Vitamin K-Dependent Carboxylase in Skin

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Vitamin K-dependent carboxylase is demonstrated in skin microsomes from humans, rats, rabbits, and mice. This enzyme converts a number of distinct protein-bound glutamic acid residues into γ-carboxyglutamic acid residues, which strongly interact with Ca^{++} ions. The enzymatic activity (expressed per mg protein) in skin is about 20% of that in liver. Vitamin K-dependent carboxylase is present in both epidermal and dermal tissue. It is demonstrated that warfarin treatment in mice results in an accumulation of noncarboxylated precursor proteins in both dermal and epidermal microsomes. Most probably this effect of warfarin is not restricted to mice, but occurs also in the skin of patients under oral anticoagulant therapy. A possible relation between vitamin K-dependent skin carboxylase and the γ-carboxyglutamic acid-containing protein in calcified nodules from patients with scleroderma and dermatomyositis is discussed. *J Invest Dermatol 87:377–380, 1986*

Vitamin K is involved in a posttranslational modification of proteins [1,2]. This modification consists of the carboxylation of a number of distinct glutamic acid residues into γ-carboxyglutamic acid residues (Gla). The presence of Gla has initially been discovered in blood coagulation factors, which are synthesized in the liver [3,4]. The Gla-rich regions are essential for the proper functioning of these coagulation factors. Coumarin derivatives, such as warfarin, inhibit the formation of Gla residues and therefore they are used as anticoagulants [5].

For a long period it has been thought that the vitamin K-dependent carboxylation was restricted to the liver. During the last decade, however, similar enzyme systems have been detected in various other tissues such as kidney, spleen, testis, lung, and vessel wall [6,7]. In vivo, the administration of low doses of warfarin affects both the hepatic as well as the nonhepatic carboxylase systems [8]. It is to be expected that all these vitamin K-dependent enzymes are involved in the production of Gla-containing proteins, but in most cases their precise function and even their place of action are unknown. Gla-containing proteins have been demonstrated in, for instance, bone [9], renal stones [10], calcified atheromatous plaques [11], calcium-containing material extruded from the skin of scleroderma patients, and in the calcified skin and subcutaneous plaques from patients with dermatomyositis [12]. Whereas it has been suggested that the bone Gla protein is synthesized by the osteoblasts [13], the origin of the Gla-containing proteins in the calcified plaques occurring in the skin diseases mentioned above has remained obscure until now. It was of interest, therefore, to examine skin tissue for the presence of vitamin K-dependent carboxylase. The results of our investigations are presented in this paper.

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Abbreviations:
CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DTT: dithiotreitol

MATERIALS AND METHODS

Materials Vitamin K_1 (konakion) was obtained from Hoffmann-La Roche (Basel, Switzerland). Vitamin K hydroquinone was prepared as described earlier [14]. 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), dithiothreitol (DTT), and warfarin were from Sigma (St. Louis, Missouri) and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was from Vega Biochemicals (Tucson, Arizona). $NaH^{14}CO_3$ (40–60 Ci/mol) was purchased from Amersham (Amersham, U.K.) and Atomlight from New England Nuclear (Dreieich, F.R.G.). Dispase protease neutrale from *Bacillus polymixa* grade II was obtained from Boehringer Mannheim (Mannheim, F.R.G.). All other chemicals were from Merck (Darmstadt, F.R.G.). Athymic, hairless mice, strain NMRI, *nu/nu* were obtained from TNO (Zeist, The Netherlands).

Methods Skin tissue was obtained from 12- to 16-week-old hairless, athymic mice, newborn Lewis rats, and newborn Hollander rabbits. The animals were sacrificed under ether anesthesia. Skin tissue from humans without known skin diseases was obtained from the Departments of Pathology in hospitals at Maastricht and Amsterdam. All pieces of skin tissue were obtained less than 3 h after death and they were immediately frozen at −80°C. For some experiments, livers, kidneys, lungs, and testes were excised from athymic mice shortly after their sacrifice. For some experiments these mice were injected i.p. with warfarin dissolved in 150 mM NaCl, pH 7.4, in a dose of 10 mg/kg body weight, 18 h before sacrifice.

Epidermal tissue was separated from the underlying connective tissue by dispase treatment. Therefore we slightly modified the method described by Kitano and Okada [15]. Skin tissue was cut into pieces of about 2 × 4 cm and incubated at room temperature with 150 ml 0.5% dispase (w/v), 0.15 M NaCl pH 7.4, during 4–6 h. After this incubation the skin could be divided into epidermis and dermis with a small spatula. To control the separation, pieces of tissue were fixed in phosphate-buffered formalin and embedded in paraffin. Four-micrometer tissue sections were stained with hematoxylin or azan and examined with a light microscope.

The microsomal fractions of the tissues were prepared in a similar way as described earlier [6]. After 3 washing cycles the microsomes were suspended in buffer A [0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, 20% (v/v) ethyleneglycol] to a concentration of 3–10 mg protein/ml and stored at −80°C.

The carboxylase activity was measured by incubating 0.25 ml

of reaction mixtures at 25°C in buffer A, containing 0.4 mg microsomal protein, 0.1 mg CHAPS, 10 mM F L E E L, 8 mM DTT, 0.2 mM vitamin K hydroquinone, 0.01 mCi $NaH^{14}CO_3$, and 1 M $(NH_4)_2SO_4$. The reaction was stopped and nonbound $^{14}CO_2$ was removed by adding trichloroacetic acid (5%) and degassing the solution for 2 min at elevated temperatures. The samples were supplemented with 10 ml Atomlight and counted in a Beckman scintillation counter. Since under the incubation conditions all reaction components are present in excess, the initial reaction rates are proportional to the respective amounts of carboxylase. To measure the amount of endogenous substrate no F L E E L was added to the reaction mixture. In all experiments parallel incubations were performed in the absence of vitamin K hydroquinone and DTT; these blank values were subtracted. It was ascertained that dispase had no effect on the carboxylase-activity measurements.

Protein concentration was determined according to Sedmak and Grossberg [16].

Gla residues were determined by alkaline hydrolysis of homogenized tissue and subsequent high-performance liquid chromatography analysis on a Nucleosil 5SB column (Chrompack) as described by Kuwada and Katayama [17].

RESULTS

Detection of Vitamin K-Dependent Carboxylase Activity Skin tissue was obtained from humans, hairless mice, and from newborn rats and rabbits. After homogenization the microsomal fractions were prepared. In all these species vitamin K-dependent carboxylase activity could be demonstrated (Table I). The rather low amounts measured in the rabbit and rat preparations may be caused by a less efficient homogenization of the tissues. All experiments described below were performed with hairless mice as their skin is most readily homogenized.

To compare the amounts of vitamin K-dependent carboxylase activity in skin and other tissues we also prepared microsomal fractions of liver, kidney, spleen, and testes from the same animals. In all microsomal preparations carboxylation proceeded in a linear way for at least 30 min. The skin carboxylase activity is comparable with that in other nonhepatic tissues, but much lower than the liver carboxylase activity (Table II). The ratio between the enzyme activities in liver, kidney, spleen, lung, and testis microsomes from these mice is in reasonable agreement with results earlier found for rats [7].

Localization of Skin Carboxylase In earlier experiments we demonstrated a considerable vitamin K-dependent carboxylase activity in bovine vessel wall. It cannot be excluded that this vascular enzyme accounts, at least partly, for the carboxylase activity demonstrated in microsomes derived from several whole tissue homogenates. Experiments were undertaken to investigate whether the skin carboxylase activity could be attributed to the vascular vitamin K-dependent carboxylase system. As the epidermis does not contain any blood vessels, skin was separated into epidermal and dermal tissue by dispase treatment. It was ascertained by histologic techniques that the epidermal cells did not contain any detectable amount of dermal tissue and vice versa (Fig 1). Carboxylase activity was detected in both microsomal preparations. The carboxylation rate was constant for about 30 min as is shown in Fig 2.

Table I. Vitamin K-Dependent Carboxylase Activity in Skin from Various Species

Species	Carboxylase Activity (dpm/min/mg protein)
Human	500
Rabbit	105
Rat	200
Mouse	590

Microsomes were prepared from about 10 g of skin tissue, and carboxylase activity was measured as described in *Materials and Methods*. Blank values without added KH_2 and DTT (15–30 dpm/min/mg protein) were subtracted. The data are the mean of duplicate measurements.

Table II. Vitamin K-Dependent Carboxylase Activity in Various Tissues from Athymic Mice

Tissue	$^{14}CO_2$ Incorporated (dpm)		Relative Carboxylase Activity (%)
	$+KH_2$	$-KH_2-DTT$	
Skin	6,470	245	100
Liver	36,200	200	580
Kidney	3,810	340	56
Lung	12,010	220	190
Spleen	2,720	370	38
Testis	5,250	290	80

The vitamin K-dependent carboxylase activities are given as the amount of $^{14}CO_2$ incorporated in 25 min in the presence and absence of vitamin KH_2 and DTT. All reaction mixtures contained 0.4 mg of microsomal protein. In the last column the activities are expressed as a percentage of the skin carboxylase activity per mg microsomal protein.

Effects of Warfarin Treatment In Vivo In liver, warfarin treatment causes the accumulation of noncarboxylated precursors of blood coagulation factors. A part of these precursors remains firmly bound to the microsomal carboxylase complex and can be carboxylated in vitro (endogenous substrate). We investigated

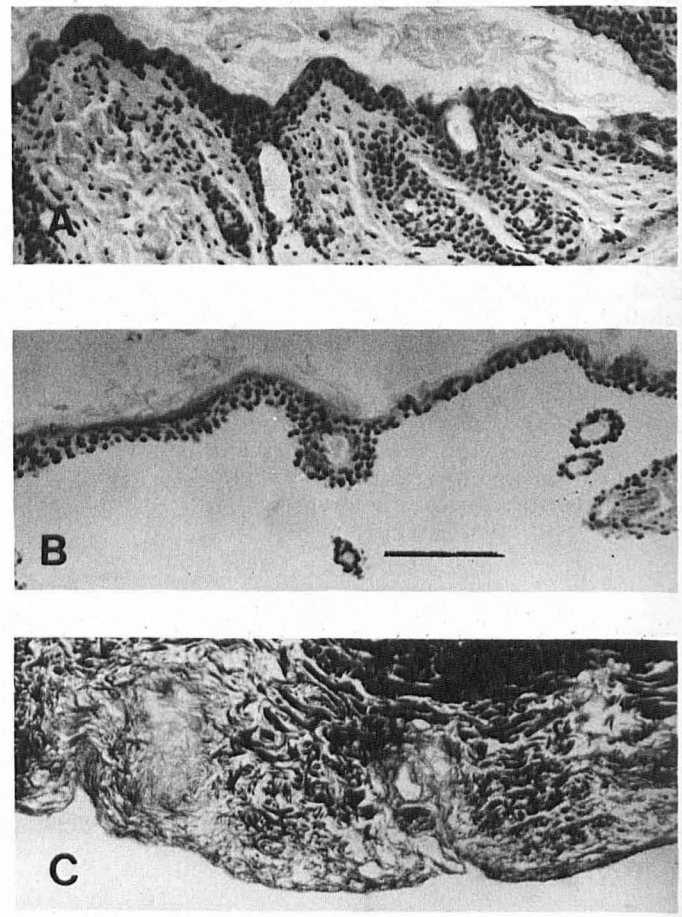

Figure 1. Skin tissue of athymic mice. *A*, Skin before dispase treatment, stained with hematoxylin. *B*, Epidermis, stained with hematoxylin. *C*, Dermis, stained with azan. *Bar* = 0.1 nm.

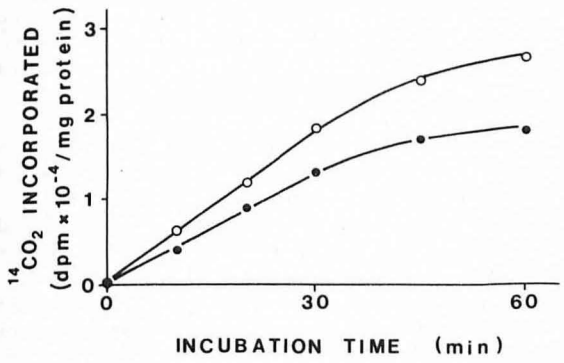

Figure 2. Time course of vitamin K-dependent carboxylation. The carboxylation was performed as described in *Materials and Methods*. Blank values without added vitamin KH_2 and DTT (200–450 dpm/incubation) were subtracted. *Solid circles,* dermis microsomes; *open circles,* epidermis microsomes.

whether a similar effect of warfarin would occur in skin tissue. Athymic mice were injected i.p. with a dose of 10 mg warfarin/kg body weight. After 24 h the mice were sacrificed, the skin was removed, and the livers and kidneys were excised. Microsomes were prepared and the amounts of endogenous substrates were measured. In all preparations an increase of noncarboxylated precursor proteins was demonstrated after warfarin treatment (Table III). This increase was comparable in skin and kidney, whereas in liver a higher level of endogenous substrate was detected. This is in agreement with earlier results obtained from bovine and rat experiments in which invariably a higher accumulation of endogenous substrate was found in liver than in other tissues [6–8]. The sum of the quantities of endogenous substrate found in the microsomes from isolated dermal and epidermal tissue was slightly lower than the amount of endogenous substrate found in total skin microsomes. This was due to the dispase treatment. Nevertheless, an increase of noncarboxylated precursor proteins was demonstrated after warfarin treatment in epidermis and dermis as well. To determine whether [^{14}C]Gla was formed in the precursor proteins, incubated samples of microsomes from warfarin-treated mice were heated at 110°C in 6 N HCl for 24 h. This treatment resulted in a loss of 59% of the bound ^{14}C in total skin microsomes, 47% in dermal microsomes, 56% in epidermal microsomes, and 54% in liver microsomes. The theoretical loss of ^{14}C from [^{14}C]Gla labeled in one of the carboxyl groups would be 50%. From these results we concluded that under our experimental conditions Gla residues had been formed by skin microsomes.

Presence of Vitamin K-Reductase In bovine liver and other nonhepatic microsomes, vitamin K-dependent carboxylase has been shown to be closely linked to another enzyme, vitamin K reductase. The latter enzyme is responsible for the reduction of vitamin K quinone to vitamin K hydroquinone, which is an essential cofactor in the carboxylation reaction. To detect the presence of vitamin K reductase in dermis and epidermis, in vitro carboxylation studies were performed with vitamin K quinone as a cofactor instead of vitamin K hydroquinone. The carboxylation rates were about 50% of the vitamin K hydroquinone-stimulated reaction for dermis and epidermis as well as for total skin microsomes. This is comparable with previous results found in bovine liver microsomes. In addition, the in vitro inhibition by warfarin of vitamin K quinone reductase was measured. The carboxylation was carried out with vitamin K quinone as a cofactor and at various warfarin concentrations. In all microsomal preparations, a warfarin concentration of 0.002 μM inhibited the carboxylation rate by 50%. From these results we concluded that vitamin K reductase is present in both dermal and epidermal microsomes.

Table III. Amounts of Accumulated Endogenous Substrate after Warfarin Treatment

Tissue	Amounts of Endogenous Substrate (dpm/60 min/mg protein)	
	Normal	After Warfarin Treatment
Liver	4,970	14,460
Kidney	770	1,540
Skin	1,260	2,560
Dermis	970	2,000
Epidermis	800	2,230

Warfarin was given i.p. in a dose of 10 mg/kg body weight to 16 athymic mice. Microsomes were prepared from the pooled tissues of normal and warfarin-treated mice, respectively, and endogenous substrate was measured as described in *Materials and Methods*. Blank values (250–500 dpm/60 min/mg protein) were subtracted. The data are the mean of triplicate measurements.

DISCUSSION

It has been well established that the vitamin K-dependent carboxylation reaction is not restricted to the liver, but that it occurs in many nonhepatic tissues as well [6,7]. In a previous study we have also demonstrated that the vitamin K-dependent carboxylases from bovine liver, kidney, lung, and testis may be regarded as a group of isoenzymes, which mainly differ in their substrate specificity [18]. In the present paper we report the discovery of vitamin K-dependent carboxylase in skin and, as in other tissues, the enzyme is located in the microsomal fraction of tissue homogenates. It remains to be investigated whether skin carboxylase is identical with one of the previously discovered carboxylases or whether it is another distinct member of the group of vitamin K-dependent carboxylases.

The Gla residues which are formed in the carboxylation reaction are known to bind strongly and selectively to Ca^{++}. The Gla-containing proteins discovered to date may be classified as follows [2]: (1) the Gla proteins in blood plasma (e.g., various coagulation factors), which are all able to bind to phospholipid surfaces via the complexed Ca^{++}; (2) the Gla proteins found in depositions of insoluble calcium salts such as bone, dentin, renal stones, and atheromatous plaques; (3) the membrane-bound Gla proteins, examples of which are found in the kidney and in the chorioallantoic membrane of chicken eggs; and (4) the proteins that do not fit into one of the 3 former groups, such as a snail neurotoxin [19] and the Gla protein in spermatozoa. Whereas most of the Gla proteins found in plasma are involved in the process of blood coagulation, the function of the other proteins is less clear at this moment.

In an attempt to estimate the physiologic importance of skin carboxylase, we have tried to identify its putative product. Obviously, this should be a Gla-containing protein and we have tried to find such a protein in KOH extracts of hair and nail, in sweat, and in normal human and animal skin homogenates. In none of these products could Gla be detected. However, skin homogenates, like liver homogenates, contain too many proteins to detect Gla without further purification. On the other hand, a Gla-containing protein has been reported to occur in the pathologic depositions of calcified material in the skin of patients suffering from scleroderma and dermatomyositis [12]. Obviously the latter protein belongs to the category mentioned under (2) (see above), but whether it is indeed produced by skin carboxylase is not certain at this moment.

Speculating about the function of the skin Gla protein we would like to mention 2 possibilities. In the first place the protein might play a role in the calcium metabolism in skin. Indications in favor of this hypothesis were obtained from clinical studies in which patients with dermatomyositis and massive depositions of subcutaneous calcific nodules were treated with low doses of warfarin. This treatment induced a profound and rapid decrease of the calcified lesions [20]. As warfarin inhibits the formation of

Gla, these data suggest a possible role for the skin Gla protein in the regulation of the calcification process.

Alternatively, a pathologic effect of warfarin on skin has been described: especially during the early stages of oral anticoagulant treatment skin necrosis may occur [21]. It has been proposed that the latter phenomenon may be the result of an increased thrombogenic activity in the capillaries induced by a protein C deficiency [22]. Protein C is a Gla-containing plasma protein, produced by the liver and indeed it has an anticoagulant activity [23]. Without doubt its synthesis is inhibited by warfarin. It is remarkable, however, that the disturbed balance between protein C and the vitamin K-dependent coagulation factors has been reported to cause ischemic necrosis of capillaries in skin only and not in other tissues. Therefore another protein may also be involved in the occurrence of this type of thrombosis. Such a protein might be the putative skin Gla protein which is produced by skin carboxylase. In this paper we have shown that coumarin derivatives directly inhibit skin carboxylase and thus the carboxylation of the skin Gla protein. This might result in a decresaed biologic activity of the latter, which may play an additional role in the observed skin necrosis.

Obviously further research is wanted to elucidate the function of the skin Gla protein and thus the physiologic importance of the vitamin K-dependent skin carboxylase.

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