Progesterone and its metabolites 5-dihydroprogesterone and 5-3-tetrahydroprogesterone decrease LPS-induced NO release in the murine microglial cell line, BV-2

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Abstract

OBJECTIVE: Microglial cells, important immunosurveillance cells in the nervous system, respond to pathogens with an increase in inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) release. Because excessive release of NO may be neurotoxic, tight regulation of nitrosative stress is required.

DESIGN AND SETTING: We cultured the murine microglial cell-line, BV-2, with bacterial lipopolysaccharide (LPS) to induce NO synthesis and quantified the impact of progesterone and its metabolites, 5α-dihydroprogesterone (DHP) and 5α-3α-tetrahydroprogesterone (THP), on NO release.

RESULTS: Our *in vitro* experiments showed that in BV-2 cells LPS-induced NO release is suppressed by progesterone and THP. Both neurosteroids decreased NO release by about 40% when used at a concentration of 10 μM. NO release was less sensitive to DHP. This neurosteroid decreased NO release only by 20% when used at a concentration of 10 μM. NO release was sensitive to N^G–methyl–L–arginine (L-NMMA), a completive inhibitor of NOS, indicating that LPS-induced NO release is mediated by NOS activity. Trypan blue exclusion experiments showed that the ratio of viable to dead cells did not vary using different concentrations of progesterone. Furthermore, progesterone did not increase apoptosis or necrosis when estimated by the distribution of DAPI-labelled condensed chromatin.

CONCLUSION: These experiments indicate that the decline in NO release is mainly due to modulation of NOS activity or expression. Because progesterone, DHP, and THP are synthesized in astrocytes and oligodendrocytes, these neurosteroids may locally suppress an immune response.

ORIGINAL ARTICLE

Introduction

Microglial cells, immune competent cells in the nervous system, are activated after detection of pathogen cues. Activation is characterized morphologically by transformation from a ramified to an amoeboid phenotype [3, 24, 25] and physiologically by an upregulation of MHC I, MHC II [14, 23] and B7 [13], proinflammatory cytokines, like TNF α, IL-1β, IL-6, IL-8, and IL-12 [14], as well as by the release of reactive oxygen species and NO [23]. In microglia, NO is predominantly synthesized by iNOS [4, 11, 20]. In contrast to the constitutive forms, neuronal NOS (nNOS) and endothelial NOS (eNOS), the inducible form, iNOS, is Ca^{2+}-independent and generates more NO than the former enzymes [6]. Despite its beneficial effects on neurotransmission and neuroplasticity, NO is also cytotoxic to neurons and oligodendrocytes [17, 19, 22]. To prevent excessive inflammatory damage to the brain, immune response of microglia has to be tightly regulated.

Neurosteroids are likely candidates to participate in this challenge. Progesterone and its metabolites, like 3α-hydroxy-allopregnane-5-20-one, are well known to modulate neural activity via regulation of $GABA_A$ receptors [1, 10, 12, 15]. However, progesterone is also a potent inhibitor of immune cells [9]. Because progesterone and its metabolites are synthesized locally in astrocytes and oligodendrocytes [1, 10], they may also modulate the immune response to pathogens locally. Interestingly, it has been shown that progesterone inhibits NO synthesis in primary microglial cells and in the microglial cell line, N9 [7, 16].

In the present study, we found that progesterone and THP equipotentially suppressed LPS-induced NO release.

Material and Methods

The immortalised microglia cell-line, BV-2 [2], was cultured in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum (Gibco/BRL Life Technologies). All experiments described below have been done on BV-2 cells cultured in media containing 10% foetal bovine serum. Cells were plated at a density of 5×10^4 cells per ml in 24-well plates for 16 hours. Then, nitric oxide release was stimulated by addition of 10 μg/ml LPS to the media for 24 hours. In a further set of experiments we evaluated the impact of progesterone, 5-α-dihydroprogesterone (DHP; = 5α-pregnane-3, 20-dione), and 5-α-3-α-tetrahydroprogesterone (THP; = 3α-hydroxy-allopregnane-5-20-one). BV-2 cells were either pre-exposed to the steroid for one hour and then co-exposed to LPS (10 μg/ml) and steroid or first incubated in LPS (10 μg/ml LPS) for one hour and, then, co-exposed to LPS (10 μg/ml LPS) and the steroid hormone for additional 24 hours. Steroids were tested at concentrations of 1μM, 10μM, and 30 μM. After 24 hours of culture in LPS or LPS and neurosteroid, cell viability was tested using trypan blue exclusion, apoptosis and necrosis was evaluated by examining nuclear distribution of DAPI-labelled chromatin, and the NO concentration in the supernatant was measured. NO production was quantified by measuring nitrite concentration in the supernatant spectrophotometrically, using a commercially available Griess assay. The Griess reaction detects nitrite, an oxidized product of NO. The absorbance was estimated at 540 nm against $NaNO_2$ standards (in μM: 0, 1, 5, 10, 20, 30, 40, and 50) and the results were expressed in μM nitrite. N^G–methyl–L–arginine (L-NMMA), a competitive inhibitor of NOS, was used to test whether LPS-induced NO release is due to NOS activity. In these experiments, cells were cultured in the presence of LPS and L-NMMA (100μM) and nitrite concentration was measured. To evaluate nuclear morphology, cells were submersed in methanol/acetic acid and nuclei were stained with the fluorescent DNA-binding dye, DAPI. Distribution of labelled chromatin was analyzed by confocal microscopy (LSM 510, Zeiss). Apoptotic cells were identified by accumulation of condensed chromatin at the nuclear periphery and necrotic cells were identified by pycnotic nuclei. Unless otherwise stated, all chemicals were purchased from Sigma – Aldrich.

Results

To investigate, whether progesterone and its metabolites, DHP and THP, suppress nitrosative stress, we quantified LPS-induced NO release in the absence and presence of these neurosteroids (Fig. 1). BV-2 cells were incubated with LPS (10μg/ml) in a serum-containing medium for 24 hours in order to stimulate NO release. In some experiments, BV-2 cells were first pre-incubated with the appropriate neurosteroid for one hour and, then, incubated in LPS and neurosteroid for additional 24 hours. In an additional set of experiments, BV-2 cells were first exposed to LPS (10μg/ml) for one our and, then, in LPS and the appropriate neurosteroid. Both approaches gave similar results. Incubation of BV-2 cells with LPS increased NO release from 0.8 ± 0,23 μM to 4.0 ± 1,39 μM ($n = 12$; $p < 0.01$). Progesterone and THP significantly inhibited LPS-induced NO release in a dose-dependent manner. Both neurosteroids were equally potent and reduced NO release by about 40% when used at a concentration of 10 μM (n = 6) (Fig. 1*A*, *B*). On the contrary, we observed only a marginal effect of DHP on NO release, which decreased NO release only by about 20% when used at a concentration of 10 μM (n = 3) (Fig. 1*C*).

In animal cells, NO derives mainly from the NOS-dependent metabolism of L-arginine to L-citrulline and NO. To verify that LPS-induced NO release is due to an increased activity of NOS, we determined whether L-NMMA, a competitive inhibitor of NOS, decreases NO release in BV-2 cells. In these experiments, cells were treated with LPS (10μg/ml) in serum-containing medium in the presence of L-NMMA (100 μM). Following incubation for 24 hours, NO was quantified. NO

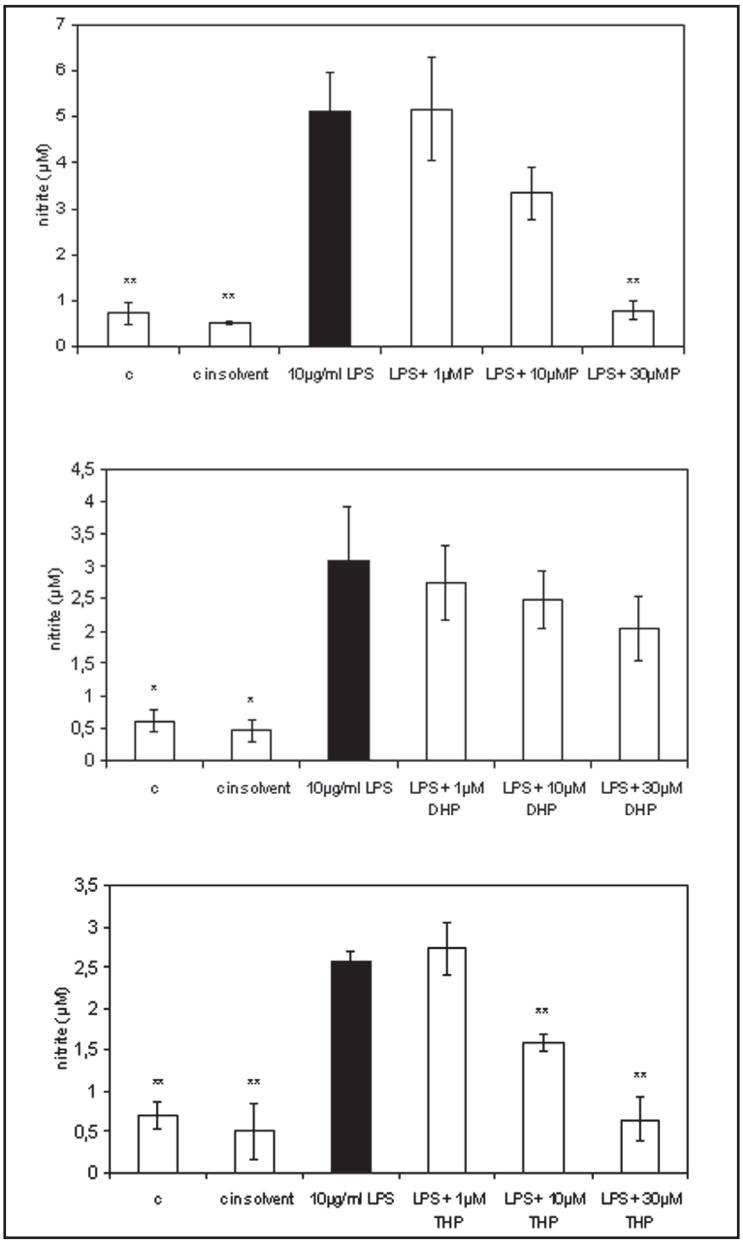

Figure 1: Progesterone (*A*), DHP (*B*), and THP (*C*) suppress LPS induced NO release in the murine microglial cell line, BV-2. The cells were first exposed to LPS (10 µg/ml) for one hour and, then, exposed to LPS (10 µg/ml) and the appropriate neurosteroid for additional 24 hours. After 24 hours, the nitrite concentration was estimated using a Griess assay. Each neursteroid has been tested at least in three independent experiments.

release under these conditions was decreased by about 2,6 µM ± 0,34 µM (n = 3, $p < 0.05$), indicating that NO production dependents on NOS activity. Despite an IC_{50} of about 7µM, the incomplete inhibition of NO synthesis is probably due to competition between L-NMMA and L-arginine, which is present in the serum-containing medium.

We next analyzed whether the decrease of NO is due to a cytotoxic effect of progesterone or its metabolites, DHP and THP. BV-2 cells were exposed to the neurosteroids for 24 hours and viability was estimated using trypan blue exclusion experiments and nuclear chromatin distribution was evaluated using DAPI labelling. In control conditions, the ratio between viable and dead cells was 0.05 (3 independent experiments) in trypan blue experiments. In the presence of progesterone (up to 30 µM), this ratio did not change (n = 3 independent experiments). Accumulation of condensed chromatin at the nuclear periphery was used as a hallmark for apoptosis, whereas pycnotic nuclei were used as an indicator of necrosis. BV-2 cells cultured in serum-free medium had about 0.4% apoptotic cells. Necrotic cells have not been detected (n = 3 independent experiments; about 300 cells each). Even when used at the highest concentration (30 µM), none of the neurosteroids used increased apoptosis or necrosis significantly.

Discussion

Consistent with previous findings, we found that progesterone suppresses LPS-induced NO release in microglial cells [7, 16]. We extended these findings showing that THP is equally and DHP is less potent than progesterone in inhibiting LPS-induced NO release in microglia.

NO has ambivalent physiological functions in the central nervous system. Depending on the amount of NO synthesis and the redox state of NO-related species,

NO is either neuroprotective or neurotoxic [17]. The neuroprotective role of NO is mediated by S-nitrosylation of NMDA receptors, which decreases the Ca^{2+}-influx, and caspases, which suppress the death pathways [17]. The neurotoxic impact of NO is mainly mediated by formation of peroxynitrite by NO and superoxide anion [17]. In contrast to constitutive forms of NOS, expression of iNOS is correlated with intense production of NO [21]. Excessive production of NO and ROS leads to nitrosative and oxidative stress, respectively, which culminates in the production of peroxynitrite and, consequently, triggers neuronal cell death [17]. NO synthesis by iNOS could be regulated in two ways. Expression of iNOS may be downregulated, and accordingly, NO synthesis declines. Alternatively, local availability of the substrate, L-arginine, may be controlled either by arginase activity or by transport activity of amino acid transporters, like the cationic amino acid transporter (CAT). Although other studies on microglia demonstrated that progesterone mediates a decline in cytoplasmic iNOS content [7], our preliminary Western blot experiments using a polyclonal antibody to mouse iNOS did not reveal a difference in iNOS content between BV-2 cultured in the presence of LPS or in the presence of LPS and the appropriate neurosteroid for 24 hours (unpublished results). This indicates that under our experimental conditions, progesterone and its metabolites may regulate NO production by controlling L-arginine availability.

Progesterone has a profound history in being anti-inflammatory. For example, during pregnancy progesterone protects the fetus from the maternal immune system by triggering the production of the progesterone induced blocking factor (PIBF), which suppresses the degranulation and perforin release in natural killer cells as well as enhances release of non-inflammatory cytokines and inhibits production of pro-inflammatory cytokines [8]. Furthermore, progesterone suppresses activation of lymphocytes [9]. Interestingly, the central nervous system expresses progesterone metabolizing enzymes. 5α-reductase, which metabolises progesterone to 5α-dihydroprogesterone, is mainly expressed in neurons, and 3α-hydroxysteroid dehydrogenase, which metabolizes DHP to THP, is mainly expressed in macroglial cells [18]. Furthermore, neurons, astrocytes, and oligodendrocytes can endogenously synthesize progesterone [1, 10]. Thus, these neurosteroids may modulate neural micromilieu and, consequently, regulate neuro-inflammatory processes.

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