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Beneficial effects of dantrolene on lipopolysaccharide-induced haemodynamic alterations in rats and mortality in mice

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Abstract

We investigated the effect of dantrolene, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, on the induction of nitric oxide (NO) synthase II by bacterial endotoxin (lipopolysaccharide) in the anaesthetised rat and on survival in a murine model of severe endotoxaemia. Injection of lipopolysaccharide (i) induced biphasic changes of rectal temperature and blood glucose: an initial increased phase (<180 min after injection of lipopolysaccharide) followed by a decreased phase (at 240–360 min), (ii) caused a fall in mean arterial blood pressure from 115 ± 3 mmHg (time 0) to 83 ± 6 mmHg at 360 min, (iii) resulted in a substantial hyporeactivity to noradrenaline (1 μg/kg i.v.), (iv) raised plasma nitrate (an indicator of NO formation) in a time-dependent manner, (v) elicited a significant increase in NO synthase II activity in the lung and (vi) caused a 80% lethality (in mice). Pretreatment of animals with dantrolene not only attenuated the delayed circulatory failure, but also prevented the overproduction of NO and the induction of NO synthase II caused by lipopolysaccharide in the rat, and improved survival in a murine model of severe endotoxaemia. Thus, dantrolene has beneficial haemodynamic effects in animals with endotoxin shock. We propose that a decrease of free cytosolic Ca^{2+} levels plays an important role in the prevention of NO synthase II induction.

Keywords: Dantrolene; Nitric oxide (NO) synthase II; Lipopolysaccharide; Mortality; Hemodynamic alteration

1. Introduction

A diminished reactivity to several vasopressor agents including Ca^{2+} occurs in blood vessels obtained from animals with endotoxic shock (Parratt, 1989) and in blood vessels exposed to endotoxin (lipopolysaccharide) in vitro (Fleming et al., 1990; Gray et al., 1990; Rees et al., 1990). The development of this hyporeactivity to Ca^{2+} under depolarizing conditions or in the presence of a Ca^{2+} ionophore suggests that post receptor mechanisms are also impaired (Bigaud et al., 1990). It has been proposed that the decreased Ca^{2+} efflux seen in sepsis is due to a decrease of intracellular Ca^{2+} in response to noradrenaline (Litten et al., 1988). Since the sarcoplasmic reticulum is a major determinant of intracellular free Ca^{2+} concentrations in vascular smooth muscle cells (Bohr, 1963; Van Breemen et al., 1986, 1995), changes in the release and uptake of Ca^{2+} from the sarcoplasmic reticulum could be a major factor associated with the decreased contractility in septic shock.

In addition, an enhanced formation of nitric oxide (NO) in response to lipopolysaccharide is also associated with the development of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxin shock (Kilbourn et al., 1990; Thiemermann and Vane, 1990; Gray et al., 1991; Wright et al., 1992; Szabo et al., 1993b). This overproduction of NO in endotoxaemic animals is due to the induction of NO synthase (i.e., inducible NO synthase or NO synthase II) (see Moncada et al., 1991). NO synthase II is different from other NO synthase isoforms, for its activation is independent of changes in free cytosolic Ca^{2+}. Park et al. (1995) have demonstrated that a rapid increase in cytosolic free Ca^{2+} is associated with the induction of NO synthase II in murine peritoneal macrophages, suggesting that the induction of NO synthase II is dependent on free cytosolic Ca^{2+} levels. Indeed, several different Ca^{2+} channel antagonists protect against cardiovascular failure and prolong survival time in various models of endotoxin shock (Bosson et al., 1985; Lee and Lum, 1986; Szabo et al., 1993a).

However, agents which directly decrease free cytosolic Ca^{2+} have not been evaluated. Dantrolene, an agent which suppresses the release of Ca^{2+} from the sarcoplasmic

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reticulum, reduces free cytosolic Ca^{2+} concentrations (Ellis and Bryant, 1972; Hainaut and Desmedt, 1974; Van Winkle, 1976). Here, we further examine the effect of dantrolene on (i) the haemodynamic alterations, (ii) the induction of NO synthase II in endotoxaemic rats and (iii) on the mortality induced by lipopolysaccharide in mice.

2. Materials and methods

2.1. In vivo experiments

Ten-week-old male Wistar-Kyoto (WKY) rats, whose stock originated from the Charles River Breeding Laboratories in Japan, were purchased from the Department of Laboratory Animal Science of the National Defense Medical Centre. Rats were anaesthetised by intraperitoneal injection of urethane (1.2 g/kg). The trachea was cannulated to facilitate respiration and environmental temperature was maintained at 24°C with an air-conditioning system. A thermometer was placed into the rectum to record the rectal temperature of animals. The right carotid artery was cannulated and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, USA) for the measurement of phasic and mean arterial blood pressure and heart rate which were displayed on a Gould model TA5000 polygraph recorder (Gould, Valley View, OH, USA). The left jugular 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, animals were given noradrenaline (1 μg/kg i.v.), and 10 min later animals received vehicle (saline) or *Escherichia coli* lipopolysaccharide (10 mg/kg i.v.) and were monitored for 360 min. The pressor responses to noradrenaline were reassessed at every hour after vehicle or lipopolysaccharide injection. Prior to (i.e., at time 0) and at every hour after vehicle or lipopolysaccharide, 0.3 ml of blood was taken to measure the changes in blood glucose and the plasma levels of nitrate. Any blood withdrawn was immediately replaced by the injection of an equal amount of saline (i.v.). In another group of animals, dantrolene (1 mg/kg i.v.) was administered at 15 min prior to the injection of lipopolysaccharide. All haemodynamic and biochemical parameters were recorded for 6 h in both of the above animal groups.

In a separate experiment, a group of rats was anaesthetised, instrumented (as above) and treated with dantrolene (1 mg/kg i.v.) only. All haemodynamic and biochemical parameters were also recorded for 6 h.

2.2. Determination of blood glucose and plasma nitrate

Before the blood sample was centrifuged (10 000 rpm for 3 min) to prepare plasma, 15 μl of whole blood was taken to measure the blood levels of glucose by means of a 'One Touch II' blood glucose monitoring system (Lifescan, Milpitas, CA, USA) and the plasma was kept in a −20°C freezer. At a later stage, plasma samples were thawed and de-proteinized by incubating them with 95% ethanol (4°C) for 30 min. The samples were subsequently centrifuged for a further 7 min at 13 000 rpm. It is noted that the nitrate concentration in plasma depicted in the study is actually the total nitrite and nitrate concentration in plasma. In this method nitrate is reduced to NO via nitrite. The amounts of nitrate in the plasma (2 μl) were measured by adding a reducing agent (0.8% VCl_3 in 1 M HCl) to the purge vessel to convert nitrate to NO which was stripped from the plasma by using a helium purge gas. The NO is then drawn into the Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate (Sigma, St. Louis, MO, USA).

2.3. Nitric oxide synthase assay

Lungs from all groups of animals were removed at 360 min after vehicle or lipopolysaccharide injection and frozen in liquid nitrogen. Lungs were stored for no more than 2 weeks at −80°C before assay. Frozen samples were homogenized on ice with an polytron PT MR 3000 homogenizer (Kinematic, Littau, Germany) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonyl fluoride 1 (pH 7.4). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as described in a previous study (Wu et al., 1995). 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 Ca^{2+} (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 (LS6000TA; Beckman, Fullerton, CA, USA). Experiments performed in the absence of NADPH determined the extent of [^{3}H]L-citrulline formation independent of a specific NO synthase activity. Experiments in the presence of NADPH, without Ca^{2+} and with EGTA (5 mM), measured the Ca^{2+}-independent NO synthase activity, which was taken to represent NO synthase II activity.

2.4. Survival studies

Survival studies were performed in ICR mice (28–32 g), whose stock originated from the Institute of Cancer Research of the National Institute of Health (Bethesda, MD, USA), were purchased from the National Animal Centre (Taipei, Taiwan). Lipopolysaccharide (60 mg/kg i.p.) was injected in the presence of vehicle or drugs and survival was monitored every 3 h until 15 h, then again at

24 h. Different groups of animals received vehicle (saline) together with lipopolysaccharide ($n = 15$) or lipopolysaccharide plus dantrolene (1 mg/kg at time 0 and 6 h after lipopolysaccharide, $n = 15$) as in a previous study (Wu et al., 1995).

2.5. Chemicals

Calmodulin, bacterial lipopolysaccharide (*Escherichia coli* serotype 0.127:B8), NADPH, noradrenaline bitartrate and Dowex 50W anion exchange resin were obtained from Sigma. Dantrolene sodium was purchased from Eaton Laboratories (Norwich, NY, USA). L-[2,3,4,5-^{3}H]Arginine hydrochloride was obtained from Amersham (Amersham, UK). Tetrahydrobiopterin (6*R*-5,6,7,8-tetrahydro-L-biopterin dihydrochloride) was obtained from Research Biochemicals International (Natick, MA, USA).

2.6. Statistical analysis

All values in the figures and text are expressed as mean ± S.E.M. of *n* observations, where *n* represents the number of animals studied. Statistical evaluation was performed by using analysis of variance (ANOVA) followed by a multiple comparison test (Scheffé's test), except for the determination of NO synthase II activity which was analysed by unpaired Student's *t*-test. The chi-square test was used for determining the significant differences in the survival rate between control and drug-treated groups. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of dantrolene on alterations of body temperature and blood glucose caused by endotoxin in *vivo*

Baseline values for temperature and blood glucose of the animal groups treated with vehicle (sham-operated), vehicle plus lipopolysaccharide (10 mg/kg i.v.), or dantrolene (1 mg/kg i.v.) plus lipopolysaccharide were between 36.7 ± 0.2 and 36.8 ± 0.4°C, and 179 ± 9 and 196 ± 15 mg/dl, respectively, which were not significantly different between groups (Fig. 1). Administration of lipopolysaccharide caused an increase of temperature and blood glucose within 120 min, which thereafter started to decline. The body temperature was significantly lower than the baseline value at 6 h after lipopolysaccharide (Fig. 1a), while blood glucose was significantly lower than the pre-lipopolysaccharide value from 240 min to 360 min after lipopolysaccharide (Fig. 1b). Pretreatment of rats with dantrolene prevented the hyperthermia, but not the hypothermia, induced by lipopolysaccharide. In contrast, the lipopolysaccharide-induced hypoglycaemia, but not the hyperglycaemia, was attenuated by treatment of rats with dantrolene. However, injection of dantrolene (1 mg/kg i.v.) alone did not significantly affect the body temperature and blood glucose in normal control animals (Table 1).

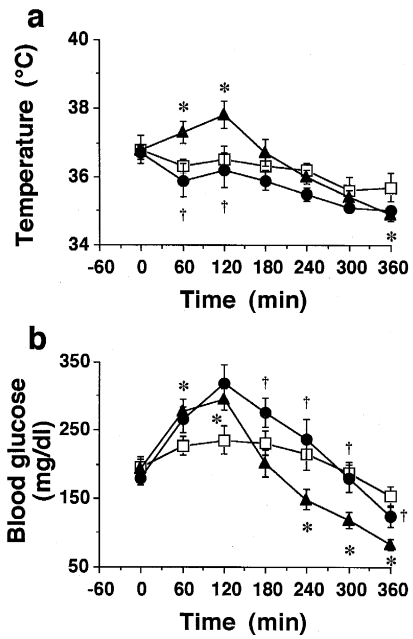

Fig. 1. Effects of dantrolene on (a) rectal temperature and (b) blood glucose in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in (a) temperature and (b) blood glucose in different groups of animals which received injection of vehicle (sham-operated rats, SOP; open squares; $n = 11$), vehicle plus LPS (10 mg/kg; solid triangles; $n = 16$), or dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (solid circles; $n = 8$). Data are expressed as mean ± S.E.M. of *n* observations. * $P < 0.05$ represents significant differences when compared to SOP control. † $P < 0.05$ represents significant differences between endotoxin rats in the absence and presence of dantrolene.

3.2. Attenuation by dantrolene of the circulatory failure caused by endotoxin in *vivo*

The mean baseline values for mean arterial blood pressure ranged from 115 ± 3 to 118 ± 3 mmHg in all animal groups studied and were not significantly different between groups. Fig. 2a demonstrates that administration of lipopolysaccharide caused a rapid fall in mean arterial

Table 1
Effects of dantrolene (1 mg/kg i.v.) on body temperature, blood glucose (BG), mean arterial blood pressure (MAP), pressor responses to noradrenaline (NA) and plasma nitrate (Nitrate) in normal control anaesthetised rats

	Time (min)			
	0	120	240	360
Body temperature (°C)	36.7 ± 0.3	36.4 ± 0.3	36.5 ± 0.4	36.0 ± 0.7
BG (mg/dl)	182 ± 14	222 ± 18	200 ± 17	166 ± 12
MAP (mmHg)	117 ± 2	113 ± 4	114 ± 4	110 ± 5
NA (mmHg)	30 ± 3	30 ± 4	28 ± 4	33 ± 3
Nitrate (μM)	8.18 ± 0.32	8.10 ± 0.36	8.29 ± 0.35	8.47 ± 0.41

Note that in these rats which did not receive lipopolysaccharide, injection of dantrolene did not cause significant changes in the above parameters. Data are mean ± S.E.M. from 5 rats.

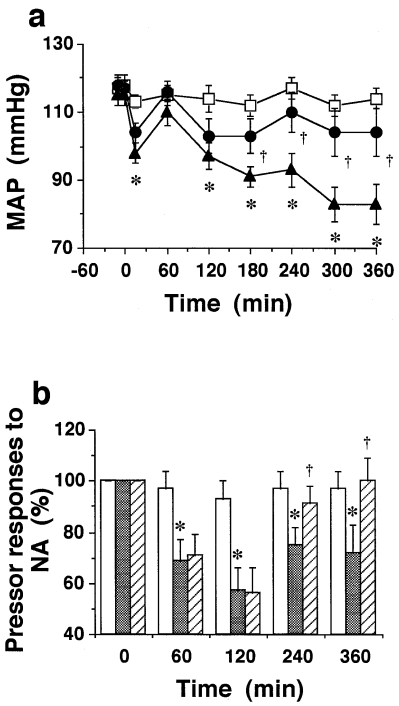

Fig. 2. Effects of dantrolene on (a) mean arterial blood pressure (MAP) or (b) pressor responses to noradrenaline (NA) in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in (a) MAP and (b) pressor responses to NA during the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; open squares; $n = 11$), vehicle plus LPS (10 mg/kg; solid triangles; $n = 16$), or dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (solid circles; $n = 8$). Data are expressed as mean ± S.E.M. of n observations. * $P < 0.05$ represents significant differences when compared to SOP control. † $P < 0.05$ represents significant differences between endotoxin rats in the absence and presence of dantrolene.

blood pressure from 115 ± 3 to 98 ± 3 mmHg ($n = 16$, $P < 0.05$) within 15 min. After 120 min after lipopolysaccharide, there was a continuous further fall in mean arterial blood pressure to 83 ± 6 mmHg at 360 min. In the sham-operated group, there was no significant change of mean arterial blood pressure during the experimental period (i.e., from 117 ± 4 at time 0 to 114 ± 3 mmHg at 360 min, $n = 11$, $P > 0.05$). In addition, the mean baseline values for the pressor responses to noradrenaline (1 μg/kg i.v.) ranged from 29 ± 2 to 32 ± 2 mmHg and were not significantly different between any of the experimental groups studied. Injection of lipopolysaccharide resulted in a substantial, time-dependent attenuation of the pressor responses elicited by noradrenaline ($n = 16$, Fig. 2b), whereas injection of vehicle rather than lipopolysaccharide had no significant effects on the noradrenaline-induced pressor responses during the 6 h experimental period ($n = 11$, $P > 0.05$).

Pretreatment of rats with dantrolene did not exert a significant effect on the mean arterial blood pressure (prior to injection of lipopolysaccharide). However, dantrolene prevented the delayed (e.g., after 120 min) fall in mean arterial blood pressure observed in lipopolysaccharide rats treated with vehicle. Thus, the mean arterial blood pressure of lipopolysaccharide rats pretreated with dantrolene ($n = 8$) was significantly higher than in the respective lipopolysaccharide control group at 180–360 min (e.g., at 360 min: 104 ± 7 vs. 83 ± 6 mmHg, $P < 0.05$; Fig. 2a). In addition, pretreatment of lipopolysaccharide rats with dantrolene enhanced the pressor responses afforded by noradrenaline (Fig. 2b). Thus, the pressor responses to noradrenaline at 240–360 min in lipopolysaccharide rats pretreated with dantrolene were significantly greater than in animals treated with lipopolysaccharide alone ($P < 0.05$; Fig. 2b).

Injection of normal control animals with dantrolene alone had no significant effects on mean arterial blood pressure and the pressor responses afforded by noradrenaline (Table 1).

3.3. Attenuation by dantrolene of the rise in plasma nitrate and NO synthase II activity in the lung caused by endotoxaemia

The mean plasma levels of basal nitrate ranged from 8.08 ± 0.45 to 8.20 ± 0.38 μM and were not significantly different between any of the experimental groups studied. Endotoxaemia for 360 min was associated with a 9.7-fold

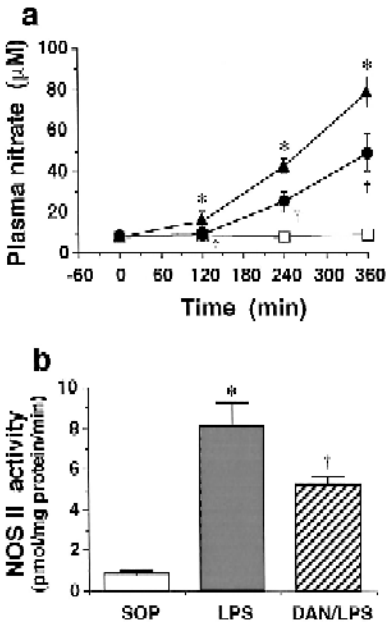

Fig. 3. Effects of dantrolene on (a) plasma nitrate or (b) inducible nitric oxide synthase activity (NOS II) in the lung obtained from rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in (a) plasma nitrate during the experimental period and (b) NOS II activity at the end of the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; open squares or open column; $n = 5–7$), vehicle plus LPS (10 mg/kg; solid triangles or grey column; $n = 7–9$), or dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (solid circles or hatched column; $n = 6–8$). Data are expressed as mean ± S.E.M. of n observations. * $P < 0.05$ represents significant differences when compared to SOP control. † $P < 0.05$ represents significant differences between endotoxin rats in the absence and presence of dantrolene.

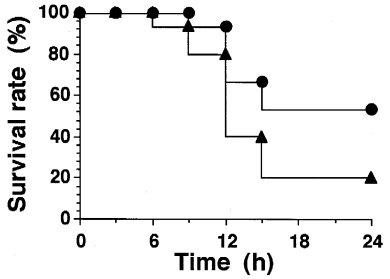

Fig. 4. Effects of dantrolene on the survival rate in mice treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes of survival during the experimental period in different groups of ICR mice which received intraperitoneal injection of LPS (60 mg/kg; solid triangles; $n = 15$) or dantrolene (1 mg/kg; solid circles; at time 0 and 6 h after LPS, $n = 15$) plus LPS. Data are expressed as percentage of mice survived at the observed time point.

rise in the plasma levels of nitrate ($P < 0.05$, $n = 9$; Fig. 3a). In addition, a small, Ca^{2+}-independent NO synthase II activity was detectable in lung homogenates obtained from animals treated with vehicle rather than lipopolysaccharide (Fig. 3b). Endotoxaemia for 360 min was associated with a substantial increase in NO synthase II activity ($P < 0.05$, $n = 7$; Fig. 3b).

The increase in plasma nitrate caused by endotoxaemia was significantly reduced in lipopolysaccharide-rats pretreated with dantrolene ($P < 0.05$, $n = 8$, Fig. 3a). Similarly, the activity of NO synthase II was also significantly reduced in homogenates of lungs obtained from lipopolysaccharide rats treated with dantrolene ($P < 0.05$, $n = 6$, Fig. 3b).

It was noted that injection of dantrolene alone had no significant effects on plasma nitrate levels (Table 1) and NO synthase II activity was almost undetectable in lung homogenates obtained from animals treated with dantrolene alone (i.e., 0.68 ± 0.22 pmol/mg protein/min, $n = 5$, $P > 0.05$ when compared to sham-operated controls).

3.4. Dantrolene increases the survival rate of mice treated with lipopolysaccharide

The administration of a high dose (60 mg/kg i.p.) of lipopolysaccharide to ICR mice was associated with a 24 h survival rate of only 20% (i.e., 3/15 of the animals). In contrast, lipopolysaccharide mice treated with dantrolene (1 mg/kg i.p. at time 0 and 6 h after lipopolysaccharide, $n = 15$) had a survival rate of 53% (i.e., 8/15 of the animals) at 24 h (Fig. 4). Thus, dantrolene significantly increased the survival rate of animals injected with lipopolysaccharide.

4. Discussion

This is the first study which demonstrates that the injection of lipopolysaccharide causes a biphasic change of body temperature and blood glucose in the anaesthetised rat, namely an initial increase (< 180 min after lipopolysaccharide) followed by a decrease (between 240 and 360 min) in temperature and blood glucose. These alterations are similar to those observed in animals or man with septic shock (Chaudry et al., 1979; Siegel et al., 1979; Chen et al., 1994). In addition, lipopolysaccharide causes a substantial decrease of mean arterial blood pressure, which is caused by or associated with (i) insensitivity to various vasoconstrictor agents such as catecholamines (this study; Chernow et al., 1982), angiotensin II (Fink et al., 1985), vasopressin (Schaller et al., 1985), serotonin (Wakabayashi et al., 1987), activators of protein kinase C (Wakabayashi et al., 1988) and depolarizing agents (Chen et al., 1994), and (ii) a reduction of peripheral vascular resistance which may be due to an overproduction of endogenous vasodilators such as prostacyclin (Pomerantz et al., 1982; Mozes et al., 1995) and NO (Thiemermann and Vane, 1990; Kilbourn et al., 1990; Wright et al., 1992). Lipopolysaccharide also causes a substantial lethality in the murine model of endotoxaemia used in this study. All our results imply that the acute rodent model of endotoxaemia used here (within 6 h) mimics most of the clinical features of sepsis and, hence, may be useful to investigate the pathophysiology of endotoxaemia and possible therapies.

Recently, a number of studies have shown that an enhanced formation of NO importantly contributes to the hypotension and vascular hyporeactivity to various vasoconstrictor agents (Moncada et al., 1991; Thiemermann, 1994). This overproduction of NO is due to an induction of NO synthase II in various cells and tissues (Nathan, 1992; Moncada and Higgs, 1993; Szabo and Thiemermann, 1995). Indeed, the present study also demonstrates that plasma nitrate (a final metabolite of NO) and NO synthase II activity are increased in animals treated with lipopolysaccharide, suggesting an overproduction of NO in the rodent model used here. It is now well established that NO synthase II tightly binds calmodulin and therefore does not need any further addition of exogenous calmodulin to exert its full biological activity. Thus, NO synthase II produces large amounts of NO and its activity is not regulated by alterations in the levels of free cytosolic Ca^{2+}. Indeed, Park and colleagues have demonstrated that a rapid increase of free cytosolic Ca^{2+} concentrations increases the induction of the NO synthase II gene in murine peritoneal macrophages (Park et al., 1995). Here, we demonstrate that dantrolene, an agent which suppresses the Ca^{2+} release from the sarcoplasmic reticulum and, hence, decreases free cytosolic Ca^{2+} concentrations, prevents the formation of NO by reducing either the induction of NO synthase II and/or the activity of NO synthase II in the lung from endotoxaemic animals. In particular, we demonstrate that dantrolene (i) attenuates the delayed fall in blood pressure and the vascular hyporeactivity to noradrenaline elicited by prolonged periods of endotoxaemia in the rat and (ii) improves survival in a murine model of severe endotox-

aemia. In addition to causing beneficial haemodynamic effects in rats with endotoxin shock, dantrolene also prevents the hyperthermia and the hypoglycaemia caused by lipopolysaccharide, which are, at least in part, due to the production of NO (Lin and Lin, 1996; Casado et al., 1996). Many effects of NO production lead to the simultaneous release of mediators (e.g., prostaglandin E_2) from the cyclooxygenase pathway. The lipopolysaccharide-induced early fever was inhibited by indomethacin (Lin and Lin, 1996), which is a more potent inhibitor of cyclooxygenase-1 than cyclooxygenase-2 (Mitchell et al., 1993). Thus, the early inhibition by dantrolene (within 2 h) of the fever may be due to inhibition of cyclooxygenase-1, which is also dependent on free cytosolic Ca^{2+} levels. However, dantrolene had no significant effects on the parameters of normal control rats which we examined in this study.

Several different Ca^{2+} channel antagonists including nifedipine also protect against the cardiovascular failure and prolong survival time in various models of endotoxin shock (Sakaguchi et al., 1984; Bosson et al., 1985; Lee and Lum, 1986; Lee et al., 1989; Szabo et al., 1993a). This protective mechanism has been related to an inhibition of cellular Ca^{2+} overload (Sakaguchi et al., 1984; Sayeed and Maitra, 1987), prevention of disseminating intravascular coagulation (Lee et al., 1989), protection against myocardial or intestinal ischemia (Bosson et al., 1985) and inhibition of the expression of NO synthase II (Szabo et al., 1993a). However, the inhibition of NO synthase II induction by nifedipine in cultured cells and in the anaesthetised rat seems not to depend on Ca^{2+} channel antagonism, as other Ca^{2+} antagonists (verapamil and diltiazem) and reduction of extracellular Ca^{2+} with EGTA had only a minor effect on the expression of NO synthase II (Szabo et al., 1993a). Nifedipine has a greater vasodilator effect than verapamil and diltiazem (Van Zwieten and Pfaffendorf, 1993), which may be due to the inhibition of the release of Ca^{2+} from the sarcoplasmic reticulum in addition to its inhibition on Ca^{2+} influx, whereas the release of Ca^{2+} from intracellular storage sites is more important in vascular beds (Bohr, 1963; Van Breemen et al., 1986, 1995).

We propose here that the observed beneficial haemodynamic effects (prevention of the delayed fall in blood pressure and development of a vascular hyporeactivity to noradrenaline) afforded by dantrolene are due to a reduction of the increase in NO synthase II activity caused by lipopolysaccharide in blood vessels. The prevention of the induction of NO synthase II by dantrolene is similar to that of other agents which also suppress the induction of NO synthase II. For instance, dexamethasone has beneficial effects on endotoxaemic rats but fails to prevent the early lipopolysaccharide-induced fall in blood pressure while attenuating the delayed hypotension (Szabo et al., 1993a). Although one could argue that dantrolene directly inhibits the activity of NO synthase II, this is unlikely as the delayed treatment of rats with dantrolene (at 5 h) did neither restore blood pressure nor the pressor response to noradrenaline (personal observations). In contrast, inhibitors of NO synthase II activity, such as aminoguanidine or N^{ω}-nitro-L-arginine methyl ester (Wu et al., 1996), also exert beneficial effects when given after (rather than before) the administration of lipopolysaccharide. Thus, our results support the notion that dantrolene prevents the expression of NO synthase II protein.

The mechanism(s) by which dantrolene prevents the induction of NO synthase II are unclear and warrant further investigation. In principle, dantrolene may have prevented the release of the proinflammatory cytokine tumour necrosis factor-α, which mediated the induction of NO synthase II caused by lipopolysaccharide in the rat (Thiemermann et al., 1993). Alternatively, dantrolene may have affected the signal transduction events leading to the expression of NO synthase II. There is now good evidence (at least from studies in cultured cells) that the expression of NO synthase II is secondary to the activation of tyrosine kinase, mitogen activated protein kinase, protein kinase C and the nuclear transcription factor κB. Thus, it is possible that dantrolene interferes with one or more of these signal transduction events. In addition, dantrolene may prevent the expression of NO synthase II by reducing the release of Ca^{2+} from the sarcoplasmic reticulum. Although there is no evidence that dantrolene affects the biosynthesis of tumour necrosis factor-α or any of the above intracellular signalling systems, there is evidence that agents which reduce the release of Ca^{2+} from the sarcoplasmic reticulum also prevent the expression of NO synthase II in macrophages. For instance thapsigargin, an agent which prevents the reuptake of Ca^{2+} into the sarcoplasmic reticulum and, hence, increases the intracellular Ca^{2+} stores, enhances the expression of NO synthase II protein caused by lipopolysaccharide in macrophages (Park et al., 1995). This finding supports our current working hypothesis that the prevention of the expression of NO synthase II afforded by dantrolene in vivo is secondary to the prevention of the release of Ca^{2+} from the sarcoplasmic reticulum in target cells, rather than due to non-specific effects of dantrolene.

In conclusion, dantrolene prevents the induction of NO synthase II and the overproduction of NO in response to lipopolysaccharide in the anaesthetised rat and improves the survival in a murine model of severe septic shock. Although the mechanism underlying the inhibition by dantrolene of the expression of NO synthase II in vivo warrants further investigation, we propose that this effect of dantrolene is due to the reduction of the release of Ca^{2+} from the sarcoplasmic reticulum and, hence, that the expression of NO synthase II in vitro (Park et al., 1995) and in vivo (this study) requires the release of Ca^{2+} from the sarcoplasmic reticulum. Our finding is of particular importance as dantrolene (dantrium) is currently in clinical use for the therapy of malignant hyperthermia (Rosenberg and Fletcher, 1987). Most notably the doses of dantrolene used in patients are higher (2–5 mg/kg) than the one used in

this study (1 mg/kg) suggesting that this agent may be useful to prevent the expression of NO synthase II in man.

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