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MODULATION OF 25-HYDROXYVITAMIN D_3-24-HYDROXYLASE BY AMINOPHYLLINE: A CYTOCHROME P-450 MONOOXYGENASE SYSTEM[1]

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SUMMARY

The chick kidney mitochondrial 25-hydroxyvitamin D_3-24-hydroxylase is a cytochrome P-450 catalyzed monooxygenase system. The administration of aminophylline [3,7-Dihydro-1,3-dimethyl-1H-purine-2,6-dione compounded with 1,2-ethanediamine (2:1)], a cyclic nucleotide phosphodiesterase inhibitor, to vitamin D_3-sufficient chicks at a dose of 78 mg/kg body weight increased the 24-hydroxylase activity by 10-fold without a corresponding increase in the kidney mitochondrial cytochrome P-450 concentration. The product of the reaction co-migrated with authentic 24,25-dihydroxyvitamin D_3 in high pressure liquid chromatography and was quantitatively sensitive to periodate oxidation cleavage. The increase in the enzyme activity occurred while serum calcium concentration fell from 14 to 9 mg%. Serum phosphate concentration remained unchanged. These results support the idea that the regulation of kidney 24-hydroxylase activity may be mediated by tissue levels of cyclic nucleotides.

Vitamin D_3 undergoes a series of biotransformations the first of which is the hydroxylation of C-25 of the secosteroid by liver microsomes to yield 25-hydroxyvitamin D_3 (25-OH-D_3).[3] Next, in the kidney, 25-OH-D_3 is utilized as a common substrate for additional hydroxylations to yield either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ by two mitochondrial enzymes designated 1α-hydroxylase and 24-hydroxylase, respectively (1). Although the in vivo production of the kidney metabolites can be predicted by manipulating dietary calcium and phosphorus

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[3]Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; cAMP, 3',5'-cyclic adenylic acid; DTT, 1,4-dithiothreitol; metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone; elipten, 1-(4-aminophenyl)-1-ethyl-glutarimide phosphate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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levels, the precise biochemical events in the mitochondrion accompanying the selective formation of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ remain unknown. While the relationship of the 24-hydroxylase to the 1α-hydroxylase is not clear, several comparisons can be made. Both enzymes utilize molecular oxygen and 25-OH-D_3 as substrates, and require NADPH for activity (2-5). The expression of 1α-hydroxylase activity has been amply demonstrated to be dependent upon the participation of mitochondrial cytochrome P-450 (6). Although the involvement of cytochrome P-450 in the 24-hydroxylase activity has not been fully demonstrated, the work of Madhok et al. (2) has presented strong evidence in support of such a role for kidney mitochondrial cytochrome P-450.

Many compounds such as prostaglandins (7), parathyroid hormone (8,9) indomethacin (7), prolactin (10), and estradiol (11,12) alter the activities of the kidney 25-OH-D_3 hydroxylases. Several of these compounds alter kidney cAMP metabolism as well (13,14). These and other data have led us to the hypothesis, described elsewhere in detail (15), that endogenous cAMP metabolism is intimately linked to the selective expression of the kidney hydroxylases. In this study we (a) provide evidence that the 24-hydroxylase is a cytochrome P-450 mediated enzyme system, and (b) demonstrate that inhibition of cyclic nucleotide phosphodiesterase activity in vivo by aminophylline results in altered hydroxylase activities in vitro.

MATERIALS AND METHODS

Animals: In this study, vitamin D-deficient and vitamin D-sufficient (normal) chicks were used. One-day old white Leghorn cockerel chicks (Northern Hatcheries, Beaver Dam, WI) were housed and maintained as previously described in detail (16). Fresh blood was collected after killing the birds by decapitation, allowed to stand at room temperature for 1 h then centrifuged at 3550g for 15 min to separate the clot from serum. Serum calcium was determined by atomic absorption spectrophotometry (17) and phosphorus by the method of Chen et al. (18).

Assay for 25-OH-D_3-1α-hydroxylase and 24-hydroxylase activities: A 25 ml Erlenmeyer flask contained 1.5 ml of 20% kidney homogenate (in 0.25 M sucrose-15 mM Tris acetate buffer, pH 7.4, 1 mM DTT and 0.1 mM EDTA), 5 mM succinate, 5 mM magnesium acetate and 504 ng 25-OH-[26(27)-methyl-^{3}H]vitamin D_3 in 10 µl of 95% ethanol with a specific activity of 196 dpm/ng. The flask was flushed with 100% O_2 for 1 min, stoppered and incubated at 37° C for 30 min with gentle shaking. The reaction was terminated by the addition of 10 ml of methanol-chloroform (2:1) and the products extracted by the procedure of Bligh and Dyer

(19). The extract was chromatographed on a column containing 20 g of Sephadex LH-20 using 35% n-hexane in chloroform as the solvent (20). A total of 80 fractions (5.5 ml each) were collected. The solvent in each fraction was evaporated under a stream of air and the radioactivity counted using a toluene scintillant (3). Tritium counting efficiency was 60% in a Packard Model 3255 spectrometer. For CO-inhibition studies, flasks containing all the reactants except the radioactive substrate were sealed with rubber septa and connected through needle outlets to a vacuum train. They were evacuated repeatedly each followed by flushing with N_2 passed over heated copper then finally filled with 25 ml of the appropriate gas mixtures as indicated. The reactions were initiated by the addition of the radioactive substrate using a micro-Hamilton syringe. The reactions were terminated and the products analyzed as above.

RESULTS

Evidence for participation of cytochrome P-450 as a terminal monooxygenase in hydroxylation reactions, in general, has been derived from (a) studies showing incorporation of one atom of $^{18}O_2$ into hydroxylase substrates; (b) characteristic drug-induced spectral changes signifying a specific hemeprotein-drug interaction which may accompany inhibition of hydroxylase activity; and (c) inhibition of hydroxylase activities by CO. The 25-OH-D_3-1α-hydroxylase has been amply demonstrated to be a cytochrome P-450-catalyzed monooxygenase (2-6). Although the requirement for a similar cytochrome in the C-24 hydroxylation of 25-OH-D_3 has not been fully demonstrated, Madhok et al. (4) have reported the 24-hydroxylase-catalyzed incorporation of isotopic oxygen atom into the substrate 25-OH-D_3. The isolated product, 24,25-$(OH)_2D_3$, contained the stable isotope in the hydroxyl function at C-24. Our present results with isolated kidney mitochondria concerning CO and drug susceptibility of the 24-hydroxylase (Table I) substantiate the participation of cytochrome P-450 in the C-24 hydroxylation of 25-OH-D_3. The rapid change in the 24-hydroxylase activity above 50% CO in oxygen is clearly attributable to the specific interaction of CO with the enzyme. In the calcium containing NADPH supplemented reaction mixture, the dependence of the mitochondrial 24-hydroxylase on respiratory chain cytochrome oxidase for the necessary reducing equivalents (NADPH) is circumvented (21). Therefore, any inhibition of hydroxylase activity by CO would be independent of cytochrome oxidase function. While it appears that loss of 24-hydroxylase activity does result upon reducing O_2 tension (80-95% N_2), the loss in 24-hy-

TABLE I

EFFECT OF INHIBITORS OF CYTOCHROME P-450 ON THE 25-OH-D_3-24-HYDROXYLASE OF ISOLATED MITOCHONDRIA

Source of reducing equivalents[a]	(n)	Gas mixture[b]	Inhibitor (M)	pmol 24,25-$(OH)_2D_3$ mg^{-1} mitochondrial protein 30 min^{-1}
Ca/NADPH	(10)	100% O_2	-	63.4 ± 2.9
"		50% CO	-	63.3 ± 4.3
"		70% CO	-	35.3 ± 2.5
"		80% CO	-	19.8 ± 1.4
"		90% CO	-	11.9 ± 0.9
"		95% CO	-	2.4 ± 0.2
"		80% N_2	-	23.3 ± 1.2
"		90% N_2	-	17.5 ± 1.2
"		95% N_2	-	10.4 ± 0.8
Succinate	(10)	100% O_2	-	63.4 ± 2.9
Succinate	(5)	100% O_2	Metyrapone 2.5×10^{-4}	43.0 ± 2.2
Succinate	(5)	100% O_2	Elipten 6.6×10^{-6}	59.5 ± 2.8
			6.6×10^{-4}	55.0 ± 2.6

[a]When 5 mM succinate was not used, NADPH generating system replaced succinate in the medium of the indicated reactions. 10 mM calcium acetate was added to swell the mitochondria to allow translocation of NADPH into the mitochondria as described previously (3). The NADPH generating system consisted of 0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM nicotinamide, and 1.5 units yeast glucose-6-phosphate dehydrogenase (Sigma Chemical Co.). The reaction mixture contained 10 mg of mitochondrial protein in a total assay volume of 1.5 ml. Mitochondria were prepared from kidneys of normal vitamin D_3-supplemented chicks as previously described (3). (n) is the number of chicks in each test.

[b]The percentage of the atmosphere not designated is oxygen.

droxylase activity in the presence of CO however is significantly greater than that observed at comparable oxygen:nitrogen ratios. The inhibition of 24-hydroxylase activity by the specific modifiers of cytochrome P-450 [metyrapone and elipten; K_i for the 1α-hydroxylase $\sim 10^{-4}$ and $\sim 10^{-3}$M, respectively (6)] provides further support for the involvement of this hemeprotein in the catalytic biotransformation of 25-OH-D_3 to 24,25-$(OH)_2D_3$.

A variety of agents which have been shown to alter the 25-OH-D_3-1α-hydroxylase and 24-hydroxylase activities also influence intracellular and extracellular cAMP levels. Since aminophylline is an inhibitor of cyclic nucleotide

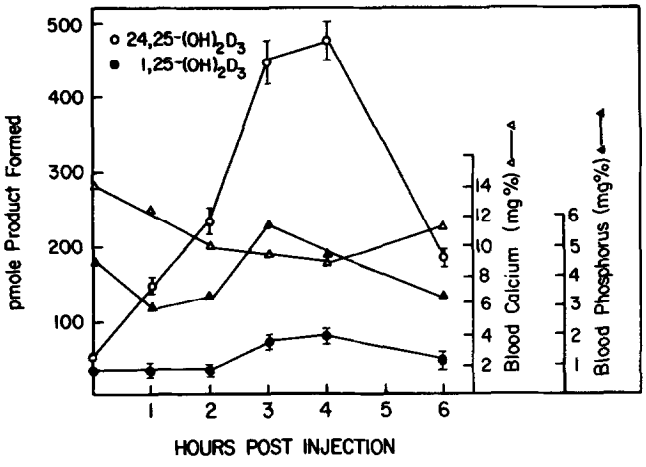

Figure 1. Effect of aminophylline on kidney 25-OH-D_3-24-hydroxylase and 1α-hydroxylase. Vitamin D_3-sufficient chicks were injected subcutaneously on the dorsal abdomen with 0.5 ml saline containing 12.5 mg aminophylline (The Vitarine Co., Inc.)(78 mg/kg body weight). Controls received saline only. At the indicated times, the animals were killed and blood collected. The kidneys were then removed and homogenized as described. The assay values are the average of several observations.

phosphodiesterase activity (22), it was of interest to study whether the inhibition of this esterase by aminophylline in vivo modulates the activity of the hydroxylases in vitro. Figure 1 shows the time course of aminophylline effect on the kidney 25-OH-D_3 hydroxylases in normal vitamin D_3-supplemented chicks. It is clear that approximately a 10-fold increase in the 24-hydroxylase activity is achieved at about 3 to 4 hours post injection. This effect on the 24-hydroxylase appears to be selective as no large changes in the 1α-hydroxylase activity are detected in the same animals. Furthermore, the rapid decrease in the serum calcium level from normal should have produced a diametric change (increase) in the 1α-hydroxylase activity, as one would have expected (23), and not the dramatic 10-fold increase in the 24-hydroxylase activity. Thus, the effect of aminophylline on the 24-hydroxylase appears to be independent of serum calcium concentration. However, it should be noted that the slight increase in the 1α-hydroxylase activity at 4 hours post injection is most likely a result of the lower serum calcium content at that point (9.04 versus 14 mg% for the control). In contrast to calcium, no specific trend was seen in serum

phosphorus values. The values varied widely within the range of reported normal serum phosphorus levels. While doubling the amount of aminophylline injected resulted in prolonging the duration of maximum level of 24-hydroxylase activity attained (by 2 hours), this larger dose, however, could not reduce the time required to achieve maximum level of activity. Moreover, the repetitive injections of 156 mg aminophylline/kg body weight in 3-hour intervals resulted in the death of the birds. On the other hand, repetitive does smaller than 78 mg aminophylline/kg body weight in 1-hour intervals were not as effective in elevating the 24-hydroxylase activity. In any event, the maximum increase in the 24-hydroxylase activity noted post injection was not accompanied by an increase in the renal mitochondrial cytochrome P-450 concentration which remained constant at 0.072 nmol cytochrome/mg protein.

While the observed effects of aminophylline on the vitamin D-sufficient chicks were dramatic, modulations in 1α-hydroxylase activity or in serum calcium or phosphorus levels did not occur in the vitamin D-deficient chicks in response to aminophylline. The 1α-hydroxylase activity remained at near control value of 605 pmol 1,25-$(OH)_2D_3$ formed in 30 min. Serum calcium and phosphorus levels also remained constant at 7.5 mg% and 5 mg% respectively.

DISCUSSION

The possibility that cAMP is involved in the selective expression of the kidney 25-OH-D_3 1α- and 24-hydroxylase activities was suggested by our recent work (15). We have advanced a hypothesis in which the central idea is that cAMP-protein kinase-dependent phosphorylation and dephosphorylation reactions involving the kidney mitochondrial P-450 cytochromes are related to the modulations in the kidney production of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$.

The results presented in this communication would suggest the following. First, there is no net gain in the total amount of enzyme molecules in response to the cyclic nucleotide phosphodiesterase inhibitor (aminophylline). This is evidenced by the 10-fold increase in 24-hydroxylase activity without a corresponding increase in mitochondrial cytochrome P-450 content. Second, the meas-

ured increase in 24-hydroxylase activity is a direct result of aminophylline-dependent increase in the renal cAMP levels.

The question whether the increase in 24-hydroxylase activity in response to aminophylline is secondary to a possible effect of the drug on serum calcium levels was dismissed based on the results shown in Fig. 1. The increase in the 24-hydroxylase activity in normal chicks receiving aminophylline occurred concurrent with the rapid decrease in serum calcium, a condition not compatible with an increase in 24-hydroxylase activity (1,23). Also, there was virtually no change in the serum calcium or phosphate concentration of vitamin D_3-deficient chicks receiving aminophylline.

Although it is not exactly clear how the aminophylline-responsive 24-hydroxylase activity is modulated relative to the 1α-hydroxylase, a role for cAMP in this modulation process can best be explained if some components of the two enzyme systems are subject to phosphorylation and dephosphorylation. Most appropriate components subject to this type of modification would be the cytochrome P-450 moieties. This assumption is based on the finding that the concentration of the ferredoxin component of the 1α-hydroxylase is not influenced by the vitamin D status of the chick (16). Studies are in progress to measure changes in amount of phosphate incorporated into the cytochromes under a variety of physiological circumstances that affect tissue cyclic nucleotide concentrations. No doubt these studies may provide exciting possibilities for future investigation in renal regulation of vitamin D metabolism.

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