Research article

The inhibitory action of butyrate on lipopolysaccharide-induced nitric oxide production in RAW 264.7 murine macrophage cells

Dipshika Chakravortty, Naoki Koide, Yutaka Kato, Tsuyoshi Sugiyama, Mya Mya Mu, Tomoaki Yoshida, Takashi Yokochi

Department of Microbiology and Immunology and Division of Bacterial Toxin, Research Center for Infectious Disease, Aichi Medical University, Nagakute, Aichi, Japan

The effect of butyrate, a natural bacterial product of colonic bacterial flora, on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 murine macrophage cells was studied. Butyrate significantly reduced NO production in LPS-stimulated RAW cells. The inhibition was abolished by the removal of butyrate. Butyrate also inhibited the expression of inducible type NO synthase (iNOS) in LPS-stimulated RAW cells. Furthermore, butyrate prevented the activation of nuclear factor (NF)-κB through the stabilization of IκB-α and IκB-β. Butyrate did not affect the phosphorylation of mitogen-activated protein (MAP) kinases by LPS. It was, therefore, suggested that butyrate down-regulated LPS-induced NO production in RAW cells through preventing the expression of iNOS, and that it was due to the inhibitory action of butyrate on the activation of NF-κB.

INTRODUCTION

Butyrate is a short chain fatty acid derived from the metabolism of intestinal contents by gut bacteria and is present in normal large intestines at a concentration range of 5–24 mM.[1] However, in conditions such as inflammatory bowel diseases, the level of butyrate seems to be elevated.[2,3] Bioactive butyrate has been shown to modulate various immune functions of the host, such as enhancement of enzyme activities,[4] production of prostaglandin[5] and gene expression of pro-inflammatory cytokines.[6–8] Moreover, butyrate is suggested to collaborate with bacterial lipopolysaccharide (LPS) in modulating immune functions of the host.[5] Butyrate synergistically acts with LPS to induce the production of pro-inflammatory cytokines and mediators.[6,9,10] Nitric oxide (NO) exhibits a wide range of immune functions, acting as a major regulatory molecule and principal cytotoxic mediator of the immune system.[11–14] LPS is well known to enhance NO production in macrophages.[14] There are no reports on the effect of butyrate on LPS-induced NO production in macrophages. In the present study, we investigated the effect of sodium butyrate on LPS-induced NO production in the RAW 264.7 murine macrophage cell line. Here we describe the mechanism of the inhibitory action of butyrate on LPS-induced NO production in RAW macrophage cells.

Received 17 April 2000
Revised 16 June 20000
Accepted 6 July 2000

Correspondence to: Takashi Yokochi MD, Department of Microbiology and Immunology, Aichi Medical University, Nagakute, Aichi 480-1195, Japan
Tel: +81 561 62 3311 ext. 2269; Fax: +81 561 63 9187
E-mail: yokochi@amugw.aichi-med-u.ac.jp

MATERIALS AND METHODS

Materials

LPS from *Escherichia coli* O55:B5 and sodium butyrate was obtained from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of LPS and sodium butyrate were dissolved at 1 mg/ml and 100 mM in distilled water, respectively. They were further diluted in culture medium for experiments.

Cell culture

The RAW 267.4 murine macrophage cell line was obtained from Health Science Resource Bank (Tokyo,

Japan) and maintained in RPMI 1640 medium (Sigma) containing 5% heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY, USA) at 37°C with 5% CO_2. The cells were washed gently with Hank's balanced salt solution (Sigma) and scraped from the dishes. The cells were counted and seeded in a 96-well or 12-well plates.

Determination of nitrite concentration

Nitrite, as the end product of NO metabolism, was measured by using the Griess reagent as described elsewhere.[15] Culture supernatants (50 µl) were mixed with 100 µl of the Griess reagent, and the nitrite concentration in the culture supernatant was measured 10 min after mixing at an absorbance of 570 nm. The nitrite concentration was determined with reference to the standard curve using sodium nitrite. All data represent the mean of triplicates ± SD from four experiments.

Immunoblotting

RAW cells were cultured with butyrate (10 mM), LPS (1 µg) or the mixture of butyrate and LPS in 35-mm culture dishes (4 x 10^5 cells/dish) for 8 h. The cells were suspended directly in a lysis buffer and boiled for 5 min at 100°C. The cell lysates containing an equal amount of protein (20 µg/lane) were loaded onto a 4–20% gradient gel, run under reducing conditions and transferred to the membrane. The membranes were treated with 5% bovine serum albumin for 1 h, rinsed and incubated with a 1:1000 dilution of rabbit anti-inducible type NO synthase (iNOS) antibody (Upstate Biotechnology, Lake Placid, NY, USA) or rabbit anti-IκB-α or anti-IκB-β antibody (New England Biolabs, Beverly, MA, USA) for 1 h. The blots were further treated with a 1:3000 dilution of peroxidase-conjugated protein G for 1 h. The immune complex on the blots was detected with enhanced chemiluminescence substrate (New England Nuclear, Boston, MA, USA) and exposed to Kodak XAR X-ray film. Rabbit antibodies against extracellular signal-regulated kinase 1/2 (Erk1/2), p38 or Jun-N-terminal kinase (SAPK/JNK) and their phospho-products (New England Biolabs) were also used.

Luciferase reporter gene assay for NF-κB activation

RAW cells (3 x 10^5/ml) were seeded in 35-mm dishes and, on the following day, transfected with 0.5 µg of luciferase reporter genes driven by 5 times tandem repeats of NF-κB (pNFκB-Luc plasmid, PathDetect system, Stratagene, La Jolla, CA, USA) and 0.5 µg of pCMV-β-gal plasmid[16] by the lipofectin method (Gibco-BRL, Gaithesburg, MD, USA) for 8 h. After removal of the plasmids, the cells were incubated for 48 h and further treated with butyrate (10 mM), LPS (1 µg/ml) or the mixture of butyrate and LPS for 8 h. The cells were lysed with 100 µl of the reporter lysis buffer (Promega, Madison, WI, USA) for measurement of the luciferase activity. The cell lysates

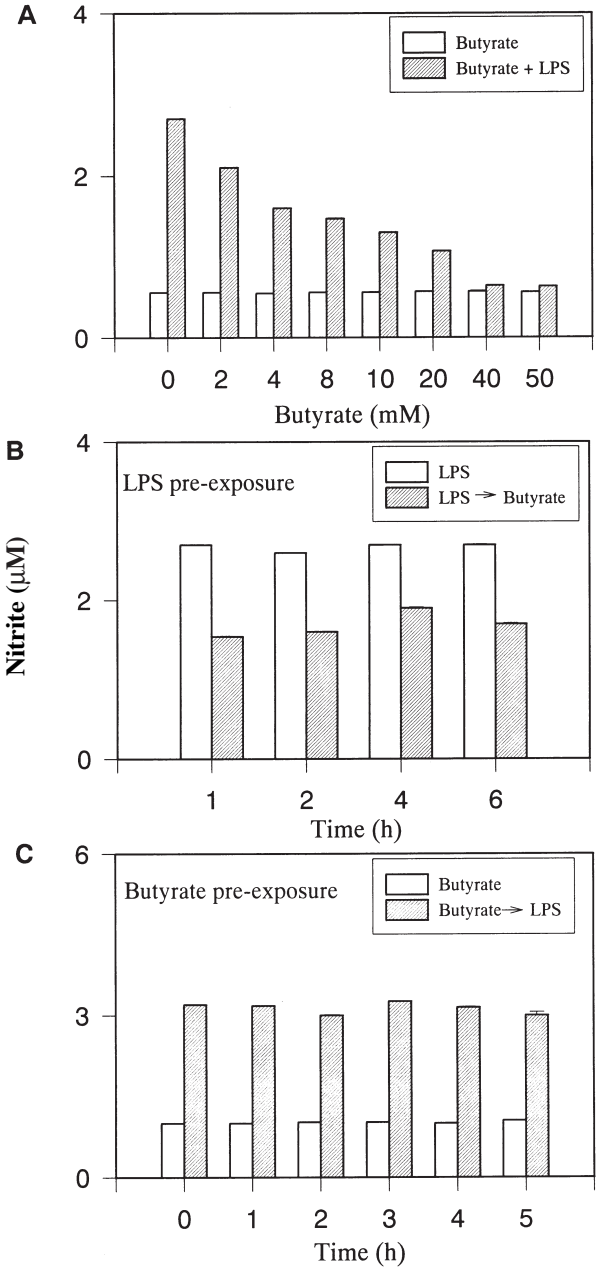

Fig. 1. Effect of butyrate on LPS-induced NO production in RAW 264.7 macrophage cells. (A) RAW cells were seeded in 96-well plates and incubated with various concentrations of sodium butyrate in combination with LPS (1 µg/ml) for 24 h. (B) RAW cells were exposed to LPS (1 µg/ml) for various times, washed to remove LPS, and further incubated with sodium butyrate (10 mM) and LPS (1 µg/ml) for 24 h. (C) RAW cells were exposed with sodium butyrate (10 mM) for various times, washed and further incubated with LPS (1 µg/ml) for 24 h. The concentration of nitrite in the culture supernatant was determined for NO production. The concentration of nitrite (µM) is expressed as the mean value of triplicates ± SD.

(10 μl) were mixed with the luciferase substrate (100 μl), and the luciferase activity was determined by a luminometer. The activity of β-galactosidase was measured using *o*-nitro phenyl galactopyranoside as substrate and used for normalizing transfection efficiencies. All bar diagrams are shown as the mean ± SD of two experiments in which each transfection was performed in duplicate.

RESULTS

Effect of butyrate on LPS-induced NO production in RAW cells

The effect of various concentrations of butyrate on LPS-induced NO production was examined by using RAW 264.7 cells (Fig. 1A). Butyrate at concentrations ranging from 2 to 50 mM reduced NO production in LPS-stimulated macrophages in a dose-dependent manner. Butyrate alone did not affect NO production at any concentration tested. In addition, butyrate alone or in combination with LPS exhibited no cytotoxic effect on RAW cells, suggesting that reduced NO production was not due to cell damage by butyrate. Next, the effect of butyrate on LPS-pretreated RAW cells was examined (Fig. 1B). RAW cells were pre-exposed with LPS (1 μg/ml) for 1, 2, 4 and 6 h, washed to remove LPS and incubated with fresh culture medium containing sodium butyrate (10 mM) for 24 h. The addition of butyrate to the cultures of LPS-pretreated RAW cells significantly reduced LPS-induced NO production. The inhibitory action of butyrate was effective even when it was added 6 h after LPS stimulation. Subsequently, we examined LPS-induced NO production in butyrate-pretreated RAW cells (Fig. 1C). RAW cells were cultured with sodium butyrate (10 mM) for 1–5 h, and then butyrate was removed from the cultures by washing. Butyrate-pretreated and untreated RAW cells were further incubated with LPS (1 μg/ml) for 24 h to determine NO production. There was no significant difference in LPS-induced NO production between butyrate-pretreated and untreated RAW cells, suggesting that an inhibitory action of butyrate on LPS-induced NO production could be reversed by the removal of butyrate. The inhibition of LPS-induced NO production by butyrate seemed to require the continuous presence of butyrate.

Effect of butyrate on LPS-induced iNOS expression in RAW cells

Since butyrate inhibited LPS-induced NO production in RAW cells, the expression of iNOS was examined in RAW cells treated with butyrate, LPS or the mixture of LPS and butyrate (Fig. 2). The immunoblotting analysis clearly demonstrated the band of iNOS protein in LPS-stimulated RAW cells. On the other hand, the expression of iNOS was not detected in RAW cells treated with the mixture of butyrate and LPS, suggesting that butyrate inhibited the iNOS expression in LPS-stimulated RAW cells.

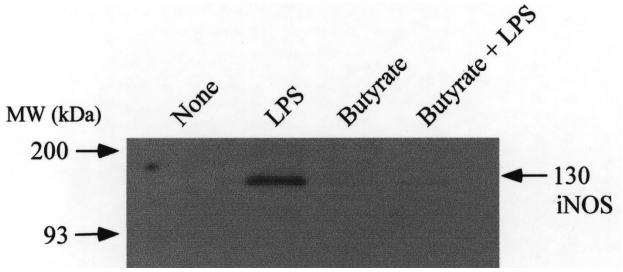

Fig. 2. Effect of butyrate on LPS-induced iNOS expression in RAW 264.7 macrophage cells. The cells were cultured with sodium butyrate (10 mM), LPS (1 μg/ml) or the mixture of butyrate and LPS for 8 h. The expression of iNOS protein was detected by an immunoblotting method.

Effect of butyrate on LPS-induced NF-κB activation in RAW cells

LPS-induced NO production via iNOS is reported to be mainly dependent on the activation and translocation of NF-κB.[17,18] Therefore, we investigated the effect of butyrate on the activation of NF-κB in LPS-stimulated RAW cells (Fig. 3). The NF-κB activation was measured by NF-κB-dependent luciferase reporter gene activity. LPS significantly augmented luciferase reporter gene activity, indicating NF-κB activation, whereas butyrate completely repressed it. Treatment with butyrate alone

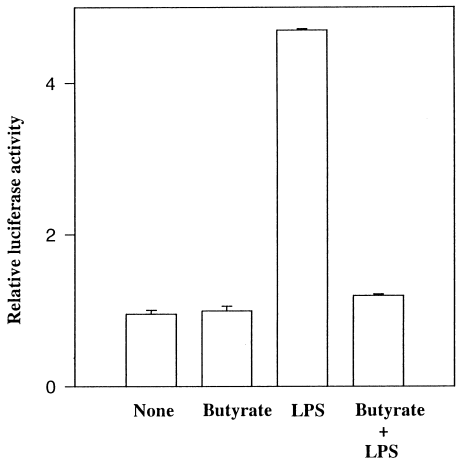

Fig. 3. Effect of butyrate on LPS-induced NF-κB activation in RAW 264.7 macrophage cells. The cells transfected with the NF-κB-dependent reporter genes were incubated with sodium butyrate (10 mM), LPS (1 μg/ml) or the mixture of LPS and sodium butyrate for 8 h. Values are expressed as the mean ± SD for two experiments in which each transfection was performed in duplicate.

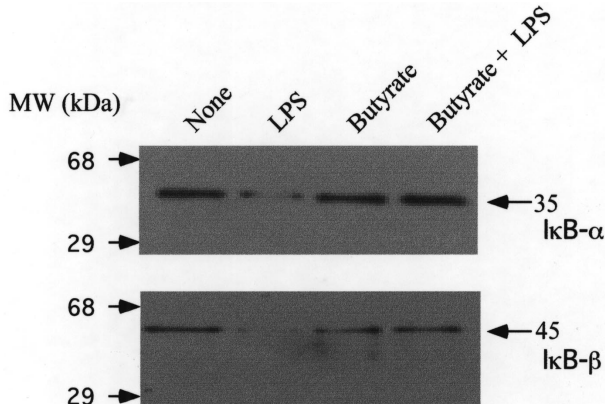

Fig. 4. Effect of butyrate on LPS-induced degradation of IκB-α and IκB-β in RAW 267.4 macrophage cells. The cells were incubated with sodium butyrate (10 mM), LPS (1 μg/ml) or the mixture of LPS and butyrate for 1 h. The expression of IκB-α and IκB-β proteins were detected by an immunoblotting method.

did not affect reporter gene activity. The inhibitory action of butyrate on LPS-induced NO production might be due to the repression of NF-κB activation.

Effect of butyrate on LPS-induced degradation of IκB-α and IκB-β in RAW cells

The effect of butyrate on IκB-α and IκB-β degradation was examined to clarify the inhibitory action of butyrate on LPS-induced NF-κB activation (Fig. 4). The expression of IκB-α and IκB-β protein was studied in RAW cells stimulated with LPS in the presence or absence of butyrate. The immunoblotting analysis demonstrated that LPS did not affect the constitutive expression of IκB-α and IκB-β in the presence of butyrate, although it markedly down-regulated IκB-α and IκB-β expression in the absence of butyrate. Butyrate seemed to inhibit LPS-induced degradation of IκB-α and IκB-β in RAW cells and stabilize them.

Effect of butyrate on the phosphorylation of various mitogen-activated protein (MAP) kinases in RAW cells treated with LPS

In the preceding section, we found that NF-κB signaling played a crucial role in the inhibition of LPS-induced NO production by butyrate. It was of interest whether MAP kinases as well as NF-κB participated in the down-regulation of LPS-induced NO production by butyrate. We studied the effect of butyrate on the phosphorylation of Erk1/2, p38 and Jun-N-terminal kinase (JNK) – MAP kinases which are closely related to LPS-induced macrophage activation.[19,20] By an immunoblotting method using anti-phospho-MAP kinase antibody, butyrate did not inhibit LPS-induced phosphorylation of Erk1/2, p38, and JNK/SAPK MAP kinase (data not shown). These results indicate that a series of MAP kinases tested might not be involved in the inhibition of LPS-induced NO production by butyrate.

Discussion

In the present study, we demonstrated that butyrate inhibited LPS-induced NO production in RAW 264.7 macrophage cells by preventing iNOS expression. As far as we know, this is the first report concerning the inhibitory action of butyrate on NO production in LPS-stimulated macrophages. Butyrate seems to be an important bacterial modulator of NO production in macrophages. Butyrate is already known to modulate various immune functions, such as the production of colony-forming factor,[7] interleukin-8,[6] prostaglandin E_2,[5] antibody,[21,22] and tumor necrosis factor (TNF)-α.[5] Butyrate is also capable of increasing lysozyme production by monocytic cells.[23] Furthermore, butyrate synergistically acts with LPS to induce the production of pro-inflammatory cytokines and mediators.[6,9,10] In the present study, however, butyrate counteracted LPS in NO production by macrophages. The synergistic action of butyrate and LPS was mostly observed in epithelial cells,[6,9,10] whereas butyrate down-regulated LPS-induced TNF-α production in Kupffer cells. The discrepancy in the actions of butyrate might be elucidated by the difference in cell types. Thus, in inflammatory bowel disease, butyrate and LPS could modulate the immune function of macrophages in a complicated fashion.

The inhibitory action of butyrate on LPS-induced iNOS expression and NO production seemed to be due to the down-regulation of NF-κB activation. Moreover, the present study demonstrated that butyrate inhibited the degradation of IκB-α and IκB-β molecules for NF-κB activation. Therefore, butyrate seems to inhibit NF-κB activation through the stabilization of IκB-α and IκB-β, although we did not confirm the inhibition of NF-κB translocation by butyrate. Based on the fact that the induction of iNOS in LPS-stimulated macrophages is dependent on NF-κB activation,[17,18] the inhibitory action of butyrate on LPS-induced NO production might be essentially caused by the stabilization of IκB. The down-regulation of NF-κB through IκB by butyrate has also been reported in a colonic epithelial cell line.[24] Furthermore, our finding is consistent with the report that butyrate inhibits NF-κB-dependent TNF-α production in LPS-stimulated Kupffer cells.[25] On the other hand, butyrate induced interleukin-8 production in intestinal epithelial cells through NF-κB activation.[6] Therefore, NF-κB, which plays a central role in regulating immune and

inflammatory response, was the important target of butyrate. In addition, it is unlikely that Erk1/2, p38 and JNK/SAPK MAP kinases might be target molecules of butyrate in the inhibition of LPS-induced NO production.

Macrophages as inflammatory cells might be exposed to butyrate and LPS in conditions of inflammatory bowel disease. It is reasonable that butyrate might down-regulate LPS-induced NO production in activated macrophages. NO is a principal cytotoxic mediator of activated immune effector cells.[11–14] As a cytotoxic effector molecule, NO has been shown to inhibit the growth and function of infectious agents.[13,14] Therefore, it is likely that butyrate, a metabolite derived from bacteria, might reduce the production of cytotoxic NO by immune effector cells to escape from the protective mechanisms of the host. On the other hand, the host might recognize the presence of LPS, a bacterial product from the outer membranes of Gram-negative bacteria, and induce the production of cytotoxic NO molecules by activated immune effector cells. The phenomenon found in the present study might indicate the delicate balance of the host-parasite relationship.

ACKNOWLEDGEMENTS

We are grateful to K. Takahashi and A. Morikawa for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and the DIAKO Foundation.

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