Betaine Promotes LKB1-AMPK Activation Inhibits UVB-Mediated Senescence of Human Epidermal Keratinocytes Through Autophagy Induction

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Abstract
Betaine demonstrates antioxidative activity, enhances organic osmolytic activity and is an important cofactor in methylation. However, the main mechanisms betaine-induced autophagy in human dermal skin cells are not yet completely understood. Therefore, we hypothesized that betaine induces autophagy. Thus, the autophagic effects exerted by betaine through activation of LKB1-AMPK signaling in human dermal fibroblasts (HDFs) and human epidermal keratinocytes (HEKs) were assessed. Betaine enhanced LKB1 and AMPK phosphorylation in HDFs, and LKB1, AMPK induced autophagy through mTOR downregulation. Betaine-induced autophagy was inhibited in cells transiently transfected with AMPK siRNA. Increased autophagosome activity was confirmed by LC3-II formation and by increased perinuclear LC3-II puncta in betaine-treated HEKs. According to our in vitro findings, and in vivo studies in HR-1 hairless mice demonstrated that betaine treatment significantly reduced the activity of the senescence-associated marker β-galactosidase (SA-β-gal) and increased p-LKB1 and p-AMPK levels compared with UVB-irradiated skin tissues. Collectively, our findings suggest that betaine-dependent autophagy diminishes mouse skin senescence and betaine may reduce HEK senescence through an LKB1-AMPK-dependent mechanism.

Keywords: Betaine; LKB1; AMPK; Autophagy; Senescence

Introduction
Aging is the major risk factor for cancer, cardiovascular disease, diabetes, and neurodegenerative disorders [1]. Several types of normal tissue and tumor cells undergo premature senescence after exposure to oxidative stress, UV radiation, and DNA damaging agents [2,3]. Autophagy, a cellular degradation pathway important in the turnover of whole organelles and long-lived proteins [4]. It is a primordial and highly conserved intracellular process that occurs in most eukaryotic cells and participates in stress management. This pathway involves the de novo formation of vesicles called autophagosomes, which can engulf entire regions of the cytoplasm, individual organelles, protein aggregates, and invading pathogens [5]. Autophagy has received more attention during the aging process because the proteasome pathway could not degrade protein aggregates in the presence of an enhanced pro-oxidant and aggregation-prone milieu, which is characteristic of aging and injured organelles [6,7]. Several lines of evidence suggest a role for AMP-activated protein kinase (AMPK), in autophagy induction, and AMPK binds to ULK1 (the mammalian homolog of yeast Atg1), and this interaction is mediated through autophagy [8-10]. Numerous reports have proposed that AMPK is regulated by upstream kinases of which the tumor suppressor, LKB1 [11,12]. The LKB1-AMPK energy pathway is a straight and secondary regulator of autophagy because it affects cell proliferation and apoptotic cell death during metabolic stress [9]. As a central regulator of protein synthesis, mTOR has to integrate a wide range of intracellular and extracellular signals to control protein translation, autophagy and cell growth. Also, mTOR can be reactivated after prolonged starvation by the autolysosomal products generated by autophagy. mTOR inhibits autophagy as part of a negative feedback loop to prevent cells from eating themselves to death. Thus, autophagy may actually regulate mTOR. [8,9,13]. Therefore, the LKB1-AMPK pathway is thought to be a significant controller of autophagy in response to nutrient status [14]. Betaine is a indeed occurring compound that is widely distributed in plants and medicinal herbs, particularly Lycium chinense, which has been established to have high levels of betaine [15]. Betaine has been described to be useful for several conditions and diseases, containing heart and liver disease [16,17]. During selection photoprotective agents based on antioxidant activity, our previous investigation focused on determining whether betaine, which exhibits antioxidant properties, may be applied for photoprotection, in addition to its known effect in traditional medicines [18]. However, the precise mechanism underlying the autophagic effects of LKB1-AMPK signaling pathway and the effects of betaine in human epidermal keratinocytes (HEKs) remain unidentified. A central focus of these studies is to regulate whether betaine can induce AMPK activation in HEKs. We furthermore examined whether betaine-induced AMPK activation can increase autophagy initiation in senescent cells and a mouse model. Now, we determine the accurate roles of autophagy facilitated signaling in HEKs and the contribution of the LKB1-AMPK signaling pathways in betaine-induced autophagy in senescent HEKs.

Materials and Methods

Reagents
Betaine (B2629), rapamycin (R8781), compound C (171260), 3-methyladenine (3-MA) (M9281) and bafilomycin A1 (11701) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HR-1 hairless mice (6-weeks old) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HR-1 hairless mice (6-weeks old) were purchased from Japan SLC (Shizuoka, Japan). HR-1 hairless mice (6-weeks old) were purchased from Japan SLC (Shizuoka, Japan).

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Received March 21, 2018; Accepted April 24, 2018; Published April 27, 2018


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pan). Antibodies specific for p-LKB1 (Ser 428) (#3482), p-AMPK α (Thr 172) (#2531), p-mTOR (Ser 2448) (#2971), p-70S6K (Thr389) (#9822), Beclin-1 (#3738), LC3B-II (#2757), LKB1 (#3050), AMPKα (p#2532), and mTOR (p#2972) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for β-actin (ab8229), p53 (ab90363), and p21 (ab227443) were purchased from Abcam (Cambridge, UK). DRAQ5, and a Senescence β-Galactosidase Staining Kit (SA-β-gal) were obtained from Cell Signaling Technology (Beverly, MA, USA). LKB1 siRNA (3 target sequence, 5’-GCCAACCGUGAGAAGGAAAT-3’ and 5’-UUCCUUCCUUUCAC-GUUGGCTT-3’. 5’-GGGUGUUAACAGCATT-3’ and 5’-UUCCGUUGUUAACACACCTT-3’. 5’-CCAAAGGCGGUUG-UAGAAAT-3’ and 5’-UUCAUAACACCGCCUGUGTGT-3’) and control siRNA (nonspecific off-target effects of siRNA) were purchased from Sigma-Aldrich unless otherwise indicated.

**Cell culture**

HEKs were purchased from Lonza (Walkersville, MD, USA) and maintained in KGM-Gold™ SingleQuots™ medium containing supplements and growth factors. The human dermal fibroblast (HDF) cell line was obtained from ScienCell (Carlsbad, CA, USA). HDFs were propagated in fibroblast medium supplemented with 5% FBS, 1% fibroblast growth supplement, and 1% penicillin and streptomycin (P/S). The human liver cell (HEP-G2) line was purchased from American Type Culture Collection (Manassas, VA, USA). HEP-G2 cells were cultured in DMEM containing 100 U/ml P/S, 10% FBS, and 3 mM glutamine. These cell lines were maintained in a humidified 5% CO₂ incubator at 37°C.

**RNA interference**

Small interfering RNAs (siRNA) against LKB1, and AMPKα, and control siRNA (Santa Cruz, CA, USA) were transiently transfected into cells using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Aliquots of 1 × 10⁴ cells/well were plated in 6-well plates on the day before transfection and grown to approximately 70% confluence. The cells were then transfected with 10 μM LKB1, AMPKα, or control siRNA plus 100 pmol of Lipofectamine for 5 h in Opti-MEM™ reduced serum medium (Invitrogen, Carlsbad, CA, USA). Following an incubation period of 24 h, the protein levels were measured by western blot analysis.

**UVB radiation**

Cells were preincubated with the indicated concentration of betaine for 24 h, washed with PBS and exposed to a UVB (20 mJ/cm²) light source using a UVP cross linker (Ultra-Violet Products Ltd, Cambridge, UK) for 10 min. Afterwards, the cells were washed with PBS, DMEM was added, and cells were further incubated for 60 min.

**Immunofluorescence analysis**

HEKs were seeded on coverslips in 35-mm glass-bottomed dishes, fixed in 4% formaldehyde, and permeabilized with 0.2% Triton X-100. Primary anti-LKB1 antibody was used at 1:100 dilution and incubated with cells overnight at 4°C. Goat anti-rabbit FITC-labeled secondary antibody (Abcam, Cambridge, UK) was used at 1:100 dilution and incubated with cells for 45 min at room temperature. Nuclei were stained with DRAQ5 (Cell Signaling, Beverly, MA, USA) at 1:1000 dilution. Fixed and immunofluorescently stained cells were imaged using a confocal microscope (Olympus, FV10i, Tokyo, Japan).

**Western blotting**

Cell lysates were prepared from HEKs (5 × 10⁶/ml) in Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris (pH 6.8), 0.21 M sodium dodecyl sulfate, and 0.3 mM bromophenol blue) and boiled for 10 min. The protein content was measured using BCA protein assay reagent (Pierce, Waltham, MA, USA). Additionally, proteins were extracted from the mouse skin tissue samples using a PreCellys 24 homogenization system (Bertin Technologies, Montigny-le-Bretonneux, France). The protein content was measured using BCA protein assay reagent (Pierce, Waltham, MA, USA). Protein samples (20 μg) were diluted with 1x lysis buffer, separated by electrophoresis (4.5% - 15% gradient), and transferred onto PVDF membranes. The membrane was then incubated with primary antibodies against p-LKB1 (1:1000), p-AMPKα (1:1000), LC3B-II (1:1000), Beclin-1 (1:1000), p-mTOR (1:1000), and β-actin (1:1000) overnight. Subsequently, the membranes were incubated with horseradish peroxide-conjugated secondary antibodies, and immunocomplexes were detected using an enhanced chemiluminescence detection system from Merck-Millipore (Beverly, MA, USA). Protein expression levels were determined by analysis of the signals captured using an image analyzer (Las-3000, Fujifilm, Tokyo, Japan).

**Senescence Associated β-Galactosidase (SA-β-gal) Staining**

SA-β-gal staining was performed as previously described. Briefly, the HEKs were seeded in 6-well plates and fixed with 4% formaldehyde for 5 min at room temperature. The cells were then washed with PBS and incubated with SA-β-gal staining solution (1 mg/ml of X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂, in 40 mM citric acid/sodium phosphate buffer (pH 6.0) for 16 h at 37°C at room temperature. The SA-β-gal- stained cells were then incubated with 1× lysis buffer, separated by electrophoresis (4.5% - 15% gradient), and transferred onto PVDF membranes. The membranes were then incubated with primary antibodies against p-LKB1 (1:1000), p-AMPKα (1:1000), LC3B-II (1:1000), Beclin-1 (1:1000), p-mTOR (1:1000), and β-actin (1:1000) overnight. Subsequently, the membranes were incubated with horseradish peroxide-conjugated secondary antibodies, and immunocomplexes were detected using an enhanced chemiluminescence detection system from Merck-Millipore (Beverly, MA, USA). Protein expression levels were determined by analysis of the signals captured using an image analyzer (Las-3000, Fujifilm, Tokyo, Japan).

**Experimental animals**

HR-1 hairless mice were acclimated for one week prior to experiments. All experimental protocols were approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee (11-061: Daejeon, Korea). The mice were housed under a constant temperature in an atmosphere containing 50% relative humidity with a 12 h light/dark cycle and provided access to food and water ad libitum. The mice were divided into the following three groups: Control (n=5), UVB-treated vehicle (n=5) and UVB-treated betaine (n=5). The mice in the UVB-treated vehicle and UVB-treated betaine groups were exposed to UV irradiation. Control mice were not exposed to irradiation. The mice in the UVB-treated betaine group were orally administered orally with 0.1 ml of water containing 100 mg betaine/kg body weight/day. As a control, the mice in the UVB-treated vehicle group were provided drinking water only, whereas animals in the control group received no treatments.

**UVB irradiation**

Mice were exposed to UVB irradiation using a UVM-225D Mineralight UV Display Lamp, which emitted radiation at a wavelength of 302 nm. The UVB irradiation was applied to the back of the mice three times each week for 12 weeks. The level of irradiation was progressively increased from 60 mJ/cm²/exposure at week 1 to 90 mJ/cm²/exposure at week 7.

**Hematoxylin and Eosin (H&E) staining**

H&E staining was performed as previously described. Briefly, mouse skins were fixed in 10% buffered formalin, embedded in
paraffin, and cut into 4μm thick sections. The sections were placed on glass slides, deparaffinized, and stained with H&E solution (Sigma, St. Louis, MO, USA).

Histological evaluation

Sections were incubated at 4°C overnight with an anti-β-gal (Abcam, MA, USA) antibody and then incubated with a biotinylated secondary antibody (Vector laboratories, CA, USA) for 1 h at room temperature. Following the addition of the detection system, the reaction was visualized using diaminobenzidine (DAB; Vector laboratories) as a substrate. Images of each section were captured and analyzed using ImageJ software (ImageJ 46a; NIH, USA). The β-gal-positive area was analyzed in 5 randomly selected fields (magnification, 200) from each section of mouse tissue, and the data are expressed as β-gal-positive area per total area.

Statistical analysis

The data are presented as the mean ± standard deviation (SD) of at least three separate experiments. Comparisons between two groups were made using the Student’s t-test in Graph Pad Software (San Diego, CA, USA); significance was established at \( P < 0.05 \).

Results

The LKB1-AMPK pathway is involved in betaine-induced autophagy in HEKs

Exposure to betaine for 12 h produced a dose-dependent increase in LKB1 and AMPK phosphorylation in HEKs (Figure 1A). Compared with control cells, betaine caused an increase in mutually LKB1 and AMPK phosphorylation (Figure 1B). Prior studies known LKB1 as a kinase upstream of AMPK. Because betaine treatment activated AMPK, we next studied whether betaine would affect LKB1-AMPK phosphorylation in fibroblast and HepG2 cells. Treatment with betaine increased the phosphorylation levels of LKB1 and AMPK over those of the control. Additionally, betaine treatment increased the protein expression levels of LC3B-II and Beclin-1, marks of autophagy, in fibroblast and HepG2 cells (Figure 1C). We confirmed examined whether betaine-induced LKB1 translocation. Our data showed that enhanced cytosolic localization of LKB1 is the mechanism responsible for betaine-motivated AMPK activation in HEK (Figure 1D). These results showed that betaine induces autophagy through the LKB1-AMPK pathway in HEKs.

Betaine prevents mTOR signaling through the LKB1-AMPK pathway in HEKs

mTOR adversely regulates autophagy therefore, we assessed whether mTOR signaling is involved in the betaine-induced activation of the LKB1-AMPK pathway. Western blot analysis showed that mTOR phosphorylation downregulated in betaine-treated cells. Moreover, phosphorylation of p70S6K, which is a downstream target of mTOR was downregulated by betaine (Figure 2A). Furthermore, as shown in Figure 2B, we found that transfection with siRNA targeting LKB1 decreased mTOR phosphorylation. We next observed the properties of LKB1 siRNA in senescent HEKs cells. As shown in Figure 2C, HEKs transfected with control siRNA exhibited a reduced number of SA-
β-gal-positive cells, and loss of LKB1 attenuated the betaine-induced decrease in SA-β-gal-positive cells. These results indicate that the activation of the LKB1-AMPK pathway induced by betaine regulates mTOR phosphorylation in HEKs.

Betaine provokes autophagy is mediated through AMPK activation

We established that the autophagic effect of betaine was facilitated through AMPK, we observed the effect of AMPK siRNA on betaine-induced autophagy induction. Betaine-induced LC3B-II and Beclin-1 protein levels were lower in cells transfected with AMPK targeted siRNA than in control siRNA-transfected cells (Figure 3A). Moreover, treatment with compound C, a specific AMPK inhibitor, reduced betaine-induced autophagy in HEKs (Figure 3B). These results indicate that AMPK plays a role in betaine-induced autophagy in HEKs.

3-MA suppresses autophagy progression in HEKs

To confirm that whether betaine-induced autophagy is characterizable, we evaluate expression of LC3B-II protein. During the autophagic progression, LC3B-II is focus in autophagosomes, and cytosolic punctate labeled LC3B-II fluorescence can serve as an indicator of autophagy. As shown in Figure 4A we found enriched LC3B-II expression in cells treated with betaine. Additionally, the numbers of LC3B-II puncta were decreased after treatment with 3-MA (initial autophagy inhibitor). Moreover, we measured the LC3B-II and Beclin-1 proteins levels via western blotting (Figure 4B) and found that 3-MA inhibited LC3B-II conversion. These results show that betaine induces autophagy in HEKs.

Bafilomycin A1 enlarged autophagy development in HEKs

We further demonstrate that whether the numbers of LC3B-II puncta were increased by bafilomycin A1 (Baf A1; a late autophagy inhibitor). Our data that the numbers of LC3B-II puncta was amplified in the cells treated with betaine and were more increased by bafilomycin A1 exposure (Figure 5A). Additionally, we assess the levels of LC3B-II and Beclin-1 proteins using western blotting (Figure 5B) and found that bafilomycin A1 increased LC3B-II conversion. These results show that betaine induces autophagy during Baf A1-treatment of HEKs.

Betaine-cause AMPK activation increases autophagy stimulation in senescent mouse Skin

Histological analyses revealed no morphological abnormalities in the skin of control mice. By contrast, skins from mice exposed to UVB irradiation exhibited epidermal hyperplasia and an increased epidermal thickness. Notably, betaine treatment reversed the morphological effects of UVB exposure, restoring histological features similar to those in the control mouse skin tissues (Figure 6A). We examined whether UVB light induced cellular senescence in dermis using immunohistochemistry to detect β-gal and found that betaine administration of betaine significantly reduced the proportion of senescent cells induced by UVB exposure (Figure 6B). Furthermore, using western blotting analysis, we confirmed that betaine-induced LKB1-AMPK activation increased autophagy induction in UVB-exposed mouse skin tissues using western blotting analysis. Betaine reduced p53, and p21 expression in the UVB-treated mouse skin tissue. Additionally, betaine significantly increased p-LKB1 and p-AMPKα expression compared with UVB-treated mouse skin tissue (Figure 6C). These results indicate that betaine-induced AMPK activation inhibits senescence caused by exposure to UVB radiation through autophagy induction in mouse skins tissue.
Figure 3: AMPK plays a vital role in betaine-induced autophagy. (A) HEKs were transfected with control siRNA or AMPK α siRNA and then treated with betaine (20 μM) for 8 h. (B) HEKs were pretreated with compound C 2 μM (comp C) for 30 min and then treated with betaine (20 μM) for 8 h. HEKs were subjected to western blot analysis to determine the levels of p-AMPK, LC3B-II and Beclin-1 proteins. The data from three independent experiments are shown. *P<0.05, versus the control. # P<0.05, versus the control.

Figure 4: Effect of 3-MA on betaine-induced autophagy. (A) HEKs were pretreated with 3-MA (5 μM) for 1 h and then treated with betaine (20 μM) for 8 h. Cells were observed with a confocal microscope for LC3B-II puncta (B) The LC3B-II puncta/cell were counted and are presented. HEKs were treated with betaine (20 μM) or pretreated with 3-MA for 1 h and then treated with betaine (20 μM) for 8 h. The protein expression of LC3B-II, Beclin-1 and β-actin protein expressions was determined by western blot analysis. **P<0.01, versus the control; *P<0.05, versus the control. *P<0.05, versus betaine alone.
Figure 5: Effect of bafilomycin A1 on betaine-induced autophagy (A) HEKs were pretreated with Baf A1 (500 nM) for 1 h and then treated with betaine (20 μM) for 8 h. Cells were observed with a confocal microscope for LC3B-II puncta (B) The LC3B-II puncta/cell were counted and are presented. HEKs were treated with betaine (20 μM) or pretreated with Baf A1 for 1 h and then treated with betaine (20 μM) for 8 h. LC3-B, Beclin-1 and β-actin protein expression was determined by western blot analysis. *P<0.05, versus the control **P<0.01, versus betaine alone. #P<0.05, versus betaine alone.

Figure 6: Betaine-induced AMPK activation prevents skin senescence induced by UVB radiation (A) Representative skin sections from UVB radiation-treated and UV + betaine-treated hairless mice. All samples were subjected for H&E staining. Magnification 400×. (B) Representative skin sections from UVB radiation-treated and UVB + betaine-treated hairless mice. All samples were stained for β-gal staining. Magnification 400×. *P<0.05 versus the control; #P<0.05 for the betaine-treated group versus the group treated with UVB radiation alone (C) Protein extracts from mouse skin tissues were subjected western blotting analysis using antibodies for p-LKB1, p-AMPKα, p53, and p21 expression. The data from three independent experiments are shown. *P<0.05, versus the control; *P<0.05, versus the UVB radiation group.
Discussion

Autophagy is a catabolic pathway utilized by cells for bulk turnover of long-lived proteins and organelles via lysosomal degradation. Autophagy is even closely related to senescence, aging, skin color formation, and also displays the ability of anti-stress and anti-infection. Most notably, it distinctly functions as the switch for controlling the cell death, as sometimes autophagy presents a cytoprotective talent, but it shifts to a fatal mechanism in some conditions [19]. On the other hand, autophagy acts as a tumor suppressor to cellular damaged organelles and accumulated growth factors and maintain chromosomal stability and autophagy process be manipulated to improve anticancer therapeutics [20,21]. In relative to the regulation of autophagy by polyphenolic compounds as a potential therapeutic strategy for cancer and cancer type specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target [22,23].

In the current study, we examined the mechanisms by which betaine induces autophagy in senescent HEKs and revealed that betaine induces autophagy of the cells through LKB1-AMPK signaling pathways. Our results showed that betaine increased LKB1 and AMPK phosphorylation in a dose-dependent manner. Also, betaine induced LC3B-II cleavage through the LKB1-AMPK pathways in fibroblast and HepG2 cells (Figure 1). This result was similar to that reported in a previous study, which demonstrated that genistein exerted antioxidant effects via AMPK activation [24]. LKB1 and AMP-activated protein kinase control of mTOR signaling and growth [14]. And our present results suggest that betaine inhibited mTOR signaling through the LKB1-AMPK pathway in HEKs and cell with transient LKB1 siRNA knockdown exhibited an enhanced number of betaine-diminished SA-β-gal-positive cells (Figure 2). Moreover, AMPK inhibition by compound C and AMPK siRNA restrain betaine-induced autophagy induction (Figure 3). These results exhibited that betaine-induced autophagy induction through activation of AMPK. Thus, we examined whether betaine induces autophagy in HEKs. Current studies have reported the observation of numerous amino acid and mTORCI were autophagy cross talk and co-operation to control cellular homeostasis [25,26]. Our findings suggest that the 3-MA-mediated a decrease in the number of LC3B puncta (Figure 4). In addition, betaine further induced autophagy during Baf A1 treatment and increased the number of LC3-positive puncta observed in cells (Figure 5). Besides, senescence-associated loss of AMPK activity was contributing to the interrupted mitochondrial function [27]. The biomarkers of cellular senescence activated by UVB irradiation include continuing cell cycle arrest, enlarged and flattened cell morphology, and SA-β-gal activity [28]. SA-β-gal activity has been measured in a variety of cells and tissues to exhibit the onset of cellular senescence [28,29].

Conclusion

In the current study, betaine treatment reversed the morphological effects of UVB exposure, restoring histological features similar to those in the control mouse skin tissues. SA-β-gal staining confirmed that betaine significantly reduced the proportion of senescent cells induced by UVB exposure. Additionally, betaine increased LKB1 and AMPKa phosphorylation in skin tissues (Figure 6). Together, these findings suggest that betaine inhibits the cellular senescence of cells in skin tissues and that modulation of AMPK activation leads to autophagy pathways. Our findings suggest that betaine-induced AMPK activation increases autophagy and protects against UVB-induced cells damage and cellular senescence as demonstrated SA-β-gal activity [30]. These results support the idea that modulate of betaine-induced autophagy could be a treatment strategy for senescence.

Acknowledgments

This work was supported by a grant (K18300) from the Korea Institute of Oriental Medicine (KIOM).

Conflict of Interest

The authors do not have any conflict of interest to disclose.

References


