antibody (Abcam, 4°C, overnight), and secondary anti-rat cyanine 3 (Cy3)-conjugated antibody (Sigma-Aldrich, room temperature, 1 h). Nuclei were stained using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, USA). Proliferating BrdU-positive cells were visualised with a Zeiss Axiovert 200M microscope (Carl Zeiss Jena GmbH, Jena, Germany) and counted automatically using Image J 1.48v software.

Assessment of HCASMC migration by scratch assay

HCASMC were starved overnight in order to minimise cell proliferation. At 100% confluence, the cell layer was injured with a small tip and cells were stimulated with E2 (10 nM) or E2 + GW9662 (1–30 µM). DMSO (0.1% (v/v)) served as control. The width of the scratch was quantified immediately with a Zeiss Axiovert 200M microscope. Microphotographic pictures were taken every 3 h until the complete closure of the scratch. The residual gap of the cell layer at any given time point was normalised to the width of the initial lesion.

Detection of reactive oxygen species by L-012 and DCF-DA assays

Production of reactive oxygen species by HCASMC was measured by L-012 chemiluminescence and 2′,7′-dichlorofluorescin diacetate (DCF-DA) staining. L-012 stock solution (Wako Chemicals GmbH, Neuss, Germany) was diluted in PBS (1 : 100) and KH-HEPES-buffer (1 : 10), containing: NaCl 99.01 mM, KCl 4.69 mM, $CaCl_2$ 1.87 mM, $MgSO_4$ 1.20 mM, NaHEPES 20.0 mM, K_2HPO_4 1.03 mM, $NaHCO_3$ 25.0 mM, D(+)glucose 11.1 mM, adjusted to pH 7.40 mM. HCASMC were stimulated with DMSO (0.1% (v/v)), E2 (10 nM), or E2 (10 nM) + GW9662 (1 µM). Cells were dissociated enzymatically with trypsin/EDTA (0.05% (w/v)), centrifuged (170 × g; room temperature; 5 min), and reconstituted in L-012 working solution. ROS catalysed a luminescent reaction that allowed for quantification in a scintillation counter (Lumat LB 9501, Berthold Technologies GmbH & Co. KG, Wildbad, Germany). Events were normalised to the cell count. Five-minute scintillation counts were used for statistical analyses.

In an additional approach, ROS were visualised by DCF-DA staining. HCASMC were stimulated with E2 (10 nM), E2 + GW9662 (1–30 µM), or DMSO (0.1% (v/v)) for 24 h. Hereafter, cells were exposed to 2 ml DCF-DA staining buffer, containing DCF-DA (10 µM) for 30 min at 37°C. Finally, cells were washed with staining buffer and microphotographic pictures were taken at an excitation wavelength of 485 nm and an absorption wavelength of 538 nm.

Statistical analysis

Data are presented as the mean ± SEM. Data were analysed using Microsoft excel software (Microsoft, Redmond, USA) and GraphPad Prism (GraphPad Software, San Diego, USA). Continuous variables of two groups were compared using unpaired Student's two-sided *t*-test. For the comparison of three or more groups, a one-way ANOVA and subsequent Bonferroni correction was performed. *P*-values < 0.05 were considered statistically significant.

Results

E2 induces PPARγ in an ERα dependent fashion

Stimulation of HCASMC with E2 led to a time-dependent increase in PPARγ mRNA expression levels. PPARγ expression increased by 1.61 ±0.27-fold (n = 5; p > 0.05) after 18 h and by 1.95 ±0.41-fold (n = 5; p = 0.0335) after 24 h of E2 stimulation (Figure 1 A). Stimulation with the ERα agonist PPT mimicked the stimulatory effect of E2 on PPARγ yielding an increase of PPARγ mRNA expression levels by 1.63 ±0.27-fold (n = 7; p = 0.0489; Figure 1 B). Concomitantly, co-stimulation with the selective ERα antagonist MPP abrogated the stimulatory effect of E2 on PPARγ expression (1.17 ±0.18-fold; n = 3; $p_{vs.\ control}$ > 0.05) (Figure 1 B). Induction of PPARγ by E2 was also recapitulated on the protein level by Western blot analyses, which showed a significant increase in PPARγ protein expression upon stimulation with E2 (2.82 ±0.51-fold; n = 6; $p_{vs.\ control}$ = 0.0044; Figure 1 C). Stimulation with the ERα agonist PPT produced a similar increase in PPARγ protein expression. However, this effect was not statistically significant (2.40 ±0.94-fold; n = 4; $p_{vs.\ control}$ = 0.0534; Figure 1 C).

E2 augments PPARγ expression in the nucleus without affecting PPARγ binding activity to PPRE

In addition to globally enhanced PPARγ protein expression levels, PPARγ protein expression was also significantly increased in the nucleus of HCASMC after 24 h of E2 stimulation (1.53 ±0.16-fold; n = 4; $p_{vs.\ control}$ = 0.0074; Figure 2 A). However, E2 did not impact on PPARγ's DNA binding activity to PPRE, as assessed by a colorimetric binding assay (0.94 ±0.11-fold; n = 4; $p_{vs.\ control}$ > 0.05) (Figure 2 B).

E2-induced transcription of PPARγ depends on PI3K/Akt signalling

Subsequently, we studied by which signalling pathway E2 alters PPARγ expression. While E2 increased PPARγ mRNA expression levels, co-incubation with the reversible inhibitor of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) pathway, LY294002, significantly diminished E2-induced PPARγ expression (0.24 ±0.09-fold; n = 3; $p_{vs.\ E2}$ = 0.0017; Figure 3). Meanwhile inhibition of p38 mitogen-activated protein kinase (MAPK) by SB203580 had no significant effect on the induction of PPARγ by E2 (1.50 ±0.26-fold; n = 3; $p_{vs.\ E2}$ > 0.05). Similarly, the effect

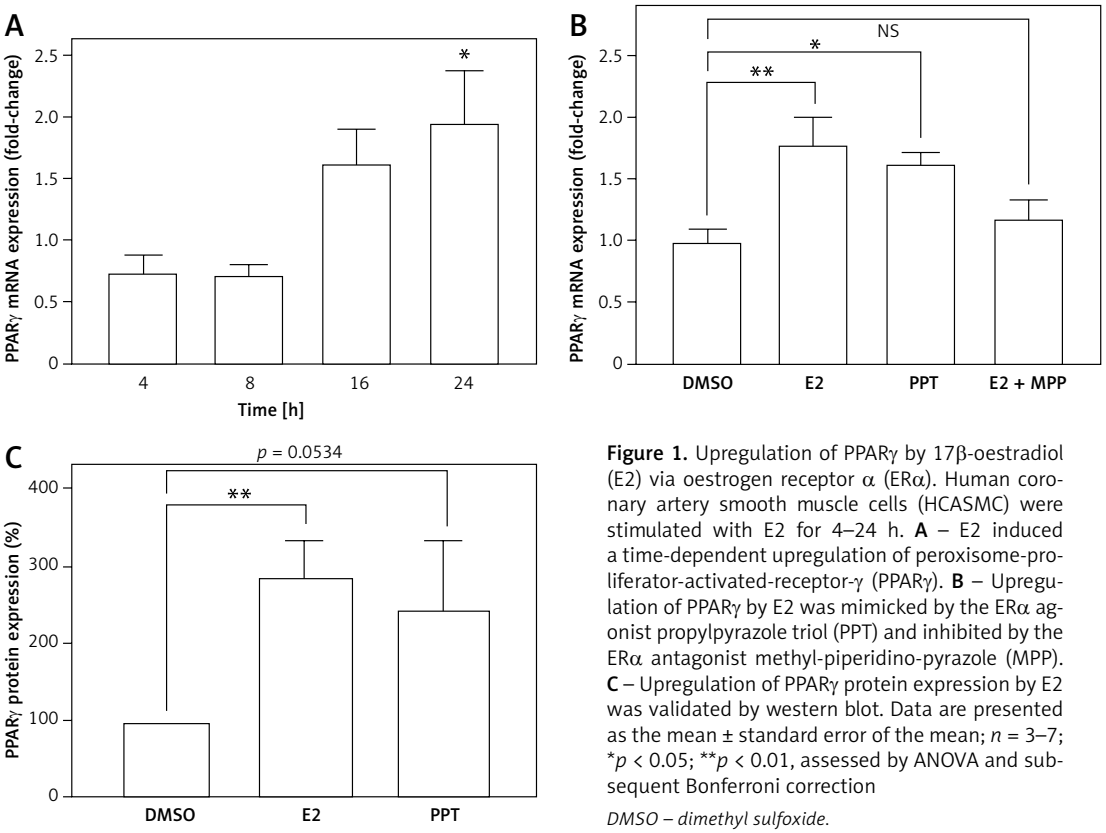

Figure 1. Upregulation of PPARγ by 17β-oestradiol (E2) via oestrogen receptor α (ERα). Human coronary artery smooth muscle cells (HCASMC) were stimulated with E2 for 4–24 h. **A** – E2 induced a time-dependent upregulation of peroxisome-proliferator-activated-receptor-γ (PPARγ). **B** – Upregulation of PPARγ by E2 was mimicked by the ERα agonist propylpyrazole triol (PPT) and inhibited by the ERα antagonist methyl-piperidino-pyrazole (MPP). **C** – Upregulation of PPARγ protein expression by E2 was validated by western blot. Data are presented as the mean ± standard error of the mean; n = 3–7; *p < 0.05; **p < 0.01, assessed by ANOVA and subsequent Bonferroni correction

DMSO – dimethyl sulfoxide.

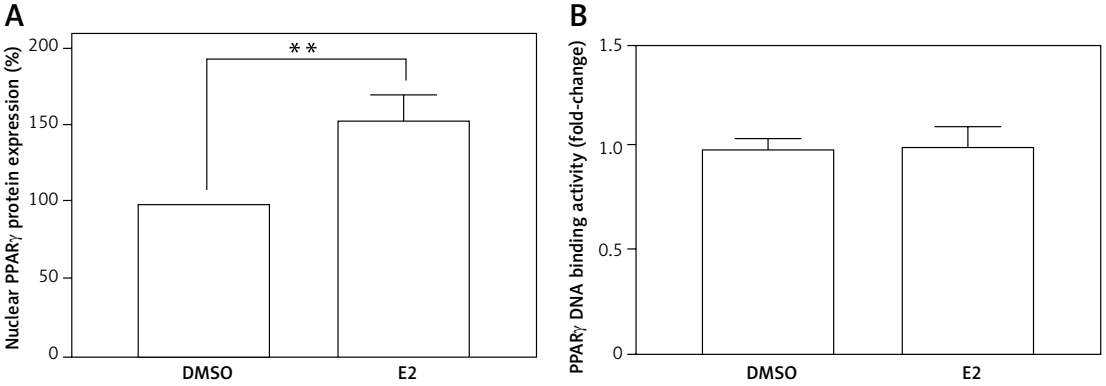

Figure 2. E2 augments nuclear expression of peroxisome-proliferator-activated-receptor-γ (PPARγ). Human coronary artery smooth muscle cells (HCASMC) were stimulated with E2 for 24 h. Nuclear protein was isolated and nuclear PPARγ protein expression was assessed by western blot analysis. 17β-oestradiol (E2) significantly increased nuclear PPARγ protein expression (**A**). DNA binding affinity of PPARγ was assessed by a colorimetric binding assay (**B**). Stimulation with E2 did not influence DNA binding affinity of PPARγ. n = 4; **p < 0.01, assessed by Student's two-sided t-test

DMSO – dimethyl sulfoxide.

of E2 on PPARγ did not depend on eNOS activity, because inhibition of eNOS with L-NNA did not significantly diminish PPARγ induction by E2 (1.62 ±0.62-fold; n = 3; $p_{vs.\ E2} > 0.05$).

E2 inhibits HCASMC proliferation in a PPARγ-dependent fashion

Because E2 is known to reduce murine experimental atherosclerosis in a PPARγ-dependent fashion, we studied whether E2 influences HCASMC cellular function by increasing PPARγ expression. Interestingly, E2 significantly reduced HCASMC proliferation as assessed by BrdU incorporation of proliferating cells. Following stimulation with E2, HCASMC proliferation was significantly diminished from 53.0 ±10.1% under control conditions to 30.8 ±2.4% under stimulatory conditions (n = 3–4; p = 0.0131; Figure 4). Inhibition of PPARγ by its selective antagonist GW9662 reversed the ef-

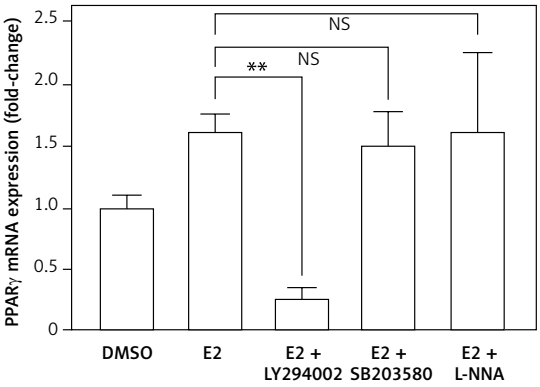

Figure 3. Upregulation of peroxisome-proliferator-activated-receptor-γ (PPARγ) by 17β-oestradiol (E2) occurs via phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) signalling. Human coronary artery smooth muscle cells (HCASMC) were stimulated with E2 for 24 h. Co-stimulation with LY294002 (inhibitor of PI3K/Akt), SB203580 (inhibitor of p38 mitogen-activated protein kinase (p38 MAPK)), L-NNA (L-N^G-nitroarginine, inhibitor of eNOS) revealed upregulation of PPARγ by E2 to depend on PI3K/Akt signalling. $n = 3$; $^{**}p < 0.01$, assessed by ANOVA and subsequent Bonferroni correction. Similarly, the effect of E2 on PPARγ did not depend on endothelial nitric oxide synthase (eNOS) activity, since inhibition of eNOS with L-NNA did not significantly diminish PPARγ induction by E2 (1.62 ±0.62-fold; $n = 3$; $p_{vs.\ E2} > 0.05$)

DMSO – dimethyl sulfoxide.

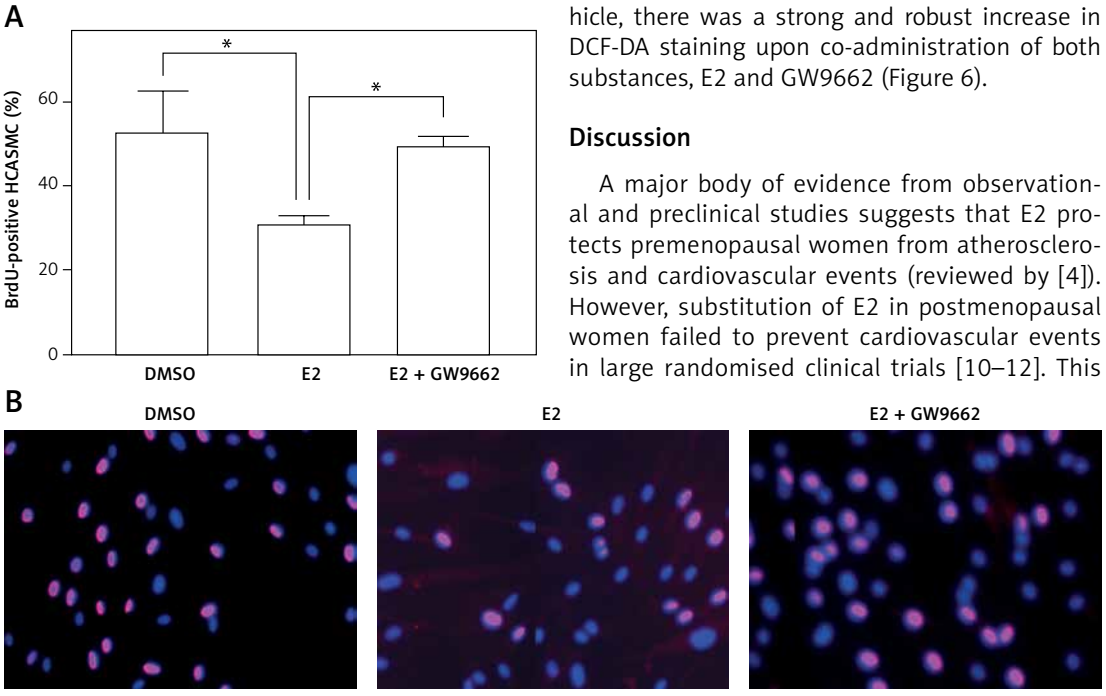

Figure 4. 17β-estradiol (E2) inhibits human coronary artery smooth muscle cells (HCASCM) proliferation via peroxisome-proliferator-activated-receptor-γ (PPARγ). HCASMC were stimulated with E2 or with E2 + PPARγ inhibitor 2-chloro-5-nitro-N-phenylbenzamide (GW9662) for 24 h. Replicating cells were tagged with 5-bromo-2'-deoxyuridine (BrdU). E2 significantly diminished the percentage of proliferating cells. Proliferation was restored by co-incubation with PPARγ inhibitor GW9662 (**A**). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), incorporated BrdU was visualised with the appropriate primary antibody and a cyanine 3 (Cy3)-conjugated secondary antibody (red; **B**). $n = 3–4$; $^{*}p < 0.05$, assessed by ANOVA and subsequent Bonferroni correction

DMSO – dimethyl sulfoxide.

fect of E2 on HCASMC proliferation (proliferation rate: 49.8 ±2.4%; $n = 4$; $p_{vs.\ E2} = 0.0250$; $p_{vs.\ control} > 0.05$; Figure 4).

E2 does not alter HCAMSC migration

The effect of E2 on HCASMC migration was assessed in an in vitro wound healing assay. Administration of E2 slightly reduced HCASMC migration, resulting in a tendency towards a more prominent gap in the cell layer after 6 h. However, this effect did not reach statistical significance (79.7 ±9.4 (E2) vs. 45.4 ±25.6 (Control); $n = 3$; $p > 0.05$; Figure 5). Antagonism of PPARγ by GW9662 tended to restore HCAMSC migration potential, again without reaching statistical significance (Figure 5).

PPARγ antagonism promotes ROS production in HCASMC

Finally, the impact of E2 and PPARγ antagonism on ROS formation was studied using the L-012 assay. While E2 failed to significantly reduce ROS production in HCASMC, co-administration of E2 and GW9662 yielded significantly increased ROS levels compared to stimulation with E2 alone (1036 ±169 RLU/s × cell vs. 561 ±99 RLU/s × cell; $n = 5–6$; $p = 0.0287$; Figure 6). This finding was corroborated by DCF-DA staining. While E2 only slightly reduced DCF-DA staining compared to vehicle, there was a strong and robust increase in DCF-DA staining upon co-administration of both substances, E2 and GW9662 (Figure 6).

Discussion

A major body of evidence from observational and preclinical studies suggests that E2 protects premenopausal women from atherosclerosis and cardiovascular events (reviewed by [4]). However, substitution of E2 in postmenopausal women failed to prevent cardiovascular events in large randomised clinical trials [10–12]. This

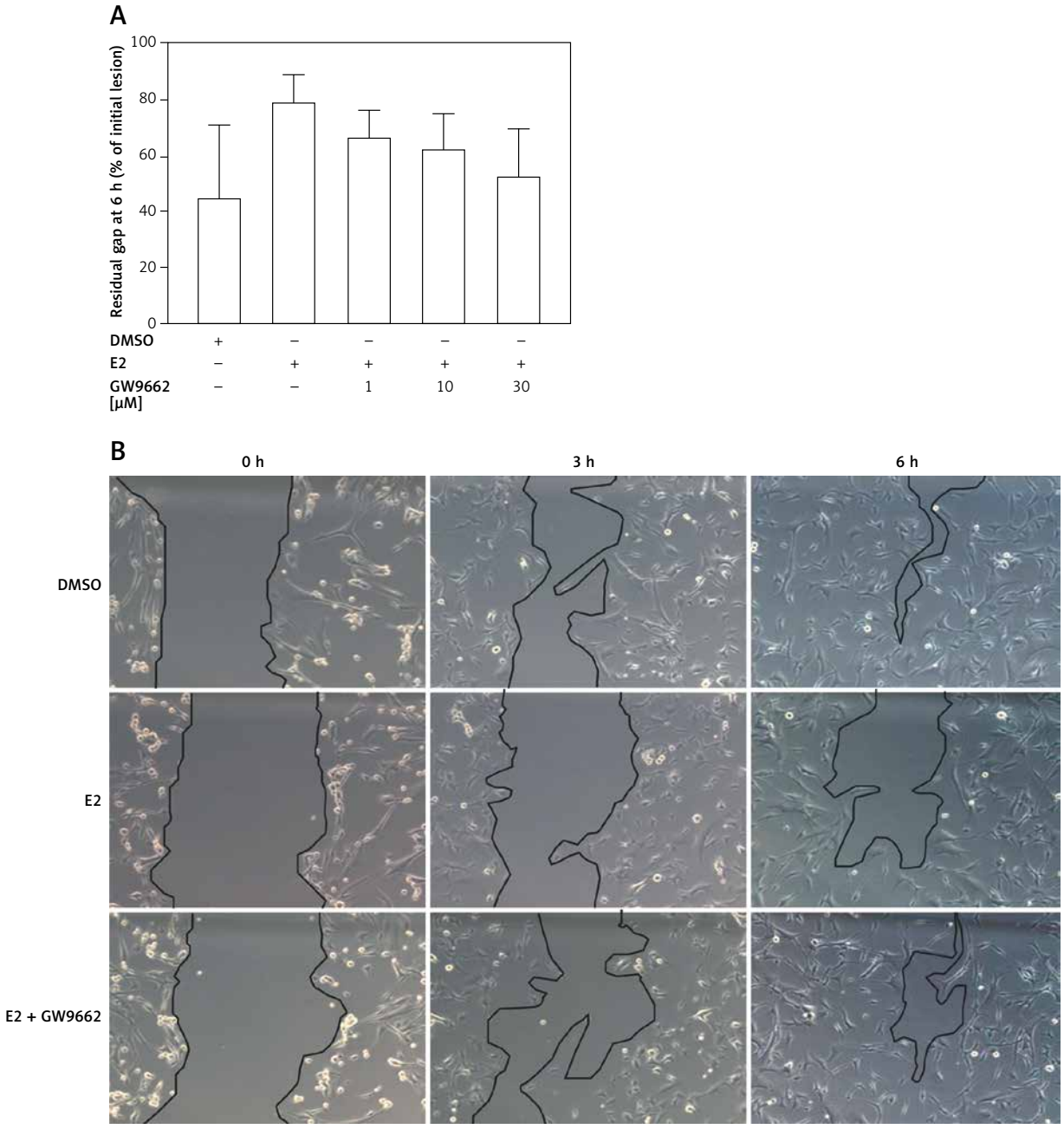

Figure 5. 17β-estradiol (E2) does not significantly alter human coronary artery smooth muscle cells (HCAMSC) migration. The HCASMC cell layer was injured with a small tip and cells were subsequently stimulated with E2 or with E2 + peroxisome-proliferator-activated-receptor-γ (PPARγ) inhibitor 2-chloro-5-nitro-N-phenylbenzamide (GW9662) (**A**). The width of the scratch was quantified at time points 0, 3, and 6 h (**B**). E2 tended to inhibit HCASMC migration and PPARγ inhibitor GW9662 tended to reverse the effect of E2. Neither effect reached statistical significance (A). $n = 3$; $p > 0.05$, assessed by ANOVA and subsequent Bonferroni correction

DMSO – dimethyl sulfoxide.

discrepancy between observational and preclinical trials on the one hand and randomised controlled clinical trials on the other hand illustrates the complexity of oestrogen signalling and underlines the necessity to identify druggable downstream targets of E2. The objective of the present work was to study whether PPARγ acts as a downstream target of E2 in HCASMC and to identify the responsible signalling pathways, as well as the subsequent implications for cellular function.

Our data demonstrate that both transcription and translation of PPARγ is induced by E2 in HCASMC. This finding corroborates the data from an earlier study by our group using ovariectomised mice with/without E2 replacement therapy; in this former approach, menopause was induced surgically by ovariectomy, which led to a decline in

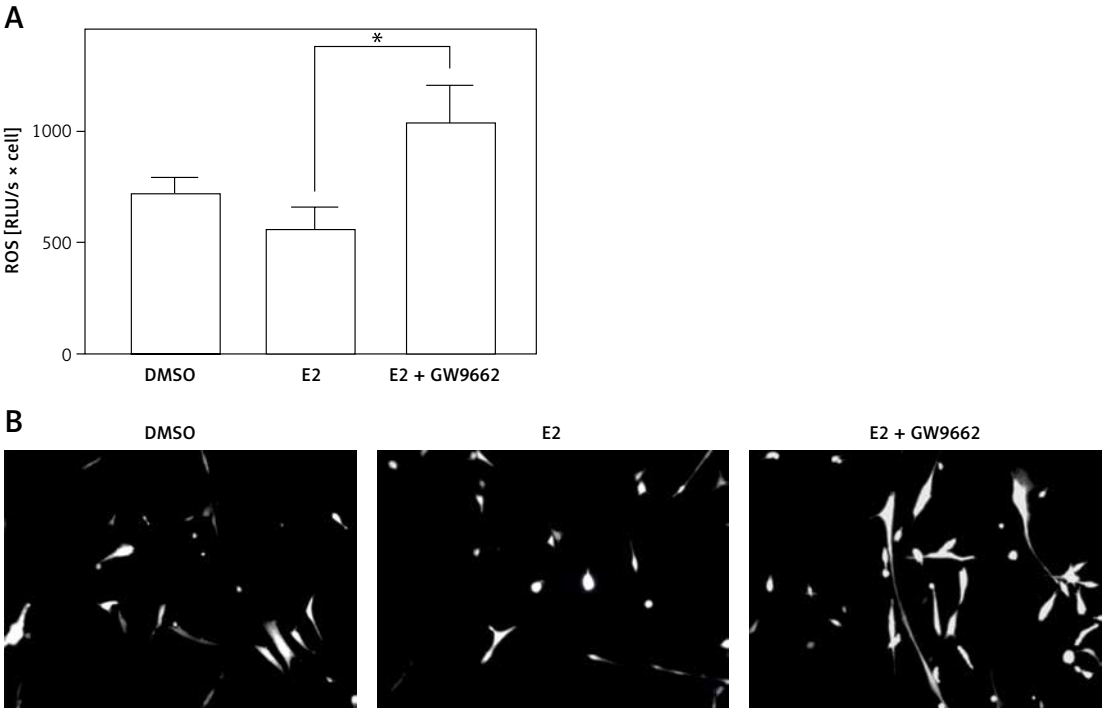

Figure 6. Peroxisome-proliferator-activated-receptor-γ (PPARγ) antagonism increases reactive oxygen species (ROS) production in human coronary artery smooth muscle cells (HCASMC). HCASMC were stimulated with 17β-estradiol (E2) or with E2 + PPARγ inhibitor 2-chloro-5-nitro-N-phenylbenzamide (GW9662) for 24 h. Reactive oxide species (ROS) were detected by L-012 luminescence (**A**) and 2′,7′-dichlorofluorescin diacetate (DCF-DA) staining (**B**). $n = 5–6$; $^*p < 0.05$, assessed by ANOVA and subsequent Bonferroni correction

DMSO – dimethyl sulfoxide, RLU – relative light units.

aortic PPARγ expression. This drop in aortic PPARγ expression could be prevented by E2 replacement after ovariectomy [15]. A few studies have already linked PPARγ- to ER signalling; crosstalk was reported for endometrial carcinoma cells [17] and in uterine leiomyoma [18]. PPARγ and ERα/β have also been demonstrated to co-dependently regulate endothelium-dependent vasorelaxation in rat aortas [19]. However, PPARγ has not been described as a downstream target of E2 signalling in vascular smooth muscle cells before.

Our current work extends the mechanistic understanding of the link between E2 and PPARγ signalling. Our data suggest that elevation of PPARγ by E2 depends on activation of ERα, given that PPARγ upregulation may be prevented by pharmacological ERα inhibition and may be mimicked by the selective ERα agonist PPT. Furthermore, we found that E2 not only increases global PPARγ expression in whole cell lysates but also that nuclear PPARγ expression is induced by E2, arguing for a functional relevance of PPARγ upregulation.

Using a small molecule inhibitor of PI3K/Akt signalling, we unravelled the significance of this signalling pathway to the E2-induced increase in PPARγ expression. PI3K and Akt are known downstream targets of non-genomic ERα signalling, i.e. of the plasma membrane-bound non-nuclear ERα receptor. Thus, non-genomic signalling by ERα might be responsible for the effects observed in this study.

Furthermore, the current study elucidates that E2 accomplishes its atheroprotective functions via PPARγ in HCASMC. Both E2 and PPARγ have independently been associated with favourable antiatherogenic effects on vascular smooth muscle cells [2, 14]. However, the present study is the first to demonstrate that E2 modulates HCASMC proliferation and ROS production via upregulation of PPARγ. These findings might open up a new therapeutic avenue for an antiatherosclerotic treatment with PPARγ agonists in postmenopausal women. PPARγ agonists such as thiazolidinediones are already commonly used to treat insulin resistance and metabolic syndrome because of their beneficial effects on glucose and fat metabolism [20]. Our data suggest that postmenopausal women, whose E2 levels decline, might profit from treatment with a PPARγ agonist to prevent vascular smooth muscle cell-mediated neointimal hyperplasia and consecutive cardiovascular events. More preclinical and clinical trials are warranted to evaluate the application of PPARγ agonists for the prevention of atherosclerosis and its clinical manifestations in postmenopausal women.

In conclusion, the present study sheds light on the link between E2 and PPARγ signalling. Our data identify PPARγ as a downstream target of

non-genomic ERα signalling in HCASMC. Beneficial antiatherogenic effects of E2 appear to depend on PPARγ. The clinical use of PPARγ agonists might broaden the therapeutic spectrum against atherosclerosis in postmenopausal women. The clinical benefit of PPARγ agonists in this patient group will have to be evaluated in future studies.

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Conflict of interest

The authors declare no conflict of interest.

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