JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 1249–1254

Received March 15, 2007
Accepted May 27, 2007
DOI 10.1211/jpp.59.9.0009
ISSN 0022-3573

Effect of a leukotriene inhibitor (MK886) on nitric oxide and hydrogen peroxide production by macrophages of acutely and chronically stressed mice

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Abstract

We evaluated the effect of a leukotriene inhibitor (MK886) on nitric oxide (NO) and hydrogen peroxide (H_2O_2) production by peritoneal macrophages of mice subjected to acute and chronic stress. Acute stress was induced by keeping mice immobilized in a tube for 2 h. Chronic stress was induced over a 7-day period by the same method, but with increasing duration of immobilization. The effects of MK886 were investigated in-vitro after incubation with peritoneal macrophages, and in-vivo by submitting mice to stress and treating them daily with MK886. Supernatants of macrophage cultures were collected for NO determination and adherent cells were used for H_2O_2 determination. Macrophages from mice submitted to acute or chronic stress showed no alterations in H_2O_2 production. However, macrophages of acutely and chronically stressed mice showed inhibition of NO after incubation with MK886 in-vitro. Administration of MK886 to chronically stressed mice increased generation of H_2O_2 and inhibited production of NO. Our data suggest an important role of leukotrienes in NO synthesis, which is important in controlling replication of several infectious agents, mainly in stressed and immunosuppressed animals.

Introduction

Stress comprises a variety of events resulting from the action of one or more stressors, which subsequently activate physiological 'fight or flight' responses, with disruption of homeostasis (Dhabhar 2002; Sahin & Gumuslu 2004). Stress results in the release of neurotransmitters, hormones and cytokines, and interactions of the central nervous system with the endocrine and immune systems, causing activation of the hypothalamic–pituitary–adrenal (HPA) axis and the autonomous nervous system (Kioukia-Fougia et al 2002).

Stressor agents may induce the production of pro-inflammatory molecules such as cytokines and lipid mediators (Webster et al 2002). Following arachidonic acid (AA) release from membrane phospholipids in response to a variety of non-specific activation stimuli, such as stress, AA is converted to prostaglandins by cyclooxygenases and to leukotrienes (LTs) by 5-lipoxygenase (5-LO) (Rocca & Fitzgerald 2002). LTs are classic mediators of inflammatory responses, and are synthesized predominantly by myeloid cells in response to several stimuli, including microbes. AA can be oxygenated by 5-LO in association with 5-LO-activating protein (FLAP). This Ca^{2+}-dependent reaction results in the formation of the unstable intermediate LTA_4, which can be hydrolysed to LTB_4 or conjugated with reduced glutathione to LTC_4. These lipids promote inflammatory processes by binding to specific surface receptors on target cells (Miller et al 1990; Peters-Golden & Brock 2001). LTs have an important role in cell activation, regulate phagocytes, have microbicidal activity and participate in the host immune response to a wide range of microbes, including bacteria (Bailie et al 1996), fungi (Medeiros et al 2004) and helminthes (Machado et al 2005).

MK886 is a potent inhibitor of LT biosynthesis, both in-vivo and in-vitro; it binds to FLAP and prevents the translocation of 5-LO from the cytosol to the membrane. MK886 does not affect any other route of AA metabolism, including the cyclooxygenase or 12-LO pathways (Gillard et al 1989; Ford-Hutchinson 1991).

Since stress affects immune and inflammatory responses, the aim of this work was to evaluate the involvement of LTs in acute and chronic stress induced in mice by immobilization.

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Basal production of hydrogen peroxide (H_2O_2) and nitric oxide (NO) by peritoneal macrophages from acutely and chronically stressed mice was analysed after incubation in-vitro with MK886, and after administration of MK886 to chronically stressed mice. Stress indicators (glucose and corticosterone) were also measured.

Materials and Methods

Animals and stress procedures

Male BALB/c mice (6–8 weeks old) were used. To induce acute stress, animals were immobilized inside a tube (restrainer) of about 50 mL capacity for 2 h. To induce chronic stress, animals were submitted to the same protocol for a 7-day period with successively increasing duration of immobilization (15, 30, 45, 60, 75, 90 and 120 min). This procedure is easily performed and causes no physical pain to the animals (Domínguez-Gerpe & Rey-Méndez 2001). This work is in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA – 17.09.2003).

MK886

MK886 (L-663,536 (3-(1-(p-chlorobenzyl-5-isopropyl-3-t-butylthioindol-2-yl)-2,2-dimethyl propanoic acid sodium salt) was a generous gift from Merck Frosst, Canada Inc. MK886 was dissolved according to the manufacturer's instructions in 100 μL absolute ethanol (Medeiros et al 2004) and then diluted in water in order to obtain the treatment solution, or in culture medium for in-vitro studies.

Peritoneal macrophages

Peritoneal macrophages were obtained by inoculation of 3–5 mL cold phosphate-buffered saline into the abdominal cavity. After gentle abdominal massage for 30 s, the peritoneal fluid was collected and put in sterile plastic Falcon tubes (BD Biosciences, San Jose, CA, USA). This procedure was repeated 3 or 4 times for each animal. The tubes were then centrifuged at 200 *g* for 10 min. Cells were stained with neutral red (0.02%) and incubated for 10 min at 37°C to achieve a final concentration of 2×10^6 cells mL^{-1} (determined by counting in a haematocytometer). Cells were resuspended in cell culture medium (RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES, 25 μM 2-mercaptoethanol (all Sigma, St Louis, MO, USA)), and cultured in 96-well flat-bottomed plates (Corning Inc., Acton, MA, USA) at a final concentration of 2×10^5 cells per well. Cells were incubated at 37°C and non-adherent cells were removed after 2 h. Adherent cells were reincubated under the conditions described below.

In-vitro and in-vivo assays with MK886

To evaluate the effect of MK886 in-vitro, mice (n = 8) were submitted to acute or chronic stress, as described above. Macrophages taken from these mice were then incubated in-vitro with medium or MK886 (1 and 10 μM) for 24 h (Mancuso et al 1998).

To evaluate the effect of MK886 in-vivo during chronic stress, animals were divided into four groups of eight animals each. The control group were treated daily with 0.5 mL physiological saline (0.9%). A second group were submitted to immobilization stress and were treated with 0.5 mL physiological saline. A third group were treated daily with MK886 (5 mg kg^{-1} in 0.5 mL) orally by gavage (Medeiros et al 2004) and the fourth group were submitted to stress and treated daily with MK886 before stress, following the same protocol. All groups had no water and food during stress procedures. After 24 h of the respective treatments, animals were killed by cervical dislocation, and macrophages were collected and incubated with culture medium for 24 h (Orsi et al 2005).

Measurement of basal H_2O_2 and NO production

Supernatants of cell cultures were collected for measurement of NO, and adherent cells were collected for measurement of H_2O_2 (Pick & Mizel 1981). Red phenol solution (100 μL) containing 140 mM NaCl, 10 mM K_2HPO_4, 5.5 mM dextrose and 5.5 mM horseradish peroxidase (Sigma, St Louis, MO, USA) was added to the adherent cells for measurement of H_2O_2. After 1 h, 10 μL NaOH was added and absorbance measured at 620 nm using an automatic ELISA reader (Labsystems, Helsinki, Finland).

NO production was determined based on the Griess reaction (Green et al 1981). Supernatants of the 24-h-stimulated cells were collected and 100 μL Griess reagent (0.1% *N*-1-naphthyl-ethylenediamine and 1% sulphanilamide in 5% H_3PO_4) added. After 10 min at room temperature, absorbance was measured at 540 nm using an automatic ELISA reader (Labsystems).

Measurement of glucose and corticosterone

Before animals were killed, blood was collected by retro-orbital puncture and glucose concentration determined immediately using a commercial kit (Accutrend, Roche-Diagnostics Corp., Indianapolis, IN, USA). Serum was stored at −20°C and concentration of corticosterone was determined by radioimmunoassay using a commercial kit (Coat-A-count, DPC, Los Angeles, CA, USA).

Statistical analysis

Analysis of variance (ANOVA) was used, followed by Tukey–Kramer multiple comparison tests. Glucose and corticosterone levels were analysed using Student's independent *t*-test. A P value of less than 0.05 was considered significant.

Results and Discussion

Effect of MK886 on H_2O_2 and NO production by peritoneal macrophages of acutely stressed mice: in-vitro assays

Stress comprises a wide variety of events, providing evidence for autonomic pathways and endocrine modulation of the immune response following stress exposure. Studies of the

involvement of LTs on the production of oxygen and nitrogen intermediates (H_2O_2 and NO) contribute to understanding of the immune response during stress. Thus, this work was carried out in order to evaluate the relationship between stress LTs and macrophages, since LTs may be produced during stress and macrophage function may therefore be affected.

Reactive oxygen and nitrogen intermediates are related to the microbicidal activity of macrophages (Macfarlane et al 1999; Riber & Lind 1999). The rate of oxygen consumption is increased during cold stress in mammals, and the most common types of reactive oxygen species are superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals (OH^-) (Ates et al 2006).

We found no differences in basal H_2O_2 production by macrophages from acutely stressed mice compared with control mice. MK886 did not have an effect on in-vitro production of H_2O_2 by peritoneal macrophages of control mice or stressed mice (Figure 1A). Basal production of NO was increased in macrophages from acutely stressed mice when compared with control mice ($P < 0.001$). NO production in control mice was inhibited by 56% and 63% after incubation with 1 and 10 μM MK886, respectively ($P < 0.01$). Moreover, NO generation was strongly inhibited (~93%) in macrophages from acutely stressed mice incubated with 10 μM MK886 ($P < 0.001$; Figure 1B).

Effect of MK886 on H_2O_2 and NO production by peritoneal macrophages of chronically stressed mice: in-vivo assays

Acute stress lasts for a period of minutes to hours, and may enhance immune function. Chronic stress persists for several hours a day, for weeks or months, and affects important behavioural, neurochemical and endocrine parameters, inducing immunosuppression (Dhabhar 2002). Evidence accumulated over the past decade strongly suggests that stress hormones differentially regulate T-helper (Th) cells, affecting Th1/Th2 patterns and cytokine secretion, suppressing the Th1 response and causing a shift towards the Th2 response (Elenkov & Chrousos 1999).

With regard to chronic stress, we observed a non-significant increase in H_2O_2 production by macrophages from chronically stressed mice compared with control mice (Figure 2A). However, no differences in NO production were seen between the groups (Figure 2B). In-vivo treatment with MK886 increased

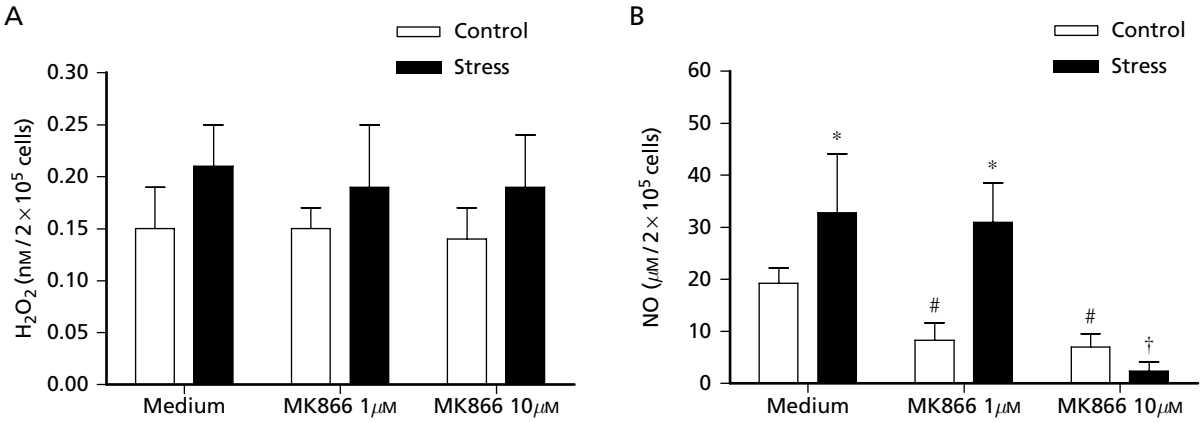

Figure 1 H_2O_2 (A) and NO (B) production by peritoneal macrophages from acutely stressed mice incubated with MK886 (1 and 10 μM) in-vitro. Data are mean ± s.d. of 8 mice. *$P < 0.05$ vs control group; #$P < 0.05$ vs control group incubated with medium; †$P < 0.05$ vs stress group incubated with medium.

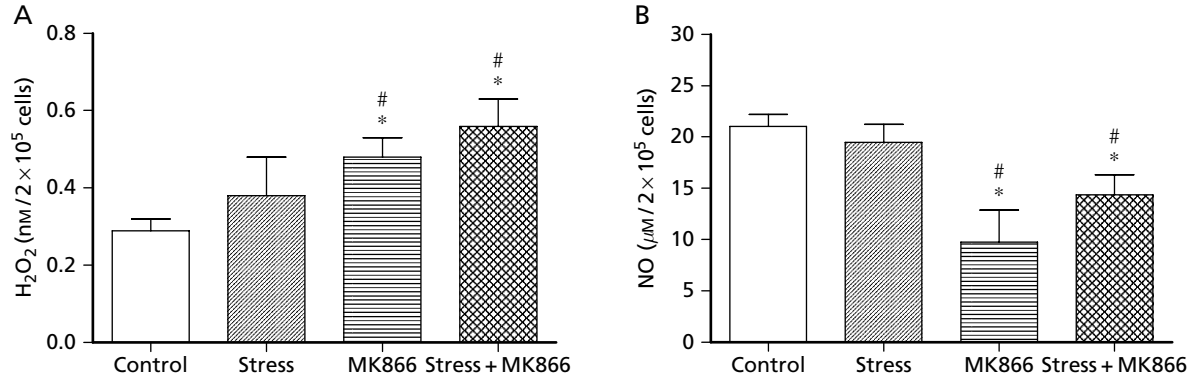

Figure 2 H_2O_2 (A) and NO (B) production by peritoneal macrophages of chronically stressed mice treated with MK886 (5 mg kg^{-1}) in-vivo. Data are mean ± s.d. of 8 mice. *$P < 0.05$ vs control group; #$P < 0.05$ vs stress group.

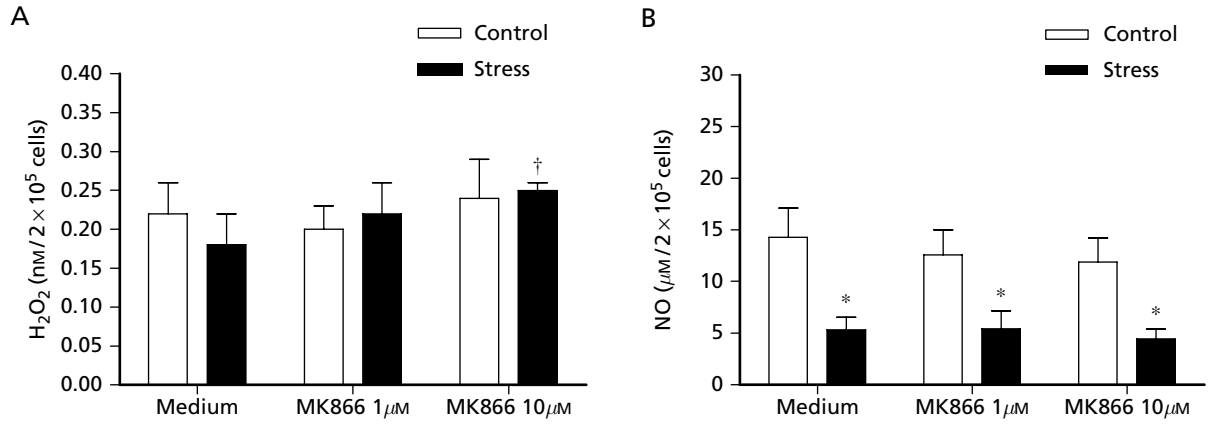

Figure 3 H_2O_2 (A) and NO (B) production by peritoneal macrophages of chronically stressed mice incubated with MK886 (1 and 10 μM) in-vitro. Data are mean ± s.d. of 8 mice. *$P < 0.05$ vs control group; †$P < 0.05$ vs macrophages from stress group incubated with medium.

H_2O_2 generation ($P < 0.01$; Figure 2A), but inhibited NO production ($P < 0.01$; Figure 2B) in macrophages from control and chronically stressed mice. In fact, it has been demonstrated that LTs may induce NO production.

For example, Talvani et al (2002) reported that LTB_4 induces NO production by peritoneal macrophages from animals infected with *Trypanosoma cruzi* in a dose- and time-dependent manner. LTB_4 was also involved in increased production of NO by macrophages in response to *Leishmania amazonensis* challenge (Serezani et al 2006). It has therefore been suggested that MK886 diminishes NO production (Chen et al 2001; Medeiros et al 2004).

Recent work has demonstrated that MK886 also acts as a selective antagonist of peroxisome-proliferator-activated receptor (PPAR)-α (Kehrer et al 2001). Although most of the actions of MK886 seem to be related to inhibition of LT synthesis, there are other important aspects to point out. The PPAR-α agonist Wy16643 stimulated the formation of reactive oxygen species in several murine and human macrophages (Teisser et al 2004). In our study, in-vivo treatment with MK886 increased H_2O_2 generation by peritoneal macrophages from both control and stressed mice (Figure 2A) but this effect was not observed after in-vitro incubation. Other PPAR-α agonists (LTB_4 and 8(S)-hydroxyeicosatetraenoic acid) increase NO production in RAW264.7 macrophages stimulated by interferon-gamma and lipopolysaccharides (Vivancos & Moreno 2002). It is not clear whether inhibition of NO production after treatment in-vivo with MK886 (Figure 2B) relates to the inhibition of LT synthesis, antagonism of PPAR-α receptors or both.

Effect of MK886 on H_2O_2 and NO production by peritoneal macrophages of chronically stressed mice: in-vitro assays

Incubation in-vitro with MK886 did not affect H_2O_2 generation by peritoneal macrophages of control mice. A slight but significant increase in H_2O_2 generation ($P < 0.01$) was observed when macrophages of chronically stressed mice were incubated in-vitro with 10 μM MK886 (Figure 3A). Peritoneal macrophages from chronically stressed mice produced significantly less NO than those from control mice ($P < 0.001$), and this was not affected by in-vitro incubation with MK886 (Figure 3B).

The combined effects of glucocorticoids and catecholamines are to inhibit innate immunity (Charmandari et al 2005). These molecules, released during stress, could explain the decreased generation of NO observed in macrophages of chronically stressed mice.

Glucose and corticosterone determination

Serum glucose levels have been used as an indicator of stress. However, we found no differences in glucose concentration after chronic stress compared with control mice (Table 1). Kioukia-Fougia et al (2002) also reported no differences in serum glucose levels of animals submitted to three different stress protocols. In fact, one may find higher glucose levels after acute stress, but these high levels may not persist.

Serum corticosterone concentrations were higher in stressed animals than in controls, but this difference was not statistically significant (Table 1). Activation of the HPA axis has an important role in behavioural and immunological responses during stress. Azpiroz et al (1999) also found that chronic stress did not affect corticosterone levels in mice, suggesting an adaptive response of the HPA axis in the presence of prolonged elevated glucocorticoid concentrations. It is possible that the end point chosen for blood collection (24 h after treatment) was not adequate for the quantification of glucose and corticosterone.

The stress system coordinates the adaptive response of an organism to stressors and plays an important role in maintenance

Table 1 Serum concentrations of glucose and corticosterone of control and chronically stressed mice

	Glucose (mg dL^{-1})	Corticosterone (ng mL^{-1})
Control	171.00 ± 17.56	127.25 ± 56.48
Stress	190.33 ± 26.41	147.76 ± 36.24

Data are mean ± s.d. of 8 animals.

of basal and stress-related homeostasis (Charmandari et al 2005). Our results show that LTs are important during stress, because they induce NO production, which is a potent mechanism of pathogen killing. The current study suggests that LTs were probably in a higher concentration, because of pro-inflammatory cytokines produced during stress, but MK886 inhibited this metabolite generation both in-vitro and in-vivo.

A better understanding of the effects of stress on production of reactive intermediates by macrophages and the mediators that regulate these functions will improve understanding of the stress–immunity axis, and open new perspectives for further investigations.

Conclusions

NO production by macrophages from acutely and chronically stressed mice was inhibited after in-vitro incubation with MK886. Administration of MK886 to chronically stressed mice increased generation of H_2O_2 and inhibited production of NO. Our data suggest an important role of LTs in NO synthesis, which is important to control replication of several infectious agents, mainly in stressed and immunosuppressed animals.

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