

# Dietary Sodium Restriction Exacerbates Age-Related Changes in Rat Adipose Tissue and Liver Lipogenesis

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To investigate the effects of prolonged dietary sodium restriction on lipid metabolism, male rats weighing 35 to 40 g (just weaned) were fed either a low-salt (LSD) or a normal salt diet (NSD) and used in metabolic experiments after 1, 2, or 3 months of diet consumption. After 2 and 3 months on the diet, LSD rats showed increased amounts of lipid in carcass and retroperitoneal tissue. In both LSD and NSD, extending the feeding period from 2 to 3 months resulted in a marked reduction in the *in vivo* rates of adipose tissue fatty acid synthesis that was accompanied by increases in liver lipogenesis and in the activity of adipose tissue lipoprotein lipase (LPL). However, these increases were more marked in LSD rats. Thus, *in vivo* rates of liver fatty synthesis and LPL activity in LSD rats, which were already higher (by about 35% and 20%, respectively) than in controls after 2 months, attained levels 50% higher than those in NSD animals after another month on the diet. Brown adipose tissue (BAT) thermogenic capacity, estimated after 2 and 3 months by the tissue temperature response to norepinephrine (NE) injection and by guanosine diphosphate (GDP) binding to BAT mitochondria, did not change in controls, but was significantly reduced in LSD rats. This raises the possibility that a decrease in overall energy expenditure, together with an LPL-induced increased uptake of preformed fatty acids from the circulation, may account for the excessive lipid accumulation in LSD rats. Taken together, the data indicate that prolonged dietary sodium restriction exacerbates normal, age-related changes in white and BAT metabolism.

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**R**ESTRICTION OF DIETARY sodium is usually recommended in the treatment of essential hypertension.<sup>1</sup> However, recent studies<sup>2-5</sup> suggest that severe (but not moderate<sup>6,7</sup>) reduction in dietary salt may adversely affect lipid (increased levels of plasma triacylglycerol (TAG), cholesterol, and low-density lipoprotein) and glucose metabolism (increased resistance insulin), raising doubts about the efficacy of this type of diet in the prevention of coronary disease and reduction of mortality.<sup>8</sup> Adipose tissue and liver metabolism were not specifically addressed in the above studies, which have been performed in patients submitted to salt restriction for relatively short (1 to 4 weeks) periods.

Except for a recent study,<sup>9</sup> no studies have been hitherto reported on the effect of low-salt diets (LSDs) on adipose tissue lipid metabolism in laboratory animals. In that study,<sup>9</sup> it was found that chronic salt restriction (12 weeks) in normotensive rats induced an increase in body weight and in the mass of abdominal adipose tissue, the animals showing a 90% increase in the amount of mesenteric adipose tissue when compared with control rats. The present study was designed to investigate the biochemical mechanisms involved in the increased fatness observed in rats submitted to an LSD for a long period, extending from weaning to adulthood. To this end, just weaned rats were maintained on an LSD, and metabolic experiments were performed after 1, 2, or 3 months of diet consumption,

including measurements of rates of liver and adipose tissue fatty acid synthesis and adipose tissue lipoprotein lipase (LPL) activity during the last 2 experimental periods. The results of experiments in which brown adipose tissue (BAT) thermogenic capacity was assessed are also reported.

## MATERIALS AND METHODS

Male Wistar rats weighing 35 to 40 g (just weaned) were fed for 1, 2, or 3 months with either an LSD (0.06% Na<sup>+</sup>) or a normal salt diet (NSD) (0.5% Na<sup>+</sup>) (TD 92141 and TD 92140, respectively, Harlan Teklad, Madison, WI). Except for Na<sup>+</sup> content, these 2 diets have the same composition and contain 25% protein, 51% carbohydrates, and 6% fat. Rats were housed in individual cages in a controlled-temperature environment (25°C ± 2°C) with a 12-hour light:dark cycle and water *ad libitum*. Food and water ingestion, 24-hour urinary volume, and body weight were measured during the 10 days that preceded the terminus of each of the periods (1, 2, and 3 months) of diet consumption, when the metabolic measurements were made. All experiments were performed between 8 AM and 10 AM.

### *In Vivo Lipogenesis Measurements*

<sup>3</sup>H<sub>2</sub>O (5 mCi) was injected intravenously through a Silastic catheter (Dow Corning, Midland, MI) inserted into the right jugular vein 2 days before the experiment, and the rats were killed by decapitation 60 minutes after label injection. The retroperitoneal fat depots and liver were rapidly removed and weighed. Total lipids from tissue samples were extracted with 2:1 chloroform:methanol by the procedure of Folch et al.<sup>10</sup> <sup>3</sup>H<sub>2</sub>O not incorporated was removed from the inferior phase (predominantly chloroform) by washing 3 times with a superior-phase mixture.<sup>10</sup> After each shaking, the tubes were briefly centrifuged to sharpen the phase boundary, and the superior phase was aspirated and discarded. Isolation and counting of <sup>14</sup>C-labeled fatty acids and glycerol from the inferior phase was as previously described.<sup>11</sup>

For calculations, it was assumed that the specific activity of intracellular water was identical to that of plasma water, which was determined directly in aliquots of diluted (20 times) plasma. Rates of tissue lipid synthesis were calculated assuming that each glycerol and each fatty acid incorporated into TAG contained 3.3 and 13.3 atoms of tritium, respectively.<sup>12,13</sup>

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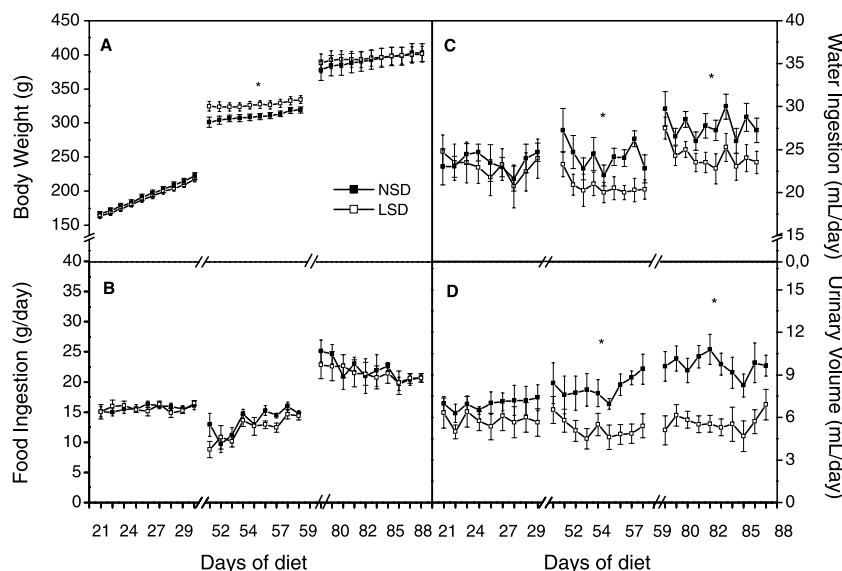


Fig 1. Effect of prolonged (1, 2, and 3 months) administration of an LSD on (A) body weight, (B) food, (C) water intake, and (D) urinary volume of rats. \* $P < .05$  v NSD.

#### LPL Activity

An anhydrous emulsion of tri- $^{14}\text{C}$ -oleoylglycerol, stabilized by lecithin, was prepared in glycerol as described by Nilsson-Ehle and Schotz.<sup>14</sup> The assay substrate solution was prepared daily by adding 2 vol of the emulsion, 2 vol of Tris buffer 0.2 mol/L (pH 8.8) containing 6% (wt/vol) bovine serum albumin and 1 vol of 36-hour fasted rat serum. After vigorous shaking in a Vortex mixer for 5 seconds, the new emulsion was ready for immediate use. Retroperitoneal adipose tissue was homogenized in 0.25 mol/L saccharose, 1 mmol/L EDTA buffer with 20 U/mL heparin. Incubations were performed at 37°C in a total volume of 0.2 mL (0.1 mL of assay substrate and 0.1 mL of tissue homogenate and/or buffer). The  $^{14}\text{C}$ -fatty acid produced during the incubations was isolated using a modification of the liquid-liquid partition system described by Belfrage and Vaughan<sup>15</sup> and counted. Enzyme activity was expressed as nanomoles of oleic acid per minute per milligram protein.

#### BAT Temperature Response to Norepinephrine Injection

Animals were anesthetized with pentobarbital (40 mg/kg, intraperitoneal [IP]), and a catheter was inserted into the right jugular vein using a Silastic tubing (no. 602-135, Dow Corning). The BAT temperature was measured by making a small surgical incision above the BAT and placing a small thermistor between the 2 lobes of the tissue. Core (colonic) temperature was measured by inserting a similar thermistor 5 cm beyond the anus (256S; Dixtal Tecnologia, Ribeirão Preto, São Paulo, Brazil). Norepinephrine (NE) was infused through the jugular vein catheter at a rate of  $2 \mu\text{g} \cdot 100 \text{ g body weight}^{-1} \cdot \text{min}^{-1}$ .<sup>16</sup>

#### Guanosine Diphosphate Binding to BAT Mitochondria

BAT mitochondria were isolated as described by Cannon and Lindberg<sup>17</sup> and suspended in 0.25 mol/L sucrose to a final protein concentration of 8 to 10 g/L. Binding of purine nucleotide to isolated mitochondria was measured with  $^3\text{H}$ -guanosine diphosphate (GDP) (Amersham, Little Chalfont, UK) as described by Nicholls,<sup>18</sup> except that  $^{14}\text{C}$ -inulin (NEN Products, Du Pont, Boston, MA) was used to correct for the amount of  $^3\text{H}$ -GDP in the trapped medium. Mitochondrial protein was determined by the method of Lowry et al<sup>19</sup> with bovine serum albumin as the standard.

#### Other Chemical Analyses

Tissue lipid content was determined as described.<sup>10</sup> After removal of the intestinal tract, the remaining carcass was weighed, autoclaved to soften the bones, homogenized in 500 to 1,000 mL water, and the lipid content was determined gravimetrically after extraction by the Folch procedure.<sup>10,20</sup> Plasma glucose was determined using a Beckman glucose analyzer (Fullerton, CA). Plasma TAG, free glycerol, and free fatty acid (FFA) levels were assayed enzymatically using commercial kits from Labtest Diagnóstica, São Paulo, Brazil and Wako Chemicals, Richmond, VA, respectively. Plasma concentration of insulin was determined by radioimmunoassay using a kit from Amersham. Sodium concentration was measured by flame photometer, and the osmolality was determined by freezing point depression.

#### Statistical Methods

Data are expressed as means  $\pm$  SEM. Differences between means were analyzed using 2-way analysis of variance (ANOVA) or Student's *t* test, as appropriate, with  $P < .05$  as the criterion of significance.

### RESULTS

Figure 1A to D shows the results of measurements made in metabolic cages during the 10 days that preceded the terminus of each of the periods (1, 2, and 3 months) of diet consumption. The data in Fig 1A and 1B show that, except for body weight values somewhat higher in LSD rats at the end of the second month, no significant difference was observed in body weight and food intake of LSD and NSD rats during the periods examined. Figure 1C and D show that, as expected, urinary volume and water ingestion were reduced in LSD rats probably because of sodium retention. These differences became more evident after 3 months of LSD consumption (Fig 1C and D). Except for a 20% decrease in LSD rats ( $129 \pm 3 \text{ mEq/L}$ , 10 rats) compared with control values ( $148 \pm 3$ , 10 rats) after the first month, the concentration of plasma sodium did not differ significantly in the 2 groups after 2 and 3 months, remaining around  $146 \pm 1.5 \text{ mEq/L}$  (average of 40 animals). Urinary sodium (mEq/24 hours) of both LSD and NSD rats did not

**Table 1. Lipid Content of Carcass, Liver, Epididymal, and Retroperitoneal Fat Pads From Rats Fed the LSD or Control (NSD) Diet for 1, 2, and 3 Months**

Diet (mo)	Total Lipids (%)			
	Carcass	Liver	Epididymal	Retroperitoneal
NSD				
1	12.6 ± 0.6	4.1 ± 0.5	81.4 ± 2.5 (2.14 ± 0.15)	83.4 ± 1.1 (1.75 ± 0.22)
2	11.8 ± 0.8	5.8 ± 0.3	84.5 ± 1.7 (3.74 ± 0.18)	86.6 ± 1.6 (2.91 ± 0.24)
3	13.9 ± 1.1	6.9 ± 0.4	83.9 ± 1.0 (6.95 ± 0.44)	85.7 ± 1.9 (6.44 ± 0.48)
LSD				
1	14.0 ± 1.7	4.4 ± 0.6	81.4 ± 0.9 (1.83 ± 0.09)	83.4 ± 1.5 (1.29 ± 0.10)
2	13.4 ± 0.6	6.7 ± 0.5	85.8 ± 1.5 (3.14 ± 0.26)	92.4 ± 1.2* (3.62 ± 0.28)
3	20.6 ± 2.2*	7.1 ± 0.4	84.0 ± 1.7 (6.57 ± 0.50)	95.6 ± 1.6* (6.04 ± 0.53)

NOTE. Data are means ± SEM of 10 animals. The weights of epididymal and retroperitoneal fat pads (g) are shown in parentheses.

\* $P < .05$  v NSD ( $n = 8$  to 10 animals).

change significantly during the 3 months of observation and was reduced in the LSD animals ( $0.33 \pm 0.02$ , average of 30 rats) to about 10% of values in rats on the NSD ( $2.75 \pm 0.15$ , 30 animals). The osmolality of the urine (mOs/ kg/24 hours) also remained relatively constant during the 3-month experimental period and was 25% lower in LSD rats ( $2,034 \pm 112$ , 30 animals) than in NSD rats ( $2,781 \pm 51$ , 30 animals). On the other hand, plasma osmolality (mOs/ kg) was not affected by the diet during any of the experimental periods, averaging  $298 \pm 0.8$ , 60 animals).

Plasma levels of glucose, FFAs, TAGs, and insulin were not affected by the LSD and did not differ significantly after 2 and 3 months in both NSD and LSD rats. Combined values for 2 and 3 months in control and LSD animals ( $n = 20$  rats) were, respectively, glucose (mg/dL),  $120.5 \pm 3.5$  and  $118.0 \pm 1.5$ ; FFAs ( $\mu$  mol/mL),  $0.53 \pm 0.03$  and  $0.52 \pm 0.02$ ; TAG (mg/dL),  $70.0 \pm 7.0$  and  $68.5 \pm 7.5$ ; insulin (ng/mL),  $3.6 \pm 0.4$  and  $4.1 \pm 0.5$ .

In the 2 experimental groups (Table 1), liver lipid content increased progressively from 1 to 3 months, but neither liver fat nor liver weight (not shown) was affected by sodium restriction. Except for a tendency ( $P = .07$ ) to an increase in the weight of LSD retroperitoneal adipose tissue after 2 months on the diet, the weight of epididymal and retroperitoneal pads did not differ significantly in the 2 groups during all experimental periods (Table 1). However, the fat content of retroperitoneal,

but not of epididymal tissue, increased significantly (about 8% to 10%) in LSD rats after 2 and 3 months on the diet (Table 1). The amount of fat in the abdominal area, difficult to isolate for quantitative comparisons, was visibly greater in LSD animals. Table 1 also shows that sodium restriction induced a 50% increase in the carcass lipid content after 3 months of diet consumption.

The results of in vivo lipogenesis measurements in liver and retroperitoneal adipose tissue after 2 and 3 months of diet consumption are shown in Table 2. The data in Table 2 show that in both LSD and NSD rats, extending the feeding period from 2 to 3 months resulted in a marked reduction in the in vivo rates of adipose tissue fatty acid synthesis that was accompanied by increased rates of tissue glyceride-glycerol synthesis. No significant difference was observed in adipose tissue lipogenesis in LSD and NSD rats (Table 2). Table 2 also shows that, in contrast to adipose tissue, extending the feeding period increased liver lipogenesis in both experimental groups. Furthermore, the increase in liver lipogenesis was more marked in LSD rats. Thus, in vivo rates of liver fatty acid synthesis in LSD rats, which were already higher (about 35%) than in controls after 2 months, increased to levels about 50% of values in NSD animals after another month on the diet (Table 2).

The data in Fig 2 show that the activity of adipose tissue (retroperitoneal) LPL changed in parallel to the changes in liver fatty acid synthesis. The activity of the enzyme increased with

**Table 2. In Vivo Triacylglycerol-Fatty Acid and -Glycerol Synthesis ( $\text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ) From  $^3\text{H}_2\text{O}$  by Retroperitoneal Adipose Tissue and Liver From Rats Fed the LSD or Control (NSD) Diet for 2 or 3 Months**

Tissue	Diet (mo)	Triacylglycerol-Fatty Acid		Triacylglycerol-Glycerol	
		NSD	LSD	NSD	LSD
Retroperitoneal	2	55.0 ± 5.1	58.6 ± 5.9	50.4 ± 5.3	50.5 ± 6.1
	3	1.8 ± 0.2*	1.6 ± 0.2*	72.5 ± 5.7*	76.4 ± 7.4*
Liver	2	2.2 ± 0.1	2.9 ± 0.3†	55.2 ± 4.9	52.2 ± 4.4
	3	3.4 ± 0.3*	7.0 ± 0.7*†	66.0 ± 7.0	56.8 ± 4.6

\* $P < .05$  v 2 months; † $P < .05$  v NSD ( $n = 8$  to 10 animals).

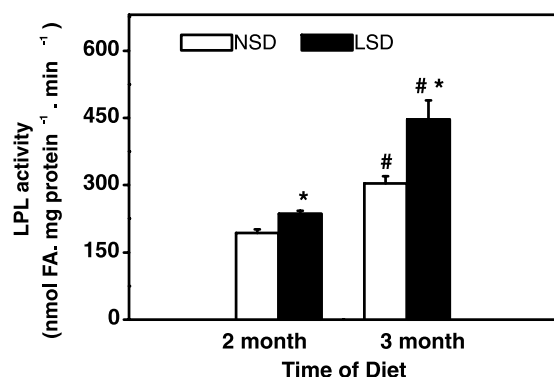


Fig 2. Effect of administration (2 and 3 months) of an LSD on the activity of LPL in retroperitoneal adipose tissue. \* $P < .05$  v NSD; # $P < .05$  v 2 months of diet.

the period of diet consumption in both LSD and NSD rats, and the increase was also more marked in LSD rats. In these animals the activity of LPL was already 20% higher than in controls after 2 months and attained levels 50% over NSD values after 3 months on the diet (Fig 2).

The results of the experiments in which the effect of sodium restriction on BAT thermogenic capacity was investigated are shown in Table 3 and Figs 3 and 4. These experiments were prompted by the observation that the accumulation of fat occurred with no change in food intake, together with the well-known role of BAT in the control of overall energy expenditure in rodents. The data in Fig 3 show that in both NSD and LSD rats, extending the feeding period from 2 to 3 months resulted in a significant ( $P < .05$ ) decrease in basal (preinjection) interscapular BAT (IBAT) temperature. Table 3 and Fig 3 show that at both time intervals the response of IBAT temperature to NE infusion was markedly reduced ( $P < .05$ ) in rats fed the LSD. After 3 minutes of infusion, maximal increases in IBAT temperature were 70% smaller in LSD rats than in controls. Maximal increases in colonic temperature were also significantly reduced in rats fed the LSD (Table 3). The results in Fig 4 show that in the 2 experimental groups, the capacity of BAT mitochondria to bind GDP was not affected by the feeding period, but was markedly reduced in BAT mitochondria from rats with dietary restriction of sodium. These signs of reduced BAT thermogenic capacity were not accompanied by changes in tissue mass ( $0.274 \pm 0.025$  and  $0.272 \pm 0.022$  g,

Table 3. Response of IBAT and Colonic Temperature to Intravenous Infusion of Norepinephrine in Rats Fed the LSD or Control (NSD) Diet

	Maximal Temperature Increase (°C)			
	2 Months		3 Months	
	IBAT	COLON	IBAT	COLON
NSD	$1.32 \pm 0.10$	$0.49 \pm 0.04$	$1.25 \pm 0.07$	$0.48 \pm 0.05$
LSD	$0.39 \pm 0.05^*$	$0.28 \pm 0.06^*$	$0.34 \pm 0.05^*$	$0.26 \pm 0.01^*$

NOTE. Values are means  $\pm$  SEM from 10 animals.

\* $P < .05$  v NSD.

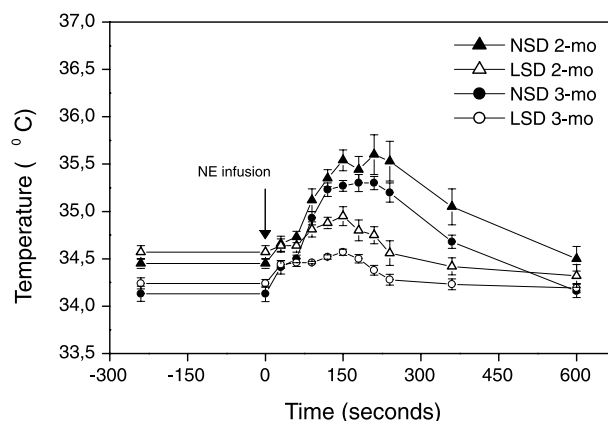


Fig 3. Effect of administration (2 and 3 months) of an LSD on the response of BAT temperature to a 3-minute infusion of NE ( $2 \mu\text{g} \cdot 100 \text{ g body weight}^{-1} \cdot \text{min}^{-1}$ ). See Results for significance ( $P$  values).

combined values [2 and 3 months] of control and LSD rats, respectively).

## DISCUSSION

Aside from the expected LSD-induced changes in urinary volume and water intake, the main findings of the present study were the alterations produced by the diet in the metabolism of adipose tissue. An adequate discussion of these alterations requires a previous analysis of the results obtained in the control rats, fed the NSD. In these animals, prolongation of the experimental period from 2 to 3 months resulted in a marked reduction in adipose tissue lipogenesis that was accompanied by significant increases in liver fatty acid synthesis and in the activity of adipose tissue LPL. It has been known for many years, and it has been repeatedly confirmed, especially in rats, that adipose tissue lipogenesis and the activity of lipogenic enzymes decrease with the age of the animals.<sup>21-25</sup> Most of the studies have been performed in rats 1 to 2 years old, and the reduction in lipogenesis has usually been considered to be related to the aging (senescence) of the animals. Also, because

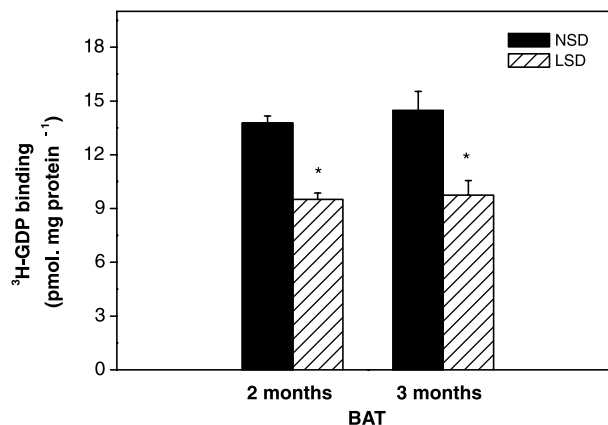


Fig 4. Effect of administration (2 and 3 months) of an LSD on GDP binding to BAT tissue mitochondria. \* $P < .05$  v NSD.

in aging rats there is a progressive increase in body fat, the reduced lipogenic activity has been considered a self-regulatory process to prevent excessive fat accumulation.<sup>25</sup> In view of the results obtained in control rats, these concepts require some qualifications. First, the animals were started on the diet immediately after weaning, and after 3 months had reached adulthood, but were not old. Therefore, the pronounced reduction (compared with levels after 2 months) in adipose tissue lipogenesis in these rats, although age-related, cannot be considered as an "aging" (senescence) process, but rather as related to the development of the animal. Secondly, the view of the decrease in lipogenesis as a self-regulatory process has been proposed and may be valid for older rats, when the reduced adipose tissue lipogenesis is accompanied by a progressive increase in body fat. However, despite the decrease in lipogenesis at the third month, there was no change in the lipid content of carcass or adipose tissue in the control rats of the present study during the 3-month experimental period. This finding also supports the idea that the reduction in lipogenesis found in the present study was part of a developmental process.

The finding in the present study that, in contrast to adipose tissue, liver lipogenesis in rats fed the NSD increased during the third month (postweaning), is at variance with studies showing that, similar to adipose tissue, lipogenesis and activity of lipogenic enzymes decrease in liver with age.<sup>26</sup> Again, these studies were performed in rats 1 to 2 years old. However, in one study using young (just weaned), adult (3 months old), and aged (1-year-old) rats, it was found that liver lipogenesis increased markedly in adult animals before decreasing in 1-year-old rats.<sup>27</sup> Hence, the possibility exists that the increase observed in control rats when the feeding period was extended from 2 to 3 months correspond to the initial phase of liver lipogenesis activation, before the subsequent period of progressive reduction.

The finding of an increase in the activity of adipose tissue LPL observed in control rats during the third month of the experimental period is in agreement with the substantial and progressive increase in the activity of this enzyme after weaning.<sup>28</sup> In view of the pattern of lipid metabolism observed in the control rats, fed the NSD, the simplest explanation for the effects of the LSD would be that the administration of this diet exacerbates developmental changes in adipose tissue and liver lipid metabolism that occur normally in the rat. This would explain the higher than normal increases in liver lipogenesis and in the activity of LPL and also the excessive accumulation of lipid in LSD rats, which would result from the increased rates of LPL-induced uptake of fatty acids from lipoprotein-TAG (chylomicrons- and/or very-low-density lipoprotein (VLDL)-TAG).

Another energy-linked metabolic process that is well known to decline with aging is the thermogenic capacity of BAT.<sup>29</sup> Most of the experiments have been performed in 1 to 2 years old rats, but a decrease in BAT activity has also been observed at the age of 6 months.<sup>29</sup> In the present study, extending the feeding period of control NSD rats from 2 to 3 months did not affect significantly BAT thermogenic capacity, as judged by the response (increase over preinjection values) of tissue temperature to NEnephine infusion (Fig 3) and by the binding of GDP to BAT mitochondria (Fig 4). However, there was a clear

decrease in BAT thermogenic activity in rats fed the NSD, evident by the significant reduction in basal, nonstimulated, BAT temperature when the feeding period was prolonged from 2 to 3 months (Fig 3). Although the mechanisms involved in this decrease in activity in normally fed animals are not known, it is interesting to speculate that it is a preindication of the reduction in BAT thermogenic capacity, which has been demonstrated, in older rats. In any case, this age-related reduction was greatly anticipated in the rats fed the LSD, in which both BAT temperature response to NEnephine and GDP binding to BAT mitochondria were already markedly reduced after 2 months of diet consumption. This raises the possibility that a decrease in overall energy expenditure and an increase in metabolic efficiency could also contribute to the exaggerated accumulation of fat in the rats consuming the LSD.

The fundamental biological mechanisms that underlie the developmental process are not known. Clearly, more information about these mechanisms is needed before any reasonable hypothesis can be advanced to explain the exacerbation induced by chronic sodium restriction on the age-related changes of liver and adipose (white and brown) metabolism. Because thermogenesis in BAT is under direct sympathetic control, the possible association between the age-related attenuation of BAT thermogenesis and a decrease in sympathetic activity has been investigated. However, the activity of the sympathetic nervous system seems to increase with age. Thus, although BAT from old rats do not maintain the capacity to respond to NEnephine with increased thermogenesis, it has been found that the sympathetic signaling to the tissue, as determined by neural recordings<sup>30</sup> and NEnephine turnover measurements,<sup>31</sup> does not decrease, but rather appears to increase with age. Hence, the age-related reduction in BAT thermogenesis seems to be due to an alteration of the cellular signal transduction, rather than to changes in neural or hormonal signaling. As reviewed,<sup>29</sup> the preponderance of the evidence suggests that BAT  $\beta$ -adrenergic receptor number and responsiveness decreases significantly with age. Verification of the effects of prolonged administration of an LSD on the transduction of sympathetic and hormonal signals in both BAT and white adipose tissue may facilitate the understanding of the mechanisms of the changes in lipid metabolism induced by sodium restriction.

In summary, the present results show that chronic administration of an LSD to rats causes an excessive accumulation of fat that is accompanied by increases in liver lipogenesis and in the activity of white adipose tissue LPL and by a reduction in BAT thermogenic capacity. The data suggest that sodium restriction exacerbates normal, age-related, developmental changes in lipid metabolism, and that the accumulation of body fat probably results from increases in the uptake of preformed fatty acids from the circulation and in the organism metabolic efficiency.

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