Effect of Loop Diuretics and Nonsteroidal Antiinflammatory Drugs on Thyrotropin Release by Rat Anterior Pituitary Cells In Vitro

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The close inverse-feedback relationship between serum free thyroxine (T_4) and thyrotropin (TSH) is altered in some patients receiving therapeutic doses of drugs such as furosemide, fenclofenac, and diphenylhydantoin. We therefore examined the effect of nonsteroidal antiinflammatory drugs (NSAID), diuretics, and diphenylhydantoin on TSH release in rat anterior pituitary cells in primary culture. TSH content of the culture medium was measured at 22 hours at 37°C either with or without thyrotropin-releasing hormone ([TRH] 10 nmol/L) in medium containing 0.5% bovine serum albumin. The mean basal TSH release by pituitary cells was 6.2 ± 1.2 ng/mL (n = 10) and was not influenced by unlabeled triiodothyronine ([T_3] 100 nmol/L) or any of the drugs tested at ≤ 400 μmol/L, except ethyacrynic acid. TRH 10 nmol/L increased mean TSH release by 346% ± 95% (n = 10). T_3 1 and 100 nmol/L inhibited TRH-stimulated TSH release by 24% and 31%, respectively ($P < .001$), whereas TRH-stimulated TSH release was inhibited by 100 μmol/L meclofenamic acid (29%), fenclofenac (28%), furosemide (24%), and diphenylhydantoin (48%) ($P < .001$ v TRH alone). Meclofenamic acid and furosemide (100 μmol/L) did not significantly alter the inhibitory effect of T_3 1 nmol/L on TRH-stimulated TSH release. These in vitro studies suggest that meclofenamic acid, fenclofenac, furosemide, and diphenylhydantoin could influence TSH release by attenuating the TSH response to TRH. This effect may influence T_4-TSH relationships when these agents are used in vivo.

IN ADDITION TO its inverse-feedback relationship with free thyroxine (T_4) and free triiodothyronine (T_3) in the circulation, thyrotropin (TSH) secretion is pulsatile, shows diurnal variation, and is inhibited by glucocorticoids and dopamine.[1] Effects of drug competitors of plasma protein binding of thyroid hormone on the secretion of TSH are incompletely documented. In the absence of specific indices of thyroid hormone action, it is difficult to establish whether acute drug-induced increases in free thyroid hormone concentrations are biologically relevant. With a drug of short half-life such as furosemide, such effects may be transient and repeated, in contrast to the new steady state observed in vivo with the long half-life nonsteroidal antiinflammatory agents (NSAID) such as fenclofenac.

The acute suppression of TSH secretion in vivo by fenclofenac in man and by the synthetic flavonoid EMD 21388 in the rat has been attributed to a transient drug-induced elevation of free thyroid hormone concentration.[2-4] However, the lack of consistent effects on TSH secretion despite a significant alteration in free T_4 in patients receiving a range of drugs such as furosemide,[5] diphenylhydantoin, carbamazepine, and valproate[6] suggests that serum TSH is influenced by factors in addition to the free thyroid hormone concentration. The lack of obvious effect of these drugs on TSH secretion in vivo could result from complex drug interactions with thyroid function, including a direct effect on TSH release by the thyrotroph.

It has been previously demonstrated that release of TSH is stimulated by thyrotropin-releasing hormone (TRH) in a mixed culture of normal rat anterior pituitary cells, and that this effect is inhibited by T_3.[7-10] In this study, we assessed the NSAID fenclofenac, meclofenamic acid, and aspirin and the diuretics furosemide, bumetanide, piretanide, and ethacrynic acid as potential modulators of TSH release by investigating their influence on TSH content of the medium using rat pituitary cells in primary culture. Diphenylhydantoin was also included, since this drug has been reported to inhibit TRH-stimulated TSH release in rat anterior pituitary cells.[10]

Our study demonstrates that furosemide, fenclofenac, and meclofenamic acid may modulate TSH release by directly inhibiting the release of TSH. This interaction may contribute to discordant TSH-T_4 relationships during treatment with these drugs.

MATERIALS AND METHODS

Drugs and Reagents

Furosemide and piretanide were obtained from Hoechst (Melbourne, Australia); ethacrynic acid from Merck, Sharp, and Dohme (Sydney, Australia); bumetanide from Astra Pharmaceuticals (Sydney, Australia); aspirin from Ajax Chemicals (Melbourne, Australia); fenclofenac from Reckitt and Colman (Hull, United Kingdom); meclofenamic acid from Warner Lambert (Ann Arbor, MI); and diphenylhydantoin from Parke Davis (Sydney, Australia). Bovine serum albumin was obtained from Sigma (St Louis, MO), T_3 from Henning (Berlin, Germany), and [^{125}I]T_3 (3,000 μCi/μg) from Amersham International (Aylesbury, Buckinghamshire, United Kingdom). Labeled [^{125}I]T_3 was purified on C-18 Sep-Pak cartridges (Millipore, Milford, MA) on the day of the experiment.[11]

Fenclofenac (0.1 mol/L) and diphenylhydantoin (0.03 mol/L) were dissolved in 100% ethanol. After dilution in culture medium, the final concentration of ethanol in the assay mixture was less than 0.7%, which had no effect on cell viability or TSH release. Meclofenamic acid (0.03 mol/L) was dissolved in 0.1 mol/L NaOH. All the drugs were diluted in culture medium without binding proteins.

Solutions used for isolation and culture of pituitary cells were obtained from GIBCO (Glen Waverley, Victoria, Australia); human serum albumin (96% pure) from Commonwealth Serum Laboratories (Parkville, Victoria, Australia); dispase (grade II) from Boehringer (Mannheim, Germany); culture plates (48-well)

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from Costar (Cambridge, MA); and TRH from Roche (Basel, Switzerland). Reagents for rat TSH radioimmunoassay (RIA) were generously provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD).

Rat Pituitary Cell Culture

Male Wistar rats weighing 200 to 250 g (12 rats per experiment) were used for the studies. Animals were killed by decapitation under carbon dioxide anesthesia. Anterior pituitary lobes were collected in calcium- and magnesium-free Hanks balanced salt solution supplemented with human serum albumin (10 g/L), penicillin (10^5 U/L), amphotericin B (Fungizone, 0.5 mg/L; Gibco, Gaithersburg, MD), and sodium bicarbonate as reported previously.[7] The pituitaries were disrupted by mincing with a sterile scalpel and then further dissociated with dispase (2.4×10^5 U/L).[7] One to two million cells were obtained from each pituitary, and viability of the cells as assessed by trypan blue exclusion was greater than 90%.

Cells (0.5×10^6) were incubated with 1 mL culture medium in a 48-well culture dish and maintained at 37°C in 95% air and 5% carbon dioxide at 95% relative humidity. The culture medium consisted of minimum essential medium supplemented with Earle salts with nonessential amino acids, sodium pyruvate (1 mol/L), 10% fetal calf serum, penicillin (10^5 U/L), Fungizone (0.5 mg/L), L-glutamine (2 mol/L), and sodium bicarbonate (2.2 g/L).[12,13] The cells were cultured for 3 days and were used for the study of TSH secretion.

TSH Secretion

On the day of the experiment, the medium was removed and replaced with culture medium (without fetal calf serum) containing 0.5% bovine serum albumin supplemented with drug (100 μmol/L) or unlabeled T_3 (100 nmol/L). After 2 hours, TRH (10 nmol/L) was added together with the drug or unlabeled T_3, and the cultures were incubated for a further 22 hours. The medium was collected and stored at −20°C for either TSH or prolactin measurement. All incubations were performed in triplicate and repeated at least twice.

The concentration of TRH used in our study was chosen on the basis of preliminary experiments (data not shown). TSH secretion by rat anterior pituitary cells increased progressively with the addition of 0.1 to 100 nmol/L TRH. A maximum TRH-stimulated TSH secretion occurred between 10 and 100 nmol/L TRH, a concentration similar to that previously reported for maximal stimulation of TSH release in cultured cells.[10] All subsequent studies were therefore performed with the addition of 10 nmol/L TRH.

Measurement of Rat TSH

Rat TSH was assayed by RIA using antibody and peptide supplied by the NIDDK pituitary program. The TSH tracer was iodinated using the iodogen method, with the resulting [^{125}I]-TSH being separated from [^{125}I]-sodium iodide by chromatography on 10-mL Bio-Rad (Hercules, CA) disposable columns. Assays were performed at an antibody dilution of 1:10,000. Incubations were performed at room temperature for 24 hours, followed by an overnight incubation at room temperature with a precipitating antibody (sheep antirabbit IgG). After centrifugation, bound tracer was estimated by gamma-counting the pellet. Sensitivity of the assay was 40 pg with respect to the reference standard, RP-2. The intraassay coefficient of variation was 6%. All samples from a given experiment were estimated in the same assay.

Measurement of Rat Prolactin

Rat Prolactin was assayed by double-antibody RIA using material supplied by the NIDDK pituitary program. The rat prolactin tracer was iodinated using the iodogen method and separated from free [^{125}I]-sodium iodide on a 10-cm DEAE-A25 column. Samples were incubated with the first antibody at a dilution of 1:2,500 at room temperature for 2 days, followed by precipitation with a second antibody (sheep antirabbit IgG) and polyethylene glycol (final concentration, 7%). Results are expressed relative to the reference standard, RP-3. Sensitivity of the assay was 17 pg, and the intraassay coefficient of variation was 6%.

Statistical Analysis

The difference between two groups was evaluated using Student's *t* test for unpaired data. All results are expressed as the mean ± SD.

RESULTS

Basal TSH Secretion

In the absence of TRH, TSH content of the medium was 6.2 ± 1.2 ng/mL (mean ± SD, n = 10). Neither the addition of unlabeled T_3 100 nmol/L nor the drugs furosemide, bumetanide, piretanide, meclofenamic acid, fenclofenac, aspirin, and diphenylhydantoin ≤ 400 μmol/L significantly altered TSH concentration in the medium. Figure 1 A is a typical representation of the lack of effect of unlabeled T_3 and a drug competitor (fenclofenac) on basal TSH release.

Addition of ethacrynic acid (100 μmol/L) resulted in a doubling of basal TSH release by rat anterior pituitary cells (ethacrynic acid 17.3 ± 5.3 ng/mL *v* medium alone 8.4 ± 3.1 ng/mL, n = 3).

TRH-Stimulated TSH Release: Effect of a Single Drug Concentration

Figure 1B shows the effect of unlabeled T_3 and fenclofenac on TRH-stimulated TSH release. Addition of TRH alone (10 nmol/L) increased TSH release from 8.3 ± 2.5 to 35.6 ± 2.8 ng/mL. Addition of T_3 (100 nmol/L) and fenclofenac (100 μmol/L) reduced TRH-stimulated TSH

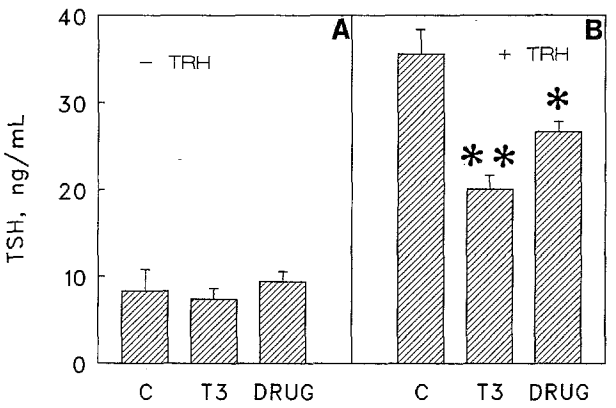

Fig 1. Effect of unlabeled T_3 and fenclofenac on TSH release in the absence (A) or presence (B) of TRH. Pituitary cells (≈500,000/well) were incubated with either T_3 100 nmol/L or fenclofenac 100 μmol/L for 2 hours followed by addition of TRH 10 nmol/L, and the cultures were incubated for a further 22 hours. *$P < .01$; **$P < .001$.

Table 1. Effect of Loop Diuretics and NSAID on TRH-Stimulated TSH Concentration

Agent	TSH Concentration (% of TRH alone)						
	Furosemide	Bumetanide	Piretanide	Ethacrynate	Meclofenamate	Fenclofenac	Aspirin
TRH alone‡	100 ± 6	100 ± 8	100 ± 4	100 ± 5	100 ± 5	100 ± 7	100 ± 9
T_3 + TRH	60 ± 9*	85 ± 1†	75 ± 10†	75 ± 10†	64 ± 14*	53 ± 6*	70 ± 10*
Drug + TRH	77 ± 5*	97 ± 8	103 ± 14	99 ± 9	71 ± 8*	72 ± 6*	100 ± 5

NOTE. Concentrations are as follows: TRH 10 nmol/L; T_3 100 nmol/L; aspirin 500 μmol/L; all other drugs 100 μmol/L.
*$P < .001$, †$P < .005$: compared with the addition of TRH alone.
‡TSH secretion in the presence of TRH 10 nmol/L ranged from 22.9 to 35.6 ng/mL.

release to 20.1 ± 1.6 and 26.7 ± 1.2 ng/mL, respectively (Fig 1B).

The effect of various drugs on TRH-stimulated TSH release is shown in Table 1. Addition of TRH (10 nmol/L) increased mean TSH secretion by 346% (n = 10). TRH-stimulated TSH release was inhibited by 31% in the presence of T_3 100 nmol/L (n = 10; Table 1). Furosemide, meclofenamic acid, and fenclofenac (all 100 μmol/L) significantly inhibited TRH-stimulated TSH release by 23%, 29%, and 28%, respectively. Bumetanide, piretanide, and ethacrynic acid (all 100 μmol/L) and aspirin (400 μmol/L) did not significantly alter TRH-stimulated TSH release (Table 1). We were able to reproduce the effect of diphenylhydantoin on TRH-stimulated TSH release described previously[10]: addition of diphenylhydantoin 100 μmol/L significantly inhibited TRH-stimulated TSH release by 48%.

Combined Effect of T_3 and Drugs on TSH Release

To study further the mechanism of action of drugs on TSH release by pituitary cells, we investigated whether meclofenamic acid and furosemide interfered with the inhibitory effect of T_3 on TRH-stimulated TSH release. The lowest concentration of T_3 that inhibits TRH-stimulated TSH release in vitro by pituitary cells is between 1 and 10 nmol/L.[10] In this experiment, we tested T_3 (1 nmol/L) either in the presence or absence of a drug competitor (100 μmol/L). Addition of T_3 1 nmol/L inhibited TRH-stimulated TSH release by 24%. Relative to T_3 alone, TRH-stimulated TSH release was not significantly altered by the presence of either meclofenamic acid or furosemide (Table 2).

Table 2. Effect of Drugs and Unlabeled T_3 on TRH-Stimulated TSH Concentration

Agent	TSH Concentration (ng/mL)	
	Plus Meclofenamic Acid	Plus Furosemide
Basal TSH (no TRH)	5.7 ± 0.3	11.6 ± 1.1
TRH 10 nmol/L	19.9 ± 0.6	32.8 ± 1.0
TRH + T_3 1 nmol/L	16.3 ± 1.7†	25.0 ± 0.9†
TRH + drug 100 μmol/L	18.4 ± 0.6*	27.5 ± 1.4†
TRH + drug + T_3	18.0 ± 0.6*‡	26.4 ± 1.9†‡

NOTE. Mean ± SD; n = 3.
*$P < .05$, †$P < .025$: *v* TRH alone.
‡Not significant *v* TRH + T_3.

Dose-Response of Meclofenamic Acid on TSH Release

We investigated the effect of increasing concentrations of meclofenamic acid on TRH-stimulated TSH release by rat anterior pituitary cells (Fig 2). The effect of drug addition was compared with TRH alone. A mean inhibition of TSH release of 7%, 13%, 25% and 29% was observed with 10, 31.6, 100, and 316 μmol/L added meclofenamic acid, respectively.

Mean prolactin concentration in the medium was increased by 23% (3.1 ± 0.14 to 3.82 ± 0.5 μg/L, n = 3, $P < .1$) in the presence of TRH. TRH-stimulated prolactin release was not affected by addition of unlabeled T_3 (100 nmol/L), furosemide, or fenclofenac (100 μmol/L).

DISCUSSION

The data presented here suggest that TRH-stimulated TSH secretion can be directly modulated by drug competitors. Inhibition of TRH-stimulated TSH release by furosemide, fenclofenac, and meclofenamic acid is similar to the effect of diphenylhydantoin previously reported,[10] suggesting that these drugs may have multiple sites of action, including direct inhibition of TSH release by the thyrotrophs.

It is interesting to reconsider the effect of fenclofenac[2] on TSH release in light of our results. It was suggested that the

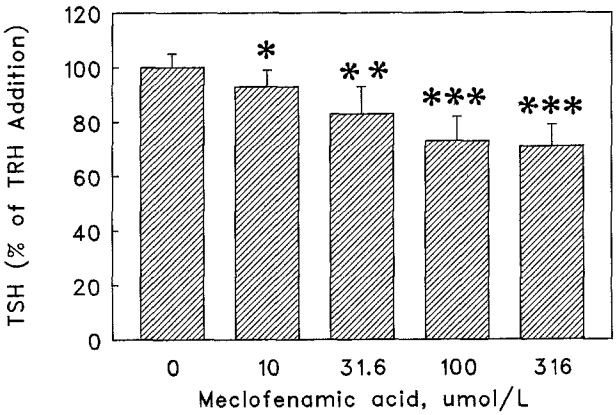

Fig 2. Effect of increasing concentration of meclofenamic acid on TRH-stimulated TSH release. Mean TSH release in the presence of TRH 10 nmol/L in three separate experiments was 28.1, 32.1, and 38.6 ng/mL. TSH release in the presence of meclofenamic acid is expressed as a percentage of values with TRH alone. *$P < .02$; **$P < .05$; ***$P < .001$.

acute displacement of T_4 from plasma protein binding by fenclofenac was responsible for the prompt suppression of serum TSH, but our present data suggest that in addition to this effect there could be direct inhibition of TSH release by the drug. This observation is supported by Taylor et al,[14] who reported that in patients on long-term treatment with fenclofenac, TRH testing continued to show impairment of TSH release. This observation, together with a normal basal TSH and a reduced serum free T_4 in these patients, suggested that fenclofenac may directly affect pituitary TSH secretion in vivo.

It is important to consider whether TSH content of the medium gives a true index of hormone release in these experiments. This assumption is supported by the demonstration that acute stimulation of TSH release into the culture medium by TRH and inhibition by T_3 or diphenylhydantoin were reflected by inverse alterations of the cellular content of TSH, with the net effect being that the total amount of TSH (cell plus medium) remained unaltered.[10]

Basal TSH release in vitro was unaffected by a large dose of unlabeled T_3. Despite the fact that serum TSH is sensitive to small changes of free hormone concentrations in vivo,[15] the lack of effect of T_3 on basal TSH release has been previously reported.[10,16] This lack of effect is probably not due to the presence of thyroid hormone in fetal calf serum in our culture medium, because in an earlier study using resin-stripped serum, basal TSH secretion was still only marginally suppressed by T_3.[10] Surks et al[10] suggested that the failure of a large dose of unlabeled T_3 to suppress basal TSH secretion may reflect cell damage as a result of enzymatic and mechanical dispersion of the pituitary lobes. An alternative explanation is that the culture conditions are not optimal for studies of TSH synthesis, as opposed to acute release of preformed TSH.

Furosemide, bumetanide, and piretanide did not affect basal TSH release in vitro, but ethacrynic acid significantly stimulated basal TSH release. The reason for this observation is unclear, but may relate to the structural differences between the four diuretics: ethacrynic acid is an alpha-beta unsaturated ketone derivative of an aryloxyacetic acid, whereas bumetanide, furosemide, and piretanide are sulfonamide-type diuretics.[17]

We have not yet examined the influence of drugs on the T_4 effect on TSH release in pituitary cells in primary culture. However, the influence of T_4 on TSH release has recently been reported.[18] With regard to the control of pituitary TSH secretion, it is important to note that T_4 is as important as T_3. Studies in rats demonstrated that although approximately 50% of intracellular T_3 in the pituitary is derived from plasma T_3, an equal proportion of pituitary T_3 comes from the intracellular conversion of T_4 to T_3.[19,20]

The effect on plasma TSH concentration in vivo of long-term administration of drugs, including anticonvulsants, NSAID, and heparin, has been reported.[6,21,22] No significant effect on basal TSH secretion was demonstrated, despite the fact that administration of diphenylhydantoin, valproate, and carbamazepine[6] significantly decreased free T_3 and free T_4 concentrations, suggesting a direct drug effect on TSH release.

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