Sustained effects of repeated restraint stress on muscle and adipocyte metabolism in high-fat-fed rats

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ZHOU, JUN, XIAOLANG YAN, DONNA H. RYAN, AND RUTH B. S. HARRIS. Sustained effects of repeated restraint stress on muscle and adipocyte metabolism in high-fat-fed rats. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R757-R766, 1999.—Repeated restraint stress 3 h/day for 3 days in rats causes a temporary hypophagia but a sustained weight loss. We investigated whether poststress changes in peripheral tissue metabolism contributed to these responses. One day after the last restraint, insulin sensitivity, measured by oral glucose tolerance test, was improved in restrained rats. Restraint and pair-fed rats weighed less than controls, but body fat content was the same in all groups. Muscle glucose uptake, measured in vitro, was not changed by treatment, whereas in vitro adipocyte glucose uptake was substantially inhibited only in restrained rats. Adipocytes from restrained rats had elevated rates of fatty acid oxidation but not fatty acid esterification, indicating a shift in energy supply from glucose to fatty acids. Five days after the last restraint, the reduced weight of restrained and pair-fed rats resulted from loss of both lean and fat tissue. These results demonstrate that restraint caused sustained, tissue-specific changes in metabolism that may contribute to changes in body composition and body weight of the rats.

glucose tolerance; glucose transport; fatty acid oxidation; body composition

STRESS IS A STATE of threatened homeostasis that causes a variety of changes in the central nervous, endocrine, and immune systems and in peripheral tissue metabolism (3, 19, 30). Repeated restraint stress is a mixed psychological and physiological stress that has been used as a model for depression and anorexia nervosa (33). We previously reported that repeated restraint stress (3 h/day for 3 consecutive days) suppressed food intake and body weight (10). This suppression of body weight was observed immediately after stress and was sustained for an extended period (10). The stress-induced reduction in food intake lasted for up to 1 wk after the stress was ended, and there was no compensatory hyperphagia, a response that is usually observed in food-deprived animals (8, 10). Because the rebound hyperphagia was absent in restrained rats, their body weight remained lower than that of control rats, even 40 days after the end of restraint stress (10). These results indicated that repeated restraint stress, in addition to causing acute responses, had sustained, poststress effects on food intake and body weight by mechanisms that have not been elucidated. Most investigations of stress-induced changes in feeding behavior have focused on central mechanisms that are both activated during stress and that are involved in the regulation food intake. These include the neurotransmitter monoamines corticotrophin-releasing hormone, neuropeptide Y (NPY), and serotonin (5, 11, 29). In a previous study, we demonstrated that hypothalamic monoamines, NPY, and peripheral corticosterone were at control levels 2 h after the end of 3 h of restraint stress (28). Therefore, changes in these systems can successfully explain the hypophagia that immediately follows restraint stress but fail to explain the prolonged hypophagia and the absence of compensatory hyperphagia during the poststress period.

Because repeated restraint stress results in sustained changes in body weight, even when food intake has returned to control levels, we hypothesized that there were poststress effects on peripheral tissue metabolism and nutrient partitioning, which could contribute to the absence of rebound hyperphagia and the reduced body weight of the rats. Therefore, we investigated the effects of repeated restraint stress on whole body glucose uptake and the utilization of glucose and fatty acids in muscle tissue and adipocytes 1 day after the end of repeated restraint stress.

METHODS

Adult (12-wk-old) male, Sprague-Dawley rats, weighing 350 g, were obtained from Harlan Sprague Dawley (Houston, TX) and were housed in individual wire mesh cages in a humidity- and temperature-controlled room (22 ± 2°C, 65-67% humidity) on a 12:12-h light-dark cycle with lights on at 0700. Body weights and food intakes were recorded daily in each of four experiments, described in detail below. All food intakes, including those of pair-fed rats, were corrected for spillage. At the end of each experiment, the rats were killed by decapitation, and serum was collected for analysis, except in experiment 3. All animal protocols were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

For the repeated restraint stress protocol, rats were placed in Perspex restraining tubes (Plas Laboratories, Lansing, MI) for 3 h in the morning for 3 consecutive days. The control and pair-fed rats were moved to the same room as the restraint rats and did not have access to food or water for the period of restraint. Pair-fed rats had diet and water ad libitum before stressed rats were restrained but were pair fed to restrained rats from the first day of restraint until the end of the experiment. Experiments involving measurement of tissue metabolism were completed with the rats subdivided into groups, and the restraint protocol was staggered over 3–4 days to ensure timely collection and handling of tissue and facilitating pair feeding to the voluntary intake of restrained animals.

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Repeated measurements of variance were used for body weights, food intakes, glucose tolerance test, glucose transport, fatty acid oxidation, and fatty acid esterification with day, time, or insulin concentration as the repeated measure. The rest of the data were analyzed by ANOVA with post hoc Duncan’s multiple range test. The SAS system version 6.12 was used for computations. Data are presented as means ± SE.

Experiment 1: Effect of Repeated Restraint Stress on Oral Glucose Tolerance Measured 1 Day After the Last Restraint

This experiment investigated poststress effects on whole body glucose clearance using an oral glucose tolerance test (OGTT) in rats fed diets of different fat content, 1 day after the last restraint in the protocol. Diet composition is indicated in Table 1. The diet designated as low fat contained 10% kcal fat and 3.66 kcal/g energy, and the high-fat diet contained 40% kcal fat and 5.00 kcal/g energy.

Thirty six rats were maintained on a low-fat diet and tap water ad libitum for 6 days and then were divided into the following two groups: one group remained on a low-fat diet for another 7 days and the other was fed a high-fat diet for the same period. Both dietary treatments were further divided into two weight-matched groups: high-fat control, low-fat control, high-fat restraint, and low-fat restraint.

One day after the end of repeated restraint, all rats were food deprived for 5 h, and a small amount of blood (300 μl) was taken by tail bleeding. Immediately after the first tail bleeding, each rat was gavaged with glucose solution (2.5 g/kg body weight). Additional blood samples were collected 15, 30, 45, and 60 min after glucose administration and were analyzed for serum insulin (Rat Insulin RIA kit; Linco, St. Louis, MO) and glucose (Sigma Diagnostic Kit 510; Sigma Chemical, St. Louis, MO). Daily measurements of food intake and body weight were ended 5 days after the last restraint.

Experiment 2: Effects of Repeated Restraint Stress on Liver and Body Composition Measured 5 Days After the Last Restraint

This experiment determined the poststress effects of repeated restraint on glucose tolerance, liver lipid and glycogen content, and body composition of rats fed a high-fat diet. An OGTT was performed 1 day after the last repeated restraint to confirm the results in experiment 1. All of the other measurements were performed 5 days after the last repeated restraint. Pair-fed rats were included in this and the following experiments. Because the results of experiment 1 indicated that the effects of stress on body weight, energy intake, and insulin sensitivity were exaggerated in rats fed a high-fat diet, this diet was used in all subsequent experiments.

Twenty-four rats were fed a high-fat diet (see Table 1) for 5 days and then were divided into three weight-matched groups: repeated restraint, pair fed, and control. One day after the last restraint, an OGTT was performed, as described above. The rats were killed 5 days after the last restraint stress, 4 days after the OGTT. Blood was collected for measurement of serum insulin, glucose, corticosterone (Corticosterone RIA; ICN Pharmaceuticals, Costa Mesa, CA), leptin (Rat Leptin RIA kit; Linco), nonesterified fatty acids (Wako NEFA C kit; Wako Chemicals), and triglycerides (Sigma Triglyceride Kit; Sigma Chemical). Livers were frozen for determination of lipid and glycogen content, and carcasses were analyzed, as described previously (7, 8).

Experiment 3: Effects of Repeated Restraint Stress on Muscle and Adipocyte Glucose Uptake and Body Composition, Measured 1 Day After the End of Stress

This experiment measured glucose transport in soleus muscle and adipocytes from control, restrained, and pair-fed rats 1 day after the last repeated restraint stress. Thirty rats were fed the high-fat diet for 11 days and then were divided into three weight-matched groups: repeated restraint stress, pair fed, and control groups.

One day after the last restraint stress, all rats were food deprived for 4–6 h and were anesthetized (90 mg/kg body wt ketamine and 10 mg/kg body wt ip xylazine). Soleus muscle from each hind leg was taken immediately for muscle glucose transport measurements, and epididymal fat was dissected, weighed, and digested to measure adipocyte glucose transport. Carcass composition was also determined.

Glucose transport in soleus muscle. Muscle glucose transport was measured using methodology described by Hansen et al. (6). Two pieces (20–30 mg) from each soleus muscle from each rat were cut from the outer edges of the muscle. All incubations were performed at 30°C with shaking and a continuous supply of gas (95% O2-5% CO2). The four samples from each rat were used for measurement of basal and insulin-stimulated 2-deoxyglucose (2-DG) uptake. Insulin (Humulin R; Eli Lilly) was added to preincubation (Kreb’s bicarbonate buffer, 10 mM HEPES, 2 mM sodium pyruvate, 5 mM glucose, and 23 mM mannitol, pH 7.5), wash (Krebs, 10 mM HEPES, 2 mM sodium pyruvate, and 28 mM mannitol, pH 7.5), and transport (Krebs, 10 mM HEPES, 2 mM sodium pyruvate, 26 mM mannitol, 0.5 μCi/ml 2-[3H]DG and 0.01 μCi/ml [14C]mannitol) media at insulin concentrations of 0, 0.25, 0.5, and 2 μU/ml. The samples were equilibrated in incubation media without insulin for 10 min, preincubated with insulin for 10 min, washed for 10 min, and finally incubated in transport media for exactly 10 min when glucose transport was stopped by transferring the tissue to ice-cold saline. Samples were dissolved in 1 N NaOH at 90°C and transferred to scintillation vials, and the amounts of 2-[3H]DG and [14C]mannitol were counted. 2-DG incorporation, corrected for extracellular fluid volume, was expressed as nanomoles glucose incorporated per milligram muscle per 10 min.

Glucose transport in adipocytes. Epididymal adipocytes were isolated by the method of Rodbell (27) and were suspended in wash buffer (Krebs, 0.1 mM glucose, and 2% BSA). Glucose uptake was measured in basal and insulin-stimulated conditions (0, 0.1, and 0.8 μM insulin/ml) by methodology based on that described by Olefsky (24). One milliliter of each cell suspension was added to 2 ml of media (1.5× wash buffer, 0.1 μCi/ml [3H]mannitol) and was incubated for

Table 1. Diet composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Low Fat (g)</th>
<th>High Fat (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>1,464</td>
<td>2,000</td>
</tr>
<tr>
<td>AIN 76 vitamin mixture</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AIN 76 mineral mixture</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Alphacel</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Corn oil</td>
<td>262.5</td>
<td>1,313</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>187.5</td>
<td>937</td>
</tr>
<tr>
<td>Starch</td>
<td>3,603</td>
<td>2,435</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3,603</td>
<td>2,435</td>
</tr>
<tr>
<td>Dietary energy, kcal/g</td>
<td>3.66</td>
<td>5.00</td>
</tr>
<tr>
<td>% kcal fat</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>% kcal protein</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

Units are g/10 kg. AIN, American Institute of Nutrition. All dietary ingredients were obtained from ICN Biochemical. Dietary energy was calculated from diet composition.
Next, 0.2 mM 2-DG (1.0 mCi/mM 2-[^3H]DG) was added, and counting by a Coulter Counter, as described previously (9). Next, 0.2 mM 2-DG (1.0 mCi/mM 2-[^3H]DG) was added, and the sample was incubated for exactly 2 min. Triplicate 200-µl aliquots of the sample were transferred to vials containing 100 µl phthalic acid dinonyl ester and were immediately centrifuged to separate cells from media. All incubation conditions were carried out in duplicate. The cell fraction was counted for 2-DG incorporation and was corrected for extracellular fluid volume. Results are expressed as nanomoles glucose incorporated per 10^6 cells per 2 min and as percentage change from basal levels.

Experiment 4: Effects of Repeated Restraint Stress
on Liver Composition, Adipocyte Fatty Acid Oxidation, and Esterification, Measured 1 Day After Last Restraint

This experiment determined adipocyte fatty acid oxidation and esterification 1 day after the last restraint stress. Thirty rats were fed the high-fat diet for 11 days and then were divided into restraint, pair-fed, and control groups, matched for average body weight.

One day after the last restraint stress, rats were food deprived for 4–6 h before decapitation. Blood was collected for measurement of insulin, glucose, corticosterone, leptin, nonesterified fatty acids, and triglycerides. Epididymal adipocyte fatty acid oxidation and esterification were measured in the presence of increasing concentrations of insulin. Liver lipid and glycogen content were determined (7).

Fatty acid oxidation and esterification. Adipocytes were suspended in wash buffer (Krebs, pH 7.5, 5.0 mM glucose, 0.5 mM palmitate, 2.0% BSA, and 0.3 µCi/ml [14C]palmitate). Cell number was determined as described above. The flasks were gassed with 95% O₂-5% CO₂, sealed with rubber stoppers carrying center wells. Cells were extracted for esterified fatty acids as described previously (9).

RESULTS

Experiment 1

Because the energy contents of low- and high-fat diets were different, the food intake data and calculated dietary energy content were used to determine energy intakes of the rats. As shown in Fig. 1, rats fed the high-fat diet gained more weight and consumed more energy than those fed the low-fat diet. Repeated restraint caused significant reductions in body weight and energy intake of both low- and high-fat-fed rats during the stress and poststress periods. OGTT results, shown in Fig. 2, indicated no significant effect of either stress or diet on serum glucose at any time point. Because neither glucose nor insulin returned to fasting levels by the end of the test, we did not measure the complete insulin response to the glucose challenge. However, it was clear that restraint stress reduced the amount of insulin released (high fat: P < 0.001, low fat: P < 0.05) during the early phases of the response to a glucose challenge and that this was adequate to produce similar rates of glucose clearance in control and restrained rats on both diets.

Experiment 2

As shown in Fig. 3, there were significant effects of treatment (P < 0.05), day (P < 0.01), and a treatment times day interaction (P < 0.01) on both body weight and food intake. Body weight was significantly reduced in both restrained and pair-fed rats compared with controls. Food intake was significantly lower in restrained and pair-fed rats than in controls from the second day of restraint and had not returned to control

![Graphs showing body weight and energy intake changes](http://ajpregu.physiology.org/)
levels by the end of the experiment. There was no significant difference in body weight or food intake of restrained and pair-fed animals. The results of the OGTT are shown in Fig. 4 and are similar to those in experiment 1. There were no significant differences in blood glucose concentrations among the three groups, but insulin was significantly lower in restrained and pair-fed groups compared with the control group \( (P < 0.05) \) after glucose administration, although they were not different before the OGTT. The results from serum assays on day 5 after the end of restraint are summarized in Table 2. All three groups of rats had similar serum glucose concentrations; however, insulin and leptin were significantly lower in restrained and pair-fed animals than controls \( (P < 0.01) \). Pair-fed rats also had significantly lower levels of nonesterified fatty acids \( (P < 0.02) \) and triglycerides \( (P < 0.01) \) than either restrained or control animals. There were no significant differences in corticosterone concentrations.

Carcass and liver composition are shown in Table 3. Carcass weights of restrained and pair-fed groups were significantly lower than those of the control group \( (P < 0.01) \), but there was no difference between pair-fed and restraint groups. Body fat content, calculated either as grams or as a percentage of carcass weight, was significantly different among the groups \( (P < 0.01) \), with controls having the highest and pair-fed rats having the lowest fat content. Carcass protein and water content in pair-fed and restraint groups tended to be reduced compared with controls, but differences did not reach statistical significance \( (P = 0.067, \text{data not shown}) \). Lean body mass (protein + water) was statistically different among the three groups \( (P = 0.05) \), with control rats having significantly more lean tissue than pair-fed rats. Liver weight was significantly reduced in pair-fed and restrained rats compared with controls \( (P < 0.01) \). Liver lipid was the same in pair-fed and restrained groups but lower than control rats \( (P < 0.01) \), and liver glycogen was the same in restrained and control rats but was reduced in the pair-fed group \( (P < 0.01) \).

**Experiment 3**

Daily body weights and food intakes of the different groups of rats showed the same pattern of response as in experiment 2 (data not shown). Figure 5 shows the results of muscle glucose transport measurements. Basal glucose uptake was the same in all three groups and was stimulated by insulin in all three groups, although the degree of insulin stimulation was not the same, indicated by a significant interaction between treatment and insulin \( (P = 0.03) \). Glucose uptake was significantly lower in the restrained than control or
pair-fed groups in the presence of 0.5 mM insulin. Adipocyte glucose uptake is shown in Fig. 6A. Basal and insulin-stimulated glucose uptake were significantly lower in restrained rats than in control or pair-fed animals (P = 0.03). There was no difference between control and pair-fed groups. Insulin-stimulated glucose transport calculated as a percentage of basal rate is shown in Fig. 6B. The percent change in glucose transport in adipocytes exposed to insulin was the same in restrained and control groups but was significantly greater in pair-fed animals (P = 0.04).

Body composition results are summarized in Table 4. Changes in daily body weights and food intakes were the same as in previous experiments (data not shown).

![Graph](image1.png)

**Fig. 4.** Glucose tolerance test results for experiment 2. Data are means ± SE for groups of 8 rats. A: serum insulin levels. Restraint and pair-fed groups had significantly lower levels of serum insulin when compared with the control group (P < 0.05). B: blood glucose concentration. There was no significant difference in blood glucose among the 3 groups.

**Table 2.** Serum assay results from experiment 2, measured 5 days after the end of repeated restraint stress

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pair Fed</th>
<th>Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>2.26 ± 0.24*</td>
<td>0.79 ± 0.14†</td>
<td>1.15 ± 0.19†</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>118 ± 4</td>
<td>125 ± 5</td>
<td>117 ± 3</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>44 ± 8</td>
<td>81 ± 17</td>
<td>42 ± 18</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>12.1 ± 1.5*</td>
<td>3.1 ± 0.4†</td>
<td>5.6 ± 0.4†</td>
</tr>
<tr>
<td>Nonesterified fatty acid, mg/l</td>
<td>1.08 ± 0.12*</td>
<td>0.62 ± 0.03*</td>
<td>1.04 ± 0.14*</td>
</tr>
<tr>
<td>Tryglycercide, mg/dl</td>
<td>104 ± 8*</td>
<td>33 ± 6†</td>
<td>100 ± 15*</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 8 rats. Statistical significance was determined by ANOVA. Differences between observations were determined by post hoc calculation of Duncan’s multiple range test at P < 0.05. Values for a specific parameter that do not share a common superscript are significantly different.

**Table 3.** Carcass and liver compositions of rats from experiment 2, measured 5 days after the end of repeated restraint stress

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pair Fed</th>
<th>Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcass weight, g/rat</td>
<td>392 ± 7*</td>
<td>364 ± 6†</td>
<td>373 ± 3†</td>
</tr>
<tr>
<td>Fat, g/rat</td>
<td>50 ± 2*</td>
<td>36 ± 2†</td>
<td>42 ± 2†</td>
</tr>
<tr>
<td>Fat, % of carcass</td>
<td>12.6 ± 0.4*</td>
<td>9.7 ± 0.4†</td>
<td>11.3 ± 0.5†</td>
</tr>
<tr>
<td>Lean body mass, g/rat</td>
<td>329 ± 5*</td>
<td>313 ± 5†</td>
<td>316 ± 4†</td>
</tr>
<tr>
<td>Liver, g/rat</td>
<td>14.2 ± 0.4*</td>
<td>10.3 ± 0.5†</td>
<td>12.8 ± 0.3†</td>
</tr>
<tr>
<td>Lipid, mg/liver</td>
<td>718 ± 26*</td>
<td>543 ± 40†</td>
<td>603 ± 22†</td>
</tr>
<tr>
<td>Glycogen, mg/liver</td>
<td>822 ± 147*</td>
<td>120 ± 23†</td>
<td>700 ± 22*</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 8 rats. Statistical significance was determined by ANOVA. Differences between observations were determined by post hoc calculation of Duncan’s multiple range test at P < 0.05. Values for a specific parameter that do not share a common superscript are significantly different.

for all three groups of rats. Weight loss in pair-fed and restrained rats was accounted for by loss of lean body mass (P < 0.05), especially water (P = 0.02, data not shown).

**Experiment 4**

Changes in daily body weights and food intakes were the same as in previous experiments (data not shown). Adipocyte fatty acid oxidation results are shown in Fig. 7. There was a significant interaction between insulin concentration and treatment (P < 0.05) in that oxidation was higher in restrained and pair-fed groups than controls in basal conditions (P < 0.02) and in the presence of 0.3 mU insulin (P = 0.02). At the highest insulin concentration, the rate of fatty acid oxidation was the same for all three groups. Insulin-stimulated fatty acid oxidation expressed as percentage change from basal also showed a significant interaction between treatment and insulin concentration (P < 0.05). Fatty acid oxidation was stimulated by insulin in adipocytes from control rats but not those from restrained or pair-fed rats, due to the high rate of oxidation in basal conditions. Adipocyte fatty acid esterification is shown in Fig. 8. There was no signifi-

**Fig. 5.** Muscle glucose transport in control, pair-fed, and restraint stress rats for experiment 3. Data are means ± SE for groups of 10 rats. Increasing insulin concentration significantly stimulated glucose transport in muscle in all 3 groups of rats. Restrained rats had significantly reduced glucose transport compared with control or pair-fed rats in the presence of 0.5 mU/ml insulin (*P = 0.03).
cant difference among treatment groups and no signifi-
cant interaction between treatment and insulin concen-
tration. There was a significant stimulatory effect of
insulin ($P < 0.01$). When the data were expressed as
percentage change from basal, the interaction between
treatment and insulin became significant ($P < 0.05$).
Adipocytes from restrained rats had a lower percentage
change from basal levels.

Serum analysis results are summarized in Table 5.
Pair-fed rats had lower serum insulin and triglyceride
concentrations than control or restrained rats ($P < 0.01$
and $P < 0.05$, respectively). Serum leptin also tended
to be lower in pair-fed rats than control and restrained
rats but did not reach statistical significance ($P = 0.1$).
Both restrained and pair-fed rats had smaller livers
than controls ($P < 0.05$), but there was no significant
difference in lipid or glycogen content among the three
groups (Table 4).

**DISCUSSION**

The objective of this series of experiments was to
determine the prolonged effects of repeated restraint
stress on peripheral tissue metabolism. We chose 1 day
after the termination of the last restraint stress to
make the measurements, as all of the acute responses
to stress, such as increased corticosterone, body tem-
perature, and changes of monoamines in the brain,
would have been reversed 24 h after the last restraint
(10, 28). Exposure of rats to a single 3-h restraint
causes a small, but sustained, weight loss and reduces
food intake during the 24 h after stress (28). Because
the response is greater when rats are exposed to
repeated restraint, we chose to use a protocol of three
periods of restraint to maximize the likelihood of
identifying statistically significant changes. In addition,
because the high-fat diet exaggerated the effects of

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Table 4. Carcass and liver composition of rats from
experiment 3 and 4, measured 1 day after the end
of repeated restraint

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pair Fed</th>
<th>Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcass weight, g/rat</td>
<td>383 ± 8</td>
<td>371 ± 6</td>
<td>368 ± 7</td>
</tr>
<tr>
<td>Fat, g/rat</td>
<td>40 ± 4</td>
<td>41 ± 2</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>Fat, % of carcass</td>
<td>8.8 ± 0.8</td>
<td>9.3 ± 0.4</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>Lean body mass, g/rat</td>
<td>341 ± 10*</td>
<td>324 ± 4†</td>
<td>323 ± 6†</td>
</tr>
<tr>
<td>Liver, g/rat</td>
<td>12.3 ± 0.4*</td>
<td>11.0 ± 0.2†</td>
<td>11.2 ± 0.3†</td>
</tr>
<tr>
<td>Lipid, mg/liver</td>
<td>289 ± 19</td>
<td>274 ± 14</td>
<td>271 ± 17</td>
</tr>
<tr>
<td>Glycogen, mg/liver</td>
<td>712 ± 165</td>
<td>362 ± 115</td>
<td>450 ± 87</td>
</tr>
</tbody>
</table>

Data are means ± SEM for groups of 10 rats. Statistical signifi-
cance was determined by ANOVA. Differences between observations
were determined by post hoc calculation of Duncan’s multiple range
test at $P < 0.05$. Values for a specific parameter that do not share a
common superscript are significantly different.
restraint stress in experiment 1, all of the animals used in subsequent experiments were fed a high-fat diet.

All rats exposed to repeated restraint lost body weight and decreased food intake or energy intake in experiments described here, consistent with our previous studies (10, 28). Inclusion of pair-fed rats allowed us to determine which changes in restrained rats were secondary to their voluntary reduction in food intake and which were specific to restraint stress. Pair-fed rats maintained almost the same level of body weight as restrained rats during the stress period although they ate less food due to spillage, suggesting that they

had an increased efficiency of energy utilization. Because the two groups of rats did not have the same intake, these animals can only be considered to be an example of animals that are forcibly food restricted compared with restrained rats that voluntarily consume less than nonstressed, ad libitum-fed controls.

Body composition, measured 1 day after the last restraint, indicated a similar body fat content in all three groups of rats, even though the restrained and pair-fed rats had reduced body weight. In contrast, restrained and pair-fed rats had significantly lower lean body mass than the controls, similar to changes observed in injury and sepsis stress (2). Our results imply that stressed and pair-fed animals protected body fat during the weight loss, and the loss of lean tissue was secondary to a reduced food intake, rather than a specific effect of restraint stress. However, food deprivation is a physiological stress that may have caused the specific loss of lean tissue in pair-fed rats. It is unclear what caused rats to maintain body fat despite a loss of lean body mass and body weight. One possible explanation is that stress activates the hypothalamic-pituitary-adrenal system and causes a transient release of the catabolic hormone corticosterone, which inhibits growth hormone, and, as a consequence, the stressed animals lose lean tissue (1, 16). Five days after the termination of repeated restraint stress, body composition measurements showed that restrained and pair-fed rats had lost both lean and fat tissue. Therefore, in the days immediately after restraint, there appeared to be a shift in metabolism to redistribute energy and restore the normal proportions of lean and fat tissue.

Measurements of liver composition indicated that liver weight, like carcass weight, was reduced in restraint and pair-fed rats 1 day after the last restraint. Liver lipid and glycogen content were no different 1 day after the last restraint, but, 5 days after the last restraint, liver lipid was lower in both restraint and pair-fed rats than controls, but glycogen was reduced only in pair-fed rats. The changes in liver and body composition can be interpreted as restraint and pair-fed rats responding differently to reduced food intake during the recovery period: restraint rats switched to using lipid as their main energy supply, reducing body fat during the weight loss, and the loss of lean tissue was secondary to a reduced food intake, rather than a specific effect of restraint stress. However, food intake, these animals can only be considered to be an example of animals that are forcibly food restricted compared with restrained rats that voluntarily consume less than nonstressed, ad libitum-fed controls.

Table 5. Serum analysis results in rats from experiment 4, measured 1 day after the end of repeated restraint.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pair Fed</th>
<th>Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>1.40 ± 0.14</td>
<td>0.98 ± 0.14</td>
<td>1.62 ± 0.10*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>118 ± 3</td>
<td>114 ± 5</td>
<td>112 ± 3</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>33 ± 9</td>
<td>39 ± 8</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>6.06 ± 0.89</td>
<td>3.63 ± 0.39</td>
<td>5.62 ± 0.97</td>
</tr>
<tr>
<td>Nonesterified fatty acid, meq/l</td>
<td>0.75 ± 0.06</td>
<td>0.65 ± 0.04</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>Tryglyceride, mg/dl</td>
<td>67 ± 9*</td>
<td>42 ± 6*</td>
<td>68 ± 5*</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 10 rats. Statistical significance was determined by ANOVA. Differences between observations were determined by post hoc calculation of Duncan's multiple range test at P < 0.05. Values for a specific parameter that do not share a common superscript are significantly different.
control and restrained rats was a significant decrease in the response to an intermediate dose of insulin. This did not appear to be insulin insensitivity, as muscle from restrained rats was responsive to a low dose of insulin, and the muscle was not insulin resistant, as the response to the highest dose of insulin was the same for all three groups of rats. Inhibition of glucose uptake into adipocytes was a specific response to the repeated restraint stress, as pair-fed rats consumed less food than restrained rats, but adipocyte glucose uptake was the same as in cells from pair-fed and control rats. The percent change in glucose uptake of adipocytes in response to insulin was not different between control and restrained rats but was relatively small, possibly because the high-fat diet had caused insulin resistance, which was reflected in a failure of insulin to translocate the insulin-sensitive glucose transporter, GLUT-4, to the membrane and promote glucose transport. Our results also show increased rates of fatty acid oxidation in adipocytes for restrained rats, leading to the conclusion that repeated restraint stress causes adipocytes to shift to using fatty acids, rather than glucose, as a primary energy source 24 h after the last restraint stress. We did not observe changes in adipocyte fatty acid esterification among the three groups, suggesting that fatty acids were not being stored in adipose tissue but were being catabolized for energy. The reduced glucose uptake and increased fatty acid oxidation in adipocytes of stressed rats may also explain why, although all of the tissue lost during stress was lean body mass, the difference in weight of control and restrained rats was accounted for by both lean and fat tissue 5 days after stress. The triggers that cause adipocytes to switch energy utilization from glucose to fatty acids after stress need to be determined.

The glucose tolerance test results in experiments 1 and 2 are repeatable and similar to those of other investigators (20, 21, 23, 32). Both restraint and food restriction increased insulin sensitivity. Although the whole body insulin sensitivity was improved in restrained rats, their glucose transport into muscle and adipocytes did not increase. The other major insulin-sensitive organ that may account for improved glucose clearance is liver, and future studies will be needed to examine hepatic glucose production and metabolism to determine whether this is also changed in rats exposed to repeated restraint. The improved insulin sensitivity in restrained rats seems contradictory to the stress-associated activation of sympathetic outflow that would be expected to impair insulin sensitivity (12, 13). However, other stress-induced hormones may play a role in the metabolic responses of rats exposed to repeated restraint. Corticosterone's catabolic effect can cause insulin resistance (26), but Ottenweller and colleagues (25) reported that repeated stress changed the rhythmic pattern of corticosterone release, advancing the phase of corticosterone release. In addition, a recent study by Tannenbaum et al. (31) has shown that high-fat feeding exaggerates restraint stress-induced corticosterone release. This change in the temporal pattern of hormone concentration may account for improved insulin sensitivity in stressed rats if corticosterone levels were lower in these rats when measurements were performed in the early afternoon, a time when corticosterone would be rising in controls with a normal release pattern. The single time point measures made in the morning in experiment 4, 1 day after the end of stress, indicated only a nonsignificant reduction in serum corticosterone concentrations of restrained rats compared with control or pair-fed animals. A thorough investigation of circadian patterns of hormone release is needed to determine whether they play a role in poststress changes in body weight and tissue metabolism.

An alternative explanation for improved insulin sensitivity in restrained rats is that whole body glucose disposal represents glucose uptake by both insulin-dependent and -independent mechanisms. It has been reported that non-insulin-mediated glucose uptake is the predominant pathway for glucose disposal in septic and nonseptic rats and that intracerebroventricular administration of either N-methyl-d-aspartate (NMDA) or kainate, agonists of excitatory amino acid, can produce metabolic alterations comparable to those observed under stress conditions (22). Also, restraint stress was reported to cause atrophy of pyramidal neurons in the C-3 region of the hippocampus not immediately after the stress but over the 3–4 wk after stress, and this effect could be blocked by NMDA receptor antagonists (17, 18). The metabolic alterations caused by NMDA include increased hepatic glucose output and elevated glucose metabolic clearance rates in peripheral tissue (14, 22). If glucose metabolic clearance rate exceeds hepatic glucose output, insulin sensitivity could be improved in a glucose tolerance test as less insulin would be required in response to a glucose challenge because non-insulin-dependent glucose clearance would be increased in restrained rats. Because most metabolic changes that we observed in this study were insulin independent and NMDA receptor mediated, glucose metabolism is also insulin independent, and it is possible that the stress-activated NMDA receptor is involved in these sustained effects of repeated restraint stress.

The results from serum analysis in experiment 4 indicated that insulin and triglyceride concentrations in pair-fed rats were lower than in control or restraint rats. There was no significant difference in serum leptin levels, supporting the observation that body fat content was the same in all three groups of animals. The same serum analysis carried out 5 days after the end of repeated restraint stress showed that restraint and pair-fed rats had lower serum insulin and leptin levels than control rats, but only pair-fed rats had lower free fatty acid and triglyceride levels. This result suggests that repeated restraint stress-increased lipid turnover after the stress was terminated such that free fatty acids and triglycerides in restraint rats were the same as in control rats, but body fat content was reduced. This would be consistent with the increased fatty acid oxidation in adipocytes of restraint rats in
experiment 3 and may explain why body fat is protected during stress but not during the poststress period.

In conclusion, repeated restraint stress can cause a variety of changes in body composition and peripheral tissue metabolism, including tissue-specific alterations in glucose transport and fatty acid oxidation. These changes are observed not only during the stress period but are also apparent during the recovery period after stress and may, at least partially, account for the sustained weight loss and absence of compensatory hyperphagia in rats exposed to repeated restraint stress.

The short-term effects of stress on body weight and food intake are well established. We have previously described the repeated restraint model in which a relatively short exposure to restraint stress causes prolonged downregulation of body weight (10, 28). The studies described here demonstrate that this disruption of body weight may be related to sustained effects of stress on tissue energy metabolism. During the recovery period, or after the termination of repeated restraint stress, the rats shifted adipocyte energy utilization from glucose to fatty acid. If tissue fuel utilization is used as a feedback signal in the regulation of energy balance, this shift in metabolism may represent an erroneous signal that prevents compensatory hyperphagia and recovery of a normal body weight. Because the pair-fed rats in this study consumed less than restrained rats, the two groups cannot be compared directly; however, the different metabolic responses suggest that stress-induced weight loss cannot be attributed exclusively to hyperphagia. Identification of mechanisms that mediate the response could contribute to trauma and sepsis research and to obesity research. Future studies may be needed to further clarify metabolic responses to repeated restraint and to identify mechanisms responsible for the shift in homeostatic equilibrium.

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