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Glycerophosphate-Dependent Hydrogen Peroxide Production by Rat Liver Mitochondria

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Summary

We studied the extent to which hormonally-induced mitochondrial glycerophosphate dehydrogenase (mGPDH) activity contributes to the supply of reducing equivalents to the mitochondrial respiratory chain in the rat liver. The activity of glycerophosphate oxidase was compared with those of NADH oxidase and/or succinate oxidase. It was found that triiodothyronine-activated mGPDH represents almost the same capacity for the saturation of the respiratory chain as Complex II. Furthermore, the increase of mGPDH activity induced by triiodothyronine correlated with an increase of capacity for glycerophosphate-dependent hydrogen peroxide production. As a result of hormonal treatment, a 3-fold increase in glycerophosphate-dependent hydrogen peroxide production by liver mitochondria was detected by polarographic and luminometric measurements.

Key words

Rat liver mitochondria • Mitochondrial glycerophosphate dehydrogenase • Triiodothyronine • Hydrogen peroxide

Introduction

Hepatocytes represent a unique mammalian cell population in which biogenesis of mitochondrial glycerophosphate dehydrogenase (mGPDH) can be stimulated by thyroid and steroid hormones (Lee and Lardy 1965, Lardy *et al.* 1995, Lotková *et al.* 2001). Flavoprotein-dependent, membrane-bound mGPDH is located in the inner mitochondrial membrane and together with the cytosolic, NADH-dependent glycerophosphate dehydrogenase (cGPDH) forms the glycerophosphate shuttle (Bucher and Klingenberg 1958). This shuttle, similarly to the malate-aspartate shuttle, is a cellular device for reoxidation of cytosolic NADH produced by glycolysis. Both shuttles thus participate in the maintenance of the high rate of glycolysis. Whereas malate-aspartate shuttle operates in all mammalian tissues, the activity of glycerophosphate shuttle varies in different tissues and the highest activity was found in brown adipose tissue, the thermogenic organ of newborn mammals and hibernators (Houštěk *et al.* 1975).

Low activity of the glycerophosphate shuttle in many mammalian tissues is due to the low activity of mGPDH. For example, the mGPDH activity in liver mitochondria is 50-100 times lower than in brown fat mitochondria (Houštěk *et al.* 1978). The reason for this

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down-regulation of mGPDH biogenesis is not yet well understood. In addition, it is not quite clear, why in hepatocytes the extremely low activity of mGPDH is 2-3 times enhanced by triiodothyronine. Therefore, using polarographic measurements we tried to evaluate the extent to which the newly formed mGPDH can saturate the respiratory chain capacity by supplying the reducing equivalents in comparison with Complex I (NADH oxidase) and/or Complex II (succinate oxidase).

We found in our previous studies that mGPDH in brown adipose tissue participates in reactive oxygen species production by mitochondria (Drahota *et al.* 2002, 2003). We speculated that this fact could be related to the down-regulation of mGPDH biogenesis in most mammalian tissues. We therefore also tested, whether the increase of mGPDH activity in triiodothyronine-treated rats correlates with the capacity for glycerophosphate-dependent peroxide production.

Methods

In our experiments, male Wistar rats (BioTest Konarovice, Czech Republic) with an initial body weight of 180-220 g were used. The animals were housed at 23±1 ºC, in a 12:12 hour light-dark regime and had free access to standard laboratory rat chow and water. Triiodothyronine (20 μg/100 g b.w., 0.5 ml solution/ 100 g b.w.) was applied intraperitoneally. The control rats were treated with an equivalent amount of physiological saline solution (0.15 M NaCl). Rats received three doses of triiodothyronine in 24-h intervals. All animals were killed 72 h after the last hormone administration.

The isolation of liver mitochondria was performed by differential centrifugation in 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA pH 7.4, according to Schneider and Hogeboom (1950) as described by Svátková *et al.* (1996).

Interscapular brown adipose tissue from newborn rats was used for isolation of mitochondria as described (Hittelmann *et al.* 1969). Isolation medium contained 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. Frozen-thawed mitochondria were used for the measurements.

The mitochondrial proteins were determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

Oxygen consumption was measured using High Resolution Oxygraph (OROBOROS, Austria) (Gnaiger *et al.* 1995) as previously described by Drahota *et al.* (2002, 2003) and Wenchich *et al.* (2003). Measurements were performed at 30 ºC in 1.5 ml of incubation medium containing 100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.2, using 0.09-0.15 mg/ml of the mitochondrial protein. OROBOROS software was used for calculations of oxygen production and for graphic presentation of experimental data. Oxygraphic curves represent the first derivation of oxygen tension changes; the height of the peak represents the rate of oxygen consumption. Oxygen consumption was expressed in pmol/s/mg protein. Cytochrome *c* oxidase was measured in the presence of 5 mM ascorbate, 0.2 mM tetramethyl p-phenylene diamine (TMPD) and 20 μM cytochrome *c*. The reaction was started by the addition of mitochondria. The enzyme activity was corrected for the rate of oxygen consumption due to autooxidation of ascorbate, TMPD and cytochrome *c*. The rate of autooxidation (pmol/s/ml medium) was subtracted from the rate after addition of mitochondria (pmol/s/ml medium) and the resulting value was divided by mg of mitochondrial protein per ml. This portion of oxygen consumption was completely inhibited by KCN. Measurements of hydrogen peroxide production by oxygraphic method was performed in the same medium in the presence of 10 mM glycerophosphate, 0.3 mM KCN and 1 mg/ml bovine serum albumine. The mitochondria protein concentration was 0.43-1.80 mg/ml. The peroxide production was started by 83 μM ferricyanide.

Hydrogen peroxide production was measured as an increase of luminescence by Luminometer 1250 from Bioorbit (Finland) as previously described (Wilhelm and Vilim 1986). The measurements were performed at room temperature in 1 ml of medium containing 50 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, 30 mM glycerophosphate, pH 7.4 and 1.5-3.2 mg of the mitochondrial protein. Luminol (5-amino-2, 3-dihydro-1, 4-phtalazionedione) concentration was 1 mM, and that of horseradish peroxidase was 2.5 units/ml. The reaction was started by addition of 83 μM potassium ferricyanide. The luminiscence peak reached maximum values within 2-3 s after addition of potassium ferricyanide. For evaluation of peroxide production the value of the peak was assessed.

Results

In isolated rat liver mitochondria from control and triiodothyronine-treated rats, the activity of

cytochrome *c* oxidase (COX), glycerophosphate oxidase, succinate oxidase and NADH oxidase was measured as the oxygen consumption. Because the COX activity represents the maximum capacity of the respiratory chain we expressed the activity of other enzymes also in relation to the COX activity.

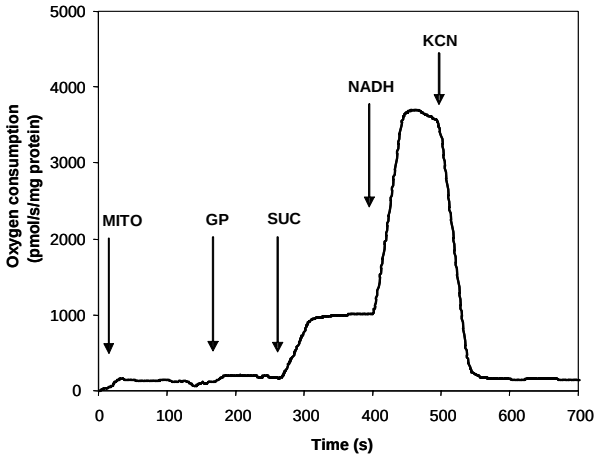

Fig. 1. Respiration of rat liver mitochondria in the presence of glycerophosphate (GP), succinate (SUC) and NADH. Oxygen consumption was measured in 1.5 ml KCl medium (100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA) with 0.1 mg protein of mitochondria/ml. Where indicated, 10 mM glycerophosphate (GP), 10 mM succinate (SUC), 0.1 mM NADH and 0.5 mM KCN were added.

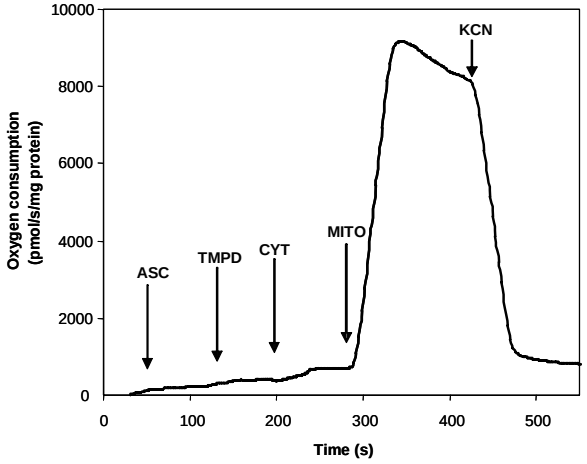

Fig. 2. Cytochrome *c* oxidase activity in rat liver mitochondria. Oxygen uptake measurement was performed in 1.5 ml KCl medium (100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA) with 0.09 mg/ml of mitochondrial protein. Additions of 5 mM ascorbate (ASC), 0.2 mM TMPD, 20 μM cytochrome *c* (CYT) and 0.3 mM KCN are indicated.

Figure 1 shows the mitochondrial respiration in the presence of glycerophosphate, succinate and NADH, whereas cytochrome *c* oxidase activity measurement as the oxygen consumption with TMPD, ascorbate and cytochrom *c* as substrates is demonstrated in Figure 2. The data on oxygen consumption with various substrates are summarized in Table 1. In agreement with previous findings (Lee and Lardy 1965, Lardy *et al.* 1995, Weitzel *et al.* 2001), we observed that triiodothyronine treatment specifically activates the mGPDH activity. The activity of other tested enzymes remained unchanged (Table 1). It is also evident that in control rats the activity of mGPDH saturates only 1 % of the cytochrome *c* oxidase capacity. After triiodothyronine treatment this value increased 3.44 fold and the mGPDH capacity was almost the same as that of succinate oxidase (Complex II), but far below the capacity of NADH oxidase (Complex I).

In our previous studies on brown adipose tissue mitochondria (Drahota *et al.* 2002, 2003) we demonstrated that mGPDH represents a new site of oxygen radical production in the mitochondrial respiratory chain. We also found that this hydrogen peroxide production can be highly stimulated when mGPDH reacts with one electron acceptor ferricyanide (Klingenberg 1970). Therefore, we tested in further experiments, whether hormonally induced expression of mGPDH biogenesis in liver mitochondria is accompanied by the increased capacity for glycerophosphate-dependent hydrogen peroxide production.

Figure 3 demonstrates hydrogen peroxide production in brown fat mitochondria with high mGPDH activity. It is quite evident that, after addition of glycerophosphate, we can detect KCN-insensitive oxygen uptake, which is highly stimulated by addition of ferricyanide. When added, ferricyanide is reduced and hydrogen peroxide production returns to original values. Similar glycerophosphate-dependent hydrogen peroxide production could be detected in mitochondria from triiodothyronine-treated rats (Fig. 4). The rate of the ferricyanide-induced glycerophosphate-dependent oxygen consumption by mitochondria from control and triiodothyronine-treated rats was 7.5 and 14 pmol/s/mg protein, respectively. Because the rates of hydrogen peroxide production were rather low, we repeated these experiments using more sensitive luminometric detection of hydrogen peroxide production. As demonstrated in Figure 5, glycerophosphate-dependent hydrogen peroxide production could be detected both in control and triiodothyronine-treated rats. Similarly to the oxygraphic measurements, the rate of hydrogen peroxide production was much higher in mitochondria isolated from triiodothyronine-treated rats (Table 1).

Table 1. Oxygen uptake of rat liver mitochondria from control and triiodothyronine-treated rats in the presence of various respiratory substrates

Respiratory chain enzymes	Control rats (A)		T3-treated rats (B)		B/A
	Oxygen uptake	%	Oxygen uptake	%	
Cytochrome c oxidase	8.46±1.33	100.0	8.35±1.50	100.0	0.97
Glycerophosphate oxidase	0.09±0.03	1.0	0.31±0.01	3.7	3.44
Succinate oxidase	0.70±0.14	8.1	0.43±0.08	5.2	0.61
NADH oxidase	2.22±0.30	25.7	2.20±0.23	26.4	0.90
Glycerophosphate + succinate + NADH oxidase	3.58±0.41	41.4	3.22±0.42	38.6	0.90

Measurements were performed in the presence of 10 mM glycerophosphate, 10 mM succinate, 0.1 mM NADH and 5 mM ascorbate, 0.2 mM TMPD and 20 μM cytochrome *c*, in the KCl medium using 0.1 mg of mitochondrial protein/ml. Oxygen consumption data were expressed as nmol/s/mg protein. Data are mean ± S.D., n = 3.

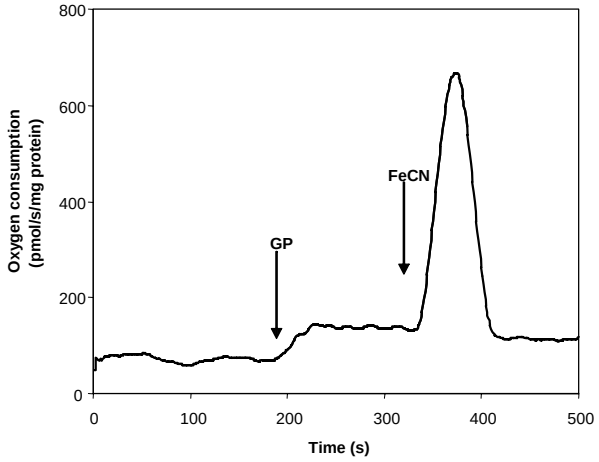

Fig. 3. Glycerophosphate-dependent ferricyanide-induced hydrogen peroxide production by brown adipose tissue mitochondria. Measurements were performed in medium described in Fig. 1 in the presence of 10 mM glycerophosphate (GP) and 0.3 mM KCN. Potassium ferricyanide (FeCN) was 83 μM and mitochondrial protein was 0.43 mg/ml.

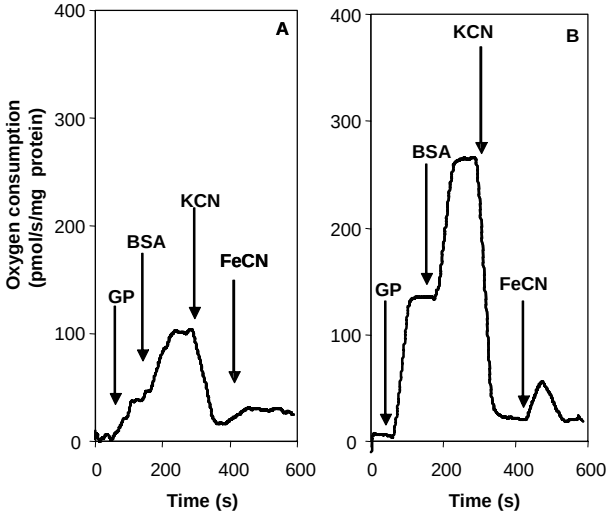

Fig. 4. Glycerophosphate-dependent ferricyanide-induced hydrogen peroxide production: (**A**) control rat liver mitochondria, (**B**) rat liver mitochondria after triiodothyronine treatment. Measurements were performed in medium described in Fig. 1 in the presence of 10 mM glycerophosphate (GP), 1 mg/ml bovine serum albumine (BSA), 0.3 mM KCN and 1.8 mg/ml of mitochondrial protein. Ferricyanide (FeCN) was 83 μM.

Discussion

Triiodothyronine is known as a hormone stimulating mitochondrial biogenesis in general (Weitzel *et al.* 2001) and it is also considered as a thermogenic hormone (Lardy *et al.* 1995). Lardy *et al.* (1989) speculated that increased flow of reducing equivalents to the respiratory chain through flavoprotein-dependent enzymes is a very efficient heat dissipating mechanism. By this pathway, hydrogen-reducing equivalents overpass the first phosphorylation site at Complex I. In this way, 30 % of energy released by respiratory substrates oxidation is not used for ATP synthesis, but dissipates as heat. As it was demonstrated in our experiments, the increase of mGPDH in liver mitochondria substantially increased the capacity of the flavoprotein-dependent pathway of the respiratory chain. In agreement with this hypothesis is also the fact that cell metabolism is more dependent on glycolytic ATP production when ATP production by mitochondrial system of energy provision decreases. This requires efficient reoxidation of NADH produced by glycolysis. Therefore, the activation of mGPDH biogenesis can participate in two processes involved in triiodothyronine-activated thermogenesis,

namely in the increase of reducing equivalents flow that overpasses Complex I and in maintenance of the high rate of glycolysis. Increased activity of mGPDH means that glycerophosphate shuttle can cooperate with malate–aspartate shuttle in NADH reoxidation and more protons are oxidized through the heat-dissipating pathway.

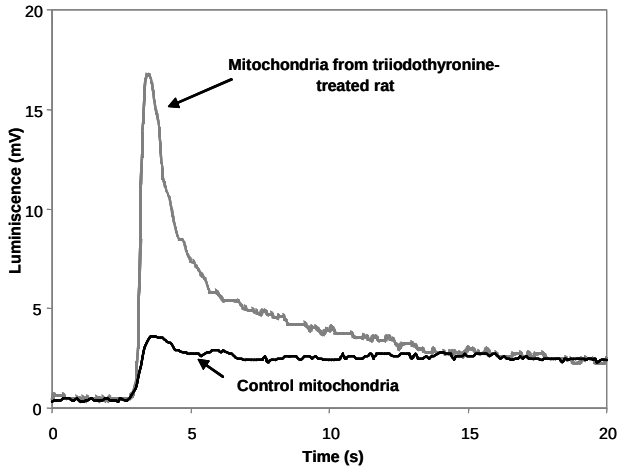

Fig. 5. Luminescence detection of glycerophosphate-dependent ferricyanide-induced hydrogen peroxide production by liver mitochondria from control and triiodothyronine-treated rats. Luminometric measurements were performed in 1 ml of medium containing 50 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, in the presence of 30 mM glycerophosphate, 1 mM luminol and horseradish peroxidase (HRP) 2.5 units/ml. The reaction was started by addition of 83 μM potassium ferricyanide (FeCN). Concentration of mitochondrial protein was 3.2 mg protein/ml - control mitochondria or 1.5 mg protein/ml - triiodothyronine-treated mitochondria.

As we found in our previous studies on brown adipose tissue mitochondria, mGPDH represents a potential risk for the cell as generator of reactive oxygen species (Drahota *et al.* 2002, 2003). Using rat liver mitochondria with triiodothyronine-induced mGPDH we further extended these findings by demonstration of glycerophosphate-dependent peroxide production in liver mitochondria. In the present experiments, we found a direct relationship between the enhanced mGPDH activity and the increased capacity for glycerophosphate-dependent hydrogen peroxide production. The potential risk of high ROS production by increased mGPDH activity could be controlled by excess of the COX capacity over capacities of the respiratory chain dehydrogenases and by decreased mitochondrial membrane potential. Under these conditions, respiratory chain oxidoreductases are shifted towards oxidized state and the risk of electron leak is minimal. Evidently, a part of these protecting mechanisms could also involve rapid elimination of increased mGPDH from the mitochondrial membrane when the hormonal signal connected with the increased thermogenic function disappears.

Recent studies of Brown *et al.* (2002a,b) have clearly indicated how essential is the function of glycerophosphate shuttle for normal cell metabolism, because knock-out mice lacking both the mitochondrial and the cytosolic GPDH died few days after birth. Other studies also showed that mGPDH could be involved in regulation of thermogenesis (Lardy *et al.* 1995), in the pathogenesis of obesity (Lardy *et al.* 1989) and diabetes mellitus (Sener *et al.* 1993). Disturbed hormonal induction of mGPDH due to immaturity of thyroid hormone metabolism and low levels of triiodothyronine in the neonatal period could be critical for normal development of preterm neonates (Houštěk *et al.* 1993, Pavelka *et al.* 1997). All these findings indicate that mGPDH and glycerophosphate shuttle play an important role in the regulation of cell metabolism, but more experimental data are needed to elucidate the mechanisms regulating expression of mGPDH and the regulatory function of the glycerophosphate shuttle in various mammalian tissues.

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Reprint requests

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