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Gender differences in the endotoxin-induced inflammatory and vascular responses: potential role of poly(ADP-ribose) polymerase activation

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List of non-standard abbreviations: ER: estrogen receptor; INO-1001 indeno[1,2-*c*]isoquinolinone–based PARP inhibitor; PARP: poly(ADP-ribose) polymerase; PAR: poly(ADP-ribose); PJ34: N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethyl-acetamide HCl

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Abstract

Activation of poly(ADP-ribose) polymerase 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-alpha (TNF-α and MIP-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 in female mice. Ovarectomy partially reverses the protection seen in female mice. Endotoxin-induced PARP activation in circulating leukocytes is reduced in male, but not female animals by pharmacological PARP inhibition, as shown by flow cytometry. Pretreatment with 17-beta-estradiol prevents endotoxin-induced hepatic injury, and reduces poly(ADP-ribosyl)ation *in vivo*. In male, but not female animals, endotoxin induces an impairment of the endothelium-dependent relaxant responses, which is prevented by PARP inhibition. *In vitro* oxidant-induced PARP activation is reduced in cultured cells placed in female rat serum, as compared to male serum. Estrogen does not directly inhibit the enzymatic activity of PARP *in vitro*. However, PARP and estrogen receptor alpha 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 alpha 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.

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Introduction

Poly(ADP ribose) polymerase (PARP) is an abundant nuclear enzyme of eukaryotic cells, which has been implicated in response to DNA injury (overviewed in 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 consuming 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 pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α) in response to pro-inflammatory 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 (Garcia Soriano et al., 2001) and angiotensin II (Szabó 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-beta-estradiol) in this process.

Methods

In vivo studies

Systemic inflammation and mortality were induced by intraperitoneal injection of *E. coli* endotoxin (lipopolysaccharide, LPS) into wild-type and PARP-1 deficient mice (Liaudet et al., 2001). In order to induce systemic inflammatory mediator production without mortality, LPS was injected at a dose of 1 mg/kg, followed by the measurement of tumor-necrosis factor alpha (TNF) and macrophage inflammatory protein-1α (MIP-1α) at 90 minutes, using ELISA. In order to inhibit the catalytic activity of PARP *in vivo*, the phenanthridinone-based PARP inhibitor PJ34 (N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide HCl, Jagtap et al., 2002) at 10 mg/kg or the indeno[1,2-*c*]isoquinolinone-based PARP inhibitor INO-1001 (Szabó 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 ovarectomized female mice, in the absence or presence of PARP inhibitor pretreatment (doses as above), followed by measurement of TNF at 90 minutes.

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 (in order to make it comparable to 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 PJ34 [30 mg/kg, i.v., for 30 min] or its vehicle, saline) were given LPS injection (10 mg/kg i.v.). Thoracic aortae were obtained 3h later and endothelium-dependent relaxant responses to acetylcholine were recorded in isolated thoracic aortic rings as described (Szabó et al., 2004).

In order to induce systemic inflammatory response and mortality, animals were injected with 55 mg/kg LPS, and mortality recorded. In one set of experiments, male mice were pretreated with estrogen (20 mg/kg 17-beta-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 ALT by a colorimetric kit at 6h after LPS injection (Sigma).

Poly(ADP-ribose) activation in circulating cells was measured by a flow cytometric method based on the 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 3h later from whole blood using Histopaque-1083 according to the users manual (Sigma, Saint Louis, MO). After the fixation and permeabilization of the cells with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Becton Dickinson, San Jose, CA), monoclonal mouse anti-PAR antibody was used as primary antibody to stain intracellular PAR (Tulip Biolabs, West Point, PA). After the fixation, all procedures were performed in Cytoperm solution. Purified mouse $IgG_{3\kappa}$ Isotype

control (anti-KLH) antibody served as isotype control (BD). FITC-conjugated anti-mouse immunoglobulin specific polyclonal antibody (multiple adsorption) was used as secondary antibody (Becton Dickinson, San Jose, CA). FCM was performed on single-cell suspension of rat leukocytes using FACSCalibur (Becton Dickinson, San Jose, CA). 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 polysine-coated slides (Fisher, Atlanta, GA), deparaffinized and rehydrated. Optimal staining was achieved with an antigen retrieval method, which was performed in 10 mmol/l citric acid for 15 minutes. Endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 15 minutes. Sections were blocked with 2% normal goat serum at room temperature for 1-2 hour, and were incubated overnight with 1:500 dilution of primary anti-poly(ADP)-ribose antibody (Tulip Biolabs Inc, West Point, PA). Specific labeling was detected with a biotin-conjugated goat anti-chicken IgG and avidin-biotin peroxidase complex (Vector Laboratories, Inc., 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 hours prior to PARP activity measurements, tissue culture medium was replaced to medium without fetal calf serum, but supplemented with 10% male of female rat serum. Measurement of PARP activity was conducted by the measurement of tritiated NAD^+ incorporation as described previously (Garcia Soriano et al., 2001; Szabó et al., 2004).

Cell-free PARP assay was conducted as previously described (Jagtap et al., 2002) using a commercially available PARP inhibition assay kit (Trevigen, Gaithersburg, MD). The assay was carried out in 96 well ELISA 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 Strep-Diluent (supplied with the kit). After incubation (1h, room temperature) plates were washed four times with PBS. Various concentrations of 17-beta-estradiol (1 pM-1 μM) were combined with 2x PARP cocktail (1.95 mM NAD^+, 50 μM biotinylated NAD^+ in 50 mM TRIS pH 8.0, 25 mM $MgCl_2$) 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 4 washes in PBS, incorporated biotin was detected by peroxidase-conjugated streptavidine (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'-GGAGTGTTGCATTCCTCTCTGGGCGCCGGGCAGGTACCTGCT-3', antisense 5'-GGAGCAGGTACCTGCCCGGCGCCCAGAGAGGAATGCAACACT-3'), 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-^{32}P]dCTP and Klenow fragment of DNA polymerase 1. Unincorporated radio-nucleotides were removed the reaction by spin column chromatography. For EMSA with purified proteins, 5 pmoles of PARP-1 (Trevigen) and/or 5 or 100 pmoles of estrogen receptor α (Sigma) 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 $MgCl_2$, 100 mM KCl, 10 mM DTT and 10% glycerol. Reactions were initiated by adding 100 nmole of 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 0.5X TBE buffer at room temperature for 2 h at 150V, dried under vacuum, and then autographed with an intensifying screen at -80^0C.

Statistical Analysis

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Results are reported as mean ± SEM. 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., 1988; 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. 1b). PARP inhibition was unable to significantly reduce TNF production in female mice even when the dose of LPS was increased in order to produce a higher level of "baseline" TNF production, in order to make it comparable to 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, while 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 minutes 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 overactomized female mice, LPS induced higher levels of TNF, when compared to regular control females (8582±1187 pg/ml vs. 5504±806 pg/ml, n=5). Furthermore, in overactomized 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, while 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-beta-estradiol exerted protective effects against the mortality induced by high-dose endotoxin (70% mortality vs. 40% mortality at 24 hours), reduced plasma markers of hepatic damage (plasma ALT 224±53 U/l in the vehicle-treated animals, vs. 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 approx. 50% (from 140±30 485±38 cpm, n=6). The inhibition of PARP activity by

estrogen is not a direct effect of the hormone on the enzyme, as no significant inhibitory effect of estrogen on PARP was noted against the purified PARP enzyme in the concentration range of 1 pM-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).

Electrophoretic mobility shift assay (EMSA) demonstrated the interaction of DNA, PARP and estrogen receptor, which was enhanced by estrogen (Fig. 5) Using purified PARP and ERα 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 lane 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 to 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 two-fold excess DNA to PARP-ERα-DNA complexes damaged the complex significantly (Fig 5b, lane 5 and 8). However, addition of 2 fold excess cold DNA to PARP-ERα-DNA complexes in the presence estrogen has very little effect (Fig 5b, lane 12 and 15). Further, 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 and colleagues (2004) and by McCullough and colleagues (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 (1) the gender difference to the LPS-induced TNF production is partially diminished in ovarectomized animals, (2) poly(ADP-ribosyl)ation is attenuated by estrogen in male animals challenged with LPS *in vivo* (3) there is a difference in the degree of PARP activation between cells incubated in male vs. female rat serum, and (4) 17-beta-estradiol pretreatment in male animals protects against LPS-induced mortality and PARP activation all point to the potential involvement of the main female sex hormone, 17-beta-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.

Estrogen receptor-α (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 associate 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 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 tercenary complex. Such a stable complex may sequester PARP to specific regions on the DNA making it difficult to 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 present study is an *in vitro* finding only, and in the present report we did not present direct evidence that such interaction also occurs in intact cells or in *in vivo* systems. 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. The concentrations of estrogen in the physiologically relevant concentration range are approximately 300 pM – 1 nM, and hormone replacement therapy in postmenopausal women generally aims to achieve plasma estrogen levels in the 100-300 pM range (Harris et al., 2002; Gavaler et al., 2002; Greenspan and Gardner, 2003).

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 pro-inflammatory 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 IL-10, or other inflammatory factors and processes (e.g. infiltration of mononuclear cells into tissues), in order 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 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 towards clinical testing for a variety of indications, including stroke and cardioprotection (Southan and Szabó, 2003; Graziani and Szabó, 2005). As stroke and myocardial infarction predominantly develops 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: potential gender differences in the up-coming 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, while 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 endothelium-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 (Schroder 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.

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Footnotes

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Legends for figures

Fig. 1. Gender differences in the anti-inflammatory effect of PARP inhibition. Top: Female animals produce less TNF in response to i.p. LPS (1 mg/kg) than age-matched males. While in male animals the PARP inhibitor PJ34 (10 mg/kg) significantly (**P<0.01) inhibits LPS-elicited TNF production (as measured at 90 min after LPS injection) in female animals the effect of the PARP inhibitor is minimal. Bottom: While PARP deficient female mice produce significantly (*P<0.05) less TNF in response to LPS (1 mg/kg i.p.) than wild-type, PARP deficient females (which produce less TNF than males) do not produce less TNF in response to LPS than wild-type female counterparts. Both PARP deficient phenotype (in males) and female gender protect against endotoxic shock associated mortality (induced by 55 mg/kg LPS i.p.). As in female mice the LPS-induced TNF production is much less than males, we have also tested the effect of PARP inhibition or PARP deficiency against TNF production (at 90 min) and mortality in response to higher doses of LPS (30 mg/kg) in females. PARP inhibition or PARP deficiency did not protect against these responses (right side of the top panel, and data not shown). Similar results were seen with the structurally different pharmacological inhibitor INO-1001 (3 mg/kg, data not shown). N=12-14 animals per group.

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 PJ34 [30 mg/kg, i.v., for 30 min] or its vehicle, saline) were given LPS injection (10 mg/kg i.v.). Leukocytes were prepared were 3h later. Cells having typical Forward Scatter and Side Scatter properties of lymphocytes were defined as Region 1 (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 case of male rats PJ-34 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 case of female rats PJ-34 pretreatment failed to alter this effect of LPS. N=6-8 animals per group.

Fig. 3. Gender differences in vascular effect of LPS: effect of PARP inhibition. Male or female Wistar rats (either pretreated with PJ34 [30 mg/kg, i.v., for 30 min] or its vehicle, saline) were given LPS injection (10 mg/kg i.v.). Thoracic aortae were obtained 3h later and endothelium-dependent relaxant responses to acetylcholine were recorded in isolated, precontracted thoracic aortic rings. The left side of the figure shows that in male animals, LPS treatment induces a significant loss of acetylcholine-induced vascular

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relaxations, which is evidenced by both a reduction of the relaxant response to acetylcholine (*P<0.05) and by a significant (p<0.01) shift to the right in the EC_{50} value to acetylcholine from $3.6\pm1.5x10^{-7}$ M to $26\pm13x10^{-7}$. These changes are prevented by inhibition of PARP with PJ34: the relaxation curve is restored ($^{+}$P<0.05) and sensitivity to acetylcholine is improved, the EC_{50} being $1.1\pm0.4x10^{-7}$ (p<0.01). The right side of the figure shows that female animals produce no endothelial dysfunction in response to LPS challenge and no change in the EC_{50} value for acetylcholine-induced relaxation ($6.2\pm2.0x10^{-7}$ vs. $5.5\pm4.1x10^{-7}$ before and after LPS). Pharmacological inhibition of PARP attenuates the maximal endothelium-dependent relaxations in female animals challenged with LPS ($^{+}$P<0.05) and shifts the EC_{50} value for acetylcholine-induced relaxations to the right to $41\pm17x10^{-7}$ (p<0.01). Values shown represent mean±SEM of n=10-12 determinations.

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 poly(ADP-ribose) (PAR), as measured 6h after LPS injection. This increased PAR staining is consistent with PARP activation. Bottom: Estrogen-pretreated animals (20 mg/kg 17-beta-estradiol) respond to LPS with a reduced degree of PARP activation. Stainings shown are representative from 4-5 animals per group are shown.

Fig. 5. Estrogen stabilizes PARP and ERα interactions with DNA.

Part A: PARP and ERα interacts with DNA cooperatively. EMSA was performed as described under "Materials and Methods." Purified PARP (5 pmoles) or ERα (5 pmoles) 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 estrogen (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] to lane 4 [PARP and estrogen receptor in the absence of estrogen]).

Part 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 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, 60 in lanes 7 and 14. Lanes 8 and 15, 9 and 16, and 10 and17 received 3 ng, 30 ng and 100 ng of sonicated plasmid DNA respectively. The final concentration of the estrogen (ED) added was 50 pmoles. In the absence of estrogen, addition of two-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 3-4 independent experiments are shown.

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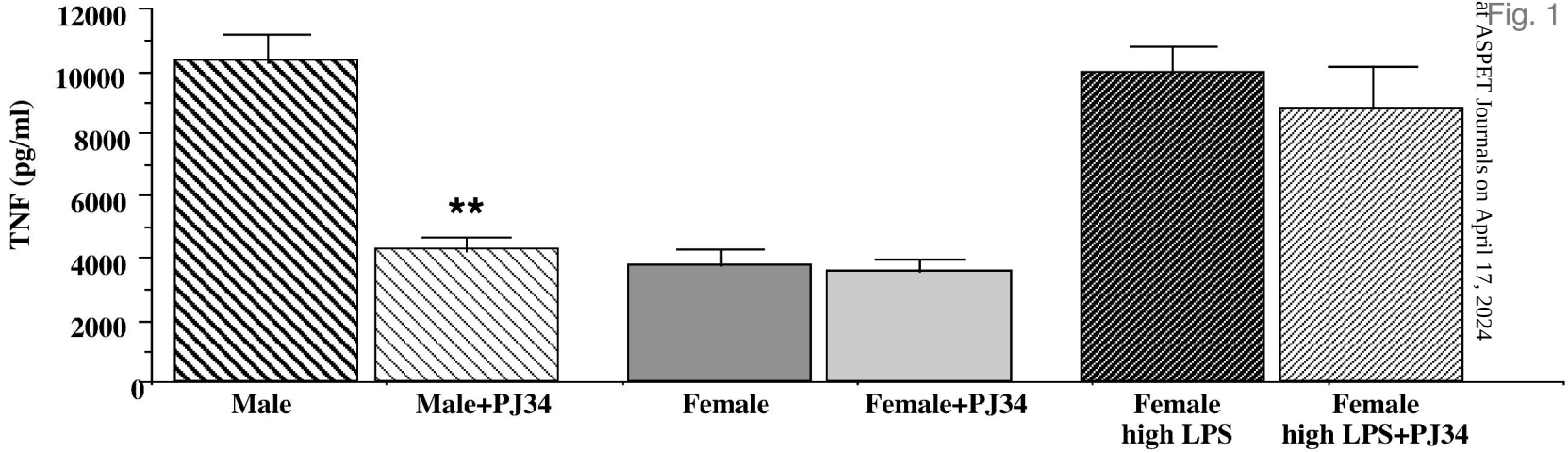
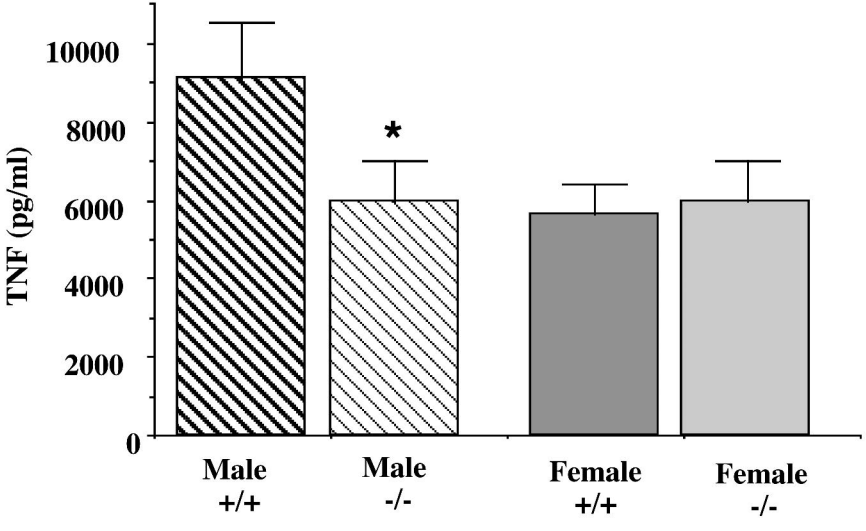
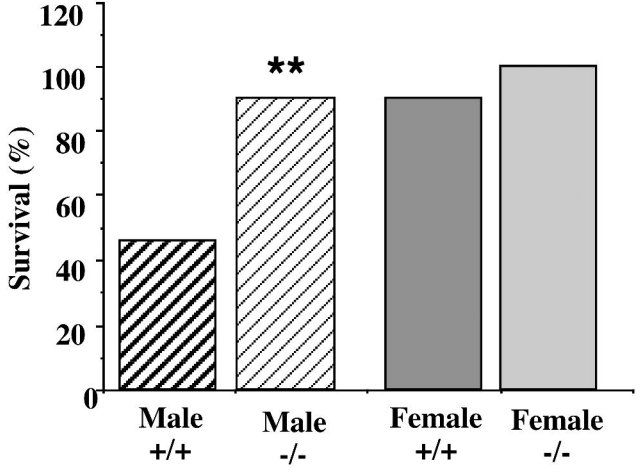
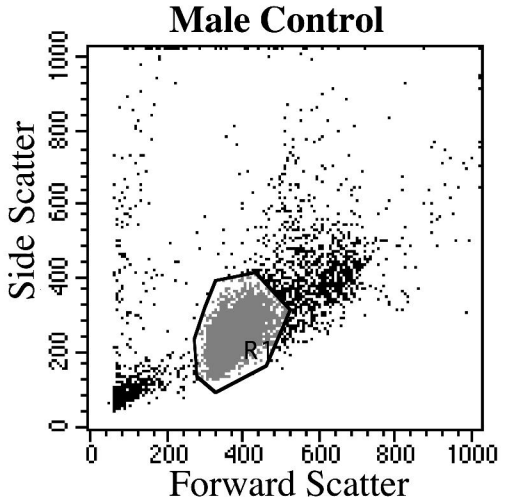
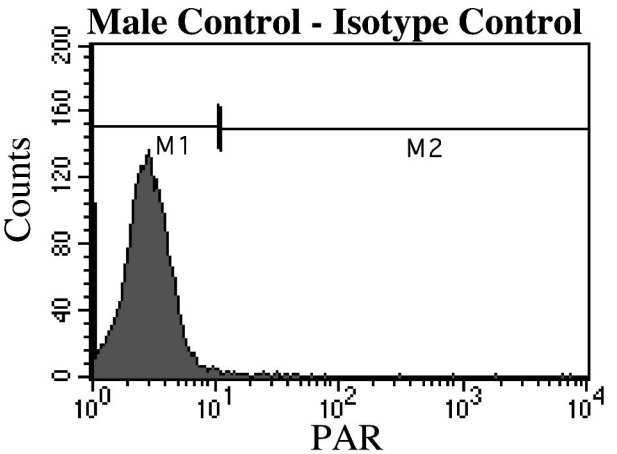
Fig. 6. Proposed model for the interaction of estrogen, estrogen receptor and PARP in conjunction with DNA single strand breakage and PARP activation. The top panel shows the situation in the absence of estrogen (PARP and ERα interact weakly, and PARP can recognize DNA strand breaks and can become catalytically activated). In the presence of estrogen (bottom panel), PARP and ERα interact strongly, which may anchor PARP to the DNA and reduce its ability to recognize DNA single strand breaks and thereby may prevent its activation.

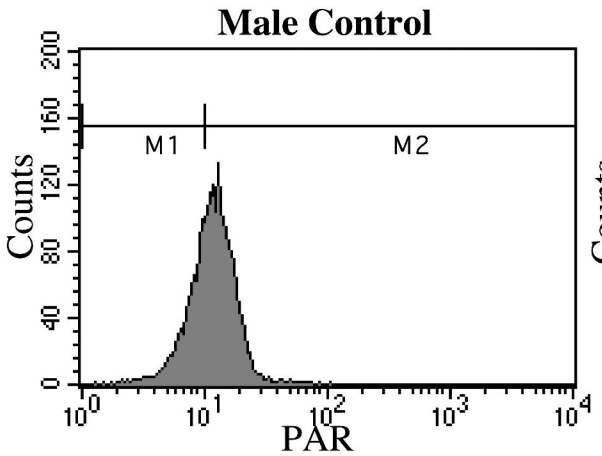


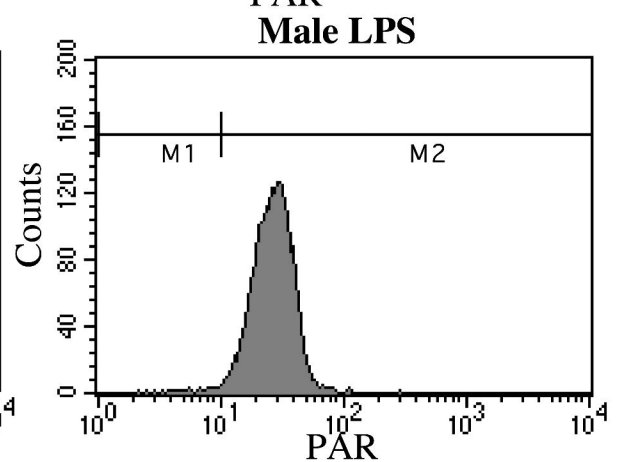


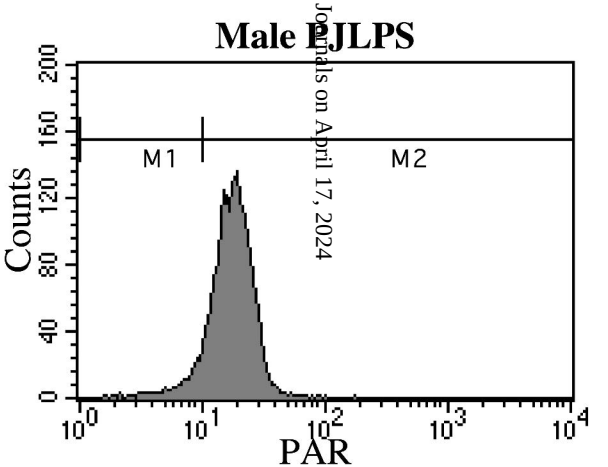


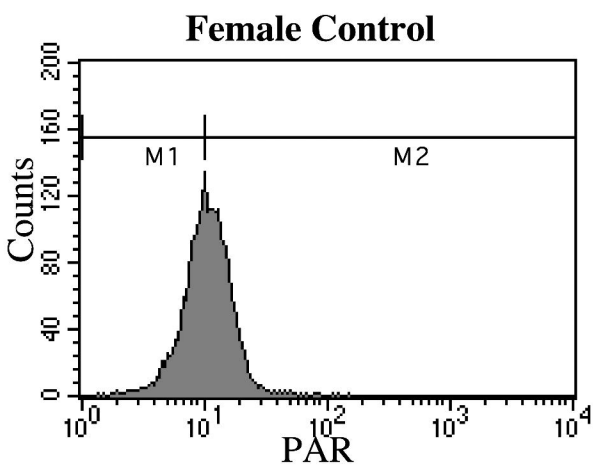


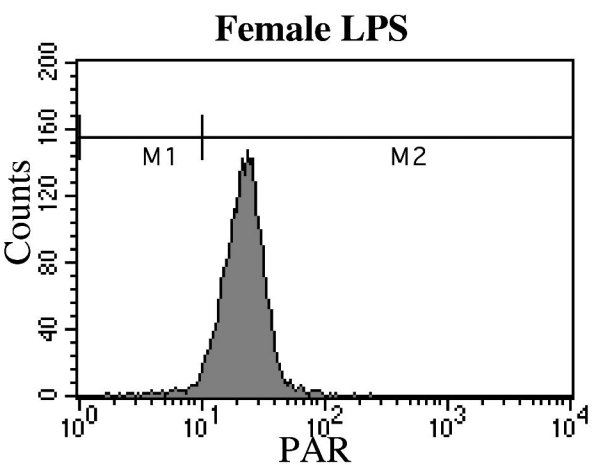












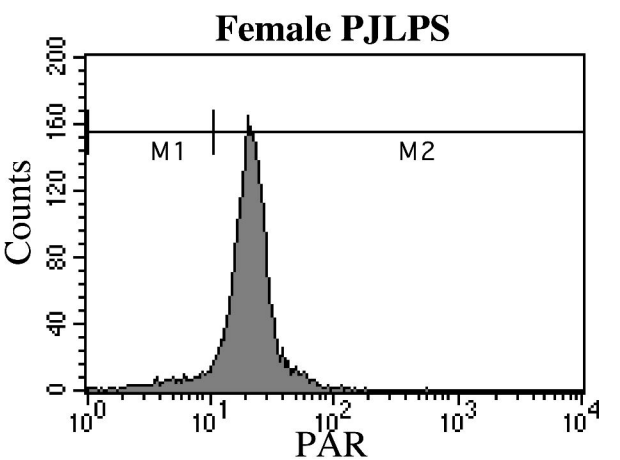
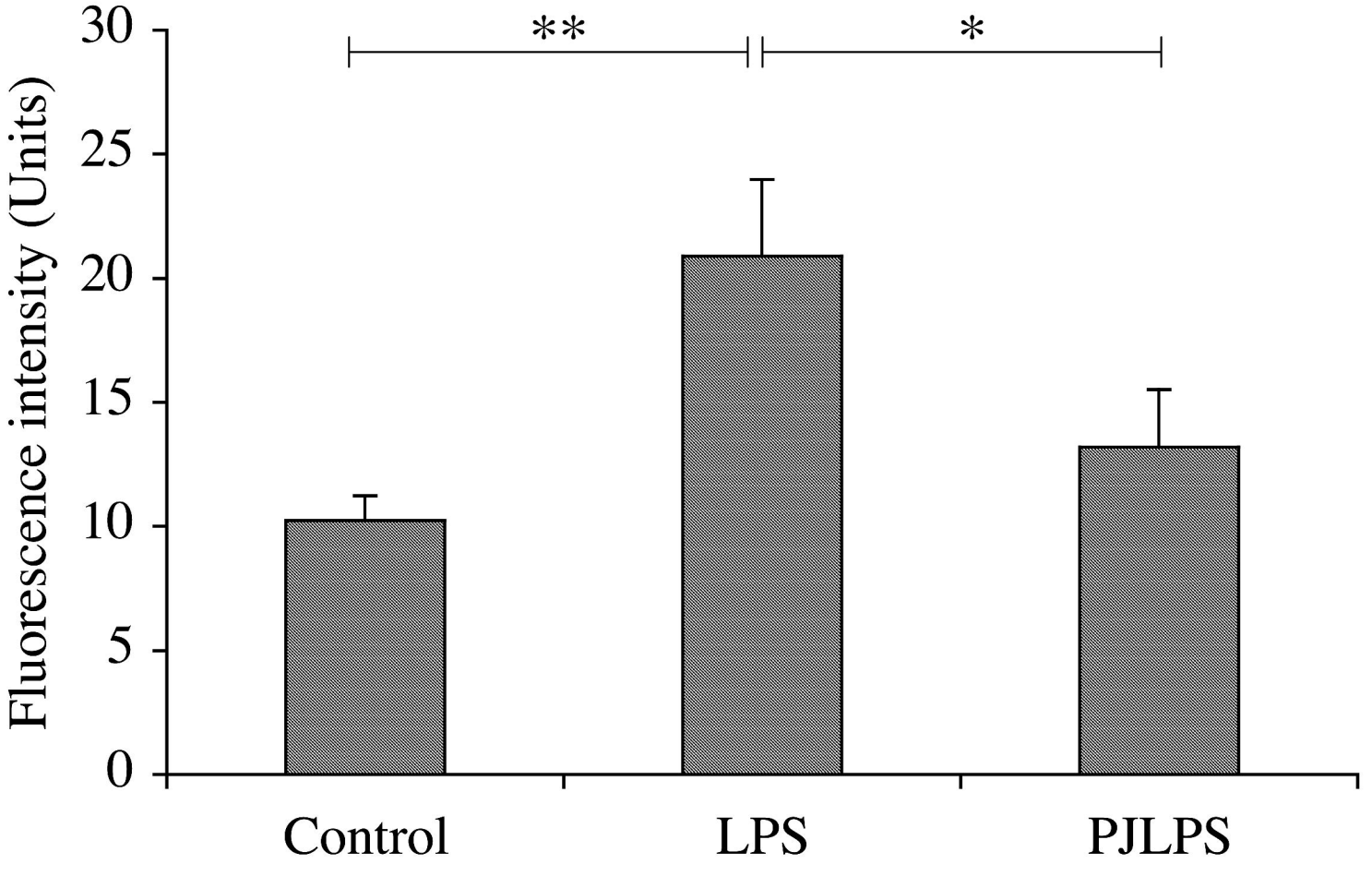
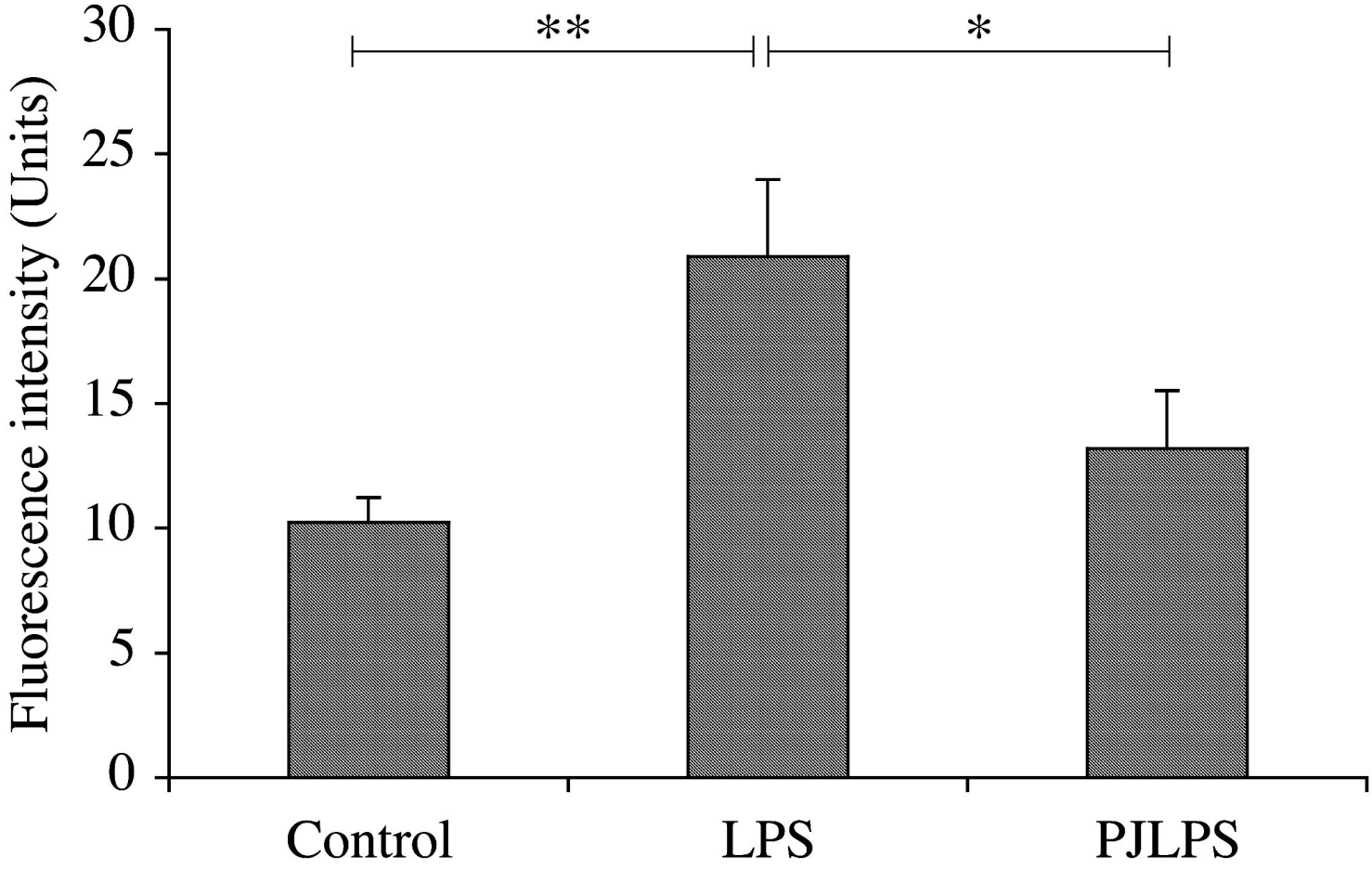
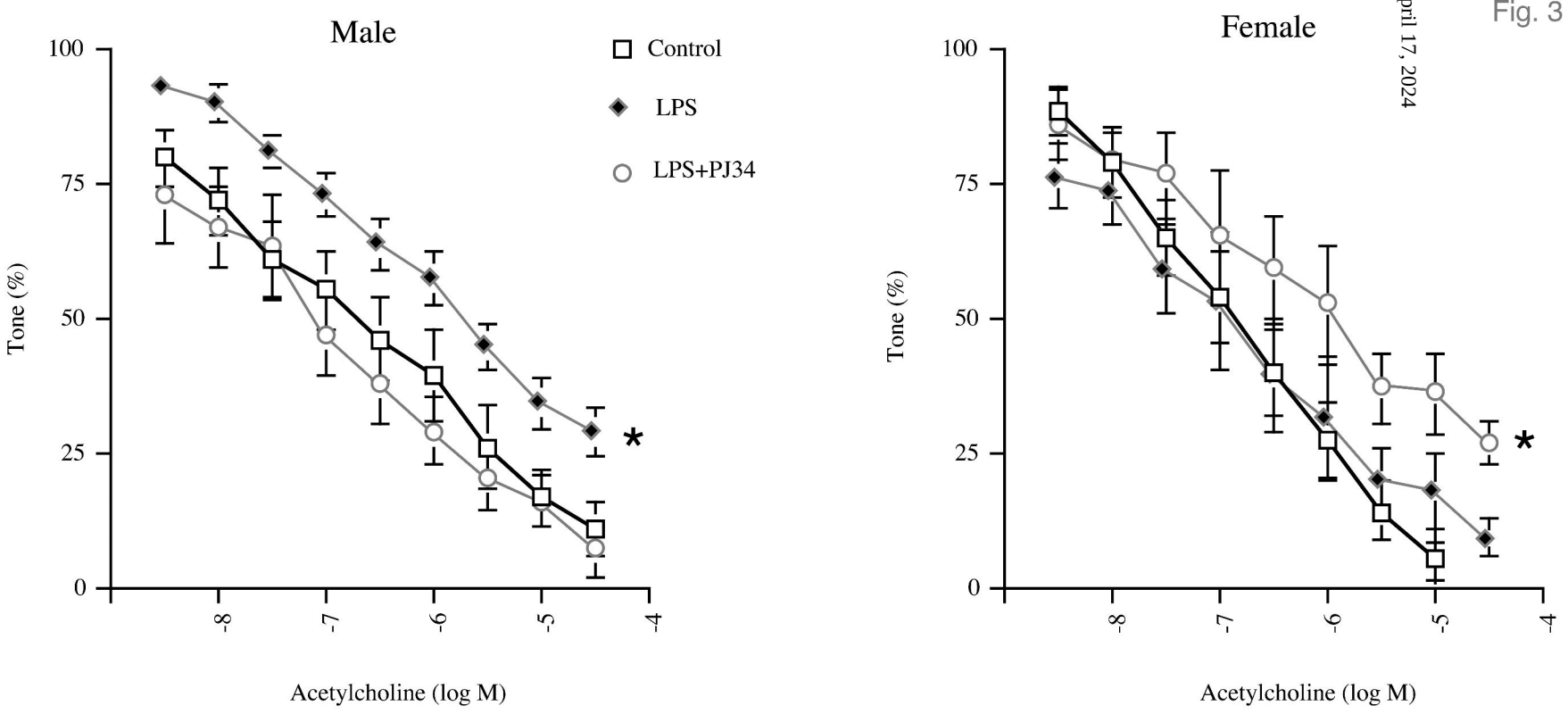


Fig. 2b







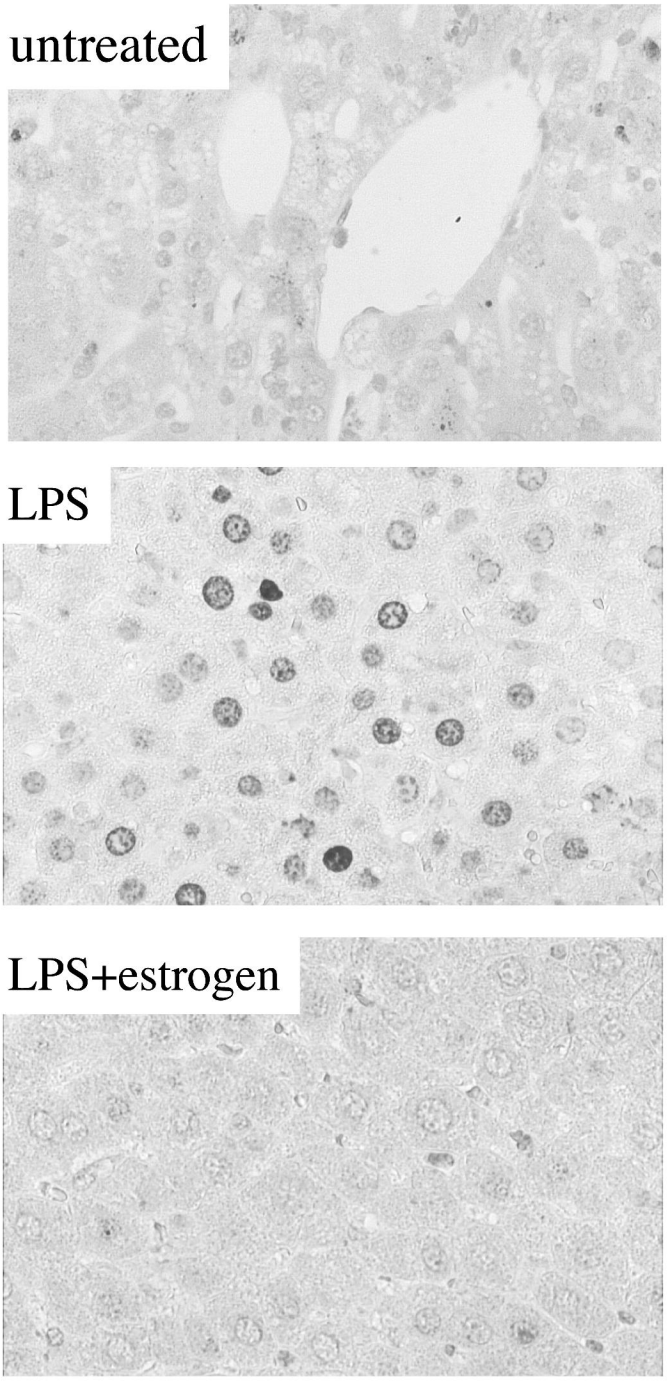
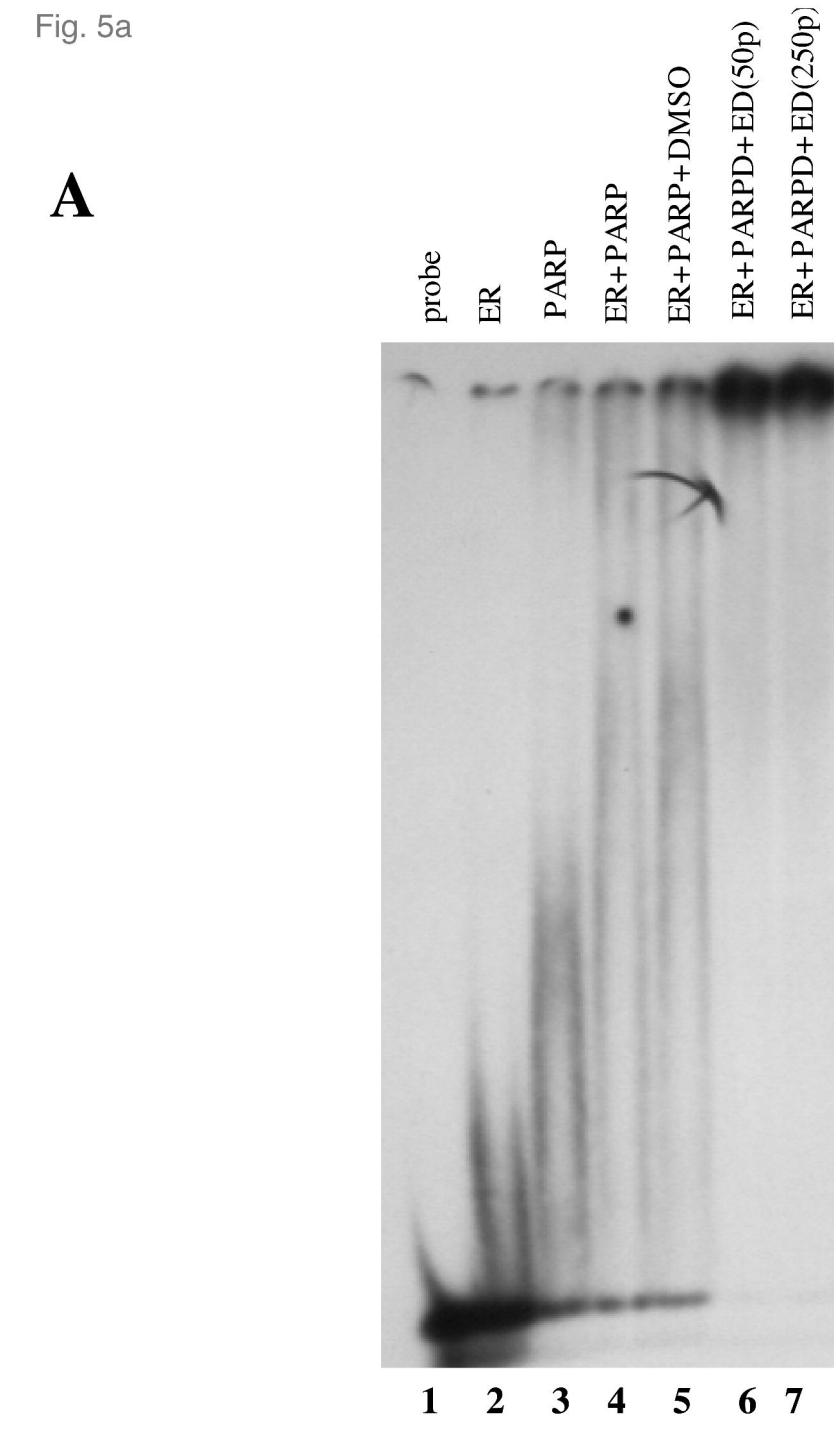

untreated
LPS
LPS+estrogen

Fig. 5a


A
probe
ER
PARP
ER+PARP
ER+PARP+DMSO
ER+PARPD+ED(50p)
ER+PARPD+ED(250p)
1
2
3
4
5
6
7

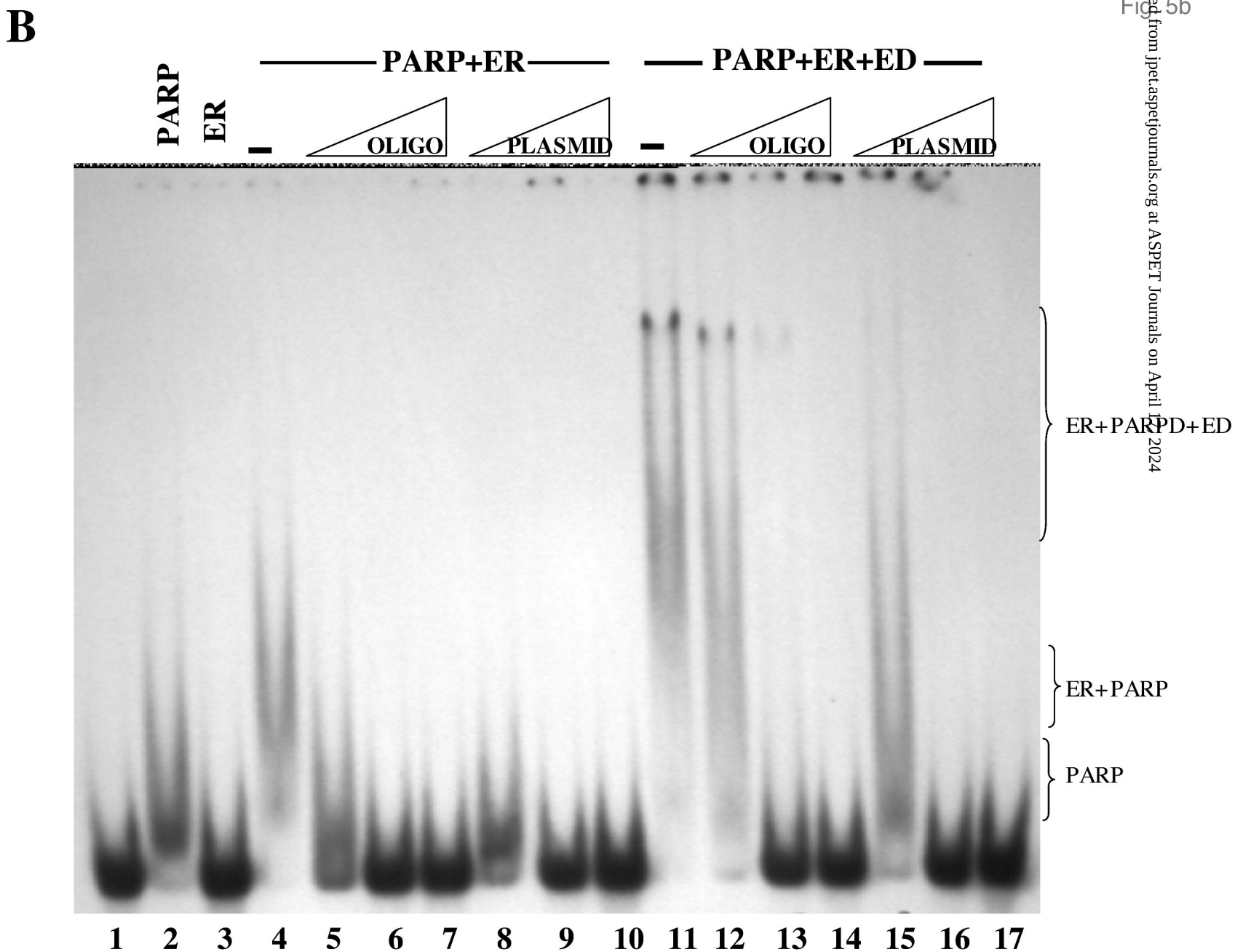


Fig. 6.