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Platelet-activating factor: the effector of protein-rich plasma extravasation and nitric oxide synthase induction in rat immune complex peritonitis

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1 The involvement of platelet-activating factor (PAF) in immune complex-induced/polymorphonuclear-mediated tissue injury was studied by use of a reverse passive Arthus (RPA) model in the peritoneal cavity of rats.

2 Extravasation of protein-rich plasma, accumulation of polymorphonuclear leukocytes (PMN), and the production of nitric oxide (NO) by resident peritoneal mononuclear phagocytes were assayed.

3 Treatment of rats with either UR-12460 or BB-823, two compounds which possess different chemical structures, but elicit the same antagonistic effect on the PAF receptor, abrogated protein-rich plasma extravasation. In contrast, they did not show any effect on the accumulation of PMN.

4 Inhibition of NO production with both N^G-mono methyl-L-arginine and N^G-nitro-L-arginine failed to prevent protein-rich plasma extravasation.

5 The production of NO by peritoneal adherent cells following RPA was measured in cells maintained for 2 to 28 h in culture, and it was significantly increased in cells removed as early as 15 min after RPA induction, as compared to controls.

6 Addition of 10 nM PAF to the culture medium reduced the generation of NO by peritoneal cells from RPA rats, whereas this mediator enhanced NO production in cells from naive control animals.

7 Treatment with either UR-12460 or BB-823 prior to the induction of RPA produced an almost complete inhibition of NO production.

8 Assay of nitric oxide synthase activity in cell homogenates from peritoneal cells showed that the activity was due to the inducible form of the enzyme.

9 Study by Northen blotting of mRNA coding for the inducible NO synthase (iNOS) showed transcription at 6 and 18 h after the induction of RPA, which was inhibited in UR-12460-treated rats.

10 These data indicate that PAF is the main mediator of the early plasma leakage observed in RPA, and also that PAF is implicated in the triggering of long-term changes via induction of specific genes, as judged from its ability to promote the expression of iNOS.

Keywords: Arthus reaction; complement; cytokines; endotoxin; immune complexes; inflammatory oedema; nitric oxide; PAF; polymorphonuclear leukocytes; shock; tumour necrosis factor; vascular endothelial-cells

Introduction

The Arthus reaction was first described at the turn of the century as an acute inflammatory and haemorrhagic reaction produced in the skin of rabbits when a local injection of horse serum was administered to previously sensitized animals (Arthus, 1903). Experimentally it is convenient to investigate the reversed passive Arthus reaction (RPA), which is elicited by a local injection of antibody and an intravenous injection of its cognate antigen. This model is a good counterpart of immune complex peritonitis, when developed in the peritoneal cavity. The sequence of events that occur in the RPA reaction includes formation of immune complexes in the microvessel wall, activation of the complement cascade, migration and adherence of polymorphonuclear leukocytes (PMN) to the endothelial cells, which results in an increase of microvascular permeability (Wedmore & Williams, 1981), and release of PAF from neutrophils which acts on endothelial cells to cause further leakage (Hellewell & Williams, 1986; Warren *et al.*, 1989a; Rossi *et al.*, 1992; Tavares de Lima *et al.*, 1992). Since tissue injury in RPA is complement-dependent and largely neutrophil-mediated, detailed attention has been dedicated to ascertain the mechanisms which in addition to generation of C5a might lead to recruitment of polymorphonuclear leukocytes. Thus, it has been shown that tumour necrosis factor-α (Warren *et al.*, 1989b), interleukin (IL)-1β (Warren, 1992), cytokines related to IL-8/neutrophil activating protein 1 (Collins *et al.*, 1991), and products of oxidative metabolism of arachidonate via 5-lipoxygenase (Zhang *et al.*, 1992) are involved.

Recent therapeutic approaches to the Arthus reaction focus on the use of recombinant soluble human complement receptor type I (Rossi *et al.*, 1992) to control the activation of the complement cascade, the utilization of a recombinant soluble form of human FcγRII (FcγR, receptor for the Fc portion of IgG) genetically engineered to interfere with neutrophil activation via FcγRII, and the ensuing release of inflammatory mediators (Ierino *et al.*, 1993); and the utilization of recombinant selectin chimeric molecules to block PMN recruitment (Mulligan *et al.*, 1993). Another step for pharmacological modulation might be the antagonism of mediators released from PMN that could be particularly active either on their own or because of their central position

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in the triggering of other mediators. Recent studies have emphasized the involement of nitric oxide in the production of tissue injury in a model of alveolitis and dermal vasculitis analogous to RPA (Mulligan *et al.*, 1991; 1992), whereas there has been some controversy regarding the possible involvement of eicosanoids in the mediation of plasma leakage in RPA (Boughton-Smith *et al.*, 1993). The purpose of this study was to assess the effect of blocking receptors for the lipid mediator platelet-activating factor on events of the RPA that occur at different times after antigen challenge.

Methods

Materials and animals

Male pathogen-free Wistar rats (200–300 g) were obtained from Valladolid Medical School Animal Facility. IgG antibodies were raised in rabbits by subcutaneous injections of ovalbumin in complete Freund's adjuvant followed by booster intramuscular injections until a positive reaction in radial immunodiffusion could be observed at a dilution of antiserum 1/16. IgG2 antibodies were purified by sequential precipitation with octanoic acid and ammonium sulphate. The PAF receptor antagonist UR-12460 (1-[(N-diphenylmethyl) aminoacetyl]-4-[(2-methyl-3-pyridyl)cyanomethyl] piperazine) was obtained from URIACH, S.A., Barcelona, Spain, and solubilized in saline solution (Carceller *et al.*, 1993). The PAF receptor antagonist BB-823 (N-4-(1-H-2-methylimidazo[4,5-c]pyridylmethyl)phenylsulphonyl-L-leucinyl ethyl ether) was a gift from British Bio-technology Ltd., Oxford, U.K., and was solubilized by stoichiometric mixture with 1N HCl to form the hydrochloride salt, and dilution with saline solution (Whittaker *et. al.*, 1992). 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), N^G-nitro-L-arginine methyl ester (L-NAME), L-NOARG (N^G-nitro-L-arginine), L-NMA (N^G-methyl-L-arginine), molsidomine and isosorbide dinitrate were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Induction of the reverse passive Arthus reaction

Rats were injected i.p. with 1 mg of specific antibody and i.v. with 10 mg ovalbumin and 20 mg kg^{-1} of Evans blue dye to study extravasation of protein-rich plasma. The doses of antibody and antigen used produced an extravasation response near the maximum, which is comparable to that observed in a passive anaphylaxis model with IgE sensitizing antibody (Pellón *et al.*, 1994). The concentration of Evans blue in the peritoneal cavity was determined spectrophotometrically at a wavelength of 620 nm in the fluid obtained by washing the peritoneal cavity with 10 ml of phosphate-buffered saline solution. The results were compared with a standard curve prepared with known amounts of Evans blue dye, and the content of each sample was expressed as μg Evans blue per ml. The experimental protocol was approved by our Institutional Review Board.

Assay of polymorphonuclear accumulation into the peritoneal cavity

Neutrophil influx was evaluated by measurement of myeloperoxidase (MPO) activity colorimetrically in the lysate of cells obtained from the peritoneal cavity. MPO activity in the cell lysate was measured by the change in optical absorbance at 460 nm resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine (Henson *et al.*, 1978). The results were expressed as number of PMN obtained by comparison with a standard curve of the MPO activity of known numbers of PMN from peritoneal exudate after 6 h of RPA (Zhang *et al.*, 1992).

Assay of NO production by peritoneal macrophages ex vivo

Peritoneal cells obtained 15 min after induction of the Arthus reaction in rats were resuspended in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin, 0.5 mM L-arginine, 2mM glutamine and 10% heat-inactivated foetal calf serum. Non-adherent cells were removed two hours after challenge, and the production of NO was assessed after different times of incubation at 37°C in an atmosphere containing 5% CO_2.

Determination of NO and nitrite

NO released from macrophage cultures was determined by the accumulation of nitrite (Green *et al.*, 1982). The cell cultures (5×10^5 cells in 1 ml of phenol red-free medium) were filled with 100 μl of a solution of 1 mM sulphanilic acid and 100 mM HCl (final concentration). After incubation for 5 min the medium was aspirated and centrifuged in an Eppendorf microcentrifuge; 50 μl of naphthylenediamine (1 mM in the assay) was added to the samples, and the reaction was completed after 15 min of incubation. The absorbance at 548 nm was compared with a standard of $NaNO_2$, and the production of NO was expressed as nmol of NO_2 mg^{-1} protein.

Assay of NOS activity

NOS activity was measured in 105,000 *g* supernatants from homogenates of 10^7 cells in a medium containing 20 nM Tris, 20 mM KCl, 100 μM EGTA, 10 μM (6R)-5,6,7,8-tetrahydrobiopterin, 0.5 mM dithioerythritol (DTT), 10 μg ml^{-1} of leupeptin and 0.5 mM phenylmethylsulphonyl fluoride, pH 7.5. After partial purification by 2',5'-ADP-sepharose chromatography (0.5 ml of gel), the activity was determined by the production of [^{14}C]-citrulline from [U-^{14}C]-arginine (Schmidt *et al.*, 1992). The reaction was started by the addition of 50 μM [U-^{14}C]-arginine (0.3 μCi) to a mixture that contained 20 mM Tris, 0.5 mM NADPH, 0.1 mM DTT, and 10 μM (6R)-5,6,7,8-tetrahydrobiopterin (200 μl of incubation volume). After 10 min of incubation at 30°C, the reaction was stopped by addition of 1 ml ice-cold 10 mM EGTA, 0.1 mM citrulline, 10 mM PIPES, pH 5.5. One ml of this mixture was applied to a 1 ml Dowex AG50W-X8 column (Na^+-form), and the [U-^{14}C]-citrulline was eluted in 2 ml of water. After counting the emerging radioactivity, the enzyme activities were calculated as the differences of product formation in the absence or presence of 0.25 mM L-NMA in the reaction mixture. The Ca^{2+}-dependence of NOS was assayed in the presence of 200 μM $CaCl_2$ and 10 nM calmodulin in the reaction mixture. One unit of NOS activity was defined as the amount of protein that released 1 nmol of [U-^{14}C]-citrulline per min.

Preparation of total RNA and Northern blot analysis of NOS

Total cellular RNA was prepared from cell cultures (10^7 cells) following the guanidium isothiocynate method (Chomczynski & Sacchi, 1987). Aliquots of total RNA (5 μg) were denatured at 65°C for 15 min in 5% formaldehyde, 50% formamide and 8% glycerol and then were size-separated by electrophoresis in a 0.9% agarose gel containing 2% formaldehyde and 3-(N-morpholine) propane sulphonic acid (MOPS) buffering system (Maniatis *et al.*, 1989). After transference of the RNA to Nytran membranes (NY 13-N; Schleicher & Schuell) with 10 × SSC (10 × SSC is 1.5 M NaCl, 0.3 M sodium citrate, pH 7.4), the membranes were prehybridized for 6 h at 42°C in 50% formamide, 0.25 M NaCl, 0.1 M sodium phosphate, 7% SDS and 0.01% of salmon sperm. An 817 bp fragment (nucleotides 1 to 817)

from the complementary DNA of macrophage NOS was random primed labelled (45% efficiency) and was used to detect the level of transcription (Xie *et al.*, 1992). The membranes were washed once with 0.1 × times standard saline citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 10 min, and twice at 50°C for 30 min, followed by exposure to an X-ray film (Kodak, X-OMAT). Quantitation of the film was performed by laser densitometry (Molecular Dynamics, Kemsing, U.K.) using the hybridization with a β-actin probe (0.6 kb EcoRI/HindIII fragment isolated from VC18 vector) as internal control to demonstrate the integrity of the RNA, the equivalence of loading, and the specificity of mRNA induction.

Statistical analyses

Data are expressed as the mean ± s.e.mean. For comparison of two groups of samples normally distributed, Student's two-tailed *t* test was used to analyze differences for significance. For comparison of two groups of samples not normally distributed, the Mann-Whitney U test was used. Changes of a variable measured during a period of time were analyzed by one way analysis of variance (ANOVA). Statistical procedures were performed using a data base and statistical package (Instat, GraphPAD Software Inc., San Diego, CA, U.S.A.), $P<0.05$ was considered significant.

Results

Protein-rich plasma extravasation in RPA

Induction of RPA produced a marked increase of the accumulation of Evans blue dye in the peritoneal cavity of rats. This was already apparent at 15 min, but maximal accumulation occurred at 30-60 min (solid columns in Figure 1). Attempts to follow extravasation at later times showed a decay of the Evans blue dye (EB) in the peritoneal lavage fluid, even when the experiment was modified to perform the injection of EB at 3 h after challenge and the collection of the peritoneal exudate carried out 1 h thereafter (4.92 ± 0.24 μg ml^{-1} in a group of 3 rats). These findings were interpreted as evidence that extravasation is an early event in RPA, that subsides within 1-2 h after RPA challenge. When the rats were pretreated with an i.v. dose of 5 mg kg^{-1} of UR-12460 10 min prior to the induction of RPA, a reduction of extravasation to levels similar to those detected in vehicle-treated animals was observed at 30 min; however, the inhibition was less marked when rats were killed 60 min after challenge. This could indicate either recruitment of other extravasation-inducing mediators or metabolism of the drug. To ascertain whether the effect of UR-12460 was a consequence of its purported PAF receptor antagonist effect, experiments were also performed with BB-823, a PAF receptor antagonist with a chemical structure unrelated to UR-12460. As indicated by the cross-hatched column in Figure 1, BB-823 showed a similar protective effect on extravasation. Treatment with the L-arginine analogues L-NOARG and L-NMA alone or in combination at doses as high as 100 mg kg^{-1}, i.p., prior to challenge with antigen and antibody gave no protection (Table 1). Moreover, to substantiate that these drugs were acting on the NO system, mean arterial pressure was measured under the conditions described by Pellón *et al.* (1994) in three rats after L-NMA treatment and found to be significantly higher than in vehicle-treated rats. Attempts to enhance extravasation by the NO-generating compounds molsidomine and isosorbide dinitrate at doses of 15 mg kg^{-1} in combination with 200 mg kg^{-1} L-arginine did not influence extravasation.

Table 1 Effect of NO synthase inhibitors on Evans blue dye extravasation

Treatment	*Evans blue dye* (μg ml^{-1} peritoneal fluid)	n
Anti-ovalbumin antibody	4.1 ± 0.6	9
RPA	14.8 ± 2.4	8
RPA + 100 mg kg^{-1} L-NOARG	15.0 ± 3.3*	4
RPA + 100 mg kg^{-1} L-NMA	13.2 ± 2.4*	4
RPA + 100 mg kg^{-1} L-NOARG + L-NMA	14.5 ± 3.0*	4

NO synthase (L-NOARG = N^G-nitro-L-arginine methylester; L-NMA = N^G-methyl-L-arginine) inhibitors were i.p. injected 10 min before the induction of RPA. The peritoneal exudate was obtained 30 min after RPA or antibody administration. Data represent mean ± s.e.mean. *Not significant as compared to RPA animals.

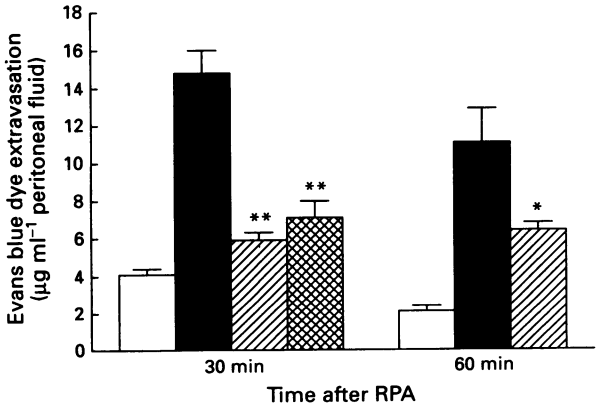

Figure 1 Extravasation of Evans blue dye in the peritoneal cavity during RPA and effect of PAF-receptor antagonists. The peritoneal exudate from rats was obtained at the times indicated after local injection of anti-ovalbumin antibody (negative control, open columns), local antibody and intravenous antigen (RPA animals, solid columns), or local antibody and intravenous antigen preceded by i.p. injection of 5 mg kg^{-1} UR-12460 10 min prior to challenge (hatched columns), or 5 kg^{-1} BB-823 (cross-hatched column). Results represent mean ± s.e.mean of 7 to 9 animals in each group. $^{**}P<0.01$, $^{*}P<0.05$.

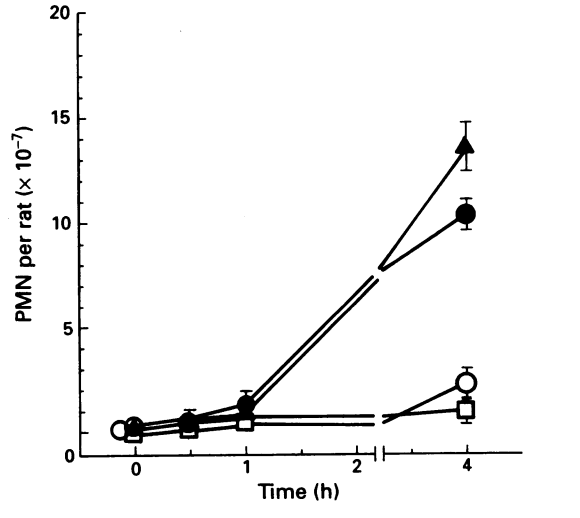

Figure 2 Effect of UR-12640 on the infiltration of PMN in the peritoneal cavity. Saline-treated rats (□), antibody-treated rats (○), RPA rats (●) and RPA rats pretreated with 5 mg kg^{-1} UR-12460 (▲) were killed at the times indicated for collection of peritoneal cells. The number of infiltrating PMN was assessed from the assay of myeloperoxidase activity.

Accumulation of PMN in the peritoneal cavity

Assay of myeloperoxidase (MPO) activity as an index of PMN accumulation in the peritoneal cavity showed that infiltration by PMN was a delayed event as compared to plasma leakage, with little infiltration of PMN up to 1 h and a significant increase at 4 h (Figure 2). Treatment with UR-12460 did not influence the number of infiltrating PMN. When PAF was injected at doses up to 10 $\mu g\,kg^{-1}$ i.p., it did not induce PMN recruitment in the peritoneal cavity, and higher doses induced haemorrhagic necrosis of the small intestine, which is most probably due to its ability to induce widespread endothelial damage (Pellón *et al.*, 1994).

Nitrite production and iNOS expression in RPA

Adherent peritoneal cells from RPA rats showed an enhanced *ex vivo* production of NO as compared to cells obtained from control animals receiving either antigen or antibody alone (Figure 3). This enhanced production occurred after 8 h in culture, with a linear increase up to 18 h and a less prominent increase up to 28 h. Both this pattern of NO production and its magnitude suggested the involvement of the inducible form of NO synthase, and would be in keeping with the above mentioned lack of effect of the modulation of NO production on the early phase of RPA. The involvement of the inducible form of NOS was confirmed by measuring the activity in the presence and absence of calcium/calmodulin. As shown in Table 2, the result was almost the same under either condition, which points to the singular involvement of the inducible form of the enzyme. To determine the optimal period of time after induction of RPA required to show an enhanced *in vitro* nitrite production, peritoneal cells were collected either 15 or 60 min after challenge. As shown in Figure 4, no significant difference was observed at either time, which indicates the dependency of enhanced NO production on events occurring within 15 min after induction of RPA. Treatment of rats prior to the induction of RPA with either UR-12460 or BB-823 showed no enhancement of nitrite production as compared to controls (Figure 3). Since these compounds possess different chemical structures, but elicit the same pharmacological effect on the PAF receptor, the results strongly suggest a role for PAF on the induction of NO synthase in RPA. Moreover, adherent peritoneal cells collected from rats that had been injected i.p. with PAF at the dose of 5 $\mu g\,kg^{-1}$ showed a production of NO of 18 ± 2 nmol mg^{-1} protein after 24 h in culture. In agreement with the production of NO by adherent cells after RPA, the levels of iNOS mRNA increased 14 fold after 18 h of culture in animals challenged with antibody plus antigen. This increase could be observed at 6 h after the initiation of the culture, but was most prominent at 18 h. The appearance of iNOS mRNA was inhibited more than 90% by pretreatment of the animals with UR-12460 (Figure 5).

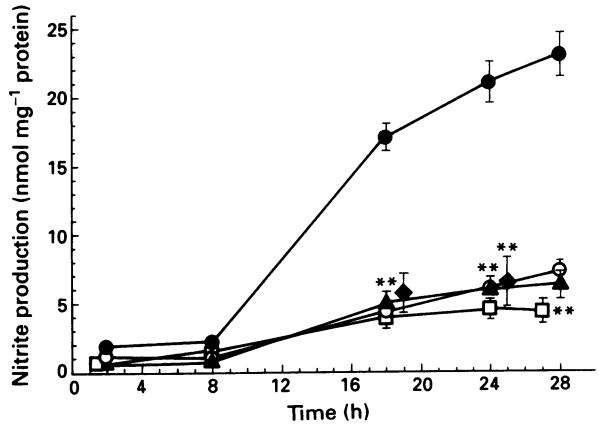

Figure 3 Production of NO by adherent peritoneal cells in culture after induction of RPA. Peritoneal cells were collected by washing the peritoneum with 40 ml of DMEM, followed by centrifugation and resuspension in 10 ml of the same medium supplemented with antibiotics. At that time cells from each rat were distributed in aliquots and maintained at 37°C to allow the collection of adherent cells. NO_2-production was assayed in duplicate samples at the times indicated. Control animals (□); rats treated with i.p. antibody alone (○); animals treated with i.p. antibody and i.v. antigen (●); RPA animals pretreated with 5 mg kg^{-1} UR-12460, i.p., 10 min before elicitation of RPA (▲); at 18 and 24 h (◆) indicate rats receiving 5 mg kg^{-1} BB-823, under similar conditions to UR-12460-treated rats. Data represent mean ± s.e.mean of 9 to 12 animals in each group. $^{**}P > 0.001$.

PAF enhances nitrite production by control peritoneal cells and abrogates the response after RPA

Experiments carried out with adherent cells from control rats showed that the production of nitrite can be elicited by PAF with a bell-shaped pattern of dose-dependency (Figure 6). This was abrogated by the inclusion of UR-12460 in the medium (Figure 6), suggesting again that PAF might induce the production of NO via the inducible form of NOS by signalling through its receptor. When adherent peritoneal cells obtained 1 h after RPA were incubated with different concentrations of PAF, (Figure 6) , a dose-dependent inhibition of nitrite production was observed. The time-dependency of this effect was studied with PAF at the dose of 10 nM.

PAF induced a diminution of nitrite formation by adherent peritoneal cells from RPA rats at all of the times

Table 2 iNOS activity in *ex vivo* cultured adherent peritoneal cells

Treatment	*NOS activity* (%) + *0.5 mM EGTA*	+ Ca^{2+}/*Calmodulin*
None	3	4
RPA	100	96
Antibody	5	5

Animals were treated as indicated and peritoneal cells were cultured for 8 h. iNOS activity was measured by the production of [^{14}C]-citrulline from [^{14}C]-arginine. Results show the means of two cultures assayed in duplicate and expressed as percentage of the maximal activity (12.1 nmol min^{-1} mg^{-1} protein).

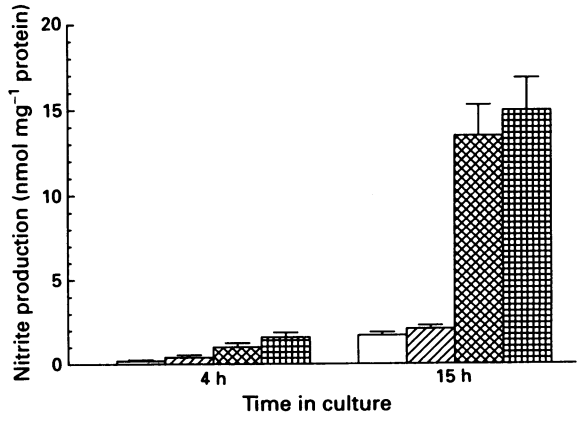

Figure 4 Influence of the time elapsed after RPA on nitrite production by rat adherent peritoneal cells. Rats were challenged with saline solution (open column), antibody (hatched columns), or antigen and antibody, and removed from the peritoneal cavity 15 (cross-hatched columns) or 60 min (checkered columns) thereafter. Nitrite production was assessed 4 and 15 h after removal of non-adherent cells.

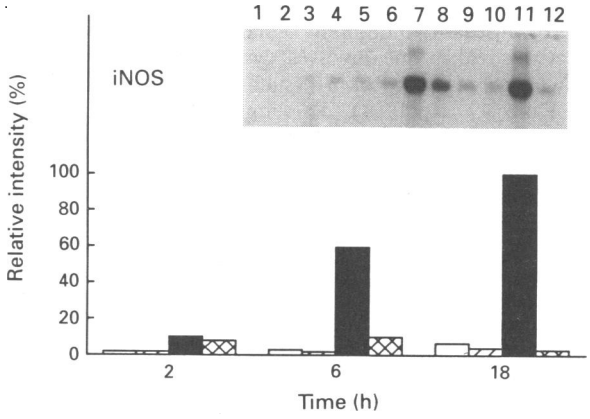

Figure 5 iNOS mRNA levels in RPA. Rats were injected with saline (lanes 1, 5 and 9), antibody (lanes 2, 6 and 10), or antibody and antigen in the absence (lanes 3, 7 and 11) or in the presence of 5 mg kg^{-1} of UR-12460 (lanes 4, 8 and 12). Adherent peritoneal cells were cultured *ex vivo* for 2 (lanes 1-4), 6 (lanes 5-8), and 18 h (lane 9-12), and used for the analysis of iNOS and mRNA using a specific probe. Quantitative analyses of mRNA levels were obtained by densitometry of the bands after normalization for the β-actin content, and are shown in the histogram.

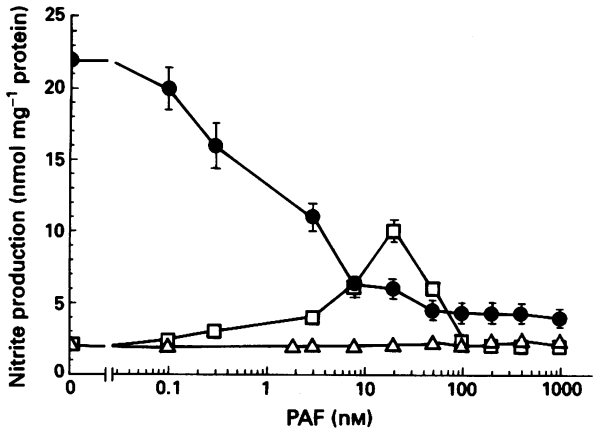

Figure 6 Effect of PAF on the production of nitrite by adherent peritoneal cells after RPA: dose-response. Rats treated to induce RPA (●); rats receiving saline solution alone (□); peritoneal cells from rats treated with saline solution incubated *in vitro* with different concentrations of PAF and 1 μM UR-12460 (△). The measurement of nitrite was carried out after 15 h in culture.

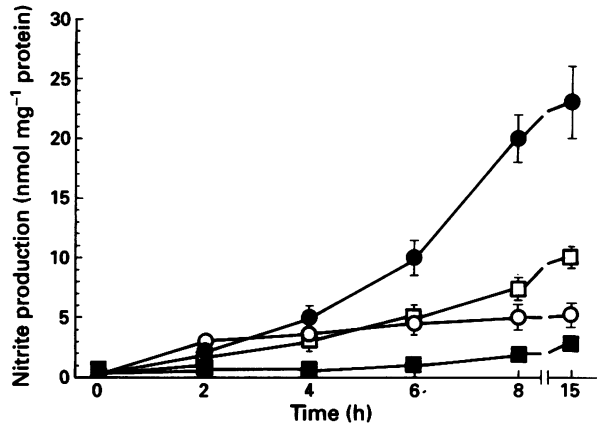

Figure 7 Effect of PAF on the production of nitrite by adherent peritoneal cells after RPA: Time course. Peritoneal cells were collected 15 min after induction of RPA (○, ●) or after saline injection (□, ■). The production of nitrite was assessed in adherent cells after culture for the times indicated in the presence of 10 nM PAF (open symbols) or vehicle (closed symbols).

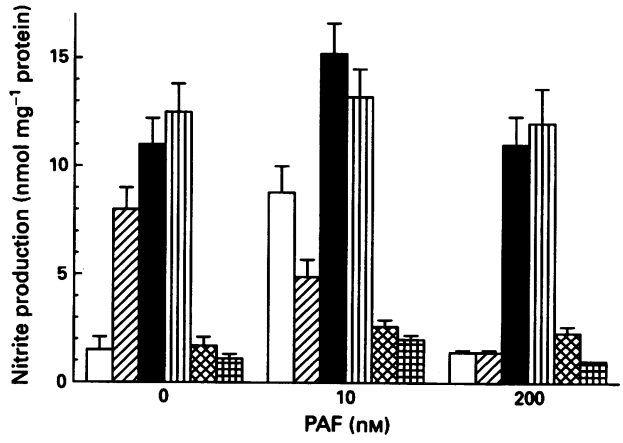

Figure 8 Effect of different agonists on the production of nitrite induced by PAF. Adherent peritoneal cells were incubated in the presence of different concentrations of PAF and the additions indicated. The production of nitrite was assessed 15 h after separation of adherent cells. No addition (open columns), 200 ng ml^{-1} phorbol dibutyrate (hatched columns), 20 μg ml^{-1} *E. coli* lipopolysaccharide (solid column), 1 nM epidermal growth factor (vertically lined columns), 1 μM UR-12460 (cross-hatched columns), 1 mM N^Gnitro-L-arginine methylester/N^G-nitro-L-arginine (checkered columns).

studied, whereas this concentration of PAF enhanced nitrite production in control saline-treated rats (Figure 7).

Some characteristics of the transduction pathway used for PAF to elicit NO production were studied with the use of other agonists, and are shown in Figure 8. Whereas phorbol dibutyrate showed a slight inhibition of the production of nitrites elicited by PAF, lipopolysaccharide (LPS) and epidermal growth factor (EGF) showed either additive effect or its own effect when PAF was used at doses that do not induce NO production.

Discussion

The data presented herein indicate a prominent role for PAF in the RPA model of rat immune complex peritonitis, which confirms earlier findings in the rabbit skin model (Hellewell & Williams, 1986; Rossi *et al.*, 1992), and in rat alveolitis (Warren *et al.*, 1989a; Tavares de Lima *et al.*, 1992). The release of PAF is an early event, and can probably be explained by the stimulation of PMN by immune complexes and/or complement-derived components in the microvasculature (Sánchez Crespo *et al.*, 1980). Endothelial cells are one of the targets for PAF, as judged from the early appearance of protein-rich plasma leakage. In fact, it has recently been reported that exposure of confluent endothelial cells to PAF results in a rapid and concentration-dependent redistribution of protein kinase C, rearrangement of cytoskeleton, and increase in the transendothelial flux of [^{125}I]-albumin (Bussolino *et al.*, 1994). On this basis, PAF-induced extravasation seems to be a clearcut component of the inflammatory response, and not a mere consequence of physical interactions between neutrophils and endothelial cells in which selectin-dependent adherent interactions seem to be involved (Lorant *et al.*, 1993). In contrast, our data are not consistent with a role for PAF on the cascade of events leading to the migration of PMN into the peritoneal cavity, and they would indirectly support current views on the

mechanisms of recruitment of PMN in this model, which involves C5a, leukotrienes and tumour necrosis factor-α (TNF-α) (Warren *et al.*, 1989b; Zhang *et al.*, 1992).

Peritoneal macrophages are another target for PAF in RPA as judged from the effect of both PAF and PAF-receptor antagonists on nitrite production under a wide array of experimental conditions including *ex vivo* experiments with adherent peritoneal cells from RPA rats and *in vitro* studies with PAF and other agonists. PAF showed a great ability to down regulate the production of NO in adherent peritoneal cells from both RPA and PAF-treated rats, whereas by itself it elicited NO production. This may be the consequence of antagonistic signalling triggered by previous exposure to an agonist, as observed in hepatocytes and peritoneal macrophages treated with LPS and phorbol ester (Hortelano *et al.*, 1992), which is explained by an effect on protein kinase C. In support of this view there may be mentioned the ability of PAF to stimulate calcium influx and phosphoinositide hydrolysis in mononuclear phagocytes at very low concentrations (Randriamampita & Tsien, 1993), the inhibitory effect observed when PAF was used in combination with phorbol ester, an agonist acting via protein kinase C; and the additive effect obtained when agonists acting on other signalling pathways were used, i.e. LPS and EGF.

The contribution of infiltrating PMN to the increased production of NO by peritoneal cells in *ex vivo* experiments seems unlikely, since the cell population was collected before infiltration of PMN had occurred, and also because the assay of nitrite production was carried out with adherent cells. In addition, the production of NO by PMN has been a matter of debate (Stuehr & Nathan, 1989; Schattner *et al.*, 1990; Stewart *et al.*, 1993), and it has been suggested that the NOS in PMN may represent a third type of enzyme intermediate between that of the inducible form and the calcium/calmodulin dependent constitutive form (Yui at al., 1990; Hiki *et al.*, 1991). Interestingly, a recent study has shown that the production of reactive nitrogen intermediates by human PMN in response to phorbol esters is greatly enhanced by azide and catalase, which strongly suggests that the alleged nitric oxide synthase activity detected under these conditions rather represents the catalase-catalyzed conversion of azide to nitrite (Klebanoff & Nathan, 1993).

Since iNOS induction is a delayed event involving nuclear signalling, this should be analyzed in the light of recent reports linking PAF receptor signalling and gene expression. Thus, PAF stimulates transcription of genes possessing the Fos-Jun/AP-1 responsive sequence (Squinto *et al.*, 1989; Mazer *et al.*, 1991), and the accumulation of collagenase type I mRNA in corneal epithelial cells (Bazan *et al.*, 1993). It has recently been shown that the level of iNOS mRNA is controlled by at least two different signalling cascades which may act synergistically, one involves cyclic AMP and the other one is triggered by cytokines such as IL-1β (Mühl *et al.*, 1994; Kunz *et al.*, 1994). This raises the question of the possible relationship of PAF-triggered iNOS induction to any of these pathways.

The biological role for NO synthase induction in RPA might be linked to either the induction of tissue damage or to the reparative phase of inflammation. Initial studies in rat immune complex alveolitis linked NO production to generation of peroxynitrite and to extravasation of protein-rich plasma, even though a diminished recruitment of PMN was not observed (Mulligan *et al.*, 1991; 1992). Our data differ from these results since we have not observed any effect on the extent of plasma extravasation by either inhibition of NO production with pharmacological tools or enhancement of NO production by NO-inducers. These discrepancies could be due to differences in either experimental conditions or properties of the tissues studied, which differ in both cellular components and vascularity. In keeping with the difficulty of assigning a damaging role to NO production in tissue injury there are a number of reports emphasizing its role as an oxygen radical scavenger (Wink *et al.*, 1993; Choi, 1993), and its protective role in hypoxia-induced intestinal injury in rats (Caplan *et al.*, 1994). Alternative roles for NO in immune-mediated inflammation could be regulation of blood flow in the reparative phase of inflammation (Moncada *et al.*, 1991; Pons *et al.*, 1993), or reduction of leukocyte adherence and emigration after the initial injury (Arndt *et al.*, 1993). Another function for NO not apparently related to inflammatory damage could be the recently reported synergistic effect with calcium in gene transcription linked to an increase in binding activity of the AP-1 transcription factor comprising the Fos and Jun family proteins (Peunova & Enikolov, 1993).

Finally, the possibility that PAF could also play a significant role in the production of NO in other models of tissue injury is strongly suggested by the finding of significant attenuation of the induction of NO synthase produced by bacterial LPS in anaesthetized rats treated with the PAF receptor antagonist WEB 2086 (Szabo *et al.*, 1993).

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References

ARNDT, H., RUSSEL, J.B., KUROSE, I., KUBES, P. & GRANGER, D.M. (1993). Mediators of leukocyte adhesion in rat mesenteric venules elicited by inhibition of nitric oxide synthesis. *Gastroenterology*, **105**, 675–680.

ARTHUS, M. (1903). Injections repetées de serum de cheval chez le lapin. *C.R. Soc. Biol. (Paris)*, **55**, 817–820.

BAZAN, H.E.P, TAO, Y. & BAZAN, N.G. (1993). Platelet-activating factor induces collagenase expression in corneal epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8678–8682.

BOUGHTON-SMITH, N.K., DEAKIN, A.M., FOLLEFANT, R.L., WHITTLE, B.J.R. & GARLAND, L.G. (1993). Role of oxygen radicals and arachidonic acid metabolites in the reverse passive Arthus reaction and carrageenin paw oedema in the rat. *Br. J. Pharmacol.*, **110**, 896–902.

BUSSOLINO, F., SILVAGNO, F., GARBARINO, G., COSTAMAGNA, C., SANAVIO, F., ARESE, M., SOLDI, R., AGLIETTA, M., PESCARMONA, G., CAMUSSI, G., & BOSIA, A. (1994). Human endothelial cells are targets for platelet-activating factor (PAF). Activation of α and β protein kinase C isozymes in endothelial cells stimulated by PAF. *J. Biol. Chem.*, **269**, 2877–2886.

CAPLAN, M.S., HEDLUND, E., HILL, N., & MACENDRICK. (1994). The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology*, **106**, 346–352.

CARCELLER, E., MERLOS, M. GIRAL, M., ALMANSA, C., BARTROLI, J., GARCIA-RAFANELL, J. & FORN, J. (1993). Synthesis and structure-activity relationships of 1-acyl-4-[(methyl-3-pyridyl) cyanomethyl]piperazines as PAF antagonists. *J. Med. Chem.*, **36**, 2984–2997.

CHOI, D.W. (1993). Nitric oxide: foe or friend to the injured brain. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9741–9743.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem.*, **162**, 156–159.

COLLINS, P.D., JOSE, P.J. & WILLIAMS, T.J. (1991). The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationships between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein. *J. Immunol.*, **146**, 677–684.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNEBAUM, S.R. (1982). Analysis of nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

HELLEWELL, P.G. & WILLIAMS, T.J. (1986). A specific antagonist of platelet-activating factor suppresses oedema formation in an Arthus reaction but not oedema induced by leukocyte chemoattractants in rabbit skin. *J. Immunol.*, **137**, 302–307.

HENSON, P.M., ZANOLARI, B., SCHWARTZMAN, N.A. & HONG, S.R. (1978). Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J. Immunol.*, **121**, 851–855.

HIKI, K., YUI, Y., HATTORI, R., EIZAWA, H., KOSUGA, K. & KAWAI, C. (1991). Three regulation mechanisms of nitric oxide synthase. *Eur. J. Pharmacol.*, **206**, 163–164.

HORTELANO, S., GENARO, A.M. & BOSCA, L. (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. Antagonism with the induction elicited by lipopolysaccharide. *J. Biol. Chem.*, **267**, 24937–24940.

IERINO, F.L., POWELL, M.S., McKENZIE, I.F.G. & HOGARTH, P.M. (1993). Recombinant soluble human Fc gammaRII. Production, characterization and inhibition of the Arthus reaction. *J. Exp. Med.*, **178**, 1617–1628.

KLEBANOFF, S.J. & NATHAN, C.F. (1993). Nitrite production by stimulated human polymorphonuclear leukocytes supplemented with azide and catalase. *Biochem. Biophys. Res. Commun.*, **197**, 192–196.

KUNZ, D., MÜHL, H., WALKER, G., & PFEILSCHIFTER, J. (1994). Two distinct signalling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 5387–5391.

LORANT, D.E., TOPHAM, M.K., WHATLEY, R.E., McEVER, R.P., McINTYRE, T.M., PRESCOTT, S.M. & ZIMMERMAN, G.A. (1993). Inflammatory roles of P-selectin. *J. Clin. Invest.*, **92**, 559–570.

MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. (1989). *Molecular Cloning: A Laboratory Manual.* pp 7.1–7.79. New York: Cold Spring Harbor Laboratory Press.

MAZER, B., DOMENICO, J., SAWAMI, H. & GELFAND, E.W. (1991). Platelet-activating factor induces an increase in intracellular calcium and expression of regulatory genes in human lymphoblastoid cells. *J. Immunol.*, **146**, 1914–1920.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–141.

MÜHL, H., KUNZ, D., & PFEILSCHIFTER, J. (1994). Expression of nitric oxide synthase in rat glomerular mesangial cells mediated by cyclic AMP. *Br. J. Pharmacol.*, **112**, 1–8.

MULLIGAN, M.S., HEVEL, J.M. MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6338–6342.

MULLIGAN, M.S., MONCADA, S. & WARD, P.A. (1992). Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis. *Br. J. Pharmacol.*, **197**, 1159–1162.

MULLIGAN, M.S., WATSON, S.R., FENNIE, C. & WARD, P.A. (1993). Protective effects of selectin chimeras in neutrophil-mediated lung injury. *J. Immunol.*, **151**, 6410–6417.

PELLON, M.I., STEIL, A.A., FURIO, V. & SANCHEZ CRESPO, M. (1994). Study of the effector mechanism involved in the production of haemorrhagic necrosis of the small intestine in rat passive anaphylaxis. *Br. J. Pharmacol.*, **112**, 1101–1108.

PEUNOVA, N. & ENIKOLOV, G. (1993). Amplification of calcium-induced gene transciption by nitric oxide in neuronal cells. *Nature*, **364**, 450–453.

PONS, F., WILLIAMS, T.J. & WARREN, J.B. (1993). Nitric oxide, but not interleukin-1, mediates the local blood flow response to lipopolysaccharide in rabbit skin. *Eur. J. Pharmacol.*, **239**, 23–30.

RANDRIAMAMPITA, C. & TSIEN, R.Y. (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature.*, **364**, 809–813.

ROSSI, A.G., NORMAN, K.E., DONIGI-GALE, D., SHOUPE, T.S., EDWARDS, R., & WILLIAMS, T.J. (1992). The role of complement, platelet-activating factor and leukotriene B_4 in the reversed passive Arthus reaction. *Br. J. Pharmacol.*, **107**, 44–49.

SÁNCHEZ-CRESPO, M., ALONSO, F, & EGIDO, J. (1980). Platelet-activating factor in anaphylaxis and phagocytosis. Release from polymorphonuclear leukocytes and monocytes during phagocytosis and by ionophore A23187. *Immunology.*, **40**, 645–655.

SCHATTNER, M.A., GEFFNER, J.R., ISTURIZ, M.A. & LAZZARI, M.A. (1990). Inhibition of human platelet activation by polymorphonuclear leukocytes. *Br. J. Pharmacol.*, **101**, 253–256.

SCHMIDT, H.H., WARNER, T.D., NAKANE, M., FORSTERMAN, U. & MURAD, F. (1992). Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Mol. Phamacol.*, **41**, 615–624.

SQUINTO, S.P., BLOCK, A.L., BRAQUET, P. & BAZAN, N.G. (1989). Platelet-activating factor stimulates a Fos/Jun/AP-1 transcriptional signalling system in human neuroblastoma cells. *J. Neurosci. Res.*, **24**, 558–566.

STEWART, A.G., DUSTING, G.J., GIARRACCA, R.G., HARRIS, T., LIM, Y.M. & SOBEY, C.G. (1993). Nitrite is produced by elicited but not by circulating neutrophils. *Med. Inflam.*, **2**, 349–356.

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**, 1543–1555.

SZABO, C., WU, C.C., MITCHELL, J.A., GROSS, S.S., THIEMERMANN, C. & VANE, J.R. (1993). Platelet-activating factor contributes to the induction of nitric oxide synthase by bacterial lipopolysaccharide. *Circ. Res.*, **73**, 991–999.

TAVARES DE LIMA, W., SIROIS, P. & JANCAR, S. (1992). Immune-complex alveolitis in the rat: evidence for platelet-activating factor and leukotrienes as mediators of the vascular lesions. *Eur. J. Pharmacol.*, **213**, 63–70.

WARREN, J.S. (1992). Relationship between interleukin-1β and platelet-activating factor in the pathogenesis of acute immune complex alveolitis in the rat. *Am. J. Pathol.*, **141**, 551–560.

WARREN, J.S., MANDEL, D.M., JOHNSON, K.J. & WARD, P.A. (1989a). Evidence for the role of platelet-activating factor in immune complex vasculitis. *J. Clin. Invest.*, **83**, 669–679.

WARREN, J.S., YABROFF, K.R., REMICK, D.G., KUNKEL, S.L., CHENSUE, S.W., KUNKEL, R.G., JOHNSON, K.L. & WARD, P.A. (1989b). Tumour necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. *J. Clin. Invest.*, **84**, 1873–1882.

WEDMORE, C.V. & WILLIAMS, T.J. 1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289**, 646–650.

WHITTAKER, M., BEAUCHAMP, C.L., BOWLES, S.A., CACKETT, K.S., CHRISTODOULOU, M.S., GALLOWAY, W.A., LONGSTAFF, D.S., McGUINNESS, G.P., MILLER, A., TIMMIS, D.J. & WOOD, L.M. (1992). BB–823, A PAF receptor antagonist with picomolar activity. *Pharmacol. Commun.*, **1**, 251–257.

WINK, D.A., HANBAUER, I., KRISHNA, M.C., DEGRAFF, W., GAMSON, J. & MOTCHELL, J.B. (1993). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9813–9817.

XIE, Q-W., CHO, H.J., CALAYCAY, R.A., MUMFORD, R.A., SWIDEREK, K.M., DEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science (Wash. D.C.)*, **256**, 225–228.

YUI, Y., HATTARI, R., KOSUGA, K., EIZAWA, H., HIKI, K., OHKAWA, S. & OHNISHI, K. (1990). Calmodulin independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J. Biol. Chem.*, **266**, 3369–3371.

ZHANG, Y., RAMOS, B.J. & JAKSCHIK, B.A. (1992). Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science, (Wash. D.C.)*, **258**, 1957–1959.

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