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Involvement of guanylate cyclase and potassium channels on the delayed phase of mouse carrageenan-induced paw oedema

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Abstract

Previous studies from this laboratory have shown that administration of nitric oxide (NO) donors reduces the early phase (which peaks at 4 h) of carrageenan-induced paw oedema. The aim of this study was to investigate the influence of NO donors on the delayed phase of the mouse paw oedema, which peaks 48 h after carrageenan injection. Treatment of animals with sodium nitroprusside (1.5, 5 and 10 μmol/kg, subcutaneously (s.c.)) 8 h after the subplantar carrageenan injection (300 μg/paw), reduced (~50%) the delayed phase of paw oedema and the delayed increase in plasma leakage, as assessed by Evans Blue extravasation. Two other NO donors, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) or glyceril trinitrate (both at 28 μmol/kg) yielded an inhibition in paw oedema similar to that of sodium nitroprusside. NO-induced inhibition of the delayed phase of paw oedema was reversed when animals were treated with 1*H*-[1,2,4]-oxadiazolo-[4,3-*a*]quinoxalin-1 (ODQ, a soluble guanylate cyclase inhibitor, 11 μmol/kg, s.c.) or with tetraethylammonium (TEA, a nonselective potassium channel blocker, 300 μmol/kg, s.c.), 30 min before the prophylactic dose of sodium nitroprusside. In conclusion, our results show that a brief exposure to NO donors, even when made several hours after the inflammatory reaction has been triggered, is still able to cause an important reduction on the delayed phase of carrageenan-induced mouse paw oedema and fluid leakage. Moreover, this long-lasting NO antiinflammatory effect appears to be dependent on guanylate cyclase and potassium channels.

Keywords: Nitric oxide; Paw oedema; Potassium channel; Guanylate cyclase; Plasma leakage; Inflammation

1. Introduction

Based on the work from a number of laboratories, it is becoming increasingly apparent that nitric oxide (NO) is an important modulator of the inflammatory cascade. In both acute and chronic inflammatory disorders, NO has been shown to influence many pathophysiological processes such as endothelial cell adhesion molecule expression (Khan et al., 1996), leukocyte–endothelial interaction (Kubes et al., 1991), increase in microvascular permeability (Kubes and Granger, 1992) and lymphocyte proliferation (Sternberg and McGuigan, 1992).

Several of NO physiological actions are mediated through its interaction with the heme iron of soluble guanylate cyclase, leading to enzyme activation and consequent increase in guanosine cyclic monophosphate. However, the classical view of cGMP as the exclusive mediator of NO activity has been supplanted by findings that guanylate cyclase is only one of many proteins that are targets for NO. In this context, another mechanism, involving the reaction between NO and free sulphydryl (–SH) groups (the so-called *S*-nitrosylation, which forms nitrosothiols, RSNO) has since been implicated in the control of a wide array of protein functions and cell activities (Stamler et al., 2001; Foster et al., 2003).

Among the several protein targets of NO, potassium channels are being characterised as one of the most important in this regard. For instance, Williams et al. (1988) and Fujino et al. (1991) reported that nitric oxide

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donors and exogenous cGMP activated potassium channels. This is consistent with observations that NO and cGMP-elevating agents hyperpolarize some arteries (Ito et al., 1978; Tare et al., 1990; Garland and McPherson, 1992). Subsequent reports showed evidence that vasorelaxation in response to NO is associated with activation of potassium channels (Hamaguchi et al., 1992; Khan et al., 1993). This activation of potassium channels by NO is mediated by cGMP (Robertson et al., 1993; Taniguchi et al., 1993; Archer et al., 1994; Hall and Wu, 1996; 1998) or by NO itself (Bolotina et al., 1994). Several types of potassium channels are expressed by inflammatory cells, suggesting that these channels may possibly be involved in the inflammatory response (Tanhehco, 2001). In this regard, it has been demonstrated that potassium channels are involved in the vascular changes to vasoactive mediators caused by NO in inflammatory conditions (Hall et al., 1996; Wu et al., 1998), and this has been confirmed by our group (Da Silva-Santos and Assreuy, 1999; Terluk et al., 2000).

Carrageenan-induced paw oedema is a useful model to assess vascular changes associated with inflammation. Subplantar injections of carrageenan in mice induce a biphasic oedema. The first phase peaks at 4 h and the second (or delayed) phase peaks at 48 h after carrageenan injection. In the early phase, there is a diffuse cellular infiltrate with predominance of polymorphonuclear neutrophils (Di Rosa and Sorrentino, 1968), whereas the infiltrate of the delayed phase is composed by macrophages, eosinophils and predominantly lymphocytes (Henriques et al., 1987). We have previously showed that a single injection of NO donors exerts an antiinflammatory effect evidenced by a reduction in vascular permeability and neutrophil infiltration on first phase on carrageenan-induced mouse paw oedema. This antiinflammatory effect appears to be dependent on cGMP and on potassium channels (Fernandes et al., 2002). These results led us to investigate the effect of NO donors on the delayed phase of carrageenan-induced mouse paw oedema.

2. Material and methods

2.1. Animals

Female Swiss mice (weighing 20–25 g) used in this study were housed in a temperature-controlled (23±2 °C) and light-controlled (12-h light/dark cycle) room, with free access to water and food. All procedures were approved by our Institutional Ethics Committee and were in accordance with NIH Animal Care Guidelines.

2.2. Paw oedema

Animals were lightly anaesthetised with ethyl ether and were injected intradermally (maximal volume 50 μl) with carrageenan (300 μg/paw) in the right hindpaw. The contralateral (left) paw received 50 μl of saline and was used as control. Carrageenan was dissolved in sterile Dulbecco's phosphate-buffered saline (PBS, in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.5, $NaHPO_4$ 8.1; pH 7.4). Paw oedema development was measured by plethysmometry, as previously described (Ferreira, 1979), at the indicated time intervals. The difference in the volume between the right and the left hindpaws was taken as paw oedema and was expressed in microliters.

2.3. Effects of NO donors on mouse paw oedema

To study the influence of NO on carrageenan-induced mouse paw oedema, the NO donors sodium nitroprusside (1.5, 5 and 10 μmol/kg), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) or glyceril trinitrate (both at 28 μmol/kg) were injected subcutaneously (s.c.) 4 h before or 8, 16 or 24 h after the inflammatory stimuli. To exclude any NO-independent effect of SNAP, its nonnitrosylated parent molecule *N*-acetyl-DL-penicillamine (NAP, 28 μmol/kg, s.c.) was injected as a control. Controls groups were run simultaneously. The doses of sodium nitroprusside and SNAP were based on our own experience (Da Silva-Santos and Assreuy, 1999; Fernandes et al., 2002), and glyceril trinitrate dose was chosen as being equipotent to sodium nitroprusside and SNAP.

2.4. Evans blue dye leakage

Animals received a single intravenous (i.v.) injection of Evans blue (80 mg/kg) 24 h after carrageenan. After another 24 h (that is, 48 h after carrageenan injection), animals were sacrificed by cervical dislocation, the paws were amputated and the tissue was minced before being incubated with formamide/water (1:1, v/v) for 48 h at 37 °C. The optical density of the supernatants was measured at 600 nm in a spectrophotometer (model U-2001, Hitachi, Japan). The concentration of dye was determined from a standard curve of Evans blue in formamide as previously described (Bertrand et al., 1993). The changes in vascular permeability were expressed as the difference in the amount of dye extravasation between the foot injected with carrageenan and that injected with saline.

2.5. Inhibition of soluble guanylate cyclase and potassium channel blockade

For this set of experiments, 7.5 h after injection of intraplantar carrageenan injection, animals were treated with 1*H*-[1,2,4]-oxadiazolo-[4,3-*a*]quinoxalin-1 (ODQ, a soluble guanylate cyclase inhibitor, 11 μmol/kg, s.c.) or with tetraethylammonium (TEA, a nonselective potassium channel blocker, 300 μmol/kg, s.c.), and 30 min later, animals received sodium nitroprusside or PBS (10 μmol/kg or 1 ml/kg, respectively, s.c.). Controls groups were run simultaneously. Paw volume and Evans blue dye leakage were measured at the indicated times after carrageenan injection,

as described above. Doses of both inhibitors were chosen based on our previous results (Da Silva-Santos and Assreuy, 1999; Da Silva-Santos et al., 2002; Fernandes et al., 2002).

2.6. Reagents

Sodium nitroprusside, *N*-acetyl-DL-penicillamine, ODQ, methylene blue, carrageenan type IV, TEA and Evans Blue were purchased from Sigma (St. Louis, MO, USA). SNAP was prepared in our laboratory by a published method (Field et al., 1978). Glyceril trinitrate (Tridil™) was a kind gift of Cristália (São Paulo, SP, Brazil).

2.7. Statistical analysis

Data are expressed as mean±S.E.M. of *n* animals. Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by *t*-test subjected to the Bonferroni correction. A *P* value of less than 0.05 was considered significant. Statistical analysis was performed using Graph-Pad Prism (San Diego, CA, USA) software.

3. Results

3.1. Effects of NO donors on second phase of carrageenan-induced paw oedema

In agreement with previous results (Fernandes et al., 2002), subcutaneous injection of sodium nitroprusside 4 h before carrageenan caused a dose-dependent reduction in the early phase of paw oedema (Fig. 1A and B). Additionally, this pretreatment with sodium nitroprusside also reduced, in a dose-dependent manner the delayed phase of mouse paw oedema (Fig. 1A and B).

When the animals received the injection of sodium nitroprusside 8 h after the inflammatory stimulus (hence, after the early peak), although the first phase of paw oedema occurred integrally (Fig. 1C and D), there was a dose-dependent reduction of the delayed phase of mouse paw oedema (Fig. 1C and D). This reduction was similar that obtained when sodium nitroprusside was injected 4 h before carrageenan.

Injection of other NO donors (SNAP and glyceril trinitrate), 8 h after inflammatory stimulus also attenuated the delayed phase of oedema, whereas *N*-acetyl-DL-penicillamine (NAP; the nonnitrosylated SNAP parent compound) was ineffective in this regard (Fig. 2).

The inhibitory effect of sodium nitroprusside was still present when it was administered 12 h after the inflammatory stimulus (Fig. 3), but not if NO donors were injected 16 h after carrageenan (Fig. 3). Experiments involving higher doses of sodium nitroprusside have confirmed that the inhibition attained by 10 μmol/kg was maximal (data not shown).

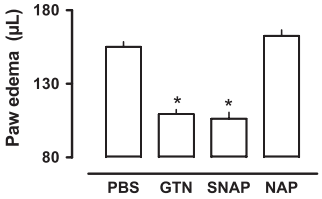

Fig. 2. Effects of therapeutic treatment with NO donors on the delayed phase of carrageenan-induced mouse paw oedema. Glyceril trinitrate (GTN), SNAP or NAP (28 μmol/kg, s.c.) or PBS (1 ml/kg, s.c.) was injected s.c. 8 h after the intraplantar injection of carrageenan (300 μg/paw). Paw oedema was quantified by plethysmometry 48 h after carrageenan injection. Each bar represents the mean of eight animals and vertical lines the S.E.M. **P*<0.05 compared to the control group (PBS). Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test.

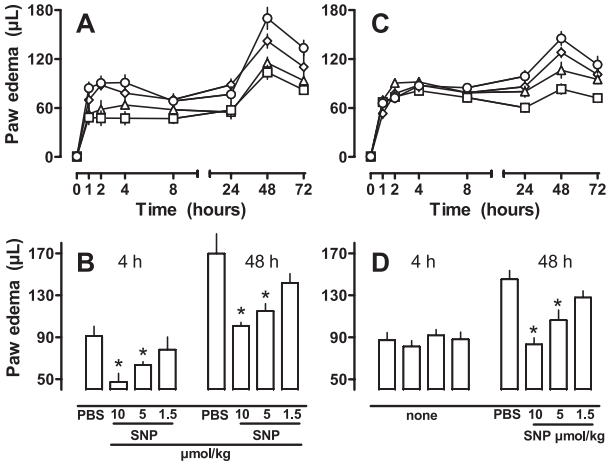

Fig. 1. Effects of treatment with NO donor sodium nitroprusside (SNP) on mouse paw oedema induced by carrageenan. (A) Sodium nitroprusside was injected s.c. 4 h before the intraplantar injection of carrageenan (300 μg/paw; ○) at the doses of 1.5 (◇), 5 (△) and 10 μmol/kg (□). (B) Paw oedema evaluated at 4 and 48 h after carrageenan. (C) Sodium nitroprusside was injected s.c. 8 h after the intraplantar injection of carrageenan (300 μg/paw; ○) at the same doses as above. (D) Paw oedema evaluated at 4 and 48 h after carrageenan. Each point or bar represents the mean of eight animals and vertical lines are the S.E.M. **P*<0.05 compared to the control group (PBS). Statistical analysis was performed using ANOVA test followed by Bonferroni's post hoc *t* test.

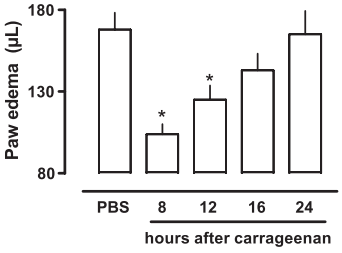

Fig. 3. Time-course of sodium nitroprusside (SNP) effect on the delayed carrageenan-induced mouse paw oedema. Sodium nitroprusside (10 μmol/kg, s.c.) or PBS (1 ml/kg, s.c.) was injected after carrageenan at the indicated times. Paw oedema was evaluated 48 h after carrageenan injection. Each bar represents the mean of 8–9 animals and vertical lines the S.E.M. **P*<0.05 compared to the control group (PBS). Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test.

3.2. Involvement of soluble guanylate cyclase and potassium channels in NO inhibitory effect on the delayed phase of carrageenan-induced paw oedema

Treatment of animals with sodium nitroprusside 10 μmol/kg s.c., 8 h after carrageenan resulted in a significant inhibition (~60%) of second phase of paw oedema (Figs. 1C and D and 4A). ODQ, administered 30 min before sodium nitroprusside (and hence 7.5 h after carrageenan), reduced the inhibitory effect of sodium nitroprusside (Fig. 4A). When injected alone (7.5 h after carrageenan), ODQ did not affect the time-course nor the intensity of the paw oedema (161.3±9.8 and 154.5±15.4 μl, without and with ODQ, respectively, n=4, measured 48 h after carrageenan). A similar inhibitory effect was also observed when TEA was injected 30 min before sodium nitroprusside (Fig. 4A). When injected alone (7.5 h after carrageenan), TEA did not change paw oedema (142.3±10.3 and 140.1±13.0 μl, without and with TEA, respectively, n=8, measured 48 h after carrageenan).

3.3. Effects of sodium nitroprusside on dye leakage

The second phase on mouse paw oedema induced by carrageenan was paralleled by plasma exudation, as assessed by Evans Blue leakage (Fig. 4B). Sodium nitroprusside (10 μmol/kg, s.c.) injected 8 h after carrageenan reduced the increase in vascular permeability (Fig. 4B). This effect was also prevented when ODQ was injected 30 min before sodium nitroprusside (Fig. 4B). ODQ by itself did not change Evans blue leakage (11.0±0.55 and 12.3±1.2 μg/ml, without and with ODQ, respectively, n=4, measured 48 h after carrageenan). A similar blocking effect was also observed with TEA (Fig. 4B). TEA alone did not change Evans Blue leakage (13.4±0.58 and 12.6±1.2 μg/ml, without and with TEA, respectively, n=6, measured 48 h after carrageenan).

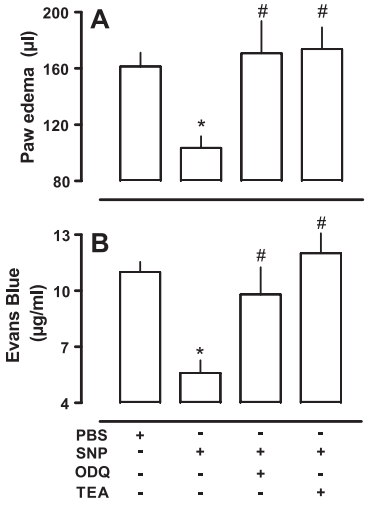

Fig. 4. Effect of 1H-[1,2,4]-oxadiazolo-[4,3-a]quinoxalin-1 (ODQ) and tetraethylammonium (TEA) on sodium nitroprusside (SNP)-induced inhibition of carrageenan delayed mouse paw oedema and Evans blue leakage. Animals were treated with sodium nitroprusside (10 μmol/kg, s.c.) or PBS (1 ml/kg, s.c.) 8 h after intraplantar injection of carrageenan (300 μg/paw). ODQ (a soluble guanylate cyclase inhibitor, 11 μmol/kg, s.c.) or TEA (a nonselective potassium channel blocker; 300 μmol/kg, s.c.) was injected 7.5 h after carrageenan (hence, 30 min before sodium nitroprusside injection; see Methods section). (A) Paw volume was measured at 48 h after carrageenan injection. (B) Evans blue exudation (expressed as μg/ml) was quantified 48 h after carrageenan. Each bar represents the mean of 5–8 animals and vertical lines the S.E.M. *P<0.05 comparing with PBS-treated animals and $^{\#}P$<0.05 comparing with SNP-treated animals. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test.

4. Discussion

The inflammatory reaction induced by injection of carrageenan in mouse paw presents two distinct phases (Henriques et al., 1987). The early phase, which peaks 4 h after the inflammatory stimulus, consists of a low-intensity oedema. This early phase in the mouse is similar to rat paw oedema elicited by carrageenan, which is characterised by the release of histamine, serotonin, bradykinin and production of prostaglandins (Di Rosa and Willoughby, 1971; Di Rosa et al., 1971). Local neutrophil infiltration also contributes to the inflammatory response (Di Rosa and Sorrentino, 1968; Vinegar et al., 1971). However, contrary to the situation prevailing in the rat, mouse carrageenan-induced paw oedema displays a second and delayed phase, which peaks around 48 h after the stimulus, and is characterised by accumulation of macrophages and lymphocytes at the inflammatory site (Henriques et al., 1987).

Because of limited utility of authentic NO gas in many experimental systems and short half-life of NO in vivo, compounds that have the capacity to release NO have been widely used as therapeutic agents and as pharmacological tools to investigate the role of NO in several systems. In a previous work, we have demonstrated that a short-term infusion of NO donors in equivalent dose to that used in the present report caused changes in the vascular reactivity to several vasoactive mediators (Da Silva-Santos and Assreuy, 1999). Additionally, we have demonstrated that similar regimen of treatment with NO donors reduced the early phase of carrageenan-induced paw oedema (Fernandes et al., 2002).

In the present work, we have demonstrated that the prophylactic treatment with NO donors reduced (~50%) both the early and the delayed phase of mouse carrageenan-induced paw oedema. To study whether the reduction of the second phase would be simply a consequence of a smaller early phase, NO donors were injected 8 h after inflammatory stimulus induction, hence after the early peak has reached its nadir. When administered in this way, NO donors reduced (~50%) of the delayed paw oedema. Therefore, NO appears to reduce independently the inflammatory events of both early and delayed phases of mouse carrageenan-induced paw oedema.

This inhibitory effect of NO donors can be ascribed solely to NO because a similar pattern of inhibition was found with sodium nitroprusside, SNAP and glyceril trinitrate, three structurally unrelated NO donors. All of them have a nitrate functionally within the molecule, and a nitroso functional group is present in all of these compounds (for review, see Yamamoto and Bing, 2000). In addition NAP, the nonnitrosylated parent compound of SNAP, was completely devoid of any effect.

When injected 8 or 12 h after carrageenan, sodium nitroprusside was able to reduce the delayed phase of carrageenan-induced paw oedema. However, when injected 16 or 24 h after the inflammatory stimulus, sodium nitroprusside was ineffective in this regard. This result shows that whatever mechanism NO is affecting to interfere with the delayed oedema development, there is a critical window for this action that remains opened until around 12 h after the onset of the inflammatory reaction. In addition, NO targets appear to be equally important for the early and the delayed phases of paw edema.

Despite of NO short half-life (seconds), reports from our laboratory have shown that some of NO effects persist for several hours after NO injection, indicating that these effects are long-lasting (Da Silva-Santos and Assreuy, 1999; Costa and Assreuy, 2002; Fernandes et al., 2002). The results shown here indicate that reduction on delayed phase on carrageenan-induced paw oedema by NO donors does seem to belong to this long-lasting category of effects because a single injection of NO donors 4 h before the inflammatory stimulus was enough to substantially reduce the delayed phase of paw oedema, which takes place 48 h after the inflammatory stimulus.

Guanosine cyclic monophosphate (cGMP) acts as a second messenger of several actions of NO (for review, see Lucas et al., 2000). Our results indicate that guanylate cyclase activation is indeed important for the inhibitory effect of NO on paw oedema because ODQ, a high selective inhibitor of this enzyme (Garthwaite et al., 1995), reduced it. This finding is consistent with other reports showing that NO-induced reduction of the inflammatory response is dependent on cGMP (Kubes, 1993; Fernandes et al., 2002). The exact intracellular mechanism by which cGMP acts for reducing paw oedema remain to be elucidated. Methylene blue, a guanylate cyclase inhibitor structurally distinct from ODQ, also displayed exactly the same profile of action (data not shown).

Vascular effects of NO are known to be, at least in part, mediated through activation of potassium channels (Hamaguchi et al., 1992; Khan et al., 1993; Taniguchi et al., 1993; Archer et al., 1994; Bolotina et al., 1994). Potassium channels have been shown to be involved in the long-lasting actions of NO (Da Silva-Santos and Assreuy, 1999; Costa and Assreuy, 2002; Terluk et al., 2000), including antiinflammatory actions (Fernandes et al., 2002). The results presented here demonstrate that NO inhibition of delayed phase of carrageenan-induced mouse paw oedema involves potassium channels because TEA reduced the NO donor inhibitory effect. Thus, in agreement with other reports, it seems that potassium channels are indeed important intermediary elements for some NO effects, especially when these effects are long-lasting. NO has been shown to activate potassium channels either directly (possibly by sulphydryl nitrosylation; Bolotina et al., 1994) or via cGMP (possibly by channel phosphorylation through cGMP-activated kinases; Taniguchi et al., 1993; Archer et al., 1994). However, we do not yet have evidence for which mechanism would be relevant in our model.

The delayed phase of oedema was paralleled by an important increase in vascular permeability. Our results indicate that at least part of the NO-induced reduction in carrageenan mouse paw oedema may result from its inhibitory effect on vascular fluid leakage. Additionally, NO-induced reduction on vascular permeability is dependent on both guanylate cyclase and potassium channels. This view is further supported by several experimental studies showing that inhalation of NO gas upon reperfusion attenuate microvascular leakage (Barbotin-Larrieu et al., 1996; Chetham et al., 1997).

In summary, our results show that a brief exposure to NO donors several hours before carrageenan injection in the mouse paw causes an important reduction of the delayed phase of oedema. This NO inhibitory effect appears to be dependent on cGMP and potassium channels. Whether the mouse delayed paw edema is a model for chronic inflammation is still an open issue. Notwithstanding, our results indicate that NO can modify an inflammatory reaction after its installation or alternatively, it can change a delayed response if administered in the proper window of opportunity. A better understanding of the relationship among NO, potassium channels, guanylate cyclase and inflammation may, in the future, lead to development of improved strategies for the clinical management of chronic inflammatory disorders.

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