Detection of Glycyrrhetinic Acid–like Factors (GALFs) in Human Urine

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Patients with the syndrome of apparent mineralocorticoid excess and those who ingest licorice show markedly decreased 11β-hydroxysteroid dehydrogenase (11β-OHSD) and 5α-reductase activity; both are important for the deactivation of glucocorticoids and other steroid hormones. Glycyrrhetinic acid (GA), present as its glycoside in licorice, is a potent inhibitor of both 11β-OHSD and 5α-reductase and, as we have also shown, confers Na⁺-retaining properties on glucocorticoids and amplifies those of aldosterone and deoxycorticosterone. We report the results of our initial studies demonstrating the presence of naturally occurring substances, which inhibit both 5α-reductase and 11β-OHSD as does GA, in partially purified extracts of urine from normotensive men and nonpregnant and pregnant women. Since these substances exhibit GA-like activity, we have termed them GA-like factors (GALFs). This "inhibitory" material is heat stable and does not react with ninhydrin; the majority is not extractable with ethyl acetate and thus is not a "free" steroid. When further purified by high-performance liquid chromatography with a methanol/water gradient, the majority of these GALFs appeared in two regions of inhibitory activity. The chemical nature of this material is currently being investigated. These experiments indicate that normal human urine contains GALFs that may play a role in Na⁺ homeostasis and regulation of blood pressure. (Hypertension 1992;20:356–360)

KEY WORDS • licorice • hydroxysteroid dehydrogenases • oxidoreductases • glycyrrhetinic acid • urine

Glycyrrhetinic acid (GA), the active principal in licorice root, has been shown to markedly inhibit 11β-hydroxysteroid dehydrogenase (11β-OHSD) and steroid 5α-reductase when incubated with these enzymes. The importance of both these enzymes became apparent after Ulick, New, Monder and coworkers demonstrated that hypertensive children with the syndrome of apparent mineralocorticoid excess (AME) lack both 11β-OHSD and 5α-reductase enzyme activity. Alteration in these inactivating enzymatic metabolic pathways was shown to result in changes in the peripheral metabolism of cortisol. It has been postulated that higher peripheral intrarenal concentrations of cortisol may then interact with mineralocorticoid receptors and promote Na⁺ reabsorption. These patients exhibit Na⁺ retention, K⁺ wasting, and increased blood pressure without measurable increases in circulating aldosterone. A pharmacological equivalent of this congenital condition results from the ingestion of licorice, which contains glycyrrhizinic acid derivatives of GA. Because of the importance of these enzymes, it is possible that other categories of hypertension may also result from less extreme alterations of their activities.

Furthermore, we have recently shown that treatment of adrenalectomized male rats with carbenoxolone sodium, a succinate derivative of GA, caused the glucocorticoids corticosterone and cortisol to display mineralocorticoid-like activity and also amplified the Na⁺-retaining properties of the mineralocorticoids aldosterone and deoxycorticosterone. These results led us to consider the possibility that endogenous or exogenous sources of GA-like "inhibitory" activity might exist in humans and possibly in certain animal models of hypertension and that these sources might play a role in altering sodium homeostasis. We have termed these inhibitors "glycyrrhetinic acid–like factors" (GALFs) without implying anything as to their chemical nature. We, therefore, have embarked on a program to determine whether human urine from a variety of sources contains chemical substances with GALF activity that when isolated could be tested in a radioenzymatic screening assay for their inhibitory activity against hepatic 11β-OHSD and 5α-reductase.

Methods

Urine Samples
Random urine samples were donated by normotensive male and female members of the laboratory and healthy pregnant women on routine visits to their obstetricians. All subjects were between 25 and 35 years of age. Aliquots were centrifuged and assayed for their creatinine concentrations using a Beckman CX3 Analyzer.
Ten milliliters of these samples were extracted onto C18 Sep-Pak cartridges (Waters Chromatography Division, Millipore Corp., Milford, Mass.), washed with 5 ml water, and eluted with 5 ml methanol. No inhibitory activity was lost in the initial water eluate. The methanol eluates were evaporated to dryness under nitrogen and then redissolved in 1 ml distilled water. Samples processed in this manner are hereafter referred to as "urine extracts."

High Performance Liquid Chromatography
Gradient Separation of Urine Extracts

Urine extracts (2 ml) were diluted with methanol to a final concentration of 30% methanol and chromatographed on a 25×0.5-cm C18 5-µm reverse-phase column (Isco Corp., Lincoln, Neb.). Components were eluted with a gradient commencing with 30% aqueous methanol that increased linearly to 40% aqueous methanol by 400 seconds, then to 60% aqueous methanol by 1900 seconds, and finally to 100% methanol by 2000 seconds. One-milliliter fractions were collected at a flow rate of 1 ml/min. Each fraction was evaporated under nitrogen and was assayed for inhibitory activity in both 5β-reductase and 11β-OHSD radioenzymatic assays. Results were expressed as relative percentage of inhibition, rather than as GA units (see below). Samples with marked inhibitory effects were also spotted onto Whatman filter paper, dried, and sprayed with ninhydrin to detect the presence of peptides or other amines or sugar amines.

Experiments With Urine Samples

Heat. Urine extracts adjusted to pH 7.4 were heated at 100°C for 10 minutes. Both heated and unheated samples were then assayed for inhibitory activity.

Solvent extraction. Urine extracts were dried under nitrogen and then reconstituted in 2 ml H2O. Each was extracted two times with an equal volume of ethyl acetate, and the aqueous portion was retained. All fractions were then repassed through Sep-Pak cartridges and again eluted with 100% methanol and assayed for inhibitory activity as above.

Assay of Inhibitory Activity

Radioenzymatic assay of 5β-steroid reductase was performed by measuring the conversion of [3H]aldosterone to its 3α,5β-tetrahydro derivative and 11β-OHSD activity by measuring the conversion of [3H]corticosterone to [3H]11-dehydrocorticosterone as previously described. 2,3 [1,2-3H]Aldosterone and [1,2-3H]corticosterone with specific activities of 53.9 and 56.4 Ci/mmol, respectively, were obtained from Dupont New England Nuclear, Boston, Mass. Their purity was checked by high-performance liquid chromatography (HPLC) before use. Methanol (HPLC grade) was obtained from Fisher Scientific, Medford, Mass. Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), Trizma base [tris(hydroxymethyl)aminomethane], NADP+, GA, corticosterone (compound B), and 11-dehydrocorticosterone (compound A) were obtained from Sigma Chemical Co., St. Louis, Mo., and aldosterone was obtained from Andard Mount, London, UK.

Both enzymes were prepared in crude form from the livers of adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.). After the rats were killed, the livers were rapidly removed and washed with ice-cold 0.25 M sucrose, and microsomal and cytosolic fractions were prepared for measurements of 11β-OHSD and 5β-reductase enzymatic activities, respectively, as described below.

Livers were homogenized in 0.25 M sucrose (1:4 w/vol) using a Teflon homogenizer, and subcellular fractions were obtained by differential centrifugation at 4°C using a Sorvall RC-2 preparative and Beckman L8—80 ultracentrifuge. The homogenate was centrifuged at 10,000g for 10 minutes, and the resultant supernatant from this and subsequent spins were centrifuged first for 10 minutes at 10,000g and then at 17,000g. The 17,000g supernatant was centrifuged at 105,000g for 70 minutes, and the microsomal pellet was rinsed with cold homogenization medium and resuspended in cold 0.25 M sucrose at a concentration of 30 mg protein per milliliter. The final supernatant (20 mg protein per milliliter) is the liver cytosol preparation containing 5β-reductase, 2 and the microsomal pellet is the source of 11β-OHSD activity. 2

Enzyme Assays

11β-OHSD assay. Liver microsomes (0.14 mg protein) were incubated at 37°C for 10 minutes with 5 µM corticosterone and [3H]corticosterone (1 µCi) as tracer in 50 mM Tris-HCl buffer (pH 8.5) containing 3.4 mM NADP+ in a total volume of 0.25 ml. Included in this volume is an aliquot of water (control), urine extract, or GA. The reaction was terminated by addition of 1 ml methanol. Synthesis of 11-dehydrocorticosterone was quantitated by HPLC.

5β-Reductase assay. Aliquots of cytosol (approximately 2–3 mg protein) were incubated with 45 µM aldosterone and [3H]aldosterone (1 µCi) in 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl2, 2.76 mM NADPH, and 2% ethanol in a final volume of 0.25 ml. Appropriate aliquots of water (control), urine extract, or GA were added. The reaction mixtures were incubated at 37°C for 10 minutes, and the reactions were terminated with 1 ml methanol. Synthesis of 3α,5β-tetrahydroaldosterone was quantitated by HPLC as described below.

Estimation of Enzyme Activity

The methanol extracts of the incubation media were centrifuged at 1,000g for 10 minutes; 0.5-ml aliquots supernatant were evaporated to dryness under nitrogen, dissolved in 20% MeOH, and chromatographed on a DuPont Zorbax C8 reversed-phase column, at 44°C with 45% aqueous methanol (isocratic) at a constant flow rate of 1 ml/min. This system separated compound B from compound A and aldosterone from 3α,5β-tetrahydroaldosterone. The radioactive metabolite peaks were detected by an on-line β-detection system (model LB-504, Berthold Instrument, Nashua, N.H.) after the HPLC column eluate was mixed with scintillation fluid (Ultima-Gold, Packard Instruments, Downers Grove, Ill.) with a 10% counting efficiency under conditions of flow. After subtraction of background radioactivity, percent percentage distribution of radioactivity among all HPLC peaks in each chromatogram was determined. The enzyme activity and inhibition was then calculated as below.
Hypertension Vol 20, No 3 September 1992

**FIGURE 1.** Standard curves show relation between amount of glycyrrhetinic acid (GA) added to the incubation mixture and the percentage of inhibition of 11β-hydroxysteroid dehydrogenase (11β-OHSD) (panel A) and 5β-steroid reductase (panel B) activity as measured under specified conditions of incubation.

**Calculation of Inhibition**

To provide a basis for the quantitation of urine inhibitory activity, aliquots of GA were added to control incubation mixtures (volume 0.25 ml) in varying amounts (0–1.2 μg for 5β-reductase and 0–0.012 μg for 11β-OHSD). Percentage of inhibition was calculated relative to controls without GA (equation 1). Standard curves were then constructed (Figures 1A and 1B).

\[
\% \text{ inhibition} = \left( \frac{P_c - P_i}{P_c} \right) \times 100
\]

where \( P \) is the product formed per milligram protein per 10 minutes, \( C \) is the control, and \( I \) is the inhibitor (GA or urine extract).

The reduction in apparent enzyme activity due to the urine extracts was then converted to μg GA (GA equivalent units) using the appropriate GA standard curve. Finally, these GA equivalent units were expressed as micrograms GA per milligram creatinine to normalize them for dilute or concentrated urine. Note that because of the different values of \( K_i \) for GA inhibition of 5β-reductase and 11β-OHSD and because the two enzymes were measured in two different bioassay systems, the magnitude of the GALF units is different for the two enzymes. Therefore, we designate them as 5β-GALF units and 11β-GALF units.

**Results**

**Measurement of Urine Inhibitory Activity**

The aim of the present experiments was to investigate whether GA-like factors (as defined above) can be detected and measured in human urine. Random urine specimens were obtained from 10 men, nine nonpregnant women, and nine women in the second and third trimesters of pregnancy. Aliquots of urine from these subjects were assayed for an ability to inhibit the two enzymes 5β-reductase and 11β-OHSD (Figure 2). Known amounts of GA standards were also added to control incubation mixtures to define two standard inhibition curves (Figures 1A and 1B) relating the amount of GA to the percentage of inhibition of each of the enzymes. From these results and the initial urinary creatinine measurements, we expressed the inhibitory activity in terms of micrograms GA per milligram creatinine (5β-GALF units and 11β-GALF units; see Figure 3) in each urine sample.

Using the hepatic cytosolic 5β-reductase radioenzymatic assay, which measures the fraction of [3H]aldosterone converted to [3H]3α,5β-tetrahydroaldosterone (Figure 3), the inhibitory activity was found to range from 2.0 to 3.0 5β-GALF units for men, from 1.6 to 4.4 5β-GALF units for nonpregnant women, and from 5.4 to 13.0 5β-GALF units in the second trimester, and from 0.0207 to 0.0441 11β-GALF units in the third trimester.
GALF Inhibitory Activity in Human Urine

There was no statistically significant difference between male and nonpregnant female 5β-GALF units, but the 11β-GALF units for nonpregnant women were slightly higher (p<0.05) than the values for men. However, both groups had significantly less inhibitory activity than any of the pregnant women (Figure 3).

Chromatographic Separation of Urinary Inhibitory Activity

The Sep-Pak-extracted inhibitory material from each group of urine specimens revealed two major regions of inhibitory activity. The first major region of activity was found in the fractions eluting between 6 and 9 minutes (34–38% methanol); a second region was found in those eluting between 17 and 18 minutes (46–47% methanol). Standards of cortisol, cortisone, and compound A all eluted at higher concentrations of methanol than either of these two regions.

Similar elution patterns of inhibitory activity were observed for urine specimens from men, women, and pregnant women that were measured using this HPLC gradient. These initial results do not as yet permit us to generalize on the relations between the inhibitory components in urine specimens from different sources.

Chemical Classification of Inhibitory Activity

Sensitivity to heat. No reduction in inhibitory activity of any of the samples occurred when urine samples, buffered to pH 7.4 with TRIS-HCl, were heated for 10 minutes in boiling water.

Reactions with ninhydrin. Unfractionated urine samples were slightly positive for ninhydrin, but fractions containing inhibitory activity eluted from the HPLC gradient were unreactive.

Extractability. Attempts to extract the total inhibitory activity from the urine samples directly into ethyl acetate were not successful; the majority of the activity remained in the aqueous phase.

Additional studies are currently being undertaken to establish the number of inhibitory components present in urine from different sources, their individual biochemical properties, and their chemical identities.

Discussion

The major finding, that ingestion of licorice mimics AME, led us to question whether licorice also mimics the effects of an as yet undescribed endogenous factor (or factors) that might likewise be involved in the regulation of steroid metabolism either by affecting the extent of 11β-hydroxyl oxidation by 11β-OHSD or Ring A-reduction by 14-steroid-5β-reductase of cortisol and possibly other adrenal steroid hormones. That is, are there circulating substances or substances that are ultimately excreted in the urine with GA-like inhibitory activity that also alter the pathways of steroid metabolism? Urine was chosen as the source since this was more easily obtained in large quantities and would permit subsequent isolation and chemical characterization of any inhibitory substance (or substances) found in these experiments.

In these initial studies we have shown that human urine from normotensive individuals contains at least two such substances or groups of substances that can be extracted and partially purified using Sep-Pak cartridges containing reverse-phase C18-Bondapak support resin. This initial purification step removes all salts and small organic molecules, such as urea, and also results in a protein-free extract. The extracted material (or materials) inhibited both cytosolic 5β-reductase and microsomal 11β-OHSD obtained from rat liver. Because the chemical identity and molecular weights of these substances are not known at this time, we have expressed the GALF inhibitory activity in units equivalent to micrograms GA per milligram creatinine based on the inhibitory effects of standards of GA on each of these two enzymes. Subsequent purification steps involving further separation by gradient HPLC reproducibly resolved the extracts from all urine specimens into at least two regions that possessed GALF inhibitory activity.

The majority of this material is water soluble and is not extractable with ethyl acetate, indicating that it is not composed of free urinary steroids, such as cortisol, that might compete as a substrate in both enzyme reactions and thus also appear as an inhibitory material. In addition, gradient HPLC showed that most inhibitory activity eluted at lower methanol concentrations than those required for cortisol, cortisone, and 11-dehydrocorticosterone. The GALF inhibitory material is heat stable, is resistant to trypsin digestion, and does not react with ninhydrin. At this preliminary stage, any further claims as to their chemical identities would be premature.

Urine specimens from normotensive men and women (nonpregnant) contained similar quantities of activity against 5β-reductase when measured as micrograms GA per milligram creatinine, but women had slightly greater amounts of 11β-OHSD inhibitory activity. By contrast, women in both the second and third trimesters of pregnancy had severalfold increases in activity against both enzymes as compared with men and nonpregnant
women. Despite differences in levels, the chromatographic profiles and other chemical properties were similar in all three groups tested.

In summary, data from these preliminary studies show that, like licorice, human urine contains GA-like inhibitory activity against two of the enzymes in the metabolic pathways important for the inactivation of adrenal steroid hormones. We cannot be sure at this time whether all of the peaks containing GALF inhibitory activity are derived from endogenously synthesized substances or whether they represent substances present in the diet. Nevertheless, we believe it is important to communicate, particularly to those interested in the mechanisms of hypertension, that these preliminary findings point the way to further interesting work: 1) to explore and elucidate the chemical structures of these interesting substances, which we have named GALFs, 2) to determine their origin, and 3) to determine whether they are endogenous or are affected by diet. It will now also be most important to measure their presence and levels in a variety of clinical states including hypertension and to determine whether they play a physiological role, are involved in the pathogenesis of hypertension, or both.

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