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Modulation of 3-hydroxy-3-methyl glutaryl CoA reductase by 2,3-diphosphoglyceric acid

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Summary. Rat liver microsomal 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase was activated by 50% at a concentration of 0.4 mM 2,3-diphosphoglyceric acid (DPG) and by 11-fold at 10 mM DPG. DPG also prevented the inactivation of HMG-CoA reductase by ATP and Mg^{++}. Rat liver microsomal HMG-CoA reductase prepared in the presence of 1 mM DPG was significantly more active than when prepared in the absence of DPG. Activation of the enzyme by DPG and protection of the enzyme against inhibition by ATP and Mg^{++} by DPG were also observed with solubilized HMG-CoA reductase.
Key words. Cholesterol; 2,3 diphosphoglyceric acid; 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase.

Red blood cell 2,3-diphosphoglycerate plays an important role in oxygen transport in mammals, binding to hemoglobin and thus reducing its affinity for oxygen[2]. Noble et al.[3] developed 2 rat strains by genetic selection based upon relative levels of red cell DPG. Starting with outbred hooded rats they produced one set of rats with high cell DPG and another with low DPG. They showed that rats with low red cell DPG had significantly higher plasma cholesterol levels than rats with high red cell DPG. Alterations in red cell DPG were also associated with parallel changes in red cell ATP. There are a number of reports showing regulation of HMG-CoA reductase by ATP and Mg^{++}[4-10]. HMG-CoA reductase is a major regulatory enzyme in the biosynthesis of cholesterol[11-14].

The present study was undertaken in order to determine whether DPG has any direct effect on HMG-CoA reductase and if there is any interrelationship between DPG and ATP with regard to HMG-CoA reductase activity.

Materials and methods. DL-[3-^{14}C]-HMG-CoA (specific activity 18.5 μCi/mol) was purchased from New England Nuclear. Unlabeled HMG-CoA (from PL Biochemicals, Milwaukee, WI) was added to the radioactive substrate to give a specific radioactivity of 1.1 μCi/mol, and this substrate was used in all the experiments. DL-[4-^{3}H] mevalonic acid was purchased from Amersham/Searle. Unlabeled mevalonic acid and 2,3-diphosphoglyceric acid were obtained from the Sigma Chemical Company.

Male Sprague-Dawley rats, 10 weeks of age, were used for all experiments. The rats were maintained ad libitum on a diet of rat chow and tap water. For 2 weeks one group of animals was subjected to the following lighting cycle: dark, 4.00 to 16.00 h local time; light, 16.00 to 4.00 h. For another group of animals this lighting was reversed. The animals were killed at the midpoint of dark phase (10.00 h) for the first group of animals and at the midpoint of the light phase (22.00 h) for the second group of animals.

The livers from these rats were homogenized in 0.04 M phosphate buffer, pH 7.2, containing 0.1 M sucrose, 0.05 M KCl and 0.01 M dithioerythritol. The homogenate was centrifuged twice at 20,000 × g for 12 min each time. The 20,000 × g supernatant was centrifuged at 105,000 × g for 1 h and the pellet suspended in the above buffer and centrifuged at 105,000 × g for a second time. The pellet from the second centrifugation was suspended in the above buffer for use in studying the effect of DPG and other compounds.

In the experiment where the microsomes were prepared in buffer containing DPG, the preparation of the microsomes was as described above except that 1.0 mM DPG was added. In the latter case the liver was divided into 2 portions; one part was homogenized in buffer only and the other part homogenized in the same buffer containing 1.0 mM DPG.

Table 1. Activation of rat liver microsomal HMG-CoA reductase activity by DPG

DPG (mM)	HMG-CoA reductase activity (pmoles mevalonic acid formed/min/mg protein)
None	179
1 mM	633
2.5 mM	933
5 mM	1320
10 mM	2050
20 mM	2110

Microsomes were prepared from the rat liver in buffer without EDTA. Assay conditions were as described under methods. Experiments were done in triplicate.

For the preparation of soluble extracts of the microsomes, the microsomal suspension was subjected to slow freezing in an ethanol bath at 0 °C. The temperature of the bath was lowered to −50 °C at a rate of 6 °C/min by adding pellet of dry ice. The frozen microsomes were stored at −60 °C and the next day allowed to thaw in a waterbath at room temperature. Then the microsomes were homogenized in the buffer (10 times the volume of the pellet) using a ground glass mortar and pestel. The homogenate was then centrifuged at 105,000 × g for 1 h, and the supernatant used as the soluble extract of HMG-CoA reductase. This method is similar to the method of Heller and Gould[15]. Assay conditions: The reaction mixture containing 100 µl of the microsomal suspension (50–200 µg of protein), NADPH (4 mM) and DPG, was preincubated at 37 °C for 30 min. At this time the ^{14}C-HMG-CoA (300 µM) substrate was added and the incubation continued for 20 more minutes. The reaction was stopped by adding 1 ml of 2 N HCl. 4-^{3}H-Mevalonic was added as an internal standard to quantitate losses during further analysis. The remaining procedure for quantitating ^{14}C-mevalonic acid formed was according to the method of Ackerman et al.[16]. Protein was determined by the method of Bradford[17].

Results and discussion. DPG had a potent activating effect on microsomal HMG-CoA reductase activity (table 1). The latter was activated by 50% at a DPG concentration of 0.4 mM and with 10 mM DPG the enzyme was activated about eleven fold. This effect was seen only if microsomes were prepared in buffer free of EDTA. If microsomes were prepared in EDTA-free buffer and later suspended in buffer containing EDTA, the DPG did not have any activating effect. Mg^{++} did not have any potentiating effect on DPG activation. The extent of activation of the enzyme by DPG was the same irrespective of the time of sacrifice of the rats, i.e. either at midphase of the dark cycle or midphase of the light cycle. ATP and Mg^{++} at a concentration of 4 mM each inhibited the enzyme by 50% during a 20 min incubation period (table 2). This inhibition was completely prevented by adding 4 mM DPG. Once the enzyme was inactivated by ATP and Mg^{++} it could not be re-activated by addition of DPG. When microsomes were prepared in the presence of DPG, HMG-CoA reductase activity was more than 2-fold higher (540 ± 81 pmole/mg/min) than in microsomes prepared without DPG (240 ± 30). The DPG used in the above experiments was the pentacyclohexylammonium salt; other forms such as the Tris and sodium salts of DPG were equally effective. Activation of HMG-CoA reductase by DPG was also observed when crude solubilized microsomal extract was used as the enzyme source (table 3). This enzyme preparation was also inhibited by ATP and Mg^{++} and the inhibition could be prevented by DPG.

The physiological relevance of the observed HMG-CoA reductase activation by DPG and protection against ATP and Mg^{++} inhibition by DPG are uncertain because the report of Noble et al.[3] indicates an inverse relationship between plasma cholesterol and red cell DPG concentration. At present it is not known whether there is any correlation between red cell DPG and liver DPG levels. The prevention by DPG of inhibition of HMG-CoA reductase by ATP and Mg^{++} may be of physiological significance, since most current work supports the view that phosphorylation-dephosphorylation is a short term regulatory mechanism for cholesterol biosynthesis[4–10], although there is some evidence to the contrary[18–19].

Mg^{++} is not required for the activation of HMG-CoA reductase. For activation by DPG, however, the enzyme must be prepared in an EDTA-free buffer. Activation by DPG is not due to chelation of Mg^{++}, because in experiments conducted with up to 80 mM Mg^{++} in the presence of 4 mM DPG the Mg^{++} did not counteract the stimulatory effect of DPG on HMG-CoA reductase. In other experiments, the addition of up to 50 mM pyrophosphate, which binds magnesium ions, did not have any stimulatory effect on HMG-CoA reductase in the absence of DPG and EDTA. The activity of HMG-CoA reductase with 10 mM DPG was 2 to 3 times higher than the activity with EDTA alone under similar conditions. When both EDTA and DPG were included in the reaction mixture the activity was 30–50% less than with DPG alone. These experiments suggest that EDTA is responsible for the loss of an additional entity from the microsomes which is required for DPG activation. The higher HMG-CoA reductase activity of microsomes prepared with DPG suggests a possible in vivo effect on DPG activation. In this activation DPG probably remains bound to the microsomes during the process of homogenization and centrifugation to produce the increased activity. Solubilized HMG-CoA reductase behaved just as the microsomal preparation with regard to activation by DPG and protection by DPG against ATP inactivation, suggesting that intact membranes are not necessary for the DPG effects.

The results presented in this report suggest that some glycolytic cycle intermediates may be directly involved in the regulation of hepatic cholesterol biosynthesis. Two other phosphate compounds – phosphoenolpyruvate and pyrophosphate, did not have any effect on HMG-CoA reductase activity, suggesting that the DPG effect could be specific. The effects of other glycolytic intermediates such as fructose 1,6 diphosphate, 3-phosphoglyceric acid and 2-phosphoglyceric acid are being tested for their ability to activate the enzyme and also to protect it against ATP and Mg^{++} inhibition. The mode of action of DPG will be studied further using the purified enzyme.

Table 2. Inactivation of HMG-CoA reductase by ATP and Mg^{++} and protection by DPG

Experiment	ATP (mM)	Mg^{++} (mM)	DPG (mM)	Specific activity (pmoles/mg/min)
1	0	0	0	350
2	4.0	4.0	0	215
3	4.0	4.0	4.0	340
4	4.0	4.0	8.0	410

Microsomes were preincubated with above constituents for 30 min at 37 °C, then NADPH (4 mM) and ^{14}C-HMG-CoA were added and the incubations continued for 20 more minutes. Experiments were done in triplicate.

Table 3. Effect of DPG on HMG-CoA reductase in the crude extract of liver microsomes

Experiment	ATP	Mg^{++}	DPG	Enzyme activity (pmoles/mg/min)
1	–	–	–	855
2	4 mM	4 mM	–	43
3	4 mM	4 mM	4 mM	551
4	4 mM	4 mM	8 mM	1600
5	–	–	4 mM	2260

Solubilized HMG-CoA reductase was prepared by slow freezing and thawing of microsomes as described under methods. 0.4 mg of protein from this extract was used in each of the experiments, each of which was done in triplicate.

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The choroid in the eye of the eel *(Anguilla anguilla)*

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Summary. By means of light and scanning electron microscopy evidence of the existence of a normally organized choroid in the eye of the eel, *Anguilla anguilla,* is presented.
Key words. Anguilla anguilla; eel, eye; eye, eel; choroid.

The eye of the eel, *Anguilla anguilla,* is of particular interest, since its retina is directly fed by the membrana vasculosa retinae[1,2], whose vessels are derived from the hyaloid artery, spread on the inner surface of the optic cup and penetrate within the retina. If one considers that only angiotic mammals and the snake *Tarbophis* show intraretinal vessels, the unusual organization of the eye of the eel is clear. Furthermore, according to several authors[3-6], the fish is unique in having no demonstrable choroid[7]. Therefore, the present work was undertaken to provide a description of the organization of the ocular layers of the eel, with particular regard to existence of the choroid.

Male eels *(Anguilla anguilla)* were collected in their natural habitat and killed by decapitation. The eyes were rapidly enucleated, cut into 2 halves and fixed in 4% glutaraldehyde in phosphate buffer (pH 7.4; 0.2 M). One half was critical point dried, gold-sputtered and examined in an ETEC Autoscan scanning electron microscope, the other was trimmed into small pieces (1–2 mm^3), post-fixed in 1% OsO_4 in phosphate-sucrose buffer (pH 7.4; 0.2 M) and embedded in Durcupan. Semithin sections were cut in a LKB Ultrotome V ultramicrotome, stained with toluidine blue-pironine[8], and examined using an Olympus BH-2 microscope.

The existence of the choroid could be demonstrated in both kinds of micrograph. In fact, when observed with the scanning electron microscope (fig. 1), the eye of the eel shows blood vessels within the neuroretina and, under the pigment epithelium layer, a regular row of wider vessels filled with erythrocytes. A semithin section of the same region (fig. 2) shows a vascular

Figure 1. Longitudinal section, obtained with a razor blade, of the optic cup of the eel. A, membrana vasculosa retinae; B, intraretinal vessels; C, choroidal vessels. × 560.

Figure 2. Semithin section of the eye of the eel. A, photoreceptors; B, pigment epithelium; C, Bruch's membrane; D, choroidal vessels; E, argentea; F, scleral cartilage; G, sclera. × 520.