Inactivation of HDAC5 by SIK1 in AICAR-treated C2C12 Myoblasts

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Abstract. Salt inducible kinase (SIK) 1, a member of the AMP-activated kinase (AMPK) family, is activated by the AMPK-activator LKB1 which phosphorylates SIK1 at Thr182. The activated SIK1 then auto-phosphorylates its Ser186 located at the +4 position of Thr182. The phospho-Ser186 is essential for sustained activity of SIK1, which is maintained by sequential phosphorylation at Ser186-Thr182 by glycogen synthase kinase (GSK)-3β. Meanwhile, SIK1 represses the transcription factor cAMP-response element binding protein (CREB) by phosphorylating its co-activator transducer of regulated CREB activity (TORC). Recently, histone deacetylase (HDAC) 5 was identified as a new substrate of SIK1. Inhibition of SIK1 or AMPK results in the stimulation of glyconeogenesis in the liver by enhancing dephosphorylation of TORC2 followed by up-regulation of peroxisome proliferator-activated receptor coactivator (PGC)-1α gene expression. However, expression of the PGC-1α gene has been found to be repressed in LKB1-defective muscle cells. Our findings show that the AMPK agonist 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR)-dependent expression of PGC-1α is diminished by inhibitors of GSK-3β or SIKs in C2C12 myoblasts. Treatment with AICAR or the overexpression of SIK1 induces nuclear export of HDAC5 followed by the activation of myogenic transcription factor (MEF)-2C. The levels of phosphorylation at Thr182 and Ser186 of SIK1 in AICAR-treated C2C12 cells are elevated, and GSK-3β enzyme purified from AICAR-treated cells shows enhanced phosphorylation activity of SIK1 in vitro. These observations suggest that GSK-3β and SIK1 may play important roles in the regulation of PGC-1α gene expression by inactivating HDAC5 followed by activation of MEF2C.

Key words: SIK1, HDAC5, AICAR, AMPK, C2C12 cells

SALT-INDUCIBLE kinase (SIK) 1 is a serine/threonine protein kinase isolated from adrenals of rats fed with a high Na⁺- or K⁺-diet [1]. SIK1 belongs to a family of sucrose nonfermenting-1 protein kinase/AMP-activated protein kinase (AMPK) that is activated by metabolic/energy stresses [2]. Although SIK1 mRNA is not induced by salt stresses in cultured adrenocortical cells [3], it is rapidly induced when the cells are treated with cAMP. The induced SIK1 then represses the transcription(s) of steroidogenic enzymes by inhibiting cAMP-responsive element (CRE) on the promoters [4]. The mechanism by which SIK1 represses cAMP-induced gene expression consists of phosphorylation of a CRE-binding protein (CREB) specific co-activator transducer of regulated CREB activity (TORC) [5, 6]. Recently, the class II histone deacetylase (HDAC) 5 was also identified as a new SIK1 sub-
strate in a neuron of *C. elegans* [7] and in muscles of mice [8]. The phosphorylation of SIK1 at Ser577 is also important for SIK1’s HDAC5-phosphorylation activity, suggesting that the kinase activity of SIK1 and its phospho-modification may play important roles in a variety of signaling cascades.

The tumor suppressor kinase LKB1 has been identified as a major upstream activator of AMPK family kinases and phosphorylates Thr182 in the A-loop of SIK1 [9]. We found that Ser186 was also phosphorylated in activated SIK1 by autophosphorylation, and glycogen synthase kinase (GSK)-β was found to phosphorylate Thr182 in a phospho-Ser186 dependent manner, which in turn depends on the LKB1 cascade [10].

A drop in cellular energy results in an increase in the efficiency of energy-producing apparatus via the activation of AMPK [2]. In muscle cells, the energy-producing apparatus varies with cell type and physiological conditions [11]. Fast-twitch muscles develop the capability of quick energy-production by means of anaerobic glycolysis. In contrast, slow-twitch muscles are rich in mitochondrion and efficiently produce energy by means of oxidative phosphorylation [12].

Endurance exercise enhances AMPK activity in muscles, which leads to the expression of peroxisome proliferator-activated receptor coactivator (PGC)-1α followed by the upregulation of mitochondrial biogenesis [13, 14]. Expression of the PGC-1α gene is regulated by a number of transcription factors and coactivators, such as CREB-TORC [15, 16], FOXO1 [17, 18] and myocyte-enhancing factor (MEF) 2/class II HDACs [19]. However, the precise mechanism by which AMPK activates *PGC-1α* gene expression has not been fully clarified yet.

In the study reported here, it was found that the treatment of C2C12 myoblasts with the AMPK agonist 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR) enhanced GSK-3β activity followed by SIK1 phosphorylation at Thr182. Disturbance of the SIK1 activity by small compounds was accompanied with reduced expression of *PGC-1α*, probably due to an impaired regulation of HDAC5 and MEF2C.

### Materials and Methods

#### Cell culture, chemicals and antibodies

C2C12 cells were maintained in Dulbecco’s Modified Eagle medium (DMEM; Sigma, St. Louis, MO) supplemented with 20% FCS and antibiotics at 37 °C in an atmosphere of 5% CO₂-95% air. Staurosporine (STS) was purchased from Calbiochem (San Diego, CA) and Indirubin-3'-monoxime (Indi) and compound C (CC) were from Sigma-Aldrich (St. Louis, MO). AICAR was purchased from Toronto Research Chemicals Inc. (ON, Canada). GSK-3β antibodies were obtained from Cell Signaling Technology (Danvers, MA). The phospho-SIK1 antibody is described elsewhere [10].

#### Expression vectors and transformation

cDNAs for rat SIK1 and GSK-3β were previous report [10]. Cells (5 × 10⁵) plated on a 10-cm dish were transformed with 3–6 μg of the expression plasmid pEBG for Glutathione-S-transferase (GST)-tagged SIK1 by using 10 μl of LipofectAMINE 2000 (Invitrogen). After 36–48 h incubation, cells were lysed in 0.7 ml of lysis buffer [20]. The GST-tagged SIK1 protein was purified with glutathione columns. Aliquots of purified SIK1 were then subjected to western blot analyses with the anti-SIK1 IgG.

The preparation of adenoviruses is detailed elsewhere [21]. Briefly, a DNA fragment for the multicloning site of the GST expression vector pGEX-6P3 (GE Healthcare, Chalfont St Giles, UK) was amplified by PCR with primers linked with the Gateway™ recognition sequence (attB1 for forward and attB2 for reverse). The amplified product was then constructed into the pDONR221 vector by using BP-clonase (Invitrogen). After introduction of cDNAs for SIK1 and GSK-3β into pDONR221-GST by using the BamH1-NotI sites, adenovirus DNAs were prepared by means of LR-reaction (Invitrogen).

#### Reporter assay and quantitative RT-PCR analysis

Reporters for the –3 kb *PGC-1α* promoter (mouse) and HDAC5-expression vector are detailed elsewhere [19]. DNA fragments for –1.1 kb and –486 bp *PGC-1α* promoters were amplified by means of PCR using primers 5'-GGGGCCTAGC(NheI)TCATTGACTGACTCAGG
AACGACA (–1.1 forward primer), 5'-GGGCTAGC (NheI)CCAGTCACATGACAAAGCTA (common antisense primer) and 5'-GGGCTAGC(NheI)AATGTATCACATGAGGAGCG (–486 forward primer/common antisense primer). A cDNA fragment for mouse MEF2C was amplified by means of PCR using primers 5'-TTTTGGATCC (BamHI)ATGGGGAGAA AAAAGATTCAGAT and 5'-TTTTTGCGGCCGC (NotI)TCATGTTGCCCATCCTTCAGAGA, digested with BamH1/NotI and ligated into the pM2 vector [4].

C2C12 cells, cultured at semi-confluence in 24-well plates, were co-transformed with a SIK1 expression plasmid and PGC-1α-promoter reporters or with the pM2-MEF2/5xGAL4-Luc reporter in the presence of an internal standard reporter, pRL-TK(Int-). When cells had reached confluence (approximately 24 h later), the medium was changed to a differentiation medium (DMEM with 2% FCS) in the presence or absence of AICAR or other compounds. After 6 h incubation, cells were harvested for a reporter assay using the Dual Luciferase assay system (Promega, Madison, WI). Quantitative RT-PCR analysis was performed with the Sybr-Green methodology for iCycler-PCR (Bio-Rad, Hercules, CA) as described previously [22]. Primer sequences for mouse 36B4 and PGC-1α were [22] and 5'-GGGAAACCTTAAGTG TGGAAAC and 5'-CACCACGGTCTTGCAAGAGG, respectively.

**Results**

AICAR activates the PGC-1α gene promoter in C2C12 myoblasts

SIK1 and AMPK reportedly suppress the promoter activity of the PGC-1α gene in hepatocytes [15]. In contrast, the AMPK agonist AICAR was shown to induce PGC-1α gene expression in muscle cells [23]. Recently, we found that the adipose-specific SIK isoform SIK2 was activated by AICAR in 3T3-L1 adipocytes [24]. To determine the possibility of involvement of SIK1 in the AICAR-induced PGC-1α gene expression in C2C12 myoblasts, we measured the
levels of PGC-1α mRNA in AICAR-treated cells in the presence or absence of small compounds which interfere with SIK1 and AMPK.

Indi is a potent inhibitor of GSK-3β that is essential for sustained activity of SIK1[10]. STS can directly inhibit SIK1 activity even at a low concentration (10 nM) [25]. As shown in Fig. 1A, both Indi and STS inhibited AICAR-induced PGC-1α gene expression. But, the combination of Indi and STS did not synergistically suppress the AICAR-induced PGC-1α gene expression, suggesting that Indi and STS might block the same pathway. In contrast, the AMPK specific inhibitor compound C strongly inhibited the action of AICAR, indicating that AMPK might up-regulate PGC-1α gene expression through at least two pathways, a direct action and SIK1-dependent pathway. Results of reporter analyses further suggested that AICAR and SIK1 predominantly modulated the PGC-1α promoter activity at a distal region to which MEF2C binds (Fig. 1B).

**AICAR and SIK1 inactivate HDAC5 in C2C12 cells**

HDAC5 suppresses transcription activity of MEF2C in muscles [26], while SIK1 is capable of eliminating HDAC5 activity by inducing its nuclear export [8]. We therefore decided to examine whether SIK1 and AICAR could change the intracellular distribution of HDAC5 in C2C12 myoblasts.

AICAR was found to weaken the nuclear accumulation of GFP-tagged HDAC5, and SIK1 strongly promoted the export of HDAC5 (Fig. 2A and B). Both Indi and STS inhibited the action of AICAR and SIK1.

Next, we examined whether SIK1 and AICAR upregulate MEF2C activity in C2C12 cells. Although numerous studies have provided evidences of strong transcriptional activities of MEF2C [27, 28], over-expression of MEF2C in our C2C12 cells resulted in suppression of the PGC-1α promoter (Fig. 3A). To monitor MEF2C activity directly, we prepared Gal4-fusion MEF2C and co-expressed it with a GAL4-luciferase reporter. Gal4-MEF2C also showed transcriptional repression activity in the C2C12 cells (dotted line in Fig. 3B), and this activity was not influenced by overexpression of HDAC5. However, co-expression with SIK1 produced transcriptional activation of MEF2C. This activation was then suppressed by wild type HDAC5 and completely shut down by the HDAC5 mutant with a disruption at the SIK1-phosphorylatable Ser residue (S259A) [8].

AICAR weakened the transcriptional repression activity of MEF2C (Fig. 3C) and synergistically upregulated transcriptional activation of MEF2C with SIK1, which was reduced by overexpression of HDAC5 or treatment with Indi. These results suggest that SIK1 may play a role in AICAR-induced PGC-1α gene expression in C2C12 myoblasts, and that GSK-3β may be involved in the activation of SIK1.
Activation of GSK-3β enhances phosphorylation of SIK1 at Thr182 in C2C12 myoblasts

GSK-3β phosphorylates Ser/Thr residues located at the fourth position ahead of the phosho-primed Ser/Thr residues. In addition, GSK-3β phosphorylates SIK1 at Thr182 by recognizing the priming-autophosphorylation at Ser186 [10]. Phosphorylation of GSK-3β at Ser9 diminishes its kinase activity by mimicking a phosho-primed substrate. AICAR has been found to induce dephosphorylation of GSK-3β at Ser9 by inhibiting the upstream kinase AKT in muscle cells [29]. To determine whether the activation of GSK-3β could enhance phosphorylation of SIK1 at Thr182 in cultured myoblasts, C2C12 cells were treated with AICAR.

The differentiation stimuli led to a reduction in the level of phosphorylation of GSK-3β at Ser9, which was further enhanced by AICAR (Fig. 4A). The level of SIK1 phosphorylation (pT182 and pS186) showed a negative correlation with that of GSK-3β Ser9. The protein level of SIK1 also seemed to have increased in cells maintained in the differentiation medium and in those supplied with AICAR.

To assess the specific level of phosphorylation at Thr182, GST-tagged SIK1 was overexpressed in C2C12 cells and the cells were treated with AICAR. AICAR treatment increased the level of phosphorylation of SIK1 at Thr182 and Ser186, while treatment with Indi lowered it (Fig. 4B). Phosphorylation of GSK-3α in our C2C12 cells did not appear to be affected by these treatments.

As we mentioned above, AKT is one of the major kinases that inactivate GSK-3β by phosphorylation at Ser9, and inhibition of AKT has been proposed to be a cause of the AICAR/AMPK-dependent activation of GSK-3β [29]. We, therefore, monitored correlations between the levels of active-AKT and phosphorylation of SIK1 in serum-stimulated C2C12 cells. As shown in Fig. 4C, when cells were stimulated with serum, the level of phospho-SIK1 was reduced. AICAR suppressed the serum-dependent reduction in the level of phospho-SIK1, which was accompanied with reduced level of active AKT (phospho-AKT) and with increased level of active GSK-3β (dephospho-GSK-3β). Inhibition of AKT by the PI3K inhibitor LY294002 strongly blocked the serum-dependent dephosphorylation of SIK1. We mention here that, in addition to the serum-dependent dephosphorylation of SIK1, the AICAR-dependent activation of SIK1 was also inhibited by the treatment with LY29002, suggesting that PI3K might play an important role in the regulation of SIK1 through AKT/GSK-3β and an unknown pathway.

To investigate whether GSK-3β enzymes purified from AICAR-treated C2C12 cells could possess enhanced SIK1-phosphorylation activity, GST-tagged

Fig. 3. SIK1 up-regulates MEF2C activity. (A) Overexpression of MEF2C suppresses PGC-1α promoter activity. C2C12 cells were co-transformed with -3k PGC-1α promoter-reporter (Fig. 1 B) and MEF2C expression vector (pM-MEF2C: 30 ng or 100 ng). Transcriptional activity was expressed as the reporter activity of the empty pGL3 vector as described in Fig. 1B. (n = 4). (B) MEF2C activity in C2C12 cells. C2C12 cells were co-transformed with Gal4-MEF2C expression plasmid (100 ng) and 5 × GAL4-luciferase reporter plasmid (200 ng)/internal standard plasmid (30 ng) together with SIK1/HDAC5 expression plasmid (100 ng/30 ng). When cells reached confluence, the medium was switched to the differentiation medium. After 6 h incubation, cells were harvested for reporter analyses. Transcriptional activity was expressed as the reporter activity of the empty pM vector. (C) C2C12 cells co-expressing Gal4-MEF2C and SIK1/HDAC5 were treated with AICAR with or without Indi as described in Fig. 1A.
GSK-3β was overexpressed in C2C12 cells and purified after treatment of the cells with AICAR. As shown in Fig. 5, GSK-3β enzymes purified from the AICAR-treated cells showed higher SIK1 phosphorylation activity in vitro than those purified from non-treated cells. This enhanced activity was accompanied by an increase in the activating phosphorylation of GSK-3β at Tyr216 [30]. These results suggest that SIK1 may be activated by GSK-3β in the AICAR-treated C2C12 cells and inactivates HDAC5.

Discussion

We recently discovered two lines of correlation between SIK-family kinases and nutritional regulators. One was that GSK-3β was important for the maintenance of SIK1 activity because it directly phosphorylated SIK1 at Thr182 and Ser186 [10]. The other was that SIK2, the adipose specific isoform of SIK1, was activated by the AMPK agonist AICAR and downregulated lipogenic gene expression [24]. In addition to our observations, the finding that GSK-3β was activated by the AICAR in the oxidative soleus muscle [29] prompted us to investigate the possible activation of SIK1 by AICAR in C2C12 myoblasts.
SIK1 downregulates glyconeogenesis in the liver by phosphorylating TORC2 which binds to CREB on the PGC-1α promoter [15]. Loss of LKB1 in the liver also reduces TORC2 phosphorylation [31] due to a decrease in the activity of AMPK, which leads to enhanced expression of PGC-1α. It has also been found that overexpression of TORCs in the muscle results in the induction of PGC-1α gene expression [16], which suggests the possible involvement of AMPK and SIK1 as negative regulators of PGC-1α gene expression in muscle cells. However, the AMPK activator AICAR has been found to be a stimulant of PGC-1α gene expression in muscle cells [14, 23]. Moreover, loss of the LKB1 gene in the muscle leads to a decrease in PGC-1α gene expression [32]. These complexities related to the PGC-1α expression suggest the presence of other downstream targets of AMPK or SIK1 on the PGC-1α promoter in muscle cells.

The results of reporter analyses of the PGC-1α promoter and recent findings of the new SIK-substrate HDAC5 suggest that AICAR-dependent activation of SIK1 upregulates PGC-1α gene expression via the inactivation of HDAC5 followed by the activation of MEF2C. Although the subtypes are different, AICAR has been reported to enhance the level of both MEF2A and MEF2D [33], which share characteristics with MEF2C as targets of HDAC5 [19].

Ca2+/calmodulin-dependent kinase (CaMK) is known to phosphorylate HDAC5 and inactivate it during myogenesis [26], while contribution of STS-sensitive kinases to the phosphorylation of HDAC5 has also been reported [34]. Activation of SIK1 was evident in C2C12 cells maintained in a differentiation medium. Moreover, it has been found that SIK1 (this gene is also known as snf1lk) is induced during myogenic differentiation [35]. These findings suggest that SIK1 may act as the HDAC5 kinase during myogenesis and may perform important roles in the regulation of PGC-1α gene expression, which in turn may be one of the causes of the down-regulation of PGC-1α expression in LKB1-defective myocytes [32].

During endurance exercise a drop in the ATP-to-AMP/phospho-creatine-to-creatine ratios activates AMPK [36] and elevates the level of PGC-1α mRNA [37] and protein [38] in muscle. In contrast, resistance training downregulates the level of PGC-1α protein, which negatively correlates with the capability of protein synthesis via the activation of the IGF-1-AKT-mTOR pathway [36]. The phosphorylation of GSK-3β at Ser9 is increased during both types of exercise, but the degree of the phosphorylation is obviously high in muscles during resistance training [38, 39]. The attenuation of the AKT-dependent inactivation of GSK-3β by AICAR has been observed in oxidative soleus muscles, but not in glycolytic skeletal muscles [29]. While, GSK-3β has been reported to constitute an anti-hypertrophy signaling pathway in myocardial cells [40], and inhibition of GSK-3β induces myogenic differentiation [41]. These observations suggest that the GSK-3β-SIK1 cascade in muscles may play a role in the determination of adaptation processes, rather than in the differentiation processes.

Finally, there are several points in this study that could not be clarified. One of these points is whether GSK-3β-mediated phosphorylation of SIK1 at Thr182 is sufficient to induce PGC-1α gene expression. The answer to this question may be negative. A recent finding that PKA inactivates HDAC5 through the induction of SIK1 expression via CREB [8] is based on the premise that PKA activation does not last for a long time, since prolonged activation of PKA results in the nuclear accumulation of HDAC5 followed by the inactivation of MEF2 [42]. The opposite effect of PKA on HDAC5 activity can be explained by the different level of phosphorylation of SIK1 at Ser577 [8]. In addition, CaMK-K, an upstream kinase of CaMK, was shown to be able to activate AMPK [43], suggesting that when CaMK phosphorylates HDAC5, the CaMK-K/AMPK pathway is able to play a similar role. Recently, AMPK was found to phosphorylate HDAC5 and activate the GLUT4 promoter via the activation of MEF2C [44], and the AMPK inhibitor compound C more potently inhibited the action of AICAR than the GSK-3β inhibitor Indi or the SIK1 inhibitor STS (Fig. 1A). We therefore assume that various cascades composed of kinases and phosphatases may be required to fully regulate PGC-1α gene expression, and that SIK1 may be involved as a component in these cascades.

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