Original Article

β-Lapachone, a substrate of NAD(P)H:quinone oxidoreductase, induces anti-inflammatory heme oxygenase-1 *via* AMP-activated protein kinase activation in RAW264.7 macrophages

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AMP-activated protein kinase (AMPK), a crucial regulator of energy metabolic homeostasis, is suggested to regulate inflammatory responses, but its precise mechanisms are not fully understood. It has been reported that pharmacological activation of AMPK induces heme oxygenase-1 (HO-1) expression. β-Lapachone (BL), a well-known substrate of NAD(P)H:quinone oxidoreductase (NQO1), has been demonstrated to stimulate AMPK activation *via* NQO1 activation, and to exert anti-inflammatory effects in macrophages. Here we examined whether AMPK activation by BL would be linked to HO-1 expression in RAW264.7 macrophages and whether HO-1 expression could mediate the anti-inflammatory effects of BL. BL treatment induced concentration- and time-dependent AMPK phosphorylation and HO-1 expression. 5-Aminoimidazole-4-carboxamide-1-β-*D*-ribofuranoside, an AMPK activator, also induced HO-1 expression. In contrast, compound C (CC), an inhibitor of AMPK activation, prevented the increase in BL-induced HO-1 expression. BL pretreatment reduced lipopolysaccharide-induced production of tumor necrosis factor-α, a pro-inflammatory cytokine, and expression of inducible nitric oxide synthase, a pro-inflammatory enzyme. These inhibitory effects BL were almost completely abolished by CC and partly by tin protoporphyrin-IX, a competitive inhibitor of HO-1. Accordingly, the present results indicate that BL induces anti-inflammatory HO-1 expression in macrophages *via* AMPK activation, providing one of possible mechanisms by which BL can exert anti-inflammatory effects.

Key Words: **β-lapachone, AMP-activated protein kinase, heme oxygenase-1, tumor necrosis factor-α, inducible nitric oxide synthase**

AMP-activated protein kinase (AMPK), an energy-sensing enzyme that regulates energy homeostasis and metabolic stress, is activated primarily by increase in cellular AMP-to-ATP ratio, which can occur in various conditions, such as glucose deprivation, heat shock, oxidative stress, and ischemia.[1] However, pharmacologically relevant molecules, such as 5-aminoimidazole-4-carboxamide-1-β-*D*-ribofuranoside (AICAR), A769662, and metformin, are also capable of activating AMPK independently of changes in the AMP-to-ATP ratio.[2–4] Recent studies have demonstrated that AMPK activation has anti-inflammatory effects in several cell types, including macrophages and endothelial cells.[5–8] However, the molecular mechanism(s) by which AMPK activation can exert anti-inflammatory effects remains unclear. Interestingly, it has been demonstrated that pharmacological activation of AMPK stimulates the expression of the anti-inflammatory heme oxygenase-1 (HO-1) in macrophages and endothelial cells.[9,10]

HO-1 is a highly inducible enzyme that degrades heme into carbon monoxide, free iron, and biliverdin.[11] Generally, HO-1 expression has been considered to be an adaptive cellular response against the toxicity of oxidative stress.[12] However, recent studies have demonstrated that HO-1 expression exhibits important anti-inflammatory functions.[12] In particular, HO-1 expression in macrophages has been shown to inhibit the excess production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and the prolonged expression of pro-inflammatory enzymes, including inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2.[12]

NAD(P)H:quinone oxidoreductase 1 (NQO1) catalyses the oxidation of NADH to NAD^+ by various quinones.[13] Several activators and substrates of NQO1 have been identified. One of the well-known NQO1 substrates is β-lapachone (BL) isolated from the bark of the *Lapacho* tree.[13–16] Recent studies have indicated that BL stimulates AMPK activation by increasing NAD^+-to-NADH ratio *via* NQO1 activation; so being considered as a novel AMPK activator.[17] In addition, BL has been shown to exert anti-inflammatory effects in macrophages,[18] but the mechanism(s) of anti-inflammatory actions of BL remains to be established.

Considering the findings that BL can stimulate AMPK activation by enhancing NQO1 activity[17] and AMPK can induce HO-1 expression,[9,10] we sought to examine whether AMPK activation by BL would be linked to HO-1 expression in RAW264.7 macrophages and, if so, whether HO-1 could mediate the anti-inflammatory effects of BL.

Materials and Methods

Reagents and antibodies. BL, lipopolysaccharide (LPS), compound C (CC), AICAR, tin protoporphyrin-IX (SnPP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), dicoumarol (DC), and Dulbecco's modified Eagle's medium (DMEM) were from Sigma-Aldrich (St. Louis, MO). A polyclonal HO-1 antibody was from StressGen Biotechnologies (Victoria, Canada), antibodies directed against AMPK, phospho (P)-AMPK,

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Acetyl-coenzyme A carboxylase (ACC), iNOS, P-ACC, and β-actin were from Cell Signaling Technology (Beverley, MA), and secondary antibodies was from Santa Cruz Biotechnologies (Santa Cruz, CA).

Cell culture. RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM *L*-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere and experiments were conducted on cells at approximately 80–90% confluence.

Cell viability assay. Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. RAW264.7 macrophages were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of BL for 24 h. Thereafter, culture medium was aspirated and 100 μl of MTT dye (1 mg/ml in phosphate-buffered saline) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

NAD^+-to-NADH ratio assay. The NAD^+-to-NADH ratio was measured from whole-cell extracts of RAW264.7 macrophages using the Biovision NAD/NADH quantization kit (BioVision, Mountain View, CA), performed according to the manufacturer's instructions.

Reverse transcriptase polymerase chain reaction (RT-PCR). The cells were harvested and total RNA was isolated using RNeasy Mini Kits according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). Two microgram of total RNA was used to synthesis the first stranded cDNA using RT-PCR kit (Invitrogen, Carlsbad, CA). For amplification of rat HO-1, the following primers were used: HO-1,5'-ACAGGTTGACAGAAG AGGCTAA-3' (sense) and 5'-AACAGGAAGCTGAGAGTG AGG-3' (antisense). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as a control in a similar way using the following primers: 5'-AGGTGGTCTCCT CTGACTTC-3' (sense) and 5'-TACCAGGAAATGAGCTTGAC-3' (antisense). For PCR amplification, the following conditions were used: 94°C for 5 min for one cycle and then 94°C for 1 min, 56°C for 30 s and 72°C for 1 min for 25 cycles. The amplified PCR products were separated with 1.5% agarose gel, and then stained with ethidium bromide.

Western blot analysis. Macrophages were washed with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were re-suspended in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% NP-40, 0.5% Tween 20, 1 mM dithiothreitol, and protease inhibitor cocktail and vortexed for 20 min at 4°C; insoluble material was removed by centrifugation. Protein (30 μg) was separated by SDS-PAGE. After transfer to nitrocellulose membrane, blots were blocked with PBS and nonfat milk (5%) and then incubated with antibodies directed against HO-1 (1:500), AMPK (1:500), P-AMPK (1:100), ACC (1:500), P-ACC (1:100), iNOS (1:500) or β-actin (1:1,000). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-mouse, or donkey anti-goat antibodies, and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL).

Enzyme-linked immunosorbent assay (ELISA). Macrophages were cultured in 24-well plates in the absence or presence of indicated compounds and LPS (1 μg/ml) for 18 h. According to the manufacturer's protocol, the concentration of TNF-α in the culture medium was measured using ELISA kits (R&D Systems, Minneapolis, MN).

Nitrite assay. The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader.

Statistical analysis. Statistical analysis was performed using one-way ANOVA combined with the Bonferroni test. Differences were considered to be significant at $p<0.05$.

Results

In RAW264.7 macrophages, BL at concentrations ranging from 1 to 4 μM had no significant effect on cell viability after 48 h (Fig. 1). These non-cytotoxic concentrations of BL were, therefore, used in all subsequent experiments.

BL stimulates AMPK activation in macrophages. Studies have demonstrated that BL is capable of stimulating AMPK activation in vascular smooth muscle cells,[15] endothelial cells[16] and myoblasts.[17] However, it is unclear whether BL could also activate AMPK in macrophages. To evaluate the effect of BL on AMPK activation, which is directly linked with its phosphorylation state, we treated RAW264.7 macrophages with BL at different concentrations (1, 2 and 4 μM) for 3 h (Fig. 2A) or at 4 μM for different times (1, 3 and 6 h) (Fig. 2B) and examined the level of AMPK phosphorylation using Western blot analysis. In macrophages, BL increased AMPK phosphorylation in a concentration- and time-dependent manner. An increase in AMPK phosphorylation was detected for 3 h with BL at 1 μM, and BL at 4 μM showed a significant increase in AMPK phosphorylation (Fig. 2A). The phosphorylation of AMPK by BL at 4 μM was first detected at 1 h, peaked at 3 h, and slightly decreased at 6 h (Fig. 2B). AMPK activation stimulates fatty acid oxidation by phosphorylating and inhibiting ACC,[19] which is referred to as a direct downstream target of AMPK. Thus, we investigated the effect of BL on ACC phosphorylation in macrophages. In accordance with its effect on AMPK activation, BL increased ACC phosphorylation in a concentration-dependent manner (Fig. 2C). We also examined whether BL-induced AMPK activation would require NQO1 activity in RAW264.7 macrophages. As expected, BL increased the cellular NAD^+-to-NADH ratio, and this was

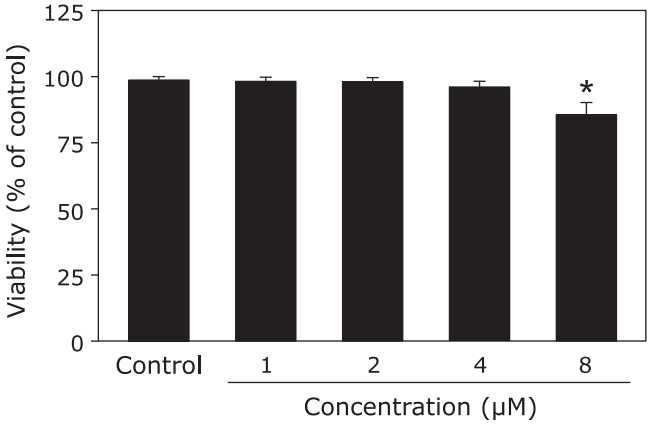

Fig. 1. Effects of BL on cell viability. RAW264.7 macrophages were incubated for 48 h with indicated concentrations of BL. MTT assay for cell viability was performed as described in the section of Materials and Methods. Data are expressed as means ± SE from 3 and 4 experiments. *$p<0.05$ with respect to the untreated control group.

significantly inhibited by DC, a specific inhibitor of NQO1 (Fig. 3A). Inhibition of NQO1 using DC abrogated the BL-induced phosphorylation of AMPK (Fig. 3B).

BL induces HO-1 expression *via* AMPK activation. It has been revealed that AMPK activation is capable of inducing HO-1 expression in human vascular cells[9] and rat pancreatic β-cells.[20] To test the effect of BL on HO-1 expression, we treated RAW264.7 macrophages with BL at different concentrations (1, 2 and 4 μM) for 12 h (Fig. 4A) or at 4 μM for different times (6, 12 and 24 h) (Fig. 4B) and examined the level of HO-1 mRNA and protein expression. BL increased HO-1 mRNA and protein expression in a concentration- and time-dependent manner. An increase in HO-1 protein expression was detected for 6 h with BL at 2 μM, and BL at 4 μM showed a further increase in HO-1 protein expression (Fig. 4A). HO-1 protein expression by BL at 4 μM was first detected at 6 h, peaked at 12 h, and slightly decreased at 24 h (Fig. 4B). To explore whether the AMPK pathway could be required for BL-induced HO-1 mRNA and protein expression, we used CC, a specific inhibitor of AMPK, to

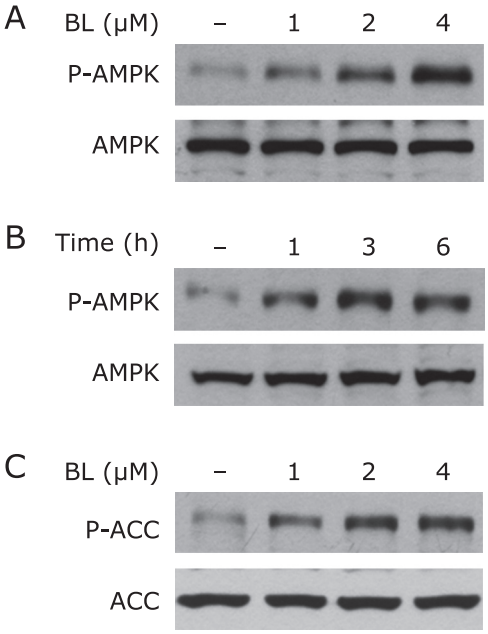

Fig. 2. Effects of BL on activation of AMPK and ACC. RAW264.7 macrophages were incubated for 3 h with indicated concentrations of BL (A and C) or for indicated times with 4 μM BL (B). Western blot analysis for AMPK phosphorylation and expression (A and B) and ACC phosphorylation and ACC expression (C) were performed as described in the section of Materials and Methods. Blots shown are representative of three independent experiments.

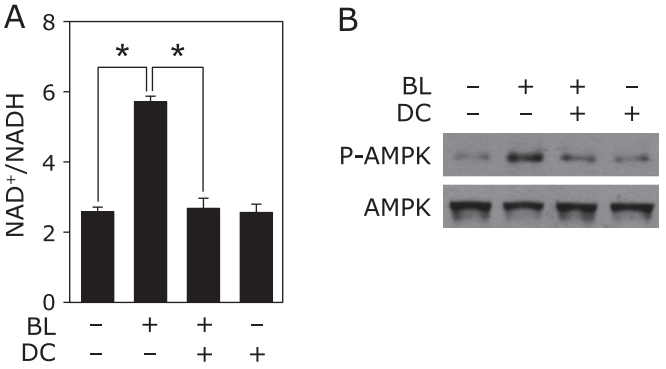

Fig. 3. Effects of DC on BL-induced activation of AMPK. RAW264.7 macrophages were incubated for 3 h with 4 μM BL in the absence or presence of 10 μM DC. DC blocked BL-induced NADH oxidation (A), and AMPK phosphorylation (B). NAD^+-to-NADH ratio assay (A) and Western blot analysis for AMPK phosphorylation (B) were performed as described in the section of Materials and Methods. Data are expressed as means ± SE from 3 and 4 experiments. *$p<0.05$. Blots shown are representative of three independent experiments.

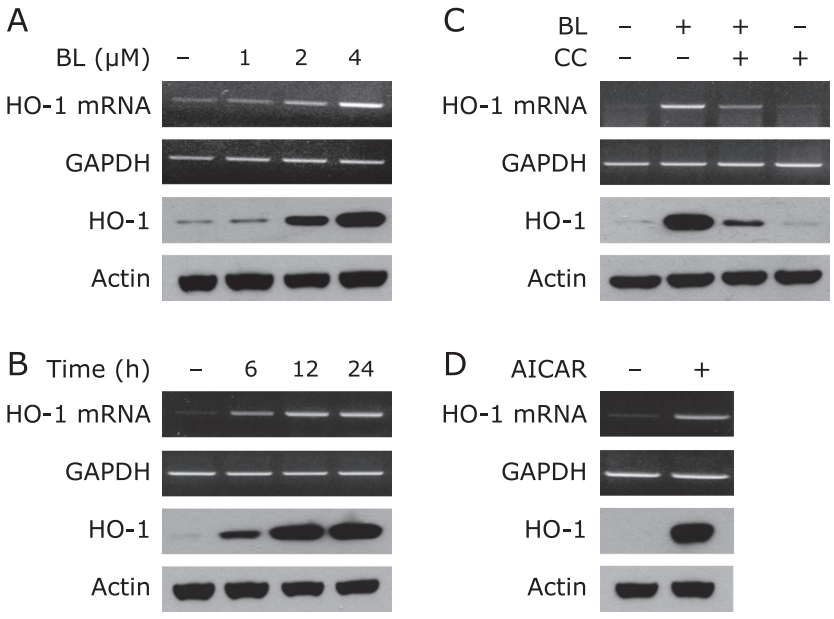

Fig. 4. Effects of BL on HO-1 mRNA and protein expression. RAW264.7 macrophages were incubated for 12 h with indicated concentrations of BL (A) or for indicated times with 4 μM BL (B). RAW264.7 macrophages were incubated for 12 h with 4 μM BL in the absence or presence of 10 μM CC (C) or for 12 h with 1 mM AICAR (D). RT-PCR for HO-1 mRNA expression and Western blot analysis for HO-1 protein expression were performed as described in the section of Materials and Methods. Blots shown are representative of three independent experiments.

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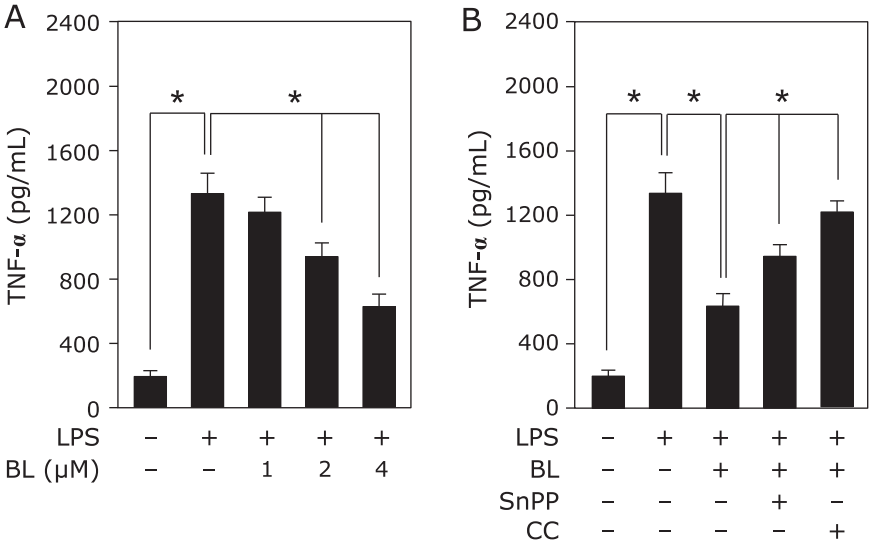

Fig. 5. Effects of BL on LPS-induced TNF-α production. RAW264.7 macrophages were pre-incubated for 12 h with indicated concentrations of BL (A) or with 4 μM BL in the absence or presence of 10 μM CC or 50 μM SnPP (B), and then activated for 18 h with 1 μg/ml LPS. ELISA for the concentrations of TNF-α was performed as described in the section of Materials and Methods. Data are expressed as means ± SE from 3 and 4 experiments. *$p<0.05$.

inhibit AMPK activation in macrophages. As shown in Fig. 4C, CC reversed BL-induced HO-1 mRNA and protein expression. Additionally, AMPK activation by AICAR, an AMPK activator, induced HO-1 mRNA and protein expression in macrophages (Fig. 4D). CC and AICAR alone had no significant effect on cell viability (data not shown).

BL inhibits LPS-induced TNF-α production *via* AMPK activation and HO-1 expression. Studies have demonstrated that LPS-induced production of pro-inflammatory cytokines, including TNF-α, in macrophages can be inhibited through AMPK activation by metformin[7] and berberine.[8] To test whether BL could also inhibit LPS-induced production of TNF-α *via* its activation of AMPK, RAW264.7 macrophages were pre-incubated for 12 h with BL at different concentrations (1, 2 and 4 μM), and then activated with LPS for 18 h. As shown in Fig. 5A, BL at 2 and 4 μM significantly inhibited LPS-induced production of TNF-α. To explore whether the AMPK pathway could be required for this inhibitory effect, we used CC to inhibit AMPK activation in macrophages. As shown in Fig. 5B, the inhibitory effect of BL on LPS-induced production of TNF-α was reversed by the AMPK inhibitor CC.

It has been revealed that HO-1 expression in macrophages exerts anti-inflammatory effects by reducing pro-inflammatory cytokine production and enzyme expression.[12] In light with this, the potential involvement of HO-1 expression induced by BL in inhibition of LPS-induced production of TNF-α was investigated by pre-incubating RAW264.7 macrophages with BL for 12 h in the presence of SnPP, a competitive inhibitor of HO-1, followed by LPS stimulation. Interestingly, SnPP treatment partially reversed the inhibitory effects of BL-induced AMPK activation on TNF-α production, as shown in Fig. 5B.

BL inhibits LPS-induced iNOS expression and NO production *via* AMPK activation and HO-1 expression. The free radical NO from iNOS activation has been implicated as an important inflammatory mediator in the process of macrophage-mediated inflammation, but uncontrolled/excess NO production by activated macrophages leads to the development of various inflammatory diseases.[21] The inhibition of NO production and/or iNOS expression, thus, is a promising strategy for reducing the potentially harmful pro-inflammatory activity of macrophages.[22] With this regard, we finally tested whether BL could also inhibit LPS-induced iNOS expression and NO production *via* AMPK activation and HO-1 expression. BL at 2 and 4 μM significantly inhibited LPS-induced iNOS expression (Fig. 6A) and NO production (Fig. 6C). These inhibitory effects of BL were almost completely abolished by CC and partially by SnPP (Fig. 6 B and D).

Discussion

The present study demonstrates that the well-known NQO1 substrate BL increased AMPK activation and HO-1 expression and also decreased LPS-induced TNF-α production and iNOS expression in RAW264.7 macrophages.

The enhancement of NADH oxidation by BL, a natural substrate of NQO1, in NQO1-present cells can stimulate AMPK activation.[17] In this study, we sought to investigate whether BL could also stimulate AMPK activation in RAW264.7 macrophages. Treatment with BL stimulated a concentration- and time-dependent increase in AMPK phosphorylation. Similarly, a concentration-dependent increase in the phosphorylation of ACC, a downstream target of AMPK,[19] was also observed when macrophages were exposed to BL, and was a good correlate of AMPK phosphorylation. Moreover, the NQO1-specific inhibitor DC almost completely blocked BL-induced AMPK phosphorylation, suggesting that BL may induce AMPK activation specifically by NQO1. These findings, therefore, indicate that BL is also capable of stimulating AMPK activation in macrophages, perhaps by enhancing NQO1 activity. The increased intracellular levels of NAD^+ have been reported to activate AMPK in skeletal muscle cells and cardiomyocytes,[23,24] but the underlying mechanism(s) is not well understood yet. Because the intracellular NAD^+-to-NADH ratio indicates the energy status of cells,[25] the increased intracellular NAD^+-to-NADH ratio that may result from enhanced NADH oxidation may mimic energy depletion status. Because the AMPK signaling pathway, a well-known energy sensing pathway, can be activated under energy depletion,[26] we speculate that BL-induced NADH oxidation in RAW264.7 macrophages may transiently activate AMPK to compensate for cellular energy depletion.

Next, we tested the effect of BL on HO-1 expression in

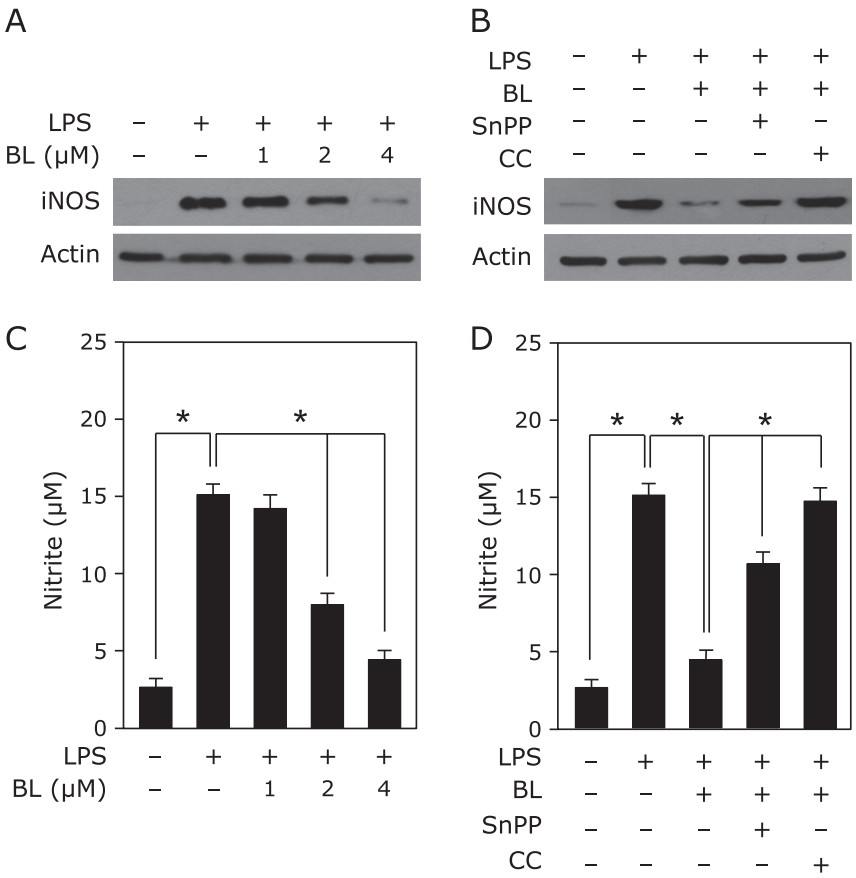

Fig. 6. Effects of BL on LPS-induced iNOS expression and NO production. RAW264.7 macrophages were pre-incubated for 12 h with indicated concentrations of BL (A and C) or with 4 μM BL in the absence or presence of 10 μM CC or 50 μM SnPP (B and D), and then activated for 6 h (A and B) or 18 h (C and D) with 1 μg/ml LPS. Western blot analysis for iNOS expression (A and B) and nitrite assay for NO production (C and D) were performed as described in the section of Materials and Methods. Blots shown are representative of three independent experiments. Data are expressed as means ± SE from 3 and 4 experiments. *p<0.05.

RAW264.7 macrophages. Treatment with BL stimulated a concentration- and time-dependent increase in HO-1 mRNA and protein expression. The induction of HO-1 by BL was dependent on AMPK activity, since CC, a pharmacological inhibitor of AMPK activation, prevented the increase in HO-1 expression. In support of this, the well-known AMPK activator AICAR was also capable of inducing HO-1 expression in macrophages, which is consistent with previous studies showing that AICAR stimulates HO-1 expression in human vascular cells[9] and rat pancreatic β-cells.[20] Our results, for the first time, indicate that BL can induce HO-1 expression through activation of AMPK in macrophages.

BL has been reported to exert anti-inflammatory properties.[18] In line with this, pretreatment of RAW264.7 macrophages with BL effectively inhibited LPS-induced production of the pro-inflammatory cytokine TNF-α and expression of the pro-inflammatory enzyme iNOS, of which activation results in excess production of the free radical NO. The inhibitory effects of BL were almost completely abolished by the AMPK inhibitor CC, implying that AMPK activation is essential for the anti-inflammatory effects of BL in macrophages. Consistent with this finding, other AMPK activators, such as metformin[7] and berberine,[8] have been shown to suppress pro-inflammatory responses in macrophages. However, it is unclear how AMPK activation can mediate anti-inflammatory responses, especially in BL-treated macrophages.

HO-1 expression has been shown to exert significant anti-inflammatory effects in a variety of cell types, including macrophages, and models of inflammatory disease.[12] In light with this, we explored the potential involvement of HO-1 in the anti-inflammatory effect of BL in RAW264.7 macrophages. BL markedly increased HO-1 expression by activating AMPK, and the expression correlated with the decrease in LPS-induced TNF-α production and iNOS expression. Inhibition of HO-1 activity by SnPP partly and not completely reversed the attenuation of pro-inflammatory cytokine release. Accordingly, these results strongly indicate that the anti-inflammatory effects of BL are associated, at least in part, with HO-1 expression. It should be noted that BL-induced AMPK activation, together with HO-1 pathway, might also activate other anti-inflammatory pathways that may be mediated by AMPK activation, and overall anti-inflammatory effects of BL could be achieved by virtue of the concerted actions of the multiple pathways being activated. The beneficial effects of HO-1 expression have been attributed to several factors, including the degradation of pro-oxidant heme, formation of biliverdin and/or bilirubin with their antioxidant properties, as well as the release of carbon monoxide, which has cytoprotective and anti-inflammatory effects.[12] Although the exact mechanisms involved in anti-inflammatory actions of the HO-1 system have not been fully elucidated, one or more of the HO-1 reaction products may mediate the anti-inflammatory effect of BL-induced AMPK activation under our experimental conditions.

In conclusion, the present study demonstrates that BL induces HO-1 expression in RAW264.7 macrophages *via* its activation of

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AMPK. In addition, we found that AMPK activation and HO-1 expression inhibits LPS-induced TNF-α production and iNOS expression. The ability of BL to induce AMPK activation and HO-1 expression may provide one of possible mechanisms by which BL, which increases the cellular NAD^+-to-NADH ratio *via* NQO1 activation,[17] can exert anti-inflammatory effects.

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Abbreviations

ACC	acetyl-coenzyme A carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1-β-*D*-ribofuranoside
AMPK	AMP-activated protein kinase
BL	β-lapachone
CC	compound C
DC	dicoumarol
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
HO-1	heme oxygenase-1
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
NO	nitric oxide
NQO1	NAD(P)H:quinone oxidoreductase 1
SnPP	tin protoporphyrin-IX
TNF-α	tumor necrosis factor-α

Conflict of Interest

No potential conflicts of interest were disclosed.

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