Emodin regulates glucose utilization by activating AMP-activated protein kinase

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* Running head: AMPK activation by Emodin

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Background: AMPK activation improves glucose tolerance and insulin sensitivity.

Results: Emodin increases glucose uptake in skeletal muscle and lowers blood glucose level via AMPK activation.

Conclusion: Administration of emodin leads to increased glucose tolerance and insulin sensitivity in vivo.

Significance: Our results highlight the potential value of emodin as a drug for the treatment of diabetes

Abstract

AMP-activated protein kinase has been described as a key signaling protein that can regulate energy homeostasis. Here, we aimed to characterize novel AMPK activating compounds that have a much lower effective concentration than metformin. As a result, Emodin, a natural anthraquinone derivative was shown to stimulate AMPK activity in skeletal muscle and liver cell. Emodin enhanced GLUT4 translocation and [14C] glucose uptake into myotube in an AMPK dependent manner. Also, emodin inhibited glucose production by suppressing the expression of key gluconeogenic genes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in hepatocytes. Furthermore, we found that emodin can activate AMPK by inhibiting mitochondrial respiratory complex I activity,
leading to increased reactive oxygen species (ROS) and Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK) activity. Finally, we confirmed that a single-dose administration of emodin significantly decreased the fasting plasma glucose levels and improved glucose tolerance in C57Bl/6J mice. Increased insulin sensitivity was also confirmed after daily injection of emodin for eight days using an insulin tolerance test and insulin stimulated PI-3K phosphorylation in wild type and HFD induced diabetic mouse models. Our study suggests that emodin regulates glucose homeostasis \textit{in vivo} by AMPK activation and that this may represent a novel therapeutic principle in the treatment of Type 2 diabetic models.

**Introduction**

AMPK is a highly conserved mammalian serine/threonine protein kinase and acts as a master energy sensor that is responsible for regulating energy homeostasis (1-2). These heterotrimeric enzymes are activated under cellular stress conditions such as starvation, exercise, oxidative damage and hypoxia. Liver kinase B1 (LKB1) and Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK) are two regulatory upstream kinases that stimulate AMPK phosphorylation (3). AMPK controls whole body energy homeostasis by regulating glucose and lipid metabolism in multiple peripheral tissues. For example, AMPK activation by AICAR stimulates glucose uptake via PI-3K independent GLUT4 translocation in skeletal muscle (4-5) and inhibits hepatic glucose production (6).

Recently, many types of AMPK activators, including cytokines, drugs and natural compounds have been identified (3). Among them, metformin (1,1-dimethylbiguanide hydrochloride) and thiazolidinediones are widely used for the treatment of type 2 diabetes. The main function of metformin is to decrease blood glucose level by inhibiting hepatic gluconeogenesis and increasing glucose uptake in skeletal muscle (7). Interestingly, the mitochondria respiratory complex is the common target of metformin, which indirectly activates AMPK (8). Inhibition of complex I activity can cause changes in the cellular nucleotide ratio (9) and increase reactive oxygen species (10), which are important intermediates to increase AMPK activity. Despite their beneficial effects to glucose utilization, metformin must be administered at high concentrations and they have adverse effects, such as gastrointestinal symptoms and relatively rare lactic acidosis \textit{in vivo} (11). Furthermore, some human studies have shown that the glucose-lowering effects of metformin are secondary to reduction in hepatic gluconeogenesis (11-13). Thus, there is growing demand to identify more effective AMPK activators, which have low dosage and affect various metabolic organs.

In the present study, we screened a drug library to identify novel AMPK activators. As a result of this screening, emodin (1,3,8-trihydroxy-6-methylanthaquinone), a natural active compound in the herbs Rheum palmatum L. was shown to stimulate the AMPK pathway. Some of the previous studies have suggested that emodin has an beneficial effect on energy metabolism, including adipocyte differentiation (14), anti-fibrotic effect on pancreatic fibrosis (15) and liver (16). However, validation of the molecular mechanism and the anti-diabetic effects of this compound has not been fully examined. Here we show that emodin can increase glucose uptake in skeletal muscle via inhibition of mitochondrial...
complex I, which then leads to activation of AMPK. In addition, emodin inhibited hepatic gluconeogenesis through AMPK. Importantly, acute intravenous administration of emodin in both wild type and high fat diet induced diabetic mice significantly decreased blood glucose levels associated with increased glucose utilization in skeletal muscle and liver. Based on the improved glucose metabolism and insulin sensitivity, this compound holds great promise for eventual use as a therapeutic agent to treat Type 2 diabetes.

**EXPERIMENTAL PROCEDURES**

**Materials** - 5-aminoimidazole-4-carboxamide-1-b-ribofuranoside (AICAR) was purchased from Toronto Research Chemical Incorporation (Toronto, ON, Canada). Emodin, Dichlorodihydrofluorescein diacetate (DCFH-DA), o-Phenylenediamine (OPD), resveratrol and Metformin were purchased from Sigma Chemical Company (St. Louis, MO). And 2-deoxy [14C] glucose was from ARC (American Radiolabelled Chemicals Inc.). compound c, an AMPK inhibitor, and SB 203580, a p38 MAPK inhibitor, were provided by the MERCK Company (RY 70-100; Rahway, NJ).

**Cell culture** - L6 myoblasts were grown in a-MEM and Mouse hepatoma Hepa1c1c7 cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 50 U of penicillin per ml and 50 µg of streptomycin per ml at 37 °C under humidified air atmosphere containing 5% CO₂. Differentiation of myoblast was induced in medium supplemented with 2% FBS within 7 days.

**Immunoblotting** - To prepare total cell lysates, myotube was washed with cold PBS and then lysed in cold lysis buffer (40 HEPES pH 7.5, 120 NaCl, 1 EDTA, 10 pyrophosphate, 10 glycerophosphate, 50 NaF, 1.5 Na₃VO₄, 1 PMSF, 5 MgCl₂, mmol/l, 0.5% Triton X-100 and protease inhibitor cocktail). Transferred membrane incubated with primary antibodies (1:1000) overnight at 4 °C. p-AMPK(Thr172), ACC, P-Akt(Ser473) (Cell Signaling Technology, MA), AMPK (Upstate, IL), p-ACC(Ser79) (Millipore, MA), PEPCK (Cayman, MI), PGC1 alpha (Abcam, UK) and monoclonal anti-Myc antibody was purchased from Millipore Corporation (Billerica, MA).

**AMPK activity assay** - Cells were serum fasted for 3hrs then treated with emodin for 5mins. After precipitation, AMPK activity was evaluated as incorporation of 32P into a synthetic SAMS peptide in kinase reaction buffer (40 HEPES pH 7.5, 80 NaCl, 1 DTT, 0.2 AMP, 0.2 ATP, 5 MgCl₂, 0.1 SAMS, 0.25 [γ-32P]ATP, mmol/l) at 30 °C for 20 min.

**Determination of 2-deoxy[14C - glucose] uptake in cells** - 2-deoxyglucose uptake was determined as described previously (17). Briefly, differentiated L6 were serum-starved for 2 h prior to the assay. Cells were then washed twice with Krebs-Henseleit buffer and were stimulated with or without the indicated agents for 60 min. Glucose uptake was measured by incubation with 0.1 mCi/ml 2-deoxy[14C - glucose] at room temperature for 10 min.

**Mitochondrial Complex I activity** - Cells were disrupted with non-denaturing detergent lauryl maltoside to maintain enzymatic activity and equilibrated with the indicated concentration of metformin and emodin for 10 min. Reaction was initiated by adding the reaction substrate - NADH (500 µmol/l) and ubiquinone-1 (50 µmol/l). A decrease in 340 nm absorbance was recorded over 10 min using UV spectrophotometer.
Fatty acid oxidation - Serum fasted myotube was incubated with emodin for 2 hour in oxidation media (0.1% lipid-free BSA, 100 µM [3H] Palmitate, vaporized for 30 minutes with N₂ gas to saturate palmitate). After oxidation, rest of free fatty acid was removed 10% TCA precipitation. Supernatant in capless-microtube was vaporized in D.W-added scintillation tube at 50 °C for 12 hours.

Mitochondrial Complex I activity - Cells were disrupted with non-denaturing detergent lauryl maltoside to maintain enzymatic activity and equilibrated with the indicated concentration of metformin and emodin for 10 min. Reaction was initiated by adding the reaction substrate - NADH (500 µmol/l) and ubiquinone-1 (50 µmol/l). A decrease in 340 nm absorbance was recorded over 10 min using UV spectrophotometer.

Glucose production assay - Serum fasted Hepa1c1c7 cells were pretreated with emodin at the indicated concentration for 10 min. After 18 hours of stimulation with forskolin (10 µmol/l)/dexamethasone (500 nmol/l), the cells were treated with emodin for additional three hours. Glucose production was measured in glucose and phenol red free DMEM, containing gluconeogenic substrate sodium lactate (20 mmol/l) and sodium pyruvate (2 mmol/l) for 8 hours.

Quantitative PCR analysis - Total RNA was isolated from hepatocyte and mouse liver tissue using the TRIzol reagent and 3µg of RNA was reverse transcribed into cDNA. Quantitative real-time PCR analysis was carried out using HotStart-IT SYBR® Green and Bio-Rad iCycler iQ. The sense and antisense primers used in PCR are listed in Table 1. The relative quantitation of mRNA was calculated by the comparative Ct method after normalization to ribosomal protein L32.

[Ca²⁺]i changes - [Ca²⁺]i was measured using the fura-2 AM method. Briefly, cells were grown on cover slip and loaded with 1 µmol/l fura-2 AM in loading buffer (125 NaCl, 5.9 KCl, 1.28 CaCl₂, 1.2 MgCl₂, 25 Glucose, mmol/l, 0.1% BSA) for 30 min. During the experiment, cells were continuously drenched with the loading buffer. For [Ca²⁺]i measurements after emodin treatment, fura-2 images were recorded using an OLYMPUS fluorescence microscope (DG4 light source), captured by a Hamamatsu’s ImagEM electron multiplier CCD (EMCCD) camera and analyzed using a MetaMorph program.

Animal experiments - All experimental procedures involving animals were approved by the POTECH Animal Use and Care Committee. C57Bl/6J male mice (6–8 weeks old) were kept in a 12 hours light/dark cycle with free access to water. To generate insulin resistance, mice received a high-fat diet with 60% Kcal fat for 10 weeks and the body weight and blood glucose levels were investigated. Emodin was dissolved in a vehicle of 10% DMSO in saline/Tween 80. After emodin was injected intravenously, the fasting glucose levels were measured using a gluco-meter (Accu-Check Active; Roche Diagnostics) after the indicated time. Serum insulin levels were determined using insulin ELISA kits. For glucose tolerance test, overnight fasted mice were injected with emodin and metformin 30 min before intraperitoneal glucose injection (2 g/kg). Blood was collected from the tail vein at 15, 30, 60, 120 min and glucose levels were measured. For insulin tolerance test (ITT), fasted mice were injected intraperitoneally with 0.75 U/kg body weight and blood glucose levels were measured at the indicated time point after injection. The liver and skeletal muscle were immediately frozen in liquid nitrogen and stored.
at -80°C after sacrifice.

**Pyruvate tolerance test** - For the pyruvate tolerance test, mice received an intraperitoneal injection of sodium pyruvate (2 g/kg) dissolved in saline after 16 hours fasting.

**Immunohistochemistry** - Paraffin-embedded skeletal muscle was sectioned and incubated with F4/80 (ab6640, abcam) and CD11b primary antibody (555386, BD Biosciences) for 4 hour at RT. A biotinylated secondary antibody and HRP-Streptavidin antibody with chromogen as a substrate were used for detection (Vectastain kit PK-6102, 6104; Burlingame, CA). The sections were counterstained in Harris Hematoxylin and Eosin (H&E) Staining.

**Statistical analysis** - All data are expressed as means ± SEM. Statistical analysis between two groups were performed by unpaired 2-tailed Student’s t test or one-way ANOVA for multiple comparisons. P values of <0.05 were considered significant.

**RESULTS**

**Emodin activates AMPK signaling pathway in L6 myotubes.** – To identify novel metabolic regulators, we carried out screening with available drug library (Supplemental Fig. 1A). and emodin (1,3,8-trihydroxy-6-methylantraquinone) was identified to be a potent AMPK activator (Supplemental Fig. 1C-G). Similar to its activators of AMPK, emodin also elevated the phosphorylation level of AMPK and ACC in differentiated myotubes (Fig. 1A). AMPK activation by emodin occurred in a concentration and time dependent manner (Fig. 1B, C). To further confirm the AMPK activation status, we performed a kinase assay with immunoprecipitated AMPK after emodin treatment. In these experiments, we observed emodin significantly enhanced AMPK kinase activity (Fig. 1D). To compare the EC\(_{50}\) of each known activators for AMPK phosphorylation, the effect of different concentrations of metformin, resveratrol and emodin on AMPK activation was studied. As a result, 0.5 µM of emodin was shown to reach half effective concentration which was much lower than metformin (~ 4 mM) and resveratrol (~ 50 µM) (Fig. 1E). Finally, there was no significant change in cell viability when cells were treated with emodin (Supplemental Fig. 1B). These data indicate that emodin activates the AMPK pathway in myotubes at much lower concentrations than any other naturally active compounds.

**Effect of emodin on glucose uptake in myotubes.** – Next, we examined the effect of emodin on glucose uptake. In this analysis, we confirmed that emodin increased 2-deoxy [\(^{14}\)C] glucose uptake into myotubes in a concentration and time dependent manner (Fig. 2A, B). To elucidate the mechanism behind this increase in glucose uptake, the level of surface GLUT4 was measured after emodin (3 µM) and metformin (10 mM) treatment by immunofluorescent microscopy. Because myc epitope is tagged at the extracellular region of GLUT4, translocation can be easily measured without any subcellular fractionation. Exposure of myc-epitope at the plasma membrane was increased by emodin (Fig. 2C), which was in agreement with the glucose uptake results. We also used the o-phenylenediamide (OPD) assay to accurately measure surface GLUT4 and observed a 1.5-fold increase at 3 µM (Fig. 2D), which was comparable with the 10 mM metformin control.

To validate the effect of emodin on the insulin-mediated signaling pathway, we assessed the
effect of co-treatment with emodin (3 µM) and insulin (25 nM) on AKT activation and glucose uptake. As shown in Fig. 2E, co-treatment did not interfere with respective signaling pathways. Finally, emodin was shown to increase maximal insulin-stimulated glucose uptake (Fig. 2F). Taken together, our findings suggest that emodin increases GLUT4 translocation and glucose uptake, and co-treatment with emodin and insulin produced additive effects on glucose uptake through independent pathway.

**AMPK mediates an increase in glucose uptake by emodin.** – To examine the importance of AMPK in emodin induced glucose uptake, we used a selective AMPK chemical inhibitor and dominant negative AMPK α2 virus, which were shown to be a major isoform in skeletal muscle (18-19). In these experiments, emodin induced AMPK activation and increase in glucose uptake were completely blocked by Compound C pretreatment without any effects on insulin mediated AKT activation and glucose uptake (Fig. 3A, B). These results indicate that the AMPK pathway is involved in emodin mediated glucose uptake. Similar to the chemical inhibitor and knockdown results (Supplemental Fig. 2A), infection with dominant negative AMPK α2 completely inhibited AMPK phosphorylation and glucose uptake induced by emodin, when compared to empty control virus (Fig. 3C, D). These findings demonstrate that AMPK is a critical mediator of emodin stimulated glucose uptake in skeletal muscle.

In addition to AMPK activation, it has also been suggested that insulin mediated PI-3K and p38 MAPK activation is involved in glucose uptake in skeletal muscle (20-21). However, emodin did not activate AKT (Fig. 3E) and glucose uptake was not inhibited in cells pre-treated with LY-294002, a PI3K inhibitor or SB-203580, a p38 MAPK inhibitor (Fig. 3F).

**Intracellular ROS and Ca2+ are involved in emodin induced AMPK activation and glucose uptake.** – Emodin could not activate AMPK directly (Supplemental Fig. 3A). A previous report suggested that emodin can increase intracellular ROS levels in tumor cell (22). In addition, ROS has been shown to activate AMPK through a LKB1 and CaMKK dependent pathway (23-25). Thus, we hypothesized that emodin induced activation of AMPK was caused by ROS. First of all, emodin was shown to stimulate ROS production (Fig. 4A, Supplemental Fig. 3B). Next, we examined changes in mitochondrial complex I activity which is major target of metformin (8) to better understand the mechanism of ROS generation by emodin. As a result, emodin dose-dependently suppressed NADH oxidation, more effectively than metformin (Fig. 4B). As shown in Fig. 4C, D emodin induced AMPK phosphorylation and *in vitro* kinase activity was largely inhibited by anti-oxidant N-acetylcysteine. In addition, the increase in glucose uptake was also blocked by N-acetylcysteine (Fig. 4E).

In order to verify which kind of upstream kinases were involved in the emodin induced AMPK activation, we used HeLa cells, which do not express LKB1 protein. As shown in Fig. 4F, emodin induced AMPK activation was also observed in the HeLa cells, which means that emodin largely depends on the CaMKK. This hypothesis was further confirmed with a CaMKK inhibitor, STO-609. Pretreatment of myotubes and HeLa cells with STO-609 decreased AMPK phosphorylation (Fig. 4F). Similarly, emodin induced glucose uptake was significantly attenuated by STO-609 (Fig. 4G). Finally, we examined change in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]), which is critical for CaMKK activation. As was observed for the
signaling kinetics, an increased Fura ratio was detected immediately, going from $0.57 \pm 0.005$ to $1.02 \pm 0.03$ (Fig. 4H). Taken together, our findings indicate that emodin decreases mitochondria complex I activity and stimulate AMPK through intracellular ROS dependent Ca^{2+}–CaMKK pathway.

Emodin affects gluconeogenic gene expression and hepatic glucose production. – In order to examine the function of emodin on gluconeogenesis, we first checked the effect of emodin on the AMPK signaling pathway in a mouse hepatoma cell line. Exposure of mouse hepatocytes to emodin increased phosphorylation of AMPK and ACC within 5-30 min (Fig. 5A, Supplemental Fig. 4A). To evaluate the effect of emodin on gluconeogenesis in hepatocyte, we measured glucose production with or without emodin. As shown Fig. 5B and Supplemental Fig. 4B, we found that emodin suppressed the increased glucose levels caused by forskolin/dexamethasone in a concentration-dependent manner. Next, we examined the effects of emodin on the expression of gluconeogenic genes. As shown in Fig. 5C and Supplemental Fig. 4C, increased transcripts level of important gluconeogenic genes was largely suppressed by emodin treatment. Finally, suppression of glucose production was also recovered by AMPK inhibition (Fig. 5D). These results show that emodin can suppress hepatic glucose production and gluconeogenic genes via stimulating AMPK activity.

Emodin decreases blood glucose concentration in wild type mice. – To investigate glucose homeostasis and insulin sensitivity after emodin treatment in vivo, we first examined AMPK phosphorylation in skeletal muscle and liver after intravenous injection of emodin (1 mg/kg). When compared to vehicle, emodin induced a significant increase in AMPK phosphorylation in both tissues, which was consistent with the in vitro results (Fig. 6A). More importantly, a single intravenous injection of emodin (1 mg/kg) significantly decreased fasting blood glucose levels in wild type mice at each indicated time point (Fig. 6B). After three hours, emodin decreased blood glucose level by 42 %, from $185 \pm 9$ mg/dl (injection start point) to $106 \pm 6$ mg/dl, whereas metformin (125 mg/kg) only decreased blood glucose levels by 28 %, from $168 \pm 12$ mg/dl to $121 \pm 6$ mg/dl. The decrease caused by administration of vehicle was 15 %, from $176 \pm 14$ mg/dl to $149 \pm 10$ mg/dl. There was no change in serum insulin levels between vehicle and emodin injection group ($1.33 \pm 0.1$ vs $1.04 \pm 0.2$ ng/ml) (Fig. 6C). These results strongly suggest that the glucose lowering effects of emodin are mediated by AMPK dependent and PI-3K independent phenomena.

To further confirm the AMPK dependency in vivo, we examined the glucose lowering effect of emodin with or without compound c. In a previous report and our result (Supplemental Fig. 5), i.v injection of compound c was shown to eliminate phosphorylation of AMPK and ACC (26). In compound c treated mice, the blood glucose levels were much higher than those in emodin alone treated group at all time points (Fig. 6D) and attenuation of the glucose lowering effect of compound c was profound at two hours (decreased by 36 % blood glucose level from $218 \pm 16$ mg/dl to $140 \pm 9$ mg/dl with emodin and 17 % from $214 \pm 12$ mg/dl to $178 \pm 11$ mg/dl with compound c co-injection). Furthermore, emodin suppressed Pepck and G6Pase gene expression (Fig. 6E). Finally, administration of emodin significantly reduced blood glucose levels in a glucose tolerance test (Fig. 6F).

Emodin stimulates glucose utilization and
increases insulin sensitivity in high fat induced diabetic mice. – We also evaluated the acute and chronic glucose-lowering effect of emodin in type 2 diabetic mouse models. Consistent with the wild type results, phosphorylation of AMPK in skeletal muscle was also significantly increased after single intravenous injection of emodin (3 mg/Kg) (Fig. 7A). In addition, these effects correlated with a decrease in fasting glucose levels, the decrease being 36 %, from 241 ± 47 mg/dl (injection start point) to 154 ± 28 mg/dl (Fig. 7B). More importantly, compound c significantly inhibited the glucose lowering effect caused by emodin, which means that the acute effect of emodin on blood glucose levels is largely dependent on AMPK activity. During the glucose tolerance tests, impaired glucose tolerance in high fat induced obese mice was markedly improved in the emodin injected group and the improvement was much more distinct than that observed in wild type mice (Fig. 7C). To examine insulin sensitivity in chronic emodin administrated mice, we investigated insulin tolerance after daily injection of emodin for eight days. During this period, there was no change in food intake and body weight (Supplemental Fig. 6A, B). Chronic treatment of emodin reduced basal blood glucose levels (Fig. 7D) and fed insulin levels (Fig. 7E). Furthermore, insulin mediated glucose lowering effects in ITT were more obvious than vehicle group (Fig. 7F) and AKT phosphorylation by insulin was significantly increased in emodin injected mice (Fig. 7G). Finally, increased blood glucose levels were largely attenuated in emodin treated mice in pyruvate tolerance test, indicating reduced gluconeogenesis in liver (Fig. 7H). Together, our results suggest that emodin activates AMPK in vivo, which is responsible for increased glucose tolerance and insulin sensitivity in multiple peripheral tissues.

Diet induced inflammatory cytokines and macrophages are attenuated after emodin administration. – It has been demonstrated that inflammatory cytokines in skeletal muscle and adipose tissue are elevated in obesity. (27). To elucidate the effect of emodin on muscle inflammatory cytokine and macrophage level, we measured transcript levels of pro-inflammatory cytokine after treatment of emodin in HFD induced obese mice. Figure 8A and Supplemental Fig. 7, indicates that the cytokine transcript levels were largely inhibited. Furthermore, infiltration of F4/80-positive macrophages was also suppressed by chronic treatment of emodin (Figure 8B). These results suggest that emodin decreases diet induced inflammation status in the skeletal muscle..

DISCUSSION

It has been demonstrated that emodin suppresses cell growth and proliferation in many types of tumor cells (22,28-29), and inhibits inflammations in vascular endothelial cells (30-31). In the present study, we found that emodin activates AMPK by modulating mitochondrial complex I activity. Through inhibiting complex I, emodin increases cellular ROS and Ca²⁺ influx, which are well known conditions for AMPK activation. Importantly, the effect of emodin was much more potent than that of metformin and extended to muscle and liver. As an anti-tumor agent, the required effective concentration of emodin in vivo is relative high and it inhibits only some types of tumors but not non-tumor cells (22,28). The emodin concentrations (up to 5 µM) used in this study does not cause cell death or apoptosis as measured using the MTT assay. In addition, a very recent paper showed that
exposure to nearly 800 mg/kg of emodin for 14 weeks through the diet did not show any developmental and testicular toxicity in wild type mice (32-33). Hence the low concentration range that we have used is reasonable with regard to a potential therapeutic treatment reagent for type 2 diabetes.

Mitochondrial complex I is the rate limiting step of the electron transport chain. In addition to cellular respiration, the importance of complex I as a diabetic target has been suggested. Metformin directly inhibits complex I, which leads to an increase in AMPK activity and insulin sensitivity (8,34). We have also observed that emodin decreased NADH conversion to NAD⁺, which means complex I activity was inhibited. This, in combination with the relationship between AMPK and mitochondria, suggest that emodin increases AMPK activity by modulating mitochondrial metabolism in skeletal muscle. One possibility is competitive inhibition between emodin and ubiquinone, a membrane embedded electron carrier (35). Because emodin (1,3,8-trihydroxy-6-methylanthraquinone) also belongs to the quinone family, it is possible that they have a common binding site in the complex I subunit. Therefore, it would be worth examining if the binding domain of ubiquinone overlaps with that of emodin.

A recent study demonstrated that chronic treatment with emodin lowered body weight in diet induced obese mouse, by suppressing 11β-hydroxysteroid dehydrogenase type I (11-HSD1) activity in liver and adipose tissue (36). 11-HSD1 is an NADPH dependent enzyme that is related to obesity and insulin resistance via the glucocorticoid signal (37). This study suggested that reduced food intake, by inhibition of the orexigenic glucocorticoid signal, may partly contribute to body weight loss. Although reduced fat mass and serum lipid contents through the inhibitory function on 11-HSD1 in liver and adipocyte tissue are important in vivo, emodin induced AMPK activation in skeletal muscle may be another major anti-diabetic mechanism. Skeletal muscle constitutes ~ 40% of body mass and is an essential tissue responsible for 75% of the whole body glucose uptake to exert glucose lowering (38). Indeed, in our study, AMPK inhibitor compound c largely attenuated glucose uptake in skeletal muscle cells, resulting in much less effective glucose lowering in a mouse model. In addition, emodin induced AMPK activation can contribute to weight loss, because AMPK is known to increase fatty acid oxidation (39-40) and mitochondria biogenesis (19), which protects against obesity (2). The data provided in this study, where AMPK was shown to increase fatty acid oxidation and related genes, supports this hypothesis (Supplemental Fig. 8 A, B). Truly, a very recent paper also reports that emodin increases AMPK and ACC in hepatocytes and adipose tissues of HFD-fed rats and improves dyslipidemia after eight weeks treatment (41-42).

In contrast to chronic exposures, many reports have suggested that modest ROS generation represents a favorable condition for glucose utilization (43). For example, ROS can decrease protein phosphatase activity, such as PTP1B (44) and PTEN (45). In addition, ROS also stimulates AMPK activity in many peripheral tissues (25,46-47), and it is well known that ROS generated during exercise and muscle contraction accelerates glucose uptake in skeletal muscle through AMPK (48-49). Metformin and epigallocatechin-3-gallate (EGCG) suppress hepatic gluconeogenesis by ROS-mediated AMPK activation in the liver (50). By using genetic models, many groups have demonstrated the acute requirement of ROS for AMPK
activation, which has many beneficial metabolic effects (47,51-52). Based on the significant inhibition of AMPK and glucose uptake by NAC, we suggest that ROS generation by emodin plays an important role in the activation AMPK. Of course, it is still possible that other mechanisms are involved in the induction of AMPK activity. Mitochondrial uncoupling proteins function with ROS to produce heat without ATP generation and we also observed a small, but statistically significant decrease in ATP content (Table 2). However, given the AMPK phosphorylation in LKB1 (-/-) cells, AMP/ATP ratio changes may take small charge of emodin induced AMPK activation, because LKB1 is a critical upstream kinase to activate AMPK under AMP enriched conditions. When we consider the importance of LKB1 in actions of metformin (53), this difference would be a clue for explaining a distinct kinetics and in vivo potency between emodin and metformin. Furthermore, some previous and our experimental results suggest that the liver is the main target tissue of metformin to show a glucose lowering effect (11,13). So when we compared to metformin, emodin does not have tissue specificity and has a comparative advantage regarding this aspect.

Blood glucose concentration is mainly regulated by glucose uptake in skeletal muscle and glucose production in liver. To determine whether emodin can also activate AMPK and decrease blood glucose in vivo, we carried out a single and prolonged intravenous administration of emodin in wild type and high fat-diet induced obese mice. Remarkably, a single injection of emodin sufficiently lowered fasting glucose levels within three hours in both in vivo models and this effect was much more potent than the effect observed in the metformin groups. Because there was no change in serum insulin levels between vehicle and emodin administration, the AMPK pathway is likely the main mechanism by which emodin regulates blood glucose levels. Also, we observed that emodin was able to improve glucose tolerance both in wild type and high fat induced diabetic mice and chronic treatment increased insulin sensitivity.

In summary, we show that emodin improves glucose utilization in wild type and diabetic mouse models. The main mechanisms of these glucose lowering effects are stimulation of glucose uptake in skeletal muscle and suppression of hepatic gluconeogenesis via AMPK phosphorylation. Furthermore, AMPK activation is mediated by inhibiting the enzymatic activity of complex I of the respiratory chain. Hence, we have identified a new mechanism whereby emodin lowers plasma glucose levels, which may have interesting implications for the treatment of Type 2 diabetes.
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**Acknowledgements**

We thank Dr Joohun Ha and his colleagues (Kyung Hee University College of Medicine, Republic of Korea) for the generous gift of adenovirus. Also, we are grateful to Dr. Amira Klip (Hospital for Sick Children; Toronto, Canada) for kindly providing the L6 GLUT4myc myoblast.

**FOOTNOTES**

* This work was supported by FPR08B1-160 of the 21C Frontier Functional Proteomics Project and the World Class University (WCU) program (R31-2008-000-10105-0), funded from the Korean Ministry of Education, Science and Technology (MEST). This study was also supported by a grant of the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111345), as well as the Swedish Research Council, the Family Erling-Persson Foundation and the Stichting af Jochnick Foundation.

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2The abbreviations used are: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; GLUT4, glucose transporter 4; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; CM/DCF-DA, 5,6-Chloromethyl-2,7-dichlorodihydrofluorescein diacetate.
Figure Legends

Figure 1. Emodin stimulates AMPK pathway in L6 myotubes.
A. L6 myotubes were prepared as described in EXPERIMENTAL PROCEDURES. The cells were treated with 10 mM metformin for 2 h, 100 μM resveratrol for 1 h and 5 μM emodin for 5 min. Cell lysates were analyzed by immunoblotting for phospho/total AMPK and ACC antibodies. B. L6 myotubes were stimulated with 3 μM emodin for an increasing amount of time. C. L6 myotube was stimulated with an increasing concentration of emodin for 5 min. The level of AMPK and ACC phosphorylation were quantified by densitometry and normalized to the total AMPK and ACC respectively. D. AMPK activity was measured by SAMS peptide phosphorylation assay using immunoprecipitated AMPK. E. Concentration-effect curve of various AMPK activators. The scales were selected so that the graphs were of roughly comparable height with the saturation point at 100. Data represent one of four independent experiments. Values are means ±S.E.M *,p<0.05 and **,p<0.01 versus untreated cells.

Figure 2. Increase of GLUT4 translocation and glucose uptake in response to Emodin.
A. L6 myotubes were equilibrated in glucose free Krebs-Ringer buffer for 1 h, and then incubated with the indicated concentration of emodin for 1 h. [14C] 2-DOG uptake was measured as described in EXPERIMENTAL PROCEDURES. B L6 myotubes were incubated with 3 μM emodin for 0 to 1 h and glucose uptake was measured. C. Immunofluorescent detection of surface GLUT4 on confluent, quiescent L6 myotube. The cells were treated with 3 μM emodin for 1 h and surface GLUT4 levels were determined. D. Quantitative analysis of surface GLUT4. Exposure of GLUT4 was detected using the anti-Myc antibody and coupled to the OPD assay as described in the EXPERIMENTAL PROCEDURES. E. Co-treatment with 3 μM emodin and 25 nM insulin for 5 min. AMPK phosphorylation was quantified by densitometry and normalized to the total AMPK. Data represent one out of four independent experiments. F. [14C] 2-DOG uptake was measured with emodin for 1 h and/or insulin for 10 min at the indicated concentration in L6 myotubes. Values are means ±S.E.M of three independent experiments performed in triplicate. *,p<0.05 and **,p<0.01 versus untreated cells.

Figure 3. Inhibition of AMPK blocks emodin induced glucose uptake.
A. After pre-incubation with 5 μM of compound c for 30 min, L6 myotubes were stimulated with 3 μM emodin or 25 nM insulin for 5 min. Data represent one of four independent experiments. B. Cells were pre-incubated with compound c and stimulated at the indicated conditions. [14C] 2-DOG uptake was measured for 10 min. C. L6 myotubes infected with either a mock or DN-AMPK α2 adenovirus for 48 h, were treated with or without emodin. DN-AMPK α2 expression was confirmed using the anti-Myc antibody. D. [14C] 2-DOG uptake was measured with either a mock or DN-AMPK α2 adenovirus. E. Emodin effect is independent of AKT. Cells were stimulated for the indicated time
period with emodin. The cell lysates were analyzed by immunoblotting for phosphor-/total AMPK and AKT antibody. Data represent one of four independent experiments. F. L6 myotubes were pre-incubated with AMPK inhibitor compound c, PI-3K inhibitor LY 294002 and p38 MAPK inhibitor SB294002 for 1 h. [14C] 2-DOG uptake was measured for 10 min. Values are means ±S.E.M of three independent experiments performed in triplicate. *,p<0.05 and **,p<0.01 compared between two groups as indicated.

Figure 4. AMPK activation and stimulation of glucose uptake is ROS-CaMKK dependent.

A. Intracellular ROS generation by emodin. Cells were serum starved for 3 h and incubated with emodin for the indicated time in the presence or absence N-acetylcystein. ROS levels were measured using DCF-DA. Increased ROS generation was visualized by fluorescence microscopy. B. Effects of metformin and emodin on mitochondrial complex I activity were measured after 10 min at the indicated concentration. The relative activity was expressed as decrease in absorbance at 340 nm. C. After pre-incubating with NAC for 30 min, L6 myotubes were stimulated with 10 mM metformin for 1 h and 3 µM emodin for 5 min. The cell lysates were analyzed for phosphorylation specific antibodies. Data represent one of four independent experiments. D. AMPK activity was measured by SAMS peptide phosphorylation assay using immunoprecipitated AMPK and NAC. E. [14C] 2-DOG uptake was measured for 10 min in the presence of compound c or NAC. F. L6 myotubes and HeLa cells were stimulated with 3 µM emodin for 5 min with or without CaMKK inhibitor STO-609. The cell lysates were analyzed by immunoblotting, using phosphorylation specific antibodies. G. L6 myotube was pre-incubated with CaMKK inhibitor STO-609 for 30 min and then treated with 3 µM emodin. [14C] 2-DOG uptake was measured for 10 min. H. Cells were loaded with Fura2-AM for 30 min and treated with emodin. Response of single cell was recorded using a fluorescence microscope, captured by an EMCCD camera. Values are means ±S.E.M of three independent experiments performed in triplicate. For the [Ca2+]i measurements, 69 cells were used. *,p<0.05 and **,p<0.01 compared between two groups as indicated.

Figure 5. Emodin phosphorylates AMPK and suppresses gluconeogenesis in hepatocyte.

A. HeLa1c1c7 cells were fasted for 12 h and treated with 3 µM emodin for various time points. AMPK phosphorylation was quantified by densitometry and normalized to the total AMPK. Data represent one of four independent experiments. B. Serum fasted cells were treated with emodin for 10 min and then stimulated with Fsk/Dex for additional 18 h. Glucose production was measured for 8 h as described in EXPERIMENTAL PROCEDURES. C. Relative changes in the gluconeogenic mRNA levels. D. Glucose production was measured with or without compound c. Values are means ±S.E.M of three independent experiments performed in triplicate. *,p<0.05 and **,p<0.01 compared between two groups as indicated.
Figure 6. Emodin activates AMPK and decrease fasting glucose level in wild type mice.

A. AMPK and ACC phosphorylation in skeletal muscle and liver of wild type C57Bl/6J mice was evaluated after a single intravenous injection of emodin. B. Acute fasting glucose lowering effect of emodin. Eight weeks old male mice were intravenously treated with metformin and emodin at the indicated dose. Blood glucose levels were measured every 60 min. C. Serum insulin levels 3 h after intravenous injection of vehicle and emodin. D. The effect of AMPK inhibitor compound c in the emodin injected groups. Mice were pretreated with compound c (2 mg/kg) for 30 min and intravenously treated with the vehicle and emodin. E. mRNA level of hepatic gluconeogenic genes in the liver were measured in mice that were intravenously treated with vehicle and emodin. F. Glucose tolerance was determined 30 min after injection of 1 mg/kg emodin. Glucose (2 g/kg) was injected intraperitoneally. Results are the means ±S.E.M of six mice per group (n =6). Statistical analysis conducted on fasting glucose lowering was performed using one-way ANOVA, Tukey's multiple comparison test. *,p<0.05 and **,p<0.01 versus vehicle treatment. #,p<0.05 versus emodin groups.

Figure 7. Emodin improves glucose tolerance and insulin sensitivity in high fat induced diabetic mice.

A. AMPK and ACC phosphorylation in skeletal muscle of high fat-diet induced C57Bl/6J obese mice after a single intravenous injection of emodin at the indicated dose. B. The effect of AMPK inhibitor compound c in emodin injected groups. Mice were pretreated with compound c (2 mg/kg) for 30 min and injected intravenously with the vehicle and emodin. C. Blood glucose levels were measured after intraperitoneal glucose injection (2 g/kg) in single i.v administration of emodin. D and E. Basal glucose levels and fed insulin levels were measured after 8 days injection of emodin. F. Insulin tolerance test (ITT) after daily i.v injection of emodin and metformin for eight days. Blood glucose levels were measured after intraperitoneal insulin injection (0.75 U/kg). G. Representative AKT phosphorylation in skeletal muscle under basal conditions was compared with that after insulin treatment in mice subjected to emodin i.v injection for eight days. H. Pyruvate tolerance test after 8 days injection of emodin. Results are the means ±S.E.M of nine mice per group (n = 9). For the ITT, five mice per group (n = 5). One-way ANOVA, Tukey's multiple comparison test was performed at fasting glucose lowering and glucose tolerance test. *,p<0.05 and **,p<0.01 versus vehicle treatment. #,p<0.01 versus emodin groups.

Figure 8. Decreased muscle pro-inflammatory cytokines and macrophages.

A. Relative mRNA level of inflammatory cytokines in skeletal muscle were measured. B. Representative F4/80 staining of macrophages in skeletal muscle. Muscle sections of vehicle or emodin treated high fat-diet induced obese mice were stained for the general macrophage marker F4/80 with magnification 40X. Magnification 20X was used for the quantification (n=10). *,p<0.05 and **,p<0.01 versus vehicle treatment. B.
### Tables

**Table 1. Target gene primers sequences for Q-PCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>G6pase</td>
<td>TCCTGGGACAGACACACAAG</td>
<td>CAACTTTAATATACGCTATTGG</td>
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<td>Pepck</td>
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<td>Pgc-1 α</td>
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Table 2. Adenine nucleotide concentrations after emodin treatment.

Effect of emodin on the intracellular AMP/ATP contents. Cell lysates were extracted with a perchloric acid and analyzed using a HPLC. Values are means ±S.E.M of two independent experiments performed in triplicate. *, p<0.05 versus untreated cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AMP (nmol/mg)</th>
<th>ATP (nmol/mg)</th>
<th>AMP to ATP ratio</th>
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<tbody>
<tr>
<td>NT</td>
<td>0.90 ± 0.09</td>
<td>34.4 ± 1.96</td>
<td>0.026 ± 0.001</td>
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<tr>
<td>Emodin (3 µM)</td>
<td>1.13 ± 0.31</td>
<td>29.2 ± 2.44*</td>
<td>0.040 ± 0.01</td>
</tr>
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</table>
Fig. 1; Song et al.

A. Samples treated with different concentrations of metformin and emodin were analyzed by Western blotting. The blots show the levels of pACC, ACC, pAMPK, AMPK, and Actin. The graph on the right shows the relative phosphorylation levels of pACC and pAMPK/AMPK in response to metformin and emodin.

B. The relative response of pAMPK/AMPK to metformin and emodin is indicated by the graph. The x-axis represents the concentration of metformin and emodin, and the y-axis represents the relative response (pAMPK/AMPK).

C. The graph shows the relative phosphorylation levels of pACC and pAMPK/AMPK in response to metformin and emodin. The x-axis represents the concentration of metformin and emodin, and the y-axis represents the relative phosphorylation levels.

D. The graph shows the relative phosphorylation levels of pAMPK/AMPK in response to different concentrations of metformin. The x-axis represents the concentration of metformin, and the y-axis represents the relative phosphorylation levels.

E. The graph shows the relative response of pAMPK/AMPK to different concentrations of metformin, resveratrol, and emodin. The x-axis represents the concentration of the compounds, and the y-axis represents the relative response (pAMPK/AMPK).
Fig. 2; Song et al.

A. 

B. 

C. 

D. 

E. 

F.
A. CC - - + + +
  Insulin - + + + +
  Emodin - + + + +

B. Control
   C C

C. Emodin NT
   Ctl virus
   AMPK DN-α-2

D. Ctl-virus
   AMPK-DN virus

E. Emodin time
   0 5 10 30 60 IS (min)

F. Emodin (3 μM) = + + + +
   Compound C = + + + +
   SB203580 = - - - -
   LY294002 = - - - -
A. (min) 0 1 2 5 10 30

B. Fig. 5; Song et al.

Relative Response (pAMPK/AMPK)

Gluco

C. Relative mRNA levels (normalized to RPL32)

D. N.S

Relative mRNA levels (normalized to RPL32)
**Figure 6; Song et al.**

A. 

**Skeletal muscle**

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Emodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACC</td>
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<tr>
<td>pAMPK</td>
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**Liver**

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Emodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACC</td>
<td></td>
</tr>
<tr>
<td>pAMPK</td>
<td></td>
</tr>
</tbody>
</table>

AMPK Phosphorylation (Fold Increase)

- Vehicle: 1.0
- Emodin: 2.0

B. 

Blood glucose (mg/dl) over time:

- Vehicle: Decrease over time
- Metformin: Decrease over time
- Emodin (1mg/Kg): Decrease over time

C. 

Serum insulin (ng/ml)

- Vehicle: 1.0
- Emodin: 0.8

D. 

Blood glucose (mg/dl) over time:

- Vehicle: Decrease over time
- Emodin (1mg/Kg): Decrease over time
- Emodin+Compound C: Decrease over time

E. 

Relative transcripts Level (Fold Increase)

- G6Pase: 1.2
- PEPCK: 0.8

F. 

Blood glucose (mg/dl) over time:

- Vehicle: Increase over time
- Emodin (1 mg/kg): Decrease over time
A. Skeletal muscle
Vehicle    Emodin

B. Blood glucose (mg/dl)

C. Blood glucose (mg/dl)

D. Basal Blood Glucose

E. Serum insulin (ng/ml)

F. Blood glucose (mg/dl)

G. Skeletal muscle

H. Blood glucose (mg/dl)
Fig. 8; Song et al.

A. Relative transcripts Level (Fold Increase)

- **MCP-1**
  - Vehicle: y
  - Emodin: y
  - Significance: *

- **IL6**
  - Vehicle: y
  - Emodin: y
  - Significance: *

- **TNF α**
  - Vehicle: y
  - Emodin: y
  - Significance: **

B. F4/80 Immunohistochemistry

- **HFD + Vehicle**
- **HFD + Emodin**

- **F4/80 positive Macrophage (Cell/Section)**
  - Vehicle: y
  - Emodin: y
  - Significance: **
Emodin regulates glucose utilization by activating AMP-activated protein kinase
Parkyong Song, Jong Hyun Kim, Jaewang Ghim, Jong Hyuk Yoon, Areum Lee,
Yonghoon Kwon, Hyunjung Hyun, Hyo-Youl Moon, Hueng-Sik Choi, Per-Olof
Berggren, Pann-Ghill Suh and Sung Ho Ryu

J. Biol. Chem. published online January 9, 2013

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