ARTICLE
Journal of Cellular Biochemistry 113:3547–3558 (2012)

Journal of Cellular Biochemistry

Promotion of Adiponectin Multimerization by Emodin: A Novel AMPK Activator With PPARγ-Agonist Activity

Zhifen Chen,[1] Lu Zhang,[1] Junyang Yi,[1] Zhuanbo Yang,[1] Zhijie Zhang,[2] and Zhen Li[1*]

[1]*MOE Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University, Beijing 100084, China*

[2]*Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*

ABSTRACT

Adiponectin is an important insulin-sensitizing adipokine with multiple beneficial effects on obesity-associated medical complications. It is secreted from adipocytes into circulation as high, medium, and low molecular weight forms (HMW, MMW, and LMW). Each oligomeric form of adiponectin exerts non-overlapping biological functions, with the HMW oligomer possessing the most potent insulin-sensitizing activity. In this study, we reported that emodin, a natural product and active ingredient of various Chinese herbs, activates AMPK in both 3T3-L1 adipocytes and 293T cells. Activation of AMPK by emodin promotes the assembly of HMW adiponectin and increases the ratio of HMW adiponectin to total adiponectin in 3T1-L1 adipocytes. Emodin might activate AMPK by an indirect mechanism similar to berberine. We also found that emodin activates PPARγ and promotes differentiation and adiponectin expression during differentiation of 3T3-L1 preadipocytes. Therefore, emodin is a novel AMPK activator with PPARγ-agonist activity. Our results demonstrate that the effects of emodin on adiponectin expression and multimerization are the ultimate effects resulting from both AMPK activation and PPARγ activation. The dual-activity makes emodin or the derivatives potential drug candidates for the treatment of type 2 diabetes and other obesity-related metabolic diseases. J. Cell. Biochem. 113: 3547–3558, 2012.

KEY WORDS: ADIPONECTIN; AMPK; EMODIN; PPARγ; MULTIMERIZATION

The adipose tissue produces and secretes a variety of adipokines, which regulate the metabolism and numerous other processes in the human body [Rajala and Scherer, 2003; Lago et al., 2007]. Adiponectin is the most abundant adipokine present in human serum at a concentration of 5–30 μg/ml. The serum level of adiponectin inversely correlates with obesity and directly correlates with insulin sensitivity [Arita et al., 1999; Hotta et al., 2000]. Additionally, serum adiponectin levels increase with weight loss, caloric restriction or thiazolidinedione (TZD) treatment [Maeda et al., 2001; Combs et al., 2003; Satoh et al., 2003; Bobbert et al., 2005]. The insulin-sensitizing effect of adiponectin is due primarily to its ability to activate AMPK [Yamauchi et al., 2002; Wu et al., 2003; Kubota et al., 2007]. Reduced expression of adiponectin or its receptors results in impaired adiponectin signaling and leads to insulin resistance [Kadowaki et al., 2006; Yamauchi et al., 2007; Iwabu et al., 2010]. Therefore, adiponectin is a promising drug target for obesity, insulin resistance, type 2 diabetes, and other obesity-related metabolic diseases.

Adiponectin is comprised four distinct domains: a signal peptide at the N terminus; a short variable region; a collagenous domain; and a C-terminal globular domain homologous to C1q [Scherer et al., 1995; Hu et al., 1996]. Adiponectin is secreted from adipocytes into circulation as low molecular weight (LMW) trimers, medium molecular weight (MMW) hexamers, and the high molecular weight (HMW) multimers consisting of 18–36 monomers [Tsao et al., 2003; Waki et al., 2003]. Each oligomeric form of adiponectin displays distinct biochemical characteristics and exerts non-overlapping biological functions, with the HMW oligomer possessing the most potent insulin-sensitizing activity [Pajvani et al., 2003; Wang et al., 2008]. The serum level of HMW adiponectin decreases in patients with obesity, type 2 diabetes, metabolic syndrome, and cardiovascular diseases [Kishida et al., 2003; Basu et al., 2007; Koenen et al.,

Zhifen Chen and Lu Zhang contributed equally to this work.

The authors of this manuscript have no conflicts of interests.

Grant sponsor: NSFC; Grant numbers: 30470354, 30570356.

*Correspondence to: Zhen Li, School of Life Sciences, Tsinghua University, Beijing 100084, China.
E-mail: lizhen@tsinghua.edu.cn

Manuscript Received: 8 April 2012; Manuscript Accepted: 11 June 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 June 2012

DOI 10.1002/jcb.24232 • © 2012 Wiley Periodicals, Inc.

2008]. The ratio of HMW adiponectin to total adiponectin correlated with TZD-mediated improvement in insulin sensitivity [Pajvani et al., 2004]. Therefore, multimerization plays an important role in regulating the numerous activities of adiponectin [Wang et al., 2008; Liu and Liu, 2010].

A major challenge in developing adiponectin as a therapeutic drug is the high abundance of adiponectin in the plasma, making further elevation rather difficult. In addition, endogenous adiponectin undergoes various post-translational modifications, such as hydroxylation and glycosylation, which are critical for its biological functions [Wang et al., 2008]. Despite the extensive research on the physiological functions of adiponectin, little is known about the molecular mechanisms of adiponectin multimerization and secretion. Ero1-Lα and DsbA-L have been found to promote the assembly of HMW adiponectin [Qiang et al., 2007; Wang et al., 2007; Liu et al., 2008]. Because adiponectin mRNA is expressed at high levels in adipocytes, optimizing the assembly and secretory pathway should be the most effective way to increase the level of HMW adiponectin in the serum. We have found that activation of AMPK by berberine or AICAR promotes adiponectin multimerization [Li et al., 2011]. Therefore, the AMPK signaling pathway plays a positive role in regulating the assembly of HMW adiponectin.

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that regulates cellular and whole body energy homeostasis in response to metabolic or non-metabolic stress [Towler and Hardie, 2007]. In addition, AMPK has been found to regulate many other cellular processes, such as mitochondrial biogenesis, autophage, cell polarity, and cell proliferation [Hardie, 2011]. AMPK, a heterotrimer composed of a catalytic α subunit that is only active after phosphorylation at Thr-172 by upstream kinases, and regulatory β and γ subunits, is activated by an increase in the AMP/ATP ratio. A number of AMPK activators have been identified, most of which activate the kinase indirectly by altering cellular AMP/ATP ratio [Hawley et al., 2010]. A-769662, a direct AMPK activator, activates AMPK by a mechanism that is distinct from AMP activation [Sanders et al., 2007; Hawley et al., 2010]. The widely used anti-diabetic drugs, such as metformin and thiazolidinediones, are also AMPK activators [Fryer et al., 2002]. They activate AMPK indirectly via inhibition of ATP synthesis and consequent increase in the level of AMP [Owen et al., 2000; Brunmair et al., 2004]. As a result, they are inhibitors of mitochondrial function. It is important to identify more specific and potent AMPK activators, which should have few side effects as therapeutics for metabolic disorders.

Emodin is a natural product and active ingredient of various Chinese medicinal herbs [Xie and Du, 2011]. Recent studies have shown that emodin exerts anti-diabetic, anti-atherosclerotic, anti-cancer, and anti-allergic effects [Heo et al., 2008; Xue et al., 2010; Lu et al., 2011; Liu et al., 2012]. It is reported that emodin can function as a PPARγ agonist with PPARγ ligand-binding activity [Yang et al., 2007; Liu et al., 2009]. In this study, we examined the effect of emodin on adiponectin multimerization in 3T3-L1 adipocytes. We found that emodin activates AMPK and promotes the assembly of HMW adiponectin. We also demonstrated that emodin activates PPARγ and promotes differentiation and adiponectin expression during differentiation of 3T3-L1 preadipocytes. Therefore, emodin is a novel AMPK activator with PPARγ-agonist activity. We provide evidence that the effects of emodin on adiponectin expression and multimerization are the ultimate effects resulting from both AMPK activation and PPARγ activation.

MATERIALS AND METHODS

MATERIALS

Emodin, berberine, rosiglitazone, GW9662, Compound C, 3-isobutyl-1-methylxanthine, dexamethasone, insulin, Oil Red O, and antibody against β-actin were purchased from Sigma. A-769662 was purchased from Ascent Scientific. Antibodies against phospho-AMPK, total AMPK, or AMPKα1 were purchased from Cell Signaling or Upstate Biotechnology, respectively. Antibody against PPARγ was purchased from Abcam. A siRNA oligonucleotide targeting the mouse AMPKα1 with the sequence ACAUAUGCUG-CAGGUGGA was synthesized at GenePharma (Shanghai, China).

REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from 3T3-L1 adipocytes and quantitative real-time PCR was used to examine the level of different transcripts as described previously [Li et al., 2011]. PCR reactions were performed in an ABI PRISM 7500 real-time PCR system. All results were obtained from at least three independent experiments. The mRNA levels of all genes were normalized using β-actin as an internal control.

CELL CULTURE, DIFFERENTIATION AND TREATMENT

3T3-L1 preadipocytes (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) at 37°C in 5% CO_2 and induced to differentiate as described previously [Li et al., 2011]. To examine the effects of emodin on differentiation, 3T3-L1 preadipocytes were induced to differentiate in the presence of different concentrations of emodin. At Day 6 post-differentiation, the cells were stained with Oil Red O as described previously [Li et al., 2011].

Mature 3T3-L1 adipocytes or 293T cells were incubated in a serum-free medium containing 0.05% BSA for 16 h before being treated with emodin alone or together with other chemicals. 3T3-L1 adipocytes were transfected with mouse AMPKα1 siRNA or control siRNA for 48 h followed by treatment with emodin.

All chemicals used in the treatment were dissolved in DMSO. The stock solution of emodin is 50 mM. There are matched vehicle controls for each treatment so that the final concentration of DMSO is identical for each experiment. GW9662 was used together with emodin or rosiglitazone at a concentration of 20 μM. Compound C was used at a concentration of 10 μM.

SDS–PAGE AND WESTERN BLOT ANALYSIS

The cell lysates of 3T3-L1 adipocytes were subjected to 2–15% gradient gel electrophoresis under non-reducing and non-heat-denaturing conditions as described [Li et al., 2011]. Adiponectin oligomers and the total amount of adiponectin were detected using antibodies against the globular domain or the N-terminal peptide of adiponectin. AMPK was detected using antibodies specific for phospho-AMPK, total AMPK, or AMPKα1. The amount of each oligomer and the total adiponectin were quantified by analyzing the

western blots using the NIH ImageJ software. All experiments were performed at least three times and the representative results were presented.

LUCIFERASE REPORTER ASSAY

293T cells were transfected with PPRE-TK-Luciferase reporter along with PPARγ and RXRα expression vectors. Twenty-four hours after transfection, the cells were treated with emodin, berberine, or rosiglitazone in the presence or absence of GW9662 or Compound C. The cells were harvested for the luciferase assay after treatment for 24 h. Luciferase activities were normalized to Renilla activities cotransfected as an internal control.

RESULTS

EMODIN ACTIVATES AMPK AND PROMOTES ADIPONECTN MULTIMERIZATION IN 3T3-L1 ADIPOCYTES

We have previously found that activation of AMPK by berberine or AICAR promotes adiponectin multimerization in 3T3-L1 adipocytes [Li et al., 2011]. To investigate whether emodin activates AMPK, we treated 3T3-L1 adipocytes with emodin and examined phosphorylated AMPK (pAMPK) by western blot with a phosphorylation-specific AMPK antibody. The level of pAMPK was increased by emodin, with the highest level of pAMPK seen at 25 μM emodin (Fig. 1A). This result indicates that emodin is a novel AMPK activator.

To investigate the effect of emodin on adiponectin multimerization, we treated fully differentiated 3T3-L1 adipocytes with emodin for 48 h. We found that the level of HMW adiponectin was increased, whereas the level of LMW adiponectin was decreased in the presence of emodin (Fig. 1B). The ratio of HMW adiponectin to total adiponectin was increased in a dose-dependent way by emodin (Fig. 1C). Therefore, emodin promotes adiponectin multimerization in 3T3-L1 adipocytes.

To demonstrate that the AMPK signaling pathway mediates the effect of emodin on adiponectin multimerization, we used siRNA to specifically knock down the expression of endogenous AMPKα1. Forty-eight hours after transfection of AMPKα1 siRNA, we treated the cells with emodin for another 48 h. In cells transfected with AMPKα1 siRNA, the level of different adiponectin oligomers was not changed significantly, whereas the level of total adiponectin was increased after emodin treatment (Fig. 1B). As a result, the HMW/total ratio was decreased, compared to the cells transfected with the control siRNA (Fig. 1C). Therefore, suppression of AMPKα1 abolished the effects of emodin on adiponectin multimerization. This result demonstrates that emodin activates AMPK and promotes the assembly of HMW adiponectin.

EMODIN ACTIVATES PPARγ AND PROMOTES ADIPOCYTE DIFFERENTIATION OF 3T3-L1 PREADIPOCYTES

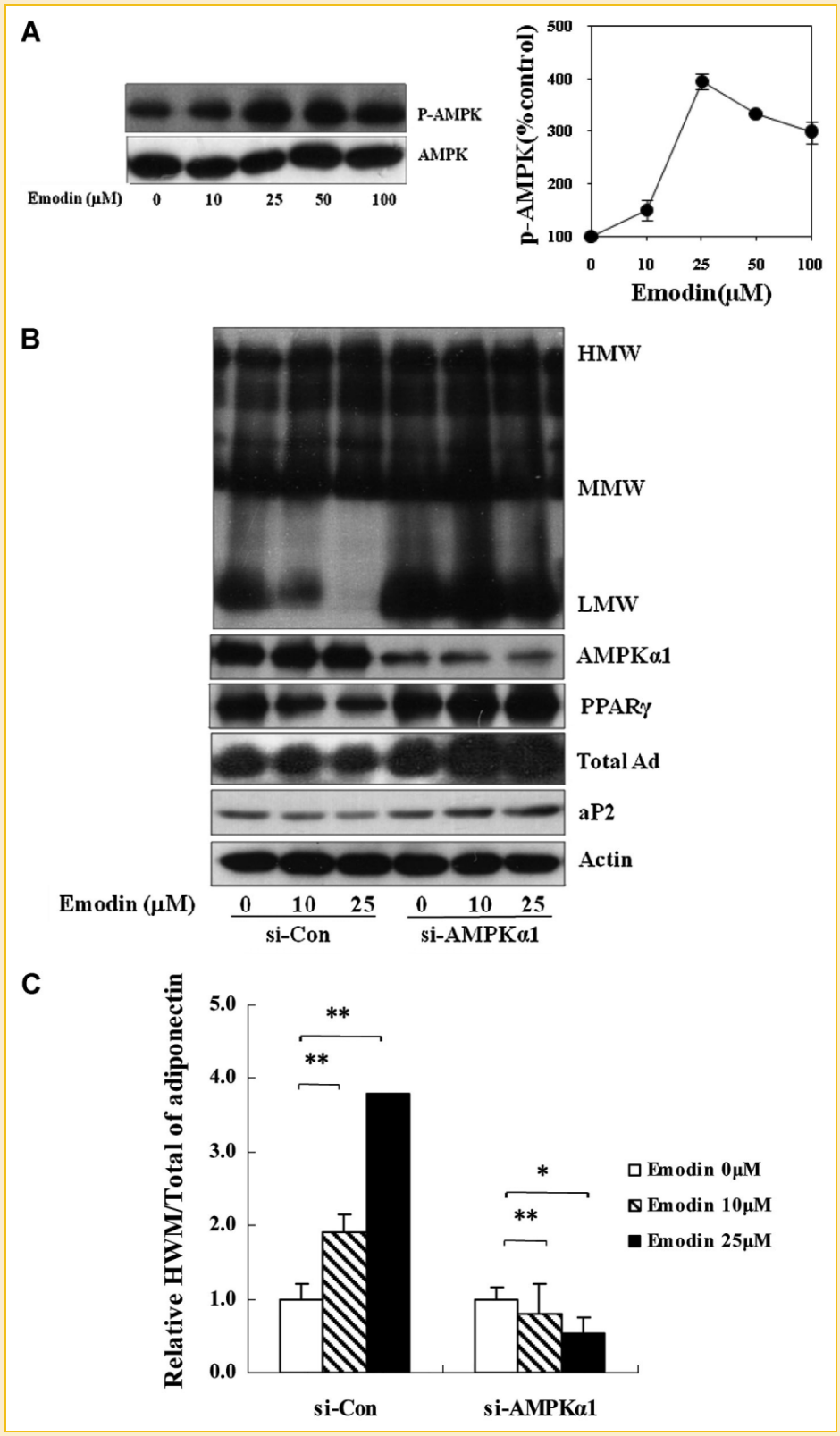
Emodin was reported to function as a PPARγ agonist with PPARγ ligand-binding activity [Yang et al., 2007; Liu et al., 2009]. We also found that emodin promoted the differentiation of 3T3-L1 preadipocytes, with the maximal effect seen at 25 μM (Fig. 2A). PPARγ is a key transcription factor regulating adipocyte differentiation [Morrison and Farmer, 1999]. aP2, a hallmark of adipogenesis, is one of the target genes for PPARγ during adipocyte differentiation [Gregoire et al., 1998]. We found that emodin up-regulated the expression of PPARγ and aP2. The expression of adiponectin, another PPARγ-responsive gene, was also increased by emodin at both mRNA and proteins levels (Fig. 2B,C). Therefore, emodin promotes differentiation of 3T3-L1 preadipocytes.

To confirm the involvement of PPARγ in the promotion of differentiation by emodin, we examined the effect of GW9662, a PPARγ inhibitor. Treatment of GW9662 clearly attenuated the effect of emodin on adipocyte differentiation, as evaluated by Oil Red O staining (Fig. 2D). Consistently, the expression of PPARγ, aP2, and adiponectin was inhibited significantly by GW9662 (data not shown). These results demonstrate that PPARγ is involved in emodin-mediated promotion of adipocyte differentiation and adiponectin expression. Thus, emodin promotes adipocyte differentiation by activating PPARγ.

THE EFFECTS OF EMODIN ON ADIPONECTIN ARE THE ULTIMATE EFFECTS RESULTING FROM BOTH AMPK ACTIVATION AND PPARγ ACTIVATION

We have previously shown that activation of AMPK by berberine or AICAR inhibits adiponectin expression in 3T3-L1 adipocytes [Li et al., 2011]. AMPK activation leads to phosphorylation of PPARγ, which decreases PPARγ expression and inhibits its transcriptional activity [Dagon et al., 2006; Lee et al., 2006]. Emodin treatment also leads to a decreased level of total adiponectin protein in 3T3-L1 adipocytes (Fig. 1B). Suppression of AMPKα1 abolished the effect of emodin and led to a dose-dependent increase in the expression of adiponectin, PPARγ, and aP2 (Fig. 3A). These results demonstrate that the inhibitory effect of emodin on adiponectin expression is the result of AMPK activation in 3T3-L1 adipocytes.

The fact that adiponectin expression is increased in AMPKα1-silenced cells suggested that the PPARγ-agonist activity of emodin also regulates adiponectin expression in 3T3-L1 adipocytes. To confirm this, we treated 3T3-L1 adipocytes with both emodin and GW9662. A further reduction in the mRNA expression of adiponectin, PPARγ, and aP2 was seen when both emodin and GW9662 were present (Fig. 3B). The level of adiponectin mRNA was reduced by approximately 80% in the presence of 20 μM GW9662 and 25 μM emodin. The protein level of adiponectin, PPARγ, and aP2 was also substantially reduced after treatment with both emodin and GW9662 (Fig. 3C). These results suggest that emodin upregulates adiponectin expression by its PPARγ-activating activity, although the effect is not as strong as that of the AMPK-activating activity in 3T3-L1 adipocytes. When the PPARγ-activating activity is inhibited by GW9662, the AMPK-activating activity is more prominent; a twofold increase in the HMW/total ratio of adiponectin was seen in the presence of 20 μM GW9662 and 25 μM emodin, compared to treatment with only 25 μM emodin (Fig. 3D). These results suggest that the PPARγ-activating activity and the AMPK-activating activity of emodin are present in 3T3-L1 adipocytes. Thus, the effects of emodin on the expression and multimerization adiponectin are the ultimate effects resulting from both AMPK and PPARγ activation.


A
P-AMPK
AMPK
Emodin (μM) 0 10 25 50 100
p-AMPK(%control)
500
400
300
200
100
0 10 25 50 100
Emodin(μM)
B
HMW
MMW
LMW
AMPKα1
PPARγ
Total Ad
aP2
Actin
Emodin (μM) 0 10 25 0 10 25
si-Con
si-AMPKα1
C
Relative HWM/Total of adiponectin
5.0
4.0
3.0
2.0
1.0
0.0
**
**
*
**
Emodin 0μM
Emodin 10μM
Emodin 25μM
si-Con
si-AMPKα1

EMODIN EXHIBITS BOTH AMPK-ACTIVATING AND PPARγ-ACTIVATING ACTIVITIES IN 293T CELLS

We further investigated the relationship between the AMPK-activating and PPARγ-activating activities of emodin in 293T cells. We first examined the effect of emodin on the expression of PPARγ. Activation of AMPK by emodin leads to decreased expression of PPARγ (Fig. 4A). This inhibition was completely abolished when the cells were treated with both emodin and Compound C, a specific AMPK inhibitor. As a matter of fact, the level of PPARγ protein was increased in the presence of both emodin and Compound C (Fig. 4A). This result suggests that the PPARγ-activating activity of emodin is more prominent when the AMPK-activating activity is inhibited. On the other hand, the level of PPARγ protein was further reduced when the cells were treated with both emodin and GW9662, compared to cells treated with only emodin (Fig. 4A). Berberine, a known AMPK activator, also inhibits PPARγ expression, however, no increase in PPARγ expression was seen when the cells were treated with both berberine and Compound C (Fig. 4B). PPARγ expression is promoted by rosiglitazone, a well-known PPARγ agonist. The level of PPARγ protein was not significantly changed in the presence of both rosiglitazone and GW9662 (Fig. 4C). Therefore, emodin regulates the expression of PPARγ in a way different from that of berberine or rosiglitazone.

We also examined the effect of emodin on PPARγ transcriptional activity in luciferase reporter assays. 293T cells were transfected with a luciferase reporter along with PPARγ and RXRα expression vectors and treated with emodin, berberine, or rosiglitazone in the presence or absence of inhibitors. Emodin dose-dependently inhibits the transcriptional activity of PPARγ, whereas inhibition of AMPK activity by Compound C leads to increased PPARγ activity (Fig. 4D). No increase in PPARγ activity was seen when cells were treated with both berberine and Compound C. Treatment with both GW9662 and emodin leads to further reduction in PPARγ activity (Fig. 4D). These results demonstrate that both AMPK-activating and PPARγ-activating activities of emodin are present to regulate the transcriptional activity of PPARγ in 293T cells.

EMODIN ACTIVATES AMPK BY A MECHANISM SIMILAR TO BERBERINE

Most of the AMPK activators identified so far activate AMPK indirectly. Berberine and metformin have been found to affect the intracellular AMP/ATP ratio by inhibiting the mitochondrial respiratory chain [Owen et al., 2000; Turner et al., 2008]. A-769662 is one of the AMPK activators which directly activate AMPK [Sanders et al., 2007]. We found that emodin also activates AMPK in 293T cells, with the highest level of pAMPK seen at 25 μM (Fig. 5A), same as in 3T3-L1 adipocytes (Fig. 1A). Berberine and A-769662 also activate AMPK in 293T cells in a dose-dependent way (Fig. 5B,C).

To investigate the mechanism by which emodin activates AMPK, we examined whether emodin has any synergistic effects with berberine or A-769662 on AMPK activation. When 293T cells were treated with both emodin and A-769662, the level of pAMPK was increased as the concentration of emodin increased (Fig. 5D). However, no such effect was seen when berberine and emodin were used to treat the cells. The highest level of pAMPK was seen at 5 μM emodin in the presence of both emodin and berberine (Fig. 5D). Similar results were seen when the experiments were performed in 3T3-L1 adipocytes (data not shown). These results suggest that emodin activates AMPK indirectly by a mechanism similar to berberine.

DISCUSSION

In this paper, we found that emodin activates AMPK in both 3T3-L1 adipocytes and 293T cells (Figs. 1A and 5A). This is the first report of the AMPK-activating activity of emodin. We also found that emodin activates PPARγ and promotes the differentiation of 3T3-L1 preadipocytes (Fig. 3). This is consistent with the previous reports [Yang et al., 2007; Liu et al., 2009]. Therefore, emodin is a novel AMPK activator with PPARγ-agonist activity.

Activation of AMPK decreases PPARγ expression and inhibits its transcriptional activity [Dagon et al., 2006; Lee et al., 2006]. Therefore, as an AMPK activator and a PPARγ-agonist, emodin has both PPARγ-inhibiting and PPARγ-activating activities, which regulate adiponectin expression in opposite ways. We demonstrate that both activities of emodin are present in 3T3-L1 adipocytes and 293T cells. Emodin promotes the differentiation of 3T3-L1 preadipocytes (Fig. 2); therefore, the PPARγ-agonist activity is more prominent than the AMPK-activating activity during differentiation. The effect of emodin on promoting adipogenesis peaked at 25 μM. When emodin is present at 50 μM, a decline in PPARγ activity was seen (Fig. 2A), probably as a result of AMPK activation by emodin at higher concentrations. In mature adipocytes and 293T cells, the AMPK-activating activity is higher than the PPARγ-agonist activity, as can be seen from decreased expression of adiponectin or PPARγ (Figs. 3B,C and 4A). On one hand, the PPARγ-agonist activity is the predominant activity when the AMPK activity is suppressed by siRNA. As a result, the level of adiponectin protein was upregulated by emodin in a dose-

Fig. 1. Emodin activates AMPK and promotes the assembly of HMW adiponectin in 3T3-L1 adipocytes. **A:** 3T3-L1 adipocytes were treated with emodin for 1 h. The cell lysates were subjected to SDS–PAGE and western blot analysis using antibodies specific for phospho-AMPK and total AMPK. The results are representative of at least three independent experiments with similar results. The amount of pAMPK and AMPK was quantified using the NIH ImageJ software. Results (mean ± SD, n = 3) were expressed as a percentage of the control (emodin 0 μM). **B:** 3T3-L1 adipocytes were transfected with AMPKα1 siRNA or control siRNA. Forty-eight hours after transfection, the cells were treated with emodin for another 48 h. The cells were harvested and subjected to 2–15% gradient gel electrophoresis under non-reducing and non-heat-denaturing conditions to detect the three oligomeric forms of adiponectin (LMW, MMW, and HMW) using anti-globular domain antibodies (top panel). The amount of total adiponectin (total Ad), PPARγ, aP2, or actin was detected with antibodies against the N-terminal peptide of adiponectin, PPARγ, aP2, or β-actin, respectively. **C:** The amount of each oligomer and total adiponectin shown in (**B**) was quantified using the NIH ImageJ software and the HMW/total ratio of adiponectin was calculated. The results are presented as the ratio of HMW/total relative to the control (emodin 0 μM) and as the mean ± SD (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

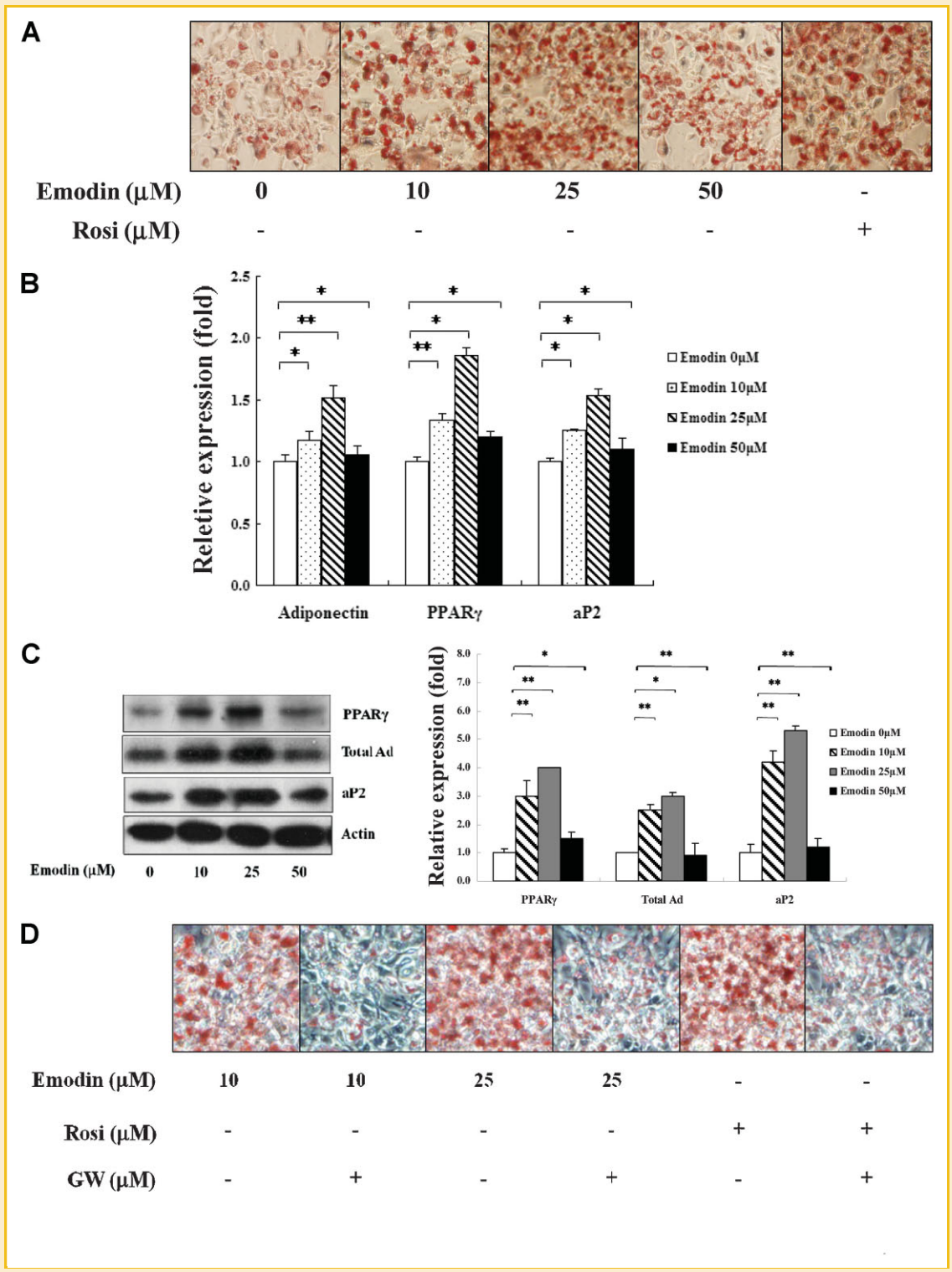


Fig. 2. Emodin activates PPARγ and promotes adipocyte differentiation and adiponectin expression during differentiation of 3T3-L1 preadipocyte. 3T3-L1 preadipocytes were induced to differentiate in the presence of 0, 10, 25, 50 μM emodin or 1 μM rosiglitazone (Rosi) for 6 days. **A:** The cells were stained with Oil Red O. **B:** The mRNA level of adiponectin, aP2, and PPARγ was examined by quantitative real-time PCR. Data are expressed relative to β-actin. **C:** The amount of total adiponectin (total Ad), PPARγ, aP2, or actin was detected with corresponding antibodies. The results were quantified and presented relative to the control (emodin 0 μM) and as the mean ± SD (n = 3). **D:** GW9662 (20 μM) was used together with emodin or rosiglitazone (1 μM) during differentiation of 3T3-L1 preadipocytes. The cells were stained as described in (**A**).

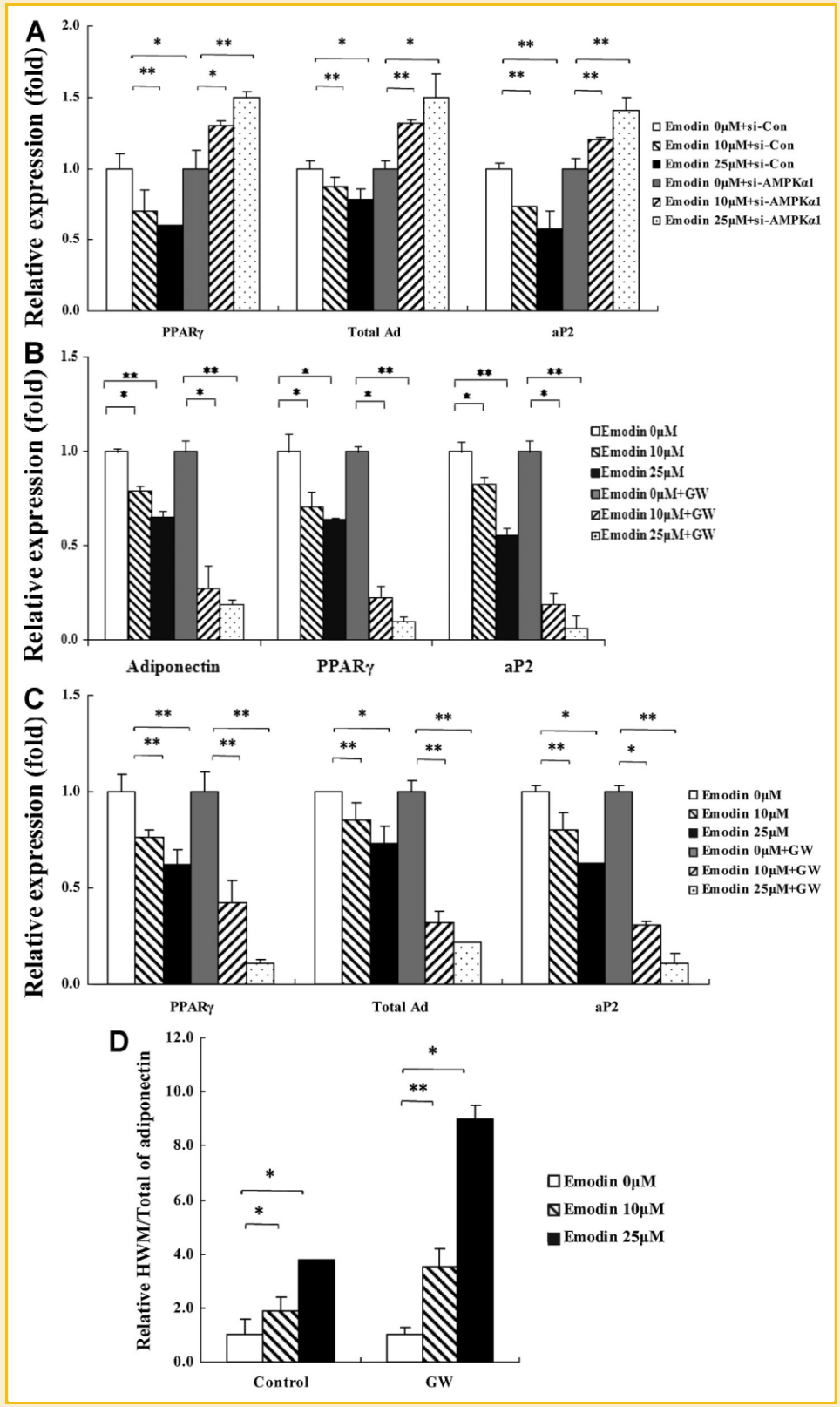


Fig. 3. The effects of emodin on adiponectin are the ultimate effects resulting from both AMPK activation and PPARγ activation. **A:** The total amount of adiponectin (total Ad), PPARγ, and aP2 shown in Figure 1B was quantified and presented as described in Figure 2C. **B:** 3T3-L1 adipocytes were treated with emodin or emodin with GW9662 for 48 h. The mRNA level of adiponectin, aP2, and PPARγ was examined by real-time PCR as described in Figure 2B. **C:** After treatment with emodin or emodin and GW9662 for 48 h, the cell extracts were subjected to western blot analysis. The amount of total adiponectin (total Ad), PPARγ, and aP2 was quantified and presented as described in Figure 2C. **D:** The HMW/total ratio of adiponectin after 48-h treatment was calculated and presented as described in Figure 1C. All the results were presented relative to the control (emodin 0 μM, with the same type of treatment).

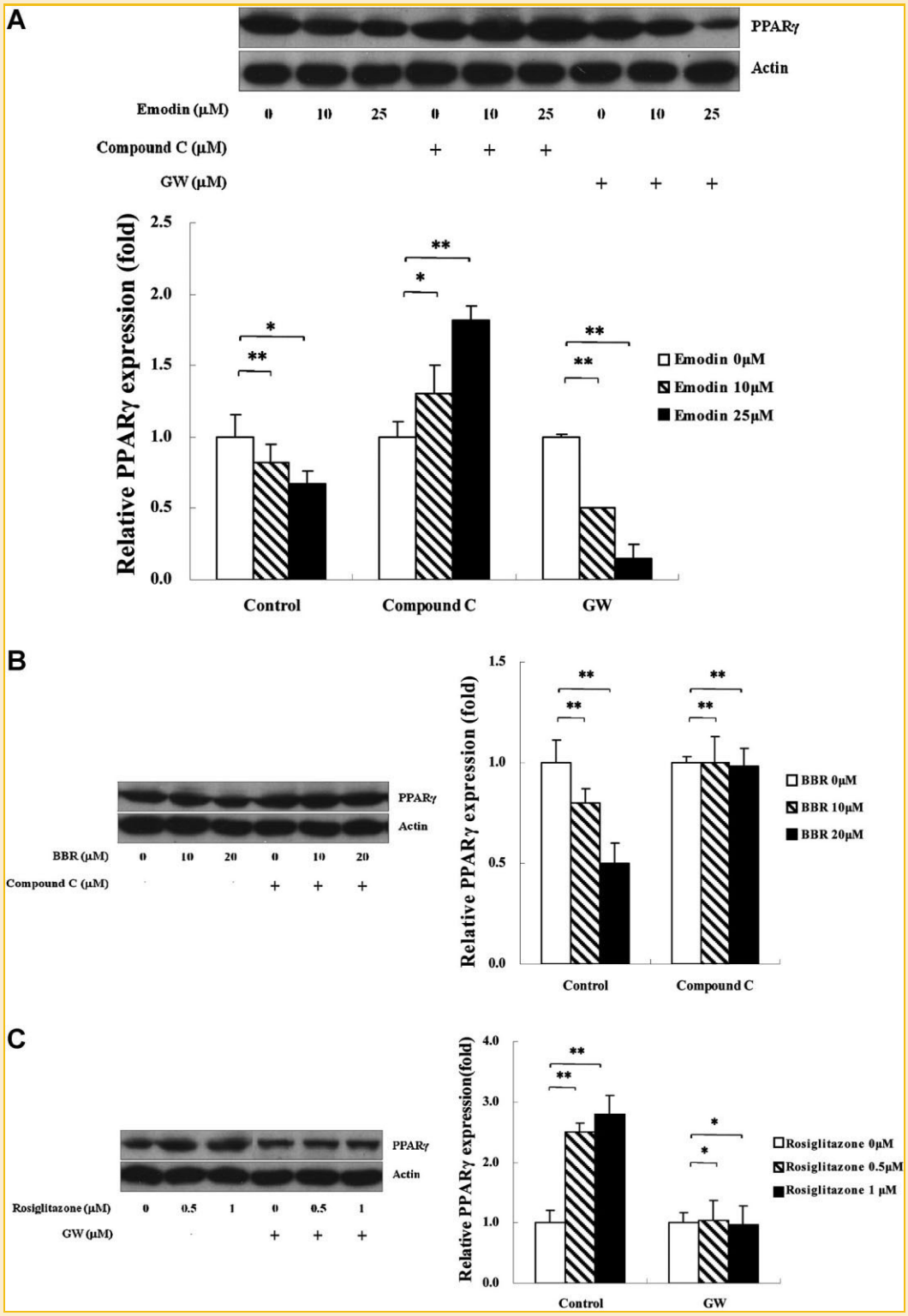


Fig. 4. (*Continued*)

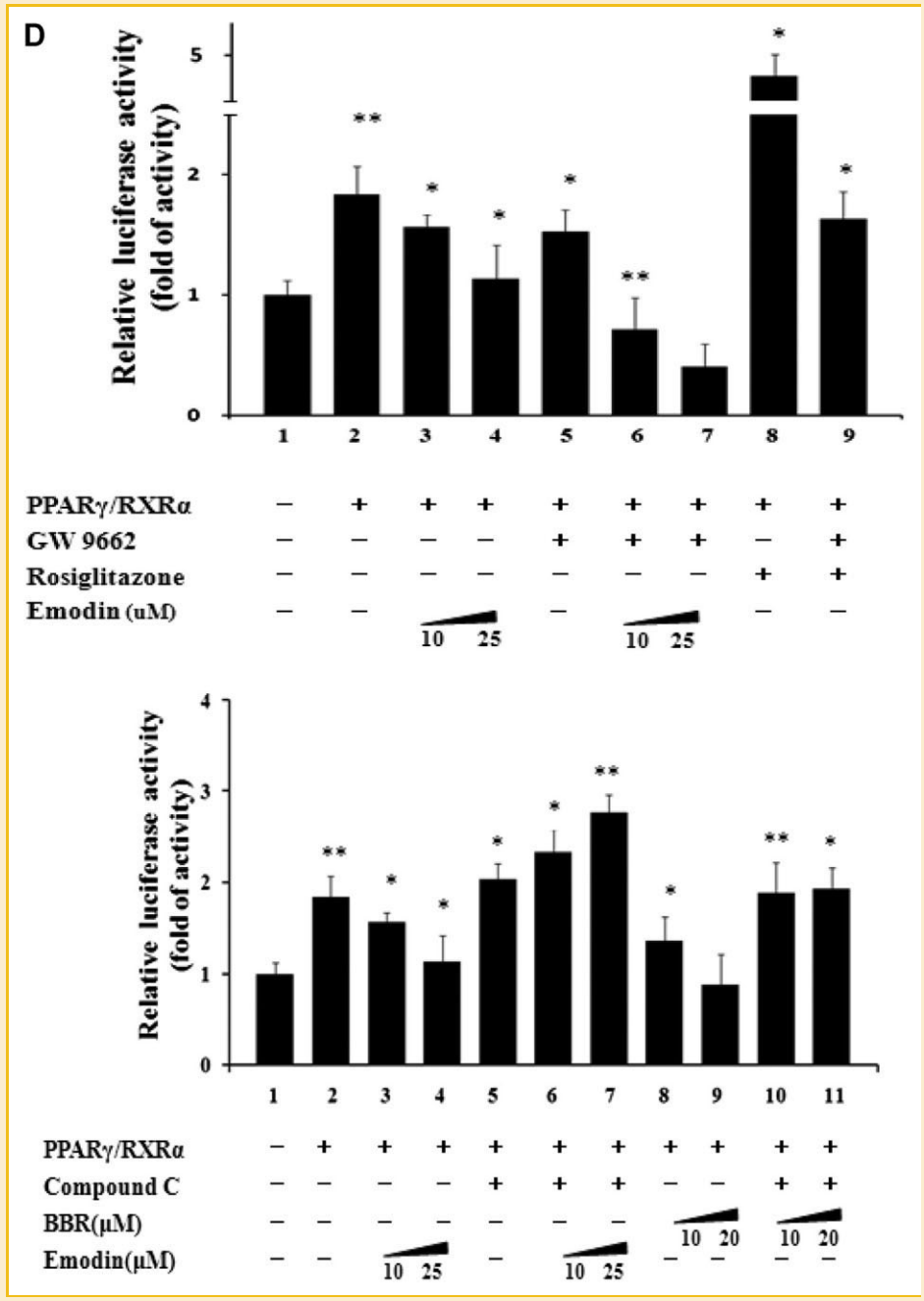


Fig. 4. Emodin exhibits both AMPK-activating and PPARγ-activating activities in 293T cells. The 293T cells were treated with emodin **(A)**, berberine **(B)**, or rosiglitazone **(C)** in the presence or absence of either Compound C or GW9662 for 24 h. Cell lysates were subjected to western blot analysis with anti-PPARγ antibody. The amount of PPARγ was quantified and presented relative to the control (emodin 0 μM, with the same type of treatment). **D:** The 293T cells were transfected with PPRE-TK-Luciferase reporter along with PPARγ and RXRα expression vectors. Twenty-four hours after transfection, the cells were treated with emodin, berberine, or rosiglitazone in the presence or absence of GW9662 or Compound C for another 24 h. The cell extracts were subjected to luciferase assay.

dependent way in cells transfected with AMPKα1 siRNA (Fig. 3A). Increased PPARγ activity was also seen in 293T cells when AMPK was inhibited by Compound C (Fig. 4D). On the other hand, the AMPK activity is more prominent when the PPARγ activity was inhibited by GW9662; we saw a more substantial decrease in both adiponectin mRNA and total protein (Fig. 3B,C), but a greater increase in HMW/total ratio as concentration of emodin increased (Fig. 3D). Therefore, both PPARγ-activating and PPARγ-inhibiting activities of emodin are present in adipocytes to regulate adiponectin expression and multimerization in 3T3-L1 adipocytes.

The dual-activity makes emodin different from other AMPK activators and rosiglitazone in certain respects. Like rosiglitazone, emodin promotes differentiation (Fig. 2A), whereas AMPK activators, such as berberine or AICAR, inhibit differentiation [Li et al.,

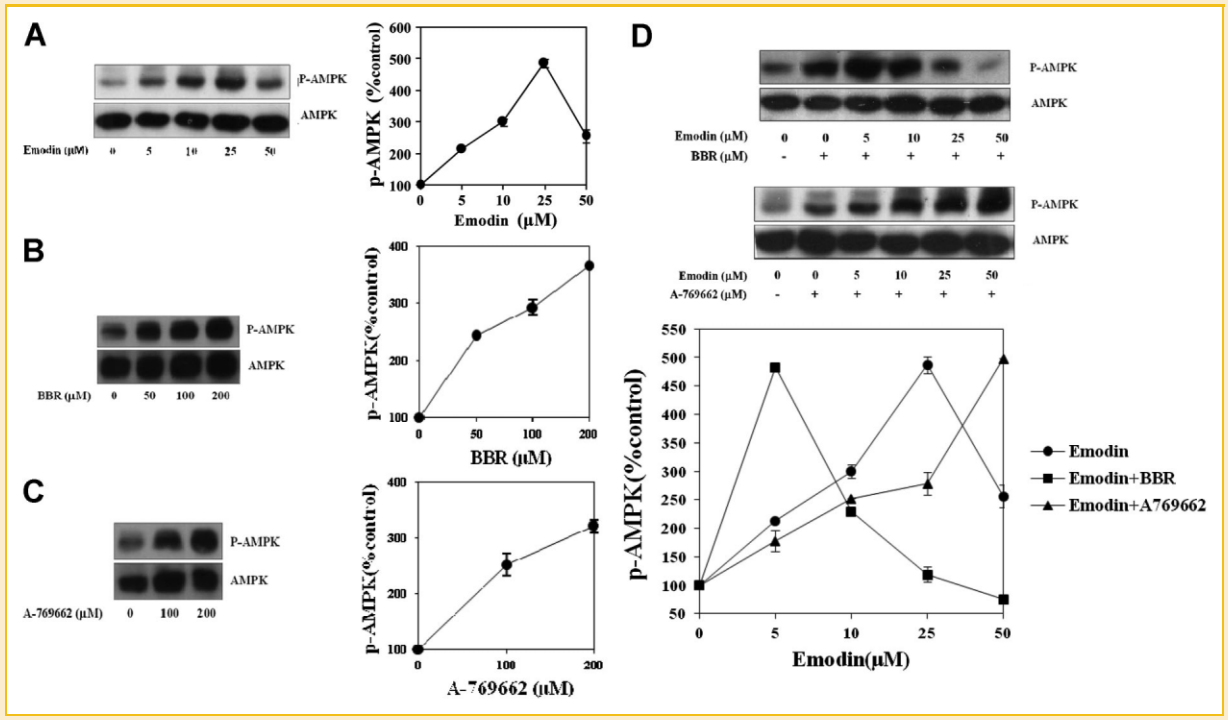


Fig. 5. Emodin activates AMPK by a mechanism similar to berberine. The 293T cells were treated with emodin (**A**), berberine (**B**), A-769662 (**C**), emodin and 200 μM berberine or emodin and 200 μM A-769662 (**D**) for 1 h. The amount of pAMPK and AMPK was quantified and presented as described in Figure 1A.

2011]. On the other hand, treatment with rosiglitazone increases not only the level of adiponectin oligomers but also the total amount of adiponectin (data not shown). Therefore, the HMW/total ratio of adiponectin was not increased by rosiglitazone. However, emodin, like berberine or AICAR, increases this ratio (Figs. 1C and 3D). In addition, we have reproducibly noticed that the effect of emodin on adipogenesis was not as potent as that of rosiglitazone; a higher concentration of emodin is required to promote differentiation, compared to that of rosiglitazone (Fig. 2A). Furthermore, the mRNA level and total protein level of adiponectin are reduced substantially by berberine or AICAR [Li et al., 2011]. However, emodin treatment only leads to a moderate decrease in the mRNA and total protein level of adiponectin (Figs. 3B,C and 1C). The AMPK-activating activity of emodin is counteracted by that of the PPARγ-agonist activity. Thus, the effects of emodin on the expression and multimerization adiponectin are the ultimate effects resulting from both AMPK and PPARγ activation.

AMPK is a key sensor and regulator of intracellular and whole-body energy metabolism [Towler and Hardie, 2007]. In this paper, we demonstrate that emodin activates AMPK and promotes the assembly of HMW adiponectin. Our previous study shows that activation of AMPK by berberine or AICAR promotes adiponectin multimerization[Li et al., 2011]. Our study reveals a novel regulatory role of the AMPK signaling pathway in adiponectin multimerization and provides more evidence that AMPK would be a prime therapeutic target for obesity-related metabolic diseases.

Most of the AMPK activators identified so far activate AMPK indirectly by inhibiting mitochondrial function [Hawley et al., 2010]. Metformin and thiazolidinedione (TZD) are currently the frontline treatment for type 2 diabetes worldwide. Both of them activate AMPK by inhibiting Complex I of the respiratory chain [Owen et al., 2000; Brunmair et al., 2004]. A-769662 directly activates AMPK independent of AMP activation [Sanders et al., 2007]. We found that emodin and A-769662 activate AMPK synergistically (Fig. 5D), suggesting that emodin activates AMPK by a mechanism different from A-769662. No such effect was seen between emodin and berberine or emodin and metformin on AMPK activation (Fig. 5D, data not shown). Therefore, emodin might activate AMPK indirectly, similar to berberine and metformin. Emodin has been reported to be an inhibitor of tyrosine kinase syk and Her2/neu [Jayasuriya et al., 1992; Zhang et al., 1995; Zhang et al., 1998; Lim et al., 2007; Lu et al., 2011]. It remains to be investigated whether the tyrosine kinase inhibiting activity of emodin is related to its function as an AMPK activator. We are currently in the process of investigating how emodin activates AMPK and how the AMPK signaling pathway regulates adiponectin multimerization.

Berberine treatment increased AMPK activity in 3T3-L1 cells, which has been demonstrated to be associated with GLUT1-mediated glucose uptake [Kim et al., 2007]. Increased glucose uptake by emodin has also been reported [Yang et al., 2007]. In addition, emodin has been shown to improve glucose tolerance and insulin sensitivity in high-fat diet-induced obese mice and low-dose STZ-induced diabetic mice [Xue et al., 2010]. In this study, we found that emodin promotes adiponectin multimerization and increases the HMW/total adiponectin ratio, which has been shown to be closely associated with peripheral insulin sensitivity [Pajvani et al., 2004]. Therefore, emodin might increase insulin sensitivity by promoting

the assembly of LMW adiponectin into HMW adiponectin. We are currently in the process of investigating the effect of emodin on adiponectin multimerization and secretion in the mouse model of type 2 diabetes. The efficiency of emodin on improving the insulin sensitivity of these mice will be examined as a way to assess the physiological significance of increasing the HMW/total ratio of adiponectin.

The expression of adiponectin was substantially inhibited by berberine and other AMPK activators we have examined, although the HMW/total ratio is increased. It is hard to predict whether an increased HMW adiponectin level can counteract a decrease in the total protein level or to predict the possible physiological relevance. Based on the fact that emodin promotes differentiation and increases the HMW/total ratio with only moderately inhibiting adiponectin expression, emodin or its derivatives might be potential drug candidates for the treatment of type 2 diabetes and other obesity-related metabolic diseases.

ACKNOWLEDGMENTS

We thank Professor David J. Mangelsdorf for providing the PPARγ and RXRα expression vectors. This work was supported by the NSFC (no. 30470354 and 30570356), the Program for New Century Excellent Talents in University (NCET), and the National Science Foundation for Fostering Talents in Basic Research of NSFC.

REFERENCES

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 257:79–83.

Basu R, Pajvani UB, Rizza RA, Scherer PE. 2007. Selective downregulation of the high molecular weight form of adiponectin in hyperinsulinemia and in type 2 diabetes: Differential regulation from nondiabetic subjects. Diabetes 56:2174–2177.

Bobbert T, Rochlitz H, Wegewitz U, Akpulat S, Mai K, Weickert MO, Möhlig M, Pfeiffer AF, Spranger J. 2005. Changes of adiponectin oligomer composition by moderate weight reduction. Diabetes 54:2712–2719.

Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhausl W, Furnsinn C. 2004. Thiazolidinediones, like metformin, inhibit respiratory complex I: A common mechanism contributing to their antidiabetic actions? Diabetes 53:1052–1059.

Combs TP, Berg AH, Rajala MW, Klebanov S, Iyengar P, Jimenez-Chillaron JC, Patti ME, Klein SL, Weinstein RS, Scherer PE. 2003. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. Diabetes 52:268–276.

Dagon Y, Avraham Y, Berry EM. 2006. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes. Biochem Biophys Res Commun 340:43–47.

Fryer LG, Parbu-Patel A, Carling D. 2002. The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem 277:25226–25232.

Gregoire FM, Smas CM, Sul HS. 1998. Understanding adipocyte differentiation. Physiol Rev 78:783–809.

Hardie DG. 2011. AMP-activated protein kinase: An energy sensor that regulates all aspects of cell function. Genes Dev 25:1895–1908.

Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM, Hardie DG. 2010. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell Metabol 11:554–565.

Heo SK, Yun HJ, Park WH, Park SD. 2008. Emodin inhibits TNF-alpha-induced human aortic smooth-muscle cell proliferation via caspase- and mitochondrial-dependent apoptosis. J Cell Biochem 105:70–80.

Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 20:1595–1599.

Hu E, Liang P, Spiegelman BM. 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 271:10697–10703.

Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H, Kubota N, Takamoto I, Hayashi YK, Yamauchi N, Waki H, Fukayama M, Nishino I, Tokuyama K, Ueki K, Oike Y, Ishii S, Hirose K, Shimizu T, Touhara K, Kadowaki T. 2010. Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+) and AMPK/SIRT1. Nature 464:1313–1319.

Jayasuriya H, Koonchanok NM, Geahlen RL, McLaughlin JL, Chang CJ. 1992. Emodin, a protein tyrosine kinase inhibitor from *Polygonum cuspidatum*. J Nat Prod 55:696–698.

Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest 116:1784–1792.

Kim SH, Shin EJ, Kim ED, Bayaraa T, Frost SC, Hyun CK. 2007. Berberine activates GLUT1-mediated glucose uptake in 3T3-L1 adipocytes. Biol Pharm Bull 30:2120–2125.

Kishida K, Nagaretani H, Kondo H, Kobayashi H, Tanaka S, Maeda N, Nagasawa A, Hibuse T, Ohashi K, Kumada M, Nishizawa H, Okamoto Y, Ouchi N, Maeda K, Kihara S, Funahashi T, Matsuzawa Y. 2003. Disturbed secretion of mutant adiponectin associated with the metabolic syndrome. Biochem Biophys Res Commun 306:286–292.

Koenen TB, van Tits LJ, Holewijn S, Lemmers HL, den Heijer M, Stalenhoef AF, de Graaf J. 2008. Adiponectin multimer distribution in patients with familial combined hyperlipidemia. Biochem Biophys Res Commun 376:164–168.

Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, Kozono H, Takamoto I, Okamoto S, Shiuchi T, Suzuki R, Satoh H, Tsuchida A, Moroi M, Sugi K, Noda T, Ebinuma H, Ueta Y, Kondo T, Araki E, Ezaki O, Nagai R, Tobe K, Terauchi Y, Ueki K, Minokoshi Y, Kadowaki T. 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 6:55–68.

Lago F, Dieguez C, Gomez-Reino J, Gualillo O. 2007. Adipokines as emerging mediators of immune response and inflammation. Nat Clin Pract Rheumatol 3:716–724.

Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB. 2006. Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. Diabetes 55:2256–2264.

Li Y, Wang P, Zhuang Y, Lin H, Liu L, Meng Q, Cui T, Liu J, Li Z. 2011. Activation of AMPK by berberine promotes adiponectin multimerization in 3T3-L1 adipocytes. FEBS Lett 585:1735–1740.

Lim BO, Lee JH, Ko NY, Mun SH, Kim JW, Kim do K, Kim JD, Kim BK, Kim HS, Her E, Lee HY, Choi WS. 2007. *Polygoni cuspidati* radix inhibits the activation of Syk kinase in mast cells for antiallergic activity. Exp Biol Med (Maywood) 232:1425–1431.

Liu DL, Bu H, Li H, Chen H, Guo HC, Wang ZH, Tong HF, Ni ZL, Liu HB, Lin SZ. 2012. Emodin reverses gemcitabine resistance in pancreatic cancer cells via the mitochondrial apoptosis pathway in vitro. Int J Oncol 40:1049–1057.

Liu M, Liu F. 2010. Transcriptional and post-translational regulation of adiponectin. Biochem J 425:41–52.

Liu M, Zhou L, Xu A, Lam KS, Wetzel MD, Xiang R, Zhang J, Xin X, Dong LQ, Liu F. 2008. A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. Proc Natl Acad Sci USA 105:18302–18307.

Liu Y, Jia L, Liu ZC, Zhang H, Zhang PJ, Wan Q, Wang R. 2009. Emodin ameliorates high-glucose induced mesangial p38 over-activation and hypocontractility via activation of PPARgamma. Exp Mol Med 41:648–655.

Lu Y, Yang JH, Li X, Hwangbo K, Hwang SL, Taketomi Y, Murakami M, Chang YC, Kim CH, Son JK, Chang HW. 2011. Emodin, a naturally occurring anthraquinone derivative, suppresses IgE-mediated anaphylactic reaction and mast cell activation. Biochem Pharmacol 82:1700–1708.

Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y. 2001. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 50:2094–2099.

Morrison RF, Farmer SR. 1999. Insights into the transcriptional control of adipocyte differentiation. J Cell Biochem Suppl 32–33:59–67.

Owen MR, Doran E, Halestrap AP. 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 348:607–614.

Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE. 2003. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem 278:9073–9085.

Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE. 2004. Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. J Biol Chem 279:12152–12162.

Qiang L, Wang H, Farmer SR. 2007. Adiponectin secretion is regulated by SIRT1 and the endoplasmic reticulum oxidoreductase Ero1-L alpha. Mol Cell Biol 27:4698–4707.

Rajala MW, Scherer PE. 2003. Minireview: The adipocyte—At the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 144:3765–3773.

Sanders MJ, Ali ZS, Hegarty BD, Heath R, Snowden MA, Carling D. 2007. Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. J Biol Chem 282:32539–32548.

Satoh N, Ogawa Y, Usui T, Tagami T, Kono S, Uesugi H, Sugiyama H, Sugawara A, Yamada K, Shimatsu A, Kuzuya H, Nakao K. 2003. Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. Diabetes Care 26:2493–2499.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 270:26746–26749.

Towler MC, Hardie DG. 2007. AMP-activated protein kinase in metabolic control and insulin signaling. Circ Res 100:328–341.

Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF. 2003. Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. J Biol Chem 278:50810–50817.

Turner N, Li JY, Gosby A, To SW, Cheng Z, Miyoshi H, Taketo MM, Cooney GJ, Kraegen EW, James DE, Hu LH, Li J, Ye JM. 2008. Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: A mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. Diabetes 57:1414–1418.

Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T. 2003. Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. J Biol Chem 278:40352–40363.

Wang Y, Lam KS, Yau MH, Xu A. 2008. Post-translational modifications of adiponectin: Mechanisms and functional implications. Biochem J 409:623–633.

Wang ZV, Schraw TD, Kim JY, Khan T, Rajala MW, Follenzi A, Scherer PE. 2007. Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. Mol Cell Biol 27:3716–3731.

Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R, Goldstein BJ. 2003. Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 52:1355–1363.

Xie W, Du L. 2011. Diabetes is an inflammatory disease: Evidence from traditional Chinese medicines. Diabetes Obes Metab 13:289–301.

Xue J, Ding W, Liu Y. 2010. Anti-diabetic effects of emodin involved in the activation of PPARgamma on high-fat diet-fed and low dose of streptozotocin-induced diabetic mice. Fitoterapia 81:173–177.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn B, Kadowaki T. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8:1288–1295.

Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K, Kadowaki T. 2007. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med 13:332–339.

Yang Y, Shang W, Zhou L, Jiang B, Jin H, Chen M. 2007. Emodin with PPARgamma ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-Ll cells. Biochem Biophys Res Commun 353:225–230.

Zhang L, Chang CJ, Bacus SS, Hung MC. 1995. Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin. Cancer Res 55:3890–3896.

Zhang L, Lau YK, Xi L, Hong RL, Kim DS, Chen CF, Hortobagyi GN, Chang C, Hung MC. 1998. Tyrosine kinase inhibitors, emodin and its derivative repress HER-2/neu-induced cellular transformation and metastasis-associated properties. Oncogene 16:2855–2863.