Free fatty acid-induced histone acetyltransferase activity accelerates lipid accumulation in HepG2 cells

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common metabolic disease triggered by epigenetic alterations, including lysine acetylation at histone or non-histone proteins, affecting the stability or transcription of lipogenic genes. Although various natural dietary compounds have anti-lipogenic effects, their effects on the acetylation status and lipid metabolism in the liver have not been thoroughly investigated.

MATERIALS/METHODS: Following oleic-palmitic acid (OPA)-induced lipid accumulation in HepG2 cells, the acetylation status of histone and non-histone proteins, HAT activity, and mRNA expression of representative lipogenic genes, including PPARγ, SREBP-1c, ACLY, and FASN, were evaluated. Furthermore, correlations between lipid accumulation and HAT activity for 22 representative natural food extracts (NExs) were evaluated.

RESULTS: Non-histone protein acetylation increased following OPA treatment and the acetylation of histones H3K9, H4K8, and H4K16 was accelerated, accompanied by an increase in HAT activity. OPA-induced increases in the mRNA expression of lipogenic genes were down-regulated by C-646, a p300/CBP-specific inhibitor. Finally, we detected a positive correlation between HAT activity and lipid accumulation (Pearson’s correlation coefficient = 0.604) using 22 NExs.

CONCLUSIONS: Our results suggest that NExs have novel applications as nutraceutical agents with HAT inhibitor activity for the prevention and treatment of NAFLD.

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development of NAFLD.

Unlike genetic changes, epigenetic modifications, like Lys acetylation, are potentially reversible and can be modified by environmental, dietary, and lifestyle factors [18]. Additionally, these epigenetic factors are closely associated with the phenotype and determine progression of NAFLD [19]. In this study, we investigated the correlation between HAT activity and protein acetylation in NAFLD and identified natural extracts (NExs) that attenuate lipid accumulation by modulating HAT activity in an NAFLD model of HepG2 cells.

**MATERIALS AND METHODS**

**Cell culture**

Cells Human hepatocellular carcinoma (HepG2) cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in a humidified atmosphere of 5% CO₂ at 37°C using high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Welgene, Daegu, Republic of Korea). To establish an NAFLD model in HepG2 cells, a non-fat BSA-conjugated combination of oleic acid (OA) (Sigma-Aldrich, St. Louis, MO, USA) and palmitic acid (PA) (Sigma) was used at a ratio of 4:1 (OPA). HepG2 cells were simultaneously treated with 200 μg/mL of natural extracts and OPA for 24 h.

**Preparation of natural extracts**

Based on 2015 Korea National Health and Nutrition Examination Survey, about 80 natural food which are frequently consumed by Korean chosen, and 22 natural food extracts among them in which experiments had been first analyzed in the current study (Table 1). The natural food used in this study purchased from a local market (Seongnam, Gyeongggi-do, Korea). A voucher specimen has been deposited in Korea Food Research Institute (KFRI). Following cleaning of specimens those were extracted in a 10-fold volume of 70% ethanol by shaking for 24 h at 25°C. Finally, the precipitate was eliminated by centrifugation at 8,000 × g for 30 min, and supernatants were lyophilized in a freeze-drier (II Shin, Dongdo-chum-Si, Korea).

**Table 1. Abbreviation of natural extracts used in this study.**

| No. | Scientific name | English name | Abbreviation used in this study |
|-----|----------------|--------------|--------------------------------|
| 1   | Allium tuberosum | Garlic chives | EAT                             |
| 2   | Morus alba       | Mulberry     | EMA                             |
| 3   | Schizandra chinensis Bail | Magnolia vine | ESC                            |
| 4   | Spinacia oleracea | Spinach | ESO                            |
| 5   | Solarium lycopersicum | Tomato | ESL                            |
| 6   | Citrus unshiu    | Citrus       | ECU                            |
| 7   | Perilla frutescens | Perilla leaf | EPF                           |
| 8   | Allium fistulosum | Green onion | EAF                            |
| 9   | Lactuca sativa   | Lettuce      | ELS                            |
| 10  | Allium cepa      | Onion        | EAC                            |
| 11  | Codonopsis lanceolata | Doduk | ECL                           |
| 12  | Cucurbita maxima | Sweet pumpkin | ECM                         |
| 13  | Helianthus tuberosus | Jerusalem artichoke | EHT                       |
| 14  | Zingiber officinale | Ginger | EZO                           |
| 15  | Brassica oleracea | Broccoli     | EBO                            |
| 16  | Dioscorea polystachya | Hemp | EDP                           |
| 17  | Capsicum annum | Bell pepper | ECA                           |
| 18  | Platycodon grandiflorus | Bellflower | EPG                               |
| 19  | Porphyra tenera | Laver        | EPT                            |
| 20  | Fragaria x ananassa | Strawberry | EFA                           |
| 21  | Allium monanthum | Wild Chive | EAM                           |
| 22  | Malus domestic | Apple        | EMD                           |

**Table 2. Primers used in this study.**

| Primer list | Primer name |
|-------------|-------------|
| SREBP-1c    | Forward: 5’-AACTCAACGGAGAGACCTAGTC-3’ |
|             | Reverse: 5’-GTGATTGCTTCTTCACTTCAGT-3’ |
| ACLY        | Forward: 5’-TACCTCAGCACTGCAAGTT-3’ |
|             | Reverse: 5’-GACCGGAAGCCAGCAAGTT-3’ |
| FAS         | Forward: 5’-AACCGGCTTCTTCTTCTTCTGATTT-3’ |
|             | Reverse: 5’-TGCAGGGGGCCAGGCTTCTTCTGATTT-3’ |
| PPARγ      | Forward: 5’-CCACCACTCGTTGGTGAAG-3’ |
|             | Reverse: 5’-TTGGTGGTGGTGGTGGTGGTGAAG-3’ |
| GAPDH      | Forward: 5’-AGTTCTGACGAGGTTGGAAC-3’ |
|             | Reverse: 5’-GAGTCACTGAGGTTGGAAC-3’ |

**SREBP-1c:** sterol regulatory element-binding protein-1c; **ACLY:** ATP-citrate lyase; **FAS:** Fatty acid synthase; **PPARγ:** peroxisome proliferator-activated receptor-γ; **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase.
**Table 3. Antibodies used in this study**

| Antibody        | Vendor                      | Local         | Country | Titer | Usage |
|-----------------|-----------------------------|---------------|---------|-------|-------|
| αH3K9ac         | Cell signaling              | Denvers, MA   | USA     | 1:1000|       |
| αH4K8ac         | Cell signaling              | Denvers, MA   | USA     | 1:1000|       |
| αH4K16ac        | Cell signaling              | Denvers, MA   | USA     | 1:1000|       |
| Total Acetyl -Lys | Cell signaling            | Denvers, MA   | USA     | 1:1000| W. B  |
| αHDAC1          | Santa cruz biotechnology    | Dallas, Texas | USA     | 1:1000|       |
| αβActin         | Enzo life science           | Farmingdale, NY | USA     | 1:10000|      |
| αTubulin        | Cell signaling              | Denvers, MA   | USA     | 1:5000|       |

HDAC: histone deacetylases

**Quantitative real-time RT-PCR**

Cells were seeded at $5 \times 10^4$ cells/well. After reaching approximately 70% confluence, cells were treated with or without OPA for 18 h. To observe the effect of HATs on lipid accumulation in HepG2 cells, C-646, a p300/CBP-specific inhibitor, was added with OPA to HepG2 cells for 18 h, and then we performed qRT-PCR as described previously [20]. The primers used in this study were listed in Table 2.

**Immunoblotting**

Following treatment, cell extracts were supplemented with lysis buffer (Cell signaling) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) for 30 min and centrifugation at 20,000 × g for 20 min at 4°C. The cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with specific antibodies as indicated in Table 3 overnight at 4°C, followed by incubation with a secondary antibody (Thermo Scientific, Rockford, IL, USA). Proteins were visualized using a ChemiDoc system (Bio-Rad) with an enhanced chemiluminescence substrate (Thermo Scientific).

**Statistical analysis**

qRT-PCR data were analyzed using *t*-tests. Correlations between HAT activity and lipid accumulation in HepG2 cells were evaluated by Pearson’s correlation coefficients. Statistical analyses were carried out in SPSS (Ver. 20; SPSS Inc., Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

**RESULTS**

The acetylation status of non-histone and histone proteins increased by OPA

To examine whether the acetylation level of non-histone proteins and histone proteins increased in the NAFLD model, the acetylation status was evaluated in HepG2 cells with or without OPA treatment. In particular, cells were treated with 400 μM of OA and 100 μM of PA (1×) or 800 μM of OA and 200 μM of PA (2×) with or without trichostatin A (TSA). As shown in Fig. 1A, the acetylation level of total proteins was higher in the samples treated with OPA and TSA than in the control group. Additionally, the acetylation level was relatively higher in the group treated with a high concentration of OPA. Next, to observe changes in the specific acetylation status of histone tails, H3K9, H4K8, and H4K16, related to the expression of genes related to lipid accumulation in liver, were extracted from HepG2 cells. Unlike total protein acetylation, H3K9, H4K8, and H4K16 were acetylated without TSA treatment and the acetylation levels increased in proportion to the concentration of OPA (Fig. 1B). Although the levels of acetylation at H3K9, H4K8, and

![Fig. 1. Oleic and Palmitic acids (OPA)-induced activation of HAT increased non-histone and histone acetylation.](image-url)
Fig. 1. continued

Fig. 2. mRNA expression of lipogenic genes is dependent on HAT activity. (A) mRNA expression of genes related lipid metabolism was measured by qRT-PCR. Oleic and palmitic acid (OPA) was treated for 24 hr in HepG2 cells, RNA was extracted, and then 2 μg RNA was synthesized to cDNA. All experiments were carried out three times independently and data are expressed as mean ± SD. * P<0.05 (student t-test). (B) C-646, a specific inhibitor of p300/CBP, blocked the OPA-induced mRNA expression of lipogenic genes. HepG2 cells were treated with OPA in the presence or absence of C-646 for 18 h. mRNA expression of ACLY, FASN, SREBP-1c, and PPAR-γ was measured by qRT-PCR. The values presented are the means±SD of three independent experiments. Data are expressed as mean±SD. * P<0.05 (student t-test). PPAR-γ, peroxisome proliferator-activated receptor-γ; SREBP1c, sterol regulatory element-binding protein-1c; ACLY, ATP citrate lyase; FASN, fatty acid synthase.
H4K16 increased in the groups treated with both OPA and TSA, similar to the patterns observed for the groups treated with OPA only, the degree of acetylation was much greater than that of groups treated with OPA only (Fig. 1B). Based on the close association between protein acetylation and HAT activity, we investigated whether HAT activity was greater in the NAFLD model than in the control. As expected, HAT activity was much greater in the group treated with OPA (Fig. 1C). Taken altogether, these results showed that the acetylation levels of both non-histone and histone proteins were positively related to HAT activity, which increased by lipid accumulation in HepG2 cells.

**Figure 3.** Effect of natural extracts (NExs) on lipid accumulation in HepG2 cells. (A) HepG2 cells were treated with oleic acid only or co-treated with oleic acid and NExs for 24 h. Cells were stained with Oil red O staining as described in Materials and Methods and were viewed by microscopy (upper panel). Quantitative Oil red O dye as fat drops and data are expressed as mean ± SD. ## control group, **P < 0.01, * P < 0.05 (student t-test) (low panel). (B) Cytotoxicity test of NExs in HepG2 cells. HepG2 cells were treated with the various NExs in the presence or absence of oleic and palmitic acids (OPA) for 24 h. The cell viability was measured using a WST-1 assay in HepG2 cells. All data are expressed as mean ± SD from the samples of each group. There was no statistical significance.
Fig. 4. Effect of natural extracts (NExs) on histone acetyl transferase activity. (A) HAT activity assays were performed with NExs as described in Materials and Methods. The result was represented with relative percentage compared to a positive control which was incubated without a NEx. Values are means ± SD for three independent experiments; # control group, ** P < 0.01, * P < 0.05 (student t-test). Epigallocatechin gallate (EGCG) was used as a negative control.

Fig. 5. There are positive correlation between lipid accumulation and HAT activity. To validate the correlation between HAT activity and lipid accumulation, Pearson’s correlation value was calculated based on the experimental data from Oil red O (Fig. 3A) and HAT assay (Fig. 4) following NExs treatment with oleic and palmitic acids (OPA). Pearson’s correlation value is 0.604.

with or without C-646, a specific p300/CBP inhibitor, in combination with OPA for 18 h. As shown in Fig. 2B, the mRNA expression of lipogenic genes, i.e., PPARγ, SREBP1c, ACLY, and FASN, was abrogated by blocking p300/CBP activity using C-646. Collectively, our data suggest that there is a correlation between the expression of lipogenic genes and HAT activity.

EMA and ESC effectively prevent OPA-induced lipid accumulation in HepG2 cells

We hypothesized that natural compounds that can block OPA-induced lipid accumulation may show relatively high anti-HAT activity. Thus, we selected 22 commonly consumed vegetables in Korea and measured their ability to prevent OPA-induced lipid accumulation in HepG2 cells. 200 μg/mL extract with OPA were treated in cells, and lipid accumulation was measured by Oil Red O staining (Fig. 3A, upper panel). To quantify it, absorbance of the dissolved Oli Red O dye in isopropanol was measured (Fig. 3A, lower panel). Lipid accumulation was greater in OPA-treated cells than in the control group, and most extracts, including the extract of Allium tuberosum (EAF), had a protective effect on lipid accumulation. In particular, the extract of Morus alba (EMA) and that of Schizandra chinensis Bail (ESC) reduced lipid accumulation by almost 30% compared to that in cells treated with OPA alone. The extracts of Platycodon grandiflorus, Fragaria x ananassa, Allium monanthum, and Malus domestica did not have preventative effects on OPA-induced lipid accumulation in HepG2 cells. We examine whether the effect of NExs on the inhibition of lipid accumulation occurred as cytotoxicity. HepG2 cells were incubated in the presence or absence of OPA, with or without NExs. As shown in Fig. 3B, NExs did not influence the viability of cells. Altogether, NExs used in this study significantly abrogates OPA-induced lipid accumulation without cytotoxicity.

ESC shows the strongest effect on HAT activity

To analyze the correlation between lipid accumulation and HAT activity, we used a cell-free system. NEs at the same concentrations used in the previous experiment were incubated with HeLa nuclear extract, and epigallocatechin-3-gallate (EGCG), which is a well-established specific HATi [21], was used for comparison. As shown in Fig. 4, anti-HAT activity was observed in 22 NEs. Among these, 10 NEs, i.e., EAT, EMA, ESC, ESO, ESI, ECU, EPC, EHT, EZO, and EPT, inhibited HAT activity by over 20% in comparison with activity in the control group (gray bars in Fig. 4). ESC demonstrated the strongest anti-HAT effect, and EMA also showed a relatively strong inhibitory effect, supporting the correlation between lipid accumulation and HAT activity.

Lipid accumulation and HAT activity are positively correlated

To further evaluate the correlation between lipid accumulation and HAT activity, Pearson’s correlation coefficients were calculated using Oil Red O absorbance data and HAT activity for each NE (Fig. 5). The correlation coefficient was 0.604 (P = 0.0035), suggesting that there is a positive correlation between lipid accumulation and HAT activity.

DISCUSSION

NAFLD is the most common liver disease worldwide. Many studies have focused on the effects of natural dietary compounds on lipid metabolism to develop new therapeutic strategies [22]. However, lifestyle influences the development
and progression of NAFLD via epigenetic regulation [23], since it is a complex disease determined, in part, by environment factors [24]. Thus, epigenetic mechanisms involved in lipid metabolism are regarded as potential preventive or therapeutic targets [25].

Our data supported the results of previous studies showing that NAFLD may be controlled by epigenetic regulatory mechanisms. In particular, the balance between histone acetylation/deacetylation at the N-termini, which are catalyzed by HAT and HDAC, respectively [26], influences gene expression profiles in NAFLD [27]. As shown in Fig. 1B, histone H3K9, H4K8, and H3K16 acetylation increased dramatically following OPA treatment, and the acetylation status increased when HDAC activity was inhibited by TSA, an HDAC-specific inhibitor, indicating that aberrant histone acetylation by an imbalance between HAT and HDAC activity is involved in the hyperacetylation of histones, resulting in the development and progression of NAFLD. In adipocytes, histone H3K56 acetylation was first identified in a genome-wide profile analysis [28]. Mikula et al. demonstrated that the acetylation of histones H3K9 and H3K18 in the TNFα and Cc2 promoters was increased in the livers of mice fed a high-fat diet [29]. Furthermore, our results indicated that the total protein acetylation status increased significantly in cells treated with OPA in combination with TSA. Unexpectedly, acetylated-Lys was not detected without HDAC inhibition in extracts of the total cell lysate, unlike that of histones (Fig. 1A), suggesting that HDACs, which are predominantly localized in the cytosol or shuttle between the cytoplasm and the nucleus, are closely involved in the regulation of acetylation on non-histone proteins related to lipid accumulation. Similarly, a previous study reported that total protein acetylation is significantly increased in the hearts of HFD-fed mice [30], suggesting that lipid accumulation-induced hyperacetylation is not limited to the liver and is involved in obesity-related diseases in various organs. In particular, SREBP1c is acetylated by p300/CREB in both cells and mouse livers, leading to increased stability and recruitment to its lipogenic target gene promoter [31]. p300/CREB hyperactivity was also associated with ChREBP acetylation and hepatic steatosis in a mouse model of obesity [17]. Accordingly, the increase in HAT activity in OPA-treated cells (Fig. 1C) was expected.

Transcription is a highly regulated process, and acetylation plays a major role in this regulation [32] by the various conformational changes of the chromatin, including change neutralization [33], consequently providing unique binding surfaces for activators of transcription [34]. PPARγ and SREBP1c regulate genes required for lipid metabolism, such as ACYL or FASN [35]. The mRNA expression of PPARγ is up-regulated in the liver of obese patients with NAFLD, reinforcing the lipogenic mechanism in response to SREBP1c induction in the development of NAFLD [36]. Considering previous studies, our data (Fig. 2A) showing the increase in lipogenic gene expression is not unexpected. However, the reduction in lipogenesis-related gene transcription following C-646 treatment was noteworthy, indicating that dynamic histone acetylation and deacetylation are also involved in the transcriptional activation of lipogenesis-related genes.

Currently, there are no approved pharmacological agents or FDA guidelines for the treatment of NAFLD [37]. Anti-obesity drugs, such as orlistat and sibutramine, are used for NAFLD but have potentially hazardous side effects [38]. Accordingly, a variety of natural phytochemical compounds to prevent NAFLD have been explored. In our study, 15 of 22 NExs prevented OPA-induced lipid accumulation in HepG2 cells (Fig. 3A). The anti-lipogenic effects of the extracts, including EMA, ESC, and EFA, are well established [39,40]. However, our aim was to elucidate the mechanisms involved in the beneficial effects of NExs on NAFLD prevention from a new perspective, i.e., epigenetics. Alterations in acetylation status associated with chronic metabolic diseases, including the abnormal regulation of hepatic lipid metabolism, have been related in the development and progression of NAFLD [2]. Certain bioactive foods may have a beneficial effect on NAFLD via their effects on acetylation. Interestingly, two NExs, EMA and ESC, demonstrated strong anti-lipogenic effects and inhibited HAT activity (Fig. 4), suggested that there is a positive correlation between lipid accumulation and HAT activity. An analysis of the correlation between these two factors supported this finding. Although the relationship was not strong (Fig. 5, Pearson’s correlation coefficient = 0.604), it was sufficient to suggest a new approach for the prevention or treatment of NAFLD based on natural foods with epigenetic effects.

The present study had many limitations. The number of preventative natural food extracts examined in the study was small. To definitively establish the correlation between lipid accumulation and HAT activity, further studies of the molecular mechanism are needed.

AVAILABILITY OF DATA AND MATERIAL

All data are contained and described within the manuscript.

AUTHORS’ CONTRIBUTIONS

H.-K.C. conceived and designed the experiment and wrote the manuscript. S.-W.C. performed the experiments and wrote a draft of the manuscript. J.-T.H. and J.-H.P. supported the experiments. H.-K.C supervised the work and critically reviewed the manuscript.

CONFLICT OF INTERESTS

The authors declare no potential conflicts of interests.

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