Anti-inflammatory substances can influence some glial cell types but not others

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**ABSTRACT**

In rat microglial enriched cultures, expressing Toll-like receptor 4, we studied cytokine release after exposure with 1 ng/ml LPS for 0.5–24 h. Dexamethasone and corticosterone exposure served as controls. We focused on whether naloxone, ouabain, and bupivacaine, all agents with reported anti-inflammatory effects on astrocytes, could affect the release of TNF-\(\alpha\) and IL-1\(\beta\) in microglia. Our results show that neither ultralow (10\(^{-12}\) M) nor high (10\(^{-7}\) M) concentrations of these agents had demonstrable effects on cytokine release in microglia. The results indicate that anti-inflammatory substances exert specific influences on different glial cell types. Astrocytes seem to be functional targets for anti-inflammatory substances while microglia respond directly to inflammatory stimuli and are thus more sensitive to anti-inflammatory substances like corticoids. The physiological relevance might be that astrocyte dysfunction influences neuronal signalling both due to direct disturbance of astrocyte functions and in the communication within the astrocyte networks. When the signalling between astrocytes is working, then microglia produce less pro-inflammatory cytokines.

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1. Introduction

Microglia are considered as immunocompetent cells of the CNS and are activated during pathological events such as stroke, ischaemia, or brain trauma to cause a neuroinflammation (Kreutzberg, 1996). In the normal brain, microglia appear as highly branched or ramified cells and thought to be quiescent. Activation of microglia alters their ramified morphology to amoeboid and proliferative with migratory behaviour. Surface molecules are expressed, cytokines are released, and growth factor synthesis show an up-regulated immunophenotype (Kettenmann et al., 2011).
Activated microglia are known to release pro-inflammatory cytokines, particularly tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and also nerve growth factors, nitric oxide and prostanoids (Chao et al., 1992). These substances are present during inflammatory reactions and they produce long-term pain or hyperalgesia. Antagonism or neutralization of these factors can reduce the pain (Marchand et al., 2005).

Several substances have been shown to exert anti-inflammatory properties especially in astrocytes at extremely low concentrations: naloxone, ouabain, and bupivacaine (Lundborg et al., 2010, 2011; Block et al., 2012). Extremely low doses, at picomolar concentrations, of opioid receptor antagonists, such as naloxone or naltrexone, have been shown to enhance the efficacy and specificity of morphine and related opioid analgesics in mice and postoperative patients (Crain and Shen, 2000). Extremely low doses of naltrexone inhibit the development of spinal morphine antinociceptive tolerance, and clinical studies demonstrated that this may be due to attenuated glial activation (Mattioli et al., 2010). Ouabain, a digitalis-derived glycoside is a well-recognized Na+/K+-ATPase inhibitor, especially pronounced at high concentrations. It also enhances LPS down-regulated iNOS activity in peritoneal macrophages (Sowa and Przewlocki, 1997). Ouabain, at extremely low concentrations, nanomolar and picomolar, stimulates Na+/K+-ATPase activity (Zhang et al., 2008), and activates complex signalling cascades in kidney cells (Holthouser et al., 2010), and in cardiac and smooth muscle cells (Manunta et al., 2010). Ouabain also decreases the release of IL-1β in astrocytes (Forshammar et al., 2011). Bupivacaine is known to block Na+ channels at high concentrations and change the excitability of action potentials. Clinically, this drug has been used in the treatment of various inflammation-related conditions and diseases (Cassuto et al., 2006), and to treat long-term pain in both cancer and non-cancer patients (Deer et al., 2002). Later it has been observed that local anaesthetics possess anti-inflammatory properties through their effects on cells of the immune system. These agents also attenuate the release of the pro-inflammatory cytokines TNF-α and IL-1β from intestinal cells (Lahav et al., 2002; Bedirli et al., 2011).

The purpose with the present study was to investigate if a number of non-steroid anti-inflammatory substances, administered within a wide dose range (10−12 M to 10−6 M) influence microglial release of pro-inflammatory cytokines.

Fig. 1  (A) Microglial cultures were stained with antibodies against OX42, or with antibodies against TLR4, revealing that the cells are microglia expressing TLR4 receptors. The two antibodies are then merged. (B) Microglia express TLR4 visualised with Western blot. Cultures were incubated with 1 ng/ml LPS for 0.5, 1, 4, 8, or 24 h. The TLR4 expression is shown as integrated density. (C) TLR4 is also shown as a band at ~70 kDa, reflecting the time exposure to LPS. Statistical analysis: The level of significance was analysed using one way ANOVA followed by Dunnett's multiple comparisons test. No significances were found.
2. Results

2.1. Characterisation

The microglial cells were stained with antibodies against the microglial specific antigen OX42, and with antibodies against TLR4, revealing that these cells do express TLR4 receptors (Fig. 1(A)).

Microglia express TLR4 visualised with Western blot (Fig. 1(B)). Cultures were incubated with LPS for 0.5, 1, 4, 8, or 24 h. TLR4 is seen as a band at approximately 70 kDa. Full length TLR4 is observed at approximately 90 kDa, but cleavage fragments have been observed at 30 and 60 kDa (Zager et al., 2007). Since no other bands were present on the membrane the TLR4 antibody was considered specific even though it did not match the full size TLR4. The TLR4 is measured as integrated density and presented as % of 0 h (Fig. 1(C)).

2.2. Cytokine release after LPS incubation

Microglia incubated with LPS for 0.5, 1, 4, 8, or 24 h released TNF-α and IL-1β in accumulating amount over time. After 4 h incubation a small release of TNF-α was observed, but it was not significant until 8 h of incubation (P<0.01; n=8), with increasing release after 24 h incubation (P>0.001; n=8) (Fig. 2(A)). IL-1β release was small after 1, 4, and 8 h, and was significantly accumulated after 24 h (Fig. 2(B)).

Microglia were treated with dexamethasone or corticosterone 30 min before the cells were incubated with a cocktail of LPS and dexamethasone or corticosterone. TNF-α release was attenuated after both dexamethasone and corticosterone treatment (P<0.001; n=9) (Fig. 3(A)). IL-1β release was attenuated after both dexamethasone and corticosterone treatment (P<0.001; n=9) (Fig. 3(B)). Dexamethasone and corticosterone were used as control substances to evaluate that the system was validated to test anti-inflammatory substances.

Microglia were treated with ultralow (10^{-12} M) or high (10^{-6} M) concentrations of naloxone, ouabain, or bupivacaine, 30 min before the cells were incubated with a cocktail of LPS and naloxone, ouabain, or bupivacaine for 24 h, respectively. Naloxone, ouabain, or bupivacaine were not able to attenuate the TNF-α release after LPS incubation (n=9). Instead naloxone and ouabain at ultralow concentration increased the TNF-α release (Fig. 3(A)). None of the different substances were able to decrease the IL-1β release (n=9) (Fig. 3(B)).

The selection of choosing one ultralow and one high concentration of the anti-inflammatory substances are due to results obtained from concentration curves, and results obtained from astrocytes. LPS-induced TNF-α release from microglia after stimulation with bupivacaine, 10^{-18}-10^{-3} M, shows that bupivacaine was not able to decrease the TNF-α release after LPS incubation, except at 10^{-3} M, where the cells died (Fig. 4). The other concentration curves for naloxone and ouabain showed similar results, (not shown). LPS-induced IL-1β release from astrocytes after naloxone and ouabain stimulation with different concentrations has earlier been published by our group (Forshammar et al., 2011), as well as with bupivacaine stimulation (Block et al., in press). The TNF-α release is very small in our astrocytes (Andersson et al., 2005).

Fig. 2 – Microglia incubated with 1 ng/ml LPS for 0.5, 1, 4, 8, or 24 h. (A) TNF-α release with accumulating amount over time. After 4 h incubation a small release of TNF-α was observed, but it was significant first after 8 h (n=8), and increased after 24 h incubation (n=8). (B) IL-1β release was small after 1, 4, and 8 h, and was significant after 24 h (n=6). Statistical analysis: The level of significance was analysed using one way ANOVA followed by Dunnet’s multiple comparisons test. Data are mean ±SEM. **=P<0.01, ***=P<0.001.

3. Discussion

After nerve injury a course of events takes place where the microglial receptor TLR4 has been implicated (Tanga et al., 2005). Signals from the surrounding milieu trigger microglial activation through this receptor, where after the cells will be activated and release pro-inflammatory cytokines. Activation of TLR4 by the inflammatory stimulus LPS (Neher et al., 2011) results in increased expression of TNF-α in microglia (Zhou et al., 2010). In our microglial cell model we see increases of both TNF-α and IL-1β after 8 h and 24 h, respectively of LPS incubation. The cells express TLR4, even at a high level before they were stimulated with LPS, which can be due to a high TLR4 protein content already at time point zero. TNF-α is released in response to inflammation or other types of insult where it can act protective to neurons (Fontaine et al., 2002), and astrocytes (Kuno et al., 2006) because it is able to
As we demonstrate, inflammatory activated microglial cells are stimulated by signals, which activate TLR4 and the cells change their release of pro-inflammatory cytokines. One tentative target to restore these processes would be to inhibit the inflammation activating cellular changes and to decrease the pro-inflammatory cytokine release. Astrocytes and microglia are known to play a role in the development, spread, and potentiation of neuroinflammation (DeLeo et al., 2004; Milligan and Watkins, 2009), and we have recently shown that Ca$^{2+}$-signalling in astrocytes is disturbed when influenced by inflammatory stimuli (Hansson, 2010). Two substances with proposed anti-inflammatory properties at extremely low concentrations, naloxone and ouabain, demonstrate an ability to limit the inflammatory induced alterations in astrocytes (Forshammar et al., 2011). We conclude that this is a note-worthy step in understanding astrocyte responses and neuroinflammatory mechanisms. There are more substances that have been proposed to have anti-inflammatory qualities and up-regulate or restore parameters related to inflammation especially at extremely low concentrations in astrocytes. In the present study we wanted to examine a number of substances, which have anti-inflammatory effects on astrocytes, and we wanted to test them in LPS-activated microglia. The substances tested were naloxone, ouabain, and bupivacaine. We also used some well-known classical anti-inflammatory substances, dexamethasone and corticosterone, as control substances. They attenuated both TNF-$\alpha$ and IL-1$\beta$ release. Glucocorticoids prevent swelling of cells and release of pro-inflammatory cytokines (Chao et al., 1992; Lekander et al., 2009), and decrease the number of activated microglia (Hinkerohe et al., 2010). These two glucocorticoids are frequently used in acute pain states (De Oliveira et al., 2011). On the other hand, glucocorticoids can also cause extracellular accumulation of glutamate, which could cause excitotoxicity and acute stress (Jacobsson et al., 2006).

Naloxone at ultralow concentration, prevented LPS induced down-regulation of Na$^+$/K$^+$-ATPase (Forshammar et al., 2011), and down-regulated LPS-induced endomorphin stimulated Ca$^{2+}$-transients in astrocytes (Block et al., 2012), as well as reversed down-regulation of the Na$^+$ dependent glutamate transporter (Tsai et al., 2009). So far naloxone has not been able to decrease the release of pro-inflammatory cytokines in LPS-activated astrocytes or microglia. Instead a small increase of TNF-$\alpha$ was observed in microglia.

Ouabain also enhances LPS down-regulated iNOS activity in peritoneal macrophages (Sowa and Przewlocki, 1997). It decreased the IL-1$\beta$ release in astrocytes (Forshammar et al., 2011), but showed a small increase of TNF-$\alpha$ in microglia. It can be speculated in if the increased release of TNF-$\alpha$ with ultralow concentrations of naloxone or ouabain might have a protective effect. Exogenous TNF-$\alpha$ as well as TNF-$\alpha$ produced by astrocytes, induces production of neurotrophic factors such as nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (Kuno et al., 2006). TNF-$\alpha$ as well as IL-1$\beta$ are considered to initiate a cascade of activation of cytokines and growth factors. Recent reports indicate that TNF-$\alpha$ can have dual actions mediated via different receptors, which can be responsible for neurotoxic and neuroprotective effects, respectively (Mika, 2008). Interestingly it was observed that both IL-1 receptor antagonists and intrathecal administration of IL-1$\alpha$ prevent neuronal

encourage the expression of anti-apoptotic and anti-oxidative proteins and peptides. It has also been demonstrated that microglia protect neurons against ischaemia through the synthesis of TNF-$\alpha$ (Lambertsen et al., 2009).
apoptosis, while such actions were not seen after IL-1β administration (Mika, 2008). Both IL-1α and IL-1β act on the IL-1 receptor. The mechanisms behind IL-1β and its receptor are still unclear regarding responses to inflammation and provision of any form of protection.

Another substance of interest is the local anaesthetic bupivacaine, which can block neural activity and prevent nerve-injury-induced spinal microglia activation (Wen et al., 2013). In our microglial cultures, pre-stimulation with bupivacaine prior to cell activation by LPS did not suppress the TNF-α or IL-1β releases. However, bupivacaine decreases the IL-1β release at ultralow concentration in astrocyte primary cultures (Block et al., in press).

As none of the tested substances, which have been shown to have anti-inflammatory effects on astrocytes in extremely low concentrations, decreased the cytokine release, we extended the study to include also treatment of microglial with high concentrations of these substances. Naloxone and ouabain both attenuated the increased TNF-α release at high concentration, but showed no ability to decrease the cytokine release compared with controls.

4. Experimental Procedures

4.1. Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) if not stated otherwise.

The experimental protocols were approved by the Ethical Committee in Gothenburg for Laboratory Animals (Nos. 205-2010).

4.2. Microglial cultures

Purified microglial cells were obtained from astroglial-enriched cultures, rat cerebral cortex, grown as previously described by Hansson et al. (1984). Confluent astroglial-enriched cultures, no more than 6 weeks old, were shaken for 30 min at 37 °C and 240 rpm on an orbital shaker in an incubator with a humidified atmosphere of 95% air and 5% CO2. Culture medium, minimum essential medium (MEM), with suspended microglial cells was collected. The microglial cell suspension was plated in six well plates (NUNC), both with or without glass coverslips, and cells were allowed to adhere for 30 min in the incubator. Together with nonadherent cells, the culture medium was removed from the wells and discarded. Additional medium containing microglia was added. This was repeated until a satisfactory amount (~50 μg total protein per well) of microglia was obtained in each well. The culture medium was then replaced with fresh, pre-warmed (37 °C) supplemented MEM, and microglial cells were kept in the incubator and allowed to rest overnight (Persson et al., 2006).

4.3. Cell treatments

Cells were incubated with lipopolysaccharide (LPS, Escherichia coli 011:B4) (1 ng/ml) for 0.5, 1, 4, 8, or 24 h. Some cells were treated with dexamethasone or corticosterone (10−6 M) for 30 min before they were incubated with LPS in a cocktail in un-supplemented MEM. Other cells were incubated with LPS and treated with naloxone (10−12, 10−6 M), ouabain (10−12, 10−6 M), or the local anaesthetics bupivacaine (10−12, 10−6 M) in the same way as above.

4.4. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (Histolab Products AB, Gothenburg, Sweden) for 10 min and washed twice with phosphate buffer saline (PBS) (Invitrogen) containing 1% BSA (PBS–BSA). The cells were permeabilised with PBS–BSA containing 0.05% saponin (PBS–BSA–Sap) for 20 min. Thereafter the cells were incubated for 1 h with a cocktail of rabbit polyclonal antibody against Toll-like receptor, TLR4, (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 and a monoclonal antibody against OX42 diluted 1:100 in PBS–BSA–Sap. The cells were washed with PBS–BSA–Sap. for 3 × 5 min and then incubated with a mixture of FITC conjugated F(ab)2 donkey anti-rabbit IgG and Texas Red conjugated F(ab)2 donkey anti-mouse IgG secondary antibodies (Jackson Immuno Research, Westgrove, USA), both diluted in PBS–BSA–Sap. The cells were washed with PBS–BSA–Sap for 3 × 5 min and finally rinsed with PBS. Controls were treated similarly except for incubations with the primary antibodies. The cover slips were mounted on microscope slides with a fluorescent mounting medium (DAKO, Glostrup, Denmark) and viewed in a Nikon Eclipse 80i microscope. Pictures were taken with a Hamamatsu CS810 colour intensified 3CCD camera.

4.5. SDS-PAGE and western blotting

Cells were rinsed twice in phosphate buffered saline (PBS) and immediately lysed 20 min on ice in cold RIPA lysis buffer. The procedure was done according to the process described by Persson et al. (2005). Separate aliquots were taken for protein concentration determination. All samples were correlated for total protein contents and an equal loading of 20 μg total protein of each sample was applied in each lane of the gel. SDS-PAGE were conducted using the Novex pre-cast gel system (Invitrogen) according to the manufacturer’s recommendations using 4–12% Bis–Tris gels (Invitrogen) at 200 V for 50 min. The separated proteins were then transferred at 30 V for 60 min to a nitrocellulose membrane (Invitrogen) using NuPAGE transfer buffer (Invitrogen) supplemented with methanol and NuPage antioxidant. The membranes were rinsed twice with distilled water and the proteins were visualised with Ponceau S solution (Sigma).

Proteins were blocked with 5% fat free skim milk (Semper AB, Sundbyberg, Sweden) in TBST (50 mM Tris–HCl, 150 mM NaCl and 0.05% Tween) for 60 min at room temperature. The membranes were then probed with primary antibodies overnight (+4 °C), washed 4 × 2 min with TBST, and subsequently probed with secondary horse-radish peroxidase (HRP) conjugated secondary antibodies for 60 min at room temperature, and finally washed several times in TBST. The primary antibody used was rabbit polyclonal TLR4 diluted 1:500. The secondary antibody used were HRP-conjugated donkey anti rabbit F(ab)2 fragment (both from Jackson Immunoresearch) diluted 1:10000. Both primary and secondary antibodies were diluted in 5% fat free skim milk in TBST. Protein was then
detected with an enhanced chemiluminescence kit (PerkinElmer Inc., Waltham, MA, USA) and visualised with a FUJI Film LAS-3000 (Tokyo, Japan).

4.6. Cytokine release

Enzyme-linked immunoabsorbent assay (ELISA) was performed with respect to TNF-α using OptEIA kits (BD Biosciences, San Jose, CA) and IL-1β using Nordic Biosite, Täby, Sweden.

Supernatants from purified microglial cell cultures were collected after microglia had been stimulated for 0.5–24 h. ELISA was performed on the supernatants according to the manufacturer’s instructions. All stimulations were performed in a total volume of 1 ml MEM. Cell lysates were produced by harvesting remaining cells with a cell scraper in 1 M NaOH, and aliquots were taken for protein determination. ELISA plates were analysed at 450 nm with a Molecular Devices VersaMax microplate reader and were analysed using SoftMax Pro 4.8, both from Molecular Devices (Sunnyvale, CA, USA).

4.7. Protein determination

The protein determination assay was performed in accordance with the manufacturer’s instructions using a DC Protein Assay (Bio-Rad, Hercules, CA, USA), based with some modifications on the method used by Lowry et al. (1951). Both standard (0–4 mg/ml BSA) and samples were mixed with the reagents, incubated for 15 min at room temperature, read at 750 nm with a Versa-max microplate reader, and analysed using SoftMax Pro 4.8.

4.8. Statistics

Differences between grouped mean values were identified using one way ANOVA followed by Dunnett’s multiple comparisons test. Error bars show standard error of the mean (SEM).

5. Conclusion

In a microglial cell culture we observed that exposure of LPS was associated with a release of both TNF-α and IL-1β, which increased over time. Dexamethasone and corticosterone attenuated these responses. Other investigated anti-inflammatory agents in this study, which previously have been shown to counter a LPS-dependent release of TNF-α and IL-1β in astrocytes, were not associated with corresponding effects in microglial cells. After inflammation, increases of pro-inflammatory cytokines are observed. Astrocytes seem to be better target cells for anti-inflammatory substances than microglia. The physiological relevance might be that communication within the astrocyte networks seems to be of importance. If the signalling between astrocytes is working, thereby the microglia show a normal and non-inflammatory state. Thus, our findings indicate that anti-inflammatory substances have a cell-type specific capacity to modulate pro-inflammatory reactions in glial tissues.

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