Inhibitors of 11β-Hydroxysteroid Dehydrogenase and 5β-Steroid Reductase in Urine From Patients with Congestive Heart Failure*

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ABSTRACT

Recently, the current authors reported the presence in normotensive male and female urines of reproducibly measurable levels of naturally occurring substances in partially purified extracts of urine with inhibitory activity like glycyrrhetic acid (GA) towards both 11β-hydroxysteroid dehydrogenase (11β-OHSD) and steroid 5β-reductase (5β-SR) in vitro. Since these substances mimic two known inhibitory activities of GA, they have been named 'Glycyrrhetic Acid-Like Factors', abbreviated as 'GALFs' or, more specifically 11β-GALF for substance(s) active against 11β-OHSD, and 5β-GALF for those inhibitory to 5β-SR. Administration of glycyrrhetic acid in man leads to cortisol-dependent mineralocorticoid hypertension, owing to impaired inactivation of cortisol by 11β-OHSD, and may be associated with increased sensitivity to mineralocorticoids owing to impaired 5β-SR. In this preliminary report, the results are described of a study on the presence of GALF factors in urines collected from patients with congestive heart failure (CHF) and mild essential hypertension. The results show that in such patients there are increased amounts of both 11β- and 5β- GALFs compared to normotensive. The possible physiological significance of these results is discussed.
Introduction

The activities of two of the major enzymes responsible for the metabolic inactivation of the adrenocorticosteroids have been shown to be markedly diminished in children with the syndrome of apparent mineralocorticoid excess (AME). These two enzymes are 11β-hydroxy-steroid dehydrogenase (11β-OHSD), which metabolizes cortisol to cortisone; and Δ4-steroid 5β-reductase (5β-SR) which catalyzes the formation of 5β-dihydro derivatives of aldosterone and cortisol, the initial step in the formation of their tetrahydro derivatives. Using measurements of urinary steroid metabolites the activities of these two enzymes were also shown to be markedly reduced in humans following the excessive ingestion of liquorice. Subsequently glycyrrhetic acid (GA), the active ingredient in liquorice, was identified as the substance which strongly inhibits both 11β-OHSD and 5β-SR.3

The importance of 11β-OHSD and possibly 5β-SR in Na+ homeostasis and hypertension led to the investigation of whether or not endogenous substance(s) exist in humans which might likewise inhibit either of these two enzymes and hence possibly play a regulatory role in the control of electrolyte balance and blood pressure (BP). In our initial experiments, it was possible to show that urines from both males and females do indeed contain measurable levels of substance(s) which inhibit both 11β-OHSD and 5β-SR.4 Since these substances mimic two known inhibitory activities of GA, the inhibitory substances found in urine have been named ‘Glycyrrhetic Acid-Like Factors’, abbreviated as ‘GALFs’ or, more specifically 11β-GALF for activity against 11β-OHSD, and 5β-GALF for activity inhibitory to 5β-SR. It was also found that the levels of both types of GALFs increased with advancing pregnancy, a condition in which there are marked changes in Na+ homeostasis and water balance. In this paper the preliminary results are described of our experiments to determine the levels of 11β-GALF and 5β-GALF in the urines of hospitalized patients with congestive heart failure (CHF) and in the urines of patients with mild essential hypertension.

Methods

Collection of Urine Samples

Twenty-four hour urine samples of known volume and creatinine content were collected from the Memorial Hospital, Pawtucket, RI (mild essential hypertension) and The Miriam Hospital, Providence, RI (congestive heart failure). All samples were kept frozen in the laboratory prior to processing and analysis. Samples were collected on a voluntary basis using the system of informed consent appropriate to each location. Both sites excluded individuals with physical or laboratory findings suggestive of secondary hypertension, liver or renal diseases, diabetes mellitus, obesity, or abuse of alcohol or drugs. All medication was withheld from all hypertensive patients from the Memorial Hospital in Pawtucket for four weeks prior to the collection of their urines.

Selection of Subjects

A. The Memorial Hospital: Fourteen patients, (12 men and 2 women), 18 to 75 years old, were recruited for a clinical research trial on the treatment of mild hypertension. Subjects conformed to the selection criteria given previously. In addition, all subjects with diastolic pressures >105 mm Hg were excluded from the study. Nine healthy adults, age 22 to 53, without known hypertension were selected as controls.
B. The Miriam Hospital: Fourteen patients with congestive heart failure (CHF), characterized, by pulmonary edema and hypoxia (9 men and 5 women), 53 to 86 years old, were recruited by a staff cardiologist. Seven healthy adults, ages 34 to 53, were used as controls in this study.

ANALYSIS OF URINE SAMPLES

Urine creatinine concentrations were assayed using a Beckman CX3 Analyzer.*

Ten ml urine samples were desalted and partially purified by extraction onto C18 Sep-Pak cartridges† and urine extracts prepared (Figures 1 to 4) as previously described.8

ASSAY OF "INHIBITORY" ACTIVITY

Radioenzymatic assay of 5β-steroid reductase was performed by measuring

* Beckman Instruments, Brea, CA

† Waters Chromatography Division of Millipore Corp., Milford, MA

Figure 1. Bar graph showing levels of 11β-GALF units (GA-equivalent units, μg/24 hr) in urine from patient with congestive heart failure (CHF) and control subjects. Values represent means ± S.E.M. *p < 0.01, n = 7.

Figure 3. Scatter plot showing levels of 11β-GALF units (GA-equivalent units, μg/24 hr) in urine from patient with mild essential hypertension and normotensive subjects. Values represent means ± S.E.M. (n = 7–14).

Figure 2. Bar graph showing levels of 5β-GALF units (GA-equivalent units, μg/24 hr) in urine from patient with congestive heart failure and control subjects. Values represent means ± S.E.M. *p < 0.01, n = 7.

Figure 4. Bar graph showing levels of 5β-GALF units (GA-equivalent units, μg/mg creatinine) in urine from patients with mild essential hypertension and normotensive subjects. Values represent means ± S.E.M. *p < 0.01, n = 7–14.
the conversion of \(^{3}\text{H}\)-Aldo to its 3α, 5β-tetrahydro derivative and 11β-OHSD by measuring the conversion of \(^{3}\text{H}\)-corticosterone to \(^{3}\text{H}\)11-dehydrocorticosterone as previously described.\(^{4,7}\) [1,2-\(^{3}\text{H}\)]-Aldosterone (Aldo) and [1,2-\(^{3}\text{H}\)]-corticosterone with specific activities of 53.9 Ci per mmol and 56.4 Ci per mmol, respectively, were obtained\(^{4}\). Their purity was checked by high performance liquid chromatography (HPLC) before use. Methanol (HPLC grade) was used.\(^{§}\) Nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH), Tris-ma base (tris-hydroxymethyl-aminomethane), NADP\(^{+}\), glycyrrhetic acid (GA), corticosterone (Compound B), and 11-dehydrocorticosterone (Compound A) were obtained\(^{1}\) as was Aldo.\(^{†}\)

Both enzymes were prepared in crude form from the livers of adult male Sprague-Dawley rats.\(^{**}\) Livers were rapidly removed, 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 using a modification of the previously described methods.\(^{7}\)

11β-OHSD ASSAY

Liver microsomes (0.07 mg protein) were incubated at 37°C for 10 min with 5 \(\mu\)M corticosterone and \(^{3}\text{H}\)-corticosterone (1 \(\mu\)Ci) 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 either water (controls), urine extracts, or GA. The reaction was terminated by addition of one ml methanol. Synthesis of 11-dehydrocorticosterone was quantitated by HPLC.\(^{7}\)

5β-REDUCTASE ASSAY

Aliquots of cytosol (approximately 2 mg protein) were incubated with 45 \(\mu\)M Aldo and \(^{3}\text{H}\)-Aldo (1 \(\mu\)Ci) in 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl\(_{2}\), 2.76 mM NADPH, and 2 percent ethanol in a final volume of 0.25 ml. Appropriate aliquots of water (controls) or urine extracts or GA were added. The reaction mixtures were incubated at 37°C for 10 min and terminated with one ml methanol. Synthesis of 3α, 5β-tetrahydro-Aldo was quantitated by HPLC.\(^{7}\)

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 to 1.2 \(\mu\)g for 5β-reductase and 0 to 0.012 \(\mu\)g for 11β-OHSD) and the percent of inhibition was calculated relative to controls (without GA) as previously described. Briefly, percent of inhibition owing to urine extract was converted to \(\mu\)g GA (GA equivalent units) using the appropriate GA standard curve. These GA equivalent units were expressed as \(\mu\)g GA per mg of 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, they have been designated as 5β-GALF Units and 11β-GALF Units.

Results

CONGESTIVE HEART FAILURE

Figures 1 and 2 show the number of units of 11β-GALF and 5β-GALF in urine from subjects with CHF as compared with controls. The mean increase for both enzymes is highly significant (p < 0.01), with 11β-GALF rising approxi-
mately 600 percent and 5β-GALF increasing to 225 percent.

**Essential Hypertension**

Figures 3 and 4, showing urinary 11β-GALF and 5β-GALF activity in essential hypertension, present a different picture. There is a significant increase (p < 0.01) in urinary 5β-GALF in the hypertensive group as compared with the normotensive group (Figure 3). By contrast, there is no statistically significant difference between the urinary 11β-GALF levels in essential hypertension as compared with the control group. However, the scatter of the results from hypertensive patients is much greater than the scatter for normotensive (figure 3). In particular, there are elevated outliers which suggest the possibility that there may be a specific subset of essential hypertensives who may indeed consistently excrete elevated levels of both 5β- and 11β-GALFs in their urine.

**Discussion**

Diminished activity of the enzyme 11β-OHSD leading to decreased rates of inactivation of cortisol to cortisone has been suggested to account for the increased Na⁺ retention, hypokalemia, and increased B.P. in patients with AME.10,13 These patients were also shown to have decreased 5β-SR activity.6 Ingestion of liquorice, which is also associated with similar clinical features, also leads to lower activities of both these enzymes.12 Since in-vitro, glucocorticoids have equal affinity for mineralocorticoid receptors as mineralocorticoids such as aldosterone, but do not normally activate these receptors in-vivo, 11β-OHSD-mediated inactivation of cortisol and corticosterone has been suggested to play a major role in protecting mineralocorticoid receptors from glucocorticoids and thereby regulating electrolyte balance and blood pressure.1,2 The finding that urine from normotensive males and females contains measurable levels of 11β-GALF and 5β-GALF, and that both of these increase significantly throughout pregnancy,8 with its marked changes in electrolyte and water homeostasis, prompted the determination of their levels in the urines of patients with CHF and essential hypertension.

In these preliminary experiments, two pilot groups, one of patients with essential hypertension and the other with CHF, were chosen. All patients with CHF showed consistent and marked increases in their urinary output of both 11β- and 5β-GALF output. The hypertensive group had had their medication withheld for eight weeks. These patients all had increased levels of 5β-GALF activity, both in terms of their concentration in the urine and also the total amount excreted in the 24 hr. urine samples. However, only a small number of these patients, possibly a physiological subset of this group, also showed increases in the concentration and total amount of 11β-GALF excreted in their 24 hr urine samples.

Although preliminary, owing to size of the two groups so far studied, the present experiments add two more groups to those conditions in which increased mineralocorticoid activity might be necessary to maintain electrolyte and water balance. Increasing the half-lifes of both glucocorticoids and mineralocorticoids by inhibiting their catabolic enzymes might provide such increased mineralocorticoid activity.

Our initial study8 and the current work have clearly shown that it is possible to measure GALF substance(s) reproducibly, which may well represent undescribed endogenous factor(s) involved in the regulation of steroid metabolism. These factors may affect the extent of (a) 11β-hydroxy oxidation by 11β-OHSD and/or (b) Ring A-reduction by 5β-SR of cortisol and possibly other adrenal steroid hormones. Our results may provide the explanation for previously observed alterations in the urinary metabolite tet-
rahydrocortisol/tetrahydrocortisone ratios in essential hypertensives and changes in the plasma half life of 11α-3H-cortisol in these patients, suggestive of 11β-OHSD deficiency in patients with essential hypertension. The presence of GALFs in these patients may be linked to changes in the handling of glucocorticoids and mineralocorticoids in many sites, including liver and kidney. Supporting evidence for such a concept is provided by our demonstration 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.

Summary

These preliminary data suggest that endogenous factors produced in excess by patients with essential hypertension may alter corticosteroid metabolism, thus amplifying tissue sensitivity to cortisol, and perhaps also to aldosterone and deoxycorticosterone, and contributing to the elevation of blood pressure in these patients. Further studies employing larger populations of CHF patients, hypertensives, and normotensive controls are now necessary to determine whether such substance(s) do indeed play a physiological role and/or are involved in the pathogenesis of hypertension.

References