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# Abnormal Activation of $K^+$ Channels Underlies Relaxation to Bacterial Lipopolysaccharide in Rat Aorta

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We have examined the role of $K^+$ channels in mediating vasorelaxation produced by bacterial lipopolysaccharide (LPS) in endothelial-denuded strips of rat aorta precontracted with phenylephrine (1 $\mu$M). *Salmonella typhosa* LPS (0.1 $\mu$g/ml) caused significant relaxation of tension which peaked at ~4hr. The $K^+$ channel blocker, tetraethylammonium chloride (TEA; 10 mM), fully reversed these relaxations whether applied before or after long term exposure to LPS. L-arginine, the substrate for nitric oxide synthase, caused large relaxations in tissues incubated with LPS that were markedly inhibited by TEA. In contrast, TEA or L-arginine had little effect on phenylephrine contractions in control tissues. Furthermore, the inducible nitric oxide synthase inhibitor, aminoguanidine (0.4 mM), reversed the effects of LPS and blocked responses to TEA. These results suggest that activation of $K^+$ channels, possibly Ca-activated $K^+$ channels, through induction of the nitric oxide synthase pathway, may well be responsible for endotoxin-mediated hyporeactivity to vasoconstrictor agents in vascular smooth muscle. 

It is well known that administration of endotoxin (lipopolysaccharide, LPS) or cytokines (tumour necrosis factor or interleukin-1) to animals produces a shock-like syndrome, characterised by low blood pressure and hyporeactivity to vasoconstrictor agents (1,2). Moreover, isolated blood vessels exposed to these agents or removed from treated animals also show diminished responses to vasoconstrictor agents (3-7). Substantial evidence now exists to suggest that hypotension and hyporeactivity to vasoconstrictors can largely be accounted for by the non-endothelial production of nitric oxide (NO) or an NO-like compound generated from the conversion of L-arginine (L-Arg) by NO synthase (NOS) (8,9). Consistent with this hypothesis is the observation that non-selective inhibitors of NOS such as $N^G$-mono-methyl-L-arginine (L-NMMA) or $N^G$-nitro-L-arginine (L-NNA), reverse much of the cardiovascular changes associated with a number of *in vivo* and *in vitro* animal models of endotoxic shock (3-5,7,10). Furthermore, LPS and cytokines induce the expression of a Ca-independent isoform of NOS (iNOS) which is capable of producing large amounts of NO in many cell types, including macrophages and vascular smooth muscle (8,9). In addition, recent data show that selective inhibitors of this enzyme are equipotent at reversing the vascular effects of LPS (6,11-13).

It appears widely accepted that NO and NO donors relax smooth muscle primarily by activating cytosolic guanylate cyclase and elevating cGMP (for review see 9,14). In septic shock models, lowering intracellular cGMP by inhibition of guanylate cyclase restores the contractile response to noradrenaline in isolated aortic strips (4,5,15). Although the mechanism by which cGMP lowers arterial tone still remains uncertain, there is good evidence that cGMP

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Abbreviations: LPS, bacterial lipopolysaccharide; PE, phenylephrine; TEA, tetraethylammonium chloride; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NMMA, $N^G$-mono-methyl-L-arginine; L-NNA, $N^G$-nitro-L-arginine; L-Arg, L-arginine; Ach, acetylchloline; $K_{ATP}$, ATP-sensitive $K^+$; $K_{Ca}$, $Ca^{2+}$-activated $K^+$.



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reduces agonist-induced rises in $[Ca]_i$ in part through membrane hyperpolarization (14). This is also consistent with observations that NO and cGMP-elevating agents do hyperpolarize some arteries (16-19). In mesenteric artery, hyperpolarization by NO is blocked by the ATP-sensitive $K^+$ ($K_{ATP}$) channel blocker, glibenclamide (17), while in a number of other vascular beds, vasorelaxation in response to NO and NO-donors appears to involve $Ca^{2+}$-activated $K^+$ ($K_{Ca}$) channels (20-22). Indeed, patch-clamp studies in isolated smooth muscle cells have found NO to activate $K_{Ca}$ channels directly in rabbit aorta (23) and indirectly *via* cyclic GMP-dependent-kinase in rat pulmonary artery (20).

Recent evidence suggests that altered $K^+$ channel function may occur in septic shock since glibenclamide reversed much of the hypotension associated with animal models of endotoxic shock (7,24,25). However, $K_{ATP}$ channels do not appear to mediate hyporeactivity directly since glibenclamide is not effective at restoring vasoconstrictor responses in isolated rat aorta from endotoxin-treated animals (7). In contrast, recent patch-clamp experiments in cultured arterial smooth muscle cells suggests that induction of the iNOS pathway is responsible for persistent activation of both $K_{ATP}$ and $K_{Ca}$ channels (26,27). Although there would appear to be good evidence that $K^+$ channels are involved in endotoxic shock, no functional evidence exists to show their direct involvement in mediating hyporeactivity to vasoconstrictors in isolated blood vessels. Thus we examined the effects of tetraethylammonium chloride (TEA), a non-selective blocker of $K^+$ channels (28) on contractions to phenylephrine (PE) in control and LPS treated rat aorta. A preliminary report of some of this work has been presented (29).

## MATERIALS AND METHODS

*Organ bath studies.* Male Wistar rats (200-250 g) were killed by cervical dislocation and the thoracic aorta removed and placed in physiological salt solution (PSS) containing in mM: 112 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 0.5 $KH_2PO_4$, 0.5 $NaH_2PO_4$, 10 glucose, 0.03 phenol red (pH 7.4 with 95% $O_2$/5% $CO_2$). The connective tissue was carefully removed and helical strips of muscle ~1.5 mm wide and ~15 mm long were cut. In most experiments the endothelium was removed by rubbing the intimal surface of the aortic strip with filter paper. The absence of functional endothelium was verified by the application of 10 $\mu$M acetylcholine (Ach; 1 min) which produces no relaxation in the absence of intact endothelium. Muscle strips were mounted in a small organ bath (0.3 ml) and tension measured using an isometric transducer (Harvard Apparatus, USA) connected to a chart recorder (Labdata Instrument Services, UK). Tissues were subjected to a basal tension of 1.25 g and perfused (1.2 ml/min) at 37 °C with PSS containing the antibiotics, penicillin (10 units/ml) and streptomycin (10 units/ml). After a 45 min equilibration period, muscle strips were precontracted with 1 $\mu$M phenylephrine (PE). Tension was measured for up to 8 hr and expressed as the percentage of the contraction measured at the time of LPS application (t=0) or an equivalent time for controls. LPS was generally added 50-60 min after the addition of PE.

*Drugs.* Phenlyephrine, acetylcholine, L-arginine, lipopolysaccharide (*Salmonella typhosa*) and aminoguanidine were all purchased from Sigma Chemical company (Poole, Dorset, UK). Tetraethylammonium chloride was purchased from Fluka Chemicals (Gillingham, Dorset, UK) and penicillin/streptomycin from Gibco (Paisley, Scotland). All stock solutions were made up in distilled water and diluted with PSS.

*Statistics.* Results in the text are expressed as mean ± standard error of the mean (S.E.M.) of n observations. Statistical significance within a group was determined using one-way analysis of variance (ANOVA) followed, where appropriate, by the Bonferoni t-test for multiple comparisons to compare means between groups. In some cases the paired Student's t-test was used. A P value $<0.05$ was considered to be statistically significant.

## RESULTS

Incubation of endothelium-denuded muscle strips with LPS (0.1 $\mu$g/ml) produced gradual relaxation of PE contractions after 2hr, which became significantly ($p<0.05$) greater than control tissues at 3hr (figure 1). The effect of LPS peaked at ~4 hr, at which point the average relaxation was 43±4.6% (n=10) compared to 16.0±2.4 % (n=7) for control tissues. Pre-treatment of muscle strips with the non-selective $K^+$ channel blocker, TEA (10mM) prevented LPS from causing substantial relaxation of rat aortic strips (figure 1). Furthermore, in the presence of TEA, tension actually declined at a slower rate than controls between 1 and 4 hr.

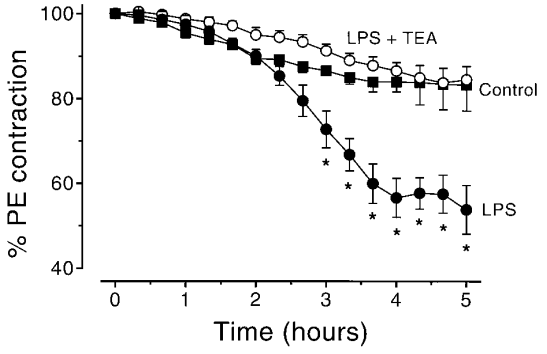


**FIG. 1.** Pre-treatment with the $K^+$ channel blocker, tetraethylammonium chloride (TEA) prevents lipopolysaccharide (LPS), induced relaxation in rat aorta. Tension was measured in endothelium-denuded helical strips precontracted with 1 mM phenylephrine (PE) under control conditions (■, 7 strips), in the presence of LPS (0.1 $\mu$g/ml; ●, 11 strips) or in the presence of LPS (0.1 $\mu$g/ml) and 10 mM TEA (LPS + TEA; ○, 8 strips). Tension, plotted against time, was expressed as the percent of the PE contraction measured at the time of LPS addition to the perfusate (t=0). On most days, LPS-treated tissues were taken from the same animal as either controls or tissues treated with LPS and TEA. Data are expressed as means ± S.E.M. and each time point represents at least 5 measurements. *$P<0.05$ when compared to control values.

Since TEA might be preventing the effects of LPS through inhibition of iNOS induction, experiments were repeated where TEA was applied after the peak response to LPS had been obtained (figure 2A). Experiments showed that TEA (10 mM) still reversed the LPS-induced decline in tension to a similar extent as pre-treatment of muscle strips (compare figure 1 & 3). A lower concentration of TEA (2 mM), which is considered to increase the selectivity of this agent towards $K_{Ca}$ channels (28), also substantially inhibited LPS relaxations (figure 3). However, higher concentrations of TEA (10 mM) always produced a greater block when applied in the same muscle ($P<0.05$). In contrast, TEA had little or no effect on PE contractions in control tissues (figure 2B & 3).

L-arginine (L-Arg; 1 mM) produced substantial relaxations in aortic strips that had been incubated with LPS for several hours (figure 2A & 3). This observation is consistent with previous observations showing the activity of iNOS (but not endothelial-derived NOS) to be partially dependent on the presence of extracellular L-Arg (5). However, the response to L-Arg was small if it was applied in the presence of TEA (figure 2C and 3) or to control tissues at 5-6 hr after the onset of PE contractions (figure 2B). Relaxations to L-Arg were never observed (n=14) if applied at the beginning of the experiment (figure 2A & B). Furthermore, the effects of LPS were also completely inhibited by the relatively selective inhibitor of iNOS, aminoguanidine (0.4 mM; figure 4). In contrast, this agent had no effect on tension (with or without endothelium) when applied at ~60 min after the onset of the PE contraction ($p=0.11$; n=7). It did, however, produce a small, but significant ($p=0.04$) contraction when applied at 5 hr (87.3±0.9 % *versus* 98.0±0.24%, n=4), indicative of some induction of iNOS in control tissues with time. Moreover, aminoguanidine prevented the contractions to TEA that were observed in LPS-treated tissues (figure 4), suggesting these two agents were affecting the same pathway.

## DISCUSSION

The above results demonstrate that TEA, a non-selective inhibitor of $K^+$ channels is able to fully reverse the LPS-induced impairment of PE contractions in isolated strips of aorta. In addition, we demonstrate that TEA substantially reversed L-Arg-induced stimulation of the

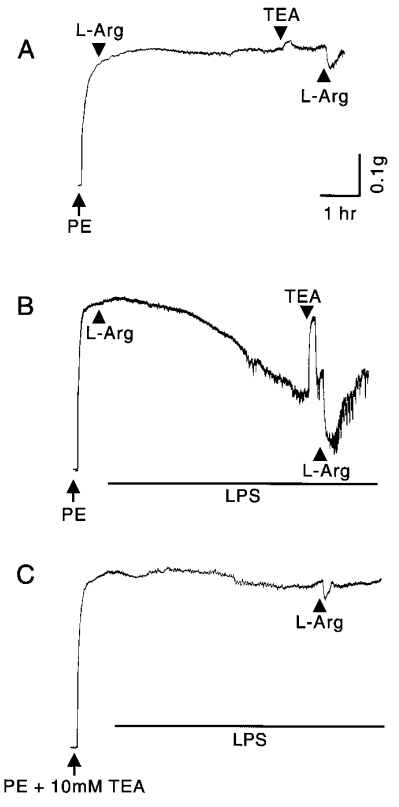


**FIG. 2.** TEA reverses the effect of LPS and reduces relaxation to L-arginine (L-Arg) in endothelium-denuded helical strips of rat aorta. The effects of L-Arg (1 mM; 5min) and TEA (10 mM; 5min), added at the time indicated by the filled triangle, on contractions to 1 mM PE in controls (A) and in tissues treated with LPS (B). In (C), the effect of L-Arg (1 mM; 5min) in tissues pre-treated with 10 mM TEA and 0.1 $\mu$g/ml LPS.

iNOS pathway. Thus we conclude that this pathway is involved in the activation of $K^+$ channels by LPS. This conclusion is also based on the observation that the iNOS inhibitor, aminoguanidine, reversed the effects of LPS, and prevented TEA from having any effect on PE contractions. Although recent data suggests that $K^+$ channel blockers might be beneficial in animal models of septic shock because of their ability to prevent the induction of iNOS either directly (7) or indirectly through inhibition of tumour necrosis factor release (30), this cannot explain the mechanism of action of TEA in these experiments. This agent was found to be equally effective regardless of whether it was given before or after tissues were exposed to LPS. Furthermore, recent data have shown that TEA (up to 10 mM) does not inhibit the induction of iNOS in macrophages, whereas the $K_{ATP}$ channel blocker, glibenclamide does (7). Moreover, the time-dependent effects of LPS and TEA were not affected if the endothelium was removed. Thus, these results provide the first direct demonstration that vasorelaxation produced by endotoxin *in vitro* involves abnormal activation of vascular $K^+$ channels.

Recent evidence suggests that activation of $K^+$ channels may contribute to impaired vasoconstrictor responses following endotoxin administration. This evidence has come from patch-clamp studies of cultured coronary arterial cells treated with LPS, where activation of iNOS with L-Arg, caused persistent activation of both $K_{Ca}$ and $K_{ATP}$ channels. Although we cannot at present be sure of the exact identity of the $K^+$ channel(s) mediating the effects of LPS in

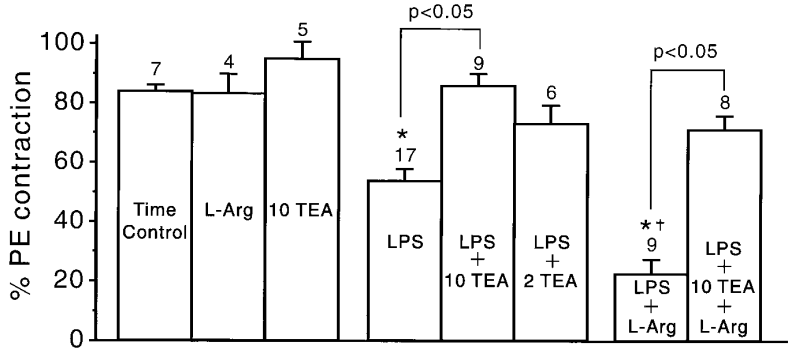


**FIG. 3.** Time-dependent effects of L-Arg (1 mM), TEA (2 and 10 mM) and LPS (0.1 $\mu$g/ml) on tension in helical strips of rat aorta precontracted with 1 $\mu$M PE. Data are expressed as means ± S.E.M. and plotted from experiments similar to those shown in figure 2. All measurements were made between 5.5 and 6.5 hr after the onset of contraction to PE. *P<0.05 represents significant effect of treatment compared to time control, and †P<0.05 when compared to LPS alone.

rat aorta, our data would favour a direct role for $K_{Ca}$ channels. There are several lines of evidence to support this conclusion: (i) TEA reversed a substantial part of the relaxation induced by LPS when applied in the low mM range (2 mM) - at this concentration TEA is a selective and potent blocker of $K_{Ca}$ channels but not other known types of vascular $K^+$ channels which require much higher concentrations (> 5 mM) to produce significant block (28); (ii) prior treatment with 4-aminopyridine (1 mM), which would effectively block the delayed rectifier and A-like $K^+$ current in vascular smooth muscle (28,31), failed to produce any inhibition of LPS-induced relaxations (n=5, data not shown); (iii) glibenclamide does not reverse the hyporeactivity to noradrenaline if applied to isolated rat aorta tissue taken from LPS-treated animals (7), thus ruling out a direct involvement of $K_{ATP}$ channels in mediating the relaxant effects of LPS; (iv) vasorelaxation to NO donors in a number of vascular tissues including aorta is significantly inhibited by charybdotoxin, a highly specific blocker of $K_{Ca}$ channels (20-23). However, we cannot rule out the possibility of contributions from other types of $K^+$ channels, since higher concentrations of TEA (which would start to block other $K^+$ channel types) were always needed to produce full inhibition of LPS effects on tension. Indeed, LPS has recently been found to activate two types of quinine-sensitive $K^+$ channels in human macrophages (32).

The intracellular mechanism whereby LPS produces activation of $K^+$ channels and vasorelax-

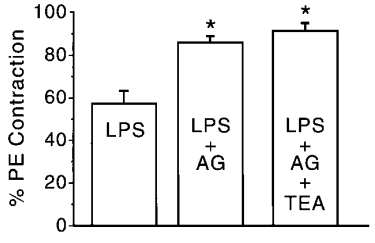


**FIG. 4.** The effect of aminoguanidine (AG; 0.4 mM, 20min) on relaxation induced by a 4hr incubation of 0.1 mg/ml LPS. A subsequent application of TEA (10 mM) in the presence of both agents had no significant effect on tension. Data are taken from 4 animals and expressed as means ± S.E.M. *P<0.05 represents significant difference (paired Bonferoni t-test) compared to LPS alone.

ation remains to be determined. There is increasing evidence that overproduction of NO within the vascular smooth muscle cell largely accounts for the vasorelaxation and hyporeactivity to vasoconstrictor agents seen when tissues are exposed to LPS for many hours (3-24hr) in the organ bath (for review see 8,9). In our experiments, the effects of LPS on tension had a lag phase of ~2h. This is similar to the lag phase for induction of iNOS, changes in tension and cGMP previously reported in endothelial-denuded rings of rat aorta (3) and presumed to be related to *de-novo* synthesis of protein. Consistent with a role for iNOS in the time-dependent relaxation of PE contractions, we found that LPS-induced effects could be fully reversed by aminoguanidine, a preferential inhibitor of iNOS (6,11,12). This agent had however, no effect on tension 1 hour after the onset of PE contractions regardless of whether the endothelium was present or absent. This is consistent with previous studies showing aminoguanidine to have no action on control rings of rat aorta with endothelium (6,12). In contrast, non-selective inhibitors of NOS like L-NMMA or L-NNA do cause contraction and membrane depolarisation in rat aorta (6,19). Furthermore, the concentration of inhibitor used in this study (0.4 mM) was similar to that used previously, where aminoguanidine was found to have no effect on endothelium-dependent relaxation to Ach in rat aorta (12).

In conclusion our study demonstrates that activation of the inducible form of NOS by bacterial endotoxin produces relaxation of vascular smooth muscle through activation of $K^+$ channels. The $K^+$ channels most likely to be involved are Ca-activated $K^+$ channels, although this remains to be determined electrophysiologically. Our results suggest that $K^+$ channel blockers might be beneficial in the treatment of septic shock through their ability to restore contractions to constrictor agents.

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