Pioglitazone increases secretion of high-molecular-weight adiponectin from adipocytes

Angela M. Bodles,* Anannya Banga,* Neda Rasouli, Fumiyo Ono, Philip A. Kern, and Randall J. Owens

The Central Arkansas Veterans Healthcare System, and Department of Medicine, Division of Endocrinology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Submitted 19 April 2006; accepted in final form 23 June 2006

Bodles, Angela M., Anannya Banga, Neda Rasouli, Fumiyo Ono, Philip A. Kern, and Randall J. Owens. Pioglitazone increases secretion of high-molecular-weight adiponectin from adipocytes. Am J Physiol Endocrinol Metab 291: E1100–E1105, 2006—Adiponectin is an adipocyte-derived serum protein that plays important roles in energy homeostasis, obesity, and insulin sensitivity. Using sucrose gradients and Western blotting of non-denaturing gels, we examined the adiponectin isoforms secreted from human adipose tissue, human and mouse adipocytes, and cell lines in response to pioglitazone added in vitro. The predominant form secreted from adipose tissue in vivo was the high-molecular-weight (HMW) isoform, with small amounts of low-molecular-weight (LMW) forms present. The addition of pioglitazone (1–3 μM) in vitro increased the secretion of the HMW isoform, with no significant effect on the other isoforms. Human adipose tissue was also examined for changes in adiponectin mRNA levels upon pioglitazone treatment. No difference was detected, suggesting that the effect of pioglitazone is not at the transcriptional level but, rather, at a posttranscriptional phase of the secretory pathway. Additional experiments were conducted to determine whether adiponectin expression was mechanistically similar in other adipocyte cells. Examination of primary human adipocytes revealed an increase in intracellular HMW isoform with a decline in LMW forms following pioglitazone treatment, with a corresponding increase in the secreted HMW form. Similar results were observed with primary mouse adipocytes, 3T3-F422A cells, and SGBS human adipocyte cells, although differences in the distribution of HMW and LMW isoforms were apparent between cell types. Although there are differences in isoforms between species, in all cases pioglitazone served to increase the secretion of the HMW form of adiponectin.

Insulin resistance is a prominent feature of diabetes and is present long before the development of fasting hyperglycemia (6, 9). Insulin resistance develops with obesity, and numerous studies have demonstrated the potential importance of adipocyte secretory proteins, or “adipokines,” in the development of insulin resistance (1). One important adipokine is adiponectin, or Acrp30 (adipocyte complement-related protein of 30 kDa), which is a major secretory product of the adipocyte, circulates at a high level in blood, and binds to receptors in numerous tissues involved in insulin resistance.

Since the discovery of adiponectin in 1995 (21), there has been a wealth of information generated regarding its structure, function, physiological activities, and correlations with obesity, insulin resistance, and type 2 diabetes. Blood levels of adiponectin are low in subjects with diabetes, insulin resistance, coronary heart disease, and other features of the metabolic syndrome (2, 10, 16). However, the precise interpretation of these measurements in blood is clouded by the complex structure of adiponectin. Adiponectin is initially synthesized as a 30-kDa monomer and is then assembled into complex isoforms that are secreted and circulate in plasma. In humans, several studies have separated circulating adiponectin isoforms by sucrose gradient centrifugation and have designated the adiponectin isoforms as low (LMW) or high molecular weight (HMW) (15). The globular head cleavage product of adiponectin monomer increases lipid oxidation and may protect susceptible mice from diabetes and atherosclerosis (5, 25), and one study found evidence for circulating globular head adiponectin in human blood (5). However, a complete characterization of adiponectin isoforms has not been performed, and no study has examined the characteristics of adiponectin secreted by human adipose tissue.

Thiazolidinediones (TZDs) improve insulin sensitivity in humans and result in a two- to threefold increase in total adiponectin levels (7, 14). Recent evidence suggested that the treatment of diabetic subjects with TZDs specifically increased the blood level of the HMW isoform of adiponectin, and the HMW isoform of adiponectin correlated strongly with the increase in insulin sensitivity (15, 22). In this study, we examined the adiponectin isoforms produced and secreted by adipocytes and their response to pioglitazone treatment in vitro. We also compared primary and continuous adipocyte cell types from human and mouse origins, to determine whether adiponectin expression in human adipose tissue explants was similar to expression in isolated adipocyte cultures. Although there are differences between adipocytes and adipose cell lines, pioglitazone overall significantly increases the secretion of the HMW adiponectin isoform.

MATERIALS AND METHODS

Subject recruitment. To obtain adipose tissue for this study, we performed subcutaneous fat biopsies on nine nondiabetic subjects (7 females) who were recruited by local advertisement. All subjects provided written, informed consent under a protocol that was approved by the Institutional Review Board of University of Arkansas for Medical Sciences (UAMS) and was conducted at the UAMS General Clinical Research Center. The baseline characteristic of subjects is summarized in Table 1. Subjects were generally healthy without any history of liver or kidney dysfunction. A history of cardiovascular disease and the use of aspirin or anti-inflammatory medications were contraindications for the study. Four subjects had impaired glucose tolerance defined as 2-h glucose of 7.77–11.06 mM following a 75-g oral glucose tolerance test.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* A. M. Bodles and A. Banga contributed equally to this work.

Address for reprint requests and other correspondence: R. J. Owens, Central Arkansas Veterans Healthcare System, 4300 West 7th St., Little Rock, AR 72205 (e-mail: OwensRandallJ@uams.edu).

E1100
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>42.0±2.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>31.56±1.84</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>39.67±2.78</td>
</tr>
<tr>
<td>2-h glucose, mM</td>
<td>7.91±0.73</td>
</tr>
<tr>
<td>Fasting glucose, mM</td>
<td>5.18±0.2</td>
</tr>
<tr>
<td>S&lt;sub&gt;0&lt;/sub&gt;, 10&lt;sup&gt;-5&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;·pM&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.31±0.49</td>
</tr>
<tr>
<td>Triglyceride, mM</td>
<td>1.09±0.17</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>5.04±0.28</td>
</tr>
<tr>
<td>LDL, mM</td>
<td>3.12±0.26</td>
</tr>
<tr>
<td>HDL, mM</td>
<td>1.42±0.2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE, where n = 9, consisting of 7 females and 2 males. S<sub>0</sub>, insulin sensitivity.

calf serum and a mixture of penicillin and streptomycin. For experiments, cells were subcultured in 12-well dishes. Confluent cultures were allowed to differentiate with the addition of DMEM containing 10% fetal bovine serum and 100 nM insulin. The cells differentiated well in 3–5 days after being switched to the differentiation medium. Differentiated cells at day 4 were treated with pioglitazone (3 μM) for 48 h before analysis.

Sucrose gradient sedimentation analysis. To study adiponectin isoforms, we separated cellular or secreted protein fractions using a sucrose gradient and then performed Western blotting with anti-adiponectin antibody of each fraction after nondenaturing gel electrophoresis. Sucrose gradients (5–20%) were formed in 5-ml centrifuge tubes (Beckman, Palo Alto, CA). Culture medium or cell lysate (1 ml) was layered on top of the gradient and spun at 50,000 rpm for 6 h at 4°C in an SW50 rotor in a Beckman L8-80 ultracentrifuge. Gradient fractions (250 μl) were successively collected from the top of the gradients.

Western blot analysis and densitometry. The gradient fractions (10 μl each) were loaded, run in 4–20% Criterion precast gel (Bio-Rad, Hercules, CA), and transferred onto Trans-Blot transfer medium (Bio-Rad). One lane on each gel contained molecular weight markers (Benchmark prestained protein ladder; Invitrogen, Carlsbad, CA), varying between 182 and 6 kDa. Anti-human or anti-mouse Acrp30/adiponectin (R&D Systems, Minneapolis, MN) and donkey anti-goat IgG horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) were used for Western blot detection. ImageQuant TL (Amer sham Biosciences, Piscataway, NJ) was used for Western blot densitometric analysis. All the bands on the gel in Fig. 1 were analyzed, summed, and expressed in relation to total adiponectin with no pioglitazone added.

Total RNA isolation and real-time RT-PCR. Total RNA from adipose tissue treated with pioglitazone (1 μM) for 24 h was isolated using an RNeasy lipid tissue minikit from Qiagen (Valencia, CA) following the manufacturer’s instructions. The quantity and quality of the isolated RNA was determined using the Agilent 2100 bioanalyzer with RNA 6000 nano chips. Total RNA (1 μg) was reversed transcribed using random hexamer primers with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Reverse-transcribed RNA was amplified with 1× SYBR Green PCR Master Mix (Applied Biosystems) plus 0.3 μM of gene-specific upstream and downstream primers during 55 cycles on a Rotor-Gene 3000 real-time thermal cycler (Corbett Research, Sydney, Australia). Each cycle consisted of denaturation at 94°C for 20s, annealing at 60°C for 20s, and extension at 72°C for 20s. Amplified 18S expression was used as standard control to normalize the differences in individual samples. All data are expressed in relation to 18S RNA, where the standard curve was generated using pooled RNA from the samples assayed. Therefore, the data represent arbitrary units that accurately compare each set of samples to each other but do not necessarily accurately compare samples between different assays. The Ct values of the PCR reactions were generally between 20 and 30 for all assays. The results are expressed as the sum of three independent experiments.

Statistics. All data are expressed as means ± SE, and Student’s paired t-test was used for statistical analysis, with a level of statistical significance set at P < 0.05.

RESULTS

HMW isofrom is the predominant secretory product of adipose tissue and is increased after pioglitazone treatment. To study the secretion of adiponectin, we cultured human adipose tissue for 24 h, as described in MATERIALS AND METHODS, and identified adiponectin isoforms in the medium using sucrose gradient density separation followed by native SDS-PAGE and Western blotting. As shown in Fig. 1A, the smaller isoforms were generally found in the lower density fractions.
and the larger forms in the higher density (bottom) of the sucrose gradient. The fractions from the bottom (right) of the gradient contained adiponectin isoforms with a molecular mass of $>300$ kDa, designated HMW, and this isoform was the predominant adiponectin protein identified on these gels. From these same fractions, a somewhat ill-defined adiponectin isoform was identified in some gels that migrated with a molecular mass of $180$ kDa. The fractions from the top (left) of the sucrose gradient yielded little adiponectin isoforms, but bands at about 90 kDa and occasional bands at 60 kDa were identified. The isoforms were designated to be LMW. Although the tissue was washed, immunoglobulin was present in the medium from residual blood and reacted with the secondary antibody, and this is indicated in Fig. 1A.

The adipose tissue pieces also were treated in vitro with pioglitazone for 24 h, at concentrations ranging from 1 to 3 $\mu$M. As shown in Fig. 1A, the addition of pioglitazone to the adipose tissue resulted in an increase in the HMW adiponectin isoforms, which migrated near the bottom of the sucrose gradient. To quantitate these changes in adiponectin isoforms, we performed densitometry on each isoform and expressed these changes relative to total adiponectin in the control (no pioglitazone) samples. As shown in Fig. 1B, there was an increase in total adiponectin secretion in a dose-dependent manner after the addition of pioglitazone, and this increase in adiponectin was entirely due to the increase in HMW adiponectin isoforms.

To determine whether pioglitazone was interacting at the transcriptional level, we measured adiponectin mRNA levels in human adipose tissue treated with and without pioglitazone (1 $\mu$M) for 24 h. Compared with control, there was no difference in message with pioglitazone treatment, demonstrating that pioglitazone is not affecting transcription (Fig. 1C).

**HMW isoform is the predominant secretory product following pioglitazone treatment in adipocytes.** The above-described experiments were performed in whole adipose tissue, which contains multiple cell types in addition to adipocytes. To examine adiponectin secretion and processing in adipocytes, we prepared isolated adipocytes from a collagenase digestion of human adipose tissue. These cells were incubated in serum-free medium for 24 h, and we used the same methodology to resolve the complex distribution of individual adiponectin isomers. In human adipocytes, four predominant isoforms of adiponectin were noted, which migrated at apparent molecular masses of 60, 90, 180, and $300$ kDa (Fig. 2). These represented the same four isoforms found in the medium surrounding adipose tissue, and although the adipocytes contained a higher proportion of LMW, the most prominent bands on these gels were the HMW isoforms, which appeared as a dense singlet. LMW isoforms were present in control adipocytes, but treatment with pioglitazone increased the amount of cellular HMW while diminishing these faster migrating isoforms. The medium from these adipocytes was analyzed in a similar manner. Under native SDS-PAGE conditions, the amount of adiponectin was too low for detection in medium from control cells, whereas pioglitazone-treated cells demonstrated only HMW isoforms in the medium (Fig. 2).

To determine whether the same adiponectin isoforms were present in other adipocyte species and whether pioglitazone affected them all in a similar manner, we examined several...
different adipose cells. In 3T3-F422A cells, the highest level of adiponectin was produced by day 4 of adipocyte differentiation (results not shown), and these cells secreted a small amount of HMW, with higher levels of LMW isoforms ranging from ~100 to 200 kDa (Fig. 3A). After treatment with pioglitazone, there was a shift in the isoforms, with a pronounced increase in the amount of HMW, although these cells continued to secrete a considerable amount of other LMW isoforms. Isolated adipocytes obtained from mouse fat pads, on the other hand, secreted HMW isoforms, along with a considerable amount of one LMW isoform, with very little detection of other isoforms (Fig. 3B). Again, the addition of pioglitazone increased the amount of HMW isoform, although these cells continued to secrete some LMW isoform (Fig. 3B). SGBS cells are a human adipocyte cell line and were treated with pioglitazone for 24 and 48 h at day 6 of differentiation (Fig. 3C). Both cells and medium were analyzed by SDS-PAGE for adiponectin isoforms. Many more isoforms were present in SGBS cells, with an abundant HMW isoform detected at ~60 kDa in both the cells and medium. Other LMW forms were detected at 90, 130, and 180 kDa, and all of these were present in both cells and medium. The most noticeable difference between SGBS cells and medium, however, was the amount of HMW isoform. SGBS cells contained very little detectable HMW, whereas these cells secreted significant amounts of HMW into the medium. As with other adipose cells, SGBS cells secreted more HMW into the medium in the presence of pioglitazone.

Adiponectin is expressed at a high level in adipose tissue and only at a very low level by other tissues and is an important adipokine associated with insulin sensitivity and protection from vascular disease (8). In both rodents and humans, including impaired glucose tolerant (IGT) subjects, the improved insulin sensitivity that resulted from treatment with TZD drugs yielded striking increases in blood adiponectin levels (13, 14, 17, 26). In addition, several studies have demonstrated that the HMW isoform of adiponectin correlates best with features of metabolic syndrome, such as insulin resistance (12, 15). They also have demonstrated, along with others, that it is the HMW isoform that is the active and predominantly secreted form in human sera (4, 15, 22).

The synthesis and assembly of adiponectin is complex. After synthesis of the adiponectin monomer, the protein is assembled into a trimer, which consists of two of the three monomers covalently linked by a disulfide bond and a third cysteine residue available to form a disulfide bond with another trimer, yielding a hexamer (23). This covalent disulfide linkage is also necessary for the formation of the HMW isoforms (23). More recently, it was shown that adiponectin undergoes further posttranslational modifications that also are necessary for the formation of HMW. The hydroxylation and glycosylation of four lysine residues in the collagenous domain had a dramatic effect on the assembly of the multimeric isoform (20). The importance of the assembly of HMW isoforms has been shown in previous studies, where the ratio of HMW to total adiponectin was highly associated with insulin sensitivity and TZDs direct an increase in blood levels, especially an increase in HMW adiponectin (3, 15). Several isoforms of adiponectin circulate in blood, yet it has not been clear whether all forms of adiponectin are secreted by adipocytes, whether there is assembly of HMW in blood, or whether the HMW form is secreted, followed by degradation in blood.

Our data demonstrate that human adipose tissue secretes predominantly the HMW form. With the addition of pioglitazone in vitro, there was an increase in adiponectin secretion in a dose-dependent manner, which was accounted for by the increase in HMW adiponectin (Fig. 1).

DISCUSSION

These studies demonstrate for the first time the secretion of adiponectin isoforms from human adipose tissue and adipocytes as well as the secretory response of adipocytes to a TZD. With the use of sucrose gradient centrifugation and nondenaturing gel electrophoresis, the medium from human adipose tissue contained predominantly the HMW form of adiponectin, along with smaller amounts of LMW isoforms. With pioglitazone treatment in vitro, there was an increase in adiponectin secretion in a dose-dependent manner, which was accounted for by the increase in HMW adiponectin (Fig. 1).
complex. Thus these data suggest that either adipose tissue secretes a constitutive low level of lower molecular weight isoforms, or, alternatively, the HMW form is the only secreted form, followed by subsequent degradation to LMW isoforms. If the latter is the case, then these data suggest that pioglitazone increases the synthesis and secretion of the HMW form. This increase in secretion occurs through posttranscriptional mechanisms, because neither the addition of pioglitazone in vitro, as shown in the present study, nor the treatment of IGT subjects with pioglitazone (19) results in an increase in adiponectin mRNA levels. Another explanation for the increase in secreted HMW isoforms is a stabilization of the HMW structure by pioglitazone, and hence an inhibition of degradation.

A comparison among adipose tissue, primary adipocytes, and continuous adipocyte cell lines from human and mouse origins was performed to determine whether adiponectin expression in adipose tissue explants was similar to expression in isolated adipocyte cultures. Other adipose cells behave similarly to whole human adipose tissue, although there are important differences. As with whole fat, treatment of human adipocytes in vitro with pioglitazone demonstrated the tendency to form mostly HMW complexes, as LMW isoforms diminished, and the secreted form of adiponectin found in the medium with pioglitazone treatment was only the HMW isoform. Similar scenarios were found when we examined the adiponectin isoforms in mouse adipocytes and two different cell lines. In all cases pioglitazone served to increase the amount and secretion of HMW adiponectin. However, other adipose cell lines synthesize and secrete other isoforms, in addition to HMW, suggesting that human adipocytes are relatively unique in their ability to secrete adiponectin only after formation of the HMW complex.

In summary, these studies demonstrated an induction of adipose tissue secretion of HMW adiponectin by pioglitazone regardless of tissue or cell type investigated. Human adipose tissue treated with pioglitazone demonstrated increases in the HMW isoform. Because the HMW form is the primary secretory product of adipose tissue, these data suggest that consid-

Fig. 3. Effect of pioglitazone treatment on adiponectin isoforms. A: representative gels of 3T3-F422A conditioned medium from day 4 differentiated cells, treated with pioglitazone for 48 h, and, after sucrose gradient, analyzed by nondenaturing gel SDS-PAGE and Western blotting. B: mouse adipocyte conditioned medium, treated with pioglitazone for 24 h and analyzed in a similar manner. C: SGBS cells and conditioned medium from day 6 differentiated cells treated with pioglitazone at the concentrations indicated for 24 and 48 h, without sucrose gradient, analyzed by nondenaturing gel SDS-PAGE and Western blotting. The top and bottom of the sucrose gradient is noted in A and B, along with identification of the adiponectin bands referred to as HMW and LMW.
erable modification of adiponectin can be influenced by the application of pioglitazone.

GRANTS

This work was supported by a grant from the American Diabetes Association (to R. J. Owens), a Merit Review Grant (to P. A. Kern) and a VISION Career Development Award (to N. R) from the Department of Veterans Affairs, General Clinical Research Center Grant M01RR14288, and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-39176 and DK-71277 (to P. A. Kern).

REFERENCES