Mechanism of the Enrichment of Phosphatidylcholine in Liver Accompanying Enzyme Induction by Phenobarbital

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Summary. The mechanism of the increase of phosphatidylcholine in liver, accompanying enzyme induction by phenobarbital, has been studied in rats. Using radioactively labeled precursors, the two main pathways of phosphatidylcholine biosynthesis—the CDP-choline pathway and the methylation of phosphatidylethanolamine—were analyzed after pretreatment with 4 doses of phenobarbital (80 mg/kg) on 3 consecutive days.

After i.v. injection of choline [Me-3H], choline [Me-14C] or NaH2[32P]O4 the specific radioactivity (sp. act.) of phosphatidylcholine (dpm/nmol) was decreased by 60%, and after methionine [Me-3H] or ethanolamine [1.2-14C] by 40% compared to control rats.

These changes are partly due to the increased concentration of phosphatidylcholine and phosphatidylethanolamine, causing the incorporated precursors to dilute, and partly to a secondary effect which leads to a reduction of the sp. act. of free choline in pretreated animals.

The concentration of glycerylphosphorylcholine, one of the metabolites of phosphatidylcholine catabolism, was also diminished by almost 50%.

From these results it may be concluded that the increase of phosphatidylcholine is due to a retardation of its breakdown rather than to an increase of its synthesis.

Key words: Phosphatidylcholine — Phenobarbital — Liver — Enzyme induction.

INTRODUCTION

The phenobarbital induced proliferation of hepatic endoplasmic membranes is characterized by an increase in both phospholipid and protein content. Whether the increase of membrane phospholipids is due to an enhanced synthesis or to a delayed catabolism, is still controversial.

Orrenius et al. (1965) found that phenobarbital leads to an increased incorporation of [32P] into phospholipids and to a rise of sp. act. of microsomal phospholipids indicating an enhanced synthesis. Holtzman and Gillette (1968) have repeated these experiments and pointed out that the increased sp. act. of microsomal phospholipids reported by Orrenius et al. could be due to a higher sp. act. of the inorganic precursor of the phospholipid phosphorus. Therefore, Holtzman and Gillette interpreted the increase of phospholipids in microsomes by a diminished catabolism. Their interpretation was confirmed by Stein and Stein (1969) who were able to demonstrate a lower rate of degradation of endogenous phospholipids by liver tissue from phenobarbital treated rats in vitro. They also studied the incorporation of choline [Me-3H] and ethanolamine [14C] into microsomal phospholipids in vivo. It was found that owing to the rise in microsomal phosphatidylcholine and phosphatidylethanolamine sp. act. were lower in pretreated than in control rats 1 h after injection of labeled precursors. These results did not support the suggestion that phospholipid synthesis was enhanced.

Young et al. (1971) and, recently, Davison and Wills (1974), however, reported an increase in the enzyme activity of S-adenosylmethionine phosphatidylethanolamine (SAME:PE) methyltransferase after phenobarbital and therefore suggested that the increase of phosphatidylcholine was related to an acceleration of the biosynthesis involving the sequential methylation of phosphatidylethanolamine.

In order to provide more detailed information on the question whether the increase of membrane phospholipids is due to an enhanced synthesis or to a slowed catabolism the incorporation of labeled choline,
ethanolamine, phosphate and methyl-groups into phosphatidylethanolamine was studied in vivo. Sp. act. of the precursors of phosphatidylethanolamine namely free choline, phosphorylcholine and phosphatidylethanolamine were also determined.

MATERIALS AND METHODS

Labeled Compounds and Chemicals. Choline \([\text{Me}^{14}\text{C}]\) (sp. act. 50 mCi/mmol), choline \([\text{Me}^{3}\text{H}]\) (sp. act. 5 Ci/mmol), ethanolamine \([\text{Me}^{2}\text{H}]\) (sp. act. 5 mCi/mmol) and ortho \([^{32}\text{P}]\) phosphate (sp. act. 1 Ci/mmol) were purchased from NEN Chemicals GmbH, West Germany; \(L\)-methionine \([\text{Me}^{14}\text{C}]\) (sp. act. 50 mCi/mmol) by the Radiochemical Centre Amersham, England. The radiochemical purity of all compounds was > 98%.

Distilled deionized water was used throughout. Chemicals were of analytical reagent quality.

Animals. Male Sprague-Dawley rats, 140–170 g, from Mus Rattus (Brunnthal, West Germany) were kept under conventional conditions and had free access to water and food (standard chow, Altromin®). On 3 consecutive days they were injected i.p. in the morning with 80 mg/kg of phenobarbital dissolved in 0.9% NaCl. A further injection was given in the evening of the first day. In one experiment (Fig. 6 and Fig. 7) the animals received a fifth injection in the morning of the fourth day. Radioactively labelled precursors dissolved in 0.9% NaCl were administered into the tail vein 24 h after the last dose of phenobarbital, with the exception of one experiment in which choline \([\text{Me}^{14}\text{C}]\) was injected 5 h after the last dose (Fig. 6 and Fig. 7). Rats were decapitated and livers dissected, weighed and chilled on ice. If the incorporation into total liver was studied, pieces of tissue were homogenized in chloroform/methanol 2:1 (v/v).

Cell Fractionation. Subcellular fractions were prepared from 1–2 g of liver, homogenized with 14 volumes of ice-cold 0.25 M sucrose containing 0.1 mM Na2-EDTA. After centrifugation at 600 g for 15 min in a Sorvall RC-2B centrifuge, the supernatant was centrifuged at 15000 g for 20 min, the pellets resuspended in 5 ml 0.25 M sucrose solution and centrifuged again for 20 min at 15000 g yielding the mitochondrial fraction. To obtain the microsomal fraction, both supernatants were combined and centrifuged for 1 h at 105000 g in a Beckman L2-65B ultracentrifuge.

Extraction and Determination of Phosphatidylcholine, Lipid-Choline and Phosphatidylethanolamine. Lipids were extracted and purified as described before (Stahl, 1967). For the separation of phospholipids thin layer silica-gel plates (E. Merck, Darmstadt, West-Germany) were used according to Skipski et al. (1964). The individual compounds were visualized by exposing the plates to iodine vapor. The area corresponding to phosphatidylcholine was scraped off, eluted and hydrolyzed in boiling saturated \(\text{Ba(OH)}_2\) solution for 1 h. Choline was separated by ion-pair extraction (Köppel et al., 1970) and an aliquot taken to measure radioactivity and choline concentration photometrically by the use of dipicrylamine as a chromophor (Schill and Danielsson, 1959). In 2 experiments (see Fig. 4 and Fig. 7), the concentration of phosphorylcholine and phosphatidylethanolamine was measured as phosphorus according to Bartlett (1959).

In some experiments lipids were not separated by TLC but hydrolyzed in toto. The hydrolyzed lipid-bound choline was separated and its concentration and radioactivity determined resulting in sp. act. of choline called lipid-choline. These values were proven to be representative for those of phosphatidylethanolamine, since almost 90% of the lipid-bound choline was recovered in phosphatidylethanolamine and, furthermore, more than 90% of the labeled phosphatidylcholine was recovered as phosphatidylethanolamine in pretreated and control rats.

To hydrolyze, separate and measure lipid-choline both of the following methods were applied which gave almost identical results: 1. lipids were hydrolyzed in ethanolic 1 N NaOH for 16 h at room temperature and neutralized with concentrated HCl. The hydrolyzed lipid-bound choline was separated by paper-chromatography according to Dross and Kewitz (1972) and the concentration determined with the eneueioidide-method (Appleton et al., 1953). 2. Lipids were hydrolysed in saturated Ba(OH)\(_2\) and choline separated and estimated by ion-pair extraction (Köppel et al., 1970) and the dipicrylamine-method (Schill and Danielsson, 1959).

Extraction and Determination of Water-Soluble Choline Compounds. Free choline, phosphorylcholine and glycerylphosphorylcholine were extracted by homogenisation of the tissue with 20 ml of 67% ethanol in \(\text{H}_2\text{O}\) according to Björnstad and Bremer (1966) and centrifuged at 600 g for 20 min. The supernatant was dried and the samples dissolved in 2 ml \(\text{H}_2\text{O}\). Lipids were removed by shaking the solution twice with 20 ml of heptane/isoamylic alcohol (1:1, v/v) and once with 20 ml of ether. An aliquot of the water soluble residue was chromatographed on paper according to Dross and Kewitz, (1972). Free choline was determined after an ion-pair extraction (Köppel et al., 1970) as described before (Schill and Danielsson, 1959), choline from glycerylphosphorylcholine after hydrolysis in boiling 1 N HCl and choline from phosphorylcholine after treatment with 1 mg acid phosphatase (Biochemica Boehringer) in 1 ml of acetate buffer pH 5.7 for 1 h at 37°C. Before choline concentrations were measured, the hydrolysed samples were chromatographed on paper and the choline further purified by ion-pair extraction.

To avoid \(^{32}\text{P}\) contamination after application of ortho \(^{32}\text{P}\) phosphate as precursor, phosphorylcholine had to be isolated from other organic phosphates. Samples were dissolved in 5 ml \(\text{H}_2\text{O}\), shaken for 10 min with 30 mg Norit A (Crane and Lipmann, 1953) and centrifuged at 600 g for 10 min. An aliquot from the supernatant was applied to descending paper chromatography with methanol/\(\text{NH}_3\) 10% (trichloroacetic acid 10% \(\text{H}_2\text{O}\) (50:15:5 :30) for 16 h (Stahl, 1967), the area corresponding to phosphorylcholine eluted with \(\text{H}_2\text{O}\) and an aliquot taken to measure radioactivity. The concentration was determined after hydrolysis with acid phosphatase as described before.

Protein was determined by the method of Lowry et al. (1951).

Determination of Radioactivity. A liquid scintillation spectrometer Packard Tricarb model 3141 was used in combination with the absolute activity analyzer Packard model 544. Samples were dissolved in 10 ml of dioxan with 10% naphthaline, 1% (w/v) 2,5-diphenyloxazole (PPO) and 0.025% (w/v) 2,2-p-phenylene-bis (5-phenyl-oxazole) (POPOP).

Statistics. To establish statistical differences Student’s t-test has been used. At a \(P\)-value < 0.05, a difference was considered significant.

RESULTS

Effect of Phenobarbital Treatment on the Concentration of Lipid-Choline, Protein and Free Choline

As indicated in Table 1, relative liver weight, total liver lipid-choline as well as microsomal lipid-choline and protein were significantly elevated in the phenobarbital treated animals. Microsomal lipid-choline was elevated to a larger degree (+ 58%) than micro-