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ORIGINAL ARTICLE

Regulation of adiponectin release and demonstration of adiponectin mRNA as well as release by the non-fat cells of human omental adipose tissue

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Objective: Adiponectin is an adipokine produced by adipose tissue. The present studies examined the *in vitro* release of adiponectin by human omental adipose tissue explants as well as the mRNA content of freshly isolated non-fat cells and adipocytes plus cultured preadipocytes and adipocytes derived from omental fat.
Results: The release of adiponectin was reduced while that of interleukin-8 (IL-8) was enhanced in tissue explants from morbidly obese women. The release of adiponectin was also reduced by one-third in explants from morbidly obese diabetic women while that of IL-8 was unaffected by diabetes. The release of adiponectin was enhanced by insulin and by inhibition of endogenous tumor necrosis factor (TNFα) using etancercept. Adiponectin was released in appreciable amounts by the undigested matrix obtained by collagenase digestion of adipose tissue. The release of adiponectin by non-fat cells (matrix + SV cells) was comparable to that by the adipocytes derived from the same amount of tissue while the adiponectin mRNA content of the pooled matrix and SV cell fractions was 40% of that in intact tissue. The adiponectin mRNA content was 48-fold greater in isolated adipocytes than in non-fat cells derived from adipose tissue. In contrast, the amount of adiponectin mRNA *in vitro* differentiated omental adipocytes was 1×10^6-fold greater than that in cultured preadipocytes while that of leptin was 3×10^4-fold greater.
Conclusion: Adiponectin mRNA is present in the non-fat cells of freshly isolated adipose tissue and release by the non-fat cells derived from a gram of adipose tissue is comparable to that by adipocytes isolated from the same amount of tissue. While leptin is only released by mature adipocytes, adiponectin is released by both the non-fat cells and the fat cells derived from human omental adipose tissue.
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Keywords: human adipose tissue; adiponectin; interleukin 8; leptin; tumor necrosis factor

Introduction

Adiponectin was discovered by Scherer *et al.*[1] in 1995 as a protein found in differentiated adipocytes, but not in preadipocytes, and also by Hu *et al.*[2] and Maeda *et al.*[3] The importance of this protein was not recognized until 1999 when Arita *et al.*[4] reported that the circulating level of adiponectin is decreased in obese humans. Subsequently, it was found that plasma levels of adiponectin are also reduced in type 2 diabetes.[5,6] To date, adiponectin is the only known hormone released by adipose tissue whose circulating levels are reduced by obesity and/or diabetes. It is now thought that adiponectin has anti-diabetic, anti-atherogenic as well as anti-inflammatory properties and is a biomarker of the metabolic syndrome.[7,8]

Adiponectin was originally described as an adipocyte-specific protein.[1–3] However, more recently adiponectin mRNA and secretion have been demonstrated in bone-forming cells,[9,10] cardiomyocytes,[11] skeletal muscle,[12,13] human liver,[14] human placental syncytiotrophoblasts[15] and human fetal tissues of mesodermic and ectodermal origin.[16] It is unclear whether tissues other than adipose tissue contribute to circulating levels of adiponectin that are some 40-fold higher than those of leptin in obese subjects. It is equally uncertain whether the non-fat cells of human adipose tissue contribute to adipose tissue release of

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adiponectin. Furthermore, it is not known how the expansion of adipose tissue that is seen in obesity results in higher release of leptin and lower release of adiponectin.

Visceral adiposity correlates with insulin resistance.[17,18] However, obesity causes enhanced accumulation of fat in all depots. But, there is a sex-linked effect in that men have relatively more visceral adipose tissue 'beer belly fat' than do women.[17] Because of the importance of visceral adipose tissue, this report focuses on *in vitro* studies using omental adipose tissue. However, we did not see any difference in adiponectin release between explants of subcutaneous as compared to omental adipose tissue.[19] Similar results have been reported with regard to adiponectin mRNA in subcutaneous versus omental adipose tissue of lean, obese and morbidly obese women.[20] However, two reports found lower levels of adiponectin mRNA in visceral omental adipose tissue as compared to subcutaneous adipose tissue;[21,22] while Motoshima *et al*.[23] found a higher release of adiponectin by adipocytes isolated from omental as compared to those isolated from subcutaneous adipose tissue.

Present studies were designed to compare the adiponectin release *in vitro* as well as the mRNA content of non-fat cells as compared to fat cells (adipocytes) of omental adipose tissue from obese women. The role of insulin and TNFα in the regulation of adiponectin release by omental adipose tissue extracts was also examined in these studies.

Materials and methods

Omental adipose tissue was obtained from women undergoing either laparoscopic adjustable gastric banding surgery or laparoscopic gastric bypass with Roux-en-y gastroenterostomy surgery for the treatment of morbid obesity as well as some women undergoing abdominoplasty. Approximately 40% of patients were age 40 or less, 40% were ages 40–49 and 20% were over the age of 50. The mean age of the controls was 37 and of the diabetics 42. Each experimental replication involved tissue from a separate individual. Fasting blood glucose values exceeding 125 mg dl^{-1} defined the 7 morbidly obese type 2 diabetic patients whose mean fasting blood glucose was 180 despite the fact that all were on diabetes medications (1 was taking only insulin, 2 insulin plus an oral agent, 3 were taking one oral agent and 1 was taking two oral agents). It should be noted that in a larger study of 58 type 2 morbidly obese diabetic women (body mass index (BMI) above 36 and age over 18), there was no correlation between age and fasting blood glucose (Pearson's correlation coefficient of $r=-0.07$ and $P=0.65$). In 58 morbidly obese non-diabetic women, there was only a small and non-significant correlation between age and blood glucose ($r=0.20$ and $P=0.12$). In the same large group of 126 morbidly obese women, there was in fact a negative correlation between age and waist circumference ($r=-0.26$ and $P=0.05$ in non-diabetic and $r=-0.27$ and $P=0.03$ in diabetic women). The study had the approval of the local Institutional Review Board (IRB) and all patients involved gave their informed written consent.

The omental adipose tissue was transported to the laboratory within 5–15 min of its removal from the donor. The handling of tissue and cells was done under aseptic conditions. The tissue was cut with scissors into small pieces (5–10 mg). All the studies utilized explants of adipose tissue that had been incubated in buffer plus albumin (3 ml g^{-1} of tissue) for approximately 1 min to reduce contamination of the tissue with blood cells and soluble factors. At the conclusion of this incubation, the tissue explants were centrifuged for 15 s at 400 *g* in order to remove blood cells and pieces of tissue containing insufficient adipocytes to float. The cut pieces of tissue were then incubated for an indicated time in suspension culture under aseptic conditions in buffer containing 17.5 mM glucose and with air as the gas phase.

Adipocytes were prepared by incubating 0.5 g of cut adipose tissue per ml of incubation medium containing 0.6 mg ml^{-1} of bacterial collagenase in a rotary water bath shaker (100 r.p.m.) for 2 h. The collagenase digest was then separated from undigested tissue matrix by filtration through 200 μm mesh fabric. Five milliliters of medium was then added back to the digestion tubes and used to wash the undigested matrix on the filter mesh. This wash solution was combined with the collagenase digest and stromovascular (SV)-cell fraction separated from adipocytes and medium by centrifugation in 15 ml tubes for 1 min at 400 *g*. The SV cells are defined as those cells isolated by collagenase digestion that do not float. The SV cells and adipocytes were each suspended in 5 ml of fresh buffer and centrifuged for 10 s at 400 *g*. This medium was removed. The undigested tissue matrix on the nylon mesh, the SV cells and the adipocytes were incubated in a volume of 5 ml for the indicated period.

The serum-free buffer for incubation of adipose tissue and adipocytes was as previously described.[24] The pH of the buffer was adjusted to 7.4 and then filtered through a 0.2 μm filter. Interleukin-8 (IL-8), leptin and adiponectin release to the medium were determined using an ELISA assay with Duoset reagents from R&D Systems of Minneapolis, MN. Lactate release was determined using lactate dehydrogenase.

Human omental preadipocytes were isolated from the SV cell fraction obtained after collagenase digestion of human omental adipose tissue as previously described.[25,26] The SV-cell pellet is suspended in omental preadipocyte medium (DMEM/F12, HEPES pH 7.4, FBS, biotin, pantothenate, insulin, dexamethasone and antibiotics) and plated in a culture flask for expansion. Preadipocytes are subcultured prior to reaching confluence and plated at a density of 6300 cells cm^{-2} for further expansion in preadipocyte media containing growth factors. Human omental preadipocytes plated at a density of 40 625 cells cm^{-2} were incubated with differentiation medium (OM–DM, Zen–Bio Inc.) to induce differentiation to mature adipocytes. Cells were incubated in

OM–DM for 7 days. The medium was then partially exchanged with adipocyte medium (AM-1, Zen–Bio Inc.) and incubated for an additional 7 days. During this period, the cells developed lipid droplets indicative of the adipocyte phenotype.

Isolation of total RNA from preadipocytes and differentiated adipocytes was performed using an RNAeasy Mini Kit according to the manufacturer's protocol (Qiagen Inc., Valencia, CA, USA). For studies involving mRNA isolation from omental adipose tissue approximately 0.5 g of tissue or the fractions derived from 1 g of tissue the matrix (SV cells or adipocytes) were homogenized using a polytron homogenizer with 5 ml of a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent from Invitrogen of Carlsbad, CA, USA). The extracts were then spun at 12 000 *g* for 10 min at 2–8°C and the fat layer on top of the extract removed using a Pasteur pipette. Total RNA was obtained from the cleared homogenate by the procedure of Chomczynski and Sacchi.[27]

The cDNA was prepared using the Transcriptor First Strand cDNA synthesis Kit from Roche Diagnostics. The quantification of mRNA was accomplished using the Roche Lightcycler 480 Real-time RT-PCR system and the Universal Probe Library of short hydrolysis locked nucleic acid (LNA) probes in combination with the primers suggested by their web-based assay design center (www.universalprobelibrary.com). Integrated DNA Technologies of Coralville, IA, synthesized the primers. Approximately 70 ng per tube of total mRNA was used for each assay. The data are obtained as crossing point values (Cp) obtained by the second derivative maximum procedure. Samples with higher copy number of cDNA have lower Cp values, while those with lower copy numbers have the reverse. Relative quantification of the data as well as the ratio of mRNA in fat cells to non-fat cells and of preadipocyte to adipocytes was based on the comparative Cp method that eliminates the need for standard curves. The arithmetic formula to calculate ratios from ΔCp is $2^{-\Delta Cp}$. This procedure is identical to the comparative C_T procedure described in the ABI PRISM 7700 Sequence Detection System user Bulletin #2 for quantitative RT-PCR except that Cp is used, since Roche uses the crossing point procedure instead of crossing threshold to calculate cycles required for detection of mRNA. The calculation of ratios assumes that the number of target molecules doubles with every PCR cycle. It is not possible to directly compare the ΔCp values for one gene with another because of the relative efficiencies of the particular primers and probes used for each gene are different.

Results

The release of adiponectin was reduced by 67%, while that of IL-8 was enhanced by 228% in explants of adipose tissue incubated for 48 h from women with a BMI of 45 as compared to women with a BMI of 32 (Figure 1a). The release of adiponectin was reduced by 34% in omental adipose tissue explants from obese diabetic women as compared to that by tissue from morbidly obese women with similar BMI values while that of IL-8 was unaffected (Figure 1b). The enhanced release of inflammatory mediators such as IL-8 by adipose tissue from morbidly obese women is in agreement with prior results.[28]

Another inflammatory mediator is TNFα whose release by human adipose tissue explants over a 4 h incubation was negatively associated (Pearson's $r = -0.60$) with the release of adiponectin.[29] Kern *et al*.[30] found a similar negative correlation between plasma adiponectin and adipose tissue TNFα mRNA as well as adiponectin mRNA content in adipose tissue versus TNFα secretion over a 2 h incubation of adipose

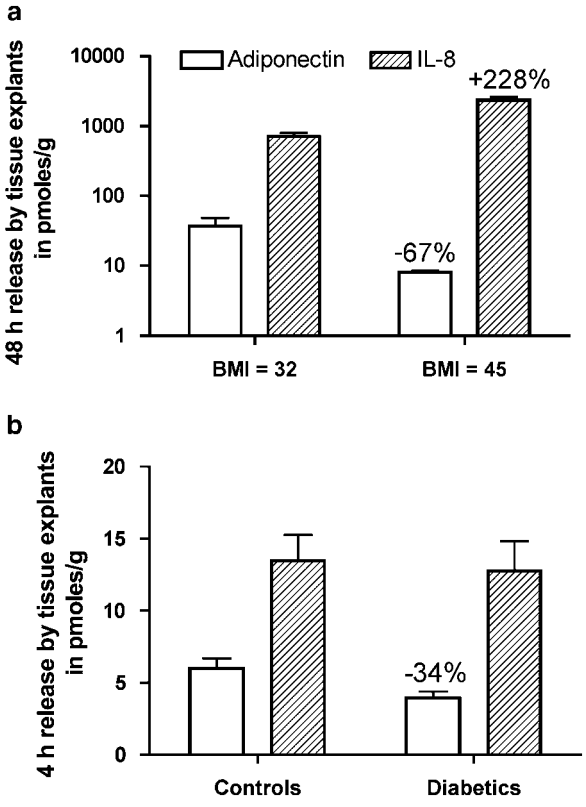

Figure 1 Adiponectin release is reduced in obesity and diabetes. The data in part a are the means ± s.e.m. (log_{10} scale) of experiments using omental adipose tissue explants from eight abdominoplasty patients with a mean body mass index (BMI) of 32 (range was 27–35) and eight gastric bypass patients with a mean BMI of 48 (range was 41–48). The percent value above the bars in the BMI = 45 group is the percent decrease (adiponectin) or increase (leptin) in release over a 48 h incubation as compared to fat from patients with an average BMI of 32 and each was statistically significant ($P = 0.001$). The data in part b are shown as the means ± s.e.m. of release over a 4 h incubation by omental-adipose tissue explants from 47 gastric bypass patients without and 7 patients with type 2 diabetes. The average BMI of the controls was 45.2 ± 1.5 and for the diabetics was 44.9 ± 3.8 as the mean ± s.e.m. The −34% over the adiponectin bar for the diabetics represents the percent decrease in adiponectin release seen in adipose tissue from diabetics and was significant with a $P < 0.02$.

tissue. Bruun *et al*.[31] reported an inhibition of adiponectin mRNA accumulation due to the direct addition of TNFα to human adipose tissue. The data in Figure 2 show that inhibition of endogenous TNFα release over a 48 h incubation of omental adipose tissue explants with etanercept (Enbrel) enhanced adiponectin release by 30%. A similar increase in adiponectin release over 48 h was seen in the presence of insulin and the effect was not additive to that of etanercept (Figure 2).

There was appreciable release of adiponectin, but not of leptin, by the matrix and SV fractions obtained by collagenase digestion of adipose tissue over 48 h (Figure 3). The release of adiponectin by adipocytes derived from a gram of tissue was only slightly higher than that by the matrix fraction derived from the same amount of tissue (Figure 3). The release of lactate is included for comparison and indicates that while the SV-cell fraction derived from a gram of tissue released as much lactate as the adipocyte fraction but only 6% as much adiponectin as the adipocytes. In contrast, adiponectin release by the matrix fraction was 86% of that by the adipocytes while lactate formation was 5-fold greater than that by adipocytes (Figure 3).

If the release of adiponectin by the adipose tissue cellular matrix fraction represented synthesis in the cells in the cellular matrix, there should be mRNA present in the matrix + SV cell fractions. The adiponectin mRNA content in the pooled matrix + SV fractions was 25% while that of leptin was 12% of that in cut pieces of omental adipose tissue (Figure 4). The adiponectin and leptin mRNA content of freshly isolated adipocytes was 20- and 6-fold greater than that of adipose tissue mRNA. However, there was virtually no detectable adiponectin mRNA (<0.03%) in preadipocytes derived from the SV cell fraction after primary culture for 7 days, while that of leptin in preadipocytes was 1% of that in omental adipose tissue (Figure 4). In contrast, the amount of adiponectin and leptin mRNA in differentiated adipocytes was 208- and 39-fold greater respectively than that of adipose tissue.

The ratio of adiponectin, leptin and perilipin mRNA contents in omental adipocytes obtained by differentiation of preadipocytes as compared to the preadipocytes was far greater than that of adipocytes as compared to the pooled matrix + SV cell fractions (Table 1). Perilipin was used as a control since it is a protein involved in lipolysis that is localized in adipocytes. PPARγ 1–2 (the probes do not

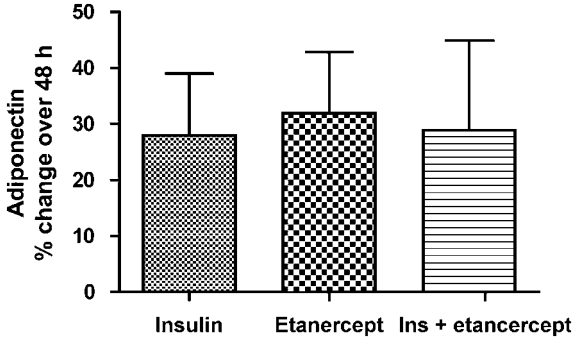

Figure 2 Insulin and etanercept stimulate adiponectin release over a 48 h incubation of omental adipose tissue explants. Explants of omental adipose tissue from ten gastric bypass patients with an average BMI of 48 ± 2.4 were incubated for 48 h either without or with 10 nM insulin, 200 ng ml^{-1} of etanercept (Enbrel) or both. The basal release of adiponectin was 7.2 pmol g^{-1} over 48 h and the data are expressed as % change due to added agents ± s.e.m. The increases in adiponectin release due to insulin and etancercept based on paired comparisons were statistically significant ($P<0.05$).

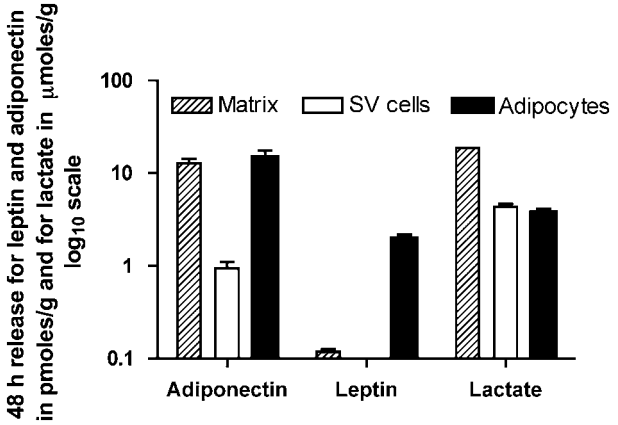

Figure 3 The matrix and SV fractions derived from human omental adipose tissue release adiponectin but not leptin. The data are expressed in pmol g^{-1} of adipose tissue from which the fractions were derived and expressed on a $\log_{10}$ scale. Adiponectin and leptin release are in pmol g^{-1} and lactate release in μmol g^{-1} ± s.e.m. of 11 experiments from as many abdominoplasty patients with an average BMI of 32.

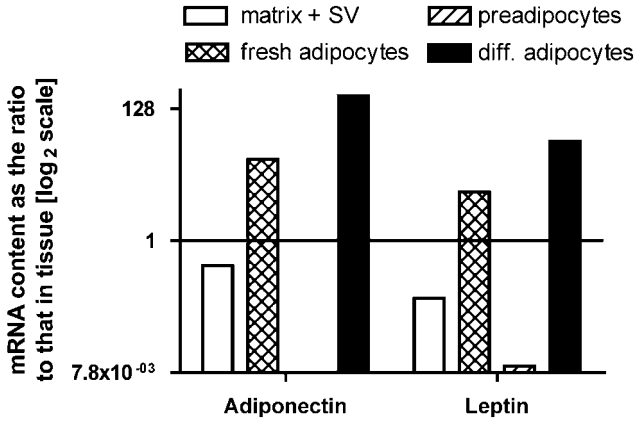

Figure 4 Relative amount of adiponectin and leptin mRNA in non-fat cells (matrix + SV fractions), adipocytes, preadipocytes and *in vitro* differentiated adipocytes as compared to that in omental adipose tissue. The data for mRNA content are expressed as the ratio of that in the four fractions to that in omental adipose tissue explants expressed on a $\log_2$ scale that is based on the ΔCp values for six experiments using isolated omental adipose tissue, adipocytes and the non-fat cells (pooled-matrix + SV fractions), four experiments using preadipocytes obtained from omental adipose tissue and three experiments using *in vitro* differentiated adipocytes.

Table 1 Comparison of mRNA in isolated adipocytes and the non-fat cells with cultured preadipocytes and differentiated adipocytes derived from omental adipose tissue

mRNA	*Ratio in cultured adipocytes/ preadipocytes*	*Ratio in freshly isolated adipocytes/ non-fat cells*
Adiponectin	1×10^6	48
Leptin	3×10^4	48
Perilipin	4×10^4	128
PPARγ	4.6	23

The data are based on 4 preadipocyte samples and 3 *in vitro* differentiated adipocyte samples derived from omental adipose tissue. The data for freshly isolated omental adipocytes and non-fat cells (the pooled-undigested matrix and SV-cell fractions) are from 6 experiments. The mRNA values were obtained by RT-PCR and expressed as the ratios derived from the ΔCp values ($2^{-\Delta Cp}$).

Table 2 Comparison of the mRNA content in the matrix as compared to the SV fraction obtained from omental adipose tissue

mRNA	*Cp matrix*	*Cp SV*	*ΔCp ± s.e.m.*	P-*value*	*Ratio*
Complement Factor C3	23.8	23.9	0.1 ± 0.1	NS	0.93
Leptin	26.5	27.8	1.3 ± 0.5	0.05	0.44
Perilipin	26.6	28.2	1.6 ± 0.3	0.02	0.44
PPARγ	26.2	27.8	1.6 ± 0.4	0.01	0.44
Adiponectin	23.8	25.9	1.9 ± 0.4	0.01	0.27

The values are the means of 8 experiments using fractions derived from omental adipose tissue from as many women. The ΔCp values are the means ± s.e.m. of the paired differences for each individual and the *P* values are based on the paired differences. The ratio of each mRNA in the SV fraction to that in the matrix fraction was calculated from the ΔCp values ($2^{-\Delta Cp}$).

distinguish between the isoforms) was found to be enriched in adipocytes, but not to nearly the same extent as perilipin, leptin or adiponectin. Interestingly, PPARγ was found to a greater extent in freshly isolated adipocytes than in adipocytes differentiated *in vitro* (Table 1).

The SV cell fraction is those cells released by collagenase digestion that do not float while the matrix is those cells remaining in adipose tissue after collagenase digestion. Preadipocytes are essentially obtained by culturing the SV cell fraction for a week in the presence of insulin and dexamethasone plus serum and other factors. The question was whether adiponectin is enriched in the SV fraction as compared to the adipose tissue matrix was examined in the studies shown in Table 2. Just the reverse was seen in that adiponectin mRNA content in the SV fraction was 27% of that in the matrix fraction. Complement factor C3 mRNA was included since it is one mRNA that is present to the same extent in both fractions, while leptin, perilipin and PPARγ1–3 mRNAs were present to a significantly lesser extent in SV than in the matrix cell fraction.

Discussion

The present results confirm the finding originally noted in murine 3T3 cells[1,2] that adiponectin mRNA is not expressed in preadipocytes and extend these findings to human omental preadipocytes. We also found some adiponectin mRNA in freshly isolated human adipose tissue SV cells; but the amount of adiponectin mRNA was 4-fold greater in the adipose tissue matrix than in SV cells. These data suggest that the release over 48 h of adiponectin by the adipose tissue matrix obtained after a 2 h digestion of tissue with collagenase represents synthesis by cells in the matrix. The matrix includes endothelial and smooth muscle cells associated with the blood vessels along with fibroblasts and other cells comprising undigested connective tissue. One problem is that mRNA levels are not always reflected in protein levels. This was the case with regard to leptin whose mRNA was present in the matrix + SV fractions of omental adipose tissue at levels 12% that in tissue but there was no release of leptin.

While the data suggest that as much as half of adiponectin release could be by the non-fat cells of adipose tissue, this conclusion is based on fractions obtained by collagenase digestion. Possibly this procedure has more deleterious effects on adiponectin release by adipocytes than by the tissue-matrix fraction.

In short-term studies with human omental adipose tissue, the release of bound adiponectin is a problem because of the relatively high-circulating concentrations of adiponectin. However, it was possible to see net release, after correcting for the change in tissue adiponectin, over 2 h of approximately 5 pmol g^{-1} of adiponectin by omental adipose tissue.[28] Approximately, the same rate of release was seen by cut pieces of adipose tissue that did not contain enough lipid to float.[28] The view that the expression of adiponectin mRNA is restricted to mature adipocytes is clearly untenable in view of the present findings and the recent reports that adiponectin is synthesized and secreted by human osteoblasts,[10] human cardiomyocytes,[11] murine skeletal muscle,[12] human skeletal muscle and placenta,[13] human liver biopsies,[14] human placental synctiotrophoblasts,[15] as well as human fetal tissues of both mesodermal and ectodermal origin.[16]

The present data using omental adipose tissue explants confirm the prior reports by Matsuzawa's laboratory that adiponectin release is reduced by obesity[4] as well as diabetes.[5,6] It is still unclear exactly why leptin release is enhanced, while that of adiponectin is reduced in obesity.[7,8] The negative correlation between IL-8 release and that of adiponectin by adipose tissue suggests that the obese state is associated with enhanced release of inflammatory cytokines in adipose tissue. A negative relationship has also been reported *in vivo* between plasma adiponectin and cytokines.[31] Kern *et al*.[30] found a similar negative relationship between TNFα release and adiponectin release by human adipose tissue *in vitro*.[30] While TNFα is a potent inhibitor of adiponectin release by murine 3T3–L1 adipocytes,[32,33] it inhibited adiponectin release by only 7% in human adipocytes.[32] However, Wang and Trayhurn[34] reported a decrease due to TNFα of adiponectin mRNA in human

adipocytes differentiated *in vitro*. Our data demonstrated that the release of adiponectin over 48 h by omental adipose tissue explants was enhanced by 30% when endogenous TNFα was blocked with etanercept, which is the soluble TNFα receptor that binds to TNFα and thus prevents TNFα from binding to cellular receptors. Fain *et al*.[35] found that etanercept alone had no effect on the release of IL-8 under the same conditions but the combination of etanercept and an antibody which binds IL-1β resulted in a 46% decrease in endogenous IL-8 release. The present data indicate that while inhibition of TNFα can enhance adiponectin, it requires blocking the effects of both TNFα and IL-1β to reduce IL-8 release.[35] Thus the primary negative regulator of adiponectin release is likely to be TNFα rather than IL-8 whose release is, in part, secondary to that of TNFα and IL-1β.

The finding that insulin also enhanced adiponectin release by omental adipose tissue explants by almost 30% over a 48 h incubation is in agreement with the report by Halleux *et al*.[36] that insulin enhances adiponectin mRNA accumulation in human omental adipose tissue explants. Thus, the reduced adiponectin release observed by adipose tissue explants from diabetic obese individuals as compared to obese individuals with the same BMI could reflect inhibition of endogenous insulin effects on adiponectin release by human omental adipose tissue.

In conclusion, the present data are compatible with the hypothesis that the reduced release of adiponectin in obesity is secondary to enhanced TNFα release and that the further decrease seen in type 2 diabetes is linked to insulin resistance. Adiponectin mRNA is found in appreciable amounts in the freshly isolated non-fat cell fractions obtained by collagenase digestion of human omental adipose tissue but not in cultured preadipocytes. However, the question of whether there is any contribution of cells other than adipocytes to circulating adiponectin in humans remains to be answered.

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