Salt Loads Raise Plasma Fatty Acids and Lower Insulin

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Some fatty acids are potent inhibitors of angiotensin binding and aldosterone production in adrenal glomerulosa cells and thereby may be involved in regulating salt and water balance. To study the possible regulation of fatty acids by salt, we measured the levels of unesterified fatty acids in plasma from patients subjected to extremes of dietary salt intake and saline infusion. Insulin and catecholamines, two known regulators of plasma fatty acids, also were measured. Infusion of 2 l saline over 4 hours caused the levels of most unesterified fatty acids to rise. Total unesterified fatty acids rose 60-100%. A high salt diet caused a smaller rise in total unesterified fatty acids (approximately 33%). In both instances, oleic and palmitoleic acids showed the greatest proportionate increases, whereas stearic acid was relatively unaffected. When salt loads were administered by either intravenous or dietary routes, plasma insulin levels fell by approximately 50%. Plasma norepinephrine increased after saline infusion but not during a high salt diet. Postsaline levels of fatty acids correlated inversely with postsaline levels of aldosterone, supporting a possible role for fatty acids as physiological regulators of the adrenal glomerulosa. A rise in plasma fatty acids and fall in insulin in response to salt loads could act in concert to increase sodium excretion, constituting a physiological mechanism contributing to salt and water balance. (Hypertension 1991;17:958-964)

While studying the interaction of angiotensin with adrenal glomerulosa cells, we found that some fatty acids are potent inhibitors of angiotensin receptors and aldosterone secretion. Among naturally occurring fatty acids, the most potent inhibitors are several unsaturated, long-chain congeners, including oleic (18:1), linoleic (18:2), and arachidonic (20:4) acids. These fatty acids inhibit aldosterone secretion in vitro at concentrations in the micromolar range. We also obtained evidence that bovine, rat, and rabbit adrenal glomerulosa cells are under tonic inhibition by endogenous fatty acids, inhibition that can be reversed by washing the cells with fatty acid–free albumin. One probable source of the inhibitory fatty acids found on adrenal cells is plasma, so we postulated that unesterified fatty acids in plasma participate in regulating salt and water balance by affecting aldosterone secretion. To learn whether salt and water balance could, in turn, regulate plasma fatty acids,* we measured unesterified fatty acids in plasma from humans who had received intravenous or dietary salt loads. We also measured two of the principal humoral regulators of plasma fatty acids, insulin and catecholamines.

Methods

Adult human subjects were studied under three protocols at Indiana University, Indianapolis; Brigham and Women's Hospital, Boston; and the University of Michigan, Ann Arbor. These protocols were designed to assess the renin-angiotensin-aldosterone axis and the sensitivity of blood pressure to salt intake. Informed consent was obtained from all subjects. In the analyses presented below, subjects in

*The term “fatty acids” in this report refers to unesterified fatty acids (UFAs) or nonesterified fatty acids (NEFAs) to distinguish them from fatty acids in triglycerides or other complex lipids. They are sometimes called “free” fatty acids (FFAs), but this term may be confusing by inferring that the fatty acids are not bound to albumin. In fact, the vast bulk of unesterified fatty acids in plasma are bound to albumin and are not “free” in the usual sense.
each protocol were pooled without regard to sex, age, race, or diagnosis. Hypertensive subjects were studied after at least 14 days without antihypertensive medication.

Details of the 3-day Indiana inpatient protocol have been published elsewhere. In brief, subjects were admitted to the General Clinical Research Center and received a diet containing 200 meq sodium/day. On the morning after admission, after an overnight fast, they received an infusion of 2 l saline over 4 hours. Blood samples were drawn just before and just after the infusion. After the infusion, subjects were fed a diet containing 200 meq sodium/day. The third sample we analyzed was drawn on the following morning, after an overnight fast, before the diuretic phase of the protocol was begun.

In the Brigham and Women’s Hospital protocol, studies were performed over a 16-day period during which outpatients first ingested a diet containing approximately 250 meq sodium/day for 7 days. The first blood sample was drawn on the eighth day, after an overnight fast. Subjects then received a diet containing 10 meq sodium/day for 9 days. A saline infusion of 2 l over 4 hours was administered to these subjects on the ninth day of the low sodium diet. The second and third blood samples were drawn, after an overnight fast, before and after saline infusion.

In the Michigan protocol, outpatients ingested diets containing either 250 or 20 meq sodium/day for 7 days. The sequence of diets was randomized, and the two experimental diets were separated by 2 weeks of ad libitum diet. Blood was drawn after an overnight fast on the day following the last (seventh) day of each diet. In all three protocols, sodium intake was verified by 24-hour urine collections.

Plasma samples were stored frozen. All fatty acid analyses were performed at the William S. Middleton Memorial Veterans Hospital, Madison, Wis. Stability of the samples and reproducibility of the assays were estimated by analyzing aliquots of a single sample after one or more freeze-thaw cycles and different lengths of storage. For example, the ratio of stearic acid levels after the second thaw compared with the first was, on average, 1.102; the ratio of arachidonic acid levels was 0.957. All samples from a given patient were analyzed on the same day and compared with a standard mixture of fatty acids tested on that day.

Plasma unesterified fatty acids were extracted with the Dole solvent system as described by Parmelee et al. The extract was treated with O-p-nitrobenzyl-N,N'-disopropylisourea to esterify the fatty acids with the nitrobenzyl chromophore. In preliminary experiments, we showed that the extraction and derivatization procedure did not cleave triglycerides into measurable fatty acids.

Individual fatty acid esters were separated by high-performance liquid chromatography (HPLC), using an 8 × 100-mm Nova-Pak C18 cartridge (Waters Chromatography Div., Millipore Corp., Milford, Mass.). The starting solvent was methanol/acetonitrile/water (72:10:18) with 100 ppm trifluoroacetic acid. Thirty-nine minutes after injection, an 18-minute linear gradient (number 6 on the Waters Model 880 solvent programmer) was run to the final solvent, methanol/acetonitrile/water (93:1:6) with 50 ppm trifluoroacetic acid. Eluate was scanned at 254 nm.

Recovery and derivatization efficiency were measured by adding known amounts of two unnatural fatty acids, nonadecanoic (19:0) and undecanoic (11:0), to each plasma sample. These also provided chromographic landmarks. Peaks were identified by running derivatized samples of standards purchased from Sigma Chemical Co., St. Louis. The elution sequence agreed with other published methods using similar solvents and a phenacyl ester. Individual fatty acids were measured by comparing peak heights with those of a standard mixture run the same day. Corrections for extraction and derivatization efficiencies and chromatographic losses were applied to the raw data. Total unesterified fatty acids were calculated by adding the individual values.

Plasma insulin and aldosterone were measured by radioimmunoassays and catecholamines by a radioenzymatic assay. Statistical methods applied to each result are described in the legends to figures and the table.

**Results**

**Analytical Technique**

Fifteen fatty acid esters were separated by our HPLC system. We did not quantitate docosatetraenoic (22:4) or docosapentaenoic (22:5) acids. Esters of all the fatty acids that we have found to inhibit bovine adrenal aldosterone production were separated from one another and from fatty acids that have no inhibitory activity. For example, palmitic acid (16:0), which essentially is inert in bovine adrenal cells, was clearly separated from oleic acid (18:1), which is a potent inhibitor. Therefore, it is unlikely that our assessment of the plasma content of inhibitory fatty acids was inflated by inadvertent inclusion of inactive congeners. Figure 1 shows typical results.

**Effects of Saline Infusion on Plasma Fatty Acids**

We tested the effects of saline infusion in two different groups of human subjects. The raw data from one experiment are shown in Figures 1A and 1B. Figure 2 shows that infusion of 2 l normal saline into human subjects over 4 hours resulted in a marked elevation of total plasma unesterified fatty acids. Fatty acids increased in 42 of the 44 subjects at the University of Indiana (panel I) and in 14 of the 15 subjects at Brigham and Women’s Hospital (panel B). The mean increase was 62% in the first group and 100% in the second. In the Indiana cohort, the mean level increased from 460 ±32 (SEM) to 744 ±44 μM and in the Brigham cohort from 229 ±33 to 457 ±40 μM. Subjects in the Brigham cohort had ingested a low salt diet for 8 days before infusion; those studied in Indiana had been on an ad libitum diet. This difference in diet may account for the different baseline
fatty acid levels between the two groups (see next section below). Panel I includes a third time point, showing a residual effect on plasma fatty acids 20 hours after saline infusion. Levels of fatty acids were 27% higher at 20 hours after infusion than at baseline.

The results of the two experiments with intravenous saline are shown in greater detail in Figure 3. Values for four individual fatty acids are depicted. Not all fatty acids changed proportionately. For example, saline infusion caused a greater percentage increase in oleic than in stearic acid.

Effects of Dietary Salt on Plasma Fatty Acids

The effects of extreme changes in dietary sodium chloride on plasma levels of unesterified fatty acids are shown in Figure 4. In the Brigham and Women's Hospital protocol (panel B), the high salt diet always preceded the low salt diet, whereas in the Michigan protocol (panel M), the sequence was random, and the experimental diets were separated from each other by a 2-week interval. A high salt diet caused increased levels of total plasma unesterified fatty acids, but the change was less consistent and smaller than that seen with intravenous saline. Among the Michigan subjects, six of nine showed higher plasma fatty acids on high salt; among the Brigham and Women's subjects, only 12 of 21 showed that difference. The mean increase with a high salt diet was 32% in the first group and 33% in the second. These differences were not all statistically significant, with p values of 0.05 for the Brigham cohort and 0.146 for the Michigan cohort.

Figure 4 also shows how oleic acid levels changed with different levels of dietary salt. Oleic (18:1) is the plasma fatty acid that best combines high inhibitory potency against aldosterone production and high circulating level; it inhibits 50% of aldosterone secre-
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FIGURE 3. Changes in individual plasma unesterified fatty acids after intravenous saline infusion (INF). Panel I summarizes results in 44 subjects studied at Indiana University; panel B summarizes results in 15 subjects studied at Brigham and Women's Hospital. The 4-hour infusion of 2 l saline is indicated on the abscissa. Panel I also includes means of levels from samples drawn 20 hours after infusion. Individual fatty acids were determined from elution patterns like those in Figure 1. Vertical brackets indicate SEM. Note the differences in scales for the individual fatty acids. The results show that plasma fatty acids change to different degrees with saline infusion.

FIGURE 4. Effects of dietary sodium chloride on plasma unesterified fatty acids. Panel M: Mean results in a group of nine patients studied at the University of Michigan; panel B: means from 21 patients studied at the Brigham and Women's Hospital. Protocols are described in the text. Total fatty acids were calculated as the sum of individual fatty acids; oleic acid alone is shown in the shaded portions of the bars. Vertical brackets indicate SEM. By matched-pair t-tests, the differences in total fatty acids and oleic acid between low and high salt diets in panel B were significant, with p values of 0.05 for total fatty acids and 0.04 for oleic acid. Means in panel M were not statistically significant, with p values of 0.15 for both total fatty acids and oleic acid.

Effects of Salt Loads on Hormones
One of the principal regulators of plasma levels of fatty acids is insulin, which inhibits fatty acid formation by lipases and accelerates esterification of fatty acids to form triglycerides. In the Indiana and Michigan groups, plasma insulin was measured before and after saline infusion or feeding (Table 1). In the Indiana subjects, saline infusion lowered plasma insulin by an average of 44%. In the Michigan group, insulin levels of subjects on the high salt diet were 47% lower than the levels of those on low salt.

### Table 1. Hormone Changes Accompanying Salt Loads

<table>
<thead>
<tr>
<th>Experiment (and site)</th>
<th>n</th>
<th>Insulin (venous) (microunits/ml)</th>
<th>Norepinephrine (venous) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Preinfusion</td>
<td>Postinfusion</td>
</tr>
<tr>
<td>Saline infusion (Indiana)</td>
<td>44</td>
<td>13.5±1.3</td>
<td>7.5±0.6</td>
</tr>
<tr>
<td>Saline infusion (Brigham)</td>
<td>16</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Dietary salt (Michigan)</td>
<td>9</td>
<td>14.5±2.2</td>
<td>7.7±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Protocols for the separate studies are described in text. Hormone levels were performed on the same plasma aliquots analyzed for fatty acids. Methods of hormone assay are described in References 7 and 9.
The data in Table 1 and the preceding figures might suggest that the rise in fatty acids and the fall in insulin are causally related. However, we did not observe a statistical correlation between these two variables when their levels in individual subjects were compared.

Another powerful group of regulators of plasma unesterified fatty acids are the catecholamines. Indeed, Michigan subjects on the low salt diet showed a direct correlation between venous levels of norepinephrine and plasma nonesterified fatty acids (data not shown). However, this correlation did not persist during the high salt diet, and epinephrine levels showed no correlation with fatty acids on either diet. Both intravenous and dietary salt loads elevated plasma fatty acids, but changes in catecholamines were not congruent; intravenous saline caused a rise in mean plasma norepinephrine, whereas a high salt diet caused a fall. In neither case did the changes in norepinephrine levels correlate with the changes in fatty acids.

To test the possible role of fatty acids in aldosterone regulation, the increase of plasma unesterified fatty acids caused by saline infusion was compared with the fall in plasma aldosterone. In the Indiana cohort, the levels of fatty acids at the end of saline infusion bore a significant inverse relation to the plasma levels of aldosterone (Figure 5.)

**Discussion**

In the experiments described in the present study, human subjects responded to a salt load, either dietary or intravenous, with an increase in plasma levels of unesterified fatty acids and a decrease in plasma insulin. These changes were seen in all three groups of subjects, studied under somewhat different circumstances in three different locales. The changes were seen in subjects of both sexes, black and white subjects, a wide range of ages, and subjects with different degrees of hypertension. Two questions present themselves: 1) What is the mechanism by which salt affects fatty acids and insulin; and 2) what relevance do these plasma changes have to normal and abnormal handling of salt?

The best-recognized regulators of plasma unesterified fatty acids are insulin and catecholamines. Although the rise in fatty acids we observed after salt loads coincided with a fall in insulin levels, there was no statistically significant correlation between these two variables. Furthermore, as shown in Table 1, the fall in insulin with dietary salt was the same as the fall with intravenous saline, but the fatty acid increase with dietary salt was much smaller. Thus, a fall in insulin does not fully explain the rise in fatty acids in response to salt loads.

Catecholamines elevate plasma fatty acid levels by stimulating hormone-sensitive lipases. Recent evidence suggests that this effect is mediated in some adipocytes by \( \beta \)-receptors whose second messenger is probably cyclic AMP. In the Michigan subjects, the levels of plasma fatty acids correlated directly with venous and arterial levels of norepinephrine while the subjects were eating a low salt diet, but no correlation was seen in the same subjects on a high salt diet. Table 1 shows another discrepancy between norepinephrine and fatty acids: saline infusion caused the average plasma level of norepinephrine to rise, whereas salt in the diet caused it to fall. Fatty acid levels rose in both cases. Changes in norepinephrine did not correlate with changes in fatty acids in either the intravenous or dietary experiments. In brief, a rise in catecholamines is unlikely to have been a predominant cause of the rise in fatty acids seen with salt loads.

Other hormones affect fatty acid levels, including corticotropin, glucagon, adrenal glucocorticoids, and thyroxine, but we found no literature describing fluctuations in these hormones with salt loads, and we did not measure them. To examine a possible role for atrial natriuretic peptide (ANP), which is released by saline infusion, we studied plasma from three normal subjects who received ANP infusions. ANP had no effect on plasma fatty acids in those subjects (data not shown). One contributor to the rise in fatty acids during saline infusion may be the 4 hours of fasting involved in the test. In fasting subjects receiving ANP and sham infusions, there was a small rise in fatty acids over the 6 hours of those experiments, but much less than the rise seen during the 4-hour saline infusion in our experiments.

Whatever the mechanism mediating the rise in fatty acid levels with a salt load, it causes variable responses among different acids. For example, palmitoleic (16:1) and oleic (18:1) acids changed more...
dramatically than stearic (18:0) in all of our studies. This probably reflects the specificity of the effects of salt on individual adipocytes or lipases.

Our results document a decline in insulin during salt loads. A similar effect was observed by Iwaoka et al.\textsuperscript{14} In the Indiana cohort, levels of C peptide fell after saline infusion, indicating that saline lowers insulin levels by suppressing insulin secretion (data not shown).

The second major question arising from our observations is what role increased plasma fatty acids or decreased insulin might have in salt and water balance. We had hypothesized that fatty acids in the extracellular fluid participate in the regulation of aldosterone secretion. This hypothesis came from observations of adrenal glomerulosa cells in vitro where some fatty acids inhibit aldosterone secretion by blocking angiotensin receptors and inhibiting post-receptor steps as well.\textsuperscript{1} In the present study, we found that fatty acids rose when aldosterone fell during salt loading, and the levels of fatty acids after infusion showed a significant inverse correlation with the levels of aldosterone. This is consistent with the hypothesis that some plasma unesterified fatty acids participate in regulating aldosterone levels.

In isolated adrenal cells, in the absence of albumin, the most potent fatty acids, such as oleic and linoleic, inhibit at concentrations in the micromolar range. Concentrations of those fatty acids in plasma certainly exceed the micromolar range, but in plasma, fatty acids are largely bound to albumin. It is difficult to predict the distribution these fatty acids assume between albumin and cell membranes and whether it is reasonable to suppose that they could inhibit adrenal cells at concentrations achieved in humans.

Another experiment that provided indirect evidence for a role of fatty acids in the regulation of aldosterone was published recently in this journal. Infusion of insulin into dogs sensitized them to the aldosteronogenic action of angiotensin II and simultaneously lowered plasma levels of fatty acids.\textsuperscript{15} That experiment, and those reported here, suggest that the sodium-retaining action of insulin may be partly indirect—a result of its ability to lower those plasma fatty acids that inhibit aldosterone secretion and angiotensin action.\textsuperscript{16}

The circumstantial evidence for the role of fatty acids in fluid and electrolyte balance thus can be summarized as follows: fatty acids inhibit aldosterone secretion in vitro; salt loads raise plasma fatty acids while they lower aldosterone; and insulin lowers fatty acid levels while it raises the aldosterone response to angiotensin.

Several reports have linked dietary unsaturated long-chain fatty acids with reductions in blood pressure, but plasma unesterified fatty acids were not measured in any of them.\textsuperscript{17-22} It is possible that diets rich in unsaturated fatty acids alter the composition of plasma fatty acids to favor those that inhibit aldosterone production.

Unesterified fatty acids inhibit Na⁺,K⁺-ATPases.\textsuperscript{23,24} Tamura et al\textsuperscript{25} and Kelly et al\textsuperscript{26} showed that a large proportion of the "digitalislike activity" of porcine and human plasma can be attributed to oleic and linoleic acids. Tamura et al\textsuperscript{25} also found that plasma oleic and linoleic acids rose when pigs were infused with saline. Thus, the rise in levels of plasma fatty acids partially accounts for the increase in digitalislike activity that follows a saline challenge.

The changes we observed in fatty acids and insulin when humans were subjected to salt loads could act in concert as an appropriate physiological response. The rise in fatty acids would favor sodium excretion by inhibiting aldosterone production and renal Na⁺,K⁺-ATPase, and the fall in insulin would favor sodium excretion by reducing its own direct effect on the kidney.\textsuperscript{27,28} This would constitute a feedback loop contributing to salt and water balance.

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