Insulin resistance has been shown to occur in hypertensive subjects and has been proposed as a metabolic link between hypertension, non–insulin-dependent diabetes mellitus, obesity, dyslipidemia, and atherosclerotic cardiovascular disease. Therefore, in hypertension treatment, consideration should be given to the effect of antihypertensive agents on insulin sensitivity (IS).

Salt restriction is recommended for antihypertensive treatment, and its effect on IS has been studied by several groups. Most of the studies showed that salt restriction lowers IS, although some of them concluded the opposite. Almost all studies from the literature analyzed the effects of short-term changes in salt consumption. However, to better understand the antihypertensive effects of salt restriction on IS, a long-term study was advisable. Recently, we have shown in rats that chronic salt restriction compared with overload is associated with lower blood pressure (BP) and decreased insulin-independent and dependent glucose uptake (GU) in epididymal isolated adipocytes. However, on the basis of the unchanged EC$_{50}$ of GU, no alteration on IS was observed. Therefore, the present study was planned to evaluate, by use of the euglycemic hyperinsulinemic clamp (EHC), IS in rats fed a high (HSD), normal (NSD), or low salt diet (LSD) from weaning to adulthood. The amount of glucose transporter (GLUT4) in insulin-sensitive tissues was measured, and effects of treatment with an angiotensin-converting enzyme inhibitor (ACE-I); captopril; or an angiotensin receptor-1 (AT$_1$) antagonist, losartan, on the insulin-stimulated GU were also evaluated.

**Methods**

All experiments reported herein were in accordance with guidelines of the Committee on Ethics of the School of Medicine of the University of São Paulo, Brazil.

**Animals**

Male Wistar rats were fed from weaning either an LSD (0.06% Na, TD 92141-Harlan Teklad), NSD (0.5% Na, TD 92140), or HSD (3.12% Na, TD 92142). Rats were housed in a controlled-temperature environment (25°C), with light on/off cycling (lights on at 6 AM) with free access to chow and tap water.

**Experimental Protocol**

Experiments were performed on male Wistar rats at the age of 3 months and when body weight (BW) ranged from 355 to 425 g. On
the day of the experiment, animals, after 5 hours fasting, were anesthetized with sodium pentobarbital (50 mg/kg BW, IP). Catheters were inserted into the left jugular vein and carotid artery and filled with heparinized saline (500 IU/mL). Animal temperature was maintained at 37°C. Experiments were started when glycemia had returned to stable levels, ≥30 minutes after the end of the surgical procedure. BP was measured through the carotid artery catheter, which was attached to a pressure transducer (Gould Statham Instruments Inc, model P23DB) that was connected to an amplifier (Stemtech Inc, GPA-4 model 2) that provided the analog BP signal, which was digitized with a computer-based monitoring system (DATAQ Instruments Inc). Recordings of carotid artery BP were performed during 5 minutes at basal levels and during 30 minutes along the steady-state (SS) period of the EHC. The mean of all values obtained during these periods was considered for calculations.

Before the EHC was applied, 600 μL blood samples were collected for basal blood glucose (BG) and plasma insulin (INS) determinations. The EHC was applied (n = 52) by continuous regular insulin infusion (2.23 pmol · kg⁻¹ · min⁻¹ – Actrapid, Novo Nordisk A/S) through the jugular line for 2 hours. Insulin was diluted in 26.61 μmol/L bovine serum albumin solution. To maintain euglycemia at levels similar to basal levels, a glucose infusion (1.11 mmol/L D-glucose) through the jugular vein was started 5 minutes after the beginning of the insulin infusion and was corrected every 5 minutes on the basis of a servocontrol negative feedback principle.¹⁰ Samples (25 μL) for BG determinations were taken through the carotid artery every 5 minutes. At 75, 90, 105, and 120 minutes of the EHC, 600-μL blood samples were taken for insulin determination. Blood samples for insulin measurements were centrifuged immediately, and the plasma was stored at −20°C until assayed. Blood cells were resuspended in saline and reinfused into the animal. During the last 30 minutes of the EHC, SS blood glucose (SSBG) and plasma insulin (SSPI) were attained, by which the glucose infusion rate equals GU in all body tissues and is therefore a measure of tissue sensitivity to exogenous insulin. Experiments in which the coefficient of variation of BG or INS during the SS was >10% were excluded. Hematocrit was determined at the beginning and end of all experiments. At the end of the experiments, animals were euthanatized and kidney mass determined. The retroperitoneal, epididymal, and mesenteric adipose tissues were excised and weighed. After carcasses were shaved and the gastrointestinal tract was removed, carcasses were warmed overnight at 45°C and homogenized in a blender. Lipids were extracted from 5-g aliquots with petroleum ether, and lipid levels were determined gravimetrically.¹¹

Five days before the EHC was applied, blood samples were collected from the tail vein in awake rats for nonesterified fatty acids (NEFA; n = 20) and plasma renin activity (PRA; n = 18) measurements.

Another group of LSD (n = 6), NSD (n = 6), and HSD (n = 6) rats were anesthetized, and epididymal white adipose tissue (WAT), skeletal muscle (SM) gastrocnemius, interscapular brown adipose tissue (BAT), and cardiac muscle (CM) were removed; immediately weighed; frozen; and stored at −70°C for further GLUT4 analysis.

**GLUT4 Determination**

Three subcellular membrane fractions of WAT were prepared as previously described.¹² Tissues were homogenized in 4 wt/vol buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 250 mmol/L sucrose; pH 7.4) and centrifuged at 3000g for 15 minutes. Fat cakes were discarded, and the infranatant, a fat-free extract fraction (FFE), was measured. One mL of FFE was kept for analysis, and the rest was centrifuged at 12 000g for 15 minutes, and then the pellet was discarded and the supernatant centrifuged at 146 000g for 75 minutes. The final pellet was resuspended as a plasma membrane fraction (PMF) in 1 mL of buffer. The supernatant was centrifuged at 28 000g for 15 minutes, and then the pellet was discarded and the supernatant centrifuged at 100 000g for 10 minutes. The supernatant was saved and the pellet was resuspended in one third of the initial volume and centrifuged at 1000g for 10 minutes. Both supernatants were centrifuged at 150 000g for 75 minutes. The final pellet was resuspended as a total membrane fraction in 1 mL of buffer.

GLUT4 protein was assessed by Western blotting analysis as previously described.¹³ Immunoblotting was performed with an antiserum developed against the COOH terminus of the rat GLUT4 protein followed by 125I–protein A (Amersham). Autoradiograms were analyzed by densiometry using Image Master 1D software (Pharmacia Biotech). Results were expressed as arbitrary units (AU) per microgram of total protein electrophoretized. The GLUT4 translocation index was calculated by use of the following formula: GLUT4 Index = PMF × 100/(PMF + MF).

**Pair Weight**

Rats on LSD (n = 6), NSD (n = 6), and HSD (n = 5) were housed in individual cages from weaning for the pair-weight study. They were weighed daily, and the amount of food was adjusted each day to maintain similar BW among the 3 experimental groups. At 12 weeks of age, the EHC was used as previously described.

**Captopril and Losartan Treatment**

AT₁ antagonist (losartan [MK954] 200 mg/L; n = 4) or ACE-I (captopril 100 mg/L, kindly provided by Dr Luiz Olympio T. Nascimento from Bristol-Meyers Squibb S.A., Sao Paulo, Brazil; n = 6) was given for 7 days in the drinking water to 11-week-old rats treated from weaning to adulthood with LSD. A third group of LSD animals (n = 5) received only water. On the seventh day, EHC was used as described above.

**Analytical Methods**

BG concentration was measured using a glucometer (Advantage, Boehringer Mannheim). INS and PRA were measured by radioimmunoassay (Diagnostic Products Corp). NEFA was assayed by NEFA C, enzymatic method (Wako Chemicals GmbH).

**Statistical Analysis**

Values are expressed as mean ± SD. Comparison between 2 means were made by unpaired Student’s t test and between ≥3 means by 1-way ANOVA followed by Tukey’s test. Null hypothesis was rejected whenever P < 0.05.

**Results**

**Baseline and EHC Data**

As shown in Table 1, BW was higher (P < 0.01) in rats on LSD than in those on NSD or HSD. Basal BG and INS did not differ among the 3 groups. SSBG was maintained at a level similar to basal values (5.6 to 5.8 mmol/L). SSPI was characterized by high insulin physiological levels (700 to 900 pmol/L) in rats on LSD, NSD, and HSD. However, in rats on LSD, SSPI was higher (P < 0.05) than in those on NSD or HSD. Despite higher SSPI levels, GU was lower in rats on LSD than in those on NSD (P < 0.05) or HSD (P < 0.001). NEFA was lower in rats on LSD than in those on NSD (P < 0.01) or LSD (P < 0.05). Percentage fat carcass content was higher in rats on LSD than in those on NSD (P < 0.05). Retroperitoneal and epididymal adipose tissue weights were higher in rats on LSD than on NSD (P < 0.05) or HSD (respectively, P < 0.01 and P < 0.05). Mesenteric adipose tissue weight was higher in rats on LSD than in those on HSD (P < 0.05). These results suggest that exogenous insulin stimulated GU decreases after long-term ingestion of LSD.

No significant difference existed in basal systolic BP (SBP) among LSD (119 ± 22), NSD (126 ± 27), and HSD (150 ± 3 mm Hg). However, in HSD rats, basal SBP was increased by 18%. Basal diastolic BP (DBP) was higher.
rat values were significantly higher ($P < 0.01$) in rats on HSD (102 ± 1) than in those on NSD (80 ± 6) or LSD (80 ± 9 mm Hg). No difference existed in SBP at SS among LSD (128 ± 10), NSD (130 ± 6), and HSD (144 ± 9 mm Hg) rats. DBP during SS was also not different among the groups (LSD, 98 ± 2; NSD, 98 ± 3; and HSD, 100 ± 5 mm Hg). Higher DBP ($P < 0.001$) during SS compared with basal period of EHC use was observed in NSD rats.

**Pair Weight**

In the pair-weight study, no differences were found in BW values (377 ± 25.6 in rats on LSD; 374 ± 23.7 in those on NSD; and 389 ± 4.2 g in those on HSD). However, the amount of food consumed was lower ($P < 0.01$) in rats on LSD (14.6 ± 1.12) than in those on NSD (19.5 ± 1.9) or HSD (19.3 ± 2.77 g/d per rat). When the EHC was used, similar data to those of the non–pair weight rats were obtained. The rate of insulin-stimulated GU was 0.6 ± 0.39 in LSD, 0.9 ± 0.2 in NSD, and 2.0 ± 1.12 mmol · kg$^{-1}$ · min$^{-1}$ in HSD rats. HSD rat values were significantly higher ($P < 0.05$) than were those in LSD and NSD rats. This indicates that higher BW is not related to lower IS induced by LSD. No differences were observed in hematocrit, measured at the beginning and end of the EHC studies.

**GLUT4**

As indicated in Table 2, WAT GLUT4 in HSD rats was higher ($P < 0.05$) in all subcellular membrane fractions compared with NSD and LSD rats. The translocation index was also increased ($P < 0.05$) in HSD rats. The GLUT4 in SM and BAT was also higher ($P < 0.05$) in rats on LSD than in NSD and LSD rats. However, no differences in CM GLUT4 levels were observed.

As expected, PRA (Table 2) was higher ($P < 0.05$) after salt restriction and lower ($P < 0.05$) after salt overload compared with NSD. Kidney mass (Table 2) was higher ($P < 0.001$) in rats on LSD than in those on NSD or LSD.

**Captopril and Losartan Treatment**

Captopril and losartan did not influence feeding and drinking behavior among the rat groups, as shown in Table 3. BW, BG

### Table 1. Characteristics of Rats Fed an LSD, NSD, or HSD From Weaning to Adulthood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LSD</th>
<th>NSD</th>
<th>HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>408 ± 26†</td>
<td>373 ± 24</td>
<td>355 ± 18</td>
</tr>
<tr>
<td>Basal glucose, mmol/L</td>
<td>5.6 ± 0.3</td>
<td>5.5 ± 0.2</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Basal insulin, pmol/L</td>
<td>208.6 ± 107.9</td>
<td>165.5 ± 43.2</td>
<td>151.1 ± 28.8</td>
</tr>
<tr>
<td>SSBG, mmol/L</td>
<td>5.58 ± 0.41</td>
<td>5.31 ± 0.10</td>
<td>5.78 ± 0.29</td>
</tr>
<tr>
<td>SSPI, pmol/L</td>
<td>944.7 ± 161.2‡</td>
<td>673.7 ± 90.30</td>
<td>729.0 ± 183.9</td>
</tr>
<tr>
<td>GU, mmol · kg$^{-1}$ · min$^{-1}$</td>
<td>0.33 ± 0.14†</td>
<td>0.57 ± 0.09</td>
<td>0.77 ± 0.21</td>
</tr>
<tr>
<td>NEFA, μmol/L</td>
<td>0.67 ± 0.12</td>
<td>0.76 ± 0.17</td>
<td>0.46 ± 0.11§¶</td>
</tr>
</tbody>
</table>

Adipose tissue mass

<table>
<thead>
<tr>
<th>parameters</th>
<th>LSD</th>
<th>NSD</th>
<th>HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal, g/100 g</td>
<td>2 ± 0.28‡]</td>
<td>1 ± 0.32</td>
<td>1 ± 0.30</td>
</tr>
<tr>
<td>Retroperitoneal, g/100 g</td>
<td>2 ± 0.43#</td>
<td>1 ± 0.41</td>
<td>1 ± 0.42</td>
</tr>
<tr>
<td>Mesenteric, g/100 g</td>
<td>2 ± 0.42∥</td>
<td>1 ± 0.44</td>
<td>0.10 ± 0.12</td>
</tr>
<tr>
<td>Carcass lipid, g/100 g</td>
<td>12 ± 3.73*</td>
<td>7 ± 0.81</td>
<td>9 ± 0.94</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 7 to 9 rats.

*$P < 0.05$ vs NSD; †$P < 0.001$ vs HSD; ‡$P < 0.05$ vs LSD; §$P < 0.05$ vs HSD; ¶$P < 0.01$ vs NSD; #$P < 0.01$ vs HSD.

### Table 2. GLUT4 in Various Tissues and PRA and Renal Mass of Rats Fed an LSD, NSD, or HSD From Weaning to Adulthood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LSD</th>
<th>NSD</th>
<th>HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-gastrocnemius, AU/μg protein</td>
<td>50.17 ± 1.43</td>
<td>52.8 ± 4.3</td>
<td>68 ± 4.8*</td>
</tr>
<tr>
<td>CM, AU/μg protein</td>
<td>102.9 ± 6.4</td>
<td>100.1 ± 9.5</td>
<td>95.1 ± 9.3</td>
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<tr>
<td>BAT, AU/μg protein</td>
<td>195.2 ± 30.5</td>
<td>203.4 ± 41.4</td>
<td>325.6 ± 20.7*</td>
</tr>
<tr>
<td>WAT-FFE, AU/μg protein</td>
<td>42.1 ± 9.5</td>
<td>42.9 ± 5.9</td>
<td>183.5 ± 48.8*</td>
</tr>
<tr>
<td>WAT-PMF, AU/μg protein</td>
<td>100.0 ± 22.1</td>
<td>110.2 ± 17.0</td>
<td>263.9 ± 62.2*</td>
</tr>
<tr>
<td>WAT-MF, AU/μg protein</td>
<td>522.7 ± 75.8</td>
<td>511.9 ± 66.8</td>
<td>858.9 ± 104.0*</td>
</tr>
<tr>
<td>Translocation index, %</td>
<td>17.9 ± 1.4</td>
<td>16.0 ± 1.0</td>
<td>24.1 ± 2.3*</td>
</tr>
<tr>
<td>PRA, μg · mL$^{-1}$ · h$^{-1}$</td>
<td>10.5 ± 4.1†‡</td>
<td>5.7 ± 0.99</td>
<td>0.4 ± 0.1†</td>
</tr>
<tr>
<td>Renal mass, g/100 g</td>
<td>0.82 ± 0.08</td>
<td>0.88 ± 0.08</td>
<td>1.12 ± 0.11§</td>
</tr>
</tbody>
</table>

Values in AU/μg protein are expressed as mean ± SD of 6 rats. GLUT4 translocation index was calculated as PMF/(PMF + MF) · 100.

*$P < 0.05$ vs LSD and NSD; †$P < 0.05$ vs NSD; ‡$P < 0.001$ vs HSD; §$P < 0.001$ vs LSD and NSD.
levels, SSBG, and SSPi were not different among the 3 groups. Basal INS was lower (P<0.01) in rats treated with losartan than in vehicle-treated rats. Compared with LSD vehicle-treated rats, GU increased 2-fold (P<0.05) in the captopril-treated group, whereas in the losartan-treated rats, no change was observed.

Basal SBP and DBP were lower (P<0.05) in the captopril- and losartan-treated groups than in the vehicle group (Table 3). The effect of both treatments on BP was similar, which indicates equal AT1 receptor activation. DBP during SS in captopril and losartan groups was lower (respectively, P<0.05 and P<0.01) than in the vehicle group. During use of the EHC, SBP increased (P=0.005) in losartan-treated rats and DBP increased (P<0.01) in vehicle-treated rats. These results of GU and BP seem to indicate that the mechanism of impairment of IS in LSD rats does not involve AT1 receptors.

Discussion

An association between long-term salt restriction and decreased IS was shown for the first time in the present study. In a study by Fliser et al.,7 healthy male humans received an LSD (20 mmol/d) or an HSD (200 mmol/d) for 7 days. IS measured by use of EHC decreased after 3 days but not after 7 days on LSD. In another study, Donovan et al.24 arrived at opposite results. IS was higher after 5 days of LSD than it was after 5 days of HSD. Taking these studies together, the effect of salt restriction on IS seems to be time dependent, at least when salt ingestion is modified over a short period. In the present study, lower IS was observed in adult rats that consumed LSD from weaning, which shows that this effect was present after long-term dietary intervention. Previously we have shown lower insulin-dependent and -independent GU in isolated adipocytes from LSD rats compared with HSD rats.9 However, in these experiments, the EECp of the dose-response curve of GU, a measure of insulin action, was not influenced by the level of salt consumption.9 So, the results reported in the present study demonstrate that, in LSD rats, decreased GU is not universal in all tissues.

The activated renin-angiotensin system or higher BW, as shown in this study; the increased plasma triglycerides reported previously14; and the events that follow insulin-receptor stimulation are some possible mechanisms that may be responsible for lower IS in response to LSD consumption in the whole animal.

Losartan treatment compared with vehicle did not change IS in LSD rats. This means that the effect of angiotensin II mediated by AT1 receptors does not participate in the insulin and glucose metabolic changes induced by LSD. Studies already have shown that treatment with losartan does not influence IS in hypertensive and normotensive offspring of hypertensive humans15,16 or in hypertensive rats.17 In addition, research has demonstrated that losartan has no effect on insulin-stimulated insulin receptor substrate (IRS)-1 phosphorylation.18 However, in fructose-treated rats, losartan improved both glucose tolerance and IS.19

Captopril partially reversed the effect of LSD on IS

Decreased AT2 receptor stimulation can explain this result, but this was not evaluated in the present study. Another effect of captopril is the increased level of kinins.17 Henriksen and Jacob20 demonstrated that captopril improves GU in SM through a bradykinin dependent process. Recently, this group21 hypothesized that bradykinin is an insulin modulator for glucose transport in SM. In fact, the circulating bradykinin elevation resulting from inhibition of bradykinin degradation22 underlies the beneficial metabolic effects of ACE-I23 that could be prevented by a selective B2-bradykinin receptor antagonist (HOE-140).24 Therefore, the effect of captopril on GU obtained in the present study is probably due to decreased kinin degradation. To confirm this hypothesis, further studies have to be done. Equal decreases in BP after captopril and losartan suggest that the differences in IS occurred despite equal levels of AT1 receptor activation.

Recent investigations have shown that GLUT4 translocation in SM25 and heart26 is stimulated by bradykinin. No measure of GLUT4 content and activity was made in LSD captopril-treated rats in this study. However, research has
demonstrated\(^\text{18}\) that bradykinin enhances insulin-induced phosphorylation of insulin receptors and insulin-stimulated association of IRS-1 and phosphatidylinositol-3-kinase in SM of aged rats, all of which are essential for insulin-mediated GLUT4 translocation and glucose transport.\(^\text{27}\) In contrast, Jacob et al\(^\text{28}\) have found that increased muscular levels of GLUT4 protein after long-term administration of ACE-I were not mediated by kinins. In the present study, GLUT4 levels were lower in rats on LSD or NSD than in those on HSD, which suggests a GLUT4 gene expression modulation. This alteration was observed in all evaluated insulin-sensitive tissues except heart, which indicates that the heart may have additional modulators. No evidence exists for a cause-effect association between the observed lower GLUT4 and the lower IS in LSD and NSD versus HSD animals. Hormonal or hemodynamic factors related to the degree of salt consumption can be responsible for alteration of both IS and GLUT4 activity. Changes in tissue perfusion are known to modify IS.\(^\text{29,30}\)

The results observed in CM suggest that GLUT4 gene expression is not regulated in the same way in all insulin-sensitive tissues. Other mechanisms, such as continuous CM contractile activity, may be involved. Future investigations should focus on the molecular mechanisms, which includes insulin signaling factors and GLUT4 translocation, whereby long-term salt restriction decreases insulin-stimulated GU. INS was 40% higher in rats on LSD than in those on NSD or HSD during the SS period of EHC usage. Decreased insulin clearance may be responsible for this phenomenon.\(^\text{31}\) Indeed, decreased blood flow to the liver as a result of angiotensin II\(^\text{32}\) can diminish insulin clearance.\(^\text{33}\) Thus, the increased insulin level during SSPI in LSD rats may be related to high plasma angiotensin II concentration.

An interesting observation was the increase in adipose tissue mass in several locations and the increase in lipid carcass content on LSD rats. This occurs in parallel with the higher BW in this group (LSD) compared with the other 2 (NSD and HSD). Obesity is well known to be related to insulin resistance,\(^\text{34}\) but this mechanism was ruled out by data from the pair-weight experiments. In these experiments, to keep the same BW in the 3 groups, LSD rats were submitted to caloric restriction. Because in this condition no study has shown higher proteolytic than lipolytic activity, it is unlikely that LSD rats submitted to pair-weight tests had increased fat mass. The present study did not aim to elucidate which mechanisms are involved in LSD-mediated increase in BW. It is possible that decreased energy expenditure occurs during long-term salt restriction. In fact, plasma NEFA concentration was higher in rats on LSD and NSD than in those on HSD. This metabolic change could be related to alterations in energy expenditure.

High plasma cholesterol and triglycerides were observed in humans\(^\text{35,36}\) and rats\(^\text{34}\) submitted to LSD. Thus, increased triglycerides levels induced by the LSD may be an additional mechanism that explains the decreased levels of insulin-stimulated GU. In fact, recently a study has demonstrated that SM insulin resistance may be in part related to elevated circulating triglycerides levels.\(^\text{37}\)

In summary, in the present study, we observed that LSD decreases insulin stimulated GU. Captopril partially reversed the low IS induced by salt restriction, by an angiotensin II–independent mechanism. IS was not related to BW in the 3 experimental groups. GLUT4 gene expression was modulated by the degree of dietary salt intake in insulin sensitive tissues except CM.

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High- or Low-Salt Diet From Weaning to Adulthood: Effect on Insulin Sensitivity in Wistar Rats
Patrícia de O. Prada, Maristella M. Okamoto, Luzia Naôko S. Furukawa, Ubiratan Fabres Machado, Joel C. Heimann and Miriam Sterman Dolnikoff

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