An oxidized derivative of linoleic acid affects aldosterone secretion by adrenal cells in vitro

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Summary Based on the clinical observation that humans with visceral adiposity have higher plasma aldosterone levels than controls, we postulated that endogenous fatty acids can be oxidized by the liver to form stimuli of the adrenal cortex. Although we could show that hepatocytes produced adrenal stimuli from linoleic acid in vitro, the yield was very small. To facilitate the elucidation of chemical structures, we incubated a large amount of linoleic acid with lipoxygenase, then treated the hydroperoxide with cysteine and iron. The major product of this process was 12,13-epoxy-9-keto-10-trans-octadecenoic acid. This epoxy-keto compound stimulated aldosterone production at concentrations from 0.5 to 15 μM. At higher concentrations, it was inhibitory. The epoxy-keto-octadecenoic acid exhibited the chromatographic characteristics of one product of the incubation of linoleic acid with hepatocytes. The results are consistent with the postulated conversion of linoleic acid to stimuli of aldosterone production. This may be a mechanistic link between visceral obesity and hypertension in humans. Published by Elsevier Science Ltd.

INTRODUCTION

Aldosterone is the most potent mineralocorticoid secreted by the adrenal cortex. When administered to an animal or human ingesting a diet replete in sodium chloride, aldosterone can cause sodium retention and potassium excretion by the kidneys, expansion of the extracellular fluid volume, and increased arterial blood pressure. Because of these properties, excessive production of aldosterone has been implicated as a cause of some types of human hypertension.1 Aldosterone secretion is normally regulated by several interacting stimuli and inhibitors, including extracellular potassium, and the hormones angiotensin, adrenocorticotropic hormone (ACTH), and atrial natriuretic peptide (ANP).2 Adrenal cells producing aldosterone in vitro can also be stimulated or inhibited by a wide variety of other substances, including serotonin, catecholamines, endothelin, antidiuretic hormone, melanocortin, somatostatin, adrenomedullin, calcium, and non-esterified fatty acids.3 It is unclear whether any of these known, or as-yet-unknown, substances exert important influences on aldosterone secretion in humans with normal or high blood pressure, but there are well-described clinical situations in which plasma levels of aldosterone cannot be explained by the classical regulators.4

While investigating the effects of non-esterified fatty acids on aldosterone production by rat adrenal cells, we found that addition of hepatocytes to the incubation altered the results. Some fatty acids that inhibited aldosterone production when added to adrenal cells alone stimulated aldosterone production when added to adrenal cells alone. This stimulatory effect was mimicked by the supernatant of an incubation containing only hepatocytes and fatty acid.5 The fatty acid we chose to explore most thoroughly was linoleic acid. The hepatocyte/linoleic acid supernatant contained a complex mixture of compounds, all in small amounts, which presented a daunting challenge to identification of the products.
Because production of stimuli from fatty acids by hepatocytes was dependent on the presence of oxygen, we postulated that the stimulatory substances were the products of fatty acid oxidation. To accumulate sufficient quantities of stimulatory products to enable chemical characterization, we subjected bulk quantities of linoleic acid to oxidation in a cell-free system. Purification of the reaction mixture yielded individual oxidation products with the same biological and chromatographic properties as the products of hepatocyte action. We report on the structure and adrenal effects of one of these oxidation products. The possible relevance of our observations to hypertension, obesity, and nutrition is discussed.

MATERIALS AND METHODS

Linoleic acid and soybean lipoxidase Type V (lipoxygenase) were purchased from Sigma, St. Louis, MO. Other chemicals were reagent grade. Antibody to aldosterone was purchased from ICN, Costa Mesa, CA, radioiodinated aldosterone from Diagnostic Products Corp, Los Angeles, CA, and \(^{14}\)C-carboxy-labeled linoleic acid from New England Nuclear, Boston, MA.

Fatty acid was oxidized in a two-step process based on a method described earlier. In the first step, 800 mg of linoleic acid were dissolved in 70 ml of water with the aid of 0.4 g of Tween 20 (Sigma) and potassium hydroxide. The volume was made up to 500 ml with 5 mM potassium borate (pH 10) to which was added \(10^6\) units of lipoygenase. The mixture was chilled in ice and aerated with oxygen for 45 min. Assuming a molar extinction coefficient of 26 770 at 234 nm, we obtained approximately 700 mg of hydroperoxy-octadecadienoic acid (HPODE). Based on the specificity of the lipoxygenase, we assumed that the major part of the product was the 13S-HPODE stereoisomer.

The first reaction was terminated by acidifying the reaction mixture to pH 4.0 with citric acid (1 N), and extracting the lipid into chloroform/methanol (2:1). The organic layer was washed twice with water and dried. The HPODE was partially purified by column chromatography through silicic acid, pH 4 (Mallinckrodt SilicAR-CC4). Acetone/hexane mixtures starting at an acetone concentration of 5% were used to elute the HPODE. Samples of Acetone/hexane mixtures starting at an acetone concentration of 5% were used to elute the HPODE. Samples of

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The second step of the reaction sequence was accomplished by dissolving approximately 250 mg of 13S-HPODE from the first reaction in 125 ml acetone, adding an equal volume of water, 390 mg of cysteine and 8 \(\mu\)mol of \(\text{FeCl}_3\), then gassing vigorously with oxygen at room temperature for 45 minutes. The organic compounds from the iron–cysteine–HPODE reaction were extracted into chloroform and the residual aqueous layer was re-extracted with chloroform/methanol (2:1). The two chloroform extracts were pooled and fractionated by an open column containing 50 g of Mallinkrodt SilicAR-CC4 eluted with eight successive mixtures of acetone in hexane ranging from 7.5 to 50% acetone and a final stripping with pure methanol. As each solvent eluted, the effluent was monitored by thin layer chromatography (TLC), and by radioactivity if labeled fatty acid had been added to the first reaction mixture. The pattern of eluted radioactivity suggested that ten separate compounds or groups of similar compounds were identifiable, and these were labeled A through J.

Fractions from the columns were tested for biological activity in rat adrenal glomerulosa cells. Capsules of adrenal glands from adult Sprague–Dawley rats (Harlan, Madison, WI) were incubated with collagenase as described, and the suspended cells counted in a hemocytometer. By inspection, there were no more than 4% fasciculata cells among the glomerulosa cells. Cells were diluted so that each assay tube contained 40 000 cells in 0.2 ml of medium. The medium was a mixture of 10% Medium 199 (Sigma 5017) in a HEPES-buffered balanced salt solution. The incubation medium contained the following ingredients in addition to the amino acids and vitamins in Medium 199: \(\text{NaCl} 116 \text{mM}; \text{KCl} 3.6 \text{mM}; \text{MgSO}_4 1 \text{mM}; \text{NaHCO}_3 2.6 \text{mM}; \text{NaHPO}_4 0.69 \text{mM}; \text{HEPES} 6.5 \text{mM}; \) and glucose 7.4 mM. The pH was 7.4 during gassing with a mixture of 95% \(\text{O}_2\) and 5% \(\text{CO}_2\). Incubations were for 120 min at 37°C in 95% \(\text{O}_2\) and 5% \(\text{CO}_2\). Aldosterone production was measured by radioimmunoassay of supernatants.

Fractions from HPLC that altered aldosterone production by rat adrenal cells were purified by a second HPLC fractionation, then analyzed by gas chromatography/mass spectrometry (GC/MS) and by nuclear magnetic resonance spectrometry (NMR).

RESULTS

The two-step oxidation of linoleic acid by lipoxygenase followed by treatment with iron–cysteine generated several substances that affected aldosterone production.
by rat adrenal cells. Of the ten distinct fractions eluted from silicic acid, fractions B–D showed the greatest stimulatory activity when diluted to 1:1 000 in the bioassay buffer. To illustrate the number of active compounds produced by oxidation of linoleic acid, these three fractions were pooled and applied to an HPLC column in the reversed phase. Figure 1 shows a profile of absorbance at 234 nm, and biological activity in the fractions from this pool. The largest amount of ultraviolet-absorbing material eluted in fractions 21 and 22, when the eluting solvent contained 68% methanol in water, a polarity index of 7.36. At the initial dilution, these two fractions showed inhibitory activity in bioassays, but when diluted further, they were highly stimulatory. This biphasic property is shown more clearly in Figure 2, depicting an experiment in which the purified material from HPLC fraction 22 was tested in serial dilutions.

Mass spectrometry was used to elucidate the structure of the purified material from fraction 22. It showed a mass spectrum consistent with a linoleic derivative containing an epoxide across the 12,13 bond, a ketone at position 9, and a double bond between carbons 10 and 11. The epoxide and double bond were assigned trans configurations. Nuclear magnetic resonance spectrometry confirmed the structure and stereoisomerism deduced from mass spectrometry.

The chromatographic characteristics of the epoxy-keto derivative were similar to those of one adrenal stimulus extracted into chloroform/methanol from the medium after incubating rat hepatocytes with linoleic acid (data not shown).

**DISCUSSION**

Oxygen-containing derivatives of arachidonic acid, the eicosanoids, display a wide range of biological activities, and have been the objects of intensive research for
lipids.12 It is probable that the reported effects of mediators, which are released in response to the obesity.5 We postulated that the increased aldosterone levels were higher in subjects with visceral obesity (also called 'abdominal', 'central', or 'upper body' obesity) than in lean subjects or those with 'lower body' obesity. We were unable to characterize the small amounts of stimulated by a clinical observation we made while studying obesity and hypertension. We found that plasma compounds on aldosterone production, sodium retention, and blood pressure. For example, linoleic acid in foods might be oxidized during preparation or storage and enter the systemic circulation from the gut. Alternatively, unmodified fatty acids might be absorbed and reach the liver via the portal vein, where they could be oxidized, just as we propose for fatty acids from visceral fat depots. Whatever their origin, oxidized products of linoleic acid present potential lipid regulators of steroid secretion and arterial blood pressure in humans.

Another possible implication of our work is the effect that oxidized unsaturated fatty acids in the diet might have on aldosterone production and blood pressure. For example, linoleic acid in foods might be oxidized during preparation or storage and enter the systemic circulation from the gut. Alternatively, unmodified fatty acids might be absorbed and reach the liver via the portal vein, where they could be oxidized, just as we propose for fatty acids from visceral fat depots. Whatever their origin, oxidized products of linoleic acid present potential lipid regulators of steroid secretion and arterial blood pressure in humans.

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REFERENCES