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Indirect inhibition of vitamin K epoxide reduction by salicylate

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Salicylate antagonizes the vitamin K-dependent biosynthesis of clotting factors in the rat and produces an elevation of the ratio of vitamin K epoxide to vitamin K in the liver. Vitamin K epoxide is reduced to vitamin K by a vitamin K epoxide reductase, and 1 mM salicylate was required to cause a 50% inhibition of the dithiothreitol-dependent in-vitro reduction of vitamin K epoxide by this enzyme. This enzyme was, however, inhibited 50% by as little as 70–80 μM salicylate when reducing equivalents for the reaction were furnished by endogenous cytosolic reductants. This effect on the cytosolic reductant supply was shown to be unrelated to a previously demonstrated inhibition of DT-diaphorase by salicylate. The concentrations of salicylate at which significant inhibitory effects are exerted in-vitro (50–100 μM) are below the 200 μM levels observed in the livers of rats given an anticoagulating dose of salicylate.

Vitamin K is required for the post-translational modification of glutamyl residues in precursor proteins to γ-carboxyglutamyl Gla residues in clotting factors II, VII, IX, X, and other proteins (Suttie & Jackson 1977). This microsomal carboxylase requires O_2, CO_2, and vitamin K hydroquinone; and carboxylation appears to be coupled to the 2,3-epoxidation of the vitamin (Suttie 1980; Larson et al 1981). Vitamin K epoxide formed by this system can be reduced by a number of enzymes which can be demonstrated in liver microsomes. A thiol-dependent vitamin K epoxide reductase (Zimmerman & Matschiner 1974) reduces vitamin K epoxide to the quinone which can be further reduced to the hydroquinone by DT-diaphorase (Martius et al 1975; Wallin et al 1978; Fasco & Principe 1982a), other pyridine nucleotide-dependent reductases (Wallin & Hutson 1982), and by thiol-dependent vitamin K reductases (Fasco & Principe 1980, 1982b; Sherman & Sander 1981). These activities appear to be involved in recycling the microsomal pool of vitamin K during action of the vitamin. It has been suggested that a single thiol-requiring enzyme catalyses the reduction of both vitamin K quinone and vitamin K epoxide (Fasco et al 1982). The metabolic pathway of in-vivo significance which supplies reducing equivalents to this reductase has not been identified, but under in-vitro conditions dithiothreitol (DTT) can substitute for this natural reductant.

Warfarin and other coumarin anticoagulants interfere with the biosynthesis of vitamin K-dependent clotting factors by inhibiting this thiol-dependent vitamin K epoxide reductase (Matschiner et al 1974; Hildebrandt & Suttie 1982) with a resultant increase in the ratio of liver vitamin K epoxide to vitamin K (Bell & Matschiner 1970). Salicylate has also been shown to interfere with the biosynthesis of the vitamin K-dependent clotting factors in man (Meyer & Howard 1943; Shapiro et al 1943; Barrow et al 1967; Clausen & Jager 1946), rats (Link et al 1943; Hildebrandt & Suttie 1983a), perfused rat livers (Owens & Cimino 1980, 1983), and rabbits (Rapoport et al 1943; Park & Leck 1981). In salicylate-treated animals, a warfarin-like accumulation of vitamin K epoxide occurs in both liver (Hildebrandt & Suttie 1983a) and plasma (Park & Leck 1981), but the in-vitro actions of salicylate are distinctly different from those of warfarin. Salicylate is a weak inhibitor of DTT-dependent vitamin K epoxide reductase activity but is a potent inhibitor of DT-diaphorase and of the pyridine nucleotide-dependent reduction of vitamin K which is at least partially dependent on this enzyme (Hildebrandt & Suttie 1983a).

Although this effect on DT-diaphorase appeared to explain the ability of salicylate to antagonize clotting factor synthesis in the marginally vitamin K-deficient rat, it did not readily explain the increase in liver vitamin K epoxide observed in these rats. This report deals with a further investigation of the biochemical basis for the salicylate-induced accumulation of vitamin K epoxide in rat liver. A prelimi-

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nary report of these results has been presented (Hildebrandt & Suttie 1983b).

METHODS

Enzyme preparations. Male Holtzman rats (200 g) were fasted overnight, decapitated, and livers rinsed and homogenized in 2 ml g^{-1} of 0·25 M sucrose/0·025 M Imidazole-HCl, pH 7·2 (SI buffer). Debris was removed by centrifugation at 10 000*g* for 10 min. Microsomes were pelleted from the supernatant by centrifugation at 105 000*g* for 60 min and surface-washed with SI buffer. The 105 000*g* supernatant (cytosol) was filtered through glass wool and, when desirable, recentrifuged at 105 000*g* for 60 min to remove residual particulates. The microsomes were washed by resuspending the pellets in the supernatant volume of 0·15 M Tris-HCl, pH 8·0, and were repelleted at 105 000*g* (DePierre & Dallner 1976). DTT-dependent vitamin K epoxide reductase activity could be measured in lyophilized microsomal preparations, but fresh microsomes prepared from the livers of non-etherized animals were used to measure epoxide reductase activity dependent upon endogenous reductants.

Cold-shocked microsomes were prepared by resuspending the washed microsomal pellets in the supernatant volume of distilled water. The suspension was brought to 30 °C for 15 min, then poured into a shallow ice-cold dish (Glaumann & Dallner 1968). The membranes were recovered by recentrifugation at 105 000*g*. Lysed microsomes were prepared by suspending microsomal pellets or Tris-washed microsomal pellets in SI buffer containing 0·2% Triton X-100 (Carlisle & Suttie 1980). In some experiments lysed membranes were recovered by ultracentrifugation and resuspended in SI buffer to remove Triton X-100.

Assay of vitamin K epoxide reductase activity. Microsomes and cytosol were used at concentrations equivalent to 0·5 g liver ml^{-1}. Microsomal pellets were resuspended using a Dounce glass homogenizer in the supernatant volume of SI buffer or cytosol. The suspension (0·4 ml) was incubated at 25 °C with 20 μM vitamin K epoxide added in 4 μl of 1% Emulgen 911 (KAO Atlas, Tokyo). Other additions were made in 0·04 ml of SI buffer, as indicated in Figure and Table legends. Reactions were stopped by the addition of 2 vol 10 mM $HgCl_2$. Buffer, no-reductant, and/or pre-mercury-treated blanks were included in the experiments when appropriate. Samples were extracted and analysed for vitamin K and its epoxide by reverse-phase hplc as previously described (Hildebrandt & Suttie 1983a).

Determination of liver salicylate. Male rats injected intraperitoneally with 100 mg kg^{-1} sodium salicylate were anaesthetized with ether and killed at various times. Blood was flushed from the livers with 30–50 ml of 0·9% NaCl (saline) via a needle inserted into the portal vein or the vena cava just anterior to the diaphragm. The perfused livers were blotted, then frozen in plastic containers placed on dry ice. Portions were homogenized in 2 ml g^{-1} of SI buffer using a polytron homogenizer (Brinkmann Instruments, Westbury, NY) and triplicate 100 μl samples of the homogenate were acidified, extracted, and analysed for salicylate content by reverse-phase hplc (Peng et al 1978). Retention times of standards were: salicylic acid 11·4 min, salicyluric acid 6·1 min. External standardization was used, and the integrated area was linear with respect to nmoles of salicylate chromatographed. Recovery of compounds following extraction and workup was determined by addition of 10 or 100 μM salicylate to liver homogenates from uninjected animals. This recovery was constant (±1% s.e.m.) on a given day.

Vitamin and drug preparation. Vitamin K (Sigma, St Louis, MO) was converted to the 2,3-epoxide as described by Fieser et al (1941). The vitamin derivatives were purified by semipreparative hplc on μBondapak C18 with methanol as solvent. Sodium salicylate and salicylamide were obtained from Sigma. The Tris salt of Ticrynafen (2,3-dichloro-4-[2-thienylcarbonyl]-phenoxyacetic acid) was a gift of Smith, Kline, and French (Philadelphia, PA).

RESULTS

The ability of an endogenous reductant to furnish reducing equivalents for the rat liver vitamin K epoxide reductase was assessed in microsomes prepared in three different ways. Microsomal vesicles were washed with a pH 8·0 buffer to remove adsorbed cytosolic proteins from the membrane surface or lysed with 0·2% Triton X-100 to make the vesicles porous. This procedure may also extract some lipids and proteins from the membrane, and hypotonic rupture of microsomes (cold shock), which allows the release of luminal contents without altering the composition of the membrane bilayer, was also assessed. Washed, cold-shocked, and lysed microsomal preparations were tested for vitamin K epoxide reduction using reductant supplied by the preparation itself, by added cytosol, or by dithiothreitol (Table 1). In the presence of dithiothreitol the rate of reduction of vitamin K epoxide was not significantly decreased by either hypotonic rupture

or detergent lysis, but was strongly inhibited when Triton was not removed from the lysate. When no exogenous reductant was added, a low rate of reduction was observed in washed microsomes which was completely abolished by either hypotonic shock or detergent lysis. The loss of activity following these treatments would suggest that some microsomal reductant is located in the lumen of the vesicles and is lost when membrane integrity is destroyed. It is also possible that both treatments somehow inactivated or inhibited the action of the microsomal reductant. The epoxide reductase was clearly able to utilize a reductant supplied by the cytosol. This ability was retained following hypotonic treatment, and greater activity was observed when cytosol was added to Triton lysed vesicles than when it was added to intact microsomes. In contrast to the results obtained when DTT was the reductant, activity measured with the cytosolic reductant was not inhibited by the presence of Triton. In all the preparations tested, the initial rates measured with the natural reductants in either liver microsomes or cytosol were slower than the initial rate observed with DTT.

Table 1. Effect of reductant on vitamin K epoxide reductase activity. Preliminary rate measurements of each preparation were conducted to verify that product formation was still linear at the time the incubations were terminated. Subcellular fractions were used at concentrations equivalent to 0·5 g liver ml^{-1} of incubation, and values are mean ± s.e.m. for triplicate incubations.

Reductant	Reaction time (min)	Microsomal preparation (nmol product formed ml^{-1}) Tris-washed	Cold-shocked	Lysed Triton removed	Lysed Triton in assay
1 mM DTT	5	3·39 ± 0·20	4·21 ± 0·07	3·13 ± 0·36	0·56 ± 0·02
None	15	0·25 ± 0·02	<0·05	<0·05	<0·05
Cytosol	30	0·98 ± 0·05	0·62 ± 0·02	1·73 ± 0·02	2·52 ± 0·07

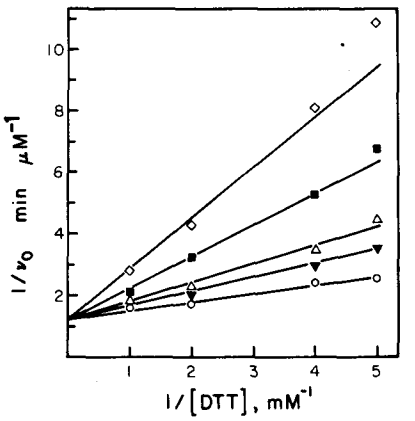

FIG. 1. Inhibition of vitamin K epoxide reductase by salicylate. Double reciprocal plot of vitamin K epoxide reductase activity, as measured by product formed at 5 min, in the presence of 0 (○), 100 (▼), 200 (△), 500 (■), or 1000 (◇) μM salicylate.

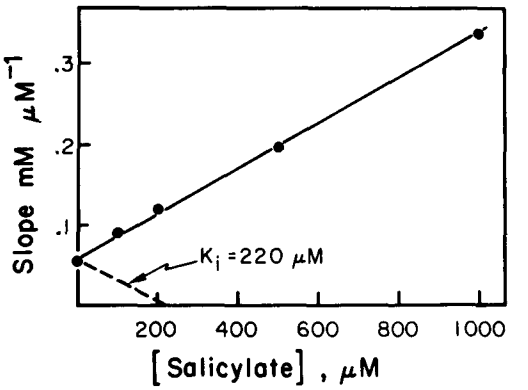

FIG. 2. Inhibition of vitamin K epoxide reductase by salicylate. Secondary plot of the data in Fig. 1. The slopes of the lines fitted to the data in Fig. 1 have been replotted as a function of inhibitor concentration. The x-intercept, shown reflected into the positive quadrant, gives the K_i.

When a microsomal suspension was prepared from lyophilized pellets, DTT-dependent vitamin K epoxide reductase activity was retained, but the endogenous reductants present in either microsomes or cytosol were no longer able to drive the reaction (data not shown). The lyophilized preparation was used to investigate the steady-state kinetics of the weak inhibitory effect of salicylate on vitamin K epoxide reduction in the presence of DTT (Fig. 1). Salicylate was clearly competitive against DTT, and a replot of the slopes (Fig. 2) gave a K_i of 220 μM for binding of salicylate to the active site of the epoxide reductase. These data confirmed the rather poor inhibition of the DTT-dependent enzyme activity by salicylate previously observed by Hildebrandt & Suttie (1983a).

Inhibition of vitamin K epoxide reduction by salicylate was also determined in the presence of the endogenous reductants in rat liver (Fig. 3). With 1 mM DTT as a reductant, the concentration of salicylate giving 50% inhibition (I50) was about 1000 μM. When the enzyme was assayed utilizing only the reductant factors present in washed microsomes, the I50 had a value of 300 μM; and when the reductant was supplied by endogenous cytosolic factors, the I50 for salicylate was reduced to only 70–80 μM. The inhibition curve obtained in the

presence of cytosol appeared to level off at approximately 20% of the control activity, and this small, apparently uninhibitable amount of product formation corresponded to the amount expected from the microsomal reductant that was present in the suspension.

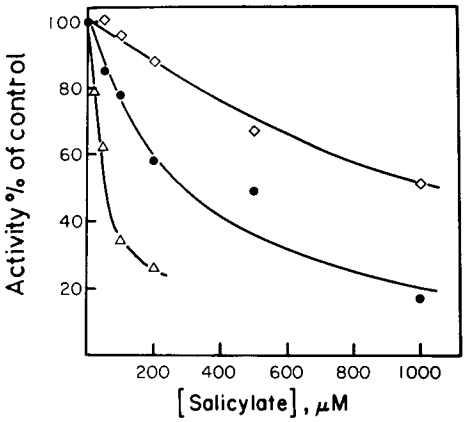

FIG. 3. Effect of reductant on the inhibition of vitamin K epoxide by salicylate. Vitamin K epoxide reductase activity was measured in the presence of 1 mM dithiothreitol for 5 min (◇), or with no added reductant for 15 min (●), or in the presence of cytosol for 30 min (△). The data points are means of duplicate incubations which differed by less than 15% (endogenous reductant) or 10% (DTT). Identical inhibition patterns were seen in two replicate experiments.

DT-diaphorase is a largely cytosolic enzyme with a sensitivity toward salicylate (Hildebrandt & Suttie 1983a) that is comparable to that of the vitamin K epoxide reductase utilizing cytosol as a reductant. This raised the possibility that DT-diaphorase was somehow involved in the salicylate sensitivity of the cytosol-dependent reduction of epoxide. Salicylate, salicylamide, and Ticrynafen (Preusch & Suttie 1983) are all strong inhibitors of DT-diaphorase, and their effects on vitamin K epoxide reduction were determined (Table 2). Under conditions where salicylate had a strong effect on the cytosol-dependent reaction, the other compounds had no effect. Thus, DT-diaphorase did not appear to be involved in the cytosol-dependent reaction. None of the three compounds had any appreciable influence on epoxide reductase activity when only the reductant supplied by the microsomal preparation itself was present.

The DTT-dependent reduction of vitamin K epoxide by cholate-treated microsomes has been shown to be stimulated by a cytosolic factor (Siegfried 1978, 1983), and the lysed microsomal preparation used in the present studies was stimulated approximately 1·5 fold when an amount of cytosol equivalent to 0·5 g liver ml^{-1} was added (Fig. 4). The addition of salicylate had a more pronounced inhibitory effect on this added increment of activity than it did on the initial reductase activity.

The concentration of salicylate in rat liver was determined at various times after a 100 mg kg^{-1}

Table 2. Effect of DT-diaphorase inhibitors on vitamin K epoxide reductase activity. Suspension of microsomes in buffer, or lysed microsomes resuspended in cytosol, were incubated at 22 °C with substrates for 15 or 25 min, respectively. Values are expressed as % of uninhibited activity which was 0·25 nmol vitamin K formed/15 min with microsomes alone, and 0·23 nmol vitamin K formed/25 min using microsomes in the presence of cytosol and are means ± s.e.m. for duplicate incubations. All salicylate incubations were significantly different ($P < 0·05$) from control incubations while there were no significant differences between salicylamide or Ticrynafen incubations and controls. Identical results were observed in a second similar study (data not shown).

		% Epoxide reductase activity	
Compound	Concentration (μM)	In washed microsomes	In lysed microsomes + cytosol
Salicylate	50	83 ± 2	60 ± 6
	200	73 ± 1	5 ± 4
Salicylamide	50	96 ± 6	97 ± 12
	200	92 ± 2	80 ± 16
Ticrynafen	50	92 ± 6	93 ± 10
	200	92 ± 8	123 ± 11

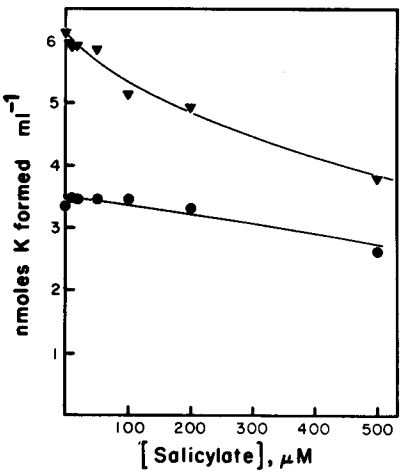

FIG. 4. Influence of salicylate on the cytosol stimulated DTT-dependent epoxide reductase activity. Lysed microsomes were resuspended in SI buffer (●) or cytosol (▼). Incubations were conducted for 10 min in the presence of 1 mM DTT. The data are uncorrected for the low amount of product formation (0·12 nmol vitamin K formed ml^{-1}) in cytosol-containing samples when dithiothreitol was omitted. The data points are means of duplicate incubations which differed by less than 10%. Identical inhibition patterns were seen in a replicate experiment.

intraperitoneal dose of the drug (Fig. 5). Despite considerable variability between individual animals, gross tissue concentrations, assuming a water content of 70%, remained between 600 and 200 μM in all animals tested. Disappearance of salicylate has been shown to be first-order in the rat (Nelson et al 1966), and data here indicated a half-time of 7·8 h for removal of salicylate from the liver. No salicyluric acid could be detected in the liver samples.

DISCUSSION

Our earlier studies (Hildebrandt & Suttie 1983a) suggested that the hypoprothrombinaemic effect of salicylate which can be observed in the hypoprothrombinaemic rat might be due to its ability to strongly inhibit the general quinone reductase, DT-diaphorase. This mechanism of inhibition would not, however, result in the increase in vitamin K epoxide noted in the plasma (Park & Leck 1981) or liver (Hildebrandt & Suttie 1983a) of salicylate-treated animals. The studies reported here have demonstrated a second potential mechanism of salicylate inhibition which is consistent with this observation. Although the DTT-dependent vitamin K epoxide reductase activity as it is normally assayed is only weakly inhibited by salicylate, the enzyme is strongly inhibited by this drug when the reductant factors in the cytosol are used to drive the reaction. When cytosol supplied the reductant for the reaction, 50% inhibition was observed at only 70–80 μM salicylate. This effect is much more potent than can be explained on the basis of an action of salicylate on the epoxide reductase alone. Rather, the results indicate a very strong effect of salicylate on the ability of the vitamin K epoxide reductase to utilize reductant factors in the cytosol. The simplest interpretation of these data is that salicylate blocks some step in an electron transport chain supplying reducing equivalents from the cytosol to the enzyme.

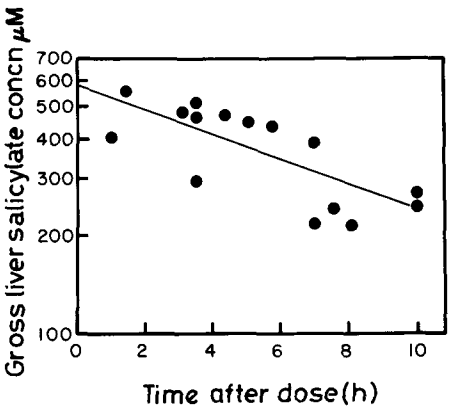

FIG. 5. Concentration of salicylate in rat liver after a 100 mg kg^{-1} dose. Liver salicylate concentrations were calculated from the amount of salicylate in duplicate extracts from homogenates of perfused livers. Each datum point represents an individual animal. The regression coefficient of the least-squares line is −0·751.

The nature of the cytosolic reductant is not known. However, the extent of reactions observed provides a minimum estimate of the amount of reductant that must be present in the preparations. The data suggest that there was at least 0·5 nmole of reductant per g of liver in the lumen of the endoplasmic reticulum and 5 nmoles of reductant per g of liver in the cytosol. These values may represent underestimates if there was incomplete recovery of these factors upon subcellular fractionation of the tissue, or if the reductant was not completely consumed when the enzymatic reaction ceased. Cytosol also stimulates the DTT-dependent reduction of vitamin K epoxide, in a manner not understood at the present time. Salicylate appeared to have some influence on the cytosol-stimulated, DTT-dependent activity, but this effect of salicylate was not as strong as its ability to inhibit the reaction dependent upon the cytosolic reductant. The present data do not distinguish whether or not interference with this cytosolic stimulatory activity contributes to the action of salicylate on the cytosol-dependent epoxide reduction.

The extreme sensitivity of DT-diaphorase to salicylate suggested the possible involvement of this enzyme in the pathway that provides cytosolic reducing equivalents to the epoxide reductase. The lack of effect of other known inhibitors of DT-diaphorase, salicylamide, and Ticrynafen on the cytosol-dependent vitamin K epoxide reduction makes it unlikely that this enzyme is involved in the salicylate-sensitive reductant pathway. The gross liver concentration of salicylate was observed to be above 200 μM for a period of 10 h after the administration of 100 mg kg^{-1} of the drug, a dose that completely blocks clotting factor biosynthesis for a period of at least 10 h in rats on a restricted vitamin K intake (Hildebrandt & Suttie 1983a). In-vitro 200 μM salicylate fully inhibited both DT-diaphorase and the cytosolic pathway supplying reductant for the vitamin K epoxide reductase. The epoxide reductase itself and the cytosolic epoxide reductase stimulatory activity could only be partially inhibited at this concentration of salicylate.

These results suggest that salicylate may interfere with clotting factor biosynthesis by blocking the reduction of vitamin K epoxide in an indirect

manner. In-vitro, this interference is only demonstrable when natural cytosolic reductants are employed in measurement of the activity, and addition of DTT bypasses the block. These findings provide an explanation for the ability of salicylate to induce the accumulation of vitamin K epoxide in the liver in-vivo, without directly inhibiting the DTT-dependent vitamin K epoxide reductase in a coumarin-like fashion in-vitro. It is also possible that inhibition of other less sensitive reactions may contribute to the block of epoxide reduction observed in-vivo.

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