*Short Communication***Dilazep Decreases Lipopolysaccharide-Induced Nitric Oxide and TNF- α Synthesis in RAW 264 Cells**Ryusuke Nakatsuka^{1,*,#}, Tadashige Nozaki¹, Mitsuko Shinohara¹, and Kiyoshi Ohura¹¹Department of Pharmacology, Osaka Dental University, Kuzuhahanazono-cho, Hirakata, Osaka 573-1121, Japan

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Abstract. Dilazep dihydrochloride (dilazep) is used to treat ischemic dysfunction, although the mechanisms underlying the anti-inflammatory effects of the drug have not yet been elucidated. The present study evaluated the anti-inflammatory effect of dilazep. Dilazep suppressed the production of nitric oxide (NO) and the expression of TNF- α mRNA by lipopolysaccharide (LPS) in RAW 264 cells. However, 1400W, an inducible NO synthase inhibitor, suppressed the production of NO but did not suppress the expression of TNF- α mRNA following treatment with LPS. Caffeine, an adenosine antagonist, restored LPS-stimulated NO synthesis, which is suppressed by dilazep. Therefore, these observations may suggest that the suppression of NO synthesis after dilazep treatment in RAW 264 cells is caused by the inhibition of TNF- α expression via adenosine receptors.

Keywords: dilazep dihydrochloride, nitric oxide, TNF- α

Neutrophils and macrophages produce inflammatory factors such as nitric oxide (NO) and TNF- α after stimulation with lipopolysaccharide (LPS) derived from bacteria. These factors promote the inflammatory process, thus resulting in serious tissue damage. However, adenosine seems to function as an anti-inflammatory factor in damaged tissues because it is able to inhibit neutrophil proliferation as well as suppress the inflammatory factors in inflammatory cells (1). Dilazep dihydrochloride (dilazep), a widely used antiplatelet agent, exerts its antiplatelet effects by increasing adenosine levels in the extracellular fluid (2). Dilazep also has a vasodilatory effect on coronary, cerebral, and renal vessels. Consequently, it is often used in patients with ischemic heart disease, cerebral ischemia, or renal dysfunction to increase the blood supply to the tissue (3, 4). Previous studies revealed that dilazep inhibits tissue factor (TF) expression in human umbilical vein endothelial cells (HUVECs) and monocytes by blocking TF expression at both the transcriptional and posttranscriptional levels (5, 6). Furthermore, its anticoagulant activity depends, in part, on the stimulation of adenosine receptors (5). TF is related to

LPS-induced inflammation via TNF- α expression in RAW 264 cells (7). While dilazep is reported to display many pharmacological activities in experimental animal models, the molecular mechanisms behind the pharmacological and biochemical effects of dilazep on macrophages have not yet been clearly elucidated. Therefore, dilazep is assumed to exert an anti-inflammatory effect via the suppression of TNF- α expression. This study evaluated the anti-inflammatory effects of dilazep by examining whether the drug suppressed LPS-induced NO and TNF- α expression in the mouse macrophage-like cell line RAW 264.

This study used RAW 264 cells, a murine macrophage cell line transformed by Abelson leukemia virus (8), provided by the RIKEN Cell Bank (Tsukuba). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 150 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂. Dilazep (Tocris Cookson, Bristol, UK), 1400W (*N*-[3-(aminomethyl)benzyl]acetamidine; Calbiochem, San Diego, CA, USA), caffeine (Maruishi Pharmaceutical Co., Osaka), and recombinant mouse TNF- α (PeproTech, Rocky Hill, NJ, USA) were dissolved in distilled water to the concentration of 10 mg/mL (dilazep), 20 mmol/mL (1400W), 1 mmol/mL (caffeine), or 0.1 mg/mL (TNF- α). LPS (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in

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PBS(-) to the concentration of 1 mg/mL. The RAW 264 cells (1.0×10^5 cells per well) were seeded onto 24-well cell culture plates in 1 mL of DMEM supplemented with 10% FCS one day before drug treatment. When the cells reached 70% confluence, they were treated with dilazep, caffeine, and/or 1400W at 24 h prior to addition of LPS or recombinant mouse TNF- α . The nitrite concentration in the culture media was colorimetrically measured by the NO₂/NO₃ Assay Kit-C II (Dojindo, Osaka) 24 h after the designated treatment. The optical density of the assay samples was measured spectrometrically at 540 nm using SPECTRA maxPLUS (Molecular Devices, Sunnyvale, CA, USA).

The cells were pelleted by centrifugation 24 h after treatment, and total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen, Stanford, CA, USA) according to the manufacturer's instructions. Total RNA (200 ng) was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo). Quantitative PCR reaction was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI7700 Real Time PCR (Applied Biosystems), and the data was quantified by the comparative C_T method. The complementary DNA was amplified using the TaKaRa Ex Taq (Takarabio, Otsu) in a GeneAmp PCR System 9700 (Applied Biosystems) for RT-PCR. The qPCR and RT-PCR were done using cDNA as a template [inducible NO synthase (iNOS), 5'-CCCTTC CGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCA GAGCCTCGTGGCTTTGG-3'; TNF- α , 5'-GCTCACT TCCTTCTCAGCATG-3' and 5'-TGGTTTCCGGTATC CTTCAGG-3'; adenosine receptors A1, 5'-ATCCCTCT CCGGTACAAGACAGT-3' and 5'-ACTCAGGTTGT TCCAGCCAAAC-3'; A2a, 5'-CCGAATTCCACTCCG GTACA-3' and 5'-CAGTTGTTCCAGCCCAGCAT-3'; A2b, 5'-TCTTCCTCGCCTGCTTCGT-3' and 5'-CCA GTGACCAAACCTTTTATACCTGA-3'; A3, 5'-ACTTC TATGCCTGCCTTTTCATGT-3' and 5'-AACCGTTC TATATCTGACTGTCAGCTT-3'; and GAPDH, 5'-AA CCTGCCAAGTATGATGAC-3' and 5'-AAGTCACA GGAGACAACCTG-3'].

RAW 264 cells were treated with dilazep and LPS, and the amount of NO released into the cell culture media was measured. The NO content in the culture media was significantly decreased following treatment with 15–20 μ g/mL of dilazep ($P < 0.05$ vs. LPS stimulation without dilazep treatment, Fig. 1A). Furthermore, iNOS mRNA expression was inhibited by dilazep treatment ($P < 0.05$ vs. LPS stimulation without dilazep treatment, Fig. 1B). In addition, TNF- α mRNA expression was up-regulated by LPS stimulation and inhibited by dilazep pre-treatment ($P < 0.05$ vs. LPS stimulation without dilazep treatment, Fig. 1C).

The effect of dilazep on cell survival was analyzed to confirm that the inhibition of NO production by dilazep does not cause cell toxicity. RAW 264 cells were treated for 48 h with increasing concentrations of dilazep (0–20 μ g/mL) and LPS (0–1 μ g/mL). The cells were analyzed by propidium iodide staining and a subsequent FACS analysis. The FACS analysis confirmed that dilazep did not induce cell death (data not shown).

Several studies have shown that iNOS expression and NO production due to LPS stimulation are involved in TNF- α signaling (7, 9). These reports raise the possibility that the inhibition of TNF- α by dilazep causes a reduction in NO synthesis. Therefore, this study examined whether the down-regulation of TNF- α expression is influenced by iNOS in the presence of dilazep. RAW 264 cells were treated with dilazep and 1400W, a selective and irreversible inhibitor of iNOS by competing with L-arginine (10–12). A previous study showed that 1400W inhibits the expression of iNOS by LPS/TNF- α in cultured human nasal microvascular endothelial cells (13). The increased NO production and up-regulation of iNOS mRNA expression by LPS treatment were suppressed by 1400W and/or dilazep treatment ($P < 0.05$ vs. LPS stimulation without Dilazep and 1400W treatment, Fig. 2, A and B). However, TNF- α mRNA expression was down-regulated by dilazep ($P < 0.05$ vs. LPS stimulation without dilazep treatment), whereas it was not significantly down-regulated by 1400W ($P > 0.1$ vs. LPS stimulation without dilazep treatment, Fig. 2C). These results suggest that the suppression of the TNF- α expression by dilazep was probably not caused by the inhibition of iNOS. Furthermore, the ability of dilazep to suppress the release of NO into cell culture media by TNF- α stimulation was evaluated to elucidate whether the inhibition of NO production was caused by the inhibition of TNF. NO production was increased by stimulating TNF- α ($P < 0.05$ vs. no treatment, Fig. 2D). This elevation in NO production was not suppressed by pre-treatment with dilazep and the NO production tended to be increased when dilazep was increased to 20 μ g/mL. However, the increased NO production was inhibited by 1400W pre-treatment ($P < 0.05$ vs. dilazep treatment, Fig. 2E). These observations suggested that the suppression of NO synthesis by dilazep was not caused by the direct inhibition of iNOS, but it was caused by the inhibition of TNF- α or/and the factors that activate TNF- α .

A previous study demonstrated that dilazep enhances adenosine receptor-mediated cellular activities by increasing adenosine levels in the extracellular fluid (5, 14). Therefore, this study determined whether the suppression of NO synthesis by dilazep was mediated by adenosine receptors. An RT-PCR analysis was performed using the specific primers for subtypes of adenosine re-

Fig. 1. Dilazep inhibits NO production and suppresses iNOS and TNF- α expression. A) LPS-induced NO production is inhibited by dilazep in a dose-dependent manner. RAW 264 cells were treated with dilazep and stimulated by 0 – 1.0 $\mu\text{g/mL}$ of LPS. Dilazep suppresses the expression of iNOS (B) and TNF- α (C) mRNA. RAW 264 cells were treated with dilazep and then stimulated by 1.0 $\mu\text{g/mL}$ of LPS; total RNA was isolated 24 h after LPS treatment. These signal intensities by qPCR product were compared with the intensity of the cells without drug treatment. Bars represent the means $\pm$ S.E.M. of 5 measurements (A) or 3 measurements (B and C). Data were evaluated by Bonferroni's multiple comparison test.

ceptors. All four adenosine receptor subtype (A1, A2a, A2b, and A3) mRNAs were expressed in RAW 264 cells (Fig. 3A). A previous study suggested that dilazep inhibits TF and TNF- α expression, and its inhibitory effect is mediated by adenosine receptors (5). Furthermore, Weber and his colleagues suggested that LPS induced nuclear activation of TF and TNF- α biosynthesis, and TNF- α up-regulates iNOS expression in RAW 264 cells (7). NO synthesis in response to LPS stimulation was restored by treatment with caffeine, which is an antagonist of adenosine, regardless of the addition of dilazep

(15) ($P < 0.05$ vs. LPS and dilazep treatment, Fig. 3B).

This study revealed that the LPS-induced expression of NO and TNF- α was suppressed by dilazep. The suppression of NO production by dilazep is probably due to the inhibition of TNF- α expression because the NO production by macrophages is involved in stimulating TNF- α . Adenosine administration blocks the LPS-induced TNF- α secretion in RAW 264 cells (16). Therefore, increasing the extracellular adenosine levels by dilazep treatment may block TNF- α secretion and down-regulate iNOS expression, consequently decreas-

Fig. 2. The suppression of TNF- α expression by dilazep was not detected by the inhibition of iNOS. Increased NO production (A) and up-regulation of iNOS expression (B) by LPS treatment were suppressed by 1400W and/or dilazep. C) TNF- α expression was not significantly down-regulated by 1400W. B and C) RAW 264 cells were stimulated by 1.0 μ g/mL of LPS, treated with 20 μ g/mL of dilazep and 50 nmol/mL of 1400W. These signal intensities by qPCR product were compared with the intensity of the cells without drug treatment. D) The elevation of NO production by TNF- α stimulation was not inhibited by dilazep. The asterisk shows a significant difference ($P < 0.05$) from the value of the TNF- α stimulation alone. E) The elevation of NO production by TNF- α stimulation was inhibited by 1400W pre-treatment. RAW 264 cells were stimulated by 10 ng/mL of TNF- α , treated with 20 μ g/mL of dilazep and 50 nmol/mL of 1400W. Bars represent the means $\pm$ S.E.M. of 3 measurements. Data were evaluated by Bonferroni's multiple comparison test.

Fig. 3. The suppression of NO synthesis after dilazep treatment is influenced by the metabolic pathways of adenosine. A) RT-PCR analyses of the expression of adenosine receptors (A1, A2a, A2b, and A3) in RAW 264 cells. The panel shows the PCR products for 30 PCR cycles. The amplicon size is displayed individually. B) The relative production of LPS-induced NO after caffeine and dilazep treatment. RAW 264 cells were treated with caffeine (0–100 μ mol/mL) and dilazep (20 μ g/mL) and/or stimulated with LPS (1.0 μ g/mL). Bars represent the means $\pm$ S.E.M. of 5 experiments. Data were evaluated by Bonferroni's multiple comparison test.

ing NO levels in RAW 264 cells. In conclusion, the inhibition of NO production and the suppression of TNF- α expression by dilazep may be caused by the anti-inflammatory effect of adenosine, which is induced by dilazep.

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