Assessment of corticoadrenal reserve through salivary steroids.

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Abstract

Recent reports have reinforced the utility of salivary cortisol in the evaluation of cortisol dynamics and in the diagnosis of Cushing´s syndrome. However,few data are available on the usefulness of salivary cortisol (SAF) and aldosterone (SAL) in response to corticotrophin (ACTH) stimulus. The aim of our study was to standardize the response of SAF and SAL to the intramuscular injection of 250 μg of synthetic human $\beta^{1\text{-}24}$ ACTH in healthy subjects. We performed this test in 21 normal adult volunteers. Salivary samples were obtained in baseline conditions and after 30,60,90 and 120 minutes after ACTH administration. The results showed that all normal volunteers achieved SAF and SAL concentrations of at least 40.0 nmol/L and 100.0 pmol/L respectively, after the stimulus. The clinical utility of the above test was confirmed when patients with already known adrenal dysfunction were studied. In 3 patients with primary adrenal insufficiency blunted SAF and SAL responses were obtained. Patients with secondary adrenal insufficiency (n:6) demonstrated subnomal or blunted SAF and normal SAL responses after ACTH stimulation.

From our data we suggest that corticoadrenal reserve can be easily, non-invasively and accurately investigated through the simultaneous measurement of salivary cortisol and aldosterone in response to corticotrophin. Salivary aldosterone was helpful in the differential diagnosis of adrenocortical hypofunction.

Introduction

The utility of salivary cortisol measurement has been recently updated [1,2]. This methodology offers the opportunity of multiple sampling avoiding the stress of flebotomy and helps to monitor the concentration of non-protein-bound biologically active serum cortisol [3]. However, few data are available using salivary cortisol measurements in the assessment of corticoadrenal reserve [2,4].Recently we reported preliminary data on the evaluation of cortisol adrenal reserve through salivary cortisol measurements and the application of this methodology in patients with chronic renal failure [5,6].

It has been reported that salivary aldosterone concentration highly correlates with plasma aldosterone levels and that it reflects the free plasma fraction [7,8,9]. However, although the biological significance of aldosterone concentration in saliva has been well defined [7,9,10,11], no recent reports on its clinical application have been found by us.

The rapid ACTH stimulation test for the assessment of corticoadrenal reserve is widely accepted [12]. The additional measurement of plasma aldosterone in response to intramuscular ACTH administration has proved to be useful in the differential diagnosis of adrenal hypofunction when a subnormal cortisol response is found [13].

The aim of our study was to evaluate corticoadrenal reserve through a non invasive methodology by the simultaneous determination of salivary cortisol and aldosterone after ACTH stimulation. With this purpose we standardized the

response of salivary steroids to synthetic human β^{1-24} ACTH stimulation (250 μg, intramuscularly) in healthy subjects. In addition salivary steroid responses were investigated in patients with diagnosis of adrenal hypofunction.

Subjects and Methods

Normal subjects

Twenty one healthy subjects (15 women and 6 men, aged 18-70 year old) were recruited from the staff faculty and medical students of the Instituto de Investigaciones Médicas Alfredo Lanari,University of Buenos Aires. All of them have recently participated as controls in a study in which serum steroid dynamics was evaluated [14].These subjects were on no medication and on a regular sodium diet. None had any endocrine disorder.

Patients

Nine patients with adrenal insufficiency were studied. Primary (I_1) and secondary (I_2) adrenal failure were diagnosed in 3 and 6 cases , respectively.

In I_1 there were two female and one male patients aged 40- 69 years old, with autoimmune adrenal and thyroid failure (Schmidt's syndrome) in one case and with TBC adrenal involvement in two other cases.Basal ACTH plasma levels in these patients ranged from 150.0 to 225.0 pg/ ml (normal range less than 50.0 pg/ml).

I_2 was constituted by 3 women and 4 men , aged 32 to 70 years old . Four male patients were on chronic steroid therapy for asthma; one female had Seehan's syndrome and two female patients were studied 3 months post successful surgical therapy for Cushing's syndrome (selective pituitary adenomectomy in one case and resection of and adrenal cortisol secreting adenoma in the other case). Basal ACTH plasma concentrations ranged from 5.0 to 25.0 pg/ml.

All patients discontinued oral replacement doses of hydrocortisone at least 24 hours before the ACTH stimulation test.

All the subjects gave their consent to participate in this study.

Methods

Saliva samples

After overnight fasting subjects were instructed to collect 3.5 ml of whole saliva in polypropylene tubes .Two hours before the sample collection they brushed their teeth and rinsed their mouth with tap water to eliminate food or debris contamination . Salivary samples were centrifuged and the supernatants were kept at -20 °C until steroids were assayed .

ACTH stimulation test

At 8.30 a.m. after 30 minutes of rest in recumbent position, baseline salivary samples were obtained . Synthetic human β^{1-24} ACTH (250 μg Synacthen, Novartis) was injected intramuscularly in the deltoid muscle . Whole saliva was obtained at 30, 60, 90 and 120 minutes after ACTH injection.

In 9 healthy donors an indwelling catheter was inserted in the antecubital vein and serum and saliva samples were obtained simultaneously before and after ACTH stimulation for the measurement of steroids.

Cortisol assay

Total serum and salivary cortisol were assessed using a solid phase radioimmunoassay commercial kit (coat a count, Diagnostic Products Corporation, Los Angeles, CA, USA obtained through Tokatlian S.A., Argentina). Serum cortisol was assayed following the kit instructions. The minimal detectable dose was 6.0 nmol/L. Coefficients of variation intraassay and interassay were below 5.0 % and 6.0 % ,respectively.

Salivary cortisol was determined using the modifications described by Raff et al [1]. Intra-assay and interassay coefficients of variation were below 6% and 13%, respectively. SAF was expressed as nmol/L. The minimal salivary cortisol concentration detected was 0.5 nmol/L. The cross reactivity of the assay for prednisolone,11-deoxicortisol, prednisone and dexamethasone was of 76%, 11.4% , 2.3 % and 0.04%, respectively.

Aldosterone assay

Total serum and salivary aldosterone concentrations were measured by competitive solid phase radioimmunoassay (Coat- A-Count, Diagnostic Products Corporation, obtained through,Tokatlian S.A., Argentina). The levels of serum aldosterone were assessed following indications from the kit manufactures, including stored frozen alicuots of the calibrators after reconstitution. The detection limit for the serum aldosterone assay was 33.0 pmol/L . The intraassay and interaassay variation was below 6.0 % and 12.0 %, respectively. Salivary aldosterone was measured using a modification of the same assay increasing the analyte volume to 400.0μl and diluting the calibrators 1:5 in distilled water. The concentration of working standards were : 27.8 pmol/L, 55.6 pmol/L, 111.0 pmol/L, 333.0 pmol/L and 660.0 pmol/L , thus aldosterone concentration was 4.0, 8.0,16.0, 24.0 and 96.0 pg /400 μl per tube respectively,according with others [8,11]. The minimal detectable dose of salivary aldosterone was 13.0 pmol/L .The intraassay and the interassay coefficients of variations were below 8% and 12%, respectively. The assay showed no cross reactivity with cortisol and it was negligible for other steroids.

Statistical analysis

Statistical significance was evaluated using Wilcoxon, Mann-Whitney and t- tests . $P < 0.05$ was considered to be statistically significant. Linear regressions were performed using the Spearman rank-order correlation test. Data are presented as mean $\pm$ SD and range.

Results

Healthy subjects

In normal subjects (N) mean baseline SAF concentrations were of 9.9 nmol/L (range, 3.5 to 19.0 nmol/L) . After ACTH stimulation mean SAF and range levels were of 34.3 nmol/L (17.0 to 80.0 nmol/L); 55.8 nmol/L (35.0 to 120 nmol/L); 64.6 nmol/L (25.0 to 120.0 nmol/L) and 53.0 nmol/L (15.0 to 110.0) at 30, 60,90 and 120 minutes respectively (Table 1). SAF was significantly higher than basal concentrations at 30,60,90 and 120 minutes after ACTH stimulation ($p \leq 0.001$). Most of the patients (48%) achieved maximal SAF values at 90 minutes post ACTH. Other patients reached peak levels at 60 (33%) and 120 minutes (19%) post ACTH stimulus. In view of the present data (Table 1) we define a normal SAFresponse to ACTH stimulation a rise in SAF levels of at least 40.0 nmol/L after intramuscular ACTH injection.

Basal mean SAL levels were of 34.5 pmol/L (range 13.0 to 65.0) and after ACTH administration (mean and range) were of 162.0 pmol/L (42.0 to 500.0 pmol/L) at 30 minutes, 193.0 pmol/L (70.0 to 566.0 pmol/L) at 60 minutes, 150.0 pmol/L (28.0 to 375.0 pmol/L) at 90 minutes and 88.0 pmol/L (10.0 a 300.0 pmol/L) at 120 minutes. SAL significantly rose at 30,60,90,120 minutes ($p \leq 0.022$) in comparison with basal values (Table 1). Peak SAL values were observed at 60 minutes post ACTH stimulus in 52% of the cases. In 29% and 19% of the patients maximal SAL concentrations were reached at 30 and 90 minutes , respectively. We define a normal SAL response to ACTH stimulus as a rise in SAL concentration of at least 100.0 pmol/L .

In 9 healthy donors we obtained simultaneous serum and saliva samples for cortisol and aldosterone assessment in baseline conditions and after ACTH stimulation (Fig. 1 A and B). In these subjects peak serum cortisol levels ≥ 552.0 nmol/L were achieved between 60 and 90 minutes after ACTH injection in coincidence with SAF values ≥ 40.0 nmol/L.Serum aldosterone levels ≥ 850.0 pmol/L were achieved at 30 and 60 minutes after ACTH administration in concordance with SAL concentrations ≥ 100.0 pmol/L.

Baseline mean total serum cortisol and aldosterone correlated positively and significantively with salivary cortisol and aldosterone (r:0.867,p:0.04 and r:0.700 ,p:0.038, respectively). When cortisol release was stimulated with ACTH the relationship between plasma and saliva concentrations became not linear ($r \leq 0.08$;p:> 0.4).On the other hand, serum aldosterone demonstrated a significantly positive correlation with saliva concentrations after ACTH stimulus ($r \geq 0.78$, $p \leq 0.016$, in all the stimulated samples).

When saliva and total serum cortisol increments after ACTH stimulation were compared, a significant higher variation was found in saliva than in total serum cortisol at 60,90 and 120 minutes after ACTH stimulus. Mean ± SD percent of variation of serum and salivary cortisol was of 179.0 ± 112.0 and 611.0 ± 359.0 at 60 minutes, respectively (p:0.003); of 155.0 ± 46.0 and 655.0 ± 385.0 at 90 minutes, respectively (p:0.001) and of 130.0 ± 41.0 and 568.0 ± 431.0 at 120 minutes, respectively (p:0.008). At 30 minutes post ACTH stimulation no difference was found between serum and saliva increments.

The comparison of aldosterone increments in serum and saliva after ACTH injection showed that the percent of variation of salivary aldosterone was significantly higher at 30 and 60 minutes post ACTH stimulus than that of serum aldosterone . The percentage of variation of serum and salivary aldosterone was of 132.0 ± 86.0 and 358.0 ± 196.0 at 30 minutes, respectively (p:0.006) and of 106.0 ± 76.0 and 451.0 ± 168.0 at 60 minutes, respectively (p: 0.0001). No differences were found between the increments in serum and salivary aldosterone at 90 and 120 minutes post ACTH injection.

Primary adrenal insufficiency

These patients showed a subnormal response of SAF levels to ACTH,as expected. A maximal SAF peak (mean) of 16.0 nmol/l was found in one case and blunted SAF response were demonstrated in the other two cases (Table 2, Fig. 2 A). Baseline mean salivary aldosterone was 13.4 pmol/L (range 13.0-13.8 pmol/L) and absence of a normal response to ACTH stimulus was demonstrated in all these subjects (Table 2,Fig. 2 B).

Mean basal SAF and SAL values were significantly lower (p:0.012 and p:0.013, respectively) than in healthy subjects. Statistically lower concentrations of SAF and SAL were found at 30,60,90 and 120 minutes after ACTH stimulation compared to controls (Table2) .

Secondary adrenal insufficiency

In I_2 mean baseline SAF was 3.6 nmol/L (range 1.4 to 6.0 nmol/L). In all patients SAF failed to rise normally after ACTH stimulus with maximal SAF levels of 30.0 nmol/L achieved in one patient at 60 minutes (Table 2, Fig. 2 A). By contrast,

baseline SAL concentration was of 28.6 pmol/l (range 13.0-80.0) and peak levels ≥ 100 pmol/l after ACTH stimulus were reached in all the subjects (Table 2 , Fig. 2 B)

In I_2 patients mean SAF values were significantly lower than in healthy subjects (p:0.003) as were the levels reached at 30,60,90 and 120 minutes after ACTH stimulus (p:0.0001). By contrast, neither mean basal SAL concentration nor its response to ACTH were significantly different than in healthy subjects.

A dissociated response in SAF and SAL was found in this group of patients demonstrating the functional indemnity of the adrenal glomerulosa.

Discussion

The present study shows the salivary response of cortisol and aldosterone to the intramuscular injection of 250 μg of synthetic β^{1-24} ACTH in healthy subjects.

As far as we know no previous reports have shown the normal salivary response in cortisol and aldosterone to corticotrophin stimulation. Our data demonstrate that all normal volunteers achieved a salivary cortisol and aldosterone concentration of at least 40.0 nmol/l and 100.0 pmol/l respectively, after ACTH stimulus. Because the above SAF and SAL concentrations were obtained between 30 and 90 minutes after ACTH stimulation, saliva sampling at 120 minutes does not seem to bring additional information. We suggest to perform a shorter ACTH stimulation test and collect saliva samples every 30 minutes during a total period of 90 minutes. We consider that this time selected sampling brings enough clinical information.

We could found, from our results,that in baseline conditions salivary cortisol and aldosterone values were positively correlated with total serum levels.However, after ACTH stimulation, the initial baseline linear correlation for cortisol disappeared. Read and Tunn [14,15] explained that this phenomenon could be due to the fact that plasma cortisol binding protein (CBG) became saturated [3,15]. On the other hand,salivary aldosterone persisted positively correlated with serum levels after acute ACTH stimulation. This data are in accordance with Atherden et al [11].The increment of variation of salivary cortisol (60, 90 and 120 minutes) and aldosterone(30 and 60 minutes) after ACTH stimulation was significantly greater than that obtained in serum. This finding was already observed for cortisol by

Umeda et al. [16] and reinforces the usefulness of the corticotrophin stimulation test in clinical practice through the determination of salivary steroids.

The clinical utility of salivary steroids in the assessment of corticoadrenal reserve was proved in our patients study group. All primary adrenal insufficient patients showed blunted salivary cortisol and aldosterone responses after ACTH stimulation.By contrast, patients with secondary adrenal insuficiency demonstrated subnormal or blunted salivary cortisol concentrations while salivary aldosterone rose normally in all the cases in response to ACTH stimulation. Thus, salivary aldosterone was useful in the differential diagnosis of adrenocortical hypofunction and it could be a practical tool when selective aldosterone deficiency is suspected.

Our prefence for the assessment of salivary steroids in response to corticotrophin is based in: 1) the advantage of evaluating biologically active serum steroids through its reflection in saliva ,2) the use on a non invasive methodology that allows the patient himself to perform the sample collection , avoiding the continous assistance of a qualified nurse who may thus supervise several tests at the same time. From our experience we may say that patients collaborate with enthusiasm in salivary sampling collection, 3) the combination of intramuscular synthetic ACTH (250 μg) injection associated to salivary steroids determination offers a less agressive procedure than when ACTH is administered intravenously. The response of salivary steroids to a lower dose of corticotrophin injected intramuscularly needs further evaluation and is currently investigated by us.

We suggest that adrenal reserve can be accurately investigated through simultaneous salivary cortisol and aldosterone measurements in response to

ACTH. Our data support the clinical utility of this practical, stress-free and non invasive test when adrenal dysfunction is suspected.

S #	SAF (nmol / L) ACTH stimulation					SAL (pmol / L) After ACTH				
	0 min	30 min	60 min	90 min	120min	0 min	30 min	60 min	90 min	120min
1	19.0	38.0	58.0	70.0	60.0	50.0	300.0	500.0	320.0	240.0
2	12.0	29.0	44.0	55.0	50.0	45.0	260.0	566.0	375.0	300.0
3	11.0	29.0	50.0	45.0	20.0	29.0	110.0	110.0	60.0	55.0
4	10.0	17.0	40.0	36.0	25.0	40.0	170.0	200.0	190.0	70.0
5	7.2	30.0	64.0	82.0	100.0	60.0	500.0	320.0	100.0	25.0
6	4.0	20.0	40.0	25.0	15.0	22.0	70.0	120.0	28.0	21.0
7	10.0	20.0	40.0	70.0	75.0	21.0	50.0	70.0	220.0	95.0
8	10.0	25.0	35.0	44.0	40.0	20.0	42.0	100.0	60.0	30.0
9	10.0	44.0	70.0	100.0	60.0	65.0	160.0	80.0	30.0	10.0
10	6.0	31.0	50.0	65.0	60.0	20.0	90.0	100.0	44.0	17.0
11	11.0	32.0	56.0	58.0	34.0	40.0	182.0	178.0	270.0	150.0
12	3.5	28.0	40.0	55.0	80.0	37.0	87.0	90.0	218.0	80.0
13	17.0	44.0	55.0	95.0	40.0	29.0	75.0	102.0	218.0	117.0
14	14.0	80.0	120.0	120.0	110.0	15.0	182.0	145.0	144.0	73.0
15	16.5	59.0	89.0	95.0	85.0	33.0	241.0	322.5	232.0	160.0
16	14.5	37.0	50.0	75.0	45.0	50.0	169.0	334.0	290.0	208.0
17	10.0	31.0	53.0	52.0	27.0	50.0	146.0	165.0	144.0	64.0
18	5.0	25.0	45.0	30.0	20.0	20.0	80.0	125.0	30.0	21.0
19	5.6	25.0	52.0	53.0	58.0	41.0	285.0	220.0	64.0	23.0
20	6.0	55.0	80.0	90.0	74.0	13.0	80.0	120.0	60.0	55.0
21	5.5	20.0	40.0	42.0	36.0	25.0	115.0	80.0	45.0	40.0
*	9.9±4.5	34.3±15.3	55.8±20.2	64.6±25.2	53.0±27.0	34.5±15.0	162.0±109.0	193.0±140.0	150.0±109.0	88.0±81.0
**	3.5–19.0	17.0-80.0	35.0-120.0	25.0-120.0	15.0-110.0	13.0-65.0	42.0-500.0	70.0-566.0	28.0-375.0	10.0-300.0
p		0.0001	0.0001	0.0001	0.0001		0.0001	0.022	0.001	0.005

S #	SAF (nmol / L) ACTH stimulation					SAL (pmol / L) After ACTH				
	0 min	30 min	60 min	90 min	120min	0 min	30 min	60 min	90 min	120min
I_1										
1	1.5	2.0	2.0	2.5	3.7	13.0	13.5	13.0	13.0	13.0
2	5.0	7.0	7.6	16.0	5.0	13.5	13.0	13.0	13.9	13.5
3	1.7	1.4	1.0	1.0	2.0	13.8	13.8	13.5	13.6	13.7
I_2										
1	4.0	8.0	14.0	12.0	6.0	80.0	150.0	160.0	70.0	32.0
2	1.7	5.5	10.0	13.0	6.0	13.0	80.0	110.0	55.6	55.0
3	6.0	23.0	30.0	28.0	25.0	22.0	110.0	85.0	50.0	80.0
4	1.4	2.0	3.3	3.1	3.0	13.8	85.0	100.0	111.0	100.0
5	5.5	11.0	14.0	18.0	25.0	13.8	55.0	65.0	120.0	100.0
6	2.8	5.0	11.0	13.0	15.0	29.0	70.0	70.0	100.0	70.0
I_1										
*	2.7±2.0	3.5±3.0	3.5±3.5	6.5±8.3	3.6±1.5	13.4±0.4	13.4±0.4	13.2±0.3	13.5±0.5	13.4±0.4
**	1.5–5.0	1.4 -7.0	1.0-7.6	1.0-16.0	2.0-5.0	13.0-13.8	13.0-13.8	13.0-13.5	13.0-13.9	13.0-13.7
p		NS	NS	NS	NS		NS	NS	NS	NS
I_2										
*	3.6±1.9	9.0±7.5	13.7±8.9	14.5±8.2	13.3±9.9	28.6±26.0	91.6±34.0	98.0±35.0	84.0±30.0	73.0±27.0
**	1.4–6.0	2.0-23.0	3.3-30.0	3.1-28.0	3.0-25.0	13.0-80.0	55.0-150.0	65.0-160.0	50.0-120.0	32.0-100.0
p		NS	NS	NS	NS		0.005	0.003	0.007	0.016

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Legends

Table1: Individual concentrations of salivary cortisol and aldosterone in response to ACTH stimulus in 21 healthy subjects.

S: subjects

*Mean ± SD

**Range

p:< 0.05 was considered significant

Table 2: Individual salivary cortisol and aldosterone concentrations after ACTH stimulus in patients with primary (I_1) and secondary (I_2) adrenal insufficiency.

S: subjects.

*Mean ± SD

**Range

p:< 0.05 was considered significant

Figure 1: Salivary and serum cortisol (A) and aldosterone (B) concentrations after ACTH stimulation (250 μg of Synacthen , i.m.) in healthy subjects.

A: * $p \leq 0.03$; ** $p < 0.02$; compared with basal values.

B: * $p \leq 0.002$; ** $p < 0.012$; compared with basal values.

Figure 2: Salivary cortisol (A) and aldosterone (B) levels after ACTH stimulation in patients with primary (I_1) and secondary (I_2) adrenal insufficiency and in 21 normal subjects (C).

A: * $p < 0.001$; compared with basal values. In I_1 and I_2 no significant differences were found between all means.

B: * $p \leq 0.022$; ** $p \leq 0.016$; compared with basal values. No significant differences were found in I_1.

Fig 1A

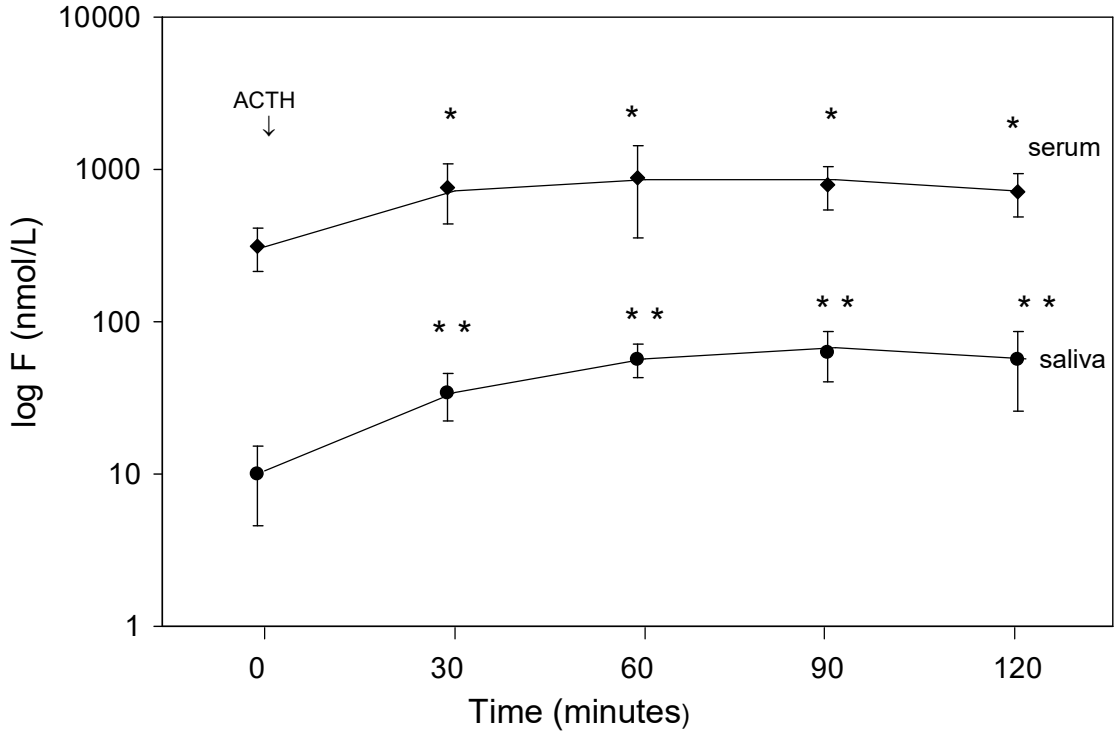

Fig 1 B

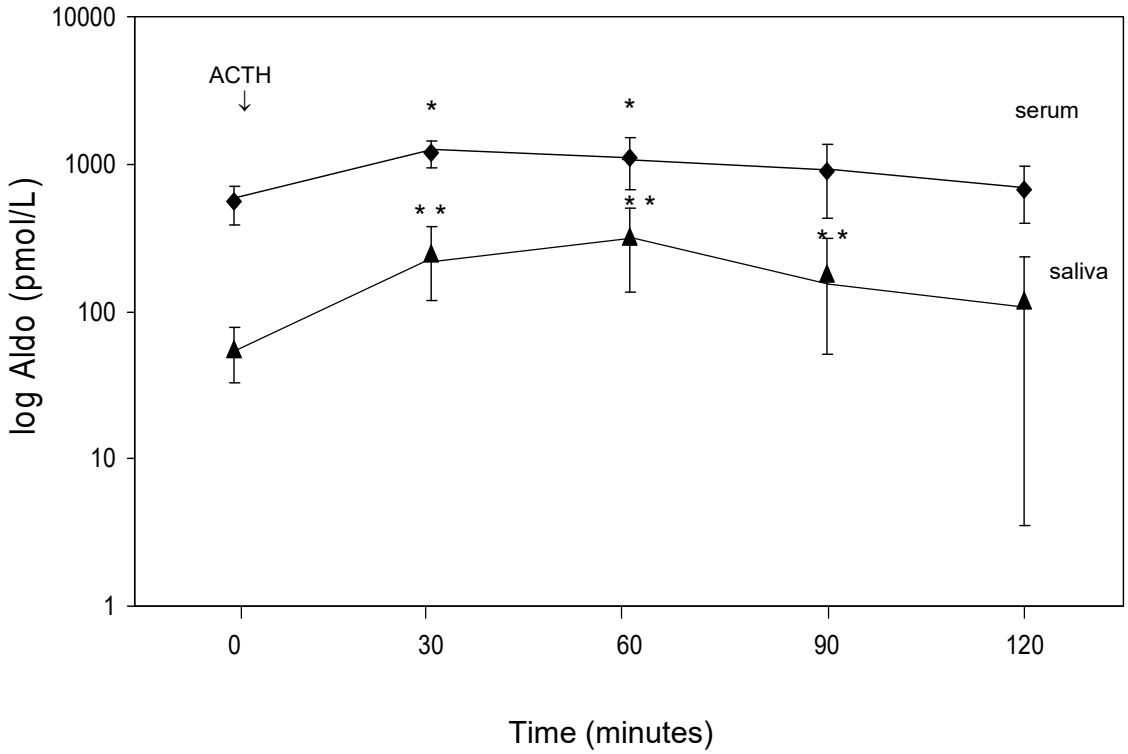
10000
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Fig2A

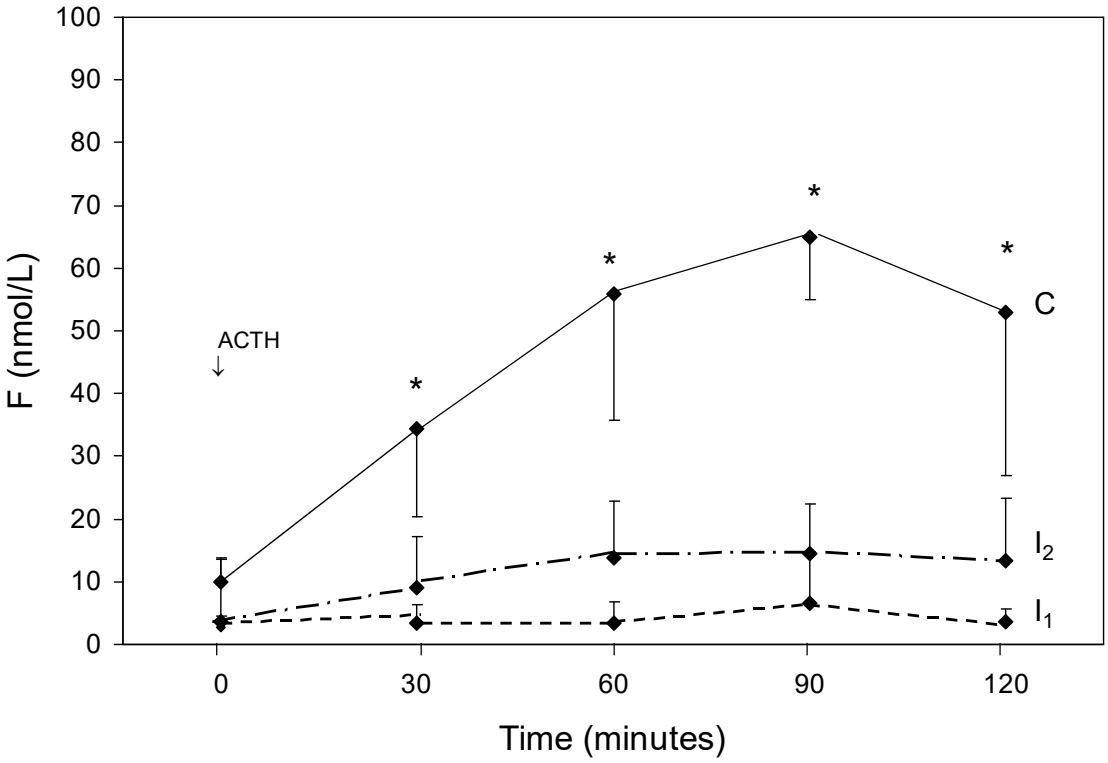
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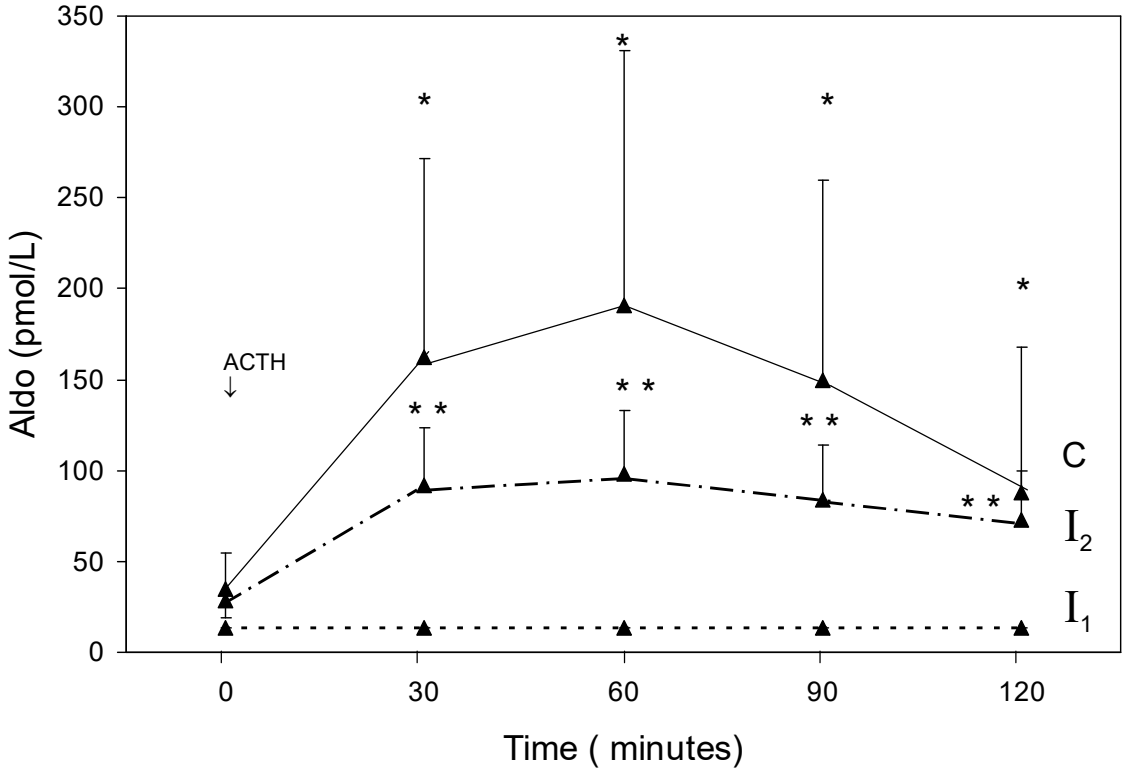
Fig 2B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