Evidence that an L-arginine/nitric oxide dependent elevation of tissue cyclic GMP content is involved in depression of vascular reactivity by endotoxin

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1 The aim of this investigation was to study the relationship between contractile responsiveness, activation of the L-arginine pathway and tissue levels of guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) in aortic rings removed from rats 4 h after intraperitoneal administration of bacterial endotoxin (*E.coli* lipopolysaccharide, LPS, 20 mg kg⁻¹).

2 LPS-treatment resulted in a reduction of the sensitivity and maximal contractile response to nor-adrenaline (NA).

3 Depression of the maximal contractile response was restored to control by 6-anilo-5,8-quinolinedione (LY 83583, 10 µM), which prevents activation of soluble guanylate cyclase.

4 Cyclic GMP levels in tissue from LPS-treated rats were 2 fold greater than cyclic GMP levels detected in tissue from control (saline-treated) rats. The LPS-induced increase in cyclic GMP content was observed both in the presence and absence of functional endothelium.

5 Addition of L-arginine (1 mM) to maximally contracted aortic rings produced significant relaxation of rings from LPS-treated rats but not rings from control animals. In the LPS-treated group, addition of L-arginine was also associated with a significant increase in cyclic GMP content. L-Arginine had no effect on the cyclic GMP content of control rings. D-Arginine (1 mM) was without effect.

6 In rings from LPS-treated rats, N^G-nitro-L-arginine methyl ester (L-NAME, 300 µM), an inhibitor of nitric oxide (NO) production, increased the contractile response to NA and prevented the LPS-induced increase in cyclic GMP content. In control rings, L-NAME increased the NA sensitivity only when the endothelium remained intact and reduced the cyclic GMP content of these rings to that of control endothelium-denuded rings.

7 These results demonstrate that LPS-induced hyporeactivity to NA occurs secondarily to activation of the L-arginine pathway and subsequent activation of soluble guanylate cyclase in vascular tissue. In addition they suggest that LPS induces the production of an NO-like relaxing factor in non-endothelial cells.

Keywords: Nitric oxide; cyclic GMP; N^G-nitro-L-arginine methyl ester (L-NAME); endotoxin (LPS); isolated arteries

Introduction

Administration of *E.coli* lipopolysaccharide (LPS) to rats causes hyporesponsiveness to several contractile agents such as noradrenaline (NA), vasopressin and angiotensin II (Fink *et al.*, 1985; Schaller *et al.*, 1985; Wakabayashi *et al.*, 1987) which persists *ex vivo* in tissue removed from rats treated with LPS.

Recently an important role in determining vascular function and reactivity has been attributed to nitric oxide (NO) formed in endothelial cells by oxidation of the amino acid L-arginine (Palmer *et al.*, 1988a). Modification of vascular reactivity by endothelium-derived NO is based on an increase in the guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) content of smooth muscle (Holzmann, 1982; Rapoport & Murad, 1983) produced by direct stimulation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986). The exact mechanisms by which cyclic GMP evokes smooth muscle relaxation are not entirely clear but increased smooth muscle cyclic GMP content is associated with reduced intracellular calcium levels (Kai *et al.*, 1987) and dephosphorylation of myosin light chain kinase (Draznin *et al.*, 1986).

Recent results from our laboratory have suggested that LPS-induced vascular hyporeactivity results from activation of an L-arginine-dependent pathway producing NO or an NO-like relaxing factor in non-endothelial cells (Fleming *et al.*, 1990a; Julou-Schaeffer *et al.*, 1990). The present study further investigates this hypothesis by the use of L-arginine, a

substrate for the production of NO (Palmer *et al.*, 1988a), and N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis (Hobbs & Gibson, 1990). The effects of these substances on contractile responsiveness to NA and tissue cyclic GMP content were compared in parallel. In addition NO-induced activation of soluble guanylate cyclase was prevented with 6-anilo-5,8-quinolinedione (LY 83583, Mulsch *et al.*, 1988).

A preliminary account of these results has been presented to the International Congress of Pharmacology (Fleming *et al.*, 1990b).

Methods

Preparation of tissue

Male Wistar rats (12 to 15 weeks, 250–300 g) were injected intraperitoneally with either LPS (20 mg kg⁻¹ in 0.15 ml 100 g⁻¹ saline) or saline. After 4 h the rats were killed by stunning and cervical dislocation. Thoracic aortae were removed, cleared of adherent connective tissue and cut into rings of approximately 2 mm in length for contractile studies or divided into five large rings, four of which were used for determination of cyclic GMP. In the latter case, 2 mm rings cut from the remaining aortic segment were also used to assess contractile responsiveness to NA and the role of the L-arginine pathway. In some rings the vascular endothelium was mechanically removed by rubbing gently with blunt forceps.

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Contraction studies

Rings were mounted, under 2 g tension, in organ baths containing physiological salt solution (PSS) of the following composition (mm): NaCl 118.4, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.3, KH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.7, at 37°C and bubbled with 95% O₂: 5% CO₂ (pH 7.4). All experiments were carried out in the presence of indomethacin (10 µM). Developed tension was measured with an isometric force transducer (Celaster, Celle l'Evescault, Lusignan, France) and visualized by computer. After an equilibration period of 90 min, during which time the PSS was changed at 15 min intervals, the basal tension was re-adjusted to 2 g. The presence of functional endothelium was verified by addition of acetylcholine (ACh, 1 µM) in arteries precontracted with noradrenaline (NA, 1 µM). The ability of ACh to induce relaxation of unrubbed rings was taken as an indication of the presence of functional endothelium. The use of this criterion has previously been verified in tissues precontracted with submaximal concentrations of NA (Julou-Schaeffer *et al.*, 1990). After a washing period of 60 min contractile responses were monitored during the stepwise cumulative addition of NA (1 nM to 10 µM). To investigate the effect of NO-induced stimulation of soluble guanylate cyclase, LY 83583 (10 µM) or solvent was added to tissues 3 to 5 min after a stable maximum response to NA was obtained and the contractile response monitored over a further 15 min. To study the effect of inhibition of NO synthesis from L-arginine, tissues were incubated with either L-NAME (300 µM) or its solvent (water, 100 µl) for 10 min prior to the cumulative addition of NA. When the stable maximum response was obtained, either L- or D-arginine (1 mM) or solvent (water, 100 µl) was added to each bath. Rings were removed, blotted dry, placed in a dessicator overnight and then weighed. Results are expressed as tension developed (g) per mg dried tissue.

Determination of tissue cyclic GMP content

Aortic segments were incubated in PSS at 37°C and oxygenated with a gas mixture of 95% O₂ and 5% CO₂. After a 90 min period during which the PSS was changed at 15 min intervals the tissues were either rapidly frozen in an aluminium clamp pre-cooled in liquid nitrogen (non-stimulated) or treated as described for contractile studies and frozen following cumulative addition of NA (NA-stimulated). Aortic segments were thawed in 400 µl of perchloric acid (1N), homogenized with a Potter glass/glass homogenizer for 30 s followed by sonication (Ultrason-Annemasse, Type 75TS, France) for 15 s. Following centrifugation at 10,000 *g* for 5 min, cyclic GMP content of the supernatant was determined by radioimmunoassay (Immunotech S.A.). DNA content was measured as described previously (Brunk *et al.*, 1976). Cyclic GMP content was expressed as fmol µg⁻¹ DNA.

Since the cyclic GMP content of control, endothelium-intact, tissues varied substantially between experiments, each experiment was performed with its own internal controls.

Drugs

Noradrenaline bitartrate (Sigma), was stored as a 1 µM stock solution in buffer containing Na₂SO₃ 7.9 mM and HCl 34 mM and diluted as required. Acetylcholine chloride (Sigma), was stored as a 10 mM stock solution in NaH₂PO₄ buffer (pH 4) and diluted as required. All other substances were freshly prepared prior to each experiment. N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), L-arginine hydrochloride (Calbiochem) and D-arginine hydrochloride (Sigma) were dissolved in deionised water, 6-anilo-5,8-quinolinedione (LY 83583, Lilly) was prepared as a 10 mM solution in dimethylsulphoxide (DMSO) and diluted in water, indomethacin (Sigma) was dissolved in 4% NaHCO₃ solution and LPS (*E. coli* 055 : B5, Difco) was dissolved in saline (0.9% NaCl).

Parameters and statistical analysis

The concentrations of agonist causing half-maximal contraction (EC₅₀) were calculated by logit-log regression and are expressed as pD₂ values (−log EC₅₀). pD₂ values, maximal contractile responses and changes in tension induced by L- or D-arginine (expressed as a percentage of the maximal contractile response to NA) were compared by Student's *t* test for unpaired data. Statistical comparisons of cyclic GMP content were made by analysis of variance (ANOVA). If significant differences were detected by ANOVA, individual means were compared with an *a posteriori* Student-Newman-Keuls test. *P* values of less than 0.05 were considered significant. Results are expressed throughout as the mean ± standard error of the mean (s.e.mean) from *n* experiments.

Results

Effect of LPS-treatment on aortic ring contractility

Compared with the response of tissues from control animals, LPS-treatment induced a rightward shift of the concentration-response curve to NA represented by a decrease in the pD₂ values (Table 1). LPS-treatment also induced a reduction of the maximal contractile response (by approximately 35%, *P* < 0.05). In rings from control rats, contractile responsiveness was modified by the presence of functional endothelium (Table 1). In contrast, contractile responsiveness in rings from LPS-treated rats was not altered by removal of the endothelium (Table 1). This is in agreement with results obtained previously (Julou-Schaeffer *et al.*, 1990).

Effect of 6-anilo-5,8-quinolinedione

LY 83583 (10 µM), which destroys NO and prevents activation of soluble guanylate cyclase (Mulsch *et al.*, 1988), caused a significant increase in recorded tension of rings from control animals only when the endothelium remained intact (the maximum contractile response being 3.71 ± 0.44 before and 4.48 ± 0.30 g mg⁻¹ tension, NS, after LY 83583 in the absence of endothelium and 3.07 ± 0.39 before and 4.42 ± 0.30 g mg⁻¹ tension, *P* < 0.05, after LY 83583 in the presence of endothelium, Figure 1).

In aortic rings from rats given LPS, LY 83583 increased the contractile response to NA towards that of control tissues both in the absence and presence of endothelium (the

Table 1 Effects of *E. coli* lipopolysaccharide (LPS)-treatment and N^G-nitro-L-arginine methyl ester (L-NAME) on contractile responses to noradrenaline

		Maximum contractile response	
Treatment		(g mg ⁻¹ dried tissue)	pD ₂
Control + E	Solvent	3.20 ± 0.28	7.34 ± 0.16
Control − E	Solvent	2.79 ± 0.18	8.04 ± 0.08*
Control + E	L-NAME	4.07 ± 0.43††	8.19 ± 0.11††
Control − E	L-NAME	3.44 ± 0.52	8.62 ± 0.17
LPS-treated + E	Solvent	2.05 ± 0.30†	6.85 ± 0.16†
LPS-treated − E	Solvent	2.24 ± 0.21††	7.20 ± 0.16††
LPS-treated + E	L-NAME	4.45 ± 0.45	7.89 ± 0.13
LPS-treated − E	L-NAME	4.49 ± 0.45	8.27 ± 0.13

Representation of maximal contractile responses and pD₂ values obtained to noradrenaline (1 nM to 10 µM) in aortic rings with (+ E) and without (− E) endothelium, from control and LPS-treated rats, in the presence or absence of L-NAME (300 µM). Results are presented as mean ± s.e.mean, *n* = 9–12. * Indicates a significant difference from the absence of endothelium (*P* < 0.05). † Indicates a significant difference from respective control (saline-treated) groups not treated with LPS or L-NAME: † *P* < 0.05; †† *P* < 0.01.

Figure 1 Histogram depicting the effect of 6-anilo-5,8-quinolinedione ($10\mu\text{M}$, hatched columns) in aortic rings, with and without endothelium, from control (open columns) and *E.coli* lipopolysaccharide (LPS) treated rats (solid columns) maximally contracted with noradrenaline ($10\mu\text{M}$). Results are presented as mean of $n = 9$, vertical lines show s.e.mean. Significant difference between groups: * $P < 0.05$; ** $P < 0.01$; NS, not significant.

maximum contractile response being increased from 1.51 ± 0.20 to $4.12 \pm 0.29\text{ g mg}^{-1}$ tension, $P < 0.0001$, in the presence of endothelium and from 2.04 ± 0.37 to $3.36 \pm 0.21\text{ g mg}^{-1}$ tension, $P < 0.005$, in the absence of endothelium, Figure 1).

Similar results were obtained when activation of soluble guanylate cyclase was inhibited with methylene blue (Fleming *et al.*, 1990a; Julou-Schaeffer *et al.*, 1990).

Effect of LPS-treatment on cyclic GMP content

Administration of LPS to rats was associated with a 2 fold ($P < 0.005$) increase in the cyclic GMP content of endothelium intact, NA-stimulated aortic tissue (Figure 2). In control tissues the level of cyclic GMP was modified by the presence of endothelium, detected levels of cyclic GMP being 9 fold greater ($P < 0.001$) in the presence compared with the absence of endothelium. In contrast, removal of the endothelium in rings from LPS-treated rats did not significantly alter cyclic GMP content (Figure 2).

Contraction studies on the fifth segment of aortae in which cyclic GMP was determined, verified that LPS-treatment resulted in a decrease in the maximal contractile response to NA, maximal contractile responses being, $2.97 \pm 0.31\text{ g mg}^{-1}$ tension in control and $1.49 \pm 0.23\text{ g mg}^{-1}$ tension in the LPS-treated group in the presence of endothelium ($n = 4-5$) and $3.81 \pm 0.27\text{ g mg}^{-1}$ tension in control and $1.76 \pm 0.17\text{ g mg}^{-1}$ tension in the LPS-treated group in the absence of functional endothelium ($n = 4-5$).

LPS-treatment produced similar results in aortic rings in the absence of any stimulation by NA (not shown).

Role of the L-arginine pathway

Effect of L- and D-arginine In NA-stimulated tissues from control and LPS-treated rats, neither the presence of solvent nor D-arginine had any significant effect on the contractile response (Figure 3) or tissue levels of cyclic GMP (Figure 4).

Figure 2 Histogram showing the cyclic GMP content in aortic rings maximally contracted with noradrenaline ($10\mu\text{M}$), from control and *E.coli* lipopolysaccharide (LPS)-treated rats, in the presence (open columns) and absence (solid columns) of functional endothelium. Results are presented as mean of $n = 6$; vertical lines show s.e.mean.

Addition of L-arginine did not cause relaxation or increase cyclic GMP levels in control tissue. However, in rings from LPS-treated rats, L-arginine produced significant relaxation ($72 \pm 7\%$, $P < 0.005$ in the presence and $72 \pm 10\%$, $P < 0.005$ in the absence of endothelium, Figure 3) and caused a 5 to 6

Figure 3 Comparative effects of solvent (hatched columns), D-arginine (1 mM, crosshatched columns) and L-arginine (1 mM, solid columns) on the contractile response of aortic rings from control and *E.coli* polysaccharide (LPS)-treated rats maximally contracted with noradrenaline ($10\mu\text{M}$, open columns) (a) in the presence and (b) in the absence of endothelium. Results are presented as mean of $n = 6$; vertical lines show s.e.mean. Significant difference between groups: *** $P < 0.005$; NS, not significant.

Figure 4 Histogram depicting the cyclic GMP content in aortic rings, (a) with and (b) without endothelium, from control and *E. coli* lipopolysaccharide (LPS)-treated rats maximally contracted with noradrenaline ($10 \mu\text{M}$) in the presence of either solvent (hatched columns), D-arginine (1 mM , crosshatched columns) or L-arginine (1 mM , solid columns). Results are presented as mean of $n = 6$; vertical lines show s.e.mean.

fold increase ($P < 0.005$) in the cyclic GMP content (from 33.4 ± 8.9 and $26.0 \pm 8.2 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$ to 201.5 ± 41.0 and $141.2 \pm 17.6 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$ in the presence and absence of endothelium respectively, Figure 4).

Effect of L-NAME Inhibition of NO production with L-NAME increased the NA-sensitivity and maximum contraction of aortic rings from control animals only when the endothelium remained intact (Table 1). This is consistent with a

Figure 5 Histogram depicting the cyclic GMP content in aortic rings, with and without endothelium, from control and *E. coli* lipopolysaccharide (LPS)-treated rats maximally contracted with noradrenaline ($10 \mu\text{M}$) in the presence (solid columns) and absence (open columns) of N^G -nitro-L-arginine methyl ester ($300 \mu\text{M}$). Results are presented as mean of $n = 5$; vertical lines show s.e.mean.

basal production of NO by endothelial cells. In the presence of L-NAME, addition of either L- or D-arginine (1 mM) to arteries maximally contracted with NA was without significant effect on contractile tension (not shown). In control tissues L-NAME ($300 \mu\text{M}$, Figure 5) reduced the cyclic GMP content of endothelium intact rings to that of endothelium-denuded rings (from $26.4 \pm 9.65 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$ to $3.28 \pm 0.12 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$, $P < 0.001$).

In tissues from LPS-treated rats, L-NAME ($300 \mu\text{M}$, Table 1) restored NA-sensitivity and maximal contractile responses to control levels. Addition of L-arginine (1 mM) in the presence of L-NAME induced a relaxation in tissues maximally contracted with NA (by $46 \pm 7\%$ in the presence and $60 \pm 10\%$ in the absence of endothelium respectively, $P < 0.01$). D-Arginine failed to produce any relaxation (not shown). L-NAME also abolished LPS-induced increases in cyclic GMP content (cyclic GMP content being reduced from 136.6 ± 27.5 and $109.4 \pm 28.2 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$ to 3.51 ± 0.67 and $5.23 \pm 0.73 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$, in the presence and absence of endothelium, respectively, $P < 0.001$, Figure 5).

Discussion

The present study demonstrates a reduced responsiveness to NA in aortic rings removed from LPS-treated rats. This is in agreement with previously published results (Wakabayashi *et al.*, 1987; Bigaud *et al.*, 1990).

Modification of vascular reactivity by endothelium-derived NO is based on an increase in the cyclic GMP content of smooth muscle (Rapoport & Murad, 1983) produced by direct stimulation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986; Mulsch *et al.*, 1987). In the present study LY 83583, which at the concentration used has been reported to destroy NO and inhibit soluble guanylate cyclase (Mulsch *et al.*, 1988; 1989a), increased contractile tension in aortic tissue from control rats only when the endothelium remained intact. This result is consistent with activation of soluble guanylate cyclase by NO basally released from the endothelium. In contrast, in aortic rings from LPS-treated rats, restoration of contractile responsiveness by LY 83583 was independent of the presence of functional endothelium. The hypothesis that soluble guanylate cyclase was activated following administration of LPS is supported by direct measurement of tissue cyclic GMP content. In endothelium-intact aortic rings from LPS-treated rats the cyclic GMP content was 2 fold greater than that of control rings.

In both non-stimulated and NA-stimulated rings from control rats, the presence of a functional endothelium was associated with a 4 to 5 fold difference in tissue cyclic GMP content. In contrast, the cyclic GMP content of tissue from LPS-treated rats was elevated to comparable levels in both the presence and absence of endothelium. Taken together, the cyclic GMP measurements and results obtained with LY 83583 indicate that, activation of soluble guanylate cyclase by a substance of non-endothelial origin precedes induction of vascular hyporeactivity by LPS.

In accordance with the results presented here, McKenna (1988) reported that inhibition of endogenous cyclic GMP production by methylene blue improved responses of aortae from septic rats to both NA and KCl. However, an elevated vascular content of cyclic GMP was not associated with this observation. The reasons for this contradictory finding are not clear but may reflect a difference between models of endotoxic and septic shock.

Since NO is known to activate soluble guanylate cyclase in smooth muscle (Arnold *et al.*, 1977) the role of the oxidative L-arginine/NO pathway in induction of the LPS-induced hyporeactivity and elevation of cyclic GMP was investigated. L-Arginine, substrate for the production of NO (Palmer *et al.*, 1988a), induced relaxation and a 5 to 6 fold increase of cyclic GMP in rings from LPS-treated but not control animals. The stereospecific nature of the pathway was demonstrated by the

lack of response to D-arginine. These observations indicate that LPS-treatment induces activation of the L-arginine pathway in vascular tissue. Continued activation of this pathway in vascular tissue *ex vivo* would result in depletion of endogenous substrate and account for the relaxation and elevated cyclic GMP observed upon exogenous addition of L-arginine. L-NAME, an inhibitor of NO biosynthesis, produced effects on control tissue that are consistent with basal production of NO by the endothelium and restored the contractile responsiveness of LPS-treated rings to control. The effect of L-NAME was reversed by L- but not D-arginine. Restoration of vascular responsiveness was concomitant with a reduction of cyclic GMP to a level comparable with that of endothelium-denuded control tissue. Thus the restoration of vascular reactivity in tissue from LPS-treated animals produced by inhibition of the L-arginine pathway, was associated with a decrease in the cyclic GMP content.

The L-arginine pathway exhibits a stereospecific requirement for L-arginine (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988), is inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (Rees *et al.*, 1989) or L-NAME (Hobbs & Gibson, 1990) and usually acts as a transduction mechanism for stimulation of soluble guanylate cyclase (Ignarro *et al.*, 1986; Mulsch *et al.*, 1987; Moncada *et al.*, 1989). Using these criteria the presence of the L-arginine pathway has been demonstrated in a number of tissues that may be activated by LPS namely, mast cells (Salvemini *et al.*, 1990a), platelets (Radomski *et al.*, 1990), neutrophils (Rimele *et al.*, 1988), macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Stuehr *et al.*, 1989) and vascular endothelium (Mulsch *et al.*, 1989b; Mayer *et al.*, 1989; Salvemini *et al.*, 1989; 1990b). Within vascular tissue removed from LPS-treated rats the cellular source of NO is unlikely to be the endothelium since the L-arginine-dependent NO producing pathway was activated in endothelium-denuded

tissue from LPS-treated rats. The efficiency of our technique of endothelium removal was demonstrated in aortic tissue from control animals both by the low level of cyclic GMP detected and by lack of responsiveness to acetylcholine.

We and others, have recently reported that incubation of endothelium-denuded rat aortae with LPS results in a hypo-reactivity to NA, identical to that described in vessels from LPS-treated rats and is associated with an increased tissue content of cyclic GMP (Fleming *et al.*, 1990a; Beasley, 1990). This observation would tend to exclude as the NO source all cell types not indigenous to vascular tissue, with the exception of blood constituents that remain attached to or which infiltrate the vessel wall on removal from the rat. Resident populations of lymphocytes and macrophages have been described within the aortic adventitia (Rhodin, 1980) and intima (Freudenberg & Riese, 1976) of untreated rats. Therefore, although the number of these cells in vascular tissue may be depleted during the endothelium denuding process, they remain possible sources of NO production. Additionally, the possibility cannot be excluded that cells such as fibroblasts or smooth muscle can be activated by LPS to produce NO. Indeed, results obtained from endothelium-denuded arterial tissue have recently been proposed as evidence for the existence of an NO-like vascular smooth muscle-derived relaxing factor (Wood *et al.*, 1990).

The results of this investigation indicate that within endothelium-denuded vascular tissue there exists an LPS-sensitive or inducible enzyme that converts L-arginine to a metabolite which activates soluble guanylate cyclase. This metabolite exhibits the characteristic features of NO generated by the oxidative L-arginine pathway.

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