British Journal of Pharmacology (1998) 124, 1099–1106
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Modulation by dantrolene of endotoxin-induced interleukin-10, tumour necrosis factor-α and nitric oxide production *in vivo* and *in vitro*

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1 Intracellular calcium has been suggested to be an important mediator of the cellular response in endotoxaemia and shock. Dantrolene is an agent that interferes with intracellular calcium fluxes resulting in a decreased availability of calcium in the cytoplasm. Here we have investigated the effect of dantrolene on lipopolysaccharide (LPS)-induced production of interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α), and nitric oxide (NO) in mice and in cultured RAW 264.7 macrophages *in vitro*.

2 In BALB/c mice, LPS-induced plasma IL-10 levels were significantly enhanced by pretreatment with dantrolene (20 mg kg^{-1}, i.p.) ($P<0.005$ at the 90 min time-point). On the other hand, dantrolene pretreatment suppressed circulating TNF-α and nitrite/nitrate (breakdown products of NO) concentrations. However, dantrolene had no effect on LPS-induced plasma interleukin-6 (IL-6) levels (67.22 ± 5.51 ng ml^{-1} in vehicle-pretreated mice and 62.22 ± 3.66 ng ml^{-1} in dantrolene-pretreated mice, $n=9$).

3 Dantrolene inhibited TNF-α and NO production in C57BL/6 IL-$10^{+/+}$ mice, as well as in their IL-10 deficient counterparts (C57BL/6 IL-$10^{0/0}$).

4 In RAW 264.7 macrophages, dantrolene (10–300 μM) reduced IL-10, TNF-α, and nitrite (breakdown product of NO) production elicited by LPS (10 μg ml^{-1}). Dantrolene (300 μM) did not affect the LPS-induced nuclear translocation of transcription factor nuclear factor κB in these cells.

5 Although LPS failed to alter the intracellullar concentration of calcium in single macrophages loaded with Fura-2, dantrolene caused a significant decrease of the basal calcium level as determined 30 min after dantrolene treatment ($P<0.005$). ATP (1 mM) caused a rapid rise in intracellular calcium levels in both dantrolene-pretreated and vehicle-pretreated cells.

6 These results indicate that unlike the secretion of TNF-α and NO, IL-10 production is differentially regulated *in vitro* and *in vivo*. The decrease of plasma levels of the pro-inflammatory mediators TNF-α and NO, and increase in circulating IL-10 concentrations by dantrolene suggest that this drug might offer a new therapeutic approach in inflammatory diseases and septic shock.

Keywords: Lipopolysaccharide; intracellular calcium; interleukin-10; tumour necrosis factor-α; nitric oxide

Introduction

Increases in intracellular calcium levels ($[Ca^{2+}]_i$) have been shown to occur in response to lipopolysaccharide (LPS) both *in vitro* and *in vivo* (Gorecka-Tisera *et al*., 1986; Baldwin *et al*., 1991; Letari *et al*., 1991; Song *et al*., 1993; Zaloga *et al*., 1993). This increase has been implicated in the pathophysiology of septic shock, as (1) low dose calcium administration increases mortality during endotoxaemia (Zaloga *et al*., 1992), (2) calcium antagonists increase survival in sepsis and ameliorate the metabolic abnormalities characteristic of endotoxin shock (Bosson *et al*., 1985; Goto *et al*., 1992; Hotchkiss & Karl, 1994). Since the pathophysiological consequences of endotoxin administration are caused by the release of a variety of inflammatory mediators, it is possible that the beneficial effects of calcium antagonists are due to interference of these drugs with the production of molecules involved in the inflammatory cascade. These molecules, produced mainly by activated monocytes/macrophages, can be classified as pro- and anti-inflammatory mediators. Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, that is recognized as a central mediator of endotoxaemia and other forms of inflammation (Beutler, 1995). In addition, the endogenous production of TNF-α is a key intermediate in the induction of nitric oxide (NO) synthesis in response to LPS (Szabó, 1995). The enhanced formation of NO by the macrophage type inducible nitric oxide synthase (iNOS) importantly contributes to the development of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxin shock (Szabó, 1995). Interleukin-10 (IL-10) was initially described as a T helper 2 product that inhibited the secretion of cytokines by T helper 1 T cell clones (Mosmann, 1994). Recently, it has been shown that monocytes and macrophages also secrete IL-10 (Fiorentino *et al*., 1991), and that macrophages appear to be a major source of circulating IL-10 in response to LPS (Barsig *et al*., 1995). It has been demonstrated that IL-10 inhibits the synthesis of various cytokines (TNF-α, interleukin-1 (IL-1), interferon-γ (IFN-γ), and interleukin-6 (IL-6)) (Fiorentino *et al*., 1991; Marchant *et al*., 1994; Mosmann, 1994) and NO (Berg *et al*., 1995) produced by monocytes/macrophages in response to activation by LPS. In experimental models of endotoxaemia and other inflammatory states, endogenous and exogenous IL-10 has been shown to be protective (Marchant *et al*., 1994; Mosmann, 1994; Berg *et al*., 1995). IL-6 is another macrophage-derived

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cytokine that is released in response to LPS, and it is a major stimulus for acute-phase protein synthesis in systemic infection (Le & Vilcek, 1989).

The release of calcium from its intracellular stores contributes to the increased $[Ca^{2+}]_i$ in inflammatory conditions (Baldwin *et al*., 1991; Letari *et al*., 1991; Song *et al*., 1993). It has been shown that dantrolene, a drug capable of reducing the mobilization of calcium from its intracellular stores, decreases the LPS-induced increase of intracellular free calcium levels in *Escherichia coli* treated HEp-2 cells (Baldwin *et al*., 1991) and in the thoracic aorta in septic rats (Song *et al*., 1993). These observations, together with the findings showing that dantrolene suppresses endotoxin-induced TNF-α and IL-1 production (Hotchkiss *et al*., 1995), have been suggested to underlie the ability of this drug to be protective in septic shock (Hotchkiss & Karl, 1994). Based on results of the above studies, we further investigated whether dantrolene is able to modulate the LPS-induced inflammatory response *in vitro* and *in vivo*. Our results demonstrate that dantrolene has substantial modulatory effects on the LPS-induced production of IL-10, TNF-α and NO.

Methods

Animals

Male BALB/c mice (20–25 g) were purchased from Charles River Laboratories (Budapest, Hungary). Male C57BL/6 IL-$10^{+/+}$ and C57BL/6 IL-$10^{0/0}$ mice (20–25 g) were obtained from the Jackson Laboratory (Bay Harbor, ME). Animals received food and water *ad libitum*, and lighting was maintained on a 12 h cycle.

In vivo *experiments*

Experimental design for plasma IL-10, TNF-α, and IL-6 measurements Animals were injected i.p. with drug vehicle or dantrolene (20 mg kg^{-1}) in a volume of 0.1 ml 10 g^{-1} body weight. Thirty minutes later, they were challenged with 4 mg kg^{-1} of LPS administered i.p. The animals were killed by decapitation under ether anaesthesia at 90 to 360 min after LPS treatment. Trunk blood was collected in ice-cold Eppendorf tubes containing EDTA and centrifuged for 10 min at 4°C. The plasma was stored at −70°C until assayed.

Experimental design for plasma nitrite/nitrate measurements Animals were injected i.p. with drug vehicle or dantrolene (as described above), followed by an i.p. LPS challenge (4 mg kg^{-1}) 30 min later. Blood was taken at 6, 9, 24 and 48 h after LPS injection, and processed in a similar way to that for determinations of IL-10, TNF-α and IL-6. In experiments using C57BL/6 IL-$10^{0/0}$ mice, blood was collected at 4 h after the LPS (80 mg kg^{-1}) injection. These conditions seemed to be optimal for the measurement of both TNF-α and nitrite/nitrate in the plasma. Since plasma TNF-α levels are not detectable in C57BL/6 IL-$10^{+/+}$ mice at 4 h post-LPS, the concentrations of this cytokine were determined at 90 min following the LPS challenge.

Cytokine assays Cytokine levels in plasma and cell culture supernatants were determined by enzyme-linked immunoassay (ELISA). Concentrations of IL-10 and TNF-α in the plasma and cell culture supernatants, and of IL-6 in the plasma were determined using ELISA kits obtained from Genzyme (Genzyme Corp., Boston, MA). Plasma samples were diluted to 1:6; 1:2; and 1:200 for TNF-α, IL-10 and IL-6, respectively. Assays were performed in duplicate as described by Haskó *et al*., (1996a) and Szabó *et al*., (1997) and according to the manufacturer's instructions. Detection limits were <15 pg ml^{-1}, <0.15 pg ml^{-1} and <5 pg ml^{-1} for TNF-α, IL-10 and IL-6, respectively. The baseline values detected in the present study and the magnitude of the LPS-induced increases in cytokine plasma levels were similar to previously reported values (Haskó *et al*., 1996a, Szabó *et al*., 1997).

Measurement of plasma nitrite/nitrate concentrations For determination of total nitrite/nitrate concentrations in plasma samples (diluted 1 : 10 in phosphate-buffered saline), nitrate was first converted to nitrite, by incubation with 60 mU nitrate reductase and 25 μM NADPH for 120 min, and then nitrite was measured by the Griess reaction (see below).

In vitro *Experiments*

Cell culture The mouse macrophage cell line RAW 264.7 was cultured in DMEM. Cells were treated with LPS (10 μg ml^{-1}) for 24 h in the presence or the absence of various concentrations (10–300 μM) of dantrolene.

Measurement of IL-10 and TNF-α IL-10 secretion 24 h and TNF-α production 6 h after LPS stimulation were measured in samples of cell culture supernatant using ELISA kits as described above.

Measurement of nitrite concentration Nitrite in culture supernatants at 24 h or in plasma (after conversion of nitrate to nitrite, see above) was measured as previously described (Szabó *et al*., 1993b), by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of medium or diluted plasma samples, respectively. The OD at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The detection limit of the assay is 1 μM. The measurements of nitrite/nitrate were performed using reagents free of nitrite and nitrate: no basal or background nitrite or nitrate levels were detected.

Measurement of mitochondrial respiration Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Szabó *et al*., 1993b). Cells in 96-well plates were incubated with MTT (0.2 mg ml^{-1}) for 60 min at 37°C. Culture medium was removed by aspiration and cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantified by measurement of OD_{550} using a Spectramax microplate reader.

Preparation of nuclear extracts and nuclear factor κB (NF-κB) Western blotting Cells were treated with LPS in the presence and the absence of dantrolene (300 μM) for 90 min. Mini-nuclear extracts were prepared as previously described (Hassanain *et al*., 1993). Briefly, cells were scraped and pellets were resuspended in 400 μl of cold buffer A (HEPES pH 7.9 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1 mM, PMSF 0.5 mM, pepstatin A 1 μg ml^{-1}, leupeptin 10 μg ml^{-1}, and aprotinin 10 μg ml^{-1} on ice for 15 min in the presence of 25 μl 1% Nonidet P-40. Then, samples were vortexed, centrifuged for 1 min at $10{,}000 \times g$, and the pellet

was resuspended with 100 μl of buffer B (HEPES pH 7.9 20 mM, NaCl 400 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, PMSF 0.5 mM, pepstatin A 1 μg ml^{-1}, leupeptin 10 μg ml^{-1} and aprotinin 10 μg ml^{-1}). After shaking on a rocker platform for 15 min at 4°C, samples were centrifuged for 15 min at 10,000 $\times g$ at 4°C. Seventy-microlitre aliquots were then treated with 150 μl of SDS-PAGE sample buffer. Western blotting was performed (as described above), with rabbit anti-mouse NF-κB as the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1 : 750 in Tween/Tris-buffered saline 0.02%).

Fluorescence measurement of $[Ca^{2+}]_i$ $[Ca^{2+}]_i$ was monitored in single cells using the calcium-sensitive dye Fura-2 acetoxymethylester. Cells were loaded with 3 μM Fura-2 acetoxymethylester at room temperature for 45 min. Images were obtained using an intensified charge coupled device camera (Photon Technology International, South Brunswick, NJ). Once loaded with the fluorescent probe, the cells were excited via a 40 $\times$ fluorine objective (Nikon) using a 75 W Xenon light and visualized in an inverted phase-contrast microscope (Nikon Diaphot TMD) at 37°C. The light beam for the appropriate excitation wavelength was produced by two monochromators for 340/380 nm. At the beginning and at the end of the experiments a brightfield image was taken at the area of interest to control the images. The actual $[Ca^{2+}]_i$ was calculated from the ratio (R) of 340 and 380 excitation wavelengths at the emission wavelength of 510 nm using the following equation:

$$[Ca^{2+}]_i = K_d\ \beta(R - R_{min})/(R_{max} - R)$$

where R_{min} is the fluorescence ratio of Ca^{2+} free Fura-2 and R_{max} is the ratio of the Ca^{2+}-bound Fura-2, the K_d is the dissociation constant, and β is the ratio of the intensity of the free-form of the dye and the bound form of the dye measured at 380 nm. The system was calibrated after the cell membrane was permeabilized with 2 μM of ionomycin to obtain R_{max}, and in the presence of EGTA (1 mM) in nominally Ca^{2+}- free medium to obtain R_{min}. Images were off-line analysed by the ImageMaster software.

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Grand Island, NY). LPS from *Escherichia coli* O55:B5, dantrolene, and all other drugs were obtained from Sigma (St. Louis, MO).

Statistical evaluation

Values in the figures and text are expressed as mean $\pm$ s.e.mean of n observations. Statistical analysis of the data was performed by Student unpaired t test or one-way analysis of variance followed by Dunnett's test, as appropriate. A P value less than 0.05 was considered statistically significant.

Results

Effect of dantrolene on LPS-induced plasma IL-10, TNF-α and IL-6 levels in mice

Intraperitoneal injection of LPS (4 mg kg^{-1}) caused an elevation of IL-10 plasma concentrations, which peaked at 90 min, started to decline thereafter and became undetectable at 300 min (Figure 1). Intraperitoneal pretreatment of animals with dantrolene 30 min before LPS injection resulted in a significant augmentation of LPS-induced plasma levels of IL-10 at 90, 180 and 240 min after the LPS challenge (Figure 1). In addition, LPS-induced TNF-α levels were significantly decreased (Figure 2a), but LPS-induced plasma IL-6 levels were not altered (Figure 2b) by dantrolene-pretreatment, as measured at 90 min following LPS injection. The 90 min time point was chosen because it represents the peak of the response for both TNF-α (Elenkov *et al*., 1995; Haskó *et al*., 1995; 1996a) and IL-6 (Haskó *et al*., 1995).

Effect of dantrolene on plasma levels of nitrite/nitrate in LPS-treated BALB/c mice

In response to LPS administered at 4 mg kg^{-1} (i.p.), a marked elevation of plasma nitrite/nitrate levels was seen at 6–24 h, which returned to baseline levels at 48 h following LPS injection (Figure 3). Pretreatment of animals with dantrolene caused a significant, partial reduction in this LPS-induced nitrite/nitrate response at 6 and 9 h (Figure 3).

Dantrolene maintains its ability to inhibit TNF-α and NO production in IL-10 deficient mice

Using IL-10 deficient mice, it was demonstrated that endogenous IL-10 down-regulates the production of both TNF-α and NO in LPS-treated mice (Berg *et al*., 1995). Therefore, we wished to examine whether the increased IL-10 production by dantrolene was responsible for the reduced plasma TNF-α and NO levels. For this purpose, we pretreated C57BL/6 IL-10$^{+/+}$ and C57BL/6 IL-10$^{0/0}$ mice with dantrolene 30 min before the LPS injection and determined IL-10, TNF-α and nitrite/nitrate production in the plasma 4 h (except in the case of TNF-α from C57BL/6 IL-10$^{+/+}$ mice, where the 90 min time-point was chosen) after the LPS challenge. In the C57BL/6 IL-10$^{+/+}$ mice, the IL-10 level rose from 2.51 $\pm$ 0.59 (n = 6) in control animals to 8.89 $\pm$ 1.01 (n = 7) in dantrolene pretreated mice ($P<0.01$). The extent of inhibition of TNF-α and nitrite/nitrate production by dantrolene was similar in C57BL/6 IL-10$^{+/+}$ and C57BL/6 IL-10$^{0/0}$ mice (Figure 4), suggesting that the increased IL-10 production does not contribute to the decreased production of TNF-α and nitrite/nitrate by dantrolene.

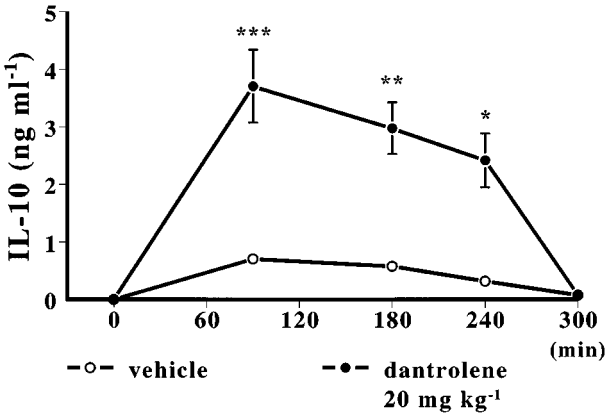

Figure 1 Effect of pretreatment with dantrolene (20 mg kg^{-1}) on LPS-induced plasma IL-10 levels at various points after i.p. administration of 4 mg kg^{-1} LPS. Data are the mean and vertical lines s.e.mean of 6 to 9 mice per group. *Indicates $P<0.05$; **indicates $P<0.01$; ***indicates $P<0.005$.

Dantrolene inhibits the production of IL-10 and TNF-α and the formation of nitrite in LPS-stimulated RAW 264.7 macrophage cells

Treatment of the RAW 264.7 cells with dantrolene (300 μM) caused a significant inhibition of production of IL-10 and TNF-α and the formation of nitrite in these cells stimulated with LPS (10 μg ml^{-1}) (Figure 5a,b,c). Dantrolene did not alter the LPS-induced suppression of mitochondrial respiration, as assessed by the reduction of MTT to formazan (Figure 5d).

Effect of dantrolene on LPS-induced nuclear translocation of NF-κB

Exposure of RAW 264.7 cells to LPS (10 μg ml^{-1}) for 90 min resulted in an increase in the nuclear translocation of the transcription factor NF-κB (Figure 6). However, pretreatment of the cells with dantrolene (300 μM) did not affect the LPS-induced nuclear translocation of this transcription factor (Figure 6).

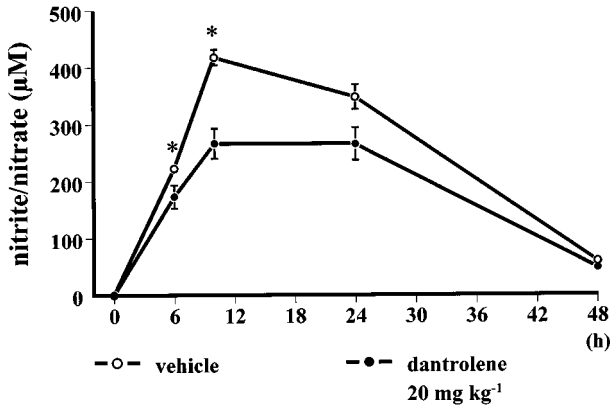

Figure 3 Dantrolene (20 mg kg^{-1}) pretreatment suppresses plasma NO levels (measured as nitrite/nitrate concentrations) at 6 and 9 h following i.p. administration of 4 mg kg^{-1} of LPS. Data are the mean and vertical lines s.e.mean of 6 to 9 mice per group. *Indicates $P<0.05$.

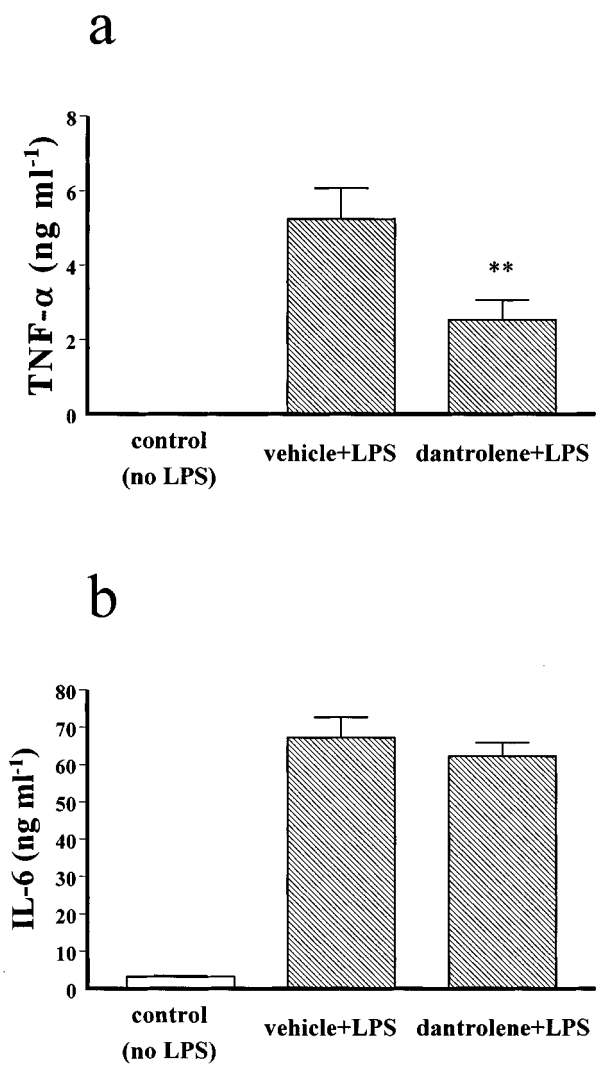

Figure 2 Effect of pretreatment with dantrolene (20 mg kg^{-1}) on plasma TNF-α (a) and IL-6 (b) concentrations 90 min after i.p. administration of 4 mg kg^{-1} LPS. Data are the mean ± s.e. mean of 6 to 9 mice per group. **Indicates $P<0.01$.

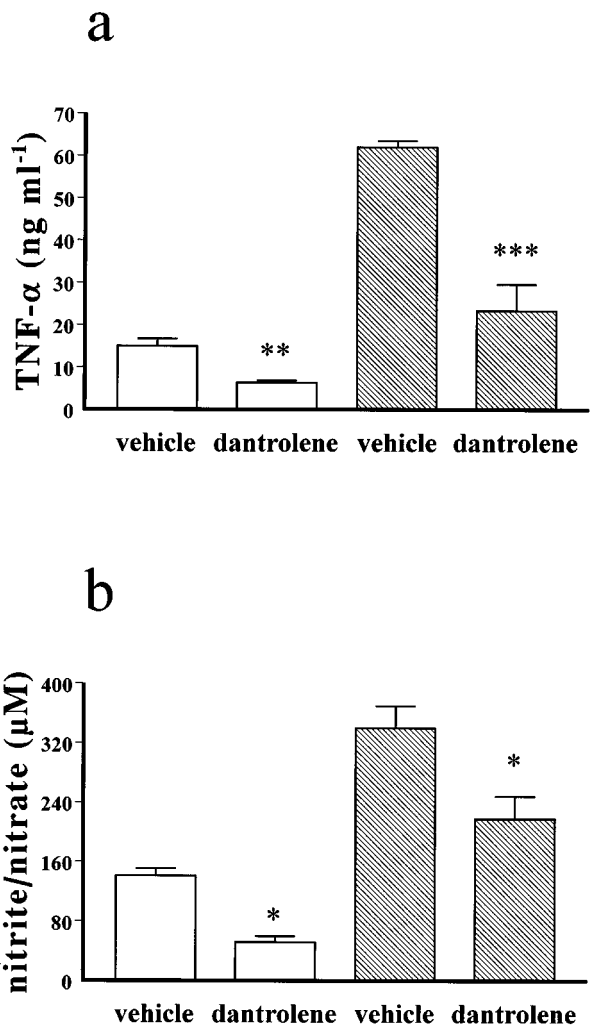

Figure 4 Effect of dantrolene (20 mg $^{-1}$, i.p.) on the production of LPS-induced plasma TNF-α (a) and nitrite/nitrate (b) concentrations in C57BL/6 IL-10$^{+/+}$ (open columns) and C57BL/6 IL-10$^{0/0}$ (hatched columns) mice. Dantrolene was injected 30 min before LPS (80 mg kg^{-1}, i.p.). Data are the mean ± s.e.mean of six to nine mice per group. *Indicates $P<0.05$; **indicates $P<0.01$.; ***indicates $P<0.005$.

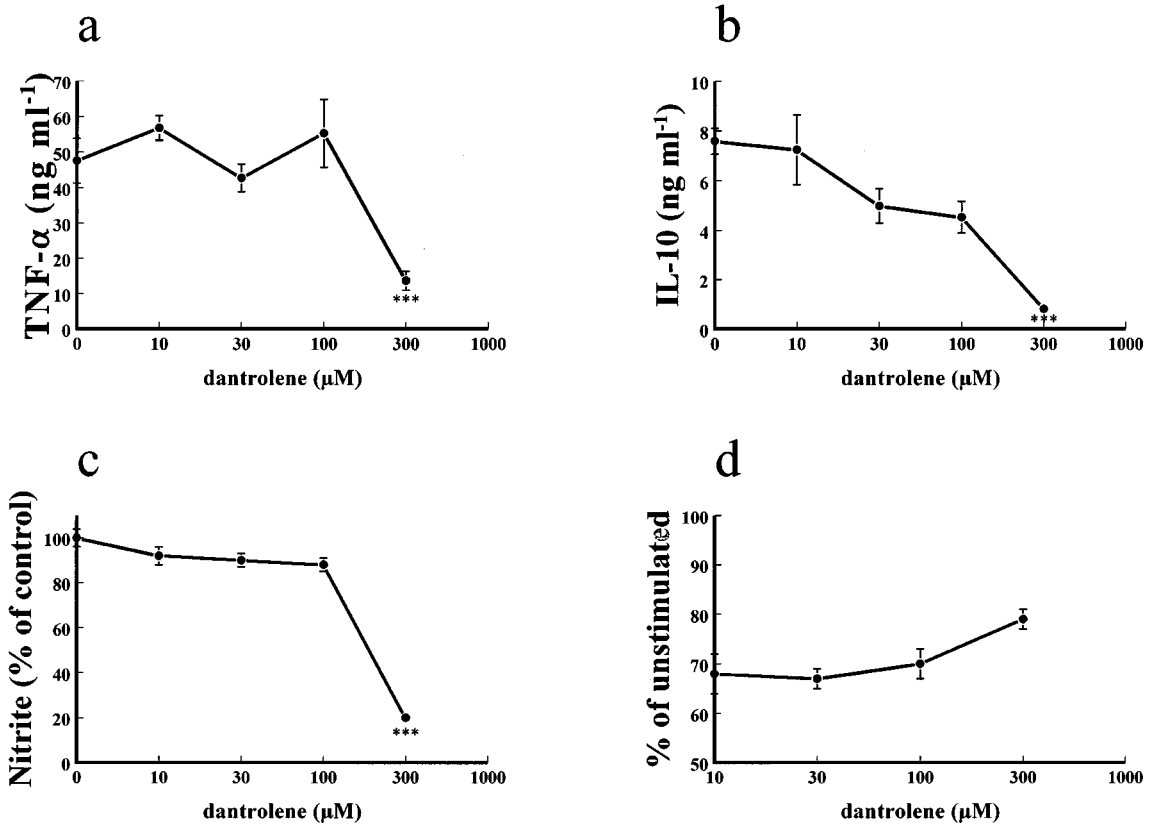

Figure 5 Dantrolene inhibits LPS-induced, TNF-α (a), IL-10 (b), and NO (measured as nitrite concentrations) (c) production by RAW 264.7 macrophages. (d) The effect of dantrolene on the mitochondrial respiration in the same cells. Cells were stimulated with LPS (10 μg ml^{-1}) for 6 (for TNF-α determination) or 24 h (for nitrite measurement) in the presence of various concentrations (10–300 μM) of dantrolene, and TNF-α, IL-10, and nitrite concentrations were determined from the supernatants. Mitochondrial respiration was measured by the conversion of MTT to formazan. Data are expressed as the mean±s.e.mean of 6 wells. ***Indicates $P<0.005$.

Dantrolene decreases basal $[Ca^{2+}]_i$

In agreement with previous studies (Drysdale *et al*., 1987; Maudsley & Morris, 1987; Hauschildt *et al*., 1990), LPS failed to influence $[Ca^{2+}]_i$ during a 60 min observation period (not shown). On the other hand, dantrolene (300 μM) caused a statistically significant decrease in $[Ca^{2+}]_i$ after a 30 min incubation time (Figure 7a). However, in vehicle control experiments $[Ca^{2+}]_i$ did not change significantly during the 30 min incubation period (data not shown). Moreover, after the 30 min exposure to dantrolene, ATP (1 mM) increased $[Ca^{2+}]_i$ in both the vehicle-treated and dantrolene-treated cells showing that the macrophages were functional in this setting (Figure 7b). These results also demonstrate that the increase in $[Ca^{2+}]_i$ by ATP is not sensitive to dantrolene, suggesting that the ATP-induced $[Ca^{2+}]_i$ elevation is not due to the release of calcium from its intracellular stores.

Discussion

The cellular signalling events leading to the systemic inflammatory response syndrome and sepsis in monocytes/macrophages activated by LPS are complex. There are a multitude of intracellular processes which are linked to (or precede) the production of inflammatory mediators from these cells. Alteration of $[Ca^{2+}]_i$ is one pathway whereby LPS may exert its activating functions with subsequent release of inflammatory molecules. LPS has been shown to increase

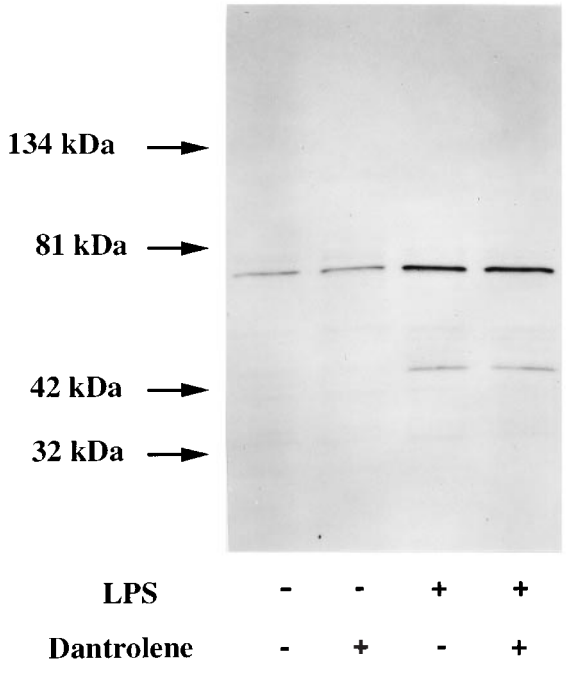

Figure 6 Dantrolene does not alter the nuclear translocation of NF-κB by LPS in RAW 264.7 macrophages. The figure shows the representative NF-κB Western blot in nuclear extracts of control RAW 264.7 cells and in cells 90 min after LPS treatment in the presence or absence dantrolene (300 μM).

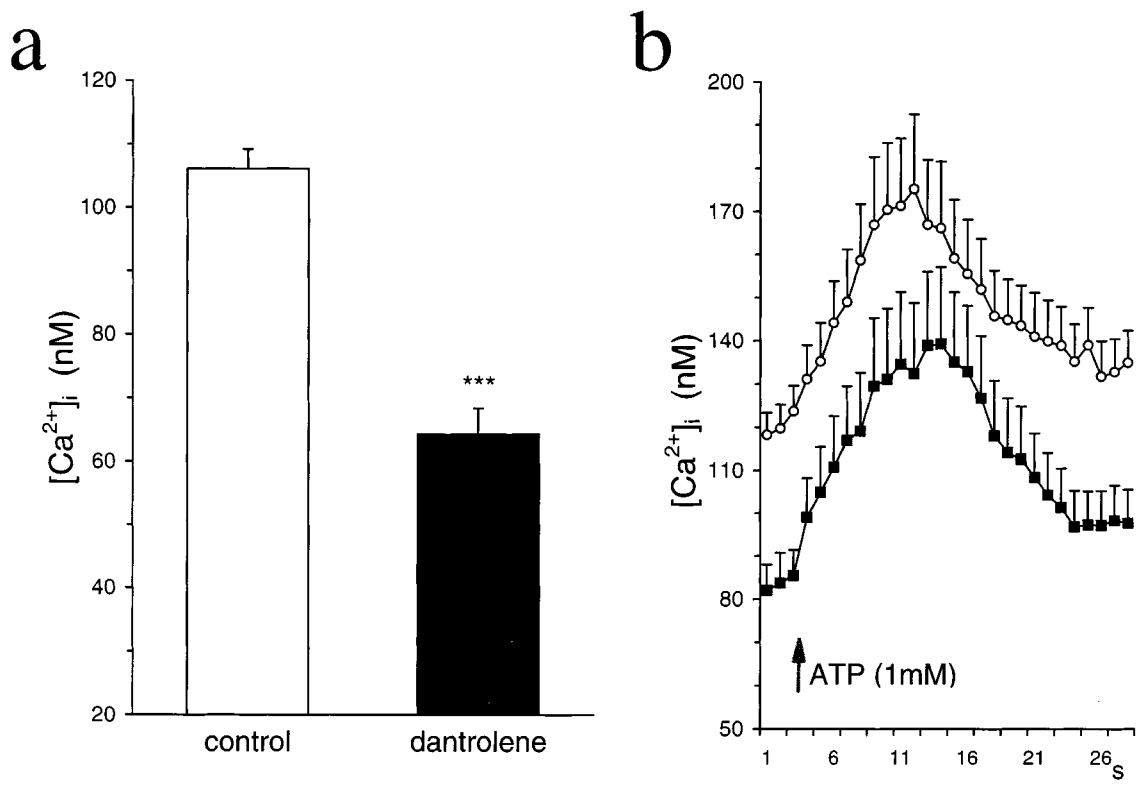

Figure 7 (a) Dantrolene at 300 μM decreases basal $[Ca^{2+}]_i$ in single macrophages loaded with Fura-2. (b) ATP (1 mM) increases the $[Ca^{2+}]_i$ in both dantrolene-treated (solid rectangles) and vehicle-treated (open circles) cells. Data are expressed as the mean and vertical lines show s.e.mean of 39–42 cells. ***Indicates $P<0.005$.

$[Ca^{2+}]_i$ in a variety of cell types both *in vitro* and *in vivo* (Gorecka-Tisera *et al*., 1986; Baldwin *et al*., 1991; Letari *et al*., 1991; Song *et al*., 1993; Zaloga *et al*., 1993). However, in the case of monocytes/macrophages, this has not been a consistent finding, as several studies describe the inability of LPS to cause an increase in $[Ca^{2+}]_i$ (Drysdale *et al*., 1987; Maudsley & Morris, 1987; Hauschildt *et al*., 1990). Interestingly, even if there was no increase in $[Ca^{2+}]_i$ in response to LPS, intracellular calcium antagonists did impair cytotoxic activity and reduced the production of a soluble cytotoxic factor in mouse peritoneal macrophages, suggesting a role for intracellular calcium in these processes (Drysdale *et al*., 1987). Despite all these inconsistencies, drugs able to inhibit the elevation of $[Ca^{2+}]_i$ have been shown to inhibit the production of TNF-α and IL-1 (Hotchkiss *et al*., 1995), and NO (Szabó *et al*., 1993a,b; 1997). Moreover, drugs that elevate $[Ca^{2+}]_i$ have been shown to stimulate the production of IL-1 (Suttles *et al*., 1990), TNF-α, nitrite, prostaglandins and leukotriens (Aderem & Cohn, 1988; Buchmüller-Rouiller & Mauël, 1991), further substantiating the importance of $[Ca^{2+}]_i$ in these processes.

Dantrolene is used in clinical practice to treat patients with the lethal malignant hyperthermia syndrome, a condition characterized by elevated $[Ca^{2+}]_i$ due to the lack of proper storage of calcium by the sarcoplasmic reticulum (Ward *et al*., 1986). This drug prevents the increase in $[Ca^{2+}]_i$ following a triggering stimulus in cells in which calcium is predominantly stored in the endoplasmic reticulum and mitochondria (Baldwin *et al*., 1991; Song *et al*., 1993; Chaudhuri & Ganguly, 1995). Moreover, dantrolene decreases basal $[Ca^{2+}]_i$ in single muscle fibres (Hainaut & Desmedt, 1974) and in rat cortical synaptosomes (Nath *et al*., 1995).

In this study, we show for the first time, that pretreatment of mice with dantrolene results in a potentiation of LPS-induced plasma IL-10 levels and a decrease in circulating concentrations of LPS-induced TNF-α and NO. In contrast, in the RAW 264.7 macrophage cell line, dantrolene inhibited the production of TNF-α and NO, as well as of IL-10. The differential regulation of IL-10 production *in vitro* and *in vivo* by a pharmacological agent is not unprecedented, as it has been shown that hydrocortisone (van der Poll *et al*., 1996a) or adenosine receptor agonists (Haskó *et al*., 1996b) enhance LPS-induced plasma IL-10 levels, but inhibit LPS-induced IL-10 production *in vitro*. A possible explanation for this phenomenon is the differential regulation of inflammatory events *in vitro* and *in vivo* under endotoxaemic conditions. LPS failed to elevate $[Ca^{2+}]_i$ *in vitro* (this study, Drysdale *et al*., 1987; Maudsley & Morris, 1987; Hauschildt *et al*., 1990) but $[Ca^{2+}]_i$ was elevated in spleen slices (Hotchkiss and Karl, 1996), aortae (Song *et al*., 1993), or hepatocytes (Rose *et al*., 1992) taken from septic animals. Therefore, it can be speculated that LPS doesn't elevate $[Ca^{2+}]_i$ in macrophages directly, but other indirect mechanisms involving circulating hormones may be responsible for the increased $[Ca^{2+}]_i$ in these cells in endotoxaemia. Sayeed & Maitra, (1987) proposed that calcium mobilizing hormones, such as adrenaline, angiotensin II or vasopressin that are known to be present at high concentrations in the circulation of septic animals (Clowes, 1988), could contribute to the increased $[Ca^{2+}]_i$ in endotoxin shock. In this case, the *in vivo* stimulating effect of dantrolene on IL-10 may be related to its ability to influence the production of these circulating hormones. In this respect, it is of note that adrenaline has been demonstrated to augment IL-10 plasma levels in

endotoxaemia (van der Poll *et al*., 1996b). On the other hand, since dantrolene decreased basal calcium concentrations in the RAW 264.7 macrophages (Figure 7), it can be suggested that in isolated macrophages the decrease in IL-10, as well as of TNF-α and NO production by dantrolene was due to the decreased basal $[Ca^{2+}]_i$.

A further important observation of our study is that the increase in IL-10 production by dantrolene did not contribute to the decreased production of TNF-α and NO. Since endogenous IL-10 is known to down-regulate TNF-α and NO production in endotoxaemic mice (Berg *et al*., 1995), it seemed possible that dantrolene may exert its inhibitory effect on TNF-α and NO by augmenting the production of IL-10. Moreover, in the case of PGE_2 (Strassman *et al*., 1994) and rolipram (Kambayashi *et al*., 1995) the decrease in TNF-α production was partly dependent on the parallel up-regulation of IL-10. However, in the present study, we provided evidence that dantrolene maintained its ability to suppress TNF-α and NO production in IL-10 deficient mice, which suggests that there is no correlation between the up-regulation of IL-10 and inhibition of TNF-α and NO production.

The formation of NO under inflammatory conditions from the guanidino nitrogen group of L-arginine is catalysed by iNOS, expressed in response to pro-inflammatory stimuli (Szabó, 1995). The mechanism of iNOS induction by LPS *in vivo* is regulated by pro- and anti-inflammatory cytokines and glucocorticoids. For instance, endogenous release of TNF-α and IL-1 are involved in the process of iNOS induction (Szabó *et al*., 1993c, Thiemermann *et al*., 1993; Szabó, 1995). Thus, it is conceivable to suggest that in our study the suppression of LPS-induced TNF-α production by dantrolene contributed to the reduction of LPS-induced NO production.

Activation of the transcription factor NF-κB is considered a crucial event in macrophage activation in response to LPS and nuclear translocation of NF-κB is involved in the initiation of transcription of several genes, including that of IL-10, TNF-α and iNOS (Müller *et al*., 1993; Szabó, 1995; Meisel *et al*., 1996). Our observation that dantrolene did not inhibit the nuclear translocation of NF-κB suggests that the mechanism of action by which dantrolene suppresses macrophage activation is not related to its interference with the activation of this transcription factor system.

In summary, it can be concluded that $[Ca^{2+}]_i$ plays an important role in the regulation of IL-10, TNF-α and NO production both *in vitro* and *in vivo*. The inhibition of TNF-α, IL-10 and NO production *in vitro* may be the consequence of the decrease in basal $[Ca^{2+}]_i$ by this drug. The observations that in LPS-treated mice, dantrolene inhibited production of the potentially harmful inflammatory mediators TNF-α (this study, Hotchkiss *et al*., 1995), NO (this study) and IL-1 (Hotchkiss *et al*., 1995), but enhanced the production of the anti-inflammatory IL-10, suggest that the therapeutic potential of this agent should be further investigated in experimental models of inflammatory diseases and/or septic shock.

This work was supported by grants from OTKA (No. T 017567 and T 016756 Hungarian Research Fund), ETT (No. 20011996) to Dr Haskó, and a Grant-In-Aid from The American Heart Association to Dr Szabó. The expert technical assistance of Mrs F. Tóth, Miss J. Benkó, Mr M. O'Connor and Mr Paul Hake is gratefully acknowledged.

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(Received October 9, 1997
Accepted April 6, 1998)