Modulation of estrogen signaling in hepatic and vascular tissue

Krom, Y.D.

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Inhibition of Neointima Formation by Local Delivery of Estrogen Receptor Alpha and Beta Specific Agonists

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ABSTRACT

Objective: Neointima formation is the underlying mechanism of (in-stent) restenosis. 17β-estradiol (E_2) is known to inhibit injury-induced neointima formation and post-angioplasty restenosis. Estrogen receptor alpha (ERα) has been demonstrated to mediate E_2 anti-restenotic properties. However, the role of estrogen receptor beta (ERβ) is not fully elucidated. In the present study, the specific role of vascular ERα and ERβ in neointima formation was assessed.

Methods and results: Neointima formation was induced by placement of a perivascular cuff around the femoral artery of male C57BL/6 mice. E_2 drug-eluting cuff significantly inhibited cuff-induced neointima formation. To address the specific roles of vascular ERα and ERβ on neointima formation, the ERα- and ERβ-selective agonists 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT) and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) were applied via a drug-eluting cuff. The ERα-specific agonist, PPT, inhibited neointima formation at low but not at high concentrations. Conversely, the ERβ-specific ligand DPN inhibited cuff-induced neointima formation dose-dependently.

Conclusions: Our data demonstrate that, in addition to ERα, specific ERβ activation inhibits neointima formation in a mouse model of restenosis. These data reveal a yet unidentified protective role of ERβ in injury-induced neointima formation.

INTRODUCTION

17β-estradiol (E_2) has been shown to have anti-restenotic properties [1-4]. Nevertheless, the anti-restenotic mechanism of action of E_2 is not fully understood and controversial results regarding its effects on vascular remodelling have been reported [5-8]. This phenomenon may be attributed to the presence of two distinct estrogen receptors (ERs) in the vasculature, ERα and ERβ. ERs are ligand-activated transcription factors [9] and although ERα and ERβ are highly homologous, activation of either one of them may lead to distinct and even opposite biological activities [10-13]. So far, studies in ER knockout mice models have revealed a putative involvement of ERα in the protective effect of E_2 on restenosis [14-17]. Nonetheless, the role of ERβ in mediating the anti-restenotic properties of E_2 has not been fully elucidated.

Recently, ERα- and ERβ-specific agonists have been developed allowing the evaluation of the specific function of each receptor. The novel ERα-specific ligand 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT) is 410-fold more potent in binding to

ERα than to ERβ [18], whereas the 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) binds to ERβ with an 72-fold higher affinity compared to ERα [19]. Therefore, these compounds provide an attractive pharmacological approach to elucidate the biological role of ERβ on neointima formation.

A well-defined mouse model of neointima formation consists of placement of a non-constrictive perivascular cuff around the mouse femoral artery [20,21]. Previously, we showed that the non-constrictive perivascular cuff to induce neointima formation can be constructed from a polymeric formulation suitable for controlled drug delivery. This novel drug-eluting cuff simultaneously induces reproducible neointima formation and allows locally confined delivery of drugs to the cuffed vessel segment [22-25].

In the present study, we assessed the respective role of vascular ERα and ERβ in the anti-restenotic properties of E_2 in a mouse model of restenosis. By local delivery of PPT, an ERα-selective agonist, and DPN, a selective ERβ agonist, we demonstrated that in addition to ERα, ERβ activation leads to neointima formation inhibition in a murine model. These data reveal a yet unidentified protective role of ERβ in injury-induced neointima formation.

RESULTS

3.1. Local delivery of E_2 using E_2-eluting cuffs

3.1.1. E_2 in vitro release profiles

In vitro release profiles of drug-eluting cuffs loaded with 1% and 5% (w/w) E_2 was determined for a three weeks period. E_2 was released in a dose-dependent manner over the 21-day period for both concentrations used (1%: 30.9±14 μg; 5%: 211±14 μg).

3.1.2. Effect of perivascular delivery of E_2 on neointima formation

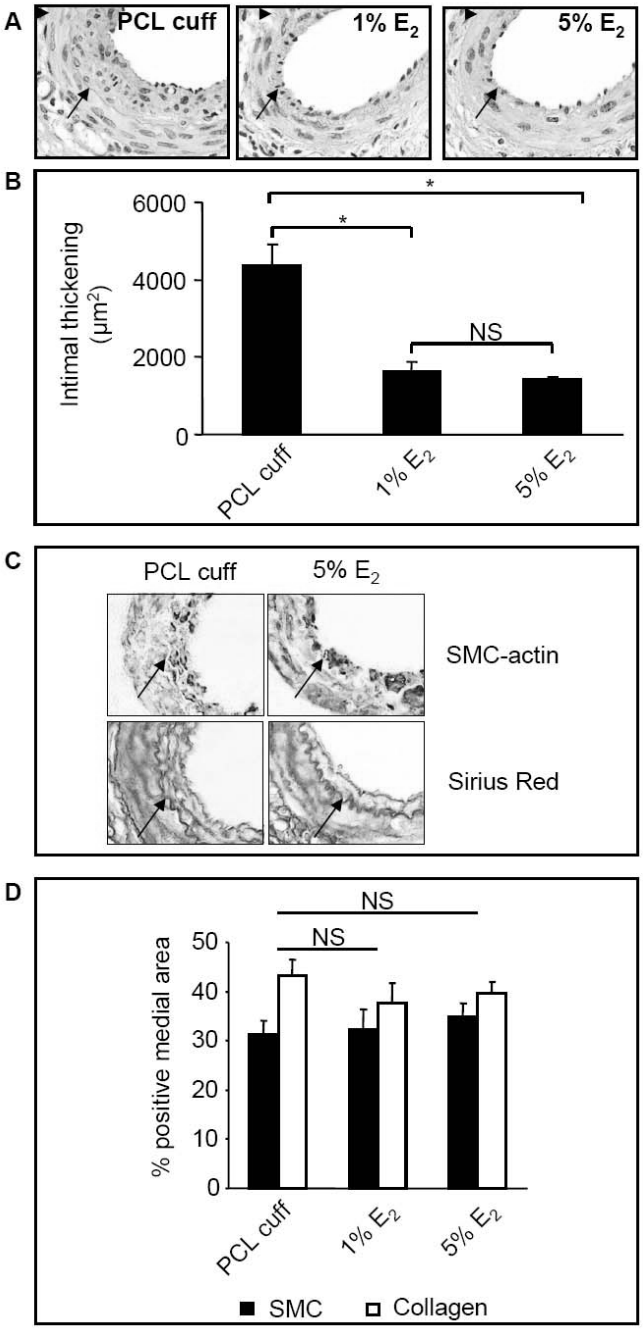

Figure 1.
A: Representative cross-sections of cuffed murine femoral arteries treated with increasing concentrations of E_2 21 days after cuff placement. HPS staining, magnification 400x (arrow indicates the internal elastic lamina; arrowhead indicates the external elastic lamina). B: Total intimal area of cuffed murine femoral arteries 21 days after E_2-eluting cuff placement. Total intimal area was quantified by image analysis using ten sections in each cuffed artery and expressed in μm^2 (mean±SEM, n=6). NS, $P>0.05$ (NS, not significant); *$P<0.05$. C: Representative micrographs of cuffed femoral arteries 21 days after placement of either a control empty cuff (Control cuff) or a 5% (w/w) E_2-eluting cuff (5% E_2). Alpha-SMC actin staining for SMC; similar α-SMC content is observed in both control- and E_2-treated cuffed segments. Sirius red stain for collagen; comparable collagen-positive area is present in both treated and untreated cuffed vessel segments. Magnification 400x (arrow indicates internal elastic lamina). D: Percentage of medial SMC- (close bars) and collagen-positive area (open bars) of cuffed femoral arteries treated with increasing concentrations of E_2 at 21 days after drug-eluting cuff placement. Medial SMC- and collagen-positive area was quantified by image analysis using six sections in each cuffed artery and expressed in μm^2. Mean ± SEM, n=6. NS, $P>0.05$ (NS, not significant).

To assess the effect of local perivascular E_2 delivery on cuff-induced neointima formation, drug-eluting cuffs were loaded with 1% and 5% E_2 and placed around the femoral artery of male C57BL/6 mice for a 21-day period. Microscopic analysis of the cuffed femoral artery

segments revealed that, after three weeks, a concentric neointima had been formed in mice receiving a control drug-eluting cuff. Animals receiving a 1% and 5% E_2-eluting cuff showed a strongly reduced cuff-induced neointima formation development (Fig. 1A). Morphometric analysis revealed a significant inhibition of cuff-induced neointima formation between mice receiving a control drug-eluting cuff and animals receiving an E_2-eluting cuff (Fig. 1B). Likewise, E_2 perivascular treatment resulted in a significant decrease in intima/media ratios for both E_2 loading dosages (Control: 0.43±0.07; 1%: 0.17±0.04, P=0.005; 5%: 0.18±0.02, P=0.003) as compared to control drug-eluting cuff. In addition, no toxic effects of local perivascular delivery of increasing E_2 concentrations on vascular integrity were found as determined by quantification of medial SMC and collagen content (Fig. 1C and 1D).

3.2. ERα and ERβ expression in cuffed femoral arteries

E_2 may exert its inhibiting effects on neointima formation via both vascular ERα and ERβ. As depicted in Fig. 2A, both ERs mRNA levels were upregulated time-dependently after the induction of the stenotic process. ERs mRNA levels showed a peak expression 7 days after cuff placement (59.5±3.9-fold increase for ERα vs. 11.4±4.2-fold increase for ERβ, both P<0.05) compared with control sham-operated arteries (T=0 days), after which the signal declined. In addition, immunohistochemical analyses showed that, both ER subtypes are present in murine femoral arteries (ERα: 19.2±0.5%; ERβ: 48.4±6.8%, Fig. 2B and 2C). Moreover, during the cuff-induced neointima formation process, cuffed femoral arteries expressed both ERα and ERβ also in intimal tissue (Fig. 2B and 2C). Altogether, ERβ is more abundantly present in vascular tissue as shown by immunohistochemistry analysis. On other hand, upregulation of ERα expression is more prominent upon vascular injury. Thus, both ERα and ERβ are present and have the potential to contribute to the anti-restenotic properties of E_2.

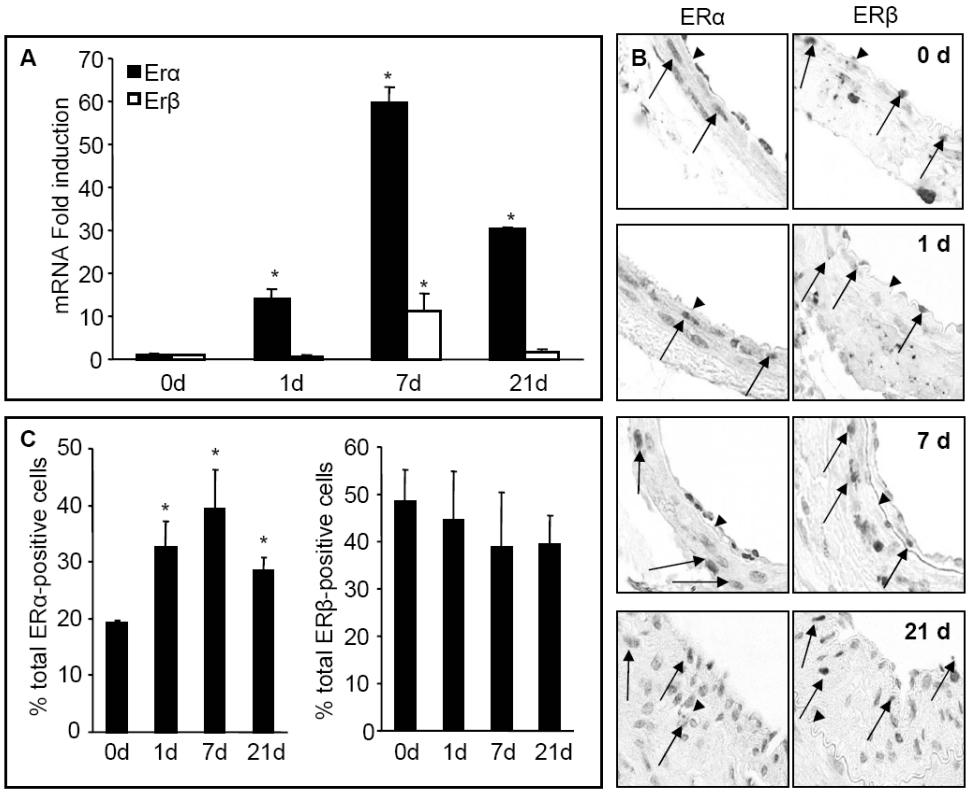

Figure 2. A: Fold induction of ERα and ERβ mRNA in cuff-induced neointima formation in time (mean±SEM, n=4; **P*<0.05 as compared to T=0 days). B: ERα and ERβ localization in cuffed murine femoral artery on diverse timepoints. Both ERα and ERβ were present on medial tissue and endothelial cell monolayer in intact arteries (0 days). During the process of neointima formation development (1, 7, and 21 days) ERs expression was also present in intimal tissue. Magnification 400x. Arrowhead indicates internal elastic lamina; arrow indicates ERs positive cells. C: Percentage of total ERα- and ERβ-positive cells of cuffed femoral arteries after cuff placement. ERα- and ERβ-labeled cells were counted in six equally spaced cross-sections from each cuffed artery and expressed as a percentage of the total number of cells (mean±SEM, n=6; **P*<0.05 as compared to T=0 days).

3.3. Local specific activation of ERα and ERβ in femoral arteries

3.3.1. PPT and DPN in vitro release profiles

To examine whether PPT and DPN were suitably loaded and released from our drug delivery device, the in vitro release profiles of 0.5%, 1%, 2.5% and 5% PPT- and 1% and 5% DPN-eluting cuffs were assessed. PPT showed a sustained and dose-dependent release for the 21-day period (0.5%: 16±0.4 μg; 1%: 36±2 μg; 2.5%: 68±1 μg; 5%: 160±6 μg). DPN was

also released from the drug-eluting cuffs in a dose-dependent manner over time. In total, 33±1 μg was released from the 1% and 83±3 μg from the 5% DPN-eluting cuffs, respectively.

3.3.2. Effect of PPT- and DPN-selective ER subtypes activation on neointima formation

To assess the role of ERα in the E_2-mediated inhibition of cuff-induced neointima formation, drug-eluting cuffs were loaded with 0.5%, 1%, 2.5%, and 5% PPT, a highly specific ERα ligand, and placed around the femoral artery of mice for three weeks. It should be noted that a broader concentration range of PPT was used as compared to E_2 and DPN. This was due to the seemingly contrasting data observed with the 1% and 5% PPT-eluting cuffs on neointima formation, as discussed below.

In animals receiving a control drug-eluting cuff a neointima had been formed. Remarkably, morphometric quantification revealed only a significant inhibition of cuff-induced neointima formation in the cuffed segments treated with the lowest PPT concentrations. Cuffed arteries locally treated with higher PPT concentrations (2.5 and 5%) did not show an inhibitory effect on neointima formation as compared with control cuffed arteries (Fig. 3A and 3C). Likewise, only intima/media ratios of the PPT-treated arteries with the lowest concentrations were significantly decreased (Control: 0.42±0.07; 0.5%: 0.13±0.01, $P<0.001$; 1%: 0.20±0.03, $P=0.008$; 2.5%: 0.34±0.05, $P=0.5$; 5%: 0.56±0.05, $P=0.2$) as compared to controls.

By placing a 1% and 5% (w/w) DPN-eluting cuff around the femoral artery of male C57BL/6 mice for 21 days, the role of ERβ on neointima formation was assessed. Morphometric analysis of the cuffed arteries of both DPN-treated groups showed a significant inhibition of neointima formation as compared to control cuffed segments (Fig. 3B and 3D). Moreover, intima/media ratios of the DPN-treated groups were also significantly decreased (Control: 0.42±0.07; 1%: 0.22±0.05, $P=0.02$; 5%: 0.15±0.03, $P=0.001$) as compared to controls.

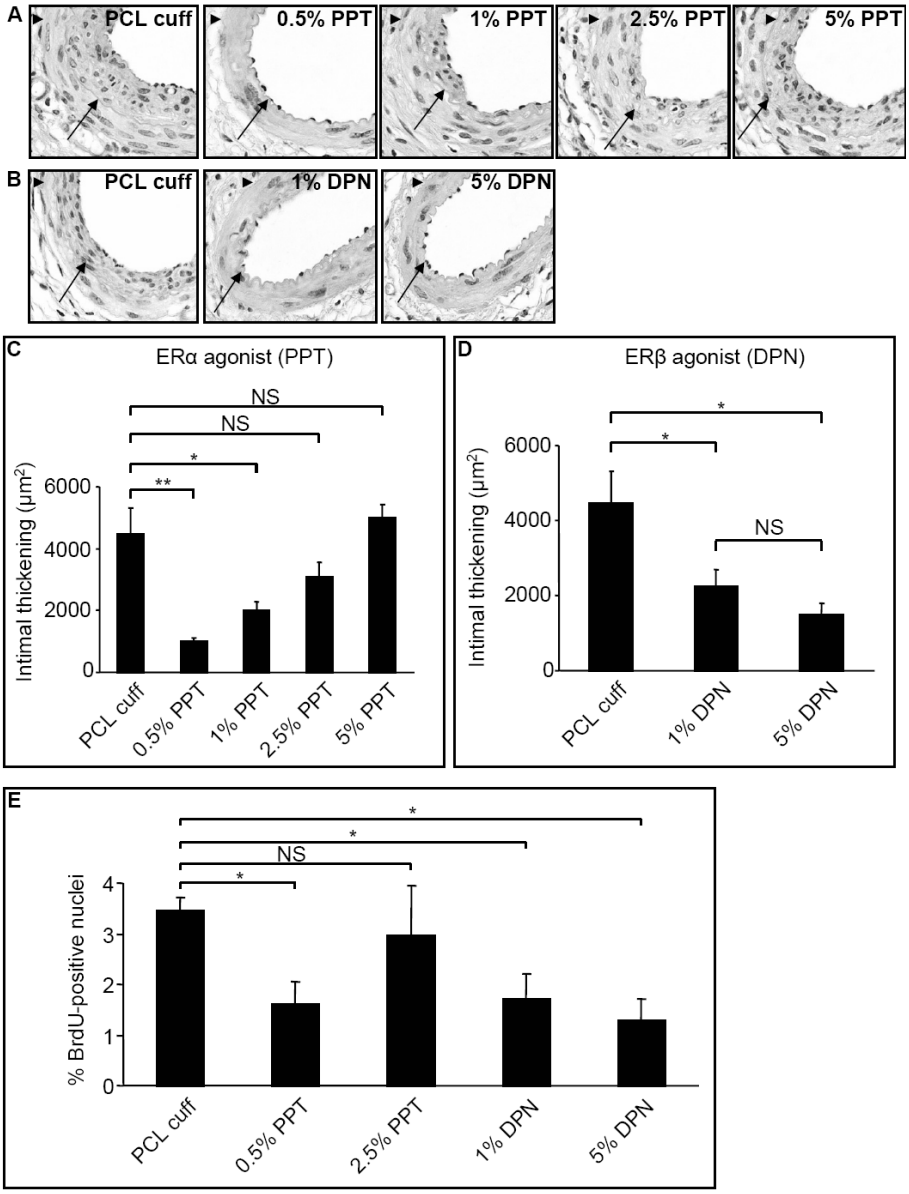

Figure 3. Representative cross-sections of cuffed murine femoral arteries treated with increasing (A) PPT and (B) DPN concentrations 21 days after cuff placement. HPS staining, magnification 400x (arrow indicates the internal elastic lamina; arrowhead indicates the external elastic lamina). Total intimal area of cuffed femoral arteries 21 days after (C) PPT- or (D) DPN-eluting cuff placement. Total intimal area was quantified by image analysis using ten sections in each cuffed artery and expressed in μm^2 (mean±SEM, n=6). NS, $P>0.05$ (NS, not significant); *$P<0.05$; **$P<0.01$. E: Percentage of BrdU-positive cells in cuffed femoral arteries treated with increasing concentrations of PPT (0.5 and 2.5%) and DPN (1 and 5%) 21 days after drug-eluting cuff placement. BrdU-labeled nuclei were counted in six equally spaced cross-sections from each cuffed artery and expressed as a percentage of the total number of nuclei. Mean±SEM, n=6. NS, $P>0.05$ (NS, not significant); *$P<0.05$.

To further investigate the apparent discrepancy on cuff-induced neointima formation between animals perivascularly treated with either an ERα or an ERβ specific agonist, DNA synthesis was evaluated. Cellular proliferation was assessed by examining incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA at 21 days after cuff placement in mice receiving either a control drug-eluting cuff, a PPT- (0.5% and 5%) or a DPN-eluting cuff (1% and 5%). As depicted in Fig. 3E, a profound incorporation of BrdU was observed 21 days after surgery in cuffed vessel segments receiving a control drug-eluting cuff (3.45±0.25%). In line with the morphometric analysis, only the 0.5% PPT-eluting cuff showed a decreased cellular proliferation but not the higher PPT dosage (0.5%: 1.62±0.43%, *P*=0.02; 2.5%: 2.95±1.01%, *P*=0.18). Conversely, cuffed artery segments of mice receiving either a 1% or a 5% DPN-eluting cuff showed a significantly decreased cellular proliferation as compared to control cuffed arteries (1%: 1.71±0.50%, *P*=0.016; 5%: 1.27±0.43%, *P*=0.016).

DISCUSSION

The present study evaluates the respective roles of vascular ERα and ERβ on neointima formation. Here we show, for the first time, the effects of specific ER subtype ligands on cuff-induced neointima formation in the mouse femoral artery. Local E_2 treatment resulted in a substantial and significant inhibition of cuff-induced neointima formation (Fig. 1). Surprisingly, mice receiving the PPT-eluting cuffs displayed a significant reduction on neointima formation only for the lower PPT concentrations (0.5%: 78±3%; 1%: 56±8%) but not for the 2.5% and 5% PPT-eluting cuffs. Conversely, perivascular delivery of DPN displayed an inhibitory effect on cuff-induced neointima formation at both low and high concentrations (1%: 50±10%; 5%: 67±7%) (Fig. 3).

E_2 has been shown to have vasoprotective properties. In rats, systemic E_2 therapy resulted in reduced vascular SMC proliferation and migration, which are fundamental steps in restenosis development [5]. In porcine coronary arteries, it has been shown that local delivery of E_2 decreases post-angioplasty restenosis due to endothelial function improvement [26-28]. Furthermore, the first short-term human pilot study using E_2-eluting stents showed low rates of restenosis [29].

E_2 mediates its effects primarily via interaction with its receptors ERα and/or ERβ. Recently, we have generated a drug-eluting polymer cuff which enables local delivery of compounds to the vasculature in an established mouse model of restenosis [22-25]. In the present study we demonstrate that both ER subtypes are expressed in cuffed femoral arteries

during the process of neointima formation (Fig 2). Therefore, both ER subtypes may be accountable for the inhibitory effect of E_2 on cuff-induced neointima formation.

Thus far, the specific role of vascular ER subtypes in the vascular wall is not fully elucidated. The current knowledge of the respective role of vascular ER subtypes derives almost exclusively from ERα- and ERβ-null mouse models [14-17]. Although ER-null mice provide interesting clues, they imply several shortcomings. Due to whole body ER deficiency, direct vascular effects of E_2 cannot be discriminated from systemic effects. In addition, potential compensatory mechanisms may have occurred during development. In the past, Pare and colleagues [17] have demonstrated very elegantly that in ERα-null mice E_2 does not have a protective effect on injury-induced vascular remodelling. However, although the concentration of E_2 used in these studies are optimal for activating ERα (mean circulating levels of 0.33 to 0.43 nmol/L) it might be suboptimal on activating the ERβ (Kd for ERα=0.2 nM and for ERβ=0.5 nM).

To dissect the contribution of ERα and ERβ in preventing neointima formation the ERα- and ERβ-selective agonists, PPT and DPN, were used. The selectivity of PPT and DPN for both receptor subtypes enables detailed analysis of the contribution of both ERs to the protective effects on neointima formation in the current experiments. PPT induces exclusively ERα mediated transcription and not ERβ (Kd for ERα=0.4 nM and for ERβ=417 nM) [18]. Thus, PPT can be stated as a highly selective ERα agonist. In vivo, we demonstrated that local release of PPT led to either anti-restenotic effects or no effect on restenosis, as low dosages inhibited neointima formation whereas high concentrations did not.

On the other hand, DPN displays ERβ specificity (Kd for ERα=80 nM and for ERβ=2.8 nM) [19]. Also in vivo DPN seems to act like a specific ERβ agonist. For example, systemic administration of the relatively high dose of 1 mg/kg/day DPN to rats does not alter uterine weight, which is regarded as a true ERα target tissue [30]. In the present study, both low and high concentrations of DPN led to an inhibition of neointima formation. Therefore we can state that, in this model, activation of ERβ seems to have a protective effect on injury-induced neointima formation.

The surprising finding that PPT does not seem to have a protective effect on cuff-induced neointima formation at higher dosages suggests a so called bell-shaped response curve, often seen when nuclear receptors are activated, might occur also in case of PPT-mediated activation of ERα. However, since E_2 activation does not show this response curve in our present studies and the Kd for the ERα for E_2 and PPT are similar (0.2 and 0.4 nM,

respectively) we do not believe this is the explanation for the observed phenomenon. Currently, the reason for the observed response curve for PPT is not fully understood.

In conclusion, while literature proposes ERα as the major receptor involved in the anti-restenotic and anti-atherosclerotic effects of E_2. Our data provide evidence for a yet unidentified protective role of ERβ in injury-induced neointima formation as well. Nevertheless, there seem to be complex and dose-dependent opposite roles for ERα and ERβ in vascular tissue.

METHODS

2.1. Drug-eluting cuffs and in vitro release profiles

E_2 was purchased from Sigma Diagnostics (St Louis, USA). PPT and DPN were obtained from Tocris Cookson Ltd. (Bristol, UK). Poly(ε-caprolactone)-based drug delivery cuffs were manufactured as previously described [22]. Drug-loaded cuffs were made from the different blended molten drug-polymer mixtures and designed to fit around the femoral artery of mice. Drug-eluting cuffs had the shape of a longitudinal cut cylinder with an internal diameter of 0.5 mm, an external diameter of 1.0 mm, a length of 2.0 mm and a weight of approximately 5.0 mg.

Drug-eluting cuffs were loaded with 1% and 5% (w/w) E_2, with 1% and 5% (w/w) DPN and with 0.5%, 1%, 2.5%, and 5% (w/w) PPT. In vitro release profiles (n=5/group) were performed by UV-VIS absorbance methods (225nm, 235nm and 257nm, respectively) as described elsewhere [22]. Calibration graphs of the different compounds were established by measuring the absorbance of a set of standards of each compound in the 0-50 μg/ml concentration range.

2.2. Femoral artery cuff mouse model

For experiments, 10-12 weeks old male C57BL/6 mice were used. Animals were fed a standard chow diet (R/M-H, Ssniff, Soest, Germany). At the time of surgery, mice were anaesthetized with an intraperitoneal injection of 5 mg/kg Dormicum (Roche, Basel, Switzerland), 0.5 mg/kg Dormitor (Orion, Helsinki, Finland) and 0.05 mg/kg Fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and loosely sheathed with a non-constrictive cuff [21,22]. Either a control empty cuff, an E_2-eluting cuff (1% and 5% w/w), a PPT-eluting cuff (0.5%, 1%, 2.5%, and 5% w/w), or a DPN-eluting cuff (1% and 5% w/w) was used (n=6/group).

All animal work was approved by TNO institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Quantification and histological assessment of intimal lesions in cuffed femoral arteries

Animals were sacrificed 21 days after cuff placement. Histological analyses were performed as described previously [21-23]. All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert´s elastin staining was used to visualize elastic laminae.

Smooth muscle cells (SMC) were visualized with α-SMC actin staining (1:800, Roche). Collagen content was determined using Sirius red stained sections. The amount of medial SMC and collagen content was determined by morphometry (Leica Qwin, Wetzlar, Germany) and expressed as the percentage of total medial area consisting of SMC actin- or Sirius red-positive area in six equally spaced serial cross-sections in all animals [23].

Incorporation of 5-bromo-2´-deoxyuridine (BrdU) into DNA as a marker of DNA synthesis was used to determine the rate of cell proliferation in cuffed vessel segments. Mice (n=6/group) were injected i.p. with 25 mg/kg BrdU (Sigma Diagnostics) three times at 72, 48, and 24 hours prior to sacrifice. Sections were incubated with a mouse monoclonal anti-BrdU antibody (1:50; DakoCytomation, Glostrup, Denmark). Specimens incubated with a mouse isotype-matched IgG diluted to the same concentration as the primary antibody were use as control. The number of BrdU-labeled nuclei per cuffed artery were counted in six equally spaced cross-sections and expressed as a percentage of the total number of nuclei.

2.4. Estrogen Receptors in femoral arteries

The presence of ERα and ERβ in cuffed vessel segments was visualized by immunohistochemistry using a rabbit and goat primary polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA) against the mouse ERα (1:600) and ERβ (1:100), respectively, according to the manufacturer's instructions.

Immunohistochemical analysis were performed in paraffin-embedded femoral artery segments at 0, 1, 7, and 21 days after cuff placement (n=6/timepoint). Specimens incubated with a mouse isotype-matched IgG diluted to the same concentration as the primary antibody was used as control. ERα- and ERβ-positive cells were counted in six equally spaced cross-sections in all mice and expressed as a percentage of the total number of cells.

2.5. Real time RT-PCR mRNA analysis

Mice underwent femoral artery cuff placement as described. Animals were sacrificed at different timepoints after surgery (0, 1, 7, and 21 days), 4 mice for each timepoint. Femoral arteries were isolated, harvested and snap frozen. Total RNA was isolated using the RNeasy Fibrous Tissue Mini-Kit (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol. Of all RNA samples cDNA was made using Ready-To-Go RT-PCR beads (Amersham Biosciences, Uppsala, Sweden).

Intron-spanning primers and TaqMan® probe were purchased from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, USA). HPRT (hypoxanthine phosphoribosyltransferase) was used as a housekeeping gene. For each timepoint RT-PCR was performed in duplicate. Per timepoint the signals were averaged and the average signal of the housekeeping gene HPRT was subtracted (ΔCt). ΔΔCt was determined as the difference between ΔCt values of the control sham-operated arteries (0 days) and the cuffed femoral arteries. Data are presented as fold induction (normalized to T=0 days), which was calculated as $2^{-\Delta\Delta Ct}$ [25].

2.6. Statistical analysis

Results are expressed as mean±SEM. Data were analyzed using the Mann-Whitney *U* test (SPSS 11.5). A value of $P<0.05$ was considered statistically significant.

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