SPANDIDOS PUBLICATIONS

GSSG-mediated Complex I defect in isolated cardiac mitochondria

CHIARA PASSARELLI[1*], GIULIA TOZZI[1*], ANNA PASTORE[2], ENRICO BERTINI[1] and FIORELLA PIEMONTE[1]

[1]Molecular Medicine Unit; [2]Laboratory of Biochemistry, Children's Hospital and Research Institute 'Bambino Gesù', Rome, Italy

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Abstract. The mitochondrial respiratory chain represents the major source of reactive oxygen species (ROS) in cells and its dysfunction may contribute to the pathogenesis of several diseases. In mitochondria, glutathione is the major redox buffer and is a good indicator for the redox environment of the cell. Indeed, overproduction of ROS decreases the ratio between reduced and oxidized glutathione leading the latter to bind to proteins by a mechanism called glutathionylation. In this study, we demonstrate that in isolated cardiac mitochondria the respiratory chain enzyme Complex I is highly susceptible to glutathionylation under conditions of oxidative stress, showing a significant dose- and time-dependent decrease of the activity after treatment with oxidized glutathione. Among respiratory chain enzymes, Complex I appears the most affected by the oxidant-mediated inactivation in isolated mitochondria. Also, in cultured cardiomyocytes CI activity was strongly inhibited after *in vivo* treatment with hydrogen peroxide. Noteworthy, HPLC analysis showed a significant increase of protein glutathionylation in oxidatively stressed cells and this rise is *in vivo* reverted after incubation of cells with anti-oxidant N-acetylcysteine. These findings take particular importance given that CI represents the entry point of electrons into oxidative phosphorylation and that the threshold at which CI dysfunction affects ATP production is lower than that of any other OXPHOS complexes, making the enzyme particularly critical for the health of cells.

Introduction

The mitochondrial respiratory chain represents the major source of ROS in cells under both normal and pathophysiologic conditions (1). This ROS production contributes to mitochondrial damage in a range of pathologies and it is also important in redox signalling from the organelle to the rest of the cell (2).

The reduced glutathione (GSH) and its oxidized form (GSSG) represent the major cellular redox buffer, therefore the ratio GSH/GSSG is a good indicator for the redox environment of the cell. Indeed, a decrease in this ratio is indicative of an increase in oxidative stress (3-5).

In mitochondria, overproduction of ROS decreases the ratio GSH/GSSG and this imbalance leads GSSG to bind to proteins by a mechanism called glutathionylation. Glutathionylation was shown to regulate, either positively or negatively, a variety of regulatory, structural, and metabolic proteins deeply influencing some important biological processes, including proliferation, apoptosis, differentiation and senescence (5,6).

The proteins of the mitochondrial electron transport chain (ETC) are rich in thiol residues and Complex I is the major component of the ETC to host protein thiols (7,8) besides being the main site of O^{-}_{2} production in the mitochondrial respiratory chain (9,10). Therefore, glutathionylation of some enzymes that are part of the respiratory chain may represent an important response to mitochondrial oxidative stress.

The aim of this study was to analyse the effect of glutathionylation on isolated cardiac mitochondria, in order to identify the redox-sensitive targets potentially involved in the regulation of mitochondrial function. Mitochondria were isolated from rat heart and treated with increasing concentrations of GSSG. The level of electron transport chain activities was monitored as a function of GSSG concentration and time of exposure.

Materials and methods

Mitochondria isolation. Rat hearts (500 mg) were thawed in isolation buffer (210 mM mannitol, 70 mM saccharose, 50 mM Tris, 10 mM EDTA, and 0.5% BSA; pH 7.4), cut in small pieces, digested for 15 min with trypsin, under agitation, and washed 2 times with isolation buffer supplemented with protease inhibitors (Complete, Roche Diagnostics, Penzberg, Germany). Tissue was then centrifuged at 4˚C for 2 min at 100 x g. Tissue was homogenized with a conical glass grinder in 1 ml isolation buffer and the homogenate centrifuged at 4˚C for 10 min at 820 x g. The supernatant was collected and centrifuged at 4˚C for 20 min at 6,800 x g. The pellet obtained was resuspended in 1 ml suspension buffer (225 mM

Correspondence to: Dr Fiorella Piemonte, Molecular Medicine Unit, Children's Hospital and Research Institute 'Bambino Gesù', P.za S. Onofrio, 4, I-00165 Rome, Italy
E-mail: piemonte@opbg.net

*Contributed equally

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mannitol, 75 mM saccharose, 10 mM Tris, and 0.1 mM EDTA; pH 7.4) and centrifuged at 4˚C for 10 min at 820 x g. The mitochondria were then pelleted by centrifuging the supernatant for 20 min at 6,800 x g and immediately used for enzyme activity assays.

Cell lines and treatments. H9c2 cell line, derived from embryonic rat heart (11), was purchased from American Type Culture Collection (ATCC, Virginia, USA). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10% (v/v) fetal bovine serum, 10% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, at 37˚C in a humidified atmosphere of 5% CO_2. Cells were fed every 2-3 days and sub-cultured once they reached 70-80% confluence, according to ATCC Product information sheet. Cells, at 80-90% of confluence, were plated at an appropriate density (1:3) to begin experiments. Stock of cells were routinely frozen and stored in liquid N_2. Cells were incubated with 100 μM H_2O_2, 10 nM rotenone and 1 mM N-acetylcysteine (NAC) for 30 min, 1 h and 1 h, respectively. Blank cells (not treated) received an equivalent amount of DMSO (the final concentration of DMSO does not exceed 0.1%). After treatment, cells were harvested and collected to perform experiments.

Measurement of reduced (RED GSH) and protein-bound (GS-Pro) glutathione. Mitochondria and H9c2 cells, differently treated, were sonicated three times for 2 sec in 0.1 ml of 0.1 M potassium-phosphate buffer, pH 7.2. After sonication, 50 μl of 12% sulfosalicylic acid were added, and GSH content on the acid-soluble fraction was determined (Red GSH). The protein pellet was dissolved in 150 μl of 0.1 N NaOH, and protein bound glutathione (GS-Pro) determined. The derivatization and chromatography procedures were performed, with little modifications, as previously reported (12).

Determination of mitochondrial enzyme activities. Heart mitochondrial preparations were stored on ice and used within 3 h of preparation. The mitochondria and H9c2 cells were resuspended in 50 μl of suspension buffer and 10 mM phosphate buffer, pH 7.4, respectively. All enzyme activities were measured spectrophotometrically with a λ25 (Perkin Elmer, Waltham, MA, USA). NADH-CoQ oxidoreductase (Complex I) activity was assayed in assay buffer containing 20 mM phosphate buffer, 0.2 mM NADH, 1 mM NaN_3, 0.1% BSA, 1 mM EDTA Na_4, 100 μg proteins by following the rotenone-sensitive initial rate of NADH oxidation at 340 nm ($\Delta\varepsilon$=6.81 mM^{-1} cm^{-1}). Rotenone (5 μM) inhibited the enzymatic activity by 70-80%. Reaction started with addition of 50 μM CoQ_1.

Succinate-Co Q oxidoreductase (Complex II) was determined by following the reduction of 2,6-dichlorophenol indophenol (DCPIP) coupled with reduction of decyl-ubiquinone (DB). The assay buffer contains 50 mM phosphate buffer (pH 7.0), 1.5 mM NaCN, 0.1 mM DCPIP and 80 μg proteins. After 5 min of incubation, reaction starts with 16 mM succinate and 50 μM DB, and it is followed at 600 nm ($\Delta\varepsilon$=19.1 mM^{-1} cm^{-1}).

CoQ-cytocrome c oxidoreductase (Complex III) was assayed in a buffer containing 50 mM phosphate buffer (pH 7.0), 2 mM NaN_3, 0.1% BSA, 50 μM cytocrome c and 80 μg proteins. Reaction starts with addition of 50 μM DBH_2 and the reduction of cytocrome c was followed at 550 nm ($\Delta\varepsilon$=18.5 mM^{-1} cm^{-1}).

Table I. Effect of GSSG on mitochondrial electron transport complex activities.

Enzyme	-GSSG	+1 mM GSSG
Complex I	733±6	492±46
Complex II	314±7	308±23
Complex III	382±9	340±7
Complex IV	5,509±25	5,234±428

Mitochondria were incubated in the absence and presence of 1 mM GSSG for 10 min at 37˚C and enzyme activities determined as described under Materials and methods. Values are expressed as nmol/min/mg of mitochondrial protein and represent the means (n=3) ±SD.

Cytochrome c oxidase (Complex IV) activity was measured by following the oxidation of cytochrome c at 550 nm ($\Delta\varepsilon$=18.5 mM^{-1} cm^{-1}). Enzymatic activity was estimated in 10 mM phosphate buffer (pH 7.0) containing 0.025% lauryl-maltoside, 80 μg proteins, 1 mg/ml reduced cytochrome c.

Citrate synthase activity was used as mitochondrial matrix enzymatic marker. Mitochondrial proteins (80 μg), 0.25 mM acetyl-coenzyme A, and 0.2 mM DTNB were added to a Tris-HCl buffer (10 mM), pH 7.4, containing 0.2% (v/v) Triton X-100. The reaction was started by the addition of 0.4 mM oxalacetate and the initial rate was measured following the reduction of DTNB at 412 nm ($\Delta\varepsilon$=13.6 mM^{-1} cm^{-1}). Protein concentrations were quantified by BCA-protein assay (Sigma-Aldrich, St. Louis, MI, USA).

Analysis of glutathione-conjugates by Western blotting. Isolated mitochondria (20 μg) were applied onto 12% SDS-polyacrylamide gel electrophoresis and proteins were transferred onto a nitrocellulose membrane overnight at 70 mA. Membrane was blocked with 5% non fat dry milk in TBST (100 mM NaCl and 10 mM Tris-HCl, pH 7.8 containing 0.1% Tween 20) for 2 h, at room temperature, and probed with monoclonal anti-GSH antibody (1/1,000) (Virogen, Watertown, MA, USA) and/or with monoclonal anti-Complex I-51 kDa subunit antibody (1/3,000) (Santa Cruz Biotechnology, CA, USA). Antibody incubation was followed by a horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies (1:25,000) (Amersham Bioscience, Buckinghamshire, UK). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). Stripping of membrane was performed in a buffer containing 25 mM glycine-HCl, pH 2.1% SDS, for 30 min, at room temperature.

Identification of glutathionylated CI by immunoprecipitation. Mitochondria (60 μg), re-suspended in 150 μl H_2O and sonicated, were incubated with 10 μl anti-GSH antibody (10 μg) overnight at 4˚C on a rocker platform. 20 μl of re-suspended volume of Protein G Plus-Agarose (Santa Cruz

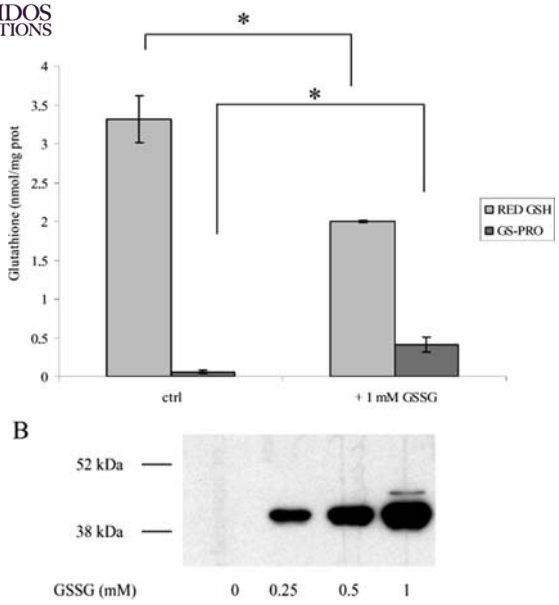

Figure 1. Effect of GSSG on protein glutathionylation of isolated cardiac mitochondria. (A) GSH bound to proteins (GS-Pro) of isolated cardiac mitochondria was determined by HPLC analysis after incubating 80 μg mitochondria for 15 min at 37˚C with 1 mM GSSG. (B) Western blot analysis of 20 μg mitochondria treated with increasing (0-1 mM) amounts of GSSG for 15 min at 37˚C, showing protein glutathionylation determined by anti-GSPro antibody. All data are represented as the mean ±SD of three experiments. *p<0.05 vs. untreated mitochondria. For details, see Materials and methods.

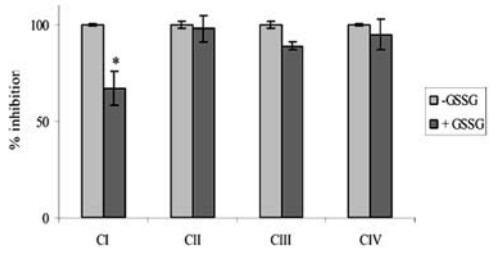

Figure 2. Effect of GSSG on the respiratory chain enzyme activities. Isolated cardiac mitochondria were incubated with 1 mM GSSG for 15 min and the respiratory chain enzyme activities were spectrophotometrically assayed as reported in Materials and methods. Data are expressed as % of inhibition of activity with respect to untreated mitochondria. *p<0.05.

Biotechnology, CA, USA) were added to the solution and incubated at 4˚C for 1 h on a rocker platform. The pellet was collected by spinning for 10 sec, at 4˚C and washed 4 times with suspension buffer. After final wash, the pellet was re-suspended in 20 μl electrophoresis sample buffer, boiled for 4 min, loaded onto reducing 8% SDS-polyacrylamide gel electrophoresis for Western blot analysis, and revealed with anti-Complex I-51 kDa subunit antibody (1:3,000).

Results

To induce protein glutathionylation, mitochondria were incubated with various amounts of GSSG (0-1 mM) at 37˚C for 10 min. As determined by HPLC analysis (Fig. 1A), total glutathionylated protein level was significantly enhanced, ranging from 0.06±0.1 to 0.41±0.1, (mean ±SD) nmol/mg prot.

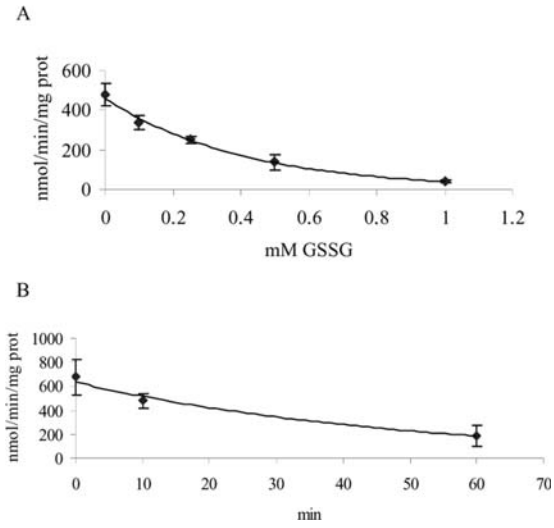

Figure 3. Dose and time-dependent GSSG inhibition of Complex I activity. (A) Isolated cardiac mitochondria (100 μg) were incubated in presence of various concentrations (0-1 mM) of GSSG for 15 min at 37˚C and the enzyme activity determined as reported in Materials and methods. (B) CI activity was measured by incubating 100 μg of isolated mitochondria with 1 mM GSSG for a time ranging between 0-60 min at 37˚C. Data are reported as the mean ±SD of three experiments and are expressed as nmol/min/mg.

after incubation with 1 mM GSSG. When reaction mixture was separated by SDS-PAGE and immunoblotted with the monoclonal antibody against glutathionylated proteins, GSSG treatment led to glutathionylation of a number of proteins whose MW ranged between 38-52 kDa and the signal was enhanced proportionally to the GSSG dose (Fig. 1B). Glutathionylation might influence respiratory chain enzymes, thus we analyzed the enzymatic activity of all complexes on GSSG-treated isolated mitochondria (Table I). As shown in Fig. 2, CI suffers more than the other respiratory chain enzymes of the inactivation by glutathionylation and the GSSG inhibition was dose- (Fig. 3A) and time-dependent (Fig. 3B), with 30-60 min incubation leading to substantial inactivation of Complex I.

Thus, in light of the inactivating effect of GSSG, we tested the possibility that one of the glutathionylated proteins observed on the isolated cardiac mitochondria after oxidation was CI. Western blot analysis of mitochondria incubated with 1 mM GSSG showed that the band recognized by the GS-Pro antibody (Fig. 4A) co-migrated with the band revealed with anti-Complex I-51 kDa subunit (Fig. 4B), after stripping the nitrocellulose and reprobing the membrane with an anti-Complex I-51 kDa subunit. The involvement of protein glutathionylation is confirmed by the decrease of the immunoreactivity in presence of the reducing agent dithitreitol (DTT) (Fig. 4A), also suggesting the reversibility of the process. The identification of CI as a sensitive target of glutathionylation was then demonstrated by immunoprecipitating the GSSG-treated mitochondria by GS-Pro antibody and analyzing by Western blotting for the 51-kDa subunit of Complex I. As reported in Fig. 4C, CI was highly glutathionylated in oxidized cardiac mitochondria and, interestingly, it appears constitutively glutathionylated even under physiological conditions.

The susceptibility of CI to the inactivation following oxidation has also been confirmed *in vivo* on cultured cardio-

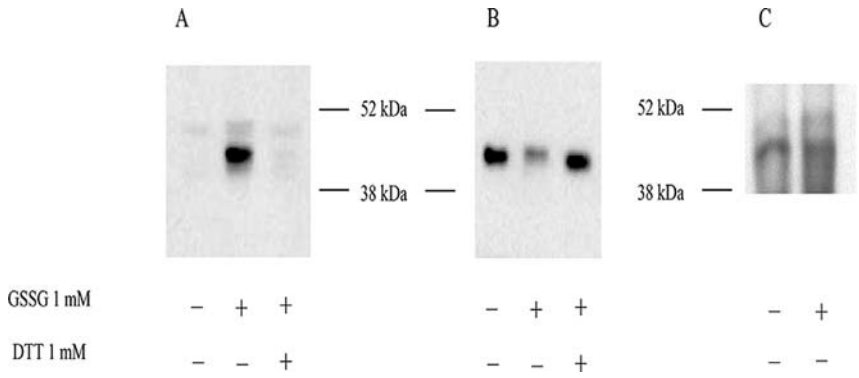

Figure 4. Identification of glutathionylated CI in isolated cardiac mitochondria. (A) Mitochondrial proteins (20 μg), incubated with and without 1 mM GSSG for 15 min at 37˚C, were separated by not-reducing SDS-PAGE and transferred to nitrocellulose. The glutathione conjugates were probed with a monoclonal anti-GSPro antibody (1/1,000). (B) Nitrocellulose was stripped and re-probed with anti-CI (51 kDa subunit) antibody (1/3,000). (C) Mitochondria (60 μg), incubated with and without 1 mM GSSG for 15 min at 37˚C, were immunoprecipitated with anti-GSPro antibody and revealed with anti-CI (51 kDa subunit) antibody. The blots are representative of one of three independent experiments. For details, see Materials and methods.

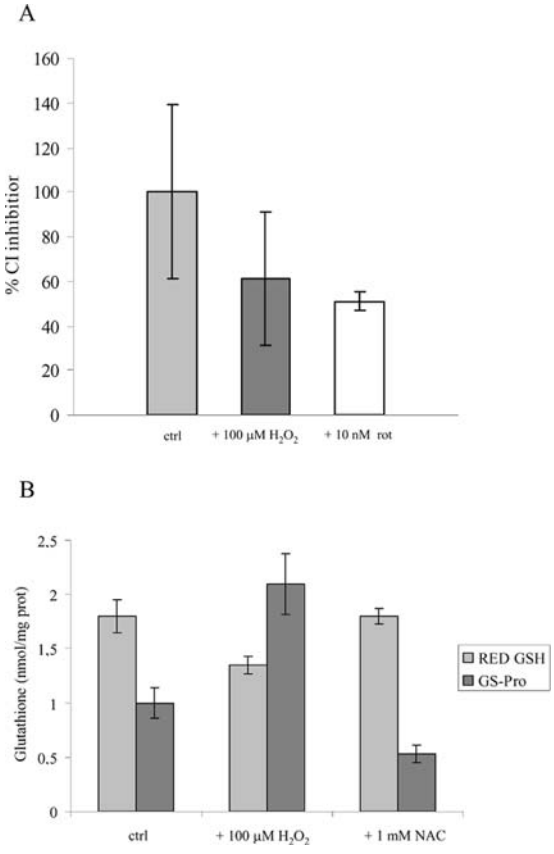

Figure 5. Effect of pro and anti-oxidant agents on H9c2 cardiomyocytes. (A) Cell lysates (150 μg), *in vivo* treated with 100 μM H_2O_2 and 10 nM rotenone for 30 min and 1 h respectively, were spectrophotometrically assayed for CI activity. Data are expressed as % inhibition of activity with respect to the untreated cardiomyocytes. (B) GSH bound to the proteins (GS-Pro) was determined by HPLC analysis on 100 μg cell lysates after *in vivo* treatment with 100 μM H_2O_2 and 1 mM NAC, for 30 min and 1 h respectively. Data are reported as the mean ±SD of three experiments and are expressed as nmol/mg.

myocytes, where CI activity was strongly (40%) inhibited after incubation for 30 min with H_2O_2 (Fig. 5). A comparable inactivation was obtained when cells were *in vivo* treated with rotenone, a specific inhibitor of CI (Fig. 5A). The *in vivo* treatment of cardiomyocytes with H_2O_2 caused a significant increase of protein glutathionylation, as measured by HPLC analysis (Fig. 5B). Noteworthy, protein glutathionylation is *in vivo* reverted after incubation of cells with the anti-oxidant N-acetylcysteine (NAC).

Discussion

Impairment of Complex I activity has been described in several neurodegenerative diseases including Parkinson's, hereditary spastic paraplegia and Huntington's disease (13,14). Oxidation of cysteine residues in Complex I may play a role in its inhibition. Indeed, Complex I is a regulable and vulnerable pacemaker in mitochondrial respiratory function (15) and several studies have provided evidence of phosphorylation of subunits of Complex I effecting on electron transfer and in superoxide production.

CI is a membrane bound multimeric enzyme comprising at least 45 subunits and catalysing NADH oxidation and ubiquinone reduction coupled to proton pumping across the inner membrane (16-19). It contains reactive thiols on the 75- and 51-kDa subunits that interacting with the mitochondrial glutathione pool may be implicated in oxidative damage in many pathologies (7,8,20,21).

In mitochondria, GSH is converted to GSSG under conditions of oxidative stress and previous studies have reported the *in vitro* glutathionylation of the purified Complex I after exposure to glutathione disulfide (GSSG) (7,22).

In our study, we showed that CI is the respiratory chain enzyme mostly affected by GSSG-induced oxidative stress in isolated cardiac mitochondria. Its enzyme activity results significantly decreased after oxidation and this finding takes particular importance given that CI represents the entry point of electrons into oxidative phosphorylation and that the threshold at which CI dysfunction affects ATP production is lower than that of any other OXPHOS complexes (~25% reduction in Complex I activity is sufficient to reduce O_2 consumption and ATP synthesis) (23).

However, despite its critical role in ETC, CI is the major source of ROS in mitochondria and its inhibition may induce collateral damage to cells, thereby amplifying the initial dysfunction of the Complex itself. In this context, gluta-

sion may become a key molecular event in redox n of mitochondrial functions (24-28). Indeed, the redox pool in mitochondria is rich of glutathione, with a physiological concentration of 5-10 mM (29). An over-production of ROS leads to an imbalance of the GSH/GSSG ratio sensitising the mitochondrial proteins to glutathionylation. By this mechanism, mitochondria could regulate redox signaling in response to the changes of glutathione pool occurring during oxidative stress and CI plays a role as a target sensitive to ROS fluctuations.

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