



Original Contribution

Protective role of benfotiamine, a fat-soluble vitamin B1 analogue, in lipopolysaccharide-induced cytotoxic signals in murine macrophages

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ABSTRACT

This study was designed to investigate the molecular mechanisms by which benfotiamine, a lipid-soluble analogue of vitamin B1, affects lipopolysaccharide (LPS)-induced inflammatory signals leading to cytotoxicity in the mouse macrophage cell line RAW264.7. Benfotiamine prevented LPS-induced apoptosis, expression of the Bcl-2 family of proapoptotic proteins, caspase-3 activation, and PARP cleavage and altered mitochondrial membrane potential and release of cytochrome c and apoptosis-inducing factor and phosphorylation and subsequent activation of p38-MAPK, stress-activated kinases (SAPK/JNK), protein kinase C, and cytoplasmic phospholipase A2 in RAW cells. Further, phosphorylation and degradation of inhibitory κB and consequent activation and nuclear translocation of the redox-sensitive transcription factor NF-κB were significantly prevented by benfotiamine. The LPS-induced increased expression of cytokines and chemokines and the inflammatory marker proteins iNOS and COX-2 and their metabolic products NO and PGE₂ was also blocked significantly. Thus, our results elucidate the molecular mechanism of the anti-inflammatory action of benfotiamine in LPS-induced inflammation in murine macrophages. Benfotiamine suppresses oxidative stress-induced NF-κB activation and prevents bacterial endotoxin-induced inflammation, indicating that vitamin B1 supplementation could be beneficial in the treatment of inflammatory diseases.

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Macrophages play a key role in inflammatory and immune reactions by releasing a variety of inflammatory markers such as cytokines, chemokines, growth factors, iNOS, and COX-2 [1]. Production of these inflammatory markers contributes to the efficient control of growth and dissemination of invading pathogens. However, the excessive levels of inflammatory markers produced by bacterial cell wall components such as LPS lead to amplified inflammatory responses and devastating illnesses characteristic of severe septic shock, which causes multiorgan failure and death [2,3]. The preceding observations suggest that the increased circulating levels of LPS lead to increased mitochondrial activity and formation of ROS resulting in disturbed redox homeostasis in macrophages that activates redox-sensitive transcription factors, such as NF-κB and AP-1 [4,5]. NF-κB and AP-1, in turn, translocate to the nucleus and transcribe various inflammatory genes causing tissue damage and dysfunction leading to apoptotic cell death of macrophages [4,6–9]. Therefore, regulation of

redox signaling during infections could be an opportunity to prevent the mortality associated with sepsis. Indeed, various antioxidants such as *N*-acetylcysteine, resveratrol, silymarin, and curcumin and vitamins such as C and E have been shown to resolve inflammation associated with bacterial endotoxins [10–19].

Vitamin B1 has a long history of use as an oral supplement without reported adverse effects. Because of its therapeutic action in some frequently observed clinical syndromes, thiamine hydrochloride has been advised and used for a long period of time [20–23]. There are no reports of adverse effects of oral thiamine, even at dosages of more than a hundred milligrams a day [22]. Benfotiamine, a unique derivative of thiamine, is the most potent of the allithiamines, a group of lipid-soluble forms of thiamine [24]. Chemically known as *S*-benzoylthiamine-*O*-monophosphate, it is found in trace amounts in roasted garlic and other herbs of the genus *Allium*. Compared to thiamine, benfotiamine has a unique open thiazole ring structure (Fig. 1), which allows it to pass directly through cell membranes, readily crossing the intestinal wall and being taken straight into the cell [24]. Compared to water-soluble thiamine salts, benfotiamine is absorbed better in the intestine, reaching maximum plasma levels of thiamine about 5 times higher; bioavailability reaches maximum at about 3.6 times as high as that of thiamine hydrochloride and other lipophilic thiamine derivatives [25]. Various studies have also suggested that benfotiamine prevents diabetic complications such as diabetic neuropathy and microangiopathy by

Abbreviations: AIF, apoptosis-inducing factor; AP-1, activator protein 1; COX-2, cyclooxygenase-2; cPLA2, cytoplasmic phospholipase A2; IκBα, inhibitory κBα; iNOS, inducible nitric oxidase; JNK/SAPK, c-Jun NH₂-terminal kinase/stress-activated protein kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; p38, mitogen-activated protein kinase p38; PARP, poly(ADP-ribose) polymerase; PGE₂, prostaglandin E2; PKC, protein kinase C; ROS, reactive oxygen species; SEAP, secretory alkaline phosphatase.

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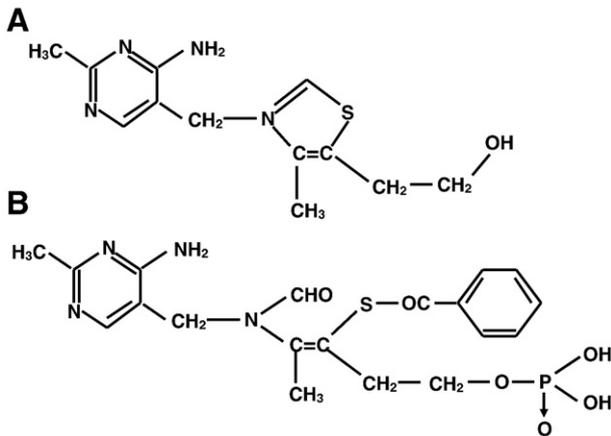


Fig. 1. Chemical structures of (A) thiamine and (B) benfotiamine. Benfotiamine contains an open thiazole ring that helps benfotiamine readily enter the cell through the plasma membrane, increasing its bioavailability. Once in the cytoplasm the ring closes and gives it a structure similar to that of thiamine.

blocking metabolic pathways such as the hexosamine pathway, the formation of advanced glycation end-products, and the diacylglycerol-protein kinase C (PKC) pathway [26–28]. Benfotiamine apparently removes glucose-derived glyceraldehyde 3-phosphate and fructose 6-phosphate through the activation of the pentose phosphate pathway

enzyme transketolase [26]. Furthermore, benfotiamine has been shown to block the activation of PKC and prevents NF- κ B activation, thereby preventing experimental diabetic retinopathy [26].

Although the relationship between vitamin B1 deficiency and bacterial infections has been noticed in animal and human studies, the mechanism by which benfotiamine supplementation prevents the inflammatory response and its involvement in inflammatory pathologies is not clear. Because vitamin B1 supplementation has been shown to be safe for human use, benfotiamine could be developed as a novel therapeutic approach for treatment of inflammatory complications. Indeed, our recent report indicated an anti-inflammatory role for benfotiamine in preventing inflammation associated with bacterial endotoxin-induced uveitis in rats [29], suggesting that benfotiamine could be anti-inflammatory. However, the molecular mechanism of the anti-inflammatory action of benfotiamine is not known. Therefore, this study investigates the effects of benfotiamine on the inflammatory signaling events induced in the murine macrophage RAW264.7 cell line by the bacterial endotoxin LPS. Our results indicate that pretreatment of macrophages with benfotiamine significantly prevents LPS-induced expression of inflammatory markers such as iNOS and COX-2, activation of PKC and NF- κ B, and expression of apoptotic proteins and decreases the mitochondrial membrane potential (MMP) and transcription of various inflammatory markers leading to apoptosis. These results suggest a possible therapeutic application of benfotiamine supplementation for the prevention of inflammatory complications.

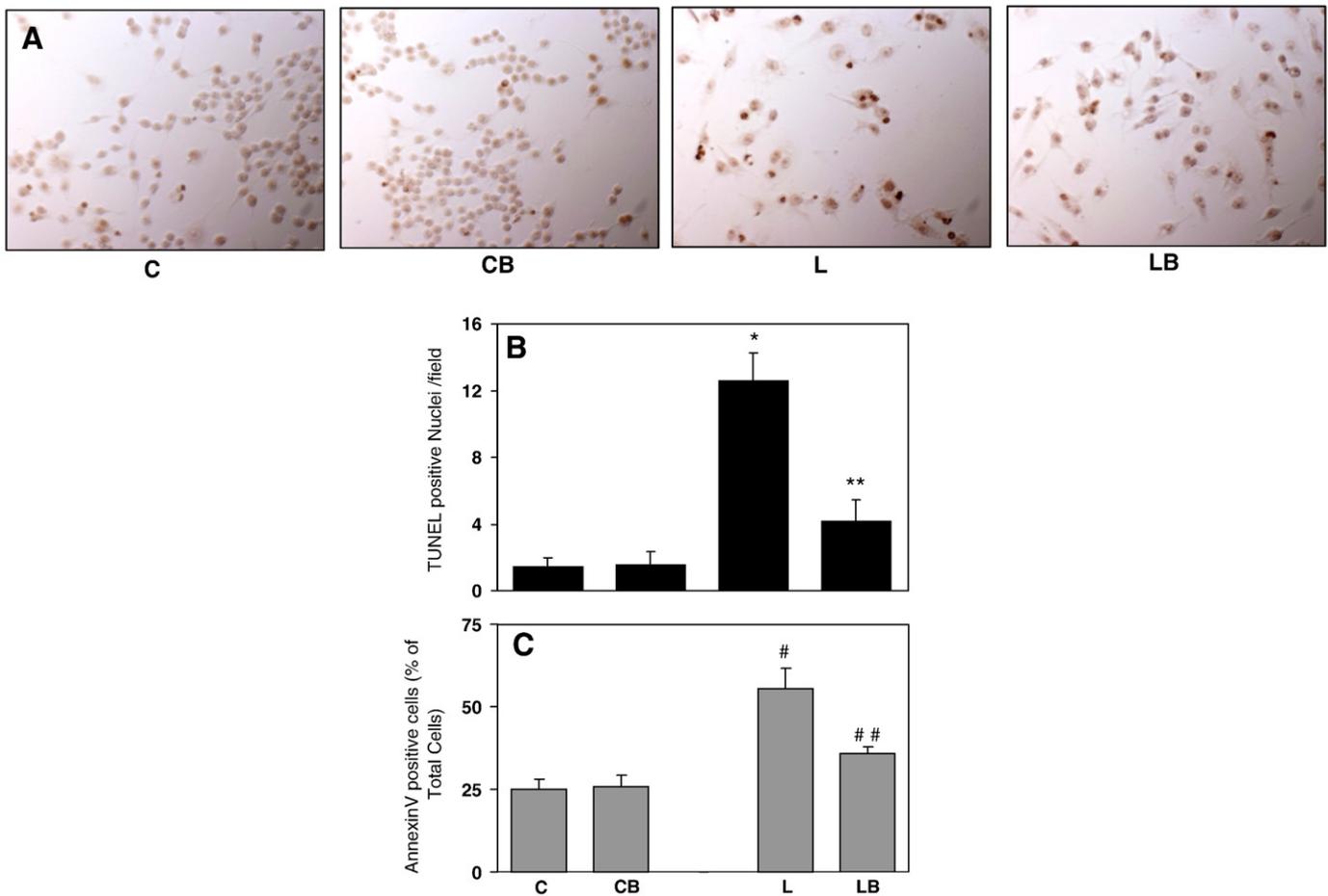


Fig. 2. Benfotiamine prevents LPS-induced apoptosis in RAW cells. (A) The RAW cells were treated with LPS (1 μ g/ml) for 24 h with or without benfotiamine, and TUNEL assay was performed. C, control; CB, control + benfotiamine; L, LPS; LB, LPS + benfotiamine. (B) The number of TUNEL-positive cells in a given field was counted to assess the apoptosis and is presented as a bar diagram; a total of six fields were counted. (C) The RAW cells were treated with LPS (1 μ g/ml) for 12 h with or without benfotiamine. Apoptotic cell death was examined using annexin V-FITC/PI and analyzed by flow cytometry. Twenty thousand events were acquired for each sample. Bars represent the percentage of total cells ($n=3$). * $P<0.0001$ vs control; ** $P<0.002$ vs LPS; # $P<0.002$ vs control; ## $P<0.006$ vs LPS.

Materials and methods

Materials and chemicals

LPS from *Escherichia coli* (Strain 0111:B4) and benfotiamine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), penicillin and streptomycin solution, trypsin/EDTA solution, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). The PGE₂ assay kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). The nitric oxide assay kit was from Oxis International (Beverly Hills, CA, USA). Antibodies against phospho-p65 (Ser536), phospho-p50 (Ser337), p65, p50, iNOS, COX-2, Bcl-2, Bax, Bak, Bad, Bcl-XL, β -actin, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against caspase-3, cytochrome c, PARP, and phospho-p38, -JNK/SAPK, -cPLA2 (Ser505), -I κ B α , -PKC β II (Ser660), -PKC β II (Thr641) -PKC δ (Ser643), and -PKC μ (Ser744/748) and against p38, JNK/SAPK, cPLA2, I κ B α , PKC β II, PKC δ , and PKC μ were from Cell Signaling Technologies (Danvers, MA, USA). Dihydroethidium (DHE) and the annexin V–FITC apoptosis detection kit were purchased from Calbiochem, EMD Chemicals (Gibbstown, NJ, USA). All other reagents used were of analytical grade.

Cell culture and LPS treatment

The murine macrophage cell line (RAW264.7) was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin and maintained at 37 °C in a humidified incubator containing 95% O₂ and 5% CO₂. The cells were pretreated with either 50 or 100 μ M benfotiamine or carrier overnight in serum-free medium and subsequently stimulated with 1 μ g/ml LPS from *E. coli* for 24 h, unless stated otherwise.

Annexin V staining and flow cytometry

Apoptotic cell death was examined using the annexin V–FITC/PI assay (Molecular Probes, Invitrogen) and analyzed by flow cytometry using LYSIS II software (FACScan; BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions as described earlier [30].

In situ detection of superoxide

The cells were rinsed with PBS and incubated with DHE (2.5 μ mol/L) at 37 °C for 15 min followed by stimulation with LPS for 1 h. Generation of superoxide in the cells was determined as described previously [30].

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Approximately 1×10^5 cells were seeded on chambered slides and starved in 0.1% serum medium with benfotiamine or carrier for 24 h. The cells were incubated with LPS (1 μ g/ml) for an additional 24 h, washed in ice-cold PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.2% Triton X-100 solution. TUNEL was performed using the DeadEnd colorimetric TUNEL system from Promega (Madison, WI, USA) essentially as described by the manufacturer. The number of TUNEL-positive cells in a given field was counted to assess the apoptosis. The images were obtained using a light microscope (EPI-800 microscope).

Measurement of MMP

The serum-starved RAW cells were washed with PBS and incubated with LPS without or with benfotiamine for 4 h. MMP was determined essentially as described by us earlier [31].

Western blot analysis

The cells were lysed in ice-cold RIPA lysis buffer containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1:100 dilutions of protease inhibitor and phosphatase inhibitor cocktails (Sigma–Aldrich) on ice. The cell lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C. In some experiments cytosolic and membrane extracts were prepared separately. The amount of protein in the lysates was determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed as described earlier [30].

Electrophoretic mobility shift assay (EMSA) for NF- κ B

The cells were pretreated with benfotiamine (100 μ M) for 24 h followed by LPS (1 μ g/ml) treatment for 1 h at 37 °C. The cytosolic and nuclear extracts were prepared as described by us earlier [30]. Consensus oligonucleotides for NF- κ B transcription factors were 5'-end labeled using T4 polynucleotide kinase, and EMSA was performed as described [30,32].

NF- κ B-dependent secretory alkaline phosphatase (SEAP) assay

The cells (2.5×10^5 cells/well in six-well plates) were transfected with the pNF- κ B-SEAP2 construct and pTAL-SEAP control plasmid (Clontech, Palo Alto, CA, USA) using Lipofectamine 2000 (Invitrogen) following the supplier's instructions. The cells were treated with benfotiamine for 24 h and then stimulated with LPS (1 μ g/ml) for 48 h. The SEAP assay was performed in the culture medium as described previously [28].

Determination of cytokines and chemokines

The levels of cytokines and chemokine in the cell culture medium were determined with a commercially available mouse-specific cytokine antibody array system according to the manufacturer's (Ray Biotech, Norcross, GA, USA) instructions. The Kodak Image Station was used for the densitometry analysis of the array.

Determination of NO and PGE₂

The level of nitric oxide in the culture medium was measured using a nitric oxide assay kit according to the manufacturer's (OXIS International) instructions. PGE₂ production was measured using an enzyme immunoassay kit following the manufacturer's (Cayman Chemical) instructions.

Statistical analysis

Data are expressed as the means \pm SD. All the data were analyzed using Student's *t* test and Microsoft Excel software. $P < 0.05$ was considered statistically significant.

Results

Effects of benfotiamine on LPS-induced macrophage cell death

Because LPS is well known to cause cell death in a variety of cells, including macrophages, the protective effect of benfotiamine on LPS-induced cell viability in macrophages was examined first. As shown in Figs. 2A and B, LPS caused cell death after 24 h of treatment, as assayed by the number of TUNEL-positive nuclei, and benfotiamine significantly prevented LPS-induced cell death. Benfotiamine alone did not alter the cell survival. Next, LPS-induced macrophage apoptosis was assayed using annexin V staining followed by FACS analysis. LPS stimulation caused more than 60% cells to undergo apoptosis as

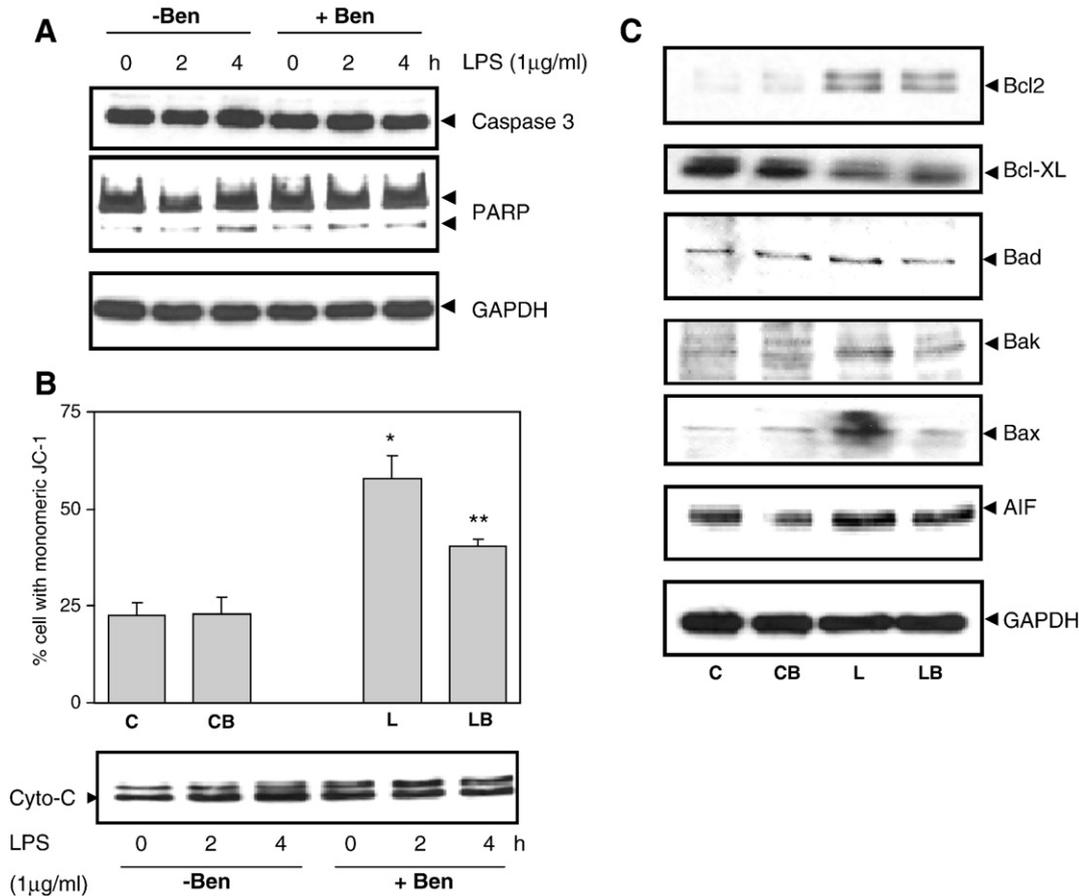


Fig. 3. Benfotiamine prevents LPS-induced activation of caspase-3, MMP, and Bcl-2 family proteins in RAW cells. (A) Growth-arrested RAW cells without or with benfotiamine were incubated with 1 µg/ml LPS for 24 h, and caspase-3 activation and PARP cleavage were determined in the cell lysate by Western blot analysis using specific antibodies. A representative blot is shown ($n=4$). (B) For assay of MMP, the growth-arrested RAW cells were treated with LPS (2.5 µg/ml) for 4 h with or without benfotiamine. The cells were harvested and washed with PBS, and MMP was evaluated by staining with JC-1 dye and analyzed with flow cytometry. Twenty thousand events were acquired for each sample. The data are means \pm SD ($n=3$). C, control; CB, control + benfotiamine; L, LPS; LB, LPS + benfotiamine. * $P<0.0007$ vs control; ** $P<0.006$ vs LPS. Cytochrome c release in the cytosol was measured after 2 and 4 h of LPS treatment by Western blot using specific antibodies. GAPDH was used as loading control. (C) The expression of Bcl-2 family proteins and AIF in the cell lysates from (A) was determined by Western blot analysis using specific antibodies. A representative blot is shown ($n=4$).

determined by annexin V-positive cells after 12 h incubation with LPS compared to approximately 25% in control. In the benfotiamine-treated group approximately 30% cells were annexin V positive after LPS challenge, which was a significant protection from LPS-induced apoptosis (Fig. 2C).

Effect of benfotiamine on LPS-induced activation of caspase-3 and mitochondrial membrane potential

To further examine the effects of benfotiamine on LPS-induced apoptosis, caspase-3 activation and PARP cleavage were determined. As shown in Fig. 3A, LPS caused increased caspase-3 in the cytosol after 4 h of treatment, which was prevented by benfotiamine. Further, PARP cleavage was also observed during the same time period, which was consistent with the caspase-3 increase in cytosol.

Because apoptosis involves changes in mitochondria that release various apoptotic intermediates, the effects of benfotiamine on the changes in MMP were investigated. As shown in Fig. 3B, LPS treatment caused an increase in the percentage of cells containing the monomeric form of the JC-1 dye, which indicates that in those cells there was a decrease in the electrochemical gradient across the mitochondrial membrane, an early event during apoptosis. In the cells treated with benfotiamine, there was significantly reduced percentage of cells with monomeric JC-1, suggesting that benfotiamine prevented the alteration in MMP in response to LPS. Because the mitochondria release cytochrome c during apoptosis as an early inter-

mediate, the level of cytochrome c in the cytosol was determined, and it increased significantly after 4 h of LPS treatment, and benfotiamine prevented the increase (Fig. 3B).

Effects of benfotiamine on the regulation of the LPS-induced Bcl-2 family of proteins

Because LPS-induced oxidative stress resulting in apoptosis is caused by altered expression of anti- and pro-apoptotic proteins, and benfotiamine prevented apoptosis in RAW cells, the effects of benfotiamine on the status of Bcl-2 family proteins under LPS challenge were examined. As shown in Fig. 3C, treatment with LPS decreased the expression of Bcl-XL protein while at the same time enhancing the expression of Bcl-2, Bax, Bad, and Bak. Benfotiamine significantly reversed the changes caused by LPS but did not affect the basal levels of these proteins when given alone. Similarly, increased expression of apoptosis-inducing factor (AIF) was also observed after 24 h incubation with LPS, which was significantly prevented by benfotiamine treatment.

Effects of benfotiamine on LPS-induced ROS in RAW264.7 cells

Because LPS-induced ROS are known to induce cell death [33], the level of ROS in RAW cells was examined by DHE staining. LPS treatment for 1 h caused a marked increase in ROS formation in RAW cells as observed by the increased red fluorescence associated with

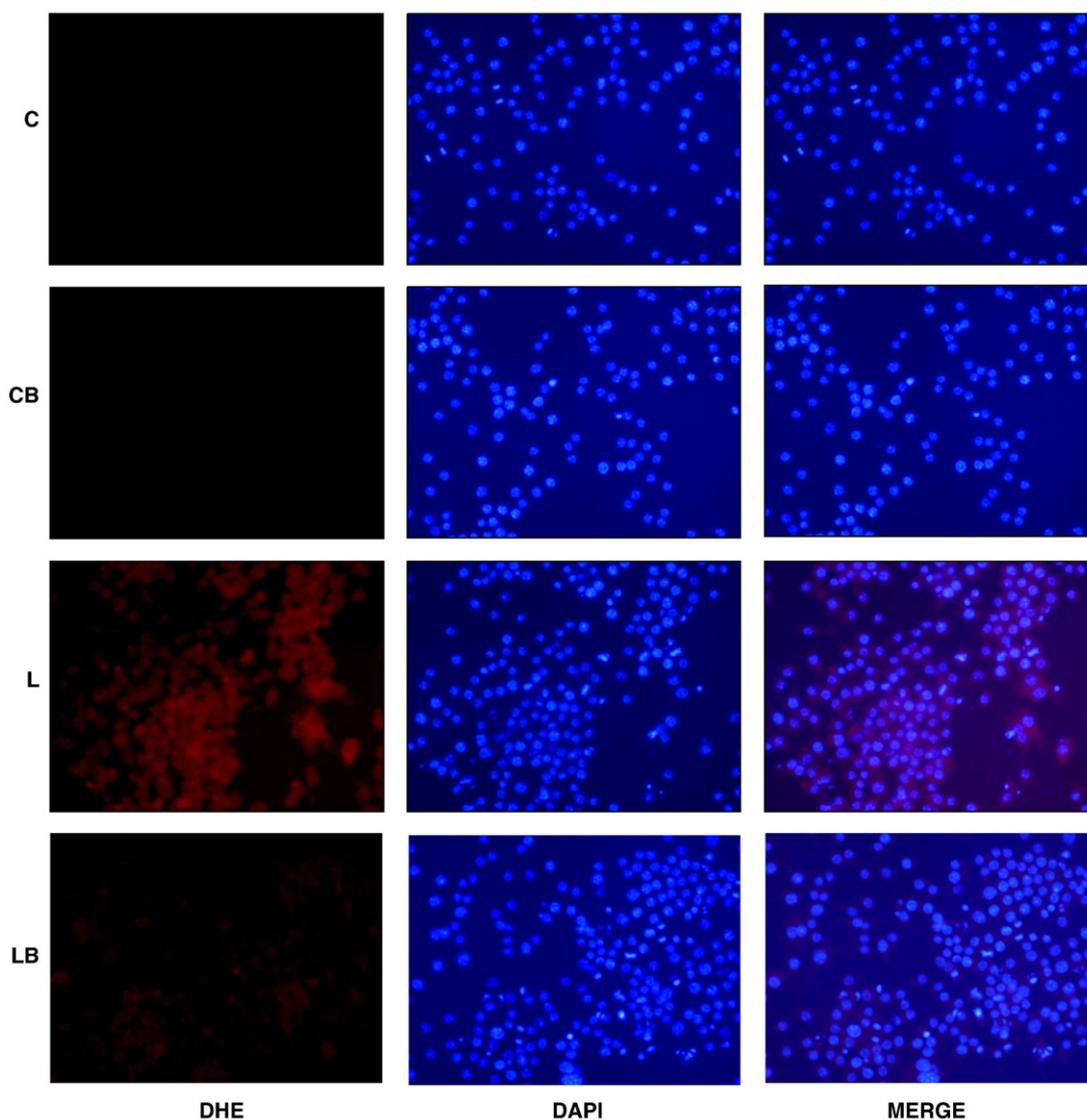


Fig. 4. Benfotiamine prevents LPS-induced ROS generation in RAW cells. RAW cells were growth-arrested by incubating in 0.1% FBS medium with benfotiamine (100 μ M) or carrier overnight followed by stimulation with LPS (1 μ g/ml) or carrier (PBS) for 16 h. The cells were washed with cold PBS and stained with the ROS-sensitive dye DHE (2.5 μ M) for 15 min at 37 $^{\circ}$ C. The cells were washed and mounted with Fluorsave mounting medium with DAPI. Photomicrographs were acquired using a fluorescence microscope (Nikon). A representative picture is shown ($n=3$); original magnification 200 \times . C, control; CB, control + benfotiamine; L, LPS; LB, LPS + benfotiamine.

DHE conversion in ethidium (Fig. 4). Preincubation of cells with benfotiamine prevented the increase in ROS levels in LPS-treated cells. These observations suggest that benfotiamine could quench the oxidative stress induced by ROS in cells.

Effects of benfotiamine on LPS-induced redox-sensitive transcription factors

LPS-induced activation of the redox-sensitive transcription factor NF- κ B is critical for the induction of cytokines, chemokines and other inflammatory markers such as iNOS and COX-2 [34,35], hence, the effects of benfotiamine treatment on LPS-induced phosphorylation, nuclear translocation, and DNA binding activity of NF- κ B in macrophages were studied. As shown in Fig. 5A, LPS caused increased phosphorylation and nuclear translocation of p65 as well as p50 subunits of NF- κ B, which was dose-dependently inhibited by benfotiamine. Next, DNA binding activity of NF- κ B was determined by EMSA, which showed approximately four-fold increased DNA binding in response to LPS, and benfotiamine inhibited it significantly (Fig. 5B). Further, to confirm the preventive role of benfotiamine on the NF- κ B

activation, a more sensitive NF- κ B-dependent SEAP reporter assay was used, which also showed that NF- κ B-dependent SEAP activity in response to LPS was significantly decreased by benfotiamine treatment (Fig. 5C). These results suggest that benfotiamine's protective role against oxidative stress-induced cell toxicity could be due to inhibition of transcription factor NF- κ B.

Effects of benfotiamine on LPS-induced NF- κ B upstream signals

Because NF- κ B activation by LPS is preceded by the phosphorylation and subsequent degradation of I κ B [35], further investigations were carried out to examine if benfotiamine treatment could inhibit the phosphorylation of I κ B. As shown in Fig. 6A, LPS stimulation induced phosphorylation and subsequent degradation of I κ B, which were prevented by benfotiamine. Corresponding to the phosphorylation of I κ B there was a reduced amount of unphosphorylated protein present in the cytosol after 30 min, suggesting degradation of phosphorylated protein, which partially recovered by 60 min. In benfotiamine-treated cells, reduced phosphorylation corresponded to increased unphosphorylated protein in the cytosol, which indicates

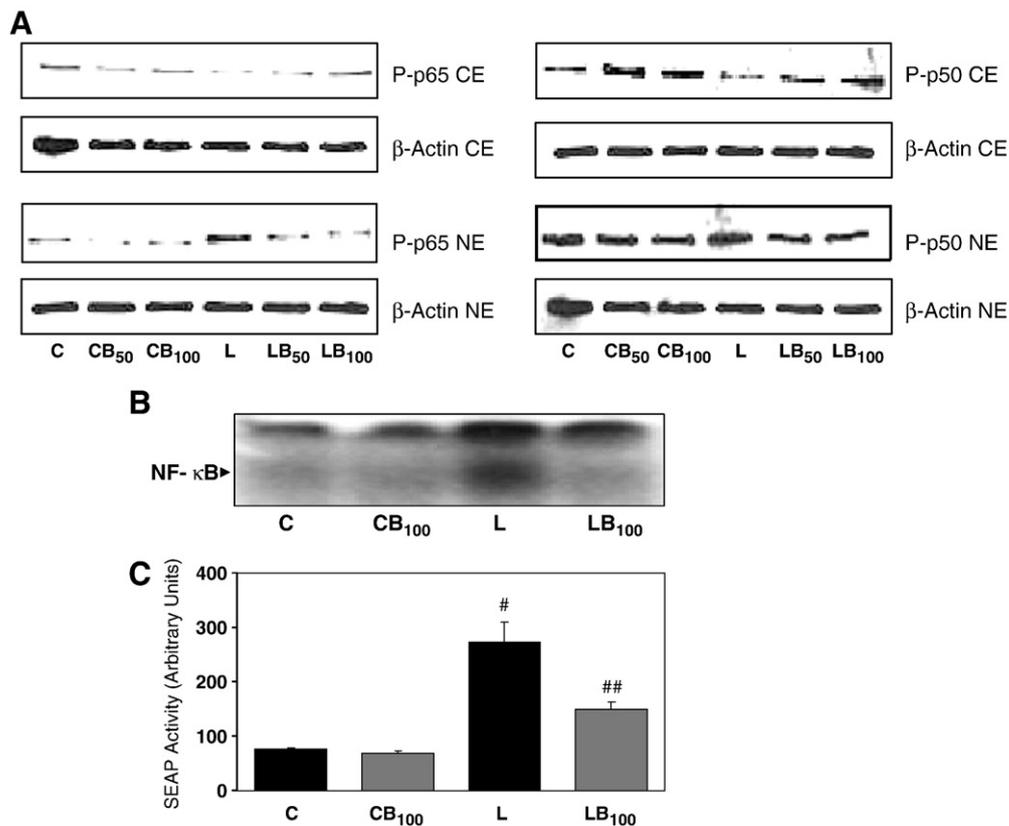


Fig. 5. Benfotiamine prevents LPS-induced activation of NF- κ B in RAW cells. The RAW cells were growth-arrested by incubating in 0.1% FBS medium with benfotiamine (50 and 100 μ M) or carrier overnight and stimulated with LPS (1 μ g/ml) for 1 h with or without benfotiamine. Nuclear extract was prepared and equal amounts of nuclear proteins were subjected to either (A) Western blot or (B) electrophoretic mobility gel-shift assays using specific NF- κ B antibodies or oligonucleotide probes, respectively. Representative blots are shown ($n = 3$). (C) The RAW cells were transiently transfected with the pNF- κ B-SEAP reporter vector. The cells were pretreated without or with benfotiamine (100 μ M) for 24 h and incubated with 1 μ g/ml LPS for an additional 24 h. The cell culture supernatants were assayed for SEAP activity using a chemiluminescence kit according to the supplier's instructions. Data represent means \pm SD ($n = 3$). [#] $P < 0.001$ vs control; ^{##} $P < 0.01$ vs LPS. C, control; CB₅₀, control + benfotiamine (50 μ M); CB₁₀₀, control + benfotiamine (100 μ M); L, LPS; LB₅₀, LPS + benfotiamine (50 μ M); LB₁₀₀, LPS + benfotiamine (100 μ M).

inhibition of degradation. These results indicate that inhibition of NF- κ B activation by benfotiamine could be via reduced phosphorylation and the resultant decrease in the degradation of I κ B.

Because p38-MAPK and JNK/SAPK have been shown to be involved in the LPS-induced release of inflammatory markers [36,37], further studies were performed to investigate whether benfotiamine would prevent the activation of these signaling kinases. As shown in Fig. 6B, LPS caused an increase in the phosphorylation of (Thr180 and Tyr182) p38-MAPK within 5 min, which returned to basal level by 90 min, whereas increased phosphorylation of JNK was observed within 15 min and returned to basal levels by 90 min. Treatment with benfotiamine significantly reduced the phosphorylation of p38-MAPK but had very little or no effect on the phosphorylation of JNK/SAPK. There was no change in the levels of unphosphorylated p38-MAPK and JNK/SAPK. Because MAPKs are known to activate ligand-dependent transcription factors, such as c-Fos and c-Jun, which constitute the transcription factor AP-1 [38], the effects of benfotiamine on the activation of c-Fos and c-Jun were investigated. As shown in Fig. 6C, LPS caused a time-dependent increase in phosphorylation of c-Fos and c-Jun proteins, which was inhibited by benfotiamine.

LPS-induced activation of the PKC β II subunit has been implicated in cytotoxicity and inflammation [26]. As they phosphorylate in response to LPS stimulus, PKCs translocate to the membrane, which indicates the activation of the kinase. As shown in Fig. 7A, when RAW cells were stimulated with LPS, there was an increased phosphorylation at the Thr641 and Ser660 residues of PKC β II within 5 min of LPS

stimulation and it increased time-dependently in the cytosolic fraction, whereas in the membrane fraction phosphorylated PKC was observed after 15 min of LPS stimulus and increased to a maximum at 30 min and decreased by 90 min after LPS stimulus (Fig. 7B). Also, total protein gradually decreased in the cytosol and correspondingly the protein level increased in the membrane fraction, which suggests membrane translocation of the phosphorylated PKC β II. In benfotiamine-treated cells, there was minimal increase in the phosphorylation at residue Thr641, whereas almost none at residue Ser660, and the total protein remained constant and did not change with time in the cytosol as well as in the membrane fraction in response to LPS (Figs. 7A and B). These results indicate that benfotiamine prevented the phosphorylation of PKC β II in response to LPS challenge in RAW cells. Similar results were also observed for the phosphorylation and membrane translocation of other PKC isozymes such as PKC α , PKC δ , and PKC μ in response to LPS challenge (data not shown).

Because cPLA2 has been shown to be induced by many factors and is involved in oxidant-induced cytotoxicity and is known to be phosphorylated by MAPK [39], activation of cPLA2 in RAW cells was determined by immunoblot analysis. There was phosphorylation of cPLA2 within 5 min of LPS stimulation and it increased with time in the cytosolic fraction (Fig. 8A), whereas in the membrane fraction phosphorylated cPLA2 was observed not before 15 min of LPS stimulus and decreased by 90 min after LPS stimulus (Fig. 8B). A gradual decrease in the cPLA2 protein level in cytosol and corresponding increase in the protein level in the membrane fraction were observed. In benfotiamine-treated cells, there was minimal increase in the

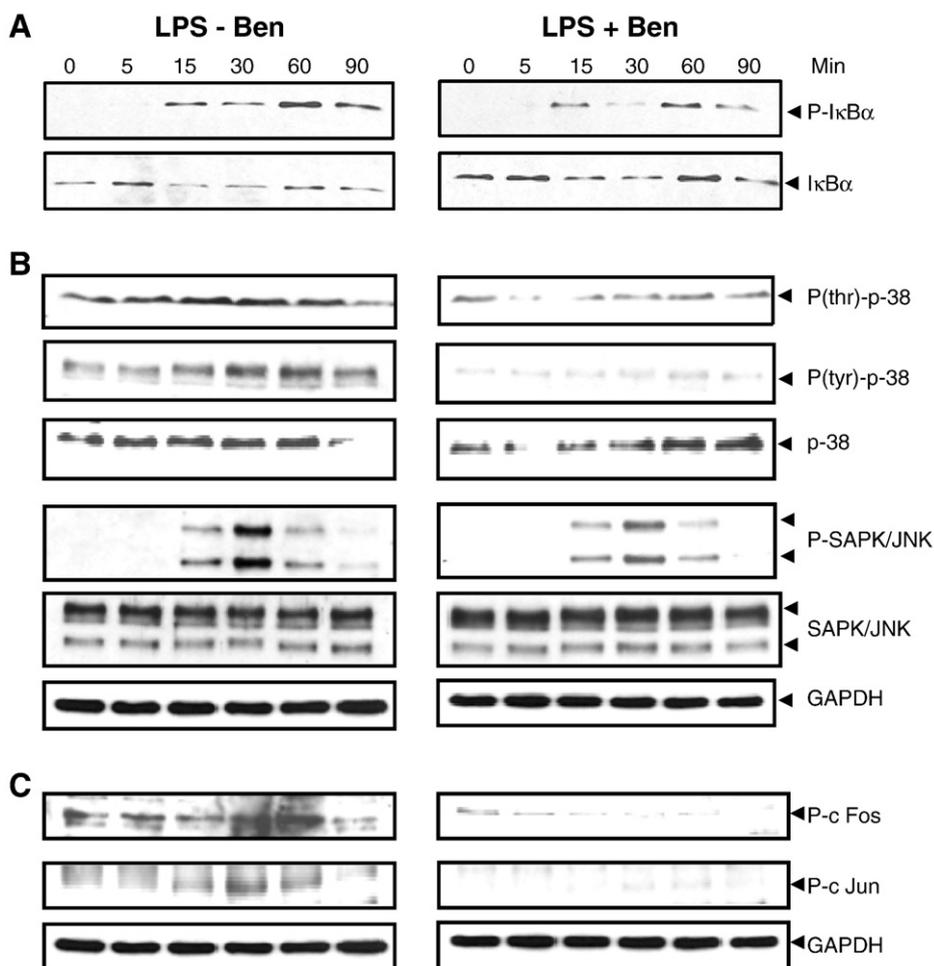


Fig. 6. Benfotiamine prevents LPS-induced activation of NF- κ B upstream signaling kinases in RAW cells. The RAW cells were growth-arrested by incubating in 0.1% FBS medium with benfotiamine (100 μ M) or carrier overnight and stimulated with LPS (1 μ g/ml) for various time intervals with or without benfotiamine. Cytosolic extract was prepared and equal amounts of cytosolic proteins were subjected to Western blot analysis using antibodies against (A) phospho-I κ B and unphosphorylated-I κ B antibodies; (B) phospho-p38 (phospho-Thr and phospho-Tyr), phospho-SAPK/JNK, unphosphorylated p38, and SAPK/JNK; and (C) phospho-c-Fos and phospho-c-Jun. GAPDH immunoblotting of the stripped membrane was done as loading control. Representative blots are shown ($n = 3$). Ben, benfotiamine.

phosphorylation of cPLA2 and the total protein remained constant in the cytosol and decreased with time in the membrane fraction in response to LPS. Because cPLA2 is involved in arachidonic acid metabolism, these data suggest that benfotiamine could modulate PGE₂ synthesis and COX-2 activation via cPLA2 in addition to a molecular signaling cascade.

Effects of benfotiamine on LPS-induced NF- κ B downstream signals

Because LPS is known to stimulate an inflammatory response in macrophages by inducing the synthesis of inflammatory mediators such as cytokines and chemokines, which cause cytotoxicity [38], next, the effect of benfotiamine on LPS-induced synthesis of various cytokines and chemokines in RAW cells was examined using a mouse-specific cytokine array system. LPS caused significantly increased synthesis of cytokines such as IL-1 α , IL-4, IL-5, IL-6, IL-12, TNF- α , and TNFRII and chemokines such as MCP5, LIX, P-selectin, C-TACK, MIP-3 α , CXCL-16, MIG, GM-CSF, GCSF, lymphotactin, and fractalkaline (Table 1). These inflammatory markers play important roles in macrophage activation during pathogen invasion and assist in recruitment, activation, and phagocytosis. The pretreatment with benfotiamine significantly prevented the release of these cytokines and chemokines (Table 1).

Further, macrophages are known to produce other inflammatory markers such as PGE₂ and NO when stimulated with LPS. Thus, the effects of benfotiamine on LPS-induced production of these inflammatory markers by RAW cells were determined. There was more than three-fold increased production of NO and approximately nine-fold increased synthesis of PGE₂ in LPS-stimulated cells, and benfotiamine treatment significantly ($P < 0.01$ and $P < 0.008$, respectively) prevented these changes (Fig. 9A). Because NO and PGE₂ are synthesized by inducible enzymes, iNOS and COX-2, respectively, in response to oxidative stress, the expression of these proteins was examined next. LPS treatment caused profound expression of iNOS and COX-2 in RAW cells compared to control cells, in which these proteins could barely be noticed by immunoblotting. Benfotiamine dose-dependently inhibited the expression of these proteins (Fig. 9B). These results suggest that benfotiamine could regulate the expression and synthesis of inflammatory markers, which could be responsible for its protective role against oxidative stress.

Discussion

This study for the first time shows that benfotiamine, a vitamin B1 analogue, prevents LPS-induced inflammatory signals leading to apoptotic cell death of murine macrophages. Alteration of the

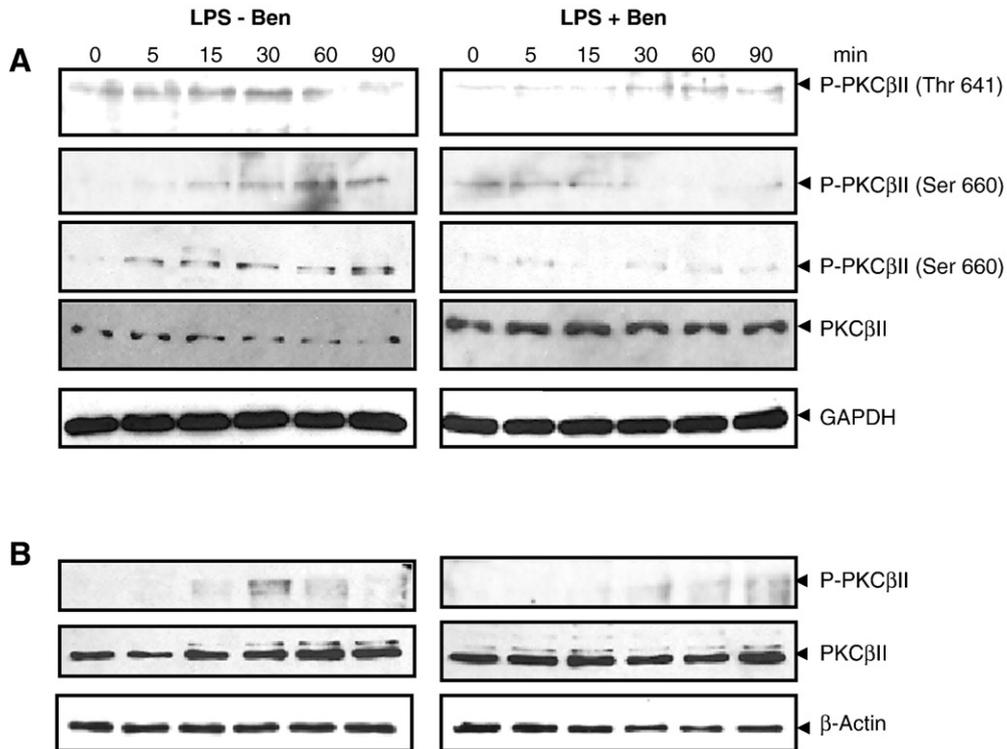


Fig. 7. Benfotiamine prevents LPS-induced activation of PKC in RAW cells. The RAW cells were growth-arrested by incubating in 0.1% FBS medium with benfotiamine (100 μ M) or carrier overnight and stimulated with LPS (1 μ g/ml) for 0–90 min. The cytosolic and membrane fractions were prepared and equal amounts of (A) cytosolic proteins (30 μ g) and (B) membrane proteins (10 μ g) were used for Western blot analysis using antibodies against phospho-PKC β II (phospho-Thr and phospho-Ser) and unphosphorylated PKC. The stripped membrane was probed with GAPDH and β -actin antibodies to depict equal loading. Representative blots are shown ($n=3$).

mitochondrial membrane potential and subsequent release of apoptotic intermediates, including cytochrome c, and activation of caspase-3 are early events during apoptosis [40]. Further, the release of inflammatory markers promotes oxidative stress, which results in programmed cell death mediated by the Bcl-2 family of proteins, including both proapoptotic proteins such as Bax, Bad, and Bak and antiapoptotic proteins such as Bcl-2 and Bcl-XL [41–43]. Inhibition of

inflammatory signals by benfotiamine could regulate apoptotic events such as activation of Bcl-2 family proteins and caspase-3, which can cause macrophage cell death.

LPS is well known to cause oxidative stress in immune cells by binding to its receptor, TLR-4, which consequently starts a myriad of signaling reactions regulated by phosphorylation and dephosphorylation of various kinases, which eventually lead to the activation of

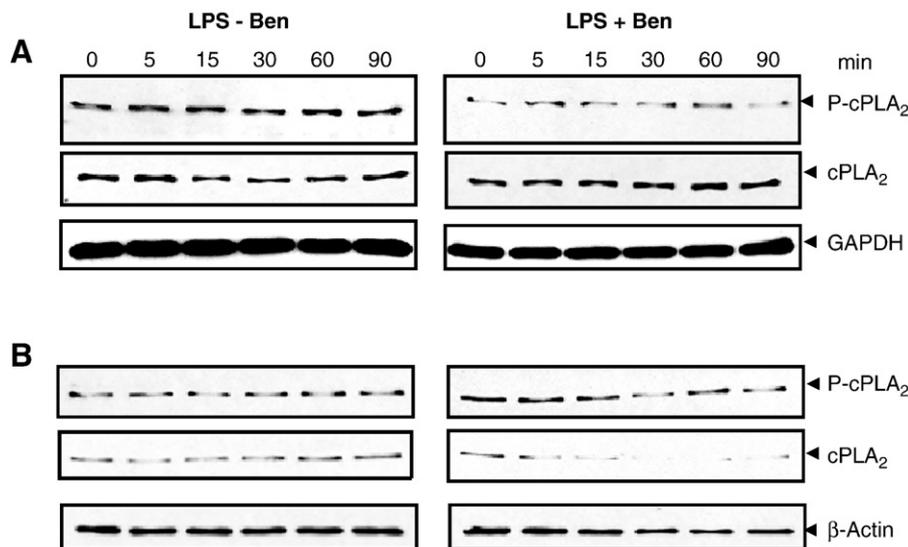


Fig. 8. Benfotiamine prevents LPS-induced activation of cPLA2 in RAW cells. The RAW cells were growth-arrested and pretreated with or without benfotiamine (100 μ M) in 0.1% serum medium for 24 h and stimulated with LPS (1 μ g/ml) for 0–90 min. Equal amounts of (A) cytosolic proteins (30 μ g) and (B) membrane proteins (10 μ g) were subjected to Western blot analysis using antibodies against phospho-cPLA2 (phospho-Thr and phospho-Ser) and unphosphorylated cPLA2. The stripped membrane was probed with GAPDH and β -actin antibodies to depict equal loading. Representative blots are shown ($n=3$).

Table 1
Benfotiamine prevents LPS-induced inflammatory cytokines and chemokines in RAW cell culture medium

Inflammatory marker	RAW cells (no treatment)	RAW cells + benfotiamine	RAW cells + LPS	RAW cells + LPS + benfotiamine
IL-1 α	2,240 \pm 730	990 \pm 200	7,140 \pm 670*	0 ^{††}
IL-4	4,920 \pm 970	4,900 \pm 520	14,540 \pm 620*	6,890 \pm 1,310 [†]
IL-5	850 \pm 210	890 \pm 160	18,610 \pm 520**	5,320 \pm 1,050 ^{††}
IL-6	0	0	158,220 \pm 4,720**	87,910 \pm 2,330 [†]
IL-12	4,540 \pm 1,220	3,500 \pm 710	8,920 \pm 2,870*	3,080 \pm 520 [†]
Lymphotactine	7,820 \pm 1,110	7,710 \pm 690	68,950 \pm 2,740**	31,200 \pm 3,680 [†]
Fractalkaline	0	0	4,410 \pm 1,290**	1,890 \pm 150 [†]
GCSF	2,260 \pm 400	0	94,800 \pm 7,130**	68,670 \pm 3,570 [†]
GM-CSF	0	0	20,580 \pm 1,610**	14,330 \pm 1,570 [†]
MIG	0	0	9,410 \pm 10**	3,780 \pm 20 [†]
TARC	2,100 \pm 70	0	13,100 \pm 6,760**	4,450 \pm 2,670 [†]
TIMP-1	670 \pm 0	0	12,350 \pm 2,990**	5,040 \pm 1,310 [†]
TNF- α	3,200 \pm 0	0	82,540 \pm 11,030**	44,870 \pm 2,490 [†]
α -TNFRII	20,510 \pm 3,180	22,620 \pm 1,000	91,670 \pm 11,540*	60,420 \pm 2,940 [†]
CXCL-16	61,870 \pm 5,280	62,960 \pm 320	76,600 \pm 3,660*	42,530 \pm 2,950 [†]
MCP5	15,430 \pm 1,240	14,500 \pm 720	50,280 \pm 11,450*	18,040 \pm 2,140 [†]
LIX	8,270 \pm 530	10,620 \pm 170	30,910 \pm 2,370**	19,390 \pm 960 [†]
P-selectin	18,580 \pm 1,490	19,010 \pm 260	37,170 \pm 450*	25,360 \pm 180 [†]
C-TACK	7,570 \pm 1,510	9,360 \pm 2,350	19,530 \pm 2,350*	7,910 \pm 1,680 [†]
MIP-3 α	0	0	7,500 \pm 510**	0 ^{††}

The RAW cells were growth-arrested and pretreated with or without benfotiamine (100 μ M) in 0.1% serum medium for 24 h and stimulated with LPS (1 μ g/ml). The culture medium was collected 24 h after LPS challenge and secreted cytokines and chemokines were determined by antibody array system as described under Materials and methods. The values represent arbitrary units of pixel density for cytokine-specific spots on the array determined by densitometry ($n = 4$). 0, not detected.

* $P < 0.01$ vs control.

** $P < 0.001$ vs control.

[†] $P < 0.01$ vs LPS.

^{††} $P < 0.001$ vs LPS.

redox-sensitive transcription factors such as NF- κ B and AP-1 [38,44,45]. The activation of NF- κ B results in the transcription of various inflammatory markers such as cytokines and chemokines and inflammatory enzymes including COX-2 and iNOS [35,46,47]. These inflammatory reactions are critical in the immune response against pathogens and foreign antigens, which are then phagocytosed and killed and removed from the body. However, excessive and persistent exposure to pathogens leads to a hyperresponse resulting in the release of an enormous amount of inflammatory markers, which cause cytotoxicity and tissue injury, requiring therapeutic intervention to contain the inflammation. Treatment with antibiotics generally kills the bacterial pathogen, but the bacterial toxins released in the body keep the inflammation recurring, causing damage to the tissues. In this scenario an anti-inflammatory and/or antioxidant therapeutic approach would block the inflammatory circuitry and give the body enough time and capacity to convalesce from the inflammation [48].

Many studies have suggested the use of various anti-inflammatory agents, which inhibit a particular kinase such as MAPK, PI3K, IKK, or PKC or transcription factor NF- κ B [49–54]. However, inhibition of one signaling kinase does not block the inflammation because an alternative signaling kinase would get activated to recompense for the inhibition of the previous kinase molecule. Therefore, a drug or agent that reduces the overall hyperresponsiveness against the pathogen and inhibits most of the inflammatory circuitry would be a better therapeutic alternative. This could be possible only by regulating the cellular metabolism in such a way that there is reduced ROS generation in response to an insult, which not only reduces the inflammation by overall inhibition of the ROS-dependent signaling cascade, as its effect would be upstream of ROS formation, but also provides an antioxidant milieu for the tissue to dispose-off ROS efficiently. Various antioxidants such as *N*-acetylcysteine, vitamin C, and vitamin E have been shown to inhibit ROS-induced inflammatory responses [55–59]. To the best of our knowledge this is one of the first reports that a lipophilic analogue of vitamin B1, benfotiamine, could prevent bacterial LPS-induced inflammatory signals and cytotoxicity in RAW cells. Previously, benfotiamine has been shown to prevent oxidative stress-induced complications through its antioxidant properties in vitro and in vivo in animal models. Nishikawa et al. have shown that

benfotiamine blocks three of the major molecular pathways leading to diabetic complications [60]. It prevents the hyperglycemia-induced increase in UDP-*N*-acetylglucosamine leading to enhanced hexosamine pathway activity and advanced glycation end-product formation [61,62]. Benfotiamine has also been shown to inhibit the hyperglycemia-induced NF- κ B pathway via PKC [26,62]. Because PKC activation has been shown to activate NADPH oxidase, which is responsible for generation of ROS, the ability of benfotiamine to inhibit PKC activation may contribute to its antioxidative effect [63,64]. Further, benfotiamine has also been shown to inhibit aldose reductase activity that regulates the glucose flux in the polyol pathway of glucose metabolism [65]. Through its antioxidant mechanism benfotiamine has been shown to prevent high-glucose-induced increase in DNA fragmentation and caspase-3 activity and consequently endothelial cell damage and apoptosis in endothelial cells and pericytes [66]. Further, benfotiamine has been shown to reduce the oxidative stress and genomic damage caused by the mutagenic model compound NQO, the uremic toxin indoxyl sulfate, and the peptide hormone angiotensin II in renal cells, indicating its direct antioxidant capacity [67]. During hyperglycemia benfotiamine has been shown to regulate the activity of the glycolytic enzyme transketolase and divert glucose utilization via the pentose phosphate pathway [20,59]. This could prevent the excessive ROS production due to glucose overutilization by mitochondria, which depends on glycolysis for reducing intermediates, NAD(P)H. Benfotiamine has been shown to be useful in treating diabetic complications such as diabetic neuropathy, microangiopathy, and nephropathy [27–29,60].

Consistent with previous studies that show benfotiamine inhibits high-glucose-induced activation of PKC and NF- κ B [27], our current observations indicate that benfotiamine also prevents the LPS-induced activation of p38-MAPK, PKC β II, PKC δ , PKC μ , and transcription factors NF- κ B and c-Fos and c-Jun. These signaling molecules are key components of the oxidative stress-induced inflammatory circuitry and potential therapeutic targets. Inhibition of these signaling molecules by benfotiamine could be responsible for the reduction in the LPS-induced inflammatory markers, such as cytokines including IL-1 α , IL-4, IL-5, IL-6, IL-12, TNF- α , and TNFRII and chemokines such as MCP-5, LIX, P-selectin, C-TACK, MIP-3 α , CXCL-16, MIG, GM-CSF,

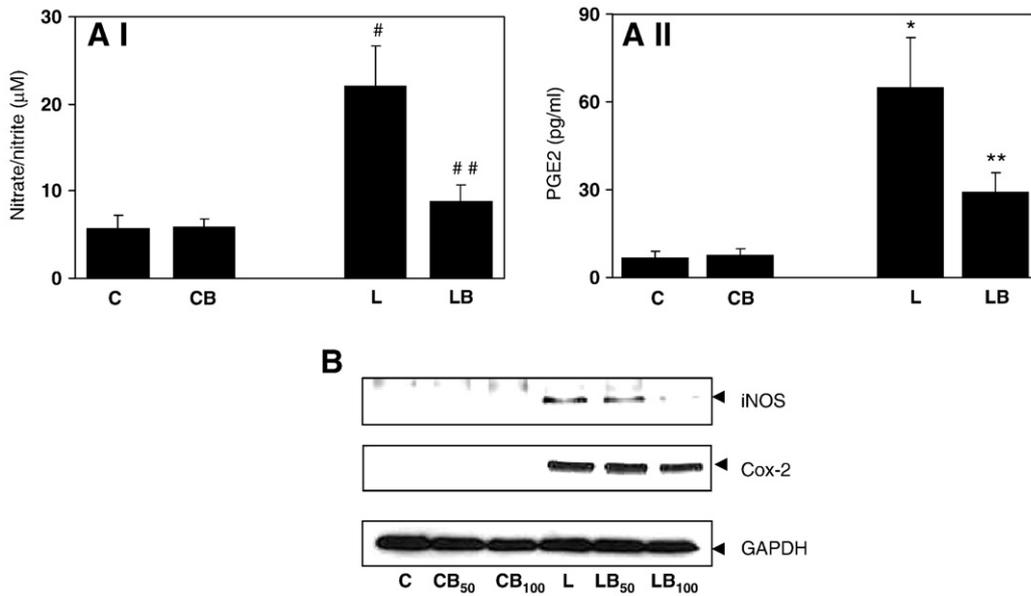


Fig. 9. Benfotiamine prevents LPS-induced NO and PGE₂ production in RAW cells. (A I and A II) NO and PGE₂ levels in the culture medium collected 24 h after LPS challenge were measured using ELISA kits as described under Materials and methods. Each value represents the mean ± SD (n = 4), [#]P < 0.005 vs control; ^{##}P < 0.01 vs LPS; ^{*}P < 0.0005 vs control; ^{**}P < 0.008 vs LPS. C, control; CB, control + benfotiamine; L, LPS; LB, LPS + benfotiamine. (B) Growth-arrested RAW cells without or with benfotiamine were incubated with 1 µg/ml LPS for 24 h. The expression of COX-2 and iNOS proteins was determined by Western blot analysis using specific antibodies. Representative blots are shown (n = 4). C, control; CB₅₀, control + benfotiamine (50 µM); CB₁₀₀, control + benfotiamine (100 µM); L, LPS; LB₅₀, LPS + benfotiamine (50 µM); LB₁₀₀, LPS + benfotiamine (100 µM).

GCSF, lymphotactine, and fractalkaline, as observed in RAW cells in this study. Also, activation of cPLA2 by LPS has been implicated in arachidonic acid metabolism, which regulates COX-2 activity and

PGE₂ synthesis [68,69]. Inhibition of COX-2 expression and reduced PGE₂ formation in benfotiamine-treated cells could be secondary to the inhibition of cPLA2 phosphorylation/activation, though

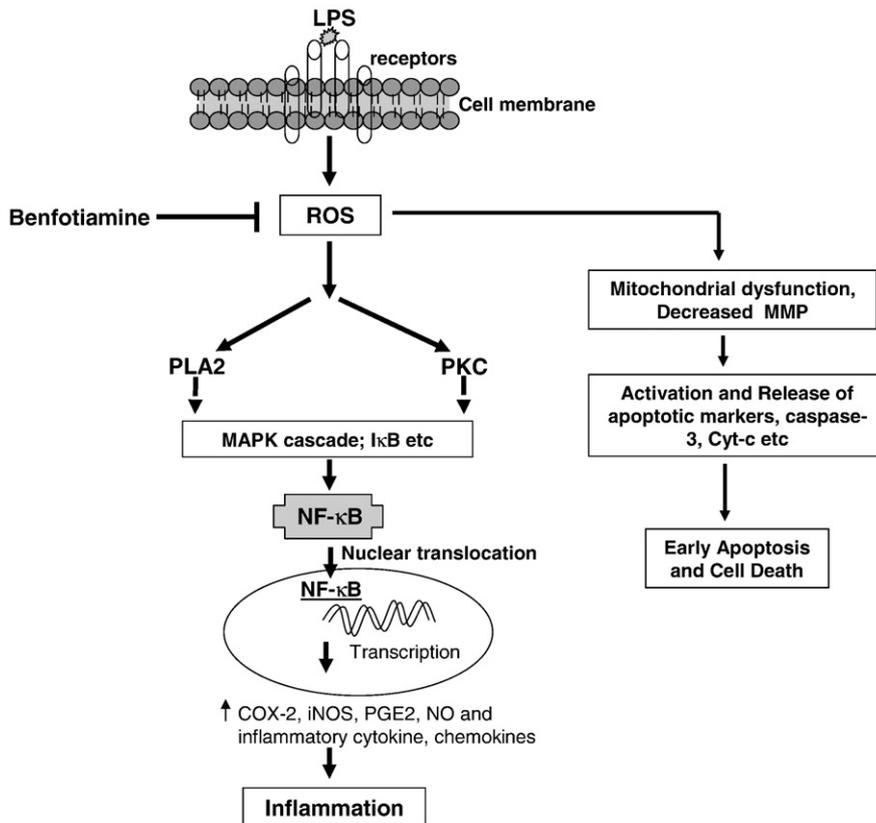


Fig. 10. The mechanism of benfotiamine-regulated inflammatory signals. Benfotiamine prevents ROS formation and thereby blocks the activation of subsequent adverse effects including mitochondrial dysfunction and activation of apoptotic markers that set an early apoptosis. Benfotiamine could also prevent the activation of protein kinases leading to activation of NF-κB, which transcribes inflammatory genes and causes inflammation.

transcriptional regulation by NF- κ B could be another route to regulate the COX-2 expression.

In conclusion, our results suggest that the fat-soluble form of vitamin B1, benfotiamine, could modulate the macrophage inflammatory response against bacterial toxin-induced inflammation by regulating signaling intermediates such as MAPK/PKC/I κ B, leading to the expression of NF- κ B-dependent inflammatory markers, which by autocrine and paracrine routes cause cytotoxicity (Fig. 10). Therefore, vitamin B1 supplementation could be beneficial in preventing inflammation-induced pathological conditions.

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