Insulin Regulates the Expression of the Enhancer of Split- and Hairy-related Protein-2 Gene via Different Pathways in 3T3-L1 Adipocytes and L6 Myotubes

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Abstract

We investigated the effect of insulin on the expression of the *enhancer of split- and hairy-related protein-2* gene in 3T3-L1 adipocytes and L6 myotubes. The level of enhancer of split- and hairy-related protein-2 mRNA was increased by insulin in both cells. While both wortmannin and LY294002 blocked the increase in 3T3-L1 adipocytes, and only PD98059 was effective in L6 myotubes. Although the increase by insulin in these cells was inhibited by treatment with actinomycin D, this was enhanced by treatment with cycloheximide. Furthermore, cyclic AMP increased the level of enhancer of split- and hairy-related protein-2 mRNA in both cells in an additive manner. Thus, we conclude that insulin and cyclic AMP induce the expression of the enhancer of *split- and hairy-related protein-2* gene in both 3T3-L1 adipocytes and L6 myotubes, and that the gene expression enhanced by insulin is regulated by the cell type-specific pathway. The former requires a phosphoinositide 3-kinase pathway and the latter a mitogen-activated protein kinase pathway.

Key words
Insulin · Signaling pathway · Gene expression · Phosphoinositide 3-kinase · Mitogen-activated protein kinase

Introduction

Most basic helix-loop-helix (bHLH) proteins bind to the E-box sequence (5′-CANNTG-3′), which is located on the promoter region of a number of genes and regulates gene transcription [1]. The rat enhancer of split- and hairy-related protein-2 (SHARP-2) belongs to the bHLH protein family [2]. Mouse and human orthologs of SHARP-2 have been cloned and termed as stimulation of retinoic acid 13 (Stra13) and differentially expressed in chondrocytes protein 1 (DEC1), respectively [3,4]. Stra13 has been reported to function as a transcriptional repressor via histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms [5,6]. The expression of SHARP-2/ Stra13/ DEC1 mRNAs are ubiquitous; levels are regulated by a variety of stimuli such as cyclic AMP (cAMP), serum starvation, transforming growth factor-β, and hypoxia [3,4,6–9]. SHARP-2/ Stra13/ DEC1 may be involved in the regulation of cell proliferation and differentiation including chondrocytes, nerve cells, fibroblasts, T cells, and adipocytes [2–5,9]; mRNA levels increase during neuronal differentiation of pheochromocytoma PC12 cells by nerve growth factor and mouse embryonal carcinoma P19 cells treated with retinoic acid [2,4]. Recent studies have also reported that DEC1 serves as a regulator of the mammalian molecular clock, and that Stra13 is a key regulator of lymphocyte activation as evidenced by an analysis of the *Stra13*-deficient mice [10,11].

We previously reported that the levels of hepatic SHARP-2 mRNA increase as the result of refeeding a high-carbohydrate diet to normal rats or the administration of insulin to diabetic rats, the increase in SHARP-2 mRNA levels by insulin is mediated

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by a phosphoinositide 3-kinase (PI 3-K) pathway in primary cultured rat hepatocytes, and that insulin stimulates the transcription of the rat *SHARP-2* gene in the liver [2].

In the present study, we examined the issue of whether the expression of SHARP-2 mRNA is regulated by insulin in other insulin-responsive cells such as 3T3-L1 adipocytes and L6 myotubes. Our findings show that SHARP-2 is also an insulin-inducible transcription factor in these cells as well as hepatocytes, but the mechanism is different between the cell types.

Materials and Methods

Materials

The Dulbecco's modified Eagle's medium (DMEM), insulin, 1-methyl-3-isobutyryl xanthine, dexamethasone, minimum essential medium (MEM), 3-, 3′-, 5-triiodo-L-thyronine, wortmannin, LY294002, rapamycin, staurosporine, PD98059, okadaic acid, 8-bromo-cyclic AMP (8-Br-cAMP), actinomycin D, cycloheximide, and TRI REAGENT were purchased from Sigma Chemical Co. (St. Louis, MO). L6 cells were purchased from the Health Science Research Resources (Osaka, Japan). The TRIZOL reagent and Superscript II were purchased from Invitrogen (Groningen, the Netherlands). The pGEM-T Easy plasmid was purchased from Promega (Madison, WI). The Big Dye terminator FS cycle sequencing kit was purchased from Applied Biosystems Japan (Tokyo, Japan). α-^{32}P-dCTP (110 TBq/ mmol) was purchased from Amersham Biosciences (Cleveland, OH). The BcaBest DNA labeling kit was obtained from TaKaRa BIOMEDICALS (Kyoto, Japan). The ExpressHyb hybridization solution was purchased from Clontech (Palo, Alto, CA).

Cells and cell culture

3T3-L1 cells were a generous gift from Dr. Tamio Noguchi (Nagoya University, Japan). 3T3-L1 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C in a 5% CO_2 incubator. Cells were plated at a density of 1×10^3 cells/cm^2 in a 10 cm dish; differentiation into 3T3-L1 adipocytes was induced according to the method of Asai et al [13]. L6 cells were maintained in MEM supplemented with 10% FBS and antibiotics at 37 °C in a 5% CO_2 incubator. Cells were plated at a density of 1×10^4 cells/cm^2 in a 10 cm dish and were differentiated into L6 myotubes according to the protocol of Printz et al [14]. These cells were treated with or without insulin in the presence or absence of inhibitors of proteins involving in insulin signaling pathways, such as 0.1 μM wortmannin, 0.1 mM LY294002, 0.1 μM rapamycin, 0.1 μM staurosporine, 25 μM PD98059, and 10 nM okadaic acid, and 1 mM 8-Br-cAMP, 0.8 μM actinomycin D, and 10 μM cycloheximide [15]. All reagents except for staurosporine and 8-Br-cAMP were dissolved in dimethylsulfoxide (DMSO). Each experiment was carried out at least three times.

Probe DNAs

A probe for SHARP-2 was prepared using the reverse transcription-polymerase chain reaction [16]. Oligonucleotides 5′-ATGGA GCGGA TCCCC AGCGC-3′ and 5′-TTAGT CTTTG GTTTC TAAGT TTAAA G-3′ were used as the primers. A 1.2-kb product was subcloned into the pGEM-T Easy plasmid to obtain pGEM-T Easy SHARP-2EM. The nucleotide sequence of the insert was confirmed by sequencing. The pGEM-T Easy 36B4 plasmid has been described previously [12]. A 0.9-kb *Eco*RI/*Mlu*I fragment of the pGEM-T Easy SHARP-2EM and a 0.9-kb *Eco*RI fragment of the pGEM-T Easy 36B4 were used as probes for SHARP-2 and 36B4, respectively. These DNA fragments were labeled with α-^{32}P-dCTP using the BcaBest DNA labeling kit.

Preparation of total RNA and Northern blot analysis

Total RNA was prepared from 3T3-L1 adipocytes and L6 myotubes using the TRI REAGENT or TRIZOL reagent, respectively. Total RNA (10 μg/lane) was subjected to a 0.8% denaturing agarose gel electrophoresis, then transferred to a Biodyne membrane (ICN Biomedicals, Inc., Glen Cove, NY), and fixed by UV-crosslinking. The ExpressHyb hybridization solution was used for prehybridization and hybridization. All procedures followed the manufacture's recommended protocol. After washing the filter with 0.1 × SSC/0.1% SDS at 50 °C, it was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected and quantified using a FUJIX BAS-2000 image-analyzing system.

Statistical analysis

The intensity of bands of SHARP-2 and 36B4 mRNAs was quantified. The ratio of the intensities between SHARP-2 and 36B4 was calculated and plotted. Data are expressed as mean and standard error. Statistical difference of the mRNA level was determined by two-tailed Student's *t*-test.

Results

The SHARP-2 gene expression by insulin in 3T3-L1 adipocytes and L6 myotubes

We initially examined effect of insulin on the expression of the *SHARP-2* gene in 3T3-L1 adipocytes using Northern blot analysis. 3T3-L1 adipocytes were treated with various concentrations of insulin for 3 h. The level of SHARP-2 mRNA increased in a dose-dependent manner (Fig. **1 a**). The optimal concentration of insulin was determined to be 100 nM and the mRNA level was 2.6-fold higher than that in the absence of insulin (Fig. **1 a**). We then analyzed the time course for the increase in SHARP-2 mRNA levels in 3T3-L1 adipocytes under optimum conditions (Fig. **1 b**). The level of SHARP-2 mRNA gradually increased, reaching a maximum level at 3 h, and decreased to the basal level after 24 h. We then examined the *SHARP-2* gene expression in L6 myotubes. L6 myotubes were treated with various concentrations of insulin for 2 h. The level of SHARP-2 mRNA increased in a dose-dependent manner and the optimal concentration of insulin was 100 nM (Fig. **1 a**). The mRNA level was 2-fold higher than that in the absence of insulin. The time course for the mRNA level of SHARP-2 in L6 myotubes was then determined (Fig. **1 b**). The level of SHARP-2 mRNA reached a maximum 2 h after the addition of insulin and decreased to the basal level within 24 h. The levels of 36B4 mRNA remained unchanged under all conditions.

Analysis of insulin-signaling pathway of the SHARP-2 gene in 3T3-L1 adipocytes and L6 myotubes

We next examined the issue of which signaling pathway is involved in the up-regulation of the *SHARP-2* gene by insulin. Both 3T3-L1 adipocytes and L6 myotubes were treated with inhibitors of protein kinases or phosphatases of insulin signaling pathway, such as wortmannin, LY294002, rapamycin, staurosporine,

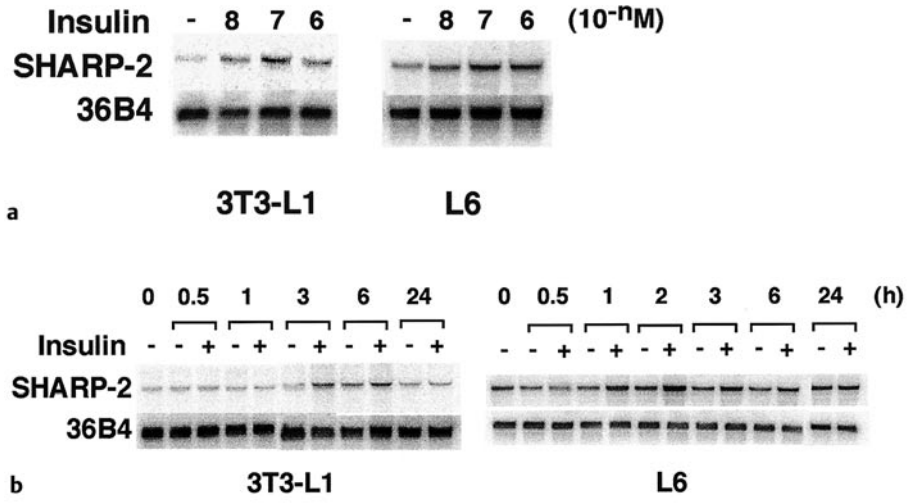
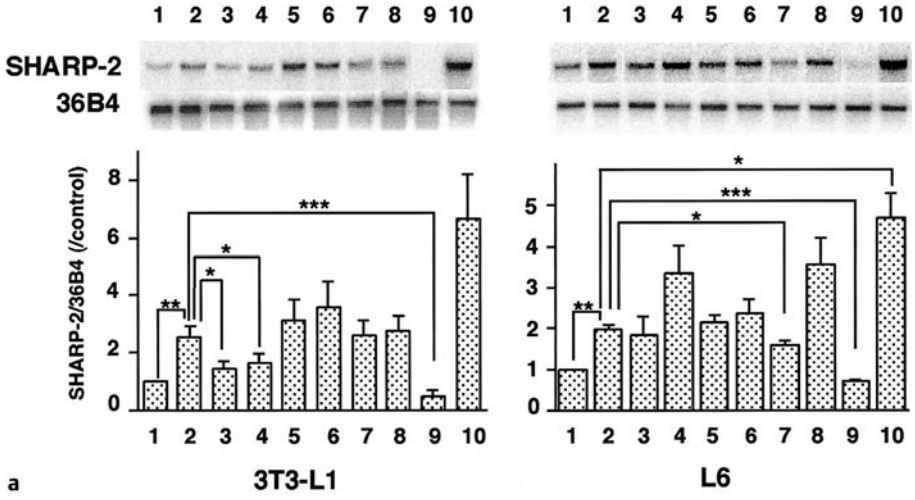


Fig. **1** Northern blot analysis of the expression of SHARP-2 mRNA in 3T3-L1 adipocytes and L6 myotubes. Total RNAs (10 µg) were analyzed. The probes used are shown on the *left*. Each result from 3T3-L1 adipocytes and L6 myotubes is shown on *left* and *right* panels, respectively. Each experiment was carried out at least three times. (**a**) Cells were treated with the indicated concentration of insulin (10^{-8} to 10^{-6} M) for 3 hours (3T3-L1 adipocytes) or 2 hours (L6 myotubes). (-), untreated. (**b**) Time course of alterations in the level of SHARP-2 mRNA by insulin. Cells were cultured in the absence (-) or presence (+) of 100 nM insulin for indicated times on *top*. SHARP-2, enhancer of split- and hairy-related protein-2; 36B4, ribosomal protein 36B4.



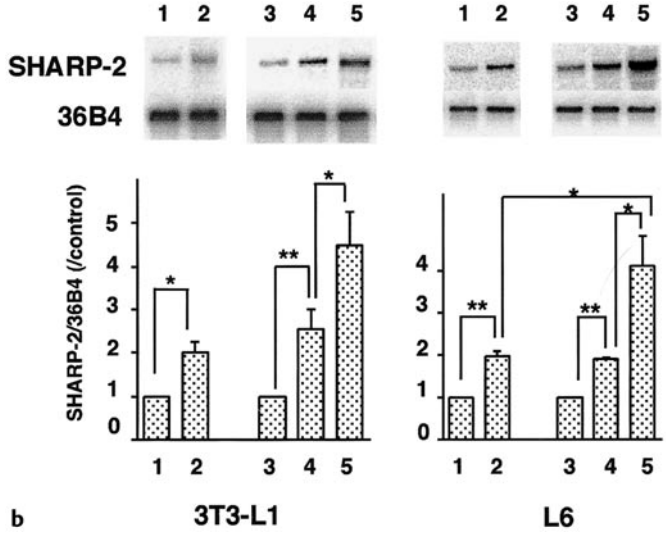


Fig. **2** Analysis of signaling pathway of the *SHARP-2* gene in 3T3-L1 adipocytes and L6 myotubes. The procedures and abbreviations are the same as those shown in the legend of Fig. **1**. Each result from 3T3-L1 adipocytes and L6 myotubes is shown on *left* and *right* panels, respectively. Each experiment was carried out at least three times. The ratio of the levels between SHARP-2 and 36B4 mRNAs was calculated *on bottom* in Fig. **2a** and **2b**. Value of the ratio in the presence of 0.1% DMSO was shown as 1. The mean and standard error from at least three independent experiments were plotted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**a**) Cells were treated for 3 hours (3T3-L1 adipocytes) or 2 hours (L6 myotubes) with various inhibitors of proteins that mediate insulin signaling pathways, actinomycin D, and cycloheximide. Lane 1, 0.1% DMSO; lanes 2 to 10, 0.1% DMSO plus 100 nM insulin; lane 3, 0.1 µM wortmannin; lane 4, 0.1 mM LY294002; lane 5, 0.1 µM rapamycin; lane 6, 0.1 µM staurosporine; lane 7, 25 µM PD98059; lane 8, 10 nM okadaic acid; lane 9, 0.8 µM actinomycin D; lane 10, 10 µM cycloheximide. (**b**) Cells were treated for 3 hours (3T3-L1 adipocytes) or 2 hours (L6 myotubes) with cAMP in the absence or presence of 100 nM insulin. Lane 1, untreated; lane 2, 1 mM 8-Br-cAMP; lane 3, 0.1% DMSO; lane 4, 0.1% DMSO plus 100 nM insulin; lane 5, 0.1% DMSO plus 100 nM insulin plus 1 mM 8-Br-cAMP.

PD98059, and okadaic acid. In 3T3-L1 adipocytes, wortmannin and LY294002 but not rapamycin, staurosporine, PD98059, or okadaic acid blocked the increase in SHARP-2 mRNA levels by insulin (Fig. **2a**). In contrast, in the case of L6 myotubes, only PD98059 blocked the increase in SHARP-2 mRNA levels by insulin (Fig. **2a**). 36B4 mRNA levels remained unchanged in these treatments.

We then examined the issue of whether *de novo* mRNA or protein synthesis is required for the increase in SHARP-2 mRNA levels by insulin. The increases in SHARP-2 mRNA levels by insulin in both

3T3-L1 adipocytes and L6 myotubes were strongly inhibited by treatment with actinomycin D (Fig. **2a**). In contrast, the increase in SHARP-2 mRNA by insulin was enhanced in both cells by cycloheximide (Fig. **2a**). 36B4 mRNA levels remained unchanged in this process. The findings indicate that *de novo* mRNA synthesis is required for the accumulation of SHARP-2 mRNA levels by insulin, and that inhibition of protein synthesis enhances the induction of SHARP-2 mRNA levels by insulin.

Effects of cAMP on the SHARP-2 gene expression in the absence or presence of insulin

Lastly, we examined whether cAMP affects the increase in SHARP-2 mRNA levels by insulin. Both 3T3-L1 adipocytes and L6 myotubes were treated with 8-Br-cAMP in the absence or presence of insulin. 8-Br-cAMP alone increased SHARP-2 mRNA levels in both cells (Fig. **2b**). In the presence of insulin, SHARP-2 mRNA levels were also increased in an additive manner by cAMP in both cells. In contrast, the 36B4 mRNA levels remained unchanged during this process.

Discussion and conclusions

The present study shows that the gene expression of SHARP-2 is induced by insulin in both 3T3-L1 adipocytes and L6 myotubes as well as hepatocytes [12]. In 3T3-L1 adipocytes, wortmannin and LY294002 inhibited the increase of SHARP-2 mRNA level ($p < 0.05$), indicating that the gene expression is mediated by a PI 3-K pathway. The downstream signal may be protein kinase B, since inhibitors of downstream atypical protein kinase C and p70S6 kinase, staurosporine and rapamycin were not affected (Fig. **2a**). These findings are consistent with those observed in hepatocytes [12]. Phosphoenolpyruvate carboxykinase (PEPCK), a known insulin-regulatable enzyme, is expressed in the liver, kidney, and adipose tissue. The hepatic expression of the rat *PEPCK* gene by insulin is downregulated via a PI 3-K pathway but not atypical protein kinase C and p70S6 kinase [17]. In addition, our previous results using primary cultured rat hepatocytes indicated that the time course for the upregulation of SHARP-2 mRNA levels by insulin is accompanied by a decrease in PEPCK mRNA levels [12]. These findings suggest that SHARP-2 may repress *PEPCK* gene expression via a PI 3-K pathway in both hepatocytes and 3T3-L1 adipocytes. In L6 myotubes, however, the mitogen-activated protein kinase (MAPK) pathway is required for the regulation of the *SHARP-2* gene expression by insulin since only PD98059 was effective ($p < 0.05$) (Fig. **2a**). We speculate that muscle-specific transcription factors, MyoD and myogenin, might be candidate genes for SHARP-2 since expressions of these genes are repressed by a MAPK pathway [18]. In any cases, these results indicate that insulin regulates the expression of the *SHARP-2* gene via different pathways in a cell type-specific manner. Expression of *the insulin receptor substrate-1* gene has been reported to be controlled by insulin via PI 3-K/ mammalian target of rapamycin (mTOR)-dependent pathway in 3T3-L1 adipocytes, but is reportedly mTOR-independent in L6 myotubes. In hepatocytes, 3T3-L1 adipocytes, and L6 myotubes [19,20], SHARP-2 mRNA levels increased as the result of insulin treatment for up to 2 to 3 h and then decreased to the basal level (Fig. **1b**, [12]). Increases in interleukin-6 mRNA levels are immediately induced by insulin in 3T3-L1 adipocytes, suggesting that interleukin-6 plays a possible role as a selectively regulated mediator of insulin resistance [21]. These findings suggest that SHARP-2 also functions at the early stage of the insulin-inducible biological process. Cycloheximide enhanced the increase in SHARP-2 mRNA levels by insulin in 3T3-L1 adipocytes and L6 myotubes (Fig. **2a**). We previously observed the same induction in primary cultured rat hepatocytes [12]. The precise mechanism remains unknown. Because turnover of SHARP-2 mRNA is rapid, cycloheximide might block synthesis of a ribonuclease for SHARP-2 mRNA.

In both 3T3-L1 adipocytes and L6 myotubes, cAMP increased the mRNA level of SHARP-2 ($p < 0.05$, $p < 0.01$) (Fig. **2b**). *SHARP-2/ Stra13/ DEC1* gene expression is reportedly induced by cAMP in many cell types, and the time course for maximum induction is apparently different among cell types. Furthermore, a consensus nucleotide sequence for the cAMP-responsive element exists at one and two sites in the promoter region of the rat *SHARP-2* and human *DEC1* genes, respectively [22,23]. These results suggest that cAMP acts at the transcription level of the gene. However, in our previous study using rat hepatocytes, no increase in SHARP-2 mRNA levels by cAMP was observed 3 h after treatment [12]. So far, whether the time of cAMP treatment was not optimal in the hepatocytes or whether cAMP is somewhat ineffective in the *SHARP-2* gene expression in hepatocytes is unclear. In addition, cAMP enhanced the mRNA level of SHARP-2 induced by insulin in both 3T3-L1 adipocytes and L6 myotubes in an additive manner ($p < 0.05$) (Fig. **2b**). Hexokinase II is reportedly an insulin-regulatable enzyme in fat and muscle cell lines; up-regulation of the gene transcription by insulin is supposedly enhanced by cAMP [24]. Therefore, it is likely that insulin does not antagonize the cAMP effect, and *vice versa*.

We conclude that SHARP-2 is an insulin-inducible transcription factor in both 3T3-L1 adipocytes and L6 myotubes as well as hepatocytes, and that the mechanism of regulation of *SHARP-2* gene expression by insulin is different for 3T3-L1 adipocytes vis-a-vis L6 myotubes, the former requiring a PI 3-K pathway and the latter a MAPK pathway. The induction by insulin requires *de novo* mRNA synthesis. Furthermore, *SHARP-2* gene expression was induced by cAMP in both the absence and presence of insulin. The biological role of the *SHARP-2* protein as an insulin-inducible transcription factor remains to be clarified. The identification of genes regulated by *SHARP-2* and an analysis of mechanism of transcriptional control of the *SHARP-2* gene will be needed to address these questions.

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