Oscillations of Fatty Acid and Glycerol Release From Human Subcutaneous Adipose Tissue In Vivo

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We sought evidence for pulsatility of lipolysis in human subcutaneous adipose tissue in vivo. Arterialized and adipose tissue venous blood samples were drawn at 2-min intervals from nine healthy subjects. This procedure was repeated during hyperinsulinemic-euglycemic clamp to remove insulin pulsatility. We found evidence for pulsatile release of both nonesterified fatty acids (NEFAs) (seven of nine subjects) and glycerol (five of six subjects) with a period of −12–14 min. This pulsatility was maintained even during the hyperinsulinemic clamp. Checks were made for spurious pulse detection, including the creation of “mock” venoarterialized differences by subtracting one subject’s arterialized concentrations from another’s venous; the peaks detected were less consistent in character than with real data (peak width, P = 0.006; peak interval, P < 0.004). Significant cross-correlations between NEFA and glycerol release also provided evidence of a real effect. Arterial norepinephrine concentrations were also pulsatile, but the period did not match that of NEFA and glycerol release. Insulin concentrations were pulsatile with a typical period of 12 min, but this was not significantly cross-correlated with lipolysis. We conclude that release from adipose tissue of the products of lipolysis is pulsatile in humans. Diabetes 54:1297–1303, 2005

It is increasingly recognized that metabolic processes do not operate at steady rates. Oscillations in glycolytic flux, synchronized across cells, have long been recognized (1). Pulsatile secretion of hormones is also well established, and for insulin, for example, there is a clear oscillatory pattern with a characteristic period of 12–13 min observable in systemic plasma (2,3). Among the metabolic processes regulated by insulin is adipose tissue lipolysis. The subcutaneous up-fat depot every 2 min at steady state. Adrenergic stimulation of lipolysis in humans is brought about mainly by β1- and β2-adrenoceptors (8), so it is not possible to block it systemically without causing cardiovascular side effects. We therefore measured plasma norepinephrine concentrations in arterial and adipose tissue venous plasma as an index of systemic and local sympathetic activity and sought relationships between these measurements and lipolysis. Because it seems intrinsically likely that oscillatory insulin concentrations will affect lipolysis, we also used a constant insulin infusion with the euglycemic-hyperinsulinemic clamp technique to remove this possible stimulus.

Rapid oscillations of insulin secretion are seen in obese as well as normal-weight subjects (3) but are attenuated in relatives of patients with type 2 diabetes (9) and absent in type 2 diabetes (10). They are somewhat restored with weight loss (10). To obtain some preliminary information on the effects of obesity on any lipolytic pulsatility observed, we included subjects with a wide range of adiposity.

RESEARCH DESIGN AND METHODS
Nine healthy subjects (five men), covering a range of adiposity levels (BMI 19.7–50.8, median 23.8 kg/m²), were recruited. Their ages ranged from 21 to 61 years (median 43). The subjects were healthy and receiving no medication. The protocol was approved by the Oxfordshire Clinical Research Ethics
Committee and the East London and City Ethics Committee, and all subjects gave written informed consent.

Subjects were studied after an overnight fast. A 10-cm, 22-gauge Hydrocat catheter (Becton Dickinson, Oxford, U.K.) was introduced over a guidewire into a superficial vein on the anterior abdominal wall and threaded toward the groin so that its tip lay just superior to the inguinal ligament. This provided access to the subcutaneous abdominal adipose tissue, which was uncontaminated by muscle drainage, with a relatively minor contribution from skin (11). A retrograde cannula was placed in a vein draining the hand, which was warmed in a hot-air box maintained at 60°C to obtain arterialized blood. The cannulae were kept patent by a slow infusion of 0.9% (wt/vol) saline. After a 30-min resting period, blood was taken with careful timing in order to ensure simultaneous sampling from the arterIALIZED and adipose tissue venous lines and was repeated every 2 min for 60 min.

After this 60-min sampling period, blood was not taken for 2 min to 60 min. Clamps were not performed in three subjects.

Adipose tissue blood flow (ATBF) was measured during the entire experiment by registering the washout of 133Xe injected into the subcutaneous adipose tissue, which was uncontaminated by muscle drainage, with a relatively minor contribution from skin (11). A retrograde cannula was placed in a vein draining the hand, which was warmed in a hot-air box maintained at 60°C to obtain arterialized blood. The cannulae were kept patent by a slow infusion of 0.9% (wt/vol) saline. After a 30-min resting period, blood was taken with careful timing in order to ensure simultaneous sampling from the arterIALIZED and adipose tissue venous lines and was repeated every 2 min for 60 min. Clamps were not performed in three subjects.

Plasma insulin was measured using a double-antibody radioimmunoassay (Pharmacia and Upjohn, Amersham, U.K.). Plasma norepinephrine was measured by high-performance liquid chromatography with electrochemical detection (15) on the 30 replicate measurements (each in duplicate) of a plasma sample described above. We used a conservative value of 5% based on experience of variation between duplicates.

We sought evidence for pulsatility in arterIALIZED and adipose tissue venous concentrations, as well as in the venoarterialized difference for plasma NEFA and glycerol. The former was measured as a simple measure of release from adipose tissue. We did not attempt to calculate absolute flux rates (venoarterIALIZED difference × blood flow) because, as described in RESULTS, measurements of ATBF over 2-min intervals turned out to be unreliable. For norepinephrine, there was generally net uptake across adipose tissue, and we calculated arteriovenous differences.

For ATBF, Mediscint software (Oakfield Instruments, Eyesham, U.K.) was used to record counts per second (cps) over 20-s intervals. We took these data into Microsoft Excel and calculated averages over 2-min periods to correspond with blood samples and over 10-min periods to calculate mean ATBF values. ATBF is proportional to the (negative) slope of the log(cps) versus time plot.

Evidence for pulsatility was sought as follows. The program Cluster 7 (http://nmjohnson.pharm.virginia.edu/pulse_xp/downloads.php) (16) was used following the guidelines supplied with the program. This was done in order to test the effect of choosing different sets of data. The latter made the program more sensitive at finding some peaks but did not consistently alter the characteristics of the peaks found (width, interval, etc.). The half-life of plasma norepinephrine in normotensive subjects was 19 ± 5 min (19,20), so for norepinephrine concentrations, we set the number of points at 1/3. Then, for each subject, Cluster 7 calculated the number of significant peaks discovered and their characteristics. This was done separately for the fasting and clamp periods (i.e., 30 points each).

We tested the reliability of this approach in a number of ways. First, we applied Cluster 7 to 30 repeated analyses of plasma NEFA from one fasting sample, and similarly to 30 repeated analyses of blood glycerol. In the latter case, 30 separate aliquots of one blood sample were extracted with perchloric acid (as described earlier) for analysis. We also created “mock” venoarterialized differences for NEFA by subtracting one subject’s arterIALIZED concentrations from the next subject’s venous NEFA concentrations and tested these for pulsatility. We created two mock datasets (subject A venous minus subject B arterialized, subject A venous minus subject C arterialized) for each real dataset (subject A venous minus subject A arterialized). This was done to reduce the impact of apparent pulses seen when pairing data from mismatched subjects (e.g., lean man with obese woman, etc.).

Second, we attempted to validate the observed pulsatility using the technique of cross-correlation analysis. Cross-correlation was also used to test for significant temporal relationships between variables. In cross-correlation, two datasets are lined up and correlated to each other. One dataset is the “mother” and one time point relative to the other (2 min in our data), and the correlation is repeated; this is repeated several times. If there are significant temporal relationships between the datasets, significant correlations will be observed with a lag (number of points moved) corresponding to the time delay between maximal coincidence. If there is a similar baseline trend in both variables (e.g., downward drift with time), then spurious significant cross-correlations will be generated. To avoid this, we transformed the data for cross-correlations by “differencing” (replacing each point with the difference between it and the previous point). We argued that if we applied cross-correlation analysis to independently measured variables, it would tell whether the variations we observed were simply random or were indeed related to some underlying process. Cross-correlations were performed using SPSS for Windows (release 11.5.0; SPSS, Chicago, IL).

The within-batch analytical variation of the methods was assessed for Cluster 7. For plasma NEFA, the coefficient of variation (CV) was 1.4% based on the 30 replicate measurements (each in duplicate) of a plasma sample described above. We used a conservative value of 3% for Cluster 7. For blood glycerol, the CV was 7.2%, again based on the 30 replicate measurements (each in duplicate) described above. We used a value of 10% for Cluster 7. For plasma insulin and norepinephrine, we used a value of 5%, based on experience of variation between duplicates.

RESULTS

Plasma and blood concentrations. Plasma and blood concentrations, averaged over the 60-min fasting and clamp periods in each individual are shown in Table 1. As expected, there was marked output of both NEFA and glycerol from subcutaneous adipose tissue in the fasting state, and this was suppressed to a large degree during the clamp. Arterialized plasma norepinephrine concentrations were not altered by the clamp.

ATBF. Detailed analysis of ATBF showed that estimates over 2-min periods have a CV >50%. It is not, therefore, possible to generate reliable results on ATBF using Cluster 7. Thus, we did not further analyze ATBF for evidence of pulsatility. Mean ATBF was not altered by the clamp (Table 1).

Evidence for pulsatility

Fasting state. Analysis with Cluster 7, as described in RESEARCH DESIGN AND METHODS, showed inconsistent evidence for pulsatile behavior of arterialized NEFA and glycerol concentrations. Not all subjects showed significant pulses, and the estimated period was extremely variable from person to person. However, there was much more consistent evidence for pulsatile release of NEFA and glycerol from the subcutaneous abdominal adipose depot, as mea-
sured by the venoarterialized difference: seven of nine and five of six subjects showed more than one peak for NEFA and glycerol release, respectively (Table 2). The period was \(~12–14\) min. Norepinephrine showed clear evidence of repeated peaks in every subject for arterialized, adipose venous, and the arteriovenous difference, with a period \(~6–8\) min (Table 2). Arterialized insulin concentrations showed clear pulsatility in all subjects, as expected, with a typical period of 12 min (Table 2).

To test whether the apparent pulsatility was indeed real for NEFA and glycerol, we examined cross-correlations between independent measurements, arguing that random fluctuations in the data would not result in significant cross-correlations. For this, we used differenced data to eliminate baseline trends as described in DESIGN AND METHODS. We examined the cross-correlation of arterialized with adipose venous NEFA concentrations in nine people. Five showed significant positive cross-correlations with no lag (\(P < 0.05\) in two subjects, \(P < 0.01\) in one subject, and \(P < 0.001\) in two subjects). For arterialized and adipose venous norepinephrine (four subjects available), three subjects showed significant cross-correlation with no lag (\(P < 0.05\) in two subjects and \(P < 0.01\) in one subject). Finally, to examine completely independent metabolic variables, we looked at the venoarterialized differences for NEFA and glycerol. Data were available in six subjects. In three subjects, there were significant positive cross-correlations with no lag (\(P < 0.05\) in one subject and \(P < 0.001\) in two subjects), and in two others, there were significant positive cross-correlations with lag \(~10\) min (\(P < 0.05\)).

We attempted to use cross-correlation analysis to show the physiological origin of the pulsatility observed. We cross-correlated venoarterialized differences for NEFA and glycerol with arterialized norepinephrine and insulin concentrations. No consistent patterns were observed for norepinephrine. There was a tendency to a positive cross-correlation between venoarterialized NEFA and arterialized insulin concentrations. Of seven people analyzed, six showed positive cross-correlations (of whom four were at \(P < 0.05\)), but one showed a significant negative cross-correlation (\(P < 0.02\)).

There was no obvious relationship between pulsatility and BMI. Pulses were observed in both lean and obese subjects (Figs. 1 and 2). If the subjects were divided into tertiles of BMI, the median number of pulses for NEFA venoarterialized difference was the same (three) in the lowest tertile (median BMI 21.1 kg/m\(^2\)) and the upper tertile (median BMI 37.3 kg/m\(^2\)).

**Checks for reliability.** We applied Cluster 7 to 30 repeated measurements of plasma NEFA and blood glycerol from a single blood sample, as described in DESIGN AND METHODS. The program did not detect any pulses, and there was no cross-correlation detected between NEFA and glycerol measurements.

Cluster 7 was also applied to the “mock” NEFA venoarterialized differences created by subtracting one subject’s arterialized concentrations from the next subject’s venous concentrations (Table 2). Cluster 7 detected fewer pulses in the “mock” NEFA venoarterialized differences than in the true data (\(P < 0.05\), Mann Whitney test), and the characteristics of the pulses found were far less consistent.
PULSATILE LIPOLYSIS IN HUMANS

TABLE 2
Analysis of pulsatility using Cluster 7 during the fasting period

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Subjects available (n)</th>
<th>&gt;1 peak found (n)</th>
<th>Median no. of peaks</th>
<th>&gt;1 peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEFA (arterialized)</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>15.4</td>
</tr>
<tr>
<td>NEFA (adipose venous)</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>11.5</td>
</tr>
<tr>
<td>NEFA (V-A difference)</td>
<td>9</td>
<td>7</td>
<td>3</td>
<td>12.0</td>
</tr>
<tr>
<td>Mock NEFA (V-A difference)</td>
<td>18</td>
<td>10</td>
<td>2*</td>
<td>14</td>
</tr>
<tr>
<td>Glycerol (arterialized)</td>
<td>6</td>
<td>3</td>
<td>2.5</td>
<td>12.7</td>
</tr>
<tr>
<td>Glycerol (adipose venous)</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol (V-A difference)</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Norepinephrine (arterialized)</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>7.1</td>
</tr>
<tr>
<td>Norepinephrine (adipose venous)</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>8.0</td>
</tr>
<tr>
<td>Norepinephrine (V-A difference)</td>
<td>4</td>
<td>4</td>
<td>6.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Glucose (arterialized)</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (arterialized)</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

V-A difference, venoarterialized difference (adipose tissue venous minus arterIALIZED concentration). Mock NEFA (V-A difference) created by subtracting one subject’s arterialized concentration from another subject’s venous (see RESEARCH DESIGN AND METHODS). *Number of peaks detected less for “mock” than for real data (P < 0.05); †significantly greater variation in peak interval compared with true NEFA (V-A difference) (P < 0.01).

(Table 2), with significantly greater variation in the reported peak width (P = 0.006) and peak interval (P < 0.004) (F tests from SPSS).

Hyperinsulinemic clamp. During insulin infusion, there was surprisingly more consistent evidence for pulsatile behavior of arterialized NEFA and glycerol concentrations, with all subjects showing more than one peak during the period of observation and with reasonably consistent periods of ~12–14 min (Table 3). There was again consistent evidence for pulsatile release from the subcutaneous abdominal adipose depot, as measured by the venoarterialized difference (six of seven and six of six subjects showing more than one peak for NEFA and glycerol, respectively). The period was again ~10–15 min. As in the baseline period, norepinephrine showed clear evidence of repeated peaks in every subject for arterialized, adipose venous, and the arterialized-venous difference, with a period ~7–10 min.

Once again, we applied cross-correlation using differentiated data during the clamp period to assess the reliability of the observed pulsatility. Cross-correlations were generally not so consistent as in the fasting state, but that was to be expected given the smaller concentrations measured. For the cross-correlation of arterialized with adipose venous NEFA concentrations, there were data from seven people, three of whom showed significant positive cross-correlations with no lag or a lag of 2 min (P < 0.05 in one subject, P < 0.01 in one subject, and P < 0.001 in one subject). For the venoarterialized differences for NEFA and glycerol, data were available in six subjects. In two, there were significant cross-correlations with no lag or 2-min lag (P < 0.05 in one and P < 0.001 in the other).

DISCUSSION

Our main finding was that there were multiple pieces of evidence for pulsatile release of NEFA and glycerol from human subcutaneous adipose tissue in vivo. In contrast, we found inconsistent evidence for pulsatility of systemic NEFA and glycerol concentrations, even though this has been found for NEFA in dogs (5). This may imply that in humans, the different adipose depots that contribute to the systemic plasma NEFA pool are not coordinated in their pulsatility. We must also consider, however, that an even more precise NEFA assay, perhaps applied over a longer period, might have revealed pulsatility of systemic concentrations. In terms of NEFA and glycerol release, we also have to remember that the transit time for blood through the tissue bed is finite and that simultaneous blood sampling (arterialized and adipose venous) might not be absolutely appropriate. However, we believe the transit time is probably short in relation to the time it takes to draw a sample: blood flow is high in relation to tissue fluid content.

We sought evidence for the main regulator of lipolytic pulsatility, but our results do not give firm conclusions in this respect. While the period of pulsatility of NEFA release was strikingly similar to that of insulin concentrations, pulsatility of NEFA release was maintained even at the suppressed levels seen during the hyperinsulinemic clamp, when insulin concentrations were steady. This seems to rule out insulin as the main stimulus. It would have been interesting to clamp insulin at fasting concentrations using somatostatin, but at this stage of investigation, we did not want to alter the secretion of other peptide hormones potentially involved. Although there would have been some residual endogenous (and potentially pulsatile) insulin secretion during the clamp, this would have been minor compared with the exogenous insulin.

Taking plasma norepinephrine concentrations as an index of sympathetic nervous system activity, there was clear and consistent evidence for pulsatility, and it would seem likely in principle that this could be a major driver of lipolytic pulsatility, as in dogs (4,5). However, the period of norepinephrine pulsatility was consistently shorter than that for lipolysis, and no strong cross-correlations were observed between the two variables. At the whole-body level, there is a correlation between plasma norepinephrine concentration and sympathetic nerve activity recorded in the peroneal nerve in the resting state (21). However, subsequent studies have shown a regional inhomogeneity of sympathetic nervous system responses to interventions such as feeding, where increased norepinephrine spillover is observed in some tissues or organs but not others (22). The lack of a relationship between oscillations in plasma norepinephrine and in venoarterialized differences for
The alternative explanation is that there is some communication between adipocytes that leads to coordinated lipolysis. This type of communication is well established in other cell types. Yeast cells in suspensions show coordinated oscillations in glycolytic flux (1). Pancreatic islets in perfusion systems or in a perfused pancreas will show coordinated oscillations in glycolysis, intracellular Ca²⁺ concentrations, and insulin secretion (23–25). However, it is difficult to imagine how signals could diffuse between adipocytes that may be several centimeters apart sufficiently rapidly to produce the periodicity observed here.

One concern in interpreting such data is the detection of spurious pulsatility. Our checks with repeated measurements of one sample gave us confidence that Cluster 7 will not always detect pulses, as did the lack of pulsatility in glycerol or NEFA indicates that any pulsatility in sympathetic nervous system outflow to abdominal subcutaneous adipose tissue is not in synchrony with the oscillations seen in arterial norepinephrine. This is not too surprising, since the major source of arterial norepinephrine is likely to be spillover from sympathetic nerves innervating the vasculature, and one would expect any physiological variation in norepinephrine release to be related to variations in blood pressure rather than in metabolic variables such as adipose tissue lipolysis.
found in plasma glucose concentration. It was also clear in the fasting state that arterIALIZED NEFA and glycerol concentrations gave less consistent pulsatility than did the corresponding venoarterialized differences. We also tried to test validity of the reported pulses by various types of randomization. For instance, we took each subject’s data (e.g., for arterialized NEFA concentrations) and randomized the time points (data not shown). We also created mock venoarterialized differences by subtracting one subject’s arterIALIZED concentrations from another subject’s venous concentration. However, we found that such randomization usually led to Cluster 7 detecting some pulses, albeit significantly less consistent in character than with the original data. The reason for this must be that a truly pulsatile dataset contains values that are significantly above or below mean values (i.e., by more than analytical variation), and even after randomization, these values will sometimes by chance associate to give peaks or nadirs. We consider the data and statistical tests suggest real pulses were present, however, because of the strong independent evidence from cross-correlation analysis. The significant cross-correlations observed between venoarterialized differences for NEFA and glycerol, for instance, can only easily be explained by true pulsatile lipolysis in the subcutaneous abdominal adipose depot.

We had hoped to test the pulsatility of ATBF, but when the data were examined in detail, it became clear that estimates of ATBF over 2-min intervals from $^{133}$Xe-washout curves are highly variable and not satisfactory for pulsatility analysis. It remains a possibility that the pulsatility of NEFA and glycerol release that we observed in fact represents pulsatility of ATBF superimposed on a constant rate of lipolysis. It will be difficult to test this with existing methodologies as the time resolution is inadequate. We did not observe any differences in pulsatility of lipolysis between lean and obese subjects. As outlined above, insulin pulsatility is retained in obesity but lost in type 2 diabetes. It would be interesting to extend these studies to diabetic patients and also to patients with disorders of lipid metabolism in future work.

We conclude that there is pulsatile release of NEFA and glycerol from human subcutaneous adipose tissue in vivo. We are unable to pinpoint the stimulus for this pulsatility, although insulin seems to be excluded because of the retention of pulsatility during a hyperinsulinemic clamp. Pulsatility of NEFA release is maintained in obese subjects, although we have not yet examined patients with type 2 diabetes. Our results have implications for understanding the regulation of lipolysis in humans.

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NOTE ADDED IN PROOF

Since the acceptance of this article, an article has appeared in Diabetes (Getty-Kaushik L, Richard AM, Corkey BE: Free fatty acid regulation of glucose-dependent intrinsic oscillatory lipolysis in perfused isolated rat adipocytes. Diabetes 54:629–637, 2005) that demonstrates oscillations in lipolysis in perfused rat adipocytes. This work suggests an intrinsic component to lipolytic oscillations that would be independent of insulin or catecholamine regulation.

REFERENCES


