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Inflammation Research

Anti-inflammatory effect of selective estrogen receptor modulators (SERMs) in microglial cells

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Abstract. *Objective:* Our aim was to study how different SERMs modulate the inflammatory responses induced by lipopolysaccharide (LPS) or unmethylated CpG-oligonucleotides in mouse and rat microglial cells.
Materials and methods: Inflammatory responses of mouse N9 microglial cells and rat primary hippocampal microglia to lipopolysaccharide (LPS) exposure were recorded by the secretion of nitric oxide (NO) and cytokine IL-6 in two models where SERM was added either 24 h before LPS addition or simultaneously or even after the LPS exposure. The responses of 17β-estradiol, tamoxifen, raloxifene and ICI 182.780 were compared. Responses were recorded by ELISA, Northern and EMSA assays.
Results: SERMs but not 17β-estradiol induced a significant, concentration-dependent anti-inflammatory response both in rat primary microglial cells and in mouse N9 microglial cells. The response was observed both in NO and IL-6 secretion as well as in total IL-6 mRNA expression. We have recently observed that histone deacetylase (HDAC) inhibitors can potentiate the LPS-induced inflammatory response. Raloxifene and tamoxifen inhibited the potentiation of LPS response induced by trichostatin A, an HDAC inhibitor, in N9 microglia. A SERM-induced anti-inflammatory response was observed in acute models where SERM was added simultaneously or even up to 6 h later than LPS exposure. In contrast, the pretreatment of N9 microglia with tamoxifen or raloxifene for 30 h before LPS exposure did not provide any protection against the LPS response. We also observed that the raloxifene-induced protection in N9 microglia was connected to a decline of LPS-induced DNA-binding activity of AP-1 but not that of NF-κB transcription factors.
Conclusions: Our results show that tamoxifen, raloxifene and ICI 182.780 induce an anti-inflammatory response in acute models of mouse and rat microglial cells. It seems that this response is not estrogen receptor -mediated but, probably, is attributable to some SERM-induced modulation of LPS-activated pro-inflammatory signalling cascades.

Key words: Estrogen – Lipopolysaccharide – Innate immunity – Neuroinflammation – HDAC

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Introduction

Recently the importance has been recognized of inflammatory mechanisms in the pathogenesis of a variety of neurodegenerative diseases, such as Alzheimer's disease (AD), and stroke [1, 2]. Microglia, the resident macrophages of brain, are pathologically activated, e.g. in AD [3]. Microglial activation and proliferation are early events occurring in AD brains, and hence they may be the cause rather than a consequence of neurodegeneration [2]. Activated microglia secrete many neurotoxic molecules, such as nitric oxide and proinflammatory cytokines IL-6, IL-1β and TNFα the levels of which are increased in AD [3]. Glial activation may also be trophic, but trophic and toxic pathways can coexist side by side and thus contribute to progressive neuronal damage [2, 3].

Some studies indicate that the use of estrogen after menopause may reduce the risk of developing AD [4, 5]. This may be due to the anti-inflammatory effect of the estrogen, which has been observed in different studies in vitro [6, 7], although other neuroprotective responses of estrogen may be involved [8, 9]. However, there is a risk of tumours and dementia associated with estrogen and progestin replacement therapy [10]. This has switched interest to the use of alternatives, such as the selective estrogen receptor modulators (SERMs) in the treatment of neurodegenerative diseases [11, 12].

SERMs, such as tamoxifen, raloxifene and ICI 182,780, are molecules which bind with high affinity to estrogen receptors (ERs), acting as estrogen agonists in some tissue and as antagonists in others [13, 14]. These agonist/antago-

nist profiles for individual SERMs may differ also among brain areas [15]. ICI 182,780 is the only SERM without any known agonist effects [16]. Both of the known estrogen receptors, ERα and ERβ, exist in microglial cells [7, 17], and are thought to mediate classic receptor-mediated responses. Furthermore, there is a vast literature demonstrating that estrogen and SERMs have several non-genomic effects, for instance, via protein kinases [18, 19].

In this study, we compared the effects of 17β-estradiol and various SERMs against lipopolysaccharide (LPS) - induced inflammation responses in rat primary microglial cells and mouse N9 microglia. We observed that tamoxifen, raloxifene and ICI 182,780 decreased the LPS-induced inflammatory response in microglial cells. This shows that SERMs have a significant anti-inflammatory potential in cultured microglial cells. However, 17β-estradiol did not provide the expected protection in parameters studied directly in microglial models. Our experiments also indicated that the direct anti-inflammatory response in microglial cells might be independent of estrogen receptors, probably mediated by the disturbing effects of SERMs on LPS-induced pro-inflammatory signalling cascades in microglial cells. ER-independent effects of SERMs, e.g. on MAPK and AKT pathways have been frequently documented in a variety of cells in vitro and in vivo models [8, 13, 14, 18, 20].

Materials and methods

Reagents

Tamoxifen, 4-hydroxytamoxifen, raloxifene and 17β-estradiol as well as trichostatin A were from Sigma (St Louis, USA). ICI 182,780 was from Tocris (Northpoint, UK). Mouse CpG oligonucleotides were purchased from HyCult Biotechnology (Uden, The Netherlands). Lipopolysaccharide used in all experiments was from *Escherichia coli* 055:B5 lyophilized powder (L 6529 from Sigma, St Louis, USA).

Murine N9 microglia

Mouse N9 microglial cell line was a kind gift from Dr. Paola Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy). The establishment of the N9 murine microglial line has been described in detail [21]. N9 cells were cultured in Iscove's Modified Dulbecco's Medium (Invitrogen, Grand Island, NY, USA) supplemented with 2 mM glutamine, 100 U/ml penicillium and 100 µg/ml streptomycin and 5% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA). N9 microglial cells were plated to 12-well plates (Nunc A/S, Roskilde, Denmark) at a density of 2×10^5 cells per well. Experiments were started 24 h after plating.

Rat primary microglial cells

Primary microglial cells matured on the astrocytes. Primary astrocytes were isolated from 1- to 2-day old Wistar rat cerebral cortices and midbrain and cultured as described by Kerokoski et al. [22]. Cell cultures for microglia were isolated as astrocytes, except that the culture flasks were not shaken. Two weeks after the isolation, the floating microglia were harvested from confluent astroglial layers by rotating the flasks on an orbital shaker for a few hours. Then the microglia were harvested from the same flasks 4–5 times, the time between each harvest being 7 days. Microglial cells were plated onto 24-well plates at a density of 5×10^4 cells/cm^2 for pure microglia culture. After 1–2 h, the medium was changed to remove nonadherent cells. Experiments were initiated 24 h after plating. The purity of the microglial cultures (over 95%) was confirmed using antisera to CD11b (OX-42, Serotec, Raleigh, NC, USA).

Northern blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to their protocol. Northern blot analysis of IL-6 mRNA expression was performed for N9 microglia as we have recently described [23]. Primer sequences, the preparation of riboprobes and the hybridization conditions were identical to our previous studies.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated and EMSA assays were performed as described earlier in detail [24]. Double-stranded oligonucleotides for the NF-κB-binding site were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The probes were labelled with T4 polynucleotide kinase (Promega, Madison, WI, USA). Non-specific binding was blocked by 2 µg of poly(DI-dC):poly(dI-dC) (Sigma, St Louis, USA). The assay conditions were as described earlier [24]. Four µg of soluble nuclear proteins were used for DNA-binding assays. DNA-bound and free probes were separated in a native 4% polyacrylamide gel. The radioactive bands were visualized with Storm 860 PhosphoImager and pixel volumes of bands were calculated with Image Quant 4.2a software (Molecular Dynamics, Sunnyvale, CA, USA).

ELISA, LDH and NO assays

The nitrite concentration in the medium was assayed by Griess reaction as described earlier [23]. LDH leakage to the medium was measured with the cytotoxicity kit obtained from Promega (Madison, WI, USA). The concentration of IL-6 in the medium was measured using OptEIA™ sets obtained from Pharmingen (BD Biosciences, San Diego, CA, USA).

Exposure models

We used two kinds of protocols to expose microglial cells to estrogen and SERMs. In the acute model, estrogen/SERMs were added at the same time as the stimulus (LPS or CpG-DNA) or even later, up to 6 h after the LPS addition. Media and cells were collected after 16–24 h depending on the experiment. The second model used was the pre-treatment (chronic) model where estrogen/SERMs were added 30 h before the LPS stimulus. This model provides the microglial cells with time to induce translational adaptations against inflammatory insults. Microglial cells were cultured during exposures either in the medium containing 5% charcoal-stripped FBS (HyClone, Logan, UT, USA) or in serum-free medium. The main observations were repeated several times in similar experiments or related experiments to verify the observations.

Statistical analysis

All values were expressed as means ± S.D (typically n = 6 in each group). The difference between control and treated groups was analyzed using Mann-Whitney *U*-test or Student's *t* test.

Results

Anti-inflammatory response of SERMs to LPS-induced microglial activation

SERMs induced a significant anti-inflammatory response both in N9 microglia (Fig. 1 and 3) and in rat primary

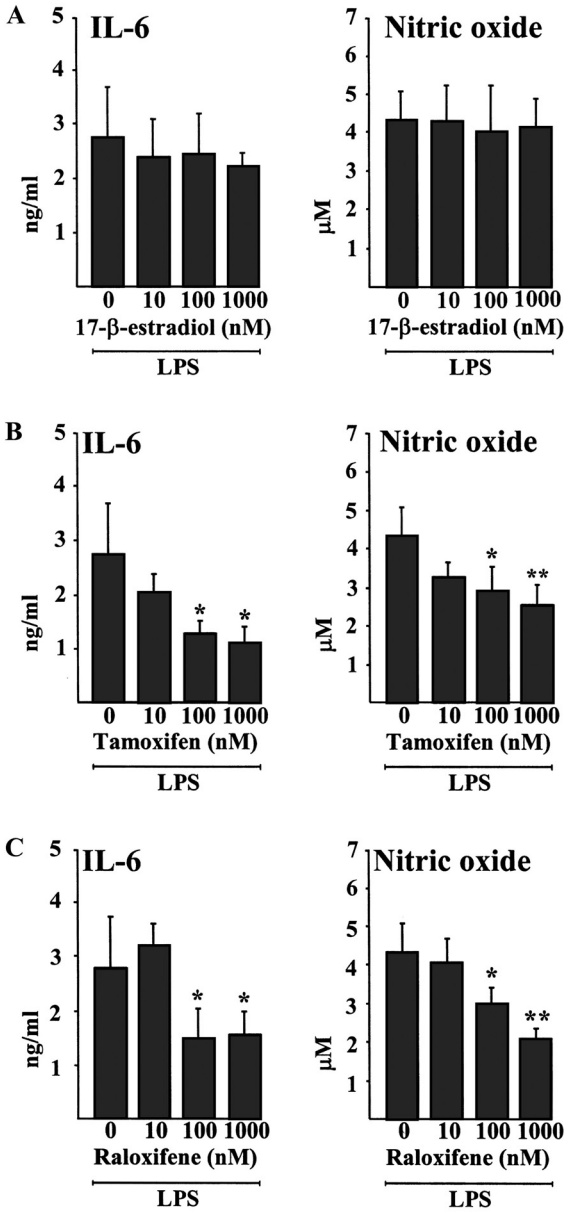

Fig. 1. Concentration-dependent anti-inflammatory response of tamoxifen and raloxifene to LPS-induced N9 microglial activation. LPS-induced IL-6 and nitric oxide secretion was assayed after 22 h exposure to LPS and either 17β-estradiol, tamoxifen or raloxifene. LPS concentration was 10 μg ml^{-1}. Values are means ± s.d. (n = 6 in each group). Statistical significance of SERM-induced reduction in LPS response: * $P < 0.01$, ** $P < 0.001$.

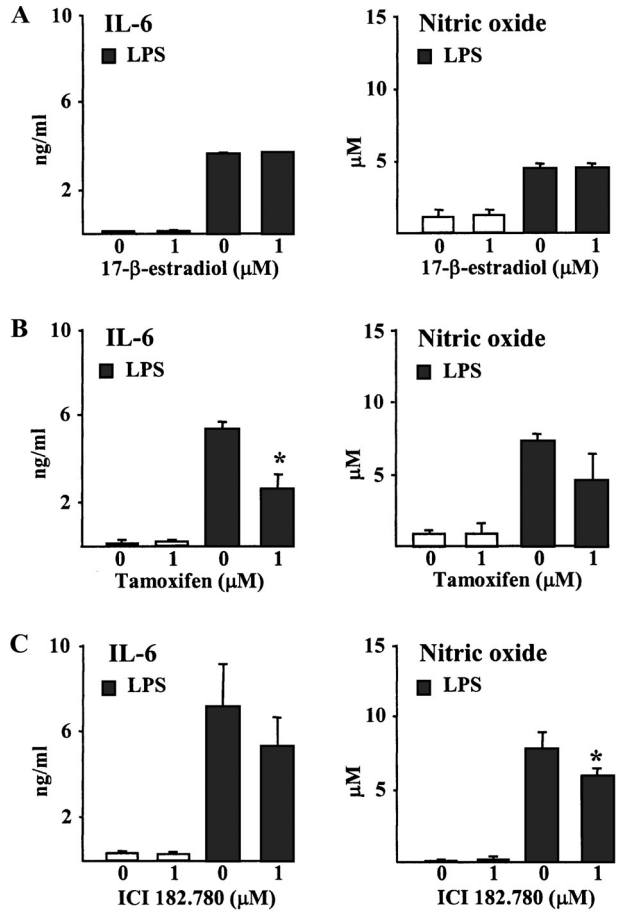

Fig. 2. Effect of 17β-estradiol, tamoxifen and ICI 182.780 on LPS-induced rat primary microglial activation. LPS-induced IL-6 and nitric oxide release after 24 h stimulation. 17β-estradiol and SERMs were added to primary microglial cells at the same time as LPS. LPS concentration was 5 μg ml^{-1}. Values represent means ± s.d. (n = 4–6). Statistical significance between LPS plus 17β-estradiol or SERM -treated groups as compared to the LPS-alone group: * $P < 0.01$.

microglia (Fig. 2). Our pilot experiments (Fig. 1) showed that tamoxifen and raloxifene induced a concentration-dependent decrease in the secretion of NO and IL-6 from N9 microglia. The decrease in IL-6 protein expression also occurred in N9 microglial cells (data not shown). The inhibitory effect appeared at the 100 nM level of tamoxifen and raloxifene in our assay conditions (Fig. 1). Interestingly, 17β-estradiol was ineffective in N9 microglial cells (Fig. 1 and Fig. 3). Tamoxifen and raloxifene did not evoke any increase in LDH release at 1 μM concentration of SERMs compared to controls in N9 microglia (Fig. 3) or in rat primary microglia (Fig. 4).

In rat primary microglia, tamoxifen (Fig. 2) and raloxifene (Fig. 4) significantly down-regulated the LPS-induced IL-6 secretion as well as ICI 182,780 (Fig. 2) and raloxifene (Fig. 4) decreased NO secretion. Interestingly, 17β-estradiol did not affect the secretion of IL-6 or NO in rat primary microglial cells (Fig. 2).

SERM responses can be either non-genomic or ER-mediated genomic responses [13, 14, 18]. LPS activates Toll-like receptor (TLR) -mediated signalling cascades in microglial cells to regulate the expression of inflammatory genes, such as cytokines and iNOS [26, 27]. Next, we studied whether SERMs could disturb the signalling cascade and hence modulate the inflammatory response. First, we exposed N9 microglia to LPS to activate the signalling cascades and later even up to 6h afterwards exposed the microglia to different SERMs (Fig. 3). This acute model revealed that tamoxifen and raloxifene induced an anti-inflammatory response in IL-6 and NO secretion which became reduced when the time between LPS activation and SERM addition was extended up to 6 h. This experiment also showed that 17β-estradiol was inefficient at inhibiting LPS-induced activation in N9 cells

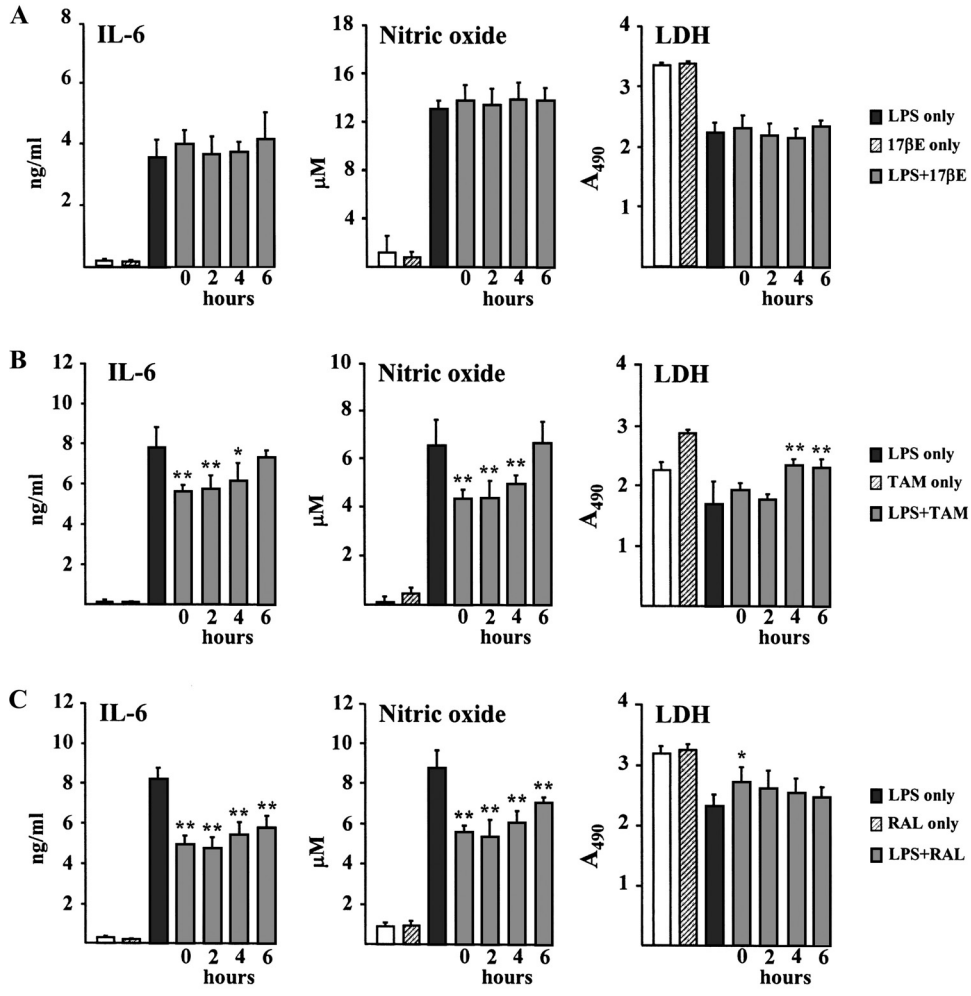

Fig. 3. Anti-inflammatory response of tamoxifen and raloxifene to LPS-induced N9 microglial activation. Microglia were exposed to 17β-estradiol or SERM simultaneously with LPS or 2 h, 4 h or 6 h later than LPS exposure for 22 h. LPS concentration was 10 μg ml^{-1} and concentrations of 17β-estradiol and SERMs were 1.0 μM. Values are means ± s.d. Statistical significances between LPS plus 17β-estradiol or SERM -treated groups as compared to the LPS-alone group: * $P < 0.01$, ** $P < 0.001$.

(Fig. 3). The final concentration of 1.0 μM of different SERMs used was non-toxic because the level of released LDH was not affected by the treatment (Fig. 3).

We also studied in rat primary microglia whether they show a similar response in the acute model as observed in N9 microglia. Figure 4 shows that in IL-6 secretion, the simultaneous addition of LPS and raloxifene produced the strongest protection which was reduced when raloxifene was added 2–6 h later than LPS. Nitric oxide secretion was reduced by raloxifene treatment independently of treatment time. Raloxifene did not increase LDH release which suggests that the treatment was not toxic (Fig. 4).

SERMs inhibit TSA-induced potentiation of LPS-stimulated microglial activation

We have recently shown that the histone deacetylase inhibitor, trichostatin A (TSA), strongly potentiates the LPS-induced inflammatory response both in N9 microglia and in rat primary microglia [23]. Figure 5 illustrates that TSA significantly potentiated the LPS-induced secretion of IL-6 from N9 microglial cells. Nitric oxide secretion was not induced by TSA treatment as such. Tamoxifen down-regulated both the LPS-induced and TSA-potentiated IL-6 secretion but interestingly, did not affect the TSA-potentiated NO secretion (Fig. 5). Raloxifene, similarly to tamoxifen, reduced the secretion of IL-6 from TSA-potentiated N9 microglia (Fig. 6C). Figure 5 shows that the ER-antagonist, ICI 182,780, reduced IL-6 and NO secretion from LPS-treated N9 microglia but interestingly, did not affect those of TSA-potentiated microglia. Figure 5 shows also that 17β-estradiol did not affect the LPS-induced IL-6 and NO secretion but slightly increased IL-6 secretion from TSA-potentiated N9 microglia. LDH release was not increased by different treatments compared to the control level (Fig. 5).

Next we studied whether SERMs could affect the mRNA levels of IL-6. Figure 6A and Figure 6B show that raloxifene inhibited the LPS-induced expression of total IL-6 mRNA in N9 microglia. Figure 6A and Figure 6B also reveal that the TSA treatment further potentiated the LPS-induced expression of total IL-6 mRNA. Interestingly, the potentiation was inhibited by raloxifene (Fig. 6A and Fig. 6B). Figure 6C

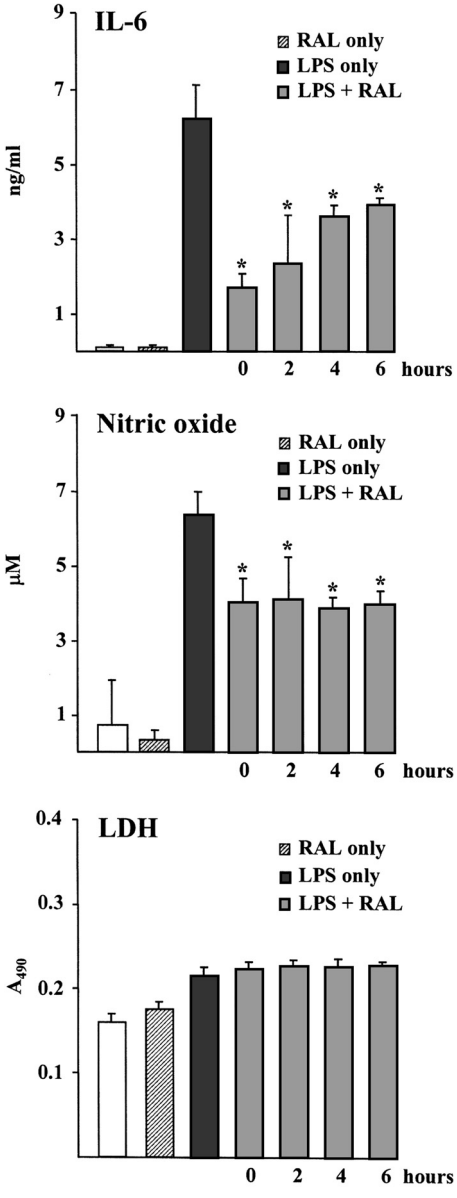

Fig. 4. Anti-inflammatory response of raloxifene to LPS-induced rat primary microglial activation. Microglia were exposed to raloxifene simultaneously with LPS or 2 h, 4 h or 6 h later than LPS exposure. Final concentrations used were LPS 5 µg ml^{-1} and raloxifene 5.0 µM. Values represent means ± s.d. (n = 6–8). Statistical significance between LPS plus raloxifene-treated group compared to LPS-alone group: *$P < 0.01$.

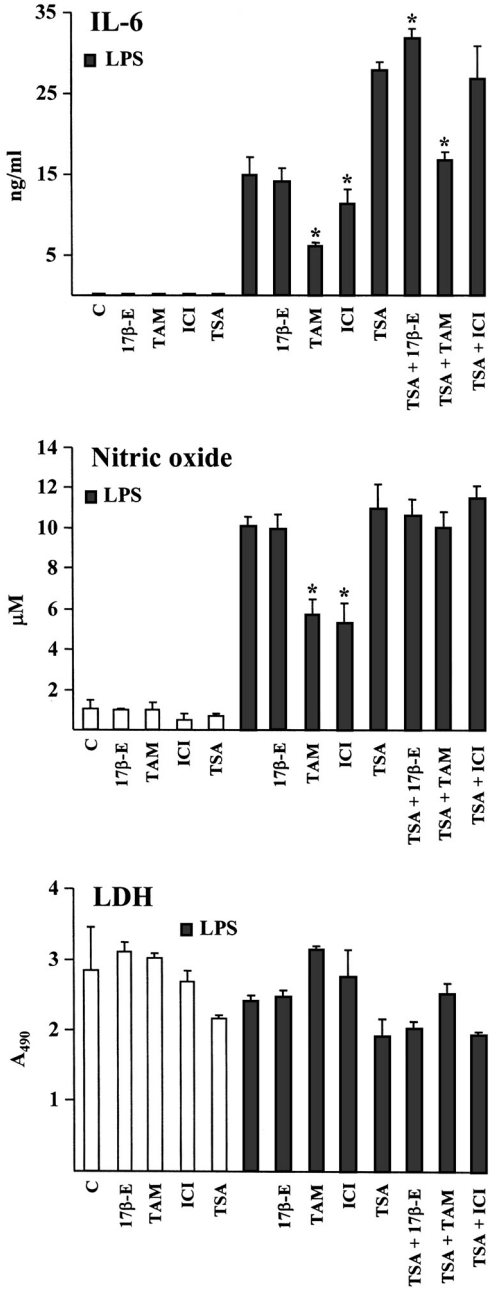

Fig. 5. Effects of 17β-estradiol, tamoxifen and ICI 182.780 on TSA-potentiated LPS response in N9 microglial cells. 17β-estradiol and SERMs were added to N9 cells at the same time as TSA and LPS and IL-6, NO and LDH release were recorded 22 h later. Final concentrations used were 10 µg ml^{-1} for LPS, 15 nM for TSA and 1.0 µM for 17β-estradiol, tamoxifen and ICI 182.780. Values represent means ± s.d. (n = 4–8). Statistical significance between LPS plus 17β-estradiol or SERM -treated groups is compared to LPS-alone or LPS plus TSA-treated group: *$P < 0.01$.

shows that the changes in total IL-6 mRNA levels correspond to the levels of IL-6 protein secreted to medium.

Anti-inflammatory effect of SERMs on CpG-induced microglial activation

LPS activates the inflammatory signalling cascades through Toll-like receptors TLR2 and TLR4 [26]. Next we studied whether SERMs could inhibit inflammatory cascades mediated by other TLRs than TLR2 and TLR4. We stimulated N9 microglia with unmethylated CpG oligonucleotides which activate TLR9 [28] and can induce neuronal injuries [29]. Figure 7 shows that raloxifene inhibited the secretion of IL-6 and NO induced by CpG. TSA prominently potentiated the CpG-induced secretion of IL-6 and NO. Raloxifene clearly inhibited the TSA-induced potentiation (Fig. 7). Raloxifene also showed a potent inhibitory capacity by reducing the IL-

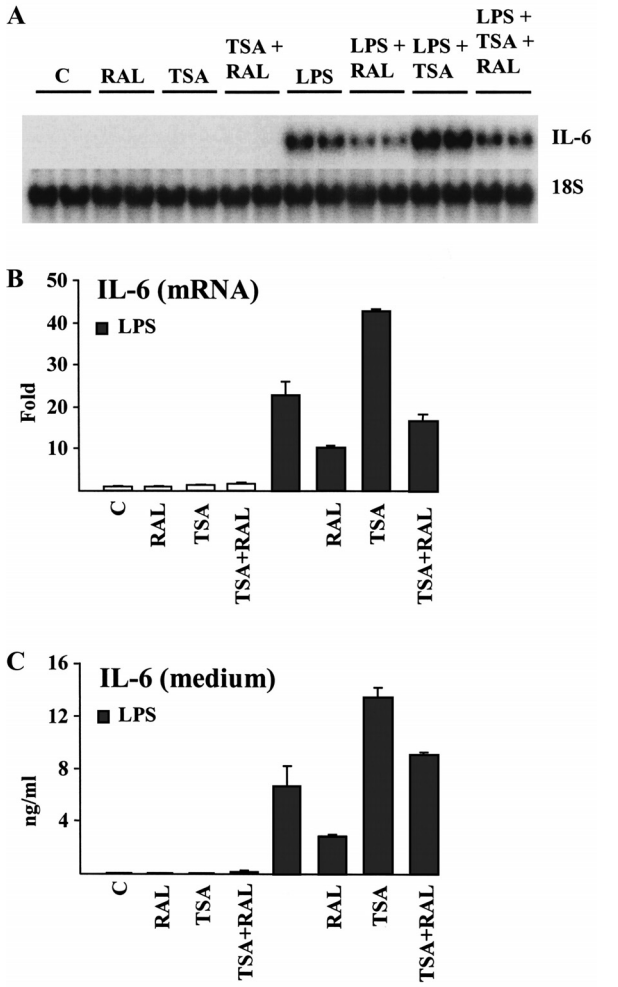

Fig. 6. Raloxifene down-regulates IL-6 mRNA expression in N9 microglia induced by LPS treatment or TSA-potentiated LPS treatment. Figure 6A shows Northern blot for total IL-6 mRNA expression compared to 18S. Treatment time was for 20 h. Figure 6B shows the changes in specific pixel values (see Methods) as folds compared control value. Figure 6C shows the IL-6 secretion assayed by ELISA in medium. Final concentrations used were 10 µg ml^{-1} for LPS, 15 nM for TSA and 1.0 µM for raloxifene. Values represent means ± s.d for samples in Figure 6A.

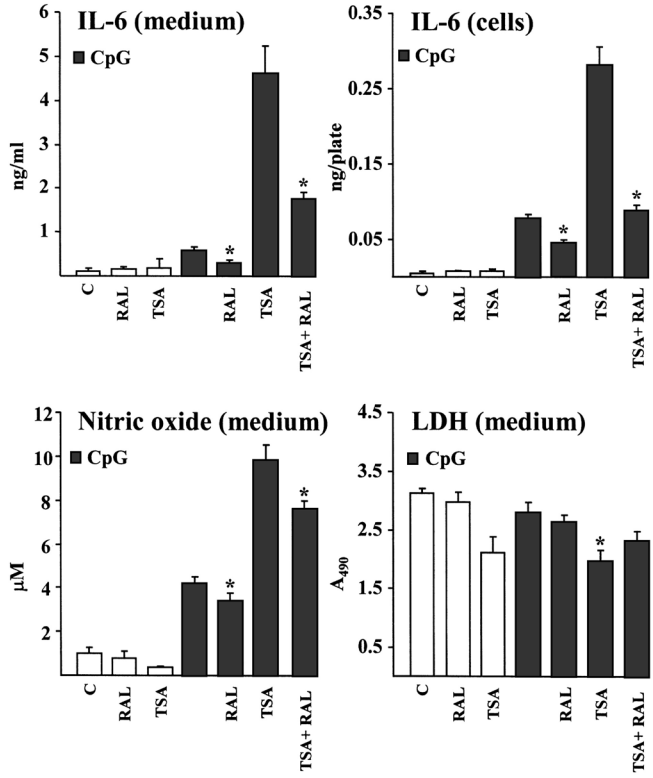

Fig. 7. Anti-inflammatory response of raloxifene to unmethylated CpG-induced N9 microglial activation. Microglia were exposed to raloxifene simultaneously with CpG or both TSA and CpG for 22 h. Final concentrations used were 1.0 µM for CpG oligonucleotides, 15 nM for TSA and 1.0 µM for raloxifene. Values are means ± s.d. (n = 4–6). Statistical significance between CpG-alone and raloxifene plus CpG or both TSA and CpG: *$P < 0.01$.

6 protein levels in N9 cells treated with CpG, especially those treated with CpG and potentiated with TSA (Fig. 7). CpG or/and TSA treatments did not show any cytotoxic effects assayed by LDH release.

Anti-inflammatory response by raloxifene is associated with down-regulation of AP-1 but not NF-κB binding activity to DNA

Estrogen and SERMs have several non-genomic effects, such as effects on MAP kinase signaling and protein kinase A and C which may have ER-independent effects [for review see 20]. Since AP-1 and NF-κB are the two major transcriptional regulators in inflammatory signalling, we studied whether the raloxifene-induced anti-inflammatory response would be associated with changes in DNA binding activities of AP-1 and NF-κB factors. Figure 8 shows that LPS treatment enhanced the DNA-binding activity of both AP-1 and NF-κB factors. However, simultaneous raloxifene and LPS treatment inhibited the DNA-binding activity of AP-1 but not that of NF-κB (Fig. 8). This result suggests that raloxifene inhibited the inflammatory response by affecting AP-1 -mediated pathway.

Pretreatment of N9 microglia with SERMs does not show any general anti-inflammatory response against LPS

SERMs modulate genomic responses mediated by ERs, those being either agonist or antagonist effects [13, 14, 18]. Next we studied whether the pre-treatment of N9 microglia with 17β-estradiol, tamoxifen, raloxifene or ICI 182,780 might induce an anti-inflammatory response against LPS stimulus, as observed in the acute exposures (see above). Figure 9A and Figure 9B show that the pre-treatment of N9 microglial cells with 17β-estradiol, tamoxifen or raloxifene for 30 h followed by LPS exposure for 22 h did not inhibit the LPS-induced secretion of IL-6 and NO. Tamoxifen pre-treatment, on the contrary, slightly elevated the secretion of IL-6 (Fig. 9A). In contrast to tamoxifen and raloxifene, ICI-182,780 reduced the secretion of IL-6. Interestingly, ICI 182,780 reduced the tamoxifen-induced increase in IL-6 secretion (Fig. 9D). As in the acute treatments (Fig. 3), 17β-estradiol

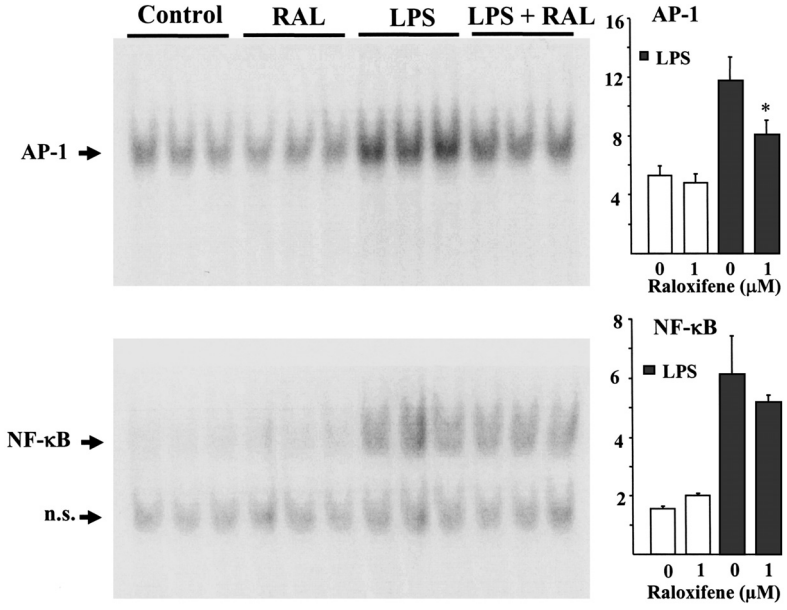

Fig. 8. Effects of raloxifene on LPS-induced up-regulation of DNA-binding activity of AP-1 and NF-κB factors in N9 microglial cells. Left panel shows specific DNA-binding activities of AP-1 and NF-κB complexes with the different treatments. Microglia were exposed to LPS (10 μg ml^{-1}) and raloxifene (1.0 μM) for 10 h. Right panel shows the pixel values (× 10^5 pixels) of Storm PhosphoImager of the specific bands (marked with arrowheads). n.s. is a non-specific band in the EMSA for NF-κB. Values are means ± s.d. Statistical significance between LPS-alone and LPS plus raloxifene: * $P < 0.05$ (Student's t test).

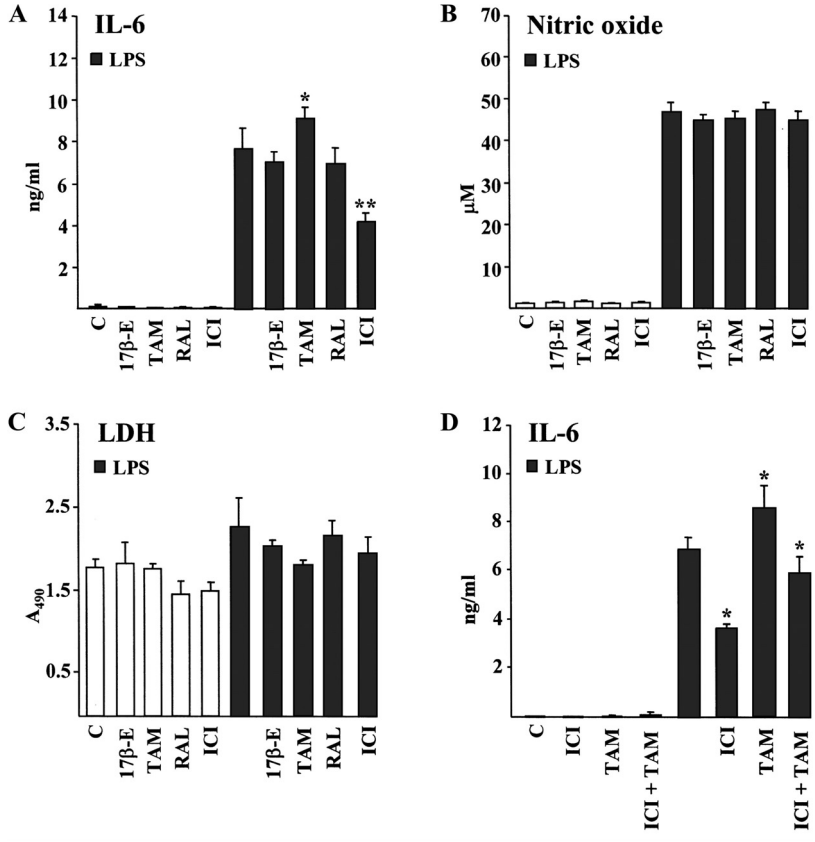

Fig. 9. Effects of pretreatment of N9 microglia for 30 h with 17β-estradiol, tamoxifen, raloxifene and ICI 182.780 on LPS-induced activation. IL-6 (Fig. 9A), NO (Fig. 9B) and LDH (Fig. 9C) release were recorded 22 h after LPS addition. Figure 9D shows the combined effects of tamoxifen and ICI 182.780 on IL-6 secretion for 22 h. Final concentrations used were 10 μg ml^{-1} for LPS and 1.0 μM for 17β-estradiol, tamoxifen, raloxifene and ICI 182.780. Values represent means ± s.d. (n = 6). Statistical significance between LPS plus 17β-estradiol/SERM-treated groups is compared to LPS-alone group or LPS plus TAM group in Figure 9D: * $P < 0.01$, ** $P < 0.001$.

did not affect the LPS-induced IL-6 and NO responses after pre-treatment (Fig. 9A and Fig. 9B). All treatments were non-toxic since they did not increase LDH leakage (Fig. 9C). The results from the pre-treatment model suggest that SERMs do not produce any classical ER-mediated genomic responses to inhibit microglial activation.

Discussion

A vast literature shows that estrogen has a complex signalling network which shows cell-type specific regulation [13, 18–20]. Traditionally, estrogen has been considered to evoke not only genomic responses mediated by estrogen receptors but also non-genomic responses e.g. via actions on second messenger systems and ion channels [19]. Selective estrogen receptor modulators, SERMs, represent non-steroid molecules which bind to estrogen receptors and show selective agonist-antagonist effects in different tissues [13, 14]. Recent studies have shown that SERMs, such as tamoxifen , raloxifene and ICI 182,780, have estrogen receptor –independent effects e.g. on MAPK and AKT signalling [8, 30, 31] which further increase the complexity of SERM regulation.

Estrogen and SERMs possess a variety of neuroprotective effects but the molecular mechanisms for these effects are still largely unknown [8]. Many of these survival effects are probably mediated by MAPK and AKT signalling pathways [8, 19]. Estrogen exerts various anti-inflammatory effects e.g. on the cardiovascular system [32] and in brain [33, 34]. Estrogen also has neuroprotective effects in cerebral ischemia which is associated with inflammation [35]. However, recent studies indicate that there is a risk of dementia and endometrial and breast cancer associated with estrogen and progestin replacement therapy [10]. The benefits of estrogen therapy in Alzheimer's disease are still controversial [36]. This has raised interest in the use of SERMs in the treatment of neurodegenerative diseases [11, 12].

There are very few studies which have investigated the role of tamoxifen, raloxifene and ICI 182,780 in inflammation. Some studies suggest that tamoxifen has anti-inflammatory potential both in human and in animal models [37, 38]. There are also observations that raloxifene can reduce the number of microglia and astrocytes in aging brain [34]. In osteoclasts, raloxifene inhibits IL-6 and IL-1β expression and decreases osteoclastogenesis [39].

In this study, we observed that SERMs possess a significant anti-inflammatory potential against the acute inflammatory response induced by LPS or unmethylated CpG-oligonucleotides. Furthermore, this protective effect was observed both in rat primary microglia and N9 microglial cells. The response was concentration-dependent being significant at 100 nM or higher levels of tamoxifen and raloxifene. Consistent reductions were recorded in IL-6 total mRNA and protein expressions as well as in IL-6 cytokine secretion. Interestingly, the anti-inflammatory response induced by tamoxifen and raloxifene is present only if these SERMs are added at the same time or even up to 4 h later than LPS stimulus but not if microglial cells are pretreated with tamoxifen or raloxifene for 24 h. This strongly suggests that the SERM-induced anti-inflammatory response is not induced by an estrogen receptor-mediated genomic response but by disturbing the inflammatory signalling cascades activated by LPS treatment.

Estrogen is known to influence the signalling through MAPK and AKT pathways [8, 14, 18, 20]. However, 17β-estradiol did not affect or had only a minor effect on the LPS-induced inflammatory responses in microglial cells recorded by IL-6 and NO secretion both in the models consisting of pre-treatment or simultaneous treatment with 17β-estradiol. Bruce-Keller et al. [17] have shown that 17β-estradiol does attenuate LPS-stimulated superoxide secretion or phagocytic activity of N9 microglial cells if the cells were treated with 17β-estradiol for 24 h before LPS exposure but no protection existed if microglial cells were treated simultaneously with 17β-estradiol and LPS. We did not record superoxide secretion or phagocytic activity. Drew and Chavis [40] observed an estrogen-induced anti-inflammatory response in NO production in N9 cells but they used 100 µM concentration. The exposure of 1 µM level of β-estradiol was inefficient against LPS-induced NO production [40], as observed in this study. Baker et al. [41] observed a prominent anti-inflammatory response by estrogen in BV-2 microglia which express only ERβ receptors but not ERα receptors. We have observed that N9 cells express both ER receptors (Johanna Ojala, unpublished results), as also observed by Baker et al. [41]. It seems that anti-inflammatory responses in microglia is dependent on microglial cell types and parameters recorded.

LPS activates the inflammatory signalling cascades through Toll-like receptors TLR2 and TLR4 [26] whereas unmethylated CpG oligonucleotides through TLR9 [28]. In microglial cells, however, the SERM-induced anti-inflammatory response was very similar whether this was stimulated by either LPS or CpG oligonucleotides. This suggests that SERMs affect downstream targets of the signalling cascades. NF-κB and AP-1 are important transcription factors regulating the activity of inflammation-related genes [14, 19]. We observed that LPS increased the DNA binding activity of both AP-1 and NF-κB factors but raloxifene appeared to decrease only the activity of AP-1 binding. This suggests that raloxifene might have influenced the MAPK pathway rather than NF-κB pathway. Clearly, it seems that the anti-inflammatory effects of raloxifene and tamoxifen are rather transient, probably signalling disturbances, since pretreatment with these SERMs did not show any anti-inflammatory response. SERMs even induced an anti-inflammatory response although added up to 6 h after LPS exposure. These observations also support the possibility that SERMs can disturb LPS-activated signalling cascades to reduce the level of inflammation.

Mandlekar and Kong [42] have recently reviewed the apoptosis mechanisms of tamoxifen which are not mediated by estrogen receptors. As well, other SERMs have a variety of estrogen receptor -independent mechanisms [43]. Tamoxifen, for instance, affects the signalling via protein kinase C, MAP kinase and AKT pathways [42]. These effects may be induced by the changes in membrane fluidity, calcium signalling or ceramide production [reviewed in 42]. In respect to anti-inflammatory responses, it may be important that the most of the SERMs induce growth inhibition in different cell types [e.g. 42, 44–46], also in N9 cells (Johanna Ojala, unpublished results). The inhibition appears both in ER-positive and ER-negative cells, and may be mediated by the

inhibitory TGF-β cytokine [see 42, 45]. Interestingly, TGF-β1 is known to activate MAPKs in microglial cells that subsequently become refractory to further stimulation by LPS [47]. Heat shock proteins, such as HSP72, involve another transient inhibitory mechanism for MAPK pathway [48]. Tamoxifen, for instance, increases HSP72 expression [49]. SERMs induce a transient, ER-independent activation of MAPKs, such as p38 and ERK1/2, in cultured cells [46, 48]. Transient MAPK activation and subsequent refractory period for LPS [see 47] could explain the decrease in AP-1 binding and LPS-induced inflammatory response. Further studies, however, are needed to elucidate the signaling pathways leading to anti-inflammatory response induced by SERMs in microglial cells.

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