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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 [^{3}H]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.

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]. 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 have been most thoroughly investigated [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]. It has also been proposed that CoQ is involved regulating the mitochondrial permeability transition pore and uncoupling proteins present in the inner mitochondrial membrane [4,5]. In addition, this lipid is our only lipid-soluble antioxidant that is synthesized endogenously [6]. CoQ dependent NADH-oxidase in the plasma membrane regulates cell growth and differentiation [7,8]. Among recent findings the most interesting are its immunological effects [9,10]. CoQ appears to enhance the expression of NFK B1-dependent genes, thereby causing release of mediators and signal

Abbreviations: CoQ, coenzyme Q; HPLC, high pressure liquid chromatography; IPP, isopentenyl pyrophosphate; GK, Goto-Kakizaki; PPAR, peroxisome proliferator-activated receptor; LXR, liver-X-receptor; RXR, retinoid-X-receptor

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substances from monocytes and lymphocytes into the blood and exerts multiple anti-inflammatory effects [11].

The individual steps in the biosynthesis of CoQ have been well characterized, in most detail in *Saccharomyces cerevisiae* [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]. One factor regulating the function of several of these enzymes is their phosphorylation state [14,15]. Recently it was proposed that the biosynthesis may be increased by dephosphorylation of Coq7 polypeptide, a hydroxylase that acts on demethoxy-Q, in yeast with a specific mitochondrial phosphatase [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]. Moreover, tissue levels of CoQ are reduced considerably in liver cancer, cardiomyopathy, Parkinson's disease and various complex myopathies [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]. There are also secondary forms of deficiency caused by mutations in genes whose products are not directly involved in CoQ synthesis [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]. The most successful results are obtained in cases involving mutations, thereby making CoQ deficiency the only mitochondrial disease that can be treated effectively today [22,23]. 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].

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]. 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 differs for these two lipids: cholesterol synthesis is inhibited because the compounds interfere specifically with oxidosqualene cyclase. In contrast, these substances upregulate CoQ synthesis at the gene level, thereby elevating the amounts of the enzymes participating in the biosynthetic pathway [25]. In the case of inhibition of cholesterol synthesis the increase in the concentration of farnesyl-PP has no effect of CoQ synthesis because of the high affinity of the branch-point enzyme (trans-prenyltransferase) for its substrate [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-[^{3}H]Mevalonolactone was synthesized employing [^{3}H]sodium borohydride (15 Ci/mmol, American Radiolabeled Chemicals) as described by Keller [27]. Risedronate was purchased from Tocris Bioscience, Ellisville, 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]. 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 6,7- and 10,11-oxidosqualenes, referred to as E2 and E3, respectively could not serve as substrate for cholesterol biosynthesis, whereas the third one, 2,3-oxidosqualene (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 and H3) 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 the 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 [^{3}H]mevalonate (3.25 Ci/mmol) was added and incubation continued thereafter for an additional 8 h. 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 trypsinization and stored at −20 °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 [^{3}H]mevalonate (3.25 Ci/mmol) was added and incubation continued for an additional 8 h 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 μmol in the case of mice and 60 μmol 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. Although, intraperitoneal injection is not commonly performed on humans, this way of administration closely resembles the patches and local application of gel by which a number of drugs, such as testosterone, estrogens, analgetics, and dilatators of heart arteries are applied.