**Effects of various squalene epoxides on coenzyme Q and cholesterol synthesis**

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ABSTRACT

2,3-Oxidosqualene is an intermediate in cholesterol biosynthesis and 2,3:22,23-dioxidosqualene act as the substrate for an alternative pathway that produces 24(S),25-epoxycholesterol which effects cholesterol homeostasis. In light of our previous findings concerning the biological effects of certain epoxidated all-*trans*-polyisoprenes, the effects of squalene carrying epoxy moieties on the second and third isoprene residues were investigated here. In cultures of HepG2 cells both monoepoxides of squalene and one of their hydrolytic products inhibited cholesterol synthesis and stimulated the synthesis of coenzyme Q (CoQ). Upon prolonged treatment the cholesterol content of these cells and its labeling with [3H]mevalonate were reduced, while the amount and labeling of CoQ increased. Injection of the squalene monoepoxides into mice once daily for 6 days elevated the level of CoQ in their blood, but did not change the cholesterol level. The same effects were observed upon treatment of apoE-deficient mice and diabetic GK-rats. This treatment increased the hepatic level of CoQ10 in mice, but the amount of CoQ9, which is the major form, was unaffected. The presence of the active compounds in the blood was supported by the finding that cholesterol synthesis in the white blood cells was inhibited. Since the ratio of CoQ9/CoQ10 varies depending on the experimental conditions, the cells were titrated with substrate and inhibitors, leading to the conclusion that the intracellular isopentenyl-PP pool is a regulator of this ratio. Our present findings indicate that oxidosqualenes may be useful for stimulating both the synthesis and level of CoQ both *in vitro* and *in vivo*.

*Keywords:*Mevalonate pathway, Squalene epoxides, Coenzyme Q induction, Cholesterol inhibition, Dolichol

**1. Introduction**

The key intermediate in the mevalonate pathway, farnesyl pyrophosphate, is utilized for the biosynthesis of cholesterol, dolichol and coenzyme Q (CoQ), as well as for isoprenylation of various proteins. The pathways involved share a common initial pathway, followed by independent terminal regulation of the biosynthesis of these individual lipids [[1](#_ENREF_1)]. The blood level of cholesterol, which is both a key membrane component and precursor of bile acids and hormones, is of considerable medical interest. The function(s) of dolichol itself, which is present in all tissues and membranes and at particularly high concentrations in endocrine organs, has not yet been established. The minor portion of this lipid that is phosphorylated serves an obligatory role in the N-glycosylation of numerous proteins.

The third major product of the mevalonate pathway, CoQ has been receiving more and more interest in recent years. Although present in all cellular membranes, the function and organization of CoQ in the inner mitochondrial membrane has been most thoroughly investigated [[2](#_ENREF_2)]. This lipid is located in the middle of the lipid bilayer, with its polar head-group oscillating between this midplane and the region in which the polar head-groups of the phospholipids are located.

CoQ plays a central role in the organization and function of mitochondrial supercomplexes and pathological changes in its level are associated with a loss of or reduction in the supramolecular organization of the respiratory chain [[3](#_ENREF_3)]. It has also been proposed that CoQ is involved regulating the mitochondrial permeability transition pore and uncoupling proteins present in the inner mitochondrial membrane [[4](#_ENREF_4), [5](#_ENREF_5)]. In addition, this lipid is our only lipid-soluble antioxidant that is synthesized endogenously [[6](#_ENREF_6)]. CoQ dependent NADH-oxidase in the plasma membrane regulates cell growth and differentiation [[7](#_ENREF_7), [8](#_ENREF_8)]. Among recent findings the most interesting are its immunological effects [[9](#_ENREF_9), [10](#_ENREF_10)]. CoQ appers to enhance the expression of NFҡB1-dependent genes, thereby causing release of mediators and signal substances from monocytes and lymphocytes into the blood and exerts multiple anti-inflammatory effects [[11](#_ENREF_11)].

The individual steps in the biosynthesis of CoQ have been well characterized, in most detail in *Saccharomyces cerevisiae* [[12](#_ENREF_12)]. At least ten genes are involved and most of the proteins are present in a biosynthetic complex. The presence of all these proteins in the complex is required for efficient biosynthesis [[13](#_ENREF_13)] .One of the factors which that is regulating the function of serveral of these enzymes is their phosphorylation state [[14](#_ENREF_14)]. Recently it was proposed that the biosynthesis may be increased by dephosphorylation of demetoxy-hydroxylase COQ7 in yeast with a specific mitochondrial phosphatase [[15](#_ENREF_15), [16](#_ENREF_16)].

The levels of CoQ in all human organs decrease during aging, a process considered to contribute to the reduced level of antioxidant protection [[17](#_ENREF_17)]. Moreover, tissue levels of CoQ are reduced considerably in liver cancer, cardiomyopathy, Parkinson´s disease and various complex myopathies [[18](#_ENREF_18)].

Interest in CoQ deficiencies due to functional mutations in the genes encoding some of the biosynthetic proteins is growing rapidly and several primary mutations of this type have been detected to date [[19](#_ENREF_19)]. There are also secondary forms of deficiency caused by mutations in genes whose products are not directly involved in CoQ synthesis [[20](#_ENREF_20)]. The clinical symptoms vary greatly, depending on the extent and distribution of the deficiency, and may involve functions performed primarily by the brain, cerebellum, muscles and kidney.

Deficiencies are treated by oral administration of CoQ [[21](#_ENREF_21)]. The most successful results are obtained in cases involving mutations, thereby making CoQ deficiency the only mitochondrial disease that can be treated effectively today [[22](#_ENREF_22), [23](#_ENREF_23)]. Children with such mutations develop normally if treatment is initiated early, i.e., before anatomical alterations in organs became apparent. The major problem with CoQ treatment is, however, that this lipid is taken up poorly when administered orally. For example, in the case of brain diseases gram quantities must be supplied daily in order to attain any effect, which indicates that effective therapy is difficult to achieve by this route [[24](#_ENREF_24)].

We reported previously that after epoxidation certain all-*trans*-polyisoprenoid lipids can alter the biosynthesis of mevalonate pathway lipids in cell cultures, either by stimulating CoQ biosynthesis and thereby elevating the cellular content of this lipid or by inhibiting cholesterol synthesis at the level of oxidosqualene cyclase [[25](#_ENREF_25)]. These are among the rare compounds that have been reported to stimulate the biosynthesis of CoQ and for this reason this line of investigation is of considerable interest. The mechanism of action is different concerning the two lipids, cholesterol synthesis is inhibited because the compounds specifically interfere with the oxidosqualene cyclase. In opposite, these substances uppregulate CoQ synthesis by gene interaction, and increase the enzymes participating in the biosynthetic pathway [[25](#_ENREF_25)]. The increase in concentration of farnesyl-PP in the case of inhibition of cholesterol synthesis has no effect of CoQ synthesis because of the high affinity of the branch-point enzyme (trans-prenyltransferase for its substrate [[26](#_ENREF_26)].

Here we have examined the influence of two epoxides of squalene (other than the natural substrate involved in cholesterol synthesis) on the biosynthesis of mevalonate lipids. We found that these epoxides, along with certain of their hydrolytic products, both induce CoQ synthesis and inhibit cholesterol synthesis. Moreover, these compounds exhibit *in vivo* effects that may be of future value for treating CoQ deficiency.

**2. Materials and methods**

*2.1. Reagents*

R,S-5-[3H]Mevalonolactone was synthesized employing [3H]sodium borohydride (15 Ci/mmol, American Radiolabeled Chemicals) as described by Keller [[27](#_ENREF_27)]. Risedronate was purchased from Tocris Bioscience, Elisville, MO. All other chemicals were procured from SIGMA.

To achieve epoxidation, squalene was first dissolved in dichloromethane and then mixed with 3-chloroperoxybenzoic acid (77%) dissolved in dichloromethane to obtain a 1:2 molar ratio of lipid:3-chloroperoxybenzoic acid [[28](#_ENREF_28)]. Following incubation at room temperature for 30 min, the solvent was removed by evaporation under nitrogen and the residue re-dissolved in hexane. Subsequently, the individual epoxides were separated by column chromatography on silica gel 60 (230-400 mesh) utilizing a gradient from hexane to a mixture of hexane and diethyl ether (96:4) for elution. Further separation of the individual compounds was obtained by elution from a second silica column with benzene, followed by repetition of this procedure. The three fractions eluted successively all contained monoepoxides with identical molecular weights as determined by mass spectroscopy. The first two fractions containing squalene monoepoxides tentatively identified as 5,6- and 9,10-oxidosqualenes and further referred to as E2 and E3, respectively could not serve as substrate for cholesterol biosynthesis, whereas the third one, 2,3-oxidoxysqualene (E1) could.

For hydrolysis, the compounds E2 and E3 were dissolved in 2 ml tetrahydrofurane, after which 100 µl 5 M HCl was added for hydrolysis and chlorination. Following incubation for 30 min at room temperature, hexane and water were added; the hexane phase evaporated off and the remaining residue dissolved in benzene. Separation of products was performed on silica columns utilizing elution with benzene. The first two fractions eluted (H2 andH3) contained isomers of the same molecular weight (as determined by mass spectrometry) substituted with one hydroxyl group and one Cl atom, while the third fraction (H1) exhibited a molecular weight corresponding to addition of two OH groups.

*2.2. Cell cultures*

Human hepatoblastoma (HepG2) cells were cultured in 10 ml DMEM medium (Invitrogen) containing 1 g glucose per 100 ml, 10% fetal bovine serum, penicillin (100 units per ml) and streptomycin (100 mg/ml). When 70% confluency was reached, the medium was changed and the compound to be investigated added in 50-100 µl ethanol to obtain a final concentration of 3 µM. Eight hours later, 0.5 mCi [3H]mevalonate (3.25 Ci/mmol) was added and incubation continued thereafter for an additional 8 hrs. In experiments in which the cells were subjected to prolonged treatment for 20 days they were split at 3, 6, 10 and 15 days of culturing and supplied with 3 µM E3 or E3H3 at each of these time-points. Finally, the cells were harvested by trypsinisation and stored at -20o C for later analysis.

For monitoring the synthesis of CoQ9 and CoQ10, the cells were cultured in the presence of mevalonate (0.39-200 µM), mevinoline (0.004-1 µM), risedronate (0.11-27 µM) or zaragozic acid A (0.11-27 µM) for 8 h. At this time-point 0.5 mCi [3H]mevalonate (3.25 Ci/mmol) was added and incubation continued for an additional 8 hrs followed by lipid extraction and determination of the radiolabeling in CoQ9 and CoQ10.

*2.3. Animals*

C57BL/6J mice, Wistar rats, C57BL/6J mice homozygous for the disrupted apoE gene (apoE–/–) and diabetic Goto-Kakizaki (GK) rats, all males, were purchased from Charles River (Belgium). The animals were housed with a 12 h light/ 12 h dark cycle at 22°C and provided *ad libitum* with standard laboratory chow and water. E3 and E3H were dissolved in ethanol and injected intraperitoneally; each injection containing 8 µmoles in the case of mice and 60 µmoles for the rats. After decapitation the blood was collected and the liver homogenized in buffer using an ultra-Turrax blender. Blood from the rats was also collected at various time-points by puncturing the saphenous vein of the leg. The method of administration, intraperitoneal injection in not commonly used in humans but this way of administration is very close to the plaster application in human medicine applied for a number of drugs such as testosteron, estrogenes, analgetics, dilatators of heart arteries etc. In both cases a gradual uptake takes place into the circulation, avoiding the gastroenteral system. Both ways effects the same target tissues and the metabolites produced are identical. In this way, intraperioneal injection is comparable to the plaster administration in human.

The white blood cells in 2 ml of total blood containing 50 mM EDTA was incubated with 1.0 mCi [3H]mevalonate at 37°C for 60 min, the mixture then treated with hemolysis buffer and the white cells collected by centrifugation and subjected to lipid extraction and HPLC analysis.

All animal experiments were pre-approved by the Northern Stockholm Ethical Committee for the Care and Use of Laboratory Animals.

*2.4. Extraction and chromatography of lipids*

Lipids were extracted from cells, blood and liver with chloroform:methanol:water (1:1:0.3), at 37o C for 1 hr with magnetic stirring [[29](#_ENREF_29)]. The extracts thus obtained were adjusted to a final chloroform:methanol:water ratio of 3:2:1 and complete phase separation then achieved by centrifugation. The lower chloroform phase was removed and evaporated to dryness under a flow of nitrogen and the resulting residue subsequently re-dissolved in chloroform. These solutions were placed onto a silica column (50 mg/1.5 ml; Extract-Clean, Alltech, Deerfield, IL) and thereafter eluted with 6 ml chloroform. After evaporation of the solvent, the neutral lipids were dissolved in chloroform:methanol (2:1).

For analysis of cholesterol and its metabolites, squalene and CoQ, the samples were subjected to reversed-phase HPLC using a Supelcosil LC-18 column (3 µm, 4.0 x 75 mm) equipped with an LC-18 Supelguard (Supelco) column [[30](#_ENREF_30)]. For separation, a stepwise gradient formed from methanol:water (9:1) in pump system A and methanol:2-propanol:hexane (2:1:1) in pump system B was employed at a flow rate of 1.1 ml/min. The gradient began at 15% solvent B, with increases to 47% after 8 min, 67% after 16 min and 100% after 24 min. The lipids in the eluent were monitored at 210 and 275 nm with a UV detector and radioactive labeling was measured with on-line detector of radioactivity. Ergosterol, CoQ6  and dolichol-23 were used as internal standards.

Following chemical epoxidation, the products were separated by silica gel column chromatography as described above. Subsequently, the individual fractions were analyzed on silica gel-60 plates (Merck) and HPTLC RP-18 plates (Merck) and the spots visualized with iodine. The solvent systems utilized for development were benzene:ethyl acetate (9:1) for the silica gel-60 plates and acetone:methanol (2:1) in the case of the HPTLC RP-18 plates.

*2.5. Mass spectrometry, NMR spectroscopy and determination of protein*

Accurate mass measurements (HR-ESI-MS) of sodiated molecular ions [M+Na]+ were performed using a MALDISynapt G2-S HDMS (*Waters Inc*) mass spectrometer equipped with an electrospray ion source and a q-TOF type mass analyzer. The instrument was controlled and recorded data were processed using *MassLynx V4.1* software package (*Waters Inc*). All results differed no more than +/- 3 ppm from the theoretical masses (Table 1). In ESI-MS spectrum of E3H3 sample the peaks corresponding to [epoxysqualene + Na]+ and [epoxysqualene + C2H5OH + Na]+  are observed.

1H-NMR spectra were obtained with Varian Unity Plus 500 MHz spectrometer at 25oC in C6D6 (D 99.5%, Cambridge Isotope Laboratories. Inc., Andover, MA, USA). 32 K data points were collected and a spectral width of 6 kHz was used.

Protein was determined with the bicinchoninic acid kit (Sigma).

*2.6. Statistical analyses*

The results obtained are expressed as means ± SD. Differences between several groups were analyzed by ANOVA, followed by Dunnett’s test. *P* values of less than 0.05 were considered statistically significant.

**3. Results**

*3.1. Structures of the oxidosqualenes and their hydrolytic products*

The structures of the three monoepoxidated derivatives of squalene of interest here are depicted in Figure 1. 2,3-Oxidosqualene (E1) is the natural substrate for oxidosqualene cyclase in the cholesterol synthetic pathway. The two other isomers contain epoxidated double bonds on the second (E2) and third (E3) isoprene residues of the polyisoprenoid chain (putative 6,7- and 10-11-oxidosqualene, respectively). A hydrolysis of the E3 resulted in three compounds, the most hydrophilic of which (E3H1) possessed two hydroxyl groups, while the other two (E3H2 and E3H3) contained one Cl atom and a hydroxyl group. The results of mass spectrometric identification are given in Table 1. The different regional isomers of the individual compounds were not separated. For this reason we have obtained the racemic mixture of derivatives which is designated in Fig 1 with a wavy bond.

1H NMR analysis confirmed the structures of E1, E2 and E3. Analysis permitted separation of signals derived from four types of methyl protons present in squalene molecule (Table 2). The appearance of the signal at δ 1.16 ppm in E1 spectrum is in line with the 2,3-oxidosqualene structure as observed previously in the literature [31a]. Differences observed in E2 and E3 spectra (signal at δ 1.05 and 1.39, ppm Table 2) clearly confirmed variable location of the epoxide ring in their molecules, ~~however the unequivocal identification of the location of epoxide ring (6,7- or 10,11-) requires detailed multi-dimensional 3D NMR study, similarly to that performed earlier for Prenol-10 [31b]~~.

~~Since biological effects of E2 and E3 seem similar (see below) such additional effort has not been undertaken. Keeping this in mind E2 and E3 notation is applied for further experiments.~~

*3.2. Synthesis of cholesterol, CoQ and dolichol*

The individual epoxidated components and their hydrolytic derivatives were added to cultures of HepG2 cells and the rate of 3H-mevalonate incorporation into cholesterol and cholesterol metabolites determined (Fig. 2A). As expected, 2,3-oxidosqualene and its hydrolytic products exerted no effect on cholesterol synthesis. In contrast, E2 inhibited cholesterol synthesis completely, resulting in accumulation of oxidosqualene, dioxidosqualene and epoxycholesterol, in agreement with inhibition of oxidosqualene cyclase. On the other hand, none of the three hydrolytic products of E2 demonstrated any inhibitory effects. E3, with the epoxide moiety on the third isoprene residue also inhibited cholesterol synthesis in a concentration-dependent manner, with 3 µM inhibiting 70%. Two of the hydrolytic products, i.e., E3H1 with two hydroxyl groups and E3H2 with a Cl atom and one OH group, had no effect; whereas E3H3 with the Cl atom and hydroxyl group in the opposite orientation, was inhibitory.

Interestingly, the same derivatives of squalene, that inhibited cholesterol synthesis, stimulated CoQ synthesis (Fig. 2B). Incorporation of 3H-mevalonate into CoQ by HepG2 cells was elevated 3-fold in the presence of E2 and E3. Of the hydrolytic products only E3H3 exerted any effect on CoQ synthesis, which was also strongly stimulatory. HepG2 cells synthesize primarily CoQ10, CoQ9 constituting approximately 20% of the total and formation of both forms was more than doubled. None of the squalene derivatives had any effect on the synthesis of dolichol, the third main lipid product of the mevalonate pathway (Fig. 2B).

*3.3. Prolonged treatment*

The quantitative changes in lipid content upon prolonged exposure of the cells to oxidosqualenes were determined. To this end, HepG2 cells were exposed to E3 or E3H3 continuously for 20 days and thereafter the levels of the different lipids and the extent of their labeling were assessed. The cellular content of cholesterol was reduced 30-35% (Fig. 3A) and the incorporation of radioactivity into this lipid was only 10-25 % of the control value (Fig. 3B).

Labeling was recovered in squalene, oxidosqualene, dioxidosqualene and epoxycholesterol after such prolonged treatment with either derivative. The total amount of CoQ rose by approximately 50 %, but the elevation in CoQ9 was considerably higher. The level of this form was increased by 5- and 8-fold in the presence of E3 or E3H3, respectively, while the corresponding values for CoQ10 were 35% with both compounds (Fig. 3C). The situation was similar when incorporation of [3H]mevalonate was monitored (Fig 3D). Following treatment of the cells with E3 and E3H3, the rate of incorporation of radioactivity into CoQ9 was enhanced 11- and 8-fold, respectively, while the corresponding values for CoQ10 were only 36% and 56 %. The prolonged treatment was performed in order to study the changes in lipid amount which cannot be expected after a short incubation. The rate of 3H-mevalonate incorporation into both CoQ9 and CoQ10 is higher in the initial period at the start of lipid synthesis. After 20 days the amount of the lipids are increased and the lower rate of incorporation indicates that the biosynthesis aims to keep up the amount of CoQ in the cells at the higher level without further increase of its amount.

In order to evaluate whether mitochondrial respiration and ATP synthesis were affected by epoxysqualene treatment, oxygen consumption rate (OCR) was measured employing Seahorse XF24 Analyzer (Seahorse Biosciences) on HepG2 cells treated with 10 µM E3 during 20 days (not shown in figure). Basal OCR was similar in the vehicle treated compared to the E3 treated cells. Neither ATP synthesis rate after the addition of oligomycine nor leak of protons through the inner mitochondrial membrane after the addition of FCCP (trifluorocarbonylcyanide phenylhydrazone) showed any significant changes. Apparently, the increase in CoQ synthesis and amount did not have major effect on the mitochondrial respiration and ATP synthesis. This type of experiments, however, will be of considerable interest in study of cells originating from children having CoQ deficiency.

*3.4. In vivo administration*

When mice were injected intraperitoneally with 9,10-oxidosqualene (E3) once daily and plasma lipids analyzed after 2, 4 and 6 days of such treatment, no alterations in the total blood content of cholesterol (free and esterified) was observed (Fig. 4A). On the other hand, the plasma level of coenzyme CoQ9 increased, becoming 75% higher than the control value after 6 days (Fig. 4B).When apo-E deficient mice were treated, the increase in CoQ9 was even higher, approximately 100% after 4 days (Fig. 4C). With diabetic GK-rats, 6 days of treatment resulted in a 75 % elevation in the blood level of CoQ (Fig. 4D). The level of CoQ10 in rodent blood is too low to be determined with certainty.

The hepatic level of cholesterol in mice remained unchanged during the 6-day period of treatment (Fig. 5A). The CoQ9 content in the liver was not modified by treatment with E3, but the amount of CoQ10 almost doubled (Fig 5 B and C). Since CoQ10 constitutes only a few percent of the total CoQ in murine liver, this latter change did not significantly alter the total content.

To determine whether the compound injected actually reaches the blood and is available for interaction with cholesterol synthesis, mice were injected intraperitoneally with E3. Five hours later a sample of total blood was incubated with [3H]mevalonate and thereafter the white blood cells were isolated. In untreated mice both the cholesterol and squalene in the white blood cells were labeled (Fig. 6A). In contrast, no labeling of cholesterol could be detected in white cells isolated from the treated mice and the presence of radioactive oxidosqualene and a high level of labeled squalene indicated blockage of cholesterol synthesis in these cells (Fig. 6B). The content and labeling of CoQ in the isolated blood cells were very low and could not be determined reliably. This experiment indicates that after i.p. injection E3 reaches the blood of mice in bioactive form.

*3.5 Biosynthesis of CoQ9 and CoQ10*

Since the ratio of CoQ9/CoQ10 varied in our experiments, the possibility that the availability and concentration of individual substrates influence this ratio was examined. In these experiments biosynthesis of the lipid was followed on the basis of incorporation of [3H]mevalonate. When HepG2 cells were cultured in the presence of 0.39 µM mevalonate, 35% of the labeling was recovered in CoQ9 (Fig. 7A). However, when the concentration of mevalonate was raised to 100 µM, only 14% was recovered in CoQ9 and the ratio of CoQ9/CoQ10 changed considerably, since the proportion of labeling in CoQ10 was elevated from 65% to 86%.Under these conditions the cellular concentration of IPP was increased as a consequence of the elevated concentration of substrate, i.e., mevalonate.

Cellular levels of mevalonate and IPP can be also modified by inhibiting HMG-CoA reductase with mevinolin [[31](#_ENREF_31)]. In control HepG2 cells 92% of the labeling was recovered in CoQ10, but this level diminished as increasing concentrations of the inhibitor were added (Fig. 7B). In the presence of 1 µM mevinolin only 77% of the labeling was in CoQ10, i.e., with reduced concentrations of mevalonate and IPP, synthesis of CoQ10 was lowered while synthesis of CoQ9 was enhanced.

Cellular IPP levels can also be altered by inhibitors that act in the vicinity of the branch-point in the mevalonate pathway. In the presence of risedronate, an inhibitor of farnesyl-PP synthase, the cellular level of IPP is expected to increase [[32](#_ENREF_32)]. Increasing concentrations of this inhibitor did not interfere with the synthesis of cholesterol or CoQ10 but drastically reduced labeling of CoQ9 (Fig. 7C).

Inhibition of the rate-limiting enzyme in cholesterol synthesis, squalene synthase, by zaragozic acid is also expected to elevate the cellular level of IPP [[33](#_ENREF_33)]. With as much as 3 µM zaragozic acid in the culture medium, the synthesis of CoQ10 and cholesterol were unaltered, whereas that of CoQ9 was strongly attenuated (Fig. 7D). In the presence of 27 µM zaragozic acid cholesterol synthesis was eliminated completely, while CoQ10 synthesis was potently stimulated.

**4. DISCUSSION**

Drugs that influence the synthesis of products of the mevalonate pathway and, in particular, reduce cholesterol synthesis are of considerable medical interest. Statins inhibit HMG-CoA reductase, and the resulting reduction in farnesyl-PP concentration preferentially lowers the activity of squalene synthase, which has a considerably lower affinity for this intermediate than do the other branch-point enzymes [[26](#_ENREF_26)]. Consequently, levels of CoQ and dolichol are influenced not at all or only to a limited extent by statins. Epoxides of all-*trans*-polyisoprenols, including squalene epoxides, inhibit a terminal enzyme in cholesterol synthesis thereby provide an alternative approach to reducing cholesterol levels.

On the other hand, no effective stimulator of CoQ synthesis is presently available and searching for such substances is of considerable interest. A number of endogenous metabolites, such as oxysterols, farnesol and its metabolites, geranylgeraniol, prenylphosphates and sterols that activate meiosis are known to influence the biosynthesis of cholesterol [[34](#_ENREF_34)]. It seems highly probable that certain as-yet-unidentified metabolites can influence CoQ biosynthesis. Nuclear transcription factors are also involved in regulating the synthesis of this lipid, but the nature of these factors has not yet been elucidated. Although not required for the basal synthesis of CoQ, cholesterol or dolichol, peroxisome proliferator-activated receptor α (PPARα) is involved in the elevated biosynthesis of CoQ caused by treatment with PPARα agonists [[35](#_ENREF_35)]. In mice with an intact PPARα, synthesis of CoQ is enhanced by treatment with clofibrate, di(ethylhexyl)phthalate or salicylic acid, but this effect is restricted to rodents and does not occur in humans [[36](#_ENREF_36)]. Liver-X-receptor (LXR), which plays a major role in cholesterol homeostasis, does not directly regulate but can influence CoQ synthesis. Thus, in LXRα-deficient mice this synthesis is reduced in the liver and elevated in the spleen, thymus and lungs [[37](#_ENREF_37)]. Moreover, in mice that lack retinoid-X-receptor α (RXRα) only in their hepatocytes, hepatic CoQ synthesis is greatly attenuated, demonstrating the involvement of this transcription factor as well [[38](#_ENREF_38)]. However, RXR functions as part of a heterodimer and the nature of its dimeric partner in this case remains unknown.

~~Aging, as well as diseases such as cancer, cardiomyopathy, Parkinson´s disease and complex myopathies afflict a considerable portion of the human population. These conditions are characterized, among other things, by reduced tissue levels of CoQ, and raising these levels might conceivably be beneficial. However, uptake of this lipid upon oral administration is very poor, so that reestablishment of the original tissue levels cannot be achieved with this approach. This is unfortunate, since the value of increasing tissue content of CoQ by dietary administration is clearly illustrated by pathogenic CoQ deficiencies due to mutations. Upon supplementation, children with this deficiency demonstrate greatly improved cognitive and physical functions and if the treatment is initiated before organ damage is apparent, the child develops normally .~~

Strategies for stimulating CoQ synthesis are highly limited today. PPARα agonists elevate the levels of this lipid in several organs, but only in rodents. Thyroxin administration, vitamin A deficiency, fluoride treatment and inhibition of squalene synthase by squalestatin-1 are employed to enhance levels of CoQ in experimental systems [[39](#_ENREF_39)]. On the other hand, the tissue content of this lipid can be increased without changing its metabolism. Since most of the cellular CoQ is located in mitochondria, the structure, number, size and distribution of this organelle greatly influence the overall content. For instance, cold adaptation and exercise increase the numbers of mitochondria in the liver and muscle and, thereby, also the total content of CoQ in these tissues [[40](#_ENREF_40), [41](#_ENREF_41)].

Certain diseases are characterized by more rapid synthesis of CoQ and elevated tissue contents, including preneoplastic nodules, Alzheimer´s and prion diseases and diabetes [[42-44](#_ENREF_42)]. Oxidative stress plays an important role in the development of these diseases, with free radicals and other reactive oxygen and nitrogen species being partially responsible for the pathogenic state. Cellular defenses include induction of antioxidative compounds and enzymes and the increase in CoQ levels is part of this process. Nonetheless, further elevation would be of value, since in connection with these diseases, both in animals and humans, positive effects are observed upon supplementation with large amounts of CoQ.

Whereas rodents synthetize mostly CoQ9 and only small amounts of CoQ10, the situation in human is the opposite. Depending on the organ in question and experimental/environmental conditions, the ratio of CoQ9/CoQ10 can vary, also in humans. In general, CoQ deficiency due to mutations involves proportional reduction in the levels of this lipid containing 9 or 10 isoprenoid side chains. One exception to this rule is the mutation in the PDSS1 gene that results in reduced activity of COQ1, the enzyme responsible for synthesizing the polyisoprenoid side-chain [[45](#_ENREF_45)]. In this case the remaining level of CoQ10 in cultured skin fibroblast is only 3% of normal, whereas the amount of CoQ9 is increased by 10-20%.

In a previous analysis of dolichol biosynthesis we found that the intracellular concentration of IPP, which can be altered by changing the concentration of this compound in the culture medium, determines the length of the polyisoprenoid chain [[46](#_ENREF_46)]. For this reason we altered the size of the IPP pool in HepG2 cells by varying the level of mevalonate in the medium and adding inhibitors of various steps of the mevalonate pathway. Lower levels of IPP result in an increased content of CoQ9, while an excess of this intermediate favors synthesis of CoQ10. It seems highly likely, that at low substrate concentrations decaprenyl-PP synthase is not saturated, which not only lowers the rate of synthesis, but also produces a shorter polyisoprenoid chain. The human mutation affecting decaprenyl-PP synthase may be considered as an indirect IPP deficiency that favors CoQ9 synthesis. The role of additional factors in regulating the length of the polyisoprenoid side-+chain remains to be examined. For example, the activity of decaprenyl pyrophosphate synthase is highly dependent on the Mn2+concentration, so that this cation may also influence the nature of the final product synthesized [[47](#_ENREF_47)].

When cholesterol synthesis is inhibited at the level of oxidosqualene cyclase, the metabolites that accumulate may not only have a direct influence on this enzyme, but also exert secondary effects. 2,3-Oxidosqualene accumulates, leading to the production of 2,3:22,23-dioxidosqualene, which in turn results in the formation of 24(S),25-epoxycholesterol. Epoxycholesterol, considered being one of the most potent oxysterol regulators of cholesterol homeostasis, inhibits cholesterol synthesis by promoting degradation of HMG-CoA reductase [[48](#_ENREF_48)]. As a ligand for LXR, epoxycholesterol may promote cholesterol efflux and reduce cholesterol uptake via the LDL receptor [[49](#_ENREF_49), [50](#_ENREF_50)]. This metabolite suppresses SREBP activation by binding to Insig, leading to down-regulation of genes whose products participate in cholesterol uptake and synthesis [[51](#_ENREF_51)]. Recently, epoxycholesterol was also shown to interfere with Seladin-1, the final enzyme in cholesterol synthesis, causing accumulation of desmosterol [[52](#_ENREF_52)]. In our experiments the two epoxides of squalene and one of the hydrolysis products effected cholesterol and CoQ biosynthesis. All these three compounds resulted in the accumulation of three identified cholesterol metabolites in our experimental system. It is possible that part or the whole biological effect on CoQ syntes is exerted by these metabolites and not the added squalene derivatives. It is also possible that other metabolites, produced during the incubation but not identified here, are responsible for the effects obtained. It will be a future task to identify, synthetize and test the biological effects of these metabolites. Thus, squalene epoxides may affect cholesterol and CoQ synthesis in several different ways and thereby regulate the homeostasis of this lipid in a complex manner.

Despite the potent inhibitory effect of 5,6- and 9,10-oxidosqualenes on cholesterol synthesis in cell cultures, the levels of cholesterol in blood and liver of mice treated with these compounds was unaltered. This was not due to lack of uptake, as demonstrated by incubation of white blood cells from these treated animals with [3H]mevalonate. The actual concentration of this inhibitor in the blood was obviously both substantial and effective. Apparently, changes in the level of one regulatory substance can be compensated for *in vivo* by alterations in the level of another. This possibility is supported by

the observations that transgenic mice exhibit substantial changes in their blood levels of oxysterols with oxidized side-chain in association with very limited alterations in cholesterol turnover and homeostasis [[53-55](#_ENREF_53)].

The induction of CoQ synthesis and increase in the content of this lipid observed here in HepG2 cells, as well as in the blood of mice and mice with disturbances in their cholesterol homeostasis and of diabetic rats, indicate that our substances may prove to be valuable in connection with the treatment of CoQ deficiencies in humans. *In vivo* administration of the oxidosqualenes elevated blood levels of newly synthesized CoQ, which is known to be produced in the liver and then excreted to the blood bound to lipoproteins [[56](#_ENREF_56)]. In the liver itself, however, only the level of CoQ10, a minor component, was elevated. It remains to be established if various types of modifications such as the way of administration, length of treatment, amount of substance supplied or administration with supporting vehicles could give an increased *in vivo* effect.

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**Table 1**

Mass spectrometry analysis of squalene epoxides synthesized as described in Material and methods. m/z values of monoisotopic pseudomolecular ions [M+Na]+ were recorded with HPLC/HR-ESI-MS.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample name | Chemical formula of molecular ion [M+Na]+ | [M+Na]+  m/z | |
| Calculated | Experimental ± error (ppm) |
| E2  Epoxy squalene | [C30H50O + Na]+ | 449.3759 | 449.3763±0.9 |
| E3  Epoxy squalene | [C30H50O + Na]+ | 449.3759 | 449.3766±1.6 |
| E3H3  Squalene chlorhydrine | [C30H51OCl+Na]+ | 485.3526 | 485.3530±0.8 |

Table 2

1H NMR analysis of the structure of squalene and its epoxides. Shown are signals corresponding to methyl protons. All the signals were recorded as singlets (s).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| proton  type | squalene | E1 | E2 | E3 |
| δ(ppm) | | | |
| CH3 | 1.56 (s)  1.60 (s)  1.61 (s)  1.67 (s) | 1.16 (s)  1.56 (s)  1.57-1.60 (3s)  1.67 (s) | 1.05 (s)  1.56 (s)  1.60-1.64 (3s)  1.67 (s) | 1.39 (s)  1.56 (s)  1.60 (s)  1.63 (s)  1.67 (s) |
|
|
|
|
|

**Figure legends**

**Fig. 1.** Putative structures of the oxidosqualenes and their hydrolytic products. E1: 2,3-oxidosqualene, E2: 6,7-oxidosqualene, E3: 10,11-oxidosqualene, E3H1: 10,11-dihydroxy-squalene, E3H2: 10-hydroxy-11-chlorosqualene, E3H3:10-chloro-11-hydroxy-squalene.

**Fig. 2.** Effects of oxidosqualenes and their hydrolytic products on the synthesis of cholesterol, CoQ

and dolichol. HepG2 cells were first cultured in the presence of 3 µM of each of the individual squalene derivatives for 8 h and thereafter with [3H]mevalonate for 8 h. Subsequently, cellular lipids were extracted and analyzed by HPLC. A, the individual columns give the radioactivity recovered in squalene, cholesterol, 2,3-oxidosqualene, 2,3:22,23-dioxidosqualene and 24(S),25-epoxicholesterol. B, the radioactivity in CoQ9, CoQ10 and total dolichol (dolichol 18-21) were determined by HPLC. The values presented are the means ± S.E. (vertical bars) of four independent experiments.\*, p <0.05; \*\*, p<0.01; \*\*\*, p< 0.001.

**Fig. 3.** Effect of prolonged treatment with 10,11-oxidosqualene or 10-chloro-11-hydroxysqualene on the synthesis and contents of cholesterol and CoQ in HepG2 cells. HepG2 cells were cultured in the presence of 3 µM 10,11-oxidosqualene (E3) or 10-chloro-11-hydroxysqualene (E3H3) and the cultures split after 3, 6, 10 and 15 days. On day 20 the content and radioactive labeling of cholesterol and CoQ were determined by lipid extraction and subsequent analysis by HPLC. A, the cholesterol contents of untreated cells and cells exposed to E3 or E3H3. B, the individual columns give the radioactivity recovered in squalene, cholesterol, 2,3-oxidosqualene, 2,3:22,23-dioxidosqualene and 24(S),25-epoxicholesterol. C, the cellular contents of CoQ9 and CoQ10 after 20 days of exposure. D. labeling of CoQ9 and CoQ10 with [3H]mevalonate following 20 days of treatment with E3 or E3H3. The values presented are the means ± S.E. (vertical bars) of four independent experiments.\*, p <0.05; \*\*, p<0.01; \*\*\*, p< 0.001.

**Fig. 4.** The influence of treatment with 10,11-oxidosqualene on blood levels of CoQ9 in control mice, apo-E deficient mice and GK-rats. Mice (35 g) were injected intraperitoneally with 8 µmoles E3 once daily for 6 days. The corresponding dose for rats (250 g) was 60 µmoles. Blood levels of cholesterol and CoQ9 were determined after 2, 4 and 6 days (A, B and D) and after 4, 8 and 12 days (C) of such treatment. A, blood levels of cholesterol in C57BL/6J mice. B, blood levels of CoQ9 in C57BL/6J mice. C, blood levels of CoQ9 in apo-A deficient mice. D, blood levels of CoQ9 in GK-rats. The values presented are the means ± S.E. (vertical bars) of three independent experiments.\*, p <0.05; \*\*, p<0.01; \*\*\*, p< 0.001.

**Fig. 5.** Hepatic levels of cholesterol and CoQ in mice liver treated with 10,11-oxidosqualene. The mice were injected intraperitoneally with 8 µmoles of E3 once daily for up to 6 days. Livers were removed after 2, 4 and 6 days of such treatment for lipid extraction and HPLC analyses. A, hepatic levels of cholesterol and cholesteryl esters. B, hepatic level of CoQ9. C. hepatic level of CoQ10. The values presented are the means ± S.E. (vertical bars) of four independent experiments.\*, p <0.05; \*\*, p<0.01; \*\*\*, p< 0.001.

**Fig. 6.** Cholesterol synthesis in the white blood cells of mice treated with 10,11-oxidosqualene. Mice were injected intraperitoneally with 8 µmoles of E3 and 6 hours later blood was collected and incubated with [3H]mevalonate for 60 min, followed by treatment with hemolysis buffer. The total white blood cell fraction was than extracted for determination of labeling in cholesterol and cholesterol metabolites. A, labeling in white blood cells from untreated mice. B, labeling in white blood cells from E3- treated mice. The values presented are the means ± S.E. (vertical bars) of three independent experiments.

**Fig. 7.** The influence of substrate and inhibitors of the mevalonate pathway on the ratio of CoQ9/CoQ10 synthesized in HepG2 cells. Cells in culture were incubated in the presence of increasing concentrations of substrate or inhibitors for 8 hrs. After addition of 0.5 mCi [3H]mevalonate the cells were further incubated for 8 h, after which time radioactivity in CoQ9 and CoQ10 was determined. A, incubation in the presence of mevalonate. B, incubation in the presence of mevinolin. C, incubation in the presence of risedondrate . D, incubation in the presence of zaragozic acid. Concentrations on the x-axis are given in a non-linear manner. The values presented are the means ± S.E. (vertical bars) of five independent experiments.

Highlights

Two mono-epoxidated squalenes and their hydrolytic products are characterized

Squalene-epoxides inhibits cholesterol synthesis and lower its amount in cell culture

Coenzyme Q (CoQ) synthesis and cellular amount is greatly increased

Injection into mice elevated blood but not liver content of CoQ

The intracellular isopentenyl-PP pool regulates the CoQ9/CoQ10 ratio

(Reviewer: The authors should proof the highlights carefully: Correct grammar in line 2 and line 5 should be written in a more active voice.)