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EFFECT OF PYRIDOXINE ON THE ALKALINE RIBONUCLEASE ACTIVITY IN THE LIVER OF DEXAMETHASONE TREATED RATS

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Summary: Alkaline ribonuclease activity (pH 7.8) is present in most mammalian cells in both the free form and bound to an inhibitor protein.
It has been recently suggested that pyridoxal–5–phosphate interacts with ε–amino groups of lysine in human pancreatic ribonuclease. There is evidence that the nuclear binding of glucocorticoid–receptor complex can be inhibited by pyridoxal phosphate.
The data presented here suggest that treatment of experimental animals with dexamethasone during a 7–day period causes a decrease of alkaline RNase activities (total, free and latent RNases). In " in vitro " experimental condition dexamethasone also decreases total and latent RNase activity, but increase the free enzyme activity.
Pyridoxine/or pyridoxal phosphate in experiments in vivo or in vitro increases the free alkaline ribonuclease activity; pyridoxine/or pyridoxal phosphate decreases inhibitor–bound activity.
In the " in vivo " experimental condition vitamin B_6 don't modulates the effect of dexamethasone but in "in vitro " experiments the effects of dexamethasone are modulated by B_6P .

Key words: Alkaline ribonuclease, dexamethasone, pyridoxine, pyridoxal phosphate, rat liver

Introduction

Alkaline ribonuclease (RNase) activity (pH 7,8) is present in most mammalian cells in both the free form and bound to the naturally occurring inhibitor protein (1–3). The equilibrium between the RNase and its inhibitor may have a role in the regulation of turnover of cytoplasmatic protein synthesis (4,5).

Pyridoxal 5'–phosphate, the active form of vitamin B_6 , is the coenzyme of a great number of enzymes acting on different specific substrates and catalysing a wide range of reactions (6,7).

It has been recently suggested that pyridoxal phosphate, in a lesser degree pyridoxal, interacts with ε–NH_2 groups of lysine in human pancreatic ribonuclease (8).

The mechanism of action of glucocorticoids has been intensively investigated. The initial interaction between steroid hormone and receptors result in the formation of a complex which undergoes conformational changes to an activated form. The transition from inactivated to activated receptor complex results in the exposure of lysine and arginine (9). There is evidence that the nuclear binding of glucocorticoid receptors can be inhibited by pyridoxal 5'phosphate; pyridoxal phosphate (PLP) acts by forming a Schiff base on the ε–NH_2 group of lysine which may be the residue appearing on the surface of the steroid – receptor complex upon activation (10).

The literature data suggest that the interaction between RNase A from human placenta and the RNase inhibitor involves the predilectively charged ε–NH_2 group of lysine–41 of ribonuclease. This interaction could result in the inactivation of the enzyme (11).

The present study was undertaken to examine the alkaline ribonuclease activity in the rat liver under the influence of pyridoxine or pyridoxal phosphate. The effect of vitamin B_6 or B_6P on the ribonuclease activity was examined alone or in the presence of dexamethasone, a synthetic glucocorticoid.

Materials and Methods

Three–month old white male rats, weighing 150–180 g. were used in the experiments. The

animals were divided into four groups, and each group included eight rats. The first group, control group–the animals received 1ml 0.9% NaCl intra–peritoneally for seven days. The second group included animals treated with dexamethasone in a single daily dose of 2mg/100g body weight intraperitoneally. The hormone was given for 7 days; the last dose was given on the 7th day 45 min before sacrificing. The third group of animals was treated with dexamethasone in the manner already described and simultaneously injected with pyridoxine hydrochloride for 7 days intraperioto–neally in a daily dose of 12.5mg/100g of body weight. The last dose of hormone and vitamin was given on 7th day 45 min before sacrificing. The animals of group IV were treated with pyridoxine hydrochloride in the manner already described for the third group. Sacrificing of animals was done by decapitation, and for examination the liver tissue homogenate (10%) was used. From the liver a fraction of heavy mitochondria was isolated by the method of Liu et al. (12). The obtained mitochondria and liver homogenate (10%) were used for the examination of enzyme activity. Activity of alkaline ribonuclease (total, free and latent–inhibitor bound) was measured spectro–photometricaly by the method of Shortman (13) with the modification that 30 mM Tris–HCl buffer pH 7.8 was used instead of veronal buffer. Proteins were measured according to the method of Lowry et al. (14).

The effect of pyridoxal phosphate and dexamethasone on the activity of alkaline ribonuclease of rat liver mitochondria was examined under "in vitro"condition. We have worked with mitochondria because they are rich with this enzyme, and effects of glucocorticoids on this organelles have not elucidated up to now.

In the first experimental group the mitochondria were preincubated at 25^{0}C for 15 min. with dexamethasone in a dose of 1x10^{-4}M/incubation medium. In the second experimental group mitochondria were preincubated at 25^{0}C for 15min. with pyridoxal phosphate ("Serva") in a dose of 0.006 mmol/incubation medium, and simultaneously with dexamethasone in a dose of 1x10^{-4}M/incubation medium. Determination of alkaline ribonuclease activity was started after 15 min of the preincubation time. In the third experimental group the rat liver mitochondria were preincubated 15 min. at 25^{0}C with pyridoxal phosphate in a dose of 0.006mmol/incubation medium.

In the attempt to explain the results obtained with mitochondria we performed the same experi–ments but instead of the isolated mitochondria we used the standard preparation of crystalline bovine pancreatic ribonuclease, purchased from "Calbiochem" in the amount of 0.0025μg/0.1ml. In "in vitro" experiment the standard solution of ribonuclease was treated with dexamethasone and pyridoxal phosphate in the same manner as in the experiments with mitochondria apart from the fact that we added a 4th experimental group. In the fourth experimental group the enzyme was preincubated with twofold higher amount of pyridoxal phosphate i.e. with 0.012mmol/incubation medium. All the analyses were performed in triplicate; the *in vitro* experiments were performed five times.

Table 1. RNase activity in whole – tissue homogenate of rat liver (U/mg prot.)

	Total RNase (+PCMB)	Free RNase (–PCMB)	Latent–inhibitor bound RNase
Control group	1.914 ± 0.031	0.825 ± 0.017	1.079 ± 0.025
Dexamethasone	1.337 ± 0.045***	0.729 ± 0.020**	0.607 ± 0.026***
Dexamethasone + pyridoxin	1.317 ± 0.045**	0.744 ± 0.031**	0.572 ± 0.017***
Pyridoxin (vit. B_6)	1.961 ± 0.051	1.095 ± 0.018***	0.866 ± 0.030***

***:P < 0.001, **:P < 0.05 } in comparrison with control group

Enzyme activity was expressed as mean values SEM. Statistical significance was estimated by the Student's t–test.

Results

The activity of alkaline ribonuclease in "in vivo" and in "in vitro" experiments is presented in Table l and 2.

Administration of dexamethasone to experimen–tal animals decreased the alkaline ribonuclease activity (total, free and latent), Table l.

Application of pyridoxine–HCl to animals increased the free alkaline RNase activity. At the same time "latent", inhibitor–bound enzyme activity decreased which influences an unchanged total

activity of alkaline ribonuclease.

The results obtained in "in vitro" experiments showed that dexamethasone decreased the alkaline ribonuclease activity of rat liver mitochondria medium of controls contained all components for enzyme assay, but exept of hormone or B_6P there are redestilated water.

Table 2. Effect of pyridoxal phosphate and dexamethasone on RNase activity of rat liver mitochondria in "in vitro" experimental conditions (U/mg prot.)

	Total RNase (+PCMB)	Free RNase (–PCMB)	Latent–inhibitor bound RNase
Control group	3.059 ± 0.059	1.570 ± 0.046	1.082 ± 0.024
Dexamethasone	2.183 ± 0.051***	1.768 ± 0.023***	0.414 ± 0.030***
Dexamethasone +pyridoxal phosphate (B_6P)	3.208 ± 0.048***	2.398 ± 0.041***	0.810 ± 0.012***
Pyridoxal phosphate (vit. B_6P)	3.823 ± 0.046***	2.154 ± 0.046***	1.668 ± 0.021***

***: $P < 0.001$ in comparrison with control group

(Table 2); pyridoxal phosphate increases the enzyme activity of the liver mitochondria.

The effect of pyridoxal phosphate (B_6P) on free alkaline ribonuclease is potent in the presence of the hormone added; pyridoxal phosphate modifies the effect of dexamethasone on the alkaline ribonuclease activity by increasing this free activity, as well as inhibitor – bound activity.

Discussion

Vitamin B_6, pyridoxine, belongs to the group of water soluble vitamins. Pyridoxal phosphate, the active form of vitamin B_6, has long been recognised as an important cofactor in transamination, decarboxylation and many other metabolic reactions (15,16). The liver is the most

Table 3. In vitro effect of dexamethasone and pyridoxal phosphate on bovine pancreatic Rnase (units/0.1 ml)

	Alkaline RNase activity Mean± SE	The increase of enzyme activity %
Control	1.0 ± 0.020	–
Dexamethasone	1.202 ± 0.059 *	20.0
Dexamethasone + B_6P	1.280 ± 0.024 **	28.0
B_6P– 0.006 mmol/inc.med. (a)	1.379 ± 0.039 ***	37.9
B_6P– 0.012 mmol/inc.med. (b)	1.385 ± 0.034 ***	38.5

*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ } in comparrison with control group

In the experimental conditions performed with standard preparation of crystalline bovine pancreatic ribonuclease dexamethasone and pyridoxal phosphate alone increased the alkaline ribonuclease activity; pyridoxal phosphate does not modulate the hormones effect. Alkaline ribonuclease activity was significantly higher than the control values at concentration of B_6P of 0.006 mmol/incubation medium; with the augmentation of coenzyme amount the enzyme activity have not been significantly higher (Table 3).

One units of RNase alkaline activity was defined as that which gave the same increase in the absorbancy of OD. at 260 nm as did 0.0025µg crystalline pancreatic RNase acting at pH 7.8(13). Under these experimental conditions incubation

active among organs in the synthesis of pyridoxal phosphate (17). Pyridoxal phosphate may form Schiff's bases with amino groups of protein (18). Recent literature data confirm that pyridoxal phosphate and their derivatives interact with both non–protonated and protonated exposed ε–amino groups of lysine residues and with α–NH_2 groups in human serum albumin and pancreatic ribonuclease (8). Ribonuclease A reacts with pyridoxal phosphate to yield three derivatives, modified at lysine residue 1, 7 and 41 respectively (19).

Metabolic labelling experiments using radioac–tive ortophosphate revealed that the 92 kDa glucocorticoid binding protein is phosphorilated in vivo. It appears that the receptor contains mostly

phosphoserine (20,21). Addition of hormone increases the phosphorilation. The significance of phosphorilation remains unclear (22).

It has been recently shown that the nuclear binding of glucocorticoid–receptor complexes can be inhibited by B_6P (9). The literature data demonstrate that pyridoxal phosphate may alter metabolic effects of glucocorticoids (10,23–26). The increasing of intracellular concentration of pyridoxal phosphate should decrease the nuclear binding of the glucocorticoid receptors (27).

A significant amount of B_6P coenzymes in the liver is located within mitochondria. In B_6P sufficient rats 20% of B_6 content is present in this organelles (28). Experiments with tRNA show that pyridoxal phosphate binds specifically to guanine at position 20 of the dihydrouridine loop and that ligation may involve a conformational change of the tRNA molecule, resulting in the inhibition of amino acid acceptance and ribosomal binding. The results suggest that translation may be modulated, in part by interaction of B_6P with tRNA (26).

The data presented here suggest that treatment of experimental animals with dexamethasone during the seven days decreases alkaline ribonuclease activity (total, free and latent RNase). Our results are in agreement with the literature data (29,30). The mechanism through which such effects take place has not been fully elucidated. Whether glucocorticoids exert their action on RNase *in vivo*, directly on the enzyme or by affecting its *de novo* synthesis is not clear.

Pyridoxine / or pyridoxal phosphate in experiments in vivo increased the free alkaline ribonuclease activity. In the same experimental condition total alkaline RNase activity has not been modified; as a consequence the inhibitor bound activity was decreased.

In "in vitro" experimental condition dexamethasone also decreases the total and latent RNase activity, but increases the free enzyme activity The results of " in vitro" experiments with mitochondria suggest that the effects of dexamethasone are modulated by pyridoxal phosphate. These results may be discussed with the literature data : pyridoxal phosphate reduces the activity of tyrosine transaminase induced by dexamethasone (27). Also, the hormonal induction of glutamine synthetase in L–cells is impaired by concentration of exogenous pyridoxine which increases the intracellular content of pyridoxal phosphate (22). Pyridoxal 5'– phosphate rapidly abolished the abilities of isoleucyl–, leucyl–, and valyl–tRNA synthetases to esterify amino acids to tRNA (31).

Our recent result which refers to the effects of dexamethasone and pyridoxine on the activity of alkaline ribonuclease in rat brain tissue suggest to the increases of total and inhibitor bound activity during the influence of pyridoxine and to the possible interaction of this vitamin with the action of hormone (25).

The present experiments "in vitro" show that pyridoxal phosphate increase the free, inhibitor–bound and total alkaline ribonuclease activity of rat liver mitochondria. The results *in* vitr*o* refer to standard preparation of bovine pancreatic ribonuclease are in agreement with the data of Seweda et al. (32) that dexamethasone causes augmentation of bovine pancreatic ribonuclease activity.

Regulation of alkaline ribonuclease activity is not well understand for now. The examination of the ribonuclease structure and catalysis suggests that the structure of the active site residues involved in the catalysis my be chemically modified; two histidine in position 12 and 119 , and one lysine in position 41 , were found to be essential to enzymatic activity. All "native" structures of RNase A have a sulphate or phosphate anion bound to the active site, lying between histidine–12 and histidine–119. The active site conformation was essentially changed with replacement of the inhibitory sulphate anion by a water molecule (33).

The increase of the activity of alkaline ribonuclease noted in our results under the influence of pyridoxal phosphate may be due to the formation of Schiff's bases between this active form of vitamin and the lysine and histidine residues present in the structure of the enzyme, as well as by the substitution of the inhibitory sulphate anion by the phosphate anion which could be a results of the increase of enzyme activity. Namely, it is well–known that the covalent modification of enzymes by phosphorylation is a central mechanism in the regulation of enzyme activity under the influence of numerous hormones (34).

Our results undoubtedly point at the significance of pyridoxine and its active form–pyridoxal phosphate on the free, functional active form of alkaline ribonuclease. The latent enzyme activity depends on an inhibitory protein (1,12) The naturally occurring inhibitor for alkaline ribonuclease from a number of mammalian tissues has been purified to homogeneity (35,36). Interaction between RNase A and RNase–inhibitor depend of amino acid lysine–4l present in ribonuclease. Polyamines and carbohydrate chains, attached to enzyme, may participate in controlling of alkaline ribonuclease activity (37,38).

The role of vitamin B_6 or pyridoxal phosphate

for alkaline ribonuclease activity in mammalian tissue both dependent or independent of the glucocorticoid hormones presence ask more scientific investigation in future time.

The evidence we have obtained points at the influence of pyridoxal phosphate on The metabolic effects of glucocorticoids but, as we have already suggested, the role of vitamin B_6 with relation to the effects of glucocorticoids is quite essential, although not transparent yet.

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UTICAJ PIRIDOKSINA NA AKTIVNOST ALKALNE RIBONUKLEAZE JETRE PACOVA TRETIRANIH DEKSAMETAZONOM

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Sažetak: Aktivnost alkalne ribonukleaze (pH 7,8) prisutna je u brojnim ćelijama sisara u slobodnom obliku ili u spoju sa specifičnim inhibitornim proteinom.
Nedavno je dokazano da piridoksal–5–fosfat, aktivna forma piridoksina, interreaguje sa ε–amino grupama lizina u humanoj pankreasnoj ribonukleazi. Istovremeno postoje dokazi da nuklearno vezivanje glukokortikoid–receptor kompleksa može da se inhibiše piridoksal fosfatom.
Rezultati prikazani u našem radu ukazuju da tretiranje eksperimentalnih životinja deksametazonom u trajanju od 7 dana izaziva smanjenje aktivnosti alkalne ribonukleaze (ukupne,slobodne i latentne) u jetri pacova. U "in vitro" eksperimentalnim uslovima deksametazon takođe smanjuje aktivnost totalne i latentne alkalne ribonukleaze, ali povećava slobodnu aktivnost enzima.
Piridoksin/ili piridoksal fosfat u "in vivo" ili "in vitro" eksperimentalnim uslovima povećava aktivnost slobodne alkalne ribonukleaze; piridoksin /ili piridoksal fosfat smanjuju inhibitorom–vezanu aktivnost,latentnu ribonukleazu.
U ekspeimentima "in vivo" vitamin B_6 ne dovodi do promene efekata deksametazona, ali u uslovima "in vitro" B_6P modulira efekte deksametazona.

Ključne reči: Alkalna ribonukleaza, deksametazon, piridoksin, piridoksal fosfat, jetra pacova

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