Berberine protects against cytokine-induced inflammation through multiple pathways in undifferentiated C2C12 myoblast cells

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Title: Berberine protects against cytokine-induced inflammation through multiple pathways in undifferentiated C2C12 myoblast cells

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Running title: Berberine reduces inflammation
Abstract

Obesity is associated with skeletal muscle insulin resistance and the development of metabolic syndrome. Undifferentiated skeletal muscle cells are sensitive to oxidative stress. Berberine hydrochloride (BBR) improves insulin resistance and exhibits anti-inflammatory properties. However, the underlying mechanism and the cell signaling pathways involved remain largely elusive. We therefore investigated the anti-inflammatory effects of BBR and the signaling pathways using skeletal C2C12 myoblast cells.

Undifferentiated C2C12 myoblast cells were treated with interleukin-1β (IL-1β) alone or in combination with tumor necrosis factor-α (TNF-α) in the presence or absence of BBR.

We found that BBR reduced the cytokine-induced expression of inducible nitric oxide synthase (iNOS) and stress-related kinases including, p-38 mitogen-activated protein kinase (p38 MAPK), NF-kB, and Stress-activated protein kinases /Jun amino-terminal kinases (SAPK/JNK) in C2C12 myoblast cells. Furthermore, BBR reversed cytokine-mediated suppression of AMP-activated protein kinase (AMPK-α), Sirtuin-1(SIRT-1), and PPAR-γ coactivator 1-α (PGC-1α). In addition, cytokine induced reduction of mitochondrial marker proteins and function were rescued after BBR treatment. Catalase, an antioxidant enzyme, was elevated after BBR treatment.

Our results demonstrate that BBR ameliorates cytokine-induced inflammation. The anti-inflammatory effect of BBR in skeletal progenitor cells is mediated through pathways including activation of the AMPKα–SIRT-1-PGC-1 α, inhibition of the Mitogen-activated protein kinase kinase 4 (MKK4)-SAP/JUNK-C-JUN as well as protection of mitochondrial bioenergetics. BBR may be a potential medication for metabolic syndrome.
Key words: Berberine, cytokine, insulin resistance, inflammation
**Introduction**

The epidemic of obesity is a global public health problem. Obesity is associated with several diseases including insulin resistance, hypertension, cardiovascular disease, and cancer. Obesity-induced infiltration of macrophages in adipose tissue produce several pro-inflammatory cytokines, or adipokines, such as tissue necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukine-6 (IL-6), and interleukine-1β (IL1-β) (Cao 2014). These cytokines play important roles in the regulation of systemic glucose and lipid metabolism, and favor the initiation and acceleration of insulin resistance (Cao 2014).

Berberine hydrochloride [18, 5, 6-dyhydro-9, 10-dimethoxybenzo (g)-1, 3 benzodioxolo (5.6-a) quinolizinium] (BBR), an isoquinolone alkaloid found in various herb plants including *Coptis Chinensis*, has drawn great attention in recent years. Anti-hyperglycemia, anti-dyslipidemia, and anti-inflammatory effects of BBR have been reported (Guo et al. 2016). Furthermore, BBR has been shown to down-regulate TNF-α, MCP-1, Interleukine-6 (IL-6), IL-1β, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in LPS, free fatty acid, and H₂O₂-treated macrophages (Jeong et al. 2009; Zhou et al. 2007). BBR is also reported to suppress the phosphorylation of p38-MAPKs, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and reduce reactive oxygen species (ROS) content in macrophages (Jeong et al. 2009). More recently BBR has been shown to ameliorate obesity-associated inflammatory response, glucose intolerance, and systemic insulin resistance (Guo et al. 2016). Several studies have demonstrated the positive modulatory effect of BBR on glucose and lipid metabolism in the liver and adipocytes of high fat diet fed animals (Brusq et al. 2006; Yin et al. 2008). Activation
of anti-oxidant and anti-inflammatory signaling pathways may contribute to the anti-diabetic and
insulin sensitive effect of BBR in those tissues (Gomes et al. 2012; Lee et al. 2006). However,
the specific underlying mechanisms and cell signaling pathways that mediate the anti-
inflammatory effect of BBR remain largely elusive.

Skeletal muscle insulin resistance, which contributes to the development of metabolic
syndrome, precedes hepatic insulin resistance and may also contribute to a predisposition for
non-alcoholic fatty liver disease (NALFD) (Petersen et al. 2007). Moreover, skeletal muscle
progenitor cells have a much lower respiratory capacity and a higher sensitivity to oxidative
stress. These undifferentiated skeletal muscle cells are therefore more sensitive to lipid or
cytokine induced toxicity than differentiated myotubes (Patková et al. 2014). The present study
therefore examined the anti-inflammatory effect of BBR and its potential underlying mechanism
using undifferentiated C2C12 myoblasts.
Materials and Method

Reagents: IL-1β and TNF-α were purchased from R&D Biosystems (Minneapolis, MN) and reconstituted as recommended. SIRT-1, phosphor-AMPKα, AMPKα, phosphor-NF-kβ, NF-kβ, PPARα, P-SAP/JNK, catalase, phosphor-C-Jun, C-Jun, SMAD4, phosphor-MKK4, MKK4, nuclear P-38 –MAPK, and total MAPK antibodies were purchased from Cell Signaling, Inc (MA). iNOS, Lamin B1, Caspases-3, PGC-1α, total oxphos cocktail, and citrate synthase antibodies were purchased from Abcam. RIPA lysis buffer and EDTA was from Thermo-scientific. C2C12 myoblast cells were purchased from ATCC (Manassas, VA). BBR and all other reagents were purchased from Sigma, and were at the highest purity.

Cell culture: C2C12 myoblast cells were grown and maintained in DMEM complete growth medium containing 4.5 g/L D-glucose and L-Glutamine, and 110 mg/L sodium pyruvate supplemented with 10% FBS and 1% penicillin and streptomycin. For cytokine treatments, cells were grown in six-well plates to 70% confluence, and then treated with IL-1β (10 ng/mL) alone or in combination with TNF- α (50 ng/mL) in the presence or absence of 10 µM BBR for 24 hours [Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2006; Guo et al. 2016]. At a concentration of 10µM, BBR didn’t exhibit toxicity in C2C12 myotubes [Xu et al. 2014].

Nuclear and cytosolic protein fraction isolation: Nuclear and cytosolic fractions were extracted as described [Zhou et al. 2007]. Briefly, cytoplasmic extraction buffer consisted of 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1mM DTT and 1 mM PMSF, adjusted to pH 7.6. Nuclear extraction buffer composed of 20 mM Tris Cl, 420 mM NaCl, 1.5 mM
MgCl2, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol, adjusted to pH 8.0. 6 x 10⁶ cells were resuspended in 150 µL of ice-cold cytoplasmic extraction buffer, incubated on ice followed by centrifugation. Supernatants were transferred to a clean micro-centrifuge tube on ice. The remaining pellet was gently re-suspended in cytoplasmic fraction wash buffer and centrifuged at 4°C. Remaining pellet were then re-suspended nuclear extraction buffer. The purity of cytoplasmic and nuclear fraction, and equal protein loading was confirmed by quantifying GAPDH and Laminin B protein levels in fractions.

**Western blotting:** Whole cell protein lysate were extracted using RIPA lysis buffer containing of protease inhibitor cocktail, sodium orothovanadate, EDTA and phenylmethylsulfonyl fluoride.. Protein concentration estimation was performed using Bradford protein assay. Forty µg proteins per sample were used for the SDS-PAGE. After electrophoresis, protein were transferred onto nitrocellulose membrane followed by blocking and incubation with primary and secondary antibodies. Chemiluminescence detection was performed using Luminata forte (EMD Millipore,). Imaging was done in LiCOR Odyssey platform. β-actin or GAPDH were used as loading control.

**Mitochondrial function:** Mitochondrial function was measured as oxidative phosphorylation in digitonin-permeabilized cells using high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as described (Doerrier et al. 2018; Ye et al. 2013). Briefly, 1 million cells were suspended in 2 ml Mir 05 buffer (0.6 mil cells/ml) at 37°C, Cell membrane was permeabilized with digitonin at a final concentration of 2 µM. Oxidative phosphorylation was measured in the presence of a combination of substrates supporting complex I (pyruvate+malate, 2.5 mM and 2 mM, respectively) and II (succinate, 10 mM) respiration.
Oxidative phosphorylation (OXPHOS) capacity was measured in the presence of 2.5 mM ADP (maximally ADP-stimulated O₂ flux). The electron transfer capacity was assessed as non-coupled state of maximal respiration with manual titration of the uncoupler carbonylcyanide-p-trifluoromethoxy-phenylhydrazone, FCCP at 0.5 M increment. FCCP collapses the proton motive force across the mitochondrial inner membrane thus stimulating maximal mitochondrial respiration. Residual oxygen consumption was recorded after sequential addition of 75 nM complex I-inhibitor Rotenone and 125 nM complex III-inhibitor antimycin A, and was subtracted from the other respiratory rates. Absolute respiration values were normalized for the total number of cells per chamber. Data was recorded using the DatLab software 5.1.0.20 (Oroboros Instruments).

**Data analysis:** All statistical analyses were performed using Graph Pad Prism 7. Blots presented are representation of at least three experiments. Group comparisons were done with Student’s unaired t-test or one-way ANOVA followed by Bonferroni post hoc testing.
Results

**BBR reduces NF-kB nuclear translocation in C2C12 myoblast cells**

Activation of the NF-kB pathway contributes to chronic inflammatory and autoimmune disease, and is linked to obesity, metabolic disorders, and diabetes (Kim et al. 2013). IL-1β and TNF-α are known to activate NF-kB pathway in several cell types (Beg et al. 1993; Green et al. 2011). To further evaluate the anti-inflammation pathways impacted by BBR, we first investigated its effect on NF-kB phosphorylation. Undifferentiated C2C12 myoblast cells were treated with 10 ng/mL IL-1β alone or in combination with 50 ng/mL TNF-α for 24 hr in the presence or absence of 10 µM BBR. An enhanced inflammatory response to cytokines was observed in cells treated with IL-1β either alone (increased by 47.2% compared to control cell, P < 0.05) or in combination with TNF-α (increased by 162% compared to control cells, p< 0.05) as evidenced by increased phosphorylated NF-kB level (Fig 1A). Co-incubation with BBR markedly abrogated the cytokine-induced inflammatory response. The combination of IL-1β and TNF-α treatment significantly induced nuclear translocation of NF-kB (induced by 47.2 % compared to control cell, p<0.05), and BBR significantly reduced cytokine-induced nuclear translocation of NF-kB in C2C12 myoblast cells (reduced by 55.9% compared to cytokines treated cells, p<0.01) (Fig 1B). However, the cytosolic distribution of NF-kB was not affected by either cytokine or BBR treatment (Fig 1C). Our findings suggest that the inhibitory effect of BBR on NF-kB activity is mediated by decreasing its nuclear migration in C2C12 cells.
BBR ameliorates cytokine-induced upregulation of SAPK/JNK pathway in C2C12 myoblast cells

Stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) cell signaling is an important inflammatory pathway involved in the development of insulin resistance in skeletal muscle (Henstridge et al. 2012). Both IL-1β and TNF-α stimulate the SAPK/JNK pathway and further activate NF-kB in skeletal muscle (Chen et al. 2002). We therefore investigated whether BBR may regulate the SAPK/JNK pathway. As shown in Fig 2A, IL-1β, either alone or in combination with TNF-α, significantly induced the phosphorylation of SAPK/JNK (increased by 111.2% compared to control cells, p< 0.05) (Fig 2A), which was significantly alleviated by the BBR treatment (reduced by 23.8% and 48.2% in IL1-β and cytokine combination treated cell, respectively, p<0.05).

We next examined the downstream signaling pathway of SAPK/JNK. Activation of SAPK/JNK and its nuclear translocation leads to activation of transcription factors including C-JUN and SMAD4 (Sprowles et al. 2005). In addition, SMAD4 plays a diverse role in inflammation and metabolic syndrome through the activation of various signaling pathways (Tan et al. 2012). We show in Figure 2 (B and C) that the phosphorylation of C-JUN and SMAD4 was significantly increased in IL-1β alone (increased 10 fold in C-JUN, and 11.5 fold in SMAD4, compared to control cells, p<0.05) or in combination with TNF-α treatment (increased 11.5 fold with C-JUN p<0.05 and increased 2.08 fold with SMAD4, p<0.05). BBR significantly decreased the cytokine-induced activation of both proteins.

The effect of BBR on the upstream SAPK/JNK pathway was also explored in our study. MAP kinases M KK4 and M KK7, two upstream SAPK/JNK pathways of mitogen-activated protein
kinase (MAPKase), are known to activate the JNK signal in vitro. Preferentially, JNK is phosphorylated on Tyr by MKK4 and on Thr by MKK7 (Tournier et al. 2001). Our study revealed that IL-1β in combination with TNFα significantly induces MKK4 phosphorylation in C2C12 cells (induced by 159% compared to control cell, p < 0.01). BBR co-treatment attenuated the cytokine-induced MKK4 phosphorylation (reduced by 47.5%, p<0.05, n=4) (Fig 2D). However, the expression of MKK7 was not affected (data not shown). Taken together, the anti-inflammatory effect of BBR may be mediated through inhibition of MKK4-SAP/JNK-C-JUN cell signaling pathway in C2C12 myoblast cells.

**BBR attenuates cytokine-induced nuclear translocation of p38 MAPK in C2C12 myoblast cells**

P38 MAPK is upregulated in response to extracellular stimuli such as inflammatory cytokines and growth factors (Zarubin and Han 2005). P38 MAPK resides in the cytoplasm of resting cells and translocates into the nucleus upon activation through phosphorylation. Inhibition of P38 MAPK results in decreased cytokine expression in skeletal muscle (Brown et al. 2015). Importantly, BBR has been reported to suppress p38 MAPK expression in several cell and tissue types (Cui et al. 2009; Jeong et al. 2009). We therefore examined the effect of BBR on cytokine-induced p38 MAPK nuclear translocation. Neither IL-1β nor BBR alone affected the nuclear translocation of the P38 MAPK. However, the combination of IL-1β and TNF-α led to a significant increase in nuclear translocation of p38 MAPK in C2C12 cells (by 91%, p<0.01) (Fig 3A). Importantly, BBR significantly attenuated this effect by 37.4% (p<0.05) (Fig 3A). Neither BBR nor combined cytokines affected cytosolic p38 MAPK expression (Fig 3B). In conclusion,
BBR reduced cytokines-induced nuclear translocation of the P38 MAPK in C2C12 myoblast cells.

**BBR attenuates cytokine-induced inflammation and activates anti-oxidative response in C2C12 myoblast cells.**

Uncontrolled production of reactive oxygen and nitrogen species (ROS, RNS) results in oxidative stress that contributes to insulin resistance. ROS activate several stress-related kinases including NF-kB, p38 MAPK, and JNK in skeletal muscle (Martins et al. 2012). IL-1β, either alone or in combination with TNF-α, has been shown to stimulate nitric oxide production in various cell types (Adams et al. 2002; Sprague and Khalil 2009). We therefore investigated the effect of BBR on iNOS-1 expression after IL-1β and TNFα treatment in C2C12 cells. Consistent with previous findings (Adams et al. 2002), IL-1β in combination with TNFα significantly induced iNOS expression (increased by 30.4%, p<0.05) (Fig 4A). BBR co-treatment attenuated cytokine-induced upregulation of iNOS by 28.8% (p<0.05) (Fig 4A). IL-1β alone did not significantly affect iNOS expression. Antioxidant pathways were also examined BBR upregulated catalase, an antioxidative enzyme, in both control and cytokine treated cells (Fig 4C), suggesting that BBR’s protective effect is mediated, at least in part, by an improvement of anti-oxidative defense in C2C12 myoblast cells.

**BBR promotes mitochondrial metabolism in cytokine treated C2C12 myoblast cells through AMPKα-SIRT1-PGC1a pathway.**
It has been suggested that AMP-activated protein kinase (AMPKα) may be an effective therapeutic target for alleviating insulin resistance and preventing T2D (Zhang et al. 2009), and has been reported to be activated by BBR in skeletal muscle (Brusq et al. 2006; Lee et al. 2006). Activation of AMPK by AMP analogs reduced gluconeogenesis and increased GLUT-4 expression and glucose uptake in skeletal muscle (Zheng et al. 2001). Furthermore, AMPK ameliorated inflammation through inhibition of NF-kB (Salminen et al. 2011) and iNOS activity (Pilon et al. 2004). We therefore examined AMPK signaling in C2C12 cells. We found that IL-1β alone or in combination with TNFα significantly suppressed phosphorylation of AMPKα at Thr-172 (Fig 5A). Their effects were synergistic, as the combination of both cytokines reduced the phosphorylation of AMPKα by 58%, (P<0.05). Cytokine-induced AMPK-α suppression was restored by BBR treatment (increased by 48.3%, compared to cytokine treated cells, p <0.05), indicating that AMPKα is an important target for the protective effect of BBR. Peroxisome proliferator-activated receptor-α (PPAR-α) is considered a BBR target and key regulator that enhances oxidative metabolism in skeletal muscle and adipose tissue (Zhou and Zhou 2007; Zhou et al. 2008). As shown in figure 5B, cytokines alone did not affect the PPAR-α expression; however, PPAR-α protein expression was elevated in C2C12 cells when co-incubated with BBR (Fig 5B). NAD+-dependent deacetylase, SIRT-1, and AMPKα activated each other to form a nutrient sensing network that promoted mitochondrial metabolism. In addition, SIRT-1 may have anti-inflammatory properties as it inhibits NF-kB activity in various cell types (Yang et al. 2012). The regulation of mitochondrial function and mass is governed by PGC-1α which is deacetylated and activated by SIRT-1 (Coste et al. 2008). We therefore examined the effect of BBR treatment on the SIRT-1-PGC-1α pathway. Our results indicate that treatment with the combination of cytokines inhibited PGC-1α by 11.3% (p<0.05) in C2C12 cells.
myoblast, and co-incubation with BBR ameliorated cytokine induced inhibition of PGC-1α (p<0.05) (Fig 5C). SIRT-1 expression was also improved by the BBR treatment (Fig 5D). Taken together, BBR stimulates mitochondrial biogenesis signal through the AMPK α-SIRT-1-PGC1α pathways.

We then investigated the mitochondrial density by assessing the amount of mitochondrial marker proteins. We found that subunits of electron transport chain complexes I, II, III and V, and the mitochondrial matrix enzyme, citrate synthase were decreased by cytokine exposure, indicating that inflammatory mediators are damaging to mitochondria, and decrease the cellular mitochondrial density. BBR treatment restored all markers of mitochondrial density (Fig 5E).

Mitochondrial function was measured as oxidative phosphorylation while the oxidation of energy substrates is coupled with ADP phosphorylation to generate ATP. We found that treatment of undifferentiated C2C12 myoblasts cells with IL-1β in combination with TNF-α causes a significant decrease in both OXPHOS capacity (maximally ADP-stimulated O$_2$ flux) and ET capacity (noncoupled state of maximal respiration). BBR has no individual effect on C2C12 myoblasts, while it improves both respiratory rates when added to cells incubated with the cytokine combination (Fig 5F).


**Discussion**

Pro-inflammatory cytokines such as IL-1β and TNF-α play important roles in inducing insulin resistance in skeletal muscle (Shoelson et al. 2006). BBR has been shown to be an effective therapeutic approach to treat insulin resistance and T2D (Pang et al. 2015). BBR has anti-inflammatory effects in macrophages, hepatocytes, and adipocytes (Choi 2016; Lou et al. 2011).

In addition, studies have revealed that BBR improves high fat diet induced hyperglycemia, glucose intolerance, and hepatic steatosis (Guo et al. 2016). However, very few studies have been done regarding the underlying mechanisms by which BBR regulates this anti-inflammatory response and promotes insulin sensitivity in skeletal muscle, particularly in undifferentiated skeletal muscle cells that are especially sensitive to oxidative stress. Our study revealed that BBR suppresses cytokine-induced inflammation through complex mechanisms in C2C12 myoblasts. Specifically, BBR activates the AMPKα-SIRT-1-PGC1α and SAP/JUNK-C-JUN pathways, and inhibits the NF-kB nuclear translocation. Importantly, BBR improves mitochondrial function in undifferentiated skeletal muscle cells, which have a lower mitochondrial respiratory capacity.

The NF-kB pathway is proposed as a critical target for the anti-inflammatory activity of BBR (Li et al. 2014). However, previous studies were designed using LPS and STZ to induce inflammation. Very few studies have been done using cytokines to trigger inflammation in
skeletal muscle, especially in skeletal muscle progenitor cells. IL-1β, either alone or in combination with TNF-α, triggers a strong inflammatory response in C2C12 myoblasts, including iNOS overexpression, NF-kB translocation, and activation of stress related kinases such as SAP/JNK and p38 MAPK, which are known to impair insulin sensitivity in differentiated C2C12 cells (Zhang et al. 2010). Nuclear translocation of P38-MAPK and NF-κB in response to cytokine treatment contributes to the development of insulin resistance (Berdichevsky et al. 2010; Zhang et al. 2010). BBR treatment reduced this negative response in our study and these results clearly indicate the anti-inflammatory and anti-oxidative role of BBR in cytokine induced inflammation in undifferentiated C2C12 cells. Our study demonstrated that cytokine induced inflammation is mediated through MKK4-SAP/JUNK-C-JUN and SMAD4 cell signaling pathways in C2C12 myoblasts. Importantly, BBR reduced all the cell signaling involved in this pathway. We identified the activation MKK4-SAP/Junk-C-Jun and SMAD 4 cell signaling pathways as important mechanisms in the cytokine induced inflammatory response in skeletal muscle progenitor cells. The components of this pathway may be therapeutic targets of BBR in treating insulin resistance.

Cytokines suppress AMPKα activity through activation of NF-kB and its target genes in skeletal muscle (Yuan et al. 2001). In contrast, the activated AMPKα reduces endoplasmic reticulum stress and inflammatory response to reactive oxygen species (Lu et al. 2010). Some studies have shown that BBR directly binds to and activates AMPKα and further inhibits stress activated kinases in macrophages, microglia, L6 my tube, and mouse skeletal muscle (Jeong et al. 2009; Lee et al. 2006). In contrast, another study suggests that BBR decreases obesity-associated inflammation in adipose tissue independently of AMPK activation (Guo et al. 2016). Our study...
showed that the effect of BBR in alleviating cytokine-induced inflammation in skeletal muscle progenitor cells is dependent on reversing AMPK inhibition. Around 80% of insulin-stimulated whole body glucose metabolism occurs in skeletal muscle. Therefore, skeletal muscle insulin resistance plays a crucial role in the development of metabolic syndrome (Petersen et al. 2007). Mitochondria, which predominately contribute to the oxidative capacity of skeletal muscle, play important roles in developing insulin resistance and hyperglycemia (Gomes et al. 2012). SIRT-1 promotes mitochondrial biogenesis (Gomes et al. 2012) via lysine deacetylation and activation of the AMPK upstream kinase, LKB1. We found that exposure to inflammatory cytokines leads to a decrease in mitochondrial marker proteins and oxidative phosphorylation rates, and that both were reversed by BBR treatment. Our results are in agreement with the concept that BBR treatment increases SIRT-1 activity and suppresses inflammation (Zhang et al. 2017), and further advances the field by identifying a novel role of BBR in preserving oxidative metabolism in skeletal progenitor myocytes via the AMPK-SIRT1-PGC1α cell signaling pathway. Although neither IL-1β nor TNF-α alone reduced PGC1-α expression, the combination of cytokines produced a synergistic effect on this transcription factor. It has also been suggested that PGC-1α is involved in the regulation of systemic and skeletal muscle inflammation (Correia et al. 2015), and prolonged exposure to proinflammatory stimuli reduces PGC-1α expression in skeletal muscle and cultured muscle cells (Remels et al. 2013; Yu et al. 2000). Cytokine incubation caused a dramatic decrease in mitochondrial markers proteins in C2C12 myoblasts indicating a decrease in mitochondrial mass, which is restored by BBR treatment, in congruence with the disinhibition of the AMPK-SIRT1-PGC1α pathway. The approximate 70% inhibition of mitochondrial function induced by cytokines was alleviated by the BBR treatment. We observed that OXPHOS capacity (maximally ADP-stimulated O2 flux) was lower than the ET capacity.
(noncoupled state of maximal respiration) in undifferentiated C2C12 myoblasts cells cultured in control media. The uncoupler, FCCP, collapses the inner membrane proton and eliminates the control of the phosphorylation apparatus (ATP synthase, phosphate carrier, adenine nucleotide translocase) on mitochondrial oxidative phosphorylation. Our data indicate that the phosphorylation apparatus contributes to the flux control in C2C12 myoblasts incubated in control media. The control of the phosphorylation apparatus on mitochondrial respiration is not affected by BBR, is lost in C2C12 myoblasts incubated with cytokine combination, and is recovered by the BBR treatment. We used a physiological substrate combination sustaining complex I (pyruvate+malate generating NADH) and II (succinate) respiration, which supports maximal ET and OXPHOS capacities due to the summative effect on supplying electrons to coenzyme Q. Therefore, our approach reveals that cytokines decrease mitochondrial function rather than identifying the specific site(s) of the mitochondrial defect. The decrease in mitochondrial function in C2C12 myoblasts matches the partially drop in mitochondrial mass.

Cytokines have been known to induce endoplasmic reticulum (ER) stress in pancreatic β-cells (Brozzi et al. 2015). Activation of JNK initiates IL-1β mediated ER stress and subsequently mediates ER-mitochondrial interaction in pancreatic cells (Verma and Datta 2010). BBR is also reported to slow the progression of non fatty liver disease in db/db mice through inhibition of ER stress (Zhang et al. 2016). In addition, BBR reduces pro-inflammatory ER stress in human intestinal epithelial cells (Hao et al. 2012). It is not clear whether proinflammatory cytokines induce ER stress in skeletal muscle, however, the possibility that BBR reduces proinflammaotry cytokine induced ER stress in skeletal muscle was not examined in the current study.
In conclusion, our study suggests that BBR ameliorates cytokine-induced activation of multiple signaling pathways in C2C12 myoblasts. The protective effect of BBR is mediated through activation of anti-oxidant and anti-inflammatory pathways, and improvement of mitochondrial metabolism. Our study provides new insight into the underlying mechanisms of BBR in treating insulin resistance, and suggests a therapeutic potential of BBR against insulin resistance and metabolic syndrome.
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Disclosure: The authors declare no conflict of interests.
Figure legends

Figure 1. BBR attenuates cytokine induced NF-kB expression in C2C12 myoblast.

C2C12 myoblasts were incubated with recombinant IL-1β (10 ng/mL) alone or in combination
with TNF-α (50 ng/mL) for 24 hours with or without BBR (10 μM). Western blotting assessed
the protein expression of NF-kB. The results of western blotting are representative of three
independent experiments. Phosphorylated NF-kB protein (A), nuclear NF-kB protein (B),
cytosolic localization of total NF-kB (C). *P<0.05, **P<0.01.

Figure 2. BBR suppresses cytokine-induced activation of SAP/JNK pathway in C2C12 myoblast.

C2C12 myoblasts were incubated with recombinant IL-1β (10 ng/mL) alone or in combination
with TNF-α (50 ng/mL) for 24 hours with or without BBR (10 μM). Phosphorylation of
SAP/JNK (A) and C-JUN (B), SMAD4 (C) and MKK4 expression (D) were measured by
western blotting. The results of western blotting are shown representative of four to five
independent experiments. *P<0.05, **P<0.01

Figure 3. BBR suppresses cytokine-induced activation of p38 MAPK pathway C2C12 myoblast.
C2C12 myoblasts were incubated with recombinant IL-1β (10 ng/mL) alone or in combination with TNF-α (50 ng/mL) for 24 hours with or without BBR (10 μM). The expression of p38 was measured by nuclear (A) and cytosolic (B) localization of total p38 by western blotting. The results of western blotting are shown representative of four independent experiments. * P<0.05, ** P<0.01

Figure 4. BBR reduces cytokine-induced inflammation and activate anti-oxidative mechanism in C2C12 myoblast.

C2C12 myoblasts were incubated with recombinant IL-1β (10 ng/mL) alone or in combination with TNF-α (50 ng/mL) for 24 hours with or without BBR (10 μM). The protein expression of iNOS (A), caspases-3 (B), catalase (C) were assessed by western blotting. The results of western blotting are shown representative of four independent experiments. * P<0.05, ** P<0.01

Figure 5. BBR promotes mitochondrial metabolism in cytokine treated C2C12 myoblast cells through AMPKα-SIRT1-PGC1-α pathway.

C2C12 myoblasts were incubated with recombinant IL-1β (10 ng/mL) alone or in combination with TNF-α (50 ng/mL) for 24 hours with or without BBR (10 μM). The expression of AMPKα (A), PPARα (B), PGC-1α (C), Sirt-1 (D), Mitochondrial marker proteins (E) were assessed by western blotting. Mitochondrial oxidative phosphorylation (F) was assessed as maximally ADP-stimulated O₂ flux capacity (OXPHOS) and noncoupled maximal electron transfer capacity (ET).
The results of western blotting are shown representative of four independent experiments. Respiratory measurements were performed in two-three independent experiments. *$P<0.05$

Figure 6. Cell signaling pathways that involved in the anti-inflammatory of BBR

Pro-inflammatory cytokine IL-1β and TNFα increase ROS production that leads to activation of stress-activated kinases such as p38, SAPK/JNK and NF-kB. NF-kB in turn activates iNOS. BBR directly reduce ROS production, inhibit stress related kinases and activates anti-oxidant system. BBR also improves the mitochondrial function and promotes the activation of AMPKα, SIRT-1 and PPARα, which results in the inhibition of inflammatory pathway by blocking iNOS and NF-kB expression.
References


Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
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Figure 6.