Modifications of Histone H3 at Lysine 9 on the Adiponectin Gene in 3T3-L1 Adipocytes

Naoko SAKURAI, Kazuki MOCHIZUKI and Toshinao GODA*

Laboratory of Nutritional Physiology, Graduate School of Nutritional and Environmental Sciences and Global COE, The University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka, Shizuoka 422–8526, Japan

(Received August 15, 2008)

Summary Modification of histone H3 at lysine 9 from methylations to acetylation is important for transactivation of genes. In this study, we found that all methylations (mono-, di-, tri-) of histone H3 at lysine 9 on the adiponectin gene decreased by stimulating adipocyte differentiation prior to increases in adiponectin gene expression and acetylation of histone H3 at the same residue on the gene during adipocyte differentiation of 3T3-L1 cells. Additionally, we revealed that decrease of adiponectin gene expression by treatment with TNFα, an inducer of insulin resistance in adipocytes, was associated with decreased acetylation of histone H3 at lysine 9 on the gene, but not methylations. Our results suggest that induction of the adiponectin gene during adipocyte differentiation is associated with modification of histone H3 at lysine 9 from methylations to acetylation, whereas reduction of the adiponectin gene in 3T3-L1 adipocytes with insulin resistance is associated with decreased acetylation at lysine 9 of histone H3.

Key Words adiponectin, histone H3 at lysine 9, acetylation, methylation, adipocyte

Adiponectin is a cytokine secreted from adipocytes and has the ability to enhance insulin sensitivity in various tissues (1–3). Recent studies have focused on the transcriptional regulation of adiponectin in adipocytes, because a higher circulating concentration of adiponectin protein is believed to improve insulin resistance, diabetes and related complications. It has also been suggested that the expression of adiponectin and other adipocyte-specific genes is induced by a coordinated cascade of sequence-specific transcription factors. It is well-known that adipocyte differentiation, as well as adipocyte-specific gene expression, is strongly induced by one of the nuclear receptors, PPARγ (4), cAMP response element binding protein (CREB) and CAAT/enhancer-binding proteins (C/EBPs) also induce expression of adipocyte specific-genes and differentiation (5, 6).

Many recent studies suggest that histone modifications such as acetylation, methylation and phosphorylation, called the histone code, play central roles in the regulation of recruitment of the transcriptional complex on the promoter/enhancer and transcriptional regions of the genes (7, 8). Among several identified histone modifications, acetylations and methylations of histone H3 are the most extensively studied, because the regulation of histone H3 modifications is related to ON/OFF switching of transcription. Previous studies have indicated that acetylation of the histone H3 tail at lysines 9 and 14, and methylation of the histone H3 tail at lysine 4 are associated with transactivation. Indeed, a recent study has demonstrated that histone H3 dimethylated at lysine 4 and acetylated at lysine 9/14, on the adiponectin gene, was associated with transactivation of the gene in adipocytes (9). On the other hand, methylation of the histone H3 tail at lysine 9 is associated with repression of transcription (10–12). It has been suggested that ON/OFF regulation of transcription is directed by switching between acetylation and methylation at lysine 9 of histone H3 on their promoter regions (13). However, it is unclear whether induction of the adiponectin gene in the process of differentiation in adipocytes, and reduction of the gene’s expression in adipocytes with insulin resistance, is associated with changes from methylation to acetylation at lysine 9 of histone H3.

In this study, we examined whether induction of the adiponectin gene during adipocyte differentiation is associated with chromatin modification changes at lysine 9 of histone H3 from methylations to acetylation, on the gene in 3T3-L1 adipocytes. Furthermore, we investigated whether reduction of the expression of the adiponectin gene by treatment with TNFα, an inducer of insulin resistance in adipocytes, is regulated by these modifications of histone H3 on the adiponectin gene.

MATERIALS AND METHODS

Chemical reagents. Dexamethasone (Dex) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and 3-isobutyl-1-methylxanthine (IBMX) from Sigma Aldrich (Tokyo, Japan), were dissolved in dimethyl sulfoxide (DMSO). The bovine insulin (Sigma Aldrich) used in this study was dissolved in PBS (phosphate buffered saline).
Cell culture. 3T3-L1 pre-adipocyte cells from the American Type Culture Collection were cultured at 37 °C in a humidified incubator with an atmosphere of 5% CO$_2$ in Dulbecco’s modified Eagle medium (DMEM) containing 10% donor serum, 2 mM glutamine, 20 mM Hepes, 50 U/mL penicillin and 50 µg/mL streptomycin sulfate. After 80% confluence, the culture medium was replaced with medium for differentiation (DMEM with 10% fetal bovine serum, 0.5 mM IBMX, 2 µM Dex and 1.7 µM insulin). After 48 h of stimulation (this time point was defined as day 0 in this study), cells were cultured with DMEM with 10% fetal bovine serum. The medium was changed every 2 d. For the experiment on the effect of TNFα on the adiponectin gene, cells were treated on day 8 with/without 20 ng/mL TNFα for 24–48 h.

Real-time RT-PCR. Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi (14). The total RNA samples were stored at −80°C before being used in quantitative RT-PCR analysis. The total RNA samples (2.5 µg) were converted into cDNA by reverse transcription using Super Script™ III reverse transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer’s instructions. To quantitatively estimate the mRNA levels, polymerase chain reaction (PCR) amplification was performed on a Light-Cycler instrument system (Roche, Tokyo, Japan). Real-time RT-PCR reactions were carried out in a total volume of 20 µL containing 400 nM each of gene-specific primers, cDNA and SYBR Premix Ex Taq (Takara, Shiga, Japan). The cycle threshold (CT)-value of each gene detected by real-time RT-PCR was

Table 1. Primer sets used for the ChIP assay.

| Region on the adiponectin gene | Fragment size | Sequence |
|--------------------------------|---------------|----------|
| −5,900                         | −5,926−5,909  | 5′-ATGGCTCAGTTGTTAAGAGCA-3′ |
|                                | −5,865−5,845  | 5′-GATGTTGTGAGCTCCTTGATGF-3′ |
| −700                           | −705−684      | 5′-ACCCTGAACTGCTCACCAC-3′ |
|                                | −628−606      | 5′-TGCTAGAGGATCCCAATCTGA-A3′ |
| −500                           | −549−528      | 5′-TGCAATGCTATTTGACACCACCA-3′ |
|                                | −502−481      | 5′-TCGATTTCCCAGCCACACAGTA-3′ |
| −300                           | −371−350      | 5′-AGGGGTCAGAGCCTCCTCTCT-3′ |
|                                | −274−253      | 5′-AGGGGTCAGAGCCTCCTCTCT-3′ |
| −100                           | −109−88       | 5′-TTCCAGACCACCCAGCTGGATTA-3′ |
|                                | −29−8         | 5′-CAACCAGTCGCAATTGC-3′ |
| 100                            | 48−68         | 5′-GGGCAATTTTCCTCATTCTC-3′ |
|                                | 84−104        | 5′-TTTGGTGTCATGTCACCTACT-3′ |
| 300                            | 226−247       | 5′-GGCAACATGTTGCGGCAATGAA-3′ |
|                                | 342−362       | 5′-GGCAACATGTTGCGGCAATGAA-3′ |
| 2,300                          | 2,251−2,271   | 5′-TGATGGTTGTTGGCGGCAATGGA-3′ |
|                                | 2,348−2,369   | 5′-TGATGGTTGTTGGCGGCAATGGA-3′ |
| 4,500                          | 4,490−4,510   | 5′-CAGGCATCTGCTCGAGACTTT-3′ |
|                                | 4,597−4,618   | 5′-TCGCCATGGAAAGATGGTG-3′ |
| 7,300                          | 7,200−7,220   | 5′-TGCTCATAAAAACCCAGAGGA-3′ |
|                                | 7,295−7,315   | 5′-TGCTCATAAAAACCCAGAGGA-3′ |
| 8,800                          | 8,714−8,734   | 5′-ATCCAGCTGCAACAGGGT-3′ |
|                                | 8,798−8,817   | 5′-ATCCAGCTGCAACAGGGT-3′ |
| 9,700                          | 9,645−9,666   | 5′-CCATCTCAATGGTGCGTACATC-3′ |
|                                | 9,730−9,750   | 5′-CCATCTCAATGGTGCGTACATC-3′ |
| over 2,000                     | 13,314−13,334 | 5′-AAAGCAGTGCTCCTTCCACC-3′ |
|                                | 13,348−13,368 | 5′-AAAGCAGTGCTCCTTCCACC-3′ |
| over 6,000                     | 17,264−17,284 | 5′-AAAGGAAGGAAGGAAGGAAGG-3′ |
|                                | 17,180−17,199 | 5′-TGAAGGTTGCGCCTTTCATGC-3′ |
converted to signal intensities by the delta-delta method, which assesses the difference of 1 CT-value as a 2-fold difference between samples (15). The formula is 

\[ \frac{\text{CT IP sample} - \text{CT Input}}{\text{CT Input} - \text{CT Total ChIP}} = 2^n \]

where \( n \) is the calculated fold change. All ChIP signals were normalized to the corresponding input signals. The formula is

\[ \frac{\text{ChIP signal}}{\text{input signal}} = 2^n \]

Changes in the adiponectin gene expression in 3T3-L1 cells during adipocyte differentiation and 3T3-L1 adipocyte treated with TNFα

To examine the change in expression of the adiponectin gene, as well as differential marker aP2 and their transcription factors (PPARγ1, PPARγ2 and CREB) during adipocyte differentiation, real-time RT-PCR was performed at several time points (pre-adipocyte, day 0, 2 and 8) after stimulation of differentiation in 3T3-L1 cells. The mRNA levels of adiponectin showed a pronounced increase from day 0 to day 8 after differentiation onset, whereas the expression of the aP2 gene showed a pronounced increase from day 0 to day 2 and its expression was continuously high by day 8 after differentiation onset. The mRNA levels of PPARγ2 increased rapidly with stimulation of differentiation and were highest on day 8 after differentiation onset. The mRNA levels of PPARγ1 and CREB did not change throughout any period (Fig. 1A).

Next, to investigate the mechanism underlying down-regulation of the adiponectin gene in adipocytes with insulin resistance, 3T3-L1 adipocytes on day 8 after differentiation onset were treated with 20 ng/mL TNFα for 24 or 48 h. Real-time RT-PCR analysis showed that adiponectin and PPARγ2 mRNA levels
were reduced significantly by the treatment with TNFα for 24 and 48 h. Expression of aP2 was reduced at 24 h (tendency) and 48 h \( (p<0.05) \) by the treatment with TNFα, but the decrease ratio was lower than that of adiponectin and PPARγ2. Expressions of PPARγ1 and CREB genes were not changed by treatment with TNFα (Fig. 1B).

Changes to histone H3 tail at lysine 9 modifications on the promoter/enhancer and transcriptional regions of the adiponectin gene during differentiation in 3T3-L1 adipocytes

To investigate whether the modifications of the histone H3 tail at lysine 9 on the adiponectin gene are associated with induction of the gene during differentiation, we first performed chromatin immunoprecipitation assays (ChIP assays) using an antibody for acetylated histone H3 at lysine 9 in 3T3-L1 adipocytes on each day of the differentiation process (pre-adipocyte, days 0, 2 and 8 after differentiation onset). A ChIP assay using the antibody enables one to observe in vivo protein binding to DNA on a chromosome. We used an antibody against acetylated lysine residue at the 9th position in the N-terminus of histone H3 and primer sets suitable for different parts on the adiponectin gene in order to detect ChIP signals (Table 1). The regions amplified using these primers represent the positions from transcription initiation site, such as upstream regions (−5,900, −700, −500, −300, −100 bp) and transcriptional regions (+100, +300, +2,300, +4,500, +7,300, +8,800, +9,700 bp), and after the transcription termination site (over +2,000, over +6,000, indicating +2,000 bp or +6,000 bp downstream of the transcription termination site) (Fig. 2A). All ChIP signals for normal IgG were less than 0.06% input. The ChIP signals of acetylated histone H3 at lysine 9 on the regions far upstream or far downstream of the adiponectin gene (−6,000, over +2,000 and

Fig. 2. Changes of histone H3 tail at lysine 9 modifications on the adiponectin gene during adipocyte differentiation in 3T3-L1 cells. 3T3-L1 pre-adipocytes and adipocytes on day 0, 2, and 8 after differentiation onset were used. The genomic DNA, which was cross-linked to nuclear proteins in pre-adipocytes and adipocytes, was sonicated and precipitated by the antibodies against acetylated histone H3 at lysine 9 (B), mono- (C), di- (D), and tri-methylated (E) histone H3 at lysine 9. Primer positions on the adiponectin gene used for PCR analysis are indicated in (A). ChIP signals were detected by quantitative real-time PCR and normalized to input signals. Means±SE for 3 wells are shown.
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over +6,000 bp) did not detect significant differences compared with those of normal IgG. On the other hand, the ChIP signals of acetylated histone H3 at lysine 9 on the regions of the promoter/enhancer (−700, −500 and −300 bp), just downstream of the transcriptional initiation site (+100 bp) and 1st intron (+300, +2,300, +4,500 and +7,300 bp) and 2nd exon (+8,800 bp) of the adiponectin gene were remarkably higher than those on the regions far upstream or far downstream. The ChIP signal levels on regions from the promoter/enhancer to the 2nd exon gradually increased during differentiation, and the levels were significantly higher on days 2 and 8 than pre-adipocyte. Acetylation of histone H3 at lysine 9 on the regions of promoter/enhancer (−700 to −300 bp) and on 1st intron (+2,300 to +4,500 bp) was particularly enhanced during adipocyte differentiation. The region just upstream of the transcription initiation site (−100 bp) was also enhanced slightly during the differentiation and it was highest on day 8, but the enhancement ratio was lower than those at the region from −700 to −300 bp and the region from +2,300 to +4,500 bp (Fig. 2B).

To investigate whether methylations of histone H3 at lysine 9 on the promoter/enhancer and transcriptional regions of the adiponectin gene were altered during adipocyte differentiation, we performed ChIP assays using specific antibodies for mono-, di-, tri-methylated histone H3 at lysine 9 in 3T3-L1 adipocytes on each day of the differentiation process (pre-adipocyte, days 0, 2 and 8 after differentiation onset). All types of methylations of histone H3 at lysine 9 on all parts of the adiponectin gene and its upstream/downstream regions tested gave high ChIP signals in 3T3-L1 pre-adipocytes, and its levels were markedly decreased by the stimulation of adipocyte differentiation (Fig. 2C–E). The ChIP signals remained at lower levels throughout adipocyte differentiation.
Effects of TNFα treatment on modifications of histone H3 tail at lysine 9 on the promoter/enhancer and transcriptional regions of the adiponectin gene in differentiated 3T3-L1 adipocytes

To investigate whether modifications of histone H3 at lysine 9 on the adiponectin gene were associated with a decrease of the adiponectin gene expression by treatment with TNFα, we performed ChIP assays using antibodies for acetylated and methylated histone H3 at lysine 9 in 3T3-L1 adipocytes treated with TNFα. Acetylation of histone H3 at lysine 9 on regions of the promoter/enhancer and transcription on the adiponectin gene was reduced by the TNFα treatment, and significant differences were shown on the promoter/enhancer (−500, −300 bp) and transcriptional (+2,300, +4,500, +7,300 bp) regions at 24 h, and on the promoter/enhancer (−700, −500, −300 bp) and transcriptional (+100, +8,800, +9,700 bp) regions at 48 h (Fig. 3A).

On the other hand, methylations of histone H3 at lysine 9 on some parts of the adiponectin gene, such as −5,900 and +100 bp for mono-methylation at 48 h after the treatment, −5,900, +100 and +7,300 bp for di-methylation at 48 h, and +300 bp for tri-methylation at 24 h, were significantly decreased by the TNFα treatment. Over all, these methylations on the adiponectin gene tended to be reduced by treatment with TNFα, but these were not notable (Fig. 3B–D).

DISCUSSION

It is well-known that expression of the adiponectin gene increases during adipocyte differentiation (1, 2, 17). It has already been established that expression of the adiponectin gene, as well as adipocyte differentiation, is induced by nuclear transcriptional factors such as PPARγ, CREB and C/EBPs (18, 19). In this study, we confirmed that adiponectin gene expression increased after stimulation of adipocyte differentiation. The induction of adiponectin gene expression was accompanied by the adipocyte differentiation because its expression followed a differential marker aP2 and PPARγ2 (a transcription factor for both adiponectin and aP2 that is known to induce adipocyte differentiation) (Fig. 1A) (4). Recent studies have shown that abrupt changes in gene expression that occur frequently in differentiating cells are regulated not only by nuclear transcription factors, but also by major chromatin structural changes triggered by modifications of the histone tail, such as acetylation, methylation and phosphorylation. Among several identified histone modifications, acetylations and methylations of histone H3 are the most extensively studied, because regulation of histone H3 modifications is related to ON/OFF switching of transcription. Recent studies suggest that ON/OFF regulation of transcription is directed by switching between acetylation and methylation at lysine 9 of histone H3 on their promoter regions, because acetylation of histone H3 at lysine 9 is associated with transactivation. On the other hand, methylation at same residue is associated with repression of transcription (7, 20). Thus we hypothesized that induction of the adiponectin gene during adipocyte differentiation is regulated by changes of histone H3 at lysine 9 from methylation to acetylation.

To provide evidence as to whether histone H3 modifications at lysine 9 on the adiponectin gene are concerned with its expression during differentiation of 3T3-L1 cells, we first performed ChIP assays using an antibody against acetylated histone H3 at lysine 9. Previous studies have demonstrated that ChIP signals on the adiponectin gene using an antibody for acetylated histone H3 at lysines 9/14, which cannot distinguish the difference of signals for histone H3 acetylated lysines at between 9 and 14, increased during differentiation (9). In this study, we found that acetylation of histone H3 at lysine 9 for the promoter/enhancer and transcriptional regions on the adiponectin gene increased during adipocyte differentiation (Fig. 2B). These results suggest that induction of the adiponectin gene in 3T3-L1 adipocytes during differentiation (Fig. 1) is concerned with abrupt inductions of histone H3 at lysine 9 on the gene. Interestingly, we detected acetylation of histone H3 at lysine 9 on the transcription region as well as promoter region at 2 and 8 d after adipocyte differentiation onset. Several studies have demonstrated that many cis-elements for transcription factor C/EBPα binding are located within the 1st intron (21). Recent studies also indicate that histone acetylation is important for recruiting complexes for mRNA elongation on transcriptional regions as well as recruiting complexes for trans-activation on promoter/enhancer regions (22). Our results indicate that enhanced acetylation of histone H3 around the 1st intron might be important for the recruitment of the transcriptional complex including C/EBPα and the elongation complex. We then performed a ChIP assay using antibodies for methylated histone H3 at lysine 9 during adipocyte differentiation of 3T3-L1 cells. Three types of methylations of histone H3 at lysine 9 have already been identified, namely mono-, di-, tri-methylation. All types of methylations of histone H3 are related to transcriptional repression. Recent studies have demonstrated that tri-methylation of histone H3 at lysine 9 is strongly associated with heterochromatin regions (7). On the other hand, mono- and di-methylation are located on both euchromatin and heterochromatin regions (23). Thus it is believed that tri-methylation is important for determination of heterochromatin regions and mono-/di-methylations are important not only for determination of heterochromatin regions, but also for transcription repression on euchromatin regions. In this study, we found that all types of methylations of histone H3 at lysine 9 on the adiponectin gene were decreased during adipocyte differentiation (Fig. 2B–D). These results suggest that all types of methylations of histone H3 at lysine 9 on promoter/enhancer and transcriptional regions on the adiponectin gene are inversely associated with the gene induction during adipocyte differentiation. Interestingly, each methylation of histone H3 at lysine 9 was rapidly reduced in 3T3-L1 adipocytes within 2 d after stimula-
tion of differentiation, whereas acetylation at lysine 9 on the gene increased gradually during adipocyte differentiation (Fig. 2). These results indicate that methylations of histone H3 at lysine 9 are independently reduced by stimulation of adipocyte differentiation prior the induction of the adiponectin gene and acetylation of histone H3 at lysine 9. Many previous studies have shown that methylations of histone H3 at lysine 9 are important for determination of heterochromatin-euchromatin regions (10, 11, 24) and that the decrease of methylations on the adiponectin gene during differential stages was closely-inversely associated with expressional changes of adipocyte differential markers aP2 and PPARγ2 compared with those of adiponectin (Figs. 1 and 2). Thus decreased methylations of histone H3 at lysine 9 on the adiponectin gene might be important for changing from heterochromatin to euchromatin when 3T3-L1 pre-adipocytes differentiate into adipocytes prior to the induction of adiponectin gene expression and acetylation of histone H3 at lysine 9.

Many recent studies suggest that adiponectin gene expression is reduced in patients developing insulin resistance, which indicates that insulin action on various tissues, including adipose, is decreased (25). TNFα is one of the major molecules involved in inducing insulin resistance in various tissues, including adipose. Indeed, TNFα reduces gene expression of adiponectin as well as PPARγ2 in adipocytes (Fig. 1B) (26). aP2 gene expression was also decreased by the treatment with TNFα for 48 h but the decrease ratio was lower than that of adiponectin and PPARγ2. These results indicate not only that TNFα treatment is more affective on mRNA levels of adiponectin and PPARγ2 than on that of aP2, but also that adiponectin expression may be strongly regulated by expressional changes of PPARγ2, especially when the adipocytes were treated with TNFα. We also demonstrated that gene expression of CREB, which is known as a transcription factor for adiponectin, was not changed by treatment with TNFα during any differential stage. These results indicate that expression of the CREB gene is independent of differentiation and insulin resistance, and that the induction of the adiponectin gene by CREB may be regulated by the changes in binding activity on the promoter/enhancer region. In this study, to explore whether the decrease of adiponectin gene expression in adipocytes with insulin resistance is caused by modification of histone H3 tail at lysine 9 on the gene, we performed ChIP assays using antibodies against acetylated or mono-, di-, and tri-methylated histone H3 at lysine 9 in 3T3-L1 adipocytes treated with TNFα. Interestingly, acetylation of histone H3 at lysine 9 on the promoter/enhancer and transcriptional regions on the adiponectin gene was decreased by TNFα treatment for 24 h and 48 h (Fig. 3). These results suggest that decreasing acetylation of histone H3 at lysine 9 is one of the factors for decreasing adiponectin gene expression in adipocytes treated with TNFα. It should be noted that no type of methylations of histone H3 at lysine 9 on the adiponectin gene changed notably with treatment with TNFα (Fig. 3). Considering that methylations of histone H3 at lysine 9 are important for determination of the heterochromatin region, TNFα treatment might not affect the region between heterochromatin and euchromatin around the adiponectin gene. It is possible that methylation of histone H3 is concerned with changing from heterochromatin to euchromatin on the adiponectin gene when pre-adipocyte differentiates into adipocytes, but might not be concerned with decreases of the adiponectin gene in adipocytes with insulin resistance, because the euchromatin-heterochromatin region is normally determined by the differentiation step and its modifications are hardly affected. Further work is needed to investigate whether the recruitment of histone acetyltransferases, histone methylases and histone deacetylases on the adiponectin gene is changed during the adipocyte differentiation and by the treatment with TNFα to adipocytes.

In conclusion, we demonstrated in this study that induction of the adiponectin gene during adipocyte differentiation is associated with induced acetylation and reduced methylation of histone H3 at lysine 9 during adipocyte differentiation. Furthermore, we revealed that decreased adiponectin gene expression by the TNFα treatment is associated with reduced acetylation of histone H3 at lysine 9. Our results suggest not only that histone H3 acetylation and methylation at lysine 9 are associated with adiponectin gene expression during differentiation, but also that the reduction of the gene’s expression in 3T3-L1 adipocytes with insulin resistance induced by TNFα is associated with histone H3 at lysine 9 acetylation, but not methylation. Further investigation is needed to determine whether developing insulin resistance reduces acetylation, but not methylation, of histone H3 at lysine 9 on the adiponectin gene in animal models and humans.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18590220, 18790171), the global COE program, the Center of Excellence for Innovation in Human Health Sciences, from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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