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The contribution of nitric oxide to endotoxin-induced ocular inflammation: interaction with sensory nerve fibres

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1 The actions of nitric oxide (NO) have been investigated in an endotoxin-evoked ocular inflammatory model in the rabbit, with particular emphasis on the relationship between NO, sensory nerves (C-fibres) and the C-fibre neuropeptides, calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase activating peptide (PACAP).

2 Endotoxin, injected intravitreally, evoked inflammatory responses, i.e. conjunctival hyperaemia, miosis and protein extravasation, reflected by the aqueous flare response (AFR). In control rabbits, the maximum AFR was 66.5 ± 9.5 (arbitrary units). Pretreatment with the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NAME, 200 mg kg^{-1}) given by intravenous injection, inhibited the endotoxin-evoked responses; the AFR was 16.5 ± 1.9 ($n=8$, $P<0.001$) and the conjunctival hyperaemia was abolished.

3 Endotoxin-evoked ocular inflammation is associated with the release of CGRP and PACAP from C-fibres. In the eyes challenged with endotoxin, the concentrations of PACAP-27, -38 and CGRP in the aqueous humour were 58.2 ± 10.9, 54.4 ± 12.4 and 5526 ± 519 (pmol l^{-1}), respectively. L-NAME inhibited the release of PACAP-27, -38 and CGRP; the concentrations were 14.3 ± 2.5, 13.5 ± 2.5 and 510 ± 67 (pmol l^{-1}), respectively ($n=8$, $P<0.01$ or 0.001).

4 Intravitreal injection of 0.3 nmol CGRP induced conjunctival hyperaemia and AFR; the maximum AFR was 140.2 ± 11.4. L-NAME suppressed the response induced by CGRP; the AFR was 23.4 ± 5.5 ($n=8$, $P<0.001$). L-NAME abolished the conjunctival hyperaemia induced by PACAP-27 and -38 (0.3 nmol) and reduced the AFR.

5 The inflammatory cells that infiltrated the uvea, cornea and aqueous humour in large numbers in response to intravitreal injection of endotoxin were found to express inducible NOS. L-NAME prevented the appearance of such cells.

6 Our findings suggest that NO plays an important role in the endotoxin-evoked ocular inflammation in the rabbit: NO activates C-fibres causing release of C-fibre neuropeptides into the aqueous humour. In addition, NO mediates some of the ocular effects of CGRP and PACAP, since L-NAME suppressed the AFR induced by these peptides.

Keywords: Nitric oxide; inducible NOS; C-fibres; calcitonin gene-related peptide; pituitary adenylate cyclase activating peptide; endotoxin; ocular inflammation; rabbit eye; transmitter release

Introduction

The inflammatory response in the eye consists of miosis, conjunctival hyperaemia and breakdown of the blood-aqueous barrier with subsequent leakage of protein into the aqueous humour. The magnitude of these responses depends on the intensity, duration and type of the noxious stimulus (Stone *et al.*, 1987; Unger, 1990; Håkanson & Wang, 1996). There is much evidence to suggest that C-fibre neurotransmitters, such as substance P and calcitonin gene-related peptide (CGRP), play a key role in the ocular response to injury (Stone *et al.*, 1987; Unger, 1990; Håkanson & Wang, 1996). Recently, pituitary adenylate cyclase activating peptide (PACAP) has been identified as a C-fibre neuropeptide, which takes part in the inflammatory responses of the rabbit eye (Wang *et al.*, 1995). As there is no barrier separating the iris and the ciliary body from the anterior chamber, any transmitter that is released from local nerve fibres will diffuse into the anterior chamber, making the eye an excellent model for studies of transmitter release.

Nitric oxide (NO) is a short-lived molecule displaying numerous bioactivities (Moncada *et al.*, 1991). It is generated from L-arginine by the enzyme NO synthase (NOS), which is inhibited effectively by analogues of L-arginine, e.g. N^G-nitro-L-arginine (L-NAME) (Moncada *et al.*, 1991). Recent observations suggest that NO may be of physiological and/or pathophysiological significance in the control of ocular function. Thus, NOS activity has been demonstrated in the anterior uvea of the rabbit (Osborne *et al.*, 1993) and NOS immunoreactivity has been visualized by immunostaining in nerve fibres in the uvea of the rat eye (Yamamoto *et al.*, 1993). Intravenous injection of L-NAME was found to reduce the regional blood flow in the uvea of the rabbit (Seligsohn & Bill, 1993). Interestingly, NO seems to play a role in the activation of ocular C-fibres in response to a minor injury (infrared irradiation of the iris) (Wang & Håkanson, 1995). In the present study, we have investigated whether NO is of importance in endotoxin-evoked ocular inflammation, with particular emphasis on the relationship between NO and sensory nerves (C-fibres).

Methods

Animals

All experiments were carried out on adult pigmented rabbits (1.5–2.5 kg) of mixed strain. The study was approved by the local Animal Welfare committee.

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Ocular inflammation induced by intravitreal injection of endotoxin, PACAP-27, -38 and CGRP

Shigella endotoxin (150 ng) or PACAP-27, -38 and CGRP (0.3 nmol each) were given by intravitreal injections (30 μl) into the corpus vitreum, 3–4 mm posterior to the limbus; the contralateral eye received the same volume of saline (Holmdahl *et al.*, 1981). The breakdown of the blood-aqueous barrier was determined by photoelectric measurement of the aqueous flare response (AFR) in the anterior chamber (Anjou & Krakau, 1961). This response is a Tyndall phenomenon in the anterior chamber reflecting protein leakage across the blood-aqueous barrier. Briefly, a narrow beam of light is passed through the anterior chamber. In the presence of large molecules (usually proteins) in the aqueous humour, light scattering (aqueous flare) occurs. A correlation between the AFR and protein concentration has been established (Anjou and Krakau, 1961; Dyster-Aas & Krakau, 1964). The AFR is expressed in arbitrary units with reference to a standard (Dyster-Aas & Krakau, 1964). Conjunctival hyperaemia was assessed visually. The pupillary diameter was measured with a transparent ruler under constant and uniform illumination.

Treatment with L-NAME

L-NAME (200 mg kg^{-1}, in 3 ml saline) was given by intravenous injection 30 min before the intravitreal injection. The injection of L-NAME was repeated every 3 h. In each study, the rabbits were divided into two groups: one group received L-NAME, the other group received vehicle (0.9% saline). Each group included eight rabbits. The dose of L-NAME was chosen based on previous findings showing that L-NAME (10–200 mg kg^{-1}) inhibits NOS in a dose-dependent manner and that 30 mg kg^{-1} inhibited the regional blood flow in rabbit uvea for a short period of time only (<20 min) (Persson *et al.*, 1991; Seligsohn & Bill, 1993; Wang & Håkanson, 1995).

Radioimmunoassay of PACAP-27, -38 and CGRP in the aqueous humour

Samples of aqueous humour were collected from the anterior chamber after the AFR had reached maximum (10 h after intravitreal injection of endotoxin). In another series of experiments, samples of aqueous humour were collected at various time intervals and analysed for PACAP and CGRP; the concentrations of the peptides were plotted against the intensity of the inflammatory response at the time of sampling (reflected by the AFR). The samples were frozen on dry ice and stored at −80°C until assayed.

Whenever aqueous humour was collected or intravitreal injections were given, the rabbits were anaesthetized by an injection of methohexital sodium (5 mg kg^{-1}) into an ear vein. The anaesthesia lasted for about 20 min, which is sufficient to prevent the discomfort to the animal induced by the intravitreal injection or by the collection of the aqueous humour. No anaesthesia was required during the rest of the experiments.

PACAP-27-like immunoreactivity (LI) was measured by use of a radioimmunoassay (RIA) kit from Peninsula. Briefly, aqueous humour samples or standard solution of PACAP-27 in RIA buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 0.1% Triton X-100, 0.01% sodium azide, pH 7.4) were incubated with 100 μl PACAP-27 antiserum at a final dilution of 1:120,000 at 4°C for 24 h. Iodinated PACAP-27, 100 μl (≈10,000 c.p.m), in RIA buffer was added. The mixture was incubated for another 24 h at 4°C. Antibody-bound tracer was separated from free tracer by addition of 100 μl goat anti-rabbit IgG serum and of 100 μl normal rabbit serum. After incubation at room temperature for 90 min, 500 μl of cold RIA buffer was added, and the samples were centrifuged at 1700 × *g* for 30 min. The tubes were gently aspirated and the radioactivity of the precipitates was measured. The ID_{50} was found to be about 4 pmol l^{-1} and the detection limit 0.8 pmol l^{-1}. The PACAP-27 antiserum cross-reacts 1% with PACAP-38 and does not recognize vasoactive intestinal peptide, substance P, neurokinin A and CGRP (Wang *et al.*, 1995).

PACAP-38 was measured with a RIA kit from Peninsula, following the same protocol as for the measurement of PACAP-27. The ID_{50} was found to be about 4 pmol l^{-1} and the detection limit is 1 pmol l^{-1}. The PACAP-38 antiserum cross-

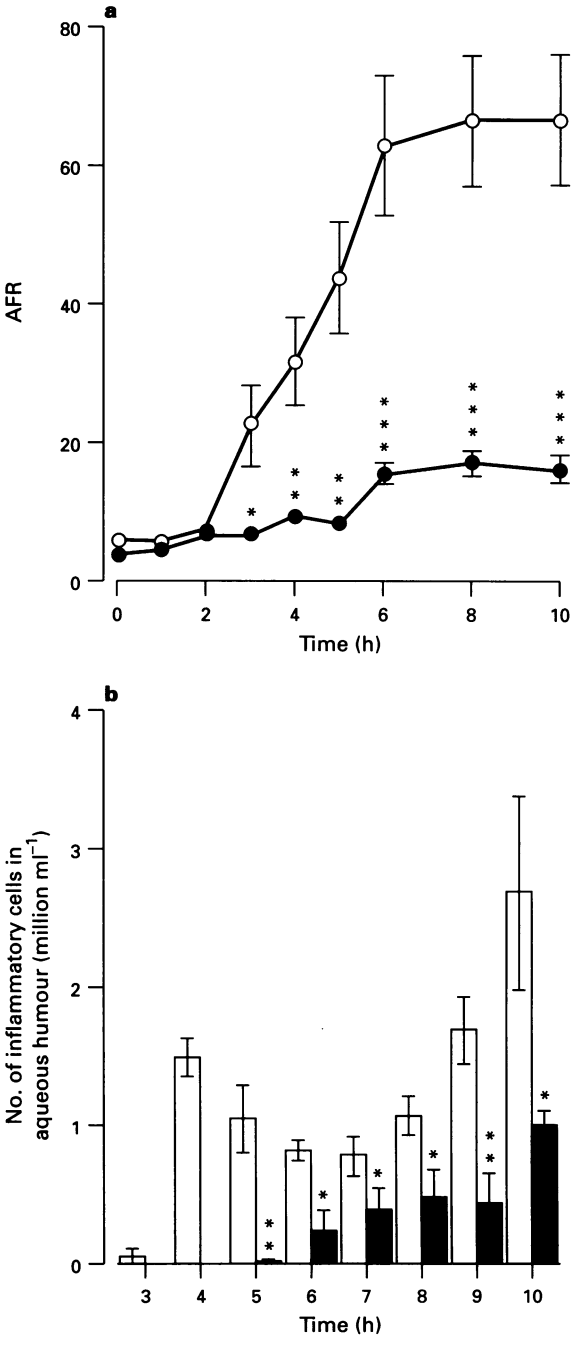

Figure 1 Intravitreal injection of endotoxin induced inflammatory responses as assessed by the aqueous flare response (AFR, see also the text) and the appearance of inflammatory cells in the anterior chamber. The nitric oxide synthase (NOS) inhibitor, L-NAME, inhibited the AFR (a) and the appearance of inflammatory cells in the anterior chamber (b) following endotoxin challenge, (●) in (a) and solid columns in (b). (○) in (a) and open columns in (b) : control rabbits not pretreated with L-NAME. Means ± s.e.mean of eight rabbits in each group are shown. Statistical differences when compared to control group are shown by $*P<0.05$, $**P<0.01$ and $***P<0.001$ at corresponding time points.

reacts 0.01% with PACAP-27 and does not recognize vasoactive intestinal peptide, substance P, neurokinin A and CGRP (Wang *et al.*, 1995).

(Tyr°) CGRP (rat) (Peninsula) was radioiodinated by conventional chloramine-T oxidation and used as tracer after purification by high performance liquid chromatography (h.p.l.c.). The specific radioactivity of the tracer was 1000–2000 μCi nmol^{-1}. Aqueous humour samples or standard solutions of rat CGRP were incubated with 200 μl antiserum (at a final dilution of 1:40,000) for 24 h at 4°C. Then, 200 μl tracer (10,000 c.p.m.) was added and the mixture was incubated for another 24 h. Free tracer was separated from bound by adding 100 μl solid phase second antibody coated cellulose suspension. After incubation at room temperature for 30 min, 500 μl cold redistilled water was added and the mixture was centrifuged at 1700 × *g* for 20 min. The radioactivity of the precipitates was measured. The ID_{50} was found to be about 860 pmol l^{-1}. The detection limit is about 100 pmol l^{-1} and the interassay variation is 10%. CGRP variants (human CGRP I and II) cross-react with the antiserum better than 100% on a molar basis, but there is no cross-reaction with calcitonin, katacalcin, C-terminal adjacent peptide, tachykinins, neuropeptide Y and vasoactive intestinal peptide (Grunditz *et al.*, 1986; Wahlestedt *et al.*, 1986; Wang *et al.*, 1995).

Immunohistochemistry and histology

The eyes were removed and placed in an ice-cold, freshly prepared solution of 4% formaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.4). The eyes were cut open about 2 mm laterally to the sclerocorneal junction. The cornea and anterior sclera and iris-ciliary body complex were immersion-fixed for 4 h, rinsed in an ice-cold solution of 15% sucrose in PBS (3 rinses over 48 h), frozen at −40°C, and stored at −70°C. Sections were cut in a cryostat at a thickness of 10 μm. They were pre-incubated in PBS with 0.2% Triton X-100 and 0.1% bovine serum albumin for 2 h, and then incubated overnight at 4°C in the presence of a mouse monoclonal antibody raised against inducible NOS (1:80; code N32020/L2, Affiniti, Nottingham, U.K.). The antibody is believed not to recognize constitutive NOS. After being rinsed, the sections were incubated with 10% goat serum in PBS for 30 min and then with fluorescein isothiocyanate (FITC)-conjugated purified $F(ab)_2$ fragments of donkey anti-mouse immunoglobulins (1:80; code 715-076-151, Jackson Immuno-Research, West Grove, PA, U.S.A.). After being rinsed again, the sections were mounted in PBS/glycerol with *p*-phenylenediamine to prevent fluorescence fading.

Samples of aqueous humour were collected from the anterior chamber at various time intervals after the intravitreal injection of endotoxin. After centrifugation, the sediments were resuspended in 0.9% NaCl solution. The cells were counted under the microscope by the use of a Bürker chamber (Kebo, Stockholm, Sweden). Alternatively, smears were prepared, air-dried and fixed for 15 min in the fixative described above. The cells were immunostained with a monoclonal antibody raised against inducible NOS following the same protocol as for tissue sections. The cells were also stained with hematoxylin and eosin.

Drugs

CGRP and PACAP-27 and -38 were purchased from Peninsula (Merseyside, St. Helens, U.K.). Endotoxin (Shigella) and L-NAME were purchased from Sigma (St. Louis, MO, U.S.A.). All chemicals were dissolved in 0.9% saline.

Analysis of results

Data are expressed as mean ± s.e.mean. Two way ANOVA and Student's *t* test (two-tailed) for unpaired groups were used for statistical analysis and a difference between samples was considered significant when $P<0.05$.

Results

Ocular responses to intravitreal injection of endotoxin

Conjunctival hyperaemia appeared 2–5 h after the injection of endotoxin and reached a maximum 1–2 h later. It was associated with a small miosis (not more than 1–2 mm reduction in pupil size). An AFR was noted 2–5 h after the injection which reached maximum after 6–10 h (Figure 1a). In rabbits pretreated with L-NAME, the endotoxin-induced AFR was small (Figure 1a) and the conjunctival hyperaemia was virtually abolished (not shown). The effect of L-NAME on the small miotic response could not be assessed properly.

Numerous inflammatory cells (neutrophilic granulocytes and monocytes, revealed by hematoxylin and eosin staining)

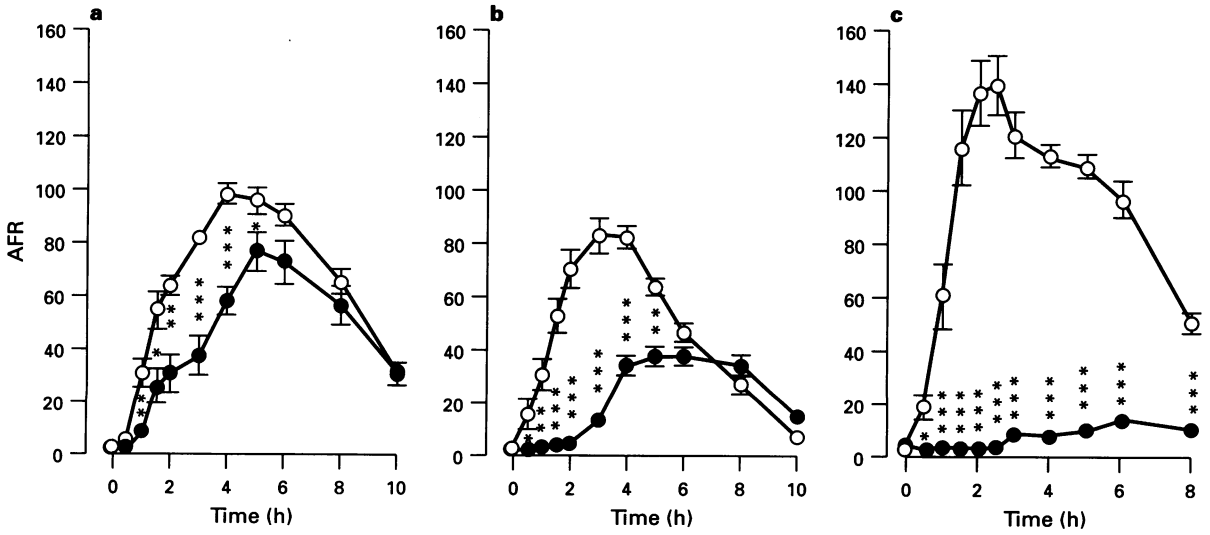

Figure 2 (a) PACAP-27-evoked aqueous flare response (AFR) was slightly inhibited by L-NAME. (b) PACAP-38-evoked AFR was almost abolished at the early stage of the response; the maximal response was attenuated by about 50%. (c) CGRP-evoked AFR was abolished by L-NAME. (●) Rabbits pretreated with L-NAME; (○) control rabbits not treated with L-NAME. Means ± s.e.mean of eight rabbits in each group are shown. Statistical differences when compared to control group are shown by $*P<0.05$, $**P<0.01$ and $***P<0.001$ at corresponding time points.

appeared in the anterior chamber, starting about 3 h after the endotoxin challenge (Figure 1b). After 8–10 h, the anterior chamber became hazy with inflammatory cells. In rabbits pretreated with L-NAME, the inflammatory cells were reduced in number and did not appear in the anterior chamber at all until about 5 h after the endotoxin challenge (Figure 1b).

Inflammatory cells were not found in the aqueous humour of the anterior chamber of control eyes (injected with saline) or of normal untreated eyes.

Ocular responses of PACAP-27, -38 and CGRP

Intravitreal injection of either PACAP-27 or PACAP-38 produced conjunctival hyperaemia, starting from 2 to 6 h after the injection. Conjunctival swelling could be observed 30 min to 2 h after the appearance of hyperaemia. A dose of 0.3 nmol was found to produce about half of the maximal AFR (Wang *et al.*, 1995).

The conjunctival hyperaemia and swelling induced by either PACAP-27 or PACAP-38 were significantly reduced by pretreatment with L-NAME. The AFR induced by PACAP-27 was slightly inhibited by L-NAME (Figure 2a) ($P<0.05$). The AFR induced by PACAP-38 was inhibited by L-NAME too ($P<0.01$); it was substantially reduced at the early stage of the response (until 2 h) and the maximal AFR seen after 4 h was attenuated by about 50% by L-NAME (Figure 2b). Interestingly, 6 h after the injection of either PACAP-27 or PACAP-38, there was no significant difference in AFR between the L-NAME-treated and control groups (Figure 2a&b)

Intravitreal injection of CGRP induced severe conjunctival hyperaemia after 30–60 min. The AFR started about 30 min after the injection and reached a maximum after 2–3 h (Figure 2c). In rabbits pretreated with L-NAME, CGRP failed to induce AFR ($P<0.0001$) (Figure 2c) and conjunctival hyperaemia (not shown).

Release of PACAP-27-, -38- and CGRP-LI into the aqueous humour

The concentration of CGRP in aqueous humour from normal untreated rabbit eyes was about 100 pmol l^{-1} (see Wang *et al.*, 1995), while PACAP-27 and -38 could not be detected. After intravitreal injection of endotoxin, the concentrations of PACAP-27-, -38- and CGRP-LI were greatly elevated in the aqueous humour. The level of CGRP-LI was approximately 100 fold greater than that of PACAP-LI. L-NAME inhibited these elevations (Figure 3).

In another series of experiments, samples of aqueous humour were collected at different stages of the inflammation. As shown in Figure 4, the progress from minimal to severe inflammation (reflected by the AFR) was accompanied by a 4–5 fold increase in the concentrations of PACAP-27- and -38-LI, while the increase in CGRP was 8 fold.

Immunohistochemistry of inducible NOS

Numerous inflammatory cells (neutrophilic granulocytes and monocytes) appeared in the aqueous humour of the anterior chamber following endotoxin challenge (Figure 5). Such cells were also observed in the uvea and cornea, particularly at the sclerocorneal junction (Figures 6 and 7a,b,). They were strongly immunoreactive to inducible NOS (Figures 5, 6 and 7a,b).

L-NAME prevented the appearance of inflammatory cells in the eye; the cells were not only reduced in number, they also displayed reduced NOS immunostaining intensity compared to cells from eyes not pretreated with L-NAME (Figure 7c).

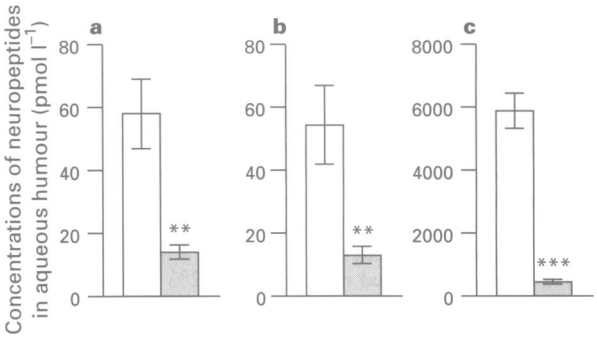

Figure 3 L-NAME inhibited the elevation in concentrations of PACAP-27 (a), -38 (b) and CGRP (c) in the aqueous humour of eyes exposed to endotoxin (hatched columns). Open columns: control rabbits not pretreated with L-NAME. Means ± s.e.mean of eight rabbits in each group are shown. Statistical differences when compared to control group are shown by $^{**}P<0.01$ and $^{***}P<0.001$.

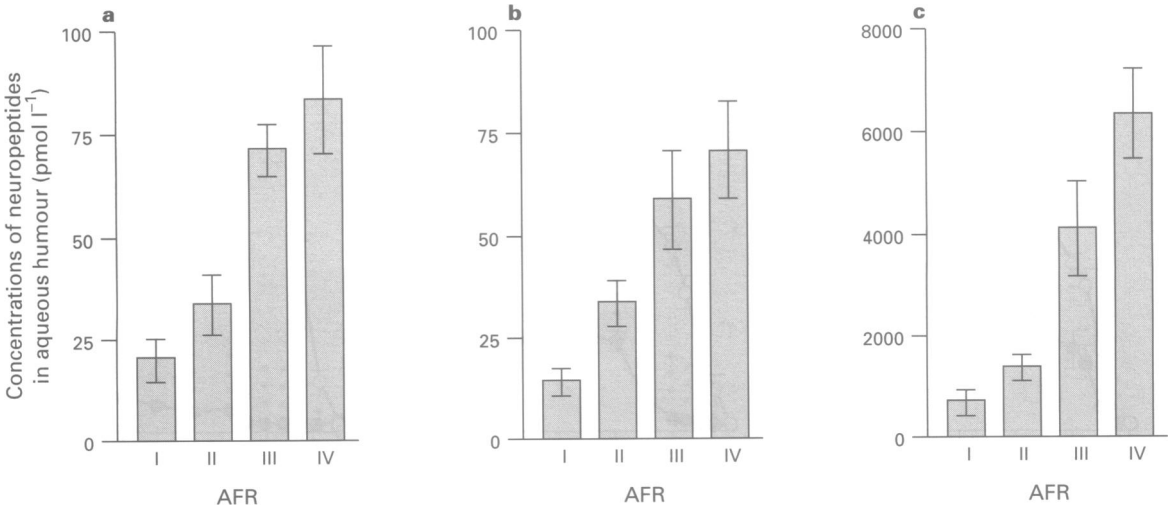

Figure 4 The concentrations of PACAP-27-(a), PACAP-38-(b) and CGRP-LI (c) in the aqueous humour were plotted against the intensity of the inflammation at the time of sampling (reflected by the aqueous flare response (AFR)), showing that the concentrations of both PACAP and CGRP increased in accord with the progress of the inflammation from minor to severe. The intensity of the inflammation (AFR) was graded as I (minimal): 1–10; II (weak): 11–30; III (moderate): 31–50; IV (strong): 51–70. Means ± s.e.mean of eight rabbits in each group are shown.

Inducible NOS could not be detected in control eyes (injected with saline) or in normal untreated eyes (not shown).

Discussion

NOS occurs as constitutive NOS and inducible NOS (Moncada *et al.*, 1991). Constitutive NOS is expressed in endothelial cells and sensory neurones (Moncada *et al.*, 1991). Inducible NOS is expressed in response to various chemical signals, including endotoxin and cytokines, in many types of cells, such as macrophages and neutrophils (Moncada *et al.*, 1991). It has been suggested that constitutive NOS produces small amounts of NO while inducible NOS generates large amounts of NO (Moncada *et al.*, 1991).

Capsaicin is known to excite C-fibres selectively (Holzer, 1991). Recently it was shown that L-NAME inhibited the capsaicin-induced (and C-fibre-mediated) increase in blood flow in rabbit skin, suggesting a role for NO in the activation of C-fibres (Hughes & Brain, 1994). In support of this view, we found that L-NAME inhibited the endotoxin-evoked release of sensory neuropeptides, CGRP and PACAP, into the aqueous humour (see also Wang & Håkanson, 1995). Thus, NO contributed to the ocular inflammation partly by releasing C-fibre

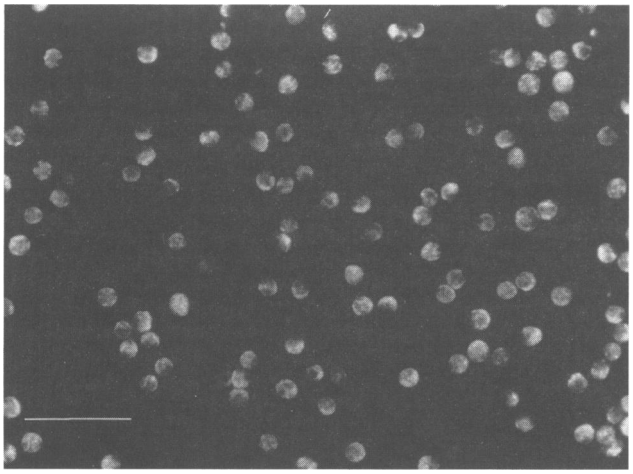

Figure 5 Aqueous humour of endotoxin-challenged eye. Numerous cells displayed strong immunofluorescence to inducible nitric oxide synthase (NOS). The cells were collected 10 h after the endotoxin challenge. No cells were seen in the aqueous humour of the control eye (without endotoxin). Fluorescein isothiocyanate (FITC)-immunofluorescence. Scale bar = 50 μm.

Figure 6 Sclerocorneal junction of endotoxin-challenged eye. Numerous cells displayed strong immunofluorescence to inducible nitric oxide synthase (NOS). In control eye (without endotoxin), such cells could not be detected. Fluorescein isothiocyanate (FITC)-immunofluorescence. Scale bar = 100 μm.

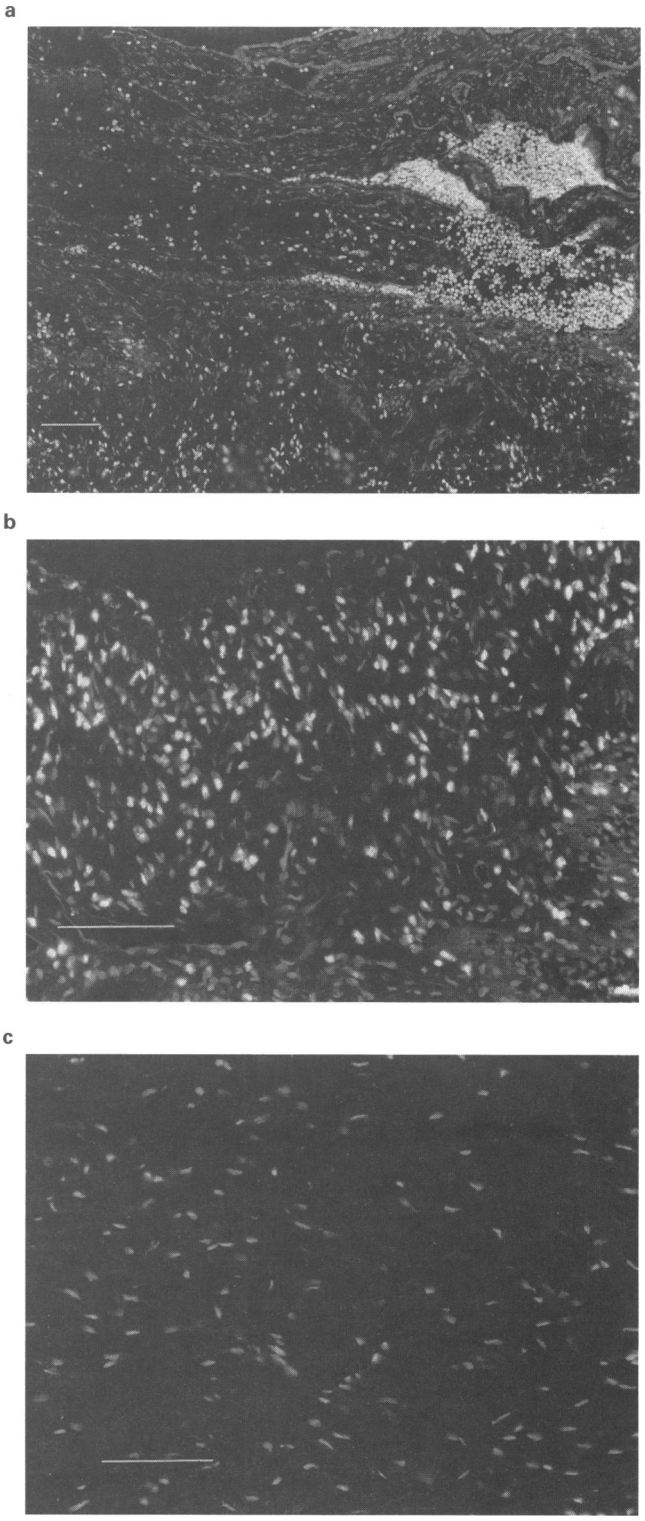

Figure 7 Endotoxin-challenged eye. (a) Iris-ciliary body complex. Many cells in stroma and blood vessels displayed strong immunofluorescence to inducible nitric oxide synthase (NOS). (b) Iris stroma, higher magnification than in (a). Many cells displayed strong immunofluorescence to inducible NOS. (c) Rabbits pretreated with L-NAME. Iris stroma: the immunofluorescent cells were reduced in number (about 20% remaining) and displayed greatly reduced NOS immunofluorescence intensity. In control eye (without endotoxin), such cells could not be detected. Fluorescein isothiocyanate (FITC)-immunofluorescence. Scale = 100 μm.

transmitters. Capsaicin releases transmitters from cultured dorsal root ganglion cells by increasing intracellular cyclic GMP levels (Holzer, 1991). Possibly, the stimulating effect of NO on sensory neurotransmitter release reflects its ability to increase cyclic GMP (Moncada *et al.*, 1991).

Besides stimulating C-fibres, NO was found to mediate the vascular effects of sensory neuropeptides; these effects include vasodilatation and protein extravasation. Possibly, vasodilatation occurs first followed by extravasation from the dilated blood vessels. The CGRP-induced AFR was abolished by L-NAME while the PACAP-induced AFR was partly inhibited by L-NAME. The possibility that NO mediates CGRP-(probably also PACAP-) induced vasodilatation and that CGRP and/or PACAP are directly responsible for the subsequent extravasation from dilated blood vessels cannot be excluded. Thus, NO has several roles to play in endotoxin-evoked ocular inflammation. Firstly, it is involved in the activation of C-fibres, causing or facilitating the release of transmitters such as CGRP and PACAP (see also Wang & Håkanson, 1995); secondly, it mediates some of the effects of these transmitters (see also Andersson, 1992). Alternatively, NO may induce vasodilatation and extravasation directly if it is being produced in large amounts (Wang & Håkanson, 1995). Hence, NO, CGRP and PACAP (possibly together with other C-fibre transmitters) may co-operate in controlling vascular functions. In addition, the L-NAME-evoked blockade of vasodilatation may help to prevent the invasion of immune cells into ocular tissues, an effect that will greatly reduce the local production of NO.

On a molar basis, the concentration of CGRP-LI in the iris was substantially higher than that of PACAP-LI and larger amounts of CGRP-LI than of PACAP-LI were released in response to noxious stimuli (Wang *et al.*, 1995). The concentrations of both PACAP and CGRP increased in accord with the progress of the inflammation from minor to severe. However, the increase in the concentration of CGRP was more dramatic than was the case with PACAP-27 or -38, possibly reflecting the fact that the two peptides play different roles in the inflammation process. In our previous study, PACAP, but not CGRP, was found to stimulate C-fibres, causing release of sensory transmitter (Wang *et al.*, 1995). We propose that PACAP is released from C-fibres in response to noxious stimuli, and that it causes release of other C-fibre neuropeptides.

We have now investigated the actions of NO in two ocular inflammatory models, namely inflammation induced by infrared irradiation of the iris (Wang & Håkanson, 1995) and by intravitreal injection of endotoxin (present study). Infrared irradiation of the iris results in a prompt, short-lasting and reversible ocular inflammation (Dyster-Aas & Krakau, 1964). The inflammatory responses (conjunctival hyperaemia and aqueous flare), which can be abolished by pretreatment with L-NAME, usually disappear within 6 h (Wang & Håkanson, 1995). It should be noted that the acute ocular response to infrared irradiation of the iris does not involve the invasion of leukocytes into the aqueous humour. In fact, neither circulating blood cells nor humoral antibodies seem to participate in this response and inflammatory cells are not recruited (Unger, 1990). Cells expressing inducible NOS could not be observed in the uvea and aqueous humour of irradiated eyes (Wang, Alm & Håkanson, unpublished observation). We suggest therefore that the NO generated in the irradiated eye reflects the activity of constitutive NOS (producing small amounts of NO). In contrast, the ocular inflammation induced by endotoxin is relatively long-lasting and characterized by infiltration of numerous leukocytes into the uvea, cornea and aqueous humour (Bito, 1977; Rosenbaum & Raymond, 1985; Howes *et al.*, 1994). In the present study, the cells that had infiltrated the ocular tissues and aqueous humour were found to express inducible NOS. Conceivably, these cells (neutrophilic granulocytes and monocytes) generate relatively large amounts of NO, that stimulates the C-fibres to release neuropeptides. Since NO is being produced continuously by the immune cells, the stimulant effect of NO on the C-fibres may be a prolonged one, contributing to a long-lasting inflammatory response (2–3 days).

In summary, our results suggest that NO plays an important role in endotoxin-evoked ocular inflammation. The inflammatory cells that infiltrate ocular tissues and aqueous humour express inducible NOS and probably generate large amounts of NO. The formation of NO is important in endotoxin-evoked inflammation as reflected by the marked effects of the NOS inhibitor L-NAME. Firstly, L-NAME prevented the C-fibre activation (reflected in inhibited transmitter release). Secondly, L-NAME blocked the vascular effects of CGRP and partly inhibited the vascular effects of PACAP. Both observations are compatible with a role for NO as an inflammatory mediator. Finally, L-NAME prevented the appearance of NOS-containing immune cells in ocular tissues, possibly because it abolished vasodilatation, suggesting that NO contributes to the recruitment of inflammatory cells.

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