

JPET #92056

**TITLE: Roflumilast Inhibits Lipopolysaccharide-Induced Inflammatory Mediators
via Suppression of NF- κ B, p38 MAP kinase and JNK activation.**

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JPET #92056

RUNNING TITLE : Inhibition of nuclear factor-kappa B, p38 and c-Jun NH₂-terminal
kinase by roflumilast

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JPET #92056

ABBREVIATIONS : PDE4, phosphodiesterase 4; NO, nitric oxide; NF- κ B, nuclear factor- κ B, TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β

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JPET #92056

ABSTRACT

Roflumilast, a potent and selective phosphodiesterase 4 (PDE4) inhibitor, has been demonstrated to be an effective anti-inflammatory agent in airway inflammatory diseases. In the present study, we investigated the mechanism of anti-inflammatory effects of roflumilast in murine macrophage cell line RAW 264.7 cells. Roflumilast inhibited NO, TNF- α and IL-1 β production via suppression of their gene expressions in lipopolysaccharide (LPS)-stimulated macrophages. To elucidate the mechanism by which roflumilast inhibits the production of inflammatory mediators, we examined the effect of roflumilast on the activation of nuclear factor- κ B (NF- κ B) in these cells. Roflumilast inhibited the DNA binding activity of NF- κ B by preventing I κ B α phosphorylation and degradation. The phosphorylation of MAP kinases including SAPK/JNK and p38 MAP kinase was also markedly inhibited by roflumilast. Similar to the effects of roflumilast, treatment of either SB203580 or SP600125, specific inhibitors of p38 MAP kinase and JNK, respectively, suppressed NO, TNF- α and IL-1 β production. Consistent with *in vitro* results, administration of roflumilast recovered the survival rate of LPS-treated mice, with concurrent suppression of plasma levels of nitrite/nitrate, TNF- α and IL-1 β . These results

JPET #92056

suggest that the inhibitory activity of roflumilast on the production of inflammatory mediators appears to be mediated via inhibition of NF- κ B, p38 MAP kinase and JNK activation in macrophages.

JPET #92056

INTRODUCTION

Roflumilast, the most potent and advanced PDE4 inhibitor so far, has been demonstrated to be an effective anti-inflammatory agent in many inflammatory diseases including asthma (Bundschuh et al., 2001). For example, roflumilast significantly inhibited airway inflammation and remodeling in antigen sensitized mice (Kumar et al., 2003), collagen induced arthritis in mice (Barsig et al., 2001) and inflammatory bowel disease in mice (Banner et al., 2004). In *in vitro* studies, roflumilast has been shown to inhibit neutrophil elastase (NE), myeloperoxidase (MPO) and MMP-9 release from TNF- α stimulated human neutrophils (Jones et al., 2005), to inhibit MUC5AC gene expression in EGF-stimulated airway epithelial cells (Mata et al., 2005), and to suppress LPS-induced TNF- α secretion in macrophages (Hatzelmann et al., 2001). Although roflumilast exhibits several beneficial effects, its downstream mechanism following PDE4 inhibition in inflammatory cells remains unclear.

Upon inflammatory stimulation, macrophages produce nitric oxide (NO) and proinflammatory cytokines such as IL-1 β and TNF- α (Zembowicz et al., 1992; Tracey et al., 1994). Overproduction of these mediators as well as their mRNA expressions has been

JPET #92056

found in macrophages of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis and hepatitis (Isomaki et al., 1997; Libby et al., 2002; Tilg et al., 1992). A transcription factor, nuclear factor- κ B (NF- κ B) is known as an important regulator of iNOS, TNF- α and IL-1 β expression in LPS-activated inflammatory cells such as macrophages and microglia (Xie et al., 1994; Colasanti et al., 1995). NF- κ B is present in the cytoplasm as a heterodimer consisting of p50, p65, and I κ B α subunits. In response to an activation signal, I κ B α is phosphorylated and degraded, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription (Pahl, 1999).

On the other hand, phosphorylation of MAP kinases (Ajizian et al., 1999) is also known to be a key component of production of NO and proinflammatory cytokines in activated macrophages. Three MAP kinase families have been identified in mammalian cells such as extracellular signal regulated kinase 1/2 (Erk1/2), SAPK/JNK and p38 (Murga et al., 1999). LPS activates ERK, SAP/JNK, and p38 MAP kinase, which mediate LPS-induced inflammatory gene expression (Ajizian et al., 1999; Bhat et al., 1998). Therefore, suppression of NF- κ B and MAP kinase activation has been used as a biomarker for the

JPET #92056

screening of anti-inflammatory activity. It was previously reported that elevation of intracellular cAMP by rolipram controlled cytokine secretion by T cells via inhibition of NF- κ B activation (Jimenez et al., 2001). In addition, rolipram inhibited the IFN- γ -stimulated phosphorylation of p38 MAP kinase in U937 monocytic cells (MacKenzie et al., 2000).

In this study, we tested whether roflumilast suppresses LPS-stimulated pro-inflammatory mediators NO, TNF- α and IL-1 β in macrophages, and whether NF- κ B and MAP kinases are involved in the actions of roflumilast. The results indicate that roflumilast suppresses NO, TNF- α and IL-1 β production by inhibiting the activation of NF- κ B, SAPK/JNK and p38. The suppressive effect of roflumilast was further confirmed by *in vivo* study in which roflumilast inhibited plasma levels of NO, TNF- α and IL-1 β triggered by LPS, with increased survival rate of septic shock.

JPET #92056

METHODS

Chemicals. RPMI-1640, fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Roflumilast was synthesized by Korea Research Institute of Chemical Technology (KRICT) (Taejon, Korea), and dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in culture media for all *in vitro* experiments. Specific antibodies against iNOS, p-SAPK/JNK, SAPK/JNK, p-p38 and p38 were the products of Santa Cruz (Santa Cruz, CA, USA), and p-I κ B α and I κ B α were purchased from cell signaling (Cell Signaling Technology Inc., Beverly, MA). Bay11-7082 {(E)-3[(4-methylphenyl)sulfonyl]-2-propenitrile}, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] and SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone] were obtained from Calbiochem (San Diego, CA, USA). Cytotox 96 nonradioactive assay kit was obtained from Promega (Road Madison, WI, USA). All other reagents were the products of Sigma Chemical unless indicated otherwise (St. Louis, MO, USA).

Animal. Male C57BL6/J (8~9 wks) mice, weighing 20~25 g, were produced and

JPET #92056

maintained in specific pathogen-free conditions at our animal breeding facility. All animal studies were performed in accordance with NIH guideline (National Academy Press, 1996).

Peritoneal macrophage isolation and cell culture. The murine macrophage cell line RAW 264.7 (American Tissue Culture Collection, Manassa, VA) cells were cultured in complete RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicilline G (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM). C57BL6/J mice were intraperitoneally injected with 1.5 ml of 4% thioglycollate broth. After 4 days macrophages were collected from the peritoneal cavity. Primary macrophages were cultured in six-well plates (4×10^5 cells/well) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Cell cytotoxicity assay. Cellular toxicity was determined by lactate dehydrogenase (LDH) release in the medium. In brief, cells (4×10^5 cells/ml) were incubated with roflumilast at concentrations of 0.1 -30 µM. After 18 h incubation, LDH activity in the media was measured by using the assay kit (Promega, Road Medison, WI). Optical density (OD) at 490 nm was measured and quantified.

JPET #92056

cAMP assay. RAW264.7 cells (1×10^6 cells/well) were incubated for 20 min in 200 μ l media alone or with roflumilast in the indicated concentrations. Ten microliters of isoprenaline (10 μ M) was added and incubated for 20 min. After washing the media, 200 μ l of lysis buffer was added as provided by biotrak cAMP EIA kit (Amersham Pharmacia, USA). The EIA kit was used according to the manufacture's instruction.

Measurement of nitrite, TNF- α and IL-1 β . Accumulation of nitrite in the medium was measured by a colorimetric assay method based on the Griess reaction. Briefly, samples were reacted with 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid at room temperature for 10 min, and nitrite concentration was determined by absorbance at 540 nm in comparison with sodium nitrite as a standard. Plasma levels of nitrite plus nitrate were determined using a nitrate assay kit (Promega, Road Medison, WI). The levels of TNF- α and IL-1 β were measured in cell culture media and plasma using BD bioscience (San Jose, CA) and R&D (R & D System, Minneapolis, MN) ELISA kits, respectively.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR).

JPET #92056

Total RNA was isolated from the cells using easy-blue reagent as suggested by the manufacturer (Intron, Seoul, Korea). Three μg of RNA was used for RT-PCR according to the manufacturer's instructions (Promega, Road Medison, WI). PCR amplification was initiated with 2 min of denaturation at 94°C followed by 30 cycles at 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec (Mastercycler, Effendorf). Primer sequences for analysis of iNOS, IL-1 β and β -actin mRNA were as follows. iNOS, 5'-CAGAAGCAGAATGTGACCATC-3' (sense) and 5'-CTTCTGGTCGATGTCATGAGC-3' (antisense); IL-1 β , 5'-TGCAGAGTCCCCAACTGGTACATC-3' (sense); 5'-GTGCTGCCTAATGTCCCCTTGAATC-3' (sense); β -actin, 5'-CCTTCTACAATGAGC-3' (sense) and 5'-ACGTCACACTTCATG-3' (antisense). After amplification, the samples were separated on a 1.5 % agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide (EtBr) and photographed. Digital photographs were assessed with image analysis software, and mRNA expression was evaluated as the ratio to β -actin.

Western blot analysis. Protein samples of whole lysate (30 μg) were mixed with an equal volume of 2x SDS sample buffer, boiled for 5 min, and then separated by 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to nylon membrane. The

JPET #92056

membranes were blocked in 5% dry milk for 1 h, rinsed, and incubated with specific antibodies against iNOS, SAPK/JNK, p-SAPK/JNK, p38, p-p38, I κ B α and p-I κ B α in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4°C. Primary antibody was removed by washing the membranes 3 times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000). Following 3 times washing in TBS-T, bands were visualized by ECL and exposed to X-ray film.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously (Jeon et al., 2000). The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instructions. The oligonucleotide sequence for NF- κ B/Rel was 5'-GATCTCAGAGGGGACTTTCCGAGAGA-3'. Double stranded oligonucleotides were end-labeled with [γ -³²P]ATP. Nuclear extracts (5 μ g) were incubated with 2 μ g of poly (dI-dC) and a ³²P-labeled DNA probe, and DNA binding activity was analyzed using a 4.8% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

JPET #92056

Induction of septic shock. Endotoxin shock was induced by administration of a single intraperitoneal (i.p.) dose of LPS/GalN (20 mg/700 mg/kg) in saline (Lehmann et al., 1987). To examine whether roflumilast has protective effects on endotoxin shock, mice were divided into four groups and treated with vehicle (0.5 % CMC, n=10), LPS/GalN (n=10), or LPS/GalN plus roflumilast (2 and 10 mg/kg, n=10, respectively). Roflumilast was administered intraperitoneally 1 h before LPS administration. Six hours later, blood sample was collected by orbital puncture and plasma was prepared by centrifugation at 12,000g for 20 min at 4°C for measuring NO_x, TNF- α and IL-1 β levels. Survival of mice was monitored for 24 h.

Statistical analysis. Data are represented as means \pm SD of more than three separate experiments performed in triplicates. The significance of difference from the respective control for each experimental test condition was assessed by using Student's *t*-test for each paired experiments except *in vivo* experiment of LPS-induced septic shock. The statistical significance of survival rate in *in vivo* experiment was determined by the Fisher's exact test. A *p* value < 0.05 was regarded as a significant difference.

JPET #92056

RESULTS

Effect of roflumilast on cAMP accumulation. We examined the effect of roflumilast on isoprenaline-induced cAMP production in RAW264.7 cells. As expected, treatment with roflumilast for 20 min augmented intracellular cAMP levels in a concentration-dependent manner with an EC₅₀ value of 0.9 nM, effects being saturated at 1 μM (Fig. 1).

Inhibition of NO production and iNOS gene expression by roflumilast. Unstimulated macrophages produced a background level of NO (about 6 μM) in the culture medium. When RAW264.7 cells were stimulated with LPS (100 ng/ml) for 18 h, the levels of nitrite, a stable oxidized product of NO, were markedly increased in the culture medium to 30-35 μM. To investigate whether roflumilast is capable of inhibiting LPS-induced NO production, RAW 264.7 cells were pretreated for 1 h with various concentrations of roflumilast followed by 100 ng/ml LPS treatment for 18 h. Roflumilast inhibited LPS-induced NO production and iNOS protein levels in a concentration-dependent manner with almost complete inhibition achieved at 30 μM (Fig. 2A and B). To examine whether the suppression of iNOS protein by roflumilast was due to reduced iNOS mRNA expression,

JPET #92056

RT-PCR analyses from LPS-stimulated cells were carried out. The results showed that roflumilast concentration dependently reduced iNOS mRNA levels (Fig. 2C). No cytotoxic effect of roflumilast was observed in this experimental condition as measured by lactate dehydrogenase (data not shown).

Inhibition of TNF- α and IL-1 β by roflumilast. We further examined whether roflumilast inhibits LPS-induced TNF- α and IL-1 β by ELISA. RAW264.7 cells were stimulated with LPS for 18 h in the absence or presence of roflumilast pretreatment. TNF- α and IL-1 β levels were increased in LPS-stimulated RAW 264.7 cells, and these elevations were significantly decreased in a concentration-dependent manner by roflumilast treatment (Fig. 3A and B). In addition, increased expression of IL-1 β mRNA by LPS was suppressed by roflumilast (Fig. 3C). Complete suppression of either TNF- α or IL-1 β by roflumilast was achieved at 30 μ M.

Inhibition of NO, TNF- α and IL-1 β by roflumilast in primary peritoneal macrophages.

Next, we examined the effects of roflumilast on NO, TNF- α and IL-1 β production in primary cultured murine peritoneal macrophages. As seen with RAW 264.7 cells (Fig. 2

JPET #92056

and 3), roflumilast (30 μ M) inhibited NO, TNF- α and IL-1 β production in primary peritoneal macrophages stimulated with LPS (Fig. 4). No cytotoxic effect of roflumilast was observed in this experimental condition as measured by lactate dehydrogenase (data not shown).

Effects of roflumilast on LPS-induced NF- κ B activation and I- κ B α phosphorylation and degradation. Previous reports have demonstrated that the transcription factor NF- κ B is involved in the LPS-induced activation of iNOS or other inflammatory cytokines (Heiss et al., 2001). To determine whether NF- κ B is an important target for the action of roflumilast in RAW264.7 cells, we performed an EMSA. As shown in Fig. 5A, the induction of specific NF- κ B DNA binding activity by LPS was decreased by roflumilast pretreatment in a concentration-dependent manner. These results suggest that roflumilast inhibits LPS-induced iNOS, TNF- α and IL-1 β expression in RAW264.7 cells, possibly by attenuation of NF- κ B activity. To gain further insight into the mechanism of roflumilast-mediated regulation of NF- κ B, we examined the effects of roflumilast on I κ B α phosphorylation and degradation. LPS treatment of RAW264.7 cells resulted in the I κ B α phosphorylation and degradation, and exposure of cells to the higher concentrations than 10

JPET #92056

μM of roflumilast for 1 h prior to LPS stimulation significantly inhibited $\text{I}\kappa\text{B}\alpha$ phosphorylation and degradation (Fig. 5B and 5C).

Effects of roflumilast on the LPS-induced activation of SAPK/JNK and p38 MAP

kinase. LPS is known to activate ERK, SAPK/JNK and p38 MAP kinase pathways in macrophages (DeFranco et al., 1995; Guha et al., 2001). The possibility was raised that roflumilast inhibited production of LPS-induced inflammatory mediators by down regulating MAP kinases. Therefore, we examined the effect of roflumilast on LPS-induced activation of MAP kinases. As shown in Fig. 6, LPS treatment induced a strong increase in the phosphorylated JNK and p38 levels. Treatment with roflumilast significantly suppressed LPS-induced activation of JNK and p38 MAP kinase concentration dependently (Fig. 6). To more closely assess the relative role of MAP kinases in LPS-induced inflammatory mediator production, we checked the effects of selective MAP kinase inhibitors on the LPS-induced NO, TNF- α and IL- β production. SP600125 (a JNK inhibitor), SB203580 (a p38 MAP kinase inhibitor) and Bay11-7082 (a NF- κB inhibitor) profoundly inhibited LPS-mediated NO, TNF- α and IL-1 β production. However, treatment with PD98059 (MEK inhibitor) had little effect (Fig. 7A, B and C). These

JPET #92056

results suggest that roflumilast-mediated inactivation of JNK and p38 MAP kinase may be, at least partly, responsible for the suppression of NO and proinflammatory cytokines in RAW264.7 cells.

Effects of roflumilast on LPS-induced lethal shock in mice. The LPS-induced murine endotoxic shock model was used to assess the protective effect of roflumilast *in vivo*. As shown in Fig. 8, the administration of LPS/GalN to the mice resulted in a survival rate of 0% within 12 h. However, pretreatment (1 h prior to LPS) of roflumilast produced a dose dependent protection against endotoxin shock. Administration of roflumilast (10 mg/kg, i.p.) significantly increased the survival rate up to 60% (6/10 mice survived). Even at the lower dose (2 mg/kg, i.p.), roflumilast increased the survival rate to 30%. These results clearly show that roflumilast is effective in protecting LPS/GalN-induced lethal shock in mice. Concurrent with its protective effects *in vivo*, roflumilast treatment markedly decreased the elevated plasma levels of NO_x, TNF- α and IL-1 β (Table 1).

JPET #92056

DISCUSSION

PDE4 inhibitors are considered as promising anti-inflammatory drugs based on their abilities to suppress the activation of many inflammatory cells. (Souness et al., 2000; Teixeira et al., 1997). However, the detailed mechanism of PDE4 inhibitors involved in the anti-inflammatory activities is largely unknown. Recently, it has been proposed that PDE4 inhibitors interfere with cellular signaling leading to activation of transcription factor, consequently blocking leukocytes activation and cytokine synthesis (Haddad et al., 2002; Matsumori et al., 2000; Jimenez et al., 2001; Wang et al., 1997). Roflumilast is a novel potent PDE4 selective inhibitor being effective in airway inflammation (Bundschuh et al., 2001; Timmer et al., 2002), and its potential usefulness in human asthma has been demonstrated in clinical studies (Bayes et al., 2002). In the present study, we examined the mechanism of anti-inflammatory action of roflumilast, and found that roflumilast inhibits the production of several inflammatory mediators such as NO, TNF- α and IL-1 β in RAW264. 7 cells via inhibition of NF- κ B activation and phosphorylation of SAPK/JNK and p38 MAP kinase.

Increased production of NO, TNF- α and IL-1 β plays a critical role in the process of

JPET #92056

macrophage activation, and is associated with acute and chronic inflammations. It was previously reported that PDE4 inhibitors, rolipram, and pentoxifylline, inhibited NO production in islet cells (Beshay and Prud'homme, 2001). In addition, rolipram was shown to inhibit the production of TNF- α on acute and chronic models of inflammation, producing anti-inflammatory activities (Beshay et al., 2001). In the present study, we have demonstrated that roflumilast inhibits NO, TNF- α and IL-1 β production in macrophages as well as in *in vivo* stimulated by LPS treatment.

The control of iNOS, IL-1 β and TNF- α transcription is mediated by the activation of NF- κ B, which is an important proximal mechanism for the overproduction of the inflammatory mediators in macrophages in response to LPS (Shapira et al., 1996). The involvement of NF- κ B in the suppressive action of roflumilast on inflammatory mediators was supported in the present study by several results including EMSA, inhibition of I κ B α phosphorylation and effects of NF- κ B inhibitor, Bay11-7082. On the other hand, the relationship between cAMP elevation and NF- κ B inhibition appears to be controversial. It was reported that elevated cAMP inhibited NF- κ B activation in transformed T cells (Chen and Rothenberg 1994; Haraguchi et al., 1995), and PDE4 inhibition by rolipram regulated transcription of particular cytokines through inhibition of NF- κ B activation in T-cells

JPET #92056

(Jimenez et al., 2001). On the contrary, inhibition of PDE 1, 3, and 4 isozymes in alveolar epithelial cells exhibited no effect on LPS-mediated translocation of NF- κ B subunits (Haddad et al., 2002). Based on the results of the present study, roflumilast caused the increased intracellular cAMP in RAW 264.7 cells, accompanied by NF- κ B inhibition, consistent with previous reports in T cells (Chen and Rothenberg, 1994; Haraguchi et al., 1995). The mechanism of action of roflumilast on NF- κ B inhibition appears to be an inhibition of I κ B- α protein phosphorylation and degradation, possibly via modulation mediated by cAMP-dependent protein kinases. However, the possibility that the anti-inflammatory action of roflumilast is mediated by the mechanism independent of cAMP elevation cannot be excluded at present.

Among various intracellular signaling pathways, MAP kinase pathway has been known to be important in the regulation of cytokine gene expression showing that SAPK/JNK and p38 MAP kinase are involved in the iNOS gene expression (Chan and Riches, 1998; Jeon et al., 2000). Recently, MacKenzie et al. (2000) reported that elevated cAMP by rolipram inhibited the IFN- γ stimulated phosphorylation of p38 MAP kinase in U937 cells. Consistent with the results, our results also demonstrated that roflumilast inhibited SAPK/JNK and p38 MAPK pathways, and the JNK inhibitor, SP600125, and p38

JPET #92056

MAPK inhibitor, SB203580, suppressed NO, TNF- α and IL-1 β production induced by LPS stimulation. These results suggest that besides NF- κ B inhibition, roflumilast may inhibit proinflammatory gene expression via an additional mechanism of JNK and p38 MAPK inactivation in RAW 264.7 cells. To our knowledge, the current data are the first demonstration of inhibitory effects of roflumilast on the activity of NF- κ B and MAP kinases. However, the possible link between the effects on NF- κ B and MAP kinases activity, and their relative contributions to cytokine regulation need to be further elucidated.

Septic shock is frequently induced in experimental conditions by lipopolysaccharide (LPS), a major constituent of Gram-negative bacterial outer membrane, with sensitization using GalN. LPS is known to stimulate production or release of inflammatory cytokines from monocytes and macrophages, thus triggering the development of septic shock. In the present study, roflumilast attenuated the lethality induced by LPS/GalN *in vivo*, concurrent with the suppression of plasma levels of NO $_x$, TNF- α and IL-1 β . These effects appear to be linked, at least in part, with the actions of roflumilast on NF- κ B, JNK and p38 kinases in macrophages, based on the *in vitro* results using RAW264.7 macrophages and primary peritoneal macrophages. Furthermore, selective inhibitors of p38 kinase (SB203580) and NF- κ B (PC-SPES) have been shown to rescue the mice from endotoxin-induced shock

JPET #92056

(Badger et al., 1996; Ikezoe et al., 2003), supporting the involvement of p38 kinase and NF- κ B in *in vivo* endotoxin-induced septic shock.

In summary, we have found that a well known PDE4 inhibitor, roflumilast, controls LPS-induced NO, TNF- α and IL-1 β production through inhibition of NF- κ B activation and SAPK/JNK and p38 MAPK pathways in RAW264.7 macrophages. In addition, our results indicate that roflumilast exerted potent *in vivo* anti-inflammatory effects and increased the survival rate of mice in sepsis model. The current data would provide a new insight into the mechanisms responsible for the anti-inflammatory and pharmacological activities of roflumilast.

JPET #92056

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JPET #92056

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JPET #92056

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JPET #92056

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JPET #92056

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JPET #92056

Legends for Figures

Fig. 1. Effects of roflumilast on intracellular cAMP levels. RAW264.7 cells (1×10^6 cell) were incubated for 20 min with media alone and indicated concentrations of roflumilast. Lysates of the cells were then assayed for cAMP using a RIA. Data shown are means \pm SD. * $p < 0.05$ versus isoprenaline-induced group. Each experiment was carried out three times in triplicates.

Fig. 2. Effects of roflumilast on NO production and iNOS expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-incubated with indicated concentrations of roflumilast (0.1-30 μ M) for 1 h, followed by the incubation with LPS (100 ng/ml). (A) Nitrite levels were measured in the cultured media by Griess reaction after incubation with LPS for 18 h. (B) Levels of iNOS protein were measured in cultured cells by western blot using antibody for murine iNOS. (C) Levels of iNOS mRNA were measured in cultured cells by RT-PCR after incubation with LPS for 10 h. Data shown are means \pm SD. * $p < 0.05$ versus LPS alone. Each experiment was carried out three times in triplicates.

JPET #92056

Fig. 3. Effects of roflumilast on production of TNF- α , IL-1 β in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were pre-incubated with indicated concentrations of roflumilast (0.1-30 μ M) for 1 h, followed by the incubation with LPS (100 ng/ml). (A) TNF- α and (B) IL-1 β concentrations were measured in the cultured media by using commercial ELISA kits after 18 h incubation with LPS. (C) Levels of IL-1 β mRNA were measured in cultured cells by RT-PCR after 10 h incubation with LPS. Data shown are means \pm SD. * p <0.05 versus LPS alone. Each experiment was carried out three times in triplicates.

Fig. 4. Effects of roflumilast on NO, TNF- α and IL-1 β production in primary peritoneal macrophages. Peritoneal macrophages isolated from mouse peritoneal cavity were pre-cultured overnight and incubated with roflumilast (30 μ M) for 1 h, followed by the incubation with LPS (100 ng/ml) for 18 h. (A) Nitrite levels were measured by Griess reaction. (B) TNF- α and (C) IL-1 β concentrations were measured by using a commercial ELISA kit. Data shown are means \pm SD. * p <0.05 versus LPS alone. Each experiment was carried out three times in triplicates.

Fig. 5. Inhibition of NF- κ B activation, I κ B α phosphorylation and degradation by

JPET #92056

roflumilast in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with indicated concentrations of roflumilast for 1 h prior to the addition of 100 ng/ml LPS, and the cells were further incubated for 1 h. (A) After isolation of nuclear extracts, EMSA was carried out. The binding specificity was determined using unlabeled wild-type probe (100-fold in excess) to compete with the labeled oligonucleotide. (B) The lysates were subjected to immunoblot analysis using I κ B α and p-I κ B α specific antibodies. Each experiment was carried out three times. (C) Densitometric analysis of I κ B α and p-I κ B α bands obtained from (B), and the results are expressed as ratio of p-I κ B α and I κ B α .

Fig. 6. Effects of roflumilast on the LPS-induced activation of SAPK/JNK and p38 MAP kinase in RAW 264.7 cells. Cells were pretreated with indicated concentrations of roflumilast for 1 h, incubated with LPS (100 ng/ml) for 1 h, and then assayed for activation of SAPK/JNK and p38 MAP kinase using western blot analysis. Each experiment was carried out three times.

Fig. 7. Effects of MAP kinase inhibitors on NO, TNF- α and IL-1 β production. RAW 264.7 cells were pre-treated with roflumilast (30 μ M), SB203580 (10 μ M), PD98059 (30

JPET #92056

μM), SP600125 (10 μM) and Bay11-7082 (10 μM) for 1 h, followed by the incubation with LPS (100 ng/ml) for 18 h. (A) Nitrite levels were measured by Griess reaction. (B) TNF- α and (C) IL-1 β concentrations were measured by using a commercial ELISA kit. Data shown are means \pm SD. * p <0.05 versus LPS alone. Each experiment was carried out three times in triplicates.

Fig. 8. Effects of roflumilast on survival of LPS/Gal/N-treated mice. Different groups of mice (n=10) were intraperitoneally treated with vehicle (■), LPS/Gal/N (20 mg/700 mg/kg) (●), LPS/Gal/N (20 mg/700 mg/kg) plus 2 mg/kg roflumilast (▲) or LPS/Gal/N (20 mg/700 mg/kg) plus 10 mg/kg roflumilast (▼). Survival rate of mice was monitored for 24 h, and the statistical significance was determined by the Fisher's exact test (* p <0.05 versus LPS alone).

JPET #92056

Table 1. Effects of roflumilast on the plasma levels of NO_x, TNF- α and IL-1 β in the LPS-injected mice

Treatment group (mg/kg)	NO _x (μ M)	TNF- α (ng/ml)	IL-1 β (pg/ml)
Vehicle	5.84 \pm 0.66	1.22 \pm 0.45	161.0 \pm 98.5
LPS/GalN (20/700)	17.8 \pm 2.41	13.7 \pm 3.37	942.4 \pm 221.3
LPS + roflumilast (2)	11.3 \pm 4.65*	7.62 \pm 3.37	672.1 \pm 235.2
LPS + roflumilast (10)	8.81 \pm 3.24*	2.83 \pm 2.72*	401.9 \pm 178.9*

Different groups of mice (n=10) were treated with vehicle (n=10), LPS/GalN (20 mg/700mg/kg) (n=10), LPS/GalN (20 mg/700 mg/kg) plus roflumilast (2 or 10 mg/kg, 1hr before LPS treatment) (n=10). Six hours later, blood samples were prepared from mice. The concentrations of plasma NO_x, TNF- α and IL-1 β were determined as described under *Methods*. Data shown are means \pm SD. *p<0.05 versus LPS alone

JPET#92056

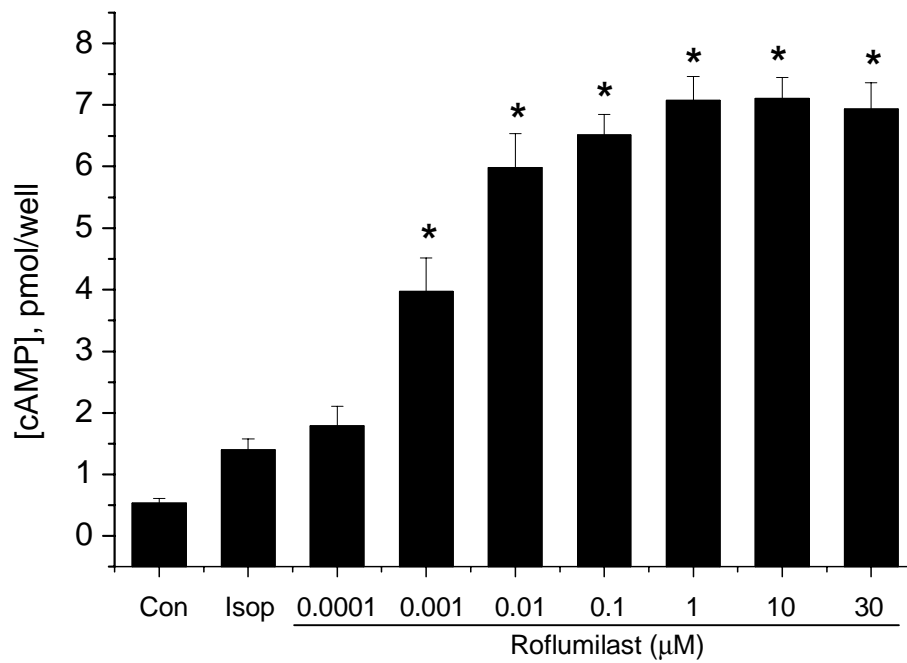


Fig. 1.

JPET#92056

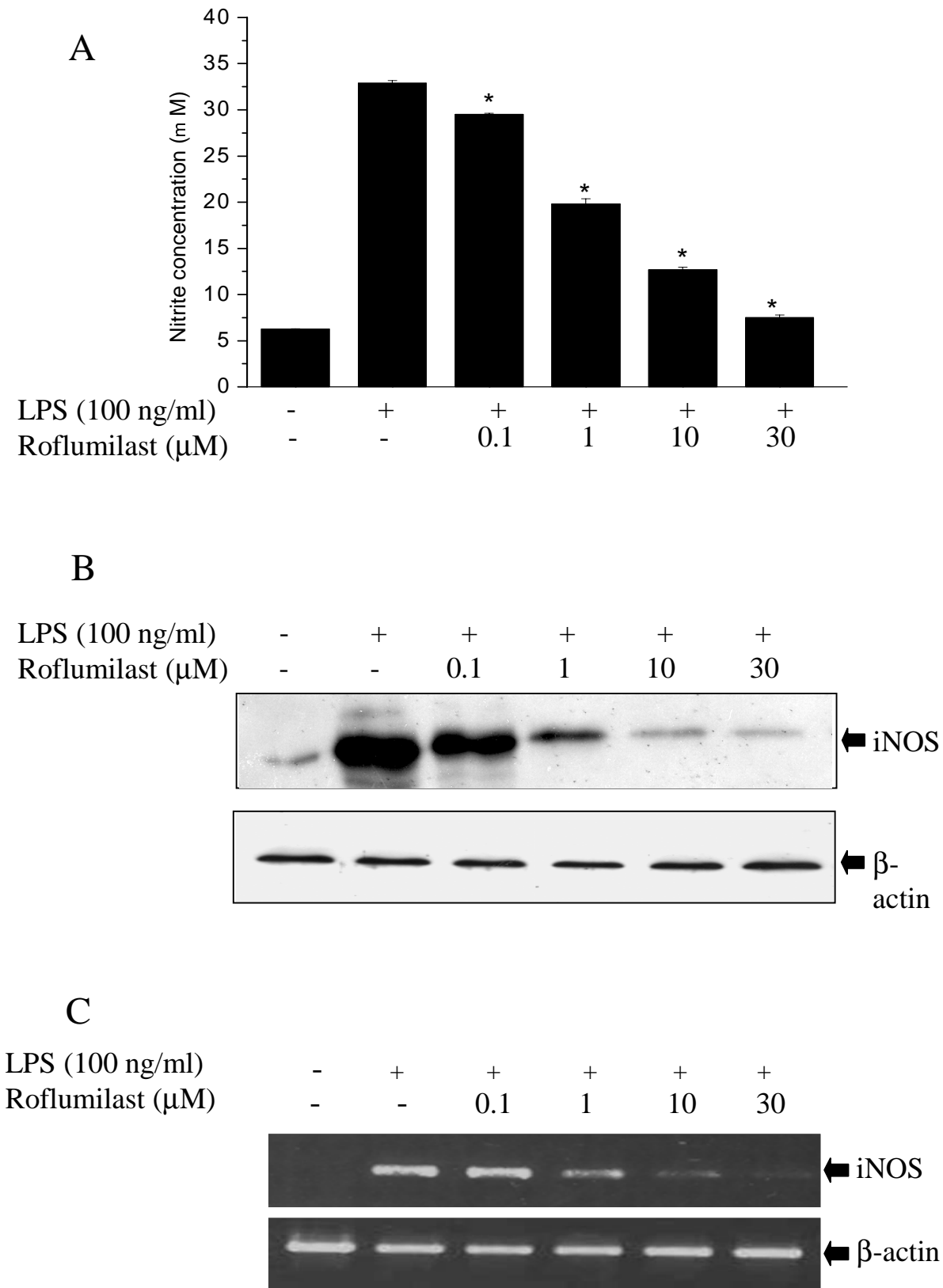
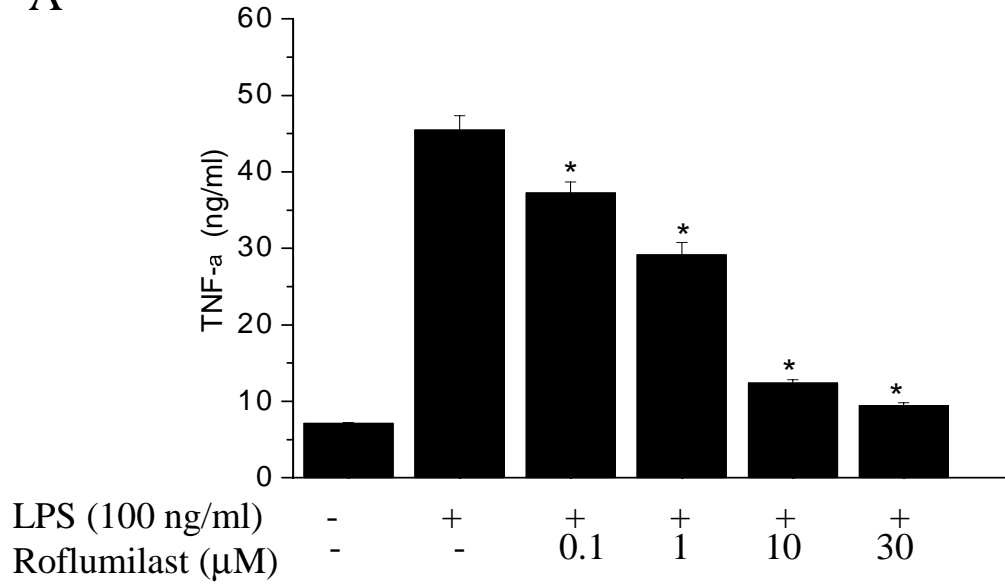


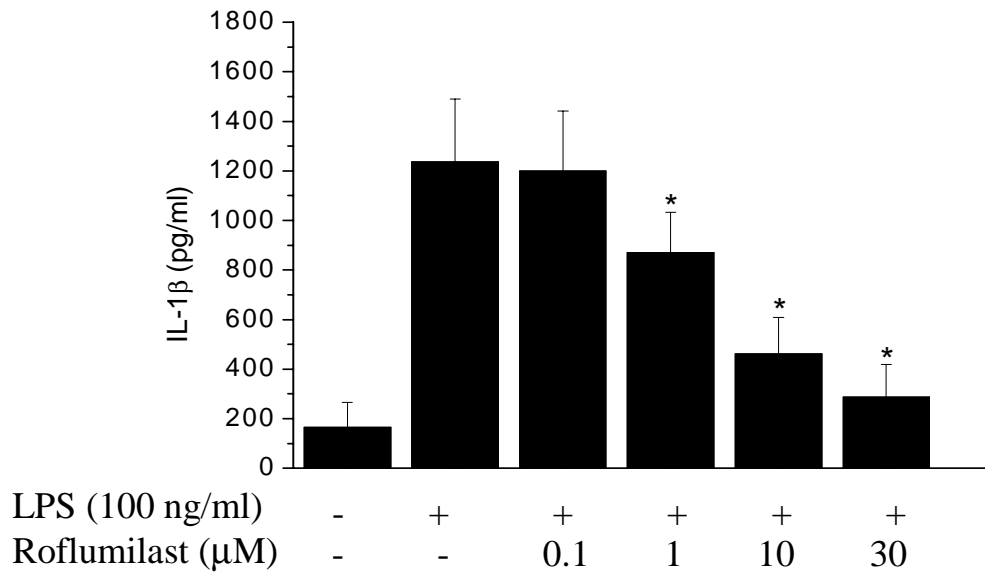
Fig. 2.

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A



B



C

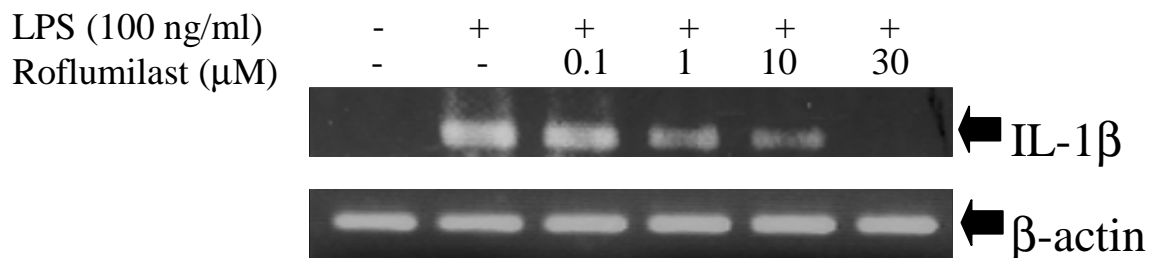
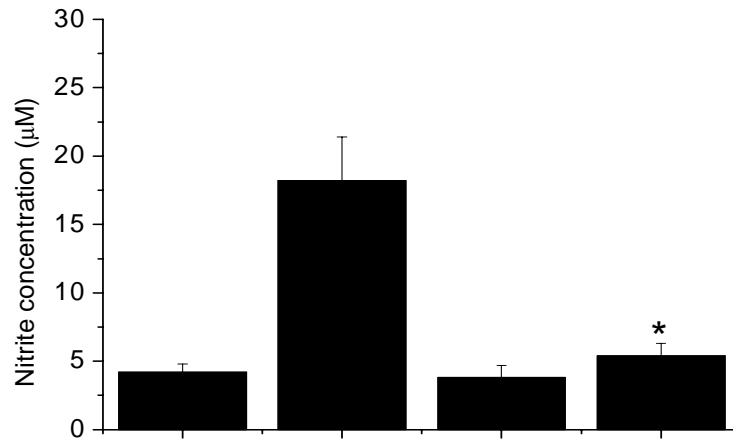


Fig. 3.

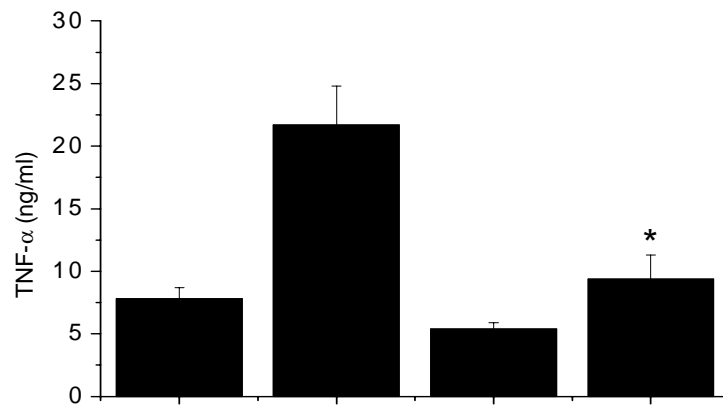
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A



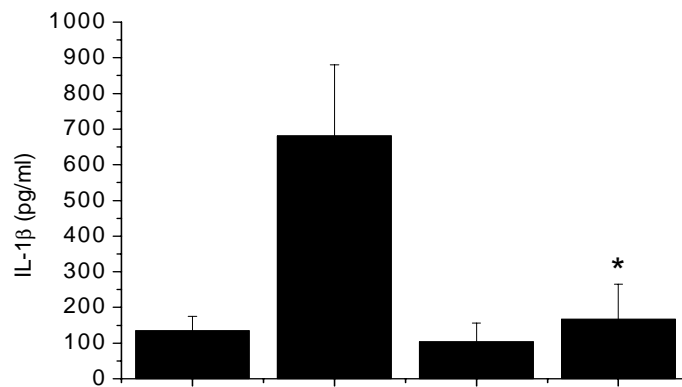
LPS (100 ng/ml)	-	+	-	+
Roflumilast (30 µM)	-	-	+	+

B



LPS (100 ng/ml)	-	+	-	+
Roflumilast (30 µM)	-	-	+	+

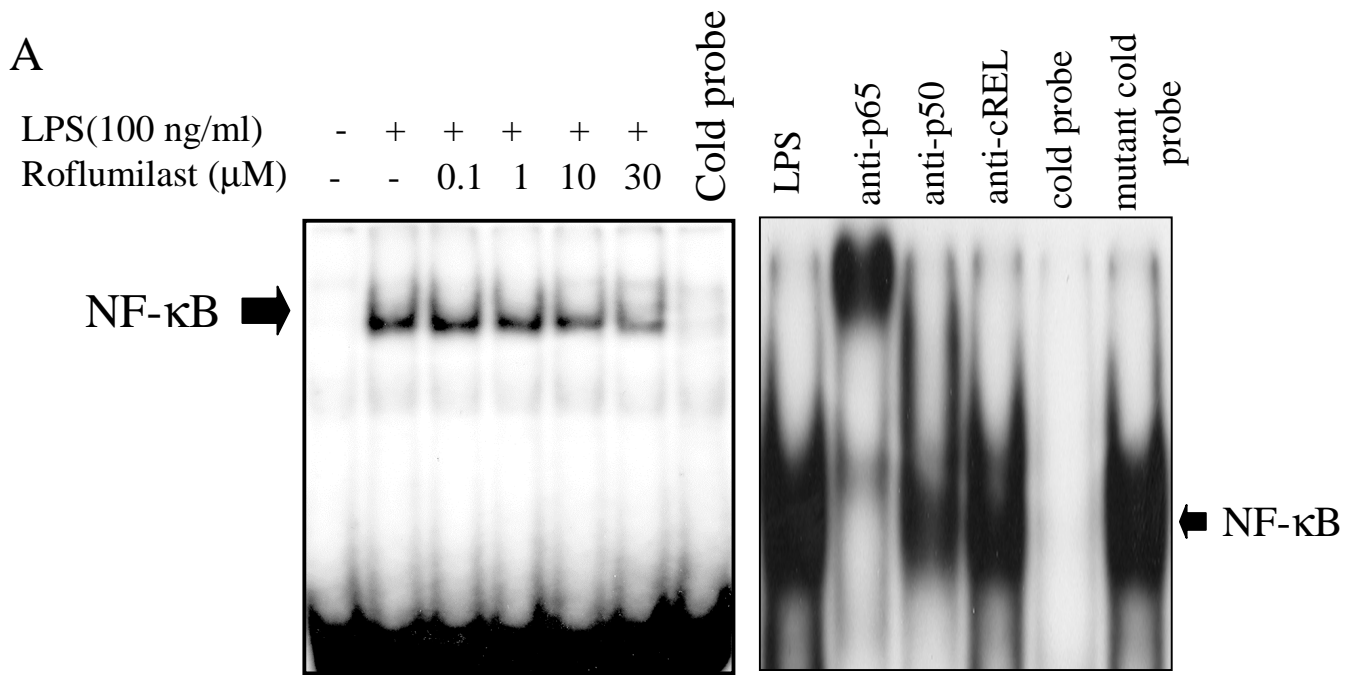
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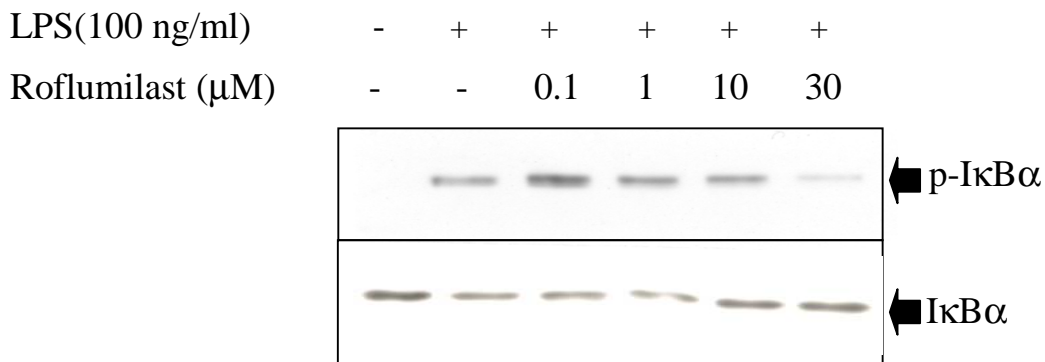
LPS (100 ng/ml)	-	+	-	+
Roflumilast (30 µM)	-	-	+	+

Fig. 4.

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B



C

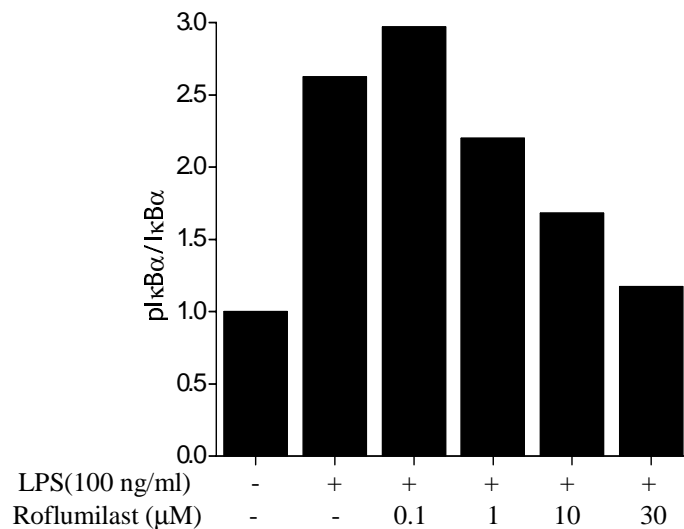


Fig. 5.

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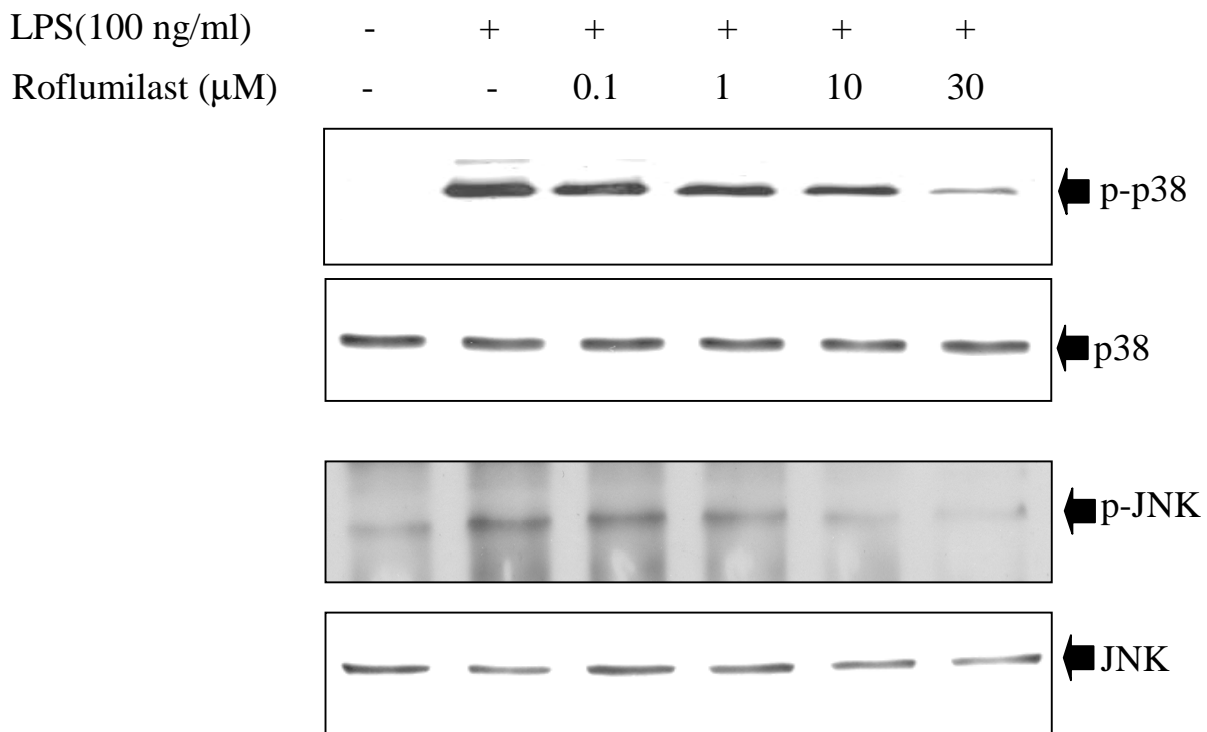


Fig. 6.

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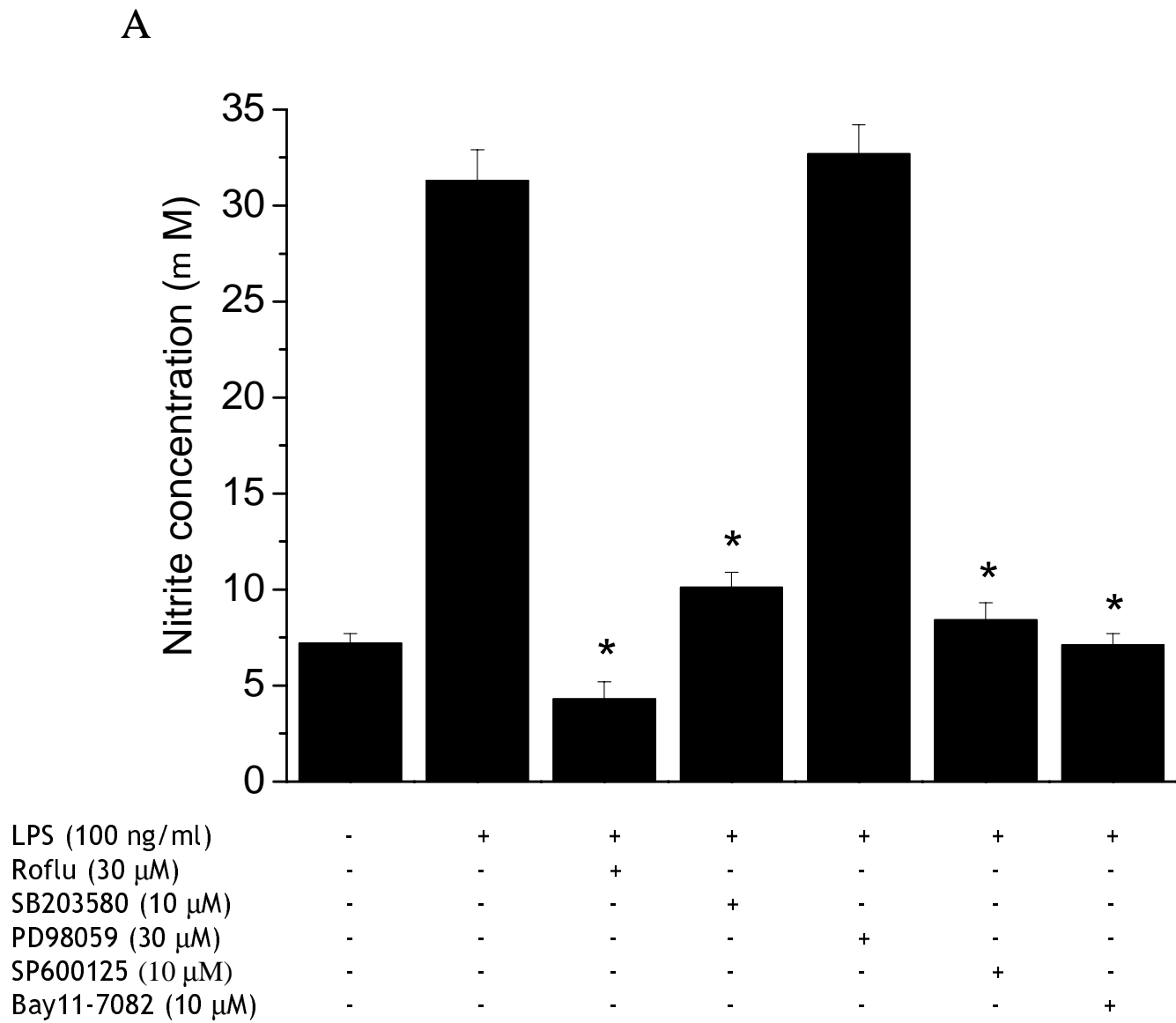


Fig. 7.

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B

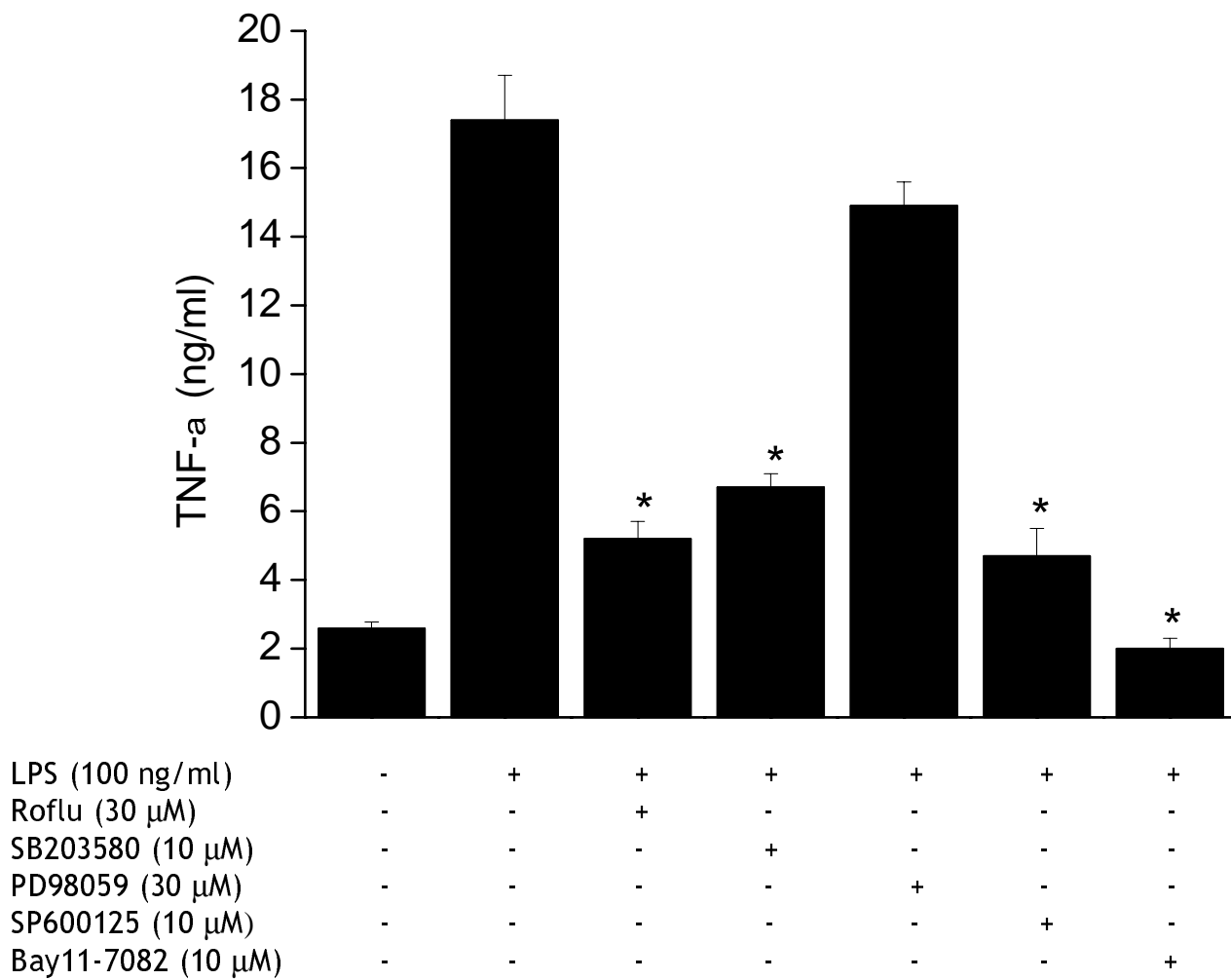
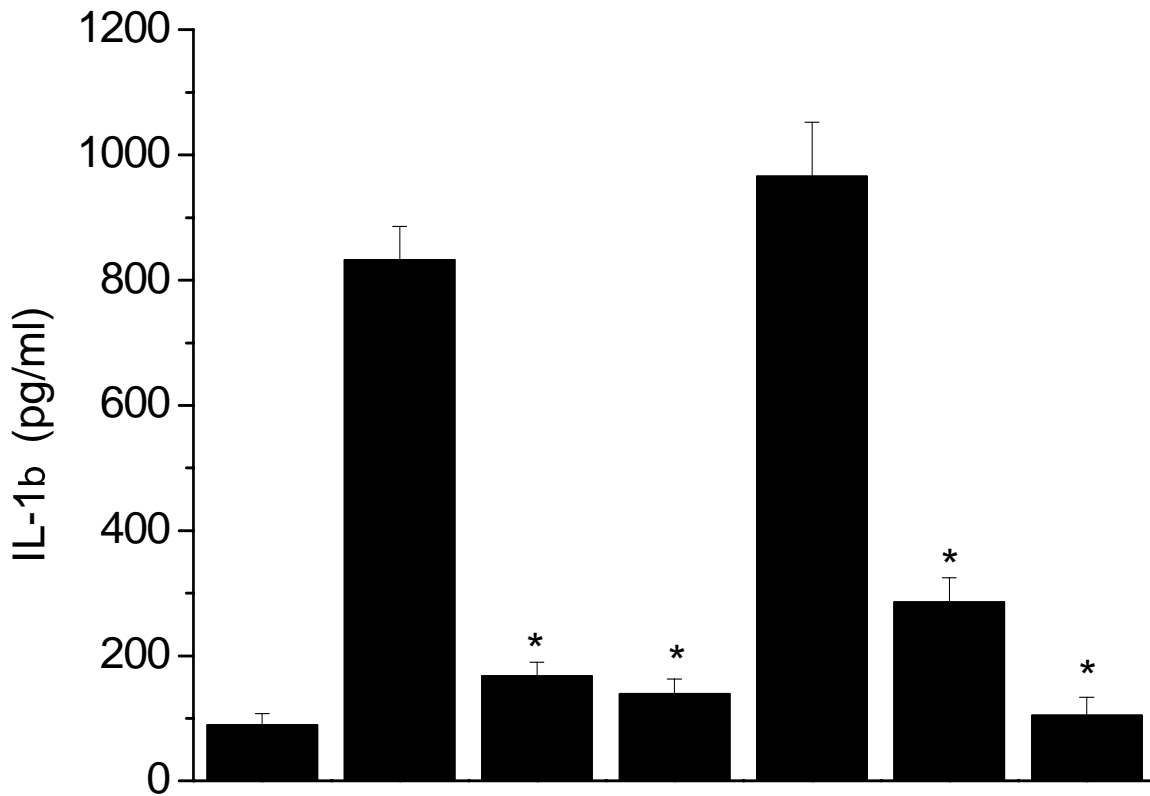


Fig. 7.

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C



LPS (100 ng/ml)	-	+	+	+	+	+	+
Roflu (30 μM)	-	-	+	-	-	-	-
SB203580 (10 μM)	-	-	-	+	-	-	-
PD98059 (30 μM)	-	-	-	-	+	-	-
SP600125 (10 μM)	-	-	-	-	-	+	-
Bay11-7082 (10 μM)	-	-	-	-	-	-	+

Fig. 7.

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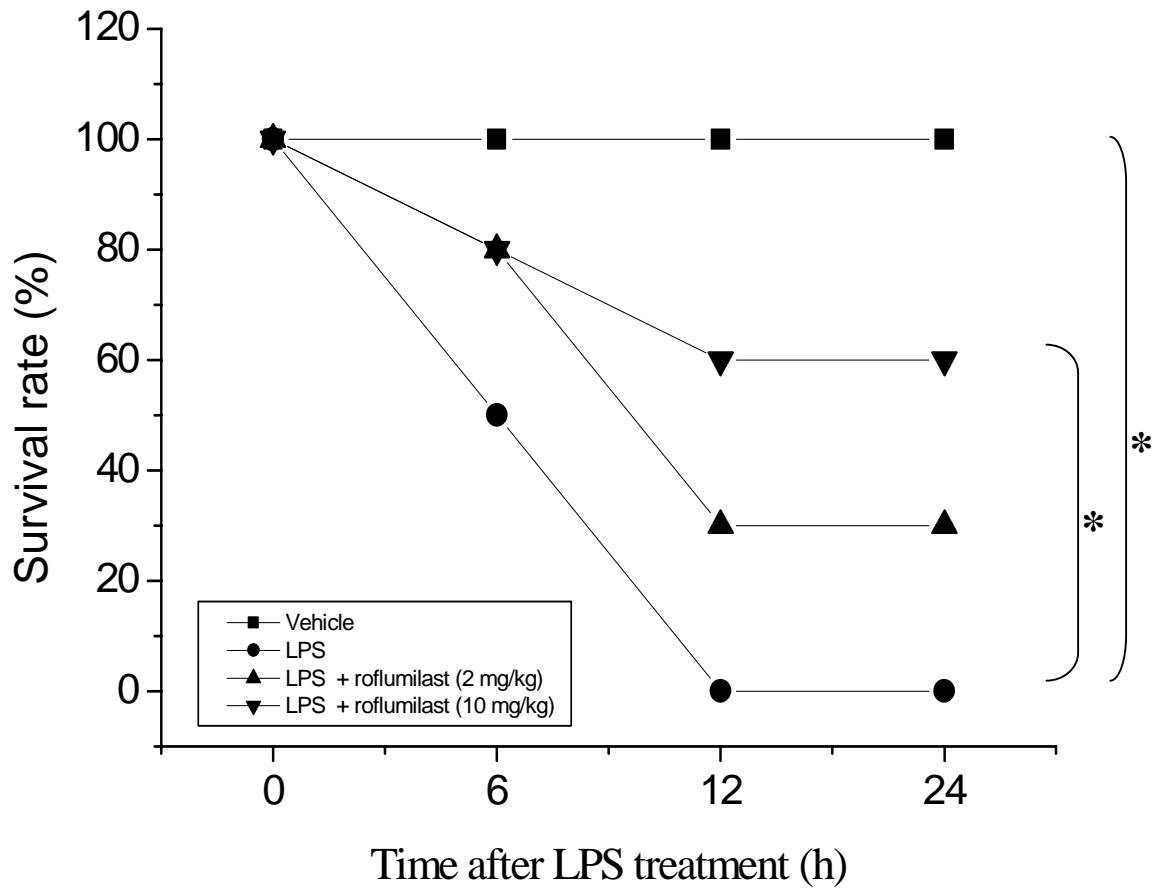


Fig. 8.