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Prostaglandin E_2 synthesis is differentially affected by reactive nitrogen intermediates in cultured rat microglia and RAW 264.7 cells

Cecilia Guastadisegni[a], Luisa Minghetti[b], Alessia Nicolini[b], Elisabetta Polazzi[b], Paola Ade[a], Maria Balduzzi[c], Giulio Levi[b,*]

[a] *Laboratory of Environmental Hygiene, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[b] *Neurobiology Section, Laboratory of Pathophysiology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[c] *Toxicology Biochemical Science Section, ENEA, Via Anguillarese 301, 00061 Rome, Italy*

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Abstract We studied the effects of nitric oxide (NO) on prostanoid production, cyclooxygenase (COX-2) expression and [^{3}H]arachidonic acid (AA) release in RAW 264.7 macrophagic cells and rat microglial primary cultures. Inhibition of NO synthesis enhanced microglial prostanoid production without affecting that of RAW 264.7 cells. Both 3-morpholinosydnonimine (SIN-1), (which, by releasing NO and superoxide, leads to the formation of peroxynitrite), and *S*-nitroso-*N*-acetylpenicillamine (SNAP), (which releases only NO), inhibited microglial prostanoid production, by preventing COX-2 expression. In contrast, in RAW 264.7 cells, SIN-1 enhanced both basal and LPS-stimulated prostanoid production by upregulating COX-2, while SNAP stimulated basal production and slightly inhibited the LPS-induced production, as a cumulative result of enhanced AA release and depressed COX-2 expression. Thus, reactive nitrogen intermediates can influence prostanoid production at distinct levels and in different way in the two cell types, and results obtained with RAW 264.7 cells can not be extrapolated to microglia.

Key words: Nitric oxide; Peroxynitrite; Prostaglandin H synthase; Cyclooxygenase; Arachidonic acid

1. Introduction

Prostaglandins (PGs), the arachidonic acid (AA) metabolites of the cyclooxygenase (COX) pathway, and nitric oxide (NO), whose formation from L-arginine is catalysed by NO synthase (NOS), play major roles in regulating inflammation, immune functions, blood vessel dilatation and neurotransmission [1]. Both COX and NOS exist in two major isoforms: the constitutive forms (COX-1 and cNOS) are present in many cells and are responsible for the production of PGs and NO involved in physiological functions; the inducible forms (COX-2 and iNOS) are rapidly up-regulated upon appropriate stimulation. COX-2 and iNOS are the major isoforms expressed in inflammatory cells and catalyse the production of high levels of PGs and NO, present in pathological conditions, such as acute or chronic inflammation [2,3].

Interactions between PGs and NO are of particular interest at inflammatory sites, where their synthesis is often elicited by the same stimuli (pro-inflammatory cytokines, bacterial endotoxin, etc.), and where they may exert synergistic as well as antagonistic actions on common targets. Modulation of the COX pathway by NO has been studied in several systems with no uniform results [4]. Yet, the results obtained with one particular system are often extrapolated to macrophages in general and microglia in particular. In a previous study we have shown that NO down-regulates COX-2 expression and PG production in microglial cells, the brain resident macrophages [5], which produce large amounts of NO and PGs in response to LPS [6,7]. In other macrophage systems, including the monocytic/macrophagic cell line RAW 264.7, NO was reported to stimulate PG synthesis [8]. This stimulatory activity has been related to an interaction of NO with the heme group of COX, which would result into an increased enzymatic activity. However, the recent finding that NO binding with the heme of COX-2 enzyme results in its inactivation [9,10], suggests that other mechanisms of interaction between NO and COX-2 may account for the increased PG production [11].

In order to understand the reason for the reported conflicting results, we analysed the alterations in the PG metabolic pathway in microglia and RAW 264.7 cells, induced by an inhibitor of endogenous NO synthesis or by compounds capable of releasing NO or peroxynitrite, a NO derivative which may account for several biological effects of NO [12]. We found that NO and affect the PG pathway at different levels and often in an opposite way in the two cell types.

2. Materials and methods

2.1. Reagents

The mouse monocytic/macrophagic cell line RAW 264.7 was kindly provided by Prof. B. Brune, University of Erlangen-Nurnberg, Germany. RPMI 1640 medium without glutamine was from HyClone (Cramlington, UK), Glutamax I supplement containing L-alanyl-glutamine was from Life Technologies (San Giuliano Milanese, Italy). LPS (serotype 026:B6), N^G-monomethyl-L-arginine (NMMA), 3-morpholinosydnonimine (SIN-1), *S*-nitroso-*N*-acetylpenicillamine (SNAP) were obtained from Sigma (Milan, Italy). [^{3}H]AA (60-100 Ci/mmol) and [^{3}H]prostaglandin E_2 (PGE_2) (171 Ci/mmol) were from Du Pont Nuclear (New England).

2.2. Cell culture and biochemical determinations

RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated foetal calf serum, 2 mM of Glutamax I (complete RPMI 1640 medium). Cells were plated at a density of 6×10^5 cells/cm^2 in 24-well plates. Microglial secondary cultures were prepared from 10–14-day mixed primary glial cultures obtained from the cerebral cortex of 1-day-old rats, as previously described [13]. They were plated on plastic in 24-well plates (1.25×10^5 cells/cm^2), allowed to adhere for 20 min, and washed to remove non-adhering cells. The cultures con-

*Corresponding author. Fax: (39) (6) 4990 2071.
E-mail: glevi@vm.iss.it

Abbreviations: NO, nitric oxide; NOS, NO synthase; LPS, lipopolysaccharide; NMMA, N^G-monomethyl-L-arginine; AA, arachidonic acid; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine

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sisted of ≥99% microglia/macrophages (positive for the macrophage marker ED1). Cells were grown in basal Eagle's medium (BME) supplemented with 10% foetal calf serum, 2 mM glutamine and 2 μg/ml of gentamicin (complete BME medium). Both cell cultures were allowed to adhere overnight, and stimulated for 24 h with the indicated inducers or inhibitors after a medium change. Then culture media were collected, centrifuged, and stored at −20°C, until tested. The level of PGE_2 released into culture media was quantified using a specific radioimmunoassay [6]. Detection limit was 25 pg/ml. Nitrite, a stable NO oxidation product, was determined using the Griess reaction [5], by a procedure modified from that of Tracey [14]. Detection limit was 0.3 μM.

For the evaluation of COX expression, RAW 264.7 and microglial proteins obtained from cell lysates [6] were subjected to western blot analysis using specific antibodies for COX-1 and COX-2 isoforms [15]. The Amersham ECL system was used to detect the antibodies. Purified COX-1 (from ram seminal vesicles) and COX-2 (from sheep placenta) were used as standard controls (0.5 μg/lane). Determination of COX-2 expression level was performed using the GS-700 Imaging densitometer (Bio-Rad). The intensity of the bands was evaluated semiquantitatively and the values measured in treated samples were always referred to the corresponding control samples, which were run in the same gel.

In order to estimate AA release, RAW 264.7 and microglial cells were incubated for 20–22 h in complete media supplemented with 0.5 μCi/ml [^{3}H]AA, washed (3 times) with complete media, and incubated at 37°C with the indicated test substances for 2 h or 5 h. The supernatants were then collected and counted for radioactivity. The incorporation pattern of [^{3}H]AA in microglial cells was as already described [6] and in RAW 264.7 cells, after a 22 h labelling period, was: phosphatidylcholine, 16%; phosphatidylethanolamine, 37%; phosphatidylinositol, 22%; phosphatidylserine, 3%; neutral lipids, 7%. Such [^{3}H]AA distribution remained identical after labelling periods of 40 and 48 h.

3. Results

3.1. Effects of endogenous NO on PGE_2 synthesis

RAW 264.7 cells spontaneously released detectable levels of both PGE_2 (0.08 ± 0.02 ng/ml, mean ± S.E.M., $n = 7$) and NO (3.23 ± 1.41 μM, mean ± S.E.M., $n = 3$, measured as accumulation of nitrite in culture media). Such basal release was significantly increased to 0.41 ± 0.09 ng/ml of PGE_2 and 16.22 ± 3.45 μM of NO in the presence of 1 μg/ml of LPS. The effect of LPS was dose-dependent and was maximal at the above concentration (not shown). Microglial cultures from both neonatal and adult rat brains are also able to produce PGE_2 and NO but compared to RAW 264.7 cells require a much lower LPS concentration (10 ng/ml) to achieve optimal PGE_2 and NO production [6,7]. The amount of nitrite produced was similar in the two cell types (around 100 nmol/mg protein), when stimulated with optimal LPS concentrations, while PGE_2 production was much higher in microglia (15.8 ± 2.4 ng/mg protein, mean ± S.E.M., $n = 4$) than in RAW 264.7 cells (2.32 ± 0.55 ng/mg protein, mean ± S.E.M., $n = 8$).

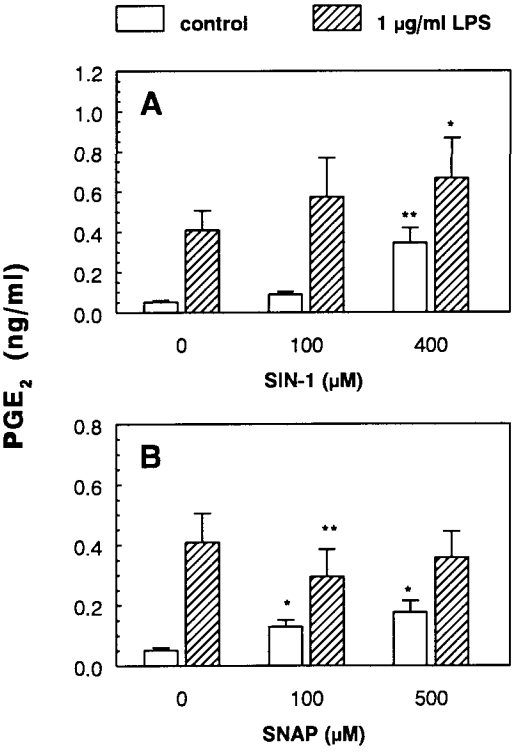

Fig. 1. Effect of the NO donors SIN-1 (100 and 400 μM) (A) and SNAP (100 and 500 μM) (B) on the basal and LPS-induced PGE_2 production in RAW 264.7 cells. The cells were incubated for 24 h in the presence of the NOS inhibitor NMMA (400 μM), without or with 1 μg/ml of LPS. Values are means ± S.E.M. of four independent experiments assayed in triplicate. $*p < 0.05$ and $**p < 0.01$ compared with the corresponding control.

To assess the regulation of PGE_2 synthesis by endogenous NO, we measured PGE_2 production in microglial and RAW 264.7 cells, after 24 h incubation with optimal LPS concentrations in the absence or in the presence of N^G-monomethyl-L-arginine (NMMA), a specific NO-synthase inhibitor (Table 1). NMMA strongly reduced NO production in both cell types, although lower inhibitor concentrations were required for microglia. Consistently with previous results [5], the inhibition of NO production in microglia was accompanied by a two fold increase of PGE_2 release. Such increase was similar with two concentrations of NMMA (20 and 200 μM) causing 70% and 90% inhibition of NO synthesis, respectively. On the other hand, NMMA (200 and 400 μM) inhibited by 70% NO

Table 1
Effect of NMMA on LPS-stimulated PGE_2 and NO production in RAW 264.7 and microglia

	NMMA (μM)	PGE_2 (% of LPS)	NO_2^-
RAW 264.7			
LPS (1 μg/ml)	200	87 ± 12	33 ± 5
	400	91 ± 8	27 ± 5
Microglia			
LPS (10 ng/ml)	20	204 ± 23	32 ± 6
	200	195 ± 22	7 ± 4

Cells were stimulated with LPS in the presence of NMMA. After 24 h, supernatants were collected and the production of PGE_2 and NO (measured as nitrite, NO_2^-) determined. LPS-induced production was taken as 100%. Values are means ± S.E.M. of three to four independent experiments, assayed in duplicate (microglia) or triplicate (RAW 264.7 cells).

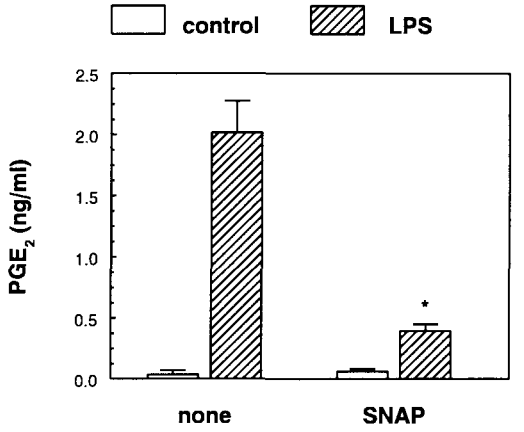

Fig. 2. Effect of the NO donor SNAP on the basal and LPS-induced PGE_2 production in microglia. Microglial cells were incubated for 24 h in the presence of the NOS inhibitor NMMA (200 μM), without or with SNAP (100 μM). Values are means ± S.E.M. of four independent experiments, assayed in triplicate. $^*p < 0.05$ as compared to the LPS-stimulated cells.

synthesis but did not affect significantly the production of PGE_2 in RAW 264.7 cells. Higher concentrations of NMMA were not tested, to avoid non-specific effects on COX activity [1]. While the LPS-stimulated PGE_2 production found in RAW 264.7 cells was comparable with that reported by others, we did not find an inhibition of PGE_2 production by NMMA as others did [8].

3.2. Effects of NO donors on PGE_2 synthesis

The effect of exogenous NO on PGE_2 synthesis in RAW 264.7 and microglial cells was evaluated by using the synthetic donors 3-morpholinosydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). The concentrations used (100 and 400 μM for SIN-1; 100 and 500 μM for SNAP), were substantially lower than those utilised in other studies [16], and produced an accumulation of nitrite in culture media which was from 2 to 10 folds higher than that induced by LPS. It is difficult to say whether the extracellular NO levels achieved in culture media may be close to those reached in vivo in the vicinity of activated macrophages or microglia in pathological conditions.

To avoid interference with the endogenously produced NO, all the experiments were performed in the presence of the inhibitor NMMA (200 μM for microglia and 400 μM for RAW 264.7 cells). In RAW 264.7 cells, SIN-1 and SNAP dose-dependently increased basal PGE_2 production, while the LPS-stimulated production was enhanced by SIN-1 and moderately decreased, with a statistically significant effect only at the lower concentration, by SNAP (Fig. 1). In microglial cultures, both 100 μM SIN-1 [5] and, even more so, SNAP (Fig. 2) inhibited the LPS-induced PGE_2 production, with no effect on basal release.

3.3. Effects of NO donors on AA release

The possibility that NO interfered with prostanoid production at the level of AA release was investigated. After 2 h exposure, LPS dose-dependently stimulated [^{3}H]AA release from prelabelled RAW 264.7 cells (50 ± 2% and 90 ± 17% over basal release with 1 and 5 μg/ml of LPS, respectively, mean ± S.E.M., $n = 4$), in agreement with previous observations in the same cell line [16] and microglial cultures [3]. This effect of LPS was not affected by NMMA (400 μM, not shown).

The two NO donors, however, affected in a different way [^{3}H]AA release in the two cell types. In RAW 264.7 cells, SIN-1 (100 μM) did not affect basal release after 2 h (Fig. 3A) or 5 h (not shown) of incubation and caused a dubious increase in the LPS-induced release (Fig. 3A), which was no longer visible at a higher SIN-1 concentration (400 μM, not shown). At variance with SIN-1, SNAP (500 μM) markedly increased (> 70%) basal [^{3}H]AA release and moderately enhanced the LPS-induced release (25%) in this cell line (Fig. 3B). Using yet another NO donor, sodium nitroprusside, both basal and LPS-induced [^{3}H]AA release were reported to be increased in RAW 264.7 cells [17]. In microglial cells, SIN-1 caused a moderate, but statistically significant decrease of both basal (10 ± 3%, mean ± S.E.M., $n = 3$) and LPS-stimulated (20 ± 5%, mean ± S.E.M., $n = 3$) [^{3}H]AA release, while SNAP was devoid of effect.

3.4. Effects of NMMA, SIN-1 and SNAP on COX-2 expression

Since COX-2 was strongly down-regulated by both endogenous and exogenous NO in microglial cells [5], we sought to analyse by western blot whether the expression of COX-2 could be differently affected in RAW 264.7 cells. As for microglial cultures [5], COX-1 was not detectable in RAW 264.7 cells by western blot analysis, both in basal conditions and after stimulation with LPS. In contrast with microglia [5], COX-2 expression was clearly detectable in unstimulated RAW 264.7 cells (Fig. 4A). Exposure of RAW 264.7 cells to

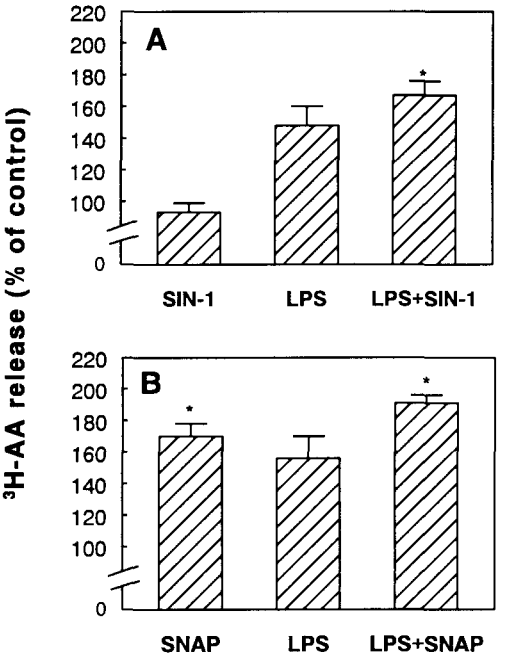

Fig. 3. [^{3}H]AA release induced by 100 μM SIN-1 (A) and 500 μM SNAP (B) in RAW 264.7 cells prelabelled with [^{3}H]AA and then incubated for 2 h with the NO donors, with or without 1 μg/ml LPS. The radioactivity released by untreated cells was taken as 100%. Values are means ± S.E.M. of four (SIN-1) and three (SNAP) independent experiments measured in triplicate. $^*p < 0.05$ compared with the corresponding control.

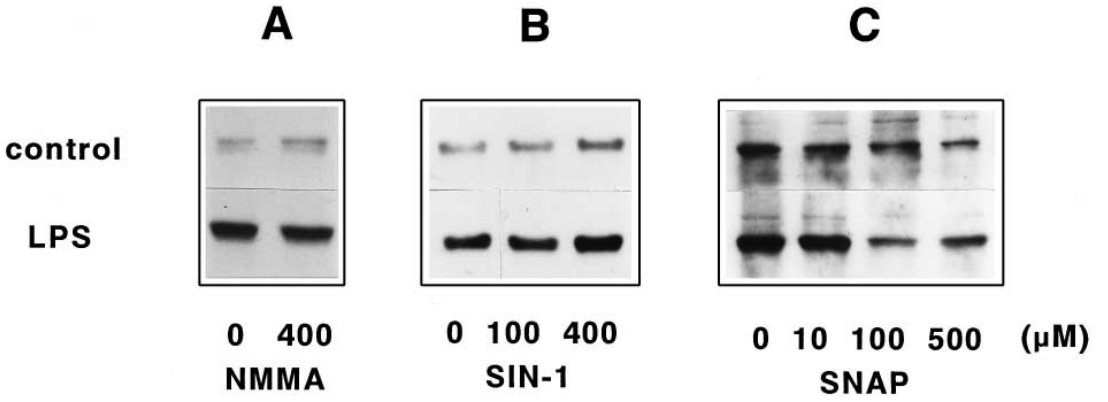

Fig. 4. Effect of NMMA, SIN-1 and SNAP on LPS-stimulated COX-2 expression in RAW 264.7 cells. Cells were incubated for 24 h in complete medium, with or without 1 μg/ml LPS, in the absence or in the presence of NMMA (A), SIN-1 (B) or SNAP (C), at the concentrations indicated. Cell lysates were prepared and equal amounts of protein (25 μg) were analysed by western blot using anti-COX-2 specific antibodies (1:500). Protein bands were visualised using horseradish peroxidase-conjugated secondary antibodies and ECL. One experiment representative of four (A) and three (B, C) is shown.

LPS (1 μg/ml) markedly enhanced COX-2 expression (4.8 ± 1.5 fold, mean ± S.E.M., $n = 4$) (Fig. 4A), and microglia behaved in a similar fashion [5]. NMMA (400 μM), which enhanced the LPS-induced expression of COX-2 in microglia [5], did not affect the basal nor the LPS-induced COX-2 expression in RAW 264.7 cells (tested in four independent experiments), in agreement with the lack of significant effect of NMMA on LPS-stimulated PGE_2 production (Table 1). The stimulatory effect of SIN-1 (400 μM) on PGE_2 production in RAW 264.7 cells (Fig. 2A) was paralleled by an increased expression of COX-2, which was evident both in basal conditions (44% ± 10) and after LPS stimulation (29% ± 5), as evaluated by densitometric analysis of western blot bands (means ± S.E.M., $n = 3$) (Fig. 4B). A lower concentration of SIN-1 (100 μM) enhanced only slightly the basal expression of COX-2 and did not affect that induced by LPS (Fig. 4B). After treatment with SNAP (which enhanced basal and depressed LPS-induced PGE_2 production in RAW 264.7 cells, see Fig. 2B), both basal and LPS-stimulated COX-2 expression were depressed in a concentration-dependent way (Fig. 4C). Once again, the effects of SIN-1 and SNAP on RAW 264.7 cells differed from those on microglia, where SIN-1 reduced by 47.5% ($n = 2$, see also ref. [5]) and SNAP almost abolished the LPS-induced expression of COX-2 (Fig. 5), while neither compound affected the basal (undetectable) expression of the enzyme.

4. Discussion

Increasing evidence indicates that NO can stimulate or inhibit PG synthesis, depending on the cell type under investigation.

By comparing the effect of NO on PGE_2 synthesis in primary microglial cultures and in RAW 264.7 cells, we have shown that NO can exert its action at multiple levels of the COX pathway. Indeed, in microglial cultures, COX-2 expression appeared to be the main regulatory step, and its down-regulation by exogenous NO, released by the synthetic donor SNAP, caused a reduction of PGE_2 synthesis. In RAW 264.7 cells the pattern of the effects of SNAP was more complex and could be explained as follows: the enhancement of basal PGE_2 production was likely to be related to a substantially increased availability of the PGE_2 precursor AA, which could counteract the inhibitory effect of the drug on COX-2 expression. In this basal condition, AA rather than COX-2 availability could be the major rate-limiting step in PGE_2 synthesis. In cells stimulated with LPS, however, the massive inhibitory effect of SNAP on COX-2 expression may not be counteracted by the small stimulation of AA release observed in this condition, with a resulting depression, albeit small, in the synthesis of PGE_2.

In addition to SNAP, which (similar to other *S*-nitrosothiols) is considered to release exclusively NO [18], we also studied the effects of SIN-1, a donor of two radicals, O_2^- and NO, which may combine to form peroxynitrite ($OONO^-$). Peroxynitrite may account for several of the biological effects of NO [12,19]. In RAW 264.7 cells, SIN-1 behaved differently from SNAP, as it enhanced both basal and LPS-stimulated PGE_2 production. Such enhancement appeared to be mainly, if not exclusively, mediated by an increased expression of COX-2.

As the main difference between SIN-1 and SNAP is the ability of the former to generate peroxynitrite, our observations favour the idea that in RAW 264.7 cells COX-2 expression is stimulated by peroxynitrite and inhibited by NO, while AA release is mainly stimulated by NO. On the other hand, in microglia LPS-stimulated COX-2 expression and PGE_2 pro-

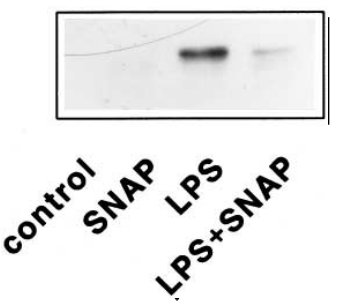

Fig. 5. Effect of SNAP on basal and LPS-stimulated COX-2 expression in microglia. Microglial cells were subcultured for 24 h, then the medium was renewed and the cells were incubated with or without 10 ng/ml LPS, in the absence or in the presence of 100 μM SNAP. After 24 h cell lysates were prepared and analysed as described in Fig. 4.

duction were both consistently inhibited by SIN-1 and SNAP. Therefore, both NO and peroxynitrite would appear to inhibit COX-2 expression, while only peroxynitrite exerts a moderate depressing effect on AA release.

In conclusion, major differences exist in the effects of nitrogen radicals on PG production between the two macrophagic cell types considered in the present study. It is difficult to say whether this is due to the adaptation of the microglial phenotype to the peculiar cerebral environment, or to the loss of the original macrophagic phenotype by the RAW 264.7 cell line. Certainly, however, results obtained with RAW 264.7 cells can not be extrapolated to microglia.

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