Concomitant expression of adrenomedullin and its receptor components in rat adipose tissues

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IT HAS BEEN WELL RECOGNIZED that adipose tissues produce and secrete a variety of biologically active molecules, so-called adipokines, such as adiponectin (38), leptin (29), resistin (34), plasminogen activator inhibitor-1 (16), heparin-binding epidermal growth factor-like growth factor (23), and tumor necrosis factor-α (TNF-α) (8). Thus adipose tissue is now considered to function not only as an energy storage tissue, but also as a variety of cells plays pluripotent roles in an autocrine/paracrine fashion. The present study was undertaken to explore the expression of AM and its receptor genes in adipose tissues, their changes during the development of obesity, and the process of preadipocyte differentiation. Both mature adipocytes and stromal vascular cells constituting adipose tissue expressed AM transcript. AM and its receptor component [calcitonin receptor-like receptor and receptor activity-modifying protein-2 (CRLR/RAMP2)] mRNAs were expressed in a variety of rat adipose tissues, including epididymal, mesenteric, retroperitoneal, and subcutaneous adipose tissue. AM mRNA levels in rat and human epididymal adipose tissue were about one-tenth of those in the kidney. Steady-state mRNA levels of AM and CRLR/RAMP2 in epididymal, mesenteric, and retroperitoneal adipose tissues in rats fed a high-fat diet for 4 wk were far greater than those in rats with normal diet accompanied by increased plasma AM levels, whereas steady-state AM mRNA levels conversely decreased in other organs, such as kidney and liver. AM mRNA expressed in a mouse preadipocyte cell line (3T3-L1) transiently decreased by day 3, returned to basal level by day 6, and then increased by day 9 during preadipocyte differentiation, which paralleled AM secretion from the cells. However, the addition of either exogenous AM or AM receptor antagonist calcitonin gene-related peptide-(8–37), to block endogenous AM did not affect lipid droplet accumulation during preadipocyte differentiation. The present study demonstrates for the first time that AM and its receptor component [CRLR/RAMP2] mRNAs were concomitantly expressed in various adipose tissues, whose tissue-specific upregulation was induced during the development of obesity. These data suggest that AM may act as a new member of adipokines, although its functional role, as well as its pathophysiologic significance in obesity, remains to be determined.

Adipokines are dysregulated when the adipose tissues are increased under the obese state (10), suggesting their pathophysiological roles in metabolic and vascular disorders.

Adrenomedullin (AM) is a potent vasodilator peptide with 52-amino acid residue originally isolated from human pheochromocytoma (14). It has been recently revealed that calcitonin receptor-like receptor (CRLR) functions as an AM receptor when receptor activity-modifying protein (RAMP)2 is coexpressed on the cellular membrane (24). Several lines of evidence have been accumulated that AM expressed by and secreted from a variety of cell types functions in an autocrine/paracrine fashion (26). Thus AM is now considered to have pleiotropic effects, including cell growth (9, 31), migration (3, 7), apoptosis (11), inflammation (6), angiogenesis (13), and hormone secretion (28). Several reports have shown that plasma AM concentrations are elevated in obesity (12, 17, 25), suggesting the involvement of AM in adipose tissue metabolism. However, the expression of AM and its receptors by adipose tissues, especially their expression in the obese state, has been poorly understood.

These observations led us to examine whether AM and its putative receptor components (CRLR/RAMP2) are expressed in human and rat adipose tissue. Furthermore, the differential gene expression of rat AM and its receptor components during the development of obesity in vivo, as well as the process of preadipocyte differentiation in vitro, was studied.

MATERIALS AND METHODS

Materials. Rat AM and calcitonin gene-related peptide [CGRP-(8–37)] were purchased from the Peptide Institute (Osaka, Japan), Dulbecco’s modified Eagle’s medium (DMEM) from Flow Laboratories (Irvine, Scotland, UK), fetal bovine serum (FBS) and calf serum (CS) from Cell Culture Laboratories (Cleveland, OH), and collagenase type I from Sigma (St. Louis, MO). PCR primers were synthesized by Bjiós (Saitama, Japan).

Sampling of human tissues. The present study was approved by the Ethics Committee of Tokyo Medical and Dental University Hospital, and informed consent was obtained from each patient. Kidney specimens were obtained from three patients undergoing nephrectomy and epididymal adipose tissue specimens from three patients undergoing testicular surgery. After surgical resection, the tissue specimens were immediately frozen in liquid nitrogen and stored at −80°C.

Animals. All experiments were conducted in accordance with the Tokyo Medical and Dental University Guide for the Care and Use of Experimental Animals. Nine-week-old male Sprague-Dawley (SD) rats (Charles River Laboratories) were randomly divided into two groups. The control group (n = 6) was fed ad libitum a standard chow diet.
containing 24.5 g of protein, 51 g of carbohydrate, and 4 g of fat/100 g. The high-fat diet group (HFD; n = 5) was fed chow consisting of 58% lard (wt/wt), 30% fish powder, 10% skim milk, and a 2% vitamin and mineral mixture (equivalent to 7.5% carbohydrate, 24.5% protein, and 60% fat; Oriental Yeast, Tokyo, Japan). Each group weighed 320 g at the start of the diet. After 4 wk, adipose tissues, aorta, kidney, heart, and liver were separated, immediately frozen in liquid nitrogen, and stored at −80°C until use. White adipose tissue was obtained from epididymal, mesenteric, retroperitoneal, and subcutaneous fat pads.

Cell culture. A mouse preadipose cell line (NIH 3T3-L1), purchased from the Health Science Research Resource Bank (Osaka, Japan), was grown in DMEM supplemented with 10% CS at 37°C in an atmosphere of 5% CO2. Confluent cells were allowed to differentiate into adipocytes in DMEM containing 10% FBS supplemented 1μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (differentiation medium) for 48 h and then with postdifferentiation medium containing 10% FBS and insulin changed every other day.

Oil Red O staining. Adipogenesis was monitored by morphological examination of the cellular accumulation of lipid droplets and Oil Red O staining. Confluent 3T3-L1 cells were treated for 2 days with DMEM supplemented with 10% FBS, followed by the treatment with differentiation medium (day 0) with or without AM (3 × 10–8 M), or CGRP-(8–37) (10–6 M), as described above. On days 0, 3, 6 and 9, cells were fixed with 10% formaldehyde, washed with phosphate-buffered saline (PBS), and stained with Oil Red O (0.3% in 60% isopropanol), followed by extensive washes, and the stained triglyceride droplets were visualized and photographed.

Isolation of mature adipocytes and stromal vascular cells. The adipose tissues from 10-wk-old SD rats were rinsed immediately in Hank’s balanced salt solution with 10 mM HEPES, and fibrous tissues and blood vessels were carefully dissected and removed. The remaining tissues were minced into small pieces and digested by 0.2% collagenase and 2% BSA for 60 min at 37°C under continuous shaking (150 rpm). The dispersed tissue was filtered through a nylon mesh sheet (pore size 250 μm) and centrifuged, thereby giving rise to the floating mature adipocytes and the sedimented stromal vascular cells.

Quantification of mRNA. Total RNA was extracted using RNA Bee (TEL-TEST, Friendswood, TX) according to the manufacturer’s protocol. Equal amounts of total RNA were reverse transcribed with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ), using random hexamers, according to the manufacturer’s instruction. mRNA levels of human and mouse AM, rat and mouse RAMP2 and CRLR, and human and mouse acid ribosomal phosphoprotein P0 (ARPP P0) were measured and shown in each figure.

Radioimmunoassay. Media from cultured 3T3-L1 and SD rat plasma from the control and the HFD groups were directly assayed using mouse or rat AM radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals, Belmont, CA), respectively. In brief, samples or standard mouse/rat AM were incubated with anti-mouse/rat AM antibody at 4°C for 24 h, followed by the addition of 125I-labeled mouse/rat AM and further incubated at 4°C for 24 h. Separation was achieved by the double-antibody method.

Statistical analysis. Data were expressed as mean ± SE. Differences between groups were examined for statistical significance using either an unpaired t-test or ANOVA with Dunn’s post hoc test, if appropriate. P values <0.05 were considered statistically significant.

RESULTS

AM gene expression in adipose tissue. We first examined AM mRNA levels in mature adipocytes and stromal vascular cells prepared from rat epididymal adipose tissue. Mature adipocytes expressed slightly greater (P < 0.05) AM mRNA than did stromal vascular cells (Fig. 1A).

AM transcript was expressed by all of the rat adipose tissues, was used as an endogenous internal control. The relative expression of any given genes from various tissues, the adipose tissue were about one-fourth of those in mesenteric, retroperitoneal, and subcutaneous adipose tissues (Fig. 1B). We next compared AM mRNA expression in rat epididymal adipose tissue with that in other organs. Steady-state AM mRNA level in rat epididymal adipose tissue was 14.7 ± 6.4% of that in the kidney, 17.5 ± 7.5% of that in the heart, and

| Table 1. Sequences of PCR primers for real-time RT-PCR |
|----------------|----------------|----------------|
| PCR Product   | Primer Sequence | PCR Product Size |
| Human AM      | F: 5′-ggtgccagctgttgtcttc-3′ | 115           |
| Rat AM        | F: 5′-caggaacagcagagcagc-3′ | 82            |
|               | T: 5′-caagcagcagcagagcagc-3′ |               |
|               | R: 5′-tctggtcgagttgtcag-3′  |               |
| Mouse AM      | F: 5′-acacgagccaggaattact-3′ | 102           |
| Rat RAMP2     | R: 5′-taggtgtgctgtttcgtctg-3′ | 152           |
| Mouse RAMP2   | F: 5′-ccggcttcgagcaaaact-3′  | 141           |
|               | R: 5′-ccgcgttgctgaacctg-3′  |               |
| Rat CRLR      | F: 5′-gccaggttgccaggctct-3′  | 148           |
|               | R: 5′-cccccagcaagaaataat-3′ |               |
| Mouse CRLR    | F: 5′-ggccgaaaacaggctctg-3′  | 119           |
| ARPP P0       | R: 5′-cgagtaagctaagcagc-3′   | 107           |
|               | F: 5′-tagagggtgtcgcaggtg-3′  |               |
|               | R: 5′-gacaaagccaggaccctttgc-3′ | 143          |
| mouse RAMP2   | F: 5′-tacggtgctgaactgctc-3′  |               |
|               | R: 5′-tcccaagtctggctctg-3′   |               |

AM, adrenomedullin; CRLR, calcitonin receptor-like receptor; RAMP2, receptor activity-modifying protein-2; ARPP P0, acid ribosomal phosphoprotein P0. F: forward primer; R: reverse primer; T: TaqMan probe.

In both the SYBR green and TaqMan real-time PCR methods, the fluorescence data were quantitatively analyzed using serial dilution of control samples included in each reaction to produce a standard curve. For verification of the PCR reaction, the PCR products were examined by 1.5% agarose gel electrophoresis, and each PCR product was confirmed to show a single band of the expected size. To compare the relative expression of any given genes from various tissues, the housekeeping gene ARPP P0, whose expression was similar in all tissues, was used as an endogenous internal control. The relative levels of each mRNA to ARPP P0 were calculated and shown in each figure.
44.8 ± 19.3% of that in the liver (Fig. 1C), whereas steady-state AM mRNA level in human epididymal adipose tissue was 11.1 ± 2.5% that in the kidney (Fig. 1D). Thus the ratio of kidney to adipose tissue levels of AM mRNA in rats and humans is comparable.

**Effect of HFD on AM gene expression in adipose and nonadipose tissues.** We next examined the changes in AM gene expression in various rat adipose tissues and other organs during the development of obesity induced by HFD (Fig. 2). As summarized in Table 2, the HFD group weighed 7.4% (P < 0.05) more than the normal-diet (control) group and developed relative hyperglycemia (P < 0.05), hyperlipidemia (P < 0.05), and hyperinsulinemia (P < 0.05). Plasma immunoreactive AM (IR-AM) levels in the HFD group were higher (P < 0.05) than those in the control group (Table 2).

Steady-state AM mRNA levels in epididymal, mesenteric, and retroperitoneal adipose tissue from the HFD group were 7.6-, 10.9-, and 5.4-fold greater (P < 0.05) than those in the control group, respectively, whereas those in subcutaneous adipose tissue from the HFD group were not significantly different (Fig. 2A). In contrast to the augmented AM mRNA expression in adipose tissue from the HFD group, steady-state AM mRNA levels in nonadipose tissues such as kidney and liver significantly (P < 0.05) decreased, and those in aorta and heart tended to decrease in the HFD group compared with those in the control group (Fig. 2B).

**Coexpression of CRLR and RAMP2 genes in adipose tissue.** We then determined gene expression of two putative AM receptor components, CRLR and RAMP2, in various rat adipose tissues and their changes after HFD. Both CRLR and RAMP2 mRNAs were expressed in all adipose tissues examined (Fig. 3). Steady-state CRLR mRNA levels were significantly (P < 0.05) greater in epididymal (14-fold), mesenteric (8.2-fold), and retroperitoneal (2.5-fold) adipose tissues from the HFD rats than those from the normal diet rats (Fig. 3A). Steady-state RAMP2 mRNA levels were also significantly (P < 0.05) greater in epididymal (13-fold), mesenteric (9.3-
fold), and retroperitoneal (1.8-fold) adipose tissues from the HFD than those from the control group (Fig 3B). Neither CRLR nor RAMP2 mRNA levels in subcutaneous fat pad differed significantly between rats fed the HFD and the normal diet. These data suggest that HFD upregulates gene expression of the putative AM receptor components (CRLR/RAMP2) in adipose tissues along with its ligand, AM.

**Differential gene expression of AM and its receptor components during preadipocyte differentiation.** Because both AM and its receptor component mRNAs were expressed in adipose tissues and upregulated during the development of obesity, we next examined the changes of gene expression of AM and its receptor components (CRLR/RAMP2) during preadipocyte differentiation using a preadipose cell line (3T3-L1). After treatment with differentiation medium, accumulation of oil droplets was observed by Oil Red O staining (Fig. 4A). During 3T3-L1 differentiation, AM mRNA level in 3T3-L1 cells transiently, but markedly decreased by day 3, returned to basal level by day 6, and then significantly increased by day 9 (Fig. 5A), whereas CRLR mRNA level also transiently decreased by day 3, but returned to basal level by days 6 through 9 (Fig. 5B). In contrast, RAMP2 mRNA level significantly increased dur-

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**Fig. 3. Changes of AM receptor component mRNA levels in different rat adipose tissues.** mRNA levels of calcitonin receptor-like receptor (CRLR; A) and receptor activity-modifying protein-2 (RAMP2; B) in adipose tissues (epididymal, mesenteric, retroperitoneal, and subcutaneous) from male rats (n = 5) after 4 wk of standard chow (control) diet (□) or HFD (■). Data are plotted as in Fig. 2. *P < 0.05 vs. each control group.

**Fig. 4. Oil Red O staining of each stage of 3T3-L1 cells during differentiation with or without AM.** Cells were fixed and stained with Oil red O at each time point (days 0, 3, 6, and 9) during preadipocyte differentiation in the absence (A) or presence (B) of AM (3 × 10^{-8} M). Magnification: (A and B, top) ×40, (A and B bottom) ×200.
AM expression and role in adipose tissue

Fig. 5. Expression of AM and its receptor component (CRLR/RAMP2) mRNAs in 3T3-L1 cells during pre-adipocyte differentiation. AM (A), CRLR (B), and RAMP2 (C) mRNA levels at each time point (days 0, 3, 6, and 9) during pre-adipocyte differentiation were measured by real-time RT-PCR. Each mRNA signal was normalized by ARPP P0. Data are expressed as percentages of baseline (day 0). Each column represents mean ± SE; n = 3. *P < 0.05 vs. control.

DISCUSSION

The present study demonstrated for the first time that the AM gene is expressed in rat and human adipose tissue and that steady-state AM mRNA levels in rat and human epididymal adipose tissue are almost comparable, being about one-tenth those in kidney. Moreover, our study showed that both mature adipocytes and stromal vascular cells are the potential sources of AM production.

In this study, steady-state AM mRNA levels expressed by various rat adipose tissues were about fourfold less in epididymal than those in mesenteric, retroperitoneal, and subcutaneous adipose tissues. Furthermore, AM mRNA levels in epididymal, mesenteric, and retroperitoneal adipose tissues from the HFD rats markedly increased compared with those in the control rats, whereas AM mRNA levels in various rat nonadipose tissues, such as aorta, kidney, heart, and liver, conversely decreased in the HFD rat. Given that adipose tissue constitutes about one-fourth of total body composition, such adipose tissue-specific upregulation and nonadipose tissue-specific downregulation of AM gene after HFD suggests differential regulation of the AM gene during the development of obesity.

Our result that plasma AM concentrations increased during the process of obesity is in accord with those of previous studies showing that plasma AM concentrations are elevated in obesity (12, 17, 25), although its origin remains elusive. Based on the adipose tissue-specific upregulation of AM gene and its secretion from 3T3-L1 cells, our data strongly suggest that adipose tissue is a potential source of AM secretion in obesity.

It has been recently reported that AM mRNA expression in 3T3-L1 cells decreased during preadipocyte differentiation by day 4 (19, 20). In contrast, our present study employing longer incubation periods (3–9 days) clearly demonstrated that AM mRNA expression as well as IR-AM secretion decreased to a very low level during the early predifferentiation phase (day 3) but markedly increased during the late phase (days 6–9). Such a U-shaped expression pattern of AM during preadipocyte differentiation is in marked contrast to that of other adipokines, such as lepin (18), adiponectin (18), TNF-α (8), and angiotensinogen (30), all of which consistently show unidirectional increases during the later phase of preadipocyte differentiation. Treatment with insulin, 3-isobutyl-1-methylxanthine, or dexamethasone alone did not induce such a U-shape pattern or preadipocyte differentiation in 3T3-L1 cells. These data suggest that the unique U-shaped pattern of AM expression is
tightly linked to the preadipocyte differentiation rather than the individual stimulation.

The present study further demonstrated that steady-state mRNA levels of AM receptor components (CRLR/RAMP2) expressed in several visceral adipose tissues other than subcutaneous adipose tissue markedly increased almost comparably to those of AM after HFD. Thus the concomitant upregulation of AM and its receptor components in visceral adipose tissue during the development of obesity suggests the possible functional link of the AM/AM receptor system in adipose tissue to the weight gain, as is the case with other adipokines, such as TNF-α, leptin, adiponectin, and so forth (10). However, the present in vitro study revealed that one receptor component (RAMP2) mRNA increased during preadipocyte differentiation in 3T3-L1 cells, whereas another receptor component (CRLR) mRNA transiently decreased during the early differentiation phase. Although the U-shaped expression pattern of CRLR appears to be similar to that of AM during preadipocyte differentiation, the functional significance of such differential gene regulation of AM and its receptor components during the process of adipocyte differentiation remains unknown.

To gain insight into the functional role of AM, the effects of AM and its receptor antagonist during preadipocyte differentiation were studied using 3T3-L1 cells. However, failure of exogenous AM and receptor antagonist CGRP-(8-37) on lipid droplet accumulation during preadipocyte differentiation makes less likely the possibility that AM may exert its direct effect on preadipocyte differentiation. Thus the significance and the molecular mechanism underlying such unique and tissue-specific gene expression of AM and its receptor components during preadipocyte differentiation remain to be elucidated.

To date, any functional role of adipose tissue-derived AM remains unknown, although the present results with the coexpression of AM and its receptor components in adipose tissue along with their concomitant tissue-specific upregulation led us to speculate on the yet-unspecified functional roles of adipose tissue-derived AM during the development of obesity. It has recently been reported that aged AM-deficient heterozygote mice showed insulin resistance and weight gain, due to increased oxidant stress, which were reversed by treatment with either antioxidant or AM supplementation (33). These data suggest that AM plays a protective role against insulin resistance and weight gain. However, the oxidant stress-induced insulin resistance was noted only in aged, not in young, AM-deficient mice, suggesting that long-term deficiency of endogenous AM increases oxidant stress with advancing age, thereby leading to impaired insulin signaling but not impaired insulin secretion.

On the other hand, it has been shown that AM inhibits insulin secretion both in vitro (isolated rat islets) and in vivo (oral glucose tolerance test in rats) and that both AM and its receptor genes are expressed in pancreatic islets (22). These data suggest that AM is involved in the negative regulation of insulin secretion in an autocrine/paracrine manner. Furthermore, several clinical studies have reported that plasma AM concentrations are elevated in patients with diabetes (4, 5, 27). In fact, it has been shown that AM infusion increased plasma glucose levels in a diabetic rat model, whereas administration of anti-AM antibody had the opposite effect and improved postprandial recovery (21). These data implicate AM as having a possible functional role in the maintenance of insulin and glucose homeostasis.

It has recently been reported that macrophages accumulated in adipose tissue in obesity appear to be responsible for excess production of a variety of proinflammatory cytokines, thereby leading to adipokine dysregulation and altered systemic metabolism (36, 37). The AM gene has been shown to be induced in inflammatory cells and by proinflammatory cytokines, which may play an immunomodulatory role in response to inflammation (1, 15, 35). Thus it is tempting to postulate that the concomitant upregulation of AM and its receptor components in visceral adipose tissue in obesity may contribute to adipokine dysregulation and the development of metabolic syndrome.

In conclusion, the present study revealed for the first time the concomitant expression of AM and its receptor components (CRLR/RAMP2) in various adipose tissues, their adipose tissue-specific upregulation during the development of obesity in vivo, and the dynamic changes of AM and its receptor components’ gene expression during preadipocyte differentiation in vitro. Our findings add a novel vasodilator hormone, AM, as a new member of adipokines, although its physiological and pathophysiological significance remains to be determined.

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