Amlodipine Inhibits Pro-inflammatory Cytokines and Free Radical Production and Inducible Nitric Oxide Synthase Expression in Lipopolysaccharide/Interferon- γ -Stimulated Cultured Vascular Smooth Muscle Cells

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ABSTRACT—Overproduction of nitric oxide (NO) from inducible nitric oxide synthase (iNOS) is importantly involved in the pathogenesis of endotoxemia and atherosclerosis. Calcium antagonists are commonly used as cardiovascular drugs and have a beneficial effect on prolonging survival in various models of endotoxin shock. The present study was to investigate the effect of a calcium antagonist amlodipine on nitrite, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) formation and iNOS induction both in lipopolysaccharide (LPS) and interferon- γ (IFN- γ)-treated rat aortic smooth muscle cells (RASMC) and in a rat model of endotoxemia. Incubation with amlodipine (0.1–10 μ M) for 24 h resulted in a significant and dose-dependent attenuation in medium nitrite, TNF- α and IL-1 β formation as well as iNOS protein expression in LPS/IFN- γ -treated RASMC. In addition, amlodipine inhibited leucigenin-induced superoxide formation in RASMC. In the rat endotoxic model, the serum nitrite/nitrate, TNF- α and IL-1 β levels as well as iNOS protein expression of lungs were also suppressed by administration of amlodipine (50 μ g/kg, i.v.). These results suggest that amlodipine may exert vascular beneficial effects by suppressing pro-inflammatory cytokines and free radical generation as well as iNOS induction in smooth muscle cells during activation of inflammatory mechanism.

Keywords: Amlodipine, Inducible nitric oxide synthase, Lipopolysaccharide, Cytokine, Free radical

It has been demonstrated that nitric oxide (NO), known as endothelium-derived relaxing factor, plays an important role in blood pressure homeostasis (1). There are at least two major isoforms of nitric oxide synthase (NOS) in the vascular system: one is constitutive Ca²⁺/calmodulin-dependent NOS, which is expressed in endothelial cells etc. NO produced by the constitutive endothelial NOS is involved in the regulation of blood pressure, organ blood flow distribution and many other important functions. The other isoform is inducible Ca²⁺/calmodulin-independent NOS (iNOS), which can be induced by endotoxin (bacterial lipopolysaccharide, LPS), oxidative stress and pro-inflammatory cytokines including interleukin-1 β (IL-1 β) tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) in a variety of cells in vitro and in vivo (2, 3). The overproduction of NO resulting from iNOS induction has been pro-

posed to be involved in the pathophysiological mechanisms leading to septic shock and inflammation (4–6).

Several different calcium channel antagonists protect against heart failure, in which NOS induction has been demonstrated (7), and prolong survival time in various models of endotoxin shock (8, 9). In addition, amlodipine, nifedipine and other dihydropyridine derivatives but not verapamil or diltiazem have been shown to cause an inhibition of the induction of NOS by LPS in macrophage cell lines (10). Although nifedipine acutely improves the hemodynamic profile of heart failure, long-term use leads to the deterioration of patient hemodynamics (11). Recent evidence indicated that amlodipine inhibits NO production and iNOS expression, as evaluated by immunohistochemistry in macrophages and heart tissues of a murine model of congestive heart failure (12). The mortality of patients with heart failure resulting from nonischemic dilated cardiomyopathy was reduced by amlodipine (13). The mechanisms involved have not been clarified but may relate to

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inhibition of overproduction of NO. To further evaluate the mechanisms involved, we investigated effects of amlodipine on pro-inflammatory cytokines and free radical formation and iNOS induction in LPS/IFN- γ stimulated rat aortic smooth muscle cells (RASMC). We next investigated these effects of amlodipine in rat model of endotoxemia.

MATERIALS AND METHODS

Materials

LPS from *Escherichia coli* (serotype 0127:B8) was purchased from Sigma (St. Louis, MO, USA). Rat recombinant IFN- γ was obtained from Genzyme (Cambridge, MA, USA). Amlodipine was a gift from Pfizer (Sandwich, UK). Reagents used in the assay of iNOS western blot were purchased from Amersham (Buckinghamshire, UK). Other reagents used in this study were purchased from Sigma.

Cell culture

RASMC were cultured and prepared as previously described (14). To examine the effects of amlodipine, RASMC were treated with LPS (100 $\mu\text{g}/\text{mL}$) and IFN- γ (100 U/mL) for 24 h in the presence or absence of amlodipine (0.1–10 μM). The culture medium of Dulbecco's Minimum Essential Medium (DMEM) without phenol red was used for nitrite and cytokines determination, and the cells were collected for iNOS induction and superoxide production assay. To assess whether amlodipine affects the iNOS activity, after RASMC were preincubated with LPS and IFN- γ for 24 h, fresh medium containing amlodipine (0.1–10 μM) and cycloheximide (4 μM) was added, and the nitrite concentration of medium was measured after a further 24 h.

In vivo study

Male Sprague-Dawley rats were anesthetized with urethane (0.6 g/kg, i.p.) and the carotid artery was cannulated for blood collection. A jugular vein was also cannulated for i.v. bolus administration of vehicle (saline) or agents. At 30 min after injection of the vehicle (saline) or amlodipine (50 $\mu\text{g}/\text{kg}$, i.v.), the rats received LPS (10 mg/kg, i.v.). Blood samples (0.5 mL) for the measurement of TNF- α and nitrite + nitrate (NO_x) were withdrawn from a catheter placed in the carotid artery at 1 and 4 h after injection of LPS, respectively. The lungs were removed at 4 h after injection of LPS for iNOS protein expression assay.

Measurement of nitrite and plasma NO_x levels

Medium nitrite and serum NO_x were determined by using the Griess reagent and a Sievers Nitrite Oxide Analyzer (Sievers 280 NOA; Sievers, Boulder, CO, USA), respec-

tively, as previously described (14).

Western blotting analysis

Cells or tissues homogenates containing 10 μg protein were denatured and an equal amount of protein were loaded on a 7.5% SDS-PAGE gel. After electrophoresis, the gel was transferred to nitrocellulose membrane using the PharmSystem (Pharmacia Biotech, Uppsala, Sweden). The membrane was blocked with 1% BSA in a Tris-buffer solution (TBS, pH 8.0) containing 0.1% Tween-20 for 2 h at room temperature. Next, the membrane was incubated overnight at 4°C with mouse monoclonal anti-iNOS antibody (1:2000 dilution; Transduction Laboratories, Lexington, KY, USA) in TBS containing 0.1% Tween-20. Then, the membrane was washed and finally incubated with a 1:1000 dilution of anti-mouse IgG conjugated to horseradish peroxidase for 1 h at room temperature. After successive washes with TBS, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (Amersham) and exposed to X-ray film for 3–5 min (15).

Measurement of cytokines level

The amount of TNF- α and IL-1 β was measured by using a rat EIA kit (Genzyme Corporation).

Measurement of superoxide formation

Production of superoxide was measured by the lucigenin-enhanced chemiluminescence response as described previously (16). The counts of RASMC ($2 \times 10^4/\text{mL}$) induced by lucigenin (0.25 mM) were obtained by luminometer (Lumicount; Packard, Meriden, CT, USA) reported by RLU (relative light units) emitted, which was integrated over 20-s intervals for 5 min. Background counts (determined in cell-free preparations) were subtracted, and values were normalized to cell number.

Cell viability

Cell viability was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. After incubation of amlodipine with cells for 24 h, the culture medium was removed and the MTT (0.4 mg/mL) was added for another 60 min incubation at 37°C. Then, the culture medium was removed by aspiration and the cells were solubilized in DMSO. The extent of reduction of MTT to formazan within the cells was quantified by measurement of OD₅₅₀. Formazan production is expressed as a percentage of the values obtained from cells treated with LPS plus IFN- γ .

Statistics

Data were expressed as the mean $\pm$ S.E.M. All results were analyzed by one-way ANOVA followed by a multiple

comparison test (Scheffe test). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of amlodipine on NO production in vitro and in vivo

Incubation with LPS (100 $\mu\text{g/mL}$) and IFN- γ (100 U/mL) in RASMC for 24 h induced a ten-fold increase in nitrite accumulation compared with that of basal cells. Amlodipine (0.1–10 μM) caused a significant and dose-dependent attenuation of nitrite accumulation in LPS/IFN- γ treated RASMC (Fig. 1A). However, if iNOS were already induced by LPS and IFN- γ for 24 h and any possible further protein synthesis was blocked by cycloheximide (4 μM), the amlodipine had no inhibitory effect on nitrite production (Fig. 1B). Similarly, in the in vivo study, pretreatment with amlodipine (50 $\mu\text{g/kg}$, i.v.) significantly inhibited the serum NO $_x$ levels compared with that of untreated LPS-rats (Fig. 1C).

Effect of amlodipine on iNOS protein expression in vitro and in vivo

The basal RASMC showed barely detectable iNOS protein. In contrast, stimulation with LPS and IFN- γ for 24 h induced a marked increase of iNOS protein expression. When amlodipine (0.1–10 μM) was added, the LPS/IFN- γ -induced expression of iNOS protein was dose-dependently attenuated (Fig. 2A). Similarly, administration with amlodipine (50 $\mu\text{g/kg}$, i.v.) reduced the iNOS expression of lungs in rat model of endotoxemia (Fig. 2B).

Effect of amlodipine on cytokines production in vitro and in vivo

In LPS/IFN- γ treated RASMC, amlodipine (0.1–10 μM) dose-dependently inhibited the TNF- α and IL-1 β production (Fig. 3). In LPS-treated rats, our unpublished data showed the injection of LPS resulted in bell-shape changes in the serum TNF- α level that reached a peak at 1 h after LPS injection and subsequently decreased slowly. Therefore, serum TNF- α was measured at the 1-h time point. Other cytokines including IL-1 β was measured at 4 h after injection of LPS. Similarly, treatment of LPS-rats with amlodipine (50 $\mu\text{g/kg}$, i.v.) significantly decreased the serum TNF- α and IL-1 β levels compared with that of untreated LPS-rats (Fig. 4).

Effect of amlodipine on superoxide formation

As shown in Fig. 5, in the RASMC, lucigenin-enhanced superoxide generation was about 3.5 times higher than resting state. Incubation with amlodipine caused a significant inhibition of lucigenin-induced superoxide formation.

Fig. 1. Effect of amlodipine on NO production in LPS/IFN- γ -treated RASMC and in LPS-treated rats. A: After incubation of RASMC with vehicle or amlodipine (0.1–10 μM), they were stimulated with LPS (100 $\mu\text{g/mL}$) and IFN- γ (100 U/mL), and their response was then measured by determining the nitrite levels in the medium. The unstimulated cells was used as the control group. B: After preincubation with LPS and IFN- γ for 24 h in RASMC, fresh medium containing amlodipine (0.1–10 μM) and cycloheximide (4 μM) was added for another 24 h. Then the medium was collected for nitrite determination. C: At 30 min after injection of saline (control) or amlodipine (50 $\mu\text{g/kg}$, i.v.), rats received LPS (10 mg/kg, i.v.). Blood was collected for serum NO $_x$ determination at 4 h after injection of LPS. Values of each group are expressed as the mean $\pm$ S.E.M. ($n = 6$, each group). ** $P < 0.01$, compared with that in the LPS and IFN- γ treated alone cells. ## $P < 0.01$, compared with that in the LPS-treated alone rats.

Effect of amlodipine on cell viability

Amlodipine alone did not significantly diminish cell respiration at the concentrations (0.1–10 μM) studied with

Fig. 2. Effect of amlodipine on iNOS protein expression in LPS/IFN- γ -treated RASMC and in lungs of LPS-treated rats. A: After incubation with vehicle (lane 2), amlodipine 0.1 μ M (lane 3), 1 μ M (lane 4) or 10 μ M (lane 5) in RASMC in response to LPS (100 μ g/mL) and IFN- γ (100 U/mL) for 24 h, the cells were harvested for iNOS Western blot analysis. Lane 1 shows undetectable iNOS expression in unstimulated RASMC. B: After injection of saline (lane 2) or amlodipine (50 μ g/kg, i.v., lane 3) for 30 min, rats received LPS (10 mg/kg, i.v.) for another 4 h. Then the lungs were removed for iNOS Western blot analysis. Lane 1 shows the iNOS Western blot of rat lungs without amlodipine and LPS treatment. The results were representative of three different experiments.

cell viability above 95% of the control cells. In addition, there was no difference of the cell viability between with or without amlodipine (0.1–10 μ M) in LPS/IFN- γ -treated RASMC.

DISCUSSION

The present study clearly demonstrates that amlodipine significantly inhibits nitrite formation in LPS/IFN- γ -treated RASMC accompanied by attenuation of pro-inflammatory cytokines as well as superoxide formation and suppressing of iNOS induction. Similarly, in the rat endotoxic model, the serum NO_x, TNF- α and IL-1 β levels as well as iNOS expression of lungs were also suppressed by administration of amlodipine.

It has been demonstrated that in response to LPS and cytokines, the iNOS of macrophages and smooth muscle cells is induced and sequentially leads to NO overproduction. Our results showed that amlodipine significantly inhibited the nitrite formation in LPS/IFN- γ -treated RASMC. To further examine whether amlodipine causes direct inhibition of iNOS activity, amlodipine was added into RASMC preincubated with LPS and IFN- γ for 24 h

Fig. 3. Effect of amlodipine on TNF- α and IL-1 β production in LPS/IFN- γ -treated RASMC. After incubation of RASMC with vehicle or amlodipine (0.1–10 μ M), they were stimulated with LPS (100 μ g/mL) and IFN- γ (100 U/mL) for 24 h, and the levels of TNF- α and IL-1 β were determined in the medium. The unstimulated cells was used as the control group. Values of each group are expressed as the mean $\pm$ S.E.M. (n=6, each group). * P <0.05, ** P <0.01, compared with that in the LPS and IFN- γ treated alone cells.

to induce iNOS expression and further protein synthesis was inhibited by cycloheximide; under this condition, no inhibitory effect of amlodipine on nitrite formation was found (Fig. 1B). Based on the temporal relationship between the different time of amlodipine added and nitrite formation, we propose that the inhibition of nitrite formation by amlodipine may be mainly due to attenuation of iNOS induction rather than direct inhibition of iNOS activity. It is strongly supported by the fact that amlodipine significantly inhibited LPS/IFN- γ -induced iNOS protein expression both in RASMC and lungs of the rat endotoxic model.

The mechanism(s) by which amlodipine prevents the induction of iNOS are unclear and warrant further investi-

Fig. 4. Effect of amlodipine on serum TNF- α and IL-1 β levels in LPS-treated rats. At 30 min after injection of saline (control) or amlodipine (50 μ g/kg, i.v.), rats received LPS (10 mg/kg, i.v.). Serum TNF- α and IL-1 β levels were determined at 1 and 4 h, respectively, after injection of LPS. Values of each group are expressed as the mean $\pm$ S.E.M. (n = 6, each group). ^{##} P < 0.01, compared with that in the LPS-treated alone rats.

Fig. 5. Effect of amlodipine on superoxide formation from RSMC. After incubation of vehicle or amlodipine (0.1–10 μ M) with RSMC in response to LPS and IFN- γ for 24 h, cells were collected for superoxide formation assay. Superoxide production was detected by lucigenin-enhanced chemiluminescence. Photon emission was averaged over 5 min and was reported as RLU per 2×10^4 cells. ^{*} P < 0.05, ^{**} P < 0.01, compared with that in the LPS and IFN- γ treated alone cells (n = 6, each group).

gation. Pro-inflammatory cytokines including TNF- α and IL-1 β etc. produced by LPS appear to be a key factor

contributing to the induction of iNOS in many cells and tissues through activation of nuclear factor- κ B (NF- κ B), an important nuclear transcription factor for iNOS and many other pro-inflammatory cytokines transcription (17, 18). This is supported by the observation that TNF- α antisera or antibodies attenuate the lethality of sepsis and endotoxin (19). Our results first demonstrated that the inhibitory effect of amlodipine on pro-inflammatory cytokines production in vitro and in vivo may account for its beneficial effects in sepsis and inflammation.

Although iNOS can bind calmodulin tightly without a requirement of elevated intracellular Ca^{2+} for iNOS activity, the role of intracellular Ca^{2+} on iNOS induction in murine macrophages or smooth muscle cells is still unclear. Previous studies have demonstrated that UTP, acting via pyrimidinoceptors, can stimulate phosphoinositide breakdown and increase intracellular Ca^{2+} levels (20). Although UTP alone had no effect, UTP potentiated the LPS-induced NO production, activation of transcription factor NF- κ B and iNOS expression in mouse J774 macrophages (21). However, when cells were treated with the intracellular Ca^{2+} chelator BAPTA/AM before stimulation with LPS plus UTP or thapsigargin, a specific and potent endoplasmic reticulum Ca^{2+} -ATPase inhibitor, and subsequently lead to increase of intracellular free Ca^{2+} , the potentiation of nitrite production by UTP or thapsigargin was completely inhibited (22, 23). Moreover, it was demonstrated that UTP or thapsigargin potentiated the LPS-induced activation of NF- κ B and iNOS induction via a Ca^{2+} /calmodulin-dependent protein kinase pathway (21). These observations indicated that the increase of intracellular Ca^{2+} has a crucial priming effect on iNOS expression, especially in the endotoxic state. Our finding, inhibition by amlodipine of iNOS induction in the endotoxic condition, is in agreement with other dihydropyridine calcium antagonists (24). However, other structurally unrelated calcium channel antagonists like verapamil and diltiazem exerted much less inhibition on NO formation than that of dihydropyridine calcium antagonists (10). These results seem to indicate that the inhibitory effect on iNOS induction may be a class effect for the dihydropyridine calcium antagonists and is independent of the action on L-type calcium channels. On the other hand, Ikeda et al. (25) reported that amlodipine increased NO synthesis in IL-1 β -stimulated cultured smooth muscle cells. It is proposed that the site of amlodipine action may be upstream of IL-1 β production. However, the true mechanism is still unknown. Many studies have shown that a synergistic action of IL-1 β , IFN- γ , TNF- α and LPS on iNOS expression was observed in smooth muscle cells and other cells (26, 27). Thus, we speculate that the discrepancy of iNOS expression by amlodipine may be due to treatment with different stimulators or combination of cytokines and LPS, and some unknown

mechanisms are involved in the effect of amlodipine on NO synthesis.

Oxidative stress has been proposed to play a key role on the NF- κ B activation and iNOS induction (28). Our results showed that amlodipine significantly inhibited the lucigenin-enhanced superoxide anion formation in RASMC, which is consistent with other dihydropyridine-type calcium antagonists such as felodipine inhibiting cytokine/LPS-induced superoxide anion production in human aortic smooth muscle cells (29). Calcium antagonists have also been shown to prevent glutathione loss (30). Intracellular thiols regulate NF- κ B activation at one or more levels in the signal transduction cascade. High intracellular thiol levels could influence protein folding or enzyme activation and thus block the activation of protein kinases (e.g., protein kinase C) that phosphorylate the I κ B (inhibitor of κ B)/NF- κ B complex and liberate activated NF- κ B (31). These results suggest the possibility that calcium antagonists inhibit NF- κ B activation. Recently, it was reported that nifedipine and amlodipine with less inhibitory potency inhibit activation of NF- κ B both in a human epithelium-like lung carcinoma cell line and the RAW 264.7 macrophage cell line (32). The fact that amlodipine inhibited induction of iNOS further support the notion that amlodipine acts as an antioxidant and NF- κ B inhibitor, which may be associated with the inhibition of LPS-induced iNOS expression.

In conclusion, this study demonstrates that amlodipine significantly inhibits NO production both in LPS/IFN- γ -stimulated RASMC and in the rat model of septic shock accompanied by suppression of iNOS induction. Furthermore, attenuation of pro-inflammatory cytokines and free radical formation by amlodipine may be involved in the attenuation of iNOS induction and NO overproduction. Our results may explain some of the beneficial effects of amlodipine on endotoxemia, cardiovascular and inflammatory diseases accompanied by overproduction of NO.

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