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Inhibition of 11β-Hydroxysteroid Dehydrogenase Obtained from Guinea Pig Kidney by Furosemide, Naringenin and Some Other Compounds

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Inhibition of 11β-hydroxysteroid dehydrogenase (11β-OHSD) can cause excess mineralocorticoid effects and hypokalemia. Several substances causing hypokalemia (glycyrrhizic acid in licorice and gossypol) inhibit this enzyme. We tested other compounds for activity to inhibit 11β-OHSD in guinea pig kidney cortex microsomes with NADP as cofactor and cortisol as substrate. Furosemide was an inhibitor while bumetanide was not, indicating a mechanism for the increased K^+ excretion caused by furosemide compared with bumetanide. Naringenin (found in grapefruit juice), ethacrynic acid, and chenodeoxycholic acid had inhibitor IC_{50} values similar to glycyrrhizic acid. We conclude that various compounds can inhibit this enzyme and may play a role in K^+ metabolism and adrenocorticosteroid action.

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INTRODUCTION

The syndrome of apparent mineralocorticoid excess, first described by Ulick, Ramirez and New in 1977 [1], has led to much research on the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD). Deficient activity of this enzyme in children leads to their inability to oxidize cortisol to inactive cortisone, providing high cortisol levels in the kidney which activate renal mineralocorticoid receptors and cause hypertension and hypokalemia. Subsequently, the mechanism of licorice-induced hypermineralocorticoidism was shown to be the inhibition of 11β-OHSD by the active principle of licorice, glycyrrhizic acid. Since then, much research has been done to explore the role that this enzyme plays in regulating the interactions of cortisol with mineralocorticoid and glucocorticoid receptors [2–6].

Gossypol, a polyphenolic constituent of cotton seed, has been studied in China as a potential oral contraceptive for men because it suppresses sperm motility and formation without affecting testosterone levels [7]. Some Chinese men who received gossypol developed hypokalemia although the cause remained obscure [7]. This is particularly remarkable since idiopathic hypokalemia, often associated with hyperthyroidism, occurs widely in China; in addition, normal Chinese men have serum potassium levels lower than men in four other countries, with 9% having values below 3.5 mmol/l [8].

In studies investigating how gossypol causes hypokalemia, we found that gossypol inhibited 11β-OHSD activity in guinea pig [9] and human [10] renal cortical microsomes. We also found that certain bioflavonoids inhibit rat liver 11β-OHSD [10]. Others have reported inhibition of the rat kidney enzyme by bile acids [11] and by steroidal and triterpenoid compounds [12], and inhibition of the rat liver enzyme by some substances in human urine [13]. We therefore decided to test a variety of compounds for their possible enzyme inhibiting effect, choosing drugs that can cause hypokalemia or sodium retention as a side effect, flavonoids from grapefruit juice that inhibit the oxidation of dehydropyridine calcium channel blocking drugs [15–17] or sterols in vegetable oils at concentrations of 100–500 mg/dl [14].

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MATERIALS AND METHODS

Chemicals and solutions

Sitosterol was a gift from Eli Lilly and Co. (Indianapolis, IN). Campesterol was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were purchased from Sigma Co. (St Louis, MO).

Most sterols, furosemide, ethacrynic acid, naringin and naringenin were dissolved in ethanol and diluted with methanol. Cholic acid, chenodeoxycholic acid, bumetanide, hydrochlorothiazide and spironolactone were dissolved in methanol. Phenylbutazone and indomethacin were dissolved in distilled water (pH 9). Glycyrrhizic acid was dissolved in distilled water. Corticosterone and hydrocortisone were dissolved in methanol (144 μmol/l) and kept at -4°C.

Enzyme preparation and measurement of 11β-OHSD activity

Kidney cortex was obtained from long-haired male Hartley guinea pigs. Tissue was homogenized in Krebs–Henseleit buffer as described previously [9], except for the use of a Tekmar Tissuemizer (Cincinnati, OH). Microsomes were prepared as described previously [9], except that they were diluted to a concentration of 1.25 mg protein/ml prior to storage at -70°C.

The enzyme activity in guinea pig kidney cortex microsomes was determined by measuring the rate of conversion of cortisol to cortisone. Five minutes before incubation, 2 μl of concentrated Triton DF-18 was added to each milliliter of the microsome suspension. The assay mixture contained 500 μl Krebs–Henseleit buffer (pH 7.2), 50 μl 5 mmol/l NADP, 40 μl of 144 μmol/l phosphate–sucrose buffer, 20–50 μl (25–63.5 μg) of microsome suspension in 0.01 M phosphate-sucrose buffer and various concentrations of each compound studied. This mixture was incubated in duplicate or triplicate. The total volume was 700 μl. Methanol concentration was kept at $< 10\%$. Control studies showed that this concentration did not inhibit the reaction. After 1 h of incubation at 37°C, the reaction was terminated by the addition of 3 ml methylene chloride and 20 μl 144 μmol/l corticosterone solution as the internal standard for assay of cortisone and cortisol.

The enzyme inhibition constant for furosemide was determined by adding furosemide in various amounts to achieve concentrations from 3.9 to 62 μmol/l in the incubation mixture and cortisol concentrations of 4, 8, and 16 μmol/l. The constants were obtained from a Dixon plot and a kinetic program (Chou J, Chou T-C: Michaelis-Menton analysis with microcomputers, Disk No. 1, Elsevier-Biosoft, 1989, Cambridge, England.

A modification of the HPLC method of Sang [9] was used to measure cortisol, cortisone and corticosterone in the microsomal incubation mixture. The steroids were extracted into methylene chloride by vortexing for 1 min, then centrifuged at 750 g for 15 min. The aqueous layer was removed by aspiration. 300 μl of 0.1 NaOH was added to the organic phase followed by vortexing for 30 s. The mixture was centrifuged and the aqueous layer removed. The organic phase was washed with 500 μl of milli-Q water (Millipore Corp., Bedford, MA). The 1.5 ml organic phase was transferred to clean glass tubes and dried by evaporation in a 45–50°C water bath. The residue was dissolved into 200 μl of methanol and 5 μl of this solution was injected into the HPLC apparatus. A standard curve for cortisol and cortisone was determined in duplicate in each enzyme experiment by using the same amount of microsome suspension after boiling to inactivate the enzyme. Standard curves were plotted as the ratio of peak height of cortisone (or cortisol) divided by the peak height of the internal standard vs steroid concentration. All unknown concentrations of cortisol and cortisone were determined from the standard curves from each experiment. The drug concentrations that inhibited the enzyme by 50% (IC_{50}) were estimated from at least 3 different concentrations of each compound evaluated by a dose–response program (Chou and Chou: Dose-effect analysis with microcomputers, Disk No. 2, Elsevier-Biosoft. 1989, Cambridge, England).

The HPLC apparatus used for quantitating the steroids consisted of a Waters Model 6000 A solvent delivery system, U6K injector, model 680 automated gradient controller, Waters 486 tunable absorbance detector and a BBC chart recorder (Model SE 120). The mobile phase contained methanol–water (30:70, v/v) at a flow rate of 1.0 ml/min. The Waters stainless steel Novapak C_{18} column (3.9 × 150 mm, 4μ) was kept at room temperature. The retention times for cortisone, cortisol and corticosterone were 6.5, 7.0 and 9.0 min, respectively.

RESULTS

The efficacy of the compounds tested to inhibit the NADP-utilizing form of 11β-OHSD from guinea pig renal cortex with cortisol as substrate is shown in Tables 1 and 2. Furosemide was the most potent inhibitor tested, with glycyrrhizic acid, naringenin, ethacrynic acid and chenodeoxycholic acid having potencies similar to each other but an order of magnitude less potent than furosemide. Data for glycyrrhizic acid, naringenin and naringin are shown in Fig. 1. The correlation coefficient (r value) for the computed values agreeing with the measured values for the potent inhibitors was 0.99 for furosemide, glycyrrhizic acid, and naringenin, 0.96 for ethacrynic acid and 0.86 for chenodeoxycholic acid. It was above 0.95 for all of the other compounds tested except for phenylbutazone which was 0.86.

The observations of enzyme inhibition by furosemide at varying concentrations of cortisol is

Table 1. Inhibition of 11β-OHSD by various compounds

Compound	IC_{50} (μmol/l)	Concentrations tested (μmol/l)
Furosemide	59	12, 50, 100, 200, 500, 1000
Glycyrrhizic acid	254	132, 246, 529
Naringenin	336	12, 25, 50, 100, 1000, 2000, 5000
Ethacrynic acid	452	50, 100,200, 400, 2000
Chenodeoxycholic acid	513	200, 400, 600, 800
Phenylbutazone	1358	167, 667, 1344
Sitosterol	1395	500, 1000, 1500
Stigmasterol	1968	500, 1000, 1500
Naringin	2373	582, 1163, 1744
Cholic acid	3529	1250, 2500, 3750, 5000

Campesterol inhibited 33% at the highest concentration tested of 1000 μmol/l. Since a second higher point could not be measured because of limited solubility of the compound, an IC_{50} was not calculated.

shown as a double reciprocal plot in Fig. 2. Most of the lines converge near the ordinate. A Dixon plot indicated that the inhibition by furosemide is competitive. The enzyme kinetic constants were: $K_m = 8$ μmol/l and $V_{max} = 30$ nmol/μg microsomal protein/h. The K_i for furosemide was 7.7 μmol/l nearly the same as the K_m for cortisol.

DISCUSSION

We have tested a number of compounds for their ability to inhibit the NADP-utilizing form of 11β-OHSD from guinea pig renal cortex with cortisol as substrate. We found that furosemide is a much more potent inhibitor than glycyrrhizic acid, and that naringenin, ethacrynic acid and chenodeoxycholic acid inhibit with a potency almost equal to that of glycyrrhizic acid.

The compounds selected for study were chosen for a variety of reasons: the diuretics because they cause potassium loss with spironolactone as a control since it does not; glycyrrhizic acid and the bile salts as reference compounds, since data about these compounds have been published and therefore they can be used in this study to evaluate relative potency of the other compounds studied; naringin and naringenin because they are active compounds in grapefruit juice that inhibit a particular pathway of drug oxidation (cytochrome P_{450} 3A4) and we were curious to see if they also inhibited this oxidation pathway (11β-OHSD); the sterols since they are present in vegetable oils and have a structure suggesting that they might inhibit 11β-OHSD; and the cyclooxygenase inhibitors because they inhibit prostaglandin formation and cause salt retention.

Table 2. Compounds that failed to inhibit 11β-OHSD

Compound	Maximum concentration tested (μmol/l)
Bumetanide	2000
Hydrochlorothiazide	8000
Indomethacin	1100
Spironolactone	2000

The maximum concentration tested was limited by the solubility of the compound.

The K_m of our enzyme preparation for cortisol (8 μmol/l) is similar to that of rat for corticosterone (2 μmol/l) found by Monder *et al.* [18]. Working with purified enzyme from rat liver (gift from Dr C. Monder), we have found an IC_{50} of 12 nmol/l for glycyrrhetinic acid [10], similar to the dissociation constant of the enzyme–inhibitor complex of 8 nmol/l reported by Monder *et al.* [18]. In a previous study from our laboratory, glycyrrhizic acid had an IC_{50} of 1994 μmol/l for guinea pig renal cortex microsomes with corticosterone as the substrate without Triton in the incubation mixture [9] compared with 254 μmol/l in the present study using Triton and cortisol as the substrate. Buhler *et al.* [12] working with rat kidney microsomes and corticosterone at 0.1 μmol/l, found an IC_{50} of 4 μM; in our study of guinea pig microsomes with a substrate concentration of 23 μmol/l we found an IC_{50} of 254 μM. Perschel *et al.* [11] working with rat kidney microsomes found cholic acid to inhibit this

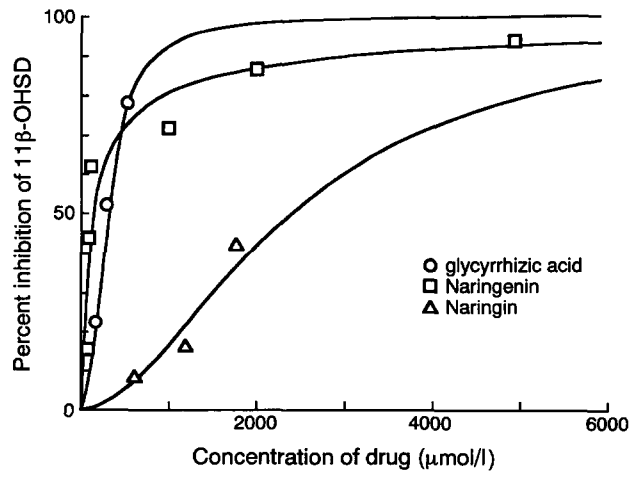

Fig. 1. Inhibition of 11[bt]β-OHSD by glycyrrhizic acid from licorice and flavonoids from grapefruit juice.

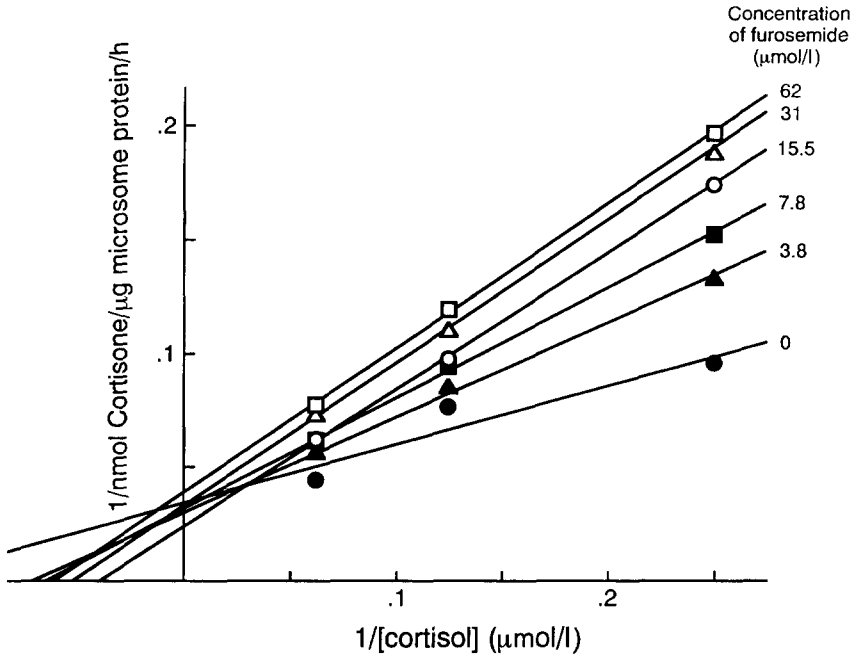

Fig. 2. Double reciprocal plot of 1/v 1/s for 11β-OHSD with varying concentrations of furosemide in incubation mixture. K_m for cortisol is 8 μmol/l. V_{max} is 30 nmol/μg microsomal protein/h. K_i for furosemide is 7.7 μmol/l.

enzyme at 1/27th the potency of chenodeoxycholic acid. We found it to be 1/7th the potency in our system.

We studied the NADP-requiring form of the enzyme that is present in most tissues rather than the NAD-requiring form that is present in the distal nephron [19–21]. Whether inhibition of the NAD-requiring enzyme is different from that of the NADP-requiring enzyme is not known. Since the mechanism of inhibition of glycyrrhetinic acid [18], gossypol [9] and furosemide (this study) is competitive, one might speculate that competitive inhibition of the NAD-requiring form of the enzyme by these compounds might also occur.

The fact that furosemide is an inhibitor of the enzyme while bumetanide is not may explain why furosemide causes more potassium excretion per unit sodium excretion than bumetanide [22–24]. It is excreted by patients with heart failure at a rate of 15–30 μg/min [25]. Assuming a 1 ml/min urine flow, the furosemide concentration would be 76 μM, compared with its IC_{50} of 59 μM in this study.

Three flavonoids: the sugar conjugates of naringenin, quercetin and kaempferol, along with some others are found in grapefruit juice [26]. These are hydrolyzed in the intestine to the aglycons which are absorbed. We found that naringenin inhibited the enzyme in this study, and previously that the flavonoids morin and quercetin were weak inhibitors [10]. The importance, if any, of these dietary constituents as *in vivo* inhibitors of this enzyme remains to be determined.

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