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European Journal of Pharmacology 303 (1996) 217–220

ejp

Short communication

Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema

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Received 12 February 1996; accepted 16 February 1996

Abstract

The role of peroxynitrite generated from nitric oxide and superoxide anion was investigated in a model of acute inflammation induced by the injection of carrageenan into the rat hind paw. Paw edema was inhibited 8 h following the administration of carrageenan by *N*-iminoethyl-L-lysine (3–30 mg/kg, $n = 6$) or aminoguanidine (30–300 mg/kg, $n = 6$), two selective inhibitors of inducible nitric oxide synthase and by recombinant human Cu/Zn superoxide dismutase coupled to polyethyleneglycol (12×10^3 U/kg, $n = 6$, $P < 0.001$). Moreover, at the same time point following carrageenan administration, intense immunoreactive staining for nitrotyrosine (a marker of peroxynitrite formation) was detected. Our results suggest that the generation of nitric oxide, superoxide anion and peroxynitrite contributes to the edema observed in this acute model of inflammation.

Keywords: Carrageenan; Inflammation; Peroxynitrite

1. Introduction

Nitric oxide (NO) generated by the inducible form of nitric oxide synthase plays important roles in tissue injury in a number of inflammatory diseases. One possible mechanism by which NO produces tissue injury is by interacting with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$, Beckman et al., 1990). Peroxynitrite is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage (Rubbo et al., 1994). However, little is known about the importance of peroxynitrite in producing inflammation in vivo. Using pharmacological and molecular tools we have assessed the roles of NO, O_2^- and peroxynitrite in producing the edema observed during the late phase of the carrageenan-induced model of paw inflammation.

2. Materials and methods

2.1. Carrageenan-induced paw edema

Male Sprague-Dawley rats (175–200 g) were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and in accordance with NIH guidelines on laboratory animal welfare. Rats received a subplantar injection of carrageenan (0.1 ml of a 1% suspension in 0.85% saline) in the right hind paw under brief (1 min) anesthesia with methoxyfluorane. All drugs were given by intravenous injection in the penile vein under brief anesthesia either 30 min (for superoxide dismutase coupled to polyethyleneglycol, PEG-rhSOD) or 1 h (for the iNOS inhibitors, *N*-iminoethyl-L-lysine, L-NIL or aminoguanidine, AG) before carrageenan administration. Paw volume (ml) was measured using a plethysmometer (Ugo-Basile, Varese, Italy) immediately before and 8 h following the injection of carrageenan. Paw edema was expressed as the increase in paw volume (ml) after carrageenan injection with respect to the pre-injection value for each animal.

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PII S0014-2999(96)00140-9

Superoxide dismutase coupled to polyethyleneglycol was obtained from DDI Pharmaceuticals (Mountain View, CA, USA) and *N*-iminoethyl-L-lysine was synthesized in house as described previously (Connor et al., 1995). All other chemicals and reagents were obtained from Sigma (St. Louis, MO).

2.2. Determination of nitrite / nitrate from carrageenan-injected rat paws

At 8 h following intraplantar injection of carrageenan, rats were killed and each paw was removed at the level of the calcaneus bone. Paws were gently centrifuged (250 × *g* for 20 min) in order to recover a sample of the edematous fluid. The volume of fluid that was recovered from each paw was measured. Blood was removed from the fluid sample by filtering through a 10000 MW cut-off filter (Millipore, Bedford, MA, USA). Nitrite/nitrate (NO_x) concentrations were measured using the diaminonaphthalene (DAN) assay as described (Misko et al., 1993a). Nitrate in the paw fluid samples (10 μl) was converted to nitrite by the incubation with nitrate reductase (14 mU) and the reduced form of nicotinamide adenine dinucleotide phosphate (1 nmol) for 10 min at room temperature. The reaction was terminated by dilution with water and addition of the diaminonaphthalene reagent. NO_x concentrations were then determined fluorometrically (Misko et al., 1993a). All determinations were performed in duplicate. Total (T) NO_x present in the entire edematous fluid of each paw was calculated as follows:

$$T = \frac{\text{pmol } NO_x \text{ in the sample} \times \text{paw edema volume (ml)}}{\text{sample volume (ml)}}$$

Results are expressed as total nmol of NO_x per paw.

2.3. Immunohistochemical localization of nitrotyrosine

At the end of the experiment (8 h), rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and then perfused through the left ventricle with 120 ml of perfusion solution (composition: Hanks' balanced salt solution containing 20 mM Hepes and 1% formaldehyde). Immunohistochemical staining for nitrotyrosine was performed using 8 μm frozen sections of hind paws obtained from these perfused rats. Tissue sections were incubated with a 1/500 dilution of either preimmune serum, an anti-nitrotyrosine polyclonal rabbit serum generated to nitrated keyhole limpet hemocyanin, or the anti-nitrotyrosine serum and excess nitrotyrosine (10 mM) in phosphate buffered saline containing 10 mM tyrosine, 1% bovine serum albumin, 0.2% powdered skim milk and 0.3% Triton X-100 at 4°C overnight. Staining was localized using a 1/200 dilution of Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and was visualized with epifluorescence.

2.4. Statistics

The results were analyzed by a two-way analysis of variance followed by a least significant procedure to determine the significance of this response. A P value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of selective inhibitors of inducible nitric oxide synthase on paw volume and NO_x

A marked increase in both paw volume (1.7 ± 0.2 ml, $n = 10$) and in the NO_x present in the paw fluid exudate (from 0.5 ± 0.05 to 50 ± 5 nmol/paw, $n = 10$, $P < 0.001$) occurred 8 h following the intraplantar injection of carrageenan. Both effects are likely due to the activity of the inducible form of nitric oxide synthase since two recently described selective inhibitors of inducible nitric oxide synthase (Misko et al., 1993b; Moore et al., 1994; Connor et al., 1995; Salvemini et al., 1995), *N*-iminoethyl-L-lysine (3–30 mg/kg, $n = 6$) and aminoguanidine (30–300 mg/kg, $n = 6$) inhibited both the edema and NO_x production (Table 1A and 1B). At the highest dose of *N*-iminoethyl-L-lysine (30 mg/kg, $n = 5$) or aminoguanidine (300 mg/kg, $n = 5$) used, no changes in mean arterial pressure were observed (from 107 ± 5 mm Hg for saline treated rats to 110 ± 3 mm Hg for rats treated with L-NIL for 8 h and 109 ± 5 mm Hg for rats treated with AG for 8 h). This indicates that these drugs did not affect the activity of the constitutive form of NOS. The increase in paw edema observed 1 h after carrageenan (0.4 ± 0.01 ml, $n = 6$) was

Table 1
Effect of *N*-iminoethyl-L-lysine and aminoguanidine on carrageenan-induced paw edema and NO_x production

(A)

L-NIL (mg/kg)	Paw volume (ml)	NO_x (nmol/paw)
0	1.7 ± 0.2	50 ± 0.05
3	1.3 ± 0.05 [a]	45 ± 1 [a]
10	1.0 ± 0.15 [b]	24 ± 1 [b]
30	0.6 ± 0.02 [b]	5 ± 0.2 [a]

(B)

AG (mg/kg)	Paw volume (ml)	NO_x (nmol/paw)
0	1.7 ± 0.2	50 ± 0.05
30	1.5 ± 0.1 [a]	40 ± 3 [a]
100	0.9 ± 0.01 [b]	20 ± 2 [b]
300	0.7 ± 0.03 [b]	7 ± 0.1 [b]

The increase in paw volume and production of NO_x in the paw fluid exudate 8 h following carrageenan was inhibited in a dose-dependent manner by *N*-iminoethyl-L-lysine and aminoguanidine. Each value is the mean ± S.E.M. for six experiments. [a] $P < 0.01$ and [b] $P < 0.005$ compared to control values.

not affected by *N*-iminoethyl-L-lysine (0.4 ± 0.03, $n = 6$) or aminoguanidine (0.5 ± 0.05, $n = 6$), but was attenuated by the non-selective constitutive and inducible NOS inhibitors, N^G-monomethyl-L-arginine or nitro-L-arginine (100 mg/kg, i.v.; from 0.4 ± 0.01 ml to 0.08 ± 0 and 0.05 ± 0 ml, respectively, $n = 6$). These findings add further support to the notion that the anti-inflammatory effects seen with *N*-iminoethyl-L-lysine or aminoguanidine 8 h following carrageenan administration are due primarily to the inhibition of inducible nitric oxide synthase.

3.2. Effects of superoxide dismutase coupled to polyethyleneglycol on paw volume

Superoxide dismutase coupled to polyethyleneglycol (12×10^3 U/kg, $n = 6$) also inhibited the increase in paw edema 8 h following carrageenan administration by approximately 60% (from 1.7 ± 0.2 to 0.6 ± 0.1 ml, $n = 6$, $P < 0.001$) indicating the involvement of O_2^- in this inflammatory response. Treatment with catalase (40×10^3 U/kg, i.p. 30 min before carrageenan; Hirschelmann and Bekemeir, 1981) or with desferrioxamine (300 mg/kg, s.c., 1 h before carrageenan, Boughton-Smith et al., 1993) had no effect on the formation of edema ($n = 4$, not shown). This indicates that neither hydrogen peroxide nor hydroxyl radicals contribute to the development of inflammation in this model.

3.3. Immunohistochemical analysis of nitrotyrosine in paws

The paws tissues were examined immunohistochemically for the presence of nitrotyrosine. Nitrotyrosine immunoreactivity was not detected in the non-inflamed paw tissue of control rats (Fig. 1A), nor was there any staining in either control or treated tissue using nonimmune serum (not shown). In contrast, marked nitrotyrosine immunoreactivity was found in the paw tissue obtained from rats 8 h following carrageenan administration (Fig. 1B). Additionally, the nitrotyrosine immunoreactivity was largely eliminated by incubating the anti-nitrotyrosine antiserum with an excess of nitrotyrosine demonstrating the specificity of staining (not shown).

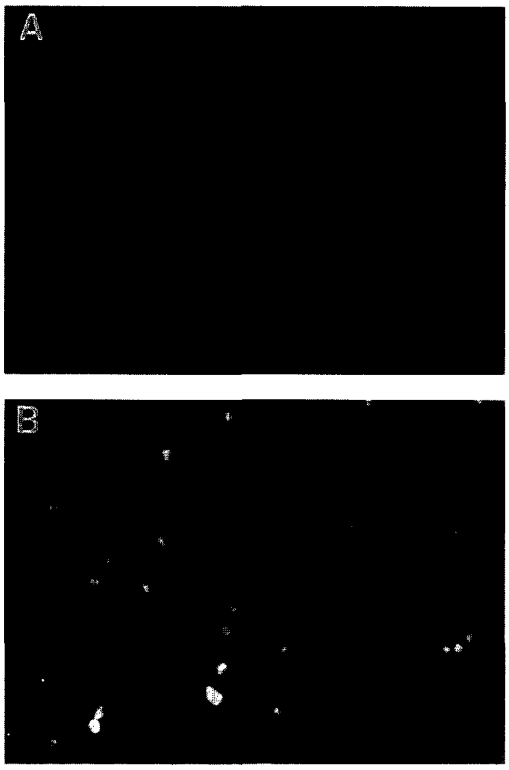

Fig. 1. Immunohistochemical localization of nitrotyrosine in inflamed paw tissue. Marked nitrotyrosine immunoreactivity was detected using indirect immunofluorescence in inflamed paws obtained from carrageenan-treated rats (B). No specific staining for nitrotyrosine was found in noninflamed control tissue (A). Specificity of the staining was confirmed by the complete elimination of staining by incubation of the nitrotyrosine antiserum with an excess of nitrotyrosine (not shown). Results are representative sections obtained from five control and carrageenan-treated rats.

4. Discussion

The generation of peroxynitrite has been proposed in a number of pathophysiological conditions that are associated with overproduction of both NO and O_2^- (Beckman and Crow, 1992, for review). At present however, there is limited data demonstrating its presence and involvement in inflammation in vivo. Our results demonstrate the generation of NO and O_2^- and suggest the involvement of peroxynitrite in edema formation during an acute inflammatory response induced by carrageenan administration. Edema was reduced by inhibition of excessive generation of NO with selective inhibitors of inducible nitric oxide synthase inhibitors and O_2^- with superoxide dismutase coupled to polyethyleneglycol, two pharmacological manipulations that should decrease peroxynitrite formation (see Beckman and Crow, 1992, for review). Furthermore, using the presence of tyrosine nitration as a specific marker of the presence of peroxynitrite, we demonstrated that the inflammatory response evoked by carrageenan involves the generation of peroxynitrite at the injured site. Although the biochemical role of peroxynitrite in producing inflammation remains to be elucidated, our data suggest that pharmacological manipulations which lower the amount of peroxynitrite formed in vivo may have significant antiinflammatory potential.

Acknowledgements

We would like to thank Dr. T.P. Misko (Inflammatory Diseases Research, G.D. Searle) for providing the antiserum to nitrotyrosine.

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