Evidence Against *in vitro* Modulation of Rat Liver Cholesterol 7α-Hydroxylase Activity by Phosphorylation-Dephosphorylation: Comparison with Hydroxymethylglutaryl CoA Reductase

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The activity of cholesterol 7α-hydroxylase in rat liver microsomes was investigated under conditions favourable for phosphorylation-dephosphorylation. The enzyme activity was similar in the presence or absence of sodium fluoride during preparation. Preincubation with ATP and magnesium did not affect the enzyme activity. Cholesterol 7α-hydroxylase was inhibited by alkaline phosphatase, but this inhibition was similar also after inactivation of the phosphatase. Under similar conditions, rat hepatic hydroxymethylglutaryl CoA reductase activity was clearly modulated in agreement with phosphorylation-dephosphorylation. The absence of such a modulation of cholesterol 7α-hydroxylase argues against involvement of phosphorylation-dephosphorylation in the regulation of this enzyme.

Cholesterol 7α-hydroxylase (EC 1.14.13.7) catalyses the initial step in the conversion of cholesterol to bile acids.[1,2] This reaction is rate limiting for bile acid biosynthesis[3,4] and cholesterol 7α-hydroxylase is subject to regulation by a number of factors including hormones, drugs, dietary components, bile diversion and lymphatic drainage.[1,2] Recently, several investigators have reported that the enzyme activity might be modulated by a phosphorylation-dephosphorylation mechanism. Addition of alkaline phosphatase was found to inhibit cholesterol 7α-hydroxylase and there was an apparent activation in the presence of ATP and protein kinase.[5,6] This would be an interesting mechanism for regulation of cholesterol homeostasis, and it has even been suggested that phosphorylation-dephosphorylation may be of importance for coordinating the activities of cholesterol 7α-hydroxylase and the rate limiting enzyme in cholesterol biosynthesis, hydroxymethylglutaryl (HMG) CoA reductase.[7] However, other authors have reported an inactivation of cholesterol 7α-hydroxylase in the presence of ATP, which was reversible in the presence of a cytosolic activator.[8,9] In a recent work, it was reported that ATP acted both as inhibitor and stimulator on cholesterol 7α-hydroxylase depending on the nucleotide concentration.[10] Again, other authors have reported a complete lack of effect by ATP or sodium fluoride on the enzyme activity.[11,12] In view of the contradictory reports, it was considered of interest to further investigate the possible role of phosphorylation on the cholesterol 7α-hydroxylase. In order to ascertain that suitable conditions for phosphorylation and dephosphorylation were present, HMG CoA reductase was included in the experiments. That this enzyme is subject to phosphorylation-dephosphorylation is well documented.[13–15] In previous works, the activity of cholesterol 7α-hydroxylase was assayed by measuring the rate of conversion of added labelled substrate. In the present work, a presumably more accurate assay based on conversion of endogenous substrate with a mass fragmentographic technique[16] was used.

Experimental procedure

Materials. Mevalonic acid lactone, HMG CoA, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol and ATP were obtained from Sigma Chemical Co, St Louis, MO. 3-^{14}C HMG CoA was a product of New England Nuclear Corp., Boston, MA., and DL-2-^{3}H mevalonic acid lactone was purchased from the Radiochemical Centre, Amersham, England. $^{2}H_{3}$ 7α-hydroxycholesterol was synthesized as described previously.[16] *E. coli* alkaline phosphatase type III was obtained from Sigma and the suspension was centrifuged at 12,000 × *g* for 45 min at 4 °C just prior to use. The supernatant was discarded and the pellet suspended in 20 mM imidazole-HCl buffer, pH 7.4.

Enzyme preparations. Male Sprague-Dawley rats with a weight of 200–250 g were used. The animals were fed standard laboratory chow and kept under a 12 h light/dark cycle (light 10 p.m. to 10 a.m.). The rats were killed at 9 a.m., the livers excised and homogenized in 9 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM EDTA and 10 mM dithiothreitol. In order to achieve different conditions during the preparation, the tissue was divided in two fractions, one being homogenized in the presence of 50 mM NaCl and the other in the presence of 50 mM NaF. The homogenates were centrifuged at 20,000 × *g* at 4 °C for 20 min and the supernatant fractions were immediately centrifuged at 100,000 × *g* at 4 °C for 60 min. The pellets were suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 10 mM EDTA as above, in the presence and absence of 50 mM NaCl or NaF. Following this procedure, the suspensions containing either NaCl or NaF were both split in two fractions, respectively, one being used for assay of HMG CoA reductase and the other one for assay of cholesterol 7α-hydroxylase. The suspensions were recentrifuged at 100,000 × *g* as above. The pellet fractions used for assay of HMG CoA reductase activity were dissolved in 20 mM imidazole-HCl buffer, pH 7.4, containing 10 mM dithiothreitol. The pellets in the tubes used for assay of cholesterol 7α-hydroxylase activity were dissolved in 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. The protein concentrations were determined as described by Lowry.[17]

Assay of enzyme activities. The activity of HMG CoA reductase was assayed essentially as described earlier[18] except that the final EDTA concentration was 2 mM. The assay for cholesterol 7α-hydroxylase was performed according to the method of Björkhem and Kallner,[16] where the product, 7α-hydroxycholesterol, is measured by isotope dilution – mass spectrometry. Alkaline phosphatase was assayed as described.[19]

Activation-inactivation experiments. The microsomal fractions were preincubated either in the presence or absence of 20 units of alkaline phosphatase or in the presence or absence of 4 mM ATP and 10 mM magnesium chloride prior to assay of HMG CoA reductase and cholesterol 7α-hydroxylase activities. The preincubation conditions chosen were 45 min at 37 °C for HMG CoA reductase and 60 min at 37 °C for cholesterol 7α-hydroxylase.

Results

When rat liver microsomes are prepared in the presence of fluoride, HMG CoA reductase activity is prevented from activation and the enzyme is mainly present in its inactive, phosphorylated form.[13–15] The results shown in Table 1 are in accordance with this concept. In contrast, the presence of fluoride during the enzyme preparation did not affect cholesterol 7α-hydroxylase activity. Incubation with MgATP did not change the activity of cholesterol 7α-hydroxylase appreciably, neither with NaCl-treated nor with NaF-treated microsomes. Addition of alkaline phosphatase did, however, decrease the enzyme activity to a similar extent in both cases. As described by others, HMG CoA reductase activity was readily affected by both MgATP and alkaline phosphatase, indicating the presence of a phosphorylation-dephosphorylation mechanism. The finding that the presence of alkaline phosphatase decreased the cholesterol 7α-hydroxylase activity, which has also been noted by others,[5,6] might however indicate a possible dephosphorylation of the enzyme. The alkaline phosphatase preparation used was, however, not homogeneous and the presence of other factors influencing the activity of both enzymes could not be ruled out. Consequently, in order to inactivate the alkaline phosphatase, the enzyme preparation was treated for 5 min at 100 °C and thereafter used in the preincubation

Table 1. Effect of ATP and alkaline phosphatase on cholesterol 7α-hydroxylase and HMG CoA reductase activity[a]

Conditions	Cholesterol 7α-hydroxylase			HMG CoA reductase		
	Microsomes prepared with NaCl/pmol · min^{-1} · mg prot^{-1}	(%)	Microsomes prepared with NaF/pmol · min^{-1} · mg prot^{-1}	(%)	Microsomes prepared with NaCl/pmol · min^{-1} · mg prot^{-1}	(%)	Microsomes prepared with NaF/pmol · min^{-1} · mg prot^{-1}	(%)
No addition	33±6	100	38± 8	115	294±28	100	105± 9	36
+ Mg ATP	35±4	106	37±10	112	181± 6	62	66± 6	22
+ Alkaline phosphatase	14±5	42	15± 8	45	520±64	177	496±43	169

[a]The buffer used for cholesterol 7α-hydroxylase was 20 mM Tris-HCl, pH 7.4. For HMG CoA reductase, 20 mM imidazole-HCl, pH 7.4, was used and potassium phosphate at a final concentration of 100 mM was added after preincubation just prior to assay. The numbers represent mean values from four experiments analyzed in duplicate, and the variation indicates the standard error of the mean. The relative numbers are expressed with reference to the respective enzyme activities with NaCl-microsomes and no additions.

Table 2. Effect of heat treatment of alkaline phosphatase on the cholesterol 7α-hydroxylase activity[a]

Conditions	Cholesterol 7α-hydroxylase activity (%)
no additions	100
+ alkaline phosphatase	40
+ heat-treated alkaline phosphatase	55

[a]After centrifugation, the alkaline phosphatase was diluted to a concentration of 100 U/ml in 20 mM imidazole-HCl buffer, pH 7.4. The enzyme preparation was then divided and one half was treated for 5 min at 100 °C. The two preparations were thereafter used in equal concentrations in the standard assay of cholesterol 7α-hydroxylase. The concentration used corresponded to 10 units per incubation of the untreated alkaline phosphatase.

procedure. During the heat treatment procedure, more than 90 % of the alkaline phosphatase activity was lost. As is shown in Table 2, the inhibitory effect on cholesterol 7α-hydroxylase activity did not differ appreciably (*cf.* Table 1) between heat-treated and control alkaline phosphatase. It was of importance to compare this effect on cholesterol 7α-hydroxylase activity with that on the HMG CoA reductase. The enzyme preparations were therefore preincubated with different concentrations of either native or heat-treated alkaline phosphatase. As is shown in Fig. 1, there was a large decrease in the activation of HMG CoA reductase following heat treatment of alkaline phosphatase, while again no difference was seen in the case of cholesterol 7α-hydroxylase.

Discussion

Several laboratories have during recent years studied the regulation of cholesterol 7α-hydroxylase. However, apparently conflicting results have been presented raising some controversy regarding the role of phosphorylation-dephosphorylation of this enzyme.[5-12] In contrast, the role of phosphorylation-dephosphorylation in the regulation of another enzyme of importance in cholesterol metabolism, HMG CoA reductase, has been well documented.[13-15] The aim of the present study was therefore to compare the influence of conditions favouring phosphorylation-dephos-

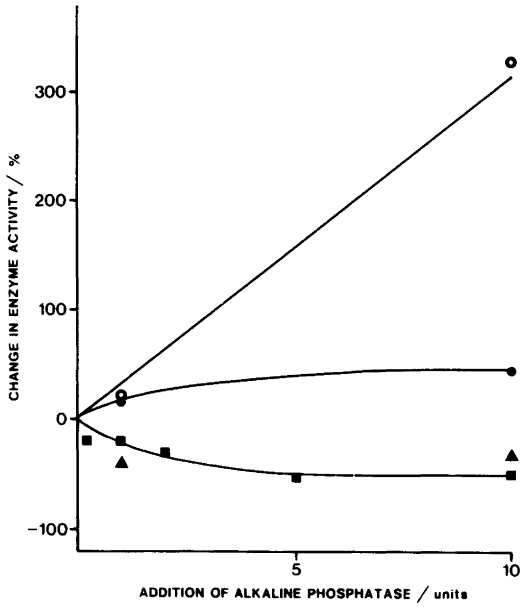

Fig. 1. Effect of preincubation with alkaline phosphatase on the activities of HMG CoA reductase and cholesterol 7α-hydroxylase. HMG CoA reductase activity after preincubation with active (○) or heat-treated (●) alkaline phosphatase. Cholesterol 7α-hydroxylase activity after preincubation with active (■) or heat-treated (▲) alkaline phosphatase. The alkaline phosphatase preparation was divided into two fractions and one fraction was treated for 5 min at 100 °C. Heat-treated and control alkaline phosphatase was added in corresponding amounts and the amount of units given represents the addition of active alkaline phosphatase.

phorylation on these two enzymes in parallel studies, in order to shed further light on the mechanisms regulating the activity of cholesterol 7α-hydroxylase.

Rat liver HMG CoA reductase is known to be activated by protein phosphatases during the preparation of microsomes, and this activation can be prevented by the inclusion of sodium fluoride.[13–15] The activity of cholesterol 7α-hydroxylase activity was virtually the same when the enzyme was prepared in the presence or absence of sodium fluoride. Furthermore, no effect of preincubation with MgATP on enzyme activity was demonstrable. The *in vitro* activation-inactivation of HMG CoA reductase was readily demonstrated under these conditions, indicating that the conditions for phosphorylation were present. These results do not support the possibility that *in vitro* modulation of cholesterol 7α-hydroxylase by phosphorylation-dephosphorylation occurs.

This hypothesis was further supported by the finding that inactivated alkaline phosphatase inhibited enzyme activity to a similar extent as the fully active phosphatase preparation. Again, a marked difference was seen between HMG CoA reductase and cholesterol 7α-hydroxylase with respect to the effect of inactivated alkaline phosphatase. The small amount (less than 10%, corresponding to less than 1 unit/incubation) of alkaline phosphatase activity remaining after heat treatment is unlikely to be sufficient to dephosphorylate cholesterol 7α-hydroxylate. Thus, at least 3 units of alkaline phosphatase have been found necessary to activate phosphatidic acid phosphatase[20] under similar conditions. There still remained, however, a small activation of HMG CoA reductase even after heat treatment of alkaline phosphatase. It must be emphasized that the alkaline phosphatase preparation used was not homogeneous and the presence of possible heat-stable activators or inhibitors can not be ruled out. The results indicate that the inhibition of cholesterol 7α-hydroxylase seen in the present experiments with alkaline phosphatase is not specific and may be due to some factor present in the phosphatase preparation.

Thus, our results do not support the contention that phosphorylation-dephosphorylation is of importance in the regulation of the cholesterol 7α-hydroxylase. It may be argued that crude preparations of the enzyme were used in the present experiments and that definite conclusions must await work with purified preparations. On the other hand, Danielsson *et al.* were not able to demonstrate specific effects of ATP on a reconstituted cholesterol 7α-hydroxylating system consisting of highly purified cytochrome P-450 and NADPH cytochrome P-450 reductase.[11,12]

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