Estrogen modulates TNF-α-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-β activation

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Xing D, Feng W, Miller AP, Weathington NM, Chen YF, Novak L, Blalock JE, Oparil S. Estrogen modulates TNF-α-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-β activation. Am J Physiol Heart Circ Physiol 292: H2607–H2612, 2007. First published January 19, 2007; doi:10.1152/ajpheart.01107.2006.—We have previously shown that 17β-estradiol (E2) attenuates responses to endoluminal injury of the rat carotid artery, at least in part, by decreasing inflammatory mediator expression and neutrophil infiltration into the injured vessel, with a major effect on the neutrophil-specific chemokine cytokine-induced neutrophil chemoattractant (CINC)-2β. Current studies tested the hypothesis that activated rat aortic smooth muscle cells (RASMCs) express these same inflammatory mediators and induce neutrophil migration in vitro and that E2 inhibits these processes by an estrogen receptor (ER)-dependent mechanism. Quiescent RASMCs treated with E2, the ERα-selective agonist propyl pyrazole triol (PPT), the ERβ-selective agonist diarylpropionitrile (DPN), or vehicle for 24 h were stimulated with tumor necrosis factor (TNF)-α and processed for real-time RT-PCR, ELISA, or chemotaxis assays 6 h later. TNF-α-stimulated and E2 attenuated mRNA expression of inflammatory mediators, including P-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, monocyte chemoattractant protein (MCP)-1, and CINC-2β. DPN dose dependently attenuated TNF-α-induced mRNA expression of CINC-2β, whereas PPT had no effect. The anti-inflammatory effects of DPN and E2 were blocked by the nonselective ER-inhibitor ICI-182,780. ELISA confirmed the TNF-α-induced increase and E2-induced inhibition of CINC-2β protein secretion. TNF-α treatment of RASMCs produced a twofold increase in neutrophil chemotactic activity of conditioned media; E2 and DPN treatment markedly inhibited this effect. E2 inhibits activated RASM proinflammatory mediator expression and neutrophil chemotactic activity through an ERβ-dependent mechanism.

inflammation; vascular smooth muscle cell; arteries

INFLAMMATION plays an important role in the pathogenesis of many forms of vascular disease, including atherosclerosis and the response to acute vascular injury (14, 37). Balloon injury of arteries has been shown to elicit accumulation of inflammatory cells, specifically granulocytes (neutrophils) and monocyte/macrophages, in the adventitia surrounding the injury site within hours after the insult (17, 28, 40). The appearance of inflammatory cells is predated by expression of inflammatory mediators, including adhesion molecules and chemokines, in acutely injured arteries (25) as well as in atherosclerotic and restenotic vessels (18, 22, 28), and is associated with activation of a variety of cell types, including adipocytes and fibroblasts, in adventitial tissues (20, 28). Since vascular smooth muscle cells (VSMCs) have been shown to produce adhesion molecules (3) and chemokines (34) when stimulated with cytokines and because our earlier studies (21) demonstrated that activation and migration of adventitial fibroblasts can be stimulated by media conditioned by VSMCs, we hypothesized that VSMCs are activated early following endoluminal injury, releasing inflammatory mediators that reach the perivascular space to recruit leukocytes and are the chief effector cells for initiation of the early inflammatory response.

Previous studies in our laboratory have characterized an early estrogen receptor (ER)-dependent anti-inflammatory effect for 17β-estradiol (E2) in this model of vascular injury (2, 25, 40). We have shown that systemic E2 administration attenuates both expression of inflammatory mediators and infiltration of leukocytes into balloon-injured carotid arteries of ovariectomized (OVX) rats at a very early time point postinjury (25, 40). In this model, we demonstrated a particularly robust effect for E2 in modulating neutrophil chemotaxis via attenuating expression of cytokine-induced neutrophil chemoattractant (CINC)-2β, a member of the cysteine-x-cysteine (CXC) chemokine family and a potent chemoattractant for neutrophils in vitro and in vivo (23, 24). Based on our hypothesis that VSMCs are the target cells that initiate the injury response, the present in vitro study was designed to define cellular and molecular mechanisms mediating estrogenic anti-inflammatory effects. We tested the estrogen receptor subtype (ERα or ERβ) dependence of the anti-inflammatory effect of E2 by employing two recently developed selective ERα and ERβ agonists. Based on published observations that J) tumor necrosis factor (TNF)-α acts as the body’s “fire alarm” (11) and induces the rapid recruitment of leukocytes from the circulation in response to many forms of stress, 2) it is elaborated from diseased bypass grafts and atherosclerotic arteries (8), and 3) our own finding that TNF-α expression is dramatically upregulated in balloon-injured rat carotid arteries and not affected by E2 administration (25), we used TNF-α as the inflammatory stimulus in the current study. Since our previous in vivo studies demonstrated robust estrogenic attenuation of injury-induced CINC-2β expression and of neutrophil infiltration into injured arteries (25, 40), we focused on neutrophil activation and chemotaxis in the current in vitro study.

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MATERIALS AND METHODS

Cell culture. Primary cultures of rat aortic smooth muscle cells (RASMCs) were derived from 10- to 14-day-old female Sprague-Dawley rats (Charles River), as previously described (31). Cells were cultured in complete medium containing phenol red-free DMEM (GIBCO) supplemented with 10% (vol/vol) FBS, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were identified as smooth muscle cells (SMCs) by their characteristic morphology and positive immunostaining for α-smooth muscle actin (α-SMA, clone 1A4, DAKO). All experiments were performed using early passage (>5) near-confluent (≈95%) cultures that were serum starved for 24 h before treatment.

Initial studies tested whether TNF-α could stimulate expression of relevant inflammatory chemokines and cytokines in RASMCs and whether pretreatment with E2 could inhibit these inflammatory responses. In these studies, cells were pretreated with E2 (10⁻⁷ M) or vehicle (ethanol at a final concentration <0.01%) for 24 h and then incubated with TNF-α (0.1–2.5 ng/ml) for an additional 6 h.

Subsequent studies assessed the ER subtype dependence of the E2 effect. Cells were pretreated with the selective ERα agonist diarylpropionitrile (DPN) (10⁻¹⁰–10⁻⁷ M) (Tocris Cookson, Ellisville, MO) or the selective ERα agonist propyl pyrazole triol (PPT) (10⁻¹⁰–10⁻⁶ M) (OBITER Research, Urbana, IL) for 24 h, and then incubated with 1 ng/ml TNF-α for an additional 6 h. Expression of the neutrophil-specific chemokine CINC-2β was assessed in these studies. To confirm ER dependence of the DPN and E2 effects, subgroups of cells from the above experiments were exposed to the nonselective ER antagonist ICI-182,780 (10⁻⁶ M) for 2 h before the E2 (10⁻⁷ M) or DPN (10⁻⁷ M) pretreatment. For CINC-2β ELISA and chemotaxis assays, cells were pretreated with E2 (10⁻⁷ M) or vehicle for 24 h, followed by TNF-α (1 ng/ml) treatment for an additional 6 h. Conditioned media were collected to assess CINC-2β protein concentration and ability to stimulate neutrophil migration.

Real-time quantitative RT-PCR analysis of inflammatory mediators. As described previously (25), total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAase I to remove genomic DNA, and then purified by using an RNA purification kit (Invitrogen). The protein- and DNA-free RNA was reverse transcribed to cDNA using the SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and specific primers (described in Ref. 25). cDNA was amplified by PCR in the iCycler for 40 cycles, and relative RNA levels were calculated using the iCycler software and a standard equation (Applied Biosystems). Unknowns were normalized using 18S rRNA and then standardized to the mRNA level of vehicle-treated RASMCs, except for CINC-2β, monocyte chemotactant protein (MCP-1), and P-selectin, which were standardized to the mRNA level of TNF-α-treated RASMCs because their mRNA was undetectable in the vehicle group.

Measurement of CINC-2β protein concentration. The CINC-2β concentration in conditioned media of vehicle control and TNF-α ± E2-treated cells was assayed with the rat GRO/CINC-2β ELISA kit (IBL, Japan). Briefly, conditioned media (4 ml) were collected and concentrated 10-fold using Centricon concentrators (Millipore, Billerica, MA). Samples were processed according to the manufacturer’s instructions. Samples were measured in duplicate, and the CINC-2β concentration was calculated from the standard curve.

Chemotaxis assays. Human myeloid leukemia HL-60 cells (ATCC, Manassas, VA) were maintained in Iscove’s modified medium (ATCC) supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, 2 U/ml penicillin, and 2 mM L-glutamine. For differentiation, cells (3 × 10⁶/ml) were incubated in the presence of 1.3% (vol/vol) DMSO for 4–6 days (Newberger PE) before assessment of their chemotactic responsiveness.

Neutrophil chemotactic activity was assayed in a 96-well modified Boyden chamber (Millipore, Billerica, MA) using differentiates HL-60 (dHL-60) cells. The bottom wells of the chamber were filled with 150 μl of conditioned medium of vehicle control or TNF-α ± E2, TNF-α ± DP neur, or TNF-α ± PPT-treated cells. To test the CINC-2 dependence of neutrophil chemoattractant activity of conditioned media from TNF-α-treated cells, anti-CINC-2α/β antibody (R&D System, Minneapolis, MN) at a final concentration of 5 μg/ml or PBS (control) was incubated with conditioned media for 30 min at room temperature; 150 μl of the conditioned media-antibody solution were added to the bottom wells of the chamber as described. A polyclonally pyrrolidone-free polycarbonate filter plate with 3-μm pores was placed over the samples, and 100 μl of the dHL-60 cell suspension (2 × 10⁶ cells/ml) were placed into the upper wells. The chambers were incubated in humidified air with 5% CO₂ at 37°C for 90 min. The upper portion was then removed, and four photomicrographs (200×) per well were digitally recorded using an Olympus IX70 microscope and Perkin-Elmer Ultraview image capture equipment. Cell counts were made from these images. For ease of comparison of results between experiments, data were standardized to a chemotactic index with cell migration to conditioned media of vehicle-treated RASMCs as a baseline (25).

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed with one-way ANOVA or Student’s t-test, as appropriate. Values of p < 0.05 were considered significant.

RESULTS

Real-time quantitative RT-PCR analysis showed that TNF-α (0.1–2.5 ng/ml) dose dependently stimulated expression of chemokines (CINC-2β and MCP-1) and adhesion molecules [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and P-selectin] (results for CINC-2β shown in Fig. 1). A TNF-α concentration of 1 ng/ml was chosen for further experiments because it was the minimum effective dose for all mediators. All mediators were expressed at low or undetectable levels in unstimulated vehicle-treated RASMCs (Fig. 2). Pretreatment with E2 (10⁻⁷ M) significantly inhibited expression of CINC-2β, MCP-1, ICAM-1, VCAM-1, and P-selectin in cells treated with TNF-α (1 ng/ml, Fig. 2). Inhibition of these chemokines and adhesion molecules expression was abolished by the nonselective ER

Fig. 1. Tumor necrosis factor (TNF-α) dose dependently increases mRNA expression of cytokine-induced neutrophil chemoattractant (CINC-2β) in rat aortic smooth muscle cells (RASMCs). Cells were grown to subconfluence (>95%) in six-well plates, deprived of serum for 24 h, and then treated with TNF-α (0.5–2.5 ng/ml) for an additional 6 h. Data, expressed as means ± SE, are from real-time quantitative RT-PCR analyses and are normalized by 18S RNA and then standardized to the mean mRNA level of the RASMCs treated with TNF-α at the 0.5 ng/ml dose. UD, undetectable; *p < 0.05 vs. vehicle-treated RASMCs.
antagonist ICI-182,780 (10^{-6} M), indicating ER dependence (Fig. 2).

The selective ER-\(\beta\) agonist DPN dose dependently inhibited TNF-\(\alpha\) (1 ng/ml)-induced expression of CINC-2\(\beta\) over the concentration range 10^{-10} to 10^{-7} M (Fig. 3). This effect was blocked by treatment with ICI-182,780 (10^{-6} M), confirming its ER dependence (Fig. 3). The selective ER-\(\alpha\) agonist PPT, over the dose range of 10^{-10}-10^{-7} M, had no effect on CINC-2\(\beta\) expression in TNF-\(\alpha\) (1 ng/ml)-treated cells (Fig. 4), suggesting that the anti-inflammatory effects of E2 in RASMCs are selectively mediated by ER\(\beta\) and not ER\(\alpha\).

CINC-2\(\beta\) protein concentration was quantified in conditioned media from RASMCs using a double sandwich ELISA technique (Fig. 5). E2 had no significant effect on CINC-2\(\beta\) protein concentration in conditioned media from vehicle-treated RASMCs. TNF-\(\alpha\) increased CINC-2\(\beta\) protein levels 2.7-fold compared with vehicle-treated control cells, and E2 pretreatment completely abolished the TNF-\(\alpha\)-induced increase in CINC-2\(\beta\) expression.

To assess the functional significance of the TNF-\(\alpha\) and E2- or DPN-induced alterations in CINC-2\(\beta\) expression, the ability of conditioned media from cells subjected to these treatments to stimulate migration of dHL-60 cells was assayed. Chemoattractant activity was normalized to conditioned media from vehicle-treated RASMCs as described in MATERIALS AND METHODS. There was no significant difference in the chemoattractant activity of conditioned media derived from vehicle-treated versus E2-, DPN-, and PPT-treated cells. TNF-\(\alpha\) treatment induced a marked (2.2-fold) increase in dHL-60 cell migration, and E2 pretreatment of RASMCs significantly attenuated the TNF-\(\alpha\)-induced effect on dHL-60 cell migration (by 48\%, \(P < 0.01\)) (Fig. 6). DPN, but not PPT, mimicked the inhibitory effect of E2 on TNF-\(\alpha\)-treatment-induced dHL-60 cell migra-
tation (by 69%, \( P < 0.01 \)). To test the CINC-2 dependence of neutrophil chemoattractant activity of conditioned media from TNF-\( \alpha \)-treated cells, conditioned media from separate groups of cells were preincubated with selective anti-CINC-2 antibody. Antibody treatment neutralized chemotactic activity of conditioned media by 63%, verifying its CINC-2 dependence.

**DISCUSSION**

This in vitro study demonstrates that isolated RASMCs express high levels of proinflammatory mediators, including the neutrophil- and monocyte-selective chemokines CINC-2\( \beta \) and MCP-1, when activated by TNF-\( \alpha \) and that estrogen inhibits this process via an ER\( \beta \)-dependent mechanism. Media conditioned by activated SMCs directed the migration of neutrophils, and this effect was partially inhibited by pretreatment with anti-CINC-2 antibody, indicating that the CINC-2\( \beta \) secreted into the conditioned media was functional. Treatment with an ER\( \beta \)-selective agonist dose dependently inhibited CINC-2\( \beta \) expression and reduced the chemotactic activity of media conditioned by TNF-\( \alpha \)-treated SMCs. Together, these data support the concept that arterial SMCs are important sources of proinflammatory mediators in the setting of acute vascular injury, triggering a robust inflammatory response that is amplified by recruitment of neutrophils and monocytes into the adventitial domain of the injured vessel and by activation and migration of adventitial fibroblasts into the neointima (25, 40).

The CXC chemokines are powerful mediators of neutrophil recruitment. A representative member of the CXC chemokine family is IL-8, which is a major chemoattractant for neutrophils in humans (29, 39). In rats, no homologue of IL-8 has been identified, and the CXC chemokines that recruit neutrophils are termed CINC (1, 26). Four CXC chemokines, including CINC (or CINC-1), CINC-2\( \alpha \), CINC-2\( \beta \), and MIP-2 (or CINC-3), have been identified in rats (10, 27, 32). CINC-2 has two splicing isoforms, CINC-2\( \alpha \) and CINC-2\( \beta \) (33), which differ in the three amino acids at the COOH terminus (27). Both CINC-2\( \alpha \) and CINC-2\( \beta \) are chemotactic for neutrophils, with an effect at a 10 \( \mu \)M concentration (80 ng/ml) (27). CINC-2 is structurally related to one another and share many functions. They have an ability to attract neutrophils and have effects on other neutrophil functions, including adhesion molecule expression, intracellular calcium influx, and phagocytosis (27, 32). Upregulation of CINC-2 has been correlated with neutrophil infiltration in many disease models, including acute right ventricular failure following pulmonary embolism (35) and ischemia-reperfusion injury of the liver (41). The present study reveals marked estrogenic modulation of CINC mRNA and protein expression in TNF-\( \alpha \)-stimulated RASMCs, consistent with our previous in vivo reports of attenuated CINC-2 expression and neutrophil infiltration in injured arteries with E2 treatment. To our knowledge, this report is the first in vitro study of CINC or CXCR2 ligand regulation by E2 in RASMCs.

The finding that antibodies to CINC-2 did not completely ablate the chemotactic activity of supernates from TNF-\( \alpha \)-treated RASMCs was not unexpected because there are undoubtedly other neutrophil chemoattractants in such supernates. These might induce leukotriene B\( \alpha \) (9) and the recently described tripeptide, PGP (38). It is, however, particularly interesting that about two-thirds of the chemotactic activity of supernates from TNF-\( \alpha \)-stimulated RASMCs is apparently due to CINC-2 and that a similar amount of chemotactic activity is inhibited by E2 (48%) or DPN (69%). This suggests that the effects of estrogen on neutrophil chemotaxis are likely due to the ability of estrogen to inhibit CINC-2 production.

In the current study, we employed the selective ER\( \beta \) agonist DPN and the selective ER\( \alpha \) agonist PPT to define the relevant ER subtypes involved in the anti-inflammatory effects of E2 on RASMCs. Because of its selective binding affinity, DPN is 30- to 70-fold more potent as an agonist of ER\( \beta \) than ER\( \alpha \) (4). Supporting its anti-inflammatory effects, DPN has been shown to suppress IL-6 promoter activity in human endothelial cancer (HEC-1) cells in vitro to the same extent as E2 (15). In a male rat model of trauma-hemorrhage, DPN, and not PPT, protected against lung injury, with associated reductions of inducible nitric oxide synthase and IL-6 expression (42), and restored cardiac function, with associated upregulation of heat shock

**Fig. 5.** E2 inhibits TNF-\( \alpha \)-induced CINC-2\( \beta \) protein expression in RASMCs. Cells were grown to subconfluence (≈95%) in six-well plates, deprived of serum for 24 h, pretreated with 10\(^{-7}\) M E2 or vehicle for 24 h, and then treated with TNF-\( \alpha \) (1 ng/ml) for an additional 6 h, and conditioned media were collected. Data, expressed as means ± SE, are from a double sandwich ELISA assay. *\( P < 0.05 \) vs. vehicle-treated RASMCs; #\( P < 0.05 \) vs. TNF-\( \alpha \)-treated RASMCs.

**Fig. 6.** Neutrophil chemotactic activity of conditioned media from TNF-\( \alpha \)-stimulated RASMCs is blocked by E2 and DPN. Cells were grown to subconfluence (≈95%) in 75-mm\(^2\) flasks, deprived of serum for 24 h, and pretreated with 10\(^{-7}\) M E2, 10\(^{-7}\) M DPN, 10\(^{-7}\) M PPT, or vehicle, respectively, for 24 h followed by TNF-\( \alpha \) (1 ng/ml) treatment for an additional 6 h. Conditioned media were collected. Selective anti-CINC-2 antibody (5 \( \mu \)g/ml) partially neutralized neutrophil chemotactic activity of conditioned media from TNF-\( \alpha \)-treated cells. Results are means ± SE normalized by vehicle-treated RASMCs. *\( P < 0.05 \) vs. vehicle-treated RASMCs; #\( P < 0.05 \) vs. TNF-\( \alpha \)-treated RASMCs.
proteins (43). A further study in this model demonstrated that DPN, in subcutaneous doses of 5 μg/kg administered during resuscitation, inhibited myeloperoxidase activity and ELISA-measured levels of CINC-1, CINC-3, and ICAM-1 in the lung, suggesting an important role in regulation of neutrophil infiltration after acute injury (44).

A related compound ERB-041 has 200-fold increased binding activity for ERβ over ERα and is orally active (16). ERB-041 in oral doses as low as 1 mg·kg⁻¹·day⁻¹ has been shown to reverse chronic diarrhea and ameliorate colonic lesions in a transgenic rat model of inflammatory bowel disease (HLA-B27) and shown to reduce joint inflammation in the Lewis rat adjuvant-arthritis model (16). In the latter model, mRNA expression profiling in the spleen, lymph nodes, and liver, and global analysis of the plasma proteome revealed disease-related alterations in a large number of genes and proteins related to immune responses that were completely or partially reversed by ERB-041 administration (12). When taken together, these findings support the current study and suggest the possibility that ERβ mediates the anti-inflammatory effects of estrogen in some tissues.

PPT is ~400 times more potent as an agonist for ERα than for ERβ (4). PPT in doses of 0.3–15 mg·kg⁻¹·day⁻¹ administered subcutaneously has been shown to evoke a number of physiologically relevant E2-induced tissue responses (uterine weight gain, prevention of OVX-induced body weight gain and loss of bone mineral density, reduction in plasma cholesterol levels, increases in brain progesterone receptor mRNA levels, and prevention of experimentally induced hot flushes) in rats (24). In the vasculature, PPT, and not DPN, has been shown to mediate estrogenic vasodilatory responses at physiological doses: Acute administration of PPT (10⁻¹³–10⁻⁷ M) to precontracted aortic rings from intact female rats dose dependently induced an ER-dependent vasodilatory response equal to that of E2, whereas DPN had no acute effect on vasoemotion (4).

Studies with knockout mice support important ERα-mediated protective effects on vascular injury (19, 30). Pare et al. (30) showed that complete ERα knockout mice (ERKO₅T) lost the protective effects of E2 in a wire injury model. In this study, the primary outcomes of change in medial area, proteoglycan deposition, and VSMC proliferation (by bromodeoxyuridine staining) were not altered by E2 administration in the ERKO₅T animals. In contrast, E2 did protect against wire injury-induced increases in vascular medial area and proliferation of VSMCs in ERβ knockout mice (18). ERα, but not ERβ, has also been shown to mediate the reendothelialization effect of E2 after electric carotid injury in mice with targeted disruptions of ER genes (6). As we have previously demonstrated, E2 restores endothelial cell function after denudation in the balloon injury model of the rat carotid artery as well (38), but this is a late event that occurs 2–4 wk postinjury (6).

In vitro studies support ER subtype and cell type-specific modulatory effects of E2 on vascular cell function (13). Selective ERβ and ERα mRNA anti-sense oligomers were used to examine the ER subtype dependence of E2-induced inhibition of PDGF-BB-induced p38 and p42/44 mitogen-activated protein kinase phosphorylation, migration, and proliferation in porcine SMCs, and endothelial cells. The inhibitory effects of E2 on porcine SMCs were abrogated by downregulation of ERβ protein expression, whereas downregulation of ERα had no effect. In contrast, downregulation of ERα expression in porcine aortic endothelial cells inhibited E2-induced p38 and p42/44 mitogen-activated protein kinase activation, whereas downregulation of ERβ had no effect. These observations are relevant to our in vivo model of balloon injury of the rat carotid artery, because the injured area is denuded of endothelium for several weeks and the early injury response is driven by activated SMCs and infiltrating leukocytes. Thus modulatory effects of ERβ activation on SMC-initiated inflammatory responses likely play an important role in inhibiting early inflammatory changes in the setting of endoluminal vascular injury.

Limitations of the current study include use of ER agonists to delineate ER subtype-specific effects in stimulated VSMCs. These agents, while the best available, are not pure ERβ or ERα subtype agonists. While we recognize that perturbation of ERβ and ERα signaling with pharmacological ER antagonists and/or siRNA strategies might theoretically provide superior approaches, no good selective ERβ and ERα antagonists are available, and small interfering RNA technology has not been successful in modulating E2 signaling in SMCs in our hands. We have not tested whether ERβ activation, as studied here, is sufficient to attenuate neointima formation in animals subjected to endoluminal injury in vivo. We are aware that other ERα-modulated processes, e.g., reendothelialization, contribute to the injury response at later time points. Further study is needed to elucidate the ER subtype-specific effects of E2 in modulating injury responses in arteries.

Taken together, our in vivo and in vitro results suggest that medial VSMCs are an important source for the proinflammatory mediators that trigger the response to acute endoluminal arterial injury. VSMCs of injured arteries are initial targets for the anti-inflammatory actions of E2. In VSMCs, ERβ appears to be the dominated ER subtype that contributes to the anti-inflammatory effects of E2.

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REFERENCES


