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20-*O*-β-D-Glucopyranosyl-20(S)-Protopanaxadiol Suppresses UV-Induced MMP-1 Expression Through AMPK-Mediated mTOR Inhibition as a Downstream of the PKA-LKB1 Pathway

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ABSTRACT

Various health effects have been attributed to the ginsenoside metabolite 20-*O*-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD); however, its effect on ultraviolet (UV)-induced matrix metalloproteinase (MMP)-1 expression and the mechanism underlying this effect are unknown. We examined the inhibitory effect of GPD on UV-induced MMP-1 expression and its mechanisms in human dermal fibroblasts (HDFs). GPD attenuated UV-induced MMP-1 expression in HDFs and suppressed the UV-induced phosphorylation of mammalian target of rapamycin (mTOR) and $p70^{S6K}$ without inhibiting the activity of phosphatidylinositol 3-kinase and Akt, which are well-known upstream kinases of mTOR. GPD augmented the phosphorylation of liver kinase B1 (LKB1) and adenosine monophosphate-activated protein kinase (AMPK), which are inhibitors of mTOR, to a greater extent than UV treatment alone. Similar to GPD, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR), an activator of AMPK, augmented UV-induced AMPK phosphorylation to a greater extent than UV treatment alone, resulting in the inhibition of MMP-1 expression. AICAR also decreased the phosphorylation of mTOR and $p70^{S6K}$. However, compound C, an antagonist of AMPK, increased MMP-1 expression. In HDF cells with AMPK knock-down using shRNA, MMP-1 expression was increased. These results indicate that AMPK activation plays a key role in MMP-1 suppression. Additionally, the cAMP-dependent protein kinase (PKA)

Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranosyl 5′-monophosphate; AMPK, AMP-activated protein kinase; GPD, 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol; HDFs, human dermal fibroblasts; LKB1, liver kinase B1; MMP-1, matrix metalloproteinase-1; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; UV, ultraviolet.

Dong Joo Shin, Jong-Eun Kim, and Tae-Gyu Lim contributed equally to this study.

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inhibitor, H-89, antagonized GPD-mediated MMP-1 suppression via the inhibition of LKB1. Our results suggest that the suppressive activity of GPD on UV-induced MMP-1 expression is due to the activation of AMPK as a downstream of the PKA-LKB1 pathway. J. Cell. Biochem. 115: 1702–1711, 2014.

KEY WORDS: AMPK; GINSENOSIDES; 20-*O*-β-D-GLUCOPYRANOSYL-20(S)-PROTOPANAXADIOL; mTOR; MMP-1; SKIN AGING; UV

Matrix metalloproteinases (MMPs) are endopeptidases that degrade extracellular matrix (ECM) components [Visse and Nagase, 2003]. MMPs play critical roles in tissue remodeling processes, such as morphogenesis and angiogenesis, and in tissue destructive processes, including tumor invasion and photoaging [Birkedal-Hansen, 1995; Fisher et al., 1996]. Among the MMPs, MMP-1 (collagenase I) functions as an interstitial collagenase and is the primary MMP that degrades native collagen [Welgus et al., 1985]. Ultraviolet (UV) promotes various pathological changes in skin such as sunburn and aging. The cleavage of collagen by UV-induced collagenases causes ECM breakdown in dermis, and this is implicated in the remodeling of connective tissues in various physiological and pathological situations, including photoaging [Birkedal-Hansen et al., 1993; Mauviel, 1993; Kahari and Saarialho-Kere, 1997; Brenneisen et al., 2002]. Therefore, UV-induced MMP-1 overexpression must be prevented in order to maintain ECM homeostasis [Brennan et al., 2003]. Previous studies using human dermal fibroblasts (HDFs) have suggested that the major mechanisms of UV-induced MMP-1 expression involve mitogen-activated protein kinases (MAPKs) [Moon et al., 2008; Shim et al., 2009; Yang et al., 2009] and phosphatidylinositol 3-kinase (PI3K)/Akt signaling [Oh et al., 2006].

The AMP-activated protein kinase (AMPK) cascade is a regulator and sensor of cellular energy status. Stresses that consume cellular ATP, such as oxidative stress, hypoxia, nutrient deprivation, and metabolic poisoning, can activate AMPK [Hardie, 2004]. AMPK is one of the most promising targets for the treatment of metabolic diseases such as cardiovascular disease, obesity, and type 2 diabetes [Oakhill et al., 2009]. In addition to its role in metabolic diseases, AMPK activation has been shown to have anti-cancer effects in a variety of human cancer cells [Hwang et al., 2006; Jin et al., 2007; Hadad et al., 2008; Johnson et al., 2008; Kim do et al., 2009a; Lee et al., 2009; Chiang et al., 2010]. In contrast, UV-induced AMPK activation has been shown to induce apoptosis in human immortalized keratinocytes (HaCaT cells) [Cao et al., 2008]. However, the role of AMPK activation in UV-induced MMP-1 expression remains unknown.

Activation of AMPK requires its phosphorylation by an upstream kinase at Thr-172 in the activation loop of the α-subunit catalytic domain [Jansen et al., 2009] . The most putative regulatory molecules of AMPK are liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) [Kahn et al., 2005]. LKB1 is activated through phosphorylation at Ser431 by cAMP-dependent protein kinase (PKA). Ca^{2+} can also activate AMPK via CaMK, independent of AMP level [Mihaylova and Shaw, 2011]. The mammalian target of rapamycin (mTOR), a possible downstream target of AMPK, is a crucial effector in cell signaling pathways known to be deregulated in cancer and diabetes [Sarbassov et al., 2005; Guertin and Sabatini, 2007]. Thus, the inhibition of signaling upstream of mTOR might offer new targets for therapeutic approaches in cancer aimed at regulating mTOR. Since AMPK hinders mTORC1 directly and indirectly, drugs that activate AMPK may be effective in treating cancer [Memmott and Dennis, 2009]. A relationship has been reported between mTOR and MMP-1 expression in HDFs. Rapamycin, an inhibitor of mTOR, reduces mTOR function and suppresses UV-induced MMP-1 expression through the inhibition of $p70^{S6K}$ in HDFs [Brenneisen et al., 2000b]. Furthermore, one previous paper has reported that treatment of rapamycin suppressed mTOR/$p70^{S6K}$ signaling pathway and subsequently MMP-1 mRNA transcriptional level was elevated by rapamycin [Lemaitre et al., 2011]. Thus, the inhibition of mTOR via AMPK activation could be used to prevent UV-induced MMP-1 expression.

Ginseng is one of the most widely used herbal medicines in Asia. Ginseng's efficacy is based on a group of triterpenoid saponins called ginsenosides. Orally administered Rb1, a protopanaxadiol-type ginsenoside, is metabolized by stomach acid and human intestinal bacteria to the ginsenosides Rg3, Rh2, and 20-*O*-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD) (Fig. 1) [Christensen, 2009]. GPD has been shown to exhibit anti-cancer [Kang et al., 2005; Chae et al., 2009; Choi and Choi, 2009], anti-inflammatory [Choo et al., 2008], and anti-diabetes activities (Fig. 1D) [Han et al., 2007; Kim do et al., 2009b]. However, the effect of GPD on UV-induced MMP-1 expression and its underlying molecular mechanisms are unknown.

Here, we report that GPD inhibits UV induced MMP-1 expression through AMPK activation and mTOR inhibition in HDFs. These findings further shed light into the mechanisms of action behind the anti-wrinkle effects of GPD.

MATERIALS AND METHODS

MATERIALS

The GPD (98%), Rb_1, Rg_3, and Rh_2 compounds were obtained from Fleton (Chengdu, China). Fetal bovine serum (FBS) and 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR) were purchased from Sigma-Aldrich (St. Louis, MO). Compound C and LY294002 were obtained from Calbiochem (San Diego, CA). H-89 was purchased from TOCRIS Bioscience (Ellisville, MO). Gentamicin and L-glutamine were purchased from Life Technologies, Inc. (Carlsbad, CA). Antibodies against phosphorylated JNK (Thr^{183}/Tyr^{185}), phosphorylated p38 (Thr^{180}/Tyr^{182}), total p38, phosphorylated Akt (Thr^{308}), total Akt, phosphorylated mTOR (Ser^{2448}), total mTOR, phosphorylated $p70^{S6K}$ (Thr^{389}), total $p70^{S6K}$, phosphorylated LKB1 (Ser^{428}), phosphorylated AMPK (Thr^{172}), phosphorylated CAMKII (T286), total CAMKII and total AMPK were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against total JNK, phosphorylated ERK1/2 (Tyr^{204}), total ERK, and total LKB1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against MMP-1 were purchased from Thermo Fisher Scientific (Fremont, CA). Active PI3K and Akt1 were obtained from Millipore (Billerica, MA). [γ-^{32}P] ATP and the

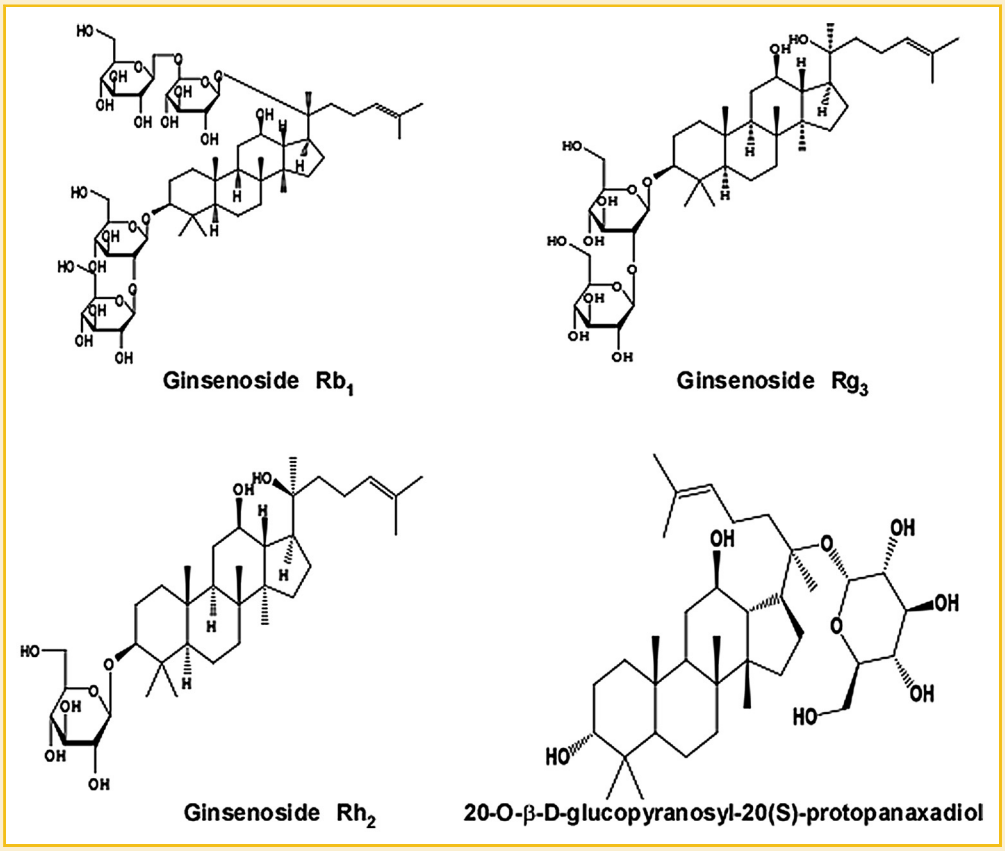

Fig. 1. Structures of the protopanaxadiol-type ginsenosides.

chemiluminescence detection kit were purchased from GE Healthcare Biosciences (Pittsburgh, PA).

CELL CULTURE

HDFs were kindly provided by Dr. Jin Ho Chung (College of Medicine, Seoul National University, Seoul, Korea). HDFs were cultured in monolayers at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mM L-glutamine.

UV IRRADIATION

A BioLink Crosslinker system (Vilber Lourmat, Marne-la-Vallée, France) with an emitting wavelength of 312 nm was used as a UV source. After serum starvation for 18 h, cells were pretreated with serum-free media containing relevant ginsenosides, chemicals, or inhibitors for 30 min or 1 h prior to UV treatment and then further cultured for various times, as indicated.

WESTERN BLOT ANALYSIS

Western blotting was performed as described previously [Lee et al., 2008]. Following culture in 6- or 10-cm dishes for 48 h, cells (5×10^4) were starved in serum-free DMEM for 18 h, then treated with chemicals for 1 h, and irradiated with 0.02 J/cm^2 of UV. The harvested cells were then disrupted in lysis buffer by vortexing and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit according to the manufacturer's instructions. The protein lysate was subjected to 6% or 10% SDS–PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Biosciences). After transfer, the membrane was blocked with skimmed milk (Sigma) for 2 h at room temperature. It was incubated overnight with specific primary antibodies at 4°C. Protein bands were visualized using a chemiluminescence detection kit (GE Healthcare Biosciences) after hybridization with a horseradish peroxidase (HRP)-conjugated secondary antibody. The bands were quantified by Image J (National Institutes of Health, MD)

GELATIN ZYMOGRAPHY

To determine the gelatinolytic activity of MMP-2 in the culture medium, equal amounts of the protein were subjected to gelatin zymography using zymogram gels containing 0.5 mg/ml gelatin as described previously [Jung et al., 2010]. Briefly, proteins were mixed with non-reducing sample buffer and then electrophoresed. The gels were rinsed with Zymogram Renaturing Buffer (Invitrogen, Carlsbad, CA) for 30 min, washed three times with Zymogram Developing Buffer (Invitrogen) for 30 min, and incubated for 24 h at 37°C. The gels were subsequently stained with a 0.5% Coomassie brilliant blue R-250 solution containing 20% methanol and 10% acetic acid for 30 min and destatined using 20% methanol and 10% acetic acid for 30 min. Areas of gelatinase activity appeared as clear bands against the blue-stained gelatin background.

CELL CYTOTOXICITY

To evaluate the cytotoxicity of ginsenosides, HDF cells were cultured to be confluent in 96-well plates. The cells were then treated with ginsenosides for 1 h and irradiated with 0.02 J/cm^2 of UV. After 6 h, cell viability was analyzed using Cell Titer96 Aqueous One Solution (Promega, Madison, WI) by incubating with 20 ml of MTS solution for 1 h at 37°C in a 5% CO_2 incubator. The absorbance was read at 492 nm.

REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Tel-Test, Inc., Friendswood, TX) following the manufacturer's instructions. The reverse transcription reaction was performed with the Superscript preamplification system (Life Technologies, Inc.). cDNA was synthesized by PrimeScript RTase (TAKARA, Otsu, Japan). Quantitative RT-PCR reaction was performed using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). cDNA was amplified in the presence of iQ(tm) SYBR(r) Green Supermix (Bio-Rad). The primers used for the amplification were: MMP-1 (NM 002424.2) sense, AGTGACTGGGAAACCAGATGCTGA; anti-sense, GCTCTTG-GCAAATCTGGCCTGTAA, GAPDH (NM 002046) sense, ATG-GAAATCCCATCACCATCTT; anti-sense, CGCCCCACTTGATTTTGG. To control variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed using comparative ΔΔCt method according to the manufacturer's instructions.

IN VITRO PI3K AND Akt KINASE ASSAYS

An in vitro PI3K assay was performed as described previously [Hwang et al., 2009]. Active PI3K (100 ng) was incubated with GPD or LY294002 for 10 min at 30°C. The mixtures were then incubated with 20 μl of 0.5 mg/ml phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) for 5 min at room temperature, followed by incubation with reaction buffer (100 mM HEPES [pH 7.6], 50 mM $MgCl_2$, and 250 μM ATP containing 10 μCi of [γ-^{32}P] ATP) for an additional 10 min at 30°C. The reaction was halted by adding 15 μl of 4 N HCl and 130 μl of chloroform:methanol (1:1). After vortexing, 30 μl of the lower chloroform phase was spotted onto a 1% potassium oxalate-coated silica gel plate that had been activated at 110°C for 1 h. The resulting ^{32}P-labeled phosphatidylinositol-3-phosphate (PI3P) was separated by thin layer chromatography (TLC); and radiolabeled spots were visualized by autoradiography.

An in vitro Akt kinase assay was performed in accordance with the instructions provided by Upstate Biotechnology. Briefly, the Akt kinase assay was performed with active Akt1, the modified crosstide contained in the assay buffer, and [γ-^{32}P] ATP solution diluted with magnesium acetate-ATP cocktail buffer. The Akt kinase assay mixtures were incubated at 30°C and then aliquots were transferred onto p81 paper and washed with 0.75% phosphoric acid. The incorporation of radioactivity was assessed using a scintillation counter. The effect of GPD (1 or 2 μM) was evaluated by incubating GPD with the Akt kinase reaction mixtures at 30°C for 10 min. Each experiment was performed in triplicate.

LENTIVIRAL INFECTION

The lentiviral expression vectors, including Gipz lentiviral shRNA for PRKAA1 (Gipz-shAMPKα) (RNAi Core Facility, BioMedical Genomic Center, University of Minnesota) and packaging vectors, including pMD2.0G and psPAX, were purchased from Addgene (Cambridge, MA). To prepare empty Gipz and Gipz-shAMPK viral particles, each viral vector and packaging vectors (pMD2.0G and psPAX) were transfected into HEK293T cells using JetPEI following the manufacturer's suggested protocols. The transfection medium was changed at 4 h after transfection and the cells were cultured for 36 h. The viral particles were harvested by filtration using a 0.45 mm sodium acetate syringe filter, then combined with 8 μg/ml of polybrane (Millipore) and infected into 60% confluent HDF cells overnight. The cell culture medium was replaced with fresh complete growth medium for 24 h and then the cells were selected with puromycin for 36 h (2 μg/ml of puromycin). The selected cells were used for the subsequent experiments.

STATISTICAL ANALYSIS

Data are expressed as the mean ± SD values. One-way analysis of variance (ANOVA) was used for comparisons in the experiments with multiple time points and concentrations. When ANOVA indicated statistical significance, Duncan's multiple range test was used to determine which means were significantly different. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

GPD EFFECTIVELY ATTENUATES UV-INDUCED MMP-1 EXPRESSION IN HDFs

To determine the most effective ginsenosides for repressing UV-induced MMP-1 expression, we evaluated the effects of several ginsenosides (Fig. 1) on UV-induced MMP-1 expression in HDFs. Our results show that GPD and Rg_3 significantly suppressed UV-induced MMP-1 expression, whereas Rb_1 and Rh_2 had little effect. Furthermore, GPD exerted the greatest inhibitory effect among the treatments. The suppressive effects of GPD and Rg_3 on UV-induced MMP-1 expression were approximately 80% and 50%, respectively, at 2 μM (Fig. 2A) without cytotoxic effect (Fig. 2B). Tissue inhibitors of metalloproteinase (TIMP) is another key factor of MMP-1 activity. There was no significant difference in the effect of ginsenosides on the expression of TIMP-1 (Suppl. Fig. 1). Therefore, we selected GPD for further study. First, we tested the effect of GPD on UV-induced MMP-1 protein and mRNA in a concentration-dependent manner. GPD inhibits UV-induced MMP-1 at both protein and mRNA levels (Fig. 2C,D), indicating these effects occur at transcriptional level.

GPD SUPPRESSES UV-INDUCED PHOSPHORYLATION OF THE mTOR/p70^{S6K} PATHWAY BUT NOT PI3K OR MAPKs

GPD exerted the strongest inhibitory effect on UV-induced MMP-1 expression in HDFs. We thus examined its molecular effects on UV-induced MMP-1 expression. The UV-activated JNKs, p38, and ERKs signaling cascades promote MMP induction [Moon et al., 2008; Shim et al., 2009; Yang et al., 2009]. We therefore investigated the effect of GPD on UV-induced phosphorylation of JNKs, p38, and ERKs in HDFs. GPD did not affect the UV-induced phosphorylation of JNKs, p38, or ERKs (Fig. 3A, Suppl. Fig. 2A). Since the PI3K pathway also functions as a regulator of MMP-1 [Brenneisen et al., 2000b;

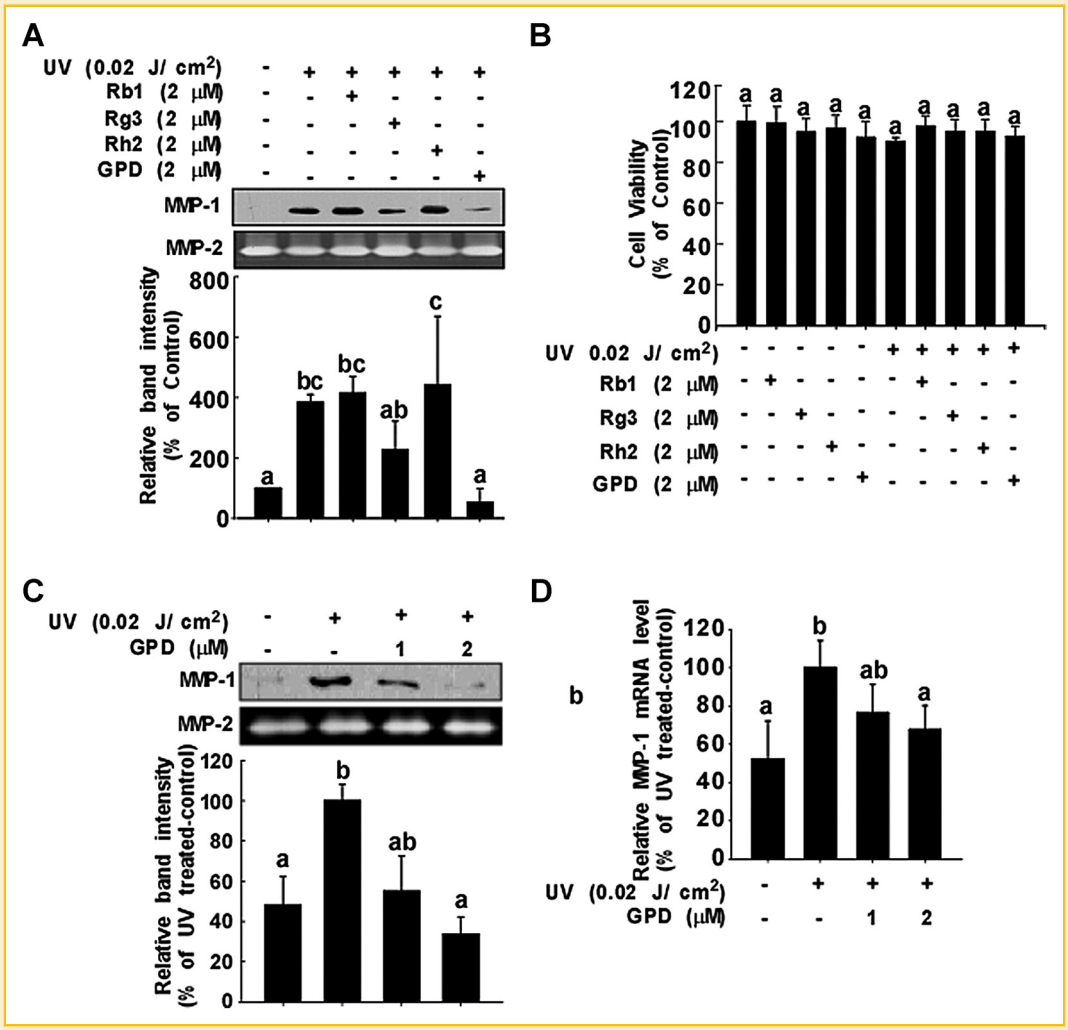

Fig. 2. GPD shows the greatest inhibitory effect on MMP-1 expression among the ginsenosides. A,B: Ginsenosides (2 μM) were added to HDFs cultures for 1 h before being exposed to 0.2 kJ/m^2 of UV and media was harvested 6 h later. Protein expression was analyzed by Western blotting (MMP-1) and zymography (MMP-2). Cell viability was analyzed as described in the Materials and Methods Section. The relative band intensity of MMP-1/MMP-2 was determined using the Image J program. C: GPD was treated at the indicated concentrations (in HDFs) for 1 h before being exposed to 0.2 kJ/m^2 of UV and media was harvested after 6 h. D: The mRNA was collected after 4 h. MMP-1 mRNA were measured as real-time PCR described in the Materials and Methods Section. Means with letters (a–c) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test.

Oh et al., 2006], we investigated the effect of GPD on the phosphorylation of Akt, mTOR, and p70^{S6K}. Our Western blot results show that GPD suppressed the phosphorylation of mTOR and p70^{S6K}, but not Akt (Thr^{308}) (Fig. 3B, Suppl. Fig. 2B–D). Since the PI3K/Akt pathway functions upstream of mTOR, we examined the effect of GPD on the in vitro kinase activities of Akt and PI3K. However, our results indicate that GPD did not inhibit the activity of Akt kinase or PI3K, an upstream kinase of Akt (Fig. 3C,D). Since the kinase activities of PI3K and Akt were not inhibited by GPD, we concluded that another upstream regulator of mTOR/p70^{S6K} may be involved.

AMPK-MEDIATED INHIBITORY EFFECTS ON MMP-1 EXPRESSION

AMPK can hinder mTOR signaling [Memmott and Dennis, 2009], which has suppressive effects on UV-induced MMP-1 expression [Brenneisen et al., 2000a]. Therefore, we hypothesized that AMPK activation by GPD could inhibit UV-induced MMP-1 expression through the dephosphorylation of mTOR. First, we verified that GPD increased the phosphorylation of AMPK in HDFs. The tumor suppressor kinase LKB1 is suggested as a possible upstream kinase that activates AMPK in the presence of GPD [Kim do et al., 2009b]. We examined the effects of GPD on the phosphorylation of LKB1 and AMPK in HDFs and found that GPD augmented the phosphorylation of AMPK and LKB1 more than UV treatment alone (Fig. 4A). GPD treatment without UV also increases phosphorylation of LKB1 and AMPK (Suppl. Fig. 3). To confirm the role of AMPK activation in UV-induced MMP-1 expression, we utilized AICAR, an activator of AMPK. Results indicate that AICAR inhibited UV-induced MMP-1 expression (Fig. 4B, Suppl. Fig. 4A). In UV-irradiated cells, AMPK phosphorylation was slightly increased and AICAR augmented UV-induced AMPK phosphorylation more than UV treatment alone. AICAR also decreased the phosphorylation of mTOR and p70^{S6K} (Fig. 4B). Since the activation of AMPK inhibited UV-induced

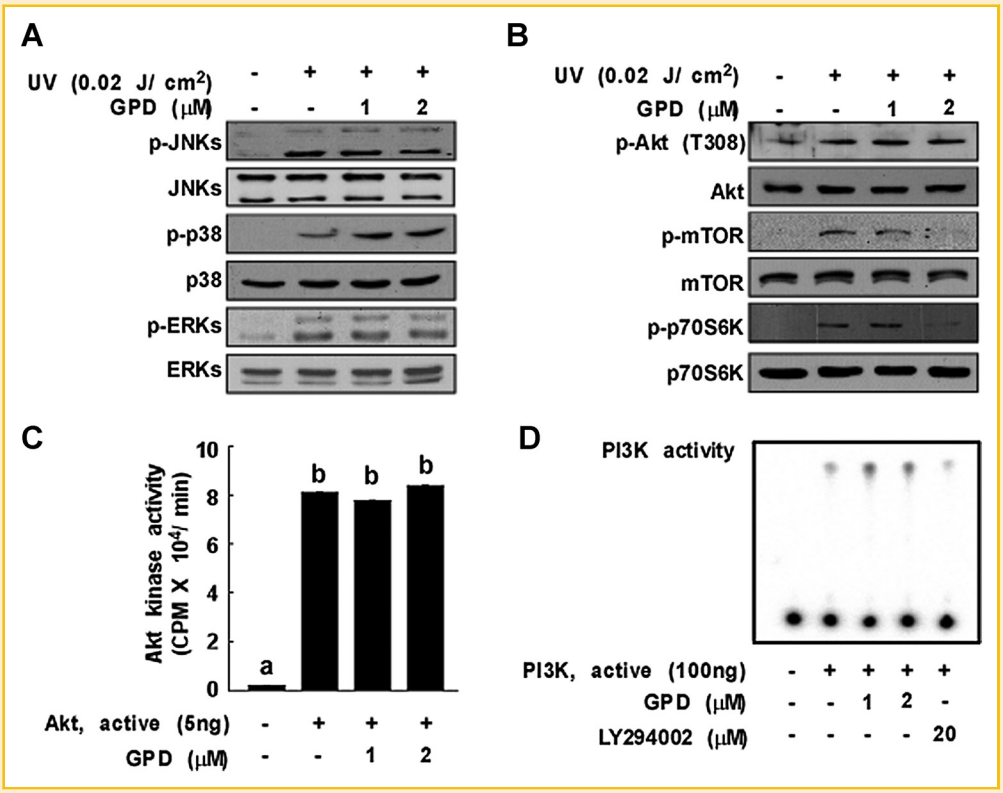

Fig. 3. GPD inhibits UV-induced MMP-1 expression through the mTOR/p70^{S6K} pathway. A,B: GPD was treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, and the cell lysates were harvested at 30 min after UV irradiation. The levels of phosphorylation and expression were determined by Western blot analysis using the indicated antibodies. C,D: GPD had no effect on Akt (C) or PI3K (D) kinase activity. Kinase assays were performed as described in the Materials and Methods Section. Means with letters (a–b) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test.

MMP-1 expression, we assumed that treatment of compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo-[1,5-a]-pyrimidine), a selective inhibitor of AMPK, and inhibition of AMPK expression using shAMPK would induce MMP-1 expression. Our results show that AMPK inhibition by chemical and genetic methods inhibited AMPK activity and induced MMP-1 expression (Fig. 4C,D). We then compared the effect of GPD between mock and shAMPK HDF groups (Fig. 4E). MMP-1 was increased in the shAMPK HDF group and MMP-1 was inhibited by GPD. However, GPD did not show inhibitory effects in shAMPK HDFs. Therefore, we concluded that the AMPK pathway is the major target pathway in the effect of GPD in HDFs.

EFFECTS OF H-89, A PKA INHIBITOR, ON GPD-INDUCED EFFECTS IN HDFs

We next focused on identifying the common upstream factor that regulates LKB1. PKA is reported to regulate LKB1 [Hutchinson et al., 2008]. Additionally, cAMP suppresses TNF-a-induced MMP-1 expression through PKA signaling [Park et al., 2010]. To investigate whether the PKA pathway is involved in GPD-mediated MMP-1 suppression, cultured HDFs were pretreated with the PKA inhibitor H-89 for 30 min at the indicated concentrations, followed by treatment with GPD for 30 min and UV stimulation. H-89 antagonized GPD-mediated MMP-1 suppression, indicating that PKA activity is critical for the effects of GPD on MMP-1 expression. Our results show that pretreatment with 5 μM H-89 completely inhibited GPD-induced MMP-1 suppression, and that pretreatment with 10 mM H-89 increased MMP-1 expression more than UV exposure (Fig. 5A). Furthermore, to confirm whether LKB1, a possible downstream target of PKA, is involved in the H-89-induced inhibitory effect on MMP-1 suppression, HDFs were pretreated with H-89 for 30 min, followed by treatment with or without GPD for 30 min and UV stimulation. Our results show that pretreatment with H-89 inhibited GPD and UV-induced LKB1 phosphorylation at a level similar to that of the controls (Fig. 5B). Since Ca^{2+}/calmodulin-dependent protein kinase (CaMK) is an alternative upstream kinase of AMPK [Kim do et al., 2009a], we studied its effect on GPD-induced MMP-1 suppression. However, STO-609, a CaMK inhibitor, did not inhibit the effects of GPD on UV-induced MMP-1 expression (Fig. 5C, Suppl. Fig. 4B) although it effectively inhibited phosphorylation of CaMK by UV (Suppl. Fig. 5). Taken together, these results suggest that the inhibition of MMP-1 expression by GPD is mediated by PKA, as an upstream kinase in the LKB1/AMPK pathway.

DISCUSSION

GPD exhibits anti-cancer, anti-inflammatory, and anti-diabetic effects via activation of JNK, caspase 8, and caspase 9, and inhibition of interleukin-1 receptor-associated kinase-1, phosphoenol-

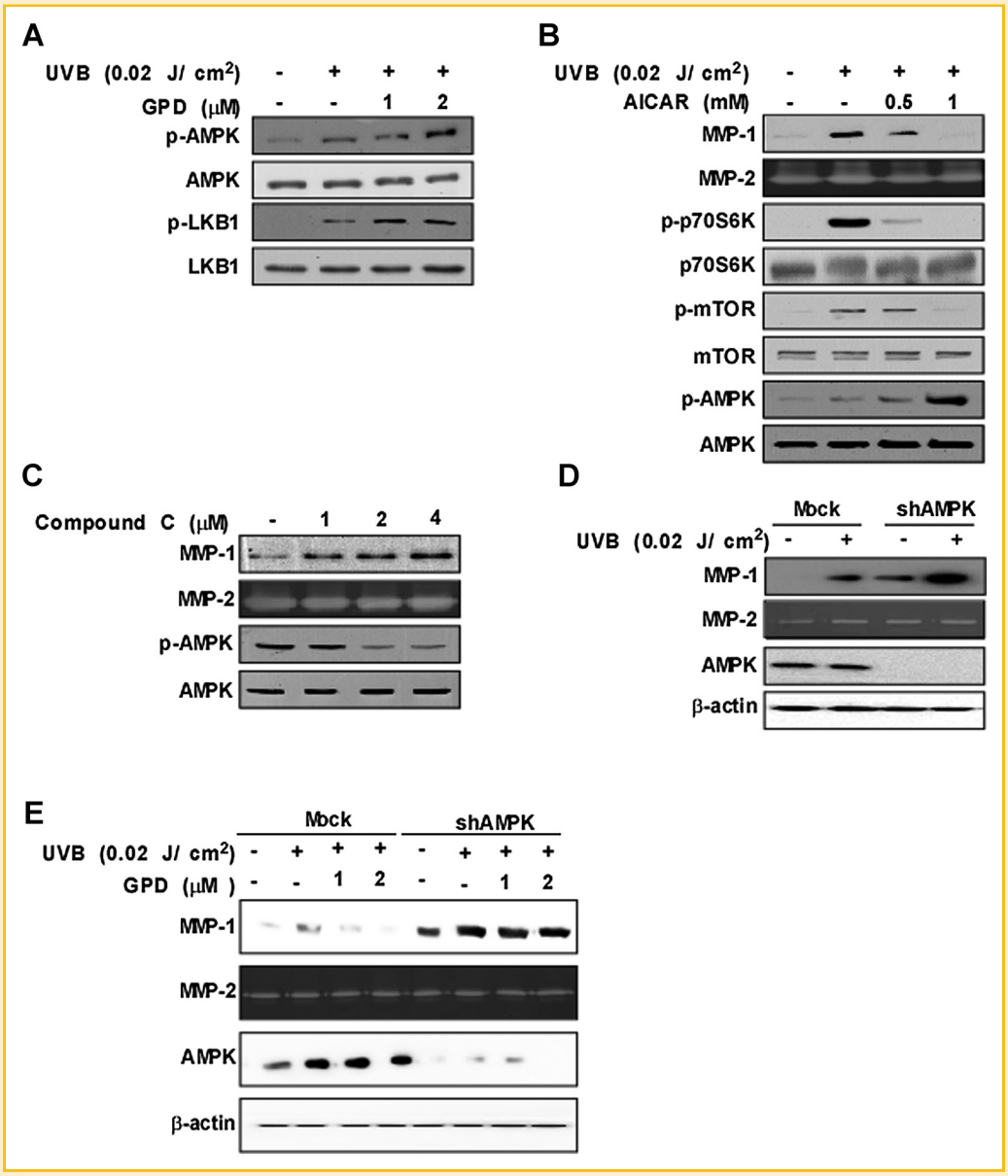

Fig. 4. GPD activates AMPK in HDFs. A: GPD was treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, and the cell lysates were harvested at 30 min after UV irradiation. B: AICAR was treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, cultured media was harvested at 6 h, and the cell lysate were prepared at 30 min after UV irradiation. C: Compound C was treated with the indicated concentrations in HDFs, cultured media were harvested at 6 h, and the cell lysate were prepared at 30 min after Compound C treatment. D,E: Knockdown of AMPK in HDFs was attained using lentiviral infection. 0.2 kJ/m^2 of UV was irradiated to HDFs, cultured media were harvested at 6 h, and the cell lysate were prepared at 30 min after UV irradiation (D). GPD was treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, cultured media were harvested at 6 h, and the cell lysate were prepared at 30 min after UV irradiation (E). MMP-1 and MMP-2 protein expression levels were measured as described in the Materials and Methods Section. The levels of phosphorylation and expression were determined by Western blot analysis using the indicated antibodies. The data are representative of more than four independent experiments that produced similar results.

pyruvate carboxykinase, and glucose-6-phosphatase [Joh et al., 2011; Li et al., 2012; Wang et al., 2012; Kim et al., 2013]. GPD inhibits UVA-induced MMP-1 expression in HDFs [He et al., 2011]. However, detailed molecular mechanisms involved in GPD action have not yet been revealed. In this study, we have shown that GPD inhibits UV-induced MMP-1 expression through transcriptional regulation, via AMPK activation and mTOR/p70^{S6K} inhibition following PKA/LKB1 activation in HDFs (Fig. 5D).

Whereas the classical activation of AMPK is dependent upon AMP concentration or AMP-to-ATP ratio (AMP-dependent activation) [Ferrer et al., 1985; Carling et al., 1989; Moore et al., 1991] , data from recent studies indicate the existence of other roles for AMPK beyond energy metabolism. AMPK is suggested to regulate the UV-induced stabilization of cyclooxygenase-2 mRNA in human keratinocytes [Zhang and Bowden, 2008]. AMPK also has the ability to counteract oxidative stress in the vascular endothelium [Colombo

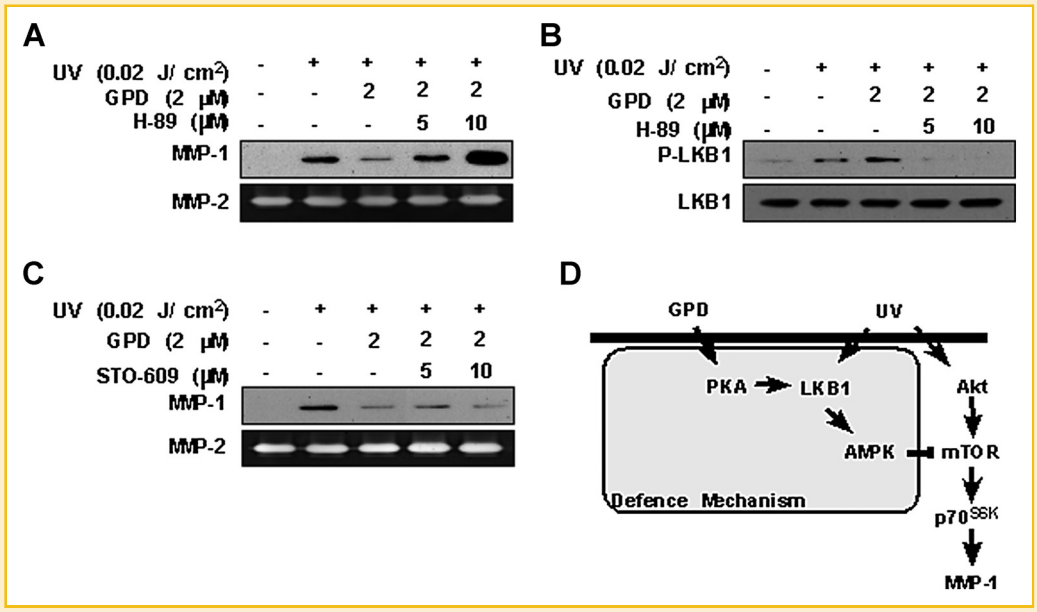

Fig. 5. Effect of the PKA inhibitor H-89 on the ability of GPD to suppress UV-induced MMP-1 expression. A,C: GPD, H-89, and STO-609 were treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, and cultured media were harvested at 6 h after UV irradiation. MMP-1 and MMP-2 expression was measured as described in the Materials and Methods Section. B: GPD and H-89 were treated with the indicated concentrations in HDFs for 1 h before being exposed to 0.2 kJ/m^2 of UV, and the cell lysates were prepared at 30 min after UV irradiation. The levels of phosphorylation and expression were determined by Western blot analysis using the indicated antibodies. The data are representative of more than four independent experiments that produced similar results. D: Proposed mechanism of GPD treatment-caused inhibition of UV-induced MMP-1 expression in HDFs.

and Moncada, 2009] and serves as a therapeutic target for vascular diseases [Nagata et al., 2004]. Recently, several studies have reported AMP-independent activation of AMPK [Petti et al., 2012; Sarre et al., 2012]. Moreover, UV exposure and H_2O_2 might activate AMPK signaling in HaCat cells [Cao et al., 2008].

In the current study, we focused on the activation of AMPK because subsequent mTOR/p70^{S6K} inhibition has been suggested as a therapeutic approach for cancer [Memmott and Dennis, 2009], and GPD enhances AMPK activity in human hepatoma cells [Kim do et al., 2009b]. In accordance with these previous literatures, we found that GPD treatment increases LKB1, AMPK, and p38 phosphorylation (Suppl. Fig. 3). Activation of p70^{S6K} is an essential step for UV-induced MMP-1 transcriptional activation in HDFs [Brenneisen et al., 2000a]. In UV-irradiated cells, AICAR pretreatment inhibited UV-induced MMP-1 expression through AMPK activation to a greater extent than UV treatment alone. These results suggest that AMPK activation plays a key role in the suppression of MMP-1. In contrast, the inhibition of AMPK by compound C and shAMPK functioned as an inducer of MMP-1 in the absence of UV treatment. GPD did not show inhibitory effects in shAMPK HDFs. Therefore, we propose that AMPK pathway is the key target pathway of GPD.

LKB1 and CaMK are the major upstream kinases of AMPK. These kinases activate AMPK in independent manner. In this study, we have shown that GPD inhibits UV induced MMP-1 expression via PKA/LKB1 activation in HDFs. However, GPD has been shown to induce apoptosis via AMPK activation in CT-26 murine colon cancer cells (CT-26) through CaMK [Hwang et al., 2013]. We propose that the mechanism of GPD induced AMPK activation is different between the two experimental models. The cell lines used in the experimental models (HDFs and CT-26) are from different species (human and mouse, respectively) and from different organs (skin and colon, respectively). UV exposure could be another reason accounting for the difference. UV can affect the influx of Ca^{2+} [Lee et al., 2012] and the CaMK kinase can be a major pathway for AMPK activation by GPD in CT-26 cells. However, if the influx of Ca^{2+} is altered by UV exposure, it could significantly affect the subsequent signaling outcomes. The specific mechanisms of AMPK activation by GPD in each experimental model should be studied further.

The anti-photoaging effects of dietary or topically applied ginseng extract have been reported in hairless mice [Kang et al., 2009; Kim et al., 2009]. However, these studies have not addressed the inhibitory effects of individual ginsenosides on MMP-1 or the molecular mechanisms involved. We investigated the effect of four ginsenosides on TIMP1. In accordance with the results shown in Figure 2A, there was no significant difference in the effect of ginsenosides on the expression of TIMP (Suppl. Fig. 1). Our study has suggested a protective role for GPD in UV-induced MMP-1 expression and indicates that AMPK activation has an important role in MMP-1 expression in HDFs. Taken together, these results suggest that ginsenoside GPD could be beneficial in preventing skin photoaging by AMPK activation.

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