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AN INVESTIGATION OF THE SEX DIFFERENCE IN THE DIURETIC RESPONSE TO ETHACRYNIC ACID IN MICE

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1 Female mice of the Hough/Porton and Tuck/TO strains were found to be more sensitive than male mice to the diuretic effects of oral and intravenous doses of ethacrynic acid.

2 The sensitivity of Hough/Porton male mice to ethacrynic acid was increased after pretreatment with stilboestrol and the sensitivity of female Hough/Porton mice decreased after pretreatment with testosterone.

3 There were no significant sex differences in the diuretic response to frusemide, acetazolamide, aminophylline, bendrofluazide, and Su 15049A although a small, but significant, increase in the sensitivity of male Tuck/TO mice to triamterene was noted.

4 The sex difference in diuretic response to ethacrynic acid may be related to an effect of sex hormone balance on its metabolism or on the sensitivity of its renal receptor.

Introduction

In a recent paper describing a method for evaluating diuretics in mice (Sim & Hopcroft, 1976) it was emphasized that this species responded to ethacrynic acid, an agent which is inactive in the rat (Beyer, Baer, Michaelson & Russo, 1965). In preliminary studies (unpublished) we noted that female mice were more sensitive to ethacrynic acid, an effect recently reported by Hill & Randall (1976). We decided to study this sex difference in greater detail and to examine possible sex differences in the response to other diuretics with differing modes of action including Su 15049A, a compound with a novel mode of action which has not been used clinically (Gaunt, Renzi, Howie, Gisoldi & Waldron, 1967).

Methods

Measurement of diuretic effects

The method was that described by Sim & Hopcroft (1976). Hough/Porton male (17 to 34 g) and female (15 to 32 g) mice and Tuck/TO male (15 to 25 g) and female (15 to 24 g) mice were used. In comparative experiments the weight range did not exceed 10 grams.

Mice, housed in groups of 35, were fasted overnight but allowed free access to water. On the morning of the experiment the mice were distributed randomly into groups of five and placed in small cages for 1 to 2 h before dosing. Oral doses of diuretics were given as suspensions or solutions in 30 ml/kg 0.25% cellosize in glass distilled water. Controls received the same volume of vehicle alone. Ethacrynic acid for intravenous doses was dissolved in sterile water with a minimal quantity of 1 N NaOH to pH 7.4 to 7.7; volumes of 5 ml/kg were injected via the tail vein. In intravenous studies all mice were given an oral load of 30 ml/kg 0.25% cellosize in glass-distilled water immediately before the intravenous dose. After dosing, mice were placed in a metabolism cage (Sim & Hopcroft, 1976) and urine was collected in a graduated tube for 3 hours. For comparison, all dose levels were tested on the same day and the experiment repeated at least four times.

Pretreatment with sex hormones

Hough/Porton mice from the same batch were distributed randomly into two groups; one group received hormone pretreatment, the other no pretreatment. Male mice were pretreated orally four days before the test with a single dose of 250 mg/kg stilboestrol suspended in 0.25% cellosize in glass-distilled water. Female mice were given 100 mg/kg testosterone subcutaneously on each of the two days before the test. The testosterone was dissolved in arachis oil to give 20 mg/ml. These large hormone doses are in the same range as those used in mice by Westfall, Boulos, Shields & Garb (1964).

Toxicity determination

Oral LD_{50} values were determined in male and female Tuck/TO mice weighing 20 to 25 grams. The mice

were fasted overnight but allowed free access to water. Ethacrynic acid in a suitable range of doses was administered to groups of 10 animals of each sex in a volume of 45 ml/kg 0.25% cellosize in glass-distilled water. Controls received the same volume of vehicle alone. The mice were observed for a period of seven days and the number of deaths noted. LD_{50} values were calculated by a probit analysis method.

Drugs

The drugs used were: acetazolamide (Diamox powder; Cyanamid of Great Britain), aminophylline B.P., bendrofluazide B.P. (Boots Company), ethacrynic acid B.P. (Merck, Sharp & Dohme), frusemide B.P. (Hoechst Pharmaceuticals), triamterene B.P. (Smith, Kline & French) and Su 15049A, the citrate of Su 15049 (2-2,6-diphenyl-4-(1-pyrrolidinyl)cyclohexyl pyridine) (Ciba-Geigy). Doses are expressed in terms of the appropriate acid or base except for Su 15049A which is expressed as the salt. Cellosize (hydroxyethyl cellulose grade QP15000) was obtained from Union Carbide.

Diethylstilboestrol and testosterone propionate were obtained from the Sigma Chemical Co. and doses are expressed in terms of the base and salt respectively.

Results

In this study sodium ion excretion has been used as an index of diuretic activity since it is a more sensitive indicator than volume excretion (Sim & Hopcroft, 1976). Potency is related to the amount of drug required to produce a given sodium ion excretion and efficacy is related to the maximal sodium ion excretion.

Sodium ion excretion in control male and female Hough/Porton mice was approximately the same but ethacrynic acid had a greater diuretic potency in female mice than in male mice after oral or intravenous administration (Table 1).

The sex difference in response to ethacrynic acid was not confined to Hough/Porton strain mice since a difference was also observed with Tuck/TO strain mice (Table 2). In contrast there was no sex difference in the diuretic response to frusemide, a high efficacy diuretic which like ethacrynic acid has its main effect on the loop of Henle (Table 2). The effects of five other diuretics were studied in male and female Hough/Porton and Tuck/TO mice using doses which were found to be submaximal in a previous study (Sim & Hopcroft, 1976). These diuretics which have differing modes of actions are all less efficacious than either ethacrynic acid or frusemide in mice (Sim & Hopcroft, 1976). There was a small but significant sex difference in the diuretic response to triamterene in Tuck/TO strain mice, in which male mice were more sensitive than female mice, but this difference was not observed in Hough/Porton mice. There were no significant sex differences in either strain in response to the four other compounds (Table 2).

We investigated this sex difference by examining the effect of exogenous sex hormones on the diuretic response. Pretreatment of male Hough/Porton mice with stilboestrol significantly reduced control sodium excretion but significantly increased the sensitivity to ethacrynic acid (Table 3). Testosterone did not significantly alter sodium excretion in control female Hough/Porton mice but significantly reduced the sensitivity to ethacrynic acid in this sex (Table 3).

Table 1 The diuretic effect of oral and intravenous doses of ethacrynic acid in male and female Hough/Porton mice

Route of administration	*Dose (mg/kg)*	*Na^+ (mmol/kg)* *Male*	n	*Female*	n
Oral	0	0.65 ± 0.11	(8)	0.52 ± 0.06	(5)
	1	0.92 ± 0.16	(9)	1.58 ± 0.18**	(10)
	10	3.35 ± 0.49	(10)	6.06 ± 0.55***	(10)
	100	6.35 ± 0.79	(10)	7.80 ± 0.48	(10)
Intravenous	0	0.60 ± 0.11	(9)	0.49 ± 0.06	(9)
	1	1.57 ± 0.16	(9)	2.24 ± 0.21**	(9)
	10	2.96 ± 0.18	(9)	5.43 ± 0.37***	(9)
	100	8.90 ± 0.56	(9)	9.51 ± 0.53	(9)

Each value is mean Na^+ excretion at 3 h ± s.e. for *n* groups of five mice. Ethacrynic acid was given orally in 30 ml/kg 0.25% cellosize in glass-distilled water, controls received the same volume of vehicle alone. Intravenous doses of ethacrynic acid were injected via the tail vein at 5 ml/kg immediately after an oral load of 30 ml/kg 0.25% cellosize in glass-distilled water. Urine was collected for 3 hours.
***$P < 0.001$; **$P < 0.01$ for differences in response between sexes at the same dose level (Student's *t* test).

Oral LD_{50} values in male and female Tuck/TO mice were 621 mg/kg and (95% fiducial limits 597 to 647 mg/kg) and 562 mg/kg (95% fiducial limits 522 to 605 mg/kg) respectively.

Discussion

These results have demonstrated a significantly increased sensitivity to the diuretic effects of ethacrynic acid in female mice compared with males in two different strains. Hill & Randall (1976) using a third strain (120/OLA) also found that female mice were more sensitive to ethacrynic acid but found no sex difference in response to other diuretics. In the present study there was also a sex difference in response to triamterene in one strain (Tuck/TO) but the difference was comparatively small and it was male mice in this case that were more sensitive than females.

Hill & Randall (1976) reported that their female mice were either in dioestrus or anoestrus at the time of their experiments. We did not determine the stage of oestrus in our mice but the animals used were four to six weeks old and were approaching sexual maturity. Our results suggest that the balance between oestrogen and androgen levels is an important factor influencing the response to ethacrynic acid since large doses of stilboestrol can increase the senstivity in male mice and large doses of testosterone can decrease the sensitivity in female mice. A hormone-dependent effect in mice has also been observed for barbiturates. Westfall *et al.* (1964) observed that the sex difference in the hypnotic response of mice to pentobarbitone could be reversed by treating male mice with stilboestrol and female mice with testosterone.

Differences in oral absorption, metabolism or in the sensitivity of the renal receptor mechanism might be responsible for the sex difference. The first possibility was excluded since the sex difference occurred after oral or intravenous administration of ethacrynic acid.

The sex difference in the hypnotic effect of barbiturates in mice may be due to differences in metabolism (Vesell, 1968) and we have, therefore, given some consideration as to whether differences in metabolism could account for our results. A major excretion product after a single dose of ethacrynic acid in rats and dogs is the cysteine adduct (EA-Cyst) (Beyer *et al.*, 1965; Klaassen & Fitzgerald, 1974). Burg & Green (1973) suggested that EA-Cyst is the major active form of the drug *in vivo* since it was 100 times as potent as the parent compound as an inhibitor of chloride transport in isolated cortical loops of Henle from rabbit kidney.

Table 2 Effects of diuretic agents on sodium excretion in male and female Hough/Porton or Tuck/TO mice

Compound	*Oral dose (mg/kg)*	n	*Strain*	*Na^+(mmol/kg)* *Male*	*Female*
Ethacrynic acid	10	6	Hough/Porton	2.39 ± 0.48	5.71 ± 0.43***
		6	Tuck/TO	2.13 ± 0.15	5.29 ± 0.53**
Ethacrynic acid	30	6	Hough/Porton	4.29 ± 0.64	8.32 ± 0.44***
		6	Tuck/TO	4.05 ± 0.44	6.87 ± 0.40***
Ethacrynic acid	100	6	Hough/Porton	5.82 ± 0.94	8.23 ± 0.30*
		6	Tuck/TO	6.23 ± 0.45	8.18 ± 0.59*
Frusemide	10	6	Hough/Porton	4.50 ± 0.37	4.50 ± 0.26
		6	Tuck/TO	4.28 ± 0.41	4.77 ± 0.62
Frusemide	30	6	Hough/Porton	7.10 ± 0.49	7.46 ± 0.31
		6	Tuck/TO	6.45 ± 0.35	6.74 ± 0.36
Frusemide	100	6	Hough/Porton	8.23 ± 0.38	8.74 ± 0.32
		6	Tuck/TO	7.69 ± 0.55	7.77 ± 0.34
Bendrofluazide	10	6	Hough/Porton	1.95 ± 0.44	2.78 ± 0.35
		4	Tuck/TO	2.03 ± 0.50	2.09 ± 0.11
Acetazolamide	10	6	Hough/Porton	1.38 ± 0.34	1.59 ± 0.32
		4	Tuck/TO	2.36 ± 0.17	2.43 ± 0.22
Triamterene	10	6	Hough/Porton	1.16 ± 0.14	1.18 ± 0.21
		4	Tuck/TO	2.85 ± 0.46	1.29 ± 0.30*
Aminophylline	30	6	Hough/Porton	1.72 ± 0.27	1.70 ± 0.12
		4	Tuck/TO	2.49 ± 0.43	2.44 ± 0.20
Su 15049A	10	6	Hough/Porton	2.12 ± 0.59	1.86 ± 0.36
		4	Tuck/TO	1.58 ± 0.33	2.10 ± 0.20

Each value is mean Na^+ excretion at 3 h ± s.e. for *n* groups of five mice. The diuretic agents were given orally in a volume of 30 ml/kg 0.25% cellosize in glass-distilled water. Urine was collected for 3 hours.
*** $P = < 0.001$; ** $P = < 0.01$; * $P = < 0.05$ for differences in response between the sexes at the same dose level (Student's *t* test).

Table 3 Effect of pretreatment with sex hormones on the diuretic response of male and female Hough/Porton mice to ethacrynic acid

	Ethacrynic acid oral dose (mg/kg)	n	*Na^+ (mmol/kg)* Untreated	Pretreated
(a) Male mice pretreated with stilboestrol	0	(9)	0.54 ± 0.12	0.20 ± 0.04*
	10	(9)	2.13 ± 0.15	3.08 ± 0.20***
	30	(9)	3.46 ± 0.14	6.16 ± 0.28***
	100	(9)	5.00 ± 0.25	8.69 ± 0.70***
(b) Female mice pretreated with testosterone	0	(3)	0.77 ± 0.13	0.58 ± 0.17
	10	(5)	6.98 ± 0.41	2.77 ± 0.22***
	30	(5)	8.36 ± 0.55	4.52 ± 0.24***
	100	(5)	8.97 ± 0.53	6.21 ± 0.18***

Each value is mean ± s.e. for *n* groups of five mice. Stilboestrol was given as a single oral dose 250 mg/kg four days before the test, testosterone at 100 mg/kg subcutaneously on each of the two days before the test. Ethacrynic acid was given in 30 ml/kg 0.25% cellosize in glass distilled water.
*** $P = < 0.001$; ** $P = < 0.01$; * $P = < 0.05$ for differences in response between untreated and pretreated mice at the same dose level (Student's *t* test)

Burg (1976) has proposed that the formation of EA-Cyst should enhance the diuretic effect but reduce the toxicity of ethacrynic acid. However, we have found no evidence in mice to support this view since the oral LD_{50} in Tuck/TO female mice was similar to that in Tuck/TO male mice. It is possible that ethacrynic acid is metabolized to some other more active derivative which is not less toxic in this species.

The nature of the renal receptor for ethacrynic acid is unknown. Various modes of action have been suggested including inhibition of Na^+K^+ adenosine triphosphatase, adenyl cyclase or glycolysis and displacement of cyclic adenosine 3',5'-monophosphate from binding sites (Jacobson & Kokko, 1976). The sex difference in response might, therefore, be due to an effect of sex hormone balance on receptor sensitivity but there is as yet no evidence to substantiate this view.

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