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Isocitrate dehydrogenase: A NADPH-generating enzyme in the lumen of the endoplasmic reticulum

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Abstract

The aim of the present study was the investigation of the occurrence of NADPH-generating pathways in the endoplasmic reticulum others then hexose-6-phosphate dehydrogenase. A significant isocitrate and a moderate malate-dependent $NADP^+$ reduction were observed in endoplasmic reticulum-derived rat liver microsomes. The isocitrate-dependent activity was very likely attributable to the appearance of the cytosolic isocitrate dehydrogenase isozyme in the lumen. The isocitrate dehydrogenase activity of microsomes was present in the luminal fraction; it showed a strong preference towards $NADP^+$ *versus* NAD^+, and it was almost completely latent. Antibodies against the cytosolic isoform of isocitrate dehydrogenase immunorevealed a microsomal protein of identical molecular weight; the microsomal enzyme showed similar kinetic parameters and oxalomalate inhibition as the cytosolic one. Measurable luminal isocitrate dehydrogenase activity was also present in microsomes from rat epididymal fat. The results suggest that isocitrate dehydrogenase is an important NADPH-generating enzyme in the endoplasmic reticulum.

Keywords: Isocitrate dehydrogenase; Endoplasmic reticulum; NADPH; Hexose-6-phosphate dehydrogenase; 11β-hydroxysteroid dehydrogenase type 1

Pyridine nucleotides constitute a major redox buffer in the lumen of the endoplasmic reticulum [1]. The maintenance of their appropriate redox balance is of vital importance. Reduced pyridine nucleotides are required for the functioning of intraluminal reductases, such as 11β-hydroxysteroid dehydrogenase type 1 [2]. This enzyme is responsible for the prereceptorial activation of glucocorticoids in several tissues [3,4]. On the other hand, effects which can alter the redox state of luminal pyridine nucleotides were shown to lead proapoptotic events in various cell types [5–8].

Hexose-6-phosphate dehydrogenase has been regarded as the main intraluminal dehydrogenase responsible for NADPH generation [9]. It has been reported that this enzyme confers reductase activity upon 11β-hydroxysteroid dehydrogenase type 1 [10]. The functional cooperativity between the two enzymes has been demonstrated in liver microsomes [11]. Genetic studies suggested that cortisone reductase deficiency can be caused by interacting mutations in the genes encoding the two enzymes [12]. Hampered activity of hexose-6-phosphate dehydrogenase due to the decreased substrate (glucose-6-phosphate) supply because of the inhibition of the glucose-6-phosphate transporter (G6PT)[1] of the endoplasmic reticulum is proapoptotic in granulocytes [5,8] and glia [6,7] cells.

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[1] *Abbreviations used:* G6PT, glucose-6-phosphate transporter; PEG, polyethylene glycol.

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However, other observations query hexose-6-phosphate dehydrogenase as a single candidate for intraluminal NADPH generation. Hexose-6-phosphate dehydrogenase knock-out mice, although 11β-hydroxysteroid dehydrogenase type 1 activity is switched from reductase to dehydrogenase direction, are devoid of serious pathological alterations [13]. Recent observations have challenged the hypothesis on the strict association of hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase type 1 in the pathomechanism of cortisone reductase deficiency [14–16]. Therefore, it can be supposed that luminal enzymes other than hexose-6-phosphate dehydrogenase contribute to NADPH generation. Besides the dehydrogenases of the strictly cytosolic pentose phosphate pathway, $NADP^+$-dependent isocitrate dehydrogenase and malic enzyme isoforms are the major NADPH generating enzymes. Since the isoforms of both enzymes are known to be present in several subcellular compartments [17–20], we supposed that they can be also represented in the lumen of the endoplasmic reticulum.

The aim of the present study was to investigate the presence of alternative NADPH-generating enzymes in the endoplasmic reticulum lumen. By investigating the possible occurrence of malate- and isocitrate-dependent NADPH generation, a remarkable $NADP^+$-dependent isocitrate dehydrogenase activity was found in the microsomes.

Materials and methods

Preparation of microsomal vesicles and other subcellular fractions from rat liver

Microsomes and other subcellular fractions were prepared from livers of overnight fasted male Wistar rats (180–230 g; Charles River Hungary, Isaszeg). The livers were homogenized in sucrose–HEPES buffer (0.3 M sucrose, 0.02 M HEPES, pH 7.2) with a glass–Teflon homogenizer. The microsomal fraction was then isolated using fractional centrifugation [21]. After centrifugation of liver homogenates for 10 min at 1000*g*, the supernatant was spun for 10 min at 12,000*g*. The 12,000*g* supernatant was spun for 60 min at 100,000*g*; the sediment (microsomes) was resuspended in MOPS–KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS, pH 7.2). Microsomal fraction was then washed by another centrifugation with the same parameters (60 min at 100,000*g*), and the final pellet was resuspended in the above mentioned MOPS–KCl buffer to give ~70 g/L protein concentration, then immediately frozen in liquid nitrogen, and kept in liquid nitrogen until use (within 6 months). The integrity of the microsomal membranes was assessed by using the mannose-6-phosphatase assay [22] and by measuring *p*-nitrophenol glucuronidation [21,23], which showed latency greater than 95%.

To evaluate the eventual entrance of cytosolic isocitrate dehydrogenase into the microsomal vesicles during the homogenization, rat livers were also homogenized in the presence of biotin-labeled cytosolic isocitrate dehydrogenase (Sigma, St. Louis, MO, USA). Biotinylation was performed by using the EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce, Rockford, IL, USA). 0.15 mg/ml biotinylated isocitrate dehydrogenase was added to the homogenization buffer; otherwise the subcellular fractionation was executed as described above. The amount of biotinylated isocitrate dehydrogenase was determined in each fraction by dot blot with ExtrAvidin Peroxidase (Sigma, St. Louis, MO, USA). Intensity of dots was evaluated by densitometric analysis with ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

In some experiments, other subcellular fractions, such as mitochondrial, cytosolic, and postmitochondrial supernatant fractions were obtained by standard differential centrifugation from rat liver homogenate [24] and were maintained in liquid nitrogen until use. The subcellular fractions (including microsomes) were characterized by measuring marker enzyme activities. The activity and distribution of cytochrome *c* oxidase, glucose-6-phosphatase, 5′-nucleotidase and catalase in the microsomal fraction was similar as measured previously [21,25–27].

The light mitochondrial fractions were prepared as reported in [20] with minor modifications. After homogenization of rat liver, the homogenate was centrifuged at 1900*g* for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 22,000*g* for 20 min. The resulting pellet was gently suspended in MOPS–KCl buffer and used as light mitochondrial fraction. Until use, it was kept in liquid nitrogen.

Preparation of rough and smooth microsomal fractions

Livers of overnight fasted male Wistar rats were perfused *in situ* with 0.25 M sucrose and quickly removed. Livers were homogenized with a Potter–Elvehjem homogenizer. The homogenates were diluted 1:1 (v/v) with 0.25 M sucrose and then centrifuged at 10,000*g* for 20 min. Five milliliters of the 10,000*g* supernatant were layered over 0.6 M sucrose (0.7 ml) and 1.3 M sucrose (3 ml), both containing 15 mM $CsCl_2$, in 10-ml polycarbonate Nalgene tubes, which were then centrifuged in a type 40 Beckman-Spinco rotor at 80,000*g* for 120 min. Smooth microsomes (at the 0.6/1.3 M sucrose interface) and rough microsomes (pellet) were separately recovered, diluted, or resuspended (10 ml, final volume) with 100 mM KC1, 20 mM NaCl, 5 mM $MgC1_2$, 1 mM KH_2PO_4, and 10 mM MOPS, pH 7.2, and centrifuged at 80,000*g* for 45 min. The resulting pellets were resuspended in the MOPS–KCl buffer as above to have about 5 mg of microsomal protein/ml, and were immediately frozen in liquid nitrogen, and kept there until use (within 6 months) [25].

Preparation of microsomal vesicles from non-hepatic tissues

Microsomes were prepared from epididymal fat pad of overnight fasted male Wistar rats (180–230 g) as reported in [28,29]. Microsomes were washed and resuspended in MOPS–KCl buffer. A cocktail of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 μM pepstatin A, 1 μM leupeptin and 1 μg/ml of aprotinin) was added to the homogenization medium, as well as to the MOPS–KCl buffer. Samples were immediately frozen in liquid nitrogen and kept there until use.

Measurement of protein concentrations

The protein concentration in microsomal samples and other subcellular fractions was determined using the Bio-Rad microprotein assay kit (Hercules, CA, USA) according to the manufacturer's instructions with bovine serum albumin as a standard.

Western blot analysis

Equal amounts of proteins of the subcellular fractions were separated by 9% SDS–PAGE and transferred to nitrocellulose membranes by electroblotting [30]. The isocitrate dehydrogenase (type 1) rabbit polyclonal antibody was used as primary antibody (Abcam, Cambridge, UK), and a horseradish peroxidase-conjugated anti-rabbit Ig (Santa Cruz Biotechnology, CA, USA) was used as secondary antibody. The primary antibody labeled a band at the expected molecular weight (40 kDa). The loading of equal amounts of proteins was also verified by densitometry analysis of the protein bands (stained with Red Ponceau) on the blot membranes.