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5-Aminoimidazole-4-carboxyamide-1-β-D-ribofranoside stimulates the rat *enhancer of split- and hairy-related protein-2* gene via atypical protein kinase C lambda

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The 5′-AMP-activated protein kinase (AMPK) functions as a cellular energy sensor. 5-Aminoimidazole-4-carbox-yamide-1-β-D-ribofranoside (AICAR) is a chemical activator of AMPK. In the liver, AICAR suppresses expression of the *phosphoenolpyruvate carboxykinase* (*PEPCK*) gene. The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is an insulin-inducible transcriptional repressor and its target is the *PEPCK* gene. In this study, we examined an issue of whether the *SHARP-2* gene expression is regulated by AICAR via the AMPK. AICAR increased the level of SHARP-2 mRNA in H4IIE cells. Whereas an AMPK inhibitor, compound-C, had no effects on the AICAR-induction, inhibitors for both phosphoinositide 3-kinase (PI 3-K) and protein kinase C (PKC) completely diminished the effects of AICAR. Western blot analyses showed that AICAR rapidly activated atypical PKC lambda (aPKCλ). In addition, when a dominant negative form of aPKCλ was expressed, the induction of SHARP-2 mRNA level by AICAR was inhibited. Calcium ion is not required for the activation of aPKCλ. A calcium ion-chelating reagent had no effects on the AICAR-induction. Furthermore, the AICAR-induction was inhibited by treatment with an RNA polymerase inhibitor or a protein synthesis inhibitor. Thus, we conclude that the AICAR-induction of the *SHARP-2* gene is mediated at transcription level by a PI 3-K/aPKCλ pathway.

Keywords: AICAR/atypical protein kinase C lambda/5′-AMP-activated protein kinase/insulin-inducible transcriptional repressor/SHARP-2.

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-Aminoimidazole-4-carboxyamide-1-β-D-ribofranoside; AMPK, 5′-AMP-activated protein kinase; aPKC, atypical PKC; cPKC, classical PKC; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGCG, (-)-epigallocatechin-3-gallete; EGTA, *O,O′-Bis* (2-aminoethyl) ethylene-glycol −*N′,N,N′,N′*-tetraacetic acid; FBS, foetal bovine serum; m.o.i., multiplicity of infection; GFP, green fluorescence protein; nPKC, novel PKC; PCR, polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-K, phosphoinositide 3-kinase; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SHARP-2, enhancer of split- and hairy-related protein-2.

Muscle, adipose tissues, and liver play a pivotal role in both carbohydrate and lipid metabolisms. Signals from various nutrients, hormones and neurotransmitters regulate these metabolisms. Of these, nutrient signals are the mechanism which is activated by sensing the energy status in the cells. The 5′-AMP-activated protein kinase (AMPK), a serine/threonine kinase, plays a central role in this regulation (*1*, *2*). AMPK is a heterotrimer which consists of three subunits, α, β and γ. Under the energy consumption, ATP is degraded to ADP in the cells, then two ADP molecules are converted to ATP and AMP by adenylate kinase, thus an AMP/ATP ratio elevates. The increasing AMP binds to γ subunit of AMPK and allosterically activates it. In contrast, the AMPK activity is also stimulated by phosphorylation of serine/threonine residues by upstream kinase(s). Adiponectin, an adipokine secreted from adipose tissues, binds to the adiponectin receptor in the liver and causes phosphorylation of AMPK by LKB1.

5-Aminoimidazole-4-carboxyamide-1-β-D-ribofranoside (AICAR) is thought to be a chemical activator of AMPK (*3*). In the cells, AICAR is metabolized to ZMP, a similar compound of AMP, and activates AMPK. It has been reported that subcutaneous administration of AICAR to genetically obese *ob/ob* mice improves the level of blood glucose to that of normal mice and this effect is against both muscle and liver (*4*). When rat H4IIE highly differentiated

hepatoma cells are treated with AICAR, expression of the *phosphoenolpyruvate carboxykinase* (*PEPCK*) gene, a gluconeogenic enzyme gene, is inhibited as well as insulin (*5*). In addition, adiponectin inhibits transcription of both the *PEPCK* and *glucose-6-phosphatase* genes *in vivo* and lowers the blood glucose level (*6*).

The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is a basic helix-loop-helix transcriptional repressor (*7*). SHARP-2 binds to the E box sequence (5′-CANNTG-3′) locating in the transcriptional regulatory region of many genes. We reported that hepatic expression of the rat *SHARP-2* gene was induced by feeding a high carbohydrate diet to normal rats or insulin administration to diabetic rats and that phosphoinositide 3-kinase (PI 3-K) pathway mediated an increase of the level of SHARP-2 mRNA by insulin in primary cultured rat hepatocytes (*8*). We also reported that a forced expression of SHARP-2 in both primary cultured rat hepatocytes and rat H4IIE hepatoma cells decreased the level of PEPCK mRNA and that SHARP-2 decreased the promoter activity of the rat *PEPCK* gene in rat MH_1C_1 hepatoma cells (*9*). In addition, insulin also stimulates the *SHARP-2* gene expression in both rat L6 myotubes and mouse 3T3-L1 adipocytes (*10*). Whereas a mitogen-activated protein kinase pathway involved in an increase in the level of SHARP-2 mRNA by insulin in L6 myotubes, a PI 3-K pathway mediated that in 3T3-L1 adipocytes. Therefore, we hypothesize that SHARP-2 is an important transcription factor involving in the regulation of blood glucose levels (*9, 10*). Recently, we have reported that SHARP-1, an isoform of SHARP-2, also involved in the regulation of gene expression by insulin (*11*).

Compounds that can increase the *SHARP-2* gene expression is useful for prevention and treatment of diabetes mellitus. We have reported that a green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), a soybean isoflavone, genistein and an isoflavone daidzein metabolite by intestinal bacteria, (*S*)-Equol, can stimulate the *SHARP-2* gene expression in H4IIE cells (*12–16*). Some signalling molecules such as PI 3-K, classical protein kinase C (cPKC), atypical protein kinase C (aPKC) λ and nuclear factor-κB mediate the stimulation of the rat *SHARP-2* gene. EGCG induces the *SHARP-2* gene expression via PI 3-K, cPKC and atypical PKC lambda (aPKCλ) (*15*). On the other hand, it has been reported that EGCG elevates AMPK activity (*17*).

In this study, we examined an issue of whether AICAR induces the rat *SHARP-2* gene expression via the AMPK. The findings indicated that the AICAR-induction was mediated by a PI 3-K/aPKCλ pathway.

Materials and Methods

Materials
Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO) and *O,O′-Bis*(2-aminoethyl) ethylene-glycol −*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Wako (Osaka, Japan). Foetal bovine serum (FBS), LY294002 and horseradish peroxidase conjugate-rabbit anti-mouse IgG antibody were purchased from Sigma Aldrich (Saint Louis, MO). Streptomycin and penicillin G were purchased from Meijiseika (Tokyo, Japan). AICAR,

Compound-C, staurosporine, actinomycin D and cycloheximide were purchased from Merck chemicals (Darmstadt, Germany). The Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA). Polyvinylidene difluoride (PVDF) membrane and Immobilon Western chemiluminescent HRP substrate were purchased from MILLIPORE (Bedford, MA). Rabbit anti-phospho-acetyl-CoA carboxylase (Ser79) antibody (#3661), rabbit anti-acetyl-CoA carboxylase antibody (#3662) and rabbit anti-rat p-PKCζ/λ (Thr 410/403) antibody (9378S) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-rat p-PKCα (Ser 657) antibody (SC-12356-R) and rabbit anti-rat PKCα (C-20) antibody (SC-208) were purchased from Santa Cruz Biotechnology (Santa Cruz,CA). Mouse anti-rat PKCλ antibody (610207) was purchased from BD Biosciences (San Jose, CA). Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody was purchased from BIOSOURCE (Camarillo,CA). TRIzol reagent was purchased from Invitrogen (Groningen, the Netherlands). High capacity RNA-to-cDNA kit was purchased from Applied Biosystems (Foster City, CA). FastStart Universal SYBR Green Master (Rox) was purchased from Roche Diagnostics (Indianapolis, IN). The Adeno-X rapid titer kit was purchased from Clontech (Palo Alto, CA).

Cells and cell culture
Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University, USA). Human HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). These cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin G at 37°C in a 5% CO_2 incubator.

One million of H4IIE cells were cultured in a 6-cm dish. After 24 h, the medium was replaced twice with serum-free DMEM and then cultured for another 24 h. The medium was replaced with the same medium. After 2 h, cells were treated with various concentrations of AICAR for various times. To analyse a signal transduction pathway(s), H4IIE cells were pretreated with various inhibitors for 30 min, then treated with 250 μM AICAR for another 2 h. Compound-C (1 μM), LY294002 (50 μM), staurosporine (0.2 μM), EGTA (1 mM), actinomycin D (0.8 μM) and cycloheximide (5 μM) were used as inhibitors.

Western blot analysis
H4IIE cells were treated with 250 μM AICAR for the indicated times, then harvested in phosphate-buffered saline. The cells were lysed with Lysis buffer (12.5 mM Tris–HCl, pH 6.8, 2% glycerol, 1% 2-mercaptoethanol and 0.5% SDS). The protein concentration was determined using the Bio-Rad Protein Assay. Whole cell lysates were resolved with either 7.5 or 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Rabbit anti-phospho-acetyl-CoA carboxylase (Ser79) antibody (1:1,000 dilution), rabbit anti-acetyl-CoA carboxylase antibody (1:400 dilution), rabbit anti-rat p-PKCα (Ser 657) antibody (1:400 dilution), rabbit anti-rat PKCα (C-20) antibody (1:400 dilution), rabbit anti-rat p-PKCζ/λ (Thr 410/403) antibody (1:400 dilution) and mouse anti-rat PKCλ antibody (1:100 dilution) were used as primary antibodies. Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (1:20,000 dilution) and horseradish peroxidase conjugate-rabbit anti-mouse IgG antibody (1: 20,000 dilution) were used as the secondary antibodies. The proteins were visualized using the Immobilon Western chemiluminescent HRP substrate and analysed with the Image Quant LAS4000 (GE Healthcare).

Preparation of total RNA and real-time polymerase chain reactions (PCRs)
Total RNA was prepared from cells using the TRIzol reagent. Total RNA (1 μg) was reverse-transcribed with the high capacity RNA-to-cDNA kit. Procedures were performed according to the manufacture's recommended protocol. Quantitative real-time PCR was carried out using the cDNA, FastStart Universal SYBR Green Master (Rox) and primers by means of an ABI 7300 Real Time PCR System. Nucleotide sequences of the primers were described as previously (*15*). Relative amounts of both SHARP-2 and 36B4 mRNA levels were determined and then the level of SHARP-2 mRNA was normalized by that of 36B4 mRNA.

Preparation of adenovirus

The Ad-green fluorescence protein (GFP) and the Ad-dn-aPKCλ were generous gifts from Drs. Jun-ichi Miyazaki (Osaka University, Japan) and Wataru Ogawa (Kobe University, Japan), respectively (18, 19). Preparation of the adenovirus was described as previously (20). The titration of the adenovirus was carried out using the Adeno-X rapid titer kit. Each adenovirus was infected with the 50 multiplicity of infection (m.o.i.) and cultured for an additional 24 or 48 h. The medium was replaced with serum-free DMEM. After 2 h, cells were treated with 250 μM AICAR then cultured for another 2 h.

Statistical analysis

All experiments were performed at least three times. Data were represented as the mean and standard error and analysed by one-way ANOVA, followed by Fisher's protected LSD multiple comparison test.

Results

AICAR induces the expression of SHARP-2 mRNA

It has been reported that AICAR stimulates the AMPK activity and the active AMPK phosphorylates acetyl-CoA carboxylase (ACC) at Ser79 (3). First, to confirm an issue of whether the AMPK in H4IIE cells was activated by AICAR, whole cell lysates were prepared from AICAR-treated H4IIE cells for western blot analyses using antibodies against phosphorylated ACC and
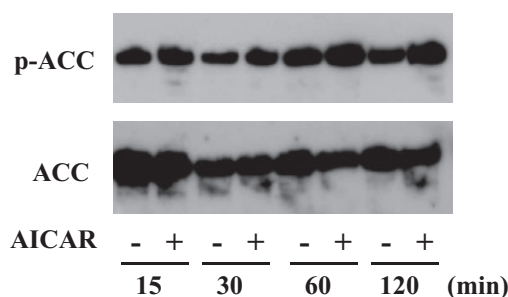
Fig. 1 Activation of AMPK by AICAR. Whole cell lysates were prepared from H4IIE cells treated with (+) or without (−) 250 μM AICAR for the indicated times. The lysates (20 μg/lane) were resolved using a 7.5% SDS-PAGE gel and transferred onto a PVDF membrane for western blot analysis. Rabbit anti-phospho-acetyl-CoA carboxylase (Ser79) antibody (p-ACC) and rabbit anti-acetyl-CoA carboxylase antibody (ACC) were used as primary antibody. Three independent experiments were performed and a representative result was shown.
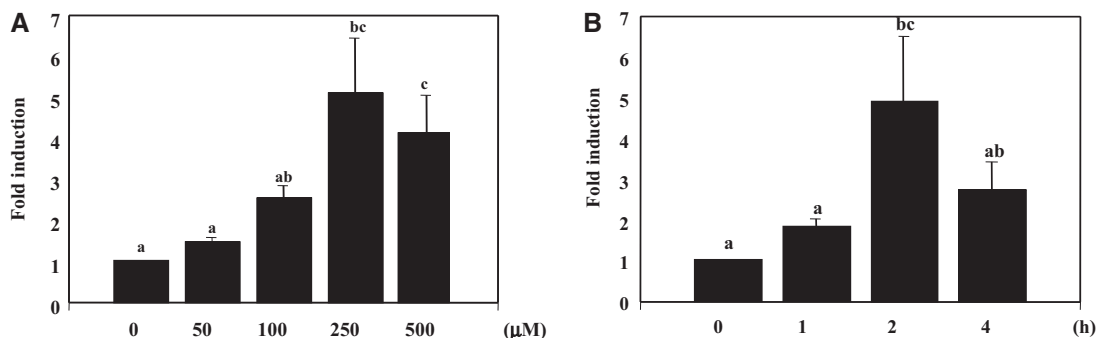
ACC. As shown in Fig. 1, a phosphorylated form of ACC was detected at 15 min post-treatment with AICAR. In contrast, the level of whole ACC protein almost remained unchanged.

This result indicates that AICAR can stimulate AMPK activity in H4IIE cells.

Next, to examine the effects of AICAR on the rat SHARP-2 gene expression, H4IIE cells were treated with various concentrations of AICAR for 2 h. The level of SHARP-2 mRNA significantly increased with AICAR treatment in a dose-dependent manner and reached a maximum level at 250 μM (Fig. 2A). Then, the time course for the increase in SHARP-2 mRNA at 250 μM of AICAR was analysed. As shown in Fig. 2B, the level of SHARP-2 mRNA increased at 2 h.

These results showed that the level of SHARP-2 mRNA was increased by AICAR.

The PI 3-K and PKC are involved in an increase of the level of SHARP-2 mRNA by AICAR

We then examined an issue of whether an increase in the level of SHARP-2 mRNA by AICAR linked to the AMPK activity. H4IIE cells were pretreated with either DMSO as a solvent or Compound-C as an AMPK inhibitor and treated with 250 μM AICAR for 2 h. Surprisingly, the induction of the level of SHARP-2 mRNA by AICAR was not inhibited by a pretreatment with Compound-C (Fig. 3A).

We previously reported that the increase in SHARP-2 mRNA level by insulin and genistein were stimulated by the PI 3-K and PKC pathways, respectively (8, 14). Then, we examined an issue of whether the induction of the level of SHARP-2 mRNA by AICAR was mediated by these pathways. H4IIE cells were pretreated with LY294002 as a PI 3-K inhibitor and staurosporine as a PKC inhibitor. The AICAR-induction of SHARP-2 mRNA was completely inhibited by both reagents (Fig. 3B).

To determine an issue of whether the AMPK activity was affected by LY294002, whole cell lysates were prepared from LY294002-treated H4IIE cells for western blot analyses using antibodies against phosphorylated

Fig. 2 Effects of AICAR on SHARP-2 mRNA level. Total RNA was prepared from H4IIE cells treated with or without AICAR. The levels of SHARP-2 and 36B4 mRNAs were determined by reverse-transcription and quantitative real-time PCR. Each column and bar represents the mean and standard error of the ratio of the levels of SHARP-2 and 36B4 mRNAs of at least three independent experiments. Expression level ratio in the absence of AICAR was normalized to one. [a,b,c]Within each graph, means without a common letter differ, $P < 0.05$. (A) H4IIE cells were treated with indicated concentrations of AICAR for 2 h. (B) Time course for alterations in SHARP-2 mRNA levels by AICAR. H4IIE cells were treated with 250 μM AICAR for the indicated times.

ACC and ACC. The AICAR-induction of the phosphorylated form of ACC at both 15 and 30 min was also shown (Fig. 4). Interestingly, a phosphorylated form of ACC was detected at 15 min post-treatment with LY294002 as well as AICAR (Fig. 4). In contrast, the level of whole ACC protein remained unchanged.

These results suggest that the AMPK activity is not parallel to an induction of the SHARP-2 mRNA level by AICAR and that the induction is mediated by both the PI 3-K and PKC pathways.

aPKCλ mediates an induction of SHARP-2 mRNA level by AICAR

PKC has many isoforms such as cPKC, novel PKC (nPKC) and aPKC (*21*). The cPKC have several isoforms and a major isoform in the liver is a PKCα. We previously reported that the PKCα was a mediator of genistein (*14*). Also, it has been reported that aPKCλ but not PKCα is activated by the PI 3-K pathway in the liver (*22, 23*).

When both PKCα and aPKCλ are activated by extracellular stimuli, serine/threonine residues of them are phosphorylated (*24*). To examine which an isoform(s) of PKC was activated in H4IIE cells by AICAR, whole cell lysates were prepared from AICAR-treated H4IIE cells for western blot analyses using antibodies against phosphorylated cPKCα, cPKCα, phosphorylated aPKCλ and aPKCλ. The levels of both an active (phosphorylated) and an inactive (non-phosphorylated) forms of cPKCα remained unchanged by a treatment with AICAR (Fig. 5). In contrast, an active (phosphorylated) form of aPKCλ was detected at 5 min post-treatment with AICAR and this level was maintained until 15 min. In contrast, the level of whole aPKCλ protein remained unchanged (Fig. 5).

These results indicate that AICAR induces activation (phosphorylation) of aPKCλ but not cPKCα.

We then determined the effect of aPKCλ on expression of the rat *SHARP-2* gene by AICAR using the adenovirus expression system. Whereas the Ad-GFP expresses the GFP, the Ad-dn-aPKCλ expresses a dominant negative form of aPKCλ (*18, 19*). Expression of these proteins was confirmed using fluorescence

microscopy and western blot analyses, respectively (data not shown). When the H4IIE cells were infected with the Ad-GFP, an induction of SHARP-2 mRNA by AICAR was not altered (Fig. 6A). In contrast, when H4IIE cells were infected with the Ad-dn-aPKCλ, that of SHARP-2 mRNA by AICAR was completely diminished (Fig. 6A).

It has reported that calcium ion is required for an activation of PKCα but not aPKCλ. EGTA is a calcium ion-chelating reagent. It has been reported that 1 mM EGTA effectively reduce cytosolic calcium levels in H4IIE cells (*25*). As shown in Fig. 6B, an increase in the level of SHARP-2 mRNA by AICAR was not inhibited by a treatment with EGTA.

These results indicate that aPKCλ but not PKCα mediates the AICAR-induction of SHARP-2 mRNA level.

AICAR acts at the transcriptional level of the rat SHARP-2 gene via de novo protein synthesis

To examine the issue of whether the induction of SHARP-2 mRNA by AICAR is required for *de novo* RNA and or protein synthesis, H4IIE cells were treated with either actinomycin D, a RNA polymerase II
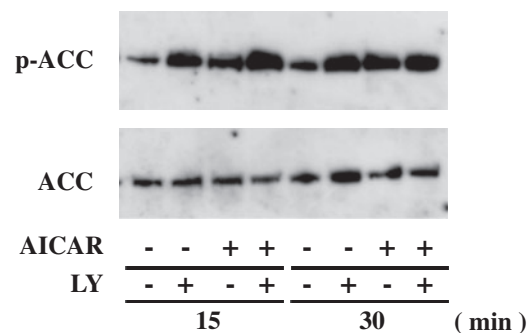

Fig. 4 Western blot analysis of the AMPK activity. H4IIE cells were pretreated with either (+) 50 μM LY294002 or (−) DMSO for 30 min and then treated with (+) or without (−) 250 μM AICAR for the indicated times. Whole cell lysates were prepared from these cells. Details for determination of the AMPK activity are described in the legend of Fig. 1. Three independent experiments were performed and a representative result was shown.
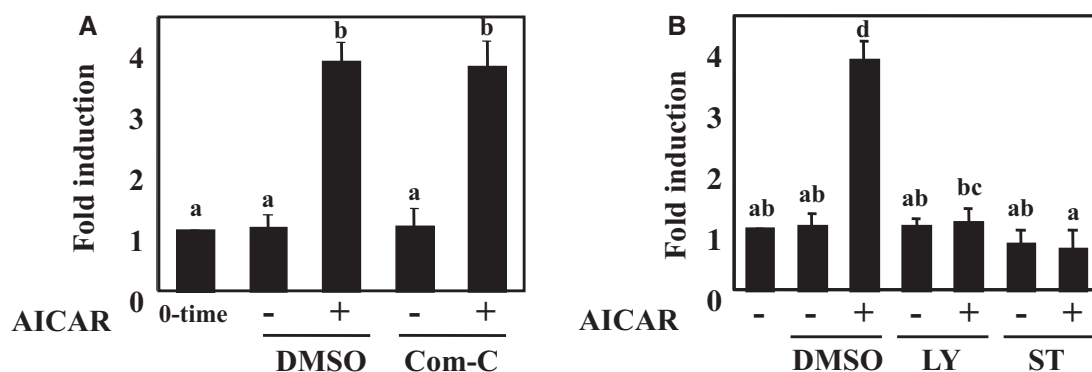

Fig. 3 Both the PI 3-K and PKC mediate the induction of SHARP-2 mRNA level. Details for determination of the levels of SHARP-2 mRNA are described in the legend of Fig. 2. Expression level ratio shown as 0-time was normalized to 1. Four independent experiments were performed. [a,b,c,d]Within each graph, means without a common letter differ, $P < 0.05$. (**A**) H4IIE cells were pretreated with DMSO or 1 μM compound-C (Com-C) for 30 min before treatment with (+) or without (−) 250 μM AICAR for 2 h. (**B**) H4IIE cells were pretreated with DMSO, 50 μM LY294002 (LY), or 0.2 μM staurosporine (ST) for 30 min before treatment with (+) or without (−) 250 μM AICAR for 2 h.

inhibitor or cycloheximide, a protein synthesis inhibitor. As shown in Fig. 7A, the induction of SHARP-2 mRNA by AICAR was partially inhibited by actinomycin D. When H4IIE cells were pretreated with cycloheximide, the level of SHARP-2 mRNA was slightly increased. However, the level was not further induced by treating with AICAR (Fig. 7B).

These results suggest that the induction of SHARP-2 mRNA by AICAR occurred at least in part at the transcriptional level of the rat *SHARP-2* gene and required for *de novo* protein synthesis.

Discussion

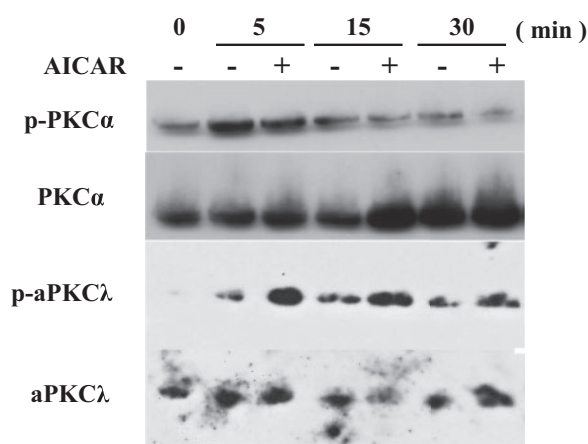We examined an issue of whether the *SHARP-2* gene expression is regulated by AICAR via the AMPK. It

Fig. 5 Activation of aPKCλ by AICAR. Whole cell lysates were prepared from H4IIE cells treated with (+) or without (−) 250 μM AICAR for the indicated times. Whole cell lysates (20 μg/lane for cPKCα or 50 μg/lane for aPKCλ) were resolved using a 10% SDS-PAGE gel and transferred onto a PVDF membrane for western blot analysis. Rabbit anti-rat p-PKCα (Ser 657) antibody (p-PKCα), rabbit anti-rat PKCα (C-20) antibody (PKCα), rabbit anti-rat p-PKCζ/λ (Thr 410/403) antibody (p-aPKCλ) or mouse anti-rat PKCλ antibody (aPKCλ) were used as primary antibody. Three independent experiments were performed and a representative result was shown.

has been reported that AICAR, an AMPK activator, decreased the level of PEPCK mRNA in H4IIE cells (5). As the *PEPCK* gene is a target gene of SHARP-2 transcriptional repressor, we hypothesized that AICAR should increase the level of SHARP-2 mRNA (9). Indeed, the level of SHARP-2 mRNA was rapidly and temporally induced in H4IIE cells treated with AICAR (Fig. 2). However, this induction was not inhibited by treating with an AMPK inhibitor, Compound-C (Fig. 3A). Metformin, another AMPK activator, did not induce the level of SHARP-2 mRNA (data not shown). In addition, the AMPK activity was enhanced by LY294002 as well as AICAR (Fig. 4). These results showed that the *SHARP-2* gene expression was not identical with a level of the AMPK activity and that AICAR might stimulate an AMPK-independent signalling pathway.

We have previously reported that the PI 3-K and PKC pathways were important for the induction of SHARP-2 mRNA by insulin, genistein and (*S*)-Equol (8, 14, 16). To determine the issue of whether these signalling pathways mediate the AICAR-induction of the SHARP-2 mRNA, these specific inhibitors were employed. Treatments with LY294002 and staurosporine completely blocked the AICAR-induction of SHARP-2 mRNA, suggesting that both the PI 3-K and PKC mediated the induction (Fig. 3B).

PKC isoforms are classified into cPKC, nPKC and aPKC (21). Phosphorylations of T497, T639 and S657 of cPKCα causes its activation (24). However, an active (phosphorylated) form of PKCα protein was not observed in AICAR-treated H4IIE cells (Fig. 5). It has been reported that insulin activates aPKCλ via PI 3-K in the liver (22, 23). As the induction of the SHARP-2 mRNA by AICAR was inhibited by a PI 3-K inhibitor, aPKCλ was one of the candidate signalling molecules involved. Indeed, an active (phosphorylated) form of aPKCλ was detected (Fig. 5). In addition to the induction of the SHARP-2 mRNA by AICAR was inhibited by overexpression of a dominant negative form of the aPKCλ, it was not inhibited by a calcium-ion-chelating reagent, EGTA (Fig. 6). Our
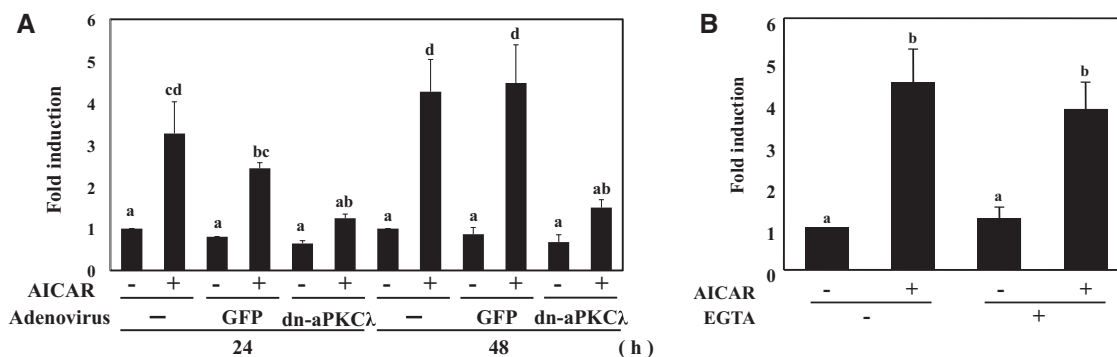

Fig. 6 aPKCλ mediates the induction of the level of SHARP-2 mRNA by AICAR. For determination of the levels of SHARP-2 mRNA from four independent experiments are described in the legend of Fig. 2. [a,b,c,d]Within each graph, means without a common letter differ, $P < 0.05$. (**A**) Expression level ratio in the absence of both AICAR and adenovirus was normalized to 1. The Ad-GFP and the Ad-dn-aPKCλ represent adenoviruses which express the GFP and a dominant negative form of aPKCλ, respectively. H4IIE cells were infected with each adenovirus at the m.o.i. = 50 for 24 or 48 h before treatment with (+) or without (−) 250 μM AICAR for another 2 h. (**B**) Expression level ratio in the absence of both AICAR and EGTA was normalized to 1. H4IIE cells were initially treated with (+) or without (−) 1 mM EGTA for 30 min before treatment with (+) or without (−) 250 μM AICAR for 2 h.
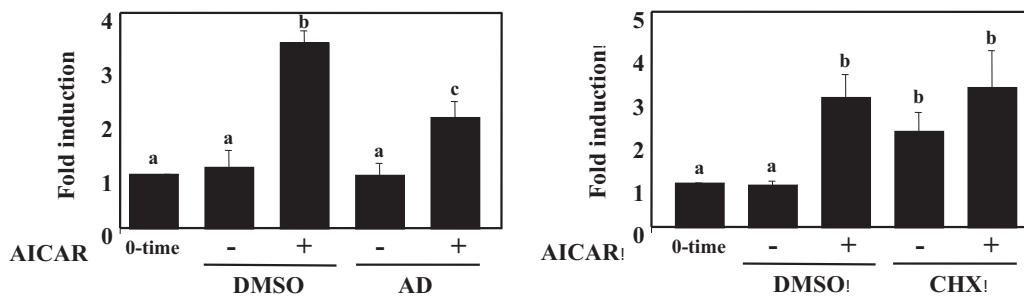
Fig. 7 Effects of actinomycin D and cycloheximide on the AICAR-induction of SHARP-2 mRNA. Details for determination of the levels of SHARP-2 mRNA from four independent experiments are described in the legend of Fig. 2. Expression level ratio at the AICAR treatment shown as 0-time was normalized to 1. [a,b,c]Within each graph, means without a common letter differ, $P < 0.05$. H4IIE cells were pretreated with DMSO, (**A**) 10 nM actinomycin D (AD) or (**B**) 5 µM cycloheximide (CHX) for 30 min before treatment with (+) or without (−) 250 µM AICAR for 2 h.
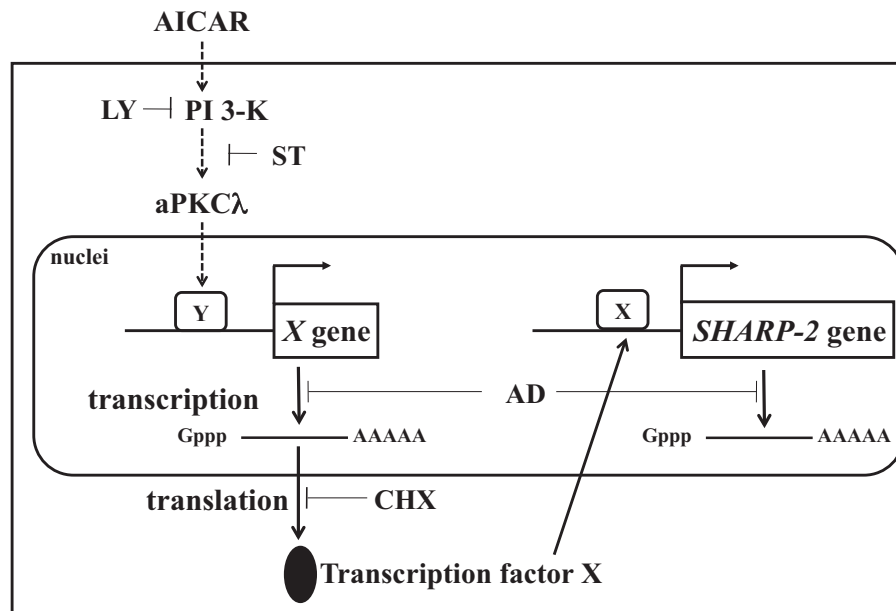

Fig. 8 A schematic drawing of the potential pathway involving in the AICAR-induced expression of the rat *SHARP-2* gene. AICAR directly or indirectly activates aPKCλ. Then, the aPKCλ activates transcription factor *Y* that induces transcription of the transcription factor *X* gene. Finally, the transcription factor *X* enhances transcription of the rat *SHARP-2* gene. LY, LY294002; ST, staurosporine; AD, actinomycin D; CHX, cycloheximide.

results showed that AICAR activated aPKCλ to increase the level of SHARP-2 mRNA in an AMPK-independent manner. Although AICAR stimulates activation of the aPKC, most cases are an AMPK-dependent manner (*26–29*). It has been reported that an effect of AICAR is different from that of metformin in rat L6 muscle cells and the mechanism is an AMPK-independent one that involves cPKC/nPKCs (*29*).

The induction of SHARP-2 mRNA by AICAR occurred at the transcriptional level of the rat *SHARP-2* gene and required for *de novo* protein synthesis (Fig. 7). There are many transcription factors regulating by AICAR (*30–33*). Most transcription factors are regulated by an AMPK-dependent mechanism (*30–32*). For example, AICAR response element-binding protein which binds to the human *PEPCK* gene promoter and inhibited its transcriptional activity

in a phosphorylation-dependent manner by AMPK (*30, 31*). It is well known that the AMPK directly phosphorylates peroxisome-proliferator-activated receptor γ coactivator 1α in muscle (*32*). In contrast, recent report showed that AICAR induced Nrf2 activation by an AMPK-independent mechanism (*33*). Although it remains to be determined the mechanism which how AICAR stimulates transcription of the rat *SHARP-2* gene, SHARP-2 could be added to a list of the AICAR-responsive transcription factors.

Thus, as summarized in Fig. 8, AICAR is an inducer of expression of the rat *SHARP-2* gene, it activates in a PI 3-K/aPKCλ pathway, then it activates transcription factor *Y* that stimulates transcription of the transcription factor *X* gene, and the transcription factor *X* elevates the promoter activity of the rat *SHARP-2* gene.

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Conflict of Interest
None declared.

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