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Adrenomedullin and ocular inflammation in the rabbit

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Abstract

Adrenomedullin administered peripherally in the rabbit (at doses of 1.25, 2.5 and 5 μg/kg) caused a dose-dependent conjunctival hyperemia accompanied by an increase of inflammatory cell number and prostaglandin E_2 concentration in the aqueous humor, and of uveal vascular response and myeloperoxidase activity. The inflammatory effect of the peptide, injected at the dose of 5 μg/kg, was abolished by pretreatment with the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methylester (50 mg/kg, i.v.). Moreover, the i.v. pretreatment with the calcitonin gene-related peptide 8–37 fragment (calcitonin gene-related peptide, CGRP-(8–37), 2.5 μg/kg), receptor antagonist of CGRP, did not inhibit the conjunctival hyperemia. In contrast, the i.v. pretreatment with the adrenomedullin receptor antagonist, adrenomedullin-(22–52) fragment (2.5 μg/kg), abolished adrenomedullin-induced ocular inflammation. These results suggest that adrenomedullin causes conjunctival hyperemia, and this effect involves the nitric oxide system acting through specific adrenomedullin receptors.

Keywords: Adrenomedullin; Inflammation; Eye; CGRP (calcitonin gene-related peptide); Nitric oxide (NO)

1. Introduction

Adrenomedullin is a vasorelaxant peptide of 52 amino acid residues isolated from extracts of human pheochromocytoma (Kitamura et al., 1993). Structurally, this peptide belongs to the calcitonin gene-related peptide (CGRP) superfamily and elicits a potent vasodilator effect inferior, however, to that induced by CGRP (Ishiyama et al., 1993). Both peptides have been shown to be involved in inflammation (Clementi et al., 1999; Raud et al., 1991). Evidence suggests that CGRP exerts an important role in inflammation also at ocular level. In fact, injected intravenously (i.v.) in rabbits, CGRP elicits hypotension and breakdown of blood–aqueous barrier, leading to leakage of plasma proteins into the aqueous humor (Andersson, 1992). Moreover, pharmacologically induced conjunctival hyperemia may be correlated to the release of CGRP (Wang et al., 1995), and mediated by nitric oxide (Wang et al., 1997). In fact, this effect was abolished by pretreatment with the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methylester (L-NAME). The hypothesis that CGRP can play an important role in inflammatory responses of the eye is supported, moreover, by the presence of CGRP receptors in the iris–ciliary body (Malminiemi and Malminiemi, 1992). Recently, immunoreactive adrenomedullin has been found in the cat iris–ciliary body and aqueous humor (Yousufzai et al., 1999) and it has been suggested that this peptide plays a role in controlling intraocular pressure in rabbit (Taniguchi et al., 1999).

These data have prompted us to verify the possible role of adrenomedullin in the inflammatory responses of the rabbit's eye. In order to gather information on the mechanism of adrenomedullin-induced ocular inflammation, we have studied the number of inflammatory cells and prostaglandin E_2 levels in aqueous humor, and myeloperoxidase activity in uveal tissue. Moreover, we have verified if these inflammatory responses involve the nitric oxide system and the CGRP or adrenomedullin receptors in the eye.

2. Material and methods

2.1. Animals

Naive New Zealand male albino rabbits, weighing 1.8 to 2.3 kg, were used. Upon receipt from the supplier

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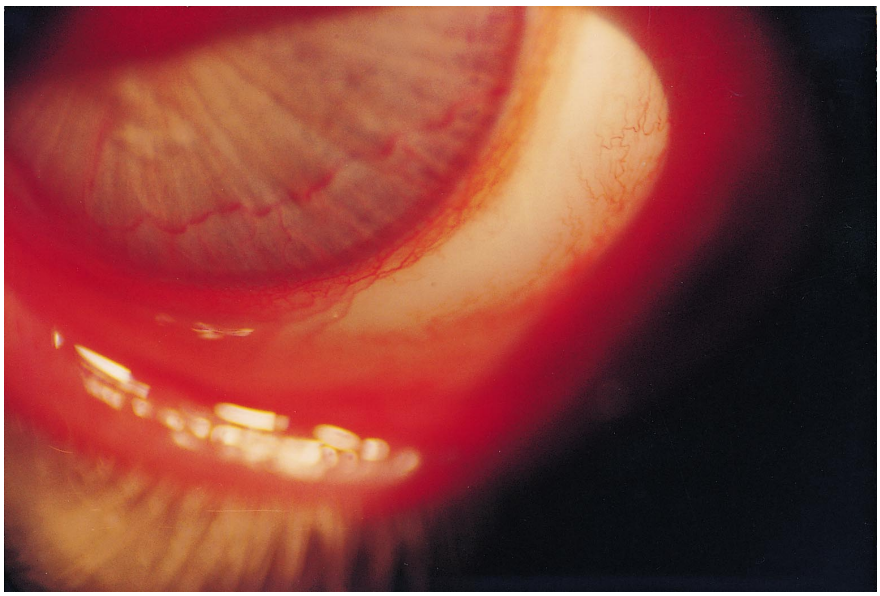

Fig. 1. Conjunctival hyperemia in a rabbit 60 min after i.v. injection of saline (grade = 0).

(Charles River, Como, Italy), the rabbits were housed in cages of one animal each, and maintained on a 12:12 h light/dark schedule. Food and water were available ad libitum. Groups of five rabbits were used and each animal was used only once in the experiments. All experiments were conducted blind to treatment in conformity with the European Communities Council Directive 86/609/EEC in agreement with the Helsinki declaration.

2.2. Drugs and treatment

Rat adrenomedullin and calcitonin gene-related peptide 8–37 fragment (CGRP-(8–37) fragment) were purchased from Peninsula Laboratories Europe (UK). N^G-nitro-L-arginine methylester and adrenomedullin-(22–52) fragment were purchased from Sigma, Italy. Solutions for i.v. injection were freshly prepared from adrenomedullin or CGRP-(8–37) fragment bulk dissolved in saline. The doses were 1.25, 2.5 and 5.0 μg/kg for adrenomedullin and 2.5 μg/kg for CGRP-(8–37) or adrenomedullin-(22–52) fragment, and 50 mg/kg for L-NAME. Control animals were injected with the same volume of saline. Adrenomedullin was injected i.v. at different doses in a total volume of 500 μl. Sixty minutes after administration, the animals were submitted to evaluation of the inflammatory parameters. Other groups of rabbits were pretreated with L-NAME,

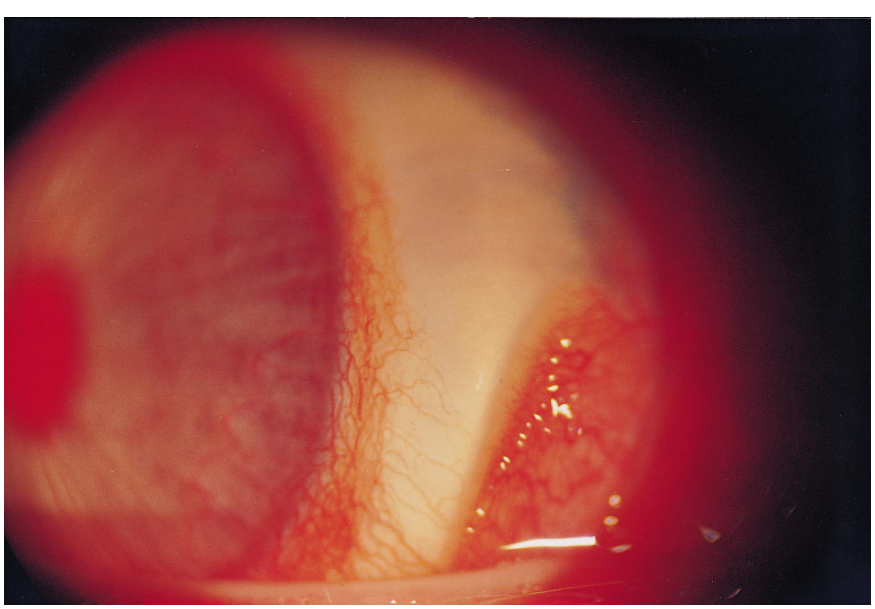

Fig. 2. Conjunctival hyperemia in a rabbit 60 min after i.v. injection of adrenomedullin 1.25 μg/kg (grade = 1).

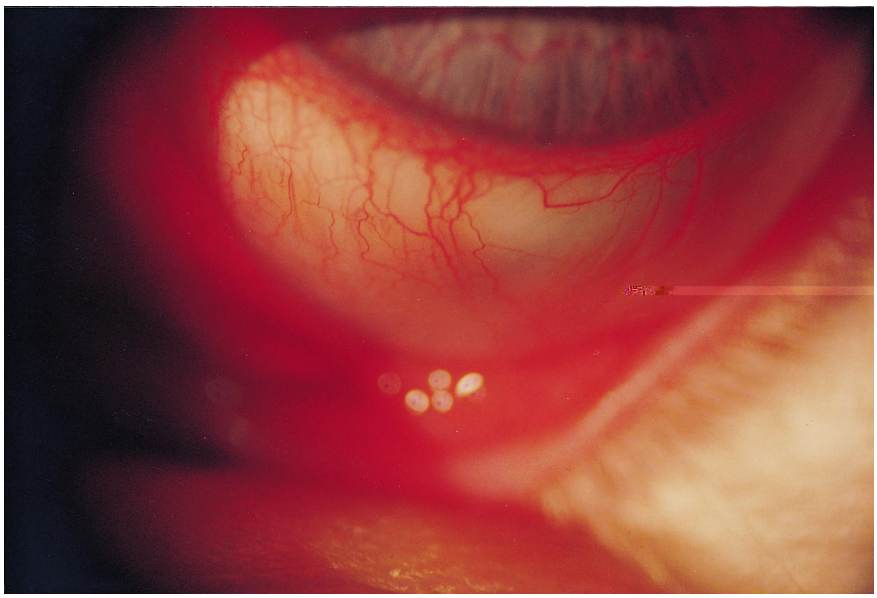

Fig. 3. Conjunctival hyperemia in a rabbit 60 min after i.v. injection of adrenomedullin 2.5 μg/kg (grade = 2).

CGRP-(8–37) fragment or adrenomedullin-(22–52) fragment 30 min prior to adrenomedullin administration and submitted to evaluation of inflammatory parameters 60 min after adrenomedullin administration. Control animals were injected with the same volume of saline. The animals were killed by barbiturate overdose and the eyes were used for calculation of inflammatory cell number, prostaglandin E_2 levels and uveal myeloperoxidase activity.

2.3. Clinical evaluation

The animals were evaluated immediately before being killed (60 min after i.v. injection of adrenomedullin or saline) by slit-lamp biomicroscopy, and the following parameters were measured: conjunctival hyperemia and chemosis, intensity of the aqueous protein (flare) and iris vascular response were graded from 0–3 + (for conjunctival hyperemia: 0 = no abnormalities visible, 1 = mild hyperemia, 2 = hyperemia and mild engorgement of vessels, and 3 = hyperemia and severe engorgement and/or conjunctival hemorrhage; for chemosis: 0 = no chemosis visible, with 1, 2, and 3, representing mild, moderate and severe chemosis; for flare: 0 = no Tyndall effect, with 1, 2, and 3, representing mild, moderate, and severe Tyndall effects, respectively; for iris vascular response: 0 = no abnormalities visible, 1 = mild hyperemia, 2 = hyperemia

Fig. 4. Conjunctival hyperemia in a rabbit 60 min after i.v. injection of adrenomedullin 5 μg/kg (grade = 3).

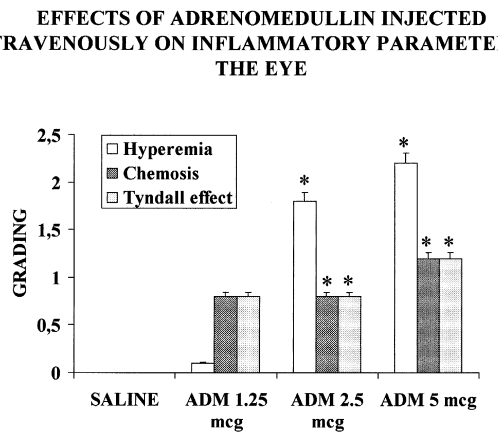

Fig. 5. Values are mean ± S.E.M. of graded evaluation of ocular inflammatory parameters, as taken from both eyes of five rabbits. Grading rates were considered as described in the text. Adrenomedullin was injected intravenously 60 min prior to ocular observation. * Significantly different as compared to saline-injected group ($P < 0.05$, Dunnett's test for multiple comparisons).

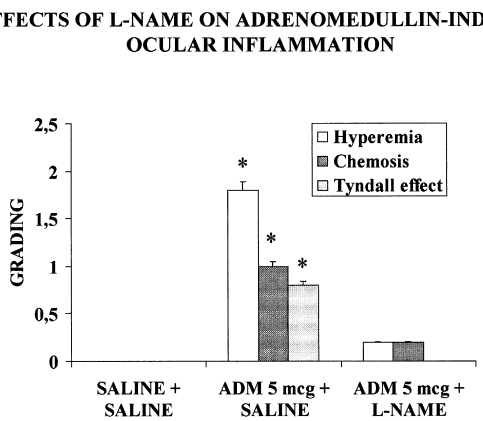

Fig. 6. Values are mean ± S.E.M. of graded evaluation of ocular inflammatory parameters, as taken from eyes of five rabbits. Grading rates were considered as described in the text. Adrenomedullin (5 μg/kg) was injected intravenously 60 min prior to ocular observation. L-NAME (50 mg/kg) was injected intravenously 30 min prior to ocular observation. * Significantly different as compared to saline-injected group ($P < 0.05$, Dunnett's test for multiple comparisons).

and mild engorgement of vessels, and 3 = hyperemia and severe engorgement and/or iris hemorrhage). Intraocular pressure was measured in conscious rabbits using an electronic pneumotonometer (Mackay Marg, USA) after surface anesthesia (0.4% benoxinate solution, one drop).

2.4. Measurement of myeloperoxidase activity

Myeloperoxidase activity, a marker for neutrophil content in uveal tissue, was assayed by the method previously described (Bradley et al., 1982). Iris and ciliary body tissue were homogenized; the homogenate was frozen and thawed three times, sonicated, and centrifuged at 40,000 × *g*. The myeloperoxidase activity was assayed spectrophotometrically by measuring the change in absorbance at 460 nm of a mixture of tissue homogenate, buffer, diansidine dihydrochloride, and hydrogen peroxide. The activity was expressed as units of peroxidase degradation per minute of reaction.

2.5. Prostaglandin levels

Aqueous and vitreous humor were acidified to pH 3.5 with 5 μl of H_3PO_4 (0.49 mol/l), extracted into six volumes of ethyl acetate (twice), dried under vacuum in a Speed-Vac concentrator, and reconstituted into 500 μl of methanol. Aliquots of the methanol reconstitute were dried under a vacuum, reconstituted in 300 μl of a modified Krebs–Henseleit buffer (pH 7.4) and assayed for prostaglandin E_2. Details of the prostaglandin E_2 assay have been described previously (Fleisher et al., 1989).

2.6. Quantitation and identification of inflammatory cells

Immediately after death, aliquots of aqueous humor were collected in heparinized syringes. Total leukocyte and erythrocyte numbers in the aqueous humor were determined by the hemocytometer method and correspondent to the total number of aqueous cells.

Table 1
Effects of adrenomedullin (5 μg/kg, i.v.) on inflammatory parameters of the rabbit's eye

	Total inflammatory cells	Aqueous prostaglandin E_2 levels (ng/ml)	Uveal myeloperoxidase (activity/mg protein)	Iris vascular response
Saline	34.2 ± 3.5	0.8 ± 0.1	1.2 ± 0.1	0.1 ± 0.0
Adrenomedullin (5 μg/kg, i.v.)	834.3 ± 45.1[a]	6.5 ± 0.6[a]	3.8 ± 0.4[a]	1.4 ± 0.1[a]

Values are mean ± S.E.M. The number of eyes is 10 (both eyes for each of five animals were considered). Adrenomedullin was injected 60 min prior to sacrifice.

[a] Significantly different as compared to saline-injected controls ($P < 0.05$, Dunnett's test for multiple comparisons).

2.7. Statistical analysis

Analysis of data was made using two-way analysis of variance two-way (ANOVA) followed by the post-hoc Dunnett's test for multiple comparisons. A P value of 0.05 or less was considered as indicative of a significant difference.

3. Results

Intravenous injection of adrenomedullin induced a dose-dependent ocular inflammation in rabbits' eyes 60 min after treatment. In particular, the effect on conjunctival hyperemia, as compared to that of saline (Fig. 1), are depicted in Fig. 2 (1.25 μg/kg), Fig. 3 (2.5 μg/kg), Fig. 4 (5 μg/kg). The quantitative effects of adrenomedullin are indicated in Fig. 5. The peptide provoked a dose-dependent conjunctival flogosis with maximum activity at the dose of 5 μg/kg. The conjunctival flogosis was accompanied by a significant increase in the number of inflammatory cells and prostaglandin E_2 levels in the aqueous humor, and of vascular response and myeloperoxidase activity in the uveal tissue (Table 1). Sixty minutes after adrenomedullin or saline injection, no significant change of intraocular pressure was observed (data omitted).

Pretreatment with L-NAME (50 mg/kg), 30 min prior to the administration of the peptide, abolished the inflammatory effect of adrenomedullin (Fig. 6). Pretreatment with CGRP-(8–37) fragment, injected i.v. at the dose of 2.5μg/kg, did not affect the inflammatory responses induced by adrenomedullin. In contrast, pretreatment with adrenomedullin-(22–52) fragment, injected i.v. at the dose of 2.5 μg/kg, was followed by a total suppression of these responses (Fig. 7).

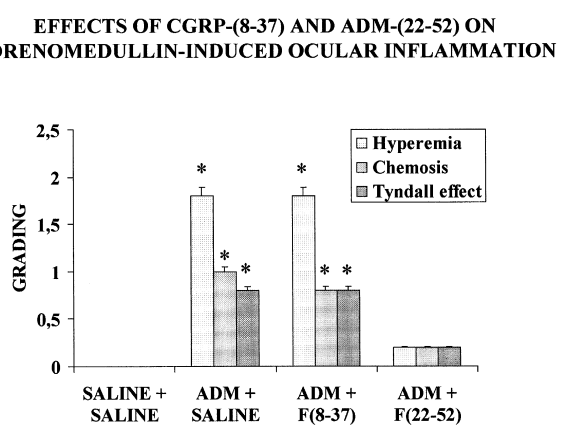

Fig. 7. Values are mean ± S.E.M. of graded evaluation of ocular inflammatory parameters, as taken from both eyes of five rabbits. Grading rates were considered as described in the text. Adrenomedullin (ADM, 5μg/kg) was injected intravenously 60 min prior to ocular observation. CGRP-(8–37) fragment or adrenomedullin-(22–52) was injected intravenously 2.5 μg/kg 30 min prior to ocular observation. * Significantly different as compared to saline-injected group ($P < 0.05$ Dunnett's test for multiple comparisons).

4. Discussion

The inflammatory sequence initiated by traumatic or noxious stimuli begins with damage to cells and connective tissue, and the subsequent release of a diverse group of inflammatory mediators that include lysosomal enzymes, histamine bradykinin, arachidonic acid metabolites, oxygen metabolites and free radicals, cytokines, chemotactic factors, and products of complement activation (Rao et al., 1987; Ward et al., 1979). Vascular permeability is changed, constituents of plasma leak through damaged interendothelial cell junctions, and the migration of neutrophils and eventually monocytes stimulated. The features and severity, however, of the ensuing inflammatory process are variable and depend largely on the nature of the underlying insult.

There is evidence indicating an involvement of the CGRP superfamily peptides in the inflammatory response of the eye. In fact, Andersson (1992) has shown that CGRP, injected i.v. induces hypotension and breakdown of the blood–ocular barrier leading to leakage of plasma proteins into the aqueous humor. The vasodilator effect of the peptide involves the nitric oxide system since the pretreatment with L-NAME blocks this effect. This evidence is not surprising since it has been suggested that nitric oxide plays an important role in the endotoxin-evoked ocular inflammation in the rabbit (Wang et al., 1997). Moreover, specific binding sites for CGRP and amylin have been shown in iris–ciliary body of the rabbit (Malminiemi and Malminiemi, 1992; Heino et al., 1998).

The present study shows that adrenomedullin also induces an inflammatory activity at ocular level. In particular, conjunctival and iris flogosis parameters appeared to be affected by adrenomedullin, while no change was observed on intraocular pressure. This finding suggests that, at least 60 min after i.v. administration, the peptide does not possess pharmacokinetic properties compatible with an effect on this last parameter. The inflammatory effect of adrenomedullin on the ocular tissues seems to be correlated with the nitric oxide system, as it is blocked by pretreatment with L-NAME. This result is not in agreement with that found by Yousufzai et al. (1999) showing that L-NAME and the cyclooxygenase inhibitor, indomethacin, have no effect on adrenomedullin-induced cAMP accumulation in the cat iris sphincter. However, it should be noted that these studies were made in vitro where only 10 μM of L-NAME and 1 μM of indomethacin were used. Interestingly, indomethacin abolished prostaglandin surge but did not affect the ocular hypotension induced by adrenomedullin in another in vivo study (Taniguchi et al., 1999). Thus, it seems that there is a substantial difference between in vivo and in vitro situation in this respect.

In adrenomedullin-induced ocular inflammation, vascular compromise is a particularly prominent feature, evidenced by the number of inflammatory cells found in aqueous humor and extravascular uveal tissues. Because

the escape of erythrocytes from the vascular compartment is a passive process that occurs in proportion to the extent of vascular injury (Hurley, 1983), this effect must be considered severe. Although vascular permeability may be altered by inflammatory cell mediators such as histamine, bradykinin, and prostaglandins (Ward et al., 1979; Sacks et al., 1978), the duration of protein leakage, the magnitude of erythrocyte extravasation seen in this study are most compatible with a direct vascular injury. The second prominent feature is the mild inflammatory response, as indicated by the relative low aqueous inflammatory cell counts, aqueous protein, prostaglandin E_2 levels, and particularly the uveal myeloperoxidase activity.

The main physiological effect of adrenomedullin seems to be vasodilatation including an increase in pulmonary blood flow (Lippton et al., 1994). The reduction of mean blood pressure after i.v. administration of the peptide is due to a decrease in peripheral vascular resistance (Nuki et al., 1993).

Given that the cardiovascular activity of adrenomedullin is inhibited after administration of the CGRP-(8–37) fragment, it has been suggested that adrenomedullin acts via CGRP receptors. Our data indicate that adrenomedullin exerts its inflammatory effect at the ocular level interfering with specific adrenomedullin receptors. In fact, the pretreatment with CGRP-(8–37) fragment did not significantly inhibit the flogistic activity of the peptide whereas pretreatment with the specific adrenomedullin receptors antagonist, adrenomedullin-(22–52), abolished it. These results are in agreement with those of Taniguchi et al. (1999) who showed that adrenomedullin decreases intraocular pressure mainly via specific adrenomedullin receptors, but not with those of Yousufzai et al. (1999) who did not find any interference of CGRP-(8–37) on adrenomedullin-induced relaxation of iris sphincter muscle in vitro. The same authors suggested that activation of adrenomedullin receptors by the peptide leads to concentration-dependant increases of cAMP accumulation and subsequent inhibition (relaxation) of smooth muscle contraction. Thus, the involvement of activated adenylate cyclase in the effects of adrenomedullin observed in the present study cannot be ruled out.

It should be noted that no attempt was made here to reverse adrenomedullin-induced flogosis with the administration of an adrenomedullin receptor antagonist. The pretreatment with an antagonist was the procedure followed by other studies, where the established effect was not attempted to treat (Taniguchi et al., 1999).

In conclusion, adrenomedullin exerts an inflammatory activity at ocular level acting through specific adrenomedullin receptors. This activity seems to involve the nitric oxide system and can be considered of a physiological meaning as adrenomedullin seems to be naturally secreted by ocular tissues (Yousufzai et al., 1999).

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