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Neuroscience Letters 381 (2005) 86–91

Neuroscience Letters

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Aspirin modulates LPS-induced nitric oxide release in rat glial cells

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Received 9 November 2004; received in revised form 12 January 2005; accepted 2 February 2005

Abstract

Nitric oxide and prostaglandins are among the numerous substances released by activated glial cells. The aim of this study was to evaluate the effect of high-level aspirin on iNOS expression in cultured rat glial cells treated with lipopolysaccharide (LPS) as pathological stimulator. Using Western Blotting, we verified that aspirin enhanced LPS-induced iNOS expression and the presence of 15-deoxy-$\Delta^{12,14}$-prostaglandin (15d-PGJ_2) suppressed this aspirin effect. However, the exposure of LPS-treated glial cells to aspirin resulted in a decrease of NO production. These results suggest that aspirin interferes with the cross-talk of prostaglandins and NO, blocking the endogenous negative control exerted by COX products on iNOS expression. On the other side, aspirin seems to act directly on iNOS reducing its activity, even if it does not completely block NO release by LPS-stimulated glial cells. Then aspirin could maintain homeostatic functions of NO, while it prevents toxic effects, corresponding to high NO concentrations.
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Keywords: Astrocyte; Microglia; Nitric oxide; Inducible nitric oxide synthase (iNOS); Lipopolysaccharide (LPS); Aspirin

Microglial cells are the resident macrophages of the central nervous system (CNS) and their activation is a natural and defensive process, occurring in most brain pathologies [17]. Damage to the CNS, by physical trauma or neurodegenerative disorders, leads these immune competent cells of the brain to proliferate, to adopt a number of potentially cytotoxic functions and to trigger consequent astrocyte reactions. Glial cell activation is one of the prominent features of Alzheimer's disease (AD) [34]. The close association of activated astrocytes and microglia with neuritic plaques, the expression of receptors for complement by glial cells and the release of cytokines strongly suggests that inflammatory processes may play a key role in the complex pathophysiological interactions that occur in AD [5]. Nitric oxide (NO) and prostaglandins (PGs), both modulators of inflammatory and immune responses in the CNS, are among the numerous substances released by activated microglial cells and reactive astrocytes [11,20]. NO, a bioactive free radical, is involved in various physiological and pathological processes: at low concentration NO has been shown to play a role in neurotransmission and vasodilation, and, at higher concentrations, it is implicated in neurodegenerative diseases [23,28]. NO is enzymatically formed from L-arginine by nitric oxide synthase (NOS). Basically, the NOS enzymes are classified in two groups: the constitutive forms (cNOS), which are typical of neurons and endothelial cells, and the inducible form (iNOS), which is rapidly expressed in glial cells and other cell types only following induction by inflammatory mediators, such as TNFα, IL-1 and bacterial lipopolysaccharide (LPS) [6,24] or by low environmental pH [3] or by the β-amyloid [2]. While cNOS is regulated predominantly at the post-transcriptional level by calmodulin in a Ca^{2+}-dependent manner, iNOS is Ca^{2+}-independent and its expression is regulated mainly at the transcriptional level [3]. Like NOS, cyclooxygenase (COX), the key enzyme for prostanoid synthesis, exists in a form expressed constitutively by most cell types (COX-1) and in a form that is rapidly inducible upon stimulation with mitogens, cytokines, and LPS (COX-2) [13,35]. Indeed, the treatment of glial cells in culture with LPS co-induces COX-2 and iNOS [21] and the activation of the transcriptional factor NF-κB seems to be critical for this process [4,26,30]. However, the mechanisms involved in the synthesis of PGs and NO, via COX-2 and iNOS respectively,

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doi:10.1016/j.neulet.2005.02.002

are complicated by their reciprocal interactions [39]. Moreover, the influence of prostanoids on NO synthesis and vice versa may vary in different cell types and with the degree of cell activation [25]. The importance of understanding the inflammatory processes observed in neurodegeneration is further strengthened by clinical and epidemiological studies that demonstrate that the use of anti-inflammatory compounds, such as nonsteroidal anti-inflammatory drugs (NSAIDs), reduces the incidence of AD [19,38]. In the present study, we have evaluated the aspirin effect on LPS-induced NO production and iNOS expression in rat cortical glial cells in primary cultures. We report that aspirin, at pharmacological and suprapharmacological doses [16], enhances LPS-induced iNOS expression in glial cells, and this effect is suppressed by the addition of cyclopentenone prostaglandin, 15d-PGJ_2. The aspirin dosing regimens are within a wide range based on the therapeutical target, and at localized environments glia may be exposed to high level of salycilates [8,41]. In this same study, aspirin inhibits LPS-induced NO production, indicating aspirin modulates multiple aspects of NO metabolism.

Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0127-B8, (Sigma) was resuspended in sterile PBS and stored at −20 °C. Aspirin was purchased from Sigma. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and polyclonal antibody anti-iNOS were purchased from Calbiochem-Novabiochem. 15d-PGJ_2 was dissolved in ethanol and, when added to cells as ethanol solution, the final concentration of ethanol never exceeding 0.1%.

Primary cultures of mixed glial cells from newborn (1–2 days) Wistar rats (Charles River, Italia) were obtained according to the slightly modified method of McCarty and de Vellis [18]. The experiments received institutional approval by the local ethics committee of the University of Camerino and the principles of laboratory animal care (NIH Publication No. 86–23, revised 1985) were followed. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Briefly, after decapitation and removal of the meningis, the cerebral cortices were collected in Falcon tubes in Dulbecco' s modified Eagle' s medium (DMEM) with Glutamax I (Gibco-brl). Following the mechanical dissociation and the passage through cell strainers (Falcon), the cells were plated in 25 cm^2 culture flasks in DMEM medium with Glutamax supplemented with 15% heat-inactivated fetal bovine serum (FBS, Gibco-brl), 100 U/ml penicillin (Gibco-brl), 100 μg/ml streptomycin (Gibco-brl) and maintained in an incubator with humified atmosphere at 37 °C and 5% CO_2. The culture medium was changed every 3 days. After 12 days in culture, confluent mixed glial cells, consisting of microglial cells seeded on top of the layer of protoplasmic astrocytes [40], were obtained.

Microglial and astrocytes were then prepared from the mixed glial cell cultures. To obtain microglial cell cultures, the culture flasks were shaken at 200 rpm for 18 h at 37 °C on day 12 of the mixed glia cultures. The medium containing detached microglial cells was collected and centrifuged at 200 × *g* for 10 min. The cells were resuspended, counted and plated. Secondary astroglial cell cultures were obtained by removing the cells attached in the culture flasks with 0.25% trypsin (Gibco-brl)/0.02% EDTA. The cells were centrifuged at 200 × *g* for 5 min, resuspended in PBS, centrifuged again at 200 × *g* for 5 min, resuspended with the culture medium and plated. Cell counting was performed following Trypan Blu staining.

Microglial–astroglial cell cocultures were obtained by seeding the microglial cells (5×10^5/flask) in the presence of astroglial cells (10×10^5/flask). This microglial/astroglial cells ratio has been chosen being the average ratio found in confluent mixed glial cells under the growth conditions used.

Twenty hours after seeding, the microglial–astroglial cell cocultures were ready to be used.

NO production in culture supernatant was evaluated by measuring nitrite, its stable degradation product, using the Griess reagent (SIGMA). Two days before the experiments, cells were replated, without shaking off microglia, onto 96-well dishes (Costar) at a density of 40,000 cells/well. The culture medium was changed to DMEM with Glutamax I and HEPES (Gibco-brl) before the cells were stimulated with LPS (1 μg/ml) for 24 h. Aspirin was used at the following concentrations: 1.25, 2.5, 5 and 10 mM, and it was added to the medium 30 min before the treatment with LPS. Briefly, 150 μl of culture supernatant from each well were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2% phosphoric acid) and incubated at room temperature for 5 min before the absorbance was measured spectrophotometrically at 570 nm in a microplate reader. Fresh culture medium served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve (0–100 μM) derived from the reaction of sodium nitrite ($NaNO_2$) in the assay.

For immunoblot analysis of iNOS expression, cells were washed with PBS and then treated in serum-free medium (DMEM with Glutamax I and HEPES). To obtain the dose–response curve, aspirin was used at the following concentrations: 0.5, 1, 5 and 10 mM, together with 1 μg/ml LPS. For the other immunoblot experiments, aspirin was used at 5 mM concentration, alone or in combination with 1 μg/ml LPS. The used concentration of prostaglandin 15d-PGJ_2 was 3 μM, alone or in combination with 1 μg/ml LPS, or in combination with 1 μg/ml LPS plus 5 mM aspirin. Aspirin and 15d-PGJ_2 were added 30 min before the addition of LPS. The pH of the medium was controlled in every experimental condition, since it has been reported that pH values of the culture medium lower than 7.2 can induce iNOS expression [3].

Following 24 h of incubation in the presence or in the absence of different stimuli, cells were washed with ice cold PBS, scraped off with hot lysis buffer (25 mM Tris–HCl pH 7.5 with 2% SDS), harvested, sonicated and boiled for 5 min. Then β-mercaptoethanol was added to each sample (5% of the volume). Lysates were stored at −20 °C before use. Equal amounts of protein (20–30 μg) from cell homogenates, estimated by bicinchoninic acid reagent (Pierce), were loaded

onto SDS–polyacrylamide gel, using 7.5% running gels. After electrophoresis, the proteins were electrotransferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore). Successively the membranes were incubated in blocking solution (5% BSA in TTBS: Tris-buffered saline/Tween 20) overnight at 4 °C. Immunoblots were probed with a polyclonal rabbit primary antibody against rat macrophage iNOS (Calbiochem-Novabiochem) (1:2000 in TTBS for 2 h) and with a secondary horseradish peroxidase-labeled anti-rabbit antibody (1:3000 in TTBS for 45 min). After each incubation, the membranes were washed extensively with TTBS. The immunoreactive bands were detected with ECL detecting reagents and developed with Hyperfilm-ECL (Amersham).

A densitometric algorithm has been developed to quantitate the Western Blot results. Each Western Blot film has been scanned (16 bits greyscale) and the obtained digital data were processed to calculate the background mean value and its standard deviation. The background-free image was then obtained subtracting the background intensity mean value from the original digital data. The integrated densitometric value associated to each band was then calculated as the sum of the density values over all the pixels belonging to the considered band having a density value higher than the background standard deviation. The ratios of band intensities were calculated within the same Western Blot. All the calculations were carried out using the Matlab environment (The MathWorks Inc., MA, USA).

The statistical significance has been calculated using a standard Student's *t* test and the one-tail/two-tails tests [36]. The dose–response curves have been analyzed using a canonical binding approach [42]. The response value *r* at each drug concentration [F] is defined as:

$$r = 1 + \frac{(r_{\max} - 1)([\mathrm{F}]/\mathrm{EC}_{50})}{1 + ([\mathrm{F}]/\mathrm{EC}_{50})} = \frac{\mathrm{EC}_{50} + r_{\max}[\mathrm{F}]}{\mathrm{EC}_{50} + [\mathrm{F}]} \qquad (1)$$

where $r_{\max}$ is the asymptotic response value at infinite drug concentration, while is the effective concentration at $r = r_{\max}/2$.

The level of the stable metabolite nitrite was measured in the culture media, to determine the effect of aspirin on NO released by activated glial cells. Aspirin inhibited in a dose-dependent manner the production of NO by glial cells stimulated with 1 μg/ml LPS. Aspirin was added, 30 min before LPS treatment, to the culture medium, at the concentration range of 1.25 mM up to 10 mM, for 24 h. The values of the nitrite levels after the treatments with aspirin plus LPS were then normalized to the values of the nitrite levels after the stimulation only with LPS. The resulting data were an index of the inhibition percentage exerted by aspirin and, when plotted against aspirin concentrations, they show a hyperbolic profile (Fig. 1A). These data were analyzed according to a canonical binding isotherm. The extrapolated EC_{50} value was $4.35 \pm .21$ mM.

As measured by Trypan Blu exclusion, viability of cells incubated for 24 h in the presence of 1.25, 2.5, 5 and 10 mM aspirin was 95%, indicating that the inhibition of NO production was not due simply to aspirin toxicity.

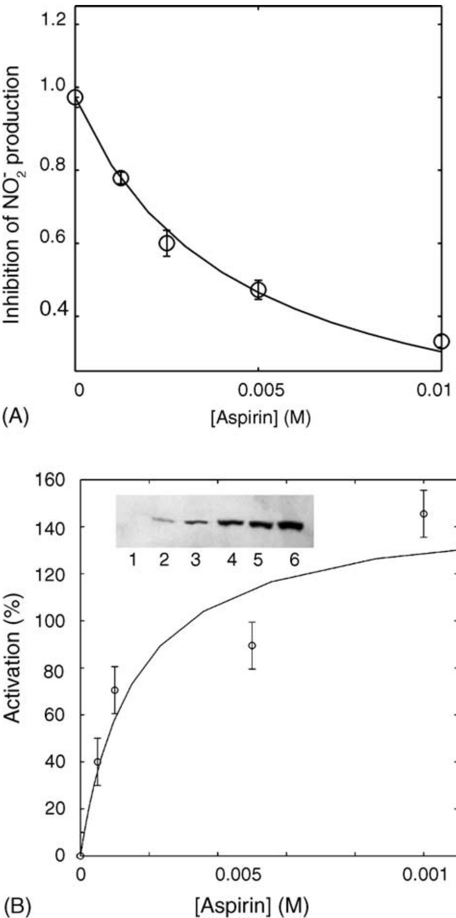

Fig. 1. (A) Concentration dependence for inhibition of LPS-induced NO production by aspirin. Glial cells were incubated with 1 μg/ml LPS and various concentrations of aspirin (1.25–10 mM) in serum-free DMEM with Glutamax I and HEPES for 24 h. The amounts of NO in the supernatants were measured. Nitrite concentrations are expressed relative to nitrite concentrations for cells stimulated with LPS alone. Each value indicates the mean ± S.E.M. ($n = 6$). (B) Dose dependency of aspirin effect on LPS-induced iNOS expression. iNOS expression levels corresponding to different aspirin concentrations (1 μg/ml LPS; LPS + 0.5 mM aspirin; LPS + 1 mM aspirin; LPS + 5 mM aspirin; LPS + 10 mM aspirin) are expressed relative to iNOS expression for cells stimulated with LPS alone. Each value indicates the mean ± S.D. ($n = 4$). The insert shows a representative image of the iNOS blots used to generate the curve (lane 1: culture medium alone; lane 2: 1 μg/ml LPS; lane 3: LPS + 0.5 mM aspirin; lane 4: LPS + 1 mM aspirin; lane 5: LPS + 5 mM aspirin; lane 6: LPS + 10 mM aspirin).

Using Western Blot analysis we compared iNOS expression levels under different experimental conditions, to verify whether the inhibitory effect of aspirin on nitrite production was due to the block of LPS-induced expression of iNOS in glial cells. We did not observe any immunoreactivity in unstimulated cells, whereas a weak band was visible in cells stimulated with 1 μg/ml LPS, and increasing aspirin concentrations strengthened in a dose-dependent manner the signal corresponding to LPS-induced iNOS. After the densitometrical analysis of the bands, the levels of iNOS following LPS

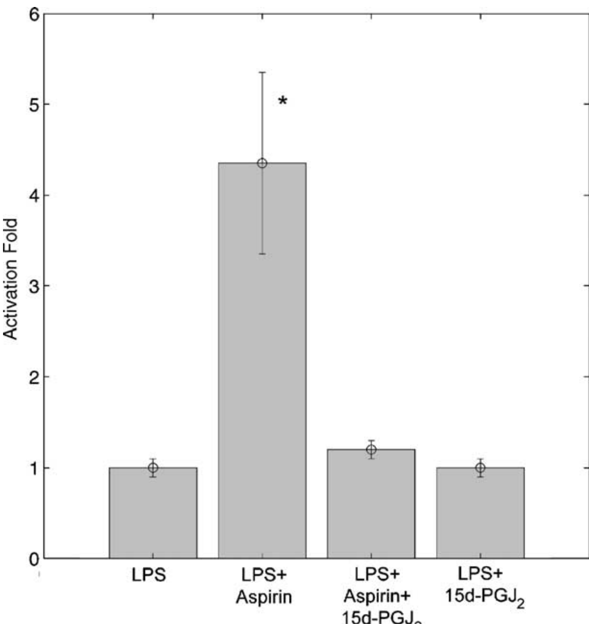

Fig. 2. 15d-PGJ$_2$ counteracts the effect of aspirin on the LPS-induced iNOS expression. iNOS expression levels corresponding to LPS, to LPS + aspirin, to LPS + aspirin + 15d-PGJ$_2$ and to LPS + 15d-PGJ$_2$ treatments are expressed relative to iNOS expression for cells stimulated with LPS alone. Each value indicates the mean ± S.E.M. ($n = 4$). $^{*}P = 0.05$, treatment with LPS + aspirin compared to LPS alone, LPS + aspirin + 15d-PGJ$_2$ compared to LPS + aspirin.

plus aspirin treatments were normalized to the levels corresponding to the treatment with LPS alone (Fig. 1B).

We compared iNOS expression after LPS treatment and after LPS plus 5 mM aspirin treatment in 10 experiments to evaluate whether the effect of aspirin, as enhancer of LPS-induced iNOS expression, was reproducible in a statistically significant number of experiments. We selected 5 mM aspirin concentration because it is about the EC_{50} calculated for aspirin effect on NO production. The iNOS protein was not detectable in control conditions or after treatment with 5 mM aspirin alone, but 1 μg/ml LPS induced iNOS expression in glial cells, even if weakly (in agreement with mRNA levels increases upon LPS addition [9]), and iNOS expression was enhanced adding 5 mM aspirin in combination with LPS to the culture medium. To test if the inhibition of prostanoids synthesis is involved on this effect of aspirin on iNOS expression, the cells were treated with 3 μM 15d-PGJ$_2$ together with 5 mM aspirin and 1 μg/ml LPS. In this condition, the effect of aspirin is suppressed by 15d-PGJ$_2$ and the level of iNOS expression is comparable to that obtained with LPS treatment without aspirin. However, 3 μM 15d-PGJ$_2$ treatment was not able to suppress iNOS expression induced by 1 μg/ml LPS alone. The values for iNOS expression, obtained with the densitometrical analysis of the bands, under the four experimental conditions (LPS, LPS + aspirin, LPS + aspirin + 15d-PGJ$_2$, LPS + 15d-PGJ$_2$) were normalized for LPS-induced iNOS and were compared in four different experiments (Fig. 2). The treatment with 15d-PGJ$_2$ alone did not induce any iNOS expression comparing to control conditions (data not shown). The difference between LPS and LPS + aspirin groups was statistically significant ($P = 0.05$) as well as the difference between LPS + aspirin + 15d-PGJ$_2$ and LPS + aspirin groups, whereas there was no statistically significant difference between LPS + aspirin + 15d-PGJ$_2$ and LPS groups and between LPS + 15d-PGJ$_2$ and LPS groups ($P = 0.05$).

Nitric oxide is an ambivalent molecular signal: it serves as substrate for the formation of toxic peroxynitrite and it stimulates the cGMP synthesis by guanylcyclase [33]. There is a rough correspondence between toxic and homeostatic functions of NO and its production in large and small quantities, respectively [23], even if the formation of toxic peroxynitrite depends also on the availability of oxygen radicals [34]. Activated glial cells represent the main source of NO in the CNS. In the present study, the modulation of NO production and iNOS expression by aspirin was investigated in rat cortical glial cells stimulated with LPS. We used cultures of mixed glial cells, consisting of microglial and astroglial cells, to evaluate the effect of aspirin maintaining the intercellular cross-talk between astrocytes and microglia. Aspirin decreased in a dose-dependent manner the production of NO by LPS-treated glial cells, most likely through a direct action on the enzymatic molecule. The viability of cells incubated with aspirin (1.25–10 mM) was always greater than 90%, in agreement with previous results in similar systems [14], indicating that the inhibition of NO production was not due to aspirin toxicity. On the other side, aspirin enhanced, in a dose-dependent fashion, LPS-induced iNOS expression. Since LPS induces both iNOS and COX-2 [39], in our experiments the low level of iNOS expression after the stimulation with LPS of glial cells could be due to a negative feedback control exerted by prostaglandins on iNOS expression. In fact, we observed that, simultaneously administrating 15d-PGJ$_2$ to the LPS- and aspirin-treated cells, the enhancement of iNOS expression promoted by aspirin was reversed, whereas that prostaglandin alone did not affect the LPS iNOS induction. Therefore, although 15d-PGJ$_2$ is known to inhibit iNOS expression in several cell types including astrocytes and microglia [30], in our experimental conditions the 15d-PGJ$_2$ treatment does not suppress directly iNOS expression induced by LPS, but 15d-PGJ$_2$ counteracts the enhancement of the iNOS levels elicited by aspirin. This hypothesis is supported by the described effect of interferon-γ as counterregulator of PG and NO synthesis mediated via COX-2 and iNOS, favoring the production of NO while inhibiting the PG cascade [11].

The anti-inflammatory properties of acetylsalicylic acid seem to be mediated by various mechanisms depending on the concentration used. Whereas 0.01–1 mM of aspirin primarily interfere with PG biosynthesis by inhibition of COX-2, higher amounts are able to suppress NF-κB activation, which is indispensable in the transcription of pro-inflammatory cytokines, in addition to the well-known mechanism of COX inhibition [10]. The regulation of iNOS is complex and previous

studies indicate that NO may function as a negative feedback regulator of iNOS enzyme activity by interacting with the enzyme-bound heme [7]. Other authors have reported that NO, generated intracellularly following iNOS induction or released from spontaneous NO donor, limits the transcriptional induction of the iNOS gene, by a negative autoregulatory feedback, in rat astroglial cells [29] and in RAW 264.7 macrophages [12]. These mechanisms could explain the opposite action on iNOS expression and nitrite accumulation exerted by aspirin. Interestingly, aspirin does not completely block NO production by LPS-treated glial cells (within the studied concentration range), so that NO homeostatic functions could be maintained, while toxic effects, corresponding to high NO concentrations, could be prevented. Other authors reported that aspirin inhibits iNOS expression [1,15,32]. Opposite actions of aspirin could depend on the cells used as model in vitro or on their stimulation: different kind of cytokines or their combination activate different intracellular signals. In addition, it is unclear which NOS form is most important for aspirin effects [27] and our results may be due to differential effects of microglia versus astrocytes. In mixed glial cells many intercellular signals could contribute to the final result. In fact there is evidence that the presence of astrocytes is essential for the inhibitory effect of TNFβ on NO production by microglial cells [40]. Several reports show NO production and iNOS expression in cultured astroglial cells [22], but primary astrocytes cultures always contain microglial elements to a varying but substantial degree and recently immunohistochemistry and histochemistry demonstrated that iNOS expression is restricted to microglial cells [31,37,40]. Thus, contaminating microglial cells seem to be responsible for NO production in astrocyte cultures. For that reason we decided to use mixed astrocytes-microglia cell cultures. In summary, we have shown that aspirin, both at pharmacological and suprapharmacological doses, enhances LPS-induced iNOS expression, whereas decreases LPS-induced NO production in glial cells. It is possible to hypothesize that aspirin interferes both with the cross-talking of prostaglandins and NO, via inhibition of prostaglandin production, and with the NO negative feedback on iNOS expression, via inhibition of NO production. Further study will be required to elucidate the involved mechanisms.

Acknowledgement

This research has been supported by a grant from Fondazione Cassa di Risparmio di Fabriano e Cupramontana.

References

[1] E.E. Aeberhard, S.A. Henderson, N.S. Arabolos, J.M. Griscavage, F.E. Castro, C.T. Barrett, L.J. Ignarro, Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene, Biochem. Biophys. Res. Commun. 208 (1995) 1053–1059.

[2] K.T. Akama, C. Albanese, R.G. Pestell, L.J. Van Eldik, Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5795–5800.

[3] A. Bellocq, S. Suberville, C. Philippe, F. Bertrand, J. Perez, B. Fouqueray, G. Cherqui, L. Baud, Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor-kappaB activation, J. Biol. Chem. 273 (1998) 5086–5092.

[4] A. Bernardo, G. Levi, L. Minghetti, Role of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and its natural ligand 15-deoxy-Delta12,14-prostaglandin J_2 in the regulation of microglial functions, Eur. J. Neurosci. 12 (2000) 2215–2223.

[5] S. Das, H. Potter, Expression of the Alzheimer amyloid-promoting factor antichymotrypsin is induced in human astrocytes by IL-1, Neuron 14 (1995) 447–456.

[6] T.O. Fischmann, A. Hruza, X.D. Niu, J.D. Fossetta, C.A. Lunn, E. Dolphin, A.J. Prongay, P. Reichert, D.J. Lundell, S.K. Narula, P.C. Weber, Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation, Nat. Struct. Biol. 6 (1999) 233–342.

[7] J.M. Griscavage, N.E. Rogers, M.P. Sherman, L.J. Ignarro, Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide, J. Immunol. 151 (1993) 6329–6337.

[8] H. Hackstein, A.E. Morelli, A.T. Larregina, R.W. Ganster, G.D. Papworth, A.J. Logar, S.C. Watkins, L.D. Falo, A.W. Thomson, Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells, J. Immunol. 166 (2001) 7053–7062.

[9] I.O. Han, K.W. Kim, J.H. Ryu, W.K. Kim, p38 mitogen-activated protein kinase mediates lipopolysaccharide, not interferon-gamma, induced inducible nitric oxide synthase expression in mouse BV2 microglial cells, Neurosci. Lett. 325 (2002) 9–12.

[10] C. Hartel, J. von Puttkamer, F. Gallner, T. Strunk, C. Schultz, Dose-dependent immunomodulatory effects of acetylsalicylic acid and indomethacin in human whole blood: potential role of cyclooxygenase-2 inhibition, Scand. J. Immunol. 60 (2004) 412–420.

[11] S.J. Hewett, Interferon-gamma reduces cyclooxygenase-2-mediated prostaglandin E2 production from primary mouse astrocytes independent of nitric oxide formation, J. Neuroimmunol. 94 (1999) 134–143.

[12] B. Hinz, K. Brune, A. Pahl, Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages, Biochem. Biophys. Res. Commun. 271 (2000) 353–357.

[13] W.D. Hirst, K.A. Young, R. Newton, V.C. Allport, D.R. Marriott, G.P. Wilkin, Expression of COX-2 by normal and reactive astrocytes in the adult rat central nervous system, Mol. Cell Neurosci. 13 (1999) 57–68.

[14] D. Kepka-Lenhart, L.-C. Chen, S.M.J. Morris, Novel actions of aspirin and socium salicylate: discordant effects on nitric oxide synthesis and induction of nitric oxide synthase mRNA in a murine macrophage cell line, J. Leukoc. Biol. 59 (1996) 840–846.

[15] H. Kim, E. Lee, T. Shin, C. Chung, N. An, Inhibition of the induction of the inducible nitric oxide synthase in murine brain microglial cells by sodium salicylate, Immunology 95 (1998) 389–394.

[16] E. Kopp, S. Ghosh, Inhibition of NF-kappa B by sodium salicylate and aspirin, Science 265 (1994) 956–959.

[17] G.W. Kreutzberg, Microglia: a sensor for pathological events in the CNS, Trends Neurosci. 19 (1996) 312–318.

[18] K.D. McCarthy, J. de Vellis, Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue, J. Cell Biol. 85 (1980) 890–902.

[19] P.L. McGeer, M. Schulzer, E.G. McGeer, Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies, Neurology 47 (1996) 425–432.

[20] L. Minghetti, A. Nicolini, E. Polazzi, C. Creminon, J. Maclouf, G. Levi, Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E2 and by cyclooxygenase inhibitors, Glia 19 (1997) 152–160.
[21] L. Minghetti, E. Polazzi, A. Nicolini, C. Creminon, G. Levi, Interferon-gamma and nitric oxide down-regulate lipopolysaccharide-induced prostanoid production in cultured rat microglial cells by inhibiting cyclooxygenase-2 expression, J. Neurochem. 66 (1996) 1963–1970.
[22] S. Murphy, Production of nitric oxide by glial cells: regulation and potential roles in the CNS, Glia 29 (2000) 1–13.
[23] C. Nathan, Inducible nitric oxide synthase: what difference does it make? J. Clin. Invest. 100 (1997) 2417–2423.
[24] C. Nathan, Q.W. Xie, Regulation of biosynthesis of nitric oxide, J. Biol. Chem. 269 (1994) 13725–13728.
[25] E. Nishio, Y. Watanabe, Aspirin and salicylate enhances the induction of inducible nitric oxide synthase in cultured rat smooth muscle cells, Life Sci. 63 (1998) 429–439.
[26] L.A. O'Neill, C. Kaltschmidt, NF-kappa B: a crucial transcription factor for glial and neuronal cell function, Trends Neurosci. 20 (1997) 252–258.
[27] M. Oka, M. Wada, A. Yamamoto, Y. Itoh, T. Fujita, Functional expression of constitutive nitric oxide synthases regulated by voltage-gated Na^+ and Ca^{2+} channels in cultured human astrocytes, Glia 46 (2004) 53–62.
[28] K. Pahan, A.M. Namboodiri, F.G. Sheikh, B.T. Smith, I. Singh, Increasing cAMP attenuates induction of inducible nitric-oxide synthase in rat primary astrocytes, J. Biol. Chem. 272 (1997) 7786–7791.
[29] S.K. Park, H.L. Lin, S. Murphy, Nitric oxide limits transcriptional induction of nitric oxide synthase in CNS glial cells, Biochem. Biophys. Res. Commun. 201 (1994) 762–768.
[30] T.V. Petrova, K.T. Akama, L.J. Van Eldik, Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-Delta12,14-prostaglandin J_2, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 4668–4673.
[31] H. Possel, H. Noack, J. Putzke, G. Wolf, H. Sies, Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies, Glia 32 (2000) 51–59.
[32] L. Sanchez de Miguel, T. de Frutos, F. Gonzalez-Fernandez, V. del Pozo, C. Lahoz, A. Jimenez, L. Rico, R. Garcia, E. Aceituno, I. Millas, J. Gomez, J. Farre, S. Casado, A. Lopez-Farre, Aspirin inhibits inducible nitric oxide synthase expression and tumour necrosis factor-alpha release by cultured smooth muscle cells, Eur. J. Clin. Invest. 29 (1999) 93–99.
[33] P. Schubert, T. Morino, H. Miyazaki, T. Ogata, Y. Nakamura, C. Marchini, S. Ferroni, Cascading glia reactions: a common pathomechanism and its differentiated control by cyclic nucleotide signaling, Ann. N. Y. Acad. Sci. 903 (2000) 24–33.
[34] Q. Si, Y. Nakamura, T. Ogata, K. Kataoka, P. Schubert, Differential regulation of microglial activation by propentofylline via cAMP signaling, Brain Res. 812 (1998) 97–104.
[35] W.L. Smith, R.M. Garavito, D.L. DeWitt, Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2, J. Biol. Chem. 271 (1996) 33157–33160.
[36] R. Sokal, F.J. Rohlf, Principle and Practices of Statistics in Biological Research, Freeman W.H. and Co, 1991, p. 887.
[37] C. Sola, C. Casal, J.M. Tusell, J. Serratosa, Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells, Eur. J. Neurosci. 16 (2002) 1275–1283.
[38] W.F. Stewart, C. Kawas, M. Corrada, E.J. Metter, Risk of Alzheimer's disease and duration of NSAID use, Neurology 48 (1997) 626–632.
[39] T.A. Swierkosz, J.A. Mitchell, T.D. Warner, R.M. Botting, J.R. Vane, Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids, Br. J. Pharmacol. 114 (1995) 1335–1342.
[40] V.A. Vincent, F.J. Tilders, A.M. Van Dam, Inhibition of endotoxin-induced nitric oxide synthase production in microglial cells by the presence of astroglial cells: a role for transforming growth factor beta, Glia 19 (1997) 190–198.
[41] K.K. Wu, Control of COX-2 and iNOS gene expressions by aspirin and salicylate, Thromb. Res. 110 (2003) 273–276.
[42] J. Wyman, S.J. Gill, Binding and Linkage, University Science Books, 1990, p. 330.