Stimulation by unsaturated fatty acid of squalene uptake in rat liver microsomes

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Abstract Supernatant protein factor (SPF) and anionic phospholipids such as phosphatidylglycerol (PG) stimulate squalene epoxidase activity in rat liver microsomes by promoting [^{3}H]squalene uptake as well as substrate translocation (Chin, J., and K. Bloch. 1984. *J. Biol. Chem.* **259:** 11735–11738). This process is postulated to be membrane-mediated and not carrier-mediated. Here we show that treatment of PG with phospholipase A_2 in the presence of bovine serum albumin abolishes the stimulatory effect of SPF on epoxidase activity. Disaturated fatty acyl-PGs are not as effective as egg yolk lecithin PG in the SPF effect. These findings suggest an important role for the unsaturated fatty acid moiety of PG. We also show that at submicellar concentrations, *cis*-unsaturated fatty acids stimulate microsomal epoxidase activity whereas saturated fatty acids do not. This effect is due to an increase in substrate uptake which in turn may facilitate substrate availability to the enzyme. **—Chin, J., and K. Bloch.** Stimulation by unsaturated fatty acid of squalene uptake in rat liver microsomes. *J. Lipid Res.* 1985. **26:** 819–823.

Supplementary key words oleate • squalene epoxidase • supernatant protein factor • anionic phospholipid

The conversion of squalene to lanosterol in liver is catalyzed by two cholesterogenic enzymes located in the endoplasmic reticulum, squalene epoxidase and squalene epoxide-lanosterol cyclase. Stimulation of these enzyme activities in preparations of liver microsomes by supernatant protein factor (SPF) and anionic phospholipid such as phosphatidylglycerol (PG) or phosphatidylserine (1–5) has been shown to be due to effects on substrate uptake (6) and the postulated substrate translocation from an inactive to an active membrane pool (7, 8). The postulated mechanism does not involve a conventional protein carrier function (9) since SPF has not been shown to bind squalene or squalene epoxide under any experimental conditions (2, 5). PG, however, which is required for SPF to be effective, binds both to SPF and to the microsomal membrane. As previously reported, lysophosphatidylserine does not substitute for phosphatidylserine in the SPF stimulation of epoxidase (2). Here we show that PG treated with phospholipase A_2 in the presence of bovine serum albumin is completely ineffective and that disaturated fatty acyl-PGs only partially substitute for normal PG in the SPF effect. We also describe stimulation of microsomal squalene uptake and conversion by submicellar concentrations of unsaturated fatty acids. The present findings suggest an important role for the unsaturated fatty acid moiety of anionic phospholipids.

MATERIALS AND METHODS

Materials

Female CD rats were supplied by Charles River Breeding Laboratories. The acetone fraction of SPF was prepared from the high-speed supernatant fraction of rat liver homogenate (6, 10). Rat liver microsomes were prepared as described (8) in 100 mM Tris-HCl, pH 7.5, – 0.2 mM EDTA and stored at −70°C. Reagents were purchased from Sigma. [^{3}H]Squalene was from New England Nuclear. Squalene epoxide was synthesized by the method of Nadeau and Hanzlik (11). Stock solutions of fatty acids were made in ethanol. For assays the solvent was evaporated under nitrogen, and then the reaction components were added. Concentrations used are indicated in the figure legends.

Assay

Squalene epoxidase activity was determined as previously described (7, 8). The reaction mixture in a final volume of 1 ml, contained [^{3}H]squalene (40,000 cpm, 15 nmol) dispersed with 0.004% Tween 80, 1.0 to 1.5 mg of microsomal protein, 0.5 mM AMO-1618 to inhibit squalene epoxide-lanosterol cyclase, 10 μM FAD, and 1 mM NADPH (7, 8) in 100 mM Tris-HCl, pH 7.5, – 0.2

Abbreviations: SPF, supernatant protein factor; PG, phosphatidylglycerol; 18:1, oleic acid; 18:2, linoleic acid; 16:0 palmitic acid.

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mM EDTA, and, when present, 200 to 250 μg of PG and 0.8 to 1.2 mg of SPF protein (acetone fraction). After 30 min at 37°C, reactions were terminated with chloroform–methanol 2:1. Substrate and product were extracted, separated by thin-layer chromatography, and analyzed for radioactivity (5). Activity is expressed as nmol of squalene epoxide formed in 30 min per mg of microsomal protein. In some experiments, PG was treated with phospholipase A_2 in the presence of bovine serum albumin according to Caras and Bloch (4). Protein was determined by the method of Lowry et al. (12).

RESULTS AND DISCUSSION

Effects of modified PG on the SPF effect

For the stimulation of microsomal squalene epoxidase and squalene epoxide-lanosterol cyclase by SPF, the prior or simultaneous presence of anionic phospholipid such as PG is required (1–6). To determine the structural features essential for the phospholipid effect, PG was treated with different phospholipases. Anionic lysophospholipids are relatively ineffective as substitutes of anionic phospholipids (2). Preincubation of PG with phospholipase A_2 in the presence of 2% bovine serum albumin completely abolished the PG effect (**Fig. 1B**). Since this loss of activity could be due to the removal of an unsaturated fatty acid moiety, the effects of disaturated fatty acyl-PG on epoxidase activity were examined. The data in **Fig. 2** show that, although there was a small stimulation with either dipalmitoyl- or distearoyl-PG alone, in the presence of SPF these saturated phospholipids were not as effective as PG in stimulating squalene epoxidase activity.

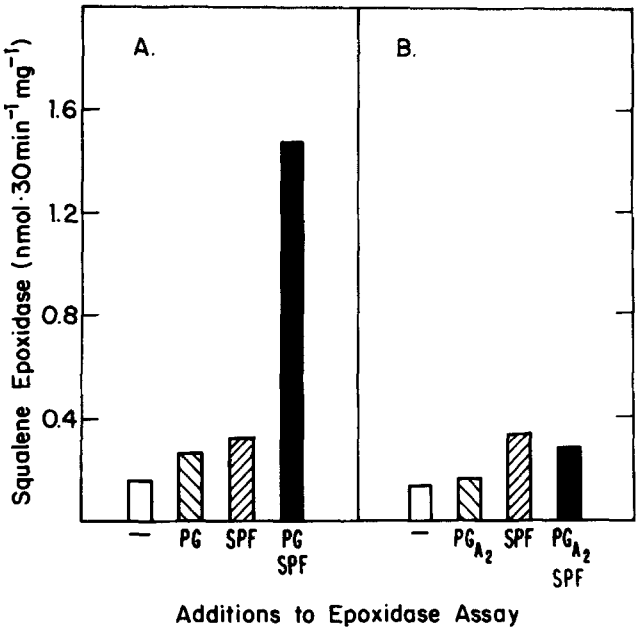

Fig. 1. Effect of phospholipase A_2-treated PG on the SPF stimulation of squalene epoxidase activity. PG was treated with phospholipase A_2 in the presence of 2 mg of bovine serum albumin per ml as described (4) and used in the assay for epoxidase activity in the presence or absence of SPF. The results are the average of duplicates which differed by less than 5%. A, microsomes with untreated PG and B, microsomes with phospholipase A_2-treated PG.

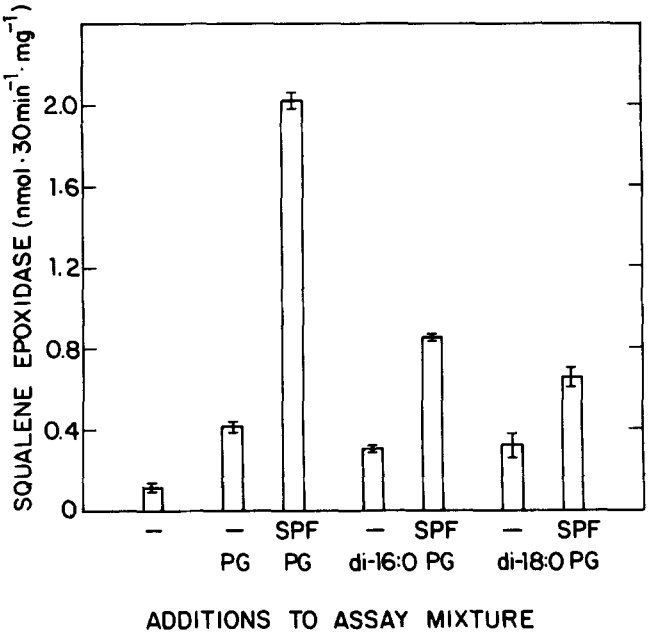

Fig. 2. Effect of PG, dipalmitoyl-pG, or distearoyl-PG in the absence and presence of SPF on squalene epoxidase activity. Phospholipid vesicles were prepared in assay buffer as previously described (5) to a concentration of 1 mg/ml. Squalene epoxidase activity is expressed as the mean of at least three determinations ± the standard error.

Effect of oleate or palmitate on epoxidase activity

Oleate alone stimulated the microsomal epoxidation of squalene to its epoxide about 2- to 4-fold at submicellar concentrations (10^{-6}–10^{-4} M) (**Fig. 3A**). The di-unsaturated fatty acid, linoleate, was also tested and had effects similar to oleate (data not shown). Submicellar concentrations of palmitate stimulated epoxidase activity only slightly (Fig. 3B).

The effects of oleate on epoxidase activity were also tested in combination with SPF, PG, or both (**Fig. 4**). There is a small additive effect with oleate and PG which might be due to the net negative charges contributed by both lipids (13). This could result in an increase in membrane permeability to substrate. In the presence of SPF, oleate shows no additive effect and vice versa. In the presence of SPF and PG, oleate inhibits slightly. The principal conclusion from the experiments is that the oleate stimulation of epoxidase, which is substantial, is not superimposable on the PG and SPF effects. While oleate appears to substitute partially for PG, SPF, or both, it apparently does not affect squalene uptake and/or squalene epoxidation by an independent mechanism.

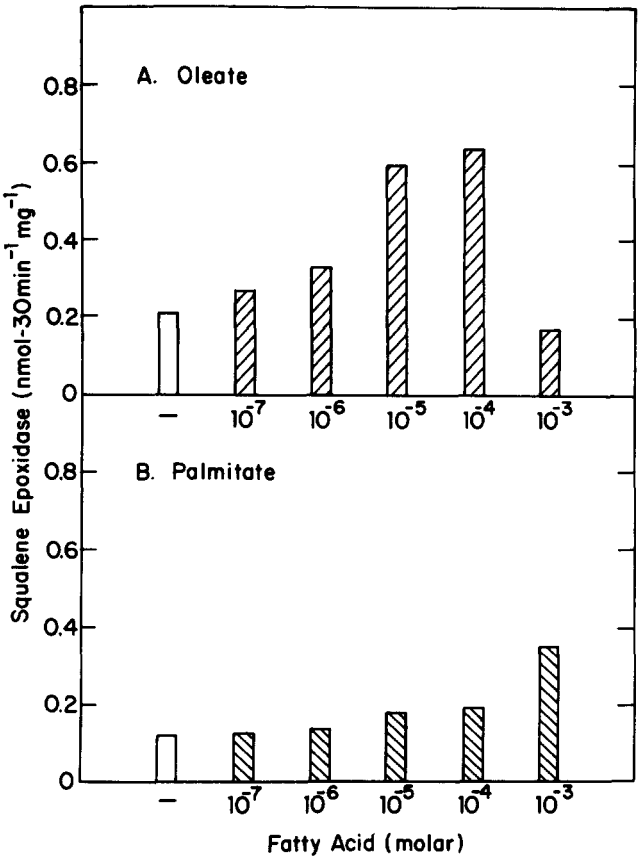

Fig. 3. Effect of oleate and palmitate on squalene epoxidase activity in microsomes. Fatty acids were added to assay tubes and dried as described in Materials and Methods. A, increasing oleate concentration and B, increasing palmitate concentrations.

Effect of temperature

The effects of SPF and PG on squalene epoxidase activity are reduced at lower temperatures (3, 7). This is consistent with the postulate that they involve membrane-associated processes (5–8). The epoxidase response to unsaturated fatty acids at both 21°C and 37°C (**Fig. 5**) is also temperature-sensitive.

Effect of oleate on substrate uptake

SPF and PG have been shown to stimulate microsomal uptake of the squalene (6), the first step in the assay of membrane-associated conversion by epoxidase and cyclase with exogenous substrate. The effect of fatty acids on substrate uptake is shown in **Table 1.** Both oleate and linoleate raise microsomal squalene incorporation about 3-fold, whereas palmitate increases uptake 1.5-fold. Concomitant with, or as a result of, increased squalene uptake, squalene epoxidase activity in these preloaded microsomes is proportionately elevated (Table 1). These results are strikingly similar to those observed with SPF and PG (6).

In order to be effective in stimulating squalene incorporation into microsomes, oleate must be present in the assay medium along with substrate. The effect is lost if particles exposed to oleate in the absence of substrate are washed and resuspended prior to squalene addition (data not shown). Thus, microsomal membranes cannot be stably primed for squalene uptake with oleate in the absence of substrate, in contrast to the results obtained with PG (6). Both oleate and palmitate are stably incorporated into the membrane fraction, but only oleate stimulates squalene uptake. The stimulation of microsomal uptake of squalene by unsaturated fatty acid may be due either to a transient effect on some membrane property or to its direct interaction with the substrate itself. In this connection, it should be noted that the oleate effect cannot be mimicked by detergents. Squalene is normally dispersed with 0.004% Tween 80. Increasing the concentration of the detergent to 0.10%, which is above its micellar concentration (0.02%), decreased squalene epoxidase activity about 3-fold (data not shown). Submicellar concentrations of Triton X-100 caused little or no stimulation of squalene conversion.

Oleic acid also has no effect on the activity of squalene epoxidase solubilized with Triton X-100 and reconstituted with phosphatidylcholine, as previously described (6, unpublished observations). This excludes the possibility that an interaction between oleate and substrate plays a

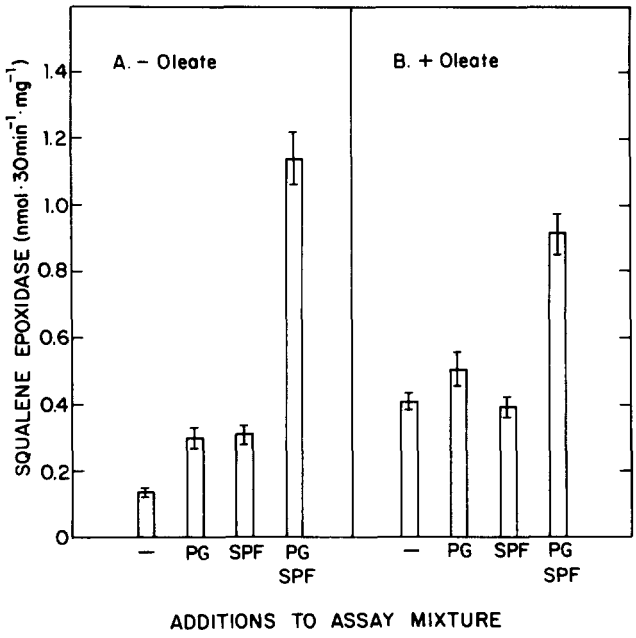

Fig. 4. Effect of oleate on squalene epoxidase activity in the presence or absence of SPF and PG. Epoxidase activity was assayed in microsomes incubated in the presence or absence of PG (200 μg/ml), SPF (1.2 mg/ml), and/or oleate (100 μM). The values presented are the means of at least four determinations ± the standard error. A, oleate absent and B, oleate present.

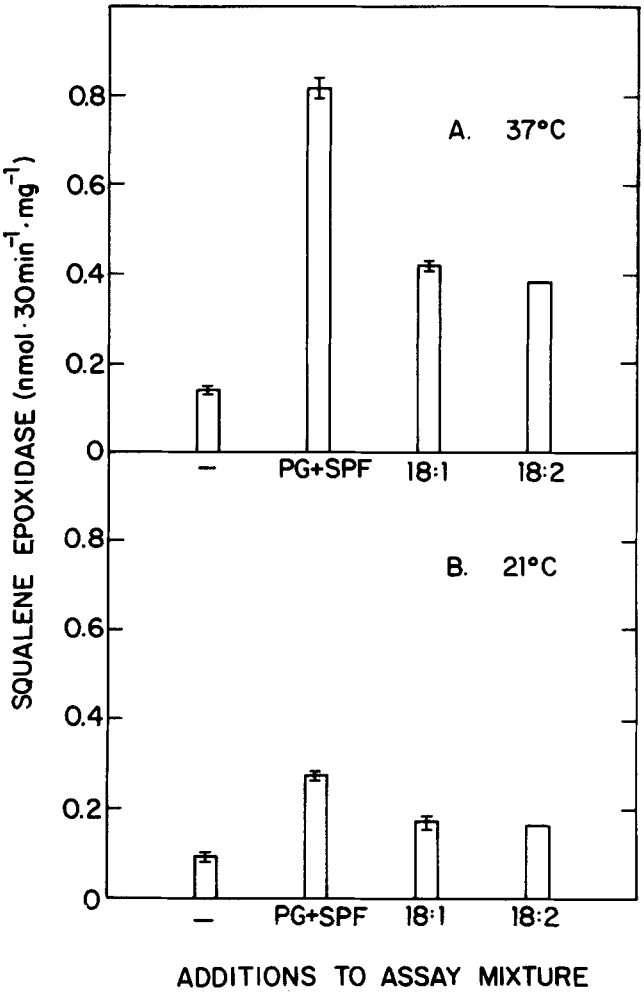

Fig. 5. Temperature dependence of the stimulation of squalene epoxidase activity by unsaturated fatty acids or SPF and PG. Squalene epoxidase activity was assayed in the absence or presence of 100 μM fatty acid as described in Fig. 4, and expressed as the mean of at least three determinations ± the standard error. The data for linoleate are expressed as the average of two determinations which differed by less than 5%. A, microsomes assayed at 37°C and B, microsomes assayed at 21°C.

role in the control of epoxidase activity per se. The possibility remains that oleate is interacting with the substrate. Even though submicellar concentrations of fatty acids and detergents were used, a mixture of oleate, squalene, and Tween 80 may have altered properties such that uptake is facilitated.

Effect of oleate on intramembrane and intermembrane transfer of substrate

For further comparison with SPF and PG, the effect of oleate on intramembrane and intermembrane transfer of squalene was investigated. To test intramembrane substrate transfer, microsomes were preloaded with squalene in the absence of cofactors, washed, and subsequently assayed for epoxidase activity (3, 7) in the presence or absence of oleate. Under these conditions, i.e., by-passing uptake of exogenous substrate, unsaturated fatty acid did not increase squalene epoxidase activity. This is in contrast to the results obtained with SPF and PG which have been interpreted as evidence for translocation of substrate from inactive to active squalene pools in the membrane (3, 7).

Oleate also had little effect on intermembrane transfer of substrate, as determined by the mixed microsome assay system. This assay measures the conversion of substrate transferred from a squalene-containing, but trypsinized, population of donor microsomes to a squalene-free, but enzymatically active, population of acceptor microsomes (7). That oleate failed to bring about intermembrane translocation of squalene was also shown by direct measurement (8) of [^{3}H]squalene in the acceptor microsomes by density gradient centrifugation. None was found (data not shown). These results also are in contrast to the ability of SPF and PG to promote substrate transfer from the donor to acceptor population (7, 8) and subsequent squalene epoxidation. The lack of an oleate effect on the translocation of substrate preloaded into microsomes suggests that oleate promotes only substrate uptake.

Free fatty acids readily enter membranes, and have been shown to affect varoius structural and functional membrane properties (14, 15). Unsaturated fatty acids may alter permeability of microsomes to solutes by increasing the net negative charge (13) or by increasing the fluidity of certain membrane domains (14). Whether oleate increases microsomal uptake of squalene by affecting some membrane property or the substrate itself is unclear at this point.

The present experiments refine the role of anionic phospholipids acting in conjunction with SPF in the

TABLE 1. Effect of fatty acids on [^{3}H]squalene uptake and its subsequent conversion

Preincubation Condition	Substrate Uptake	Squalene Exposidase
	cpm/mg of protein	*nmol · 30 min^{-1} · mg^{-1}*
	7300	0.19
Palmitate	11000	0.32
Oleate	21400	0.61
Linoleate	21600	0.67

Rat liver microsomes were loaded with [^{3}H]squalene in the presence or absence of palmitate, oleate, or linoleate (25–28 μg/ml). The suspension was flushed with nitrogen and cofactors were omitted. After 10 min at 37°C, the mixture was centrifuged at 105,000 *g* for 1 hr at 4°C. The high-speed supernatant was removed and the pellet was resuspended and rewashed. Duplicate aliquots of the resuspended pellet and supernatant were used to determine the content of [^{3}H]squalene and protein. Duplicate aliquots of the pellet fraction were also assayed for squalene epoxidase activity after admission of air and addition of cofactors. The values are the averages of duplicates which differed by less than 5%.

squalene epoxidase system. An unsaturated fatty acyl moiety in PG is clearly beneficial if not essential in this process.

This work was supported by grants-in-aid from the National Institutes of Health (HL-02477), and the National Science Foundation (PCM-03499). Jean Chin was supported by a National Research Service Award, 1 F32 HL-6714.

Manuscript received 21 September 1984.

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