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EVIDENCE FOR A ROLE OF A SERUM FACTOR STIMULATED BY METOCLOPRAMIDE IN REGULATING ALDOSTERONE SECRETION

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Summary: The stimulatory effect of metoclopramide upon aldosterone secretion is independent of the known aldosterone-regulating mechanisms (renin,potassium,adrenocorticotropic hormone),is unrelated to its effect on prolactin and is absent when metoclopramide is directly added to isolated adrenal zona glomerulosa cells.To examine the possibility of a "humoral" mediation of aldosterone stimulation by metoclopramide, we evaluated the effect of serum of 10 normal subjects injected with metoclopramide (10 mg i.v.) on aldosterone production by collagenase-dispersed calf adrenal zona glomerulosa cells.Whereas no effect was observed with serum collected before the injection,serum collected from 5 to 30 min after the injection stimulated aldosterone production.The effect was seen 2.5 min after the injection,was significant at 5 min (P 0.05),10,15,20 and 30 min (P 0.01).The effect disappeared 40 min after the injection,when plasma aldosterone in subjects was still elevated (P 0.01).The biological half-life of the factor ($t_{1/2}$) is about 12.5 min. A significant correlation was found between the maximal aldosterone response to metoclopramide *in vivo* and the maximal effect of serum *in vitro* (r^2=0.69;P 0.01).
We suggest that metoclopramide stimulates aldosterone production *in vivo* by the increase in serum of a factor which,in turn,stimulates aldosterone and whose physiological significance remains to be evaluated.

After we showed (1) that metoclopramide stimulates aldosterone production in man,a series of studies followed with the aim to establish the mechanism by which metoclopramide acts.From these studies it became evident that the stimulatory effect of metoclopramide on aldosterone secretion is independent of the known regulators of aldosterone secretion (2,3).Particularly metoclopramide does not stimulate adrenocorticotrophic hormone (ACTH)(1-4),does not increase serum potassium (1-6) and its effect is not abolished by angiotensin converting enzyme inhibitors,ruling out the possibility of an increase of angiotensin II (4).Further,the metoclopramide

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effect on aldosterone production is not due to its action on hypophysis (1,7,8) nor to a decrease of the metabolic clearance rate of aldosterone (9).Any mediation of the sympathetic nervous system is also unlikely since ganglioplegic drugs do not modify its effect (10).Finally metoclopramide has consistently failed to stimulate aldosterone secretion when tested in vitro on adrenal zona glomerulosa cells (11,12),at concentrations similar to those attainable in human serum after i.v. injection (13),or when injected into the adrenal artery of sheep with adrenal autotransplant (14).

To evaluate the possibility of a humoral mediation of the metoclopramide effect on aldosterone production,we tested the effect of serum of normal subjects injected with metoclopramide on aldosterone secretion by calf adrenal zona glomerulosa cells (13,15).

MATERIALS AND METHODS

We obtained metoclopramide from Richter (Lepetit SpA,Milan). Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) synthetic,human sequence and Angiotensin II Antagonist (Sar^1-Ala^8 Angiotensin II) were obtained from Calbiochem-Behring Corp.(La Jolla,Ca);Adrenocorticotropic hormone (ACTH) human synthetic from Sigma Chem.Co (St. Louis,MO).Collagenase (Clostridium Hystolyticum) was obtained from Boehringer Mannheim GmbH; other compounds were obtained from standard chemical suppliers.
Serum and plasma (EDTA-Na) were collected by 10 healthy subjects (5 male and 5 female;age range 22-50 years) maintained on a 2000 Cal diet containing 130 meq sodium and 60-80 meq potassium daily for 7 days before the experiment. After an overnight fast all subjects were kept recumbent 3 hours before the test and during the test.At 0900 h an indwelling needle was inserted into an antecubital vein and kept patent with a slow infusion of 0.9% NaCl in water.Metoclopramide (10 mg in saline) was injected i.v.in 2-3 min at time 0.Blood specimens were drawn at frequent intervals and promptly centrifuged.Plasma for hormonal determinations was kept at -20°C until assay,whereas serum was used immediately in the experiments with the cells.
Calf adrenal zona glomerulosa cells were obtained with slight modifications of the previously described procedures (13,15).Briefly,adrenals of 2-3 male calves freshly slaughtered,were collected and placed into ice-cold 10 mM HEPES buffer containing glucose (1 mg/ml).Extraneous tissue was dissected from the adrenals which were bisected:the capsular zona glomerulosa layer was separated by gently scraping off the medullary and the inner cortical layers.Only the capsular layer was used for cell suspension.The tissue pieces were washed and incubated in Krebs Ringer buffer with collagenase (0.3% wt/wt) at 37°C for 60 min under 95% oxygen and 5% carbon dioxide. During the incubation the pieces were maintained in continuous agitation by a gentle stream of oxygen carbon dioxide.The filtered cells were centrifuged at 100 x g for 10 min at 20°C.The pellets resuspended in the same buffer without collagenase were centrifuged again and the final cell pellets were pooled and resuspended in the final Krebs Ringer buffer (K^+ = 4.8 mM). Cells were counted and examinated for viability under light microscopy using a Neubauer hemocytometer at 1:2 dilution (cell suspension Trypan blue).

The mean number of cells for incubation was $3 \pm 0.6 \cdot 10^5$/ml. In each experiment 0.8 ml sample of the cell suspension was added to 0.1 ml of serum samples and to 0.1 ml of buffer containing various drugs.The cell suspensions were incubated for 60 min at 37°C in a water bath under oxygen and carbon dioxide. The supernatant obtained by centrifugation at 1500 x g for 10 min at 4°C was stored at -20°C until assay (16,17).
Statistical analysis was performed with ANOVA.

RESULTS

As shown in fig. 1 metoclopramide injection was follwed by a prompt rise of plasma aldosterone that was significant at 5 min (P 0.05) and peaked at 15 min (P 0.01).Aldosterone secretion remained elevated until 70 min (P 0.05).The sera of patients collected before the injection of metoclopramide did not significantly affect the aldosterone production by calf adrenal zona glomerulosa cells (fig.1).However after the injection of metoclopramide the sera of the patients displayed a stimulatory activity that was detectable but not significant at 2.5 min after the injection,was present at 5 min (P 0.05) and 10,15,20,and 30 min (P 0.01).The activity was no more detectable 40 min after the injection.

The effect of serum was still present when Sar^1Ala^8Angiotensin II was added to the cells (Tab 1).Serum drawn 15 min after the injection failed to affect cortisol production by the cells (Tab 1).The maximal increase of

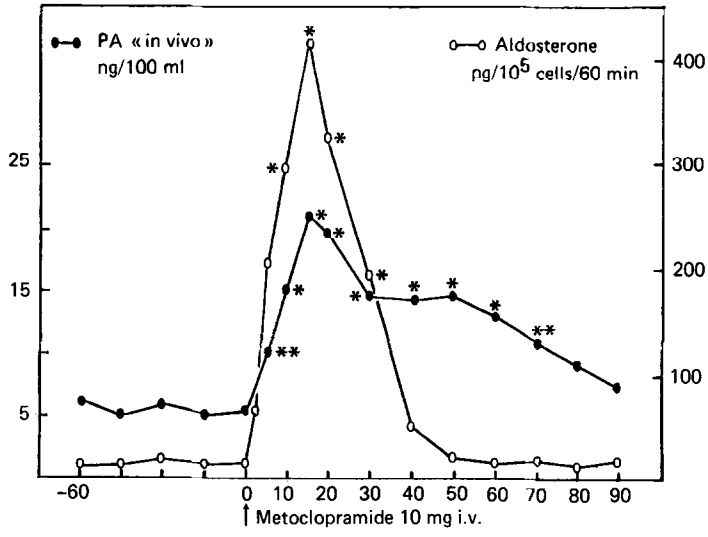

Figure 1 - Time course of the changes in plasma aldosterone (PA) in human beings after metoclopramide injection and of in vitro aldosterone production by calf adrenal zona glomerulosa cells in the presence of the sera of the same subjects.
** P 0.05 * P 0.01 vs basal values (ANOVA)

Table 1 - Effect of various agents on aldosterone production by calf adrenal zona glomerulosa cells in the presence of serum of 4 normal subjects before (A) or after (B) the injection of metoclopramide (mean±SE) *P 0.05 vs B.

	aldosterone pg/10^5cells/hr	cortisol ng/10^5cells/hr
A	24±8	0.5±0.4
B	530±110	1.0±0.8
B plus angiotensin II (1nM)	1025±250*	15.0±3.1*
B plus Sar^1Ala^8 A II (10nm)	575±115	2.1±0.5
A II (1 nM)	710±58	19.1±0.8
A II (1 nM)+Sar^1Ala^8AII(10nM)	210±45	3.2±1.1
metoclopramide 1nm	25±7	1.0±1.1
A plus metoclopramide	32±7	2.1±1.3
B plus metoclopramide	487±78	3.1±2.1
ACTH 100 pM	490±11	151.4±22.1

aldosterone in in vitro studies in the presence of serum was correlated with the maximal increase observed in vivo (fig.2).

The activity observed in serum 15 min after the injection of metoclopramide in the subject who showed the highest activity (maximal response over basal values:850 pg/10^5cells/60 min) demonstrated a dose-dependency (Tab 2).Metoclopramide failed to increase aldosterone production when added in vitro (0.1-10 nM) in the presence or not of serum of normal subjects (Tab 1).

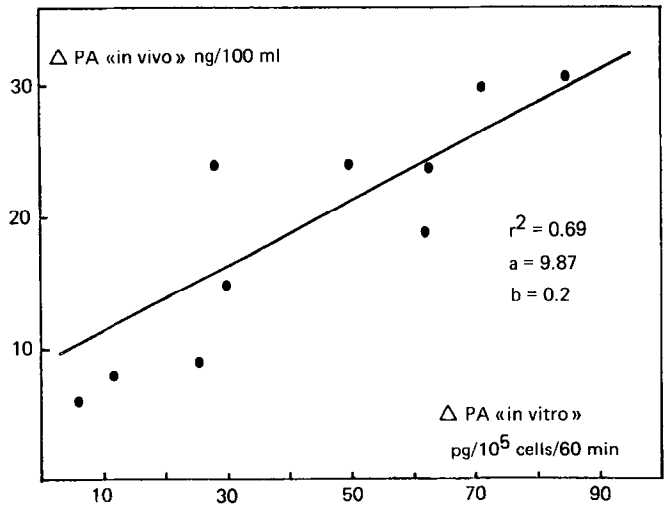

Figure 2 - Relationship between the "maximal" in vivo response of plasma aldosterone to metoclopramide and the"maximal" in vitro aldosterone production by calf adrenal zona glomerulosa cells in the presence of the serum of the subjects injected with metoclopramide.

Table 2 - Effect of dilution of the serum with buffer on the stimulatory activity of the serum on aldosterone production by calf adrenal zona glomerulosa cells (serum of the subject with the highest activity).

	aldosterone pg/10^5cells:hr	cortisol ng/10^5cells/hr
serum 1:1	850	1
" 1:2	490	1
" 1:4	210	2
" 1:8	10	2
" 1:16	not obs.	2

DISCUSSION

Metoclopramide elevates plasma aldosterone concentration in man by increasing the secretion of aldosterone (for a review see Ref. 14). The mechanism mediating this effect has not so far been elucitated and has been object of recent intensive investigations,often yelding conflicting results (14,9,10).

In this study we show that serum collected in subjects injected with metoclopramide contains a factor which elicites an increase in aldosterone production by calf adrenal zona glomerulosa cells.This effect observed *in vitro* is highly correlated with the increase of aldosterone seen *in vivo*,which suggests that metoclopramide stimulates aldosterone in man by eliciting the increase of a humoral factor which in turn stimulate aldosterone secretion.

Previous studies by our group (1,6) and by other workers (2-5) have demonstrated that the effect of metoclopramide is independent on ACTH, renin and on modifications of sodium and potassium.That an increase of Angiotensin II is not responsible of the stimulatory effect of serum post-metoclopramide is also suggested by the fact that the competitive inhibitor of Angiotensin II, Sar^1Ala^8Angiotensin II,is unable to suppress the stimulatory activity of serum.Although metoclopramide *per se*,at the doses usually found in serum after injection,does not stimulate aldosterone secretion *in vitro* (11,12,13,present study),the possibility exists that serum of subjects injected with metoclopramide contains a metabolite of metoclopramide which stimulates aldosterone.However the rapidity of the action of metoclopramide does not support this hypothesis.By inspecting the fig. 2 it seems reasonable to suggest that the factor that stimulates aldosterone production by cells has a half-life of about 12 min.

Recent investigations have indicated that,besides sodium,potassium, angiotensin and ACTH,other factors (18) are involved in the control of aldosterone secretion.Their physiological significance and their identification with the factor released by metoclopramide remains to be established. In conclusion we show here that metoclopramide stimulates aldosterone secretion in human beings through an indirect mechanism consisting in the increase in serum of a factor which stimulates the secretion of aldosterone.

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