Comparison between Effects of Dantrolene and Nifedipine on Lipopolysaccharide-Induced Endotoxemia in the Anesthetized Rats

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Abstract

Intracellular calcium is an important mediator for regulating the cellular response in endotoxemia. In this study, we investigated the effects of dantrolene and nifedipine, two agents of reducing intracellular calcium levels, on bacterial endotoxin (lipopolysaccharide, LPS; 10 mg/kg i.v.)-induced production of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) as well as hemodynamic changes in the anesthetized rat. Injection of LPS (i) induced biphasic changes of blood glucose and rectal temperature: an initial increased phase (<180 min after injection of LPS) followed by a decreased phase (at 240 or 360 min), (ii) caused a significant fall in mean arterial blood pressure from 119±3 mmHg (at time 0) to 73±67mmHg (at 360 min) with a concomitant increase of heart rate, (iii) resulted in a substantial hyporeactivity to norepinephrine (NE) (1 μg/kg i.v.), (iv) increased plasma nitrate (an indicator of NO formation) in a time-dependent manner, and (v) induced bell-shape changes in plasma TNF-α levels which reached a peak at 60 min. Pretreatment of animals with dantrolene (1 mg/kg i.v. at 20 min prior to LPS) or nifedipine (20 μg/kg i.v. infusion for 20 min at 20 min prior to LPS) not only attenuated the delayed circulatory failure (e.g. delayed hypotension and vascular hyporeactivity to NE), but also prevented the overproduction of NO caused by LPS in the rat. However, the prevention of NO overproduction by dantrolene, but not by nifedipine, was associated with an inhibition of TNF-α production elicited by LPS. Thus, both dantrolene and nifedipine have beneficial hemodynamic effects, although through different mechanisms, in animals with endotoxic shock.

Key Words: dantrolene, nifedipine, nitric oxide, tumor necrosis factor-α, lipopolysaccharide

Introduction

Calcium is an important modulator of the cellular response occurring during endotoxemia and severe bloodstream borne infection, i.e. sepsis (24, 33). Studies demonstrate that several different calcium channel antagonists including dantrolene (7, 30) and nifedipine (9, 25) protect against cardiovascular failure and prolong survival time in various models of animals treated with endotoxin (lipopolysaccharide, LPS). This protection has been attributed to inhibition of cellular calcium overload (22), prevention of disseminating intravascular coagulation (10), and/or reduction of nitric oxide (NO) synthase (NOS) induction (25, 30).

It is clear that (i) a diminished reactivity to several vasopressor agents including calcium occurs in blood vessels obtained from animals with endotoxic shock (19) and in blood vessels exposed to LPS *in vitro* (5, 21), (ii) the development of this hyporeactivity to calcium under depolarizing conditions or in the presence of a calcium ionophore suggests that post receptor mechanisms are also impaired (1), and (iii) the decreased calcium efflux seen in sepsis is due to a decrease of intracellular calcium in response to norepinephrine (NE) (12), however, the intracellular signal responses are not well clarified. It has been shown that LPS induces the release of cytokines such

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as interleukin-1, tumor necrosis factor-α (TNF-α) and γ-interferon, which contribute to the hypotension and lethality associated with endotoxemia. In addition, an enhanced formation of NO in response to LPS is also associated with the development of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock (6, 8, 28, 29). This overproduction of NO in endotoxemic animals is due to the induction of NOS II which can be induced by many cytokines, in particular TNF-α (15). NOS II is different from other NOS isoforms, for its activation is independent of changes in intracellular calcium. A recent study demonstrates that the depletion of the intracellular calcium pool is linked to the induction of NOS II in murine peritoneal macrophages (18), suggesting that the induction of NOS II is dependent on intracellular calcium levels. Indeed, both dantrolene and nifedipine have improved the circulatory failure in rats with endotoxemia by preventing the induction of NOS II (25, 30). However, what mechanisms by dantrolene and nifedipine prevented the induction of NOS II have not been fully evaluated.

In this study, we compare the effect of dantrolene with that of nifedipine on rats treated with endotoxin and further investigate what mechanism(s) by both agents contribute to the prevention of induction of NOS II in endotoxemic rats.

Materials and Methods

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 Deparment of Laboratory Animal Science of the National Defense Medical Center. Rats were anesthetized by intraperitoneal injection of thiobutabarbital (80 mg/kg) with urethane (0.4 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) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Gould model TA5000 polygraph recorder (Gould Inc., Valley View, Ohio). 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 hemodynamic parameters, animals were given NE (1 μg/kg i.v.), and 10 min later animals received vehicle (saline) or *Escherichia coli* LPS (10 mg/kg i.v.) and were monitored for 360 min. The pressor responses to NE were reassessed at every hour after vehicle or LPS injection. Prior to (i.e. at time 0) and at every hour after vehicle or LPS, 0.3 ml of blood was taken to measure the changes in blood glucose and the plasma levels of nitrate and TNF-α. Any blood withdrawn was immediately replaced by the injection of an equal amount of saline (i.v.). In a separate experiment, dantrolene (1 mg/kg i.v.) and nifedipine (20 μg/kg i.v. infusion for 20 min) was administered at 20 min prior to the injection of LPS. All hemodynamic and biochemical parameters were recorded for 6 h in all of the above animal groups.

Determination of Blood Glucose and Plasma Nitrate and TNF-α

Before the blood sample was centrifuged (7,200 g 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 Inc., Milpitas, CA) and the plasma was kept in -20 °C freezer.

At a later stage, plasma samples (50 ml) were thawed and de-proteinized by incubating them with 95% ethanol (4 °C) for 30 min. The samples were subsequently centrifuged for a further 5 min at 14,000 *g*. It is noted that the nitrate concentration in plasma mentioned 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 1N 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 Inc., Boulder, CO). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate (Sigma Chemical Co., St. Louis, MO) as previously described (30).

For the determination of TNF-a, the rest plasma samples (100 ml) were diluted 1:2 and TNF-a was measured in duplicate with by an enzyme-linked immunoadsorbent assay (ELISA) kit (Genzyme Co., Cambridge, MA) as previously described (32).

Chemicals

Bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), nifedipine, norepinephrine bitartrate, and sodium nitrate were obtained from Sigma Chemical Co. (St. Louis, MO). Dantrolene sodium was purchased from Eaton Laboratories Inc. (Norwich, NY).

Statistical Analysis

All values in the figures and text are expressed as mean ± standard error of mean of *n* observations, where *n* represents the number of animals studied. Statistical evaluation was performed by using ANOVA followed by a multiple comparison test (Scheffe's test). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Effects of Dantrolene and Nifedipine on Changes of Blood Glucose and Rectal Temperature Caused by Endotoxin in Vivo

Baseline values for blood glucose and rectal temperature of the animal groups treated with vehicle (sham-operated, SOP; n=8), vehicle plus LPS (10 mg/kg i.v.; n=15), dantrolene (1 mg/kg i.v.; n=10) plus LPS, or nifedipine (20 μg/kg i.v. infusion for 20 min; n=8) plus LPS were between 89±9 and 97±8 mg/dl, and 36.6±0.3 and 36.8±0.4 °C, repectively, which were not significantly different among groups (Fig. 1). Administration of LPS caused an increase of blood glucose and rectal temperature within 120 min, which thereafter started to decline. The blood glucose was significantly lower than pre-LPS value from 240 min to 360 min after LPS (Fig. 1A), while the rectal temperature was significantly lower than the baseline value at 360 min after LPS (Fig. 1B). The LPS-induced hypoglycemia, but not the hyperglycemia, was attenuated by pretreatment of rats with either dantrolene or nifedipine (Fig. 1A). In addition, pretreatment of LPS-rats with dantrolene prevented the hyperthermia, but not the hypothermia, while on contrast, nifedipine prevented the hypothermia, but not the hyperthermia (Fig. 1B).

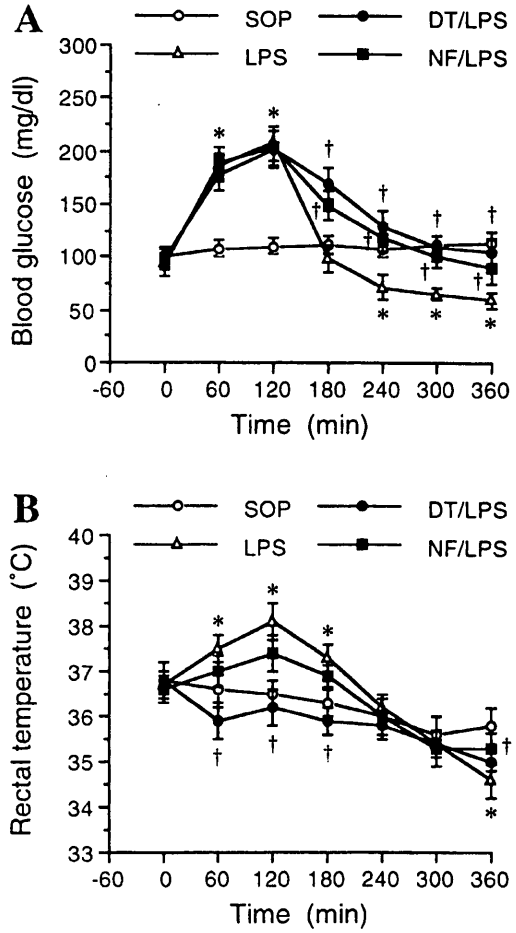

Fig. 1. Effects of dantrolene and nifedipine on (a) blood glucose and (b) rectal temperature in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in (a) blood glucose and (b) rectal temperature in different groups of animals which received injection of vehicle (sham-operated rats, SOP; n=8), vehicle plus LPS (10 mg/kg; n=15), dantrolene (1 mg/kg, at 20 min prior to LPS) plus LPS (n=10), or nifedipine (20 μg/kg, i.v. infusion for 20 min at 20 min prior to LPS) plus LPS (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 or nifedipine.

Dantrolene and Nifedipine Mitigate the Delayed Fall of Blood Pressure Caused by Endotoxin in vivo

The mean baseline values for MAP ranged from 118±4 to 122±3 mmHg in all animal groups studied and were not significantly different among groups. Figure 2A demonstrates that administration of LPS caused a rapid fall in MAP from 120±3 to 80±6 mmHg (n=15, p<0.05) within 15 min. After 60 min after LPS, there was a continuous further fall in MAP to 73±7 mmHg at 360 min. In the SOP group, there was no significant change of MAP during the experimental period (i.e. from 119±3 at time 0 to 115±2 mmHg at 360 min, n=8, p>0.05). In addition, the mean baseline values for the HR ranged from 323±18 to 330±12 beats/min and were not significantly different between any of the experimental groups studied. Figure 2B demonstrates that administration of LPS resulted in an increase of HR (tachycardia), whereas in the SOP group, there was no significant change of HR during the experimental period.

Pretreatment of rats with dantrolene or nifedipine did not exert a significant effect on the MAP (prior to injection of LPS). However, both dantrolene and nifedipine prevented the delayed (e.g. after 180 min) fall in MAP observed in LPS-rats treated with vehicle. Thus, the MAP of LPS-rats pretreated with either dantrolene (n=10) or nifedipine (n=8) was significantly higher than in the respective LPS-con-

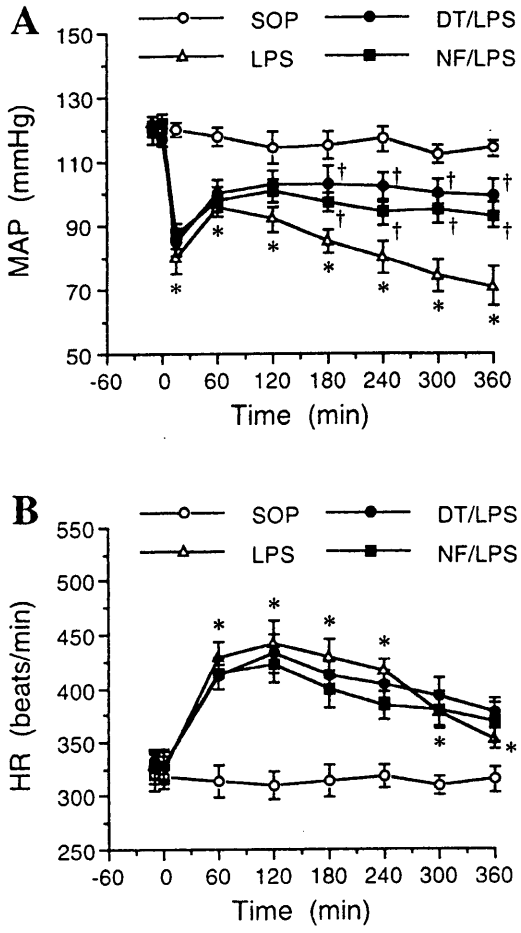

Fig. 2. Effects of dantrolene and nifedipine on (a) mean arterial blood pressure (MAP) or (b) heart rate (HR) in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in (a) MAP and (b) HR during the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; n=8), vehicle plus LPS (10 mg/kg; n=15), dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (n=8), or nifedipine (20 μg/kg, i.v. infusion for 20 min at 20 min prior to LPS) plus LPS (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 or nifedipine.

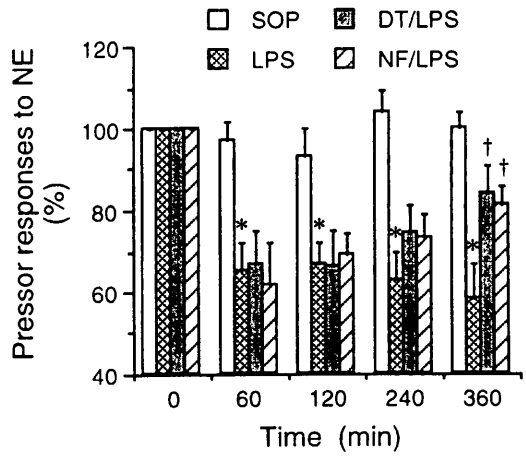

Fig. 3. Effects of dantrolene and nifedipine on pressor responses to norepinephrine (NE) in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in pressor responses to NE during the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; n=8), vehicle plus LPS (10 mg/kg; n=15), dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (n=10), or nifedipine (20 μg/kg, i.v. infusion for 20 min at 20 min prior to LPS) plus LPS (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 or nifedipine.

trol group at 180 to 360 min (Fig. 2A). However, the pretreatment of dantrolene or nifedipine had no signifcant effect on tachycardia induced by endotoxin.

Dantrolene and Nifedipine Attenuate the Vascular Hyporesponsiveness to NE in Rats with Endotoxic Shock

The mean baseline values for the pressor responses to NE (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 LPS resulted in a substantial, time-dependent attenuation of the pressor responses elicited by NE (n=15, Figure 3), whereas injection of vehicle rather than LPS had no significant effect on the NE-induced pressor responses during the 6-h experimental period (n=8, p>0,05).

Pretreatment of LPS-rats with either dantrolene or nifedipine enhanced the pressor responses afforded by NE (Figure 3). Thus, the pressor response to NE at 360 min in LPS-rats pretreated with either dantrolene (n=10) or nifedipine (n=8) was significantly greater than that in animals treated with LPS alone (p<0.05; Figure 3).

Dantrolene and Nifedipine Reduce the Nitrate Level in Plasma Obtained from Rats with Endotoxemia

The mean plasma levels of nitrate ranged from 7.18±0.65 to 8.02±0.54 μM and were not significantly different between any of the experimental groups studied. Endotoxemia for 360 min was associated with a 31.3-fold rise in the plasma level of nitrate (p <0.05, n=15; Fig. 4). However, there was no significant change of plasma nitrate level during the experimental period in the SOP group.

In contrast, the increase in plasma nitrate elicited by endotoxaemia was significantly reduced in LPS-rats pretreated with either dantrolene (n=10) or nifedipine (n=8) (p<0.05, Fig. 4). In addition, the inhibitory effect of dantrolene on plasma nitrate levels seems greater than that of nifedipine.

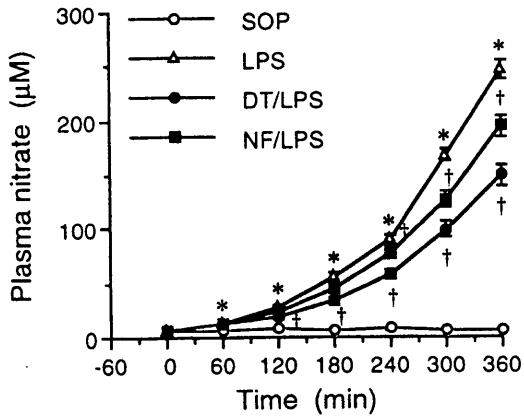

Fig. 4. Effects of dantrolene and nifedipine on plasma nitrate in rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes in plasma nitrate during the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; n=8), vehicle plus LPS (10 mg/kg; n=15), dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (n=10), or nifedipine (20 μg/kg, i.v. infusion for 20 min at 20 min prior to LPS) plus LPS (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 or nifedipine.

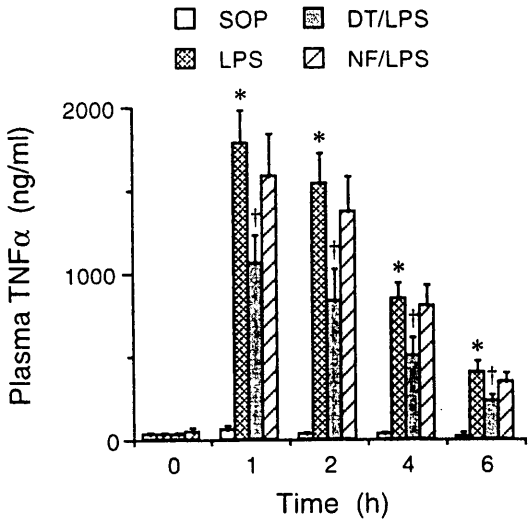

Fig. 5. Effects of dantrolene and nifedipine on tumor necrosis factor-a (TNF-α) levels in plasma from rats treated with endotoxin (lipopolysaccharide, LPS). Depicted are the changes of TNF-α levels during the experimental period in different groups of animals which received injection of vehicle (sham-operated rats, SOP; n=3), vehicle plus LPS (10 mg/kg; n=5), dantrolene (1 mg/kg, 20 min prior to LPS) plus LPS (n=4), or nifedipine (20 μg/kg, i.v. infusion for 20 min at 20 min prior to LPS) plus LPS (n=4). Note that each value of TNF-α is the mean of duplicate plasma samples from the same animal. 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.

Dantrolene, but not Nifedipine, Supressed the Plasma TNF-α Level in Rats with Endotoxemia

The basal plasma levels of TNF-α were almost undetectable and were not significantly different between any of the experimental groups studied (Fig. 5). The injection of LPS resulted in bell-shape changes in the plasma levels of TNF-α which reached a peak at 1 h after LPS injection and subsequently decreased slowly ($p<0.05$, n=5). In the SOP group (n=3), no significant amounts of TNF-α were detectable during the experimental period, indicating that the surgical procedure alone did not result in a significant rise in plasma TNF-α.

Pretreatment of LPS-rats with dantrolene (n=4), but not nifedipine (n=4), significantly decreased the TNF-α level in plasma. In other words, the peak value of plasma TNF-α was significantly reduced in LPS-rats pretreated with dantrolene (Fig. 5).

Discussion

The current study confirms previous studies and demonstrates that the injection of LPS causes a biphasic change of blood glucose and rectal temperature in the anesthetized rat, which is similar to those observed in animals or man with septic shock (2, 3, 23, 30). In addition, LPS causes a substantial decrease of MAP, which is associated with insensitivity to various vasoconstrictor agents such as catecholamines (this study, 4). The vascular hyporeactivity to NE has been shown to be related to an overproduction of endogenous vasodilators such as prostacyclin (16, 20) and NO (28, 31). Indeed, the present study demonstrates that plasma nitrate (a final metabolite of NO) levels are increased in rats treated with LPS, suggesting an overproduction of NO in this rodent model. Thus, this acute rodent model of endotoxemia mimics most of clinical features of sepsis and, hence, is applicable to investigate the pathophysiology of endotoxemia.

A number of studies show that an enhanced formation of NO importantly contributes to the hypotension and vascular hyporeactivity to various vasoconstrictor agents (15, 27). This overproduction of NO which is due to an induction of NOS II by cytokines in various cells and tissues. Here, we demonstrate that both dantrolene and nifedipine, which decrease the intracellular calium concentrations, prevent the delayed hypotension and the formation of NO by reducing either the induction of NOS II and/or the activity of NOS II in endotoxemic animals.

Although dantrolene and nifedipine both were

intracellular calcium-reducing agents and had very similar protection on the delayed circulatory failure (e.g. delayed hypotension and vascular hyporeactivity to NE) and metabolic effects (e.g. blood glucose, rectal temperature, and plasma NO levels) in rats with endotoxemia, the mechanism by which contribute to these beneficial effects was not quite the same. For instance, the mechanism of prevention of NOS II induction by dantrolene seems to be related to the reduction of TNF-α production in microphages in vitro (11) and in plasma levels *in vivo* (Figure 5). In addition, dantrolene has also been shown to suppress the poduction of interleukin-12 and γ-interferon in mice (17) which may also induce the expression of NOS II. However, in addition to TNF-α (this study) and γ-interferon (17), many cytokines also play a role in the induction of NOS II (15). Thus, the inhibitory effect of dantrolene on NO production is not solely due to the inhibition of TNF-α production. In other words, the inhibitory action of dantrolene on the release of these cytokines may lead to a reduction of NOS II induction and result in an inhibition of NO production. As for the mechanism of prevention of NOS II induction by nifedipine, it may also be due to an inhibition of LPS-stimulated cytokines release. Indeed, after stimulation of monocytes with LPS, nifedipine inhibits interleukin-1 production in these cells (13). Although the antioxidant effect of nifedipine may be also related to the inhibition of NO production, the level of hydroxyl radicals in culture medium of endothelial cells (14) and the content of superoxide anion in plasma of rats (unpublished data) were not affected by nifedipine. Our present results suggest that the prevention of NOS II induction by nifedipine is, at least, not associated with the TNF-α production. However, the inhibition of NOS II induction by nifedipine in cultured cells and in the anesthetized rat were supposed not to depend on calcium channel antagonism, as other calcium antagonists (verapamil and diltiazem) and reduction of extracellular calcium with EGTA had only a minor effect on the expression of NOS II (25), suggesting that this is a unique action of nifedipine on NOS II induction.

Thus, we propose here that the observed beneficial hemodynamic effects (prevention of the delayed fall in blood pressure and the development of a vascular hyporeactivity to NE) afforded by dantrolene and nifedipine are due to a reduction of the increase in NOS II expression caused by LPS, which is associated with an overproduction of NO in endotoxemia. In addition, datrolene augments interleukin-10 levels in plasma of animals treated with endotoxin (26) and nifedipine possesses lipid antiperoxidative activities which prevented the glutathione decrease caused by inhibition of peroxide generation (14). Although these effects are not directly associated with the inhibition of NOS II induction, they may somehow also contribute to the beneficial effect of dantrolene and nifedipine on animals with endotoxemia.

Acknowledgments

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