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A STUDY ON THE EFFECT OF ALDOSTERONE ON THE EXTRAMITOCHONDRIAL ADENINE NUCLEOTIDE SYSTEM IN RAT KIDNEY*

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SUMMARY

The *in vivo* tissue contents of ATP, ADP, AMP, P_i, phosphoryl creatine (PC), creatine (C), lactate (L), pyruvate (Py), dihydroxyacetone phosphate (DAP), and 3-phosphoglycerate (3-PG) were measured in the *in situ* freeze-clamped kidneys of rats (a) in the normal state, (b) in the adrenalectomized state and (c) in the adrenalectomized state, 2–3 h after injection of a physiological dose of aldosterone (2 μg per 100 g b w).

No significant differences in the ATP, ADP, AMP, or P_i contents, or in the ratio ATP/ADP $\times$ P_i could be observed between the 3 states.

Assuming[11] that large changes of the concentration ratio ATP/ADP $\times$ P_i in the cytoplasm would be accompanied and rendered demonstrable by changes in the ratio of the contents $Py \times$ DAP/L $\times$ 3-PG, this latter ratio was monitored. The ratio Py/L increased under adrenalectomy and decreased to a normal level on injection of aldosterone; no significant effect of the hormone on the ratio $Py \times$ DAP/L $\times$ 3-PG could be shown, however.

The ratio PC/C decreased to half the normal value under adrenalectomy. This change was reversed by injection of aldosterone. Since in rat kidney creatine kinase is located in the extramitochondrial space only, the latter data may indicate an increase in response to aldosterone of the phosphorylation of extramitochondrial adenine nucleotide in this tissue.

PREVIOUS experiments have shown that the response of the urinary Na/K ratio to aldosterone in adrenalectomized rats is accompanied by a 3–4-fold increase in reduction of the intramitochondrial free nicotinamide adenine dinucleotide system in the kidney; that is, a shift from NAD^+ to NADH[1], as indicated by the ratios of the tissue contents of β-hydroxybutyrate and acetoacetate as well as glutamate, α-oxoglutarate and ammonia. These changes were followed by an approximately 2-fold increase in the free cytoplasmic $NADH/NAD^+$ ratio as indicated by the ratio of the tissue contents of lactate and pyruvate. The effect of aldosterone on the redox state of the kidney was to restore the $NADH/NAD^+$ ratio to that found in normal rats and was elicited by a physiological dose of the hormone: 2 microgram aldosterone per 100 g rat. Furthermore, the redox shift of the mitochondrial free NAD appeared at an early time, practically indistinguishable from the time of onset of the effect on the urinary Na/K ratio. The aldosterone dependent increase in the $NADH/NAD^+$ ratio was eliminated by antimineralocorticoid doses of spirolactone. These findings are compatible with the concept of aldosterone action advanced by Edelman and coworkers, namely that aldosterone enhances mitochondrial NADH production, thereby increasing the mitochondrial energy output and consequently the supply of high energy compounds to the sodium transport system[2, 3].

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This concept implies, that in response to the hormone, the level of ATP or the ATP/ADP ratio should increase at the site of the effector sites in the cell either at the apical or at the basal-lateral borders or both. Studies on the response of high energy phosphate levels to aldosterone, however, have not given unequivocal results as yet.

There are some difficulties in approaching this question:

(i) A considerable percentage of the nucleoside tri- and di-phosphates present *in vivo* might be lost during the extraction procedure. This source of error should be obviated by applying the freeze-quenching technique.

(ii) The initial effect of the hormone might become obscured by secondary metabolic effects later in the time-course after the onset of the mineralocorticoid effect. For instance, observations by Jørgensen[4] as well as our experiments suggest, that activation of the (Na–K)–activated ATPase occurs as a secondary effect after prolonged administration of aldosterone in rat kidney and in toad bladder mucosa. We tried to eliminate this problem by using low doses of the hormone and studying the effect as early in the time course as practicable.

(iii) A third difficulty is the limited interpretability of "over-all" tissue levels, due to (a) intracellular compartmentalization and (b) the heterogeneity of the tissue.

Recent evidence indicates that in rat liver[5] the redox state of cytoplasmic NAD controls the extramitochondrial phosphorylation state of the adenine nucleotide system via the glyceraldehyde phosphate dehydrogenase (GAPDH) and the phosphoglycerate kinase (PGK) reactions. As these enzymes are present at high activity, this finding provides a method of assaying cells for cytoplasmic ATP/ADP $\times P_i$ ratios, thereby circumventing to some extent the problem of compartmentalization. In view of the high activity of GAPDH, PGK and triose isomerase (TIM) in rat kidney we used the substrate ratio technique–assuming that these reactions are near equilibrium *in vivo*–to obtain information on the phosphorylation state of the extramitochondrial adenine nucleotide system in response to aldosterone. In the original method described by Veech *et al.*[5], glyceraldehyde phosphate was measured. In the present study, we determined the renal content of dihydroxyacetone phosphate (DAP) instead of glyceraldehyde phosphate (GAP), as the content of the latter is very low in kidney ($< 0{\cdot}5$ nanomoles per gram wet weight), and incorporated K_{TIM}, the equilibrium constant of the triose isomerase reaction[6], into the equation:

* $$\frac{[ATP]}{[ADP]\times[HPO_4^{--}]_{em}} = \frac{[Pyruvate]\times[Glyceraldehyde\text{-}3\text{-}phosphate]\times K'}{[Lactate]\times[3\text{-}Phosphoglycerate]}$$

$$K' = \frac{K_{GAPDH}\times K_{PGK}}{K_{LDH}} \qquad (a)$$

$$\frac{[ATP]}{[ADP]\times[HPO_4^{--}]_{em}} = \frac{[Pyruvate]\times[Dihydroxyacetone\ phosphate]\times K''}{[Lactate]\times[3\text{-}Phosphoglycerate]}$$

$$K'' = \frac{K_{GAPDH}\times K_{PGK}\times K_{TIM}}{K_{LDH}}. \qquad (b)$$

*Abbreviations: GADPH = glyceraldehyde phosphate dehydrogenase, PGK = phosphoglycerate kinase, TIM = triose isomerase, LDH = lactate dehydrogenase. The index em means "extramitochondrial".

Another set of metabolites suitable for indicating the phosphorylation state of the extramitochondrial adenine nucleotide system in kidney are the reactants of the creatine kinase reaction, phosphoryl creatine and creatine.

In kidney, creatine kinase is extramitochondrial in location [7]. The enzyme activity content is relatively high; thus a near equilibrium state of the reactants may be expected.

The kidneys of anesthetized rats – pretreated as indicated in the tables – were freeze-clamped in situ with metal tongs precooled in liquid nitrogen. The tissue platelets of 0·5–1 mm thickness were powdered, weighed, covered with 0·4 N perchloric acid in 30% ethanol, and sonicated. The sonicates were centrifuged and the pellets were reextracted with perchloric acid-ethanol. The pooled supernatants were neutralized with 2 M K_2CO_3, 0·5 M triethanolamine chloride. The neutral extract was immediately used for the determination of the metabolites by enzymatic optical methods [8–12].

In Table 1, the metabolite contents of kidneys of normal, adrenalectomized, and adrenalectomized, aldosterone-substituted rats are compared. The latter kidneys were freeze-quenched 2–4 hr after injection of 2 μg of aldosterone in 0·5 ml of 0·9% saline per 100 g rat, or of the diluent alone. There was a decrease in lactate and increase in pyruvate content after adrenalectomy and a reversal of these effects in response to the hormone. The DAP content fell and the 3-PGA content rose after adrenalectomy; these changes were also reversed following administration of aldosterone (Table 1).

In addition, the ATP, ADP, AMP and P_i contents of the freeze-quenched kidneys were determined by conventional enzymatic and analytic techniques [9, 12]. These results are shown in Table 2. In Table 3, the metabolite ratio method is compared to the direct analytical method for determining the ATP/ADP $\times$ P_i ratio. Neither by direct analysis of the "over-all" ratio ATP/ADP $\times$ P_i nor with the metabolite ratio method (c.f. Eq. b) was there a significant change after adrenalectomy or administration of aldosterone.

In contrast, the ratio phosphoryl creatine/creatine showed a decrease to

Table 1. Contents of Lactate, Pyruvate, Dihydroxyacetone phosphate, and 3-Phosphoglycerate in kidneys of normal, adrenalectomized, and aldosterone treated adrenalectomized rats. Units: Nanomoles per g fresh weight. The kidneys were freeze clamped in situ under anesthesia (8 mg Inactin per 100 g body weight). The dose of aldosterone (line 3) was 2 μg per 100 g body weight, injected 2–4 h prior to the freeze clamping

	Lactate	Pyruvate	Dihydroxy acetone phosphate	3-Phophoglycerate
Normal	264 ± 19	30 ± 1·6	20·6 ± 2·5	134 ± 9·3
	(11)	(11)	(10)	(10)
		P < 0·001		P < 0·001
Adren X	234 ± 25	46 ± 2·6	14·8 ± 1·7	188 ± 12
	(12)	(12)	(11)	(12)
	P < 0·05			
Adren X	338 ± 29	32 ± 2·7	20·1 ± 2·6	175 ± 18
+ Aldo	(11)	(11)	(11)	(10)

The P values refer to the difference between their neighbor values in the vertical columns.

Table 2. Contents of ATP, ADP, AMP and orthophosphate in kidneys of normal, adrenalectomized, and aldosterone treated adrenalectomized rats. Nanomoles per g wet wt.

	ATP	ADP	AMP	ATP + ADP + AMP	Orthophosphate
Normal	1990 ± 50 (11)	439 ± 35 (11)	221 ± 35 (11)	2704 ± 142 (11)	2329 ± 100 (9)
Adren X	1900 ± 39 (12)	389 ± 50 (11)	164 ± 24 (11)	2458 ± 91 (11)	2443 ± 347 (8)
Adren X + Aldo	1868 ± 39 (11)	378 ± 43 (10)	160 ± 24 (10)	2391 ± 53 (10)	2163 ± 231 (10)

Table 3. Ratios between the contents of Lactate (L) and pyruvate (Py), dihydroxy acetone phosphate (DAP) and 3-phosphoglycerate (3-PG), and ATP, and inorganic phosphate (P_i) in kidneys of normal, adrenalectomized, and adrenalectomized, aldosterone substituted rats. The ratio in column 3 is calculated from columns 1 and 3 and, according to Eq. (b) represents the ratio $ATP/ADP \times P_i$ in the extramitochondrial space. Column 4 is calculated from the "over-all" tissue contents as shown in Table 2. There is a discrepancy of 1 order of magnitude between the "extramitochondrial" ratio and the "over-all content" ratio (column 4)

	$\frac{L}{Py}$	$\frac{DAP}{3\text{-}PG}$	$\frac{Py \times DAP \times K^*}{L \times 3\text{-}PG}$	$\frac{ATP \times 10^3}{ADP \times P_i}$
Normal	8·8 ± 0·5 (11)	0·17 ± 0·04 (10)	2·14 ± 0·42 (9)	2·43 ± 0·43 (11)
	P 0·001			
Adren X	6·0 ± 0·7 (9)	0·09 ± 0·01 (8)	2·04 ± 0·40 (8)	2·38 ± 0·43 (10)
	P 0·001			
Adren X + Aldo	11·1 ± 1·1 (11)	0·12 ± 0·02 (9)	1·34 ± 0·36 (9)	2·85 ± 0·63 (9)

$$^*K = \frac{K_{GAPDH} \times K_{PGK} \times K_{TIM} \times 10^{22}}{K_{LDH}} = 1{\cdot}16 \times 10^2$$

Table 4. Creatine (C) and phosphoryl creatine (PC) in kidney of normal, adrenalectomized and aldosterone treated adrenalectomized rats. The kidneys were freeze clamped in situ under an anestesia by injection of 8 mg inactin (promonta GmbH, Hamburg) per 100 mg body weight. Contents given in nmoles per g wet wt ± SEM. P values refer to the difference between their neighbor values in the vertical

	Creatine	Phosphoryl creatine	PC/C
Normal	598 ± 112 (8)	401 ± 32 (11)	0·65 ± 0·08 (8)
			P < 0·005
Adren X	1094 ± 369 (8)	263 ± 49 (7)	0·31 ± 0·06 (7)
			P < 0·025
Adren X + Aldo	513 ± 65 (9)	317 ± 44 (9)	0·65 ± 0·12 (8)

about half of the normal value in the adrenalectomized state, and a significant increase after injection of aldosterone (Table 4).

The discrepancy between the methods used to characterize the extramitochondrial state of phosphorylation of the adenine nucleotides (i.e. metabolite ratio method vs. PC/C ratio) could be due to one of the difficulties mentioned above. For instance, the problem of cellular heterogeneity has not been avoided in this study. It is possible that the contribution of the aldosterone-non-responsive cells to the over-all pool of the glycolytic metabolites used in Eqs. (a) and (b) is greater than their contribution to the over-all pool of phosphoryl creatine and creatine.

In view of the absence of a mitochondrial creatine kinase in rat kidney, the increase of the ratio PC/C in response to aldosterone suggests an increase in phosphorylation of the extramitochondrial adenine nucleotide system in response to the hormone in this tissue. The inconsistency in the results obtained by different methods, however, and the need for support from comparable experiments with other target organs of aldosterone, means that no final conclusions can be drawn, as yet.

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DISCUSSION

Leaf: On which tissue were these last studies, in which you calculated the creatine phosphate and creatine and ATP/ADP ratios, done?

Kirsten: Rat kidney.

Crabbé: May I ask you how you have prepared your animals? If I followed you correctly, the adrenalectomized rats were given salt-rich water to drink. Did you treat your control rats the same way? Another point, regarding the changes you have reported for phosphocreatine/creatine ratios: did you find analogous changes in tissues other than the kidney? My question amounts to knowing whether your observations apply only to organs that are influenced by aldosterone (considered as mineralocorticoid).

Kirsten: My animals were treated with 0·9% saline.

Crabbé: Did the normal animals get the same?

Kirsten: No, just water. We did the phosphocreatine/creatine ratio studies only on the kidney and I think it should be done now also on toad bladder and other target organs.

Crabbé: I would be even more interested in data from organs that do *not* respond to aldosterone in terms of sodium transport.

Edelman: Although there are sound criteria for classifying a tissue as a target for aldosterone, it is difficult to prove that a particular tissue is not a target. Swaneck *et al.* (*Nephron* **6** (1969) 297) found stereospecific cytosol and nuclear binding proteins in liver, spleen and brain, as well as in the kidney and intestinal mucosa. Aldosterone may have effects on these organs that have not yet been properly measured.

De Weer: Is it permissible to talk here about a tissue which is not responsive to aldosterone?

Edelman: Yes.

De Weer: For the last couple of years I have been working on a tissue which is insensitive to aldosterone, at least judging from the absence of effect on sodium transport, namely the giant axon of the squid. I would like to show some interesting data which, I think, might hint at a possible mechanism for the mode of action of aldosterone.

First of all, from data obtained by Brinley and Mullins on internally dialyzed squid axons (*J. Gen. Physiol.* **52** (1968) 192, Fig. 7) it appears that the dose-response relationship between ATP levels and active sodium transport is rather flat. One must reduce the ATP levels 100-fold to obtain a mere 50% reduction in sodium pumping! From the point of view of the cell this would be a very inefficient system indeed for regulating sodium transport, because I doubt that any cell could suffer a 100-fold reduction of ATP levels without seriously endangering other biochemical pathways. A second point of interest is that the *behaviour* of the sodium pump can be controlled by intracellular ADP levels or ATP/ADP ratios, as follows. In normal conditions sodium extrusion from the giant axon is, as in many other cells, stimulated by external potassium. However, if one raises the intracellular ADP level by micro-injection, then sodium efflux is stimulated by external *sodium*. It appears then that the sodium pump, when exposed to a high ATP/ADP ratio, will exchange internal sodium for external potassium whereas it will simply shuttle sodium when exposed to *low* ATP/ADP ratios. (*J. Gen. Physiol.* **56** (1970) 583). Hence regulation of the ATP/ADP ratio might be a good mechanism for controlling net sodium transport.

I would like to propose the following hypothetical mode of action of aldosterone (Fig. 1). The effect of aldosterone is not so much to raise ATP levels *per se*, but rather to lower the ADP concentration by, say, a factor of two or more, and hence drastically alter the ATP/ADP ratio, which in turn would shift the pump operation from a sodium/sodium exchanging mode to a sodium/potassium exchanging mode. In a resting cell you would have reasonably low ATP/ADP levels because the citric acid cycle enzymes are not stimulated. Now if the sodium pump of target organs behaves like the one we find in giant axons we would have mostly sodium/sodium exchange and little net transport. Next you expose the cell to aldosterone, reduce the ADP level, raise the ATP/ADP ratio and consequently shift from sodium/sodium exchange to sodium/potassium exchange. This might be a very sensitive system for controlling transepithelial transport.

Edelman: Dr. Kirsten, did you want to comment on this?

Kirsten: No.

Edelman: Dr. De Weer's findings are most interesting. In most cells the ATP/ADP ratio is about 4:1 or 5:1. Thus an increase in ATP synthesis sufficient to

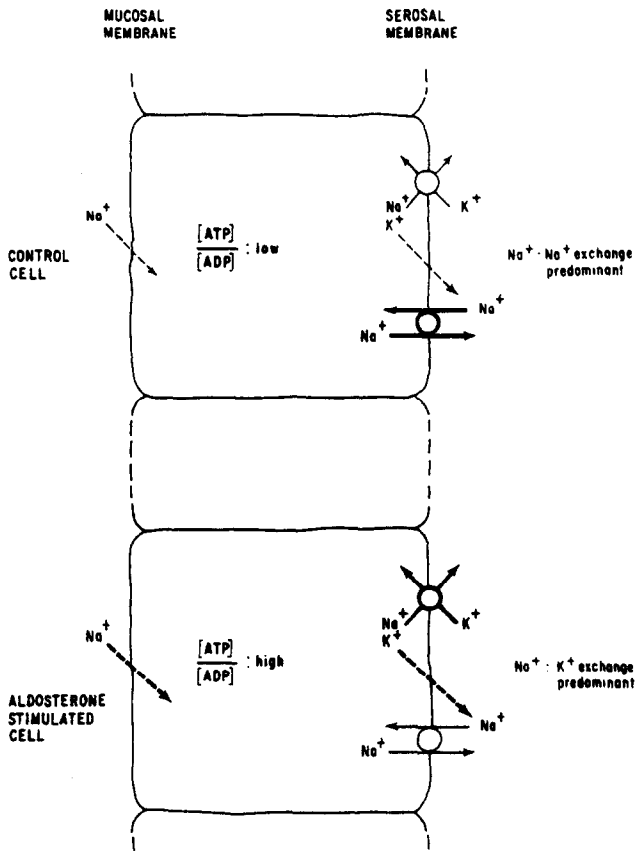

Fig. 1.

produce a 10% increase in ATP concentration would result in a 50% decrease in ADP concentration. From the standpoint of control mechanism ADP might be of crucial importance.

Snart: Does the $NAD^+/NADH$ ratio in mitochondria go up to the same extent as outside the mitochondria?

Kirsten: Yes, we measured total extractable NAD, NADH, NADP and NADPH of isolated mitochondria, and also indirectly by redox metabolite pairs the redox state of free NAD in both cytoplasm and mitochondria. In response to aldosterone the degree of reduction of free NAD in the mitochondria increased earlier and the change was greater than in the cytoplasm. Also the total extractable mitochondrial dinucleotides showed an increase in reduction under aldosterone.