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Induction of the SHARP-2 mRNA level by insulin is mediated by multiple signaling pathways

Yukiko Kanai[1], Kosuke Asano[2], Yoshiko Komatsu[1], Katsuhiro Takagi[1,2], Moe Ono[3], Takashi Tanaka[3], Koji Tomita[3], Ayumi Haneishi[2], Akiko Tsukada[2] and Kazuya Yamada[1,2,*]

[1]*Graduate School of Health Science, Matsumoto University, Matsumoto, Japan;* [2]*Faculty of Human Health Science, Department of Health and Nutritional Science, Matsumoto University, Matsumoto, Japan;* [3]*Faculty of Pharmacy, Laboratory of Molecular Biology, Osaka Ohtani University, Tondabayashi, Japan*

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The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is an insulin-inducible transcription factor which represses transcription of the rat *phosphoenolpyruvate carboxykinase* gene. In this study, a regulatory mechanism of the SHARP-2 mRNA level by insulin was analyzed. Insulin rapidly induced the level of SHARP-2 mRNA. This induction was blocked by inhibitors for phosphoinositide 3-kinase (PI 3-K), protein kinase C (PKC), and mammalian target of rapamycin (mTOR), actinomycin D, and cycloheximide. Whereas an adenovirus infection expressing a dominant negative form of atypical PKC lambda (aPKCλ) blocked the insulin-induction of the SHARP-2 mRNA level, insulin rapidly activated the mTOR. Insulin did not enhance transcriptional activity from a 3.7 kb upstream region of the rat *SHARP-2* gene. Thus, we conclude that insulin induces the expression of the rat *SHARP-2* gene at the transcription level via both a PI 3-K/aPKCλ- and a PI 3-K/mTOR- pathways and that protein synthesis is required for this induction.

Key words: SHARP-2; insulin; phosphoinositide 3-kinase; mammalian target of rapamycin; atypical protein kinase C lambda

Two members of the rat enhancer of split- and hairy-related protein (SHARP) family, SHARP-1 and SHARP-2, are basic helix-loop-helix transcriptional repressors.[1)] It has been reported that these also function as the molecular clock and that circadian rhythm in glucose metabolism was delayed in SHARP-1-, SHARP-2-, or double-knockout mouse.[2,3)] While both the SHARP-1 and the SHARP-2 mRNAs are expressed ubiquitously, their gene expressions are regulated in a cell type-specific manner by multiple extracellular stimuli.[1,3)] We reported that the levels of both SHARP-1 and SHARP-2 mRNAs were induced by insulin in the rat liver and either primary cultured rat hepatocytes or highly differentiated H4IIE rat hepatoma cells, and that both members of the SHARP family repress transcription of the rat *phosphoenolpyruvate carboxykinase (PEPCK)* gene which encodes a gluconeogenic enzyme.[4–6)] Therefore, we hypothesize that members of the SHARP family are involved in a decrease of the blood glucose level by insulin. Both a phosphoinositide 3-kinase (PI 3-K)/atypical protein kinase C lambda (aPKCλ)/Jun N-terminal kinase (JNK)- and a PI 3-K/Rac/JNK-signaling pathway mediate an induction of the level of SHARP-1 mRNA by insulin.[6)] However, that of SHARP-2 mRNA remains to be determined except for an involvement of PI 3-K.[4)]

Compounds that can induce both the SHARP-1 and SHARP-2 gene expression are useful for prevention and treatment of diabetes mellitus. We have reported that food constituents such as genistein, (-)-epigallocatechin-3-gallate (EGCG), and (*S*)-Equol, which lower blood glucose level *in vivo,* can stimulate these gene expression in H4IIE cells.[7–11)] PI 3-K, classical protein kinase C (cPKC), aPKCλ, and nuclear factor-κB mediate regulatory expression of the rat *SHARP-2* gene by them.

In this study, we investigated the effect of insulin on *SHARP-2* gene expression in H4IIE cells and identified the regulatory mechanism.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, insulin, LY294002, and horseradish peroxidase conjugate-goat antirabbit IgG antibody were purchased from Sigma-Aldrich Co. (Saint Louis, U.S.A.). Streptomycin and penicillin G were purchased from Meijiseika (Tokyo, Japan).

*Corresponding author. Email: kazuya.yamada@matsu.ac.jp
Abbreviations: aPKCλ, atypical protein kinase C lambda; cPKC, classical protein kinase C; DMEM, Dulbecco's modified Eagle's medium; EGCG, (-)-epigallocatechin-3-gallate; IEGs, immediate-early genes; JNK, Jun N-terminal kinase; mTOR, mammalian target of rapamycin; PCR, polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-K, phosphoinositide 3-kinase; PKC, protein kinase C; PVDF, polyvinylidene difluoride; SHARP, enhancer of split- and hairy-related protein.

Wortmannin, staurosporine, rapamycin, PD98059, okadaic acid, JNK inhibitor II, Rac1 inhibitor, actinomycin D, and cycloheximide were purchased from Merck chemicals (Darmstadt, Germany). The Adeno-X rapid titer kit was purchased from Clontech (Palo Alto, U.S.A.). The TRIzol reagent was purchased from Invitrogen (Groningen, the Netherlands). High Capacity RNA-to-cDNA Kit was purchased from Applied Biosystems Japan (Tokyo, Japan). FastStart Universal SYBR Green Master (Rox) and GenoPure Plasmid Maxi kit were purchased from Roche Diagnostics (Indianapolis, U.S.A.). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, U.S.A.). Polyvinylidene difluoride (PVDF) membrane and Immobilon Western chemiluminescent HRP substrate were purchased from MILLIPORE (Bedford, U.S.A.). Mouse anti-rat PKCλ antibody (610207) was purchased from BD Biosciences (San Jose, U.S.A.). Rabbit anti-rat p-mTOR (Ser2448) antibody (#2971) and rabbit anti-rat mTOR antibody (#2972) were purchased from Cell Signaling Technology (Danvers, U.S.A.). The pGL4.11, pGL4.20, and phRL-CMV plasmids, and Dual Luciferase Reporter Assay System were obtained from Promega (Madison, U.S.A.).

Cells and cell culture. Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University, U.S.A.). Adenoviruses, the Ad-GFP and the Ad-dn-aPKCλ, were generous gifts from Drs. Jun-ichi Miyazaki (Osaka University, Japan) and Wataru Ogawa (Kobe University, Japan), respectively.[12,13] Cell culture and treatments with insulin, various inhibitors, or adenoviruses were previously described.[6,7,10] Briefly, for analysis of a signaling pathway(s), 50 μM LY294002, 0.2 μM wortmannin, 0.1 μM staurosporine, 0.1 μM rapamycin, 25 μM PD98059, 10 nM okadaic acid, 10 μM JNK inhibitor II, 50 μM Rac1 inhibitor, 0.8 μM actinomycin D, or 10 μM cycloheximide were added to the medium at 15 min before the addition of 10 nM insulin. The adenovirus was infected with the 500 multiplicity of infection and cultured for an additional 24 h.

Preparation of total RNA and real-time polymerase chain reactions (PCRs). Preparation of total RNA from various H4IIE cells, reverse transcription, and real-time PCRs were previously described.[8,9,11]

Preparation of whole cell lysates and Western blot analysis. Procedures for preparation of whole cell lysates from H4IIE cells and Western blot analysis were previously described.[14] Briefly, whole cell lysates (20 μg) were resolved with 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane for Western blot analysis. The PKCλ antibody (1:100 dilution), the phospho-mTOR antibody (1:1000 dilution), and the mTOR antibody (1:1000 dilution) were used as primary antibody. Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (1:20,000 dilution) was used as the second antibody. Visualization and analysis of the proteins were also previously described.[14]

Transient DNA transfections and luciferase reporter assays. The pSHARP2/Luc-3700 plasmid was previously described.[10] Plasmids were prepared using the GenoPure Plasmid Maxi kit. A calcium-phosphate method was employed for transfection into H4IIE cells.[15] H4IIE cells were co-transfected with 10 μg of a reporter plasmid and 0.2 μg of the phRL-CMV plasmid. After transfection, the medium was replaced with serum-free DMEM. After 24 h, cells were treated with or without 10 nM insulin for various times. Firefly and sea pansy luciferase assays were carried out using the Dual Luciferase Reporter Assay System. Procedures were performed according to the manufacture's recommended protocol. Luciferase activities were determined by a Berthold Lumat model LB 9507 (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

Statistical analysis. Data were represented as the mean and standard error and analyzed by a one-way ANOVA followed by Fisher's protected LSD multiple comparison test. All experiments were repeated at least three times.

Results

Insulin induces the level of SHARP-2 mRNA in H4IIE cells

We reported that the level of SHARP-2 mRNA was induced via a PI 3-K within 2 h in primary cultured rat hepatocytes treated with 10 nM insulin.[4] Firstly, we investigated an issue of whether the level of SHARP-2 mRNA was induced by insulin in H4IIE cells. When H4IIE cells were treated for 2 or 4 h with 10 nM insulin, all the levels of SHARP-2 mRNA were increased by insulin (Fig. 1(a)). In contrast, the level of PEPCK mRNA was decreased by insulin (Fig. 1(b)).

These results indicate that insulin rapidly increases the level of SHARP-2 mRNA in H4IIE cells as well as primary cultured hepatocytes.

Effects of various inhibitors on expression of SHARP-2 mRNA induced by insulin

We then examined an issue of which signaling molecule(s) mediates the insulin-induction of SHARP-2 mRNA. H4IIE cells were treated with various inhibitors for the insulin signal transduction pathways before insulin treatment. Cell viability was not impaired by a treatment with all inhibitors. As shown in Fig. 2(a) and (b), LY294002, wortmannin, staurosporine, and rapamycin blocked the insulin-induction of SHARP-2 mRNA.

These results suggest that PI 3-K, protein kinase C (PKC), and mammalian target of rapamycin (mTOR) pathways mediate the accumulation of SHARP-2 mRNA by insulin.

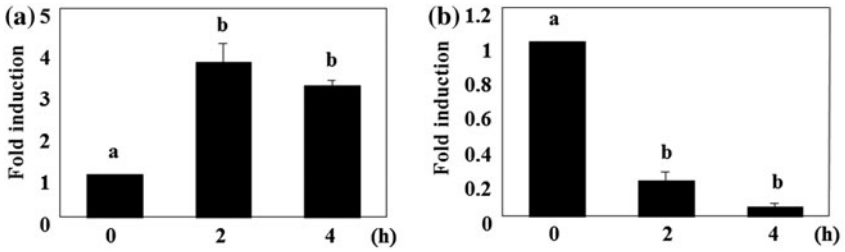

Fig. 1. Effects of insulin on expressions of SHARP-2 and PEPCK mRNAs.

Notes: The levels of SHARP-2, PEPCK, and 36B4 mRNA were determined. Each experiment was carried out three times. Each column and bar represents the mean and standard error of the ratio of the levels of either a, SHARP-2 mRNA or b, PEPCK mRNA. The value of the ratio in the absence of insulin was set to 1. H4IIE cells were treated for 2 or 4 h with 10 nM insulin. [a,b]Within each graph, means without a common letter differ, $p < 0.05$.

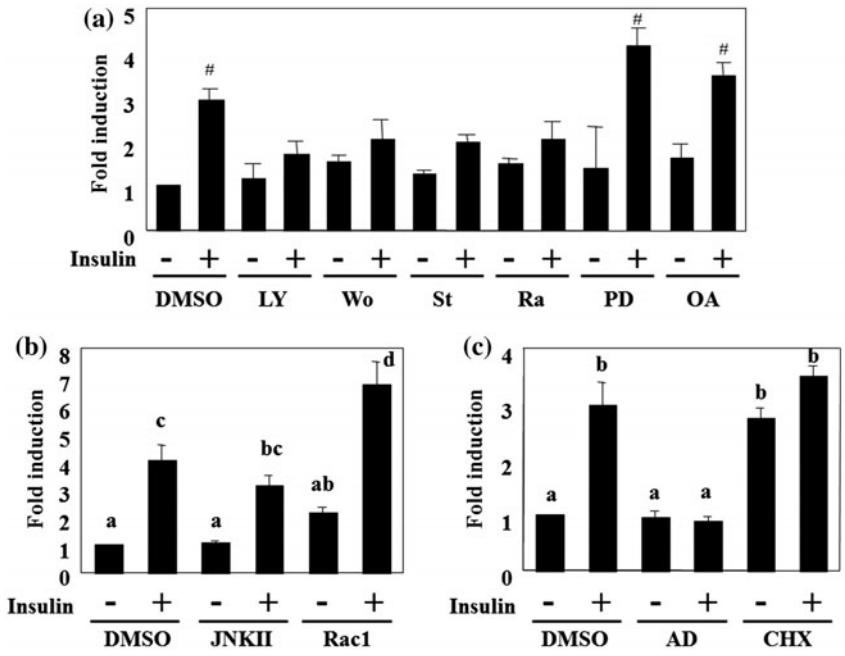

Fig. 2. Effects of various inhibitors on expression of SHARP-2 mRNA induced by insulin.

Notes: H4IIE cells were pre-treated for 15 min with (a) LY294002 (LY), wortmannin (Wo), staurosporine (St), rapamycin (Ra), PD98059 (PD) or okadaic acid (OA), (b) JNK inhibitor II (JNKII), or Rac1 inhibitor (Rac1), (c) actinomycin D (AD) or cycloheximide (CHX) indicated on the *bottom* and then treated with (+) or without (−) 10 nM insulin for 2 h. Each experiment was carried out at least four times. Details for determination of the levels of SHARP-2 mRNA are described in the legend of Fig. 1. [#,a,b,c,d]Within each graph, means without a common letter differ, $p < 0.05$.

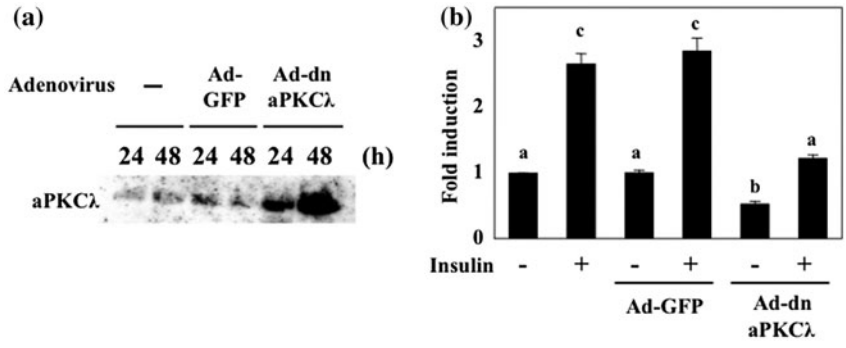

Fig. 3. aPKCλ mediates the induction of the SHARP-2 mRNA level by insulin.

Notes: (a) H4IIE cells were infected with each adenovirus. Whole cell lysates were prepared from H4IIE cells at the indicated times shown on the *top*. The anti-aPKCλ antibody was used as the primary antibody. (b) H4IIE cells were infected with each adenovirus, cultured for another 24 h, and treated with (+) or without (−) 10 nM insulin for 2 h shown on the *bottom*. Details for determination of the levels of SHARP-2 mRNA are described in the legend of Fig. 1. [a,b,c]Within each graph, means without a common letter differ, $p < 0.05$.

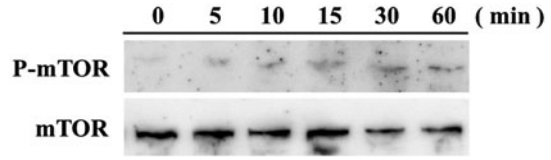

Fig. 4. Activation of mTOR by insulin.

Notes: Whole cell lysates were prepared from H4IIE cells treated with 10 nM insulin for the indicated times shown on the *top*. The anti-phospho-mTOR antibody (top) or anti-mTOR antibody (bottom) was used as the primary antibody.

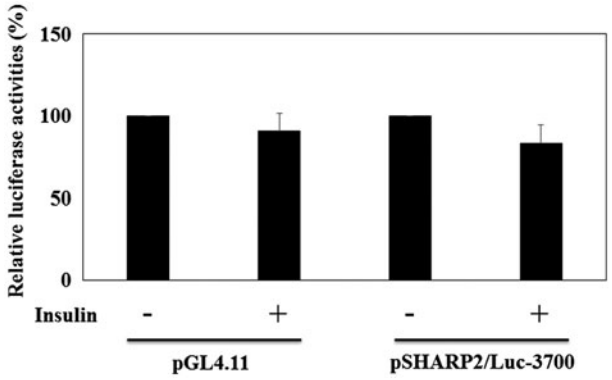

Fig. 5. An effect of insulin on transcriptional activity of the rat *SHARP-2* gene.

Notes: Either the pGL4.11 or pSHARP2/Luc-3700 plasmid shown on the *bottom* was used as the reporter plasmid. These transfected cells were cultured for 24 h in the absence (−) or presence (+) of 10 nM insulin. A value of 100 was assigned to the promoter activity from each reporter plasmid in the absence of insulin. Each column and bar represents the mean and standard error of four independent experiments.

The insulin-induction of SHARP-2 mRNA is required for both de novo *RNA and protein synthesis*

Next, to examine an issue of whether the induction of SHARP-2 mRNA by insulin is required for *de novo* RNA and/or protein synthesis, either actinomycin D or cycloheximide was used. The induction of the SHARP-2 mRNA level by insulin was completely inhibited by both reagents (Fig. 2(c)). Cycloheximide alone caused a "super-induction" of the level of SHARP-2 mRNA, but insulin did not enhance that of SHARP-2 mRNA (Fig. 2(c)).

These results suggest that the induction of SHARP-2 mRNA by insulin is regulated at the transcription level of the rat *SHARP-2* gene and that protein synthesis is required for the induction.

Effects of a dominant negative form of aPKCλ on insulin-induction of the SHARP-2 mRNA level

PKC has many isoforms such as cPKC, novel PKC, and aPKC.[16] The aPKCλ is one of the downstream PI 3-K pathway signaling molecules of insulin action.[17] Indeed, we reported that aPKCλ mediates an increase in the level of SHARP-2 mRNA by (*S*)-Equol and 5-aminoimidazole-4-carboxyamide-1-β-D-ribofranoside, respectively.[10,14] As transfection efficiency to H4IIE cells is quite low, a use of siRNA is impossible. Then, to examine the involvement of the aPKCλ in the insulin-induction of the SHARP-2 mRNA level, adenovirus expressing either the green fluorescence protein (Ad-GFP) or a dominant negative form of aPKCλ (Ad-dn-aPKCλ) was infected with H4IIE cells.[12,13] Expression of Ad-GFP was observed with a fluorescence microscope. That of Ad-dn-aPKCλ was confirmed by western blot analysis (Fig. 3(a)). Whereas the level of SHARP-2 mRNA by insulin was not altered in the Ad-GFP-infected cells, that of SHARP-2 mRNA by insulin was decreased to 41.9% in the Ad-dn-aPKCλ-infected cells (Fig. 3(b)).

These results indicate that aPKCλ plays a pivotal role in the expression of the rat *SHARP-2* gene by insulin.

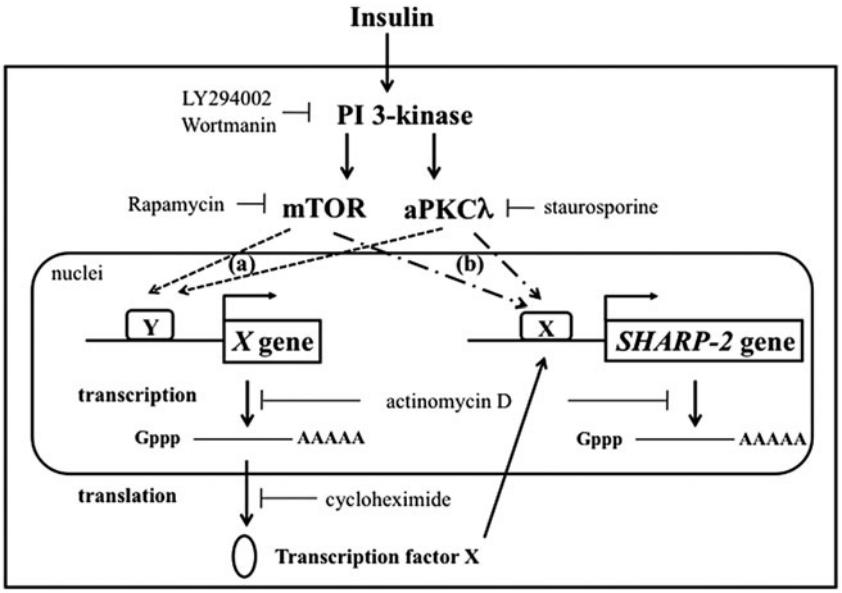

Fig. 6. Schematic drawing of regulatory expression of the rat *SHARP-2* gene by insulin.

Notes: Insulin indirectly (a) or directly (b) activates transcription factor X via a PI 3-K/aPKCλ- and/or a PI 3-K/mTOR- pathway. The X enhances transcription of the rat *SHARP-2* gene.

Activation of mTOR by insulin

Thus far, adenovirus which expresses a dominant negative form of mTOR is not available. Then, to confirm an issue of whether mTOR in H4IIE cells was activated by insulin, whole cell lysates were prepared from insulin-treated H4IIE cells. Western blot analysis was performed using antibodies against phosphorylated mTOR or mTOR. The level of active/phosphorylated form of mTOR was elevated at 5 min post-treatment with insulin (Fig. 4). In contrast, the level of whole mTOR protein remained unchanged (Fig. 4).

These results indicate that insulin rapidly activates mTOR before the increase of the level of SHARP-2 mRNA by insulin.

Effects of insulin on transcriptional activity of the rat SHARP-2 *gene*

Finally, we examined the effect of insulin on the transcriptional activity of the rat *SHARP-2* gene. We employed two reporter plasmids. One is the pGL4.11 firefly luciferase reporter plasmid which has no promoter and the other is the pSHARP2/Luc-3700, in which the nucleotide sequences between −3700 and +265 of the rat *SHARP-2* gene were inserted into a firefly luciferase reporter plasmid. The phRLuc-CMV plasmid expresses renilla luciferase driven by a cytomegalovirus enhancer/promoter. H4IIE cells were co-transfected with each reporter plasmid and the phRLuc-CMV plasmid, then cultured in the absence or presence of insulin. However, these luciferase activities remained unchanged by a treatment with insulin (Fig. 5).

These results indicate that there is no insulin-responsive element in the nucleotide sequence between −3700 and +265 of the rat *SHARP-2* gene.

Discussion

We examined the mechanism of which insulin induces the level of SHARP-2 mRNA. Insulin rapidly induced the SHARP-2 mRNA level in H4IIE cells as well as primary cultured rat hepatocytes (Fig. 1(a)). It has been reported that both aPKCλ and mTOR are independent downstream signal molecules of PI 3-K in the liver.[17–19] Indeed, a dominant negative form of aPKCλ blocked the insulin-induction and mTOR was activated at 5 min by insulin in H4IIE cells (Figs. 3 and 4). Thus, the insulin-induction of the SHARP-2 mRNA level is mediated by both a PI 3-K/aPKCλ pathway and a PI 3-K/mTOR pathway. The PI 3-K/aPKCλ pathway was identical with one of the signaling pathways of up-regulation of the SHARP-2 mRNA level by (*S*)-Equol.[10] It suggests that (*S*)-Equol partially can mimic the insulin action. We reported that an induction of the level of SHARP-1 mRNA by insulin mediates both a PI 3-K/ aPKCλ/JNK- and a PI 3-K/Rac/JNK-signaling pathway.[6] However, inhibitors for Rac1 and JNK had no effects on the insulin-induction of the SHARP-2 mRNA level, indicating that signaling molecules of downstream of PI 3-K were quite different between these genes (Fig. 2(b)). Insulin also induced the level of SHARP-2 mRNA in rat L6 myotubes and mouse 3T3-L1 adipocytes: the former requires a PI 3-K pathway and the latter a mitogen-activated protein kinase pathway.[20] Therefore, the regulatory expression of the rat *SHARP-2* gene by insulin is dependent on a tissue-specific manner.

As the induction of the level of SHARP-2 mRNA by insulin was completely blocked by pre-treatments with actinomycin D or cycloheximide, the insulin effect was controlled at the transcription level of the rat *SHARP-2* gene and synthesis of a transcription factor(s) was required for this induction (Fig. 2(c)). We examined an issue of whether 3.7 kb upstream region from a transcription initiation site of the rat *SHARP-2* gene exhibits an insulin responsiveness. However, transcriptional activity of this region did not enhance by insulin (Fig. 5).

Immediate-early genes (IEGs) including a transcriptional factor such as *c-fos* and *egr1* are rapidly and transiently activated in response to a wide variety of cellular stimuli.[21] A cycloheximide treatment alone increases their mRNA levels. The level of SHARP-2 mRNA increased by a cycloheximide treatment alone (Fig. 2(c)). SHARP-2 may be one of the IEGs and a late-inducible gene in them.

As shown in Fig. 6, insulin activates a PI 3-K pathway. Then either aPKCλ or mTOR or both activates transcription factor Y that induces transcription of the transcription factor *X* gene or directly activates transcription factor X. Finally, the transcription factor X enhances transcription of the rat *SHARP-2* gene. To evaluate this model, further studies will be required for analysis of the transcriptional activation mechanism of the rat *SHARP-2* gene by insulin.

In summary, insulin induces the expression of the rat *SHARP-2* gene at the transcription level via both a PI 3-K/aPKCλ- and a PI 3-K/mTOR- pathways and that protein synthesis is required for this induction.

Author contribution

YK, KA, and YK performed the experiments and collected data. KT, AH, and AT analyzed and interpreted the results. MO, KT, and TT prepared and determined a titer of the adenoviruses. KY designed the study. All the authors contributed to the critical revision of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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