Gender Differences in the Endotoxin-Induced Inflammatory and Vascular Responses: Potential Role of Poly(ADP-ribose) Polymerase Activation

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ABSTRACT

Activation of poly(ADP-ribose) polymerase (PARP) is an important factor in the pathogenesis of various cardiovascular and inflammatory diseases. Here, we report that the gender-specific inflammatory response is preferentially down-regulated by PARP in male animals. Female mice produce less tumor necrosis factor-α and macrophage inflammatory protein-1α in response to systemic inflammation induced by endotoxin than male mice and are resistant to endotoxin-induced mortality. Pharmacological inhibition of PARP is effective in reducing inflammatory mediator production and mortality in male, but not female, mice. Ovariectomy partially reverses the preferential modulation by PARP in male animals. Female mice produce less tumor necrosis factor (TNF)-α and macrophage inflammatory protein-1α in response to DNA injury (for review, see Virág and Szabó, 2002). PARP is also involved in the up-regulation of proinflammatory mediators such as tumor necrosis factor (TNF)-α in response to proinflammatory stimuli (Virág and Szabó, 2002). PARP inhibition or genetic deficiency of PARP-1 (the major PARP isoform) down-regulates inflammation and protects against reperfusion injury in many experimental models of disease (Virág and Szabó, 2002; Jagtap and Szabó, 2005).

There are many pathophysiological factors that induce oxidative or nitrosative stress, DNA strand breaks, and subsequently activate PARP, including elevated circulating glucose and oxidant-induced PARP activation is reduced in cultured cells placed in female rat serum compared with male serum. Estrogen does not directly inhibit the enzymatic activity of PARP in vitro. However, PARP and estrogen receptor α form a complex, which binds to DNA in vitro, and the DNA binding of this complex is enhanced by estrogen. Thus, estrogen may anchor PARP to estrogen receptor α and to the DNA and prevent its recognition of DNA strand breaks and hence its activation. In conclusion, the gender difference in the inflammatory response shows preferential modulation by PARP in male animals.

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme of eukaryotic cells, which has been implicated in response to DNA injury (for review, see Virág and Szabó, 2002). Free radical and oxidant-induced cell injury involves the activation of PARP (Ha and Snyder, 1999; Virág and Szabó, 2002; Jagtap and Szabó, 2005). When activated by DNA single-strand breaks, PARP initiates an energy-consumption cycle by transferring ADP-ribose units from NAD+ to nuclear proteins. The result of this process is a rapid depletion of the intracellular NAD+ and ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction, ultimately culminating in cell necrosis (Ha and Snyder, 1999; Virág and Szabó, 2002). PARP is also involved in the up-regulation of proinflammatory mediators such as tumor necrosis factor (TNF)-α in response to proinflammatory stimuli (Virág and Szabó, 2002). PARP inhibition or genetic deficiency of PARP-1 (the major PARP isoform) down-regulates inflammation and protects against reperfusion injury in many experimental models of disease (Virág and Szabó, 2002; Jagtap and Szabó, 2005).
cose (Garcia Soriano et al., 2001) and angiotensin II (Szabol et al., 2004). Much less is known about endogenous regulatory factors or gender in modulating the activity of this enzyme. In the present report, we demonstrate that there is a gender difference in the inflammatory response and show that PARP inhibitors preferentially modulate the response in male animals in vitro and in vivo. We also present preliminary data implicating the potential role of estrogen (17-β-estradiol) in this process.

Materials and Methods

In Vivo Studies. Systemic inflammation and mortality were induced by intraperitoneal injection of *Escherichia coli* endotoxin [lipopolysaccharide (LPS)] into wild-type and PARP-1-deficient mice (Liaudet et al., 2000). To induce systemic inflammatory mediator production without mortality, LPS was injected at a dose of 1 mg/kg, followed by the measurement of TNF-α and macrophage inflammatory protein (MIP)-1α at 90 min, using enzyme-linked immunosorbent assay. To inhibit the catalytic activity of PARP in vivo, the phenanthridine-based PARP inhibitor PJ34 (Jagtap et al., 2002) at 10 mg/kg or INO-1001 (Szabol et al., 2004; Jagtap et al., 2005) at 3 mg/kg were given i.p., as a 30-min pretreatment prior to the injection of LPS.

In some experiments, LPS (1 mg/kg i.p.) was given to ovariectomized female mice in the absence or presence of PARP inhibitor pretreatment (doses as above), followed by measurement of TNF at 90 min. In another set of experiments (in female mice), the dose of LPS was increased to 30 mg/kg to induce a more robust TNF production (to make it comparable with the TNF response seen in male animals).

In a separate subset of experiments, the effect of LPS was compared in male and female animals in its ability to induce an impairment of endothelium-dependent vasorelaxation ex vivo. Male or female Wistar rats (either pretreated with 30 mg/kg i.v. PJ34 for 30 min or its vehicle, saline) were given LPS injection (10 mg/kg i.v.). Thoracic aortae were obtained 3 h later, and endothelium-dependent relaxant responses to acetylcholine were recorded in isolated thoracic aortic rings as described previously (Szabol et al., 2004).

To induce systemic inflammatory response and mortality, animals were injected with 55 mg/kg LPS, and mortality was recorded. In one set of experiments, male mice were pretreated with estrogen (20 mg/kg 17-β-estradiol i.p.), followed by the injection of 55 mg/kg LPS. In one subset of experiments, mortality was detected, and in another subset, LPS-induced liver damage was quantified by measurement of plasma concentrations of alanine aminotransferase by a colorimetric kit at 6 h after LPS injection (Sigma-Aldrich, St. Louis, MO).

Poly(ADP-ribose) activation in circulating cells was measured by a flow cytometric method based on the immunohistochemical detection of the product of the enzyme, poly(ADP-ribose). Wistar rats were either pretreated with PJ34 (30 mg/kg i.v. for 30 min) or its vehicle, saline, and were given LPS injection (10 mg/kg i.v.). Circulating leukocytes were isolated 3 h later from whole blood using Histopaque-1083 according to the user’s manual (Sigma-Aldrich). After the fixation and permeabilization of the cells with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA), monoclonal mouse anti-poly(ADP-ribose) (PAR) antibody was used as primary antibody to stain intracellular PAR (Tulip Bioslabs, West Point, PA). After the fixation, all procedures were performed in Cytoperm solution. Purified mouse IgG2a Isotype control (anti-KLH) antibody served as isotype control (BD Biosciences). Fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin-specific polyclonal antibody (multiple adsorption) was used as secondary antibody (BD Biosciences). Flow cytometry was performed on single-cell suspension of rat leukocytes using FACSCalibur (BD Biosciences). Region 1 (R1) was defined to contain cells having typical forward scatter and side-scatter properties of lymphocytes. Isotype control-stained cells served as negative control for each sample. Fluorescence data were collected using logarithmic amplification until we reached 10,000 counts of R1 cells. On the PAR histograms, the gate was R1.

Poly(ADP-ribose) activation in tissue sections in vivo was measured by the immunohistochemical detection of the product of the enzyme, poly(ADP-ribose), as described previously (Garcia Soriano et al., 2001; Jagtap et al., 2002). Briefly, paraffin sections (5 μm) were loaded onto polystyrene-coated slides (Fisher Scientific Co., Pittsburgh, PA), deparaffinized, and rehydrated. Optimal staining was achieved with an antigen retrieval method, which was performed in 10 mM citric acid for 15 min. Endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 15 min. Sections were blocked with 2% normal goat serum at room temperature for 1 to 2 h and were incubated overnight with 1:500 dilution of primary anti-poly-(ADP)-ribose antibody (Tulip Bioslabs). Specific labeling was detected with a biotin-conjugated goat anti-chicken IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). The enzymatic reaction product was enhanced with nickel cobalt to give a black precipitate, and the sections were counterstained with nuclear fast red.

In Vitro Studies. The pulmonary epithelial cell line A549 cells and murine RAW macrophages were grown in 96- or 6-well plates in RPMI 1650 medium supplemented with 10% fetal calf serum. PARP activation was induced with 300 μM hydrogen peroxide or 600 μM peroxynitrite for 30 min in the presence or absence of 10% rat serum (obtained from adult male or female Wistar rats). Cells were grown in 10% fetal calf serum, and 24 h prior to PARP activity measurements, tissue culture medium was replaced to medium without fetal calf serum but supplemented with 10% male or female rat serum. Measurement of PARP activity was conducted by the measurement of tritiated NAD+ incorporation as described previously (Garcia Soriano et al., 2001; Szabol et al., 2004).

Cell-free PARP assay was conducted as described previously (Jagtap et al., 2002) using a commercially available PARP inhibition assay kit ( Trevigen, Gaithersburg, MD). The assay was carried out in 96-well enzyme-linked immunosorbent assay plates following manufacturer’s instructions. Briefly, wells were coated with 1 mg/ml histone (50 μl/well) at 4°C overnight. Plates were then washed four times with PBS and then blocked by adding 50 μl of Strep-Diluent (supplied with the kit). After incubation (1 h, room temperature), plates were washed four times with PBS. Various concentrations of 17-estradiol (1 PM–1 μM) were combined with 2× PARP cocktail (1.95 mM NAD+, 50 μM biotinylated NAD+ in 50 mM Tris, pH 8.0, and 25 mM MgCl2) and highly specific activity PARP enzyme (both supplied with the kit) in a volume of 50 μl. Reaction was allowed to proceed for 30 min at room temperature. After four washes in PBS, incorporated biotin was detected by peroxidase-conjugated streptavidin (1:500 dilution) and TACS Sapphire substrate.

Electrophoretic mobility shift assay (EMSA) was conducted in a cell-free system as follows: two synthetic oligonucleotides (sense, 5'-GGAGGTGTGGATTCTCTTCGGCGCCGGGACGGTACTCCGTC- T3'; and antisense, 5'-GGAGGAGGGATTGCCTGGGCCCCAGAGGAGATGGCACCACCT3'), corresponding to that of chicken TnT gene promoter region, were annealed to make a DNA substrate for PARP gel mobility shift assay. After annealing, DNA probe containing 5' overhangs was labeled with [α-32P]dCTP and Klenow fragment of DNA polymerase 1. Unincorporated radioactivity were removed from the reaction by spin column chromatography. For EMSA with purified proteins, 5 pmol PARP-1 (Trevigen) and/or 5 or 100 pmol estrogen receptor α (Sigma-Aldrich) were incubated on ice for 10 min in a final volume of 50 μl of DNA binding buffer containing 20 mM Tris-HCL, pH 7.9, 10 mM MgCl2, 100 mM KCl, 10 mM diithiothreitol, and 10% glycerol. Reactions were initiated by adding 100 nmo1 labeled probe and incubated for 10 min at room temperature. For competition experiments, a 2- or 20- or 60-fold molar excess of unlabeled double-stranded oligonucleotide or 3 or 30 or 100 ng of sonicated plasmid DNA was added, and reactions were incubated for
another 10 min at room temperature. The DNA-protein complexes were analyzed by electrophoresis on a 4% or 6% polyacrylamide gel (60:1 acrylamide/bisacrylamide ratio) in 0.5× Tris borate-EDTA buffer at room temperature for 2 h at 150 V, dried under vacuum, and then autographed with an intensifying screen at −80°C.

Statistical Analysis. Results are reported as mean ± S.E.M. Analysis of variance with Bonferroni’s correction or Student’s t test was used to compare mean values, as appropriate. Differences were considered significant when P < 0.05.

Results

Female Animals Are Protected against the Systemic Effect of LPS and Are Less Responsive to PARP Inhibition in Vivo. In agreement with previous reports (Schroder et al., 1998; Angele et al., 2000), female animals produced less TNF-α and were resistant to endotoxin-induced mortality (Fig. 1). Inhibition of the catalytic activity of PARP by PJ34 (Fig. 1) or INO-1001 (not shown) reduced TNF production and protected against endotoxin-induced mortality in male animals but did not further reduce TNF production or mortality in female animals (Fig. 1). PARP inhibition was unable to significantly reduce TNF production in female mice even when the dose of LPS was increased to produce a higher level of baseline TNF production to make it comparable with the level seen in LPS-treated male animals (Fig. 1). In addition, PARP-1-deficient male mice were resistant to LPS-induced TNF production and mortality, whereas in female mice (which were already resistant to these responses), genetic inactivation of PARP-1 failed to produce additional benefit (Fig. 1).

The gender difference in inflammatory factor production and the gender difference in the ability of PARP inhibitors to suppress inflammatory mediator production were also confirmed on the example of another mediator, the chemokine (MIP-1α), which was also measured in the plasma at 90 min after LPS. In the male mice, LPS increased MIP levels to 4205 ± 197 ng/ml, which was inhibited by the PARP inhibitor INO-1001 or by PJ34 to 2484 ± 391 and 3356 ± 171 ng/ml, respectively (n = 8–11, P < 0.05). In contrast to the male animals, in females, lower levels of MIP were produced in response to the same dose of LPS, and this MIP production was only slightly reduced by PARP inhibition. For instance, MIP levels after LPS in the absence or presence of INO-1001 pretreatment in female animals amounted to 2928 ± 134 and 2787 ± 114 ng/ml, respectively (n = 8–11).

In ovariectomized female mice, LPS induced higher levels of TNF compared with regular control females (8582 ± 1187 versus 5504 ± 806 pg/ml, n = 5). Furthermore, in ovariectomized animals, a restoration of the sensitivity of the animals to inhibition of TNF production by PARP inhibitors was seen. Pharmacological inhibition of PARP reduced LPS-induced TNF production to 4668 ± 1187 (n = 5, P < 0.05).

There was no difference between male and female animals in basal PARP activity, as detected in circulating leukocytes by flow cytometry. LPS stimulation induced significant increases in PARP activation both in male and female animals.
However, pharmacological inhibition of PARP with PJ34 only reduced PARP activity in male animals, but not in females (Fig. 2).

There was a significant degree of reduction in the endothelium-dependent relaxant ability of the vascular rings in response to LPS treatment in male animals but not in female animals (Fig. 3). Pretreatment with the PARP inhibitor PJ34 prevented the development of this LPS-induced endothelial dysfunction in the male rats, whereas it tended to attenuate the relaxant response in LPS-treated female animals (Fig. 3).

**Estrogen Exerts Protective Effects and Inhibits PARP Activation in Vivo.** Pretreatment of male rats with 17-β-estradiol exerted protective effects against the mortality induced by high-dose endotoxin (70% mortality versus 40% mortality at 24 h), reduced plasma markers of hepatic damage (224 ± 53 U/l plasma alanine aminotransferase in the vehicle-treated animals versus 48 ± 17 U/l in the estrogen-treated animals, n = 10, P < 0.05), and attenuated the immunohistochemical staining of poly(ADP-ribose), indicative of inhibition by exogenous estrogen administration of tissue PARP activation in vivo (Fig. 4).

**Potential Mechanisms Responsible for the Observed Gender Difference.** In cultured RAW murine macrophages and human A549 epithelial cells, peroxynitrite or hydrogen peroxide were used to activate PARP. This effect is due to induction of DNA single-strand breakage and recognition of these breaks by the zinc fingers of the enzyme (Virág and Szabó, 2002). The extent of PARP activation was markedly less pronounced in cells cultured in the presence of 10% female rat serum, as opposed to cells in male rat serum. For example, in A549 cells, 500 μM hydrogen peroxide induced a marked increase in PARP activity (from 117 ± 9 to 1171 ± 51 cpm, n = 6) in cells incubated in male serum, but the response was diminished in female serum by approximately 50% (from 140 ± 30 to 485 ± 38 cpm, n = 6). The inhibition of PARP activity by estrogen is not a direct effect of the hormone on the enzyme because no significant inhibitory effect of estrogen on PARP was noted against the purified PARP enzyme in the concentration range of 1 pM to 1 μM. For example, PARP activity in a cell-free assay was 92 ± 5% of control in the presence of 1 μM estrogen (n = 6).

EMSA demonstrated the interaction of DNA, PARP, and estrogen receptor, which was enhanced by estrogen (Fig. 5). Using purified PARP and estrogen receptor (ER) α and labeled synthetic duplex DNA containing specific PARP binding sequences from chicken TnT gene promoter, a mobility shift consistent with weak interaction was noted between PARP and DNA or PARP and ERα alone (Fig. 5a, lanes 2 and 3). The addition of 50 pM estrogen to PARP-DNA and ERα-DNA complexes did not change the migration pattern significantly (data not shown), but addition of these two proteins together (Fig. 5b, lane 4) enhanced the protein-DNA complex formation, and these complexes migrated slower than PARP-DNA and ERα-DNA complexes, indicating cooperative interactions. The addition of estrogen (ED) together with PARP and ERα to DNA markedly enhanced the complex formation, and this complex migrated much slower than PARP-ERα-DNA complex (compare Fig. 5b, lane 11 with lane 4). Even addition of estrogen as low as 5 pM significantly increased the complex formation (data not shown). Together, these results establish that PARP and ERα interact cooperatively to increase their association with the DNA, and these interactions are further strengthened by the presence of estrogen.

Next we examined the stability of PARP-ERα-DNA complexes in the presence and absence of estrogen. In a series of EMSA experiments, PARP-ERα-DNA complexes were chased with unlabeled DNA. In the absence of estrogen, addition of 2-fold excess DNA to PARP-ERα-DNA complexes damaged the complex significantly (Fig. 5b, lanes 5 and 8). However, addition of 2-fold excess cold DNA to PARP-ERα-DNA complexes in the presence of estrogen has very little effect (Fig. 5b, lanes 12 and 15). Furthermore, addition of 20- or 60-fold excess cold DNA completely destroyed the complex even in the presence of estrogen (Fig. 5b, lanes 6, 7, 9, 10, 13, 14, 16, and 17). Thus, our data indicate that estrogen can mediate a significant enhancement or stabilization of the binding of PARP and/or ERα.

**Discussion**

The current studies demonstrate the interrelated regulation of the endotoxin-induced inflammatory and vascular responses by gender and PARP. The production of the inflammatory mediators TNF-α and MIP-1α, the LPS-induced mortality, and the development of LPS-induced endothelial dysfunction were all markedly attenuated in female mice, and pharmacological inhibition of PARP failed to provide further protection in the female animals. On the other hand, in male mice, pharmacological inhibition reduced TNF and MIP-1α production, reduced mortality, and prevented the development of endothelial dysfunction. PARP inhibition in male animals and female gender provided a comparable degree of protection against the various inflammatory/cardiovascular parameters investigated in the current study. Consistent with these findings, we observed that in circulating leukocytes, the pharmacological PARP inhibitor PJ34 only inhibited LPS-induced PARP activation in males, but not in females.

Gender differences with respect to pathophysiological responses and PARP have recently been observed by Hagberg et al. (2004) and by McCullough et al. (2005). It was demonstrated that male mice are preferentially protected against stroke in the absence of functional PARP-1 or by pharmacological PARP inhibition (as opposed to female animals, in which PARP inhibition offered was no benefit in the outcome of ischemic stroke).

It is well known that estrogen exerts a variety of cardiovascular protective effects. The protective role of endogenous estrogen is lost after menopause (nevertheless, hormonal replacement therapy in postmenopausal women fails to reduce cardiovascular risk; Rosano and Panina, 1999; Nelson et al., 2002; Wenger, 2003). The current findings may provide an additional mode of action whereby estrogen exerts its physiological protective and anti-inflammatory effects. The findings that the gender difference to the LPS-induced TNF production is partially diminished in ovariectomized animals, poly(ADP-ribosyl)ation is attenuated by estrogen in male animals challenged with LPS in vivo, there is a difference in the degree of PARP activation between cells incubated in male versus female rat serum, and 17-β-estradiol pretreatment in male animals protects against LPS-induced mortality and PARP activation all point to the potential...
Fig. 2. Gender differences in PARP activation in circulating leukocytes in response to LPS and in the effect of PARP inhibition. 

a, representative flow cytometry plots of PAR-stained leukocytes. Male or female Wistar rats (either pretreated with 30 mg/kg i.v. PJ34 for 30 min or its vehicle saline) were given LPS injection (10 mg/kg i.v.). Leukocytes were prepared 3 h later. Cells having typical forward scatter and side-scatter properties of lymphocytes were defined as R1. For each sample, isotype control-stained cells served as negative control. On the PAR histograms, R1 was set as gate. 

b, effect of LPS and PARP inhibition with PJ34 on PARP activation in circulating leukocytes in male rats. Mean fluorescence intensity of R1 cells (lymphocytes) stained with anti-PAR antibody in male rats. LPS treatment of the animals resulted in significant increase of the PAR content of these cells ($**$, $P < 0.001$). In the case of male rats, PJ34 pretreatment significantly reduced this effect of LPS ($*$, $P < 0.05$). 

c, effect of LPS and PARP inhibition with PJ34 on PARP activation in circulating leukocytes in female rats. Mean fluorescence intensity of R1 cells (lymphocytes) stained with anti-PAR antibody in female rats. LPS treatment of the animals resulted in significant increase of the PAR content of these cells ($*$, $P < 0.05$). In the case of female rats, PJ34 pretreatment failed to alter this effect of LPS. $n = 6$ to 8 animals per group.
The involvement of the main female sex hormone, 17-β-estradiol, in the observed effects.

The finding that estrogen does not directly inhibit the catalytic activity of PARP in a cell-free assay implicates an indirect mode of action. Although estrogen can act as an antioxidant, this effect generally occurs at fairly high concentrations in vitro (Leal et al., 1998; Prokai et al., 2003). Nevertheless, the contribution of an antioxidant effect of estrogen to the presently reported findings cannot be excluded.

ERα is a well-known potent activator of transcription (Barkhem et al., 2004; Turgeon et al., 2004). ERα modulates transcription through its interaction with components of basal transcription machinery, chromatin modifiers, and regulatory proteins. In the absence of ligand, ERα binds to the corepressor complex containing histone deacetylases and remains inactive. However, in the presence of estrogen ligand, ERα associates with coactivator complex containing histone acetylases and activate transcription. Furthermore, the MAPK-dependent phosphorylation of ERα serine residues within the AF-1 domain also recruits coactivators and activates transcription through ligand-independent mechanism. Using an in vitro gel shift assay, we have provided direct evidence that PARP and ERα cooperatively interact with the DNA, and these interactions are further reinforced by the presence of estrogen. One can, therefore, propose a model of interaction between PARP and ERα (Fig. 6). PARP or ERα or PARP and ERα (on their own) interact with DNA, and these interactions are weak and reversible. In addition, PARP-ERα complex is as active as PARP alone and moves freely on the DNA.

![Fig. 4. Estrogen treatment inhibits the activation of PARP in vivo in response to LPS in male rats. Top, normal control livers show low basal PARP activity. Middle, LPS (55 mg/kg i.p.) induces an increase in the immunohistochemical staining of PAR, as measured 6 h after LPS injection. This increased PAR staining is consistent with PARP activation. Bottom, estrogen-pretreated animals (20 mg/kg 17-β-estradiol) respond to LPS with a reduced degree of PARP activation. Stainings shown represent four to five animals per group.](image-url)
DNA and repairs the DNA damage sites. However, the presence of estrogen ligand alters the conformation of ERα and forms a more stable ERα-PARP-DNA ternary complex. Such a stable complex may sequester PARP to specific regions on the DNA, making it difficult for its zinc fingers to access and recognize DNA breakpoints (without which its activation would be inhibited). Such a model would be consistent with our findings that estrogen is not a direct inhibitor of the enzymatic activity of the purified PARP enzyme but is a potent inhibitor of the activation of PARP in vivo in estrogen-pretreated animals. However, we must note that the estrogen receptor/PARP interaction demonstrated in the

![Fig. 5. Estrogen stabilizes PARP and ERα interactions with DNA. A, PARP and ERα interacts with DNA cooperatively. EMSA was performed as described under Materials and Methods. Puriﬁed PARP (5 pmol) or ERα (5 pmol) alone or in combination were incubated with labeled double-stranded oligonucleotides at room temperature for 10 min. For cooperative interactions, proteins were incubated with or without estrogen for 10 min prior to the addition of DNA probe. Samples were fractionated through a 6% polyacrylamide gel. The DNA-protein complexes were visualized by autoradiography. Please note that a weak interaction was noted between PARP and DNA or PARP and ERα alone, as reﬂected in a slight change in the migration pattern (lanes 2 and 3). The addition of 50 pM estrogen to PARP-DNA and ERα-DNA complexes did not change the migration pattern signiﬁcantly (data not shown), but addition of PARP and ERα together (lane 4) enhanced the protein-DNA complex formation, and these complexes migrated slower than PARP-DNA and ERα-DNA complexes, indicating cooperative interactions. However, very pronounced effects were seen after the addition of ED together with PARP and ERα to DNA. Under these conditions, there was a marked enhancement of the complex formation, and this complex migrated much slower than PARP-ERα-DNA complex (compare lane 11, PARP and estrogen receptor in the presence of estrogen, with lane 4, PARP and estrogen receptor in the absence of estrogen). B, estrogen stabilizes PARP-ERα interactions with the DNA. The PARP-ERα-DNA complexes (lanes 4–10) or PARP-ED-ERα-DNA complexes (lanes 11–17) were challenged with unlabeled duplex oligonucleotides or plasmid DNA as indicated. Reaction mixtures were incubated with unlabeled competitor DNA for a further 10 min before separating through a 4% polyacrylamide gel. The ratio of competitor to probe is 2 in lanes 5 and 12, 20 in lanes 6 and 13, and 60 in lanes 7 and 14. Lanes 8 and 15, 9 and 16, and 10 and 17 received 3, 30, and 100 ng of sonicated plasmid DNA, respectively. The ﬁnal concentration of the ED added was 50 pmol. In the absence of estrogen, addition of 2-fold excess DNA to PARP-ERα-DNA complexes damaged the complex signiﬁcantly (lanes 5 and 8). Addition of excess cold DNA to PARP-ERα-DNA complexes in the presence estrogen has very little effect (lanes 12 and 15). Addition of 20- or 60-fold excess cold DNA completely destroyed the complex even in the presence of estrogen (lanes 6, 7, 9, 10, 13, 14, 16, and 17). These ﬁndings suggest that estrogen can mediate a signiﬁcant enhancement or stabilization of the binding of PARP and/or ERα. Representative gels from three to four independent experiments are shown.

![Image of gel and chart]

1 2 3 4 5 6 7

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
The concentrations of estrogen in the physiologically relevant concentration range are approximately 300 pM to 1 nM, and hormone replacement therapy in postmenopausal women generally aims to achieve plasma estrogen levels in the 100 to 300 pM range (Gavaler, 2002; Harris et al., 2002; Greenspan, 2003). It is never straightforward to correlate the concentrations required to induce a pharmacological effect in vitro (especially in artificial subcellular model systems) with the in vivo responses.

We wish to point out that in the current study we only measured two inflammatory mediators (TNF-α and MIP-1α), one being a cytokine and the other being a chemokine. Additional parameters would be interesting to study in the future, not only other proinflammatory mediators (e.g., the expression of the inducible isoform of NO synthase by measuring plasma nitrite/nitrate levels at later time points after LPS), but also anti-inflammatory cytokines such as interleukin-10 or other inflammatory factors and processes (e.g., infiltration of mononuclear cells into tissues) to determine the broader applicability of the current findings.

Likewise, further work is required between linking the in vitro findings to cell-based and in vivo mechanisms. It is possible that the actions of estrogen or gender in modulating PARP activation involve more than one regulatory mechanism. It is also possible that in different cell types, different regulatory mechanisms may be involved. Likewise, the identification of the specific domains of PARP and ERα involved in recognizing each other remains a subject of further investigation.

Pharmacological inhibitors of PARP move toward clinical testing for a variety of indications, including stroke and cardioprotection (Southan and Szabo, 2003; Graziani and Szabo, 2003). Because stroke and myocardial infarction predominantly develop in men or in postmenopausal women, the current results do not discourage the clinical testing of the therapeutic effect of PARP inhibitors in both males and females. However, we believe that careful analysis should be conducted, and potential gender differences in the upcoming clinical trials should be examined.

It is interesting to note that many cell-based experiments are being conducted in tissue culture medium containing various concentrations (typically 10%) fetal calf serum, which contains detectable amounts of maternal estrogen. Based on the present data, one may wonder whether the results derived from such studies reflect artificial conditions in which estrogen receptors are engaged and PARP may be partially inhibited.

A recent study demonstrates that the neuroprotective effect of PARP-1 deficiency is gender-dependent. In female animals, PARP-1 deficiency fails to produce protective effects, whereas in male animals, it is protective (Hagberg et al., 2004). Similar results were subsequently reported by another independent laboratory (McCullough et al., 2005), where pharmacological inhibition of PARP even resulted in a worsening of the outcome of stroke in the female animals. Our current finding that PARP inhibition in endotoxin-treated female animals tends to worsen endothelial-dependent relaxations may parallel these latter findings.

A gender difference has also been shown in the susceptibility of patients to systemic inflammatory response and septic shock (Schröder et al., 1998). Recent studies also demonstrate gender differences in sensitivity of cells to oxidative injury in vitro (Du et al., 2004). The current results identify gender, and possibly endogenous estrogen, as modulators of PARP activation. Our findings may have diverse implications for physiology and pathophysiology, and the mechanisms identified in the current study may explain some of the gender differences in pathophysiological responses reported in some of the earlier studies.

References

![Diagram](image-url)

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