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# Regulation of adipolin/CTRP12 cleavage by obesity

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ABSTRACT

Obesity is highly associated with the development of insulin resistance and type 2 diabetes. Recently we found that adipolin/CRTP12 is an adipocytokine that exerts beneficial actions on glucose metabolism. Here we investigated the regulation of circulating adipolin under conditions of obesity and assessed its potential mechanisms. Both full and cleaved forms of adipolin were observed in mouse plasma. Diet-induced obese (DIO) mice showed a significant reduction of plasma levels of full and total (full and cleaved) adipolin compared with control mice, resulting in an increase in the ratio of cleaved to full isoform. In vitro gene transfection studies using HEK293 cells revealed that a deletion mutant of adipolin gene (Δaa90–93) caused a reduction of cleaved production of adipolin in media. A bioinformatics analysis of adipolin amino acid sequence indicated the potential involvement of the family of proprotein convertases (PCs) in cleavage of adipolin. Treatment of 3T3-L1 adipocytes with an inhibitor for PCs abolished the expression of cleaved adipolin form in the media. The expression of furin, the member of PCs, was increased in adipose tissue of DIO mice. Furin expression was also increased in cultured adipocytes by treatment with an inducer of inflammation. These data suggest that obesity states facilitate the cleavage of adipolin presumably through upregulation of furin in adipose tissue.



## 1. Introduction

Obesity is a major risk factor for the development of various metabolic disorders including insulin resistance and type 2 diabetes [1,2]. Accumulating evidence indicates that adipose tissue is a secretory organ releasing various bioactive molecules, also referred to as adipocytokines or adipokines [3–5]. Obesity leads to upregulation of numerous pro-inflammatory adipocytokines including TNFα that promote metabolic dysfunction, whereas it also contributes to in downregulation of several adipocytokines that are protective against obese complications [3,4]. Adiponectin is an adipocytokine whose concentration in blood stream is reduced by obesity [6–8]. We have demonstrated that adiponectin exerts protective actions on insulin resistance and cardiovascular disorders with anti-inflammatory properties [7–11]. C1q/TNF-related proteins (CTRPs) are conserved paralogs of adiponectin and function to regulate metabolic and cardiovascular function [12–15]. It is well-established that dysregulated production of adipocytokines under conditions of obesity causes various metabolic and cardiovascular diseases.

Recently we have identified adipolin/CTRP12 as an adipocytokine that is downregulated by obesity [16]. We also found that adipolin ameliorates glucose metabolism in obese mice via suppression of inflammatory responses in adipose tissue [16]. Consistent with these findings a more recent report demonstrated that adipolin improves insulin sensitivity and lowers glucose levels in obese and diabetic mice [17]. Adipolin expression is suppressed in cultured adipocytes by the inducers of inflammation and ER stress [16]. Adipolin expression is also reduced in adipose tissue in rodent models of obesity and diabetes [16,17]. Thus, these data suggest that obesity-induced pathological conditions lead to reduction of adipolin expression in the adipose tissue. Circulating adipolin is reported to exist as full and proteolytically cleaved protein forms [17]. However, the regulation of adipolin production is not fully clarified. Here we investigated whether obese states modulate the cleavage of adipolin, and assessed its potential mechanisms.

## 2. Materials and methods

### 2.1. Materials

An inhibitor for proprotein convertases (PCs), Dec-RVKR-CMK was purchased from Calbiochem (CA, USA). TNFα and rabbit anti-

*Abbreviations:* DIO, diet-induced obese; CTRP, C1q/TNF-related protein; TNF, tumor necrosis factor; PC, proprotein convertase.

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FLAG polyclonal antibody (F7425) were purchased from Sigma Chemical Co (MO, USA). The polyclonal antibody against mouse adipolin was generated by immunizing rabbits with synthetic peptide (CRLKGPVLVDKKTLVEL: amino acid residues 162–178) (Immuno-Biological Laboratories Co., Ltd, Tokyo, Japan).

### 2.2. Animal model

Wild-type (WT) mice in a background of C57BL6/J were purchased from Charles River Laboratories. To generate diet-induced obese (DIO) mice, WT mice at the age of 9 weeks were maintained on a high-fat/high-sucrose (HF/HS) diet (Oriental yeast, F2HFHSD, 30% fat) for 17 weeks. Age-matched WT mice fed a normal chow diet (CLEA CE-2, 4.8% fat) served as control mice. Study protocols were approved by the Institutional Animal Care and Use Committees at Nagoya University.

### 2.3. Mutagenesis of mouse adipolin

The cDNA encoding mouse full-length adipolin was subcloned into pShuttle-CMV vector (Qbiogene) as previously described [16]. Deletion mutants in cleavage site of mouse adipolin were introduced by PCR-based megaprimer method [18] with some modifications using high fidelity polymerase, KOD plus ver.2 (TOYOBO, Osaka, Japan). The reverse primers containing deletion mutations are shown below (A–D). At first, megaprimers were obtained by PCR from pShuttle-CMV containing full-length adipolin cDNA using forward mouse adipolin primer (E) and mutated primers (A–D). Then, full-length deletion mutant genes were amplified using purified megaprimer and reverse adipolin primer (F). FLAG epitope was added to C-terminal of each mutated fragment. The PCR products were digested with BgI II and Xho I, and then subcloned into pShuttle-CMV vector. The entire regions of the PCR-derived fragments were sequenced to verify the introduced mutation and to exclude unwanted additional mutations.

Primers for deletion mutant adipolin (A–D): (A) Δ88–91 mouse adipolin Rev: 5′-GGCCTCGCGAGCCACGACAC-3′, (B) Δ90–93 mouse adipolin primer Rev: 5′-CTGAGAGGCCGTCCCGGCCA-3′, (C) Δ93–96 mouse adipolin Rev: 5′-CTGGGAGACCCGACTTCTTG-3′, (D) Δ92,93 mouse adipolin Rev: 5′-CTGAGAGGCCCTTCTTGTCC-3′, Full-length primers for mouse adipolin (E, F): (E) BgI II-mouse adipolin primer Fwd: 5′-CTGAGATCTGCCATGTGGGCCTGGGGCT-3′, (F) Xho I-mouse adipolin-FLAG primer Rev: 5′-CTGCTCGAGTCACTTGTCATCGTCG TCCTTGTAGTCGGTACCCAAAAGCATTCCCG-3′.

### 2.4. Cell culture

HEK293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. HEK293 cells were transfected with wild-type or mutant adipolin vectors by lipofectamine LTX (Invitrogen). In some experiments, cells were treated with Dec-RVKR-CMK (10 μM, Calbiochem 344930) for 48 h. Mouse 3T3-L1 cells (ATCC) were maintained in DMEM with 10% FBS and differentiated into adipocytes by treatment with DMEM supplemented with 5 μg/ml of insulin, 0.5 mM 1-methyl-3-isobutyl-xanthin, and 1 μM dexamethasone [16]. At day 7 after differentiation, 3T3-L1 adipocytes were treated with Dec-RVKR-CMK (10 μM), TNFα (10 ng/ml) or vehicle for the indicated lengths of time.

### 2.5. Western blot analysis

Cell and blood samples were prepared, and equal amounts of proteins or plasma (1.2 μl) were separated with denaturing SDS–PAGE. Following transfer to membranes, immunoblot analysis was performed with the indicated antibodies followed by incubation with secondary antibody conjugated with horseradish peroxidase. ECL plus Western Blotting Detection kit (GE Healthcare) was used for detection. Relative protein levels were quantified by Image J program.

### 2.6. Quantification of mRNA levels

Gene expression levels were quantified by real-time PCR (RT-PCR). Total RNA was prepared using a Qiagen kit. cDNA was produced using SuperScript reverse transcription-PCR Systems (Invitrogen). PCR was performed on Real-Time PCR Detection System (Bio-Rad) using Power SYBR Green 1 as a double standard DNA specific dye as described previously [16]. Primers were: Fwd: 5′-GC GAATGGGTCCTAGAGA-3′ and Rev: 5′-GAACGAGAGTGAACTTGGT-3′ for mouse furin/PC3, Fwd: 5′-CCAACTACGATTCCTATGC-3′ and Rev: 5′-TTGCTGGCGTCATATCTC-3′ for mouse for mouse PACE4/PC6, Fwd: 5′-CGTTCAACAAGCACTATCA-3′ Rev: 5′-ATCCACTGTCTT CCCATC-3′ for mouse PC7, Fwd: 5′-CATCTTCTCAAAATTCGAGTGA CAA-3′ and Rev: 5′-TGGGAGTAGACAAGGTACAACCC-3′ for mouse TNFα, Fwd: 5′-GCTCCAAGCAGATGCAGCA-3′ and Rev: 5′-CCGGA TGTGAGGCAGCAG-3′ for mouse 36B4.

### 2.7. Statistical analysis

All data are expressed as means ± SEM. Differences were analyzed by Student's unpaired *t* test. A value of $P < 0.05$ was accepted as statistically significant.

## 3. Results

### 3.1. Expression of full and cleaved adipolin forms in plasma in lean and obese mice

To test whether obesity modulates the cleavage of adipolin in vivo, we measured full and cleaved forms of adipolin in plasma in lean control wild-type mice fed a normal diet and HF/HS diet-induced obese (DIO) mice by Western blot analysis using anti-adipolin antibodies that recognize both forms of adipolin. Both full and cleaved forms of adipolin were observed in plasma of control and DIO mice (Fig. 1A). DIO mice had a significant decrease in full and total (full and cleaved) adipolin expression levels in plasma compared with control mice, resulting in an increase in the ratio of cleaved to full adipolin isoform (Fig. 1B). In contrast, the plasma levels of cleaved forms of adipolin are not different between control and DIO mice. These data indicate that obesity states facilitate the conversion from full to cleaved adipolin form.

### 3.2. Involvement of the family of proprotein convertases in adipolin cleavage

It has been reported that adipolin contains a protease cleavage motif 90-KKSR-93 and that adipolin is predicted to be cleaved between 91-K and 92-S by N-terminal sequencing [17]. To test whether 90-KKSR-93 participates in adipolin cleavage, HEK293 cells were transfected with wild-type (WT) adipolin or a deletion mutant form of adipolin (Δ90–93) (Fig. 2A). The cleaved and full forms of adipolin were observed in media in WT adipolin-transfected cells (Fig. 2B). In contrast, the expression of full, but not cleaved adipolin, was detected in media of cells transfected with adipolin deletion mutant (Δ90–93) (Fig. 2B and C). We also generated other deletion mutant forms of adipolin (Fig. 2A). Transfection of HEK293 cells with adipolin deletion mutants (Δ92,93 or Δ93–96) did not affect the expression of cleaved adipolin in media (Fig. 2C). On the other hand, transfection with adipolin deletion mutant (Δ88–91) enhanced the expression of cleaved adipolin (Fig. 2C). These

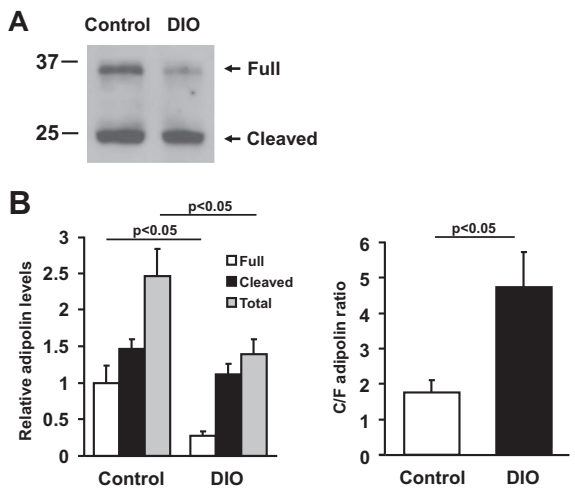


**Fig. 1.** Conversion from full to cleaved adipolin form in obese states. (A) Expression of adipolin in plasma (1.2 μl) in control and diet-induced obese (DIO) mice. Adipolin levels in plasma were determined by Western blot analysis using anti-adipolin antibody. (B) Quantitative analysis of full, cleaved and total (full and cleaved) adipolin expression levels (left) and the ratio of cleaved to full (C/F) adipolin (right) in plasma in control and DIO mice (mean ± SEM, $n = 4–5$).

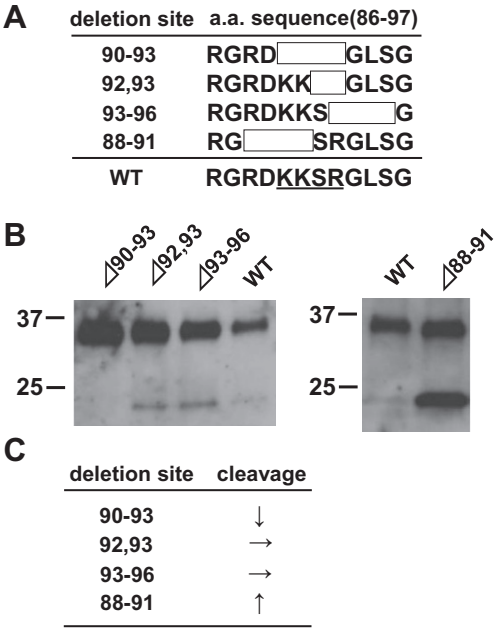


**Fig. 2.** The motif 90-KKSR-93 is necessary for cleavage of adipolin. (A) Deleted amino acid sites of each deletion mutant form of adipolin. Amino acid (a.a.) sequence of wild-type (WT) adipolin (86–97) is also shown. The underline indicates the motif 90-KKSR-93. (B) Expression of full and cleaved adipolin in media from HEK293 cells following transfection with wild-type (WT) or deletion mutant forms of adipolin (Δ90–93, Δ92, 93, Δ93–96, Δ88–91). Protein levels in media were determined by Western blot analysis using anti-FLAG antibody. (C) Summary of effectiveness of transfection with each adipolin deletion mutant in adipolin cleavage.

data indicate that the motif 90-KKSR-93 is necessary for generation of cleaved fragment of adipolin.

The findings that many basic amino acids (K or R) exist on the N-terminal side of the predicted cleavage site allowed us to speculate the potential involvement of the members of the family of proprotein convertases (PCs) in proteolytic cleavage of adipolin.

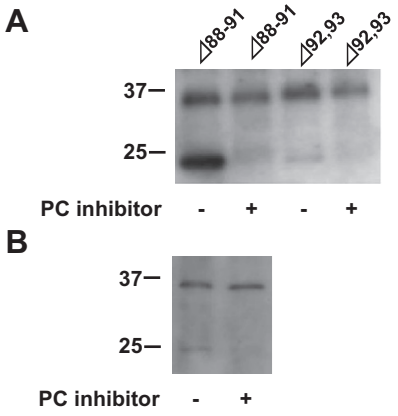


**Fig. 3.** The family of proprotein convertases is involved in cleavage of adipolin in vitro. (A) Expression of full and cleaved adipolin in the media of HEK293 cells transfected with adipolin deletion mutants (Δ88–91 or Δ92,93) in the presence of the PC inhibitor (10 μM) or vehicle. Protein levels were determined by Western blot analysis using anti-FLAG antibody. (B) Expression of full and cleaved forms of endogenous adipolin in media of 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were treated with the PC inhibitor (10 μM) or vehicle for 24 h. Protein levels were determined by Western blot analysis using anti-adipolin antibody.

A bioinformatics analysis of adipolin amino acid sequence using ProP 1.0 program (<http://www.cbs.dtu.dk/services/ProP/>) [19] revealed that adipolin can be cleaved between 91-K and 92-S by furin, the member of PC family. To investigate whether PCs contribute to cleavage of adipolin, HEK293 cells were treated with the inhibitor of PCs or vehicle. Treatment with the PC inhibitor abolished the expression of cleaved adipolin in the media of HEK293 cells transfected with adipolin deletion mutants (Δ88–91 or Δ92,93) (Fig. 3A). Furthermore, treatment of 3T3L1 adipocytes with the inhibitor of PCs completely blocked the cleavage of adipolin (Fig. 3B). These data indicate that PCs play a crucial role in regulation of adipolin cleavage in vitro.

### 3.3. Furin is upregulated in adipose tissue of obese mice

It has been shown that expression of furin/PC3, PACE4/PC6 and PC7 is observed in epididymal white adipose tissues of C57BL6 mice and is increased during differentiation of 3T3L1 cells into adipocytes [20]. Thus, we investigated the transcript levels of these PCs in adipose tissue of control and DIO mice by quantitative RT-PCR methods. Furin mRNA levels were significantly increased in fat tissue of DIO mice compared with that of control mice (Fig. 4A). In contrast, mRNA levels of PC6 and PC7 did not differ between two groups of mice.

Obesity-inducible metabolic dysfunction is attributed to increased inflammation in adipose tissue [21–23]. TNFα mRNA expression was 9.4 ± 2.4-fold higher in adipose tissue of DIO mice than in that of control mice. Thus, to test whether inflammatory states affect the expression of PCs in adipocytes, 3T3L1 adipocytes were treated with TNFα. Treatment of adipocytes with TNFα led to a significant increase in furin mRNA expression (Fig. 4B). In contrast, treatment with TNFα had no effects on mRNA expression of PC6 and PC7 in adipocytes. These data indicate that adipose furin is positively regulated by obesity-induced inflammatory states.

## 4. Discussion

The present study demonstrated that obese states accelerate the conversion from full to cleaved adipolin form in blood stream, at least in part, through induction of furin in adipose tissue. DIO mice showed reduced plasma levels of full and total (cleaved and

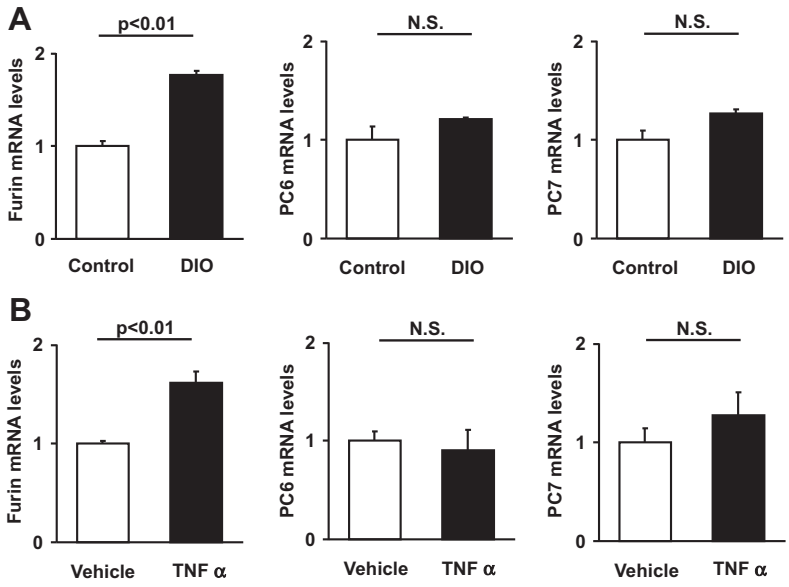


**Fig. 4.** Furin is upregulated in adipose tissue of obese mice. (A) The transcript levels of furin, PC6 and PC7 in epididymal adipose tissue of control and diet-induced obese (DIO) mice. The transcript levels were determined by RT-PCR method and expressed relative to 36B4 levels (mean ± SEM, $n = 4–5$). (B) The mRNA expression of furin, PC6 and PC7 in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were treated with TNF (10 ng/ml) or vehicle for 24 h. The transcript levels were determined by RT-PCR method and expressed relative to 36B4 levels (mean ± SEM, $n = 4$).

full) adipolin compared to control mice, whereas circulating cleaved forms of adipolin did not differ between two strains of mice. Of importance, the cleaved/full adipolin protein ratio was significantly increased in DIO mice. Treatment of adipocytes with an inhibitor for furin and other PCs resulted in reduction of cleaved adipolin form. Furin expression was elevated in fat tissue of obese mice, and was also increased in cultured adipocytes by treatment with TNFα. Although adipolin is abundantly expressed in adipose tissues, obese states result in downregulation of adipolin in fat tissue [16,17]. Therefore, these data suggest that adipose furin is upregulated under obesity-induced pathological conditions and that this induction facilitates the cleavage of adipolin released from fat tissues despite reduced production of adipose adipolin, thereby leading to an increase in circulating cleaved adipolin relative to full form.

In this study we found that a deletion mutant of adipolin (Δaa90–93) causes a reduction of cleaved production of adipolin. A bioinformatics analysis suggests the involvement of furin in cleavage of adipolin between 91-K and 92-S. Furthermore, inhibition of furin activation abolished the expression of cleaved adipolin in vitro. These results are consistent with a recent study showing that furin is the major PC present in cultured adipocytes and fat tissues and acts as an endogenous regulator of cleavage of adipolin in cultured adipocytes [24]. Here we extended these observations by showing that obesity-induced fat inflammation is associated with enhancement of adipolin cleavage in vivo.

Adipolin is the newly identified adipocytokine, which exerts salutary actions on insulin sensitivity and glucose metabolism [16,17]. A recent study demonstrated that full, but not cleaved form of adipolin promotes insulin-induced glucose uptake in adipocytes [24], suggesting that the full and cleaved forms of adipolin have different insulin-sensitizing effects. Our data showed that obesity is linked with the reduced expression of full forms of plasma adipolin. Thus, it is plausible that obesity enhances the development of insulin resistance via suppression of insulin-sensitizing full form of adipolin. However, it remains unclear whether the full adipolin and its cleaved isoform have distinct biological properties in vivo, and this investigation requires future studies.

Obesity is characterized by a chronic inflammatory state that contributes to the development of insulin resistance. TNFα is a pro-inflammatory adipocytokine that promotes insulin resistance under conditions of obesity [23]. Active form of TNFα is released from cells after cleavage by TNFα converting enzyme (TACE) [25,26]. Furin-mediated maturation of TACE is a crucial step of TACE enzymatic activation [25,26]. Therefore, furin may enhance the release of TNFα from fat tissue via activation of TACE. Taken together with our current findings, these results suggest that furin can modulate the activity of adipocytokines including adipolin and TNFα under conditions of obesity, thereby contributing to exacerbation of vicious cycle of inflammatory response and insulin resistance.

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