Regulation of squalene epoxidase activity and comparison of catalytic properties of rat liver and Chinese hamster ovary cell-derived enzymes

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Abstract  Squalene epoxidase activity has been studied in cell-free preparations of Chinese hamster ovary (CHO) cells and rat liver. In contrast to rat liver microsomal squalene epoxidase, the enzyme of CHO cells is only slightly activated by the autologous cytosolic fraction, whereas phosphatidylglycerol or rat liver cytosolic preparations are potent stimulators of this enzyme. Triton X-100, a known stimulator of the hepatic squalene epoxidase, has no activating effect on the enzyme of CHO cells. The squalene epoxidase activity of both rat liver and CHO cells varies significantly according to the lipid content of the growth medium or diet. The changes in enzyme activity are shown to be entirely due to altered microsomal enzyme per se and not to changes in the activating properties of the soluble fraction. These results further support the proposed regulatory role of squalene epoxidase in cholesterogenesis.


Supplementary key words cholesterologenesis • 2,3-oxidosqualene • supernatant protein factor • carrier protein

The first oxygen-dependent step in the biosynthesis of sterols is the conversion of squalene to 2,3-oxidosqualene, a reaction catalyzed by the microsomal enzyme squalene epoxidase (EC 1.14.99.7, squalene 2,3-monooxygenase). The rat liver enzyme requires both microsomes and high speed supernatant cytosolic fraction (S100) for full activity (1, 2). This requirement for S100 can be satisfied by a combination of FAD, anionic phospholipids, and a supernatant protein factor (SPF)(3,4). In addition, the two latter components can be replaced by Triton X-100 (5). The exact mechanism by which SPF stimulates the epoxidation of squalene is still not known, but it is proposed to promote uptake and translocation of the substrate between nonavailable and available membrane pools (6).

In contrast to the rat liver enzyme, only little is known about squalene epoxidase in extrahepatic cells. However, in such cells, the epoxidation of squalene has been suggested to represent a secondary regulatory step in cholesterogenesis (7-9). We have previously shown that, in Chinese hamster ovary (CHO) cells, squalene epoxidase activity is subjected to regulation concomitant to the extent of cholesterogenesis. Enhanced sterol synthesis due to an increase in squalene epoxidation was observed in cells grown in the presence of lipid-depleted serum (LDS) as compared to cells grown with lipoprotein-containing serum (10).

In this report we compare the catalytic requirements of microsomal squalene epoxidase of rat liver and CHO cells. We also show that both the hepatic and the extrahepatic enzyme undergo changes in activity at various cholesterogenic states. These results, together with our previous observations in intact CHO cells (10), further support the proposed regulatory role of squalene epoxidase in cholesterogenesis.

MATERIALS AND METHODS

Materials

DL-[2-14C]Mevalonate (51 mCi/mmol) was purchased from the Radiochemical Center, Amersham. [14C]-Squalene was biosynthesized in our laboratory from [14C]mevalonate according to the method of Popjak (11) with slight modifications (7). Mevalonate, cholesterol, and cholestyramine were from Sigma. Mevinolin was kindly provided by Dr. A. W. Alberts from Merck Sharp

Abbreviations: CHO, Chinese hamaster ovary; S100, cytosolic fraction; SPF, supernatant protein factor; LDS, lipid-depleted serum; MVA, mevalonate; FCS, fetal calf serum; PG, phosphatidylglycerol; N-diet, normal chow diet; CM-diet, cholestyramine-mevinolin-containing diet; S-diet, cholesterol production-suppressing diet; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
and Dohme. Fetal calf serum (FCS) and F-12 medium were from Maagar, Israel. Lipid-depleted serum (LDS) (44.5 mg of protein/ml) was prepared according to the method of Cham and Knowles (12). All chemicals were of analytical grade.

Animals

Female rats (~150 g) were adapted to a 10-hr dark/14-hr light illumination regimen for over 2 weeks before being given one of the following diets: i) normal rat chow (N); ii) rat chow containing 5% (w/w) cholestyramine for 7 days plus 3 days on chow supplemented with 5% (w/w) cholestyramine plus 0.15% (w/w) mevinolin (CM); or iii) rat chow containing 3% (w/w) cholesterol for 7 days followed by an intraperitoneal injection of 1 ml of 0.5 M mevalonate 1 hr prior to killing the rats (S, sterol-supplemented diet). All animals were killed 5 hr after onset of the dark period.

Cells

CHO cells were grown in suspension culture at 37°C in F-12 medium containing 10% FCS; penicillin, 100 units/ml; streptomycin, 100 μg/ml; and nystatin, 12.5 units/ml. In order to grow the cells in the presence of various sera, 400 ml of cell suspension (approximately 1 x 10⁷ cells) was centrifuged at 200 g for 5 min and washed with F-12 medium. Thereafter, the cells were suspended in 10 ml of F-12 medium and equally divided into identical suspension culture flasks containing 700 ml of medium with either 10% FCS (CHO-FCS) or 10% (CHO-LDS).

Preparation of microsomes and supernatant fractions

For each fractionation, 500 ml of CHO cell suspension (ca. 1 x 10⁸ cells) was centrifuged at 200 g for 5 min, washed with phosphate-buffered saline (PBS) and with hypotonic buffer, containing 1 mM Tris-HCl, pH 7.5, and 1 mM EGTA (buffer K). The cell pellet was suspended in 1.5 ml of ice-cold buffer K and then homogenized in a glass Teflon homogenizer. Sucrose was added to the homogenate to give a final concentration of 0.3 M. After additional homogenization, the homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was recentrifuged at 105,000 g for 60 min to give a soluble fraction of the FCS-grown cells (CHO-FCSs₁₀₀), and LDS-grown cells (CHO-LDSS₁₀₀). The sediments of the corresponding centrifugation were suspended in PBS containing 0.25 M sucrose to give a final protein concentration of 12–14 mg/ml. All fractions were stored frozen in liquid N₂.

Rat liver microsomes and supernatant fractions were prepared according to Yamamoto and Bloch (1) except that the working buffer was 10 mM Tris HC1, pH 7.4, containing 0.3 M sucrose. The soluble fractions of livers from rats maintained on N-diet (NS₀), CM-diet (CMS₀), and S-diet (SȘ₀), as well as the corresponding microsomal preparations, were stored in liquid N₂.

Assay of squalene epoxidase

The squalene epoxidase assay was essentially the same as described by Tai and Bloch (2) except for some modifications. The standard assay mixture contained, in a total volume of 500 μl, the following reagents: Tris-HCl buffer, 0.1 M, pH 7.4; NADPH, 1 mM; EDTA, 1 mM; AMO 1618, an inhibitor of squalene 2,3-oxide lanosterol cyclase (13), 0.4 mM; FAD, 0.01 mM; 100,000 dpm of [14C]squalene (sp act 100 Ci/mol) dispersed in 40 μl of 0.3% Tween 80 in acetone; and enzyme as indicated in the legends of the tables and figures. When present, phosphatidylglycerol (PG) and Triton X-100 concentrations were 0.1 mg/ml and 0.1%, respectively.

Unless otherwise noted, the incubations were agitated in open test tubes at 37°C for 1 hr, and stopped by addition of 0.5 ml of 10% methanolic KOH. After 30 min, 20 μg each of squalene, lanosterol, and cholesterol were added to the tubes as nonlabeled carriers. Acetone (0.5 ml) was added and the nonsaponifiable material was extracted three times from the mixture with 1 ml of hexane. The combined extracts were evaporated under a stream of N₂. The residue, taken up in a small volume of hexane and ethyl acetate, was spotted on thin-layer silica gel plates which were then developed to a height of 10 cm with 0.5% ethyl acetate in benzene as the solvent system. The plate was divided into 0.5-cm zones and each zone was scraped into a vial and counted for radioactivity. The amount of AMO 1618 used in the reaction did not completely inhibit the conversion of 2,3-oxidosqualene to sterols; the sterols formed varied between 0–10% of total products. Epoxidase activity is therefore expressed either in terms of dpm of 2,3-oxidosqualene or the sum of dpm of 2,3-oxidosqualene and sterols formed per h (see Figure Legends).

Assay of HMG-CoA reductase

HMG-CoA reductase activity was assayed according to a modification (14) of the method of Shapiro et al. (15) using 60,000 dpm of 14C-labeled HMG-CoA (4.5 μCi/μmol) as substrate.

RESULTS AND DISCUSSION

Comparison between cofactors required for activity of squalene epoxidase from CHO cells and rat liver

Differential effects of PG and cytosolic fraction on microsomal epoxidases derived from CHO cells and rat liver. Rat hepatic squalene epoxidase is known to require the combination of microsomes and cytosolic supernatant fraction for full activity (1, 2). This requirement was observed in our experiments as well (Fig. 1, panel B). Since CHO-derived microsomal squalene epoxidase has not been previously studied, we were interested to find out whether this extrahepatic enzyme has similar requirements for the presence
Fig. 1. Effect of increasing concentrations of cytosolic fractions on CHO and rat liver microsomal squalene epoxidase activity. Squalene epoxidase activity was determined in 0.25 mg of CHO-FCS microsomes (panel A) and in 0.08 mg of rat liver N-microsomes (panel B) in the presence of increasing concentrations of both CHO-FCS (〇) and rat liver (〇) cytosolic fractions. The assay was performed as described in the Methods section. The enzyme activity is expressed as dpm in products (2,3-oxidosqualene) formed per hr.

of a cytosolic fraction. The effect of increasing amounts of cytosolic fractions on CHO-FCS microsomal squalene epoxidase activity can be seen in Fig. 1, panel A. As shown, CHO-FCS_{105} had only a slight activating effect on the CHO-FCS microsomal enzyme (~2-fold at 1.72 mg CHO-FCS_{105}) whereas addition of rat liver cytosolic fraction (N-S105) resulted in a significant increase in the enzyme activity (~14-fold increase at 1.72 mg of N-S105). These results suggest that the low response of CHO epoxidase to the CHO-FCS_{105} might be due to the lack of one or more of the soluble factors, required for enzyme activity. The CHO-FCS_{105} preparation was also ineffective in activating squalene epoxidase in rat liver N-microsomes, yet the same enzyme was effectively activated by rat liver N-S105 (see Fig. 1, panel B). The similar high response of microsomal CHO-FCS and rat liver enzymes to N-S105 indicates that there are no profound differences between the requirements of the two enzymes for cytosolic factor(s).

Anionic phospholipids, including PG, play an important role in squalene epoxidation. PG is known to bind tightly to rat liver microsomes and was suggested to be involved, together with cytosolic protein factor(s), in the uptake and intermembrane translocation of squalene (6). We, therefore, monitored the effect of PG on the activity of microsomal epoxidases derived from CHO cells and rat liver. Table 1 shows that both enzymes are activated by PG. In fact, PG not only could replace the CHO autologous S_{105} in stimulating squalene epoxidation but was even more effective. Furthermore, the combination of both the CHO-FCS_{105} and PG did not result in either an additive or any synergistic effect on the activity of the CHO-FCS microsomal epoxidase.

By contrast, rat liver squalene epoxidase is stimulated maximally by autologous S_{105}, and to a lesser extent by PG alone (Table 1). Taken together with the results presented in Fig. 1, it can be concluded that a possible deficiency in an anionic phospholipid in CHO-FCS_{105} does not account for the relatively low activating properties of this cytosolic fraction. Rather, it may be suggested that the differences between S_{105} from CHO cells and rat liver

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>No Addition</th>
<th>PG* (A)</th>
<th>Autologous S_{105}† (B)</th>
<th>(A + B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-FCS microsomes' (1.0 mg/ml)</td>
<td>562</td>
<td>1990 (3.5)</td>
<td>1129 (2.0)</td>
<td>1825 (3.2)</td>
</tr>
<tr>
<td>Rat liver N-microsomes (0.2 mg/ml)</td>
<td>1010</td>
<td>13010 (13)</td>
<td>36276 (36)</td>
<td>ND*</td>
</tr>
</tbody>
</table>

The numbers in parentheses express the fold activation.
*Conditions for maximal activation were determined from activity versus PG concentration curves. For CHO-FCS microsomes, maximal activation was obtained at 0.10 mg/ml of PG and for rat liver N-microsomes at 0.06 mg/ml of PG.
†Conditions for maximal activation were determined from curves of activity versus increasing amounts of autologous S_{105}. For CHO-FCS microsomes, maximal activation was obtained at 3.0 mg CHO-FCS_{105}/ml and for rat liver N-microsomes at 5.0 mg N-S_{105}/ml.
‡Mean of three experiments.
§Not determined.
reside in protein factor(s) of these fractions. This may not be surprising since the cholesterogenic pathway in the liver is continuously active and, therefore, the need for high levels of specific carrier protein(s) is expected. Such high levels of carrier protein(s) may also be needed for the efficient utilization of dietary squalene as substrate for cholesterol synthesis in the liver (16). In extrahepatic cells, on the other hand, the cholesterol requirement is satisfied mainly by the uptake of plasma lipoproteins and the cholesterol biosynthetic pathway is down-regulated.

The effect of Triton X-100 on squalene epoxidase activity of CHO and rat liver. The nonionic detergent Triton X-100, which has been used to solubilize rat liver squalene epoxidase, has also been shown to stimulate the activity of the enzyme (5). In the presence of Triton X-100, the hepatic enzyme no longer requires the cytosolic protein (SPF) and phospholipid for expressing its activity. The explanation given for this observation was that disruption of the microsomal membrane by the detergent renders the epoxidase less restricted conformationally and thus more accessible to the substrate (6).

In Fig. 2 we compare the effect of Triton X-100 on epoxidase activities in rat liver and CHO microsomes. As can be seen, the detergent has no stimulatory effect on squalene epoxidase of CHO-FCS while rat liver enzyme activity is greatly enhanced. Moreover, Triton X-100 inhibits CHO squalene epoxidase activity when added together with PG (data not shown). Since we have not studied the effect of other detergents on these enzymes, we cannot determine whether the results point to some structural differences in the microsomal membranes of rat liver and CHO cells or to intrinsic differences between the enzymes. Whatever the explanation, these results may indicate possible differences in substrate accessibility to these enzymes. In light of the above discussed catalytic behavior of CHO-derived squalene epoxidase, it is interesting to note that the activity of microsomal epoxidase from Saccharomyces cerevisiae is not stimulated by autologous S105 fraction (17) and Candida albicans epoxidase is inhibited by Triton X-100 (18). In these respects, CHO-derived squalene epoxidase appears to resemble the epoxidase of these primitive eucaryotes more than that of the hepatic enzyme.

Regulation of the microsomal squalene epoxidase activity of CHO cells

We have previously shown that there is an increased squalene epoxidase activity in intact CHO cells grown in the presence of 10% LDS, as compared to cells grown in the presence of 10% FCS (10). Therefore, it was of interest to study whether such differences in squalene epoxidase activity could also be demonstrated in cell extracts and, if so, to localize the cellular fraction in which it occurs.

Effect of growth conditions on microsomal squalene epoxidase activity of CHO cells. When epoxidase activity in CHO-FCS and CHO-LDS microsomes is compared, a relatively large increase in enzyme activity (~3 fold) is observed in CHO-LDS microsomes (Fig. 3). This increase was observed in the presence of either PG or autologous S105 preparations. These results are in agreement with our previous report showing induction of squalene epoxidase activity in intact CHO cells (10), and indicate regulation of the metabolic step catalyzed by squalene epoxidase.

![Fig. 3](image_url)

**Fig. 3.** Squalene epoxidase activity in microsomes of CHO cells grown in the presence of FCS or LDS. CHO cells were grown for 48 hr in the presence 10% FCS or 10% LDS. Microsomes and soluble fractions were prepared from both treatment groups as described in the Methods section. Squalene epoxidase activity was determined under standard assay conditions using 1.1 mg/ml microsomes from FCS grown cells (hatched bars) or LDS grown cells (open bars). Microsomal squalene epoxidase activity was determined without any additions (A); and in the presence of 0.1 mg/ml PG (B); or 2.6 mg/ml autologous S105 (C). The enzyme activity is expressed as dpm in products (2,3-oxidosqualene and sterols) formed per hr.

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Soluble cytosolic factors, such as squalene and sterol carrier protein (SCP), have been suggested to function as regulators of the cholesterol biosynthetic pathway. In human fibroblasts (19) and in rat liver (20) changes in the level of these carrier proteins were reported to occur concomitantly with variations in cholesterogenesis. Since variations in epoxidase activity occur as a response to a change to cholesterogenic state in CHO cells, we decided to investigate the role of soluble cytosolic factors in the observed regulation of this activity as well. Table 2 shows the effect of PG, CHO-FCSs(105), and CHO-LDSs(105) on epoxidase activity in CHO-FCS microsomes. As expected, PG is more effective than either of the soluble preparations in enhancing the epoxidase activity in CHO-FCS microsomes. In this, and in other experiments, we could not observe higher activity in the presence of CHO-LDSSs(105) in comparison to CHO-FCSs(105) (compare #3 and #2). We certainly could not attribute the relatively high activity in CHO-LDS microsomes (Fig. 3 and #5 in Table 2) to some effect of residual cytosolic factor since the same cytosolic preparation failed to activate the CHO-FCS microsomes. Therefore, we can conclude that the increased squalene epoxidase activity observed in LDS-grown cells, as demonstrated in Fig. 3, is entirely due to a change in the microsomal enzyme activity rather than to changes in the activation properties of the soluble cytosolic fraction. Induction of epoxidase activity in response to increase of cholesterogenesis indicates that this enzyme, together with other enzymes in this pathway (21–23), is coordinatively regulated in CHO cells.

**Regulation of rat liver microsomal squalene epoxidase activity**

*Effect of dietary conditions on the activity of hepatic squalene epoxidase.* Coordinative regulation of enzymes of the cholesterol biosynthetic pathway has been reported for some of the liver enzymes involved in this pathway (24, 25). In light of this and the results observed in CHO cells,

**Table 2. The effect of CHO-FCSs(105) and CHO-LDSs(105) on squalene epoxidase activity of CHO-FCS and CHO-LDS microsomes**

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Additions</th>
<th>DPM in Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-FCS</td>
<td>CHO-FCSs(105)</td>
<td>1276</td>
</tr>
<tr>
<td>CHO-FCS</td>
<td>CHO-LDSS(105)</td>
<td>2068</td>
</tr>
<tr>
<td>CHO-FCS</td>
<td>PG</td>
<td>1636</td>
</tr>
<tr>
<td>CHO-LDS</td>
<td>PG</td>
<td>2463</td>
</tr>
<tr>
<td>CHO-LDS</td>
<td>PG</td>
<td>8144</td>
</tr>
</tbody>
</table>

Squalene epoxidase activity was determined in microsomes derived from cells grown for 48 hr in the presence of 10% FCS or 10% LDS. The epoxidase assay was performed under standard assay conditions using 1.1 mg/ml microsomal protein. The concentration of PG used was 0.1 mg/ml and the final protein concentrations of the S(105) preparations were 2.6 mg/ml. The enzyme activity is expressed as dpm in products (2,3-oxidosqualene and sterols) formed per hr.

we investigated a possible correlation between hepatic epoxidase activity and various cholesterol regimens in the diet. The diets used were reported to alter the activity of the main regulatory enzyme, HMG-CoA reductase (14, 26). The effect of the various diets on the induction of activity of both squalene epoxidase and HMG-CoA reductase is shown in Fig. 4. As previously reported (27), CM-diet induces and S-diet suppresses hepatic reductase activity in comparison to the activity of the enzyme in rats maintained on N-diet (see inset). As seen, there is a similar dietary effect on the activity of the liver epoxidase in the three groups. The difference in epoxidase was most pronounced when assayed at high protein concentrations due to nonlinearity of activity under the assay conditions. This nonlinearity was due mostly to the inhibitory effect of Triton X-100 on the epoxidase activity at low protein concentrations. Despite the above, it is clearly seen that dietary induction and suppression of squalene epoxidase activity follows the same pattern observed for HMG-CoA reductase. Thus, we conclude that hepatic squalene epoxidase activity is also coordinatively regulated in response to sterol requirements and dietary supply.

**Fig. 4.** The effect of dietary conditions on hepatic microsomal squalene epoxidase and HMG-CoA reductase activity. Squalene epoxidase activity was determined in the presence of 0.1% Triton X-100 at increasing concentrations of S (●), N (○), and CM (▲) microsomes. The assay was performed as described in Methods except that duration of the incubations was 30 min. The enzyme activity is expressed as dpm in products (2,3-oxidosqualene and sterols) formed. Inset: HMG-CoA reductase activity was determined in presence of S (●), N (○), and CM (▲) microsomes as described in the Methods section. Activity is expressed as dpm mevalonate formed.

**Fig. 5** depicts the activating effect of liver cytosolic extracts derived from the three dietary groups on epoxidase activity in CM and S microsomes. The arguments for these experiments are the same as given above for the CHO enzyme. Again, the involvement of cytosolic factors in the regulation of the epoxidase is tested. As shown, the squalene epoxidase of S microsomes was activated to the same extent regardless of the source of the cytosolic extract. Similarly, the enzyme in CM microsomes was
equally stimulated by the different cytosolic preparations. Therefore, we suggest that, as in CHO cells, the changes in squalene epoxidase activity caused by dietary manipulations are entirely accounted for by changes in the microsomal enzyme per se and not by changes in the properties of the cytosolic activating factors.

We conclude that the activity of the ER-associated squalene epoxidase is regulated both in rat liver and in CHO cells and varies at different states of cholesterologenesis. Further studies using antibodies to purified squalene epoxidase should be performed in order to determine whether the observed regulation is at the level of enzyme synthesis.

Although HMG-CoA reductase is the major control step in sterol biosynthesis, several other enzymes are also subject to, and function in, regulatory control (22, 28). The importance of squalene epoxidase in regulation might be crucial since it could determine the level of certain oxysterols reported to be important in the regulation of HMG-CoA reductase synthesis (29-31).

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References


