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The role of prostaglandin E_2 and nitric oxide in cell death in J774 murine macrophages

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Abstract

We investigated the role of prostaglandin E_2 (PGE_2) and its interactions with nitric oxide (NO) on cell death and NO-mediated cytotoxicity in the murine macrophage cell line J774. Stimulation of the J774 cells with lipopolysaccharide together with interferon-γ resulted in a dose-dependent cytotoxicity and production of PGE_2 and NO, measured as nitrite. Our results showed a linear correlation between PGE_2 release and cytotoxicity. The cyclooxygenase (COX) inhibitor indomethacin completely inhibited PGE_2 biosynthesis, without affecting NO production or cell death. This supports previous reports suggesting that overproduction of endogenous PGE_2 is mainly the consequence of cell death and does not cause it. In contrast, the NO synthase inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) gave a significant, though incomplete suppression of NO release and cell death. This points to the presence of other cytotoxic factors besides NO. To evaluate the toxic effect solely due to NO, macrophages were exposed to the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Incubation with SNAP also resulted in a concentration-dependent cell injury and PGE_2 production. When exogenously added, PGE_2 protected against SNAP-mediated cytotoxicity and simultaneously increased PGE_2 release into the medium, without inducing COX-2. The cytoprotection and the stimulation of PGE_2 release were both reversed by indomethacin. In conclusion, PGE_2 biosynthesis may represent a mechanism by which inflammatory macrophages protect themselves against the cytotoxic effects of NO.

Keywords: Nitric oxide (NO); Prostaglandin E_2; Lipopolysaccharide; Cytotoxicity; Macrophage

1. Introduction

The radical nitric oxide (NO) is produced by cells of the immune system, besides other mediators, and acts as a potent bactericidal and tumouricidal agent (Stuehr and Nathan, 1989). Murine macrophages produce NO from L-arginine by the inducible NO synthase, an enzyme that also exists in constitutive isoforms in other cells (Moncada and Higgs, 1993). Expression of inducible NO synthase after stimulation by bacterial endotoxin and other cytokines is accompanied by the release of other mediators such as prostaglandin E_2 (PGE_2) and prostacyclin via the cyclooxygenase (COX) pathway (Nathan, 1987; Salvemini et al., 1993). This synergistic production has been subject of several studies (Salvemini et al., 1996; Di Rosa et al., 1996), and a recent work suggests a crucial role for the link between the NO synthase and COX pathways in certain pathological conditions such as nephrosis, sepsis, or rheumatoid arthritis (Salvemini et al., 1993). The focus of most studies, however, has been on the role of NO in the expression and/or activity of COX (Salvemini et al., 1993; Swierkosz et al., 1995), and the effect of eicosanoids on the NO synthesis by the activation of the inducible NO synthase (Marotta et al., 1992; Bulut et al., 1993; Milano et al., 1995).

After stimulation with lipopolysaccharide, macrophage-derived NO diffuses to the neighbouring cells where it exerts cytostatic and/or cytolytic activity (Nathan and Hibbs, 1991). Confirming other studies (Drapier and Hibbs, 1988; Albina et al., 1989; Tucker et al., 1991), we have previously shown that apart from its cytotoxicity to tumour cells and other micro-organisms, the endogenously produced NO is cytotoxic to the macrophages themselves (Zamora et al., 1997). Given the increasing body of evidence suggesting that prostaglandins, mainly PGE_2, are major eicosanoids produced during immunostimulation and

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inflammatory events, we investigated the role of PGE_2 and its interactions with NO on cell death and NO-mediated cytotoxicity.

2. Materials and methods

2.1. Materials

S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was kindly provided by Dr. Martin Feelisch (Schwarz Pharma, Monheim, Germany). Reduced glutathione, *N*-acetyl-D,L-penicillamine, 3-amino-7-dimethyl-2-methylphenazine hydrochloride (neutral red), PGE_2 and lipopolysaccharide (phenol-extracted *Salmonella typhosa*) were purchased from Sigma (St. Louis, MO, USA). Sodium nitrite was from Merck (Darmstadt, Germany). N^{ω}-monomethyl-L-arginine (L-NMMA) was a gift from Dr. S. Moncada (The Wellcome Research Laboratories, Beckenham, UK). Recombinant rat interferon-γ was from Holland Biotechnology (Leiden, The Netherlands). *S*-methylisothiourea sulphate was from Fluka (Buchs, Switzerland). Indomethacin was given by Merck, Sharp and Dohme (Rahway, NJ, USA). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was from Alexis (Läufelfingen, Switzerland). Rabbit antisera to murine COX-2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). All cell culture media and supplements were from Gibco (Paisley, UK).

2.2. Cells and culture medium

Murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium containing 1000 mg/l glucose and supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 U/ml polymyxin B. Cells were seeded in 24-well plates (1 ml) or in 96-well microtiter plates (250 μl) at a concentration of 10^6 cells/ml and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter, medium was aspirated to remove non-adherent cells and replaced by fresh medium containing various concentrations of lipopolysaccharide plus interferon-γ or the NO-donors as described in Section 3. Following another 24 h incubation the medium was collected, centrifuged and stored at −20°C for later nitrite (NO_2^-) and prostaglandin determination.

2.3. Determination of NO production

Nitrite (NO_2^-) accumulation in the cell-free supernatant (indicating NO formation by stimulated cells or from SNAP decomposition) was measured in a microtiter assay using the Griess reagent as previously described (Zamora et al., 1997). The detection limit was 1 μM.

2.4. Prostaglandin production

PGE_2 was measured by radioimmunoassay (RIA). Dose interpolation was done with a four parameter logistic function (Dudley et al., 1985), using the IBM-PC RIA data reduction package provided by M.L. Jaffe (Silver Spring, MD, USA). The detection limit was 150 pg/ml. Similarly, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the breakdown product of prostacyclin) was assessed by a specific and sensitive (detection limit 80 pg/ml) RIA as previously described (Bult et al., 1985). Increase in the endogenous PGE_2 release in supernatant of cells exposed to SNAP plus various concentrations of exogenous PGE_2 was calculated using the formula:

$$PGE_2 \text{ concentration (fold)} = \left[C_{\text{sample}} - \left(C_{\text{SNAP}} + C_{\text{exog. PGE}_2}\right)\right] / \left(C_{\text{SNAP}} - C_{\text{control}}\right)$$

where C_{sample} represents the PGE_2 concentration in the sample supernatant; C_{SNAP}, the PGE_2 concentration in supernatant of cells exposed to SNAP alone; $C_{\text{exog. PGE}_2}$, the concentration of the exogenous PGE_2 added; and C_{control}, the concentration of PGE_2 in supernatant of control cells.

2.5. Cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red by viable cells as previously described (Löwik et al., 1993). Cells were incubated at 37°C with neutral red (0.01%) for 1–1.5 h. Culture medium was removed by inverting the plates, and after washing the cells (2×) with phosphate-buffered saline, the dye was extracted with 0.05 M NaH_2PO_4 in 50% ethanol. The extent of incorporation of neutral red was quantified by the measurement of OD_{540} in 96-well microtiter plate. Background absorbance (blank) was determined from wells containing extracting solution only. The viability and cytotoxicity were calculated according to the following formulas:

$$\text{Viability (\%)} = \left[\left(OD_{540} \text{ sample} - OD_{540} \text{ blank}\right) / \left(OD_{540} \text{ control} - OD_{540} \text{ blank}\right)\right] \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - \text{Viability (\%)}$$

The proteins of the cell monolayers were precipitated with 5% trichloroacetic acid, dissolved in 0.1 N NaOH plus 0.5% sodium dodecyl sulphate, and then quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) as previously described (Smith et al., 1985).

2.6. Western blotting

Expression of COX-2 protein was measured by Western blotting as described by Pang and Hoult (1997a). The density of immunoreactive bands corresponding to COX-2 (70 kDa) was quantified by laser densitometric scanning with an Ultrascan XL (Pharmacia-LKB, Uppsala, Sweden).

2.7. Statistics

All values are expressed as mean ± S.E.M. of *n* experiments performed in duplicate or triplicate. One-way analysis of variance (ANOVA) followed by the Bonferroni's test or Student's unpaired *t*-test, as appropriate, were used for statistical determinations. Unless indicated, a *P*-value less than 0.05 was considered significant.

3. Results

3.1. Nitrite production by stimulated macrophages: effect of L-NMMA and indomethacin

J774 cells were incubated with different concentrations (0.001–100 μg/ml) of lipopolysaccharide plus interferon-γ (100 U/ml) for 24 h. Immunostimulation resulted in a dose-dependent increase of NO_2^- (Fig. 1). As can be seen,

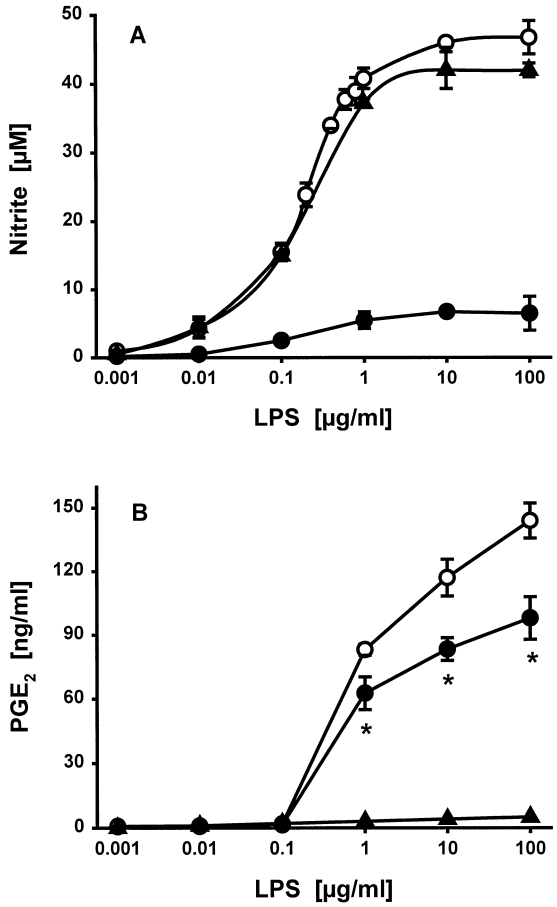

Fig. 1. Accumulation of NO_2^- (A) and PGE_2 (B) in culture supernatant of immunostimulated J774 macrophages. Cells were incubated with various concentrations of lipopolysaccharide plus 100 U/ml interferon-γ in the presence of 0.5 mM L-NMMA (closed circles) or 10 μM indomethacin (closed triangles). After 24 h the supernatant were collected for NO_2^- and PGE_2 determination. Control cells (open circles) were incubated in culture medium alone. Data are expressed as the mean ± S.E.M. ($n = 3$, $* P < 0.05$).

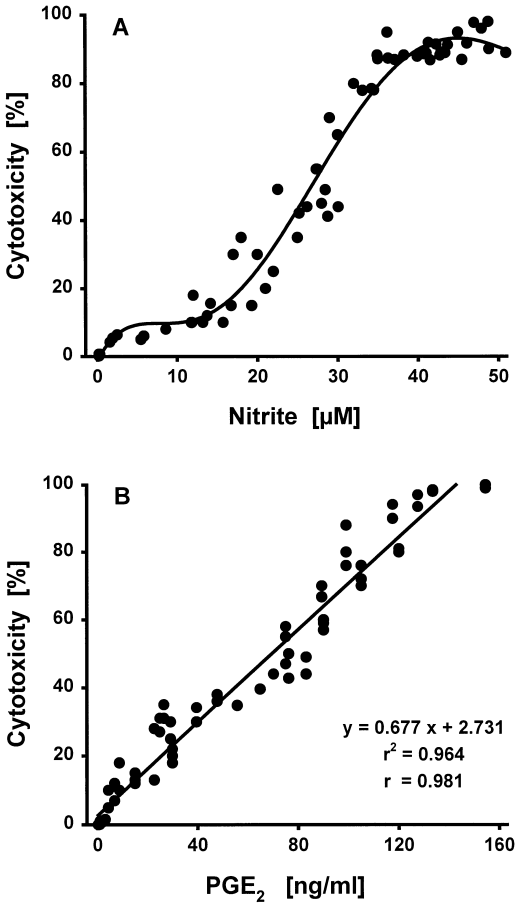

Fig. 2. Correlation between cytotoxicity, NO_2^- production (A) and PGE_2 release (B) in immunostimulated macrophages. After 24 h stimulation with lipopolysaccharide (0–100 μg/ml) plus interferon-γ (100 U/ml) the supernatant were collected for PGE_2 and NO_2^- determination and medium containing 0.01% neutral red was added to culture plates for 1.5 h. The dye was then extracted for measuring OD_{540} and the cytotoxicity was calculated as described in Section 2. The cytotoxicity for each triplicate culture in two representative experiments was plotted directly against the corresponding PGE_2 and NO_2^- values.

NO_2^- concentrations do not significantly increase at lipopolysaccharide concentrations above 1 μg/ml. NO_2^- production was strongly reduced (about 75–80%) by addition of the inducible NO synthase inhibitor L-NMMA 30 min before stimulation. Addition of the non-selective COX inhibitor indomethacin (10 μM) has no significant effect on NO_2^- production (Fig. 1). A significant inhibition of about 30–40% could only be achieved with a very high concentration (230 μM) of indomethacin (not shown, $n = 2$).

3.2. Prostaglandin production by stimulated macrophages: effect of indomethacin and L-NMMA

Stimulation of the macrophages with different concentrations of lipopolysaccharide plus interferon-γ (100 U/ml), resulted in a concentration-dependent production of PGE_2 (Fig. 1) and prostacyclin (not shown). Inhibition

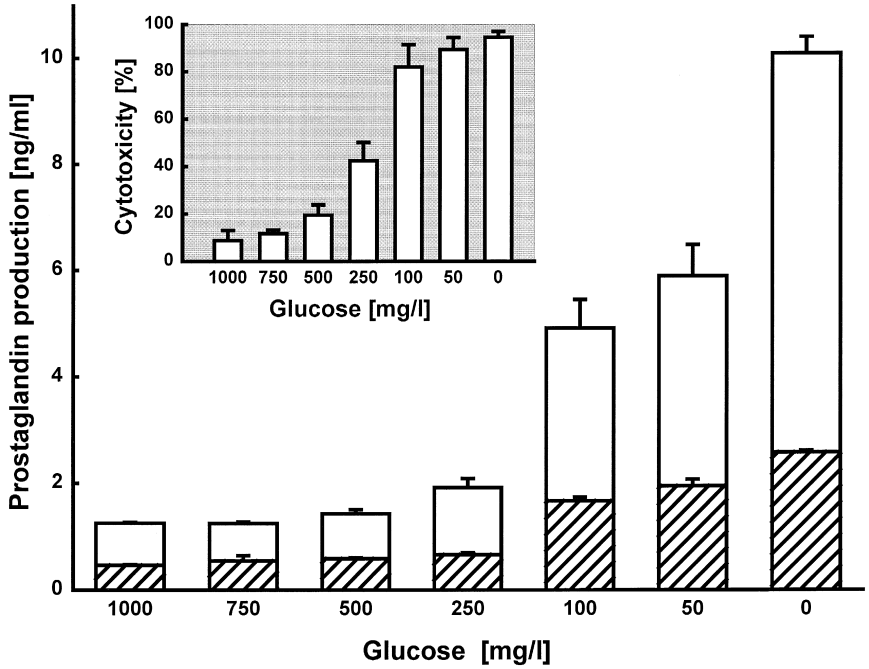

Fig. 3. Effect of decreasing glucose concentrations on SNAP-induced prostaglandin production and cytotoxicity in J774 macrophages. Cells were incubated with 100 μM SNAP in medium containing different glucose concentrations (0–1000 mg/l) for 24 h. The culture supernatant were then collected for prostaglandin determination. Open bars represent PGE_2 and hatched bars represent prostacyclin measured as 6-keto-$PGF_{1\alpha}$. The inset shows the effect of glucose on cytotoxicity. Results are the mean ± S.E.M. of an experiment performed in triplicate.

of COX activity by indomethacin (10 μM) completely inhibited the basal and lipopolysaccharide plus interferon-γ-induced PGE_2 production by the J774 cells. The inducible NO synthase inhibitor L-NMMA (0.5 mM) also inhibited the release of PGE_2 but to a much lesser extent than indomethacin (Fig. 1). Similar results were obtained with another inducible NO synthase inhibitor, *S*-methylisothiourea (not shown).

3.3. Cytotoxic effects of immunostimulation

Macrophages were stimulated with different concentrations of bacterial lipopolysaccharide for 24 h. Immunostimulation caused a concentration-dependent cytotoxicity and increase in NO_2^- and PGE_2 production. The relationships between NO_2^-, PGE_2 and cytotoxicity were studied. The cytotoxicity was plotted directly against the corresponding NO_2^- (Fig. 2A) and PGE_2 (Fig. 2B) values. There was a statistically significant positive correlation between the levels of PGE_2 in the culture medium and the degree of cytotoxicity (Fig. 2B). Though the relationship between NO_2^- production and cytotoxicity was sigmoidal, it appeared to be linear between 15 and 35 μM NO_2^- (Fig. 2A). Addition of the inducible NO synthase inhibitor L-NMMA (0.5 mM) prior to stimulation with lipopolysaccharide (1 μg/ml) plus interferon-γ (100 U/ml) significantly diminished cytotoxicity: from 85 ± 3 to 29 ± 3% ($n = 3$). In contrast, incubation with 10 μM indomethacin did not affect lipopolysaccharide-induced cell death: from 82 ± 6 to 76 ± 10% ($n = 3$).

3.4. Effect of the nitrosothiol S-nitroso-N-acetyl-D,L-penicillamine on cell viability and prostaglandin production

In 1000 mg/l glucose-containing medium, incubation with SNAP (0.1–1000 μM) for 24 h did not result in significant cell death and even at 1000 μM SNAP cells maintained about 80% viability. Assuming that NO-ex-

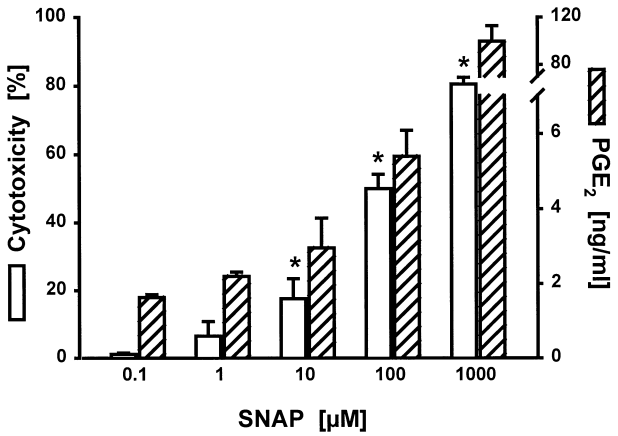

Fig. 4. SNAP-mediated cytotoxicity (open bars) and PGE_2 release (hatched bars) in J774 macrophages. Cells were incubated in 250 mg/l glucose-containing medium plus SNAP (0.1–1000 μM) for 24 h. The culture supernatant were then collected for PGE_2 determination, and fresh medium containing 0.01% neutral red was added to the culture plates. After 1.5 h, the dye was extracted for measuring OD_{540} as described in Section 2. Cytotoxicity values are expressed as the mean ± S.E.M. ($n = 3$, $* P < 0.05$).

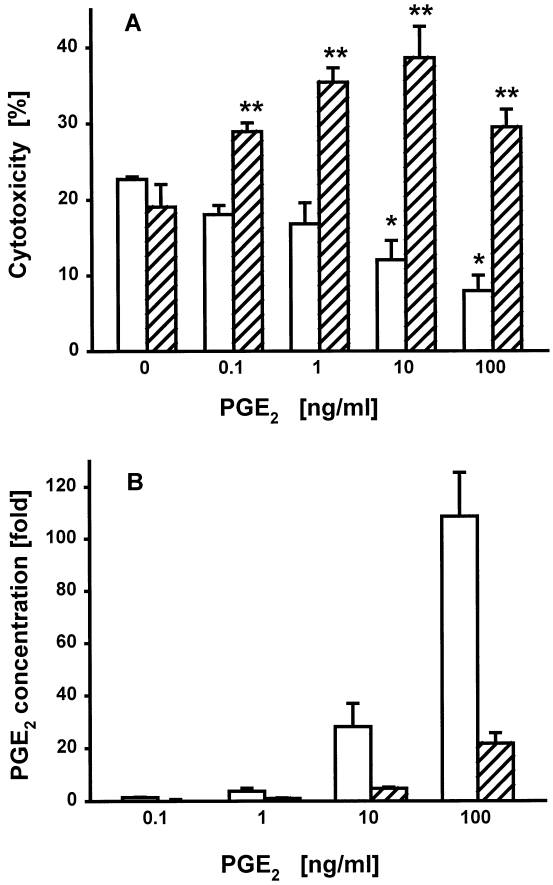

Fig. 5. Effect of exogenous PGE_2 and indomethacin on SNAP-mediated cytotoxicity (A) and PGE_2 production (B) in J774 macrophages. Cells were exposed for 24 h to 100 μM SNAP plus different concentrations of PGE_2 in the absence (open bars) or presence (hatched bars) of 10 μM indomethacin. The culture supernatant were then collected for PGE_2 determination, and fresh medium containing 0.01% neutral red was added to the culture plates. After 1.5 h, the dye was extracted for measuring OD_{540}. Cytotoxicity and increase of PGE_2 production were calculated as described in Section 2. Values are expressed as the mean $\pm$ S.E.M. ($n = 3$, $* P < 0.05$ vs. control without indomethacin, $** P < 0.01$ between groups with and without indomethacin).

posed cells may survive because of the sufficient supply of glucose for glycolysis and ATP production, cells were exposed to SNAP in low glucose medium. The toxic effects of SNAP were enhanced by decreasing the glucose content of the medium. This also resulted in a glucose concentration-dependent release of PGE_2 and smaller amounts of prostacyclin (Fig. 3). Since glucose concentrations below 250 mg/l dramatically decreased the viability of SNAP-treated cells, as well as of control cells, further experiments were performed in 250 mg/l glucose-containing medium. When the glucose content of the medium was reduced to 250 mg/l, cytotoxicity increased in a dose-dependent manner and only about 20% of the cells remained viable at 1000 μM SNAP (Fig. 4). SNAP also caused a concentration-dependent increase in the PGE_2 production. Addition of similar concentrations of *N*-acetyl-D,L-penicillamine or the SNAP metabolites *N*-acetyl-D,L-penicillamine disulphide and NO_2^- did not affect the viability ($n = 2$). The cytotoxic effect of SNAP was significantly inhibited by the NO scavenger (Akaike et al., 1993) carboxy-PTIO. Addition of the latter (100 μM) together with SNAP (100 or 1000 μM) decreased cytotoxicity from 50 ± 5 to $7 \pm 5\%$ and from 82 ± 4 to $59 \pm 2\%$, respectively ($n = 3$). Addition of 10 μM indomethacin before SNAP (100 μM) decreased the cytotoxicity only by about 10%, whereas the PGE_2 production was inhibited by 95% (not shown, $n = 3$). However, when cells were exposed to SNAP (100 μM) in the presence of a very high concentration of indomethacin (230 μM), cytotoxicity was completely abolished. Apparently, such a high concentration of indomethacin has no effect on the NO_2^- production from SNAP, because there was no significant difference between NO_2^- values in the supernatant of SNAP-exposed cells in the presence (44 ± 2 μM) or absence (46 ± 0.2 μM) of indomethacin ($n = 3$).

3.5. Effect of exogenous prostaglandin E_2 and indomethacin on S-nitroso-N-acetyl-D,L-penicillamine-mediated cytotoxicity

To test the effect of exogenous PGE_2 on NO-mediated cytotoxicity, cells were exposed to 100 μM SNAP in the presence of increasing concentrations of PGE_2. Addition of PGE_2 alone (0.1–100 ng/ml) has no significant effect on cytotoxicity. In contrast, when cells were exposed to PGE_2 in the presence of SNAP (100 μM), there was a small but significant decrease of SNAP-mediated cytotoxicity (Fig. 5A). Interestingly, the addition of exogenous PGE_2 to the cell cultures potentiated the SNAP-mediated

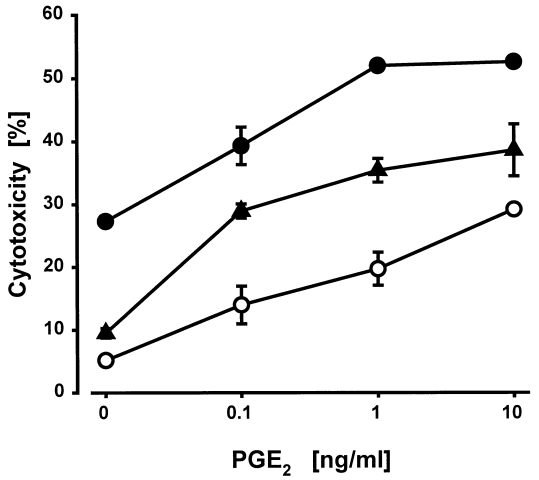

Fig. 6. Effect of indomethacin on cytotoxicity induced by SNAP and exogenous PGE_2 in J774 macrophages. Cells were exposed for 24 h to 100 μM SNAP plus different concentrations of PGE_2 in the presence of 1 μM (open circles), 10 μM (closed triangles), and 50 μM (closed circles) indomethacin. The supernatant was removed and replaced by fresh medium containing 0.01% neutral red. After 1.5 h, the dye was extracted for measuring OD_{540}. Cytotoxicity was calculated as described in Section 2. Values are expressed as the mean $\pm$ S.E.M. ($n = 3$).

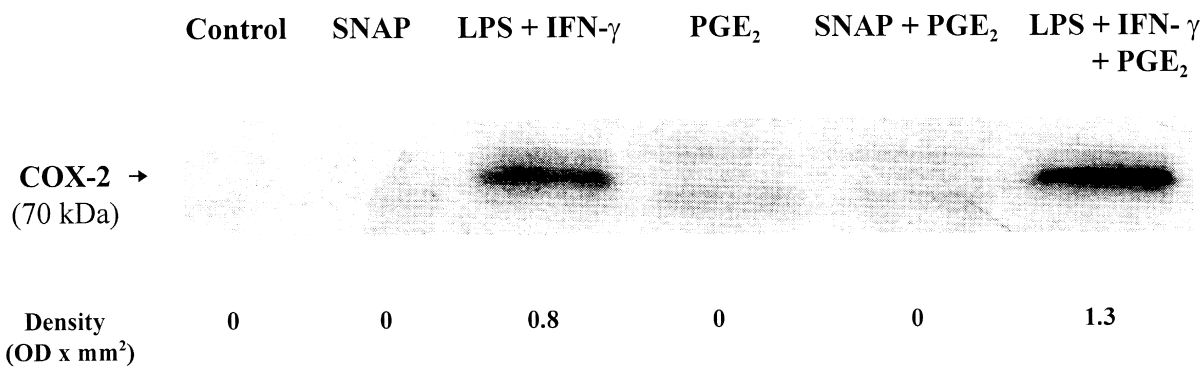

Fig. 7. Effect of SNAP (100 μM), PGE_2 (10 ng/ml) and lipopolysaccharide (LPS, 1 μg/ml) plus interferon-γ (IFN-γ, 100 U/ml) on the expression of COX-2 in J774 macrophages. Cells were exposed to the test compounds at the concentrations indicated for 24 h. Cell lysates were prepared and equal amounts of protein (20 μg) were analysed by Western blot using anti-COX-2 antibodies (1:2000). Protein bands were visualised using horseradish peroxidase-conjugated secondary antibodies and ECL. The position and molecular weight of COX-2 was validated by reference to molecular mass markers (Rainbow™, Amersham).

release of endogenous PGE_2 in a dose-dependent manner: PGE_2 values in culture supernatant of cells exposed to SNAP increased from about 2-fold for 0.1 ng/ml exogenous PGE_2 to more than 100-fold for 100 ng/ml PGE_2 (Fig. 5B).

In contrast, addition of indomethacin (10 μM) together with PGE_2 showed the opposite effect. Whereas addition of PGE_2 reduced the cytotoxic effects of SNAP, in the presence of indomethacin this phenomenon was reversed, resulting in a potentiation of SNAP-induced cell death (Fig. 5A). Moreover, the effect of indomethacin was dependent on both the concentration of the PGE_2 added and the concentration of the inhibitor (Fig. 6). The exogenous PGE_2-mediated potentiation of SNAP-induced PGE_2 release was, as could be expected, significantly reduced by indomethacin (Fig. 5B). It should be noted, however, that the accuracy of the reported PGE_2 concentrations (for 100 ng/ml exogenous PGE_2) is restricted due to this large amount of PGE_2 that required several dilutions for the RIA measurements.

3.6. Western blotting

In order to test whether exogenous PGE_2 induced the expression of COX-2 in SNAP-treated macrophages, cells lysates were analysed by Western blotting using anti-COX-2 specific antibodies. COX-2 protein was not detectable by Western blot analysis, either in basal conditions or after treatment with SNAP (100 μM) in the presence or absence of PGE_2 (10 ng/ml). In contrast, COX-2 expression was clearly detectable in lipopolysaccharide (1 μg/ml) plus interferon-γ (100 U/ml)-stimulated cells shown as positive control (Fig. 7).

4. Discussion

Macrophages produce NO by the L-arginine/NO pathway (Moncada and Higgs, 1993). After macrophage stimulation with bacterial endotoxin plus interferon-γ, induction of inducible NO synthase and NO production is also accompanied by the release of prostaglandins via the COX pathway (Nathan, 1987; Salvemini et al., 1993). Most studies have mainly focused on whether the L-arginine/NO and COX pathways regulate each other, but the role of prostaglandins (and their interactions with NO) in cell death and NO-mediated cytotoxicity has not been fully investigated. The present work was designed to study the relationships between the simultaneous production of PGE_2 and NO, and the cytotoxic effects of immunostimulation in J774 macrophages. Since exposure of macrophages to NO donors, e.g., SNAP, also results in activation of COX enzymes (Salvemini et al., 1993) and cell injury (Szabó et al., 1996; Zamora et al., 1997), the role of PGE_2 on SNAP-mediated cytotoxicity was also investigated.

The increase of inducible NO synthase activity following immunostimulation in macrophages is a well-established phenomenon (Nathan, 1992). In this study, we also found that incubation with bacterial lipopolysaccharide plus interferon-γ led to a dose-dependent production of NO_2^- in J774 cells, an effect prevented by the inducible NO synthase inhibitor L-NMMA. Addition of the COX inhibitor indomethacin was without significant effect on the NO_2^- production. It indicates that the products of the COX pathway do not play a major role in the regulation of NO synthase activity, and confirms previous studies showing the endogenous release of prostanoids from the RAW 264.7 and J774.2 macrophages being insufficient to affect the activity of NO synthase (Salvemini et al., 1993; Swierkosz et al., 1995). However, the effects of prostaglandins on NO synthase activity are still controversial. Low concentrations of indomethacin have been reported to significantly reduce NO formation (Milano et al., 1995) and the amount of inducible NO synthase protein (Pang and Hoult, 1996) in lipopolysaccharide-stimulated J774 macrophages. In our study, an inhibition of the NO_2^-

formation of about 30–40% could only be found when indomethacin was used in a very high concentration (230 μM). Recently, several anti-inflammatory drugs, like aspirin and sodium salicylate have been shown to inhibit induced NO production by immunostimulated RAW 264.7 cells at the high end of therapeutic concentrations. Moreover, this effect was not simply the result of inhibition of prostaglandin synthesis because exogenous PGE_2 failed to overcome the effects of both drugs (Brouet and Ohshima, 1995; Kepka-Lenhart et al., 1996). Thus, the inhibitory effect of the high concentration of indomethacin in the present study might not be conclusive for answering the question of the involvement of prostaglandins in the regulation of NO synthase.

In contrast to the controversial role of prostaglandins on NO production, the stimulatory effects of NO on COX activity are generally accepted. We found that stimulation of the J774 cells with lipopolysaccharide together with interferon-γ resulted in a dose-dependent production of PGE_2, most likely following the induction of COX-2 as shown by Western blotting. Indomethacin completely inhibited, as expected, the large PGE_2 production by lipopolysaccharide plus interferon-γ-stimulated macrophages. The inducible NO synthase inhibitor L-NMMA also inhibited the release of PGE_2, but to a much lesser extent than the COX inhibitor indomethacin. This finding confirms the results of other investigators in RAW 264.7 (Salvemini et al., 1993) and J774 macrophages (Swierkosz et al., 1995). These studies, however, differ from our present work in that they used as stimulus lipopolysaccharide alone, and the experiments were performed under conditions where viability was not affected. Moreover, when the cytotoxicity was plotted against the corresponding supernatant PGE_2 concentrations, there was a significant positive correlation between the actual levels of PGE_2 and the degree of cytotoxicity. Thus, measurement of PGE_2 production in lipopolysaccharide plus interferon-γ-stimulated macrophages appears to be a marker for cytotoxicity in effector cells. Interestingly, a previous study in human fibroblasts showed stimulation of PGE_2 production by sodium arsenite, a stress response inducer (Salzman and Bowman, 1992). Determination of the arsenite cytotoxicity indicated a high correlation of stimulation of prostaglandin release with cytotoxicity, but it appeared to be a correlative rather than causative occurrence in the stress response (Salzman and Bowman, 1992). Similarly, a recent study with J774 cells and mouse peritoneal macrophages showed the toxic effects of tetrandrine, an alkaloid used in China for the treatment of silicosis, and that cell death was accompanied by the release of large quantities of PGE_2. Moreover, inhibition of prostaglandin biosynthesis with indomethacin did not affect drug-induced toxicity (Pang and Hoult, 1997a) and it was concluded that prostaglandin overproduction occurs as a consequence of cell death and does not cause it. In our experiments, inhibition of COX activity by indomethacin did not affect lipopolysaccharide-induced cell injury. This further confirms the former findings that PGE_2 production is mainly the consequence of cell activation and cell death, but does not contribute significantly to the cytotoxic processes.

The macrophage-derived NO is also cytotoxic to the effector cell itself (Drapier and Hibbs, 1988; Albina et al., 1989; Tucker et al., 1991). In one of these studies, Albina et al. (1989) showed the effect of increasing concentrations of L-arginine on NO_2^- production and viability of rat resident peritoneal macrophages in culture. Loss of viability correlated with the accumulation of NO_2^- in the culture supernatant, and L-NMMA reduced both effects. In contrast, another study with a NO-sensitive tumour cell showed the absence of correlation between the lipopolysaccharide-dependent NO production by immunostimulated macrophages and the cytotoxic activity of NO against the effector cells themselves (Mateo et al., 1996). The release of cytotoxic mediators, other than NO, may account for this lack of correlation. The fact, that the inducible NO synthase inhibitor L-NMMA gave a significant, but incomplete inhibition of cell death, supports previous suggestions of the existence of additional pathways that contribute to the immune-mediated cell injury in this cell line (Zamora et al., 1997; Szabó et al., 1996). Whether the synthesis of NO or NO-related compound(s) induces the formation of other cytotoxic agents also warrants further investigation. The exact relationship between NO_2^- production and cytotoxicity over a wide range of stimulus-concentration remains controversial. Here, we also found that activation with lipopolysaccharide plus interferon-γ produced an increase in NO_2^- production and concentration-dependent cell death, supporting a direct relationship between NO and toxicity.

Since L-NMMA significantly inhibited lipopolysaccharide-induced cell death, we therefore investigated the effect of PGE_2 on a cytotoxic process only mediated by NO. Exposure of J774.A1 murine macrophages to the NO-donor SNAP resulted in a concentration-dependent loss of viability. The lack of effect of *N*-acetyl-D,L-penicillamine, its disulphide or NO_2^- alone and the prevention of cell injury by carboxy-PTIO, a known inhibitor of NO-mediated biological responses (Akaike et al., 1993), pointed to NO or a NO-derived species as mediator of the SNAP-induced cell death. The toxic effect of SNAP was significantly enhanced under glucose-limiting conditions, confirming the importance of the presence of glucose in the culture medium for cell survival. It has been previously shown that macrophage-injured tumour cells rapidly die if glucose is exhausted from their medium (Granger et al., 1980; Hibbs et al., 1990). Given that NO can target enzymes in the mitochondria, which contribute to the synthesis of ATP, it might be argued that glucose simply provides an alternative source of ATP synthesis through glycolysis. However, an additional role of glucose is to provide a source of cytosolic reducing equivalents, in the form of NADPH that are necessary for the conversion of oxidised

glutathione to the reduced form (Albina and Mastrofrancesco, 1993). The latter is an effective antioxidant and in glutathione-depleted macrophages the toxic effects of SNAP are greatly enhanced (Zamora et al., 1997). Thus, glucose may play a role in maintaining the intracellular redox milieu. There is no evidence for direct chemical trapping of NO by glucose, but this cannot be ruled out. The mechanisms underlying the protective role of glucose on NO-mediated cytotoxicity require further investigation.

SNAP also caused a concentration-dependent increase of the PGE_2 production (and in a lesser degree of prostacyclin), that was directly proportional to the decrease in the content of glucose and loss of macrophage viability. Again a low concentration of indomethacin was only slightly cytoprotective, whereas the PGE_2 production was almost completely inhibited. Interestingly, when cells were exposed to SNAP in the presence of a very high concentration of indomethacin (230 μM), cytotoxicity was completely abolished. This was apparently not due to a suppression of the NO production, because there was no significant difference between NO_2^- values in supernatant of SNAP-exposed cells in the presence and absence of indomethacin. At present, we do not have any explanation for this effect. Such a high concentration of indomethacin (230 μM) has been previously used to inhibit COX activity in lipopolysaccharide-stimulated bovine aortic endothelial cells and J774.2 macrophages as well (Akarasereenont et al., 1995). However, the fact that this concentration is at least 20 times higher than the most commonly used concentration in pharmacological studies (10 μM) may question the clinical or therapeutic relevance of the afforded protection against SNAP-mediated cytotoxicity.

It is known that PGE_2 is a regulator of macrophage functions and displays a functional dualism in immunoinflammatory conditions (Bonta and Ben-Efraim, 1993). A protective role for exogenous, as well as endogenous PGE_2 has already been described (Koyama et al., 1991; Dennery et al., 1992). A recent study showed that lipopolysaccharide (0.5 μg/ml) induces a mild cytotoxicity of J774 cells that is suppressed in a dose-dependent fashion after pretreatment with exogenous PGE_2 for 2 h (Pang and Hoult, 1997b). However, the role of PGE_2 on NO-mediated cytotoxicity has not received special attention in the literature so far. Here, we show that exogenous PGE_2 has also a small but significant protective effect against SNAP-mediated injury. Although in our experiments the cells were not pre-exposed to PGE_2, the results are in agreement with the above mentioned study. Surprisingly, the protection was accompanied by a synergistic potentiation of NO-mediated PGE_2 release and it was dependent on the concentration of the exogenous PGE_2 added. This suggest a different type of regulatory role for PGE_2 in macrophages, involving a mechanism by which increasing concentrations of extracellular PGE_2 enhance the NO-mediated synthesis of the intracellular prostaglandin and prevent cell injury. Such high prostaglandin concentrations can be found in some malignant neoplasms, presumably produced by tumour cells to suppress the activity of immunocompetent cells (Bonta and Ben-Efraim, 1993; Owen et al., 1980). The mechanism for the overproduction of PGE_2 is not known, but it was not due to the induction of COX-2 as shown by Western blotting.

The fact that addition of indomethacin reversed the protective effects of exogenous PGE_2 and augmented SNAP-induced cell death cytotoxicity may point to the involvement of the COX pathway in the observed cytoprotection. However, this does not necessarily exclude other toxic effects of indomethacin such as uncoupling of oxidative phosphorylation in the mitochondria (Brune et al., 1976) or other mechanisms reviewed elsewhere (Flower, 1974). Further experiments will be thus required to clarify whether indomethacin contributes in some other way to NO-induced cell death.

In summary, our results demonstrate that in lipopolysaccharide plus interferon-γ-activated J774 macrophages, there is linear correlation between PGE_2 release and cytotoxicity against the effector cells themselves. Although the cytotoxicity is also related to the induction of inducible NO synthase and NO production, release of mediators other than NO are involved in the immunostimulation-mediated cell injury as well. The lack of protection observed when incubation was done in the presence of indomethacin contrasts with the generally assumed cytoprotective role for prostaglandins. The role of prostaglandins on macrophage cell death may, therefore, vary in different experimental models depending on the character of the stimulus, the amount and activity of COX enzymes present, and the presence of other cells, such as tumour cells. In this respect, we showed that exogenous PGE_2 has a concentration-dependent protective effect against SNAP-mediated cytotoxicity. PGE_2 potentiated the NO-mediated release of endogenous PGE_2 and indomethacin reversed both the cytoprotection by exogenous PGE_2 and the endogenous prostaglandin production. This may represent a mechanism by which intratumoural inflammatory cells such as macrophages, respond to increasing amounts of PGE_2 and NO produced by tumour cells by producing high amounts of PGE_2, which in turn might inhibit tumour cell metabolism and growth. Indeed, evidence exist for the inhibition of tumour growth in vivo and in vitro by E-type prostaglandins (Bonta and Ben-Efraim, 1993).

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References

Akaike, T., Yoshida, M., Miyamoto, Y., Sato, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S., Maeda, H., 1993. Antagonistic action of imidazolineoxyl N-oxides against EDRF/NO through a radical reaction. Biochemistry 32, 827–832.

Akarasereenont, P., Mitchell, J.A., Bakhle, Y.S., Thiemermann, C., Vane, J.R., 1995. Comparison of the induction of cyclooxygenase and nitric oxide synthase by endotoxin in endothelial cells and macrophages. Eur. J. Pharmacol. 273, 121–128.

Albina, J.E., Mastrofrancesco, B., 1993. Modulation of glucose metabolism in macrophages by products of nitric oxide synthase. Am. J. Physiol. 264, C1594–C1599.

Albina, J.E., Mills, C.D., Henry, W.L. Jr., Caldwell, M.D., 1989. Regulation of macrophage physiology by L-arginine: role of the oxidative L-arginine deiminase pathway. J. Immunol. 143, 3641–3646.

Bonta, I.L., Ben-Efraim, S., 1993. Involvement of inflammatory mediators in macrophage antitumour activity. J. Leukocyte Biol. 54, 613–626.

Brouet, I., Ohshima, H., 1995. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. Biochem. Biophys. Res. Commun. 206, 533–540.

Brune, K., Glatt, M., Graf, P., 1976. Mechanisms of action of anti-inflammatory drugs. Gen. Pharmacol. 7, 27–33.

Bult, H., Herman, A.G., Laekeman, G.M., Rampart, M., 1985. Formation of prostanoids during intravascular complement activation in the rabbit. Br. J. Pharmacol. 84, 329–336.

Bulut, V., Severn, A., Liew, F.Y., 1993. Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP. Biochem. Biophys. Res. Commun. 195, 1134–1138.

Dennery, P.A., Walenga, R.W., Kramer, C.M., Alpert, S.E., 1992. Prostaglandin E_2 attenuates hyperoxia-induced injury in cultured rabbit tracheal epithelial cells. Pediatr. Res. 32, 87–91.

Di Rosa, M., Ialenti, A., Ianaro, A., Sautebin, L., 1996. Interaction between nitric oxide and cyclooxygenase pathways. Prostaglandins Leukotrienes Essent. Fatty Acids 54, 229–238.

Drapier, J.-C., Hibbs, J.B. Jr., 1988. Differentiation of murine macrophages to express non-specific cytotoxicity for tumour cells results in L-arginine-dependent inhibition of mitochondrial iron–sulphur enzymes in the macrophage effector cells. J. Immunol. 140, 2829–2838.

Dudley, R.A., Edwards, P., Ekins, R.P., Finney, D.J., McKenzie, I.G., Raab, R.M., Rodbard, D., Rodgers, R.P.C., 1985. Guidelines for immunoassay data processing. Clin. Chem. 31, 1264–1271.

Flower, R.J., 1974. Drugs which inhibit prostaglandin biosynthesis. Pharmacol. Rev. 26, 33–67.

Granger, D.L., Taintor, R.R., Cook, J.L., Hibbs, J.B. Jr., 1980. Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. J. Clin. Invest. 65, 357–370.

Hibbs, J.B., Jr., Taintor, R.R., Vavrin, Z., Granger, D.L., Drapier, J.-C., Amber, J.J., Lancaster, J.R., Jr., 1990. Synthesis of nitric oxide from a guanidino nitrogen of L-arginine: a molecular mechanism that targets intracellular iron. In: Moncada, S., Higgs, E.A. (Eds.), Nitric Oxide from L-arginine: A Bioregulatory System. Elsevier, Amsterdam, pp. 189–223.

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

Koyama, S., Rennard, S.I., Claassen, L., Robbins, R.A., 1991. Dibutyryl cAMP, prostaglandin E_2, and antioxidants protect cultured bovine bronchial epithelial cells from endotoxin. Am. J. Physiol. 261, L126–L132.

Löwik, C.W.G.M., Alblas, M.J., Van de Ruit, M., Papapoulos, S.E., Van de Pluijm, G., 1993. Quantification of adherent and non-adherent cells cultured in 96-well plates using the supravital stain neutral red. Anal. Biochem. 213, 426–433.

Marotta, P., Sautebin, L., Di Rosa, M., 1992. Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774. Br. J. Pharmacol. 107, 640–641.

Mateo, R.B., Reichner, J.S., Albina, J.E., 1996. NO is not sufficient to explain maximal cytotoxicity of tumouricidal macrophages against an NO-sensitive cell line. J. Leukocyte Biol. 60, 245–252.

Milano, S., Arcoleo, F., Dieli, M., D'Agostino, R., D'Agostino, P., De Nucci, G., Cillari, E., 1995. Prostaglandin E_2 regulates inducible nitric oxide synthase in the murine macrophage cell line J774. Prostaglandins 49, 105–115.

Moncada, S., Higgs, E.A., 1993. The L-arginine–nitric oxide pathway. New Engl. J. Med. 329, 2002–2012.

Nathan, C.F., 1987. Secretory products of macrophages. J. Clin. Invest. 79, 319–326.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6, 3051–3064.

Nathan, C.F., Hibbs, J.B. Jr., 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. 3, 65–70.

Owen, K., Gomolka, D., Droller, M.J., 1980. Production of prostaglandin E_2 by tumour cells in vitro. Cancer Res. 40, 3167–3171.

Pang, L., Hoult, J.R.S., 1996. Induction of cyclooxygenase and nitric oxide synthase in endotoxin-activated J774 macrophages is differentially regulated by indomethacin, enhanced cyclooxygenase-2 protein expression but reduction of inducible nitric oxide synthase. Eur. J. Pharmacol. 317, 151–155.

Pang, L., Hoult, J.R.S., 1997a. Cytotoxicity to macrophages of tetrandrine, an antisilicosis alkaloid, accompanied by an overproduction of prostaglandins. Biochem. Pharmacol. 53, 773–782.

Pang, L., Hoult, J.R.S., 1997b. Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E_2 and other cyclic AMP stimulants in J774 macrophages. Biochem. Pharmacol. 53, 493–500.

Salvemini, D., Misko, T.P., Masferrer, J.L., Seibert, K., Currie, M.G., Needleman, P., 1993. Nitric oxide activates cyclooxygenase enzymes. Proc. Natl. Acad. Sci. USA 90, 7240–7244.

Salvemini, D., Seibert, K., Marino, M.H., 1996. PG release, as a consequence of NO-driven COX activation, contributes to the proinflammatory effects of NO. Drugs News Perspect. 4, 204–219.

Salzman, J., Bowman, P.D., 1992. Independent regulation of prostaglandin production and the stress response in human fibroblasts. J. Cell. Physiol. 152, 626–631.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Proverzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klerk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Stuehr, D.J., Nathan, C.F., 1989. Nitric oxide, a macrophage product responsible for cytostasis and respiratory inhibition in tumour target cells. J. Exp. Med. 169, 1543–1545.

Swierkosz, T.A., Mitchell, J.A., Warner, T.D., Botting, R.M., Vane, J.R., 1995. Co-induction of nitric oxide synthase and cyclooxygenase, interactions between nitric oxide and prostanoids. Br. J. Pharmacol. 114, 1335–1342.

Szabó, C., Day, B.J., Salzman, A.L., 1996. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. FEBS Lett. 381, 82–86.

Tucker, S.D., Sivaramakrishnan, M.R., Klostergaard, J., Lopez-Berestein, G., 1991. Independence of the pattern of early cytokine release from autoregulation by nitric oxide. J. Leukocyte Biol. 50, 509–516.

Zamora, R., Matthys, K.E., Herman, A.G., 1997. The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells. Eur. J. Pharmacol. 321, 87–96.