European Journal of Pharmacology, 212 (1992) 93–96

EJP 21005

Short communication

Effect of endotoxin on circulating cyclic GMP in the rat

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Received 31 December 1991, accepted 7 January 1992

The aim of this study was to investigate the possible use of plasmatic cyclic GMP as an index of L-arginine/nitric oxide (L-Arg/NO) pathway activation by *E. coli* endotoxin in vivo. Endotoxin (20 mg kg^{-1} i.p.) caused a time-dependent increase in plasmatic cyclic GMP in anaesthetised rats which corresponded with the time course of L-Arg/NO pathway activation in aortas from the same rats, but was not prevented by a specific inhibitor of this pathway, N^G-nitro-L-arginine methyl ester (1 mg kg^{-1} or 20 mg kg^{-1} h^{-1} i.v.). Elevated plasmatic cyclic GMP was however also associated with an increased plasma concentration of atrial natriuretic peptide (ANP) in endotoxin-treated rats. We conclude that plasma cyclic GMP cannot be used as a direct marker of L-Arg/NO pathway activation by endotoxin but may instead be a reflection of an endotoxin-induced increase in plasma ANP activity.

Endotoxin; cGMP; L-Arginine/nitric oxide pathway; ANP (atrial natriuretic polypeptide)

1. Introduction

Recent studies have linked activation of a pathway producing nitric oxide (NO) from the amino acid L-arginine (L-Arg) to endotoxin-induced hypotension (Thiemermann and Vane, 1990) and vascular hyporesponsiveness to constrictor agents (Julou-Schaeffer et al., 1990) suggesting that this pathway may play an important role in the development of septic shock.

In a previous study we showed that stimulation of soluble guanylate cyclase and the resultant formation of tissular cyclic GMP, can be used as an index of L-Arg/NO pathway activation by endotoxin in rat aorta (Fleming et al., 1991). Since extrusion of cyclic GMP plays a role in regulation of cellular cyclic GMP content (Schini et al., 1989) the present study aimed to determine if plasmatic cyclic GMP could serve as an early marker of L-Arg/NO pathway activation by endotoxin in vivo. The concentration of cyclic GMP in the plasma and the effect of N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NO formation from L-Arg, were measured at various times after injection of *E. coli* endotoxin. This was compared to the time course of L-Arg/NO pathway induction in endothelium-denuded aortas from the same rats, as assessed by the appearance of relaxant responses to L-Arg (Julou-Schaeffer et al., 1990) and by measurement of tissue cyclic GMP content (Fleming et al., 1991).

NO increases cyclic GMP by stimulation of soluble guanylate cyclase, but cyclic GMP may also be produced via stimulation of particulate guanylate cyclase, which is the principle cellular target of atrial natriuretic peptide (ANP, Winquist et al., 1984). For this reason the effect of endotoxin on the plasmatic concentration of ANP was also studied.

2. Materials and methods

2.1 Animal preparation and blood sampling

Endotoxin (*E. coli* 055:B5, Difco, 20 mg kg^{-1} in 0.1 ml 100 g^{-1} 0.9% saline) or its vehicle were administered to male Wistar rats (10–13 weeks) by i.p. injection. At varying times from 1 to 4 h following injection the rats were anaesthetised (sodium pentobarbital, 60 mg kg^{-1}) and a cannula (containing heparin, Sigma, 150 I.U. ml^{-1} in saline) placed in the jugular vein for removal of blood samples. Blood samples (0.5 ml for cyclic GMP and phosphodiesterase, 3 ml for ANP) were taken into syringes containing ice cold EDTA (1%), heparin (200 I.U. ml^{-1}) or aprotinin (24000 I.U. ml^{-1}) for the measurement of cyclic GMP, cyclic GMP phosphodiesterase and ANP respectively. The blood

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was immediately centrifuged to separate the plasma, which was stored frozen at −20°C until required for assays.

2.2. Assay of cyclic GMP, ANP and cyclic GMP phosphodiesterase

Plasma cyclic GMP and ANP were measured by radioimmunoassay (kits from Immunotech and Amersham). Cyclic GMP phosphodiesterase activity was determined by the method of Wells et al. (1975) using a substrate concentration of 1 μM [^{3}H]cyclic GMP. Incubation time and enzyme concentration were adjusted so that no more than 15% of the substrate was hydrolysed. Phosphodiesterase activity is expressed in μmol of cyclic GMP hydrolysed in 30 min by 1 l of plasma.

2.3. Determination of the relaxant response to L-Arg

After removal of blood samples, the animals were killed and the thoracic aortas removed. The aortae were washed, rubbed to remove their endothelium and placed under 2 g tension in organ baths containing 10 ml Krebs solution as previously described (Julou-Schaeffer et al., 1990). L-Arg (Calbiochem, 1 mM) was added to arteries precontracted with 1 μM noradrenaline (NA, Sigma). Since contractions produced by NA did not significantly differ between groups, L-Arg induced relaxation is expressed as a % of NA-induced contraction. Methylene blue (MeB, 10 μM), an inhibitor of soluble guanylate cyclase was added to each bath at the end of the experiment.

2.4. Studies with L-NAME

L-NAME (Sigma), in 0.9% saline, was administered i.v. either as a bolus of 1 mg kg^{-1} or over 2 h as an infusion of 20 mg kg^{-1} h^{-1}, to rats anaesthetised 3 h after i.p. injection of endotoxin or saline. Blood samples for determination of cyclic GMP were removed before L-NAME, at 10 min after the bolus dose and at various times during the infusion (see Results). Thoracic aortas were removed from some rats after completion of infusion of L-NAME or its solvent, washed and stored at −20°C until determination of cyclic GMP content (Fleming et al., 1991).

2.5. Statistical analysis

The results are expressed as the means ± S.E.M. Results were compared using analysis of variance (ANOVA). Where ANOVA showed significant differences ($P < 0.05$) the results were further analysed using an a posteriori Student–Newman–Keuls test.

3. Results

The cyclic GMP content of venous plasma was significantly increased (from 9.3 ± 0.9 to 14.6 ± 1.4 nM, $P < 0.05$, $n = 6$) within 1 h of endotoxin (fig. 1, upper, 20 mg kg^{-1} i.p.) administration and continued to increase until 3 h post injection to 64.1 ± 4.0 nM, in endotoxin treated ($n = 8$), compared to 7.2 ± 0.3 nM in control rats at the same time ($P < 0.001$, $n = 8$). This was despite a concomitant rise in the plasma cyclic GMP phosphodiesterase activity (from 2.6 ± 0.5 to 5.1 ± 0.7 μM cyclic GMP hydrolysed per 30 min per l of plasma, $P < 0.05$) in endotoxin-treated rats. In control rats the cyclic GMP phosphodiesterase activity 3 h after saline injection was 2.6 ± 0.4 μM cyclic GMP hydrolysed per 30 min per l of plasma.

L-Arg induced a significant relaxation in aortas precontracted with NA from endotoxin-treated (fig. 1, lower) but not from control rats (not shown). The relaxations were 16.2 ± 4.8% ($n = 6$) in aortas removed 1 h after endotoxin and increased in a time-dependent manner to 30.8 ± 5.2% ($n = 9$) in aortas removed 4 h after endotoxin. In all experiments tension was returned to control level by subsequent addition of MeB (10 μM). The cyclic GMP content of aortas removed 3 h after endotoxin was significantly higher (9.02 ± 0.61 fmol/μg DNA) than that of aortas from control rats (3.17 ± 0.59 fmol/μg DNA, $P < 0.01$, $n = 6$ per group).

The increase in plasma cyclic GMP at 3 h after endotoxin was not reduced 10 or 20 min after adminis-

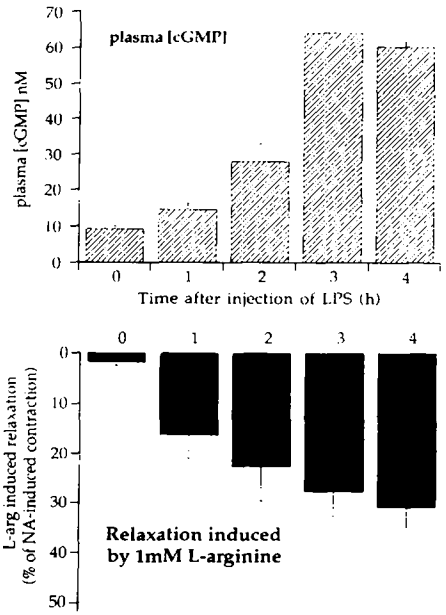

Fig. 1. Cyclic GMP concentration in the plasma (*upper*) and relaxation induced by 1 mM L-Arg in NA (1 μM)-precontracted aortas (*lower*) taken from the same rats, before and at hourly intervals after injection of endotoxin (20 mg kg^{-1} i.p.), * $P < 0.01$ vs. time 0, $n = 8$. No significant change in either parameter was seen in control rats studied over the same period (see text).

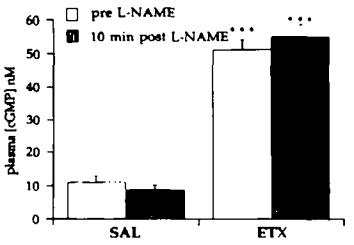

Fig. 2. The effect of L-NAME (1 mg kg^{-1}) on the plasma cyclic GMP concentration in endotoxin (ETX)- and saline (SAL)-treated rats (n = 6). Samples were taken from rats anaesthetised 3 h after i.p. injection of endotoxin (20 mg kg^{-1}) or SAL. *** $P < 0.01$ vs. control.

tration of L-NAME (1 mg kg^{-1}, fig. 2). Infusion of L-NAME (20 mg kg^{-1} h^{-1}) over 2 h, when compared to solvent infusion over the same period, also failed to significantly modify plasmatic cyclic GMP in endotoxin-treated rats (not shown). Neither protocol for L-NAME administration significantly altered plasmatic cyclic GMP in control rats. The cyclic GMP content of aortas from endotoxin-treated rats (4.24 ± 1.28 fmol/μg DNA, n = 4) was not significantly increased over that of controls (5.12 ± 1.06 fmol/μg DNA, n = 4) after infusion of L-NAME over 2 h.

In a separate study endotoxin evoked an increase in the circulating concentration of ANP (134.5 ± 11.8 pg ml^{-1} in endotoxin treated vs. 66.8 ± 6.4 pg ml^{-1} in control, $P < 0.001$). This was associated with a concomitant rise in the plasma cyclic GMP concentration (57.1 ± 3.7 and 18.6 ± 4.3 nM 3 h after endotoxin or saline administration respectively).

4. Discussion

The principle finding of the present study was that endotoxin evokes a time-dependent increase in circulating cyclic GMP in anaesthetised rats. This occurred despite a concomitant increase in plasma phosphodiesterase activity, possibly derived from platelets aggregating in response to endotoxin. The origin of cyclic GMP in the plasma is unknown but it is likely to be derived from cells which extrude it while regulating their intracellular cyclic GMP concentration (Schini et al., 1989). Following endotoxin administration, vascular tissue cyclic GMP content is increased secondary to activation of the pathway producing NO from L-Arg (Fleming et al., 1991). The hypothesis tested in the present study was that the increase in plasmatic cyclic GMP could be a reflection of tissular cyclic GMP and thus be a useful clinical indicator of L-Arg/NO pathway activation in septicaemia.

Consistent with L-Arg/NO/cyclic GMP pathway activation (Julou-Schaeffer et al., 1990) L-Arg induced a relaxation of precontracted aortas from endotoxin-treated rats which was reversible by the soluble guanylate cyclase inhibitor methylene blue. Sensitivity to L-Arg increased as a function of time after endotoxin administration likely due to induction of the NO synthase enzyme in the vessel wall (Knowles et al., 1990). Moreover, this was paralleled by an increased tissue cyclic GMP content and a time-dependent augmentation of cyclic GMP content in plasma from the same rats. However despite this similarity, increases in plasma cyclic GMP were not modified by L-NAME either when given in a bolus dose which reverses endotoxin-induced hyporeactivity to noradrenaline in vivo (Gray et al., 1991) or when given as a continous infusion over 2 h. The latter excludes the possibility that plasmatic cyclic GMP was maintained, after L-NAME bolus administration, due to liberation of preformed NO or extrusion of preformed cyclic GMP. The increase in tissue cyclic GMP associated with endotoxin administration was succesfully suppressed in rats receiving an infusion of L-NAME.

It has previously been reported that plasma cyclic GMP may be a marker of the biological activity of ANP (Hamet et al., 1986) which stimulates particulate guanylate cyclase (Winquist et al., 1984). Here we show that plasmatic ANP is increased after endotoxin administration. Furthermore, in a small number of experiments (not shown) continued infusion of endotoxin further increased both ANP and cyclic GMP. Thus it is possible that endotoxin increased cyclic GMP via ANP, either by stimulating the release or reducing the clearance of the peptide. Confirmation of the role of ANP would require further experiments using specific antibodies directed against ANP since no specific antagonists are currently available.

In conclusion, the results show that endotoxin caused a time-dependent increase in the activity of the L-Arg/NO pathway but do not support the hypothesis that plasmatic cyclic GMP might represent a direct index of this activity. They do however show that endotoxin caused an increase in circulating ANP and they suggest that this effect could participate in endotoxin-induced elevations in plasmatic cyclic GMP.

Acknowledgements

The authors would like to thank Mme Brandenberger, Laboratoire de Physiologie et de Psychologie Environmentales du CNRS, Strasbourg for determination of plasmatic ANP concentrations and Dr. Claire Lugnier for advice on the measurement of phosphodiesterase activity. This work was supported by a grant (ST2J-0457-6) from the Commission of the European Communities.

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