



## Original Contribution

## Anti-inflammatory effects of benfotiamine are mediated through the regulation of the arachidonic acid pathway in macrophages

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## ABSTRACT

Benfotiamine, a lipid-soluble analogue of vitamin B1, is a potent antioxidant that is used as a food supplement for the treatment of diabetic complications. Our recent study (U.C. Yadav et al., *Free Radic. Biol. Med.* 48:1423–1434, 2010) indicates a novel role for benfotiamine in the prevention of bacterial endotoxin, lipopolysaccharide (LPS)-induced cytotoxicity and inflammatory response in murine macrophages. Nevertheless, it remains unclear how benfotiamine mediates anti-inflammatory effects. In this study, we investigated the anti-inflammatory role of benfotiamine in regulating arachidonic acid (AA) pathway-generated inflammatory lipid mediators in RAW264.7 macrophages. Benfotiamine prevented the LPS-induced activation of cPLA2 and release of AA metabolites such as leukotrienes, prostaglandin E2, thromboxane 2 (TXB2), and prostacyclin (PGI2) in macrophages. Further, LPS-induced expression of AA-metabolizing enzymes such as COX-2, LOX-5, TXB synthase, and PGI2 synthase was significantly blocked by benfotiamine. Furthermore, benfotiamine prevented the LPS-induced phosphorylation of ERK1/2 and expression of transcription factors NF- $\kappa$ B and Egr-1. Benfotiamine also prevented the LPS-induced oxidative stress and protein-HNE adduct formation. Most importantly, compared to specific COX-2 and LOX-5 inhibitors, benfotiamine significantly prevented LPS-induced macrophage death and monocyte adhesion to endothelial cells. Thus, our studies indicate that the dual regulation of the COX and LOX pathways in AA metabolism could be a novel mechanism by which benfotiamine exhibits its potential anti-inflammatory response.

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Overwhelming inflammatory responses accompanied by deregulated immunological and coagulation functions result in multiple organ failure leading to septic shock, a major cause of mortality [1]. Although treatment with antibiotics kills bacteria and impedes the infection, the bacterial debris, which contains large amounts of endotoxins, could trigger the inflammatory responses [2]. Bacterial lipopolysaccharide (LPS)<sup>1</sup>, the structural component of the Gram-negative bacterial outer cell wall, is a potent initiator of the inflammatory response during most commonly seen bacterial infections [3]. Binding of LPS to its cognate CD14 receptor on the monocyte/macrophage cell membrane induces the release of various proinflammatory cytokines and chemokines [4–6], which are implicated in the pathogenesis of the major inflammatory complications such as sepsis [7]. Although a number of nonsteroidal

anti-inflammatory drugs and antioxidants have recently emerged as potential therapeutic targets for intervention of sepsis complications [8], their success rate in translational studies is low [9–11]. Therefore, identification of novel therapeutic compounds with both antioxidative and anti-inflammatory properties could yield potential therapeutic approaches to sepsis and other related inflammatory complications.

Thiamine (vitamin B1) has been used for decades for the treatment of several disorders such as neurological, diabetic, and cardiovascular complications [12–15]. Because thiamine is water soluble, it cannot be stored in the body for long and is rapidly excreted in the urine. The bioavailability of orally administered thiamine salt is comparatively low compared with fat-soluble analogues [16–18]. In 1954, a variety of lipid-soluble thiamine derivatives were discovered; subsequently they were named as allithiamines because they belong to the Allium family of vegetables such as crushed garlic, onions, leeks, etc. [19]. These lipid-soluble thiamine derivatives have much higher absorption and bioavailability than water-soluble thiamine salts [20,21]. Benfotiamine, one of the lipid-soluble derivatives of vitamin B1, was found to be the most effective antioxidant and was developed to improve bioavailability for pharmacological administration [22,23]. Benfotiamine contains an open thiazole ring, which allows it to pass through the cell membrane, and the open ring is closed by undergoing a reduction reaction making biologically active thiamine [21]. Oral administration of benfotiamine raises thiamine levels

**Abbreviations used:** AA, arachidonic acid; LPS, lipopolysaccharide; HNE, 4-hydroxy-2-nonenal; PGE2, prostaglandin E2; PGI2, prostacyclin; TXB, thromboxane; LTB, leukotriene; cPLA2, cytosolic phospholipase A2; COX-2, cyclooxygenase-2; LOX-5, lipoxygenase-5; ERK, extracellular signal-regulated kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; Egr-1, early growth response factor 1; ROS, reactive oxygen species; NSAID, Nonsteroidal anti-inflammatory drugs.

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in blood and tissues to a much higher degree than that of the water-soluble salts [20,21]. We have recently shown that benfotiamine prevents LPS-induced NF- $\kappa$ B-dependent expression of inflammatory cytokines and chemokines in macrophages. We have also shown that benfotiamine prevents LPS-induced generation of reactive oxygen species (ROS) and macrophage death in vitro [24]. Further, we have also demonstrated that benfotiamine prevents the LPS-induced ocular inflammatory complication, uveitis in rats [25]. Thus, our previous studies identified benfotiamine as a novel anti-inflammatory compound. However, the molecular mechanisms through which benfotiamine exerts its anti-inflammatory effect are not clear.

Recent studies indicate that LPS stimulation of macrophages leads to the activation of phospholipase A2 (cPLA2), which causes the release of AA from the cell membrane [26–28]. AA (20:4, *n*-6) is esterified in glycerol in the lipid bilayer of the cell membrane at the *sn*-2 position [26]. Once AA is released it is further metabolized via the COX-2 and LOX-5 pathways. The COX-2-catalyzed AA pathway leads to the generation of prostanoids such as prostaglandins, prostacyclins (PGI2), and thromboxanes (TXBs), which have been shown to be most potent inflammatory mediators. The LOX-5-catalyzed AA pathway results in the generation of leukotrienes (LTBs) involved in several inflammatory diseases such as asthma pathogenesis [29,30]. Even though the specific inhibitors of COX-2 and LOX-5 have been shown to be anti-inflammatory, they are not much effective, because they only stunt either the COX or the LOX pathway, moving AA metabolism towards the other pathway [31]. Therefore, to identify a possible mechanism by which benfotiamine acts as an anti-inflammatory agent, in this study, we have examined the efficacy of benfotiamine in regulating the AA pathway-generated inflammatory lipid mediators in RAW264.7 macrophages. Our results suggest that benfotiamine drastically inhibits the release of AA metabolites by inhibiting the activation of cPLA2. Benfotiamine also prevented the LPS-induced COX-2 and LOX-5 enzymes and generation of PGE2, TXB, and LTB4. Further, benfotiamine also prevented LPS-induced protein–HNE adduct and lipid hydroperoxides formation in macrophages. Thus, our results indicate that benfotiamine could be a novel anti-inflammatory agent that can ameliorate endotoxin-induced inflammation by preventing both COX and LOX pathways in AA metabolism.

## Materials and methods

### Materials

Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose, phosphate-buffered saline (PBS), penicillin/streptomycin solution, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen Gibco (Grand Island, NY, USA). [5,6,8,9,11,12,14,15- $^3$ H(N)]Arachidonic acid was purchased from NEN Life Science (Boston, MA, USA). RIPA cell lysis buffer, antibodies against LOX-5, PGI2 synthase, and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against cPLA2 were obtained from Cell Signaling (Beverly, MA, USA). Antibodies against thromboxane synthase and COX-2 were obtained from Abcam (Cambridge, MA, USA). Thromboxane B2 and leukotriene B4 EIA kits and REV 5901 were obtained from Cayman Chemical (Ann Arbor, MI, USA). PGE2 and PGI2 assay kits were obtained from Assay Designs (Ann Arbor, MI, USA). Benfotiamine, lipopolysaccharide (*Escherichia coli* O111:B4), indomethacin, and the reagents used in Western blot analysis were obtained from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

### Cell culture

RAW264.7 macrophages obtained from the American Type Culture Collection (Manassas, VA, USA) were grown in DMEM containing 10%

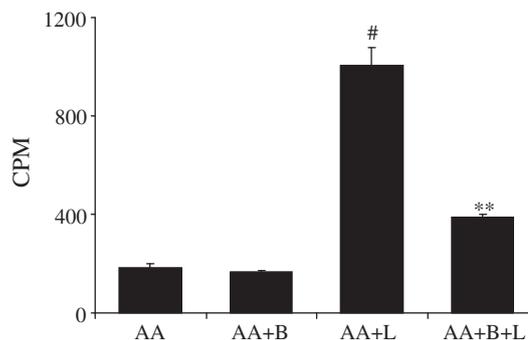
FBS, 1% penicillin/streptomycin, in a 95% air/5% CO<sub>2</sub>-humidified atmosphere at 37 °C. Macrophages were pretreated with benfotiamine at either 50 or 100  $\mu$ M or carrier overnight in serum-free medium and subsequently stimulated with 1  $\mu$ g/ml LPS for the indicated time periods.

### Release of arachidonic acid and its metabolites in RAW264.7 cell culture medium

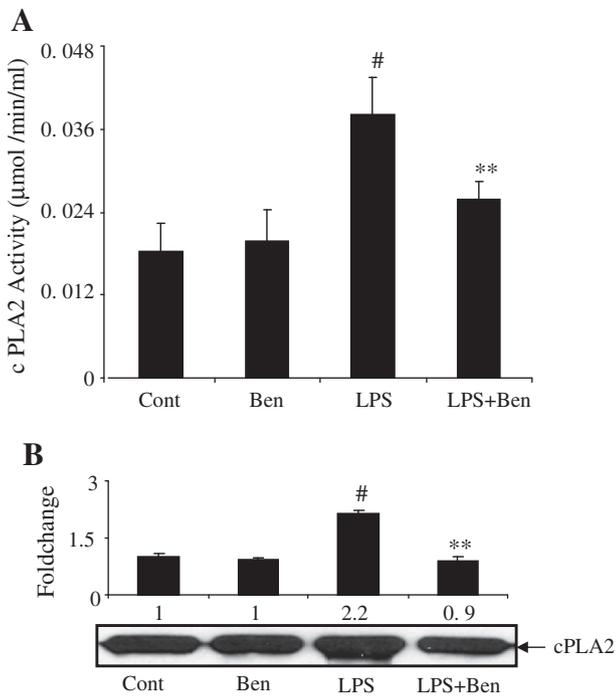
RAW264.7 cells were seeded in 12-well plates at the density of  $\sim 0.35 \times 10^6$  cells/well, without or with benfotiamine (100  $\mu$ M) in the complete growth medium. The medium was removed and replaced with 1 ml of serum-free DMEM containing 0.1  $\mu$ Ci/ml [ $^3$ H]arachidonic acid and incubated for 16 h. The cells were washed twice with PBS containing 0.1% bovine serum albumin to remove unadsorbed arachidonic acid and stimulated with 1  $\mu$ g/ml LPS for 1 h. Subsequently, the cells were incubated with fresh medium containing benfotiamine (100  $\mu$ M) for an additional 18 h. The culture medium was collected and centrifuged for 15 min at 10,000 rpm and supernatant was used to measure the radioactivity using a Beckman liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).

### Western blot analysis

The confluent macrophages were incubated without or with benfotiamine (50 or 100  $\mu$ M), followed by treatment with 1  $\mu$ g/ml LPS for 18 h. The cells were washed twice with PBS and lysed in an ice-cold RIPA lysis buffer. The crude lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C. Equal amounts of cell lysates (30–50  $\mu$ g) were separated on 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA). The membranes were incubated in blocking solution containing 5% w/v dried fat-free milk and 0.1% v/v Tween 20 in Tris-buffered saline. Subsequently, the membranes were incubated with specific antibodies against cPLA2, COX-2, LOX-5, TXB synthase, and PGI2 synthase. The blots were then washed and exposed to HRP-conjugated secondary antibodies (1:5000 dilution) for 1 h, and the antigen–antibody complex was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The membranes were stripped and probed with antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal protein loading. Fold changes in the band intensities were quantified by densitometry analysis using Kodak Image Station software.



**Fig. 1.** Benfotiamine prevents the release of arachidonic acid (AA) metabolites from LPS-stimulated RAW264.7 cells. The RAW264.7 cells were incubated with [ $^3$ H]AA (0.1  $\mu$ Ci/ml) as described under Materials and methods and stimulated with 1  $\mu$ g/ml LPS for 1 h. The cells were washed and incubated for 18 h in fresh medium containing benfotiamine (100  $\mu$ M). The release of metabolites into the medium was determined by measuring the radioactivity in the culture medium. Data represent means  $\pm$  SE ( $n=6$ ). # $P<0.0001$  compared with AA alone, \*\* $P<0.0001$  compared with AA + LPS-treated cells. L, LPS; B, benfotiamine.



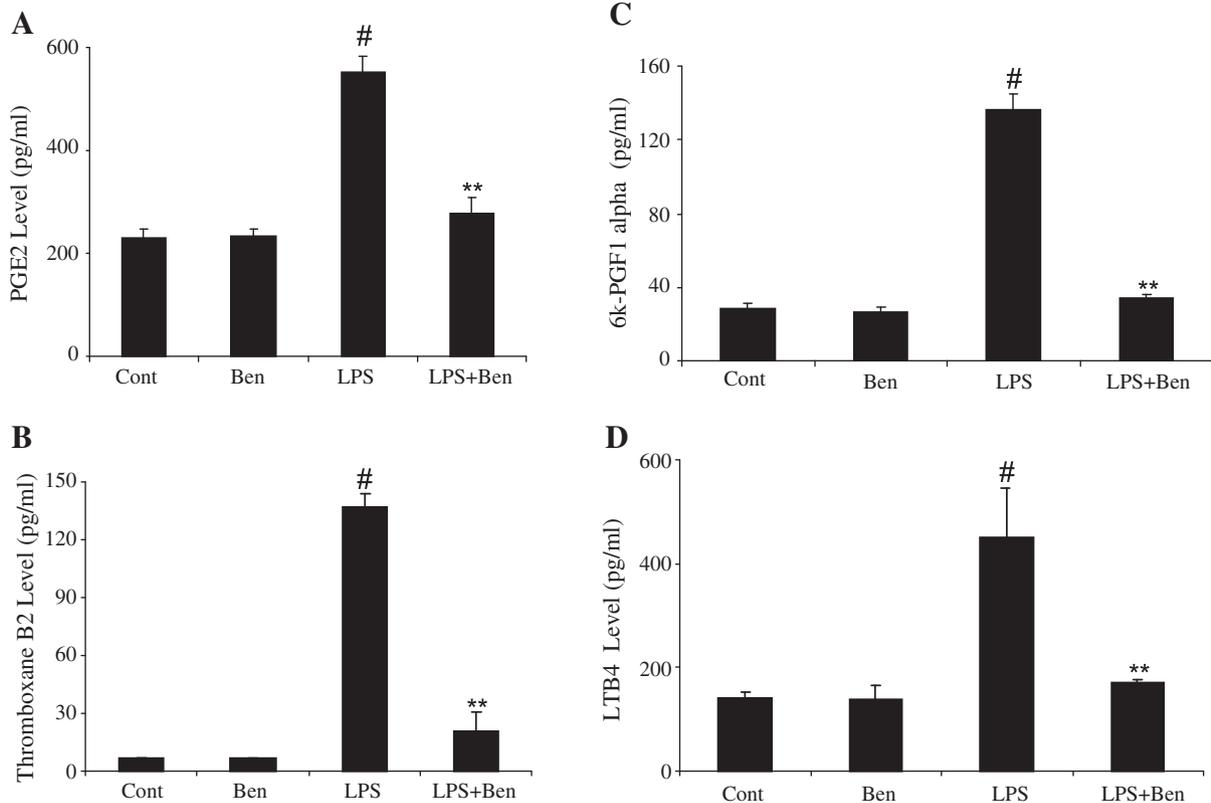
**Fig. 2.** Benfotiamine prevents LPS-induced generation of cPLA2 in RAW264.7 macrophages. The RAW cells were growth-arrested in medium containing 0.1% serum without or with benfotiamine (100 μM) and challenged with 1 μg/ml LPS. The cPLA2 (A) levels and (B) expression were determined in cell homogenates using the monoclonal enzyme immunoassay kit and Western blotting, respectively. Data represent means ± SE (n = 6). <sup>#</sup>P < 0.0001 vs control; <sup>\*\*</sup>P < 0.0001 vs LPS-treated cells. Cont, control; Ben, benfotiamine.

#### Determination of PGE2, 6k-PGF1α (PGI2), TXB2, LTB4, and cPLA2 levels

The RAW cells (~0.3 × 10<sup>6</sup> cells/well in six-well plates) were growth-arrested in serum-free medium without or with benfotiamine (100 μM) followed by incubation with 1 μg LPS/ml for another 18 h. The medium was collected from each well and cleared by centrifugation (5000 rpm; 5 min). The levels of PGE2 and PGI2 were determined using the respective assay kits according to the manufacturer's instructions (Assay Designs). For determining the levels of TXB2, LTB4, and cPLA2, specific ELISA kits were used according to the manufacturer's instructions (Cayman Chemical).

#### Reverse transcription-polymerase chain reaction (RT-PCR)

The RAW264.7 cells were preincubated without and with benfotiamine overnight followed by incubation with 1 μg/ml LPS for an additional 6 h. Total RNA was isolated using the RNeasy Mini Kit from Qiagen (Valencia, CA, USA). Equal amounts of RNA were used for RT-PCR using the Qiagen One-Step RT-PCR kit. The gene-specific primer (purchased from Sigma; Sigma Genosys, USA) sequences were COX-2, sense 5'-GGTTACAAAAGCTGGGAAGC-3', antisense 5'-GGGGTTCCGAGTTTATACT-3'; LOX-5, sense 5'-GGCACCAGACTACATCTAC-3', antisense 5'-CAATTTTGACGTCATCCC-3'; TXB synthase, sense 5'-ATCAGCCAAGCCTGTGAACT-3', antisense 5'-CCTCTCTCTGCTGCTGCT-3'; and GAPDH, sense 5'-TCCACTCACGGCAAATTCAACG-3', antisense 5'-TAGACTCCACGACATACTCAGC-3'. The PCR was carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min; and then 72 °C for 5 min for the final extension. PCR products were electrophoresed with 1% agarose-



**Fig. 3.** Effect of benfotiamine on LPS-induced production of PGE2, LTxB4, TXB2, and 6 k-PGF1α and in RAW264.7 macrophages. (A–D) The RAW cells were growth-arrested in Dulbecco's modified Eagle's medium containing 0.1% serum with or without benfotiamine (100 μM) overnight and challenged with 1 μg/ml LPS for 18 h. The levels of PGE2, LTxB4, TXB2, and 6 k-PGF1α were determined in the culture medium by using the monoclonal enzyme immune assay kits as per the manufacturer's instructions. Data represent means ± SE (n = 6). <sup>#</sup>P < 0.0001 vs control cells; <sup>\*\*</sup>P < 0.0001 vs LPS-treated cells. Cont, control; Ben, benfotiamine.

1× TAE gels containing 0.5 µg/ml ethidium bromide. The band densities were analyzed using a Kodak image station.

*Determination of lipid hydroperoxides*

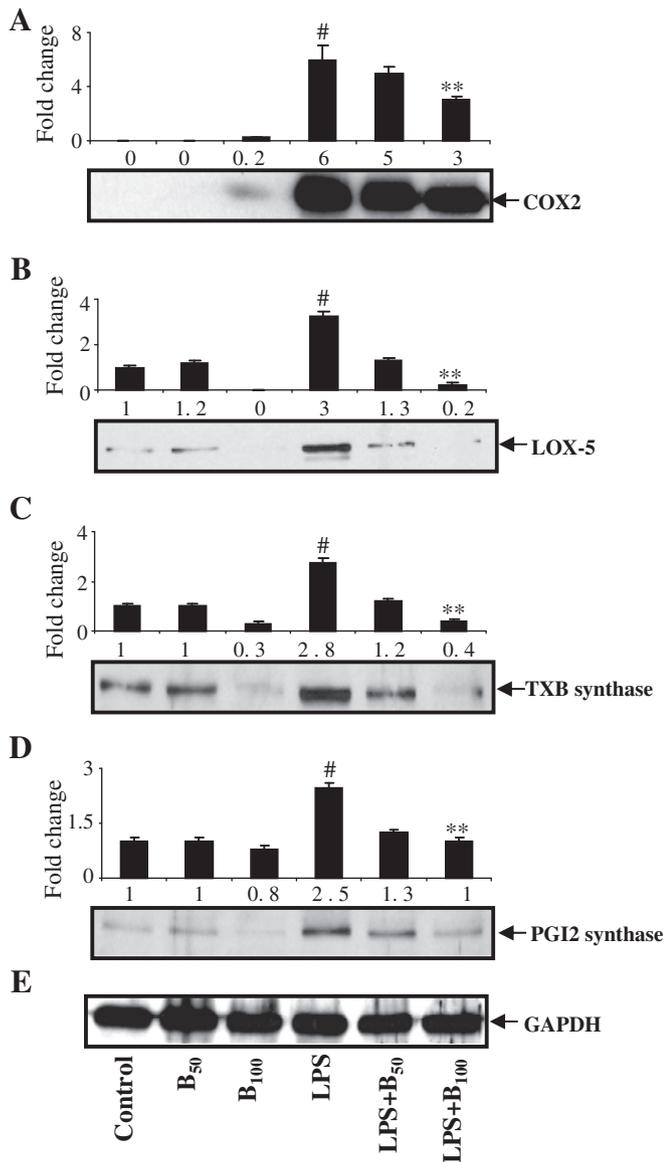
The lipid hydroperoxides were determined colorimetrically using a lipid hydroperoxide assay kit obtained from Cayman Chemicals as per the supplier's instructions. Briefly, the lipid hydroperoxide kit measures the hydroperoxides directly utilizing redox reactions with ferrous ions. Lipid hydroperoxides were extracted from the samples into 450 µl of chloroform-methanol solvent. Fifty µl of chromogen was added to the solvent, kept at room temperature for 5 min, and absorbance was read at 500 nm using a 96-well plate reader.

*Protein-HNE adduct formation*

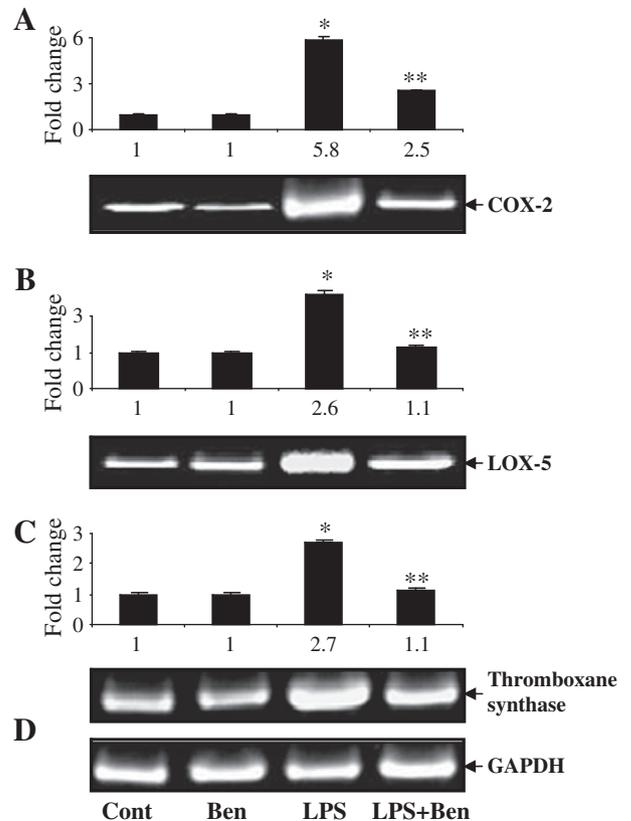
RAW264.7 cells were seeded on glass chamber slides without or with benfotiamine (100 µM), followed by incubation with 1 µg LPS/ml for 18 h. The slides were fixed with 4% paraformaldehyde for 30 min. After the slides were thoroughly washed with Tris-buffered saline containing 0.1% Triton X-100, they were incubated with anti-HNE antibodies overnight at 4 °C followed by washing and incubation with 1:160 diluted secondary antibodies (goat anti-rabbit IgG conjugated with FITC) for 1 h. The slides were mounted and photographs were taken with a Nikon fluorescence microscope.

*Macrophage viability and U-937 cell adhesion to human umbilical vein endothelial cells (HUVECs)*

The RAW cells (0.5 × 10<sup>6</sup> cells/well in 6-well plates) were growth-arrested in serum-free medium without or with benfotiamine (100 µM), indomethacin (COX-2 inhibitor, 10 µM), or REV 5901 (LOX-5 inhibitor, 10 µM) overnight, followed by incubation with 1 µg/ml LPS for another 24 h. Cell viability was determined using a Millipore cell counter. For cell adhesion HUVECs were plated in 96-well plates, grown to 70% confluence, and treated without or with benfotiamine, indomethacin, or REV 5901 overnight in the absence or presence of 1 µg/ml LPS. The cells were rinsed twice with serum-free medium and U-937 cells were added to each well followed by incubation with 1 µg/ml LPS



**Fig. 4.** Benfotiamine prevents LPS-induced expression of COX-2, LOX-5, TXB synthase, and PGI2 synthase in RAW cells. The RAW cells were growth-arrested by incubating in 0.1% FBS medium without or with benfotiamine (100 µM) and stimulated with 1 µg/ml LPS for 18 h. Cytosolic extracts were prepared and equal amounts of cytosolic proteins were subjected to Western blot analysis using antibodies against (A) COX-2, (B) LOX-5, (C) TXB synthase, (D) PGI2 synthase, and (E) GAPDH. A representative blot from each group is shown. Data represent means ± SE (n = 3). #P < 0.0001 vs control; \*\*P < 0.0001 vs LPS-treated cells. B<sub>50</sub> and B<sub>100</sub>, benfotiamine 50 and 100 µM.



**Fig. 5.** Effect of benfotiamine on LPS-induced COX-2, LOX-5, and TXB synthase at the transcriptional level in RAW macrophages. Macrophages were growth-arrested in Dulbecco's modified Eagle's medium containing 0.1% serum with or without benfotiamine (100 µM) overnight and challenged with 1 µg/ml LPS for 6 h. The total RNA was isolated and reverse transcriptase-PCR analysis was carried out using specific primers for COX-2, LOX-5, and TXB synthase. Equal amounts of PCR products were electrophoresed with 1% agarose-TAE gels containing ethidium bromide. GAPDH served as control in the reverse transcriptase-PCR analysis. A representative blot from each group is shown. Data represent means ± SE (n = 3). \*P < 0.0001 vs control; \*\*P < 0.0001 vs LPS-treated cells. Cont, control; Ben, benfotiamine.

for an additional 12 h. MTT assay was performed to determine cell adhesion, recording the absorbance at 562 nm.

#### Statistical analysis

Data presented as means  $\pm$  SE ( $n = 6$ ) and  $P$  values were determined by unpaired Student's  $t$  test using Microsoft Office Excel 2007 software.  $P < 0.05$  was considered statistically significant.

## Results

### Benfotiamine prevents release of arachidonic acid metabolites in LPS-induced macrophages

To investigate the anti-inflammatory effect of benfotiamine against LPS-induced toxicity, at first, we examined the metabolism of AA in macrophages. Macrophages were incubated with [ $^3$ H]AA followed by stimulation with LPS with or without benfotiamine. Results shown in the Fig. 1 indicate that in the LPS-treated macrophages the release of prelabeled AA and its metabolites was significantly increased approximately five-folds compared to unstimulated macrophages. Benfotiamine significantly ( $\sim 90\%$ ) prevented the LPS-induced

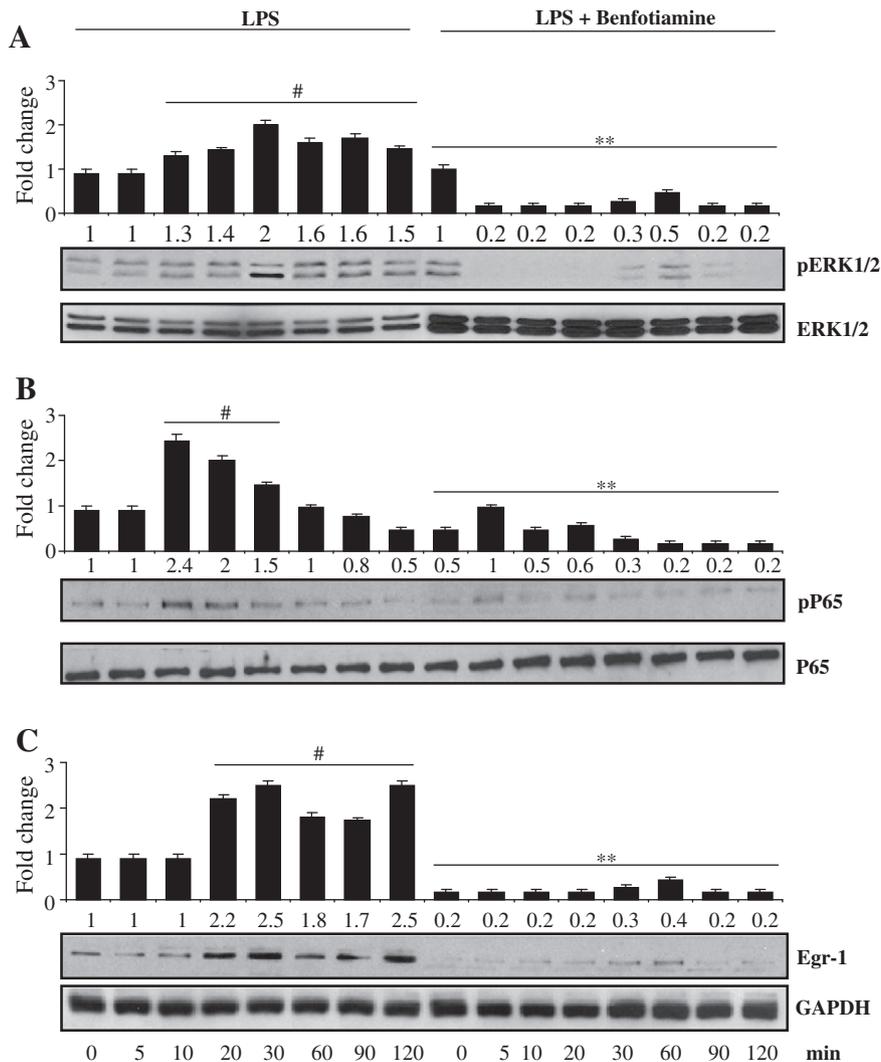
formation of AA metabolites. Thus, our studies indicate that benfotiamine prevents LPS-induced AA metabolism in macrophages.

### Benfotiamine prevents LPS-induced activation of cPLA2 in macrophages

Phospholipase A2, especially cPLA2, is well known to mediate the release of AA from membrane phospholipids. We next examined the potential of benfotiamine to prevent the activation of cPLA2 in LPS-induced macrophages. Stimulation of macrophages with LPS caused an approximately twofold increase in cPLA2 activity and preincubation of macrophages with benfotiamine significantly ( $>70\%$ ) prevented the LPS-induced activity of cPLA2 (Fig. 2A). Further, the results shown in Fig. 2B indicate that LPS-induced an approximately twofold increase in cPLA2 expression. The LPS-induced increase in the expression of cPLA2 was significantly ( $>90\%$ ) prevented by benfotiamine. Thus, our results suggest that benfotiamine prevents LPS-induced AA metabolism by preventing the activation of cPLA2.

### Effect of benfotiamine on LPS-induced production of AA pathway-generated lipid mediators in RAW264.7 macrophages

Because prostaglandins, TXBs, LTBs, and 6 k-PGF1 $\alpha$  are AA pathway-generated lipid mediators that play major roles in inflammatory



**Fig. 6.** Effect of benfotiamine on ERK1/2 phosphorylation and activation of p65 and Egr-1. Growth-arrested murine macrophages were treated with LPS without and with benfotiamine (100  $\mu$ M) for the indicated times at 37  $^{\circ}$ C. Cell lysates were prepared and equal amounts of protein were subjected to Western blot analysis using antibodies against (A) ERK1/2, (B) p65, and (C) Egr-1. A representative blot from each group is shown. Data represent means  $\pm$  SE ( $n = 3$ ). # $P < 0.0001$  vs control; \*\* $P < 0.0001$  vs LPS-treated cells.

complications, we next examined the effects of benfotiamine on LPS-induced generation of PGE<sub>2</sub>, TXB<sub>2</sub>, 6-k-PGF<sub>1</sub>α, and LTB<sub>4</sub> in cell culture medium. LPS caused >3-fold and >20-fold increases in the levels of PGE<sub>2</sub> and TXB<sub>2</sub>, respectively, in the macrophages compared to untreated macrophages. The increases in PGE<sub>2</sub> and TXB<sub>2</sub> levels were significantly brought down by benfotiamine (Figs. 3A and B). Because PGI<sub>2</sub> has a very short half-life and is readily converted to 6-k-PGF<sub>1</sub>α, we next determined 6-k-PGF<sub>1</sub>α levels in RAW264.7 macrophages. Macrophages treated with LPS showed a significant (>4-fold) increase in the levels of 6-k-PGF<sub>1</sub>α, which was significantly prevented by benfotiamine (Fig. 3C). Further, we also measured the effect of benfotiamine on LPS-induced LTB<sub>4</sub> levels in macrophages. As shown in Fig. 3D, RAW264.7 cells stimulated with LPS showed a >3-fold increase in the level of LTB<sub>4</sub>, which was significantly (>60%) reduced in cells treated with benfotiamine. These results suggest that benfotiamine could efficiently prevent LPS-induced production of AA pathway-generated inflammatory metabolites in RAW264.7 macrophages.

#### Benfotiamine prevents LPS-induced activation of AA pathway enzymes in macrophages

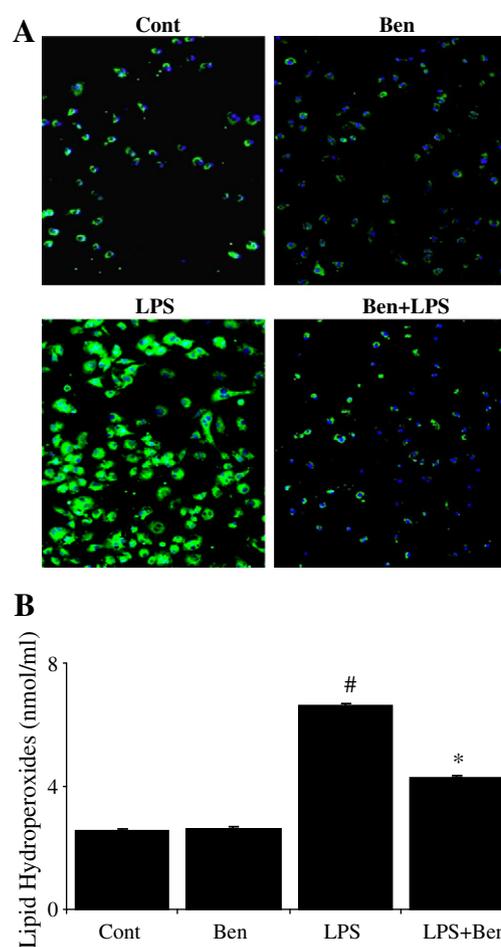
Because PGs, TXBs, LTBs, and 6-k-PGF<sub>1</sub>α are catalyzed by COX-2, TXB synthase, PGI<sub>2</sub> synthase, and LOX-5, respectively, in the AA metabolism pathway, we next examined the effect of benfotiamine on LPS-induced expression of these proteins in macrophages. Our results shown in Figs. 4A–D indicate that LPS-induced an approximately 6-fold increase in COX-2, >3-fold in LOX-5, 3-fold in TXB synthase, and 2.5-fold in PGI<sub>2</sub> synthase expression in macrophages. The increases in COX-2, LOX-5, TXB synthase, and PGI<sub>2</sub> synthase were significantly abolished in the presence of benfotiamine by 50, 95, 95, and 90%, respectively. Further, to examine the effect of benfotiamine on LPS-induced AA metabolism enzymes at the transcriptional level, we next measured the effect of benfotiamine on the LPS-induced mRNA levels of AA pathway enzymes. The results shown in Figs. 5A–C indicate that LPS caused a significant 6-, 3.6-, and 3-folds increase in the levels of COX-2, LOX-5, and TXB synthase mRNA, respectively. However, in the macrophages pretreated with benfotiamine followed by LPS treatment, the increase in the mRNA levels of COX-2, LOX-5, and TXB synthase were significantly (60 to 90%) decreased. Thus, our studies indicate that benfotiamine prevents the expression of AA pathway enzymes at their transcriptional level.

#### Effect of benfotiamine on phosphorylation of ERK1/2 and expression of transcription factors NF-κB and Egr-1

Because transcription factors such as NF-κB and Egr-1 are known to regulate the transcription of AA pathway enzymes, we next examined the effect of benfotiamine on LPS-induced activation of these transcription factors. Our results shown in Figs. 6A–C indicate that benfotiamine significantly prevented the LPS-induced activation ERK1/2 and the transcription factors NF-κB and Egr-1, which transcribe genes for COX-2 and mPGES-1, respectively [32]. Thus, our results suggest that benfotiamine prevents LPS-induced expression of AA pathway enzymes by preventing their transcriptional activation.

#### Effect of benfotiamine on LPS-induced protein-HNE adduct and lipid hydroperoxide formation

Because transcription factors such as NF-κB and Egr-1 are known to be activated by oxidative stress-generated reactive oxygen species, we next examined the effect of benfotiamine on LPS-induced oxidative stress. The levels of protein-HNE adducts and lipid hydroperoxides were used as markers for increased oxidative stress. Our results shown in Fig. 7A indicate that benfotiamine ameliorated protein-HNE adduct formation in LPS-induced macrophages. Further, macrophages challenged with LPS showed significantly (>2.5-fold)

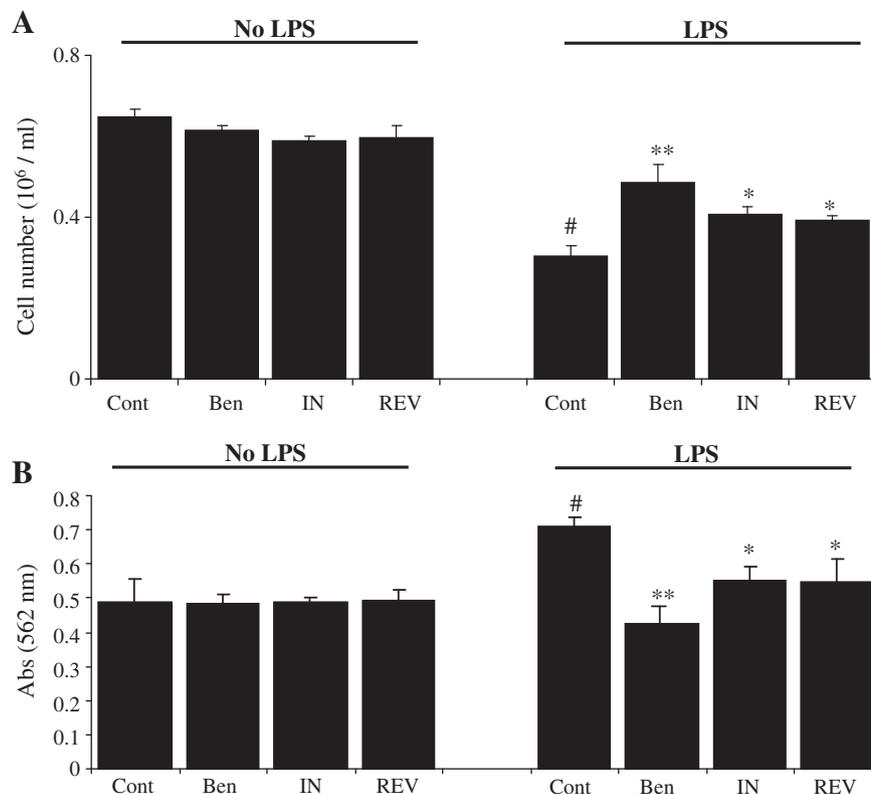


**Fig. 7.** Effect of benfotiamine on LPS-induced protein-HNE adduct and lipid hydroperoxide formation. Macrophages were growth-arrested in 0.1% FBS and with or without benfotiamine (100 μM). Subsequently, the cells were exposed to LPS (1 μg/ml) for 24 h. (A) Protein-HNE adduct formation was evaluated using rabbit anti-protein-HNE antibodies and FITC-labeled anti-rabbit IgG in goat. Representative images are shown (200× original magnification). (B) Lipid hydroperoxides were measured using a microplate assay kit according to the manufacturer's instructions. Data represent means ± SE (n = 6). <sup>#</sup>P < 0.0001 vs control cells; <sup>\*</sup>P < 0.0001 vs LPS-treated cells. Cont, control; Ben, benfotiamine.

increased lipid hydroperoxide levels, which were significantly (60%) decreased in the macrophages pretreated with benfotiamine (Fig. 7B), suggesting that benfotiamine prevents LPS-induced activation of redox-sensitive transcription factors by decreasing cellular oxidative stress in macrophages.

#### Effect of benfotiamine and specific inhibitors of COX-2 and LOX-5 on LPS-induced macrophage viability and U-937 cell adhesion to HUVECs

Because our previous results indicated that benfotiamine prevents both COX-2 and LOX-5 enzymes, we next examined the dual inhibitory activity of benfotiamine on LPS-induced macrophage death and compared it with the specific inhibitors of COX and LOX enzymes. Benfotiamine, indomethacin (COX-2 inhibitor), and REV 5901 (LOX-5 inhibitor) were preincubated with macrophages followed by stimulation with LPS for another 24 h and cell viability was measured using an automatic electronic cell counter (Scepter; Millipore). LPS caused a significant cell death and all three inhibitors prevented the LPS-induced cell death (Fig. 8A). However, of all three agents, benfotiamine significantly prevented (~70%) LPS-induced cell death, followed by COX-2 inhibitor (40%) and LOX-5 inhibitor (30%). Further, benfotiamine significantly prevented the LPS-induced monocyte adhesion (~70%) to



**Fig. 8.** Effects of benfotiamine and specific inhibitors of COX-2 and LOX-5 on LPS-induced macrophage viability and monocyte adhesion to endothelial cells. (A) Growth-arrested macrophages were preincubated in the presence or absence of benfotiamine (100  $\mu$ M), COX-2 inhibitor indomethacin (10  $\mu$ M), and LOX-5 inhibitor REV 5901 (10  $\mu$ M) overnight at 37  $^{\circ}$ C. (A) Cell viability was determined by trypan blue exclusion. (B) Serum-starved HUVECs were pretreated with benfotiamine (100  $\mu$ M), COX-2 inhibitor indomethacin (10  $\mu$ M), or LOX-5 inhibitor REV 5901 (10  $\mu$ M) for 12 h in the absence or presence of LPS (1  $\mu$ g/ml). The cells were washed and U-937 monocytes were added to each well and the incubation continued for another 12 h. Cell adhesion assays were performed by MTT assay. Data represent means  $\pm$  SE ( $n = 4$ ). <sup>#</sup> $P < 0.001$  vs control; <sup>\*</sup> $P < 0.01$  and <sup>\*\*</sup> $P < 0.001$  vs LPS-treated cells.

endothelial cells compared to COX-2 or LOX-5 inhibitor (Fig. 8B). Thus, our results indicate that benfotiamine could be a novel anti-inflammatory agent compared to specific COX-2 and LOX-5 inhibitors, because it could efficiently prevent LPS-induced generation of inflammatory lipid mediators by inhibiting both the COX and the LOX enzymes.

## Discussion

During Gram-negative bacterial infections, the pathological condition of inflammation is initiated by bacterial outer cell wall components, especially endotoxins such as LPS [33]. LPS can directly activate macrophages by triggering the production of proinflammatory cytokines such as TNF [34], IL-1, IL-6, and IFN; chemokines such as MCP-1 and MIP1 [35]; leukotrienes [36]; and other molecules, such as nitric oxide and prostaglandins [37]. Overproduction of these inflammatory markers amplifies the host immune response and leads to inflammatory complications such as sepsis [38]. Therefore, pharmacological intervention in the LPS-induced production of inflammatory mediators by macrophages is an essential event to control a variety of inflammation-related disorders. In this study, we have for the first time shown that benfotiamine, a fat-soluble analogue of vitamin B1, modulates the production of AA pathway-generated inflammatory lipid mediators.

Several studies indicate that the use of natural anti-inflammatory products provides an attractive and safe alternative to modulate various inflammatory disorders [39–41]. Because of its therapeutic action in some frequently observed clinical syndromes, thiamine hydrochloride has been advised and used over a long period of time [42–45]. There are no reports of adverse effects of oral thiamine, even at dosages of several hundred milligrams a day [44]. Benfotiamine, a

unique derivative of thiamine, is the most potent of the allithiamines, a group of lipid-soluble forms of thiamine [46]. Compared to water-soluble thiamine salts, benfotiamine is absorbed better in the intestine, reaching maximum plasma levels of thiamine about five times higher, and increases the active metabolite thiamine diphosphate to 120-fold. The bioavailability is at maximum about four times as high as that of thiamine hydrochloride and other lipophilic thiamine derivatives [47]. In fact, a recent randomized, double-blind, placebo-controlled clinical study in Germany indicated that benfotiamine at a dose of 600 mg/day and higher had no side effects [48]. A number of studies have revealed its potential to alleviate diabetic microangiopathy, neuropathy, and other oxidative stress-induced pathological conditions in various experimental animal models [49–53]. However, no reports are available that show that benfotiamine supplementation prevents sepsis complications in animal or human studies. We have recently reported that the antioxidative and anti-inflammatory properties of benfotiamine prevent LPS-induced cytotoxicity in macrophages as well as preventing ocular inflammation in rats [24,25]. The present study investigates the potential efficacy of benfotiamine in preventing AA pathway-dependent inflammatory signals in macrophages. This is an important advance for the use of benfotiamine as an anti-inflammatory food supplement for treating inflammatory disease conditions.

COX-2 and LOX-5 are the critical enzymes of AA metabolism that catalyze AA to prostaglandins and LTBs, major lipid mediators involved in various inflammatory diseases [32]. In this study, we have investigated the effect of benfotiamine on LPS-induced AA pathway-generated inflammatory lipid mediators. Our results demonstrate that benfotiamine significantly prevented the AA pathway-generated inflammatory lipid mediators as well as the expression of enzymes such as COX-2 and

LOX-5, which catalyze the formation of lipid mediators. Thus, inhibition of COX-2 and LOX-5 seems to be responsible for the decreased biosynthesis of proinflammatory prostaglandins and LT<sub>B4</sub>. Septic patients and animals treated with LPS have increased expression of proinflammatory cytokines and PG<sub>I2</sub> synthase [54]. PG<sub>I2</sub>, once formed rapidly, is converted into 6 k-PGF<sub>1</sub> $\alpha$  and is involved in vasodilation, resulting in reduced blood pressure, and can ultimately lead to sepsis [55]. In this study, benfotiamine significantly prevented 6 k-PGF<sub>1</sub> $\alpha$  levels and PG<sub>I2</sub> expression, suggesting that benfotiamine could efficiently prevent PG<sub>I2</sub>-induced vasculopathy upon bacterial infection.

Lipoxygenases catalyze the oxygenation of AA [56]. It is known that leukotrienes are important regulators of cancer cell proliferation and cause apoptosis in noncancer cells [57–61]. Therefore, we examined whether benfotiamine could regulate the expression and synthesis of LOX-5. Our results demonstrate that LPS-induced expression and synthesis of LOX-5 in RAW264.7 cells were significantly prevented by benfotiamine (Figs. 4 and 5B). Our results thus indicate that benfotiamine prevents the expression of two key regulatory enzymes of AA metabolism, i.e., COX-2 and LOX-5. Indeed, we observed that, compared to specific COX-2 and LOX inhibitors, benfotiamine is more potent in preventing LPS-induced macrophage death as well as monocyte adhesion to endothelial cells. During bacterial infections and systemic inflammatory pathologies such as severe sepsis, increased production of ROS causes oxidative stress leading to alterations in protein functions and increased susceptibility to protein degradation [58]. In addition to proteins, polyunsaturated fatty acids such as AA present in membrane lipids are highly susceptible to ROS-mediated damage [59]. Peroxidation of membrane lipids results in the fragmentation of polyunsaturated fatty acids resulting in the production of various cytotoxic and highly reactive aldehydes, such as malonaldehyde and HNE [60]. Because benfotiamine prevents the formation of AA metabolites by preventing the activation of cPLA<sub>2</sub>, we next examined if benfotiamine prevents AA-induced generation of HNE. Our results indicate that benfotiamine prevents lipid peroxidation as well as subsequent protein–HNE adduct formation. These results suggest that antioxidative properties of benfotiamine could prevent LPS-induced lipid peroxidation and HNE formation.

LPS-induced inflammatory signals are known to mediate through ROS, resulting in the activation of protein kinase C and mitogen-activated protein kinase [60,62], which can cause inflammation by activating transcription factors such as NF- $\kappa$ B. It has been shown earlier that various antioxidants such as vitamin C and vitamin E inhibit ROS-induced inflammatory responses by preventing the activation of NF- $\kappa$ B [63,64]. Our current studies indicate that benfotiamine prevents activation of NF- $\kappa$ B as well as Egr-1 and phosphorylation of ERK1/2. Thus, prevention of LPS-induced AA-metabolizing enzymes and formation of inflammatory lipid mediators by benfotiamine could be mediated, regulating redox-sensitive transcription factors.

In summary, this study shows that AA pathway-generated inflammatory lipid mediators in response to endotoxin exposure could be prevented by a dietary supplement, benfotiamine. Further, benfotiamine inhibited LPS-induced NF- $\kappa$ B and Egr-1 and expression of key regulatory enzymes of AA metabolism such as cPLA<sub>2</sub>, COX-2, and LOX-5 in murine macrophages. Our studies indicate that benfotiamine is a more potent anti-inflammatory compound than specific LOX and COX inhibitors. Thus, dual regulation of the COX and LOX pathways by benfotiamine could be a novel mechanism by which benfotiamine exhibits its anti-inflammatory response, and benfotiamine supplementation could be a potential therapeutic approach to preventing inflammatory complications such as sepsis.

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