The pore-forming subunit of the K_{ATP} channel is an important molecular target for LPS-induced vascular hyporeactivity *in vitro*

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1 ATP-sensitive K^+ (K_{ATP}) channel activation is implicated in the vascular hyporeactivity occurring in septic shock. However, channel inhibition with the sulphonylurea receptor (SUR) antagonist, glibenclamide (Glib) fails to reverse lipopolysaccharide (LPS)-induced vascular hyporeactivity *in vitro*. We investigated whether inhibitors that act by binding to the K_{ATP} channel pore could be effective.

2 Ring segments of endothelium-intact rat mesenteric artery were incubated with LPS in culture media for either 6 or 20 h before contractile responses to phenylephrine were assessed in the absence or presence of K_{ATP} channel inhibitors.

3 The pore-forming subunit inhibitors barium chloride ($BaCl_2$; $300\,\mu M$) and PNU-37883A ($1\,\mu M$) significantly reversed hyporeactivity at both time points, although less so at 20 h. In contrast, the SUR inhibitors, Glib ($10\,\mu M$), tolbutamide (Tolb) ($1\,mM$) and PNU-99963 ($1\,\mu M$) were ineffective. In LPS-incubated tissues, Glib and Tolb antagonised contractions to the thromboxane A2 mimetic, U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$) ($10^{-7}\,M$), whereas the pinacidil-derived inhibitor, PNU-99963, did not.

4 Contractions to 60 mM KCl were unaffected by LPS at 6 h, but were significantly depressed by LPS at 20 h, suggesting that K^+-channel-independent pathways contribute to hyporeactivity at the later time point.

5 The inducible nitric oxide synthase (iNOS) inhibitor, 1400 W ($10\,\mu M$) and Tolb inhibited the production of nitrite induced by LPS, whereas $BaCl_2$ and PNU-37883A had no effect.

6 In conclusion, K_{ATP} channels contribute to LPS-induced vascular hyporeactivity *via* the iNOS pathway in rat mesenteric artery. The effectiveness of pore inhibitors over SUR inhibitors of the K_{ATP} channel suggests altered SUR function following LPS administration, which cannot be explained by thromboxane receptor inhibition.

Introduction

Sepsis is a leading cause of mortality and morbidity in the critically ill. Septic shock, the most severe clinical manifestation, is associated with profound hypotension and a marked vascular hyporeactivity to norepinephrine and other adrenergic vasoconstrictors. This can be mimicked both *in vivo* and *in vitro* by administration of endotoxin, a lipopolysaccharide (LPS) component of the Gram-negative bacterial cell wall (Deitch, 1998).

LPS-induced hypotension and vascular hyporeactivity is associated with overproduction of nitric oxide (NO) within the blood vessel wall. This involves activation of both inducible (iNOS) and constitutive (eNOS) isoforms of NO synthase (Julou-Schaeffer *et al.*, 1990; Thiemermann, 1994; 1997). NO activates soluble guanylyl cyclase causing vascular smooth muscle relaxation through the formation of guanosine $3'$–$5'$ cyclic-monophosphate (cGMP). Inhibition of NO synthase (NOS) isoforms or soluble guanylyl cyclase has been shown to reverse vascular hyporeactivity both *in vivo* and *in vitro*, either partially (Yen *et al.*, 1995; Mitolo-Chieppa *et al.*, 1996; Wu *et al.*, 1998) or completely (Julou-Schaeffer *et al.*, 1990; Hall *et al.*, 1996; Scott *et al.*, 1996). In an organ culture model of vascular hyporeactivity, we have recently demonstrated that the degree of reversal with NOS inhibitors is time-dependent (O'Brien *et al.*, 2001). Thus, activation of NOS appeared to fully account for hyporeactivity in the short term (6 h), but only partially in the long term (>20 h), suggesting the involvement of additional mechanisms at the later time points. Prostaglandins could play a role since LPS induces expression

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of cyclo-oxygenase-2 (COX-2), which is associated with increased synthesis of a variety of prostanoids including prostacyclin, thromboxane A_2 (TXA_2) and prostaglandin E_2 (Cirino *et al.*, 1996; Bishop-Bailey *et al.*, 1997). Recent evidence suggests that TXA_2 may serve to counteract the vascular hyporesponsiveness to α-adrenergic agonists in sepsis through inhibiting expression of iNOS (Yamada *et al.*, 2003). This is consistent with previous observations showing worsening of vascular hyporeactivity with TXA_2 antagonists (Cirino *et al.*, 1996).

ATP-sensitive K^+ (K_{ATP}) channels are also likely to play an important role in sepsis-induced vascular hyporeactivity and the development of septic shock. In canine, porcine and rat *in vivo* models of LPS-induced shock, the K_{ATP} channel inhibitor glibenclamide (Glib) restored blood pressure without having any effect in control animals (Landry & Oliver, 1992; Vanelli *et al.*, 1995; 1997; Wu *et al.*, 1995; Gardiner *et al.*, 1999; Sorrentino *et al.*, 1999). Glib also increased the magnitude of vasopressor responses to phenylephrine (PE) in LPS-treated rats (Sorrentino *et al.*, 1999), suggesting that K_{ATP} channels mediate both hypotension and hyporeactivity *in vivo*. Yet, in all *ex vivo* organ bath studies to date, Glib had either no effect on vascular hyporeactivity (Wu *et al.*, 1995; Taguchi *et al.*, 1996) or even further reduced contractions (Sorrentino *et al.*, 1999), despite partial reversal of LPS-induced hyperpolarisation with Glib observed in the same vessel (Chen *et al.*, 2000). The reason for this may relate to real differences in the mechanisms of LPS-induced vascular dysfunction *in vivo* versus *in vitro*, or to the 10- to 100-fold higher concentrations of Glib used in the *in vivo* studies (Wu *et al.*, 1995). An intriguing possibility to consider is that LPS alters the pharmacology of the K_{ATP} channel such that agents inhibiting the channel *via* the sulphonylurea receptor (SUR) become less effective (Wilson & Clapp, 2002). Metabolic stress in cardiac muscle of the heart has previously been reported to render SUR inhibitors ineffectual (Findlay, 1993). In this respect, it is worth noting that the pressor effect of Glib is transient *in vivo* (Landry & Oliver, 1992; Vanelli *et al.*, 1995; 1997; Gardiner *et al.*, 1999; Sorrentino *et al.*, 1999) despite its long half-life in plasma (Wu *et al.*, 1995). Moreover, the use of Glib as a tool with which to probe K_{ATP} channel function is complicated by its known inhibitory action at thromboxane receptors (Delaey & Van de Voorde, 1995). Such a mechanism would be predicted to worsen hyporeactivity. Thus, it would be pertinent to investigate the mechanism of vascular hyporeactivity using more potent and selective K_{ATP} channel inhibitors as well as agents with a different mode of action (Cui *et al.*, 2003).

The K_{ATP} channel is an octomeric complex consisting of four pore-forming subunits (Kir6.x) and four SUR subunits (Bryan & Aguilar-Bryan, 1999). The pore is thought to confer ATP inhibition and determine conductance, while the SUR is considered the primary target for sulphonylureas, potassium channel openers (KCOs) and nucleotide diphosphates. The SUR subunit can be inhibited by agents such as Glib, tolbutamide (Tolb) and PNU-99963 (Khan *et al.*, 1997; Bryan & Aguilar-Bryan, 1999; Cui *et al.*, 2003). The latter is a pinacidil-derived inhibitor, which potently blocks relaxation to the K_{ATP} channel opener, pinacidil with an IC_{50} of 18 nM in rat aorta (Khan *et al.*, 1997). The pore-forming subunit can be inhibited by barium chloride ($BaCl_2$) or the vascular selective K_{ATP} channel inhibitor, PNU-37883A (Nelson & Quayle, 1995; Surah-Narwal *et al.*, 1999; Wellman *et al.*, 1999; Cui

et al., 2003). Using a previously characterised model of vascular hyporeactivity in mesenteric artery (O'Brien *et al.*, 2001), we specifically investigated whether agents acting on the pore-forming subunit of the K_{ATP} channel could reverse the effects of LPS. In addition, we wished to determine whether loss of function of SUR inhibitors in this *in vitro* model was related to inhibition of thromboxane receptors or was associated with a generalised effect of LPS on the pharmacology of the channel. Preliminary results have been presented in abstract form (O'Brien *et al.*, 2002a, b).

Methods

Male Sprague–Dawley rats (280–300 g body weight) were killed *via* cervical dislocation. This procedure conforms to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The mesenteric artery was dissected out and placed in sterile Hank's balanced salt solution (Invitrogen Ltd, Paisley, U.K.). The artery was cleaned of connective tissue and cut into eight rings. For fresh controls, rings were immediately mounted in the organ bath. In all other experiments, rings were incubated in sterile Dulbecco's modified Eagle's medium (DMEM; Invitrogen Ltd) supplemented with 10% (v v^{-1}) foetal bovine serum (low endotoxin; Invitrogen Ltd) for 6 or 20 h in an atmosphere of 95% air/5% CO_2. LPS (*Salmonella typhosa*; 1 μg ml^{-1}) was added to some of the segments for either 6 or 20 h. Following incubation in culture medium, tissues were transferred to 25 ml organ bath chambers containing physiological saline solution (PSS) with the following composition in mM: 112 NaCl, 5 KCl, 25 $NaHCO_3$, 1 $MgCl_2$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 glucose, 1.8 $CaCl_2$ and 0.03 phenol red, gassed with 95% O_2/5% CO_2 at 37°C. In selected experiments, KCl replaced NaCl in equimolar amounts in the PSS, with the concentration of all other salts remaining the same.

Rings of mesenteric artery were attached to an isometric force transducer (FT-03; Grass Instrument Company, West Warwick, RI, U.S.A.) coupled to a chart recorder and subjected to a tension of 1.25 g. These were permitted to relax to a resting tension of 0.8 g, the optimum tension found in preliminary experiments. An equilibration period of 1 h was allowed during which time tissues were washed with PSS at 15 min intervals. In rings pretreated with LPS, a similar concentration of LPS was added to the organ baths for the duration of the experiment. Endothelial function was assessed by monitoring relaxation to acetylcholine (5 μM) in rings precontracted with 1 μM PE. Contractions were allowed to plateau before the effects of acetylcholine were assessed. Relaxation was then calculated as a percent of the PE contraction, measured just before acetylcholine was applied. The magnitude of the relaxation induced by acetylcholine in control tissues did not alter with time in culture medium, being $80.6 \pm 0.2\%$, $n = 13$ *versus* 79.0 ± 9.7, $n = 8$, at 6 and 20 h, respectively. Cumulative concentration–response curves were constructed to PE (10^{-9}–10^{-5} M) with increasing doses added at 5 min intervals. At the end of those experiments where tissues were incubated with LPS for 20 h, the TXA_2 mimetic, U44619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$) was added (10^{-7} M).

Experimental protocols

At least six mesenteric rings, taken from a minimum of three animals, were used in each experimental group.

(a) Effect of K$_{ATP}$ channel inhibitors: Various inhibitors of the K$_{ATP}$ channel were individually added 25 min prior to the addition of the lowest dose of PE. These included the SUR inhibitors, Glib (10 μM), Tolb (1 mM) and PNU-99963 (1 μM) or the pore-forming subunit inhibitors BaCl$_2$ (300 μM) and PNU-37883A (1 μM). As Tolb and PNU-99963 were dissolved in 100% dimethyl sulphoxide (DMSO), and Glib in 50% DMSO and 50% polyethylene glycol, 25 μl of the solvent mixture was added to control tissues to ascertain any effect on contractile function.

(b) Nitrite assays: Tissues were incubated for 20 h in the presence or absence of LPS with or without the addition of (i) a K$_{ATP}$ channel inhibitor or (ii) the highly specific iNOS inhibitor, *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W). Following this, 150 μl of the culture medium was mixed with an equal quantity of Griess reagent in a 96-well microtitre plate. A standard curve was prepared each time. NO$_2^-$ concentration was determined colorimetrically by subtracting the absorbance at 540 nm from that obtained at the reference wavelength (620 nm). To normalise NO$_2^-$ concentration for tissue mass, the protein content of the tissue was measured. Tissues were dissolved in 0.5 M NaOH and the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, U.S.A.) kit used to determine protein content.

(c) Concentration–response curves to the NO donor, S-nitroso-N-acetyl-D, L-penicillamine (SNAP): Concentration–relaxation curves to SNAP (10^{-10}–10^{-5} M) were constructed in fresh tissues precontracted with 1 μM PE. The K$_{ATP}$ channel inhibitors, BaCl$_2$ and PNU-37883A, or the guanylyl cyclase inhibitor, ODQ (1H-(1,2,4) oxadiazolo (4,3-*a*)quinoxalin-1-one) (3 μM), were added 25 min prior to the addition of PE. Once contraction to PE had reached a plateau, doses of SNAP were added in cumulative fashion every 5 min.

Reagents

Sterile Hank's balanced salt solution was supplemented with 10 mM HEPES (pH 7.4) and 2 mM NaHCO$_3$. DMEM was supplemented with 2 mM L-glutamine (Invitrogen Ltd) and 10% foetal calf serum (Invitrogen Ltd). LPS (*S. typhosa*), acetylcholine, Glib, BaCl$_2$, Tolb, sodium nitrite and PE were all obtained from Sigma Chemical Company (Poole, Dorset, U.K.). U46619 was obtained from Affiniti-Research, (Exeter, U.K.), while 1400 W and SNAP were from Alexis Corporation (Nottingham, U.K.). PNU-99963 and PNU-37883A were kindly donated by Pharmacia-Upjohn (Kalamazoo, U.S.A.). Further PNU-37883A was obtained from Affiniti-Research (Exeter, U.K.). Glib, Tolb, PNU-99963 and PNU-37883A were dissolved in DMSO and diluted into PSS. The highest final concentration of DMSO used in experiments was 0.1%.

Statistics

All data are represented as the mean ± standard error of the mean (s.e.m.) of *n* observations. Statistical analysis was performed using the SigmaStat (Jandel corporation, Chicago, U.S.A.) software program. Two-way ANOVA with repeated measures was used and, where appropriate, corrected for multiple comparisons against the control group (Bonferroni) or all groups (Student–Newman–Keuls). Student's *t*-test was used for comparisons between two groups. The concentration of agonist causing a 50% contraction or relaxation of the maximal response (E_{Max}) is expressed as the mean pEC$_{50}$ value. Individual pEC$_{50}$ values were obtained from individual experiments and calculated using the sigmoidal curve fitting routine in Origin 6.0 (Microcal, Northampton, MA, U.S.A.). Comparisons between pEC$_{50}$ values were made using one-way ANOVA (with Bonferroni or Student–Newman–Keuls correction as appropriate). A *P*-value < 0.05 was considered statistically significant.

Results

Effect of endotoxin on the contractile responses to PE

Typical cumulative concentration–response curves to PE in rat mesenteric artery incubated in the absence and presence of 1 μg ml^{-1} LPS are shown in Figure 1. While time of incubation in culture medium did not alter the maximal contraction to PE in control tissues, a significant shift in the potency of PE was observed (top traces of Figure 1a and b). In contrast, marked hyporeactivity to PE was observed at 6 h ($P < 0.001$, $n = 14$) and 20 h ($P < 0.001$, $n = 17$) in the presence of LPS. In addition, LPS caused a rightward shift of the pEC$_{50}$ value at 6 h, which was associated with a marked reduction in the maximal contraction (E_{Max}) at 10^{-5} M PE ($P < 0.001$; Table 1). An accurate pEC$_{50}$ value could not be calculated at 20 h as the contractile responses to PE in the presence of LPS were too small.

Effect of K$_{ATP}$ channel inhibitors on LPS-induced vascular hyporeactivity to PE

The SUR inhibitors, Glib (10 μM), Tolb (1 mM) and PNU-99963 (1 μM) all failed to reverse LPS-induced vascular hyporeactivity either at 6 or 20 h (Figure 2 and Table 1). Indeed, Glib further reduced PE contractions at 6 h ($P = 0.002$ compared to LPS alone). Lack of reversal of PE contraction with SUR inhibitors could not be attributable to solvent
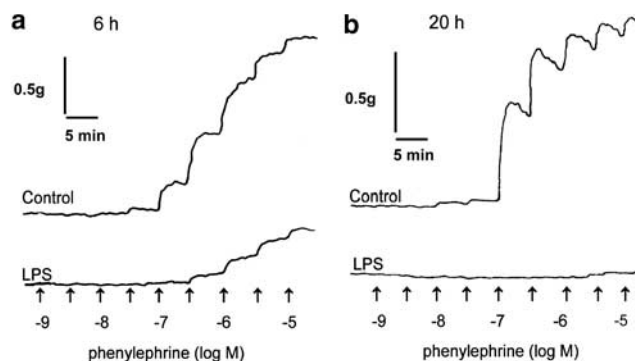

Figure 1 Concentration–response curves to PE in rat mesenteric artery incubated for (a) 6 h and (b) 20 h in the absence (control) and presence of 1 μg ml^{-1} LPS. PE was added cumulatively in half log units at the time indicated by the arrows.

Table 1 Effect of various K_{ATP} channel blockers on PE contractions in tissues previously incubated for 6 h in culture medium containing LPS

Rat mesenteric artery	pEC_{50}	E_{Max} (g)	n
Control	6.53 ± 0.10	1.53 ± 0.05	18
LPS	5.71 ± 0.18**	0.50 ± 0.05**	14
LPS + Glib (10 μM)	5.16 ± 0.38**	0.17 ± 0.03**, #	10
LPS + Tolb (1 mM)	5.99 ± 0.08	0.64 ± 0.13**	8
LPS + PNU-99963 (1 μM)	5.80 ± 0.13*	0.49 ± 0.10**	6
LPS + PNU-37883A (1 μM)	6.01 ± 0.12	1.14 ± 0.11*, #	6
LPS + BaCl₂ (30 μM)	6.03 ± 0.05	0.65 ± 0.07**	6
LPS + BaCl₂ (300 μM)	6.41 ± 0.07	1.46 ± 0.16#	8

Comparison of pEC_{50} values and maximal contraction (E_{Max}) obtained against contractions to 10^{-5} M PE in the absence (control) and presence of LPS with or without channel inhibitors. Data are expressed as the mean ± s.e.m. of n observations. *$P < 0.05$, **$P < 0.001$ when compared to control. # $P < 0.05$ when compared to LPS alone.
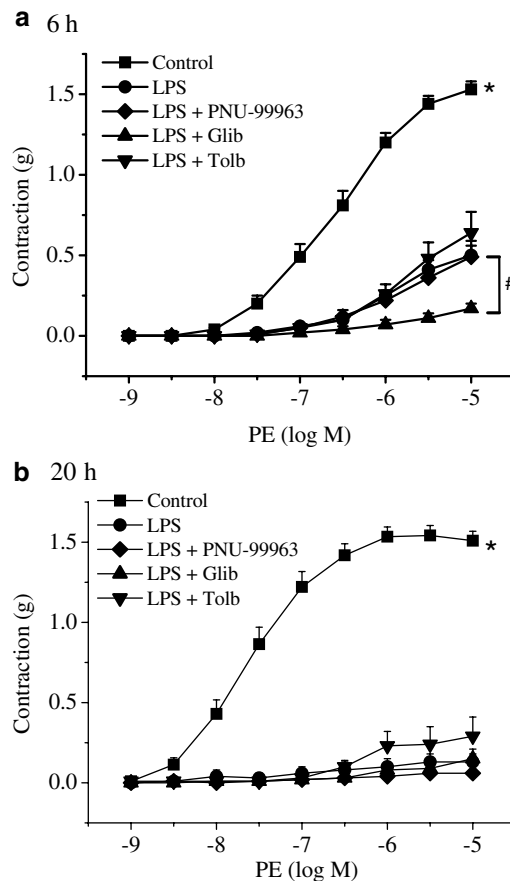

Figure 2 Effect of the K_{ATP} channel inhibitors, Glib (10 μM), Tolb (1 mM) and PNU-99963 (1 μM) on LPS-induced vascular hyporeactivity. Tissues were incubated at 37°C for 6 h (a) or 20 h (b) in culture medium (DMEM) containing serum in the absence (control) or presence of 1 μg ml⁻¹ LPS. Data are expressed as the mean ± s.e.m. of eight to 12 observations from eight animals. *$P < 0.001$ when control is compared to various LPS treatments and #$P < 0.05$ when LPS alone is compared to Glib in the presence of LPS.

Figure 3 Effect of BaCl₂ and PNU-37883A on LPS-induced vascular hyporeactivity. Tissues were incubated at 37°C in culture medium containing serum for 6 h (a) or 20 h (b) in the absence (control) or presence of LPS. Data are expressed as the mean ± s.e.m. of eight to 12 observations from eight animals. *$P < 0.001$ and #$P < 0.05$ when compared to LPS administration alone.

effects since DMSO (0.1%) failed to significantly ($P = 0.34$) alter hyporeactivity to PE induced by LPS at 20 h (max contraction in the presence of DMSO was 0.23 ± 0.01 g, $n = 4$ compared to 0.13 ± 0.05 g, $n = 17$ with LPS alone).

To investigate the effect of inhibiting the pore-forming subunit of the K_{ATP} channel, we used BaCl₂ (300 μM) and PNU-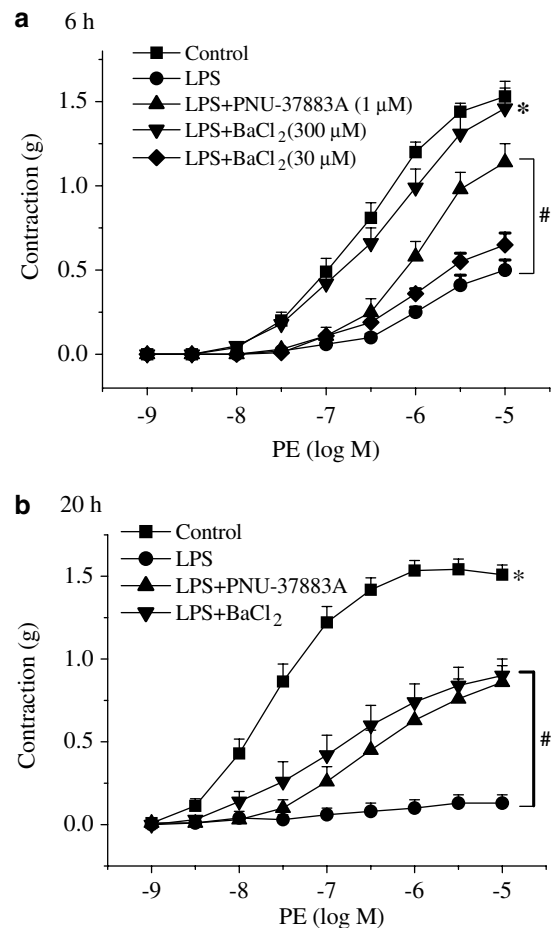37883A (1 μM). At 6 h, BaCl₂ fully reversed LPS-induced hyporeactivity, while PNU-37883A produced a 75% reversal ($P = 0.035$; $n = 8$) (Figure 3a). However, at 20 h incubation with LPS, less reversal was achieved with either BaCl₂ or PNU-37883A (Figure 3b). In contrast, no effect was seen with 30 μM BaCl₂ (Figure 3a), a concentration sufficient to inhibit

strong inwardly rectifying K^+ (Kir) channels (Nelson & Quayle, 1995), suggesting that these channels do not contribute to LPS-induced hyporeactivity. To rule out the possibility that pore inhibitors blocked basal K_{ATP} channel activity, thereby increasing the sensitivity to PE independently of LPS, dose–response curves to PE were performed in control tissues that had previously been incubated in culture medium for 6 h. In the absence and presence of PNU-37883A, dose–response curves for PE were essentially superimposable, with pEC_{50} values being 7.03 ± 0.07 and 7.22 ± 0.03, respectively ($P = 0.11$; $n = 7$). Similarly, $BaCl_2$ did not affect E_{Max} ($P = 0.71$), although it caused a small, but significant ($P < 0.05$) increase in the pEC_{50} value (7.34 ± 0.13; $n = 4$) compared to control.

Effect of LPS on contractions to 60 mM KCl

The degree of reversal of LPS-induced hyporeactivity with PNU-37883A and $BaCl_2$ suggests that the mechanism of relaxation predominately involves opening of K^+ channels at 6 h, but additional pathways at 20 h. To test this, we investigated the effect of LPS on contractions induced by 60 mM KCl, where relaxation cannot be promoted by K^+ channel opening due to a lack of transmembrane driving force on K^+ ions (Gurney & Clapp, 1994). Whereas 6 h incubation with LPS did not significantly ($P = 0.102$, $n = 16$) reduce contractions to 60 mM KCl, the contractile response was significantly depressed ($\sim 40\%$) at 20 h ($P < 0.001$; $n = 10$) (Figure 4). Thus, lack of effect of LPS on KCl contractions at 6 h is consistent with a predominately K^+ channel mechanism at 6 h, while additional mechanisms contribute at the 20 h.

Effect of U46619 on contractile responses to PE

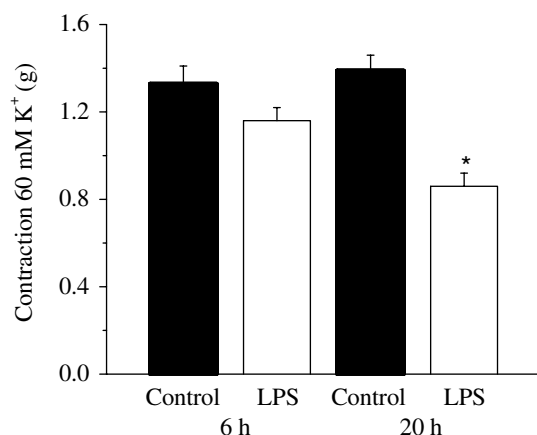Sepsis upregulates the production of thromboxane (Bernard et al., 1997), although LPS weakly affects contractile responses to thromboxane agonists in our organ bath model (O'Brien et al., 2001). Thus, competitive antagonism of thromboxane receptors by the SUR inhibitors Glib and Tolb (Cocks et al., 1990) may underlie their inability to reverse hyporeactivity. No data exist for the potent pinacidil-derived inhibitor, PNU-99963. We observed a significant contraction following addition of the TXA_2 mimetic, U46619 (10^{-7} M) when applied to PE-hyporeactive tissues previously incubated with LPS for 20 h (Figure 5a). While tissues pretreated with either Glib (10 μM) or Tolb (1 mM) failed to contract to U46619, a large contraction was observed with PNU-99963 (1 μM) pretreatment (Figure 5a and b). In rings not exposed to LPS, U46619 only caused a small contraction (from 1.48 ± 0.06 to 1.78 ± 0.17 g; $n = 5$), presumably because the contractile response to PE at 10^{-5} M was close to the maximum tension the tissue could generate.
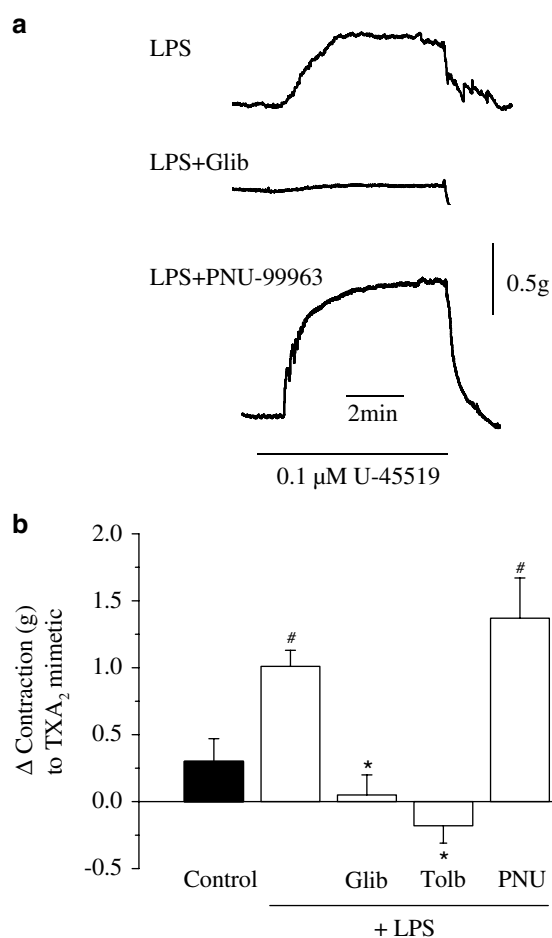

Figure 5 Effect of SUR inhibitors on contractions to the TXA_2 mimetic, U46619 following LPS administration. Tissues were first incubated for 20 h at 37°C in DMEM containing serum in the absence (control) and presence of LPS with or without either Glib (10 μM), Tolb (1 mM) or PNU-99963 (PNU; 1 μM). U46619 (10^{-7} M) was added to the organ bath at the end of the concentration–response curve to PE. Traces from some individual experiments are shown in (a) and averaged data from all experiments shown in (b). Responses are expressed as the absolute change in contraction measured in the continued presence of 10^{-5} M PE and presented as the mean $\pm$ s.e.m. of four to six observations from four animals. *$P < 0.001$ compared to LPS or LPS plus PNU-99963 and #$P < 0.05$ when compared to control.

Figure 4 LPS induces hyporeactivity to contractions with 60 mM KCl following 20 h incubation but not 6 h. Tissues were incubated at 37°C in culture medium (DMEM) containing 10% serum in the absence (control) or presence of LPS for either 6 or 20 h before being contracted with KCl as per methods. Data are expressed as the mean $\pm$ s.e.m of 10 to 16 observations from six to eight animals. *$P < 0.001$.

Effect of K$_{ATP}$ channel inhibitors on LPS-induced nitrite accumulation

Some of the observed effects of the K$_{ATP}$ channel inhibitors could be related to non-specific actions such as inhibition of iNOS induction or activity. Nitrite (NO$_2^-$), a metabolite of NO, is often used as a marker of NO production (Thiemermann, 1997). Compared to control supernatant of rat mesenteric rings, NO$_2^-$ levels doubled in the presence of LPS (1 μg ml^{-1}) after a 20 h coincubation ($P < 0.001$). This rise was completely inhibited by the specific iNOS inhibitor, 1400 W (10 μM) and by 70% with Tolb (1 mM). Neither Glib (10 μM), PNU-37883A (1 μM) nor BaCl$_2$ (300 μM) significantly affected LPS-induced nitrite production at the concentrations used in the tension studies (Figure 6), although there was a trend to lower nitrite production in the presence of BaCl$_2$.

Effect of K$_{ATP}$ inhibitors upon concentration–relaxation curves to SNAP

To determine if K$_{ATP}$ channels contribute to NO-mediated relaxation under control conditions, the effect of the pore-forming subunit inhibitors PNU-37883A (1 μM) and BaCl$_2$ (300 μM) were investigated in mesenteric rings mounted within an hour of being taken out of the animal. Neither agent had any effect on the concentration-dependent relaxation of PE contractions (1 μM) observed with the NO donor, SNAP (Figure 7). In contrast, the guanylyl cyclase inhibitor, ODQ (3 μM), fully inhibited relaxation to SNAP.

Discussion

This study is the first demonstration that the K$_{ATP}$ channel is involved in mediating LPS-induced vascular hyporeactivity *in vitro*. Importantly, only inhibitors of the pore-forming
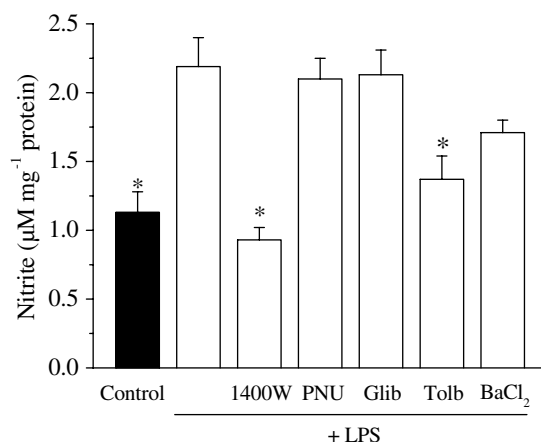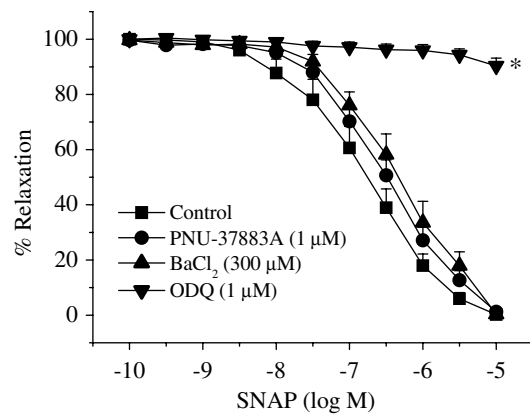
Figure 7 Concentration–response curves to the NO donor, SNAP in rings of fresh rat mesenteric artery precontracted with 1 μM PE. The inhibitors were added 25 min prior to the application of PE. Data are expressed as the mean $\pm$ s.e.m. of six to eight observations from six animals. *$P < 0.001$ compared to control.

subunit, but not the SUR subunit, were effective in reversing this hyporeactivity. Indeed, Glib further depressed contractile responses to PE in the presence of LPS, possibly as a direct result of inhibition of thromboxane receptors rather than any effect on the SUR. However, such a mechanism cannot explain the ineffectiveness of other SUR inhibitors since PNU-99963, the most potent SUR2 inhibitor available (Cui *et al.*, 2003), failed to inhibit contractions to the TXA$_2$ mimetic. In addition, restoration of vascular reactivity that was observed with the K$_{ATP}$ channel pore inhibitors cannot be explained by inhibition of NO production, since these agents did not reduce nitrite accumulation induced by LPS. The suppressed contractile response to 60 mM KCl seen after 20 h exposure to LPS, but not 6 h, implies that mechanisms independent of K$^+$ channel activation also contribute to hyporeactivity at this later time point.

The interpretation of our experimental data relies upon the specificity of the K$_{ATP}$ inhibitors used at the stated concentrations, that is, that BaCl$_2$ (300 μM) and PNU-37883A (1 μM) are specific inhibitors of the pore-forming subunit of the K$_{ATP}$ channel. In vascular smooth muscle, BaCl$_2$ inhibits the K$_{ATP}$ channel with an IC$_{50}$ of 100 μM and Kir channels with an IC$_{50}$ of $\sim$2–5 μM (Nelson & Quayle, 1995; Bradley *et al.*, 1999). As we observed no effect at 30 μM BaCl$_2$, a concentration that effectively inhibits vascular relaxation associated with Kir channels (Quayle *et al.*, 1997; Edwards *et al.*, 1998), we conclude that hyporeactivity induced by LPS is unlikely to result from activation of these channels. However, the greater degree of reversal obtained with Ba^{2+} at 6 h could suggest K$_{ATP}$-channel-independent mechanisms since we did observe a small increase in potency to PE in control tissues preincubated with Ba^{2+} but not PNU-37883A and a small reduction in nitrite levels. Alternatively, other Ba^{2+}-sensitive K$^+$ channels may contribute to vascular hyporeactivity, a notion consistent with previous data showing full reversal of hyporeactivity with the nonselective K$^+$ channel blocker, tetraethylammonium chloride (Hall *et al.*, 1996). Furthermore, mRNA for Kir1.1 and Kir3.1 has been identified in rat arteries (Michelakis *et al.*, 2001), subunits which encode weak (ROMK1) and G-protein-gated (GIRK) inwardly rectifying K$^+$ channels. Both channel types would be expected to be blocked by Ba^{2+} in the high

Figure 6 The effect of K$_{ATP}$ channel inhibitors and the selective iNOS inhibitor, 1400 W on LPS-induced nitrite accumulation. Mesenteric rings were incubated for 20 h at 37°C in culture medium (DMEM) containing 10% serum in the absence (control) or presence of LPS. PNU-37883A (PNU; 1 μM), Glib (10 μM), BaCl$_2$ (300 μM), Tolb (1 mM) or 1400 W (10 μM) were present throughout the LPS incubation. Data are expressed as the mean $\pm$ s.e.m. of four to six observations from six animals. *$P < 0.05$ when compared to LPS.

micromolar range ($>100\,\mu$M). Nonetheless, PNU-37883A is considered to be a highly selective inhibitor of the K$_{ATP}$ channel (Humphrey, 1999). It antagonises the vasorelaxant effects of pinacidil, levcromakalim and minoxidil sulphate in rabbit mesenteric artery (IC$_{50} \sim 1\,\mu$M), while not significantly affecting the vasorelaxant responses to forskolin, nitroglycerin or the Ca^{2+} channel blocker, D-600 (Meisheri *et al.*, 1993). Likewise in patch-clamp studies, PNU-37883A blocks pinacidil- or levcromakalim-activated K$_{ATP}$ currents (IC$_{50} = 3.5\,\mu$M) in isolated rat arterial smooth muscle cells, while only marginally inhibiting ($>10\%$) voltage-activated (K$_v$) or Kir currents at $10\,\mu$M (Wellman *et al.*, 1999). Thus, we have good pharmacological evidence for the involvement of K$_{ATP}$ channels in mediating LPS-induced vascular hyporeactivity. Additional evidence comes from the observation that relaxations to K$_{ATP}$ channel openers such as levcromakalim are potentiated in both *in vitro* and *ex vivo* models of LPS-induced hyporeactivity (Sorrentino *et al.*, 1999; Chen *et al.*, 2000; Wilson & Clapp, 2002). The iNOS pathway probably mediates this since both 1400 W and, to a lesser extent, ODQ reversed potentiation (Wilson & Clapp, 2002). We can exclude an effect upon the generation of NO, as PNU-37883A did not affect nitrite production in the culture media following LPS administration. Likewise, we have previously shown that inhibition of the inducible COX pathway did not reverse vascular hyporeactivity in our organ culture model (O'Brien *et al.*, 2001), ruling out a contribution from the prostaglandin pathway.

We observed that the dose–response curve to PE in control tissues shifted to the left at 20 h compared to that at 6 h. This is consistent with previous findings using the same organ culture model (compare Figure 5a and b in O'Brien *et al.*, 2001). While we have no precise explanation for this, removal of the endothelium does increase responsiveness to PE (O'Brien *et al.*, 2001), suggesting that loss of endothelial function may be occurring over time. Against this, however, we found that endothelium-dependent relaxation to acetylcholine was similar at 6 and 20 h, suggesting that alterations in the release of endothelial-derived relaxing factors *per se* cannot account for these changes. Alternatively, increased basal production of contracting factors may be occurring over time, which, in turn, might enhance sensitivity to PE. In rat mesenteric artery, organ culture increases the potency of ET-1 and this is associated with the appearance of contractile ET$_B$ receptors after 24 h (Adner *et al.*, 1998).

We have previously shown that both iNOS and guanylate cyclase inhibitors fully reverse hyporeactivity in mesenteric arteries treated with LPS for 6 h *in vitro* (O'Brien *et al.*, 2001). Thus, the NO/cGMP pathway is likely to be responsible for K$_{ATP}$ channel activation at this time point. Indeed, other studies have demonstrated activation of K$_{ATP}$ channels in response to NO in mesenteric artery (Garland & McPherson, 1992; Murphy & Brayden, 1995). In our study, neither BaCl$_2$ nor PNU-37883A inhibited relaxations to the NO donor, SNAP in fresh control tissues. Similarly, relaxation to NO in endothelium-denuded aortic tissue was only weakly affected by either Ba^{2+} or 4-aminopyridine, suggesting that activation of K$^+$ channels does not appear to be the primary mechanism of relaxation for authentic NO (Wilson & Clapp, 2002). Thus, additional factors associated with incubation of LPS may aid NO activation of K$_{ATP}$ channels in this model. Consistent with this notion, relaxation to SNAP

(or forskolin) in rat aorta was only inhibited by PNU-37883A in the presence of LPS (Wilson & Clapp, 2002). However, the degree of reversibility achieved by the K$_{ATP}$ channel inhibitors declined at 20 h. This mirrors our previous finding of decreased responsiveness to iNOS inhibitors over time (O'Brien *et al.*, 2001). We postulate that either bioenergetic failure and/or disruption of the actin cytoskeleton contributes to vascular hyporeactivity. Such a mechanism may also account for the reduced contractile response to 60 mM KCl at 20 h.

Different mechanisms of K$_{ATP}$ channel activation may underlie the hypotension and vascular hyporeactivity induced by LPS, perhaps due in part to circulating hormones not present *in vitro*. For example, calcitonin gene-related peptide, a known activator of vascular K$_{ATP}$ channels (Quayle *et al.*, 1997), is increased in septic shock (Arden *et al.*, 1994). Alternatively, K$_{ATP}$ channels may open secondary to a fall in tissue ATP, oxygen tension or pH, conditions associated with severe forms of shock (Clapp & Tinker, 1998). However, the extent to which these metabolic defects occur in sepsis remain controversial (Singer & Brealey, 1999) and has not been investigated in *in vitro* models. Nonetheless, overproduction of NO *via* iNOS is an integral part of both hypotension and hyporeactivity (Thiemermann, 1994; 1997).

The fact that Glib had no effect *ex vivo* may be related to the use in previous studies of aorta (Sorrentino *et al.*, 1999; Wu *et al.*, 1995; Taguchi *et al.*, 1996), a conduit vessel that makes little contribution to blood pressure. We previously reported that Glib was also ineffective at preventing iNOS-mediated relaxation induced by L-arginine in rat aorta (Wilson & Clapp, 2002). However, in the present study, we found that Glib also failed to reverse hyporeactivity in the mesenteric artery. Another consideration may be the counter-balancing effect of Glib on the contractile effects of thromboxane (Cocks *et al.*, 1990). Thus, inhibiting thromboxane-induced vasoconstriction could negate any reversal of vascular hyporeactivity that might have occurred through blocking the K$_{ATP}$ channel. It is worth noting that cytokine-induced hyporesponsiveness of aorta to PE was significantly augmented in TXA$_2$ receptor knockout mice (TP$^{-/-}$), suggesting a protective role for TXA$_2$ against the development of vascular hyporesponsiveness (Yamada *et al.*, 2003). Although we demonstrated that Glib and Tolb antagonised thromboxane-induced contractions in mesenteric artery, PNU-99963 did not. We thus conclude that lack of reversal of SUR inhibitors on LPS hyporeactivity is unrelated to thromboxane inhibition.

It is conceivable that Glib could be acting on the pore-forming subunit *in vivo*. The IC$_{50}$ for inhibition of the SUR by Glib is $\sim$10–200 nM (Fujita & Kurachi, 2000), whereas at a 1000-fold higher concentration, Glib appears to interact with the pore directly (Gribble *et al.*, 1998; Bryan & Aguilar-Bryan, 1999). The calculated peak plasma concentration of Glib for a dose of 1 mg kg^{-1} equates to approximately 60 μg ml^{-1} (or 120 μM) (Wu *et al.*, 1995). At this concentration, Glib would be expected to inhibit the pore directly. In fact, many investigators have used much higher doses in *in vivo* rat models, for example, Gardiner *et al.* (1999) used 20 mg kg^{-1} i.v., while Sorrentino *et al.* (1999) used 40 mg kg^{-1} intraperitoneally.

The striking difference between the effectiveness of Glib and PNU-37883A could relate to their different sites of action (SUR *versus* pore). It has previously been reported that actin

filament disruption with cytochalasin D abolishes high-affinity Glib binding to the SUR subunit in rat aortic rings, whereas KCO binding is unaffected (Loffler-Walz & Quast, 1998). Similar effects on Glib binding can be achieved with metabolic inhibition or hypoxia (Loffler & Quast, 1997). Furthermore, NO can cause cytoskeletal disassembly either through actin nitration (Banan *et al.*, 2001) or through inhibition of the RhoA kinase pathway by cGMP (Sauzeau *et al.*, 2000). Thus, LPS may cause NO- or ATP-dependent disruption of the cytoskeleton, rendering SUR agents ineffective, while leaving pore blockers relatively unaffected. Indeed, we have previously shown that Glib is significantly less effective than PNU-37883A at inhibiting levcromakalim responses in the presence of LPS (Wilson & Clapp, 2002).

In summary, we have shown that the K$_{ATP}$ channel is an important mediator of LPS-induced vascular hyporeactivity in an *in vitro* model. Moreover, we have demonstrated for the first time that inhibitors binding to the K$_{ATP}$ channel pore rather than the SUR are effective at reversing vascular hyporeactivity. Whether this relates to a structural change or functional uncoupling between K$_{ATP}$ channel subunits remains to be determined.

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