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Distinct Roles of Estrogen Receptor-α and β in the Modulation of Vascular Inducible Nitric-Oxide Synthase in Diabetes

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ABSTRACT

Estrogen is known to affect vascular function and diabetes development, but the relative contribution of estrogen receptor (ER) isoforms is unclear. The aim of this study was to determine how individual ER isoforms modulate inflammatory enzymes in the vascular wall of control and streptozotocin (STZ)-injected rodents. Primary cultures of rat aortic smooth muscle cells (SMCs) were stimulated with inflammatory agents in the presence or absence of increasing concentrations of the ERα and ERβ-selective agonists 4,4′,4′′-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT) and diarylpropionitrile (DPN), respectively. The production of inducible nitric-oxide synthase (iNOS), a classical indicator of vascular inflammation, was significantly reduced by PPT in control but not diabetic SMCs, whereas it was further enhanced by DPN treatment in both groups. This distinct action profile was not related to changes in ER transcriptional activity. However, extracellular signal-regulated kinase 1/2 signaling was activated by DPN but not by PPT in cytokine-treated SMCs. In cultured aortic rings from both normoglycemic and STZ-diabetic mice, pharmacological activation of ERα attenuated cytokine-driven iNOS induction by 30 to 50%. Vascular iNOS levels were decreased consistently when adding 1 nM 17β-estradiol to aortic tissues from ERβ- but not ERα-knockout mice. These findings suggest a possible role for ERα-selective ligands in reducing vascular inflammatory responses under normo- and hyperglycemic conditions.

Recent large-scale clinical trials found a significantly lower incidence of diabetes in postmenopausal women on hormone replacement therapy despite no improvement in vascular outcomes (Kanaya et al., 2003; Margolis et al., 2004). The mechanisms accounting for this outcome, however, are largely unknown, although estrogen is increasingly recognized as an important regulator of glucose homeostasis (Barros et al., 2006; Le May et al., 2006). Because the endogenous hormone binds to its receptors with identical affinity, the metabolic effects of individual estrogen receptor (ER) isoforms, ERα and ERβ, are hard to differentiate and appear to be tissue- and species-specific. In accordance, there is little information as to how ERα and ERβ affect the course and timeline of diabetic vascular dysfunction, which ultimately results in macrovascular complications of clinical relevance. We previously demonstrated that anti-inflammatory activity of estrogen is impaired in vascular smooth muscle cells (SMCs) from streptozotocin (STZ)-diabetic rats, which display ERβ overexpression with respect to normoglycemic controls (Maggi et al., 2003). The biological significance of ERβ overexpression in vascular cells from diabetic rats is unclear. There have been concerns that ERβ-selective agonists may be diabetogenic (Barros et al., 2006), whereas they are effective anti-inflammatory agents in selected in vivo models of inflammation (Harris, 2007).

Inducible nitric-oxide synthase (iNOS) is a well established marker of vascular inflammation (Bardell and MacLeod, 2001; Nagareddy et al., 2005). 17β-Estradiol (E_2), a nonselective ER agonist, regulates iNOS protein expression via ER (Binko and Majewski, 1998; Zancan et al., 1999). In double ER-null mice,

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ABBREVIATIONS: ER, estrogen receptor; SMC, smooth muscle cell; STZ, streptozotocin; iNOS, inducible nitric-oxide synthase; E_2, 17β-estradiol; PPT, 4,4′,4′′-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol; DPN, diarylpropionitrile; ERK, extracellular signal-regulated kinase; PD98059, 2′-amino-3′-methoxyflavone; KO, knockout; FCS, fetal calf serum; M199, medium 199; ANOVA, analysis of variance; MEK, mitogen-activated protein kinase kinase.

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iNOS expression in unstimulated aortic tissues is decreased as compared with wild-type mice (Liang et al., 2003). Data from our group indicate that moderately selective ERβ agonists induce the expression of inflammatory enzymes, including iNOS, in rat vascular SMCs (Cignarella et al., 2006). Conversely, there is evidence that ERα mediates protective anti-inflammatory effects in vascular (Darblade et al., 2002; Ardelt et al., 2005) and nonvascular tissues (Vegeto et al., 2003; Ghisletti et al., 2005), including pancreatic β-cells (Le May et al., 2006). ERα-selective, but not ERβ-selective, agonists improve vascular function by mediating vessel relaxation at concentrations where receptor selectivity is maintained (Bolego et al., 2005). Therefore, the role of ER isoforms in the modulation of vascular responses in settings of inflammation and diabetes is controversial. Based on this knowledge, we set out to assess the relative contribution of ER isoforms to vascular dysfunction in experimental insulin-deficient rodents using a comprehensive physiological and pharmacological approach.

Materials and Methods

Chemicals and Antibodies. E_2, lipopolysaccharide, and STZ were purchased from Sigma-Aldrich (St. Louis, MO); 4,4′,4′′-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT), diarylpropionitrile (DPN), and PD98059 were from Tocris Bioscience (Bristol, UK). The rabbit polyclonal anti-ERK antibody was from Santa Cruz Biotechnology, Inc., whereas the anti-iNOS antibody was from BD Biosciences (San Jose, CA). The peroxidase-coupled secondary antibody was obtained from Vector Laboratories (Burlingame, CA). Recombinant rat and mouse cytokines were obtained from Tebu-Bio (Magenta, Italy). Unless otherwise specified, chemicals were purchased from Merck (Darmstadt, Germany).

Animals. ERα- and ERβ-KO mice have a C57B6 genetic background. Generation of these animals was described previously (Dupont et al., 2000). Male mice were used in the present study. Male Sprague-Dawley rats weighing 200 to 250 g (Charles River Italia, Calco, Italy) were kept in temperature-controlled facilities on a 12-h light/dark cycle and fed normal chow. At the time of cell or tissue harvest, animals were sacrificed by asphyxia using carbon dioxide. All procedures conformed to the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Diabetes Induction. Diabetes was induced in 8-week-old wild-type or ER-KO mice littermates (n = 32–35) by a single intraperitoneal injection of 150 mg/kg STZ freshly dissolved in 50 mM citrate buffer, pH 4.5. In rats, STZ was given at 65 mg/kg i.p. Animals were diabetic for 3 weeks before aortic harvest and vascular cell isolation. Blood glucose was monitored after STZ injection using Glucurtest (Roche Diagnostics). Only animals with glycemia levels above 300 mg/dl on day 21 were used for experiments.

Cell Culture. SMCs obtained from aortic intimal-medial layers of Sprague-Dawley rats express ERs (Zancan et al., 1999; Maggi et al., 2003). These SMCs were isolated and cultured as described previously (Zancan et al., 1999; Maggi et al., 2003). In brief, SMCs at passage 6 were switched to phenol red-free M199 with 10% charcoal-stripped FCS for 2 days. Thereafter, they were synchronized in medium containing 0.4% FCS for 24 h and incubated for a further 24 h with a cytokine mixture comprising 10 ng/ml interleukin-1β, 10 ng/ml interferon-γ, and 25 ng/ml tumor necrosis factor-α plus 1 μg/ml lipopolysaccharide. Such a mixture consistently induces iNOS protein formation in SMCs (Zancan et al., 1999). ER agonists were added at the same time as cytokines.

Transient Transfections. For these experiments, 10^5 SMCs were seeded in 24-well plates in minimal essential medium 199 without phenol red supplemented with 500 μl/well 10% FCS and 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate. On the day of transfection, culture medium was replaced with Dulbecco's modified Eagle's medium without phenol red supplemented with 10% FCS, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, 0.2 g/l glucose, and 20 mM L-glutamine. Transient transfections were performed using the reporter plasmid ERE-TATAluc (0.8 μg), in which luciferase expression is controlled by an estrogen-responsive element (from Dr. M. J. Tsai, Baylor College of Medicine, Houston, TX), in the absence or presence of pCMV-ERα or pCMV-ERβ plasmids (0.02–0.2 μg) coding for human ERα or ERβ plasmids (from Dr. P. Chambon, Institut National de la Santé et de la Recherche Médicale, Illkirch, France). SMCs were transfected using the Lipofectamine reagents, according to the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany). The day after transfection, culture medium was replaced with 500 μl/well Dulbecco's modified Eagle's medium without phenol red supplemented with 1% FCS, and cells were incubated for 24 h before adding E_2. After 24 h, cells were rinsed once with PBS, and a protein extract was prepared and used to determine luciferase enzymatic activity as described previously (Ciana et al., 2001). Each experiment was performed on triplicate samples and repeated at least three times.

Tissue Culture. At sacrifice, the aorta was excised from the mice, carefully cleaned, transferred to ice-cold phosphate-buffered saline, and cut into 2-mm rings. Tissues from two mice were pooled and placed into a 30-mm culture dish containing phenol red-free M199 medium with 10% charcoal-stripped FCS for 2 days. At the end of this time, aortic tissues were stimulated with cytokines and treated with estrogenic compounds under conditions as described for cultured SMCs.

Western Blotting. Cultured SMCs or aortic tissues were washed twice with phosphate-buffered saline and extracted directly into lysis buffer as described elsewhere (Idel et al., 2002). Protein samples were pooled from at least six aortic rings isolated from three different animals. At least 30 μg of cell protein were loaded onto 10% SDS-acrylamide gels. At the end of the run, proteins were transferred onto Hybond-ECL membranes (GE Healthcare, Chalfont St. Giles, UK). The membranes were incubated in blocking solution for 2 h and then incubated with primary antibodies (1:1000) overnight at room temperature. After washing, the peroxidase-conjugated secondary antibody (1:1000) was applied for 1 h at room temperature. After extensive washing, the blots were developed using an enhanced chemiluminescence kit (GE Healthcare). Sample loading control was performed through β-actin immunodetection.

Nitrite Assay. SMC culture medium was collected and centrifuged at 12,000 rpm for 5 min. Next, 250 μl/well medium was treated with 20 μl of 6.5 M HCl and 20 μl of 37.5 mM sulfanilic acid in a 96-well plate. After incubation for 10 min, 20 μl of 12.5 mM *N*-(1-naphthyl)-ethylenediamine was added. Optical density was read at 550 nm after 15 min. Nitrite values were expressed as micromoles of nitrite per milligram of cell protein.

Statistical Analysis. All data are presented as mean ± S.E. and represent unpaired data. Cell culture data were obtained from at least four independent experiments, each value representing mean ± S.E. of duplicate or triplicate determinations. Statistical evaluation was performed using unpaired Student's t test or one-way ANOVA with Fisher analysis when more than two groups were compared. Values of $P < 0.05$ were considered significant.

Results

Differential iNOS Modulation by ER-Selective Agonists in Cultured SMCs. With the aim to discern the role of individual ER isoforms in vascular inflammatory responses in control or hyperglycemic conditions, we used the isoform-selective ER agonists PPT and DPN (Bolego et al., 2006) at concentrations known to selectively activate ERα and ERβ, respectively (Bolego et al., 2005, 2006; Harris, 2007). These compounds were first analyzed in primary culture of SMCs prepared from control or STZ-diabetic rat aortas and tested

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for the ability to regulate the expression of iNOS, an established marker of vascular inflammation. Treatment with the ERα-selective agonist PPT significantly reduced cytokine-driven iNOS protein induction in aortic SMCs from control rats at 0.1 and 1 μM (Fig. 1A), the maximal effect being a 30% reduction in iNOS levels. The negative regulation of iNOS expression after PPT incubation was not observed in aortic SMCs from STZ-diabetic rats (Fig. 1B). Consistent with this finding, PPT at the highest concentration tested reduced specific iNOS-mediated nitrite accumulation (Maggi et al., 2003) in the culture medium of SMCs from normoglycemic but not from STZ-diabetic rats (Fig. 1, C and D). These results are in agreement with previous data showing that E_2 blocks iNOS induction in aortic SMCs from control but not in those from STZ-diabetic rats, where nitrite production is reduced at baseline (Maggi et al., 2003). By contrast, the ERβ-selective agonist DPN raised iNOS protein levels in both cytokine-treated control and diabetic SMCs (Fig. 2, A and B). This effect was concentration-dependent but was not associated with higher nitrite levels recovered in the culture medium (Fig. 2, C and D). PPT and DPN in the absence of cytomix did not induce either iNOS synthesis or NO release in cultured SMCs (data not shown). These results demonstrate that ERα- and ERβ-selective ligands differentially regulate iNOS production in vascular SMCs.

ER Signaling in Cultured SMCs. To explore whether the distinct action mediated by ER isoforms in diabetic SMCs could be ascribed to altered transcriptional activity, we transfected SMCs from control and diabetic animals with a reporter plasmid carrying the luciferase gene under the control of an estrogen-responsive promoter. E_2 activity could not be detected in SMCs transfected with the reporter plasmid alone (Fig. 3), suggesting that expression levels of endogenous ER isoforms might not be sufficient to induce detectable levels of reporter protein. Thus, we separately transfected either ER isoform and observed that both were transcriptionally active in a similar dose-dependent manner in SMCs from healthy and diseased animals, as shown in Fig. 3. It is interesting that the basal, unstimulated transcription activity of the two isoforms was higher in diabetic than in control SMCs. The experiment was repeated using different reporter/receptor ratios with superimposable results (data not shown). Thus, we conclude that diabetes did not impair ER transcriptional activity in SMCs. Therefore, we investigated whether ER isoforms could differentially affect the phosphorylation state of ERK1/2, an important component of cytokine recep-

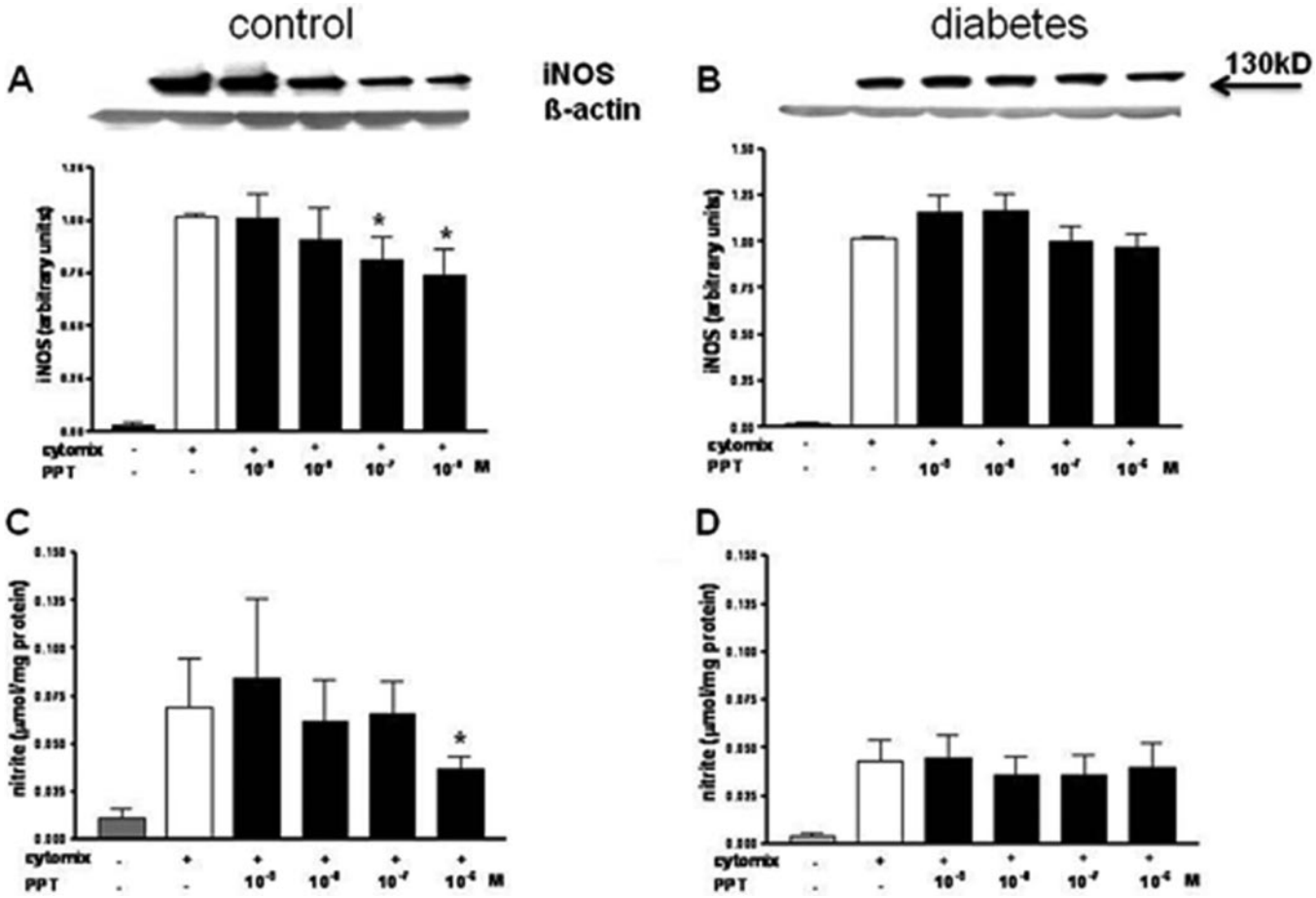

Fig. 1. Effects of the ERα selective agonist PPT on iNOS expression in aortic SMCs. Cells were prepared from control (A and C) and STZ-diabetic (B and D) rats and then challenged with a cytokine mix for 24 h in the presence of vehicle or increasing concentrations of PPT. Cell lysates were tested for iNOS protein levels by Western blot (A and B). Graphs represent the scanning densitometry analysis whereby the intensity of the cytomix band was set arbitrarily to 1 (representative Western blots are shown). β-Actin was used as an internal control for sample loading. The culture media were harvested, and samples thereof were assayed for nitrite accumulation using the Griess reaction (C and D). Data are expressed as mean ± S.E.M. of eight to 10 independent experiments. *, $P < 0.05$ compared with cytokines alone, ANOVA.

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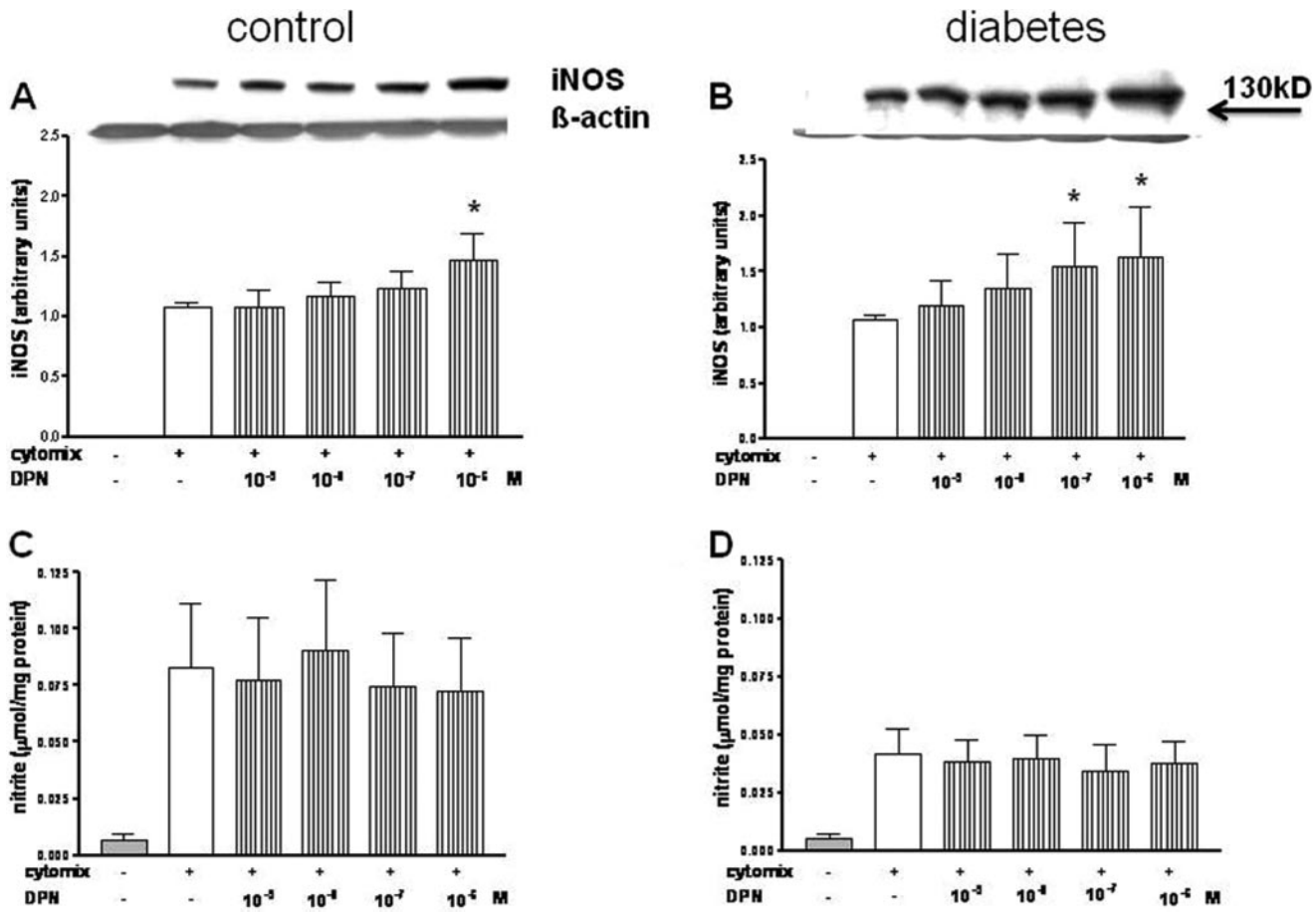

Fig. 2. Effects of the ERβ-selective agonist DPN on iNOS expression in aortic SMCs. Cells were prepared from control (A and C) and STZ-diabetic (B and D) rats and then challenged with a cytokine mix in the presence of vehicle or increasing concentrations of DPN. Western blot analysis and high-output NO release assays were performed as described in the legend to Fig. 1. Data are expressed as mean ± S.E.M. of eight to 10 independent experiments. ∗, $P < 0.05$ compared with cytokines alone, ANOVA.

tor intracellular signaling linked to vascular inflammation (Siow et al., 2007). Whereas PPT had no effect on this pathway under the same conditions as those showing iNOS inhibition (Fig. 4, A and B), a significant increase in the amount of phosphorylated ERK1/2 was observed in control SMCs after DPN treatment ($P < 0.05$; Fig. 4C), and this effect was abolished by the specific MEK-1 inhibitor PD98059. On the other hand, ERK activation was not enhanced by DPN in SMCs from STZ-diabetic rats (Fig. 4D), possibly because of overactivation of ERK1/2 as compared with the nondiabetic state. Total ERK1/2 levels were unchanged in these experiments (data not shown). These results suggest that ERβ activation is linked to the induction of the specific signaling pathway in SMCs.

Pharmacological Activation of ER Isoforms and iNOS Expression in Aortic Rings from Wild-Type Mice. We next determined to what extent selective ER activation affected cytokine-driven iNOS production in a model system more reminiscent of the physiological setting, namely tissue cultures of aortic sections freshly isolated from mice 3 weeks after injection of STZ or vehicle. Tissues were incubated with a mixture of inflammatory cytokines in the presence or absence of ER agonists (Kauser et al., 1998). The iNOS protein was not detectable by Western analysis in untreated aortic rings (data not shown). Consistent with the above isolated SMC findings, coadministration of 1 nM E_2 and cytokines significantly reduced iNOS levels in tissues from normoglycemic mice stimulated with cytokines ($n = 5$; Fig. 5A). The ERα-selective agonist PPT essentially mimicked E_2 action, whereas treatment with the ERβ-selective agonist DPN tended to increase arterial iNOS expression, although this effect did not reach statistical significance ($n = 4$; Fig. 5A). It is interesting that this regulatory profile was retained in aortic rings from STZ-diabetic mice ($n = 4$; Fig. 5B), where E_2 and PPT reduced iNOS induction, whereas ERβ activation again resulted in a trend to increasing iNOS production ($n = 4$, Fig. 5B). These data support the relevance of ER-selective agents in the pharmacological control of vascular iNOS under both physiologic and hyperglycemic conditions.

Physiological Relevance of ER Isoforms on Glycemic Control and Vascular iNOS Expression in Control and STZ-Injected Mice. To conclusively demonstrate that ERα and ERβ mediate distinct actions in the aorta, we used ERα or ERβ gene-deleted (KO) mice (Dupont et al., 2000) and assessed the effect of E_2 on vascular iNOS levels in aortic tissues normoglycemic and STZ-diabetic mice. Preliminary characterization of the effects of ER gene ablation on glucose homeostasis was performed by measuring baseline fasting glucose levels. In fasted animals, circulating glucose levels were significantly higher in ERα-KO than in wild-type or ERβ-KO mice (Fig. 6A), in agreement with previous studies (Le May et al., 2006). Despite this difference at baseline,

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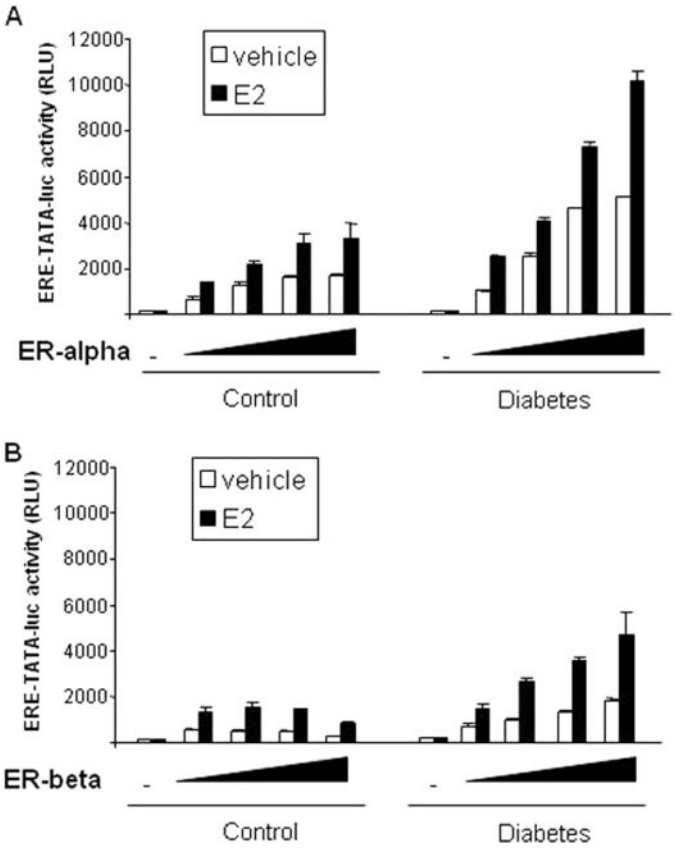

Fig. 3. Transcriptional activity of ER isoforms in aortic SMCs. Cells were transfected with increasing concentrations of ERα and ERβ and treated with 1 nM E_2 for 24 h. The ERE-TATA-luc activity was measured as relative luciferase units (RLUs). Data are expressed as mean ± S.E.M. of three independent experiments performed in triplicate. All E_2 samples were significantly different from vehicle samples ($P < 0.05$); all diabetes samples were significantly different from the corresponding control samples ($P < 0.05$) except nontransected samples (N.S.).

glycemic levels after STZ injection tended to be higher in ERα-KO and ERβ-KO as compared with wild-type mice (Fig. 6B), yet blood glucose did not differ significantly among groups after 3 weeks at sacrifice. We also noticed ERβ deletion predisposed to STZ toxicity as shown by the lower cumulative survival rate compared with ERα-KO and wild-type mice (data not shown).

We next prepared aortic ring cultures from these mice groups and evaluated iNOS production after cytokine treatment in the absence or presence of 10^{-9} M E_2. In healthy animals lacking ERα, E_2 increased cytokine-driven iNOS protein accumulation (Fig. 7A), whereas it significantly reduced iNOS protein content in ERβ-KO mice ($n = 3–4$; Fig. 7C). Consistent with ER-selective agonist data, these results indicate that ERα mediates the inhibitory activity of E_2 on inflammatory markers in the intact vessel wall, whereas ERβ is associated with opposite effects. It is interesting that in STZ-treated ERβ-KO mice, E_2 treatment still resulted in a significant reduction in iNOS accumulation ($n = 3–5$; Fig. 7D), demonstrating a prominent role of ERα in vascular inflammation also in insulin-deficient mice. E_2 unexpectedly did not modify iNOS induction upon ERα ablation (Fig. 7B), showing that selective activation of ERβ by E_2 did not correlate with increased iNOS stimulation. Based on these findings, we conclude that ERα, but not ERβ, is involved in the protective effects of E_2 on the inflammatory response of the vessel wall in normoglycemic and STZ-diabetic rodents.

Discussion

In the present study, combined application of specific pharmacologic and genetic tools provides novel insights into beneficial effects of ERα-selective agonists in specific aspects of vascular inflammation associated with diabetes. The first finding of our study is the direct demonstration that ERα reduces, whereas ERβ increases, cytokine-driven iNOS induction in vascular cells and, most importantly, that ERα anti-inflammatory activity is retained in the arterial wall of diabetic mice. This leads to the second relevant observation of our study that ERα-selective agonists inhibit specific aspects of vascular inflammation under both control and diabetic conditions. Because this effect was observed in whole aortic rings but not in isolated SMCs from diabetic animals, ERα activation requires the more physiological yet complex context of the vascular tissue, comprising the endothelium, SMCs, and adventitial cells, to blunt inflammatory enzyme action. Of note, the transcriptional machinery of both ER isoforms in response to E_2 was not impaired in SMCs from diabetic rats (Fig. 3). In line with our findings, previous studies showed the selective involvement of ERα in glucose uptake in the skeletal muscle and pancreas (Barros et al., 2006; Le May et al., 2006) and in reducing iNOS expression in endothelium-denuded aortic rings (Zhu et al., 2002). Whether ERα function is affected by macrovascular complications developing at more advanced stages of diabetes remains to be investigated. As to the specific involvement of ERβ activation, our study provides indication but no clear-cut evidence for a proinflammatory action in the arterial wall. For instance, DPN effects on cytokine-induced iNOS levels in mouse aorta only approached statistical significance; similarly, E_2 did not affect cytokine activity in aortic rings from STZ-injected ERα-KO mice (Fig. 7B). These data suggests that the mechanism of action of ERβ in vivo, particularly in diabetes, is more complex and needs further investigation. It should be noted that ERs may also control NO-related pathways by modulating either the expression of iNOS monomer, which is without enzymatic activity, or the production of enzymes other than iNOS. For instance, arginase is increased in diabetic blood vessels, and the deficit in NO is normalized by adding an arginase inhibitor (Romero et al., 2008). Because estrogens positively regulate arginase expression and activity in nonvascular tissues (Traish et al., 2003), some of our results on NO production could be interpreted by differences in ERα- versus ERβ-mediated differences in arginase expression.

A further contribution of this work is the partial definition of isoform-specific mechanisms that appear to be affected differentially and distinctly in vascular cells. Our present observation that ERβ, but not ERα, affected the phosphorylation state of ERK1/2, at least in nondiabetic SMCs, suggests that the signaling of the two receptor isoforms is diversified, leading to distinct profiles of intracellular actions in vascular wall cells (Fig. 8). In fact, induction of the ERK1/2 pathway may lead to transcriptional activation of target genes such as iNOS (Siow et al., 2007). Although the link between ER control of iNOS induction and the mitogen-activated protein kinase pathway is not definitely proven, the

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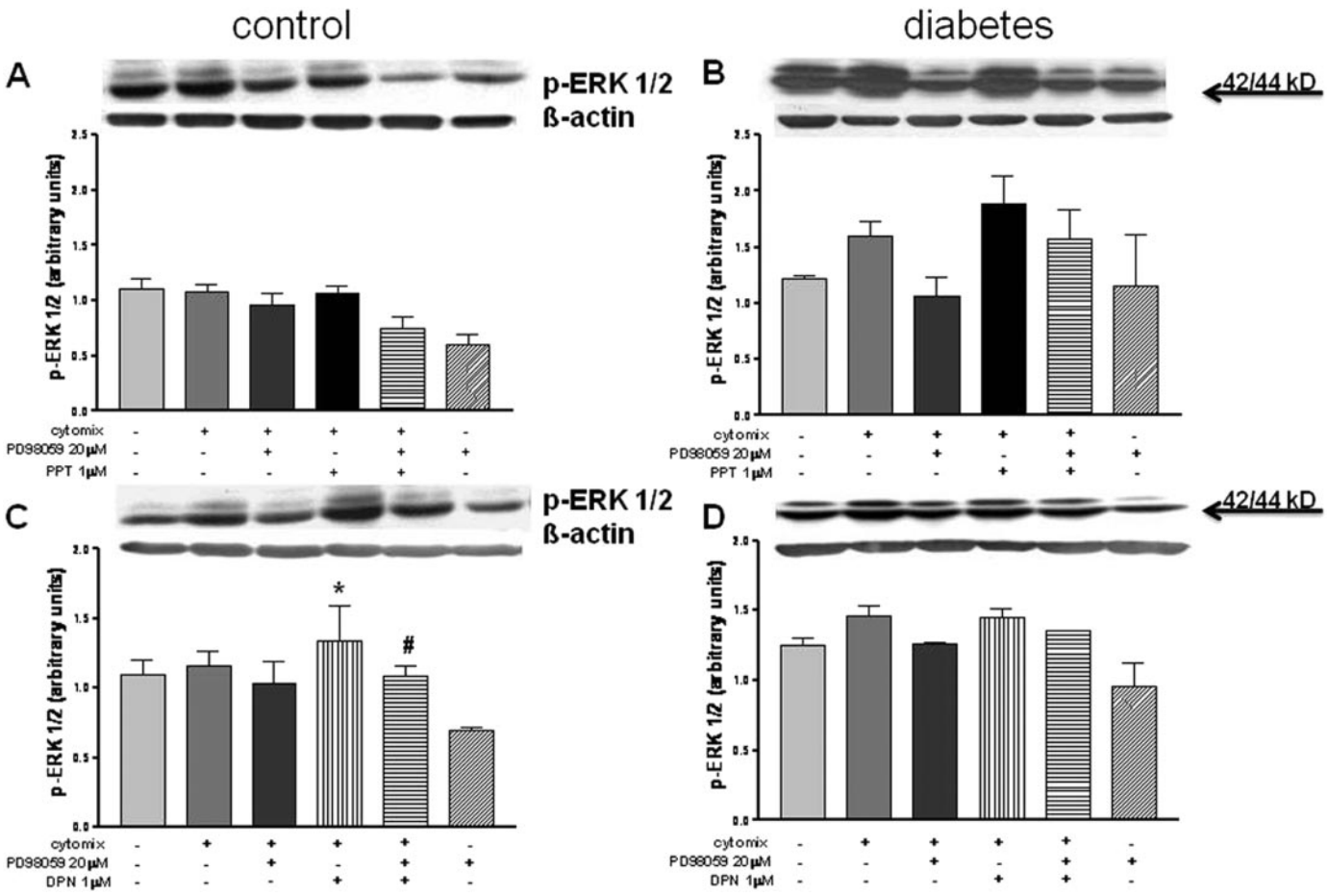

Fig. 4. Regulation of the mitogen-activated protein kinase system by ERα- and ERβ-selective agonists in aortic SMCs from control (A and C) and STZ-diabetic (B and D) rats. Cells were grown and treated as described in the legend to Fig. 1. The MEK-1 inhibitor PD98059 (20 μM) was added where indicated 30 min before cytokines and PPT or DPN (both 1 μM). For phosphorylated ERK 1/2 quantitation, the intensity of the control band was set arbitrarily to 1. Data are expressed as mean ± S.E.M. of three to five independent experiments. *, $P < 0.05$ versus cytokines; #, $P < 0.05$ versus cytokines + DPN.

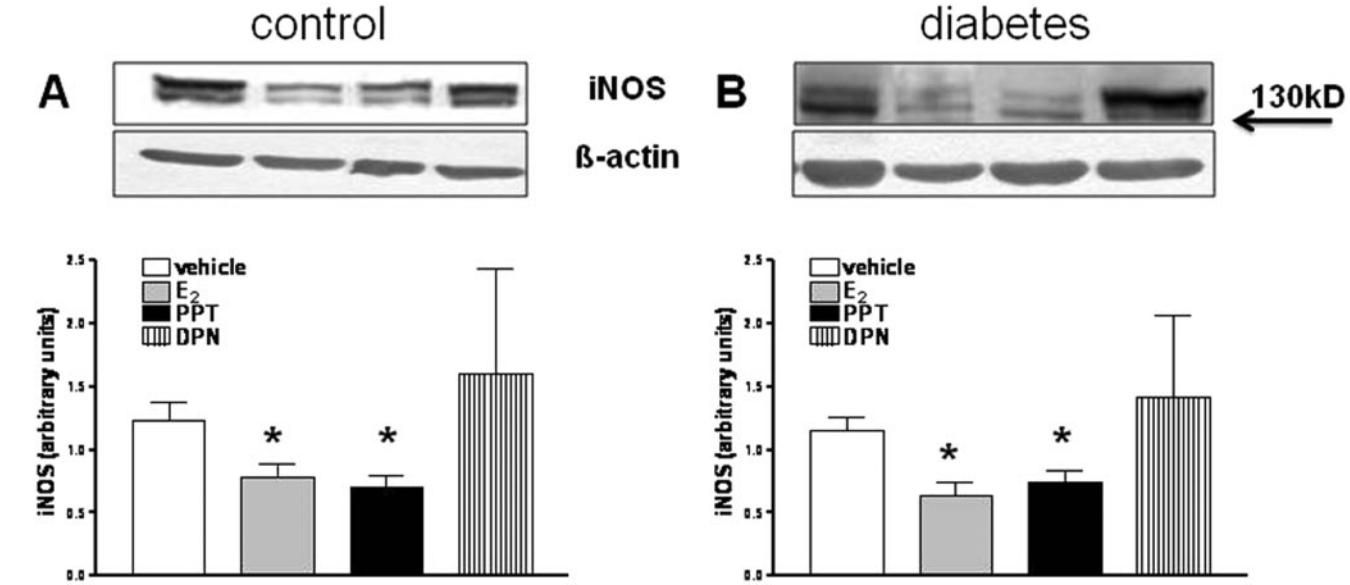

Fig. 5. ER-selective agonists and iNOS expression in whole aortic tissues of wild-type mice. Aortic rings from control (A) and STZ-diabetic (B) mice were stimulated as described in the legend to Fig. 1 in the presence of vehicle, 1 nM E_2, the ERα-selective agonist PPT, or the ERβ-selective agonist DPN (both 1 μM; $n = 4–5$). Representative Western blots are shown. The amount of iNOS in tissue lysates was measured by scanning densitometry of the upper iNOS-specific band, whereby the amount of iNOS in cytomix treatment alone was set arbitrarily to 1. *, $P < 0.05$ versus cytokines alone, ANOVA.

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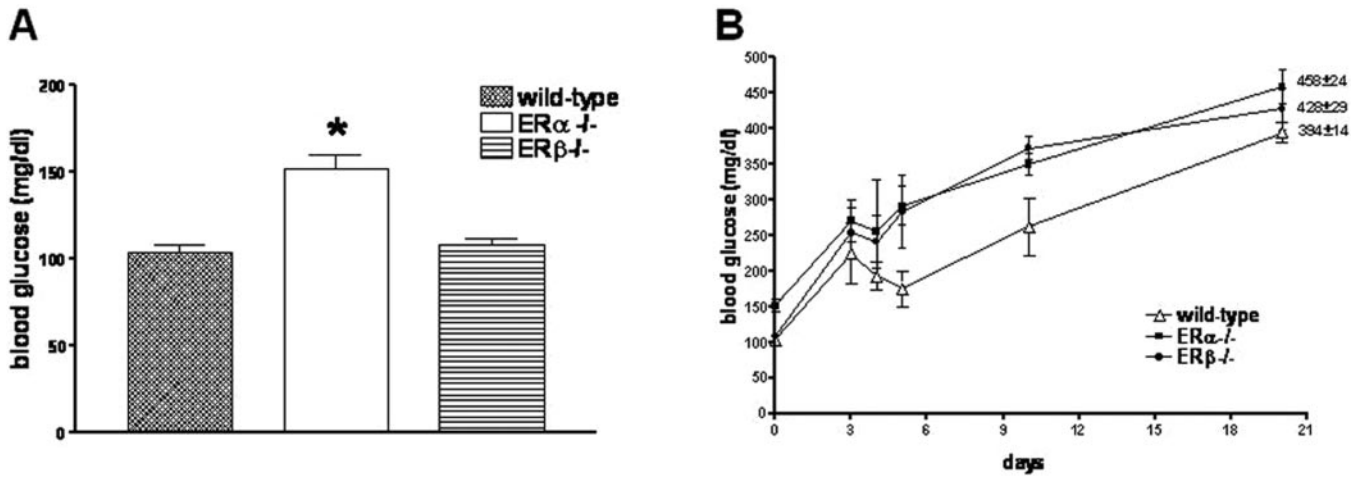

Fig. 6. Blood glucose levels in wild-type, ERα-KO, and ERβ-KO mice. Fasting blood glucose levels were evaluated in control animals (A; $n = 17$) or as a time course assay of random-fed blood glucose (B) in wild-type, ERα-KO, and ERβ-KO mice after streptozotocin injection. *, $P < 0.01$ versus wild-type and ERβ-KO, one-way ANOVA.

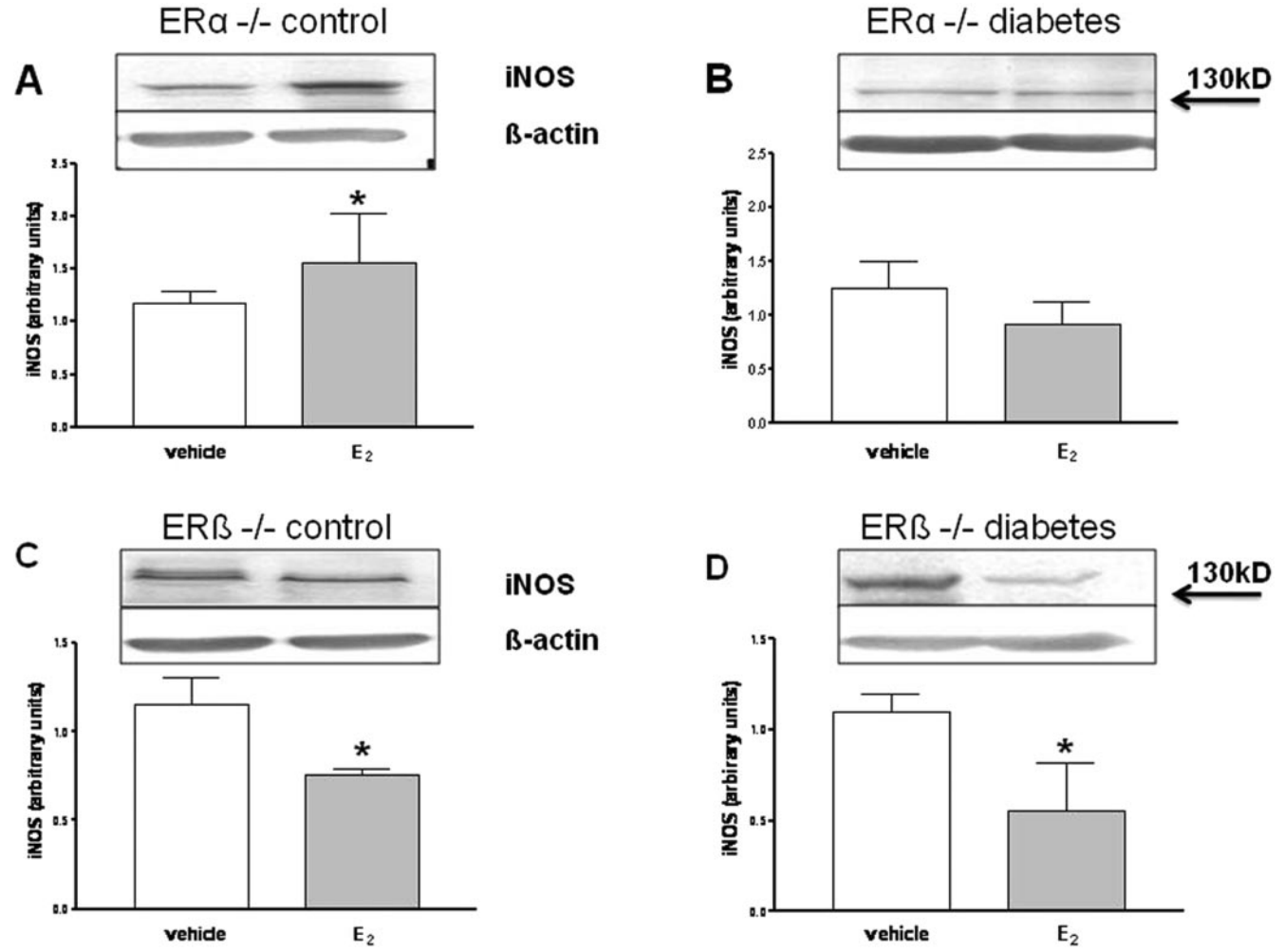

Fig. 7. Western blot analysis of iNOS in aortic tissues from ER-KO mice. Aortic rings were from control (A and C) and STZ-diabetic (B and D) mice with an ERα-KO (A and B) or ERβ-KO (C and D) genotype. Tissue cultures were challenged with a cytokine mix as described in the legend to Fig. 1 in the presence of 1 nM E_2 or its vehicle ($n = 3$ to 5). Graphs represent the scanning densitometry analysis whereby the intensity of the cytomix band in each animal group was set arbitrarily to 1. Representative Western blots are shown. *, $P < 0.05$ versus cytokines alone, ANOVA.

mitogen-activated protein kinase system is sensitive to ER-specific regulation, as previously demonstrated in mouse microglia (Baker et al., 2004) and vascular cells (Haas et al., 2007), and may be involved in cytokine-driven iNOS activation. Preliminary data (data not shown) suggest that ER signaling affects additional pathways related to iNOS induc-

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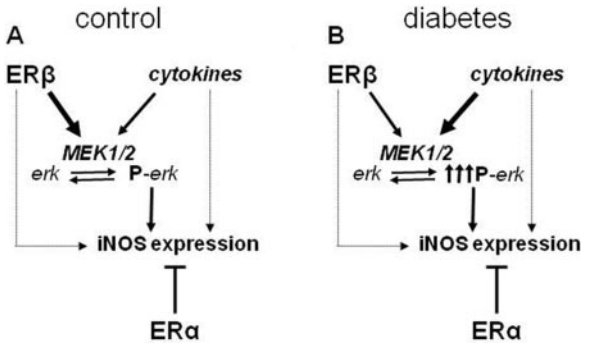

Fig. 8. Proposed scheme of differential ER action on iNOS expression in the vascular wall. ERα activation negatively regulates cytokine-driven iNOS accumulation in aortic tissues from normo- (A) and hyperglycemic (B) rodents. By contrast, ERβ activation increases iNOS levels. This may occur through induction of MEK-1-dependent phosphorylated ERK 1/2 generation in tissues from normoglycemic rodents (A). ERβ activation may increase iNOS levels also through enhanced cytokine signaling via phosphorylated ERK 1/2, which is generally overactivated in diabetes (B). Thick arrows, enhanced pathways; dashed arrows, general regulatory pathways of iNOS expression.

tion in vascular SMCs. To our knowledge, this is the first study demonstrating that in a given cell ERβ, but not ERα, may differentially interact with cytoplasmic or nuclear proteins. The identification of molecular mechanisms of ER activity on vascular inflammation holds promise for improving our understanding of estrogen action in the vasculature.

Further implications of the present work arise from the distinct role of ERα and ERβ in regulating vascular function because an imbalance in ERα versus ERβ pathways may predispose to vascular dysfunction and complications (Barros et al., 2006). In fact, ER expression profile has been shown to vary in association with physiologic or pathologic conditions including hypoestrogenicity and inflammation (Lindner et al., 1998; Marriott et al., 2007; Pinna et al., 2008). Because ER activity is strictly dependent upon the interaction with coregulators and enzymes (Harrington et al., 2003), alterations in expression or activity of these intracellular partners might impinge on receptor activity as well. Thus, it is reasonable that alterations in the ratio or activity of ER isoforms affect the outcome of estrogen action. In addition, an unfavorable ERα/ERβ ratio may foster heterodimerization between the two isoforms (Pettersson et al., 1997), hindering ERα protective activity. Our data are in agreement with a recent study showing opposing functions for ERα and ERβ in regulating distinct sets of genes in the mouse aorta in response to E_2 (O'Lone et al., 2007). Although the observed effects on iNOS protein may not be reflective of inflammatory effects overall, the present findings support our hypothesis that ER-selective ligands represent safer compounds for the vessel wall integrity with respect to currently used nonselective hormone agents (Bolego et al., 2006).

This and other studies point to an emerging role for ERs as regulators of glucose homeostasis and metabolic disease (Barros et al., 2006; Bryzgalova et al., 2006; Deroo and Korach, 2006; Le May et al., 2006). Over the last few years, several reports disclosed the relevance to glucose metabolism and insulin sensitivity of other members of the nuclear receptor superfamily, including liver X receptor and farnesoid X receptor (Cao et al., 2003; Laffitte et al., 2003; Zhang et al., 2006). Although adding to the well established role of PPARs in the field, these observations raise the question of whether molecular pathways shared by activated nuclear receptors (if any) govern the integrated coordination of lipid and glucose metabolism. Candidate pathways or targets may be identified eventually that are amenable to developing novel solutions in drug discovery.

From a pharmacological perspective, the finding that a selective ERα agonist, avoiding ERβ activation, preserves its anti-inflammatory action even in the presence of persistent hyperglycemia highlights potential benefits of isoform-selective ER ligands. In fact, the nonselective ER agonist E_2 may trigger conflicting effects in those tissues where ERα and ERβ are coexpressed, thereby outweighing potential therapeutic benefits mediated by either isoform. The availability of isoform-selective ER compounds thus represents a powerful investigational tool that will help unravel the details of ERα and ERβ interactions among each other and with other cellular components in the vasculature and, hopefully, will enable us to exploit potential therapeutic action of ER agonists, particularly in the therapy of disorders associated with the postmenopause.

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