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Original Contribution

S-D-Lactoylglutathione can be an alternative supply of mitochondrial glutathione

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ABSTRACT

The mitochondrial pool of GSH (glutathione) is considered the major redox system in maintaining matrix redox homeostasis, preserving sulfhydryl groups of mitochondrial proteins in appropriate redox state, protecting mitochondrial DNA against mitochondrial-derived ROS, and in defending mitochondrial membranes against oxidative damage. Despite its importance in maintaining mitochondrial functionality, GSH is synthesized exclusively in the cytoplasm and must be actively transported into mitochondria. In this work we found that SLG (*S*-D-lactoylglutathione), an intermediate of the glyoxalase system, can enter the mitochondria and there be hydrolyzed from mitochondrial glyoxalase II enzyme to D-lactate and GSH. We demonstrated SLG transport from cytosol to mitochondria by incubating substrates with radioactive compounds that showed two different kinetic curves for SLG or GSH substrates, indicating different kinetic transport. The incubation of functionally and intact mitochondria with SLG showed increased GSH levels in normal mitochondria and in artificially uncoupled mitochondria, demonstrating transport not linked to ATP presence. Also mitochondrial-swelling assay confirmed SLG entrance into organelles. Moreover we observed oxygen uptake and generation of membrane potential probably linked to D-lactate oxidation which is a product of SLG hydrolysis. The latter data were confirmed by oxidation of D-lactate in mitochondria evaluated by measuring mitochondrial D-lactate dehydrogenize activity. In this work we also showed the presence of mitochondrial glyoxalase II, the enzyme that catalyzes SLG hydrolysis, in intermembrane space and mitochondrial matrix. In conclusion, this work showed new alternative sources of GSH supply to the mitochondria by SLG, an intermediate of the glyoxalase system.

Introduction

GSH (reduced glutathione) is present in cells of all organisms and it is not homogeneously distributed among cellular compartments; in fact, in confluent cells, most cellular GSH is found in the cytoplasm (80–85%) whereas in mitochondria an independently controlled redox pool is present at 10–15% of total GSH. [1]. Since cellular respiration occurs in mitochondria, they are the major source of ROS (reactive oxygen species) and, in turn, they are the organelles most exposed to damage by oxygen radicals [2]. Although mitochondria are exposed to this constant generation of oxidant species, they remain functional thanks to the presence of an efficient antioxidant defense system [3]. Especially, the main redox buffering system in mitochondria is constituted of glutathione, glutaredoxin, and thioredoxin [4]. In particular the GSH/GSSG couple is considered the most important redox system in maintaining mitochondria matrix redox homeostasis, in preserving the redox state of mitochondrial proteins, and in defending the integrity of mitochondrial DNA [5–7]. mGSH (mitochondrial GSH) is also the primary defense against oxidative damage to mitochondrial membranes by ensuring the reduction of hydroperoxides on phospholipids and other lipidic peroxides. Moreover, because mitochondria lack catalase, the neutralization of the hydrogen peroxide is mainly assigned to mGSH [8–10]. Through its involvement in the metabolism of oxidant species and

Abbreviations: AA, antimycin; ASC, ascorbate; BSA, bovine serum albumin; CN^-1, cyanide; D-LDH, D-lactate dehydrogenase; DCIP, dichloroindophenol; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; $\Delta\psi$, membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Glo I, glyoxalase I; Glo II, glyoxalase II; GSH, glutathione; mGSH, mitochondrial glutathione; MG, methylglyoxal; P/O ratio, the ratio of moles of ATP synthesized to moles of oxygen atoms reduced to water during oxidative phosphorylation; RLM, rat liver mitochondria; ROS, reactive oxygen species; ROT, rotenone; SLG, *S*-D-lactoylglutathione; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Tx100, Triton X-100

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maintenance of the appropriate redox state and of the mitochondrial protein sulfhydryl groups, mGSH is critical in maintaining organelle functionality and therefore cell survival [11,12]. It is important to underline, as previously shown, that selective depletion of mitochondrial GSH induced necrosis and apoptosis, while depletion of cytoplasmatic GSH is not a threat for cell survival if mitochondrial GSH level is sustained [13–15].

Since mitochondria do not have the enzymes for GSH synthesis it must be transported into mitochondria from cytosol. The charged nature of the GSH molecule suggests that it cannot passively diffuse across the mitochondrial inner membrane. In fact, GSH is a negatively charged molecule at physiological pH and the matrix space is negative compared to the cytoplasm so GSH must be transported actively or be exchanged for the other anion [16,17]. To date, two anion carriers in the membrane (dicarboxylate, DIC Slc25a10; and 2-oxoglutarate, OGC Slc25a11) are shown to play a role in the mitochondrial uptake of GSH from the cytoplasm [18,19]. DIC and OGC together accounted for only 45–50% of total glutathione uptake in liver mitochondria, compared to 70–80% contribution in kidney mitochondria [13,20,21]. These studies suggest tissue-specific differences in the role of carriers in glutathione uptake into mitochondria and also raise the possibility of the existence of some yet "unknown" glutathione transporters in mitochondria.

Numerous approaches can and have been used to alter concentrations of GSH in cells. The most common approach to increasing cellular GSH concentrations is to incubate cells with GSH, but as it cannot cross the cell membrane, it is first broken down into amino acids and then resynthesized in the cell by the consecutive actions of gamma-glutamylcysteine (GCS) and GSH synthetases. However, since GCS is inhibited by feedback from GSH, achievable levels of GSH inside cells have an upper limit. Many researchers have proposed the use of novel molecules able to raise the intracellular GSH level. These pro-GSH molecules can be either a GSH carrying a hydrophobic group to make cellular entry easier or a source of thiol groups from which GSH is synthesized intracellularly (*N*-acetylcysteine, GSH monoethylester, *S*-acetylglutathione, *N*-butanoyl GSH, etc.). But if one wants to specifically increase GSH content in a particular subcellular organelle (e.g., the mitochondria), providing the extracellular medium with GSH or GSH precursors will not be very effective [22–24]. For these reasons it is important to study the mitochondrial supply of GSH and in this work we considered SLG (*S*-D-lactoylglutathione), as a candidate molecule to supply GSH in mitochondria.

SLG, an intermediate of the glyoxalase system, is a GSH ester synthesized in living cells. The glyoxalase pathway is involved in cellular detoxification of α-ketoldehydes and includes Glo I (EC 4.4.1.5., GlxI, glyoxalase I, lactoyglutathione lyase) that catalyzes the formation of SLG from hemithioacetal (MeCOCH (OH)-SG) formed nonenzymatically from MG (methylglyoxal) and GSH, and Glo II (EC 3.1.2.6., GlxII, glyoxalase II, hydroxyacylglutathione hydrolase) that catalyzes the hydrolysis of SLG in D-lactate, regenerating the GSH consumed in the first reaction [25–27]. In this study we hypothesized SLG entry in mitochondria from cytosol and its hydrolysis by mitochondrial Glo II with consequent GSH release into mitochondria. The aim of this work was to establish if GSH transport by means of SLG from cytoplasm into mitochondria can be alternative to the way already described.

Materials and methods

Chemicals

All chemical reagents were obtained by Sigma Aldrich (Sigma, St Louis, MO, USA). Rotenone and FCCP were dissolved in ethanol. *S*-D-Lactoylglutathione was synthesized and purified as described [28]. [Glycine-2-^{3}H]glutathione (specific activity: 20–50 Ci (740–1850 GBq)/mmol) was purchased from PerkinElmer (Watham, MA, USA). *S*-Lactoylglutathione labeled with [glycine-2-^{3}H]glutathione was synthesized in a mixture reaction containing MG and 5 UI Glo I in buffer potassium phosphate 75 mM, pH 6.8. *S*-Lactoylglutathione labeled with [^{14}C]methylglyoxal was synthesized in a mixture reaction containing GSH and 5 UI Glo I in buffer potassium phosphate 75 mM, pH 6.8. Carboxy-H_2DCFDA (C400) and JC1 were supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA). All culture reagents were obtained by Euroclone.

Animals and mitochondrial preparation

Rat liver mitochondria (RLM) ($n=25$) were isolated from adult male Wistar rats (200–250 g body weight) supplied by INRCA (Istituto Nazionale Ricerca e Cura Anziani, Ancona, Italy). The purification of mitochondria from rat liver was carried out as follows: aliquots of liver tissue (4–5 g) were homogenized 1:10 (w:v) in ice-cold buffer, pH 7.5, containing 75 mM sucrose, 225 mM mannitol, 1 mM EDTA, 5 mM Hepes, and 0.5 mg/ml fatty acid-free bovine serum albumin. The homogenate was centrifuged for 10 min at 600 *g* at 4 °C. Sediment was discarded and the supernatant was centrifuged for 20 min at 1200 *g* at 4 °C. The mitochondrial pellet obtained was washed twice and purified mitochondria were suspended in the same medium to obtain 60–70 mg of proteins/ml. The mitochondrial protein content was determined by the Lowry method [29]. This investigation was carried out in conformity with the *Guide for the Care and Use of Laboratory Animals* [30] and was approved by the local Ethics Committee. Purity of isolated mitochondria was checked by measuring Glo I activity, a cytosolic marker, in the mitochondrial suspension.

Mitoplast isolation

Mitoplasts were isolated using RLM from adult male Wistar rats isolated in medium as described above. The final mitochondrial pellet was suspended in 5 ml of medium to which 5 ml of digitonin solution (6 mg/ml in medium) was added. The mixture was shaken at 0 °C for 15 min, and 0.5% of BSA was added to the medium. Mitoplasts collected by centrifugation (10,000 *g* for 10 min) were subsequently suspended in this medium, centrifuged again under the same conditions, and finally resuspended in maximum volume of 1 ml. Supernatants of the first and second centrifugations were centrifuged (105,000 *g* for 60 min, 4 °C) to obtain intermembrane space and mitochondrial-outer membrane fractions. The absence of intact mitochondria in the sample was checked by assaying adenylate kinase (EC 2.7.4.3); a marker enzyme of the intermembrane space and mitoplast integrity were confirmed by verifying the lack of glutamate dehydrogenase (EC 1.4.1.3) activity in the suspension.

LSG mitochondrial transport

In order to follow the entry of SLG or GSH into mitochondria after incubation of substrates, the solution with radioactive compounds was diluted in 10 ml H_2O. SLG quantity was determined in mitochondrial homogenates by measuring the radioactivity after 1, 2, 5, 10, and 20 min of incubation. Isolated mitochondria were in state 1 (without additional substrates) and three different mitochondrial suspensions were combined with either ^{14}CLSG or ^{3}HLSG or ^{3}HGSH. Radioactivity of the samples was determined using a liquid scintillation counter (Beckman LS-6500). Radioactivity of each sample was calculated using the calibration of sample quenching, radioactive decay, and background.

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