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Inhibition of ERK1/2 Pathway Suppresses Adiponectin Secretion via Accelerating Protein Degradation by Ubiquitin-Proteasome System: Relevance to Obesity-related Adiponectin Decline

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Abstract

Objective—Predominantly secreted by adipose tissue, adiponectin possesses insulin-sensitizing, anti-atherogenic, anti-inflammatory, and anti-angiogenic properties. Paradoxically, obesity is associated with declined plasma adiponectin levels; however, the underlying mechanisms remain elusive. In this study, we investigated the mechanistic involvement of MEK/ERK1/2 pathway in obesity-related adiponectin decrease.

Materials/Methods—C57 BL/6 mice exposed to a high-fat diet (HFD) were employed as animal obesity model. Both fully-differentiated 3T3-L1 and mouse primary adipocytes were used in the *in vitro* experiments.

Results—Obesity and plasma adiponectin decline induced by prolonged HFD exposure was associated with suppressed ERK1/2 activation in adipose tissue. In adipocytes, specific inhibition of MEK/ERK1/2 pathway decreased intracellular and secretory adiponectin levels, whereas adiponectin gene expression was increased, suggesting that MEK/ERK1/2 inhibition may promote adiponectin protein degradation. Cycloheximide (CHX)-chase assay revealed that MEK/ERK1/2 inhibition accelerated adiponectin protein degradation, which was prevented by MG132, a potent proteasome inhibitor. Immunoprecipitation assay showed that intracellular MEK/ERK1/2 activity

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

DG contributed to design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. ZW contributed to data collection and analysis, data interpretation, and manuscript writing. XD, XZ, SL, LV, and TY contributed to data collection and analysis. ZS contributed to data interpretation, and editing manuscript. All authors approved the final version of the manuscript.

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was negatively associated with ubiquitinated adiponectin protein levels. Consistently, long-term HFD feeding in mice increased ubiquitinated adiponectin levels in the epididymal fat pads.

Conclusions—Adipose tissue MEK/ERK1/2 activity can differentially regulates adiponectin gene expression and protein abundance and its suppression in obesity may play a mechanistic role in obesity-related plasma adiponectin decline.

Keywords

ERK1/2; adiponectin; PPAR- γ ; obesity; ubiquitin

1. Introduction

Adiponectin is a 244-amino-acid protein homologous to collagen VIII and X and complement factor C1q [1], possessing insulin-sensitizing, anti-atherogenic, anti-inflammatory, and anti-angiogenic properties [2–4]. Although predominantly expressed in and secreted by adipose tissue, unlike most other adipokines, plasma adiponectin is paradoxically decreased in patients with obesity and associated metabolic disorders [5, 6].

Although the physiological effects of adiponectin have been investigated intensively, the mechanisms underlying obesity-related decrease in adiponectin production are still poorly understood. Adiponectin is secreted from adipose tissue into the circulation as three oligomeric isoforms, including trimeric, hexameric, and the high-molecular weight oligomeric complex. In general, both transcriptional and post-transcriptional/translational mechanisms are involved in the regulation of adiponectin production. For instance, proinflammatory cytokines, such as TNF- α [7, 8] and interleukin (IL)-6 [9, 10], have been shown to reduce adiponectin gene expression. Conversely, increases in adiponectin expression have been reported during adipocyte differentiation, and transcriptional factors implicated in adipogenesis, including peroxisome proliferator activated receptor- γ [7] and CCAAT/enhancer-binding protein (C/EBP) α [11, 12], have been shown to upregulate adiponectin gene expression. Moreover, posttranslational modification, including disulfide bond formation, hydroxylation, and subsequent glycosylation, has been reported to play an important role in adiponectin oligomerization [13].

Ubiquitin-proteasome system (UPS) plays a pivotal role in the regulation of intracellular protein degradation. In addition to above-mentioned regulatory mechanisms, recent studies from both us and others demonstrated that UPS system is responsible for cellular adiponectin degradation. Treatment of with MG-132, a potent 26S proteasome inhibitor, prevented intracellular adiponectin degradation, thereby increasing intracellular adiponectin abundance [14, 15]. It was estimated that the half-life of adiponectin was around 4 hours [16, 17]. In line with these observations, we recently reported that 4-hydroxynonenal, a major lipid peroxidation product from n-6 polyunsaturated fatty acids, suppressed adiponectin production from both 3T3-L1 and primary mouse adipocytes via accelerating its proteasome degradation [18].

Using primary mouse adipocytes, we previously reported that MEK/ERK1/2 pathway inhibition decreased adiponectin concentration in culture media [19]. Here we conducted both in vivo and in vitro experiments to further understand the underlying mechanisms involved in this process and examine its potential implication in obesity-related adiponectin decline. We demonstrate that the obesity-related adiponectin decrease is associated with decreased adipose tissue ERK1/2 phosphorylation status. Inhibition of MEK/ERK1/2 pathway lowered adiponectin production via accelerating its proteasome degradation.

2. Materials and methods

2.1. Animal care and feeding

Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA; 8 weeks old) weighing 25 ± 0.5 g (means $\pm$ SD) were housed in Biologic Resources Laboratory at the University of Illinois at Chicago. The studies were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. For diet-induced obesity experiments, ten mice were randomly assigned into 2 groups and started on either control diet (Con) or high-fat diet (HF). Both control diet (D12450B, 10% calories as fat) and high-fat diet (D12451, 45% calories as fat) was obtained from Research Diets Inc. (New Brunswick, NJ 08901). All animals accessed to diet and water ad libitum. Mice were maintained on the treatments for 8 weeks before being killed. At the end of the experiment, the mice were anesthetized with 5% isoflurane after 4-hour fasting, and plasma and epididymal fat pad samples were harvested for biochemical assays.

2.2. Cell culture and induction of differentiation in 3T3-L1 cells

Mouse embryo fibroblast 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and 1% antibiotics (Cellgro, Manassas, VA) until confluence and induced to differentiation. Briefly, 2 days postconfluence (day 0), cells were exposed to differentiation medium containing 0.5 mM isobutylmethylxanthine, 1 μ M dexamethasone, 1.67 μ M insulin (MDI; Sigma, St. Louis, MO), and 10% fetal bovine serum (FBS) for 3 days. Then, cells were transferred to DMEM with 1.67 μ M insulin and 10% FBS and re-fed every 2 days. Maturation of adipocytes was confirmed by Oil Red O staining of lipid droplet on day 7.

2.3. Isolation and culture of mouse primary adipocytes

Primary adipocytes were isolated from male C57BL/6 mice (8–9 weeks old) as described by us previously [19].

2.4. Enzyme-linked immunoabsorbent assays (ELISA) for adiponectin

Adiponectin levels in plasma or in the culture medium were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit for mouse full-length adiponectin (Linco Research, St. Charles, MO) according to the manufacturer's instruction.

2.5. Transient transfection

The eukaryotic expressing vector plasmid containing human MEK1 gene sequence, designated as pcDNA3.1/hCD36, was kindly provided by Dr. John Blenis (Department of Cell Biology, University of Massachusetts) [20]. The pcDNA3.1(+) plasmids was purchased from Invitrogen (Grand Island, NY). Fully differentiated 3T3-L1 adipocytes were transfected with 0.8 μ g/well of the expression vector pcDNA3.1/hMEK or empty vector control pcDNA3.1(+) in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's guidelines. Twenty four hours after transfection, ERK1/2 protein phosphorylation status was determined by Western blot analysis.

2.6. Protein degradation assay

Adiponectin protein degradation was assayed by CHX-chase analysis. After 2-hour pretreatment with cycloheximide (5 μ g/ml), 3T3-L1 adipocytes were exposed to U0126 (10 μ M) (a MEK/ERK1/2 pathway inhibitor) and MG132 (20 μ M) (a Ubiquitin-Proteasome

System inhibitor) for indicated periods and the total proteins were isolated to detect intracellular adiponectin protein abundance via Western blot.

2.7. Reciprocal Immunoprecipitation

The total protein from 3T3-L1 adipocytes or adipose tissue were extracted with immunoprecipitation buffer (IP buffer: 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% IGEPAL CA630) and diluted into a concentration of 1 mg/ml. Polyclonal antibodies against adiponectin (ab3455, Abcam, Cambridge, MA) or ubiquitin (cat#:3936, Cell Signaling, Danvers, MA) were added to this mixture at a dilution of 1:100, and the samples were incubated overnight at 4 °C with gentle agitation. At the end of incubation, 50 µl of protein A/G-Sepharose bead suspension (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each sample and gently mixed for 1 hour at 4 °C. Samples were centrifuged at 12,000 rpm for 30 seconds; the supernatant was aspirated, and the beads were washed three times in 1ml of IP buffer. The isolated beads were re-suspended in 1× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated to 95 °C for 5 minutes, vortexed, and flash-centrifuged, and the supernatants were loaded onto a 10% SDS-polyacrylamide gel for electrophoretic separation and immunoblotting.

2.8. Quantitative real-time RT-PCR

Total RNA, from either 3T3-L1 adipocytes or adipose tissue, was isolated with a phenol-chloroform extraction. For each sample, 1.0 µg total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in MicroAmp Optical 96-well reaction plates with a SYBR Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems Prism 7000 sequence detection system. Relative gene expression was calculated after normalization by a house-keeping gene (mouse or human 18S rRNA).

2.9. Western blotting

Treated 3T3-L1 preadipocytes were lysed in RIPA buffer and isolated proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to 0.45 µm polyvinylidene difluoride membrane. After transfer, membranes were blocked in 5% bovine serum albumin in PBS with 0.1% Tween 20 and probed with antibodies from Cell Signaling Technology (Danvers, MA) against total-ERK1/2 (cat#: 4370), phospho-ERK1/2 (cat#: 4344), phospho-Akt (cat#: 4056), ubiquitin (cat#: 3936), phospho-Threonine-Proline (cat#: 9391), and anti-adiponectin protein antibodies (BAF1119, R&D System, Minneapolis, MN). Horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit were used in detection of specific proteins.

2.10. Statistical analysis

All data were expressed as means ± SD. Statistical analysis was performed using a one-way ANOVA and was analyzed further by Newman-Keuls test for statistical difference. Differences between treatments were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Decreased plasma adiponectin levels in obese mice are associated with suppressed MEK/ERK1/2 and Akt pathways in adipose tissue

Male C57BL/6 mice were fed with either control diet (Con) or high-fat diet (HFD) for 8 weeks. As shown in Fig. 1, long-term exposure to HFD led to obvious obesity in mice, evidenced by significant increases in both body weight and epididymal fat pad mass (Fig. 1A & B). In comparison to these in Con group, the mice fed HFD showed significantly

reduced gene expressions of adiponectin and PPAR- γ in epididymal fat pad (Fig. 1C). Consistently, both plasma and intracellular adiponectin levels were decreased in HFD group (Fig. 1D & E). Interestingly, adiponectin declines were closely associated with suppressed ERK1/2 and Akt phosphorylations in epididymal fat pads (Fig. 1E).

3.2. Inhibition of ERK1/2 activation, but not Akt kinase, decreases adiponectin secretion

The effects of either ERK1/2 or Akt pathway inhibition on adiponectin secretion were next examined by treating cultured adipocytes with either U0126 (20 μ M) or PD98059 (20 μ M), two specific inhibitors for MEK/ERK1/2 pathway. Adiponectin levels in the media were measured 16 hours later. As shown in Fig. 2, inhibition of MEK/ERK1/2 pathway lowered adiponectin concentrations in the media from both fully differentiated 3T3-L1 (Fig. 2A) and mouse primary adipocytes (Fig. 2B). However, the inhibitor of Akt kinase, LY294002 (20 μ M), had no effect on secretory adiponectin levels (Fig. 2C).

3.3. Inhibition of MEK/ERK1/2 pathway increases adiponectin gene expression via activating PPAR- γ

To determine if decreased adiponectin secretions by MEK/ERK1/2 inhibitors were resulted from suppressed gene expression, we next examined the effect of U0126 on adiponectin mRNA levels. Both 3T3-L1 adipocytes and primary mouse adipocytes were treated with U0126 (20 μ M) for 16 hours. The total RNAs were isolated and adiponectin mRNA determined by real time RT-PCR. As shown in Fig. 3A, adiponectin gene expressions were unexpectedly increased after U0126 exposure in both types of adipocytes.

Since PPAR- γ is a dominant transcription factor in the regulation of adiponectin gene expression, we subsequently examined the effect of U0126 on PPAR- γ expression. As shown in Fig. 3B, PPAR- γ gene expression was markedly increased by U0126 in both 3T3-L1 adipocytes and primary mouse adipocytes. To further confirm the critical role of PPAR- γ activation in the upregulation of adiponectin gene expression by U0126, we then treated 3T3-L1 adipocytes with T0070907 (10 μ M), a PPAR- γ antagonist, prior to U0126 exposure. As shown in Fig. 3C, inhibition of PPAR- γ transactivation abolished U0126 induced increase in adiponectin gene expression.

3.4. MEK/ERK1/2 activity regulates intracellular adiponectin protein abundance

The observation that MEK/ERK1/2 inhibition differentially regulates adiponectin gene expression and secretion prompted us to further examine the effect of MEK/ERK1/2 activity on intracellular adiponectin protein abundance. Fully differentiated 3T3-L1 adipocytes were treated with either U0126 (20 μ M) or PD98059 (20 μ M) for 16 hours. Intracellular adiponectin protein abundance was determined by both ELISA and Western blot assays using whole cell lysates. As shown in Fig. 4A, both inhibitors significantly decreased intracellular adiponectin concentrations. Time course effects of U0126 on both intracellular and secreted adiponectin levels were shown in Fig. 4B. To determine whether the enhancement of MEK/ERK1/2 activation can *visé versa* increase intracellular adiponectin abundance, fully differentiated 3T3-L1 adipocytes were transiently transfected with a plasmid containing MEK1 DNA sequence. ERK1/2 activation and intracellular adiponectin abundance were measured by Western blot. As shown in Fig. 4C, MEK1 overexpression enhanced ERK1/2 activation. In line with this, intracellular adiponectin protein concentrations were significantly increased.

3.5. MEK/ERK1/2 inhibition accelerates adiponectin proteasome degradation

The above observations clearly demonstrated that whereas MEK/ERK1/2 inhibition increased adiponectin gene expression, both intracellular and secretory adiponectin proteins

were significantly decreased. These findings raised the possibility that inhibition of MEK/ERK1/2 pathway may accelerate adiponectin protein degradation. To test our hypothesis, we first examined the effect of U0126 on adiponectin protein degradation in 3T3-L1 adipocytes treated with cycloheximide (CHX), an inhibitor of protein synthesis. As shown in Fig. 5A, U0126 treatment resulted in accelerated adiponectin degradation in the presence of cycloheximide. In adipocytes without U0126 exposure, adiponectin protein degradation became obvious in 4 hours after CHX treatment, while it became obvious at 2 hour time point in adipocytes exposed to U0126. To determine if proteasome was involved in this process, MG-132, a potent proteasome inhibitor, was added before CHX and U0126 treatments. As shown in Fig. 5B, MG132 prevented U0126 induced adiponectin degradation, indicating that MEK/ERK1/2 inhibition accelerated adiponectin degradation by proteasome.

3.6. MEK/ERK1/2 activity regulates adiponectin protein ubiquitination

Ubiquitination is the obligatory step for proteins destined to be degraded in proteasome. To determine whether MEK/ERK1/2 activity regulates adiponectin ubiquitination, we first examined ubiquitin levels of immunoprecipitated adiponectin protein in 3T3-L1 adipocytes treated with or without U0126. As expected, U0126 treatment significantly elevated ubiquitinated adiponectin protein abundance (Fig. 6A). We then examined the effect of MEK/ERK1/2 activation on adiponectin ubiquitination via stimulating 3T3-L1 adipocytes with EGF (200 ng/ml), a potent activator of MEK/ERK1/2 pathway. As shown in Fig. 6B & C, EGF-induced rapid ERK1/2 activation was concomitant with decreased adiponectin ubiquitination. To further confirm our observations, we conducted reciprocal immunoprecipitation by using anti-ubiquitin antibody for immunoprecipitation, followed by immunoblotting with anti-adiponectin antibody. As shown in Fig. 6D, similar to our previous observations, U0126 treatment increased ubiquitinated adiponectin abundance; whereas EGF stimulation led to decreased adiponectin ubiquitination. Taken together, these results indicate that inhibition of MEK/ERK1/2 pathway reduces adiponectin protein levels by enhancing ubiquitin-proteasome mediated protein degradation.

3.7. The regulation of MEK/ERK1/2 activity on adiponectin protein turnover is independent on adiponectin phosphorylation

ERK1/2 is a subfamily of Serine/threonine protein kinases phosphorylating the OH group of serine or threonine inside a protein. Previous reports indicate that the ERK-specific phosphorylation motif features a Ser-Pro or Thr-Pro phosphorylation site in the vicinity of an ERK-binding site [21]. Since both human and mouse adiponectin protein contains ERK-specific phosphorylation motifs (Mouse: 3; Human: 1) (Fig. 7A), we examined if adiponectin is a direct substrate by immunoprecipitation and immunoblotting using anti phospho-Ser/Thr-Pro antibody. As shown in Fig. 7B, adiponectin phosphorylation could not be detected under either basal, U0126, or EGF-stimulated situation, confirming that increased adiponectin protein degradation under ERK inhibition did not involve its phosphorylation status change.

3.8. Long-term HF diet feeding increases adiponectin ubiquitination in epididymal fat pad

To determine the in vivo relevance of our observations from cell culture studies, we measured ubiquitination status of adiponectin protein in epididymal fat pads from mice fed either control diet or HF diet. As shown in Fig. 8A, ubiquitination levels of adiponectin proteins in adipose tissue were significantly elevated by long-term HF diet feeding, suggesting that increased adiponectin protein turnover contributes, at least partially, to obesity-related plasma adiponectin decline. To certify the role of ERK1/2 inhibition in HF diet-induced elevation of adiponectin ubiquitination, primary adipocytes from control group were isolated and treated with U0126 for 16 hours. Intracellular total and ubiquitinated

adiponectin levels were determined by Immunoblotting and Immunoprecipitation, respectively. As shown in Fig. 8B, while the inhibition of MEK/ERK1/2 pathway with U0126 decreased intracellular total adiponectin levels, it increased adiponectin ubiquitination.

4. Discussion

In this study, we provide evidence indicating that inhibition of adipose tissue ERK1/2 pathway contributes to obesity-related decline in adiponectin secretion. Long-term high-fat diet feeding led to increased body weight gain and decreased plasma adiponectin levels, which were concomitant with suppressed ERK1/2 activation in adipose tissue. Using adipocytes, we demonstrated that intracellular adiponectin protein abundance was positively correlated with MEK/ERK1/2 activity. Whereas MEK/ERK1/2 pathway inhibition increased adiponectin gene expression, both intracellular and secretory adiponectin levels were significantly decreased, indicating that MEK/ERK1/2 activity plays a critical role in controlling adiponectin protein turnover. Further investigation reveals that MEK/ERK1/2 activity controls adiponectin protein turnover in adipocytes via the ubiquitin-proteasome pathway, without the involvement of direct adiponectin phosphorylation. Concurrently, we show that in adipose tissues of high-fat diet fed mice, ubiquitinated adiponectin protein levels are significantly higher than that in corresponding control animals, suggesting that increased adiponectin degradation may mechanistically contribute to obesity-related adiponectin decline.

Adiponectin is predominantly produced and secreted by adipose tissue. Circulating adiponectin levels are regulated by complex and multiple intracellular mechanisms involving in gene expression, post-transcriptional/translational modification, and trafficking/secretion process [22, 23]. While it is well documented that the cellular and serum levels of adiponectin are negatively correlated with obesity, the precise underlying mechanisms remain unclear. Both increased production of proinflammatory cytokines, such as TNF- α and IL-6 [7–10, 24], and adipose tissue ER stress [25, 26] in obese individuals has been attributed to reduced adiponectin gene expression. Emerging evidence suggests that regulatory steps involved in the post-transcriptional/translational modification and trafficking process may play a more important role in controlling plasma adiponectin levels [22]. Several proteins have been discovered recently to critically involve in regulating adiponectin's trafficking and secretion at post-transcriptional/translational level [27]. In addition to above-mentioned regulatory mechanisms, a very recent study from our laboratory revealed that enhanced proteasome degradation contributed to 4-hydroxynonenal, a major product of lipid peroxidation, induced decrease in adiponectin production, providing initial evidence that altered adiponectin protein turnover rate may critically involve in obesity-related adiponectin decline [18].

ERK1/2 is a member of the MAPK family, whose activation results in cell growth, proliferation, survival, and inflammation. ERK1/2 pathway plays a critical role in regulating stability of a variety of intracellular proteins with important biological functions, such as p53, Bim, surviving, p300, GATA3 [28], to name a few; however, whether this pathway is also involved in the regulation of adiponectin protein turnover has never been examined. In this study, we identified that intracellular MEK/ERK1/2 activity plays a critical role in the regulation of adiponectin stability via ubiquitin-proteasome system. This notion was supported by the following observations: (i) Specific inhibitors for MEK/ERK1/2 activation decreased both intracellular and secretory adiponectin levels in both primary and 3T3-L1 adipocytes; (ii) MG132, a potent S26 proteasome inhibitor, prevented adiponectin decline induced by the inhibition of MEK/ERK1/2 pathway; (iii) Inhibition of MEK/ERK1/2 pathway was associated with increased intracellular ubiquitinated adiponectin levels; (iv)

Activation of MEK/ERK1/2 pathway by either EGF stimulation or MEK1 overexpression elevated intracellular adiponectin protein abundance and EGF stimulation led to decreased intracellular ubiquitinated adiponectin levels. Our study provided first-line evidence that MEK/ERK1/2 cascade is an important intracellular signaling pathway in controlling adiponectin production.

The detailed mechanisms underlying the regulation of adiponectin protein turnover by ERK1/2 remain elusive. ERK1/2 activation leads to either increased protein stability or accelerated degradation, which may be cell type-, stimulus-, and protein-specific. In general, two regulatory modes are involved in ERK-regulated protein turnover. First of all, direct phosphorylation of proteins at serine or threonine residues by ERK1/2 can foster the interaction between proteins and specific E3 ligases, leading to increased ubiquitination and eventual proteasome degradation. Secondly, ERK1/2 activity can directly regulate gene expression and enzyme activities of certain E3 ligases, such as MDM2, thereby changing protein turnover rate. Once activated, ERK1/2 can directly phosphorylate proteins containing the minimal consensus phosphor-acceptor motif Ser/Thr-Pro. Since adiponectin protein contains ERK1/2-specific phosphorylation motif (Fig. 7A), we initially hypothesized that adiponectin might be a direct substrate of ERK. However, the hypothesis was not supported by our Western blot analysis using phospho-Ser/Thr-Pro antibodies. At present, the E3 ligase that controls the ubiquitination of adiponectin hasn't been identified. Considering the critical role of adiponectin decline in obesity-related metabolic disorders, the identification of the ligase is of great clinical significance. In this context, the future investigation in this aspect is certainly warranted.

Mechanisms underlying the regulation of adiponectin gene expression are multifactorial, involving both positive regulators, such as PPAR- γ and CCAAT/enhancer-binding protein (C/EBP) [7, 29] and negative transcriptional control, including activating transcription factor 3 [30], CHOP (C/EBP homology protein) [31, 32], and Snail [33]. Among multiple factors, PPAR- γ plays a dominant role in controlling adiponectin gene expression [7]. In consistent with many previous studies, our data showed that high-fat diet feeding decreased gene expressions of both PPAR- γ and adiponectin in adipose tissue. Interestingly, but controversially, in cell culture study we clearly demonstrated that adiponectin mRNA levels were significantly increased when MEK/ERK1/2 pathway was inhibited. The discrepancy between adipose tissue of high-fat diet animals and U0126-treated adipocytes in terms of adiponectin gene expression may derive from the involvement of more complicated mechanisms in the *in vivo* environments, including proinflammatory cytokines. Increased adiponectin gene expression by ERK1/2 inhibitors was concomitant with increased PPAR- γ gene expression and the pretreatment with T0070907, a PPAR- γ antagonist, abolished adiponectin mRNA increases induced by MEK/ERK1/2 inhibitors, implying that PPAR- γ transactivation contributes to observed enhancement of adiponectin gene expression. This assumption was consistent with previous studies showing that ERK1/2 could phosphorylate PPAR- γ at serine residue 82, thereby inhibiting its translocation to the nucleus [29], although the phosphorylation status of PPAR- γ was not directly detected in this study.

The effects of obesity on adipose tissue ERK1/2 activity were controversial. The discrepancy could derive from differences in model selection, feeding duration, fat contents, as well as cell types studied [30, 31]. In this study, data obtained from cell culture investigations were supported by animal experiments. Using a diet-induced obesity mouse model, we showed that obesity induced by long-term high fat diet feeding was associated with decreased ERK1/2 activation in epididymal fat pad. More importantly, we demonstrated for the first time that the ubiquitination levels of adiponectin proteins were significantly higher in adipose tissue from obese mice, corroborating the notion that

accelerated adipose tissue proteasome degradation of adiponectin protein contributes to obesity-related plasma adiponectin decline.

In conclusion, our study provides the first-line evidence supporting the existence of one more layer of regulatory mechanism in controlling adiponectin production. Our data clearly showed that suppression of adipocyte MEK/ERK1/2 pathway accelerates intracellular adiponectin protein degradation via ubiquitin-proteasome system, contributing to obesity-related plasma adiponectin decline. Although we failed to detect the occurrence of adiponectin protein phosphorylation in an attempt to elucidate the mechanism involved in increased adiponectin ubiquitination after ERK1/2 pathway inhibition, our findings support that the specific E3 ligase that controls the ubiquitination of adiponectin could be a potential clinical target for preventing adiponectin decline. Identification of the specific E3 ligase in this process will pave the way for the discovery of the new therapies for the treatment of obesity-related diseases.

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Abbreviations

ERK1/2	extracellular signal-regulated kinases 1 and 2
HFD	High-fat diet
UPS	Ubiquitin-proteasome system
TNF-α	tumor necrosis factor-alpha
I PPAR-γ	peroxisome proliferator-activated receptor gamma

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Fig. 1.

Obesity-related adiponectin decline is associated with suppressed ERK1/2 and AKT activation in adipose tissue. Male C57BL/6 mice were exposed to high-fat diet for eight weeks. (A) body weight; (B) epididymal fat mass; (C) adiponectin and PPAR- γ gene expressions in epididymal fat pad; (D) adiponectin levels in plasma; (E) adiponectin, ERK1/2 and AKT (phosphorylated and total) levels in epididymal fat pad. Data are means $\pm$ SD (n = 5). * $p < 0.05$. HF: high-fat diet.

Fig. 2.

Inhibition of MEK/ERK1/2 decreases adiponectin secretion. Fully differentiated 3T3-L1 and mouse primary adipocytes were exposed to inhibitors for either MEK/ERK1/2 (U0126 and PD98059 at 20 µM, respectively) or Akt kinase (LY294002 at 20 µM). Cell culture media were collected 16 hours later. Adiponectin concentrations were measured by ELISA kit. Inhibition of MEK/ERK1/2 pathway lowered intracellular adiponectin secretion (A & B), whereas Akt kinase inhibition had no effect on secretory adiponectin levels (C). Data are means $\pm$ SD ($n = 3$). * $p < 0.05$ when compared with UT. UT: untreated.

Fig. 3.

Inhibition of MEK/ERK1/2 pathway increases adiponectin gene expression via activating PPAR- γ . Fully-differentiated 3T3-L1 adipocytes and mouse primary adipocytes were treated with U0126 (20 μ M) for 16 hours. Total RNA in each group was isolated to determine the gene expressions of adiponectin and PPAR- γ via real time RT-PCR. Both adiponectin and PPAR- γ gene expressions were significantly increased after ERK1/2 suppression with U0126 (A & B). Data are means $\pm$ SD ($n = 3$). * $p < 0.05$ when compared with the untreated group (UT). (C) Inhibition of PPAR- γ activation abolished U0126-induced elevation of adiponectin expression. After pretreatment with T0070907 (10 μ M), a PPAR- γ antagonist, for 2 hours, 3T3-L1 adipocytes were exposed to U0126 (20 μ M) for 16 hours. Data are means $\pm$ SD ($n = 3$). Bars with different characters differ significantly, $p < 0.05$.

Fig. 4.

MEK/ERK1/2 activity regulates intracellular adiponectin protein abundance. A. Inhibition of MEK/ERK1/2 decreased intracellular adiponectin protein abundance. Differentiated 3T3-L1 adipocytes were treated with U0126 (20 μ M) or PD98059 (20 μ M) for 16 hours. The total proteins were extracted to determine intracellular adiponectin protein abundance via ELISA assay and Western blot. Data are means $\pm$ SD (n = 3). * $p < 0.05$ when compared with the untreated group (UT). B. Time course effect of U0126 on both intracellular and secreted adiponectin levels. C. Activation of ERK1/2 pathway increases intracellular adiponectin levels. Fully differentiated 3T3-L1 adipocytes were transfected with 0.8 μ g/well of the expression vector pcDNA3.1/hMEK or empty vector control pcDNA3.1(+) in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's guidelines. Whole cell lysates were harvested 24 hour later and subjected to Western blot for phosphorylated ERK1/2 and adiponectin.

Fig. 5.

MEK/ERK1/2 inhibition accelerates adiponectin proteasome degradation. (A) U0126 treatment resulted in accelerated adiponectin degradation in the presence of cycloheximide. After 2-hour pretreatment with cycloheximide (5 μ g/ml) (time 0 hour), an inhibitor of protein synthesis, 3T3-L1 adipocytes were exposed to U0126 (20 μ M) for 2, 4 or 8 hours and the total proteins were isolated to detect intracellular adiponectin protein abundance via Western blot. Relative protein abundance was calculated as the fold change of the ratio between adiponectin and corresponding actin. One was used as the value at time 0 hour. Data are means $\pm$ SD (n = 3). * $p < 0.05$ when compared with CHX alone treatment at same time point. (B) MG132 prevented U0126 induced adiponectin degradation. MG132 (20 μ M), a potent proteasome inhibitor, was added to the medium 2 hours before cycloheximide (5 μ g/ml) treatment. Two more hours later (time 0 hour), 3T3-L1 adipocytes were exposed to U0126 (20 μ M) for 2, 4 or 8 hours and intracellular adiponectin protein abundance was detected via Western blot.

Fig. 6.

MEK/ERK1/2 pathway regulates adiponectin protein ubiquitination. The effect of ERK1/2 activity on adiponectin ubiquitination was determined by reciprocal immunoprecipitation. (A) U0126, a specific inhibitor of MEK/ERK1/2 pathway, increased ubiquitinated adiponectin protein levels. Ubiquitin levels of immunoprecipitated adiponectin protein were measured in 3T3-L1 adipocytes treated with or without U0126 in the presence of proteasome inhibitor, MG132. The pull-down proteins were subjected to Western blot and probed with anti-ubiquitin antibody to determine adiponectin-conjugated ubiquitin adduct levels. (B & C) EGF-induced quick ERK1/2 activation is concomitant with decreased adiponectin ubiquitination. Fully differentiated 3T3-L1 adipocytes were stimulated with EGF (200 ng/ml) for indicated times. Phosphorylated ERK1/2 proteins were determined by Western blot. Ubiquitin levels of immunoprecipitated adiponectin protein were measured in 3T3-L1 adipocytes treated with or without EGF in the presence of proteasome inhibitor,

MG132. The pull-down proteins were subjected to Western blot and probed with ubiquitin to determine adiponectin-conjugated ubiquitin adduct levels. (D). MEK/ERK1/2 pathway regulates adiponectin protein ubiquitination. Adiponectin level of immunoprecipitated ubiquitinated protein was measured in 3T3-L1 adipocytes treated with or without U0126 or EGF in the presence of proteasome inhibitor, MG132. The pull-down proteins were subjected to Western blot and probed with anti-adiponectin antibody to determine adiponectin-conjugated ubiquitin adduct levels.

Fig. 7.

Direct adiponectin phosphorylation is not involved in the regulation of MEK/ERK1/2 activity on adiponectin protein turnover. The ERK-specific phosphorylation motif features a Ser-Pro or Thr-Pro phosphorylation site in the vicinity of an ERK-binding site. Both mouse and human adiponectin protein contains Thr-Pro phosphorylation site (mouse: 3; human: 1) (A). 3T3-L1 adipocytes were exposed with either U0126 (20 μ M) or EGF (200 ng/ml) for 2 and 4 hours, immunoprecipitation assay could not detect adiponectin phosphorylation (B).

Fig. 8.

MEK/ERK1/2 inhibition is involved in long-term HF diet feeding-induced elevation of adiponectin ubiquitination in epididymal fat pad. **A.** Long-term HF diet feeding increased ubiquitinated adiponectin proteins in adipose tissue. Total proteins from epididymal fat pads of mice fed either control diet or HF diet for 8 months were subjected to immunoprecipitation and immunoblotting assays for ubiquitinated adiponectin protein levels. For quantification, NIH imageJ software was used and proteins between 25–75 kD MW were scanned as the ubiquitinated adiponectin, followed by the standardization by corresponding input. Data are means $\pm$ SD ($n = 5$). * $p < 0.05$. HF: high-fat diet. **B.** Inhibition of MEK/ERK1/2 in primary adipocytes leads to increased adiponectin ubiquitination. Primary adipocytes were isolated from epididymal fat pad of control mice and treated with U0126 for 16 hours. Total proteins were isolated and subjected to immunoprecipitation and immunoblotting assays for total ubiquitinated adiponectin protein levels. Data are means $\pm$ SD ($n = 3$). * $p < 0.05$ when compared to UT. For quantification, NIH imageJ software was used and proteins between 25–75 kD MW were scanned as the ubiquitinated adiponectin, followed by the standardization by corresponding input.