

Clofibrate and di(2-ethylhexyl)phthalate increase ubiquinone contents without affecting cholesterol levels*

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Induction studies were performed on liver, muscle, heart, brain and blood by feeding Sprague-Dawley rats a diet containing a peroxisome proliferator, clofibrate or di(2-ethylhexyl)phthalate. Ingestion of these drugs resulted in an increase in the amount of two different types of ubiquinone homologues UQ₉ and UQ₁₀ found in rat. Phthalate proved to be the more effective drug, leading to a highly increased amount of ubiquinone in the liver. Increases were also found in all the above-mentioned organs except the brain. The UQ₉ levels were raised to 400, 200, 120 and 120%, of the respective normal values. The antioxidant and hypolipidemic agent, probucol, was used as a control to evaluate whether the increased ubiquinone level constituted a response to the elevated hydrogen peroxide pressure, resulting from the induced increase in fatty acid β -oxidation. In the presence of probucol, ubiquinone levels were decreased in all the above-mentioned organs except heart and brain. Probuco-
l had limited effects on the amount of cholesterol and did not significantly alter the amount of dolichol. The two peroxisome proliferators differed in their effects on cholesterol, as well as on dolichol levels which was induced by phthalate but not by clofibrate.

The possible mechanisms involved, and the importance of low toxicity drugs which could elevate ubiquinone levels in various tissues, are discussed.

Ubiquinone (UQ) is a neutral lipid containing an isoprenoid moiety. The ring precursor is condensed with an isoprenoid tail which can be of different chain-length depending on the biological membrane in question. In humans the side-chain consists predominantly of 10 isoprene units, with smaller amounts of the quinone with 9 isoprene units. In rat most of the ubiquinone has 9 isoprene units, but variable amounts of ubiquinone-10 are also present in all organs [1, 2]. Yeast and many bacteria contain ubiquinone homologues with isoprenoid side-chains of shorter chain-length, usually only one such homologue per specie.

Ubiquinone's first recognized function was to act as an obligatory redox component in mitochondria, participating in the electron-transport chain localized in the mitochondrial membrane [3]. The ubiquinone of other organelles is also believed to be involved in redox reactions. For example, Golgi vesicles contain a large amount of ubiquinone (per mg protein) and it has been suggested that electron-transport systems are also present at this location [4, 5]. This is also the case with plasma membranes [6].

In addition to its electron-transporting function, the reduced form of ubiquinone, UQH₂,

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has been shown in several different experimental systems to exert a protective action [7–13], due to its ability to quench free radicals which can severely damage biological membranes. Alternatively, UQH₂ can prevent formation of free radicals and thus interfere with initiation of lipid peroxidation. Recently, it was observed that most of the ubiquinone in both rat and human tissues was present in the reduced form [14, 15]. It has also been shown that cells prefer to use ubiquinol as an antioxidant, rather than vitamin E [10]. Ubiquinone is also present in most tissues in rather large amounts and can function independently of vitamin E.

These observations, together with an increasing number of clinical reports on the beneficial effects of ubiquinone, have given rise to new experiments designed to study the function of this substance in more detail. The uptake of ubiquinone seems to be limited to the liver and spleen [16]. No uptake of ubiquinone into various peripheral organs is observed upon its dietary administration. These findings emphasize the need for drugs which can elevate the levels of ubiquinone in peripheral organs without elevating the level of cholesterol.

RESULTS

Outline of the methods applied. The rapid extraction procedure used, with petroleum ether/MeOH as solvent system, has made it possible to measure the mevalonate pathway lipids such as cholesterol, dolichol and ubiquinone in a single HPLC run. Methanol, 18 ml and petroleum ether, 12 ml, (b.p. 40–60°C) was mixed with 1 ml of 20% (w/v) tissue homogenate. This mixture was vortexed in a 50 ml screw-capped glasstube. The neutral lipids are

recovered in the upper, petroleum ether phase, while phospholipids and protein partition into the lower MeOH phase. With this procedure adsorption chromatography on silica gel is not required. The use of a convex gradient consisting of MeOH/H₂O, 9:1, v/v (solvent A) and MeOH/*n*-hexane, 2:1:1, v/v (solvent B) allows separation of the lipids present in the upper phase by HPLC. In this system the mevalonate pathway lipids are eluted in the order: cholesterol, UQ₉, UQ₁₀, and, finally, dolichols, appearing individually in the order of increasing chain-length. In this way, the same extraction procedure was used to isolate ubiquinone, cholesterol and dolichols. Since the lipids were well separated on the same HPLC run the peaks could be integrated and used for quantitation by employing internal standards. In the system used cholesterol was eluted at 7–8 min, reduced UQ₉ at 15–16 min, oxidized UQ₉ at 19–20 min, reduced UQ₁₀ at 18 min, oxidized UQ₁₀ at 23–24 min, and dolichols between 37–41 min. In the latter case dolichol gives the sum of dolichols with chain lengths of 17–22 isoprene units.

Ergosterol, UQ₆ and dolichol-23 were used as internal standards. Complete separation was obtained within 48 minutes.

Neutral lipid content. In all tissues studied, cholesterol was the most abundant product of the mevalonate pathway (Table 1). In brain 30% of total lipid consisted of cholesterol, while in liver the amount of this lipid was 4 times smaller. Heart contained 15% of the brain level, while muscle and blood had the lowest levels (0.8 mg and 1.5 mg ubiquinone per g and ml, respectively). Earlier studies have shown that ubiquinone exists in mammals as two different homologues. In rat, the homologue with 9 isoprene units dominates, but the one with 10

Table 1
Amounts of lipids in various tissues.

Aliquots of tissue homogenates and blood were extracted with petroleum ether/MeOH and individual lipids quantified by reversed phase HPLC. The values are the means ± S.D. of 6 experiments.

| Lipid | Liver | Heart | Muscle | Brain | Blood |
|---------------|---------------------------|---------------------------|-------------------------|-------------------------|--------------------------|
| Ubiquinone-9 | 143.1 ± 15.7 ^a | 264.3 ± 34.8 ^a | 48.2 ± 4.4 ^a | 43.0 ± 4.8 ^a | 0.90 ± 0.11 ^b |
| Ubiquinone-10 | 22.2 ± 2.6 ^a | 18.8 ± 2.0 ^a | 3.3 ± 0.3 ^a | 20.0 ± 2.0 ^a | n.d. |
| Cholesterol | 2.5 ± 0.2 ^c | 1.4 ± 0.2 ^c | 0.8 ± 0.1 ^c | 9.9 ± 1.0 ^c | 1.51 ± 0.19 ^d |
| Dolichol | 21.5 ± 2.8 ^a | 5.8 ± 0.5 ^a | 1.4 ± 0.2 ^a | 18.2 ± 2.3 ^a | 0.05 ± 0.05 ^b |

^aµg/g, ^bµg/ml, ^cmg/g, ^dmg/ml

Clofibrate

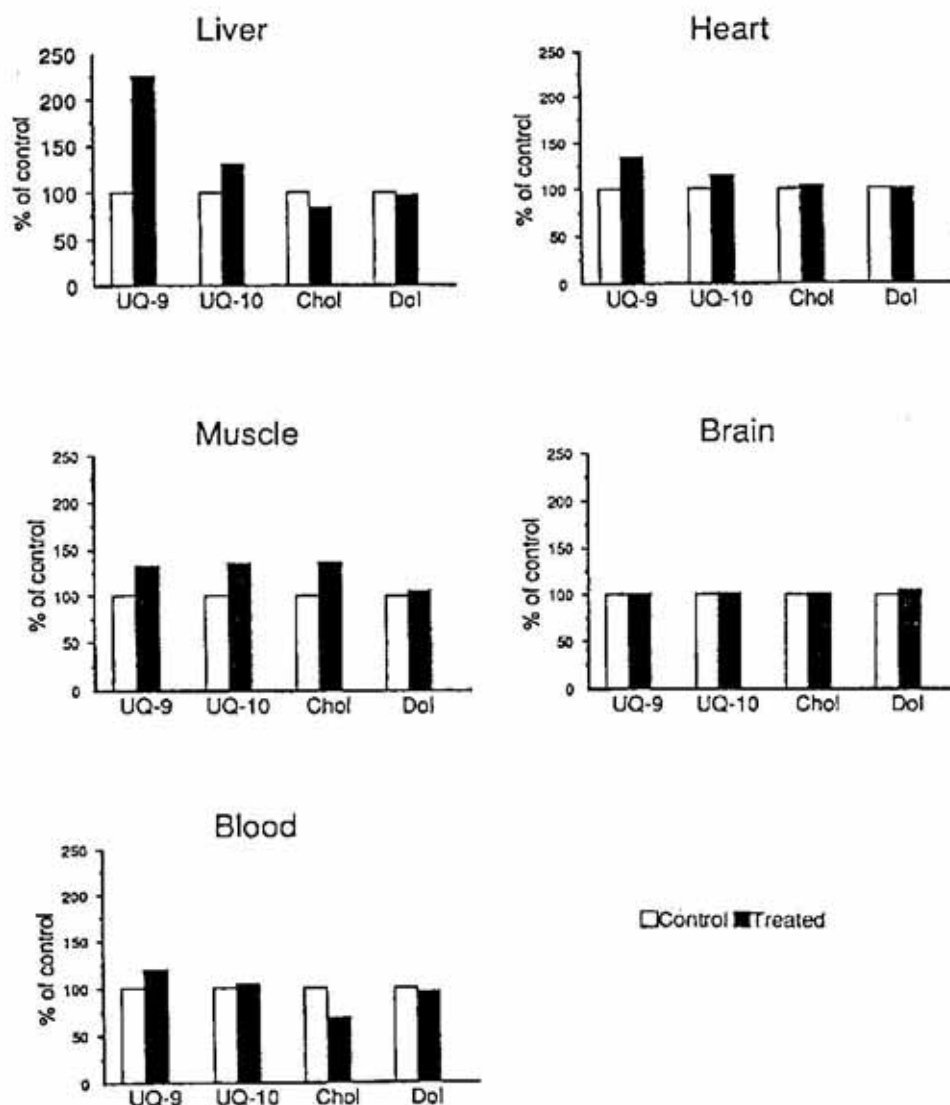


Fig. 1. Amounts of cholesterol, ubiquinone and dolichol in rat tissues after 6 weeks of treatment with a diet containing 0.6% clofibrate.

In Figs. 1-3, mean values from 5-6 experiments are shown, S.D. not exceeding $\pm 15\%$. The ubiquinone levels are the sum of the oxidized and reduced form.

isoprene units is also present in all organs. UQ₁₀ comprises 30% of total ubiquinone in brain, 13% in liver and 7% in muscle and heart.

Dolichol was the less abundant mevalonate pathway lipid, and the tissues studied contained a family of individual dolichols with 17-22 isoprene units (85-110 carbon atoms). The highest content of dolichol was found in liver and brain. About 3-4 times lower amounts were recovered in heart and even lower

amounts in skeletal muscle. Very small amounts of dolichol associated with high density lipoproteins were found in the blood. The level of dolichyl esters in blood was not determined. As in most tissues, this level was much lower than that of its unesterified counterpart.

Levels of neutral lipids after exposure to clofibrate, di(2-ethylhexyl)phthalate (DEHP) and probucol. The effects of three different drugs, the peroxisome proliferators clofibrate and

Phthalate

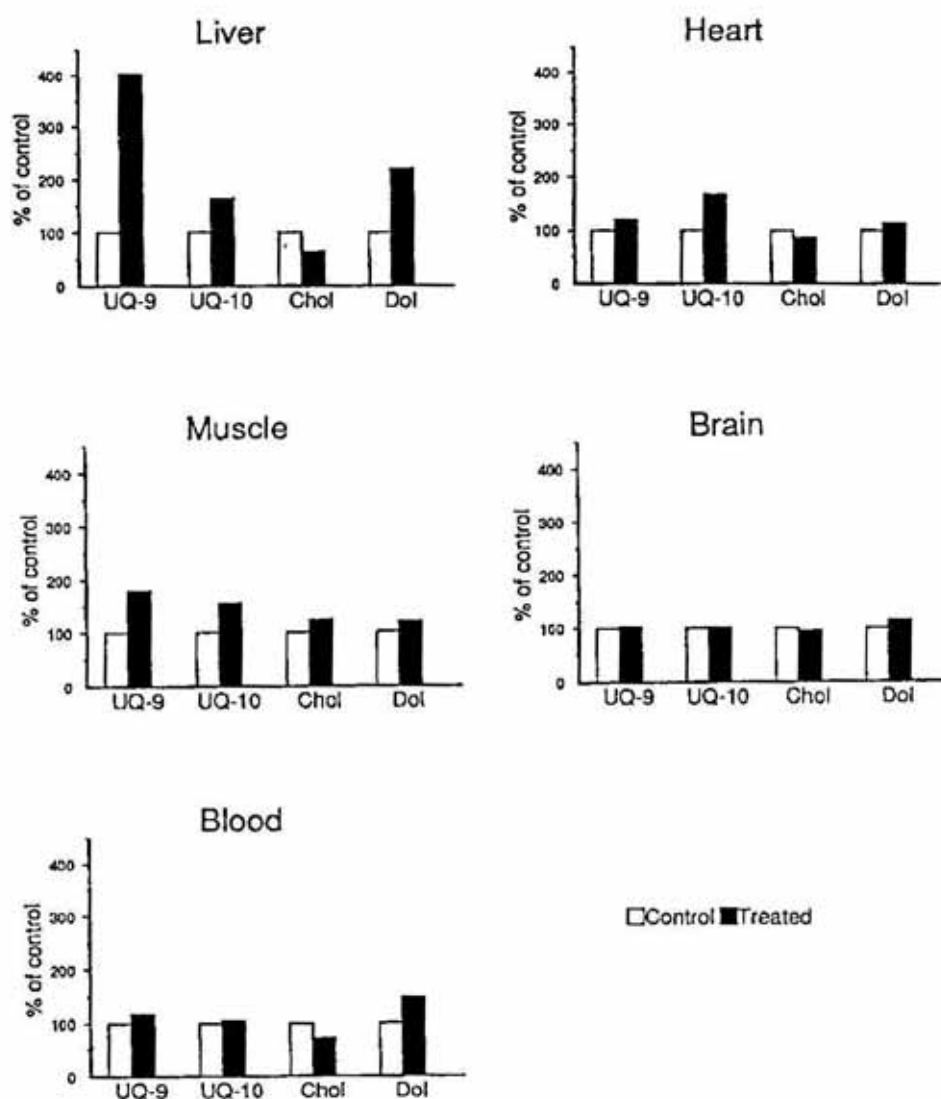


Fig. 2. Amounts of cholesterol, ubiquinone and dolichol in rat tissues after 6 weeks of treatment with a diet containing 2% phthalate.

DEHP, as well as the hypolipidemic and antioxidative agent, probucol were tested. Treatment of rats with a diet containing 0.6%, 2% and 0.25%, respectively, of these drugs resulted in different changes in the content of neutral lipids monitored, as documented in Figs. 1–3. An extensive response was achieved after 6 weeks of treatment. Five different organs were chosen for closer investigation.

With all the drugs applied, the level of neutral lipids in the brain tissue remained essentially unchanged. Clofibrate, a well-known hypoli-

pidemic agent used clinically, increased the level of UQ₉ in the liver more than two-fold. UQ₁₀ level was also elevated, but to a much lesser extent. Total cholesterol content in liver was somewhat diminished, while the level of dolichol was practically unchanged. In heart the changes were much less pronounced than those observed in liver, e.g., about 40% elevation in UQ was seen. In this organ changes in cholesterol and dolichol upon treatment were practically absent.

Probucol

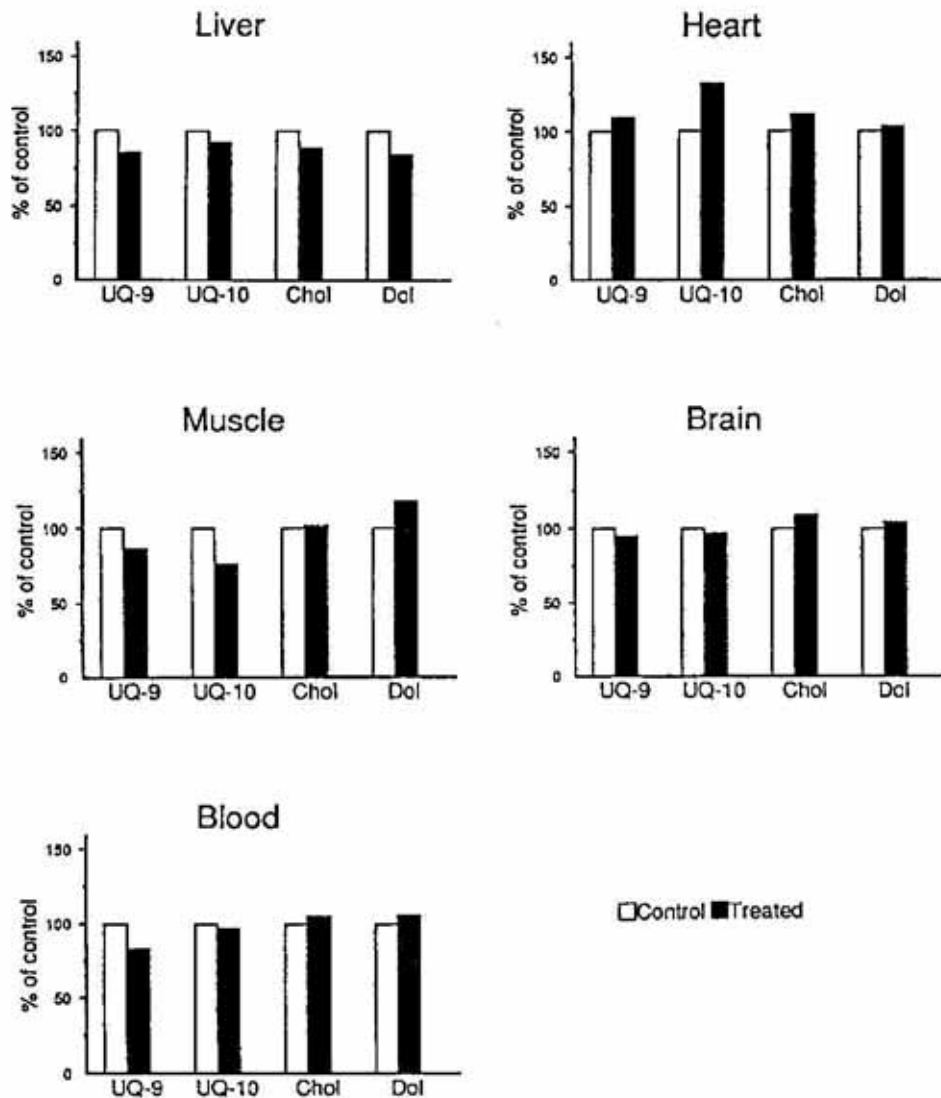


Fig. 3. Amounts of cholesterol, ubiquinone and dolichol in rat tissues after 6 weeks of treatment with a diet containing 0.25% probucol.

The increase in the ubiquinone content of skeletal muscle was similar to that in heart, but in the former case the total cholesterol level also demonstrated an increase. On the other hand, the dolichol content was not altered. Blood demonstrated a pattern of changes similar to that in liver, but distinctly less extensive, except the decrease in cholesterol level.

Treatment with the plasticizer DEHP (Fig. 2) gave results comparable to those obtained with clofibrate, although the increase in ubiquinone content was more pronounced i.e., 300% in the

liver (mainly UQ₉), compared to a doubling in the case of clofibrate. With DEHP, the dolichol level was increased in all organs, in contrast to the situation after clofibrate treatment. In heart and muscle the ubiquinone content was also increased, while the cholesterol level was decreased in liver, blood and heart and increased in muscle.

Probucol, an antioxidant and hypolipidemic agent, decreased the ubiquinone contents in all organs except the heart. The cholesterol level was increased in all organs with the exception

of liver and muscle. A slight increase in the dolichol content was also evident in most cases upon probucol treatment.

DISCUSSION

The membrane bilayer contains phospholipids, and neutral lipids with isoprenoid structures. The basic building blocks of the mevalonate pathway lipids, cholesterol, ubiquinone and dolichol, are short-chain isoprenoid pyrophosphates, which, upon *cis* or *trans* condensations, yield the characteristic polyisoprenoid structure. These isoprenoids are produced by a common initial pathway involving the main regulatory enzyme, β -hydroxy- β -methylglutaryl-CoA (HMG-CoA) reductase. The influence of this reductase on the rates of synthesis of cholesterol, ubiquinone and dolichol is obvious.

Those lipids have important multiple functions in the membrane. Cholesterol, the major product of the mevalonate pathway, is most abundant in the Golgi system, in lysosomes, and in plasma membranes. It affects considerably the membrane properties [17], decreasing fluidity, mobility and permeability, probably as a result of its localization between the fatty acyl moieties of phosphatidylcholine molecules. Enzyme activities such as Na^+, K^+ -ATPase, Ca^{2+} -ATPase and HMG-CoA reductase are inhibited by cholesterol, however stimulation of other enzymes has also been demonstrated. Steroid hormone synthesis relies on the availability of cholesterol as precursor. An important pathophysiological role of this lipid is its involvement in the process of arteriosclerosis. Cholesterol is associated with lipoproteins, mainly low-density lipoproteins. High blood levels of cholesterol and, especially, oxidation of cholesterol by free radicals are considered to be the main causes of deposition of this lipid in arteries [18]. Maintenance of normal levels of cholesterol in the blood seems, therefore, to be of utmost importance for physical well-being.

In its phosphorylated form dolichol functions as an intermediate in glycoprotein synthesis. No major function has yet been assigned to non-phosphorylated dolichol, which, in model membranes, increases phospholipid fatty acid fluidity and decreases the membrane stability [19]. It is possible that the role of dolichol is to

counteract the effects of cholesterol on membranes. Dolichol is present at highest concentrations in Golgi vesicles and lysosomes.

Biophysically, the properties of ubiquinone are similar to those of dolichol [20], and both these lipids are positioned in the hydrophobic phase between the membrane leaflets. It is accepted that ubiquinone can pass across the membrane, thereby translocating protons, in accordance with the basic function of this electron carrier in the inner mitochondrial membrane, mediating between electron-transfer complexes. UQ seems to be present in mitochondria in great excess with respect to other respiratory chain components [3]; this could suggest that, in addition to its involvement in oxidative phosphorylation, this lipid has another function.

Ubiquinone is also abundant at locations other than inner mitochondrial membranes, e.g., in outer mitochondrial membranes, Golgi vesicles and lysosomes [5, 21]. The observations that ubiquinone is found in all biological membranes including those of plants, and that *in vivo* it is present to a large extent as ubiquinol, which can quench free radicals, suggests a protective, antioxidant role. A part of the ubiquinone in mitochondria may, in fact, function, as an antioxidant.

There is a direct correlation between the rates of electron-transport and radical production. Electrons are thought to leak from the electron-transport chain generating the superoxide radical $\text{O}_2^{\cdot-}$. This radical can dismutate to H_2O_2 and, in the presence of iron, give rise to the very reactive species OH^{\cdot} [22]. The excess UQ in the inner mitochondrial membrane, easily reduced by the enzymes of the respiratory chain, could thus serve as a scavenger preventing peroxidative damage.

The short half-life of ubiquinone in comparison to those of other membrane lipids also emphasizes the importance of this compound [23]. Additional indications are the large alterations in the ubiquinone content which occur under different conditions. These effects include an increase in UQ content during development and a decrease during aging [24]. Treatment with peroxisome proliferators, such as clofibrate and DEHP, or the carcinogenic *N*-nitrosodiethylamine, elevates UQ content [25]. Preneoplastic liver noduli exhibit high UQ contents, which is also the case with brain tissue

from Alzheimer's patients [26, 27]. Hepatocellular cancer is associated with a large decrease in the level of this lipid [28].

The results presented in this paper demonstrate that blood and tissue levels of ubiquinone can be elevated without being accompanied by extensive changes in the cholesterol and dolichol levels. As mentioned above, oxidized cholesterol may be responsible for the deposition of this lipid in the arterial wall. Drugs which are able to increase the level of the endogenous lipid antioxidant, ubiquinone, without affecting cholesterol levels, could thus be of great clinical importance. No substantial increase in the ubiquinone contents in various organs occurs when this substance is supplied in the diet. Peroxisome proliferators, e.g., clofibrate and phthalate esters, increase the amounts of ubiquinone in all organs with the exception of the brain, while leaving cholesterol levels essentially unchanged. The difference between the influence of these two compounds are their effects on dolichol concentration. Clofibrate has no effect, whereas phthalates increase the dolichol levels in most organs studied. One possible explanation for this is that a metabolite of DEHP influences *cis*-prenyltransferase activity or, alternatively, slows down the breakdown of dolichol. Further studies on the biosynthesis and half-life of this lipid should provide answers to these questions.

Increased ubiquinone levels after treatment of rats with peroxisome proliferators might be explained by the increase in the number of mitochondria. It appears, however, that the greater recovery of mitochondria after such treatment does not reflect an induction of mitochondrial protein, but rather a higher recovery of these organelles in the mitochondrial fraction. The increased recovery of mitochondria after phthalate treatment has been documented by Lundgren *et al.* [29]. Peroxisome proliferators stimulate β -oxidation of fatty acids but not catalase activity. This could result in an increased cellular level of H_2O_2 , which may cause oxidative stress. The increased UQ levels would then be necessary for quenching of the radicals produced in larger amounts. This concept might also explain the good correlation observed between increases in fatty acid β -oxidation and UQ levels. These observations are

also in agreement with other experimental findings [30, 31].

An important question is whether peroxisome proliferators affect humans. Some free fatty acids are known to act as peroxisome proliferators, also in humans. We are also exposed to plasticizers such as DEHP, which are abundant in the environment.

Dietary administration of probucol to animals was used here to investigate whether an increase in UQ content can be elicited by oxidative stress. Probuco's pharmacological action is considered to be both antioxidative and hypolipidemic [32, 33]. The decreased level of ubiquinone pointed to a diminished demand for endogenous tissue antioxidant after probucol administration. On the other hand, the action of probucol is not yet fully understood. Previous investigations have shown that this compound lowers the level of cholesterol in blood. In our experiments on Sprague-Dawley rats, the cholesterol content was decreased by probucol only in the liver, but not in heart, muscle, brain or blood. Thus, it is possible that the effects described previously apply only to Wistar rats.

Several reports have appeared concerning the protective effects of ubiquinone in *in vitro* and *in vivo* systems as well as in clinical trials. Since the uptake of this compound affects its levels only in liver and blood, it is of great interest to search for drugs of low toxicity which can elevate the concentrations of ubiquinone in a number of other target organs. In our experiments clofibrate elevated the ubiquinone levels in muscle and heart. In degenerative muscle diseases and cardiomyopathies, the level of this lipid is decreased and it has been suggested that this lowering of UQ may be of etiological importance in the development of those conditions. Identification of new drugs which increase UQ synthesis in specific target tissues may, consequently, be of considerable interest.

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