WARFARIN AND VITAMIN K ACCELERATE PROTEIN AND GLYCOPROTEIN SYNTHESIS IN ISOLATED RAT LIVER MITOCHONDRIA *IN VITRO*[1]

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Summary: Protein and glycoprotein synthesis is accelerated by vitamin K_1 and warfarin in isolated rat liver mitochondria and slightly accelerated in rat liver microsomes and brain mitochondria *in vitro*. At 2×10^{-3} M vitamin K_1, protein synthesis increased 5 fold and glycoprotein synthesis 7 fold in isolated rat liver mitochondria. There is no concomitant increase in RNA and DNA synthesis in the isolated rat liver mitochondria with vitamin K_1 or warfarin. Cycloheximide has no effect on this increase in mitochondrial protein and glycoprotein synthesis in the rat liver mitochondria but chloramphenicol completely abolishes the increase. Conversely, L5178Y mouse leukemia cell macromolecular synthesis is greatly inhibited by vitamin K_1 and warfarin.

Earlier workers have thought that either the liver mitochondria or microsomes may be associated with the production of prothrombin and other clotting factors *in vitro* and *in vivo* (1-3). Studies involving vitamin K in protein synthesis, at the level of ATP generation, have been reported (4) but the evidence for a molecular action of vitamin K is contradictory (5). Recent reports showed the reversal of cycloheximide inhibition of prothrombin biosynthesis *in vitro* (6) and *in vivo* (3,7) with the addition of vitamin K. The present report shows an acceleration of protein and glycoprotein synthesis in rat liver and brain mitochondria and rat liver microsomes by vitamin K_1 (Aquamephyton^R) and sodium warfarin (Coumadin^R).

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Mitochondria are capable of the autonomous synthesis of nucleic acids (8-10), lipids (11, 12), glycolipids (13, 14), proteins (14 - 18) and glycoproteins (14, 19). Although these macromolecules are presumably synthesized for the mitochondria's own use, it is not inconceivable that the mitochondria provide some precursor type of protein or glycoprotein for the formation or activation of other macromolecules (e.g. proteins, glycoprotein, or enzyme) that the intact cell later secretes, e.g. prothrombin. Recent studies (20, 21) have shown that cycloheximide does not inhibit liver mitochondrial protein or glycoprotein synthesis but does inhibit liver microsomal protein synthesis, and that chloramphenicol does inhibit protein and glycoprotein synthesis in liver mitochondria but has no effect on microsomal protein synthesis. These findings initiated the present study on the effects of vitamin K_1 on rat liver and brain mitochondrial and microsomal synthesis of protein and glycoprotein.

Materials and Methods

The rat liver mitochondria were isolated by a modification of the method of Schneider and Hogeboom (22) as previously described (19). Rat liver microsomes were prepared by a modification of the method of Schneider and Kuff (23). pH 5 enzymes prepared separately were precipitated by the addition of 1 M acetic acid to the 140,000 x g supernatant.

Intraneural mitochondria of the rat were prepared by a modification of the method previously described (14).

Uridine diphosphate glucose-^{14}C (sp. ac. 200 C_i/Mole), glucosamine-^{14}C (sp. ac. 10 C_i/Mole), l-fucose-^{3}H (sp. ac. 10 C_i/Mole), uridine-^{3}H (10 C_i/mMole), thymidine-^{3}H (15 C_i/mMole) and l-leucine-^{14}C (sp. ac. 250 C_i/Mole) were purchased from New England Nuclear Corp.

Protein determinations were made by the procedure of Lowry et al. (22). Protein and glycoprotein synthesis was determined as previously described (14, 19).

Results and Discussion

Liver mitochondria showed the greatest acceleration of protein and glyco-

Table 1. Acceleration of incorporation of leucine-^{14}C and monosaccharide-^{14}C into protein and glycoprotein by isolated mitochondria and microsomes. Data are given as pmoles/mg protein incorporated.

System:	Liver Mitochondria			Liver Microsomes		Brain Mitochondria	
Synthesis:	Protein	Glycoprotein		Protein	Glycoprotein	Protein	Glycoprotein
Precursor:	(1)	(2)	(3)	(1)	(2)	(1)	(2)
Control	8.4	6.8	2.7	4.4	5.6	12.0	1.2
"0" time	0.4	0.2	0.2	0.3	0.3	0.6	0.1
Boil Control	0.2	0.2	0.1	0.2	0.4	0.5	0.1
+2 x 10^{-3}M Warfarin	25.9	14.0	6.0	4.9	5.6	12.0	1.2
1 x 10^{-3}M Warfarin	14.1	9.6	5.0	4.6	5.6	12.0	1.2
2 x 10^{-4}M Warfarin	10.5	8.0	4.0	4.4	5.6	12.0	1.2
2 x 10^{-5}M Warfarin	8.8	6.8	2.7	4.4	5.6	12.0	1.2
2 x 10^{-6}M Warfarin	8.4	6.8	2.7	4.4	5.6	12.0	1.2
2 x 10^{-7}M Warfarin	8.4	6.8	2.7	4.4	5.6	12.0	1.2
+2 x 10^{-3}M Vitamin K_1	40.2	42.2	15.1	5.2	12.4	28.0	2.2
+1 x 10^{-3}M Vitamin K_1	24.4	31.2	12.3	4.6	8.2	21.0	2.0
+2 x 10^{-4}M Vitamin K_1	18.3	22.1	9.2	4.4	5.9	18.0	1.4
+2 x 10^{-5}M Vitamin K_1	17.1	16.0	4.5	4.4	5.6	14.0	1.2
+2 x 10^{-6}M Vitamin K_1	14.2	6.9	2.7	4.4	5.6	12.0	1.2
+2 x 10^{-7}M Vitamin K_1	8.5	6.8	2.7	4.4	5.6	12.0	1.2
100 µg/ml chloramphenicol	2.4	5.3	---	4.4	5.6	---	---
250 µg/ml chloramphenicol	2.1	5.0	---	4.5	5.8	---	---
2 x 10^{-3}M Warfarin +100 µg/ml chloramphenicol	4.4	7.0	---	4.8	5.7	---	---
2 x 10^{-3}M Warfarin +250 µg/ml chloramphenicol	2.9	6.0	---	4.7	5.6	---	---
2 x 10^{-3}M Vitamin K_1 +100 µg/ml chloramphenicol	6.2	21.0	---	5.1	12.3	---	---
2 x 10^{-3}M Vitamin K_1 +250 µg/ml chloramphenicol	4.9	19.0	---	5.1	12.4	---	---
100 µg/ml cycloheximide	8.4	6.8	---	2.2	4.5	---	---
250 µg/ml cycloheximide	8.5	6.9	---	1.8	4.0	---	---
2 x 10^{-3}M Warfarin +100 µg/ml cycloheximide	26.1	14.1	---	2.5	4.5	---	---
2 x 10^{-3} Warfarin +250 µg/ml cycloheximide	25.9	14.0	---	2.3	4.2	---	---
2 x 10^{-3}M Vitamin K_1 +100 µg/ml cycloheximide	41.0	40.9	---	2.6	9.6	---	---
2 x 10^{-3}M Vitamin K_1 +250 µg/ml cycloheximide	40.3	42.3	---	2.3	9.0	---	---

(1) L-leucine-^{14}C#, (2) glucosamine-^{14}C+, (3) UDP-glucose-^{14}C++

Table 1. In each instance the mitochondria or microsomes were incubated in the designated system with the indicated labeled compound for 1 hour at 37°C.

*The medium contained 10 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.6), 50 mM Tris (hydroxymethyl) amino methane (pH 7.6), 5 mM phosphoenol pyruvate, 20 µg of pyruvic kinase, 2 mM adenosine triphosphate, 2 mM EDTA, 22.5 mg/ml of a complete amino acid mixture minus leucine, 10 µl of the labelled compound, 4 to 10 mg of mitochondrial or microsomal protein, and 0.154 M KCl to a final volume of 240 µl. The liver microsomal assays also contained guanosine triphosphate (0.2 mM) and approximately 2 mg of pH 5 enzymes to a final volume of 300 µl. #Uniformly labeled L-leucine-^{14}C (0.2 μC_i). +Uniformly labeled glucosamine-^{14}C (0.2 μC_i). ++Uniformly labeled uridine diphosphate glucose-^{14}C (0.2 μC_i). Dashed lines indicate the experiment was not performed.

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protein synthesis (Table 1) with vitamin K_1. At 2 x 10^{-3} M vitamin K_1 a 5-fold increase in the incorporation of l-leucine-^{14}C into protein and a 7-fold increase in glucosamine-^{14}C and UDP-glucose-^{14}C incorporation into glycoprotein was found. These increases followed a dose response curve; higher concentrations of vitamin K_1 showed only a slight increase in liver microsomal l-leucine-^{14}C incorporation and a 2-fold increase in liver microsomal glucosamine-^{14}C incorporation and a 2-fold increase in both l-leucine-^{14}C and glucosamine-^{14}C incorporation in brain mitochondria. Thus the vitamin K_1 accelerates protein and glycoprotein synthesis with the greatest acceleration in the isolated rat liver mitochondria.

Warfarin, which presumably acts in vivo as a competitive inhibitor of vitamin K, also accelerated protein and glycoprotein synthesis in rat liver mitochondria (Table 1). The increases with warfarin compared to similar concentrations of vitamin K are not as great; only a 3-fold increase in l-leucine-^{14}C incorporation and a 2-fold increase in monosaccharide incorporation occurred. No effects of warfarin occurred in the liver microsomes or brain mitochondria.

Chloramphenicol inhibits mitochondrial protein and glycoprotein synthesis (20) but has little effect on microsomal protein synthesis. The present data indicate that the inhibition of isolated rat liver mitochondrial protein synthesis caused by chloramphenicol cannot be reversed substantially by vitamin K_1 or warfarin but 50 percent of the response of glucosamine-^{14}C incorporation with high levels of vitamin K_1 was obtained in the presence of chloramphenicol

(Table 1). No effect on rat liver microsomal protein or glycoprotein synthesis was observed with chloramphenicol and the 2-fold increase in microsomal incorporation of glucosamine-^{14}C with vitamin K_1 was unaffected.

Cycloheximide at concentrations of 100 µg/ml to 250 µg/ml does not effect the acceleration of protein or glycoprotein synthesis found with warfarin and vitamin K_1 in rat liver mitochondria. These concentrations of cycloheximide inhibit microsomal protein and glycoprotein synthesis and depress the microsomal acceleration of glycosamine-^{14}C incorporation by vitamin K_1 by 50 percent (Table 1).

The time course of acceleration of protein and glycoprotein synthesis by vitamin K_1 and warfarin in isolated rat liver mitochondria was observed along

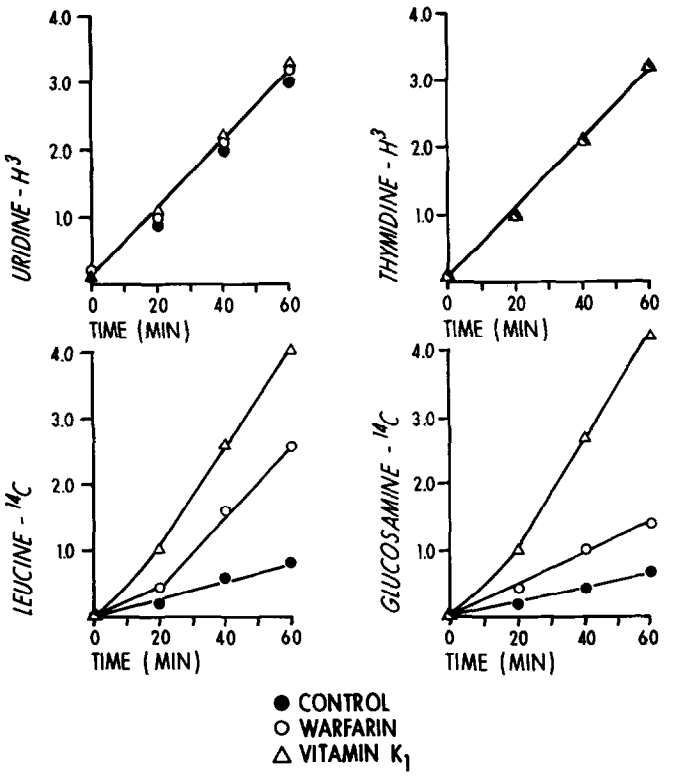

Figure 1. Time Course of Acceleration of Protein and Glycoprotein Synthesis by Vitamin K_1 and Warfarin in Isolated Rat Liver Mitochondria. Data are p moles/mg protein. Warfarin and vitamin K_1 final concentrations were 2 x 10^{-3} M. The same system for macromolecular synthesis was employed as described in Table 1. Concentrations of labelled compounds were as given in Table 1 for leucine-^{14}C and glucosamine-^{14}C. 0.5 μC_i of uridine-^{3}H and 0.5 μC_i of thymidine-^{3}H were added to the complete system for RNA and DNA synthesis. Macromolecular bound radioactivity was determined as described in Materials and Methods.

with the effect of vitamin K_1 and warfarin on rat liver mitochondrial RNA and DNA synthesis (Fig. 1). Leucine-^{14}C and glucosamine-^{14}C incorporation in isolated rat liver mitochondria by warfarin and vitamin K_1 occurred as early as the first 20 minutes of incubation and occurred in a more or less linear fashion thereafter (Fig. 1).

The data presented in Table 2 indicate that in L5178Y mouse leukemic cells, macromolecular synthesis was severely inhibited by high concentration of vitamin K_1 and warfarin. L5178Y cells were grown in Fischer medium plus 10 percent horse serum in suspension culture as previously described (25, 26). The cells, harvested in logarithmic growth, were resuspended in Fischer medium plus the required radioactively labeled precursor, in the presence or absence of warfarin or vitamin K_1. The results in Table 2 indicate that RNA, DNA, protein and glycoprotein synthesis was inhibited by concentrations of 10^{-2} to 10^{-3} of warfarin or vitamin K_1. In general the vitamin K_1 was more toxic at

Table 2. Inhibitory Effect of Warfarin and Vitamin K_1 on Macromolecular Synthesis in L5178Y Cells. Data are pmoles/mg protein.

Precursor	Uridine-H^3	Thymidine-H^3	Leucine-^{14}C	Fucose-H^3
Control	12.4	101	40	4.0
1×10^{-2}M Warfarin	2.6	9	16	2.1
5×10^{-3}M Warfarin	5.8	12	20	2.6
2×10^{-3}M Warfarin	7.9	68	25	3.7
1×10^{-3}M Warfarin	11.9	101	36	4.1
1×10^{-2}M Vitamin K_1	0.4	4	3	1.6
5×10^{-3}M Vitamin K_1	0.5	5	5	1.7
2×10^{-3}M Vitamin K_1	1.6	8	12	1.8
1×10^{-3}M Vitamin K_1	2.3	16	12	2.4

Table 2. Inhibitory effect of warfarin and vitamin K_1 on macromolecular synthesis in L5178Y cells. Data are expressed as p moles/mg protein. Logarithmic L5178Y cells (10^5 cells/ml) were incubated at 37°C for 60 minutes in Fischer medium plus the indicated labeled compound to a final volume of 0.5 ml. The amount and specific activity of each radioactive compound was: leucine-^{14}C (0.3 μC_i), l-fucose-^3H (0.5 μC_i), uridine-^3H (1.0 μC_i) and thymidine-^3H (1.0 μC_i). Macromolecular bound radioactivity was determined as given in the Materials and Methods.

equimolar concentrations than the warfarin.

The results presented herein indicate that vitamin K_1 and its analogue warfarin accelerate protein and glycoprotein synthesis but not DNA or RNA synthesis in isolated rat liver mitochondria; vitamin K_1 had a greater effect than warfarin. The fact remains that in a normal (i.e. not a vitamin K deficient) rat liver mitochondrial system, vitamin K_1 at 2×10^{-3} M increases protein synthesis 5 fold and glycoprotein synthesis 7 fold; the implications of this are of great importance to the mechanism of vitamin K action, mitochondrial autonomy, and extracytoplasmic protein and glycoprotein synthesis. That the effects reported herein were due to vitamin K and warfarin was shown by assaying the suspending vehicles for the two compounds and photo-inactivated Aquamephyton in each of the systems. In every instance these assays did not significantly differ from the controls. Thus the results reported are due to the vitamin K_1 and warfarin molecules.

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