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SHORT COMMUNICATIONS

The role of hypoxia in selenium metabolism

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Selenite biotransformation to dimethylselenide involves a six valance reduction to H_2Se and subsequent methylating reactions, and it has been studied in whole animals [1], in isolated hepatocytes [2] and in cell free systems [3]. Work with the latter model provided basic information about these metabolic pathways, but an extrapolation to *in vivo* systems was complicated by the fact that oxygen almost completely prevented dimethylselenide formation and anaerobic conditions were employed in most experiments. In the more *in vivo*-like hepatocyte model this effect of oxygen did not become apparent; aerobic conditions in the hepatocyte model gave similar rates of dimethylselenide formation as anaerobic conditions in the cell free system [2]. However, there was a lag period of about 1 hr or more before dimethylselenide production started.

A possible explanation to these differences can be derived from the assumption that Se metabolites entered redox cycles in the hepatocytes [2]. It can, for example, be anticipated that the block in metabolism during the lag period was due to an autooxidation of reduced Se metabolites, and that this oxygen consuming process eventually induced hypoxia.

In order to elucidate the role of hypoxia in selenium metabolism we have developed a method to monitor the oxygen tension continuously in a suspension of selenite metabolizing hepatocytes, and the results will be reported in this paper. In previous publications it has been shown that selenite increased the rate of oxygen consumption in hepatocytes [2, 4].

Materials and methods

Hepatocytes were isolated by the collagenase perfusion technique as described previously [5]. Male Sprague–Dawley rats (180–270 g, food and water *ad libitum*) were used as donor animals.

Collagenase (from *Clostridium histolyticum*) was purchased from Boehringer Mannheim, ^{75}Se-labelled sodium selenite (2–20mCi/mg Se) from Amersham, sodium selenite from Merck, L-tyrosine and L-methionine from Sigma. Other chemicals were obtained from local commercial sources.

Cell viability was measured either by the trypan blue exclusion test or by the NADH penetration assay [5].

After isolation, viability was determined and the hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4) supplemented with 13 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulphonic acid (HEPES). Methionine (0.5 mM) was also included since this amino acid has been shown to stimulate dimethylselenide formation (2). The suspended cells (4×10^6 cells/ml: in 50 ml) were incubated in a rotating 100 ml roundbottom flask at 37°. Carbogen gas (93.5% O_2: 6.5% CO_2) was supplied to the surface, unless otherwise indicated.

Formation of dimethylselenide was measured as the volatilization of ^{75}Se from the incubate. Dimethylselenide has been shown to be formed and evaporate from the isolated hepatocyte system and the volatilization of ^{75}Se may be used as method to quantitate its formation [2]. 2.5 μCi of $^{75}SeO_3^{2-}$ was used per 50 ml incubate. ^{75}Se accumulation in the hepatocytes was measured in cell pellets from aliquots of 1 ml incubate centrifuged at 50 *g* for 2 min.

The oxygen concentration in the incubate was monitored polarographically with a Clark type electrode. The cell incubate was continuously pumped with a peristaltic pump (Watson-Marlow, 502 S/R with pumphead 501 R), through a flow cell (Beckman Instruments Inc.) to which the electrode was attached, and the oxygen level recorded. Before the addition of selenite, cells were preincubated until a stable reading was achieved. Samples were taken in a three-port valve with a syringe.

The oxygen concentration was also monitored by measuring the accumulation of the tyrosine metabolite *p*-hydroxy-phenyl-pyruvate (pHPP) in the incubate. This metabolite has been shown to accumulate at low oxygen tensions, due to the relatively high $K_{m_{O_2}}$ value of the enzyme pHPP-oxidase (EC 1.13.11.27) [6]. At the time of selenite addition tyrosine (0.22 mM) was added and samples (2 ml) were taken and immediately injected into tubes containing 0.3 ml 2 N H_2SO_4, centrifugated (5 min, 1000 *g*) and extracted 3 times with 4 ml ethyl acetate. The ethyl acetate fractions were then extracted with 3 ml 0.2 M potassium phosphate buffer (pH 8.0) and the pHPP assayed by the method of Diamondstone [7]. 0.1 ml 10 N NaOH was added to 1.6 ml of each sample and the absorbance read at 331 nm after 30 min.

Data presented in this paper have been taken from typical experiments. All experiments were repeated at least three times with different cell batches.

Results

In a first series of experiments Se volatilization was studied in hepatocyte suspensions equilibrated with air instead of carbogen. It was found that air shortened the lag period, but that the effect varied with the concentration of selenite. At a low selenite concentration (20 μM), which gives a long lag period [2], the effect was marked (Fig. 1), while with higher selenite concentration (50 μM) the effect was less pronounced but reproducible (not shown).

When the oxygen tension was monitored in suspensions of hepatocytes equilibrated with carbogen a stable level was recorded for at least 2 hr in the absence of selenite, and there was no marked effect on cell viability by the pumping. When selenite, in concentrations known to increase the oxygen consumption (75 μM; Fig. 2a), was added the oxygen tension started to fall. Within 1 hr it approached a zero level. It then started to rise again and usually higher tensions than those recorded before selenite addition were recorded during the second hour. The effect of selenite on the oxygen tension was concentration dependent and 10 μM had no marked effect. Inhibitors of selenite induced oxygen consumption and selenite metabolism, such as *p*-tert-butyl-benzoic acid and $HgCl_2$ [4] prevented a fall in oxygen tension. When $HgCl_2$ was added 20 min after selenite, the oxygen tension started to rise and soon reached control levels (Fig. 3). After about 90 min the oxygen level started to fall again. When ^{75}Se was used and the volatilization measured (Fig. 2b) it was found that there was a strict relationship between oxygen tension and Se volatilization in that volatilization only started when the oxygen tension was at a low level. With low doses of selenite, that did not affect the oxygen tension, there was no volatilization. The amount of ^{75}Se accumulated in the cells was also measured, and it was found that it peaked at

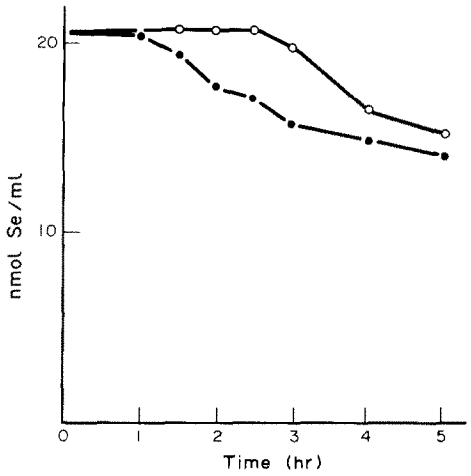

Fig. 1. Selenite metabolism in isolated hepatocytes. ^{75}Se-selenite (20 μM) was added to a suspension of hepatocytes at zero time, and the volatilization measured in aliquotes withdrawn at times indicated. The cells were incubated with either carbogen gas (○) or air (●).

about 1 hr and then decreased (as previously shown in ref. 2).

To confirm the results on a decreased oxygen tension and to possibly document cellular hypoxia during the metabolism of selenite we also measured the accumulation of the tyrosine metabolite *p*-hydroxy-phenyl-pyruvate (pHPP) (Fig. 2c). Under control conditions only a slight increase of pHPP could be detected (not shown). In selenite exposed cells the accumulation of pHPP coincided with the decrease in the oxygen level in the incubate, and it reached its maximum at about 1 hr.

Under the experimental conditions described here for oxygen tension recordings 10 μM selenite was nontoxic during a 4-hr incubation period while incubations with 75 μM usually resulted in a decreased cell viability after about 2 hr. 40 μM selenite was usually nontoxic, but in case of toxic effects there was no indication that a decreased cell viability recorded after 2 hr influenced the results obtained during the first 2 hr. There was no effect of the pre-incubation on the length of the lag period or the rate of volatilization.

Discussion

The described technique enabled us to show changes in oxygen tension in selenite metabolizing cells. In other experiments, where intermittently withdrawn samples were analysed with an oxygen electrode, no such changes could be documented, possibly because the balance between oxygen consumption and supply was changed during the sampling. The accumulation pattern of pHPP supports the oxygen tension data. It also indicates that the oxygen tension recordings reflected cellular hypoxia and not merely extracellular oxygen levels. The rate of oxygen consumption, as reported previously, changed in a reversed way as compared to the oxygen tension, that is samples taken at 1 hr had the highest rate [4]. If toxic concentrations were used, a rate lower than the control rate was recorded after 120 min [4]. It may thus be concluded that the changes in oxygen tension reported here were induced by metabolic changes in the cells. The effect of the inhibitors also supports this conclusion.

Only under hypoxic conditions could selenite biotransformation to dimethylselenide be shown to occur. To account for this correlation it may be suggested that, for example, H_2Se mainly entered redox-cycles during the lag period, and that this reaction depleted oxygen. Eventually, at a sufficiently low oxygen level, the methylating reaction became competitive and dimethylselenide formation started. This drained the cells of autooxidizable Se metabolites and allowed the oxygen levels to rise again. Also compatible with this explanation are the results presented in Fig. 1 and the effect of $HgCl_2$, which is a potent trapping agent of H_2Se [8].

We have previously shown that an increased selenite concentration [2] as well as an increased hepatocyte con-

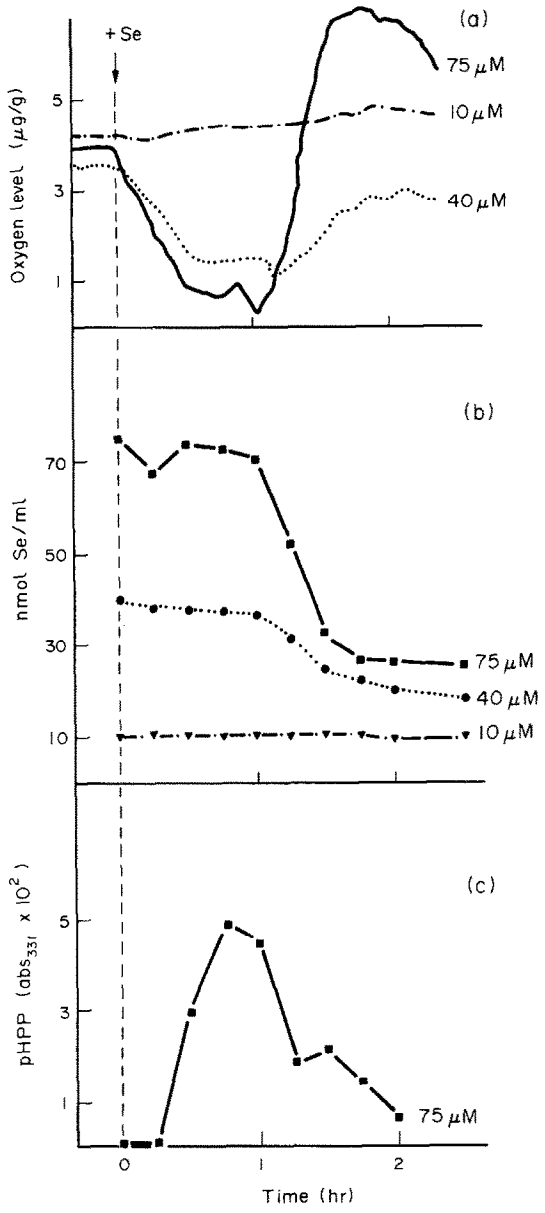

Fig. 2. Selenite induced changes in oxygen level and selenite metabolism in isolated hepatocytes. Suspensions of hepatocytes were preincubated for about 1 hr, and ^{75}Se-selenite was added at zero time. The oxygen level was recorded continuously throughout the experiments (a). The volatilization of Se (b) and the accumulation of pHPP (c) were measured in aliquots withdrawn at times indicated. The results from three experiments are shown, one for each selenite concentration. In one experiment (75 μM) all three parameters were monitored and in two experiments (10 μM and 40 μM) two parameters were monitored.

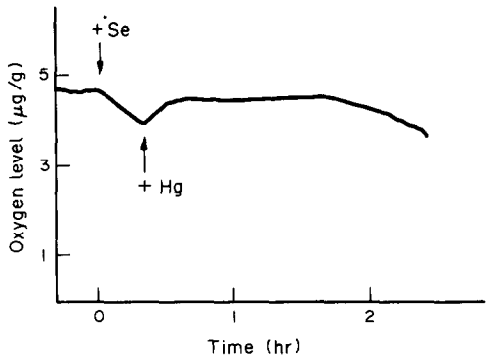

Fig. 3. The effect of $HgCl_2$ on selenite induced decrease in oxygen level. A suspension of hepatocytes was pre-incubated for about 1 hr, and selenite (75 μM) was added at zero time. $HgCl_2$ (15 μM) was added 20 min thereafter. The oxygen level was recorded continuously throughout the experiment.

centration [9] can shorten the lag period in selenite metabolism. We have now shown that the use of air instead of carbogen has the same effect. These data, when combined with the rest of the results presented here, indicate that an essential quality of the hepatocyte model was its marked tendency to become hypoxic. In non-respiring systems, like that used in ref. 3, the tendency to become hypoxic should be less pronounced. This circumstance may explain the inability of a cell free system to produce dimethylselenide under oxygenated conditions.

In summary, in order to elucidate the role of hypoxia in selenium metabolism we have developed a technique to continuously monitor the oxygen tension in a suspension of isolated hepatocytes. It could be shown that in cells, incubated under conditions which permitted selenite metabolism, selenite induced hypoxia. It was also found that dimethylselenide was not formed until hypoxia had been induced. The lag period, which preceded dimethylselenide formation, was shortened by equilibrating the hepatocyte suspension with air instead of carbogen. These data support the hypothesis that selenium metabolites may enter redox cycles and suggest that oxygen acted as modulator of dimethylselenide formation.

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Metabolism of alkoxyphenoxazones by channel catfish liver microsomes: Effects of phenobarbital, Aroclor 1254 and 3-methylcholanthrene

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Cytochrome P-450-dependent monooxygenases (MOs) are biologically ubiquitous enzymes integral in the metabolism of a multitude of organic xenobiotics. In mammals and fishes, there are multiple forms of hepatic microsomal cytochrome P-450, each with slightly different, but usually overlapping, specificities for many substrates [1, 2]. Because of this overlap, substrates that are somewhat specific for a certain cytochrome P-450 are particularly valuable for the elucidation of mechanisms governing changes in the activity of this enzyme system. Unfortunately, relatively few of these types of substrates have been developed.

In mammals, it is well established that certain hepatic microsomal cytochromes P-450 are induced, and associated MO activities increased, by treatment with a variety of compounds [3]. Phenobarbital, a drug, and 3-methylcholanthrene, a polycyclic aromatic hydrocarbon, are representative of two major classes of inducers of cytochrome(s) P-450. It has been known for some time that the rate of O-dealkylation of 7-ethoxyresorufin (7-ethoxyphenoxazone) is increased preferentially up to 50-fold by 3-methylcholanthrene-type compounds, indicating that at least one of the cytochromes P-450 induced by this class of compounds is highly specific for ethoxyresorufin [4]. Recently, a highly specific substrate for cytochrome(s) P-450 induced by phenobarbital-type compounds also has been reported. The rate of O-dealkylation of 7-pentoxyresorufin (7-pentoxyphenoxazone) by rat or mouse hepatic MOs is increased up to 250-fold by phenobarbital-type inducers [5–7]. Hence, pentoxyresorufin should be a powerful tool in the determination of induction by phenobarbital-type compounds.

Hepatic cytochrome P-450-dependent MOs in fishes are functionally quite similar to those in mammals [8, 9]; however, there appear to be differences in their responses to inducers. Although it is well established that hepatic MO activities in fishes are increased greatly by 3-methylcholanthrene-type inducers, there is some question as to the effects of phenobarbital-type compounds. Some reports indicate that hepatic MO activities in fishes are increased by phenobarbital-type inducers [10–12]; however, most studies have shown that these compounds do not affect