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ORIGINAL PAPER

Estrogen replacement regimen and brain infusion of lipopolysaccharide differentially alter steroid receptor expression in the uterus and hypothalamus

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Abstract The regimen of estrogen replacement can alter the consequences of estrogen therapy and stressors. To determine the long-term effects and interaction of these systems on the brain and periphery, adult female rats were infused with lipopolysaccharide (LPS) into the fourth ventricle of the brain for 4 weeks, and ovariectomized rats were administered either constant or pulsed regimens of estrogen replacement (17β-estradiol) until sacrifice at 8 weeks. Constant, but not pulsed, estrogen replacement reduced ERα and increased HSP90, HSP70, and PR_B uterine protein levels. Both estrogen regimens increased ERβ, HSP27, and PR_A uterine proteins. Both regimens reduced hypothalamic levels of ERα, but not ERβ, HSP, or PR. No changes were observed in the hippocampus. Long-term brain infusion of LPS activated microglia and reduced body weight, but did not alter corticosterone or nitrotyrosine levels. LPS infusion into intact rats suppressed uterine weight, increased ERα and decreased HSP90 in the uterus. LPS did not alter uterine weight in ovariectomized rats treated with constant or pulsed estrogen. Together, these data suggest the timing of estrogen replacement and neuroinflammatory stressors can profoundly affect uterine and hypothalamic steroid receptor expression and may be important parameters to consider in the post-menopausal intervention with estrogen.

Keywords
Estrogen receptor (ER; ERα (ESR1), ERβ (ESR2)) · Heat shock protein (HSP; HSP90 (HSPAA1), HSP70 (HSPA1B), HSP27 (HSPB2)) · Progesterone receptor (PGR; PR_A, PR_B) · Lipopolysaccharide (LPS) · Estrous cycle · Fluctuating regimen

Introduction

Hormone replacement is used to treat undesirable symptoms associated with menopause and has been explored as a possible preventative therapy for diseases such as Alzheimer's disease, stroke and cardiovascular disease (for review [1]). Results from observational studies have conflicted with randomized controlled trials in all three disease states (for review [1]), which has raised caution concerning the use of estrogens and progestins. Clinical trials, meta-analyses, and animal studies have attempted to reconcile these findings to determine the factors influencing the outcome of hormone therapy in post-menopausal women.

The timing of estrogen initiation and replacement regimen appear to be two factors that contribute significantly to the efficacy of hormone therapy [2]. Initiating hormone therapy closer to menopause has been explored as a means of altering the risk of cardiovascular disease [3]. Studies in rats support the importance of estrogen timing as constant estradiol replacement enhanced working memory when initiated immediately after ovariectomy but not after prolonged hormone deprivation [4]. Thus, the timing of constant hormone therapy initiation may be important for

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prevention, but does not appear to be effective if disease processes have already begun (for review [5]).

The regimen of estrogen replacement also appears to play a role and studies have begun to address whether pulsed administration of estrogens may be more efficacious [6–8]. Quality of life in women is improved by pulsed, but not constant estrogen therapy [9]. Moreover, pulsed estrogen replacement reversed cognitive impairments in primates and ovariectomized rats, whereas constant estrogen had no effect [10, 11]. The differential response produced by constant and pulsed estrogen regimens may be mediated, in part, by the ability of pulsed estrogen to better activate rapid signaling pathways important for neuroprotection and cognition [12].

Estrogen receptors (ER) mediate many of these rapid signaling pathways and ERα plays an important role in protecting the brain, cardiovascular and immune systems from inflammatory insults [13–15]. Brain inflammation is a component of several diseases present in the post-menopausal population and hormones may alter disease progression through their effects on inflammation (for review [16]). For example, ERs are expressed on inflammatory cells, such as microglia and monocytes, and estrogen can block inflammatory signaling and gene expression induced by inflammatory stressors in the brain, such as lipopolysaccharide (LPS; for review [16]). In turn, inflammatory processes may alter the efficacy of hormone therapy in post-menopausal women [17]. Thus, the regimen of constant estrogen replacement or its combined presence with inflammation may underlie some of the differences observed between experimental animal models and recent clinical trials.

We previously reported that infusion of LPS into the female rat brain simulates a low-grade neuroinflammatory response which can alter the effects of estrogen replacement measured after 6 days [18]. Brain infusion of LPS produced an unexpected increase in uterine weight in rats treated with constant, but not pulsed, estrogen that was concomitant with a 90% reduction in ERα, consistent with reports that ERα knockout mice may be more sensitive to inflammatory insults [19]. Reductions in ERα combined with HPA axis-induced elevations in corticosterone or progesterone are thought to play a role in stress-induced increases in uterine weight [19, 20]. Thus, we hypothesized that LPS stimulates the HPA axis to produce elevated progesterone and corticosterone, which can enhance uterine growth if uterine ERα levels are suppressed. To test whether activation of the HPA axis is necessary to increase uterine growth, the current study infused LPS into the brain for several weeks to maintain a neuroinflammatory reaction (i.e. activated microglia) without the involvement of the HPA axis, which returns to baseline in response to chronic stressors. Thus, despite reduced levels of uterine ERα, we hypothesized that brain inflammation would be insufficient to increase uterine weight if the HPA axis was no longer activated. Moreover, we hypothesized that if constant estrogen can alter ERα levels, longer durations of replacement would be necessary to reduce ERα levels in brain regions important for endocrine homeostasis or learning and memory, such as the hypothalamus or hippocampus, respectively. The purpose of the current study was to compare the long-term effects (8 weeks) of constant versus pulsed estrogen replacement on steroid-related protein levels and determine whether inflammatory stressors in the brain alters the physiological consequences of these regimens.

Results

Downregulation of ERα in the uterus and hypothalamus by constant estrogen

The current study compared the effects of long-term constant and pulsed estrogen replacement on ER levels in the brain and uterus. The relationship between a chronic inflammatory stressor and estrogen regimen was also examined. ERα protein in the uterus was not altered from intact levels by 8 weeks of OVX ($P = 0.94$). Likewise, ERα protein levels were not altered by pulsed estrogen replacement despite a non-significant trend suggesting an increase ($P = 0.07$). In sharp contrast to pulsed estrogen replacement, constant estrogen produced a 85% decline in uterine ERα that was significantly lower than all other treatment groups (0.15× of OVX controls; $F_{(4,76)} = 9.71$, $P < 0.0001$, Fig. 1a). Brain infusion of LPS increased ERα levels 2.1 fold in intact rats compared to CSF ($t_{(15)} = 2.88$, $P < 0.02$) and these levels were higher than all other treatment groups (all $P < 0.05$). OVX abolished the ability of LPS to increase ERα in the uterus, as protein levels did not differ from CSF controls ($P = 0.63$).

ERα protein levels in the hypothalamus were also affected by estrogen replacement (Fig. 1b). ERα levels were not altered by OVX ($P = 0.94$), but in contrast to the uterus, both replacement regimens resulted in a ∼35% reduction in hypothalamic ERα (0.7× and 0.6× of OVX controls for pulsed and constant estrogen, respectively; $F_{(4,51)} = 9.311$, $P < 0.0001$, Fig. 1b). LPS had no effect on ERα levels in the hypothalamus ($P = 0.60$). Furthermore, no changes in ERα protein levels were observed in the hippocampus by immunoblot ($P = 0.52$; data not shown) or [^{3}H]-17β-estradiol radioligand binding measured from the contralateral hippocampus ($F_{(4,97)} = 1.025$, $P = 0.399$; Table 1).

Induction of uterine ERβ by both estrogen regimens

OVX reduced ERβ protein in the uterus 62% from intact levels ($F_{(2,83)} = 10.20$, $P = 0.0001$, Fig. 1c) which was

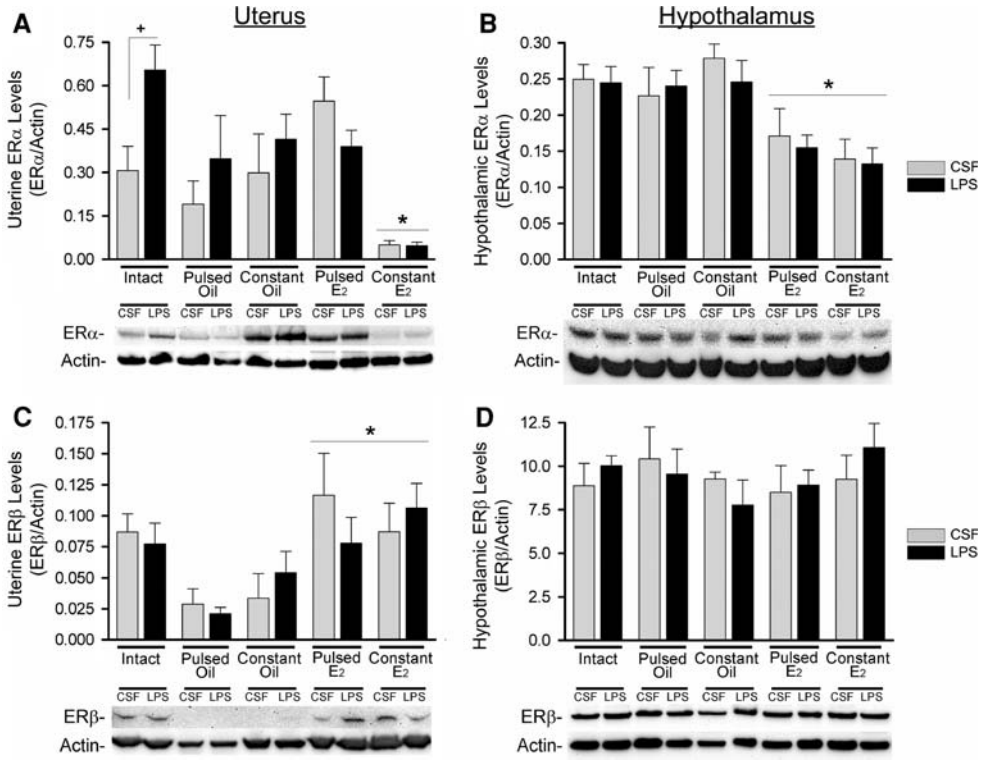

Fig. 1 Constant and pulsed estrogen replacement regimens had contrasting effects on ERα and ERβ levels in the uterus and hypothalamus. (**a**) ERα levels in the uterus were not altered by OVX or pulsed estrogen, but constant estrogen produced a >85% reduction in ERα that was significantly different from all other groups (*, all $P < 0.01$). Intact rats had 2.1× higher ERα levels when infused with LPS than CSF (+, $P < 0.02$) and these levels were higher than all other treatment groups (all $P < 0.05$). The ability of LPS to increase ERα from control levels was abolished by OVX ($P = 0.63$). (**b**) ERα levels in the hypothalamus were reduced ~35% by both constant and pulsed estrogen replacement ($P < 0.0001$). ERα levels were not altered by OVX ($P = 0.94$) or LPS ($P = 0.60$). (**c**) OVX reduced ERβ levels by 62% in the uterus which were restored to intact levels by both constant and pulsed estrogen replacement regimens (*, both $P < 0.001$). (**d**) ERβ protein levels were not altered in the hypothalamus by either hormone treatment ($P = 0.73$) or LPS infusion ($P = 0.72$). ER subtype/actin values were normalized between blots (see Methods), statistical differences determined by two-way ANOVA and Bonferroni post-tests. Values expressed as group mean ± SEM

Table 1 Biochemical markers after 8 weeks of treatment

Group			Circulating estradiol (pg/ml)	Circulating progesterone (ng/ml)	Estrogen binding sites in the hippocampus (fmol/mg protein)
Intact		CSF	9.0 ± 4.0[a]	26.9 ± 5.9[a]	30.1 ± 5.2
		LPS	20.8 ± 6.6[a]	15.6 ± 4.3[a]	27.2 ± 4.9
OVX	Pulsed oil	CSF	18.3 ± 5.3[a]	7.9 ± 3.0[b]	20.7 ± 5.5
		LPS	7.5 ± 3.0[a]	6.2 ± 1.2[b]	29.8 ± 4.7
	Constant oil	CSF	12.4 ± 7.0[a]	6.6 ± 1.5[b]	29.3 ± 5.5
		LPS	11.9 ± 2.4[a]	4.6 ± 0.9[b]	27.5 ± 5.2
	Pulsed estrogen	CSF	27.9 ± 6.1[b]	5.7 ± 0.7[b]	19.4 ± 5.2
		LPS	11.0 ± 1.8[a]	5.2 ± 1.0[b]	31.1 ± 4.2
	Constant estrogen	CSF	27.0 ± 5.5[b]	8.9 ± 2.9[b]	33.7 ± 5.5
		LPS	31.5 ± 6.5[b]	8.7 ± 1.5[b]	31.3 ± 4.5

Significant differences in circulating estradiol and progesterone levels are denoted by superscript letters after SEM (a–b, all $P < 0.05$). ChAT in the anterior cortex and hippocampal ERs were unchanged by estrogen regimen or LPS infusion. Statistical differences were determined by two-way ANOVA and values reflect group mean ± SEM

restored by both estrogen treatments (2.7× and 2.9× of OVX controls for pulsed and constant estrogen, respectively; $F_{(4,83)} = 5.299$, $P < 0.001$). ERβ levels were insensitive to LPS in the uterus ($P = 0.80$) and were unaltered in the hypothalamus ($P = 0.68$) or hippocampus ($P = 0.99$) with any treatment (Fig. 1d).

Regulation of uterine heat shock proteins (HSP90, HSP70, and HSP27)

Heat shock proteins (HSP) are estrogen-induced molecular chaperones that play a protective role in stress and estrogen receptor trafficking [21, 22]. Eight weeks of OVX reduced HSP90 in the uterus 34% from intact levels ($F_{(2,85)} = 11.86$, $P < 0.0001$; Fig. 2a). Constant, but not pulsed, estrogen increased uterine HSP90 1.8 fold from OVX levels ($F_{(4,85)} = 7.491$, $P < 0.0001$) and the two estrogen regimens differed significantly ($P < 0.05$). LPS treatment to intact rats reduced HSP90 levels to 0.7× of CSF levels ($t_{(16)} = 2.83$, $P < 0.02$). There was no change in HSP90 protein levels in the hypothalamus by LPS ($P = 0.59$) or estrogen treatment ($P = 0.90$, data not shown).

Uterine HSP70 levels were not altered by OVX ($P = 0.75$) or pulsed estrogen ($P = 0.34$), but increased 27% following 8 weeks of constant estrogen replacement (1.3× of OVX controls; $F_{(4,85)} = 3.359$, $P < 0.02$; Fig. 2b). LPS treatment did not alter HSP70 levels overall ($P = 0.29$) or within intact rats ($P = 0.07$). Likewise, there was no change in HSP70 protein levels in the hypothalamus by LPS ($P = 0.99$) or estrogen treatment ($P = 0.51$, data not shown).

HSP27 levels did not differ between intact and OVX rats ($P = 0.83$), but increased significantly following constant or pulsed estrogen replacement (3.7× and 3.5× of OVX controls, respectively; $F_{(4,38)} = 9.95$, $P < 0.0001$; Fig. 2c). HSP27 strongly correlated with uterine ERβ levels ($r_{(36)} = 0.66$, $P < 0.0001$), consistent with recent reports that HSP27 is an ERβ-associated protein involved in its signaling and localization in vitro [23]. Uterine HSP27 levels were not altered by LPS ($P = 0.96$) and HSP27 levels in the hypothalamus were undetectable using either a rabbit polyclonal or mouse monoclonal antibody (385877 and EMD-35, respectively; Calbiochem, San Diego, CA, data not shown).

Induction of uterine PRs

The progesterone receptor gene is estrogen-regulated and increases in the uterus in response to estrogen (for review, see [24]). As expected, constant and pulsed estrogen

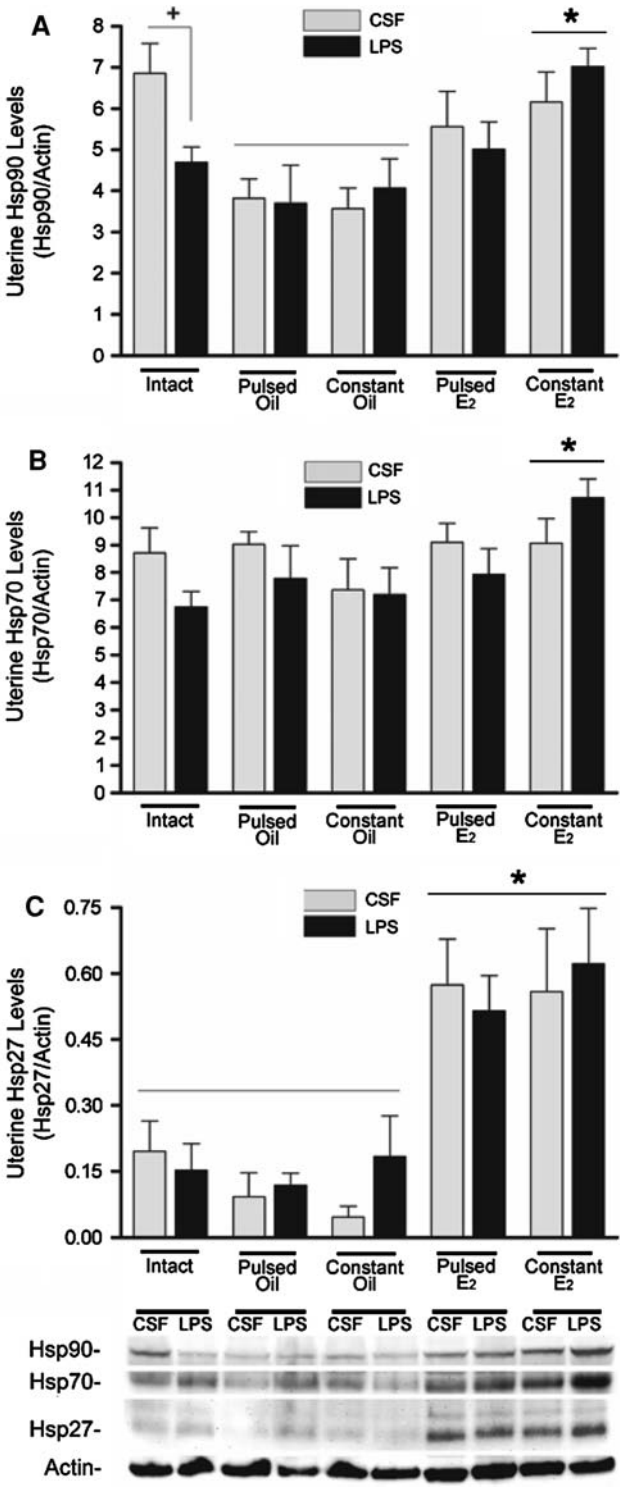

Fig. 2 Constant estrogen replacement increased HSP90, HSP70, and HSP27 (formally known as HSP90AA1, HSPA1B, and HSPB2, respectively). (**a**) Eight weeks of OVX reduced HSP90 levels ($P < 0.0001$) which were increased 1.8× by constant, but not pulsed, estrogen replacement ($P < 0.0001$). HSP90 levels were 34% higher in intact rats infused with CSF than LPS (+, $P < 0.04$). (**b**) Uterine HSP70 levels were not altered by OVX ($P = 0.75$) but increased 1.3× following constant, but not pulsed, estrogen replacement (*, $P < 0.02$). Brain infusion of LPS did not alter HSP70 levels in intact rats ($P = 0.07$). (**c**). Uterine HSP27 levels were not altered by OVX ($P = 0.83$) but increased ~3.6× following both estrogen replacement regimens (*, $P < 0.0001$). HSP27 strongly correlated to the increase in uterine ERβ (see Results). LPS had no effect on uterine HSP27 ($P = 0.96$). HSP/actin values were normalized between blots (see Methods), statistical differences determined by two-way ANOVA and Bonferroni post-tests. Values expressed as group mean ± SEM.

replacement increased uterine PR_A from OVX levels (2.0× and 1.7×, respectively; $F_{(4,62)} = 5.068$, $P < 0.002$, Fig. 3a). In contrast, only constant estrogen replacement increased uterine PR_B from OVX levels (2.2×, $F_{(4,62)} = 3.857$, $P < 0.01$, Fig. 3b). LPS had no effect on PR_A ($P = 0.55$) or PR_B levels in the uterus ($P = 0.93$). Moreover, PR levels were not altered in the hypothalamus by LPS or estrogen replacement ($P = 0.87$ and $P = 0.42$ for hormone effect on PR_A and PR_B, respectively; data not shown).

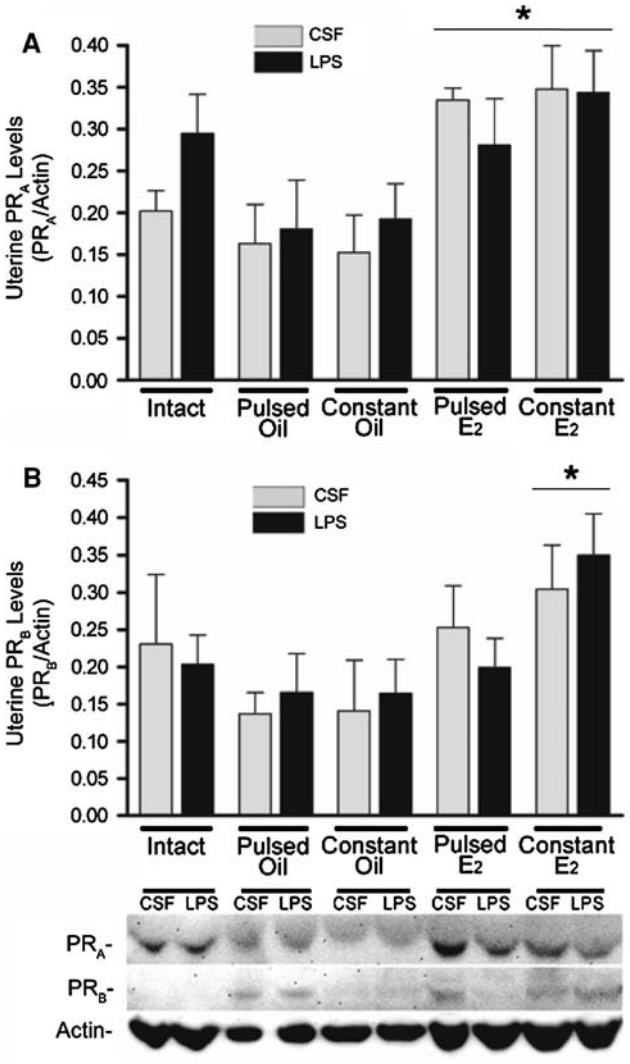

Fig. 3 (**a**) Progesterone receptor A (PR_A) increased in the uterus following both estrogen regimens (*, $P < 0.002$). LPS had no effect on PR_A levels of intact rats ($P = 0.14$). (**b**) Uterine PR_B levels increased 2.2× in rats receiving constant, but not pulsed, estrogen replacement (*, $P < 0.01$). PR/actin values were normalized between blots (see Methods) statistical differences determined by two-way ANOVA and expressed as group mean ± SEM

Brain infusion of LPS suppressed uterine weight in intact rats

Eight weeks of OVX resulted in low uterine weights (0.14 ± 0.05 g) that increased with pulsed and constant regimens of estrogen replacement (0.53 ± 0.05 g and 1.02 ± 0.05 g, respectively; $F_{(4,97)} = 46.106$, $P < 0.001$, see Fig. 4a). The increases in uterine weight correlated to vaginal cytology ($r_{(96)} = 0.75$, $P < 0.0001$), circulating estradiol ($r_{(93)} = 0.22$, $P < 0.03$), ERα levels in the uterus and hypothalamus ($r_{(76)} = -0.45$, $P < 0.0001$ and $r_{(50)} = -0.41$, $P < 0.003$, respectively), both uterine PRs ($r_{(61)} = 0.26$, $P < 0.05$) and uterine HSP90 ($r_{(84)} = 0.44$, $P < 0.0001$). Constant estrogen produced the largest increase in uterine weight (7.3× of OVX controls, $P < 0.001$); however, LPS infusion did not augment uterine growth in these rats ($P = 0.15$). In contrast, the effects of long-term brain infusion of LPS were limited to intact rats ($F_{(4,97)} = 3.004$, $P = 0.023$) who showed a 68% reduction in uterine weight when infused with LPS (0.19 ± 0.07) compared to CSF (0.60 ± 0.08 g; $P < 0.001$). The suppressive effect of LPS on uterine weight observed in intact rats correlated to increases in activated microglia quantified in the brain ($r_{(8)} = -0.68$, $P < 0.05$). Moreover, intact rats infused with LPS were predominantly in diestrus at the time of sacrifice (7/10) compared to only 1/9 rats infused with CSF

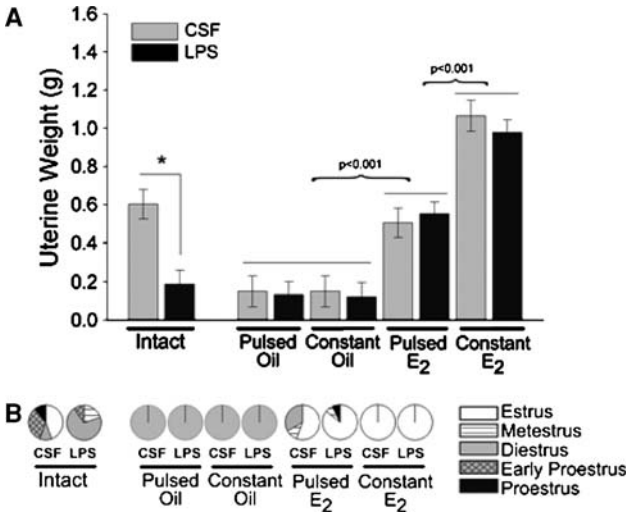

Fig. 4 Brain infusion of LPS suppressed uterine weight in intact rats. (**a**) Uterine weight increased in OVX rats after pulsed and constant regimens of estrogen administration (both $P < 0.001$); however, LPS did not augment uterine growth in constant treated rats. In contrast, uterine weights of intact rats were reduced 68% by LPS infusion into the brain (*; $P < 0.001$). Statistical differences determined by two-way ANOVA and values expressed as group mean ± SEM. (**b**) Percentage of rats in each phase of the estrous cycle at the time of sacrifice. Intact rats infused with LPS were predominantly in diestrus at the time of sacrifice (7/10) compared to only 1/9 rats infused with CSF

(Fig. 4b), consistent with previous reports demonstrating lowest uterine weights occurring in diestrus rats and the suppressive effects of inflammatory stressors on the estrous cycle [25, 26]. The remaining 3/10 intact rats infused with LPS were in early proestrus (1/10) and metestrus (2/10), both stages characterized by the presence of leukocytes. In contrast, 44% of CSF-infused intact rats had vaginal smears characterized by leukocytes, such as diestrus (1/9) and early proestrus (3/9), in addition to estrus (4/9) and proestrus (1/9) phases also being represented. Uterine weights are lowest during diestrus and highest at estrous [27]. Most intact control rats were in estrus at sacrifice and we observed a 3.6-fold swing between the highest and lowest uterine weights within this normally cycling intact control group.

Brain infusion of LPS altered circulating estradiol and progesterone levels

Consistent with the low uterine weights and diestrus vaginal cytology, OVX rats had low estradiol levels (12.4 ± 2.7 pg/ml) that increased 2.4× by constant, but not pulsed, estrogen replacement (29.6 ± 3.6 and 17.9 ± 3.2 pg/ml, respectively; $F_{(4,85)} = 3.75$, $P < 0.008$; Table 1). Estradiol levels were 61% lower in pulsed estrogen-treated rats infused with LPS (11.0 ± 1.8 pg/ml) compared to CSF (27.9 ± 6.1 pg/ml, $F_{(4,94)} = 2.606$, $P < 0.05$), possibly due to LPS effects on the cytochrome p450 system [28]. In contrast, LPS had no effect on estradiol levels in rats receiving constant estrogen ($P = 0.63$) or in ovary-intact rats ($P = 0.17$). Circulating estradiol levels were assayed twice in triplicate with similar results and estradiol levels correlated with increases in uterine weight ($r_{(93)} = 0.22$; $P < 0.03$) as well as the reductions in ERα protein levels in the uterus ($r_{(74)} = -0.33$; $P < 0.004$) and hypothalamus ($r_{(48)} = -0.35$; $P < 0.02$).

Circulating progesterone levels were significantly reduced from intact levels by 8 weeks of OVX (21.0 ± 3.7 and 6.3 ± 0.9 ng/ml, respectively; $F_{(2,96)} = 21.8$, $P < 0.0001$; Table 1) and were not restored by estrogen replacement ($P = 0.12$) or LPS infusion ($P = 0.44$) in OVX rats. Intact rats also showed no difference in progesterone levels when infused with LPS ($P = 0.14$). Circulating progesterone and corticosterone levels correlated ($r_{(96)} = 0.42$, $P = 1.99 \times 10^{-5}$), consistent with the finding that progesterone is produced by the adrenal gland as a biosynthetic precursor to corticosterone [29]. Moreover, removal of ovarian sources of progesterone by OVX resulted in a stronger correlation with a steeper slope between the two variables ($r_{(76)} = 0.77$, $P = 3 \times 10^{-16}$) than observed in intact rats ($r_{(17)} = 0.51$, $P < 0.03$).

Discussion

The current study begins to address the molecular basis underlying differences observed between pulsed and constant estrogen replacement regimens in post-menopausal women. We demonstrate that long-term constant and pulsed estrogen regimens differentially regulate steroid receptor levels in the brain and uterus (see Table 2). A striking difference between constant and pulsed estrogen treatments was observed in their ability to regulate ER subtypes in a tissue- and time-specific manner. Eight weeks of pulsed estrogen replacement was sufficient to maintain uterine ERα protein levels at intact levels. In contrast, a similar total dose of estrogen administered in a constant manner downregulated uterine ERα levels by more than 85%, suggesting that uterine ERα levels are sensitive to the regimen of estrogen replacement even when administered immediately after ovariectomy, the window when constant estrogen replacement may be most beneficial [30]. Pulsed estrogen may have produced different protein levels if quantified at other timepoints after the estrogen pulse. We find it of interest that both estrogen regimens increased uterine ERβ protein, suggesting independent regulation of ER subtypes within the same tissue. Others have shown

Table 2 Protein levels assessed by immunoblot in rats treated with pulsed or constant estrogen for 8 weeks

Arrows (↓, ↑) denote markers significantly changed ($P < 0.05$) and the fold change from OVX controls (see Results for details). Markers that did not change (–), were undetectable (und.) or were not determined (n.d.) are also indicated

	Uterus		Hypothalamus		Hippocampus	
	Pulsed E2	Constant E2	Pulsed E2	Constant E2	Pulsed E2	Constant E2
ERα (ESR1)	–	↓, 0.2×	↓, 0.7×	↓, 0.6×	–	–
ERβ (ESR2)	↑, 2.7×	↑, 2.9×	–	–	–	–
PR_A (PGR)	↑, 1.7×	↑, 2.0×	–	–	n.d.	n.d.
PR_B (PGR)	–	↑, 2.2×	–	–	n.d.	n.d.
HSP90 (HSP90AA1)	–	↑, 1.8×	–	–	n.d.	n.d.
HSP70 (HSPA1B)	–	↑, 1.3×	–	–	n.d.	n.d.
HSP27 (HSPB2)	↑, 3.5×	↑, 3.7×	und.	und.	n.d.	n.d.
Actin (ACTB)	–	–	–	–	–	–

that the alteration of the ratio of ERα to ERβ can affect NPY regulation in the hypothalamus [31] and pancreatic cancer proliferation [32]; therefore, understanding the effect of estrogen regimens on this ratio may become increasingly important.

We expected to observe differences in hippocampal ER levels, consistent with the regimen-dependent effects on spatial working memory [11]; however, no changes in levels of either ER subtype were observed by immunoblot or radioligand binding in the current study, consistent with results seen after 6 days of treatment [18]. In the hypothalamus, several studies reported conflicting effects of estrogen replacement on ER levels [33–37], possibly due to nuclei sampled, the timing of intervention or regimen of estrogen replacement. We observed no change in either ER subtype after 6 days [18]; however, both estrogen regimens reduced ERα, but not ERβ, protein by 35% following 8 weeks of treatment. The hypothalamus plays a major role in endocrine homeostasis and these actions are mediated predominantly by ERα [38]. Thus, systems that utilize ERα-dependent induction of hormonal signals, such as the HPG and HPA axes [38, 39], may be particularly sensitive to long-term estrogen replacement.

The *Pgr* gene is regulated in an ER-dependent manner [40] and increases in the uterus in response to estrogen administration [24]. PR_A and PR_B expression are controlled by two different promoters that are both estrogen-regulated [41] and consistent with this notion, uterine PR_A increased following both regimens whereas only constant estrogen was sufficient to increase PR_B. PR_B expression can be stimulated via ERα, but not ERβ [42]; although, it is unclear why PR_B levels were higher in constant estrogen-treated rats who had reduced levels of uterine ERα. PR_A can repress PR_B [43], which functions as a more potent activator of transcription of PR target genes [44]. Thus, it is possible that differences in promoter usage by PR_A and PR_B may play a role in the ability of estrogen regimens to differentially alter these isoforms.

Heat shock proteins play an essential role in cellular trafficking, protein folding and protecting cells from stress (for review [45]). Uterine HSP70 and HSP90 levels increased in the current study by constant, but not pulsed, estrogen replacement. These HSPs and their co-chaperones can alter ERα stability and signaling [46–48] as well as play a role in ER ligand binding [49]. In contrast to HSP70 and HSP90, both estrogen regimens increased uterine HSP27 levels by 350%. HSP27 strongly correlated to increases in uterine ERβ in the current study, consistent with the finding that HSP27 can associate with ERβ to act as a co-repressor of estrogen signaling [50].

Many steroid receptors can directly modulate inflammatory processes [16, 51, 52] and it has been suggested that the regimen of constant estrogen or its combined presence with inflammation may influence the efficacy of estrogen replacement in post-menopausal women. We infused LPS into the brain to test the effect of low-grade inflammation on ER, PR, and HSP expression during estrogen replacement. While all rats showed LPS-induced increases in activated microglia, we were surprised to find that only intact rats showed LPS effects on ERα and HSP90 expression. Fasting has been shown to alter ERα expression [53], but no correlation was observed between body weight and any of the steroid proteins measured in the current study (data not shown). ERα plays a role in modulating inflammatory processes [13–15, 54] and, in response to the inflammatory cytokine, TNFα, can be recruited to the *Tnf* promoter with HSP90 as part of a transcriptional complex [55].

Uterine reproductive physiology and decidual growth are directly regulated by HSP90 and its co-chaperones [56], which can be altered by LPS [57, 58]. Stress can disrupt ovarian cyclicity (for review [59]) and inoculation of the uterus with inflammatory stressors, such as LPS or bacteria, can increase inflammatory cytokines, alter circulating hormone levels and prolong diestrus [25, 26]. Consistent with these findings, most intact LPS-infused rats were in diestrus after 8 weeks and had reduced uterine weight that correlated to activated microglia in the brain. While others have documented uterine effects within 2 weeks of inflammatory stressor initiation [25, 26], we report that these physiological changes can persist weeks after cessation of LPS infusion. These data highlight the long-term impact of inflammatory stress on reproductive function.

The peripheral effects of brain infusion of LPS could be mediated by the HPA axis, the sympathetic nervous system or LPS leakage from the brain [60]. We found no evidence of inflammatory cytokines (IL1β, TNFα) in the blood or brain of male rats after brain infusion of LPS (G.L. Wenk, unpublished observations); however, sex steroids can alter blood–brain-barrier permeability [61]. Downregulation of ERα combined with HPA axis-induced elevations in corticosterone or progesterone is hypothesized to mediate uterine growth [18–20]. Therefore, our use of chronic LPS, which attenuates the HPA response [62] allowed us to test whether the corticosterone and progesterone components of the LPS-induced inflammatory response were necessary for the increased uterine growth. Consistent with our hypothesis, the 85% decline in uterine ERα with constant estrogen was not sufficient to alter uterine weight in LPS-infused rats, as the HPA response was similar to CSF controls. It remains to be determined whether injections of corticosterone or progesterone would be sufficient to increase uterine weight in constant estrogen-treated rats; however, our work suggests that these factors may contribute significantly to the uterine response. Other factors that may play a role include ER chaperones and thyroid hormones,

which can crosstalk with estrogens and are affected by stressors (for reviews, see [63, 64].

The timing of hormone therapy after menopause is being increasingly recognized as an important factor in the success of the therapy [30]; however, the data presented here suggest that the regimen of estrogen replacement also plays a role. Constant and pulsed estrogen regimens altered steroid-related proteins in a tissue- and time-dependent manner, even when administered at the time of OVX. Many of these proteins are also affected by inflammatory stressors, suggesting that stress and estrogen regimen may become important factors in the post-menopausal intervention with estrogen.

Materials and methods

Subjects

Ninety-eight virgin female F-344 rats (Harlan Sprague–Dawley), aged 3 months, were housed in a temperature-controlled room (21°C) and maintained on a 12-h dark:light cycle with lights on at 07:00 h. Food and water were available ad libitum and rats were allowed to adjust to their new environment for 1 week following arrival. Rats were randomly assigned to one of ten treatment groups (see Table 3) and housed in triplicate with other rats undergoing the same experimental treatment to reduce the impact of housing on estrous cycle [65]. All procedures were in accordance with IACUC regulations at the University of Arizona.

Table 3 Experimental groups and numbers

Inflammation	Intact	Hormone group ovariectomized (OVX)			
		Pulsed oil	Constant oil	Pulsed estrogen	Constant estrogen
CSF	9	8	8	9	8
LPS	10	11	9	14	12

Brain infusion of LPS

Rats were anesthetized and underwent brain surgery as previously described [18, 66]. Briefly, artificial cerebrospinal fluid (CSF) or lipopolysaccharide (LPS, 1.0 μg/μl dissolved in CSF; *E. coli*, serotype 055:B5, TCA extraction; Sigma) was slowly infused into the 4th ventricle via an osmotic minipump (0.25 μl/h). All tubing was prefilled with CSF to delay infusion of LPS (24 h) until estrogen was present (see Fig. 5). The dose of LPS and 4-week osmotic minipump infusion activates microglia and induces a behavioral impairment in ovariectomized female rats when tested 8 weeks after surgery [66]. The effectiveness of the LPS infusion was verified after 8 weeks (see Table 3; methods detailed below) and, consistent with previous findings, LPS infusion increased the number of activated microglia in the thalamus ($F_{(1,97)} = 7.155$, $P < 0.009$) and reduced body weight compared to CSF controls, a difference maximal between 8 and 14 days after surgery ($P < 0.05$). Rats whose body weights dropped > 20% were syringe-fed a nutritional supplement (1.5 cc animal Stat; PRN; Pensacola, FL). All rats gained weight over the 8 week period but body weights at sacrifice remained lower in rats infused with LPS (147.8 ± 4.7 g and 177.1 ± 5.4 g for LPS and CSF, respectively; $F_{(1,97)} = 35.957$, $P < 0.001$; see Fig. 2). Body weights were not affected by ovariectomy or hormone replacement. Moreover, consistent with numerous reports demonstrating reduced inflammatory products following chronic stressors [62], long-term brain infusion LPS had no effect on cortical nitrotyrosine ($F_{(4,30)} = 2.257$, $P = 0.14$) or circulating corticosterone levels ($P = 0.13$; Table 4). Corticosterone levels are normally elevated in F344 rats [67] and were quantified from samples collected in the afternoon (12:00–5:00 p.m.) when circulating levels are highest [68]; however, LPS-stimulated corticosterone levels declined from 6 days [18] and no longer differed from CSF controls (Fig. 6).

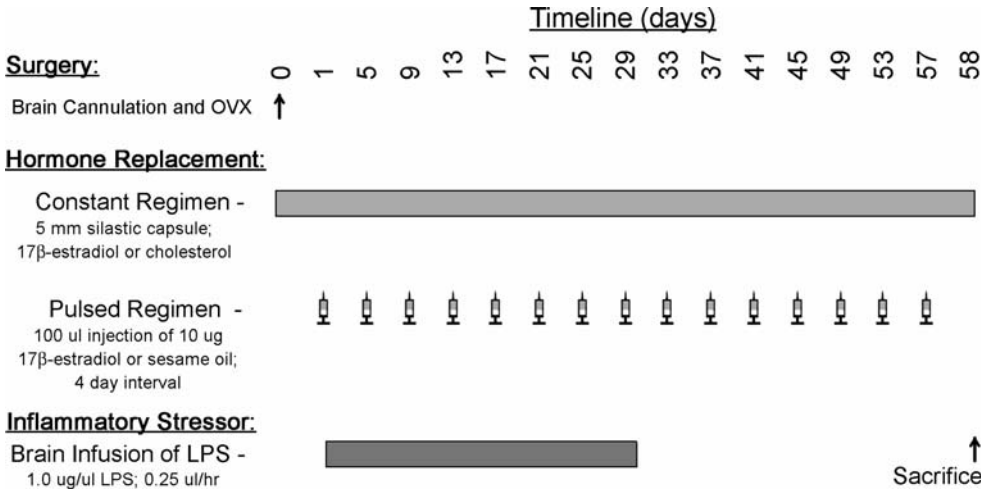

Fig. 5 Schematic representation of experimental timeline

Table 4 Verification of LPS effectiveness

	CSF (mean ± SEM)	LPS (mean ± SEM)	Significance
Activation of thalamic microglia	90.2 ± 5.3 nmol/mg protein	109.1 ± 4.6 nmol/mg protein	$F_{(1,97)} = 7.155$, $P < 0.01$
Body weight	177.1 ± 5.4 g	147.8 ± 4.7 g	$F_{(1,97)} = 35.957$, $P < 0.001$
Circulating corticosterone	564.4 ± 54.0 ng/ml	693.3 ± 67.3 ng/ml	n.s., $P = 0.13$
Cortical nitrotyrosine	4.01 + 0.08 nM	3.84 + 0.08 nM	n.s., $P = 0.14$

OVX and estrogen replacement regimens did not significantly alter these markers; therefore, all intact and OVX groups were collapsed into rats receiving CSF or LPS. Statistical differences were determined by two-way ANOVA and values reflect group mean ± SEM

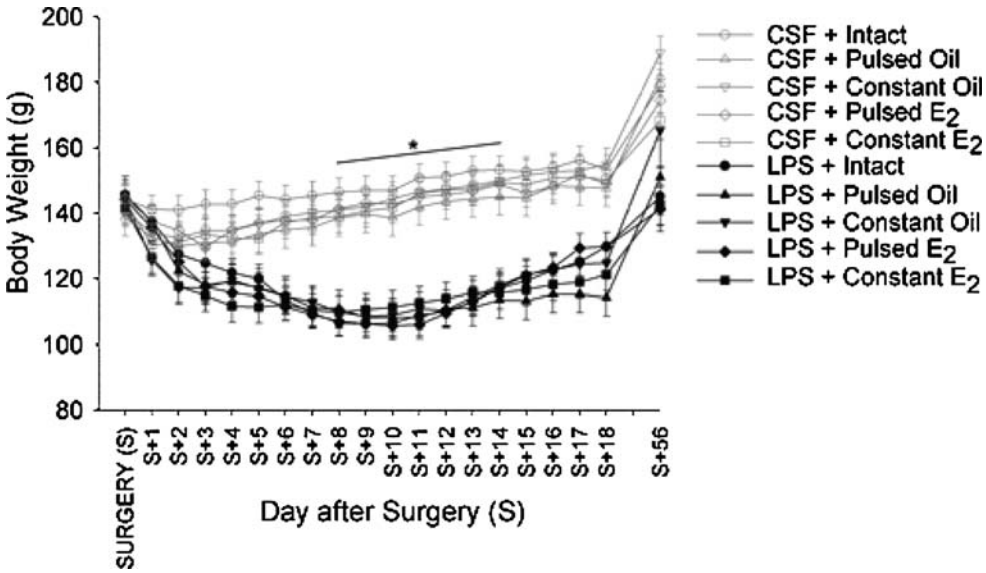

Fig. 6 Brain infusion of LPS significantly decreased body weight ($P < 0.001$). The rate of weight loss was sharpest in the first 6 days after surgery and the difference between rats infused with LPS and CSF was maximal between 8 and 14 days after surgery. Although rats in both treatment groups gained weight over time, LPS-infused rats weighed less at the time of sacrifice (Day 56) than rats infused with CSF ($P < 0.001$). Statistical differences were determined by two-way ANOVA and expressed as group mean ± SEM

Estrogen regimens

Immediately after implantation of the cannula, rats were bilaterally ovariectomized (OVX) or left intact. OVX rats were assigned to either a constant or pulsed estrogen replacement regimen (see Fig. 5). Rats receiving the constant replacement regimen were implanted (s.c.) with 5 mm silastic capsules containing estrogen (25% 17β-estradiol and 75% cholesterol) or oil (100% cholesterol) at the time of surgery, as previously described [18, 66]. Rats receiving the pulsed estrogen replacement regimen received one injection every 4 days beginning the morning after surgery. All injections were administered between 10:00 a.m. and 12:00 p.m. and consisted of either estrogen (10 µg 17β-estradiol dissolved in 100 µl sesame oil; Sigma, St. Louis, MO) or oil vehicle (100 µl sesame oil). The injection paradigm was selected to mimic the proestrus peak seen in the normal rat estrous cycle and has previously been used to enhance learning and memory [11]. Serial blood sampling from jugular catheters previously verified both estrogen regimens and determined that estradiol levels resulting from these injections returned to baseline within 24 h [18]. Approximately the same amount of estradiol was delivered by both estrogen regimens, as determined by area under the curve calculations extrapolated from serial sampling (data not shown). Serum levels of estradiol, progesterone and corticosterone were quantified using ^{125}I radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories; Webster, TX), according to the manufacturer's instructions.

Experimental design

Rats were exposed to experimental conditions described above for 8 weeks, consistent with our previous work investigating these factors on spatial working memory [66]. After 8 weeks (24 h after the last estrogen injection and between 12:00–5:00 p.m.), each rat was weighed, assessed for vaginal cytology, anesthetized with isoflurane and sacrificed by decapitation. Trunk blood was collected, serum isolated, and stored (−70°C) until analysis. Uterine

weights were quantified and brain dissections were performed as previously described [18]. Briefly, the entire hippocampus was taken bilaterally, the thalamus was isolated from between ∼−2.12 and −6.5 mm from Bregma and a hypothalamic sample was taken (∼3 mm width inferior to the anterior commissure between −2.12 and −0.8 mm from Bregma) which contained several hypothalamic nuclei including the medial preoptic and anterior hypothalamic areas, suprachiasmatic, periventricular, lateroanterior and paraventricular nuclei as well as the tuber cinerum. All results were analyzed by two-way ANOVA and Bonferroni post-tests (GraphPad Prism) unless described otherwise. Pearson's product-moment correlation coefficient was used to determine the association between variables (GraphPad Prism).

Brain chemistry

[^{3}H]PK11195 binding Activated microglia were quantified in the thalamus by in vitro [^{3}H]PK11195 filtration binding (1 nM; specific activity, 85.5 Ci/mmol; displaced by 20 μmol/l diazepam [18, 69]). The thalamus is sensitive to brain infusion of LPS in female rats [18, 66] and activation of thalamic microglia correlates to LPS-induced behavioral impairments on a Morris water maze task (L.K. Marriott, unpublished observations).

Estrogen receptor quantification Hippocampal ERs were quantified by [2,4,6,7-^{3}H(N)]-17β-estradiol radioligand binding (1 nM; specific activity, 95 Ci/mmol; displaced by 1 μM diethylstilbestrol [18, 70, 71]). The entire right hippocampus was assayed to ensure consistency between samples, as the density and subtype of the estrogen receptor varies between dorsal and ventral aspects of the hippocampus [72, 73]. ER levels were verified via immunoblot (see "Immunoblotting" below) in a subset of these rats using the contralateral hippocampus.

Nitrotyrosine levels A solid-phase ELISA (HK501, Cell Sciences, Canton, MA) was used to determine levels of nitrotyrosine, a marker of inflammation produced in the presence of nitric oxide [74] in samples of right posterior cortex as previously described [18].

Immunoblotting

Hypothalamic, uterine and left hippocampal tissues were homogenized in immunoprecipitation buffer, sonicated, centrifuged and electrophoresed onto pre-cast 4–12% Bis–Tris NuPage gels (Invitrogen; Carlsbad, CA) as previously described [75]. Equal amounts of protein (55 μg for hypothalamus; 30 μg for uterus; 10 μg for hippocampus) were loaded as determined by bicinchoninic acid assay (Pierce Biotechnology Inc., Rockford, IL). One animal per treatment group was included on each gel, and at least four gels were run per region. Protein was transferred to polyvinylidene diflouride membranes (Westran PVDF, 0.2 μM; Fisher) and blocked in 5% nonfat dry milk (NFDM) as described previously [75]. Primary antibodies [ERα (AB15, 1:500; Neomarkers, Fremont, CA); ERβ (PA1310B, 1:1000; Affinity Bioreagents, Golden, CO); heat shock protein (HSP)90 and HSP70 (formally known as HSP90AA1 and HSPA1B, respectively; 1:2000, Becton-Dickinson, Franklin Lakes, NJ), HSP27 (formally known as HSPB2; 1:2000, 385877, Calbiochem, San Diego, CA); progesterone receptor (PR, formally known as PGR; AB13, 1:1000, Neomarkers); and actin (formally known as ACTB; 1:5000, Sigma)] were incubated (overnight at 4°C) in 5% NFDM/Tris-buffered saline containing 0.1% Tween-20 and 0.02% sodium azide to prevent bacterial growth. Appropriate secondary and tertiary antibodies conjugated to horseradish peroxidase were used [18], and bands were visualized and quantified as previously described [75]. Recombinant human ERα (10 pg) or ERβ (100 pg) proteins (Affinity Bioreagents, Golden, CO) were loaded as positive controls. Blots were stripped (Reblot; Chemicon; Temecula, CA) and reprobed for each protein as described above.

Protein loading was controlled using actin; values did not differ between treatment groups in any region tested (data not shown). Band intensity was determined by subtracting out the background for each band and dividing by protein levels (i.e. actin). Blots were normalized to each other by dividing the maximal band intensity of all blots by the maximal band intensity of each individual blot. All treatment groups were equally represented on each gel and exposed to the same experimental conditions.

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