Short Communication

Inhibitory Effect of Trimidox on Lipopolysaccharide-Induced Nitric Oxide Production in RAW 264.7 Macrophages

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Abstract. We examined the effect of trimidox (3,4,5-trihydroxybenzamidoxime) on the production of nitric oxide (NO) by lipopolysaccharide (LPS) in mouse RAW 264.7 macrophages. Trimidox (50 – 300 µM) concentration-dependently inhibited NO production by LPS (0.01, 0.1, or 1 µg/ml) after incubation for 24 h. LPS-induced expression of inducible NO synthase (iNOS) and degradation of IκBα were prevented by trimidox. The protective effect against NO production by LPS was not only observed in prior incubation but also later incubation with trimidox until iNOS was activated by LPS. These results suggest that trimidox has a predominantly protective effect against LPS-induced production of NO via iNOS expression.

Keywords: trimidox, nitric oxide, macrophage

Ribonucleotide reductase (RR) is a rate-limiting enzyme for the de novo formation of deoxyribonucleotides and plays an important role in DNA synthesis. Inhibition of RR is considered a specific target for cancer chemotherapy because the activity of this enzyme is greatly increased in neoplastic cells (1). A novel group of inhibitors of RR are polyhydroxy-substituted benzoic acid derivatives (2). Trimidox (3,4,5-trihydroxybenzamidoxime) was shown to be one of the most potent in this series of compounds (3). Our lab has reported that trimidox induced apoptosis in human leukemia cell lines (4).

Lipopolysaccharide (LPS, endotoxin), a highly conserved outer membrane component of Gram-negative bacteria, triggers many biological responses such as fever, septic shock, and even death (5). Murine and human macrophages exhibit a particularly vigorous response to endotoxin, which induces the production of inflammatory modulators such as nitric oxide (NO). NO is generated by inducible NO synthase (iNOS), which is expressed in response to various inflammatory stimuli and causes a large amount of NO to be produced by macrophages during the inflammatory process (6, 7); therefore, suppression of the production of NO using drugs might be useful for the treatment of inflammatory diseases and endotoxin shock. Several polyhydroxy-substituted benzoic derivatives reportedly inhibit LPS-induced NO production by blocking iNOS gene expression (8). Accordingly, trimidox may affect LPS-induced inflammatory stimulation. In this study, we examined the effect of trimidox on LPS-induced NO production in mouse RAW 264.7 macrophages.

Trimidox was synthesized as described by van’t Riet et al. (2). LPS (Escherichia coli serotype 0127:B8) and other reagents were supplied by either Sigma (St. Louis, MO, USA) or Nacalai Tesque (Kyoto) and were of the highest grade available. All cell culture reagents were obtained from Invitrogen Corp. (Carlsbad, CA, USA).

Mouse macrophage RAW 264.7 cells were obtained by the American Type Culture Collection (Manassas, VA, USA). Cells were routinely kept in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin G (100 U/ml) / streptomycin (100 µg/ml) at 37°C in a humidified 5% CO2 – 95% air incubator under standard conditions (9). RAW 264.7 cells were cultured at 4 × 105 cells/ml in 100-µl amounts in separate wells of 96-well plates to measure cytotoxicity and nitrite concentration or in 10-ml amounts in 100-mm dishes for other assays, and they were incubated with
drugs.

Cytotoxicity was assessed by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (9). The absorbance of each well was determined at 590 nm using an Inter-med model NJ-2300 Microplate Reader. Survival (%) was calculated relative to the control.

To measure the concentration of NO produced in RAW 264.7 macrophages, the stable conversion product of NO, nitrite (NO$_2^-$), was measured using Griess assay. Briefly, after 24 h of LPS treatment in RAW 264.7 macrophages with or without trimidox, medium and Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H$_3$PO$_4$] were placed in a 96-well plate and incubated for 10 min at room temperature. Nitrite levels were determined colorimetrically at 540 nm using a sodium nitrate standard curve.

The expression of iNOS, IkBα, proteins was detected by Western blotting, modified by our previous method (4). Briefly, cells were harvested and extracted by lysis buffer. Samples of 30 µg of each protein were loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to a PVDF membrane, blocked with blocking solution for 4 h and reacted with antibody overnight at 4°C and then incubated with horseradish peroxidase-linked secondary antibody for 1 h. After another wash, the levels of protein were analyzed by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, Arlington Heights, IL, USA).

Statistical analysis of the results was performed by Student’s t-test for two-group comparisons and one-way analysis of variance followed by Dunnett’s test or Williams’ type multiple comparison test. A P-value of less than 0.05 was considered significant.

First, we examined the cytotoxic effect of trimidox in RAW 264.7 cell incubation after 24 h (Fig. 1a). There was no effect until incubation with trimidox at 200 µM and slightly decreased cell survival at 300 µM (78.4 ± 5.6% of control), but it has no significant effect. This concentration had been shown to produce cell damage in several types of leukemia cells in our previous report (4). Incubation of the cells with a combination of trimidox (300 µM) and LPS (1 µg/ml) for 24 h had no significant effect on cell viability, as estimated by MTT assay (data not shown). We used maximum concentrations of trimidox at 300 µM for the subsequent experiments. The effect of trimidox on LPS-induced NO production in RAW 264.7 cells was investigated by measuring the accumulation of nitrite in culture medium. Trimidox at 300 µM did not interfere with the reaction between nitrite and Griess reagents (data not shown). Unstimu-

Fig. 1. Effect of trimidox-induced cytotoxicity or inhibitory effect of trimidox on LPS-induced production of nitric oxide (NO) in mouse RAW 264.7 macrophages. a: Cells were incubated with trimidox for 24 h and then cytotoxicity was estimated by MTT assay. b: Cells were incubated for each indicated period with LPS at 1 µg/ml in the absence or presence of 300 µM trimidox. c: Cells were incubated for 24 h with LPS (0.01, 0.1, or 1 µg/ml) in the absence or presence of trimidox (50, 100, 200, or 300 µM). Trimidox was added 1 h before incubation with LPS. Nitrite concentrations in medium were determined using Griess reagent. Each value represents the mean ± S.E.M. for three different experiments performed in triplicate. *P<0.05 vs LPS alone incubation groups.
lated cells, after 24 h of incubation in culture medium, produced background levels of nitrite. When cells were incubated with trimidox alone, the background concentration of nitrite in the medium was similar to that in unstimulated samples. There was no increase in the levels of nitrite by single incubation with 1 µg/ml LPS up to 6 h, but nitrite levels time-dependently increased after incubation for 8 h (Fig. 1b). These increases in the nitrite levels were strongly inhibited by pre-incubation for 1 h with 300 µM trimidox. As shown in Fig. 1c, after treatment with LPS (0.01, 0.1, or 1 µg/ml) for 24 h, the release of nitrite in the medium increased concentration-dependently (20.8 ± 1.8, 43.2 ± 2.6, and 58.2 ± 3.2 µM, respectively) compared to the control (2.4 ± 0.4 µM). When RAW 264.7 cells were incubated with different concentrations of trimidox (50 – 300 µM) for 1 h followed by incubation with LPS (0.01, 0.1, or 1 µg/ml) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected at more than 100 µM trimidox (LPS 0.01 µg/ml, 7.6 ± 0.8 µM; LPS 0.1 µg/ml, 21.6 ± 4.2 µM; LPS 1 µg/ml, 40.2 ± 3.4 µM) and was extremely attenuated by 300 µM trimidox (LPS 0.01 µg/ml, 6.8 ± 0.6 µM; LPS 0.1 µg/ml, 8.8 ± 0.8 µM; LPS 1 µg/ml, 14.1 ± 1.8 µM). This result suggests that trimidox has a potent preventive effect against LPS-induced inflammatory stimulation.

Next, we examined the effect of trimidox on LPS-induced increases in the expression of iNOS by Western blotting (Fig. 2: a and b). There was little effect of the iNOS expression by single incubation with 1 µg/ml LPS until 4 h, but the iNOS protein level was time-dependently increased by incubation for 6, 12, and 24 h in RAW 264.7 cells (Fig. 2a). Trimidox at 50 µM showed almost no change, but 100, 200, or 300 µM trimidox concentration-dependently suppressed the expression increase in iNOS protein (Fig. 2b). All lanes exhibited equivalent intensities of β-actin, assayed by Western blotting as the loading control.

The iNOS expression is transregulated NF-κB, which is present in the cytosol bound to inhibitory protein, IκBα (6). To clarify one of the mechanisms through which trimidox prevents iNOS expression, we examined the effect of trimidox on LPS-induced change of IκBα using Western blotting (Fig. 2: c and d). The degradation of IκBα could be seen to have peaked at 15 min, and resynthesis of IκBα could be noted at 45 min by incubation with LPS (Fig. 2c). In the subsequent incubation with LPS for 15 min (16.3%, compared with the no treatment group), trimidox at 200 or 300 µM slightly prevented IκBα degradation by 31.6% (n = 4, P<0.05) or 34.9% (n = 4, P<0.05), respectively (Fig. 2d). These results suggest that trimidox might inhibit iNOS expression through partially inhibiting LPS-induced...
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degradation of IκBα. What is the critical mechanism for the effect of trimidox on iNOS expression? This phenomenon has not been elucidated. Some possible ways for trimidox to exert its effect are as follows: it may inhibit translation of the iNOS gene, reduce iNOS stability, or inhibit the activity of iNOS. Further experiments should explore the inhibitory property of trimidox in iNOS expression.

Finally, to examine the timing of trimidox incubation, cells were incubated with 300 μM of trimidox at 6, 4, and 2 h before and after incubation with LPS at 1 μg/mL (Fig. 3). Interestingly, the protective effect was predominantly observed with both prior incubation and incubation at up to 4 h after addition of LPS. As shown in Fig. 2a, there was no difference in iNOS expression by LPS until incubation with LPS for 4 h, but there was an increase at 6 h after LPS addition. These results suggest that trimidox cannot reduce iNOS expression after LPS induces an increase in its expression. Therefore, the inhibitory mechanism of trimidox on the production of NO induced by LPS depends on the inhibition of iNOS expression.

In conclusion, trimidox inhibited LPS-induced production of NO in RAW 264.7 macrophages. High levels of NO have been described in a variety of pathophysiological processes including various forms of septicemia, endotoxin shock, circulatory shock, and inflammation. Trimidox reportedly has anti-tumor effects. Our data suggest that trimidox might effectively attenuate these NO-involved diseases, even under cancer chemotherapy situations.

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References