Plasma Nonesterified Fatty Acids in the Dahl Rat Response to Salt Loading

RALPH A. KELLY, JANICE M. PFEFFER, WILLIAM E. MITCH, AND THOMAS W. SMITH

SUMMARY The link between dietary salt intake and the development of hypertension in the salt-sensitive Dahl strain of rats remains elusive. There is evidence that Dahl salt-sensitive rats (DS) produce less vasodilator and natriuretic prostaglandins in response to salt loading than do control salt-resistant rats (DR), although the reason for this blunted response is unknown. We examined the effects of chronic dietary salt loading on the plasma levels of nonesterified fatty acids in DS and DR. Animals were fed the same chow containing either 0.4% or 4% NaCl (wt/wt). At 12 weeks, 75 µl of tail capillary blood was obtained from restrained, conscious rats, and principal nonesterified fatty acids were measured by high performance liquid chromatography. Total nonesterified fatty acids rose in the 15 DR on high salt diets compared with values in 11 rats eating low salt (0.57 ± 0.05 vs 0.35 ± 0.01 mM; p < 0.001). The greatest changes occurred in levels of arachidonic acid (+ 287%) and in the arachidonic precursors, linoleic (+ 89%) and linolenic (+107%) acids. In marked contrast, there was no change in levels of plasma nonesterified fatty acids in DS fed 4% NaCl compared with DS fed 0.4% NaCl. These observations suggest that defective production of natriuretic and vasodilator prostaglandins by DS may be due in part to an inability to produce or release eicosanoid precursors from phospholipid stores in response to dietary salt. (Hypertension 10:198-203, 1987)

KEY WORDS fatty acids hypertension Dahl rats prostaglandins phospholipase A2 arachidonic acid

THE mechanism by which dietary NaCl contributes to the etiology of hypertension in the Dahl rat remains a topic of considerable debate.1-6 It has been proposed that the propensity of Dahl salt-sensitive rats (DS) to become hypertensive is related to their inability to produce vasodilator and natriuretic prostaglandins in response to a high salt diet7-9 or to modulate renal prostaglandin receptor number in response to changes in dietary salt.10 An alternative hypothesis holds that, in response to salt loading, the DS produce more of a humoral "hypo-
sinogenic" factor than do Dahl salt-resistant rats (DR). One proposed candidate for this transferable factor is the putative natriuretic hormone, or endogenous ouabainlike substance, which, by inhibiting Na⁺,K⁺-adenosine triphosphatase, might have a saluretic action and might act on peripheral resistance vessels to raise blood pressure.2,3,11-13 Recent evidence from our own laboratory and that of others suggests that much of the ouabainlike activity detected in mammalian plasma can be attributed to lipids, specifically, free fatty acids and lysophospholipids.14-16 Indeed, Tamura et al.16 have recently shown that the increased ouabainlike activity they detected in hogs that were volume-expanded with saline was due to oleic and linoleic acids.

Therefore, we sought to determine 1) whether plasma nonesterified fatty acids (NEFAs) increased in response to dietary salt loading in rats genetically susceptible to hypertension, the Brookhaven Dahl strain, and 2) whether there was a difference in the plasma NEFA response to dietary salt between the salt-sensitive and salt-resistant animals. All of the principal long-chain fatty acids in plasma were measured to de-
termine whether an increase in NEFAs reflected generalized lipolysis or a more specific release of eicosanoid precursors into the circulation.

Materials and Methods

Animals

Selectively bred, female weanling DS and DR were obtained from Dr. J. Iwai of the Brookhaven National Laboratory (Upton, NY, USA) and thereafter maintained in the animal facility of Dr. J. Pfeffer. Seventeen DS and II DR were fed a 0.4% NaCl chow, and 14 DS and 15 DR were given the same chow but supplemented to contain 4% NaCl. All animals were given tap water to drink. Systolic blood pressures were obtained in triplicate on each of 3 separate days in conscious animals using an indirect tail-cuff method; these data then were averaged. After 12 weeks, plasma levels of NEFAs were measured.

Protocol

For measurement of plasma NEFAs, 75 μl of capillary blood from the tail was obtained from conscious, restrained DS and DR. The animals had been trained to lie quietly in the restraints for serial blood pressure determinations; at least 1 minute elapsed between the time the animal was placed in the restraints and blood sampling was begun. Tails were warmed in water at 37°C, the tip was cut with a scalpel, and the tail was gently milked to avoid hemolysis. The blood was collected in heparinized capillary tubes and centrifuged immediately; the plasma was separated and stored at −20°C overnight.

Determination of Plasma Nonesterified Fatty Acids

Plasma samples were never left frozen for more than 18 hours before extraction. To 15-μl aliquots of plasma, 3 mmol of heptadecanoic acid (C_{17}0; Sigma Chemical Corp., St. Louis, MO, USA) in water was added as an internal standard (final volume, 500 μl). Then, 1 ml of methanol was added, and the samples were vortexed. Next, 3 ml of chloroform containing butylated hydroxytoluene, 5 mg/dl, was added, and the samples vortexed for 1 minute and centrifuged at 3000 rpm for 10 minutes. The organic layer was removed, and the aqueous layer was washed once in 4 ml of chloroform/methanol (3:1). The pooled organic phases were dried under nitrogen, reconstituted in 1 ml of chloroform, and applied to aminopropyl, bonded-phase disposable columns (Bond Elut; Analytichem International, Harbor City, CA, USA) to separate NEFA from neutral lipid and phospholipid, according to the technique of Kaluzny et al. Fractions containing NEFAs were eluted with 2% acetic acid in diethyl ether, dried under nitrogen, and reconstituted in aceton. The recovery of saturated and unsaturated NEFA exceeded 90%, as determined by the recovery of radiolabeled NEFA standards. NEFAs were quantified by reverse-phase high performance liquid chromatography (HPLC) following derivatization with 4-bromomethyl, 7-acetoxy coumarin (Br-Mac). Br-Mac was synthesized as detailed by Tsujiya et al., following the final crystallization, the compound had a melting point of 182 ± 1°C. Extracted NEFAs were derivatized by adding 50 μl of a Br-Mac stock solution (approximately 2 μmol/ml) to 50 μl of dibenzo-18-crown-6-acetone (40 nmol/50 μl) and 2 to 3 mg of a mixture of potassium bicarbonate and sodium sulfate in a 1-ml glass ampul. The ampul was sealed, covered with aluminum foil, and incubated at 50°C for 30 minutes in a shaking water bath. Derivatized NEFAs were stable for over 2 months at −20°C. Twenty-five microliters of this mixture was injected onto the HPLC column.

NEFAs derivatized with Br-Mac were separated on a Waters Nova Pak C_{18} radial compression column (particle size, 4 μm; Waters, Milford, MA, USA) at room temperature using a gradient elution technique with increasing quantities of CH₃CN to methanol and water over 70 minutes. After postcolumn, on-line alkaline hydrolysis, the fluorescence of Br-Mac was measured. Fluorescence was monitored with a Waters Model 480 fluorescence detector (excitation filter, 365 nm; emission filter, 460 nm). The coefficient of variation was less than 5% with derivatized samples containing at least 1 pmol of NEFAs per microliter injected onto the column. The detection limit was approximately 10 fmol. All HPLC runs were reviewed by an investigator initially blinded as to the identity of each sample; a chromatogram was excluded only if an internal standard (heptadecanoic acid, C_{17}0) could not be adequately resolved. The average of duplicate or triplicate chromatograms was used to determine the final concentration of NEFAs in each plasma sample. Examples of typical chromatograms of derivatized NEFAs obtained from DS and DR consuming diets containing 4% NaCl are given in Figure 1.

Statistical Analysis

Statistical significance was determined by analysis of variance (ANOVA). The null hypothesis was rejected if the p value was less than 0.05, as calculated using the Bonferroni test for multiple group comparisons. All data are given as the means ± SEM.

Results

The blood pressures of DS fed a 4% NaCl diet were significantly higher than those of DS fed a 0.4% NaCl diet or of DR fed either NaCl intake (Table 1). There were no significant differences among the groups in body weight. As illustrated in Figure 2, the plasma levels of total NEFAs were elevated by about 40% in DR consuming the high salt chow as compared with the DR eating normal (0.4% salt) chow. In contrast, there was no difference in the level of plasma NEFAs between DS consuming a 0.4% or 4% NaCl diet. In fact, there was no difference in plasma total NEFA levels between DR fed 0.4% salt and DS fed either concentration of salt in the diet (see Figure 2).

In addition to the increases in total NEFAs, the ratio of unsaturated to saturated fatty acids in plasma was significantly higher in DR eating the high salt diet compared to DS fed the same diet (p<0.03; Table 2).
When fed the normal salt (0.4%) chow, both DR and DS had identical proportions of unsaturated to saturated fatty acids.

We also calculated the differences in the plasma levels of individual fatty acids between DS fed high and low salt diets and between DR eating either the 4% or the 0.4% salt diet. These data are shown in Figure 3. There were significant differences in plasma levels of most of the major species of NEFAs from DR fed high salt chow compared to DR fed normal salt chow. In each case, the level of NEFAs was highest in DR eating a 4% salt diet. The largest percentage differences were in arachidonic acid (287%; Figure 4) and the arachidonate precursors, linoleic (89%) and linolenic (107%) acids. In contrast, plasma NEFA levels tended to fall in DS on a 4% NaCl intake as compared with those in DS rats fed 0.4% NaCl, although this fall was statistically significant (ANOVA) only for palmitoleic acid.

Discussion

These data are consistent with the observations of Tamura et al.\textsuperscript{16} that short-term salt loading results in an increase in plasma levels of oleic and linoleic acids in mammals. Our results show that long-term salt loading also causes an increase in plasma fatty acids. We found that all NEFAs increased with dietary salt loading, but that the most dramatic increases occurred in plasma levels of arachidonic acid and its precursors (see Figures 3 and 4). However, the most important finding of the present study was that this increase in plasma levels of NEFAs in response to dietary salt loading was abolished in the hypertensive, salt-sensitive Dahl animal. Although there was a tendency for total plasma NEFA values to be lower in DS fed 4% NaCl as compared with those fed 0.4% NaCl, this difference did not reach statistical significance by ANOVA (see Table 2). Regardless of these changes in total plasma NEFAs, the unsaturated to saturated fatty acid ratios were not dif-

<table>
<thead>
<tr>
<th>Variable</th>
<th>DS</th>
<th>DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>241 ± 3</td>
<td>232 ± 6</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>162 ± 4\textsuperscript{*}</td>
<td>181 ± 4\textsuperscript{†}</td>
</tr>
</tbody>
</table>

Values are means ± SEM. There were no significant differences in weight among the groups. \textsuperscript{*}p < 0.005, compared with values in DS fed 4% NaCl. \textsuperscript{†}p < 0.001, compared with values in DR fed either diet and with DS fed a 0.4 NaCl diet.
FIGURE 2. Total plasma nonesterified fatty acids (FAs) were measured on plasma samples taken from tail capillary blood of conscious, lightly restrained DR and DS at 12 weeks of age. Single (p < 0.02) and double (p < 0.005) asterisks indicate significant differences compared with values for DR fed a 4% NaCl diet.

Different for the two groups of DS, nor was there a tendency for plasma levels of arachidonate and arachidonate precursors to increase more than those of other NEFAs, in contrast to the data for DR (see Table 2).

Do these observations offer any insight into the genetic link between salt intake and hypertension in this animal model? It is unlikely that the differences in plasma NEFAs between DR and DS are simply a result of hypertension. If this were true, then hypertensive DS fed a low salt diet (see Table 1) should have had lower plasma levels of total and omega-6 fatty acids than the normotensive DR fed 0.4% NaCl (see Table 2). As the renal cortex derives a high proportion (~50%) of its energy needs from the metabolism of long-chain fatty acids, it is possible that the changes in plasma NEFAs reflect an alteration in the kidney's rate of oxidation of these lipids. According to this hypothesis, the renal cortex of DS extracts more NEFAs than the cortex of DR in response to salt loading. This difference might explain the data of Dahl and others that the susceptibility for hypertension can be transferred to a DR by transplantation of a kidney from a DS. However, this would not explain why salt-loaded DR have a higher ratio of unsaturated to saturated NEFAs.

A more likely explanation for our data is that the increased plasma levels of NEFAs are the result of renal and vascular production of natriuretic and vaso-dilator eicosanoids in DR fed a 4% salt diet. Support for this hypothesis includes the following: First, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) synthesis in the renal papilla of DS has been shown to be diminished by as much a 60% compared with that in DR regardless of salt intake. Second, a lower intrarenal PGE\textsubscript{2} level in DS is also implied by the recent demonstration by Limas and Limas that PGE\textsubscript{2} receptor density in DS kidneys is higher than that in DR. This difference was attributed to an up-regulation of receptor number. Third, the urinary excretion of two endogenous metabolites of prostaglandin I\textsubscript{2} (PGI\textsubscript{2}) by DR has been shown to increase by 50% with salt loading; the excretion of the PGI\textsubscript{2} metabolites actually declined in DS fed a high salt diet. Finally, Tobian et al. have shown that a diet high in the arachidonate precursor linoleic acid caused a threefold rise in PGE\textsubscript{2} concentration in the renal papillae of DS, reaching a level comparable to that found in DR fed normal chow. Importantly, this diet more than doubled the time required to develop hypertension in DS fed a high salt diet, and the final blood pressure was significantly lower than that of DS fed a high salt diet without supplemental linoleic acid. Although supplements of dietary arachidonate precursors could only delay, not prevent, hypertension in the DS, taken together, these observations suggest that an abnormality of prostaglandin production in DS may be causally related to their hypertension. Our data suggest that a mechanism for the blunted prostaglandin response of DS to salt loading is related to decreased production of prostaglandin precursors.

The striking increase in plasma levels of arachidonate and arachidonate precursors that we found in DR fed a high salt diet suggests that the genetic defect of DS could involve an inability to release these lipids from phospholipid storage pools. This possibility is suggested by the report that dietary linoleic acid significantly raised PGE\textsubscript{2} production in the renal papillae of DS\textsuperscript{8} and that PGI\textsubscript{2} production was normal in aortic rings of DS only if they were exposed to exogenous arachidonate. This possibility does not appear to explain why a DR parabiont joined to a DS should become hypertensive, nor why DS fed a low salt diet become hypertensive when infused with small amounts of serum from hypertensive DS fed a high salt diet. The fact that transplantation of a kidney from a DS to a DR leads to a significant rise in blood pressure in the recipient directly implicates the kidney in the pathogenesis of hypertension in this experimental model. However, it may be that the transferable "hypertensinogenic" factor in the DS does not directly
TABLE 2. Plasma Nonesterified Fatty Acids in DS and DR Fed High or Normal NaCl Diets

<table>
<thead>
<tr>
<th>Plasma NEFAs (µm)</th>
<th>0.4% NaCl</th>
<th>4% NaCl</th>
<th>0.4% NaCl</th>
<th>4% NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>2.47 ± 0.34</td>
<td>3.28 ± 0.62</td>
<td>2.15 ± 0.29</td>
<td>3.96 ± 0.61*</td>
</tr>
<tr>
<td>14:1</td>
<td>10.34 ± 0.95</td>
<td>9.13 ± 1.16</td>
<td>8.62 ± 1.62</td>
<td>13.27 ± 2.28</td>
</tr>
<tr>
<td>18:3</td>
<td>3.13 ± 0.35</td>
<td>2.1 ± 0.28</td>
<td>1.44 ± 0.34</td>
<td>2.99 ± 6.0</td>
</tr>
<tr>
<td>20:4</td>
<td>28.63 ± 2.74</td>
<td>25.89 ± 2.29</td>
<td>22.80 ± 0.94</td>
<td>38.76 ± 4.22*</td>
</tr>
<tr>
<td>16:1</td>
<td>16.40 ± 2.25</td>
<td>6.71 ± 1.21†</td>
<td>7.35 ± 1.22</td>
<td>16.12 ± 2.52*</td>
</tr>
<tr>
<td>18:2</td>
<td>35.03 ± 3.94</td>
<td>23.91 ± 0.59</td>
<td>25.35 ± 2.11</td>
<td>47.92 ± 6.26*</td>
</tr>
<tr>
<td>20:3</td>
<td>4.76 ± 0.42</td>
<td>5.24 ± 0.61</td>
<td>4.78 ± 0.45</td>
<td>8.95 ± 1.04*</td>
</tr>
<tr>
<td>18:0</td>
<td>119.4 ± 11.82</td>
<td>95.25 ± 9.37</td>
<td>90.53 ± 5.58</td>
<td>146.7 ± 13.20*</td>
</tr>
<tr>
<td>18:1</td>
<td>51.02 ± 5.42</td>
<td>42.6 ± 5.41</td>
<td>43.33 ± 3.55</td>
<td>76.96 ± 10.14*</td>
</tr>
<tr>
<td>18:0</td>
<td>145.63 ± 13.15</td>
<td>114.03 ± 8.40</td>
<td>116.67 ± 5.29</td>
<td>160.37 ± 9.48*</td>
</tr>
<tr>
<td>Total</td>
<td>410.66 ± 23.19</td>
<td>347.07 ± 25.2</td>
<td>347.47 ± 11.52</td>
<td>567.38 ± 46.97*</td>
</tr>
<tr>
<td>NEFAsₙₙ</td>
<td>0.558 ± 0.04</td>
<td>0.481 ± 0.03</td>
<td>0.559 ± 0.11</td>
<td>0.660 ± 0.06†</td>
</tr>
</tbody>
</table>

Values are means ± SEM in µm. NEFAs = nonesterified fatty acids; NEFAsₙₙ = ratio of unsaturated to saturated NEFAs. The value given as total NEFAs is larger than the sum of the individual NEFAs listed in each column due to other NEFAs present in plasma at lower concentrations.

*p < 0.01, compared with values in DR fed 0.4% NaCl (by ANOVA).
†p < 0.025, compared with values in DR fed 4% NaCl (by ANOVA).

FIGURE 3. The difference in the plasma levels of nonesterified fatty acids (NEFAs) listed in Table 2 in DR fed a high (4%) NaCl diet as compared with DR fed a low (0.4%) NaCl diet (open bars) is contrasted with the differences in plasma levels of individual NEFAs between DS fed 0.4% NaCl and 4% NaCl (closed bars). Although plasma levels for most NEFAs appear to fall in DS fed 4% NaCl as compared with those in DS rats fed 0.4% NaCl, in only one instance (C₁₆:1) was the decrease statistically significant. Single (p < 0.01) and double (p < 0.005) asterisks indicate significant difference, by ANOVA.
increase peripheral resistance or vascular reactivity. Instead, it could act to interrupt the normal sequence of events that leads to release of vasodilator and natriuretic eicosanoids. For example, it could act by inhibiting, directly or indirectly, the activation of phospholipase A₂, which results in the production of substrate for eicosanoid production. The successful cloning of both human and rat lipocortin, a potential inhibitor of phospholipase A₂, suggests that this possibility may occur.

Acknowledgments

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Figure 4. Plasma levels of arachidonic acid in DR and DS fed a high (4%) or low (0.4%) NaCl diet. The percentage rise in plasma arachidonic acid in DR fed 4% NaCl was the highest of all nonesterified fatty acids measured (287%); there was no significant change in arachidonate levels in the DS fed 4% NaCl.
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