Biochemical Pharmacology, Vol. 33, No. 9, pp. 1560–1562, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Vitamin D metabolites in avian plasma after a single, large dose of vitamin D_3

(*Received* 8 *August* 1983; *accepted* 16 *November* 1983)

Vitamin D and vitamin A are two vitamins with known toxicity [1]. Ingestion or administration of large amounts of vitamin D results in toxicity which is characterized by hypercalcemia, bone demineralization and soft-tissue calcification [2]. Vitamin D itself is inactive at physiological concentrations and must be converted to more active metabolites in liver and kidney. The conversion of vitamin D to 25-hydroxyvitamin D in the liver is only partially regulated while the synthesis in the kidney of the most active metabolite, 1,25-dihydroxyvitamin D, is strictly controlled [2]. Pharmacological doses of vitamin D, however, are able to override 25-hydroxyvitamin D 1α-hydroxylase [3] and cause a transient increase in $1,25(OH)_2D$* concentration in plasma and in target tissues [4].

The present experiment was designed to examine the regulation of synthesis of the major vitamin D_3 metabolites during several weeks after an intramuscular injection of a single, large dose of vitamin D_3. The results were also intended to give information on the selection of plasma metabolite determinations during follow-up of vitamin D intoxication or therapy with large doses.

Materials and methods

Animals. Six adult roosters were treated with an intramuscular injection of 15 mg/kg of vitamin D_3 in sesame oil into leg muscles. Blood samples were taken into heparinized syringes before the injection and one, three and seven weeks after the injection. Animals were fed *ad libitum* with a standard diet (Kasvatus-Tipu, Hankkija, Finland) during the experiment.

Chemicals and instruments. Crystalline vitamin D_3 was purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Crystalline 25-hydroxyvitamin D_3 and 24R,25-dihydroxyvitamin D_3 were generous gifts from Hoffman La Roche (Basel, Switzerland). Radioactive [1α,25(n)^{3}H]D_3 (7.4 Ci/mmole), 25(OH)-[26,27^{3}H]-D_3 (22.3 Ci/mmole) and 24R,25$(OH)_2$-[23,24(n) ^{3}H]D_3 (82 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, U.K.). All solvents and analytical grade chemicals were supplied by E. Merck AG (Darmstadt, F.R.G.). For HPLC, hexane and propan-2-ol were dried by molecular sieving and filtered under vacuum. PPO and POPOP were purchased from NEN Chemicals (Frankfurt am Main, F.R.G.). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

The HPLC-system consisted of an Altex high-performance liquid chromatograph (Altex Scientific Inc., Berkeley, CA, U.S.A.) equipped with a Model 110A pump, a pressure filter, a Model 153 UV detector with a 8 μl flowthrough cell and a Rheodyne Model 7125 20 μl-loop injector. A stainless steel column (20 cm × 4.0 mm, i.d.) prepacked with 7 μm spherical microparticulate silica (Nucleosil 50-7) and equipped with a precolumn (5 cm × 4.0 mm, i.d.) packed with Polygosil 60-30 precolumn packing were obtained from Macherey-Nagel (Düren, F.R.G.). A stainless steel column (20 cm × 4.0 mm, i.d.) prepacked with microparticulate octadodecyl silica (Nucleosil 7-C_{18}) was also supplied by Macherey-Nagel and was equipped with a precolumn (3 cm × 4.0 mm, i.d.) packed with Bondapak C_{18}/Corasil (Waters Associates Inc., MA, U.S.A.). A Model Ultrobeta 1210 liquid scintillation counter (LKB Wallac, Turku, Finland) was used at 15° for scintillation counting. ^{3}H-labelled fractions were counted for recovery estimations with about 40% counting efficiency in a toluene scintillant containing 4 g of PPO and 50 mg of POPOP per liter of toluene.

Extraction of plasma samples. To each aliquot of plasma (1–5 ml) were added 1800 cpm of vitamin [^{3}H]D_3, 2000 cpm of 25(OH)[^{3}H]D_3 and 1900 cpm of 24,25$(OH)_2$ [^{3}H]D_3 each in 100 μl of ethanol to monitor the analytical recovery of the assay. After vortex mixing, the plasma samples were allowed to equilibrate for 30 min. The lipids were extracted according to Bligh and Dyer [5]. Briefly, 3.75 vol of chloroform/methanol (1:2, v/v) were added; and after shaking vigorously the mixture was allowed to stand for 30 min. Phase separation was achieved by adding 1.25 vol of chloroform. The lower chloroform layer was collected and the upper aqueous layer was re-extracted twice with an additional 1.25 vol of chloroform. The combined chloroform layers were then washed with an equal volume of saturated aqueous sodium chloride solution and evaporated under reduced pressure on a rotary evaporator.

Chromatography on Sephadex LH-20. The lipid residue from the chloroform/methanol extraction was solubilized in hexane/chloroform/methanol (9:1:1, by vol.) and chromatographed on a column (1 × 10 cm) containing 2.5 g of Sephadex LH-20 in the same solvent. The 2.0–7.5 ml fraction was collected for vitamin D_3 determination, the 7.5–15 ml fraction for 25(OH)D_3 determination and the 17–27 ml fraction for 24,25$(OH)_2D_3$ determination. The vitamin D_3 fraction was further purified on a second Sephadex LH-20 column (1 × 34 cm) with the same solvent as above. The 11–17 ml fraction was collected for further analysis. The 25(OH)D_3 fraction was purified on a second Sephadex LH-20 column (1 × 10 cm) with a solvent of hexane/chloroform (1:1, v/v). The 6–15 ml fraction was collected for further analysis.

High-performance liquid chromatography. Final quantification of plasma 25(OH)D_3 was accomplished by HPLC on a Nucleosil 50–7 column equilibrated in propan-2-ol/hexane (5:95, v/v) at a flow rate of 1 ml/min. The 5–8 ml fraction was collected into a counting vial.

Vitamin D_3 and 24,25$(OH)_2D_3$ fractions were further purified on a Nucleosil 50-7 column equilibrated in propan-2-ol/hexane 2.5:97.5 (v/v) and 10:90 (v/v), respectively. Final quantification of vitamin D_3 and 24,25$(OH)_2D_3$ was achieved by reverse-phase HPLC on a Nucleosil 7-C_{18} column equilibrated in water/methanol 5:95 (v/v) and 10:90 (v/v) at a flow rate of 1.0 and 0.7 ml/min, respectively. The absorbance of the eluate was continuously monitored at 254 nm. The vitamin D_3 (14–17 ml) and 24,25$(OH)_2D_3$ (7–10 ml) fractions were collected into counting vials. 25-Hydroxyvitamin D_3-26,23-lactone is a normal vitamin D_3 metabolite in the chick [6] and its plasma concentration is increased after pharmacological doses of vitamin D_3 [7, 8]. Using the 10:90 (v/v) propan-2-ol/hexane solvent system, however, it is probably eluted separately from the 24,25$(OH)_2D_3$ peak with the normal phase column [7] and

* Abbreviations: 25$(OH)D_3$, 25-hydroxyvitamin D_3, calcidiol; 24,25$(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3, (24R)-hydroxycalcidiol; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3, calcitriol; HPLC, high-performance liquid chromatography; PPO, 2,5-diphenyloxazole; POPOP, p-bis2(5-phenyl-oxazolyl)benzene.

Table 1. Concentration of vitamin D_3 and its major metabolites in chick plasma after an intramuscular injection of 15 mg/kg of vitamin D_3 (means ± S.D., N = 6)

Days after injection	Vitamin D_3 (nmoles/l)	25(OH)D_3 (nmoles/l)	24,25$(OH)_2D_3$ (nmoles/l)	Sum: vitamin D_3 + 25(OH)D_3 + 24,25$(OH)_2D_3$ (nmoles/l)	Vitamin D_3/ 25(OH)D_3 ratio	25(OH)D_3/ 24,25$(OH)_2D_3$ ratio
0	30.2 ± 5.5	57.1 ± 6.6	10.6 ± 3.6	97.8 ± 14.7	0.52 ± 0.06	5.78 ± 1.48
7	532.0 ± 95.2*	137.8 ± 16.7*	55.7 ± 9.7*	725.6 ± 97.0*	3.91 ± 0.90*	2.50 ± 0.21*
21	446.2 ± 40.7*	191.1 ± 23.9*	74.8 ± 24.1*	712.1 ± 23.8*	2.38 ± 0.51*	2.74 ± 0.85*
49	450.1 ± 70.5*	210.9 ± 64.3*	63.6 ± 16.2*	724.5 ± 80.4*	2.32 ± 0.90*	3.36 ± 0.78*

* Differs from 0-day value at $P < 0.001$.

therefore not quantified with 24,25$(OH)_2D_3$ in reverse-phase HPLC.

Vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ fractions were evaporated and counted in the toluene scintillant along with the initial portions of labelled metabolites. The heights of the metabolite peaks were divided by the percentage recovery to yield corrected peak heights. These were related to standard curves to calculate the amounts of the metabolites in the original plasma samples.

Results and discussion

A single, large intramuscular dose of vitamin D_3 resulted in marked elevations of plasma concentrations of vitamin D_3 and its major metabolites 25(OH)D_3 and 24,25$(OH)_2D_3$ within 7 days of injection (Table 1). The plasma concentration of vitamin D_3 remained at a level about two to three times higher (450–530 nmoles/l) than the level of 25(OH)D_3 (140–210 nmoles/l) throughout the 7-week experiment. The plasma concentration of 24,25$(OH)_2D_3$ increased to a level of approximately 55–75 nmoles/l in 7 days and remained at this level throughout the experiment. The correlation of the plasma concentrations of vitamin D_3 and 25(OH)D_3 during the whole experiment was $r = 0.65$ ($P < 0.001$) and the correlation between plasma concentration of 25(OH)D_3 and 24,25$(OH)_2D_3$ was $r = 0.86$ ($P < 0.001$). The sum of vitamin D_3 and its two major metabolites in plasma remained at a constant level of about 720 nmoles/l throughout the one to seven week follow-up period. The vitamin D_3/25(OH)D_3 ratio increased four to seven times over the initial value and was at its highest at one week. The 25(OH)D_3/24,25$(OH)_2D_3$ ratio decreased to about half of the initial value and was at its lowest also at one week.

The administration of large, pharmacological doses of vitamin D_3 through oral, subcutaneous, intramuscular or intravenous routes has been examined in several studies [3, 4, 9, 10]. However, in those studies, a limited number of vitamin D_3 metabolites or only a single time-point have been examined. The most common form of vitamin D_3 therapy consists of a large dose administered as an intramuscular oil depot injection. This results in a rather slow liberation of vitamin D_3 from the injection site [10]. The present results indicate that in the chick the plasma concentration of the vitamin D_3 itself remains at a high level for several weeks after a single, large intramuscular injection of vitamin D_3. The next abundant metabolite is 25(OH)D_3, but its concentration did not reach the concentration of its precursor (vitamin D_3) during the 7-week experiment. This suggests that the 25-hydroxylation reaction in the liver is under relatively strict regulation and that even at these comparatively high plasma levels of vitamin D_3 there is negligible contribution of the mitochondrial high-K_m hydroxylase [11] to the overall substrate hydroxylation. Similarly, the plasma concentration of 24,25$(OH)_2D_3$ did not rise above a level of approximately 75 nmoles/l in spite of a continuous supply of the substrate. The order of magnitude of increase from the initial plasma levels was vitamin D_3 > 24,25$(OH)_2D_3$ > 25(OH)D_3. Therefore, high plasma levels of vitamin D_3 and 24,25$(OH)_2D_3$ after an intramuscular injection are even more sensitive indicators of vitamin D_3 intoxication in the chick than those of 25(OH)D_3.

Acknowledgements—This investigation was supported in part by grants from the Sigrid Jusélius Foundation, The Research Council for Natural Sciences of the Academy of Finland, and the Finnish Cultural Foundation. We wish to thank Mrs. Hanna Heikkinen for her competent technical assistance.

Department of Biochemistry
University of Kuopio
P.O. Box 6, SF-70211 Kuopio 21
Finland

RAILI LAPPETELÄINEN
KARI SAVOLAINEN
PEKKA MÄENPÄÄ

REFERENCES

1. H. F. DeLuca, in *The Fat-Soluble Vitamins* (ed. H. F. DeLuca), Vol. II, pp. 69–132. Plenum, New York (1978).
2. A. W. Norman, J. Roth and L. Orci, *Endocr. Rev.* **3**, 331 (1982).
3. T. A. Reinhardt and H. R. Conrad, *J. Nutr.* **110**, 1589 (1980).
4. C. Ratzkowski, F. Fine and S. Edelstein, *Isr. J. Meds. Sci.* **18**, 695 (1982).
5. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
6. R. L. Horst and E. T. Littledike, *Comp. Biochem. Physiol.* **73B**, 485 (1982).
7. R. L. Horst, *Biochem. biophys. Res. Commun.* **89**, 286 (1979).
8. R. M. Shepard and H. F. DeLuca, *Archs. Biochem. Biophys.* **202**, 43 (1980).
9. R. L. Horst and E. T. Littledike, *J. Dairy Sci.* **62**, 1746 (1979).
10. M. P. Whyte, J. G. Haddad, Jr., D. D. Walters and T. C. B. Stamp, *J. clin. Endocr. Metab.* **48**, 906 (1979).
11. I. Björkhem, I. Holmberg, H. Oftebro and J. I. Pedersen, *J. biol. Chem.* **225**, 5244 (1980).

Biochemical Pharmacology, Vol. 33, No. 9, pp. 1562–1564, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Effect of exogenous *S*-adenosyl-L-methionine on phosphatidylcholine synthesis by isolated rat hepatocytes

(*Received* 8 *August* 1983; *accepted* 16 *November* 1983)

The possible uptake of *S*-adenosyl-L-methionine (SAM)* by rat hepatocytes has been investigated by different authors [1, 2]. While Pezzoli *et al.* [1] concluded that SAM crosses the cell membrane of isolated rat hepatocytes with an apparent K_m of *ca* 8 μM, Hoffman *et al.* [2] reported that the hepatocytes do not take up significant amounts of SAM when rat liver is perfused with 50 μM SAM.

In the present experiments we have studied the effect of the addition of SAM to isolated rat hepatocytes on the conversion of phosphatidylethanolamine to phosphatidylcholine in cells prelabelled with [^{3}H]ethanolamine. The addition of up to 5 mM SAM to isolated rat hepatocytes has no effect on the conversion of phosphatidylethanolamine to phosphatidylcholine. However, if the hepatocytes are treated with 3-deazaadenosine (C^3-Ado), adenosine (Ado) or 3-deazaaristeromycin (C^3-Ari) to inhibit phospholipid methylation by decreasing the ratio SAM/SAH, the addition of SAM restores this reaction to normal values. This effect, however, is only observed with pharmacological concentrations of SAM (concentration $\geq$ 1 mM). These results indicate that exogenous SAM can cross the cell membrane of isolated hepatocytes at pharmacological doses. At physiological doses, SAM is either unable to cross the cell membrane or is rapidly metabolized by the cell. This argues against a physiological function for the uptake of SAM by hepatocytes. Our results are consistent with the data of Hoffman *et al.* [2], who concluded that the hepatocytes do not take up significant amounts of SAM when the rat liver is perfused with micromolar concentrations of SAM.

Materials and methods

Materials. C^3-Ado, C^3-Ari and SIBA were a generous gift from Dr. Peter Chiang (Walter Reed Army Institute of Research, Washington, DC). Adenosine was from Boehringer, and homocysteine and authentic phospholipids standards were from Sigma. *S*-Adenosyl-L-methionine was from Europharma. [1-^{3}H]Ethanolamine (8.8 Ci/mmole) and [methyl-^{3}H]choline were from Amersham.

Isolation of rat hepatocytes. Hepatocytes from normally fed Wistar rats (200–250 g) were prepared by the collagenase perfusion method as previously described [3, 4]. Hepatocytes obtained under these conditions respond to a variety of hormones [3–5], indicating that these cells maintain most of their biochemical integrity.

Phospholipid methylation by isolated hepatocytes. Isolated rat hepatocytes (2–6 × 10^6 cells/ml) were incubated for 10 min at 37° with 5 μCi per ml [1-^{3}H]ethanolamine in a medium containing 118 mM NaCl, 4.75 mM KCl, 1.18 mM KPO_4H_2, 1.18 mM $MgSO_4$, 24.9 mM $NaCO_3H$, 10 mM glucose and 2.5 mM $CaCl_2$ at pH 7.4, the gas phase being 95% O_2 and 5% CO_2. At the end of this period, cells were washed three times and resuspended again at the same cell density. C^3-Ado, C^3-Ari, Ado or SIBA was then added. All these compounds were dissolved in dimethyl sulfoxide. Control cells received the same amount of dimethyl sulfoxide (final concentration 1%). C^3-Ado, C^3-Ari and Ado were added together with 200 μM homocysteine thiolactone. After 10 min (C^3-Ado, SIBA) or 30 min (Ado, C^3-Ari) incubation at 37°, SAM was added and 20 min later the cells were centrifuged for 0.5 min at 900 *g*. The cell pellet was immediately frozen in dry-ice–acetone and the phospholipids were extracted as described in ref. [3]. Control samples were obtained 30 and 50 min after the addition of dimethyl sulfoxide to account for the different incubation times applied with the different adenosine analogues. After extraction, the phospholipids were separated by thin-layer chromatography (TLC) [3, 6]. The plates were scraped into 0.5 cm bands and the amount of radioactivity incorporated into the total methylated phospholipids (phosphatidylcholine + phosphatidyl-*N*,*N*-dimethylethanolamine + phosphatidyl-*N*-monomethylethanolamine) was determined [3, 6]. Results are expressed as the amount of radioactivity incorporated into methylated phospholipids per 10^7 cells.

Incorporation of labelled choline into phosphatidylcholine. Isolated rat hepatocytes (2–6 × 10^6 cells/ml) were preincubated for 15 min at 37°. At the end of this period, C^3-Ado, Ado, C^3-Ari or SIBA was added as described above. Then SAM was added and 20 min later cells were treated with 0.2 μCi/ml [methyl-^{3}H]choline. After 15 min incubation the cells were centrifuged and the phospholipids extracted and analysed by TLC as described above. Control experiments were carried out as described above. Under these conditions, more than 90% of the radioactivity associated to lipids migrated as authentic phosphatidylcholine [6]. Results are expressed as the amount of radioactivity incorporated into phosphatidylcholine per 10^7 cells.

* Abbreviations: C^3-Ado, 3-deazaadenosine; Ado, adenosine; C^3-Ari, 3-deazaaristeromycin; SIBA, 5′-deoxy-5′-isobutylthioadenosine; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.