Recent studies have found that angiotensinogen is expressed in white and brown fat pads, and adipocytes have been implicated as a primary source of angiotensinogen in several other tissues. The functional significance of this unexpected expression is unknown. To address this, we studied angiotensinogen messenger RNA (mRNA) expression and angiotensinogen secretion in adipose tissue and isolated adipocytes comparing fasted and refed rodents and those with genetic obesity with normal controls.

Control 2-month-old Sprague-Dawley rats, those fasted for 3 days, or those fasted for 2 days and refed for 6 days were killed, and adipocytes were isolated from epididymal fat pads using collagenase digestion. Angiotensinogen mRNA was reduced to 14.6 ±2.3% of control levels under fasted conditions and increased to 228 ±53% of control levels after refeeding. Angiotensinogen release from adipocytes was reduced to 33% of control levels by fasting and increased to 183% by refeeding. These effects of fasting and refeeding on angiotensinogen regulation were tissue specific since liver angiotensinogen mRNA and serum angiotensinogen concentrations were unaffected. Systolic blood pressure, however, was modulated by fasting and refeeding in a manner parallel to adipocyte angiotensinogen expression. In related experiments, angiotensinogen secretion per epididymal fat pad of the ob/ob mouse model of obesity was increased an average of 3.4-fold compared with control. We conclude angiotensinogen expression in white adipocytes is regulated nutritionally in a tissue-specific manner. We propose that adipocyte angiotensinogen could play a previously unrecognized role in regulating adipose tissue blood supply and thereby fatty acid efflux from fat. The existence of such an adipocyte influence on vascular resistance might offer a pathological mechanism for fasting hypotension and hypertension of the obese.

Angiotensinogen (AT), the unique precursor of angiotensin I in the classic renin-angiotensin system (RAS) for regulation of aldosterone production and systemic blood pressure, is known to be produced most abundantly by liver. However, a large body of evidence has accumulated in the past decade suggesting that the components of the RAS are made and regulated in organs outside the classic liver-kidney-lung axis (for review, see Reference 1). One such “nonclassical” tissue now known to express AT is adipose tissue. In addition to white and brown fat depots, the cells producing AT in aorta, atria, mesentery, adrenals, and lung have been identified by microdissection and cell dispersion or in situ hybridization as being adipocytes or fibroblast-like cells, suggested to be preadipocytes. High levels of AT messenger RNA (mRNA) are expressed in fat pads from multiple anatomic sites, and AT mRNA has been shown to increase after nephrectomy and treatment with dexamethasone, ethynylestradiol, and triiodothyronine. AT secretion from whole fat pads has also been demonstrated in vitro. That this represents a differentiated function of adipocytes is strongly suggested by the appearance of AT synthesis and secretion upon differentiation of the 3T3-L1 and 3T3-F442A preadipocyte cell lines. AT mRNA expression in these cells increases over a time course of differentiation coordinate with other markers of adipocyte function.

The physiological or pathological significance of adipocyte AT production has yet to be determined. We therefore undertook studies to determine whether AT expression was regulated by several physiological or pathological states that predictably and markedly alter adipocyte function. In particular, we sought to determine whether adipocyte AT expression is altered in a specific manner in starvation and refeeding and in models of genetic obesity.

**Methods**

**Tissue**

Two- to three-month-old (weights given in each figure) male Sprague-Dawley CD strain (CD) rats (Charles River Laboratories, Wilmington, Mass.) fed ad libitum were fasted for 3 days (with 0.45% saline supplementation in experiments measuring AT or blood pressure) or were fasted for 2 days and refeeding for 6 days and then were killed by CO₂ asphyxiation. Blood was...
obtained by cardiac puncture for subsequent serum assays. Epididymal fat pads and liver were surgically removed and frozen in liquid N₂ or on solid CO₂ (for mRNA) or minced (for AT determinations) in Dulbecco's modified Eagle's medium (DMEM) plus 2% bovine serum albumin (BSA). The adipose tissue was digested in 1.5 mg/ml collagenase (Worthington Biochem Corp., Freehold, N.J.), and isolated adipocytes were prepared by flotation as previously described.⁸

Ad libitum–fed ob/ob or db/db mice (The Jackson Laboratory, Bar Harbor, Me.) and their age-matched (C57 BL/6J and C57 BL/KsJ) controls were treated similarly except that tissue was collected and incubated in 4% BSA. The minced tissue was washed with 1 L of 0.9% saline at 37°C, collected on a 250-μm nylon mesh, and used for AT protein determinations directly without preparation of isolated adipocytes.

**Angiotensinogen Messenger RNA**

RNA from fresh or flash frozen tissue was extracted with guanidinium isothiocyanate before CsCl centrifugation as previously described.⁹ Equal amounts of total RNA or RNA from equal numbers of cells was electrophoresed on agarose gels, blotted to nylon filters, and hybridized as previously reported with a phosphorus-32-labeled full-length AT¹0 complementary DNA (cDNA) probe, a gift from Dr. K. Lynch. Human β-actin¹¹ used as a control for liver preparations was a gift from Dr. P. Ponte.

**Angiotensinogen Protein**

The amount of AT was determined by exhaustive conversion with exogenous renin using standard conditions¹² followed by measurement of angiotensin I asayed in duplicate using a commercial radioimmunoassay (RIA) kit (New England Nuclear, Boston, Mass.). Briefly, isolated cells or minced tissue were incubated at 37°C with vigorous shaking in DMEM containing 2% bovine serum albumin maintained under 5% CO₂. Aliquots were removed every 30 minutes and frozen at −20°C. For rats, 0.002 units purified porcine renin was added and incubated for 1 hour. For mice, mouse submandibular gland renin was prepared by the method of Jacobsen and Poulsen¹³ to the acid precipitation step. The enzymatic activity was assayed by serial dilution, of Jacobsen and Poulsen¹³ to the acid precipitation step. The adequacy of renin conversion and inhibition of the angiotensin I degradation was confirmed in each mouse experiment by adding 2 nM AT (porcine, Sigma Chemical Co., St. Louis, Mo.) to one of duplicate samples and ensuring that the same amount was recovered. The assay was found to be linear with time and amount of tissue. Preliminary experiments using tetrodotoxin or EDTA to inhibit renin and angiotensinases during the tissue incubation step were not found to be beneficial, and these agents were not used routinely. There was essentially no detectable angiotensin I in the tissue incubation media without addition of exogenous renin.

**Cell Counting**

Isolated cells or fat pads were fixed with osmium, passed through a 250-μm nylon mesh, and collected on a 250-μm nylon mesh as previously published.¹⁴ Cell number was then determined by counting in a Coulter counter (model ZM, Coulter Corp., Hialeah, Fla.).

**Blood Pressure**

Rat systolic blood pressure was measured by tail plethysmography (model 59 with model 146 Artifit filter; IITC, Woodland Hills, Calif.); at least three determinations were averaged.

**Reagents**

All chemicals were reagent or analytical grade. [³¹P]dCTP and ¹²⁵I-angiotensin I were purchased from New England Nuclear.

**Results**

Figure 1 demonstrates the level of AT mRNA in total RNA from equal numbers of isolated epididymal adipocytes of 2-month-old CD rats fasted 3 days or fasted 2 days with 6 days of refeeding. Liver RNA (25 mg, equivalent to the amount of adipocyte RNA in the control lane) is run on an adjacent lane for comparison. Perhaps the most striking initial finding is that AT mRNA is more abundant relative to total RNA in isolated adipocytes than in control liver.

After 3 days of fasting, adipocyte AT mRNA is dramatically reduced to 14.6% ± 2.3% (mean ± SEM) of control animals as measured by densitometric analysis (n = 4 experiments). This effect of fasting appeared to be fully reversible. After 2 days of fasting and 6 days of ad libitum refeeding, AT mRNA was not only restored but was enhanced to 228% ± 53% of the control fed animals (n = 4 experiments). Thus, refeeding induces an approximately 16-fold increase in AT mRNA per cell compared with the fasted state.

To determine if these changes in AT mRNA were reflected in the adipocyte secretion of AT, adipocytes from a similar experiment using ad libitum fed, fasted...
FIGURE 2. Scatter plot shows effect of fasting and refeeding on adipocyte angiotensinogen (AT) expression. Male Sprague-Dawley, CD strain (CD) rats, initially weighing 200–225 g, were fed standard lab chow ad libitum (Control Fed), fed ad libitum for 5 days then fasted for 3 days (Fasted), or fasted 2 days then re-fed for 6 days (Refed). Epididymal adipocytes were isolated and incubated in Dulbecco’s modified Eagle’s medium with 2% bovine serum albumin. AT levels are expressed as the amount of angiotensin I (AI) produced from exhaustive conversion of AT with exogenous renin. Fed and refed conditions represent pooled adipocytes from two rats. Starved condition represents data from four pooled rats. Similar results were obtained in three other experiments.

TABLE 1. Nutritional Influence on Rat Serum Angiotensinogen

<table>
<thead>
<tr>
<th>Condition</th>
<th>AT (µg Ang I/ml)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>3.4±0.9</td>
<td>4</td>
</tr>
<tr>
<td>Fasted</td>
<td>3.3±1.0</td>
<td>4</td>
</tr>
<tr>
<td>Refed</td>
<td>3.0</td>
<td>2</td>
</tr>
</tbody>
</table>

Values are the mean±SEM for the indicated number of experiments. AT, angiotensinogen; Ang I, angiotensin I.

Second experiment) of control fed animals. These findings are consistent with the nutritional regulation of AT mRNA presented above.

In an attempt to assess whether the effect of fasting was tissue specific, livers of control, fasted, and re-fed rats were assayed for AT mRNA (Figure 3). By densitometry, no statistically significant effect of fasting or refeeding was observed on liver AT or β-actin mRNA. However, if normalized to actin mRNA, AT mRNA was overexpressed (p=0.0026) in fasting versus refeeding, in contrast to the dramatic decline in adipocytes. Since the liver is the predominant source of AT under basal conditions, it is not surprising that the serum levels of AT were similar in fed, fasted, and re-fed rats (Table 1). Similarly, serum renin assays performed on the NaCl-supplemented, fasted rats were not statistically different from the nonsupplemented fed or the re-fed animals (data not shown).

Since acute nutritional changes had such a marked effect on adipocyte AT production, chronic overnutrition was also evaluated by comparing genetically obese (ob/ob) mice with their lean controls. The results with the obese mice were similar to those seen during acute refeeding of fasted normal rats as seen above. As demonstrated in Figure 4, expressed per cell, there is an approximately twofold increase in AT production by minced epididymal fat from ob/ob mice compared with their C57 controls. However, this marked difference may underestimate the physiological or pathological significance of adipocyte AT secretion.

FIGURE 3. Northern blot shows liver expression of angiotensinogen (AT) messenger RNA (mRNA) in fasting and refeeding. Male Sprague-Dawley, CD strain (CD) rats weighing 270–305 g were fed ad libitum (Control), fed ad libitum for 5 days then fasted for 3 days (Fasted), or fasted 2 days then re-fed for 6 days (Refed). Upon asphyxiation, their total RNA was isolated and analyzed for AT or human β-actin (Actin) mRNA by Northern analysis. Each lane represents 5 µg of total RNA from one rat. The ethidium bromide (EtBr)–impregnated gel was photographed to confirm equal loading. No statistically significant effect of fasting or refeeding upon hepatic AT or β-actin mRNA expression was observed by densitometry, but the AT/actin ratio was statistically higher in fasted versus refed animals. The absence of a direct influence of fasting on AT mRNA but its overexpression relative to actin mRNA was confirmed in a second experiment.
FIGURE 4. Scatter plot shows epididymal fat angiotensinogen (AT) production in ob/ob mice and controls. Minced epididymal fat from five 4-month-old ob/ob (56–63 g) or control (33–36 g) mice were pooled and incubated at 37°C in Dulbecco's modified Eagle's medium with 4% bovine serum albumin. Samples of media were removed at the indicated times and analyzed for AT, expressed per total fat pad in the lower panel and per cell in the upper panel. AT, angiotensin I.

when the adipocyte hyperplasia of this model is taken into account, expressed per fat pad AT production is 3.4-fold higher in ob/ob mice (n=4 experiments). Similar preliminary studies with older db/db obese-diabetic mice revealed a 2.4-fold increase in AT per cell (data not shown) and a 4.4-fold increase in AT production per fat pad (n=2 experiments).

Because the RAS plays a significant role in blood pressure regulation, the observed nutritional effects on adipocyte AT were correlated to their effects on blood pressure. As Figure 5 demonstrates, animals that were fasted beginning day 1 or day 6 demonstrated a prompt fall in weight and blood pressure. On refeeding fasted rats (beginning day 3), blood pressure rose to levels that were ultimately significantly higher than control fed animals by the time the animals were killed. These observations are consistent with a potential role for adipocyte angiotensinogen modulation in the nutritional regulation of blood pressure.

Discussion

Previous investigations have suggested that AT mRNA is expressed in fat in a differentiation-dependent manner as preadipocytes become adipocytes in culture. In the present study we confirmed that AT mRNA is expressed in isolated white adipocytes and demonstrated that this mRNA is translated into the biologically active protein. Further, we documented that AT mRNA is more relatively abundant in adipocytes than in liver, the primary source of AT under basal conditions.

More significantly, both AT mRNA and protein expression in adipocytes are dramatically decreased in fasting and significantly elevated on refeeding, correlating with the effect of fasting and refeeding on blood pressure. This nutritional regulation appears to be specific for adipose tissue and is not reflected in liver AT mRNA or apparently in serum AT concentration. To evaluate the effect of chronic overnutrition, we used the ob/ob mouse model. This extensively investigated model of obesity involves an as yet undetermined autosomal recessive mutation on chromosome 6. These mice are characterized by progressive obesity, hyperphagia, defective thermogenesis, insulin resistance, hyperglycemia, hyperinsulinemia, and fat pad hyperplasia as well as hypertrophy. We found that in these mice, AT secretion per cell is doubled and is more pronouncedly increased per fat pad. The same appears to be true for the db/db mouse model, a chromosome 4 recessive mutation sharing many features with the ob/ob model but whose serum insulin falls dramatically after the third month, resulting in more marked hyperglycemia.

Although these results strongly suggest a physiological role for adipocyte AT production, at this point the molecular mechanisms responsible for its regulation and the purpose of its local regulated secretion are unknown. However, interpreted in light of other studies, the findings presented here are consistent with a hypothetical model. The K_d of renin for AT is approximately equal to the physiological concentration of AT implying that changes in either renin or AT concentration will be sensitively reflected in angiotensin I produced. As a simple hypothesis, we suggest that adipocyte AT
Fatty acid efflux into the circulation causes increased reesterification in the perfusate, contrasting markedly with their production exerts its effects through local changes in angiotensin I and (since the converting enzyme is not usually rate limiting) angiotensin II (Ang II). Since Ang II is a potent physiological regulator of arterial constriction, adipocyte AT may thus affect (at least) local blood flow.

Studies by Nakamaru et al. using the isolated perfused superior mesenteric artery system, a tissue where the AT synthesis is documented to be from fat, provided evidence 1) that a complete local RAS exists in the isolated preparation and 2) that the local system, including AT presumably synthesized by adipocytes, can have an effect on vascular resistance. Further studies demonstrated that conditions that altered Ang II production and vascular resistance were unrelated to renin levels in the perfusate, contrasting markedly with their findings in perfused kidney where secreted Ang II and renin are linearly correlated. The simplest explanation for their findings is that the regulation of Ang II levels and vascular resistance are mediated by alterations in the local secretion of AT by adipocytes.

A potential physiological role for AT in the regulation of fat pad arterial constriction can be envisioned from investigations of Belfrage et al. In perfused canine subcutaneous adipose tissue, exogenous Ang II had an effect similar to partial surgical ligation of the feeding artery: inhibiting glucose uptake and decreasing the outflow of fatty acids on sympathetic stimulation. Subsequent work in the same tissue by Bulow and Madsen demonstrated that the free fatty acid efflux from fat was correlated to blood flow over the physiological range.

Leibel, Hirsch, and colleagues have recently provided data suggesting that adipose tissue blood flow may be physiologically relevant in humans. Previous studies have documented that acute glucose and insulin concentration substantially decreased blood flow in humans and rat fat pads. They found that in fasted humans, infusion of glucose with or without insulin caused a marked reduction in plasma free fatty acid concentration due not to a significant change in lipolysis but to increased fatty acid reesterification. This nutritional increase in reesterification was lost under in vitro conditions of adipocyte isolation and dispersion. The authors concluded that these results are consistent with reesterification rates being proportional to the local extracellular concentration of fatty acids from recent lipolysis, which is dependent on local blood flow.

In light of these studies, the data presented here can be interpreted within the following novel hypothesis (Figure 6). Under fasting conditions, adipocyte AT and thus local Ang II are markedly reduced. This results in the observed increased fat pad perfusion and fatty acid efflux into the systemic circulation, facilitating a fatty acid-based metabolism. Under conditions of refeeding and genetic obesity, the increased adipocyte AT production leads to increased Ang II production, arterial constriction, and thus decreased fat pad perfusion. This leads to opposing effects on the lipid economy of the fat pad: 1) decreased fatty acid efflux, facilitating a glucose-based intermediary metabolism in other tissues and reduced fat pad depletion, and 2) a decreased glucose uptake, an effect that would tend to inhibit further lipid accretion thus resisting fat pad accretion. Al, angiotensin I.

The hypothesis presented here raises questions about the potential impact of angiotensin converting enzyme (ACE) inhibitors on adipocyte metabolism and energy homeostasis. Although the drugs as a class are not viewed as having an impact on the adipocyte, it is interesting that in some studies in humans, use of ACE inhibitors has been associated with significant weight loss. Similarly, in spontaneously hypertensive rats and Sprague-Dawley rats, use of these drugs has inhibited normal weight gain, in the latter case despite control levels of food intake. In the proposed framework it would be anticipated that ACE inhibitors might prevent the effect of enhanced AT production by overnutrition, leading to increased adipose tissue blood flow under these conditions. Increased blood flow would be anticipated to result, however, in compensatory changes: 1) increasing glucose and fatty acid uptake...
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(adipose tissue accretion) and 2) increasing fatty acid efflux (adipose tissue depletion). The literature cited suggests the latter effect (increased fatty acid efflux) predominates, but effects on weight might be partially opposed by increased adipocyte nutrient uptake.

Should the hypothesis presented be correct, this physiological process might contribute to some known pathological phenomena. Since fat surrounds many of the muscular arteries (e.g., the mesenteric artery used by Nakamaru et al19-20), paracrine or local endocrine changes in AT and thus Ang II might have effects on systemic vascular resistance. Specifically the changes in adipocyte AT production observed here, presumably also occurring in perivascular fat, may substantially contribute to the effect of caloric restriction to reduce blood pressure (Figure 5),32 and to hypertension of the obese.33

In summary, results presented here support the concept that AT is not only synthesized but is also physiologically regulated in adipocytes. Its production is reduced in fasting and enhanced in states of overfeeding. In conjunction with previous studies, a model is proposed suggesting that this is a homeostatic mechanism to cause facilitation from a glucose- to fatty acid-based metabolism in fasting and resist further obesity in states of overfeeding. We also speculate that this could have pathological significance in the hypotension of fasting and in hypertension of the obese. Further studies are clearly needed to investigate these hypotheses and determine the molecular mechanisms of adipocyte AT regulation.

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