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Calcium Channel Blockers Inhibit Lipopolysaccharide-induced NO and TNF-α Production in Macrophages

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Background: We aimed to investigate the immunosuppressive effects of several calcium channel blockers as potential new anti-inflammatory drugs. We examined whether calcium channel blockers can inhibit cytokine secretion in the lipopolysaccharide (LPS)-stimulated macrophage cell line J774A.1. Results: Significant increases in the production of nitric oxide (NO) and tumor necrosis factor α (TNF-α) were found after the application of LPS for 24 h. Nifedipine, nicardipine and verapamil significantly inhibited LPS-induced NO secretion without causing changes in intracellular free calcium concentration. Diltiazem could not decrease the LPS-induced NO or TNF-α secretion levels. Only the dihydropyridine analogs, nifedipine and nicardipine, were able to reduce LPS-induced TNF-α secretion. The inhibition of NO secretion could not be antagonized by the application of cAMP and cGMP analogs or serine/threonine protein kinase inhibitors. However, the inhibition of TNF-α secretion could be reduced by the application of the protein kinase inhibitors nifedipine and nicardipine. Thus, different intracellular pathways may be involved in the regulation of production and secretion of NO and TNF-α in endotoxin-activated macrophages. **Conclusions:** Because nifedipine and nicardipine can inhibit the induction of NO as well as TNF-α our results suggest that these are potential anti-inflammatory drugs. Thus, some calcium channel antagonists could be developed for use clinically as anti-inflammatory drugs.

Key words: calcium channel blockers, lipopolysaccharide, nitric oxide, tumor necrosis factor, macrophage

INTRODUCTION

Nitric oxide (NO) is released by a variety of cells following the conversion of arginine to NO and of citrulline by nitric oxide synthase (NOS)[1]. Two isoforms are responsible for NO production[2,3], constitutive NOS (cNOS) in vascular endothelial cells and neurons, and inducible NOS (iNOS) in epithelial cells and various inflammatory cells. Nitric oxide released by endothelial cells or peripheral nerves acts as a smooth muscle relaxant, but the role of NO released at the site of inflammation is not completely understood[4].

Some organs (such as the lung) have a particularly high ratio of endothelial and inflammatory cells, which may make them more susceptible to changes in NO concentration. Indeed, iNOS is constantly expressed in the normal respiratory epithelium, but is essentially absent in the airway epithelium of patients with cystic fibrosis[5]. Furthermore, production of endogenous NO is higher in both the exhaled air and induced sputum of patients with inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease, than in normal individuals[6]. NO is not just a marker of airway inflammation, but has anti-inflammatory and proinflammatory effects *per se*[7]. Besides elevated NO levels, elevated tumor necrosis factor -α(TNF-α) production in activated macrophages is involved in many pathophysiological conditions during acute and chronic inflammation[8]. TNF-α has important biological effects on a variety of cells, mostly related to immunomodulatory and inflammatory processes[9]. Clinically, TNF-α has been shown to participate in inflammatory lung diseases[10-13].

The traditional rationale for administering calcium channel blockers is based primarily on their effects on smooth muscle contractility, as well as certain aspects of cardiac conductivity[14-16]. Moreover, calcium channel blockers might have benefits other than their cardiovascular effects[17]. Several calcium channel antagonists have been shown to inhibit the lipopolysaccharide (LPS)-mediated induction of NOS in cultured macrophages and to decrease the

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production of NO[18,19]. Studies have also attempted to determine the influence of calcium channel blockers on the immune system, as well as their potential implications in tissue transplantation and in endotoxin shock[20,21]. In this study, we examined the immunosuppressive effects of several calcium channel blockers as potential new anti-inflammatory drugs. Intracellular inhibitory pathways that downregulate inflammation were also investigated.

METHODS

Materials

Fetal bovine serum and culture media were purchased from Gibco BRL (Gaithersburg, MD, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

General Procedures

A control experiment was conducted by adding different concentrations of LPS from Escherichia coli, serotype 026:B6, to the culture medium of J774A.1 macrophages. The concentrations of nitrite and TNF-α in the culture medium were measured after LPS treatment for 24 h. The cell viability of the LPS-treated macrophages was also assayed to evaluate cell survival after treatment. In other experiments, LPS-treated macrophages were exposed to vehicle or calcium channel blockers (nifedipine, nicardipine, verapamil, or diltiazem), or pretreated with a protein kinase inhibitor (H-7), a cAMP analog (8-bromo-cAMP), or a cGMP analog (8-bromo-cGMP) before exposure to the calcium channel blocker. The levels of nitrite and TNF-α in culture medium were measured in all groups after 24 h LPS treatment.

Cell Culture

The mouse macrophage cell line J774A.1 was cultured, and suspensions were prepared according to American Type Culture Collection recommendations. Macrophages were suspended in culture medium (DMEM with 4 mM L-glutamine, 1.5 g/L $NaHCO_3$, 4.5 g/L glucose, 1 mM sodium pyruvate and 10% fetal bovine serum) and then plated in 35-mm round culture dishes. Cells were incubated in a humidified incubator with 5% CO_2 plus 95% air at 37°C. Every 72 h, the culture medium was removed and replaced with fresh medium. Experiments were conducted when cells reached 90% of confluence.

Nitric Oxide Determination

Nitric oxide secretion was evaluated by measuring nitrite, a stable oxidation breakdown product of nitric oxide[22]. Nitrite levels were analyzed using the Greiss reagent, which converts nitrite into a purple-colored azo compound and produces a red dye. In brief, 100 μl of culture medium was mixed with 100 μl of Greiss reagent (1 part 1% sulfanilamide in 60% acetic acid plus 1 part 0.1% naphthylenediamine dihydrochloride in distilled water) for 10 min at room temperature. The optical density of the mixed solution was read using a microplate reader (ELX 800 Universal Microplate Reader, Bio-Tek Instruments Inc., Winooski, VT, USA) with the wavelength set at 550 nm.

Cell Viability Assays

The survival of macrophages after drug treatment was evaluated by the examination of cellular morphology under phase-contrast microscopy and by the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay[23]. In general, after drug treatment, each culture well was filled with 0.5 mg/mL MTT culture medium and incubated for 30 min in the dark. The culture medium was then aspirated, and the remaining cells were lysed thoroughly with 50% DMSO. The optical density of the lysate at 595 nm was measured using a microplate reader. A positive correlation was found between this optical density and the cell density in our experiment ($r = 0.997$). The cell density (X) was calculated by the formula, $X = (24 \times 1.8) \times 10^6$ (where Y = optical density from the MTT assay). The cell densities were also checked sporadically by microscopic examination using a hemocytometer. Total agreement between both methods was obtained throughout our experiments.

Measurement of TNF-α Level

TNF-α levels were measured in cell culture supernatants using an enzyme-linked immunosorbent assay (Quantikine M mouse TNF-α immunoassay, R&D Systems Inc., Minneapolis, MN, USA). A polyclonal anti-mouse TNF-α antibody was used in this system. The supernatant in each culture well was collected and measured immediately after each experiment. The absorbance at 450 nm was detected using a microplate reader.

Measurements of Intracellular Calcium Concentrations

Intracellular calcium concentration was monitored by Fura-2 measurement. Fura-2 is a widely used UV-excitable fluorescent calcium indicator. Upon calcium binding, the fluorescent excitation maximum of the indicator undergoes a blue shift from 363 nm (Ca^{2+}-free) to 335 nm (Ca^{2+}-saturated); the fluorescence emission maximum is around 510 nm. First, macrophages grown on coverslips were

Table 1. Viable cell densities and the nitrite production levels in macrophages treated with different concentrations of lipopolysaccharide (LPS)

Treatment μ g/ml	Control	LPS 0.1	LPS 0.5	LPS 1.0	LPS 5.0	LPS 10.0	LPS 25.0
Cell densityb 10^6/mL	17.8±0.8	12.6±2.1	11.7±0.8	13.6±0.6	13.2±0.9	11.5±0.7	12.2±0.7
Nitritec μM	2.4±0.2	46.4±1.1	47.1±1.1	50.8±0.9	51.8±1.6	53.9±2.0	53.3±2.0

a LPS concentrations are given as micrograms per milliliter. b Cell densities were measured by the 3(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) reduction assay. c Nitrite productions were measured by Greiss assay. Data are presented as the mean±SER.

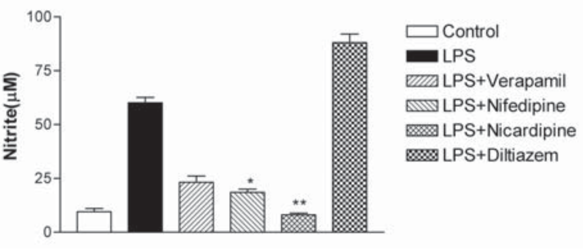


Fig. 1 Nitrite production induced by lipopolysaccharide (LPS) (1 μ g/mL) in combination with calcium channel blockers in macrophages. The concentration of each calcium channel blocker was 100 μM. * P < 0.05, ** P < 0.01 compared with LPS treatment.

loaded with the acetoxy methyl ester of Fura-2 in Tyrode's solution (137 mM NaCl 05 mM $MgCl_2$, 1.8 mM $CaC1_2$, 5.4 mM KCl, 10 mM glucose, 10 mM HEPES) with 3μM indicator, 5 mg/mL bovine serum albumin and 0.5% DMSO at 37°C for 20 min. After loading, the coverslips were mounted in a recording chamber and perfused with Tyrode's solution at a rate of 20 mL/min. All recordings were made at room temperature (20-25°C). The imaging system used in this study consisted of a Nikon Diaphot 300 microscope, a Dage-MTI cooled-CCD camera in combination with a Dage-MTI Gen II Sys image intensifier (a software package from Compix, Inc., Cranberry, PA, USA) and a 75 W Xenon lamp-based monochromator light source (Applied Scientific Instrumentation, Eugene, OR, USA). Spectra of the indicator under basal and stimulated conditions in situ and/or in Ca^{2+} (Mg^{2+})-free medium were recorded. Binding of Ca^{2+} was estimated from the ratio of Fura-2 fluorescence excited at 340 nm and 380 nm.

Statistical Analysis

Unless otherwise stated, results are expressed as the mean±standard error of mean. Statistical evaluation was performed using one-way ANOVA followed by Dunnett's test. Differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad PRISM version 2.0 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The MTT reduction assay showed that cell density decreased after a long-term, 24 h exposure to different concentrations (from 0.1 to 25 μg/mL) of LPS treatment (Table 1). However, the cell densities of macrophages treated with different concentrations of LPS did not differ significantly. On average, (70±5)% of macrophages treated with different dosages of LPS survived. A significant increase in nitrite concentration was found in macrophages treated with LPS for 24 h. The nitrite levels in culture medium of control cells and cells treated with LPS at 0.1, 0.5, 1.0, 5.0, 10 and 25 μ g/mL were 2.4 ± 0.2, 46.4 ±1.1, 47.1 ± 1.1, 50.8 ± 0.9, 51.8 ± 1.6, 53.9 ± 2.0 and 53.3 ± 2.0 μM, respectively. There were no dose-dependent increases in the production of nitrite at LPS concentrations greater than 0.5 μg/mL (Table 1).

A significant inhibition of LPS-induced nitrite production in macrophages was found after the administration of calcium channel blockers (Fig. 1). The nitrite level in culture medium of macrophages treated with vehicle control was 9.5 ± 1.5μM. The nitrite production of macrophages treated with 1 μg/mL LPS increased to 60.1 ± 2.5 μM in 24 h. Nitrite levels in medium of macrophages treated with 1 μ g/mL LPS combined with 100 μ M verapamil, 100μM nifedipine, 100μM nicardipine, or 100μM diltiazem were 23.2±2.9 μ M, 18.5±1.5 μ M, 8.1±0.8 μ M and 88 ±4 μ M, respectively.

Only nifedipine and nicardipine were able to reduce the secretion of TNF-α in macrophages induced by LPS (Fig. 2). The control level of TNF- α in the culture medium of macrophages was 148±32 pg/mL. In the presence of 1μM /mL LPS, TNF-α level increased to 4613 ± 73 pg/mL in 24 h. Combining 100 μ M nifedipine, verapamil, nicardipine, or diltiazem with the LPS decreased TNF-α levels to 2171±54, 2508±145, 639±134 and 5343±80 pg/mL, respectively.

The inhibition of nicardipine on LPS-induced nitric oxide production was not affected by pretreating macrophages with inhibitor or stimulators of protein kinases (Fig. 3). The control level of the nitrite production in the culture medium of macrophages was 9.5±1.5 μ M. Adding 1 μ g/ mL LPS increased nitrite level to 60.1±2.5 μ M. Adding LPS with 100 μ M nifedipine or 100 μ M nicardipine the

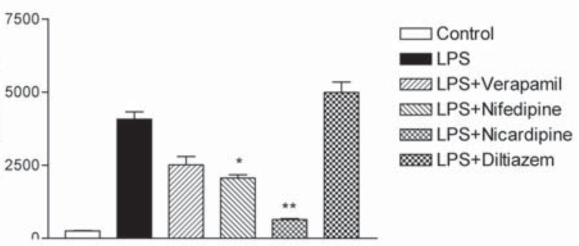


Fig. 2 The TNF-α production of macrophages induced by lipopolysaccharide (1 μg/mL) in combination with calcium channel blockers. The concentration of each calcium channel blocker was 100 μM.
* P < 0.05, ** P < 0.01 compared with LPS treatment.

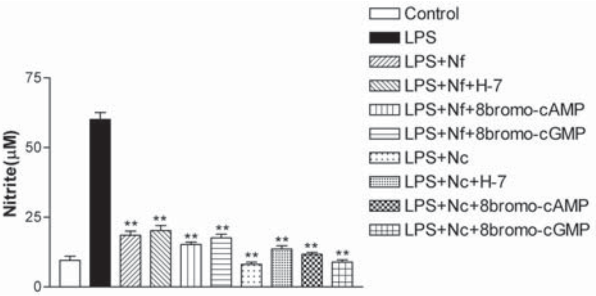


Fig. 3 The nitrite production of macrophages induced by lipopolysaccharide (LPS, 1 μg/mL) in combination with nifedipine (Nf, 100 μM) or nicardipine (Nc, 100 μM). The concentrations of H-7 and 8 bromo-cAMP were 0.1 mM and 0.5 mM, respectively.
** P < 0.01 compared with LPS treatment.

decreased nitrite level to 18.5±1.5 or 8.1± 0.8 μM, respectively. Treatment of LPS-induced macrophages with 0.1 mM H-7, 0.5 mM 8-bromo-cAMP, or 1 mM 8-bromo-cGMP before 100 μM nifedipine decreased nitrite level to 20.2±1.8 μM, 15.2 ± 0.9 μM, or 17.6±1.3 μM, respectively. Treatment of LPS-induced macrophages with 0.1 mM H-7, 0.5 mM 8-bromo-cAMP, or 1 mM 8-bromo-cGMP before 100 μM nicardipine decreased nitrite levels to 13.6 ± 1.2 μM, 11.7±0.7 μM, or 8.89±0.8 μM, respectively.

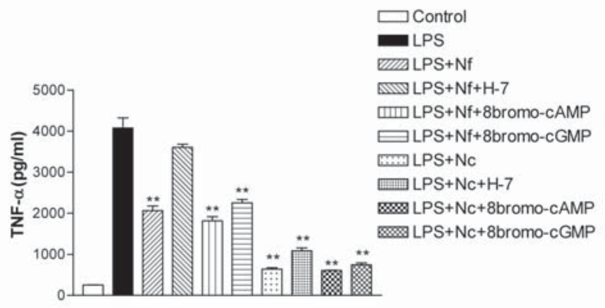


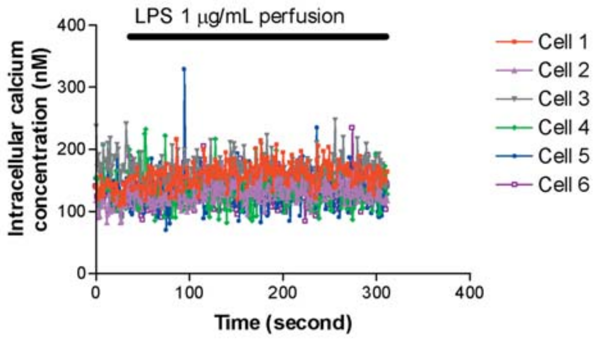
Fig. 4 The TNF-α production of macrophages induced by lipopolysaccharide (LPS, 1 μg/mL) in combination with nifedipine (Nf, 100 μM) and nicardipine (Nc, 100 μM). The concentrations of H-7 and 8 bromo-cAMP were 0.1 mM and 0.5 mM, respectively.
** P < 0.01 compared with LPS treatment.

The inhibition of nicardipine on LPS-induced TNF-α production was lessened by pretreating macrophages with inhibitors and stimulators of protein kinases. The control level of LPS-induced TNF-α production in the culture medium of macrophages was 252±3 pg/mL. Adding 1 μg /mL LPS increased the TNF-α level to 4074±248 pg/mL. Adding LPS with 100μM nifedipine or 100μM nicardipine decreased TNF-α levels to 2063±113 and 639±32 pg/mL, respectively. Treatment of LPS-induced cells with 0.1 mM H-7, 0.5 mM 8-bromo-cAMP or with 1 mM 8-bromo-cGMP prior to 100 μM nifedipine decreased TNF-α levels to 3606±75 pg/mL, 1816±100 pg/mL and 2250± 86 pg/mL, respectively. Treatment of LPS-induced cells with 0.1 mM H-7, 0.5 mM 8-bromo-cAMP, or 1 mM 8-bromo-cGMP before treating with 100 μM nicardipine decreased TNF-α levels to 1085±71 pg/mL, 607±6 pg/mL, or 745±45 pg/mL, respectively.

LPS treatment did not change the intracellular free calcium concentration in macrophages (Fig. 5, upper panel). The calcium concentration was 142±29 nM at baseline and 141±25 nM during the first 4 min after adding LPS (Table 2). In another set of experiments, the basal intracellular free calcium concentration of macrophages was 162±29 nM (Fig. 5, lower panel). After nicardipine treatment for 30 s, the intracellular free calcium concentration was 165 ± 31 nM. After adding LPS, the mean calcium concentration was 161±26 nM during the first 4 min (Table 2).

DISCUSSION

Exposure of macrophages to LPS can significantly decrease cell viability. It had been hypothesized that LPS-induced apoptosis of macrophages occurs via two independent mechanisms: first, through the autocrine secretion of TNF-α (early apoptotic events); second, through the production of NO (late phase of apoptosis)[24]. Our experiments showed that different concentrations of LPS induced similar nitric oxide levels and extent of cell death; these results do not contradict the hypothesis that NO induces apoptosis of macrophages. All LPS concentrations used in our experiment caused similar increases in NO. This suggests that NO production in long-term LPS-stimulated mac-



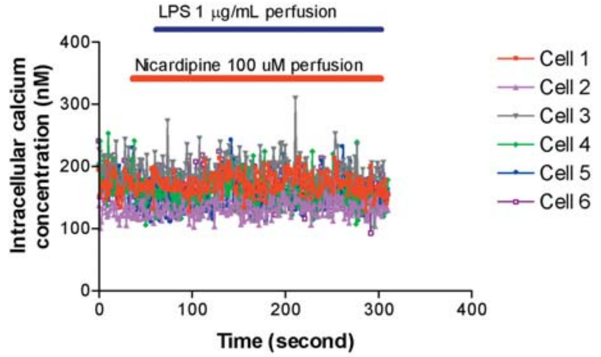


Fig. 5 The intracellular free calcium concentrations of macrophages during lipopolysaccharide (LPS, 1 µg/mL) perfusion with or without nicardipine (100 µM) pretreatment. Measurements were recorded from six individual cells.

Table 2. Intracellular calcium concentrations of macrophages before and after treatment with lipopolysaccharide (LPS) with or without the calcium channel blocker, nicardipine

Treatment and time	course	[Ca^{2+}i nM]
Control		142±29
LPS		
	5 sec	137±27
	1 min	142±25
	4 min	141±24
Control		162±29
Nicardipine	30 sec	165±31
Nicardipine +LPS		
	5 sec	154±26
	1 min	164±26
	4 min	157±24

Macrophages were preloaded with the Ca^{2+} indicator Fura-2. The cells were perfused with Tyrode's solution during all pretreatment and recovery periods. The LPS concentration was 1 µg/mL and the nicardipine concentration was 100 µM. The control calcium levels were measured after cells had been stabilized in Tyrode's solution for 15 minutes. Data are presented as the mean±SER.

rophages plateaus at LPS concentrations greater than 5 µg/mL. In addition, the increase in nitrite and TNF-α by higher concentrations of LPS was not overcome by the induced decrease in cell viability, because we still observed increases in both NO and TNF-α production.

Calcium channel blockers are all functionally similar compounds. However, here they inhibited LPS-induced nitrite and TNF-α accumulations in macrophages to different degrees, with nicardipine being the most potent inhibitor. These effects are not likely to result from the changes on cell viability, because treatment with the same concentration of each calcium channel blocker did not significantly affected the cell viability of macrophages in our preliminary study. Nifedipine, nicardipine and verapamil all decreased NO production in activated macrophages. These results are in agreement with other reports that dihydropyridine antagonists, most likely through the inhibition of iNOS expression, inhibit the production of NO in activated macrophages[18,19,25]. However, we also found (as reported previously) that this inhibition might not be caused by the decrement of intracellular calcium[26]. In addition, this inhibition might not involve the cAMP, cGMP, or protein kinase A and C pathways. Nevertheless, diltiazem did not inhibit but stimulated the production of NO in our experiments. Therefore, individual calcium channel blockers must have their own pharmacological characteristics. The drug-specific intracellular processes affecting iNOS expression clearly need further investigation.

The calcium channel blocker manidipine decreases the transcription rates of interleukin 1-α (IL-1-α) in human mesangial cells stimulated with the platelet-derived growth factor BB isomer[27]. Nilvadipine has also been shown to decrease TNF-α and IL-6 concentrations in activated T lymphocytes28. Additionally, amlodipine, but not diltiazem or nifedipine, inhibits the production of IL-1-α IL-1-α, and IL-6 induced by ouabain[29]. However, in our study, both nicardipine and nifedipine significantly diminished the production of TNF-α in activated macrophages. Our results suggest that the inhibition may not be caused by suppression of calcium channels, but by stimulation of protein kinase A or C activity. In addition, this inhibition may not relate to the cAMP or cGMP pathways. However, diltiazem and verapamil had no effect on LPS-induced TNF-α production. Thus, the inhibitory effect of nicardipine and nifedipine on TNF-α production in endotoxin-activated macrophages was a unique feature. Further study is needed to elucidate the inhibitory pathways and the involvement of protein kinases in these pathways.

TNF-α and other cytokines generated by activated immune cells can stimulate an increase in NO concentration via iNOS expression[30,31], and NO stimulates TNF-α production *in vivo*[32]. In our experiments, the production of NO increased significantly after treating activated macrophages with diltiazem, but the concomitant TNF-α production was unchanged. This implies that NO-induced TNF-α production, if it existed in our system, is not important. Furthermore, protein kinases A and C produced different effects on nicardipine- and nifedipine-mediated inhibition of NO or TNF-α production. Our results suggest that the regulation of iNOS and TNF-α expression are not identical in activated macrophages.

In summary, NO and TNF-α secretion patterns in endotoxin-activated macrophages are regulated by different intracellular pathways. Both nifedipine and nicardipine exerted significant anti-inflammatory effects through unspecified pharmacological pathways, which may be regulated by protein kinase A and/or C pathways. Therefore, the clinical use of calcium channel blockers to treat many inflammatory conditions should be considered.

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