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Glibenclamide-induced inhibition of the expression of inducible nitric oxide synthase in cultured macrophages and in the anaesthetized rat

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1 We have investigated whether glibenclamide, an inhibitor of ATP-sensitive potassium channels, influences the induction of the calcium-independent isoform of nitric oxide synthase (iNOS) in cultured J774.2 macrophages activated by bacterial endotoxin (*E.coli* lipopolysaccharide; LPS), as well as in the lung and aorta of rats with endotoxic shock.

2 Pretreatment of J774.2 macrophages with glibenclamide (10^{-7} to 10^{-5} M for 30 min) dose-dependently inhibited the accumulation of nitrite caused by LPS (1 $\mu g\,ml^{-1}$). In contrast, pretreatment of macrophages with tetraethylammonium (10^{-4} to 10^{-2} M for 30 min), a non-selective inhibitor of potassium channels, did not affect the rise in nitrite caused by LPS. At the highest concentration (10^{-5} M) used, cromakalim, an opener of ATP-sensitive potassium channels, caused a small, but significant inhibition of nitrite formation in macrophages activated with LPS, while lower concentrations (10^{-7} to 3×10^{-6} M) were without effect.

3 The inhibition by glibenclamide (3 μM) of the increase in nitrite induced by LPS in J774.2 macrophages was weaker when glibenclamide was given several hours after LPS, indicating that glibenclamide inhibits the induction, but not the activity, of iNOS. In contrast, the degree of inhibition of nitrite formation caused by the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was similar when this agent was given up to 10 h after LPS.

4 In anaesthetized rats, LPS caused a fall in mean arterial blood pressure (MAP) from 120 ± 4 (time 0) to 98 ± 4 mmHg at 180 min ($P<0.05$, $n=6$). Treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS) caused a rapid and sustained rise in MAP (e.g. MAP at 180 min after LPS: 122 ± 4 mmHg; $n=6$, $P<0.05$ when compared to LPS-rats). The maximum of the rise in MAP produced by glibenclamide (1 mg kg^{-1}, i.v.) was similar when the drug was given either at 60 or 180 min after LPS. However, the duration of the pressor response was significantly longer when glibenclamide was given at 60 min, rather than at 180 min after LPS.

5 LPS-treatment caused a significant reduction of the pressor responses elicited by noradrenaline (NA, 1 $\mu g\,kg^{-1}$, i.v.) from 35 ± 2 to 19 ± 1 mmHg at 60 min and 20 ± 2 mmHg at 180 min ($P<0.05$). Treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 60 min) caused a significant restoration of the pressor responses elicited by NA from 19 ± 1 mmHg at 60 min (prior to glibenclamide injection) to 29 ± 3 mmHg at 180 min ($P<0.05$).

6 Endotoxaemia for 180 min resulted in a significant increase in a calcium-independent NOS activity (which was taken to represent iNOS activity) in the lung from 0.17 ± 0.1 (control, $n=4$) to 6.21 ± 0.48 pmol $mg^{-1}\,min^{-1}$ ($n=6$, $P<0.05$). Injection of glibenclamide (1 mg kg^{-1}, i.v.) at 60 min after LPS attenuated the increase in iNOS activity caused by endotoxaemia in the lung by $43 \pm 7\%$ ($n=6$, $P<0.05$). In contrast, injection of glibenclamide at 180 min after LPS did not result in a significant inhibition of iNOS activity ($n=6$, $P<0.05$).

7 Thoracic aortae obtained from rats at 180 min after LPS showed a significant reduction in the contractions elicited by noradrenaline (NA, 10^{-9} to 10^{-6} M). Treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS) significantly alleviated this LPS-induced hyporeactivity to NA *ex vivo*. In contrast, when aortic rings from LPS-rats were incubated *in vitro* with glibenclamide (10 μM for 20 min), glibenclamide did not reverse the vascular hyporeactivity to NA. However, L-NAME (300 μM for 20 min) significantly enhanced the contractile response to NA in aortic rings obtained from LPS-rats ($P<0.05$, $n=6$).

8 No significant amounts of tumour necrosis factor-α (TNFα) were detectable in the plasma before the injection of LPS. Endotoxaemia for 90 min resulted in a significant rise in plasma TNFα levels (0.05 ± 0.05 ng ml^{-1} at time 0, 3.78 ± 0.24 ng ml^{-1} at 90 min, $n=6$, $P<0.05$). Treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 15 min prior to LPS, $n=5$) did not significantly reduce the rise in plasma TNFα levels caused by endotoxin.

9 Thus, glibenclamide inhibits the induction, but not the activity, of iNOS *in vitro* and *in vivo*. This inhibition of iNOS induction may contribute to the beneficial haemodynamic effects of glibenclamide in endotoxic shock.

Keywords: Nitric oxide synthase; glibenclamide; lipopolysaccharide, endotoxic shock

Introduction

Nitric oxide (NO) is a potent, endogenous vasodilator produced from L-arginine by NO synthase (NOS). Three different isoforms of NOS have been isolated, cloned, sequenced, and expressed. Under physiological conditions, the release of NO by the constitutive NOS present in the vascular endothelium (eNOS) dilates blood vessels, and, in

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concert with vasoconstrictors such as catecholamines, regulates blood vessel diameter, organ blood flow and blood pressure. Immunological stimuli including cytokines and endotoxin cause the expression of an inducible isoform of NOS (iNOS) which, once expressed, produces large amounts of NO. When produced in high local concentrations e.g. by cytokine-activated macrophages, NO is a cytotoxic molecule which kills bacteria and tumour cells. Thus, induction of iNOS in macrophages plays an important role in host defence (see Moncada & Higgs, 1993 for review).

Circulatory shock is characterized by severe hypotension, hyporeactivity of the vasculature to vasoconstrictor agents (vascular hyporeactivity), myocardial dysfunction, maldistribution in organ blood flow, and reduced tissue oxygen extraction, which ultimately lead to multiple organ failure and death (see Altura *et al.*, 1983). There is now good evidence that an enhanced formation of NO due to activation of eNOS (acute phase of shock) and induction of iNOS (delayed phase of shock) in the vascular smooth muscle makes an important contribution to hypotension and vascular hyporeactivity to catecholamines in various animal models of septic shock (see Thiemermann, 1994 for review). The induction of iNOS in septic shock is secondary to the stimulation by endotoxin (lipopolysaccharide, LPS) of the cytokines tumour necrosis factor-α (TNFα; Pittner & Spitzer, 1992; Thiemermann *et al.*, 1993) and interleukin-1 (IL-1; Moldawer *et al.*, 1993; Szabo *et al.*, 1993d) as well as the lipid mediator platelet-activating factor (PAF; Szabo *et al.*, 1993e), which either alone or in concert cause the expression of iNOS *in vivo*. The beneficial haemodynamic effects in animal models of septic shock of various drugs including dexamethasone (Wright *et al.*, 1992), monoclonal antibodies to TNFα (Thiemermann *et al.*, 1993), the endogenous IL-1 receptor antagonist (Szabo *et al.*, 1993d), the PAF receptor antagonist WEB 2086 (Szabo *et al.*, 1993e), and dihydropyridine-type calcium channel antagonists (Szabo *et al.*, 1993c) are, at least, in part due to the inhibition by these agents of the induction of iNOS *in vivo*.

Glibenclamide, an inhibitor of ATP-sensitive potassium (K^+_{ATP}) channels, exerts beneficial haemodynamic effects in dogs with septic shock (Landry & Oliver, 1992). Here, we investigate the effects of glibenclamide on the induction of iNOS caused by LPS in cultured macrophages and in the anaesthetized rat. To elucidate whether some of the observed effects of glibenclamide are due to the inhibition of K^+_{ATP} channels, we have compared the effects of glibenclamide on iNOS induction in macrophages activated with LPS with those elicited by tetraethylammonium, a non-selective inhibitor of potassium channels (see Robertson & Steinberg, 1990) and cromakalin, a potent activator of K^+_{ATP} potassium channels (Sanguinetti *et al.*, 1988).

An account of some of this work was recently presented to the British Pharmacological Society (Wu *et al.*, 1994).

Methods

Cell culture

The mouse macrophage cell line J774.2 was cultured and prepared as previously described (Szabo *et al.*, 1993d).

Nitrite production

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774.2 macrophages as described previously (Gross *et al.*, 1991). Briefly, the cells were cultured in 96-well plates with 200 μl of culture medium until cells reached confluence (approximately 60,000 cells per well). In order to induce iNOS, fresh culture medium containing LPS (1 μg ml^{-1}) was added. Nitrite accumulation in the medium was measured at 24 h after the application of LPS. To assess their effects on nitrite production, glibenclamide (10^{-7} to 10^{-5} M), tetraethylammonium (10^{-4} to 10^{-2} M) or cromakalim (10^{-7} to 10^{-5} M) was added at 30 min prior to LPS to cells.

In order to elucidate whether the inhibition of nitrite formation by glibenclamide in J774.2 macrophages activated with LPS is due to inhibition of iNOS induction or inhibition of iNOS activity, separate experiments were performed in which glibenclamide (3 μM) was given either together with LPS (1 μg ml^{-1}) or at 2, 4, 6 or 10 h after LPS. Agents which inhibit the induction of iNOS lose over time their ability to inhibit the increase in nitrite formation afforded by LPS, because the expression of iNOS in these cells is maximal after 6 to 10 h (Szabo *et al.*, 1993a). For comparison, we have also investigated the effect of the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), on the formation of nitrite by LPS-activated J774.2 macrophages. In these experiments, L-NAME (300 μM) was also given either together with LPS (1 μg ml^{-1}) or at 2, 4, 6 or 10 h after LPS.

Nitrite was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of medium. The optical density at 550 nm (OD_{550}) was measured with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Cell respiration

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Gross & Levi, 1992). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and cells were solubilized in dimethylsulphoxide (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550}.

Measurement of haemodynamic changes

Male Wistar rats (240–320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.). The trachea was cannulated and connected to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent). The right carotid artery was cannulated to a pressure transducer (P23XL, Spectramed, Statham, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. After recording baseline haemodynamic parameters, the pressor response to noradrenaline (NA 1 μg kg^{-1}, i.v.) was recorded. Ten minutes after injection of NA, animals were given *E.coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) as a slow injection over 10 min. The pressor responses to NA were reassessed at 60 min (immediately prior to injection of vehicle or glibenclamide), 120 min and 180 min. At 60 min after the injection of LPS, vehicle (10% dimethylsulphoxide, $n = 6$) or glibenclamide (1 mg kg^{-1}, i.v., $n = 6$) was administered to the rat as a single bolus injection and the haemodynamic parameters were monitored for another 120 min. A similar dose of glibenclamide exerts beneficial haemodynamic effects in dogs with endotoxic shock (Landry & Oliver, 1992). Assuming a blood volume of 60–70 ml kg^{-1} (16.8–19.6 ml of blood per rat), the injection of 1 mg kg^{-1} of glibenclamide should result in peak plasma concentrations of the drug of approximately 50–60 μg ml^{-1}. This plasma concentration of glibenclamide

is more than 100 fold higher than the one reported in patients with non-insulin-dependent diabetes mellitus treated with glibenclamide (2.5 mg day^{-1}, orally) (Ikegami *et al.*, 1986).

In a separate set of experiments, the magnitude and the duration of the rise in blood pressure elicited by glibenclamide (1 mg kg^{-1}, i.v.) were determined in rats treated with LPS (10 mg kg^{-1}, i.v.) for 180 min. To provide an indication of the duration of the pressor responses afforded by glibenclamide when given either at 60 or 180 min after LPS, we have defined the biological 'half life' ($t_{1/2}$) of the pressor response as the time which elapsed until the blood pressure had returned to 50% of the maximal response.

Organ bath experiments

At 180 min after the injection of LPS, thoracic aortae were obtained from sham-operated controls as well as from rats treated either with vehicle or with glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS). The vessels were cleared of adhering periadventitial fat and the thoracic aortae were cut into rings of 3–4 mm width. The endothelium was removed by gently rubbing the intimal surface. The lack of a relaxation to acetylcholine (1 μM) following precontraction of rings with noradrenaline (NA; 1 μM) was considered as evidence that the endothelium had been removed. The rings were mounted in 10 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Indomethacin (5.6 μM) was added to prevent the production of prostanoids. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA, U.S.A.) and recorded on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). A tension of 2 g was applied and the rings were equilibrated for 60 min. Dose-response curves to NA (10^{-9} to 10^{-6} M) in the presence or absence of L-NAME (300 μM) or glibenclamide (10 μM) were obtained in all experimental groups.

Nitric oxide synthase assay

Lungs from LPS-treated rats given vehicle (control) or glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS) were removed at 180 min and frozen in liquid nitrogen. In separate experiments, rats were treated with LPS (10 mg kg^{-1}, i.v.) for 180 min and then received glibenclamide (1 mg kg^{-1}, i.v.). After evaluating the $t_{1/2}$ of the pressor response elicited by glibenclamide (approx. 25 min), these animals were killed and the lungs were removed for the measurement of NOS activity. Lungs from sham-operated rats were also prepared for determination of baseline NOS activity. Lungs were stored for no more than 2 weeks at −80°C before assay. Frozen lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described by Szabo *et al.* (1993d). Briefly, tissue homogenates (30 μl, approx. 60 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with 5 mM EGTA, measured the calcium-independent NOS activity, which was taken to represent iNOS activity.

Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), with bovine serum albumin used as standard.

Measurement of plasma levels of tumour necrosis factor-α (TNFα)

Rats were anaesthetized and instrumented as above. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. Thereafter, 0.6 ml of blood was collected from a catheter placed in the carotid artery to measure the plasma levels of TNFα. Animals were treated with vehicle (10% dimethylsulphoxide) or glibenclamide (1 mg kg^{-1}, i.v.). At 15 min after the injection of drugs, LPS (10 mg kg^{-1}, i.v.) was administered as a slow injection over 10 min. Another blood sample (0.6 ml) for the measurement of plasma TNFα levels was obtained at 90 min after the injection of LPS, a time point at which the rise in TNFα after LPS is maximal (Lechner *et al.*, 1993). The amounts of TNFα in the plasma were measured by an enzyme linked immunoadsorbent assay (ELISA) as previously described (Lechner *et al.*, 1992).

Materials

Calmodulin, bacterial lipopolysaccharide (*E.coli* serotype 0.127:B8), NADPH, acetylcholine chloride, cromakalim, noradrenaline bitartrate, N^{ω}-nitro-L-arginine methyl ester, tetraethylammonium acetate and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset). Glibenclamide was purchased from Research Biochemical International (Natick, MA, U.S.A.). The Factor-Test-X Mouse TNFα ELISA Kit (Code 80-2802-00) was purchased from Genzyme Co. (Cambridge, MA, U.S.A.). Glibenclamide and cromakalim were dissolved in dimethysulphoxide then further diluted with saline or distilled water (final concentrations of dimethylsulphoxide are less than 10%). All other solutions were made in saline or distilled water. L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire). Tetrahydrobiopterin (6R-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical evaluation

All values in the figures and text are expressed as mean ± s.e.mean of n observations, where n represents the number of animals or plates (3 wells in each plate) studied. A one-way or two-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni's test for multiple comparisons was used to compare means between groups. A P value less than 0.05 was considered to be statistically significant.

Results

Glibenclamide reduces the increase in nitrite caused by LPS in the supernatant of cultured macrophages

Activation of J774.2 macrophages with LPS (1 μg ml^{-1}) resulted in a significant increase in nitrite concentration in the cell supernatant from 1.5 ± 0.4 μM (control cells treated with vehicle, but not LPS) to 50.8 ± 2.5 μM at 24 h after addition of LPS. Pretreatment of cells with glibenclamide (10^{-7} to 10^{-5} M at 30 min prior to LPS) reduced the increase in nitrite formation caused by LPS in a dose-dependent manner (Figure 1a). In contrast, pretreatment of macrophages with tetraethylammonium (10^{-14} to 10^{-2} M at

30 min prior to LPS) did not affect the increase in nitrite concentration in the supernatant of cells activated with LPS (Figure 1b). Pretreatment of cells with the highest dose of cromakalim used (10^{-5} M), caused a small, but significant inhibition of the increase in nitrite concentration afforded by LPS, while lower concentrations of this activator of K^+_{ATP} potassium channels (10^{-7} to 3×10^{-6} M) were without effect (Figure 1c). Glibenclamide, tetraethylammonium or cromakalim did not affect the viability of J774.2 macrophages (as determined by the MTT assay) when given alone or in combination with LPS (data not shown).

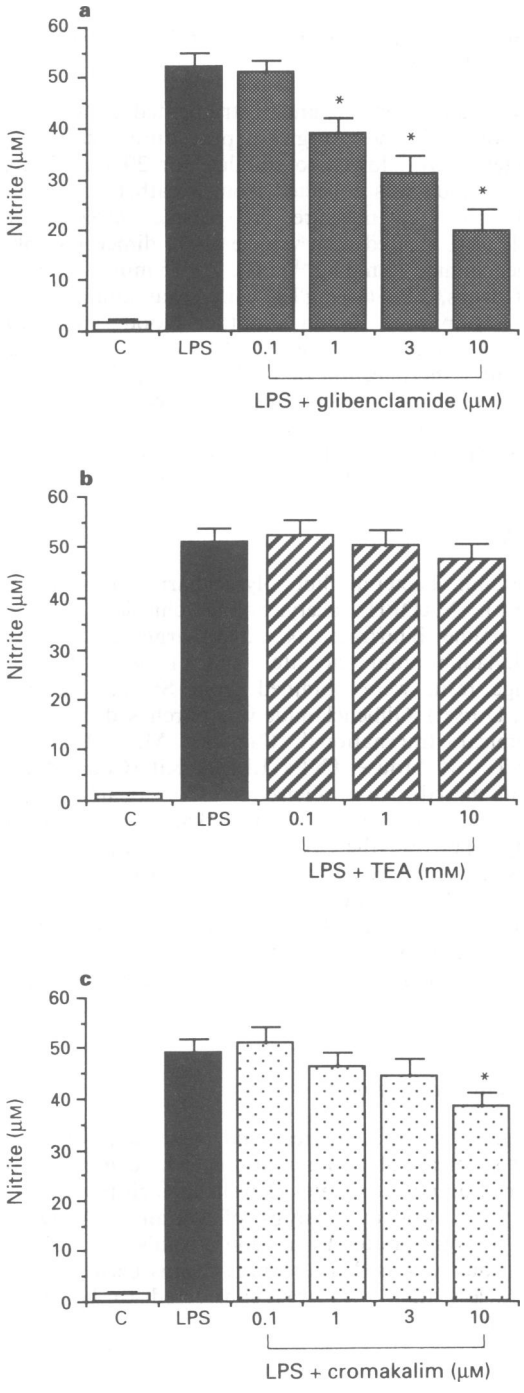

Figure 1 Glibenclamide causes a dose-dependent inhibition of nitrite formation caused by *E.coli* lipopolysaccharide (LPS). Nitrite formation was measured in J774.2 macrophages which were cultured in 96-well plates to confluence and incubated with LPS (1 μg ml^{-1} for 24 h). Depicted is nitrite formation by J774.2 macrophages incubated with culture medium alone (control, C; open columns; $n = 5$) for 24 h or cells treated with LPS alone (solid columns; $n = 5$) or with LPS plus treatment with glibenclamide (a: stippled columns; 10^{-7} to 10^{-5} M at 30 min prior to LPS, $n = 5$), tetraethylammonium (b: TEA; hatched columns; 10^{-4} to 10^{-2} M at 30 min prior to LPS, $n = 5$), or cromakalim (c: dotted columns; 10^{-7} to 10^{-5} M at 30 min prior to LPS, $n = 5$). Data are expressed as mean ± s.e.mean of $n = 5$ independent experiments. $^*P < 0.05$ represents significant difference between cells subjected to LPS with and without glibenclamide or cromakalim.

Glibenclamide inhibits the induction of iNOS, but not its activity

The inhibition by glibenclamide (3 μM; approx. EC_{50}) of the increase in nitrite formation became progressively weaker when glibenclamide was added to the cells at 2, 4, 6 or 10 h after LPS (Figure 2a). Although the addition of glibenclamide at 2 or 4 h after LPS still caused a partial inhibition of the accumulation of nitrite, addition of glibenclamide at 6 or 10 h after LPS had no effect on the increase in nitrite in the supernatant of macrophages activated with LPS (Figure 2a). In contrast, the NOS inhibitor L-NAME caused a similar degree of inhibition of nitrite accumulation when given either together with or at 2, 4, 6 or 10 h after LPS (Figure 2b).

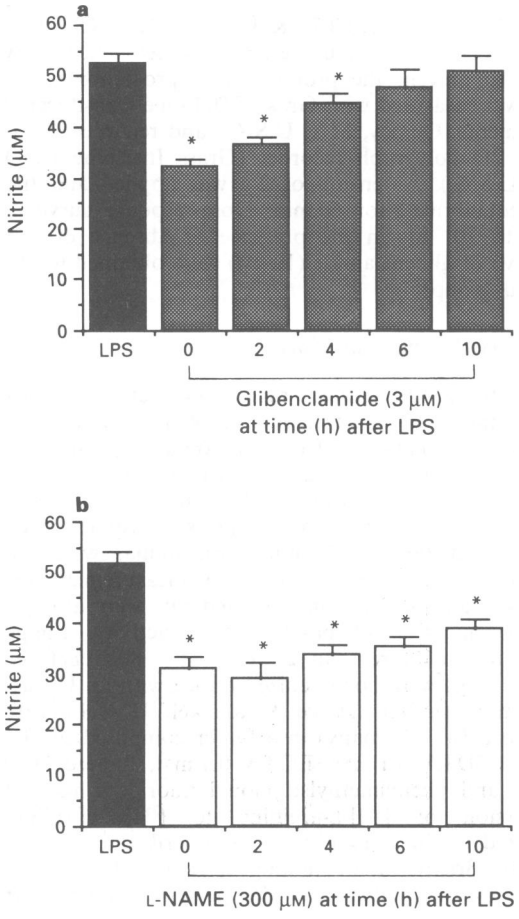

Figure 2 The inhibition by glibenclamide of the nitrite formation produced by *E.coli* lipopolysaccharide (LPS) is time-dependent. Nitrite formation was significantly increased in J774.2 macrophages incubated with LPS (1 μg ml^{-1} for 24 h; solid column; $n = 5$). In separate experiments, glibenclamide (a: 3 μM; stippled columns) or N^G-nitro-L-arginine methyl ester (L-NAME) (b: L-NAME, 300 μM; open columns) was given either together with LPS, or at 2, 4, 6 or 10 h after LPS ($n = 5$ at each time point). Data are expressed as mean ± s.e.mean of $n = 5$ independent experiments. $^*P < 0.05$ represents significant difference between cells treated with LPS with and without glibenclamide or L-NAME.

Glibenclamide exerts beneficial haemodynamic effects in rats with endotoxic shock

Baseline values of MAP ranged from 120 ± 4 to 126 ± 4 mmHg and heart rate from 375 ± 25 to 388 ± 15 beats min^{-1} and were not significantly different between any of the experimental groups studied. The injection of LPS (10 mg kg^{-1}, i.v.) resulted in a rapid fall of MAP from 120 ± 4 mmHg (time 0, prior to the injection of LPS) to 83 ± 5 mmHg at 10 min ($P<0.05$, $n=6$). Thereafter, MAP remained significantly lower than baseline in LPS-rats and hence, was 98 ± 4 mmHg at 180 min after the injection (Figure 3a). Three hours of endotoxaemia was, however, not associated with a significant tachycardia (time 0: 375 ± 25, time 180 min: 399 ± 21 beats min^{-1}, $P>0.05$, $n=6$). In addition, endotoxaemia resulted in a substantial attenuation of the pressor responses elicited by NA (1 μg kg^{-1}, i.v.) from 35 ± 2 mmHg (before LPS) to 17 ± 1 mmHg at 60 min and 20 ± 2 mmHg at 180 min after LPS injection ($P<0.05$, Figure 3b).

Administration of glibenclamide (1 mg kg^{-1}, i.v.) at 60 min after the onset of endotoxaemia resulted in a rapid (within 5 min) and sustained increase in MAP (Figure 3a). Thus, the MAP of LPS-rats treated with glibenclamide was significantly higher than in the LPS-control group at 65, 90, 120, 150 and 180 min. Although glibenclamide caused a small rise in heart rate, the heart rate of LPS-rats treated with glibenclamide was not significantly different from the heart rate in vehicle-treated LPS-rats (the former: 406 ± 11 beats min^{-1}, the latter: 364 ± 29 beats min^{-1}, $P>0.05$, $n=6$). In the glibenclamide-treated group, injection of LPS also caused an attenuation of the pressor responses elicited by NA from 38 ± 2 mmHg (before LPS) to 19 ± 1 mmHg at 60 min (before glibenclamide). Treatment of LPS-rats with glibenclamide enhanced the pressor responses afforded by NA at 180 min, but not at 120 min after injection of LPS (Figure 3b). Thus, the rise in MAP caused by NA at 180 min in LPS-rats treated with glibenclamide was significantly greater than in animals treated with LPS alone ($P<0.05$). When compared to the pressor responses elicited by NA prior to injection of LPS, however, the increase in MAP caused by NA at 180 min after LPS in rats treated with glibenclamide was still significantly reduced ($P<0.05$, Figure 3b).

In order to evaluate whether, and to what degree, the rise in MAP afforded by glibenclamide is due to inhibition of iNOS induction, rats were treated with LPS for 180 min, since this period of endotoxaemia results in a near maximal induction of iNOS activity in the lung of the rat (Szabo *et al.*, 1993d). In these experiments, the injection of LPS caused a fall in MAP from 122 ± 4 to 94 ± 6 mmHg by 180 min. The subsequent injection of glibenclamide (1 mg kg^{-1}, i.v.) resulted in a rapid, but relatively transient rise in MAP. The maximum of the rise in MAP afforded by glibenclamide when given at 180 min after LPS (23 ± 3 mmHg, $n=5$) was not different from the maximal pressor response caused by this agent when given at 60 min after LPS (25 ± 4 mmHg, $n=6$, $P>0.05$). However, the duration of this pressor response was significantly longer when glibenclamide was given at 60 min (biological half life, $t_{1/2}>120$ min) rather than at 180 min after the injection of LPS ($t_{1/2}$: 22 ± 2 min, $P<0.05$).

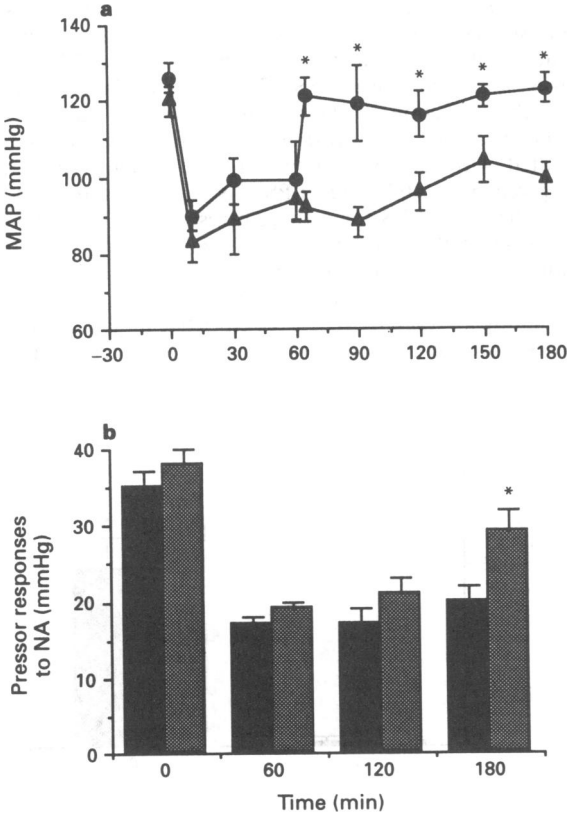

Figure 3 Glibenclamide ameliorates the delayed hypotension caused by endotoxin in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure (MAP) and (b) pressor responses to noradrenaline (NA; 1 μg kg^{-1}, i.v.) in rats treated with *E.coli* lipopolysaccharide (LPS) (10 mg kg^{-1}, i.v. at time 0). Different groups of animals were treated with vehicle (10% dimethylsulphoxide, ▲ or solid column; $n=6$) or glibenclamide (1 mg kg^{-1} i.v., ● or stippled column; $n=6$) at 60 min after LPS. Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ represents significant difference when compared to LPS-controls at the same time point.

Glibenclamide attenuates the induction of iNOS in lungs from rats with endotoxic shock

A small calcium-independent iNOS activity was detectable in lung homogenates obtained from sham-operated animals. Endotoxaemia for 180 min was associated with a significant increase of iNOS activity in lung homogenates. However, the iNOS activity in lung homogenates obtained from LPS-rats which had received glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS) was significantly lower than that in rats treated with LPS alone. In contrast, the injection of glibenclamide at

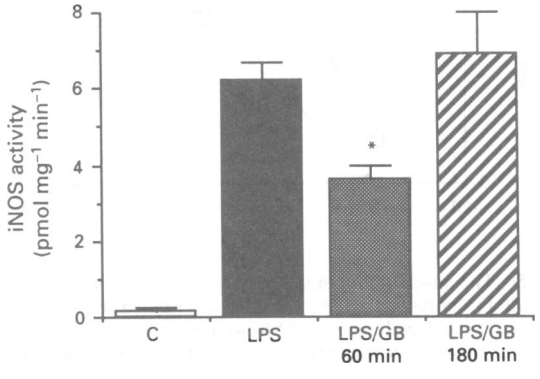

Figure 4 Treatment of rats with glibenclamide inhibits the induction of a calcium-independent iNOS activity in lung homogenates obtained from rats with endotoxaemia. Calcium-independent iNOS activity was measured in lung homogenates obtained from sham-operated control rats (C, open column; $n=4$) or rats treated with *E.coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) for 180 min. Different groups of LPS-rats were treated with vehicle (LPS, solid column; $n=6$) or with glibenclamide (LPS/GB; 1 mg kg^{-1}, i.v.) given either at 60 min (stippled column; $n=6$) or at 180 min (hatched column; $n=6$) after LPS. Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ represents a significant reduction in iNOS activity when compared to LPS-rats.

180 min after the onset of endotoxaemia did not reduce the degree of iNOS activity in the lung when compared to rats treated with LPS alone (Figure 4).

Treatment of rats with glibenclamide attenuates the vascular hyporeactivity of rat aortic rings to noradrenaline ex vivo

In rat aortic rings obtained from sham-operated control rats, NA (10^{-9} to 10^{-6} M) caused a dose-related increase in vascular tone (Figure 5). The contractions induced by NA were significantly reduced in aortic rings obtained from rats at 180 min after the injection of LPS (Figure 5). In contrast, treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 60 min after injection of LPS) caused a partial, but not complete, reduction of this vascular hyporeactivity to NA ($P<0.05$, when compared to LPS-controls).

Treatment of rat aortic rings obtained from sham-operated control animals with either glibenclamide (10 μM) or the NOS inhibitor, L-NAME (300 μM) *in vitro* did not affect the contractions elicited by NA (data not shown). Incubation of rat aortic rings obtained from LPS-rats with glibenclamide (10 μM for 20 min) *in vitro* did not enhance the contraction to NA in these vessels and hence, did not affect the vascular hyporeactivity caused by LPS. In contrast, L-NAME (300 μM for 20 min) significantly enhanced the contractile response to NA in aortic rings obtained from LPS-rats ($P<0.05$, Figure 5b).

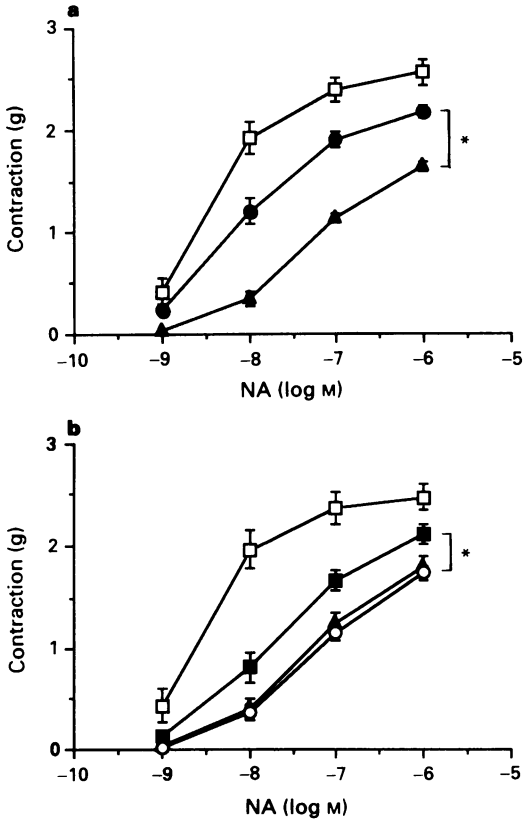

Figure 5 The vascular hyporeactivity to noradrenaline (NA) caused by *E.coli* lipopolysaccharide (LPS) is attenuated by treatment of LPS-rats with glibenclamide. (a) Shows dose-response curves to NA (10^{-9} to 10^{-6} M) in aortic rings without endothelium obtained from sham-operated rats (□; $n=6$), from rats treated with LPS (10 mg kg^{-1}, i.v.) for 180 min (▲; $n=6$) or from LPS-rats treated with glibenclamide (1 mg kg^{-1}, i.v. at 60 min after LPS, ●; $n=6$). (b) Depicts dose-response curves to NA (10^{-9} to 10^{-6} M) in aortic rings obtained from sham-operated rats (□; $n=6$), from rats treated with LPS (10 mg kg^{-1}, i.v.) for 180 min which were subsequently treated *in vitro* with vehicle (▲; $n=6$), glibenclamide (10 μM for 20 min, ○; $n=6$) or N^G-nitro-L-arginine methyl ester (L-NAME) (300 μM for 20 min, ■; $n=6$). Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents significant differences between LPS-rats treated with vehicle and those treated with glibenclamide (a) or between aortic rings obtained from LPS-rats treated with vehicle and L-NAME *in vitro* (b).

Glibenclamide does not affect the rise of TNFα levels caused by endotoxaemia

The surgical procedure alone did not result in a significant rise in plasma TNFα levels, because no significant amounts of TNFα were detectable at the end of the stabilization period. Endotoxaemia for 90 min, however, resulted in a significant rise in plasma TNFα levels (Figure 6). Treatment of LPS-rats with glibenclamide (1 mg kg^{-1}, i.v. at 15 min prior to LPS) did not affect the rise in plasma TNFα levels caused by endotoxin (Figure 6).

Discussion

Here we demonstrate that glibenclamide, an inhibitor of K^+_{ATP} channels, inhibits the induction of iNOS caused by LPS in cultured macrophages and in the anaesthetized rat. In addition, we demonstrate that the beneficial haemodynamic effects produced by glibenclamide in rats with septic shock are, in part, due to inhibition of iNOS induction.

Activation of J774.2 macrophages with LPS results in the accumulation of nitrite, one of the metabolites of NO, in the supernatant of these cells. This accumulation of nitrite reflects an enhanced formation of NO due to the induction of iNOS by LPS in these cells (Szabo *et al.*, 1993a). This study demonstrates that glibenclamide dose-dependently inhibits the accumulation of nitrite in the medium of J774.2 macrophages activated with LPS. Thus, glibenclamide inhibits either the induction or the activity of iNOS. The inhibition by glibenclamide of the formation of nitrite by macrophages activated with LPS was, however, lost when glibenclamide was given 6 or 10 h after LPS. As the exposure of J774.2 macrophages to LPS for 6 to 10 h results in a near maximal expression of iNOS in these cells (Szabo *et al.*, 1993a), this finding shows that glibenclamide inhibits the induction, but not the activity, of iNOS. This conclusion is supported by our finding that L-NAME, a competitive inhibitor of NOS activity (Moore *et al.*, 1990), caused a similar degree of

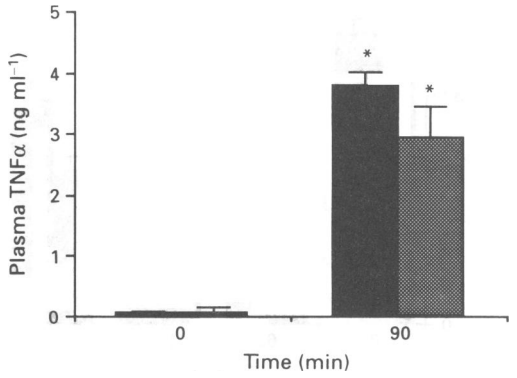

Figure 6 The increase in plasma tumour necrosis factor α (TNFα) levels caused by endotoxin is not affected by pretreatment of *E.coli* lipopolysaccharide (LPS)-rats with glibenclamide. Depicted are the changes in plasma TNFα levels in rats treated with LPS (10 mg kg^{-1}, i.v.). Different groups of animals were pretreated either with vehicle (10% dimethylsulphoxide, solid columns; $n=6$) or glibenclamide (1 mg kg^{-1}, i.v. at 15 min prior to LPS, stippled columns; $n=5$). Note that injection of LPS causes a significant increase in the plasma levels of TNFα; and that this increase was not significantly affected by pretreatment of rats with glibenclamide. Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents significant differences when compared to LPS-controls at time 0.

inhibition of nitrite accumulation in macrophages activated with LPS when given either together with LPS or at 2, 4, 6 or 10 h after LPS. Similarly, other inhibitors of iNOS induction including dexamethasone (Szabo *et al.*, 1993a), interleukin-10 (Cunha *et al.*, 1992), dihydropyridine-type calcium channel antagonists (Szabo *et al.*, 1993c) or spermine (Szabo *et al.*, 1994b) are also less potent in inhibiting the formation of nitrite by cells activated with LPS or cytokines when given several hours after the stimulus of iNOS expression.

Prolonged periods of endotoxaemia in the anaesthetized rat result in the induction of iNOS in several organs including the lung (Knowles *et al.*, 1990; Szabo *et al.*, 1993b), and the consequent enhanced formation of NO by iNOS in the vascular smooth muscle contributes importantly to the delayed hypotension and the vascular hyporeactivity to vasoconstrictor agents (Szabo *et al.*, 1993b). Injection of glibenclamide at 60 min after LPS attenuated the increase in iNOS activity in the lung seen in animals treated with LPS alone. This effect of glibenclamide is due to inhibition of iNOS induction rather than inhibition of iNOS activity, as injection of glibenclamide at 180 min after LPS, a time point at which a near maximal induction of iNOS has already occurred (Szabo *et al.*, 1993b), did not reduce iNOS activity in the lung. In contrast, inhibitors of NOS activity including N^G-methyl-L-arginine (Szabo *et al.*, 1993a), aminoguanidine (Wu *et al.*, 1995) or S-methyl-isothiourea sulphate (Szabo *et al.*, 1994a) also inhibit iNOS activity in the lung of rats when given several hours after the injection of LPS. In addition, treatment of rats with glibenclamide *in vivo* (at 60 min after LPS) also prevented the vascular hyporeactivity to noradrenaline in rat aortic rings *ex vivo*. Again, this effect of glibenclamide is due to inhibition of iNOS induction, rather than activity, because treatment *in vitro* of aortic rings obtained from LPS-treated rats with the NOS inhibitor, L-NAME, but not with glibenclamide, partially restored the contractile response caused by NA.

There is a good correlation between the degree of iNOS induction in the lung and the magnitude of the fall in blood pressure caused by endotoxaemia in the rat (Szabo *et al.*, 1993e). Moreover, the beneficial haemodynamic effects in animal models of septic shock given by dexamethasone (Wright *et al.*, 1992), PAF-receptor antagonists (Szabo *et al.*, 1993e), antibodies to TNFα (Thiemermann *et al.*, 1993), IL-1 receptor antagonists (Szabo *et al.*, 1993d) or dihydropyridine-type calcium channel antagonists (Szabo *et al.*, 1993c) are partly due to inhibition of iNOS induction *in vivo*. Thus, the beneficial haemodynamic effects caused by glibenclamide may be, at least in part, due to inhibition of iNOS induction. This hypothesis is supported by our findings that (i) the duration of the rise in blood pressure caused by glibenclamide in rats with septic shock was significantly longer (> 120 min) when the potassium channel antagonist was given at 60 min, rather than at 180 min after LPS; (ii) that glibenclamide prevented the induction of iNOS when given at 60 min, but not when given at 180 min after LPS; and (iii) that glibenclamide restored the pressor responses to NA at 180 min after LPS *in vivo*. There is now a substantial amount of evidence that this delayed (at 180 min after LPS) vascular hyporeactivity to NA is due to an enhanced formation of NO due to the induction of iNOS (see Thiemermann, 1994). Moreover, agents which prevent the induction of iNOS including dexamethasone (Szabo *et al.*, 1993b), antibodies to TNFα (Thiemermann *et al.*, 1993), IL-1 receptor antagonists (Szabo *et al.*, 1993d), PAF receptor antagonists (Szabo *et al.*, 1993e) or dihydropyridine-type calcium channel antagonists (Szabo *et al.*, 1993c) prevent the development of this delayed vascular hyporeactivity to NA. Thus, the inhibition of iNOS expression contributes to the improved haemodynamics (rise in blood pressure, restoration of pressor responses to NA) caused by glibenclamide at 180 min after LPS injection.

In addition, our findings (i) that injection of glibenclamide at 180 min after LPS did not attenuate the induction of iNOS in the lung, but still caused a transient rise in blood pressure and (ii) that at 60 min after injection of glibenclamide (120 min after LPS) the blood pressure was rapidly elevated, but the vascular hyporeactivity to NA was unaffected, clearly demonstrate that the early rise in blood pressure caused by glibenclamide in septic rats is partly due to effects other than inhibition of iNOS induction. This is not surprising, since (i) septic shock causes inadequate tissue perfusion, anaerobic metabolism and acidosis (Hopkins *et al.*, 1965; Kaufman *et al.*, 1984; Keung & Li, 1991); (ii) a decrease in intracellular ATP (anaerobic metabolism) (Noma, 1983; Deutsch *et al.*, 1991) or intracellular acidosis (Davies, 1990; Cuevas *et al.*, 1991) facilitates the activation of K^+_{ATP} channels; and (iii) activation of K^+_{ATP} channels results in vasodilatation (Standen *et al.*, 1989). Thus, it has been suggested that the beneficial haemodynamic effects of glibenclamide in dogs with septic shock are due to inhibition of the activation of K^+_{ATP} channels occurring secondary to lactic acidosis (Landry & Oliver, 1992). Interestingly, the rise in systemic vascular resistance caused by glibenclamide is significantly greater in dogs with septic shock than in dogs with lactic acidosis caused by hypoxia (Landry & Oliver, 1992), although both conditions lead to lactic acidosis and, hence, presumably the activation of K^+_{ATP} channels. In contrast to septic shock, ischaemia of the kidney which also causes local lactic acidosis does not result in the induction of iNOS in the rat (Cristol *et al.*, 1993). Thus, it is conceivable that the rise in systemic vascular resistance and, hence, blood pressure caused by glibenclamide in animals with septic shock (Landry & Oliver, 1992; this study) is due to a combination of inhibition of iNOS induction and inhibition of K^+_{ATP} channels. The relatively small and transient increase in systemic vascular resistance afforded by glibenclamide in dogs with hypoxic lactic acidosis (Landry & Oliver, 1992) or in rats with prolonged periods (> 180 min) of endotoxaemia, on the other hand, may well be due to inhibition of K^+_{ATP} channels.

Endotoxaemia in the rat causes the expression of cyclooxygenase-2 (COX-2) (Swierkosz *et al.*, 1994), which results in an enhanced formation of prostaglandins (PGs) and/or thromboxane A_2. Interestingly, glibenclamide (0.3 μM) attenutated the vasodilator effects of prostacyclin, PGE_2 and PGD_2 in the isolated perfused heart of the rat (Jackson *et al.*, 1993; Bouchard *et al.*, 1994). In isolated ring segments of the circumflex coronary artery of the dog, glibenclamide (1 to 30 μM) also caused a concentration-dependent reduction in both spontaneous isometric force and contractions induced by the thromboxane A_2-mimetic, U46619, suggesting that glibenclamide is a relatively potent and selective antagonist of thromboxane A_2 receptors (Cocks *et al.*, 1990). Thus, it is possible that some of the beneficial effects of glibenclamide in animal models with septic shock are due to the inhibition by glibenclamide of either the vasoactive effects of vasodilator PGs and/or thromboxane A_2 or even prevention of COX-2 induction.

What is the mechanism by which glibenclamide inhibits the induction of iNOS *in vitro* and *in vivo*? One could argue that this effect of glibenclamide is due to inhibition of K^+_{ATP} channels. This, however, cannot be the case, for (i) tetraethylammonium, a non-selective inhibitor of potassium channels (Robertson & Steinberg, 1990), did not inhibit the induction of iNOS caused by LPS in cultured macrophages; and (ii) cromakalim (at the highest concentration used), an activator of K^+_{ATP} channels (Sanguinetti *et al.*, 1988), caused a small, but significant inhibition of iNOS induction in these cells. Thus, the inhibition of iNOS induction afforded by glibenclamide *in vitro* is independent of the effects of this agent on K^+_{ATP} channels. Another possibility by which glibenclamide might inhibit the induction of iNOS afforded by LPS *in vivo*, would be the prevention of the release of cytokines such as TNFα and IL-1, because these cytokines mediate the induction of iNOS caused by LPS in the rat (Szabo *et al.*, 1993d; Thiemermann *et al.*, 1993). We demonstrate here, however, that pretreatment of rats with gliben-

clamide did not attenuate the rise in plasma TNFα levels caused by LPS. Taken together with our *in vitro* results, we speculate that glibenclamide inhibits the transcription or translation of iNOS.

Thus, glibenclamide inhibits the induction of iNOS caused by LPS in cultured macrophages and in the anaesthetized rat. The mechanism of this effect is unclear, but is not due to inhibition by glibenclamide of (i) K^+_{ATP} channels or (ii) the release of TNFα caused by LPS *in vivo*. The beneficial haemodynamic effects caused by glibenclamide in rats with septic shock are, in part, due to inhibition of iNOS induction.

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