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RESEARCH PAPER

Atorvastatin inhibits inflammatory hypernociception

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Background and purpose: Atorvastatin is an inhibitor of the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A reductase used to prevent coronary heart disease. We have studied the analgesic effect of atorvastatin in inflammatory models in which a sequential release of mediators (bradykinin, (BK), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and the chemokine, KC/CXCL) links the stimulus with release of directly acting hypernociceptive mediators such as prostaglandin E_2 (PGE_2).
Experimental approach: The effects of orally administered atorvastatin on inflammatory mechanical hypernociception in mouse paws were evaluated with an electronic pressure-meter. Cytokines and PGE_2 were measured by ELISA and RIA.
Key results: Treatment with atorvastatin for 3 days dose-dependently reduced hypernociception induced by lipopolysaccharide (LPS) or that following antigen challenge in sensitized animals. Atorvastatin pre-treatment reduced hypernociception induced by bradykinin and cytokines (TNF-α, IL-1β and KC), and the release of IL-1β and PGE_2 in paw skin, induced by lipopolysaccharide. The antinociceptive effect of atorvastatin on LPS-induced hypernociception was prevented by mevalonate co-treatment without affecting serum cholesterol levels. Hypernociception induced by PGE_2 was inhibited by atorvastatin, suggesting intracellular antinociceptive mechanisms for atorvastatin. The antinociceptive effect of atorvastatin upon LPS- or PGE_2-induced hypernociception was prevented by non-selective inhibitors of nitric oxide synthase (NOS) but not by selective inhibition of inducible NOS or in mice lacking this enzyme.
Conclusions and implications: Antinociceptive effects of atorvastatin depend on inhibition of cytokines and prostanoid production and on stimulation of NO production by constitutive NOS. Our study suggests that statins may constitute a novel class of analgesic drugs.
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Keywords: atorvastatin; hyperalgesia; IL-1β; mevalonate; nitric oxide; pain

Abbreviations: BK, bradykinin; CFA, complete Freund's adjuvant; HMG-CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; KC/CXCL1, keratinocyte-derived chemokine; LPS, lipopolysaccharide; mBSA, methylated bovine serum albumin; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2

Introduction

The statins are a well-known class of cholesterol-lowering drugs that inhibit the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Grundy, 1988). They are the class of drugs most widely used for the prevention of primary and secondary coronary heart disease (Corsini *et al*., 1995; Bhatnagar, 1998; MacMahon *et al*., 1998; Heerey *et al*., 2000). Today, 25 million people worldwide are taking statins and they may account for the highest expenditure on prescription drugs in the United States (Topol, 2004). Among the statins, one of the most prescribed is atorvastatin (Youssef *et al*., 2002). Recent clinical trials showed that statins reduce the risk of atherosclerosis-induced cardiovascular diseases even in the absence of a significant decrease in the blood cholesterol level (Downs *et al*., 1998; Ridker *et al*., 1998). Thus, the benefits of statin therapy may be ascribed, at least in part, to their actions on non-lipid factors (Furberg *et al*., 1994; Vaughan *et al*., 1996; Hebert *et al*., 1997; Rosenson, 1999; Koh, 2000). For instance, statins inhibit the inflammatory process in the vessel wall, an important feature of atherosclerosis (Inoue *et al*., 2000). Statins also slow the progression of atherosclerosis by inhibiting monocyte activation, metalloprotease synthesis in the vessel wall and the production of proinflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-1β (Ferro *et al*., 2000; Solheim *et al*., 2001). Further evidence for

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an anti-inflammatory effect of statins is provided by their inhibition of cyclooxygenase-2 (COX-2) expression in a rabbit model of atherosclerosis and in cultured vascular smooth muscle cells stimulated by cytokines (Hernandez-Presa *et al*., 2002). Moreover, statins inhibit inflammatory responses in different models of autoimmune disease such as collagen- and complete Freund's adjuvant (CFA)-induced arthritis and experimental encephalomyelitis (Aktas *et al*., 2003; Leung *et al*., 2003; Barsante *et al*., 2005). In the CFA-arthritis model, atorvastatin also reduced joint hypernociception (Barsante *et al*., 2005). Independent of their effects on cholesterol synthesis, statins also upregulate the expression and the activity of endothelial nitric oxide synthase (eNOS), which accounts for their beneficial effect in preventing cardiovascular diseases (Massy *et al*., 1996; Maron *et al*., 2000; Wagner *et al*., 2000).

Pain is a major symptom of inflammatory disease. The sensitization of primary afferent nociceptors is a common denominator of all kinds of inflammatory pain, leading to a state of hyperalgesia and/or allodynia, better described as hypernociception in animal models (Millan, 1999; Parada *et al*., 2003). Hypernociception is induced by the direct action of inflammatory mediators, such as prostaglandins (PGs) and sympathetic amines, on the peripheral nociceptors (Ferreira and Nakamura, 1979; Nakamura and Ferreira, 1987; Khasar *et al*., 1999). These direct acting hyperalgesic mediators are ultimately released in the inflamed tissue following a cascade of cytokines (TNF-α, IL-1β and chemokines) released by the resident and migratory cells (Cunha *et al*., 1992, 2005; Cunha and Ferreira, 2003).

Experimental evidence suggests that the intracellular mechanism involved in nociceptor sensitization results from an activation of neuronal signalling pathways, beginning with cyclic adenosine 3′,5′ monophosphate (cAMP) production and, ultimately, to modulation of the activity of tetrodotoxin-resistant Na^+ channel (Coutaux *et al*., 2005). On the other hand, increased levels of cGMP in nociceptive neurons might decrease nociceptor excitability (Ferreira and Nakamura, 1979). Indeed, substances that increase neuronal cAMP such as dibutyryl cAMP and prostaglandin E_2 (PGE_2) induce hypernociception, whereas substances that increase cGMP such as nitric oxide (NO) donors cause antinociception (Ferreira and Nakamura, 1979; Taiwo *et al*., 1989; Duarte *et al*., 1992). Some analgesics such as morphine and dipyrone are able to block directly ongoing nociceptor sensitization by stimulation of the neuronal L-arginine/NO/cGMP pathway (Ferreira *et al*., 1991; Sachs *et al*., 2004). This mechanism was supported by the observation that the peripheral antinociception achieved with these analgesics was inhibited by inhibitors of NOS or by inhibitors of guanylyl cyclase (Ferreira *et al*., 1991; Duarte *et al*., 1992; Sachs *et al*., 2004). There is overwhelming evidence that the peripheral analgesic effect of opioids also depends on inhibition of cAMP formation and of Ca^{2+} channels in the peripheral nociceptive neurons (Levine and Taiwo, 1989; Stein *et al*., 2003).

Taking into account the anti-inflammatory and immunomodulatory properties of statins, the aim of this paper was to investigate the anti-hypernociceptive effect of atorvastatin and the mechanisms underlying such an effect.

Materials and methods

Animals

All experiments were carried out with Swiss mice (male, 25–30 g weight), except for those studying the effect of deleting the gene for inducible NOS (iNOS). In these, the wild-type (WT) mice were of the C57BL/6 strain (male, 25–30 g weight), as were the iNOS (−/−) mice (University of São Paulo, Ribeirão Preto, Brazil). Mice were housed in the animal care facility of the School of Medicine of Ribeirão Preto. Mice were taken to the testing room at least 1 h before experiments and were used once. The animal care and handling procedures were in accordance with the International Association for the Study of Pain Guidelines on the use of animals in pain research, and they were approved by the Animal Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo).

Mechanical nociception test

We use the term hypernociception rather than hyperalgesia or allodynia to define the decrease in the withdrawal threshold of nociceptive behaviour (Parada *et al*., 2003). Mechanical hypernociception was tested in mice as reported previously (Cunha *et al*., 2004). In a quiet room, mice were placed in acrylic cages ($12 \times 10 \times 17$ cm) with wire grid floors 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anaesthesiometer, IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5-mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching behaviour. After the paw withdrawal, the intensity of the pressure was automatically recorded. The value for the threshold of paw withdrawal was obtained by averaging three measurements. The animals were tested before and after treatments. The results are expressed by the difference in withdrawal thresholds (in grams) calculated by subtracting the zero-time mean value from the mean values at fixed times after injection of the hypernociceptive agents. Withdrawal threshold was 10 ± 0.3 g (mean $\pm$ s.e.m.; $n = 30$) at zero time, that is, before injection of the hypernociceptive agents. Quantification of hypernociception was performed by an observer, unaware of the treatment given to the animals (treatment-blinded).

Serum cholesterol assay

Total serum cholesterol levels were measured in blood drawn from the orbital plexus of mice just before the killing by a standard enzymatic end point method according to the manufacturer's instructions (Lab test, Brazil).

Active sensitization

Swiss mice were immunized as described previously (Brackertz *et al*., 1977). Briefly, on day 0 and day 7, the animals received a single subcutaneous (s.c.) injection of methylated bovine serum albumin (mBSA; 500 μg per animal) in 0.1 ml

of an emulsion containing 0.05 ml phosphate-buffered saline (PBS) and 0.05 ml CFA. Control mice were injected s.c. with 0.1 ml of an emulsion containing equal volumes of PBS and CFA (false immunized group). The mBSA-immunized and control animals were challenged by intraplantar (i.pl.) injection with mBSA (90 μg per paw) dissolved in 30 μl saline or saline alone on day 21.

*Measurement of IL-1*β *in paw skin*
At the times shown after the injection of the inflammatory stimulus, animals were killed and the skin tissues were removed from the injected and control paws (saline and naive). The samples were homogenized in 500 μl of the appropriate buffer containing protease inhibitors and IL-1β levels were determined by enzyme-linked immunosorbent assay as described elsewhere (Safieh-Garabedian *et al*., 1995; Cunha *et al*., 2005). The results are expressed as picograms IL-1β per paw. As a control, the concentrations of this cytokine were determined in PBS-pretreated mice and paws injected with saline.

Measurement of PGE_2 in paw skin
Tissue was removed from the injected and control paws (saline) of mice as described above. The PGE_2 was extracted from tissue as described by Wallace *et al*. (1988) and determined by radioimmunoassay. The results are expressed as picograms PGE_2 per paw. As a control, the concentrations of this PG were determined in PBS-pretreated mice and animals injected i.pl. with saline.

Experimental protocols
Effect of atorvastatin on hypernociception induced by LPS or antigen challenge. To investigate the effect of atorvastatin on lipopolysaccharide (LPS)-induced inflammatory hypernociception, mice were pretreated orally with either atorvastatin, at doses of 1, 3, 10, 30 and 90 mg kg^{-1} or vehicle (PBS) once a day for 3 consecutive days. At 2 h after the last dose of atorvastatin, mice received an i.pl. injection of LPS (100 ng paw^{-1}) or saline (vehicle for LPS). The animals were also treated with atorvastatin (30 mg kg^{-1}) for 1 or 2 days before LPS challenge. The hypernociceptive responses were assessed 0.5, 1, 3, 5, 7 and 24 h after LPS or saline i.pl. injections.

In addition, we investigated the effect of atorvastatin on the immune inflammatory hypernociception in mice sensitized to mBSA and challenged with antigen. The animals were pretreated orally with atorvastatin (30 mg kg^{-1}) or PBS once a day for 3 consecutive days. At 2 h after the last dose of atorvastatin, mice received an i.pl. injection of mBSA (90 μg paw^{-1}) or saline. In the control group, mBSA was injected into the paws of the false immunized mice (see above). Mice were fasted for 8 h receiving atorvastatin or PBS. The hypernociceptive responses were assessed 1, 3 and 5 h after challenge with antigen.

Effect of atorvastatin on hypernociception induced by bradykinin, cytokines or PGE_2. In this set of experiments, the effect of atorvastatin was investigated on mechanical hypernociception induced by bradykinin (BK) (500 ng paw^{-1}), TNF-α (50 pg paw^{-1}), IL-1β (1 ng paw^{-1}), keratinocyte-derived chemokine (KC/CXCL) (20 ng paw^{-1}) and PGE_2 (100 ng paw^{-1}). The animals were pretreated for 3 days with atorvastatin (30 mg kg^{-1}, peritoneally (p.o.)) or PBS, as described above. Hypernociception was assessed 3 h after injection of the inflammatory stimulus (or saline) in the paw.

*Effect of atorvastatin on IL-1*β *and PGE_2 production induced by LPS.* To investigate whether the antinociceptive effect of atorvastatin depended on the inhibition of IL-1β and PGE_2 production induced by LPS, the levels of these mediators were measured in the paw skin of mice pretreated for 3 days with atorvastatin (30 mg kg^{-1} p.o.) or PBS, as described above. The levels of these mediators in paw skin were determined 3 h after injection of LPS or saline into the paw.

Influence of NOS inhibitors on the antinociceptive effect of atorvastatin. To assess the contribution of NO to the antinociceptive effect of atorvastatin, animals were pretreated with the statin, as described above. One hour before the injection of LPS or PGE_2 into the paw, mice received an NOS inhibitor, either L-arginine analog *N*-nitro-L-arginine methyl ester (L-NAME) (90 mg kg^{-1}, i.p.), L-NMMA (90 mg kg^{-1}, i.p.) or 1400W (1.5 mg kg^{-1}, i.v.). In a different series of experiments, using the mice lacking iNOS (iNOS −/−) and the relevant WT mice, we assessed the effect of atorvastatin (given as described) on LPS-induced hypernociception. In both sets of experiments, hypernociception was assessed 3 h after injection of LPS- or PGE_2-i.pl.

Role of products of HMG-CoA reductase on the antinociceptive effect of atorvastatin. To investigate whether the antinociceptive effect of atorvastatin reflected decreased levels of the products of HMG-CoA, two types of experiments were performed. In one, the total serum cholesterol concentration was determined in mice treated with atorvastatin at a dose of 30 mg kg^{-1} day^{-1}, or PBS, for 3 days and then injected i.pl. with LPS or saline. In the other, the HMG-CoA reductase product, mevalonate, was given (10–90 mg kg^{-1}) at the same times as atorvastatin. Hypernociception and cholesterol levels were determined 3 h after i.pl. LPS or saline.

Statistical analysis
Results are presented as means ± s.e.m. for groups of five animals (for *in vivo* experiments) or four animals (for *in vitro* experiments), and they are representative of two independent experiments. The differences between the experimental groups were compared by analysis of variance and, in the case of statistical significance, individual comparisons were subsequently made with Bonferroni's *post hoc* test. The level of significance was set at $P<0.05$.

Materials
The following materials were obtained from the indicated sources. Recombinant murine TNF-α and IL-1β were provided by the National Institute for Biological Standards and Control (South Mimms, Hertfordshire, UK). Recombinant murine KC/CXCL was purchased from PeproTech (Rocky

Hill, NJ, USA). Mevalonate, L-NMMA, PGE_2, incomplete Freund's adjuvant, CFA and mBSA were purchased from Sigma Chemical Co. (St Louis, MO, USA). Atorvastatin (Pfizer Inc., Guarulhos, SP, Brazil; prescription formulation). Bacterial endotoxin from *Escherichia coli,* referred to here as lipopolysaccharide (LPS-Difco Laboratories Ltd, West Molesey, Surrey, UK) 1400W, was purchased from Cayman (Ann Arbor, MI, USA).

Results

Atorvastatin inhibited mechanical inflammatory hypernociception and the release of cytokines and PGE_2

LPS has been often used as a stimulus to investigate some features of the inflammatory process, including hypernociception. In the present study, the i.pl. administration of LPS (100 ng paw^{-1}) induced mechanical inflammatory hypernociception assessed over 24 h after its administration. The hypernociception was significant 1 h after LPS injection reaching a maximum between 3 and 5 h and decreasing thereafter (7–24 h). The hypernociceptive effect of LPS was limited to the injected paw as the nociceptive threshold of the contralateral paw was not altered (withdrawal thresholds of the contralateral paws of mice injected with LPS or saline were 9.6±0.32 and 9.34±0.3, respectively). Pretreatment of the animals with atorvastatin (1–90 mg kg^{-1}, p.o.) for 3 consecutive days reduced, in a dose-dependent manner, the mechanical inflammatory hypernociception induced by LPS (Figure 1a). Although atorvastatin inhibited LPS-induced inflammatory hypernociception, it did not alter the nociceptive base line of the animals (withdrawal threshold baseline of PBS- and atorvastatin-treated (30 mg kg^{-1}) mice was 9.8±0.10 and 9.6±0.13 g, respectively, $P>0.05$). It should be noted that pretreatment of the animals for only 1 or 2 days with atorvastatin (30 mg kg^{-1}) did not reduce LPS-induced hypernociception measured at 3 h after its injection (Figure 1b). The dose of LPS used (100 ng paw^{-1}) also induces neutrophil migration into the injected paw, assessed by increased myeloperoxidase enzyme activity, but atorvastatin treatment did not affect this inflammatory parameter (data not shown).

As statins exert immunomodulatory effects, we also investigated the effect of atorvastatin upon hypernociception induced by antigen challenge in previously immunized mice. Again, pretreatment of mice with atorvastatin (30 mg kg^{-1} p.o.) for 3 consecutive days reduced the mechanical hypernociception induced by mBSA (Figure 1c). Treatment of the sensitized mice with atorvastatin for a longer time (5 days) also reduced hypernociception on antigen challenge, but this effect was not different from that observed with 3 consecutive days of treatment (data not shown).

Inflammatory hypernociception is generally mediated by the release of a cascade of inflammatory mediators and we therefore investigated the effect of atorvastatin upon hypernociception induced by a variety of such mediators. As shown in Figure 2, BK, TNF-α, IL-1β and the chemokine CXCL induced approximately the same intensity of hypernociception and pretreatment with atorvastatin (30 mg kg^{-1}

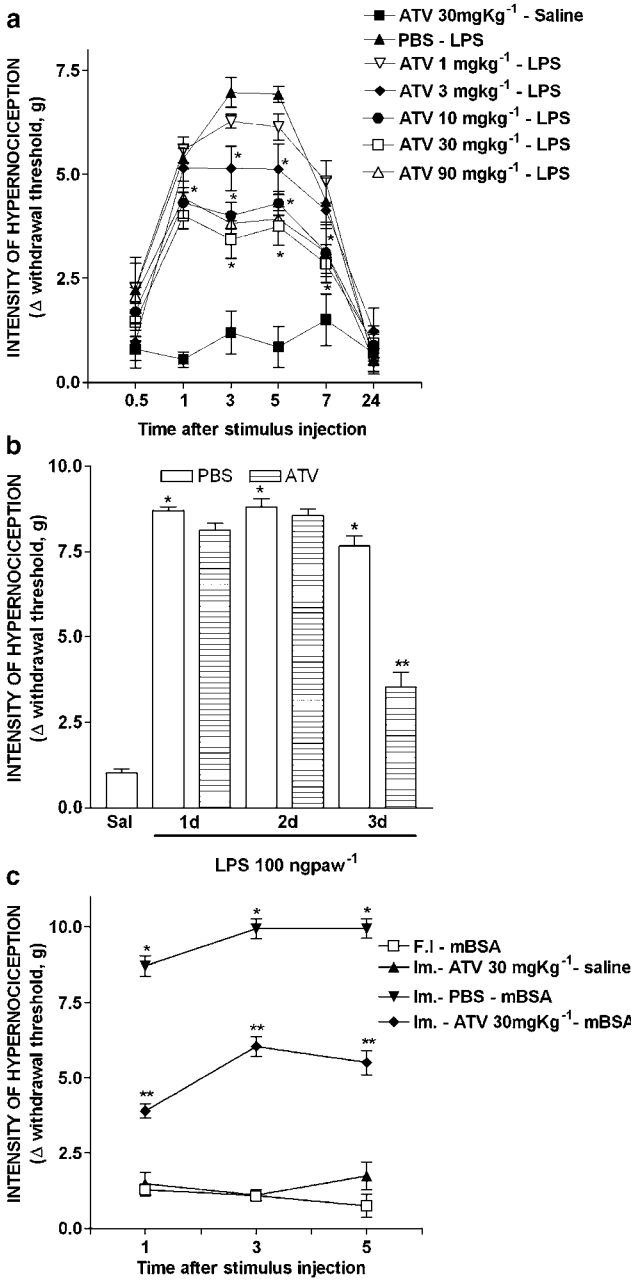

Figure 1 Effect of atorvastatin (ATV) on hypernociception induced by LPS or antigen challenge. (**a**) The mice were pretreated for 3 consecutive days with ATV (1, 3, 10, 30 and 90 mg kg^{-1} p.o.) or PBS, once a day. The last dose of ATV was administered 2 h before i.pl injection of LPS (100 ng paw^{-1}, 25 μl) or saline. (**b**) Mice were pretreated for 1, 2 or 3 consecutive days with ATV (30 mg kg^{-1} p.o.) or PBS once a day. Two hours after last the dose of ATV, LPS (100 ng paw^{-1}) or saline were i.pl injected. (**c**) Sensitized mice (Im.) were pretreated for 3 consecutive days with ATV (30 mg kg^{-1} p.o.) or PBS once a day. The last dose was administered 2 h before antigen challenge. Results from the false immunized mice are shown as the F.I group. In all panels, the mechanical hypernociception was determined at fixed times between 0.5 and 24 h after stimulus injection. The results are expressed as the mean±s.e.m. of five animals per group. The withdrawal threshold base line of the animals pretreated for 3 days with PBS before the i.pl injection of the hypernociceptive stimuli was 9.8±0.10 g ($n=10$). *Significant difference compared with the paws injected with saline and **with PBS-treated group ($P<0.05$).

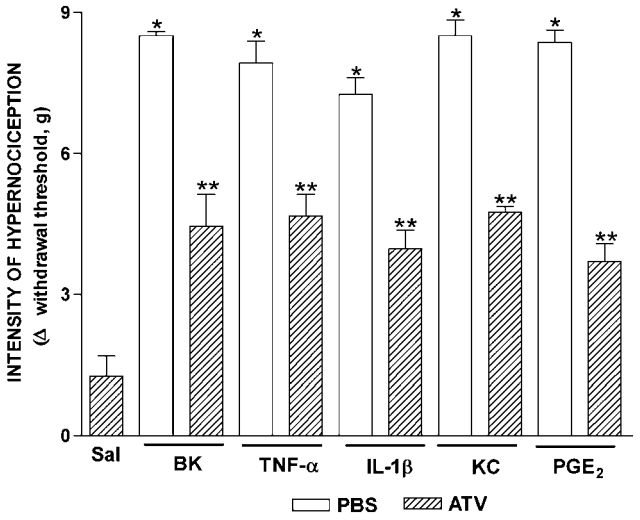

Figure 2 Effect of atorvastatin (ATV) on BK, TNF-α, IL-1β-, keratinocyte-derived chemokine (KC)- and PGE_2-induced hypernociception. The animals were pretreated for 3 days with ATV (30 mg kg^{-1} p.o.) once a day. The last dose was administered 2 h before injection of the hypernociceptive stimuli. Mechanical hypernociception was determined 3 h after stimulus injection. *Statistically significant difference compared with the paws injected with saline and **with PBS pretreated group ($P<0.05$).

p.o.) reduced, to about the same level, each of these hypernociceptive states. This figure also shows that pretreatment with atorvastatin decreased the hypernociception induced by PGE_2. All assays were carried out 3 h after the administration of the inflammatory mediators.

Because hypernociception induced by many inflammatory stimuli depends on the biosynthesis of cytokines and PGs, we assessed the effect of atorvastatin on the production of IL-1β and PGE_2 in the paw. In Figure 3, we show that statin pretreatment did inhibit the amount of IL-1β and PGE_2 synthesized in the mouse paw, after injection of LPS.

NO derived from constitutive NOS mediates the antinociceptive effect of atorvastatin

We showed that atorvastatin reduced PGE_2-induced hypernociception (Figure 2) and other established analgesics, such as morphine and dipyrone, and also inhibit PGE_2-induced hypernociception, via the release of NO. We therefore evaluated the involvement of this free radical in the antinociceptive effects of atorvastatin. In our experiments, pretreatment with L-NAME or L-NMMA, non-selective inhibitors of NOS, prevented the antinociceptive effects of atorvastatin in LPS- and PGE_2-induced hypernociception (Figure 4). However, the selective inhibitor of iNOS, 1400W, or deletion of the gene for iNOS (iNOS−/− strain) did not interfere with the effect of atorvastatin (Figure 4a).

Antinociceptive effect of atorvastatin is mediated by inhibition of HMG-CoA reductase

To investigate whether the antinociceptive effects of atorvastatin were also linked to the inhibition of HMG-CoA reductase activity, we measured the total serum cholesterol levels after atorvastatin and the effect of adding exogenous

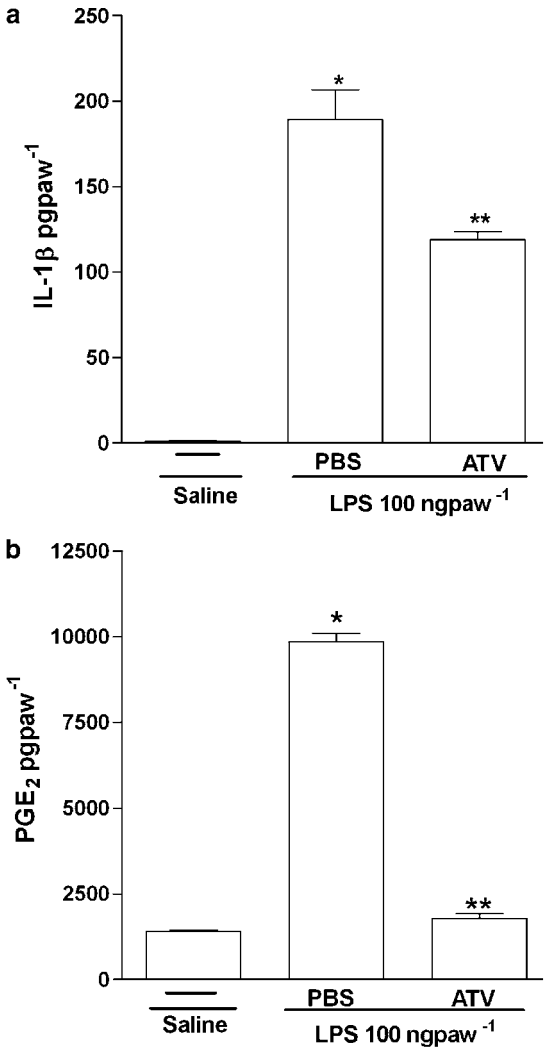

Figure 3 Effect of atorvastatin (ATV) on IL-1β and PGE_2 production induced by LPS in paw skin. The animals were pretreated for 3 days with ATV (30 mg kg^{-1} p.o.) once a day. The last dose was administered 2 h before LPS i.pl. injection. IL-1β (**a**) and PGE_2 (**b**) levels were measured 3 h after LPS injection. The results are expressed as the mean ± s.e.m. of five animals per group. *Statistically significant difference compared with the paws injected with saline and **with PBS-treated group ($P<0.05$).

mevalonate, a product of HMG-CoA reductase, upon the antinociceptive effect of atorvastatin. Total serum cholesterol levels did not decrease after 3 days of treatment with 30 mg kg^{-1} of atorvastatin, the same protocol used for hypernociception studies (Figure 5a). To investigate whether the disruption of mevalonate synthesis accounted for the antinociceptive effect of the statin, we treated the animals with exogenous mevalonate and atorvastatin at the same time. Our results (Figure 5b) showed that mevalonate (10–90 mg kg^{-1}) inhibited the antinociceptive effect of atorvastatin in a dose-dependent manner.

Discussion

Generally, immune and non-immune inflammatory diseases are accompanied by hypernociception. Because statins

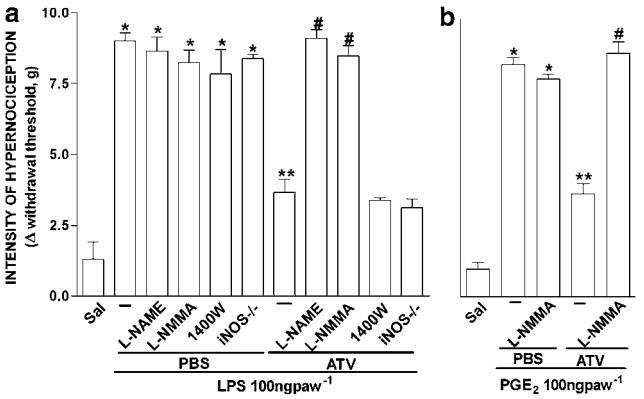

Figure 4 Effect of NO synthesis inhibition on atorvastatin (ATV)-induced antinociception. The antinociceptive effect of ATV (30 mg kg^{-1} p.o.) was determined in animals pretreated with L-NAME (90 mg kg^{-1} i.p.), L-NMMA (90 mg kg^{-1} i.p.), 1400W (1.5 mg kg^{-1} i.v.) and in iNOS-deficient (−/−) mice. Mechanical hypernociception was assessed 3 h after injection of LPS (**a**) (100 ng paw^{-1}) or PGE_2 (**b**) (100 ng paw^{-1}). The results are expressed as the mean ± s.e.m. of five animals per group. *Statistically significant difference compared with the paws injected with saline and **with PBS-treated group and $^{\#}$with ATV-pretreated group ($P<0.05$).

produce anti-inflammatory and immunomodulatory effects (Weitz-Schmidt, 2002; Blanco-Colio *et al*., 2003), in the present study we investigated whether atorvastatin exerted antinociceptive actions. We chose atorvastatin because it is the most widely prescribed statin and presents one of the most favourable safety profiles of the available statins (Youssef *et al*., 2002). We found that treatment of animals over 3 or 5 days with atorvastatin inhibited the mechanical hypernociception induced by LPS or antigen challenge. However, treatment of the animals for 1 or 2 days was ineffective, suggesting that a short period of treatment is not sufficient to bring about the changes involved in the antinociceptive actions of this statin.

Inflammatory hypernociception induced by LPS or antigen challenge involves the release of several inflammatory mediators. Those hypernociceptive mediators considered to be directly acting (PGs and sympathetic amines) act on their specific metabotropic receptors present on the primary sensory neurons, triggering a cascade of intracellular events responsible for lowering the nociceptive threshold (Ferreira and Nakamura, 1979; Nakamura and Ferreira, 1987; Taiwo *et al*., 1989; Khasar *et al*., 1999). The release of these hypernociceptive mediators is generally preceded by the initiation of a cytokine cascade (Cunha *et al*., 1991, 1992, 2005). In rats, the release of TNF-α is preceded by the generation of BK (Ferreira *et al*., 1993). Thus, BK stimulates the release of TNF-α, which in turn stimulates two distinct hypernociceptive pathways. TNF-α stimulates IL-1β production and that induces the expression of COX-2, responsible for prostanoid biosynthesis. TNF-α also stimulates release of CXC chemokines (CINC-1/IL-8) that induce the release of sympathomimetic amines (Ferreira *et al*., 1988; Cunha *et al*., 1991, 1992; Lorenzetti *et al*., 2002). In mice, IL-1β and KC/CXCL are responsible for the release of prostanoids and sympathetic amines, respectively (Cunha *et al*., 2005). As the

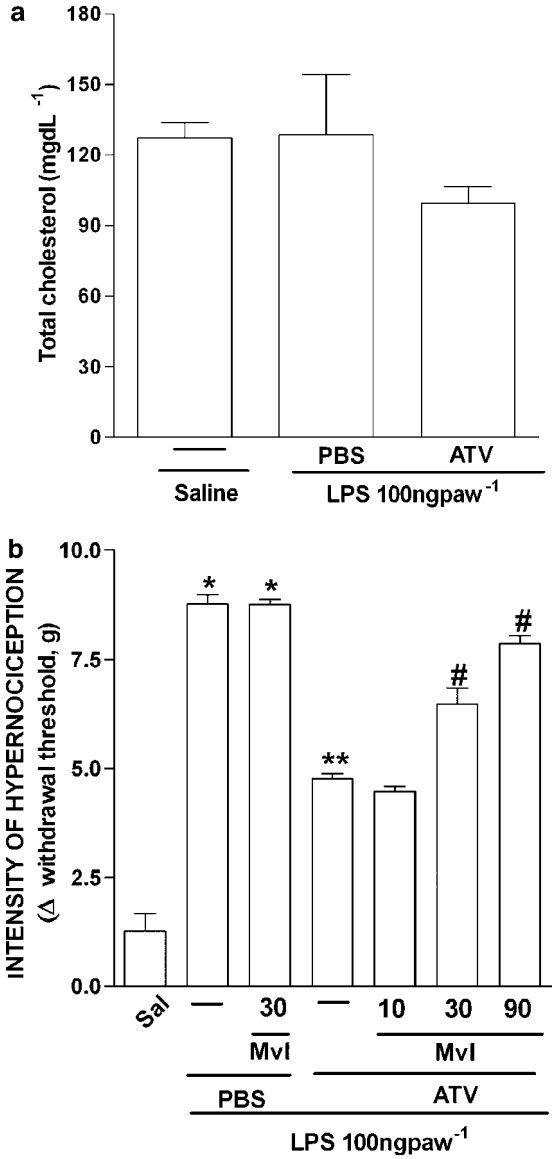

Figure 5 Role of HMG-CoA-reductase enzyme products in the antinociceptive effect of atorvastatin (ATV). (**a**) Serum cholesterol levels were measured after pretreatment for 3 days with atorvastatin (30 mg kg^{-1} p.o.) or PBS once a day. (**b**) The animals were simultaneously pretreated for 3 days with mevalonate (Mvl – 10–90 mg kg^{-1} i.p.) and ATV (30 mg kg^{-1} p.o.) once a day. Mechanical hypernociception was determined 3 h after LPS (100 ng paw^{-1}) injection. *Statistically significant difference compared with the paws injected with saline and **with PBS-treated group and $^{\#}$with ATV-pretreated group ($P<0.05$).

anti-inflammatory effects of statins have been attributed to their capacity to inhibit the production of the proinflammatory cytokines and PGs, we investigated whether atorvastatin would inhibit the hypernociceptive effects of BK, TNF-α, KC and IL-1β, effects, which depend, as described above, on a sequential release of cytokines and of PGs. Effects of atorvastatin on the release of IL-1β and PGE_2 prostanoids were also assessed. We found that atorvastatin inhibited the mechanical hypernociception induced by BK, TNF-α, IL-1β and KC. Furthermore, atorvastatin also inhibited the release

of IL-1β and PGE_2 in the mouse paw skin treated with LPS. Therefore, the antinociceptive effect of atorvastatin upon inflammatory hypernociception seems to be owing to the inhibition of the release of cytokines and PGs.

We did not investigate the mechanism by which atorvastatin inhibited the release of cytokines and PGs in our model, but it might involve the inhibition of nuclear factor-kappa B (NF-κB) activation. This possibility is supported by the finding that the release of proinflammatory cytokines and also COX-2 induction are largely dependent on NF-κB transcription pathway (Li and Verma, 2002; Ali and Mann, 2004; Wu, 2005). There is also evidence that statins diminish the activity of NF-κB owing to decreased phosphorylation and degradation of the NF-κB inhibitor protein IkappaB and it is dependent on inhibition of mevalonate synthesis (Hilgendorff *et al*., 2003; Lin *et al*., 2005; Planavila *et al*., 2005; Prasad *et al*., 2005).

With regard to the antinociceptive effect of atorvastatin on hypernociception induced by antigen challenge, we cannot discard the possibility that atorvastatin inhibited antigen presentation, as there is evidence that statins reduce macrophage major histocompatibility complex (MHC) class II expression (Kwak *et al*., 2000; Weitz-Schmidt *et al*., 2001; Youssef *et al*., 2002). Among the statins, atorvastatin was the most potent in downregulating MHC class II antigens (Kwak *et al*., 2000; Fehr *et al*., 2004). Corroborating this finding, statins have been shown to be effective in treating experimental autoimmune disease such as encephalomyelitis as well as collagen and CFA-induced arthritis in mice and rats, respectively (Aktas *et al*., 2003; Leung *et al*., 2003; Barsante *et al*., 2005). In contrast with these results, we did not observe inhibition of neutrophil migration to LPS-injected paws of mice by atorvastatin treatment. It may be that IL-1β (atorvastatin treatment reduced IL-1β production), which has been described as chemotactic to neutrophils (Cunha and Ferreira, 1986; Moser *et al*., 1989), does not play a major role in neutrophil migration induced by LPS in mice.

Although atorvastatin might be considered as a drug that prevents nociceptor sensitization by inhibiting the production of proinflammatory mediators such as IL-1β and PGE_2, this statin also inhibited PGE_2-induced hypernociception. The latter result suggests a direct antinociceptive action on nociceptors similar to that of diclofenac, dipyrone or morphine acting on peripheral sites (Ferreira *et al*., 1991; Tonussi and Ferreira, 1994; Sachs *et al*., 2004). A peripheral site for the antinociceptive action of atorvastatin is more likely as this statin does not cross the blood–brain barrier (Sparks *et al*., 2002). However, atorvastatin acting peripherally may also lead to a reduction of central sensitization as reduction of peripheral nociceptive input to the central nervous system could reduce central sensitization (Millan, 1999).

Some analgesic drugs that inhibit PG-induced hypernociception, such as peripherally acting opioids and dipyrone, act, at least in part, by stimulating the L-arginine/NO/cGMP pathway. Also, s.c. injection of NO donors inhibited PGE_2-induced hypernociception (Ferreira *et al*., 1991; Duarte *et al*., 1992) and statins induce upregulation of eNOS expression in vascular endothelial cells (Endres *et al*., 1998; Amin-Hanjani *et al*., 2001). Other studies have shown that statins could also upregulate the expression of inducible NOS through inhibition of small G proteins of the Rho family in vascular smooth muscle cells, airway epithelial cells, fibroblasts and cardiac myocytes (Chen *et al*., 2000; Muniyappa *et al*., 2000; Ikeda *et al*., 2001). Our finding that atorvastatin inhibited PGE_2-induced hypernociception leads to the hypothesis that increasing the bioavailability of NO by upregulating NOS would account for the antinociceptive effect of atorvastatin. In support of this hypothesis, non-selective inhibitors of NOS prevented the antinociceptive effect of atorvastatin in LPS- and PGE_2-induced hypernociceptive states However, selective inhibition or deletion of iNOS was ineffective. This suggests that the antinociceptive effect of atorvastatin was associated with an increase of NO derived mainly from the constitutive NOS. Although increased NO synthesis seems to be the main mechanism by which atorvastatin produced antinociception, we cannot discard the possibility that the statin was also downregulating expression of PG receptors on sensory neurons. Atorvastatin is known to decrease expression of EP receptors in human carotid atherosclerotic plaques and monocytic cells (Gomez-Hernandez *et al*., 2006).

The increase of NO production by statins can be both dependent on and independent of cholesterol inhibition (Laufs, 2003), and we found that a lowering of serum cholesterol was not necessary for the antinociceptive effect of atorvastatin. Inhibition of the enzymic activity of HMG-CoA reductase depletes downstream isoprenoids such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate. The synthesis of these compounds is dependent on mevalonate but sterol-independent (Hernandez-Perera *et al*., 1998). These isoprenoids not only serve as intermediates for cholesterol biosynthesis, but modify proteins to facilitate their attachment to cell membranes (Amin-Hanjani *et al*., 2001). We observed that exogenous mevalonate reversed the antinociceptive effect of atorvastatin, suggesting that a product downstream of HMG-CoA reductase, but not cholesterol, was involved in modulating the hypernociception. Inhibition of isoprenoid production by statins has been shown to increase NOS expression and activity in culture, with consequent production of NO, which has anti-hypernociceptive activity (Laufs and Liao, 1998; Laufs, 2003). One possible reason why we did not find significant reduction in cholesterol levels is that the duration of treatment of the animals was too short to achieve significant reduction of this product. Reduction of cholesterol levels by atorvastatin required over 4 weeks of treatment (Bustos *et al*., 1998; Endres *et al*., 1998). Taken together, our results suggest that the antinociceptive activity of atorvastatin depends on the inhibition of the synthesis of cytokines and eicosanoids and on increased NO production by constitutive NOS.

This study demonstrated the antinociceptive effect of atorvastatin in two different models of mechanical inflammatory hypernociception in mice. This antinociceptive effect involves inhibition of cytokine and prostanoid release and stimulation of NO production by constitutive NOS. In view of the widespread use of statins, these results might have broad clinical implications. In conclusion, this study suggests that statins could provide a novel class of analgesic drugs.

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Conflict of interest

The authors declare no conflict of interest.

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