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Metformin Sensitizes Insulin Signaling Through AMPK-Mediated PTEN Down-Regulation in Preadipocyte 3T3-L1 Cells

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ABSTRACT

Insulin resistance is the primary cause responsible for type 2 diabetes. Phosphatase and tensin homolog (PTEN) plays a negative role in insulin signaling and its inhibition improves insulin sensitivity. Metformin is a widely used insulin-sensitizing drug; however, the mechanism by which metformin acts is poorly understood. To gain insight into the role of PTEN, we examined the effect of metformin on PTEN expression. Metformin suppressed the expression of PTEN in an AMP-activated protein kinase (AMPK)-dependent manner in preadipocyte 3T3-L1 cells. Knock-down of PTEN potentiated the increase in insulin-mediated phosphorylation of Akt/ERK. Metformin also increased the phosphorylation of c-Jun N-terminal kinase (JNK)-c-Jun and mammalian target of rapamycin (mTOR)-p70S6 kinase pathways. Both pharmacologic inhibition and knock-down of AMPK blocked metformin-induced phosphorylation of JNK and mTOR. Knock-down of AMPK recovered the metformin-induced PTEN down-regulation, suggesting the involvement of AMPK in PTEN regulation. PTEN promoter activity was suppressed by metformin and inhibition of mTOR and JNK by pharmacologic inhibitors blocked metformin-induced PTEN promoter activity suppression. These findings provide evidence for a novel role of AMPK on PTEN expression and thus suggest a possible mechanism by which metformin may contribute to its beneficial effects on insulin signaling. J. Cell. Biochem. 112: 1259–1267, 2011.

KEY WORDS: ADIPOCYTE; AMPK; INSULIN; METFORMIN; PTEN

Metformin is an oral anti-diabetic drug of the biguanide class. Metformin originates from the French lilac (Galega officinalis), a plant known to reduce the symptoms of diabetic mellitus [Despres, 2003; Bailey and Day, 2004; Bailey, 2008; Kooy et al., 2009]. Among the known actions of metformin are an increase in insulin sensitivity in muscles and liver [Borst and Snellen, 2001; Teranishi et al., 2007], a decrease in hepatic glucose production [He et al., 2009], an increase in peripheral glucose utilization [Yoshida et al., 2009], positive effects on insulin receptor expression and tyrosine kinase activity [Wiernsperger, 1999; Pryor et al., 2000; Wang et al., 2007], and lipolysis and oxidative stress in adipose tissue [Abbasi et al., 1998; Anedda et al., 2008]. The precise molecular mechanisms which determine the effect of metformin are unknown.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism. AMPK, a heterotrimeric complex comprised of a catalytic subunit and two regulatory

Abbreviations used: ACCacetyl-CoA carboxylaseAICAR5-aminoimidazole-4-carboxy-amide-1-D-ribofuranosi-deAMPKAMP-activated protein kinasePTENphosphatase and tensin homologmTORmammalian target of rapamy-cinJNKc-Jun N-terminal kinase

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subunits, is activated when cellular energy is depleted [Hardie and Carling, 1997]. Upon activation by allosteric binding of AMP or phosphorylation at Thr^{172} of the catalytic subunit by AMPK kinase, AMPK accelerates ATP-generating catabolic pathways, including glucose and fatty acid oxidation [Makinde et al., 1997; Ai et al., 2002; Zong et al., 2002], while simultaneously reducing ATP-consuming anabolic pathways, including cholesterol, fatty acid, and triacylglycerol synthesis [Henin et al., 1995]. In addition to its roles in energy homeostasis, AMPK also has been shown to regulate insulin signaling. In vitro studies have demonstrated that the AMPK agonist, 5-aminoimidazole-4-carboxy-amide-1-D-ribofuranoside (AICAR), enhances insulin-mediated glucose transport [Fisher et al., 2002; Ju et al., 2007]. An in vivo study has also demonstrated that AICAR improves insulin action in muscle and liver of insulin-resistant rats fed a high-fat diet [Iglesias et al., 2002]. These properties of AMPK make it an attractive target for diabetic research.

Phosphatase and tensin homolog (PTEN) is a dual specificity protein/3-lipid phosphatase that functions as a tumor suppressor and negative growth regulator. The PTEN protein modifies other proteins and lipids by removing phosphate groups. Based on this activity, PTEN specifically catalyses the dephosphorylation of the 3′ phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 [PtdIns(4,5)P2]. This dephosphorylation is important because it results in inhibition of the Akt signaling pathway. PtdIns(3,4,5)P3 and its dephosphorylated product, PtdIns(3,4) biphosphate, formed by 5′-lipid phosphatases, are membrane-associated lipids that regulate cell processes and promote survival, growth, mitosis, protein synthesis, and motility. The structure of PTEN consists of a phosphatase domain, and a C2 domain; the phosphatase domain contains the active site which carries out the enzymatic function of the protein, while the C2 domain binds the phospholipid membrane. Thus, PTEN has the potential to negatively regulate or terminate insulin signaling by metabolizing second messengers generated by the insulin-dependent activation of PI3-K. Several studies have examined the impact of knock-out PTEN in insulin-sensitive tissues. Liver-specific deletion of PTEN results in enhanced insulin sensitivity [Stiles et al., 2004]. PTEN-deletion in adipocytes resulted in decreased expression of resistin [Kurlawalla-Martinez et al., 2005]. Protection of muscle PTEN-depleted mice from insulin resistance caused by a high-fat diet is associated with enhanced insulin-stimulated Akt action [Wijesekara et al., 2005]. Thus, PTEN, which negatively regulates insulin action, represents a potentially new and interesting target by which to treat diabetes.

In the current study, we investigated the effects of metformin on PTEN expression in preadipocyte 3T3-L1 cells to gain an understanding of the insulin signal regulation role. We showed that metformin down-regulated PTEN through the AMPK pathway, and further demonstrated that the activities of c-Jun N-terminal kinase (JNK)/mammalian target of rapamycin (mTOR) were involved in metformin-induced PTEN regulation. These findings provide novel insight into the manner by which metformin contributes to PTEN regulating functions in preadipocyte 3T3-L1 cells via the AMPK pathway.

MATERIALS AND METHODS

REAGENTS

Anti-phospho-AMPK (Thr^{172}), anti-AMPK, anti-PTEN, anti-phospho-mTOR, anti-mTOR, anti-phospho-p70S6 kinase, anti-p70S6 kinase, anti-phospho-JNK, anti-JNK, anti-phospho-c-Jun, anti-c-Jun, anti-phospho-Akt, anti-Akt, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA). Anti-phospho-ACC (Ser^{79}), and anti-ACC antibodies were purchased from Millipore (Billerica, MA). Anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Metformin, rapamycin (mTOR inhibitor), SP600125 (JNK inhibitor), insulin, compound C, and AICAR were obtained from Calbiochem (San Diego, CA).

CELL CULTURE

Mouse preadipocyte 3T3-L1 cells were cultured in a humidified atmosphere at 37°C in an incubator with 5% CO_2. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; GIBCO™,

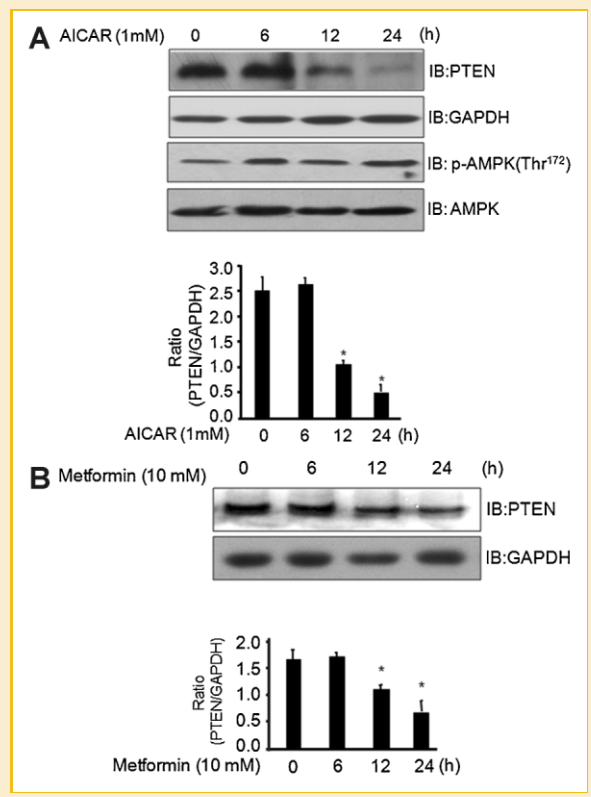

Fig. 1. AICAR and metformin down-regulate the level of PTEN protein in 3T3-L1 cells. A: 3T3-L1 cells were stimulated for the indicated times with 1 mM AICAR. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN, anti-phospho-specific AMPK, and anti-AMPK antibodies. Blotting with anti-GAPDH antibody was conducted as a protein loading control. The results shown are representative of three independent experiments. B: 3T3L1 cells were stimulated for the indicated times with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN antibody. Blotting with anti-GAPDH antibody was conducted as a protein loading control. The results shown are representative of three independent experiments.

Auckland, NZ) with 0.584 g/L of L-glutamate and 4.5 g/L of glucose, 100 μg/ml of gentamicin, 2.5 g/L of sodium carbonate, and 10% heat-inactivated fetal bovine serum (FBS).

IMMUNOBLOT ANALYSIS

Cells were grown on 6, 12-well plates and serum-starved for a minimum of 6 h prior to treatment with the indicated agents. Following treatment of the cells, the media was aspirated and the cells were washed twice in ice-cold PBS and lysed in 60 μl of lysis buffer. The samples were then briefly sonicated, heated for 5 min at 95°C, and centrifuged for 5 min. The supernatants were electrophoresed on SDS–PAGE (8%) gels, and transferred to polyvinylidene difluoride membranes. The blots were incubated overnight at 4°C on a shaker with primary antibodies, then washed six times in

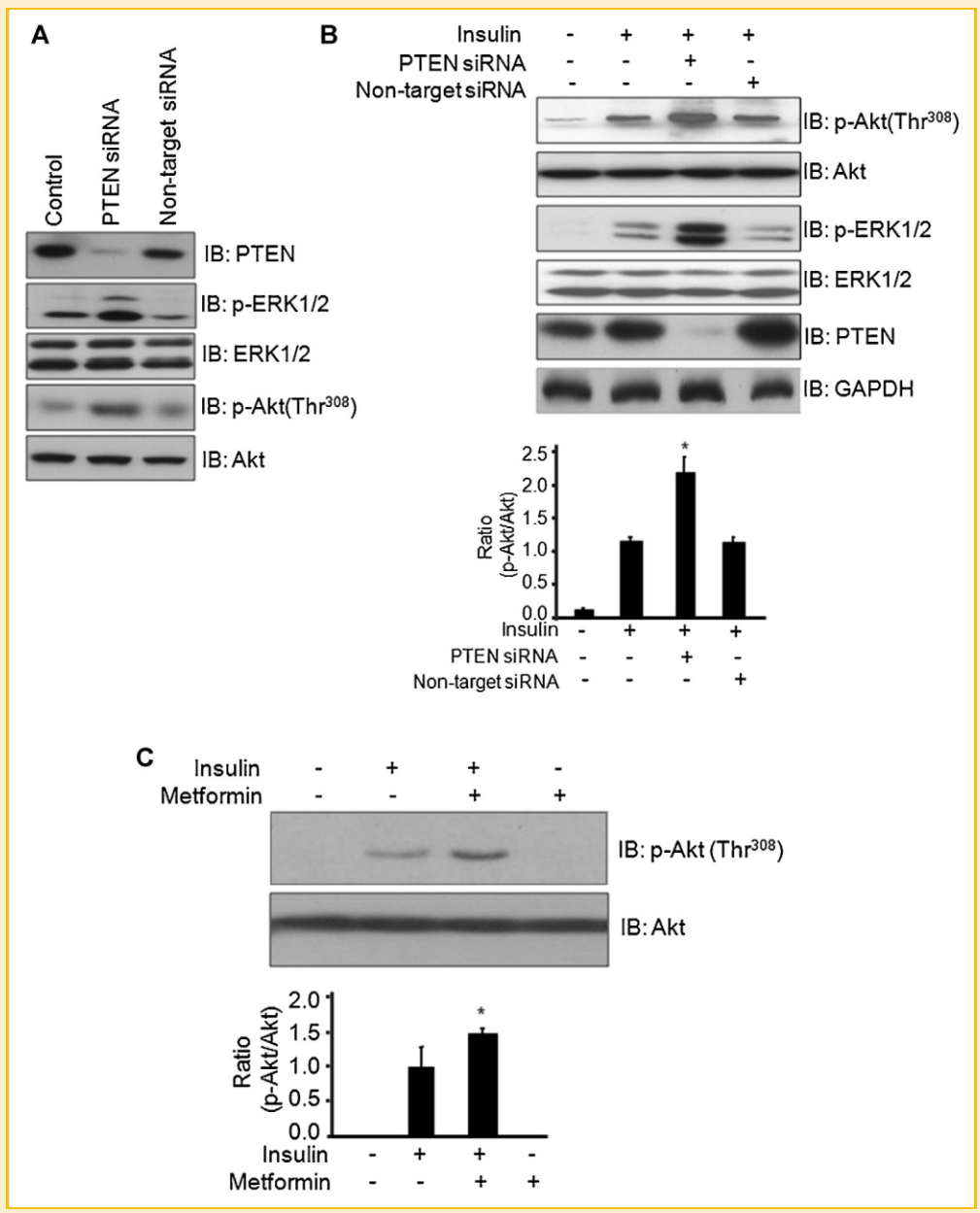

Fig. 2. PTEN knock-down enhances the insulin-mediated signaling pathway. A: 3T3-L1 cells were transfected for 24 h with either 50 nM PTEN siRNA or non-target siRNA. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN and anti-phospho-ERK1/2/Akt antibodies. Blotting with anti-ERK1/2/Akt antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. B: 3T3-L1 cells were transfected with either 50 nM PTEN siRNA or non-target siRNA for 24 h, then stimulated for 1 h with 100 nM insulin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-Akt/ERK1/2, and anti-PTEN antibodies. Blotting with anti-GAPDH and anti-Akt/ERK1/2 antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. C: 3T3-L1 cells were pretreated with 10 mM metformin for 24 h, then stimulated for 1 h with 100 nM insulin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-Akt antibody. Blotting with anti-Akt antibody was conducted as a protein loading control. The results shown are representative of three independent experiments.

Tris-buffered saline/0.1% Tween-20 prior to 1 h of incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then visualized via ECL (Amersham Biosciences, Buckinghamshire, UK). In some cases, the blots were stripped and reprobed using other antibodies.

SILENCING AMPKα2 AND PTEN

Mouse preadipocyte 3T3-L1 cells were seeded in six-well plates and allowed to grow to 70% confluence for 24 h. Transient transfections were performed with transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, AMPKα2 (NM_001013367; Dharmacon, CO) and PTEN were purchased from Dharmacon. Fifty nanomolar of siRNA and 6 μl of transfection reagent (Lipofectamine 2000; Invitrogen) were each diluted first with 100 μl of reduced serum media (Opti-MEM; Invitrogen), then mixed. The mixtures were allowed to incubate for 10 min at room temperature and then added dropwise to each culture well containing 1 ml of reduced serum media (Opti-MEM; final siRNA concentration, 50 nM). Four hours after transfection, the medium was changed with fresh complete medium. Cells were cultivated for 24 h and were lysed, and the expression of AMPKα2 protein was assayed with Western blotting.

LUCIFERASE ASSAY

After transfection with PTEN-Luc (Professor DS Min, Busan National University, Busan, Korea), cells were treated under the indicated conditions and harvested. The extracts were prepared using reporter lysis buffer (Promega, Madison, WI), and the cell lysates were analyzed for luciferase activity using a dual luciferase assay kit (Promega) and an illuminometer (VICTOR; PerkinElmer, Waltham, MA). Each extract was assayed three times.

DATA ANALYSIS

Data are expressed as the mean ± SEM. One-way ANOVA was used, followed by the Holm–Sidak multiple-range test for between groups comparisons. P values <0.05 were considered statistically significant.

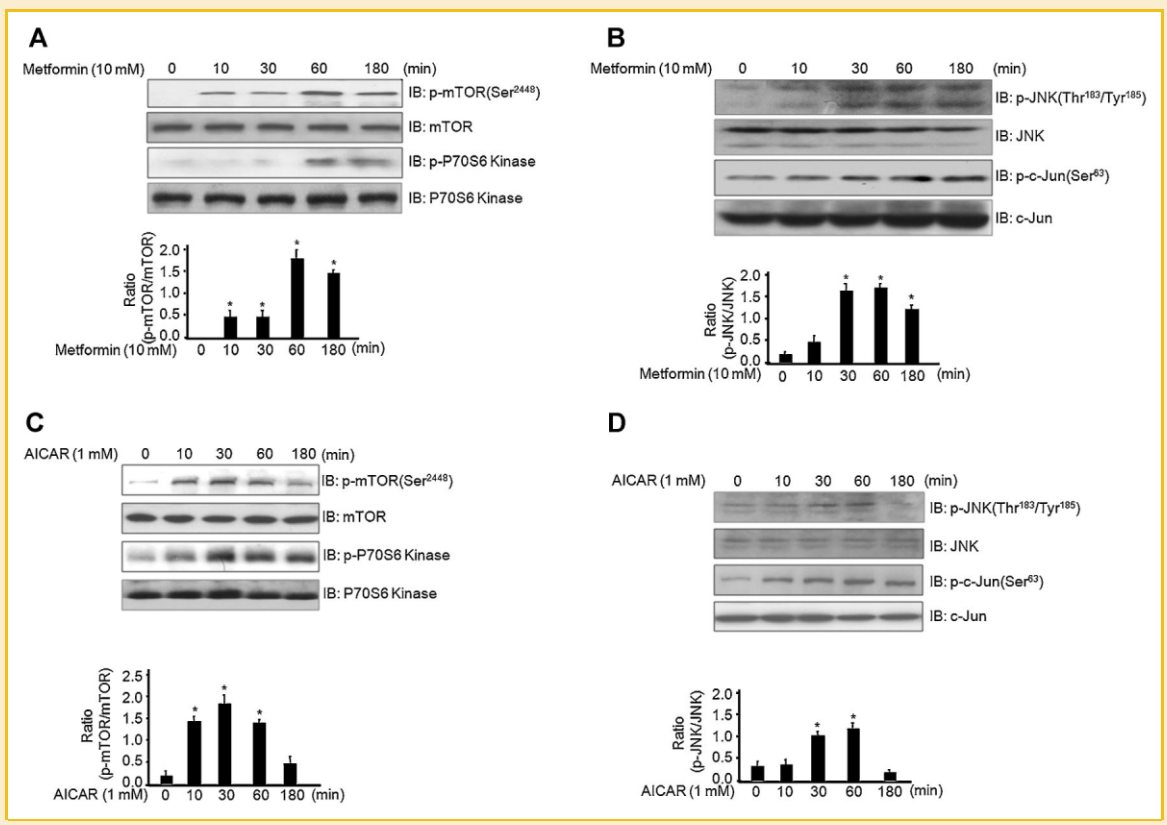

Fig. 3. Metformin and AICAR stimulate mTOR/JNK. A: 3T3-L1 cells were stimulated for the indicated times with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-mTOR/p70S6 kinase antibodies. Blotting with anti-mTOR/p70S6 kinase antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. B: 3T3-L1 cells were stimulated for the indicated times with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-JNK/c-Jun antibodies. Blotting with anti-JNK/c-Jun antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. C: 3T3-L1 cells were stimulated for the indicated times with 1 mM AICAR. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-mTOR/p70S6 kinase antibodies. Blotting with anti-mTOR/p70S6 kinase antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. D: 3T3-L1 cells were stimulated for the indicated times with 1 mM AICAR. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-JNK/c-Jun antibodies. Blotting with anti-JNK/c-Jun antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments.

RESULTS

AICAR AND METFORMIN DOWN-REGULATE PTEN PROTEIN LEVELS IN 3T3-L1 CELLS

To gain insight into the role of metformin in insulin-mediated signaling, we evaluated the effect of AICAR, an AMPK activator, on the expression of PTEN, a negative regulator of insulin signaling. The administration of AICAR induced a time-dependent decrease in PTEN expression in 3T3-L1 cells (Fig. 1A). An increase in AMPK phosphorylation validated the effect of AICAR. To provide physiologic relevance, we used an AMPK activator and well-known hypoglycemic agent, metformin. The expression of PTEN was also suppressed by metformin (Fig. 1B). These results demonstrate that metformin may suppress the expression of PTEN in 3T3-L1 cells through AMPK.

PTEN KNOCK-DOWN ENHANCES THE INSULIN-MEDIATED SIGNALING PATHWAY

To corroborate the roles of metformin in PTEN expression, we assessed the effects of PTEN knock-down on insulin signaling pathways. To this end, 3T3-L1 cells were transfected with PTEN siRNA and insulin signaling was compared between control

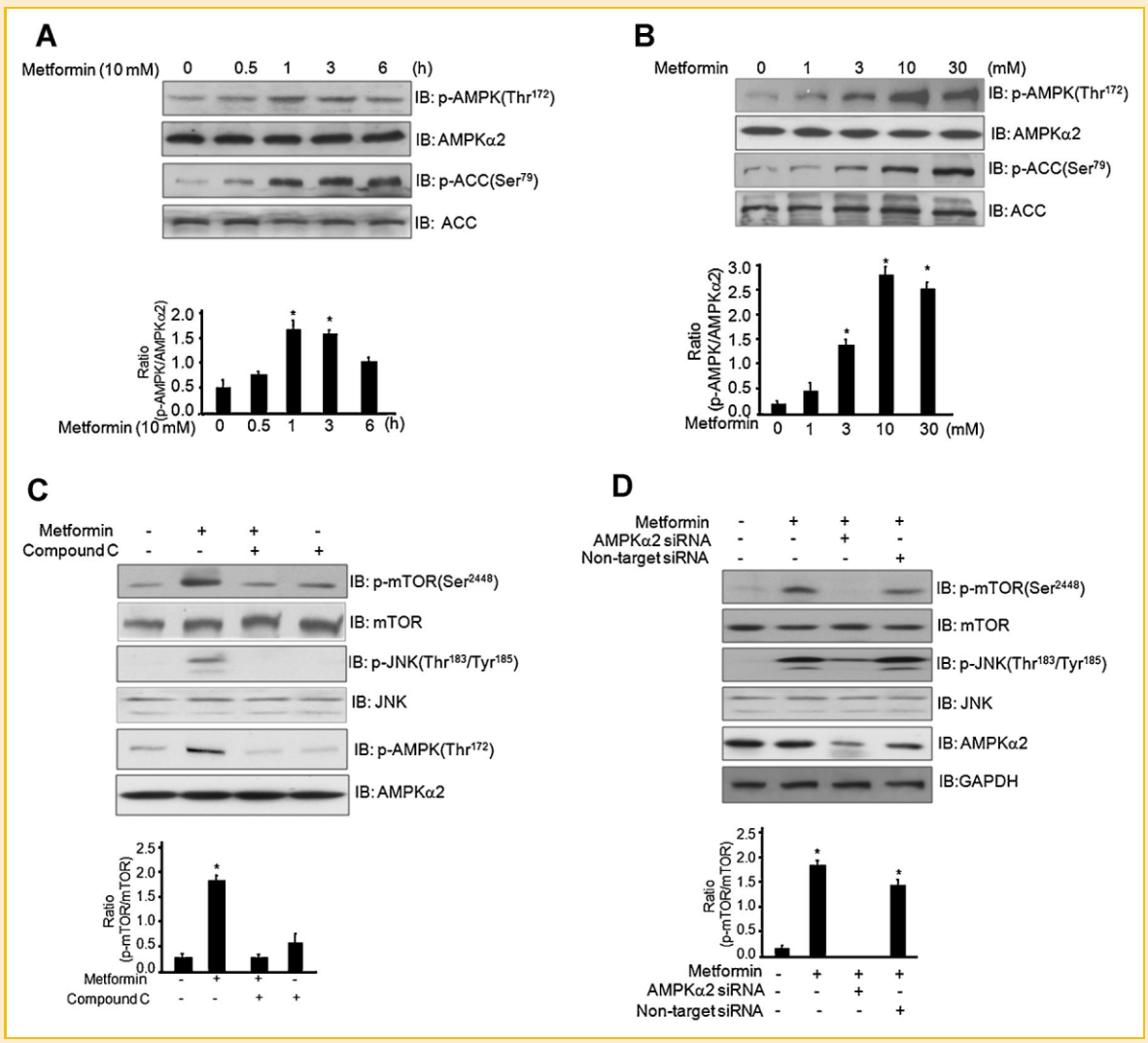

Fig. 4. AMPK is involved in metformin-mediated mTOR/JNK phosphorylation. A: 3T3-L1 cells were stimulated for the indicated times with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-ACC/AMPK antibodies. Blotting with anti-ACC/AMPKα2 antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. B: 3T3-L1 cells were stimulated for the indicated dose of metformin during 1 h. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-ACC/AMPK antibodies. Blotting with anti-ACC/AMPKα2 antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. C: 3T3-L1 cells were stimulated with 10 mM metformin after pretreatment of 2 μM compound C for 10 min. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-mTOR/JNK/AMPK antibodies. Blotting with anti-mTOR/JNK/AMPKα2 antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments. D: 3T3-L1 cells were transfected with either 50 nM AMPK siRNA or non-target siRNA during 48 h and starved for 4 h, then stimulated for 15 min with 30 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-phospho-mTOR/JNK, and anti-AMPK antibodies. Blotting with anti-mTOR/JNK antibodies and anti-GAPDH antibody was conducted as a protein loading control. The results shown are representative of three independent experiments.

and PTEN knock-down cells. We confirmed that PTEN siRNA down-regulated the expression of PTEN, while non-target siRNA did not show such an effect. The level of phosphorylation of Akt, the downstream target of insulin signaling, was increased by PTEN knock-down (Fig. 2A), indicating that PTEN may be involved in insulin-mediated signaling. Phosphorylation of extracellular signal-regulated kinase (ERK), another downstream molecule of insulin, was also enhanced in PTEN knock-down cells. In addition, insulin potently induced Akt/ERK phosphorylation under PTEN down-regulation (>90%) by siRNA treatment (Fig. 2B) compared to insulin-treated control cells. To gain insight into the role of metformin-mediated PTEN down-regulation, we measured Akt phosphorylation after insulin treatment in the absence or presence of metformin. Pretreatment with metformin potentiated the insulin-induced Akt phosphorylation (Fig. 2C). Together, our findings indicate that the insulin signal is potentiated by PTEN knock-down.

METFORMIN AND AICAR STIMULATE mTOR AND JNK

The mechanism underlying PTEN expression is complex. mTOR [Vinciguerra et al., 2008] and c-Jun [Tamguney and Stokoe, 2007] are known to be key negative regulators of PTEN. To test the hypothesis that AMPK regulates PTEN through these regulators, we investigated the effect of metformin on the phosphorylation of mTOR and JNK. The administration of metformin induced a time-dependent increase in mTOR and JNK phosphorylation in 3T3-L1 cells (Fig. 3A,B). The level of phosphorylation of mTOR reached a maximum level 1 h after treatment, then decreased to basal levels at 3-h. Consistent with the increase in mTOR activity, the phosphorylation of p70S6kinase, the best-characterized phosphorylation site by mTOR, increased after metformin administration. In addition, metformin stimulated the phosphorylation of JNK (Fig. 3B). Phosphorylation of c-Jun, a well-known substrate of JNK, was also increased by metformin. This phosphorylation was also observed by the AMPK activator, AICAR (Fig. 3C,D). Taken together, these results demonstrate that metformin and AICAR increase the phosphorylation of mTOR and JNK.

AMPK IS INVOLVED IN METFORMIN-MEDIATED mTOR AND JNK PHOSPHORYLATION

To characterize the molecular mechanisms of PTEN suppression, we evaluated the effects of metformin on the phosphorylation of AMPK, a key metabolic sensor kinase. The administration of metformin induced a time- and dose-dependent increase in AMPK phosphorylation in 3T3-L1 cells (Fig. 4A,B). The level of phosphorylation of Thr^{172}, which is in the active site of the AMPK-alpha subunit, and is essential for enzyme activity, reached a maximum level 1-h after treatment, then decreased to basal levels at 6 h. Consistent with the increase in AMPK activity, the phosphorylation of ACC (Ser^{79}), the best-characterized phosphorylation site by AMPK, increased after metformin administration. Inhibition of AMPK with compound C, an AMPK chemical inhibitor, and AMPK down-regulation by approximately 50% with siRNA treatment, decreased the phosphorylation of mTOR and JNK by metformin (Fig. 4C,D). These results suggest that metformin suppresses PTEN expression via an AMPK-mediated pathway.

AMPK KNOCK-DOWN RECOVERS METFORMIN-MEDIATED PTEN DOWN-REGULATION

To verify the roles of AMPK in the metformin-mediated signaling pathway, we assessed the effects of AMPK knock-down on PTEN expression. The expression of PTEN was decreased by metformin. This down-regulation of PTEN was recovered by AMPK down-regulation approximately 90% (Fig. 5A). Pharmacologic inhibition

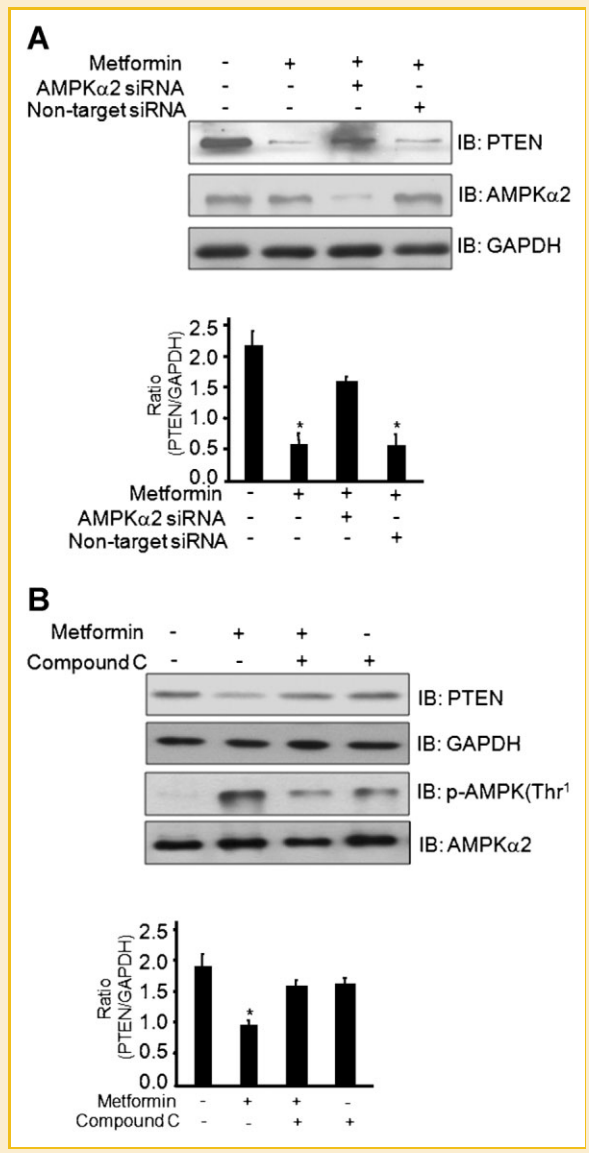

Fig. 5. AMPK knock-down restores metformin-mediated PTEN down-regulation. A: 3T3-L1 cells were transfected with either 50 nM AMPK siRNA or non-target siRNA for 24 h and stimulated for 24 h with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN/AMPKα2 antibodies. Blotting with anti-GAPDH antibody was conducted as a protein loading control. The results shown are representative of three independent experiments. B: 3T3-L1 cells were stimulated with 10 mM metformin for 24 h after pretreatment of 2 μM compound C. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN and anti-phospho-AMPK antibodies. Blotting with anti-GAPDH/AMPKα2 antibodies was conducted as a protein loading control. The results shown are representative of three independent experiments.

of AMPK with compound C also recovered the metformin-mediated PTEN down-regulation (Fig. 5B). These results suggest that metformin suppressed PTEN expression via AMPK.

METFORMIN SUPPRESSES THE PROMOTER ACTIVITY OF PTEN IN A mTOR AND JNK-DEPENDENT MANNER

To determine whether or not AMPK regulates PTEN transcription, we used a reporter plasmid in which the luciferase gene is driven by the PTEN promoter. We observed that AICAR suppressed the activity of the PTEN promoter in 3T3-L1 cells (Fig. 6A). Inhibition of AMPK with a chemical inhibitor restored the metformin-induced suppression of PTEN promoter activity (Fig. 6B), suggesting the involvement of AMPK in metformin-induced PTEN regulation. Finally, to confirm the involvement of mTOR and JNK in metformin-induced PTEN down-regulation, we used pharmacologic inhibitors. Inhibition of mTOR by rapamycin and JNK by SP600125 blocked metformin-induced PTEN promoter suppression (Fig. 6C). Chemical inhibitors were used to demonstrate that the inhibitory effect of metformin on the expression of PTEN is reversed by inhibition of mTOR and JNK. Pharmacologic inhibition of mTOR and JNK reversed the expression of metformin-induced PTEN down-regulation (Fig. 6D). Taken together, our results indicate that the inhibitory effects of metformin on the PTEN promoter are mediated via the mTOR and JNK pathway.

DISCUSSION

The principal finding of this study was that metformin is involved in PTEN expression in preadipocyte 3T3-L1 cells. Specifically, we demonstrated that AMPK is instrumental in metformin-mediated signaling in these cells. The primary assertion based on this study is that AMPK mediates some regulatory role for the lipid phosphatase, PTEN. As PTEN negatively regulates the insulin signal, PTEN down-regulation is likely to increase the cellular response to insulin. Collectively, our results indicate that AMPK may play a crucial role in metformin-mediated signaling.

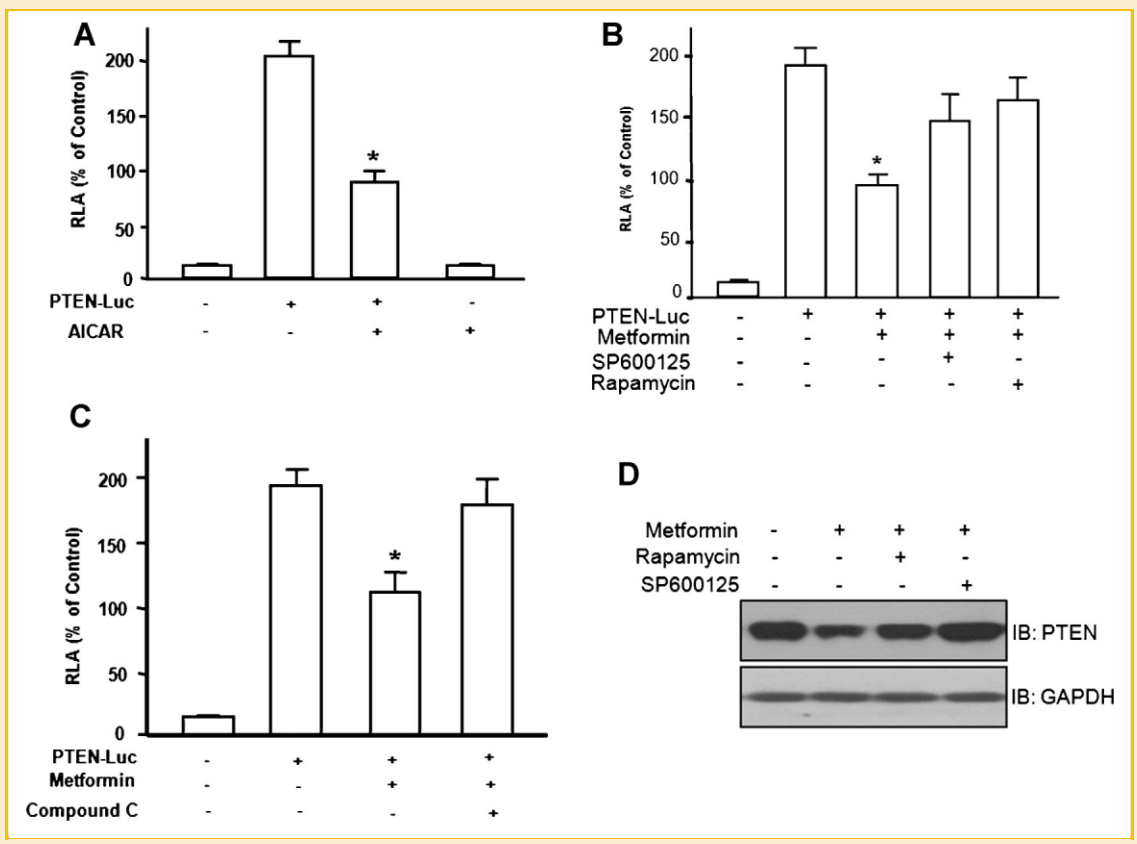

Fig. 6. Metformin suppresses the promoter activity of PTEN in mTOR/JNK-dependent manner. A: 3T3-L1 cells were transfected with PTEN promoter reporter plasmid for 24 h and starved for 4 h, then stimulated for 1 h with 1 mM AICAR. The cell lysates (20 μg) were analyzed via the luciferase assay and normalized with the β-galactose level. B: 3T3-L1 cells were transfected with PTEN promoter reporter plasmid for 24 h and starved for 4 h, then stimulated for 1 h with 10 mM metformin. For the last sample, 2 μM of compound C was pretreated for 10 min. The cell lysates (20 μg) were analyzed via a luciferase assay and normalized with the β-galactose level. C: 3T3-L1 cells were transfected with PTEN promoter reporter plasmid for 24 h and starved for 4 h, then stimulated for 1 h with 10 mM metformin. The cells were pretreated with 40 nM rapamycin and 40 μM SP600125 for 15 min. The cell lysates (20 μg) were analyzed via a luciferase assay and normalized with the β-galactose level. The relative luciferase activity (RLA; mean of triplicate measurements) is shown. $^{*}P < 0.05$ versus PTEN-Luc alone. D: 3T3-L1 cells were pretreated with either rapamycin or SP600125 for 20 min and then stimulated for 24 h with 10 mM metformin. The cell lysates (20 μg) were analyzed via Western blotting for anti-PTEN antibody. Blotting with anti-GAPDH antibody was conducted as a protein loading control. The results shown are representative of three independent experiments.

The object of the present study was to ascertain whether or not PTEN is regulated by metformin and, if so, to determine which molecules are involved in this process. Regulation of PTEN expression is very complex. Specifically, regulation of PTEN expression is positively and negatively regulated at the level of transcription, as well as the post-transcriptional level by phosphorylation. PTEN is positively regulated at the transcriptional level by EGR1 [Virolle et al., 2001], PPARγ [Patel et al., 2001], p53 [Stambolic et al., 2001], and human sprouty homolog 2 (SPRY2) [Edwin et al., 2006]. Negative regulation of PTEN transcription has been shown in mitogen-activated protein kinase kinase-4 (MKK) [Xia et al., 2007], TGFβ [Chow et al., 2007], and Jun [Hettinger et al., 2007]. PTEN expression is also regulated by phosphorylation. For example, CK2 and GSK3β are known to phosphorylate PTEN and to enhance protein stability [Al-Khouri et al., 2005]. PTEN stability is controlled by interaction with other proteins. Through its c-terminal PDZ-domain binding motif, PTEN interacts with members of the membrane guanylate-kinase inverted (MAGI) family, such as MAGI2, which results in PTEN stabilization [Wu et al., 2000a,b]. There are several factors that affect PTEN promoter activity as an activator or suppressor [Tamguney and Stokoe, 2007]. For example, PPARγ, SMAD, ATF2, and EGR1 are activators of PTEN and JUN, TGFβ, mTOR, and NFκB act as suppressors. Among these factors, the principal finding of this study was that metformin increased the phosphorylation of mTOR and JNK. This result demonstrated that the inhibitory effect of metformin on PTEN expression was mediated via the mTOR and JNK. It is important to note that we did not determine the effect of all other activators and suppressors on PTEN regulation, thus we cannot rule out the involvement of these activators and suppressors in metformin-induced PTEN regulation. Our data have revealed a novel role for mTOR and JNK downstream of AMPK activation by metformin. Overall, although the mechanism by which AMPK influences insulin signaling remains unknown, the findings in this report suggest that metformin suppresses PTEN through the AMPK pathway as a part of the process of metformin-mediated insulin signaling.

Various reports have suggested the importance of PTEN in insulin signaling [Stiles et al., 2004; Kurlawalla-Martinez et al., 2005; Wijesekara et al., 2005]. The accumulated amount of PIP_3 due to PTEN down-regulation has the potential to enable metformin to function as an insulin signal modifier. In addition, the LKB-AMPK axis in cancer suppresses tumor growth [Huang et al., 2008]. Thus, modulation of the LKB-AMPK pathway might be beneficial for cancer treatment. At the same time, the use of an AMPK activator, such as metformin, may be useful for the treatment of or prevention of cancer. Cancer cells lacking the ability to control their energy levels through the AMPK pathway may be more susceptible to stress. Together, the clinical implication is that regulation of AMPK for the treatment of tumors in which the mTOR pathway is activated is highly undesirable.

In conclusion, we have determined that metformin activates AMPK in preadipocyte 3T3-L1 cells and stimulates mTOR/JNK as well. We further demonstrated that the AMPK pathway exerts a profound influence on metformin-mediated PTEN expression. PTEN dysregulation is implicated in several pathophysiologic situations, including airway inflammation [Kim and Lee, 2008], cyclin D expression [Diao and Chen, 2007], and carcinogenesis [Leslie et al., 2010]. Further studies should be focused on the mechanism underlying control of PTEN expression and elucidating the relationship between AMPK and PTEN within the context of a metformin-mediated signaling pathway. In fact, we are currently investigating this relationship.

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