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Sulforaphane induced adipolysis via hormone sensitive lipase activation, regulated by AMPK signaling pathway

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ABSTRACT

Sulforaphane, an aliphatic isothiocyanate derived from cruciferous vegetables, is known for its antidiabetic properties. The effects of sulforaphane on lipid metabolism in adipocytes are not clearly understood. Here, we investigated whether sulforaphane stimulates lipolysis. Mature adipocytes were incubated with sulforaphane for 24 h and analyzed using a lipolysis assay which quantified glycerol released into the medium. We investigated gene expression of hormone-sensitive lipase (HSL), and levels of HSL phosphorylation and AMP-activated protein kinase on sulforaphane-mediated lipolysis in adipocytes. Sulforaphane promoted lipolysis and increased both HSL gene expression and HSL activation. Sulforaphane suppressed AMPK phosphorylation at Thr-172 in a dose-dependent manner, which was associated with a decrease in HSL phosphorylation at Ser-565, enhancing the phosphorylation of HSL Ser-563. Taken together, these results suggest that sulforaphane promotes lipolysis via hormone sensitive lipase activation mediated by decreasing AMPK signal activation in adipocytes.

1. Introduction

Obesity characterized by excessive lipid accumulation in adipose tissue as well as in ectopic localizations (especially muscle and liver) has been shown to increase the risk of developing insulin resistance, type 2 diabetes mellitus, hypertension, cardiovascular diseases, several types of cancer, and other chronic diseases [1].

In general, obesity is related to the extent of adipocyte differentiation, intracellular lipid accumulation, and lipolysis [2,3]. Adipose triacylglycerol lipase initiates lipolysis by specifically removing the first free fatty acid (FFA) to produce a diacylglycerol (DG), which is then hydrolyzed by a hormone-sensitive lipase (HSL) and subsequently by a monoacylglycerol lipase [4]. Perilipin also functions as a major regulator of lipolysis [5,6]. Under basal conditions, perilipin protects lipid droplets. Upon agonist stimulation, perilipin is phosphorylated PKA, which leads to a change in the perilipin coating that enables the recruitment of HSL from the cytoplasm to the surface of the lipid droplet [7,8].

AMP-activated protein kinase (AMPK) is a αβγ-heterotrimer complex that acts as a sensor of cellular energy status [9]. It is activated by energy stress stemming from an increase in the cellular AMP:ATP ratio and causes increased phosphorylation of the catalytic alpha subunit on Thr172, thereby stimulating the kinase activity and inducing a conformational change that inhibits the deactivation by phosphatases [10,11]. An immediate consequence of enhanced AMPK activity is the phosphorylation of HSL at Ser-565, and subsequently blocks activation of HSL at Ser-563 [12–14]. Previously, it was reported that phosphorylation of HSL on Ser-565 suppresses phosphorylation on Ser-563, thus decreasing HSL activity. Therefore, through a negative feedback mechanism involving AMPK, when PKA activation is triggered stimulation of adipocyte lipolysis is increased [15].

CPT1 (carnitine palmitoyltransferase 1) is the key regulatory enzyme in fatty acid oxidation, and committed to β-oxidation in the mitochondria by traversing the inner mitochondrial membrane [16]. Free fatty acids have a well established role as a mediator of adipocyte dysfunction, including insulin resistance and activation of inflammatory responses [16].

Sulforaphane is converted from glucoraphanin, a major glucosinolate in broccoli and cabbage, and is one of the most potent naturally occurring inducers of cytoprotective enzymes [17,18]. Recently, the effects of sulforaphane were shown to attenuate LPS-induced increases of IL-1, IL-6, and TNF-α expression in microglia. Furthermore, sulforaphane has been shown to attenuate LPS-induced activation of NF-κB and activator protein-1 (AP-1) [19].

In this study, we investigated the effects of sulforaphane on lipolysis and oxygen consumption, which was observed to know the pathway that lipolysis increases. Our results showed that sulforaphane treatment increased lipolysis and stimulated HSL

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phosphorylation at Ser-563, and inhibition of AMPK phosphorylation occurred as a consequence sulforaphane treatment.

Our results suggest that sulforaphane stimulates lipolysis in adipocytes, which is also associated with HSL phosphorylation, by inactivating AMPK phosphorylation at Thr-172.

2. Materials and methods

2.1. Cell culture and differentiation

3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and antibiotics (100 μg/ml gentamycin and 100 μg/ml penicillin–streptomycin). To induce differentiation, 2-day post-confluent 3T3-L1 cells were incubated in MDI induction media (DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μm dexamethasone and 1 μg/ml of insulin) for 2 days. Two days after MDI (day 2) the media was changed to insulin media. Detection of glycerol release was performed on day 6.

2.2. Adipolysis assay

Glycerol release was measured using the commercially available Adipolysis Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, differentiated adipocytes in a 96-well plate were stimulated with sulforaphane or an isoproterenol solution as a positive control for 24 h. After stimulation, cell culture supernatants were collected from each well and stored until use at −20 °C. Free glycerol assay reagent (100 μl) was added to 25 μl of supernatant. After incubation for 15 min at room temperature, absorbance was measured at 540 nm.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 3T3-L1 cells treated with sulforaphane using the Easy-spin™ total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). cDNA synthesis was carried out following the instructions of the TaKaRa Prime Script TM 1st strand cDNA synthesis kit (TaKaRa Bio, Tokyo, Japan). For qRT-PCR, 1 μl of gene primer with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA) in a 20 of reaction volume was applied. The sequences of the primers used for the Real-time PCR are as follows:

HSL (forward 5 volume was applied. The sequences of the primers used for β-actin (forward 5′TGAGAGGGAAATCGTGCGTGAC3′, reverse 5′GCTCGTTGCCAATAGTGATGACC3′) CPT1A (forward 5′TCC ACATTTGACTCCACATTTCC3′, reverse 5′GCAGCACCCTCACATATCC3′ β-actin (forward 5′TGAGAGGGAAATCGTGCGTGAC3′), reverse 5′GC TCGTTGCCAATAGTGATGACC3′).

All reactions with iTaq SYBR Green Supermix (Bio-Rad Laboratories) were performed on the CFX96 real-time PCR detection system (Bio-Rad Laboratories).

2.4. Western Blot

3T3-L1 cells treated with sulforaphane were lysed in a lysis buffer (25 mM HEPES; pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 0.1 mM dithiothreitol, and protease inhibitor mixture). Equal amounts of lysate protein were electrophoretically resolved on a 10–15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and the resolved proteins were transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. Images were captured using the Fusion FX7 acquisition system (Vilbert Lourmat, Eberhardzell, Germany). The density of the signal bands was analyzed using Bio-1D (Vilber Lourmat, Marne La Vallee, France).

2.5. Immunocytochemistry

Immunocytochemical analyses were performed on 3T3-L1 differentiated adipocytes with anti-phospho-HSL (Ser-563 and Ser-565) (Cell Signaling Technology), a rabbit polyclonal antibody against channel isoforms diluted to 1:250 (Ser-565) or 1:800 (Ser-563). Cells were cultured on Slide Glass (Nalge Nunc International, Naperville, IL). Cells were washed in sterilized TBST for 10 min, then blocked for 15 min with 5% FBS in TBST, and then incubated overnight at 4 °C with the primary antibodies diluted with 5% FBS in TBST. Alexa Fluor 488-labeled donkey anti-rabbit IgG antibody diluted 1:1000 (Molecular Probes, A21206) was used to visualize channel expression using fluorescence microscopy.

2.6. AMPK activity assay

AMPK was immunoprecipitated from lysated 3T3-L1 adipocytes and assayed using the preferred substrate and a generic detection system, AMPK KinEASE TM FP-645 nm FarRed Assay kit (Upstate, USA). In this assay, a phosphorylated peptide has been labeled with a red fluorescent dye. The results expressed by fluorescence polarization value were analyzed using Envision Multilabel Reader (Perkin Elmer, USA).

2.7. Statistical evaluation

All data are expressed as mean ± SEM, and the data were compared using the Student's *t*-test. Results were considered significant for values of $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{\#}p < 0.005$.

3. Results

3.1. Sulforaphane stimulates lipolysis in differentiated adipocytes

To examine whether sulforaphane modulates lipolytic activity, differentiated adipocytes were incubated for 24 h in the presence of sulforaphane. Lipolysis was measured by quantifying glycerol release into the medium (Fig. 1A). These data showed that sulforaphane increased lipolysis, with the highest glycerol release levels at 10 μM sulforaphane. Lipolysis in adipocytes is modulated in a step-wise fashion by Perilipin, ATGL and HSL. After ATGL initiates lipolysis by releasing fatty acids from TAG, HSL acts on DAG, and finally two additional FAs and glycerol are released. We also investigated whether the expression of HSL, Perilipin and ATGL mRNAs were affected by the various concentrations of sulforaphane. Compared with non-treated adipocytes, HSL mRNA levels in sulforaphane-treated adipocytes were increased (Fig. 1B), but ATGL and Perilipin mRNA levels were not affected (Fig. 1C and D). It has been suggested that HSL could regulate the mobilization of FFA by sulforaphane in adipocytes.

Increasing lipolysis, saturated fatty acids stimulates macrophages which lead to inflammation in adipose tissue [20]. The oxidation of adipocytes was measured by analyzing mRNA expression of carnitine palmitoyltransferase 1A (CPT1A), the enzyme involved in the transport of fatty acids across the mitochondrial membrane for fatty acid β-oxidation. Treatment of adipocytes with sulforaphane for 24 h induced an increase in the expression of CPT1A. Sulforaphane led to increased lipolysis as well as increased HSL mRNA expression in a dose-dependent manner, indicating sulforaphane enhanced fatty acid β-oxidation in adipocytes (Fig. 1).

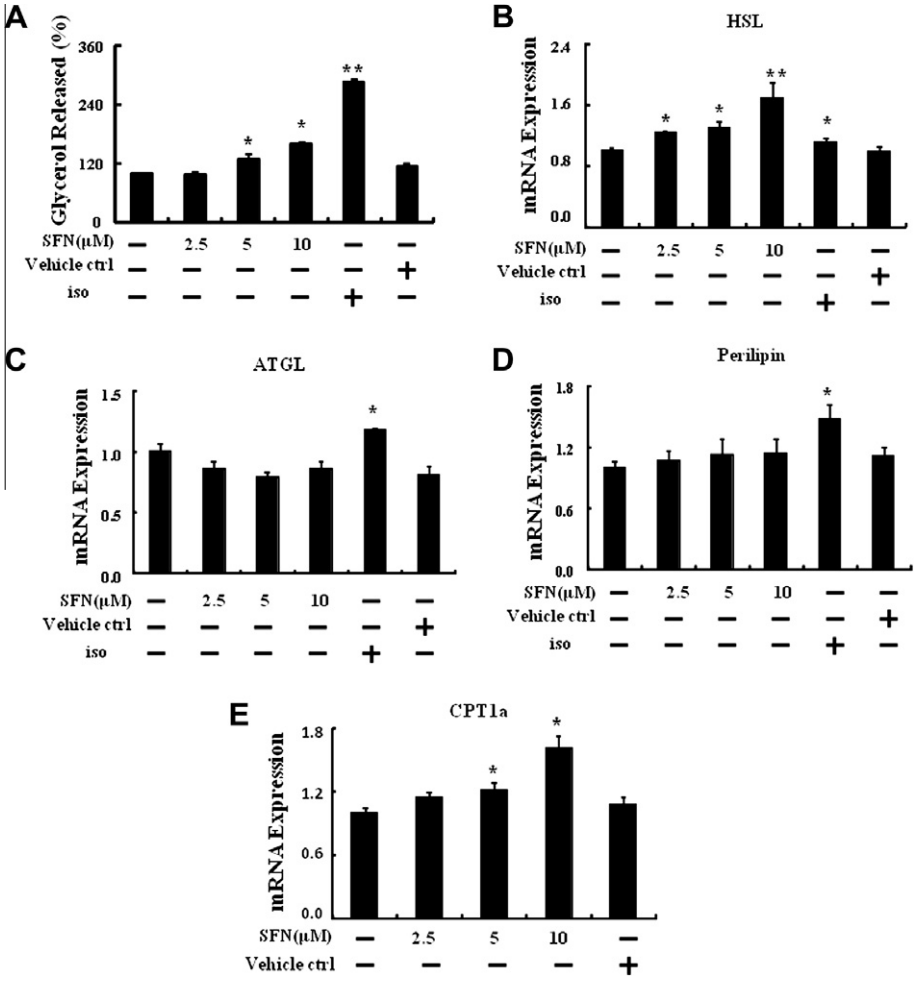

Fig. 1. Effects of sulforaphane on lipolysis in adipocytes. Adipocytes were incubated for 24 h in the presence of 2.5 μmol/l, 5 μmol/l or 10 μmol/l sulforaphane (SFN). Lipolysis was measured by quantifying glycerol released in the medium (A). The mRNA levels were determined by quantitative real-time RT-PCR for HSL, ATGL, Perilipin and CPT1A as described in Section 2(B, C, D, E). These results are representative of two independent experiments. $^{*}p < 0.05$ or $^{*}p < 0.01$ compared to basal conditions.

3.2. Sulforaphane regulates protein levels of HSL phosphorylation

We wanted to determine whether sulforaphane affects HSL activity as part of a major molecular mechanism involved in regulating lipolysis (Fig. 2). PKA phosphorylates Ser-563 on HSL leading to translocation of HSL to the lipid droplet and enhancing lipolysis. HSL can also be phosphorylated at Ser-565, preventing subsequent phosphorylation of other HSL residues and thus decreasing HSL lipase activity. While sulforaphane increased phosphorylation of HSL at Ser-563, phosphorylation of HSL at Ser-565 was decreased by sulforaphane treatment.

Changes with sulforaphane occurred in a dose-dependent manner as shown in the density graphs. HSL phosphorylation was also confirmed by immunocytochemistry. Whereas immunoreactive HSL phosphorylation on Ser-565 was suppressed by sulforaphane compared to untreated or DMSO-treated sulforaphane, phosphorylation of HSL Ser-563 was higher than in untreated or DMSO-treated sulforaphane in adipocytes.

3.3. Sulforaphane stimulates lipolysis through AMPK pathway

Activation of AMPK is mediated through its major regulatory phosphorylation site, Thr-172, located within the activation loop on the α-subunit [21]. AMPK inhibits lipogenesis and increases lipid oxidation through a decrease in malonyl-CoA content due to inhibition of acetyl-CoA carboxylase (ACC) activity by phosphorylating its Ser-79 [22]. AMPK is known to prevent lipolysis by inhibiting hormone-sensitive lipase (HSL) activity by phosphorylating Ser-565 [23]. In order to discern whether AMPK phosphorylation in adipocytes was related to an increase in adipocytes, we determined whether AMPK phosphorylation of Thr-172 decreased under the same conditions with Fig. 2. Phosphorylation of the AMPK Thr-172 site was suppressed by treatment with sulforaphane, and these results showed the lowest protein levels at 10 μM sulforaphane (Fig 3A). And also AMPK activity was reduced in dose-dependently (Fig. 3B). These data suggest that sulforaphane stimulates lipolysis via an AMPK inactivation pathway.

3.4. Enhanced lipolytic activity by sulforaphane is AMPK-dependent

Having established that AICAR upregulates AMPK activation in adipocytes, we needed to determine whether sulforaphane is strongly associated with AMPK phosphorylation. We investigated the effects of sulforaphane on AMPK activity by AICAR, a well-known AMPK activator and compound C, an AMPK inhibitor [24]. Sulforaphane exposure led to an increase in glycerol release levels from adipocytes (Fig. 1). Interestingly, when sulforaphane was added with AICAR to adipocytes, glycerol release into the medium decreased, but when AICAR was added alone, glycerol release increased (Fig. 4A). Western Blot analysis was performed to test AMPK activation responses by AICAR (Fig. 4B). As expected, AMPK phosphorylation was stimulated by AICAR and also, when adipo-

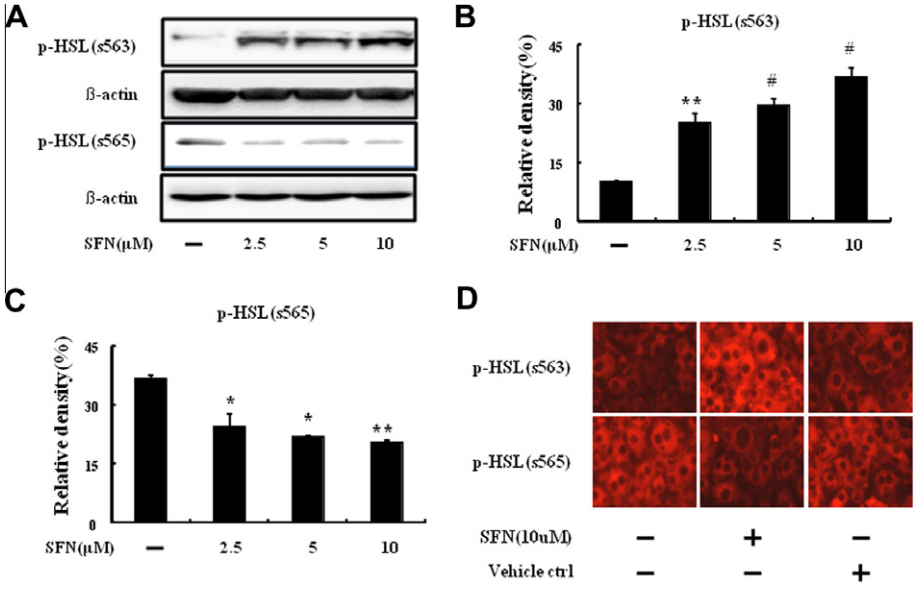

Fig. 2. Effect of sulforaphane on HSL phosphorylation in adipocytes. Adipocytes were incubated for 24 h in the presence of 2.5 μmol/l, 5 μmol/l or 10 μmol/l sulforaphane. Total adipocytes extracts were prepared and analyzed by Western Blotting for P-HSL$^{Ser-563}$ and P-HSL$^{Ser-565}$ (A). Density value was calculated as expression level versus control. Adipocytes were analyzed by immunocytochemistry for P-HSL$^{Ser-563}$ and P-HSL$^{Ser-565}$ (B). These blots are representative of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{\#}p < 0.005$ compared to basal conditions.

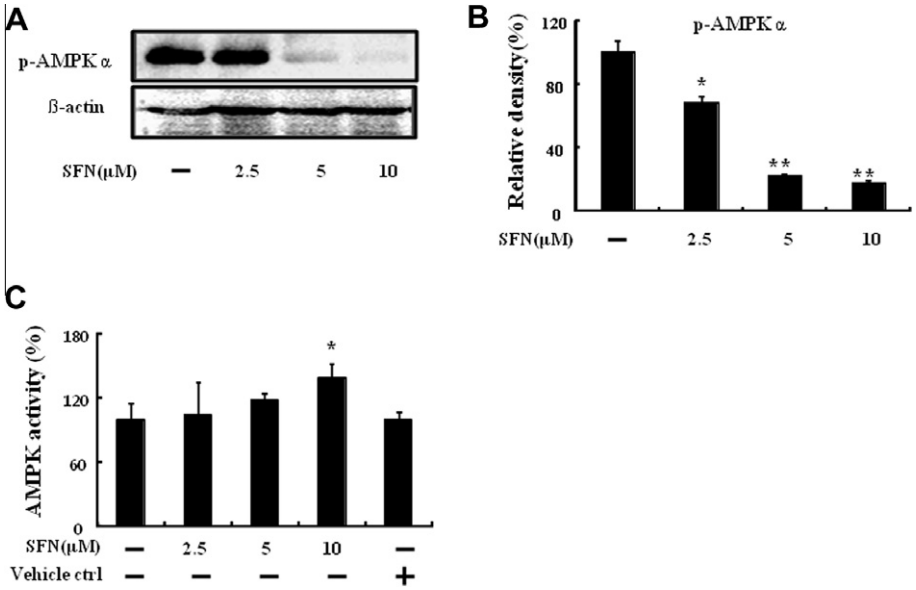

Fig. 3. Effect of sulforaphane on AMPK activity in adipocytes. Adipocytes were incubated in the presence of 2.5 μmol/l, 5 μmol/l or 10 μmol/l sulforaphane. Total adipocytes extracts were prepared and analyzed by Western Blotting for P-AMPK$^{thr-172}$ (A). Density value was calculated as expression level versus control (B). AMPK activity was measured as described in Section 2 (C). These results are representative of two independent experiments. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to basal conditions.

cytes were treated with both AICAR and sulforaphane, phosphorylation of AMPK increased. These data indicate that increased AMPK phosphorylation by AICAR was suppressed by sulforaphane treatment, suggesting an inhibitory effect on AMPK phosphorylation. These results demonstrate that sulforaphane has an influence on HSL phosphorylation, suggesting AMPK as a possible part of the mechanism of lipolytic activity.

4. Discussion

Sulforaphane is a naturally occurring isothiocyanate derived from cruciferous vegetables [25], and previous studies have shown chemopreventive effects of sulforaphane against various cancers [17]. Its effects as an anti-obesity drug, however, are unclear. Although a relationship between sulforaphane and obesity has been identified in adipocytes, it was only in relation to adipogenesis. One study indicated that sulforaphane may specifically affect mitotic clonal expansion to inhibit adipocyte differentiation, which may be associated with cell cycle arrest at the G(0)/G(1) phase through upregulation of p27 expression [26]. Sulforaphane has not yet been reported to regulate lipolysis. Therefore, we examined lipolysis by quantifying glycerol released into the cell medium and found that sulforaphane increased glycerol release in a dose-dependent manner, suggesting that sulforaphane increases lipolysis.

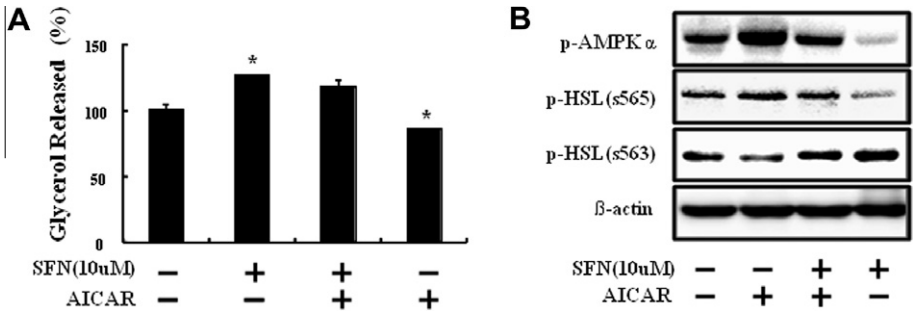

Fig. 4. Effect of sulforaphane on lipolysis in adipocytes in the presence of AMPK activator (AICAR). Adipocytes were incubated for 24 h in the presence of 5 μmol/l sulforaphane and 500 μmol/l AICAR. Lipolysis was measured by quantifying glycerol released into the medium (A). Total adipocyte extracts were prepared and analyzed by Western Blotting for P-HSL$^{Ser-563}$ and P-HSL$^{Ser-565}$ and P-AMPK$^{thr-172}$ (B). These results are representative of two independent experiments. $^{*}p < 0.05$ compared with basal conditions.

Hormone sensitive lipase (HSL) is a key enzyme in the regulation of lipid metabolism [27]. While ATGL selectively performs the first step in TG hydrolysis, HSL is the most active enzyme against DG, which are hydrolyzed faster than TG [28,29]. HSL is thought to be a potential target for treating lipid disorders and obesity [30,31]. Under basal conditions HSL is almost exclusively found in the cytosol of adipocytes. Upon phosphorylation and activation by PKA, HSL translocates from the cytosol to the surface of the lipid droplet to participate in lipolysis [27,32]. HSL activity is regulated primarily by serine phosphorylation, which is stimulatory at Ser-563 and Ser-659 via PKA and inhibitory at Ser-565 via AMPK [12,14,33]. Our results show higher HSL expression in adipocytes in the presence sulforaphane, indicating that sulforaphane stimulates lipolysis by increasing gene expression of HSL. Whereas HSL phosphorylation on Ser-563 was stimulated by sulforaphane, HSL phosphorylation on Ser-565 was suppressed in a dose-dependent manner. These data indicate that HSL activity is upregulated by sulforaphane, leading to downregulation of HSL phosphorylation at Ser-565 and suggesting that sulforaphane may be associated with the AMPK pathway.

AMPK acts as a fuel sensor in regulating glucose and lipid homeostasis in adipocytes, and its activation leads to numerous metabolic changes. AMPK activation of lipolysis could contribute to the conservation of energy by controlling the lipolytic process in adipocytes [34]. Our study concentrated on the correlation between AMPK and lipolysis.

Interestingly, our results indicate that stimulation of AMPK activity by AICAR leads to a decrease in lipolysis, an increase in HSL phosphorylation on Ser-565, and a suppression of phosphorylation on Ser-563. When adipocytes were treated with both AICAR and sulforaphane, lipolysis increased more than with AICAR treatment alone, which strongly suggests that AMPK is associated with sulforaphane activity and indicates that AMPK activation is a general process.

In skeletal muscle and liver, AMPK stimulates fatty acid oxidation to provide ATP as a fuel. In contrast, AMPK activation suppresses *de novo* lipid synthesis and fatty acid oxidation in adipocytes, [9,35]. Inhibition of AMPK activation also significantly increased palmitate oxidation indicating an increase in CPT1A activity [36].

In conclusion, we investigated sulforaphane stimulation of HSL activity via AMPK, and our results suggest that sulforaphane may be a promising therapeutic tool for obesity and obesity-related disorders.

Acknowledgments

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