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Short report

Glutathione metabolic status in the aged rabbit aorta☆

Domenico Lapenna *, Giuliano Ciofani, Maria Adele Giamberardino

Dipartimento di Medicina e Scienze dell'Invecchiamento, and Laboratorio di Fisiopatologia dello Stress Ossidativo, Centro Scienze dell'Invecchiamento e Medicina Traslazionale (CeSI-MeT), Center of Excellence on Aging, Università degli Studi "G. d'Annunzio" Chieti Pescara, 66100 Chieti, Italy

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ABSTRACT

It is not known whether aging alters glutathione metabolic status of the mammalian arterial tissue favoring vascular oxidative stress and dysfunction. Thus we assessed total, reduced and oxidized glutathione (TG, GSH and GSSG, respectively), the glutathione redox ratio (GRR, namely [GSSG] / [GSH + 2GSSG] × 100), and the activities of the glutathione status-regulating enzymes glutathione reductase (GSSG-Red), γ-glutamylcysteine synthetase (γ-GCS) and γ-glutamyl transpeptidase (γ-GT) in the aortic tissue of 9 young adult control rabbits (YACR, about 4 months old) and 9 aged rabbits (AR, about 4.5 years old); aortic lipid and protein oxidation and H_2O_2 were also determined as oxidative stress indicators. Vascular function was assessed on aortic ring preparations. TG and GSH concentrations, together with γ-GCS and γ-GT activities, were significantly lower, while GSSG content and the GRR higher, in the AR than in the YACR aortas; GSSG-Red activity did not differ significantly between the two groups. Heightened levels of lipid and protein oxidation and H_2O_2 occurred in the AR aortas, indicating age-dependent vascular oxidative stress. Moreover, in the whole population of 18 rabbits, the aortic values of GSH and related enzyme activities were inversely and significantly correlated with those of lipid and protein oxidation and H_2O_2, highlighting the antioxidant role of GSH and related enzymes in the vascular tissue. Aortic endothelium-dependent vasodilation was lower in the AR than in the YACR. In conclusion, glutathione metabolic status is altered in the aged rabbit aorta reflecting depressed γ-GCS- and γ-GT-related GSH biosynthesis and GSSG burden eventually favoring vascular oxidative stress and dysfunction.

1. Introduction

Glutathione, essentially in its reduced form (GSH), serves several vital functions such as acting as antioxidant and radical scavenger, detoxifying electrophiles, maintaining the thiol status of proteins, providing a reservoir for cysteine, and modulating critical cell processes including DNA synthesis, immune function, and cellular proliferation (Meister and Anderson, 1983; Lu, 2009). Cell glutathione levels are controlled by the balanced activity of specific enzymes, namely glutathione reductase (GSSG-Red), which regenerates GSH from oxidized glutathione (GSSG), and γ-glutamylcysteine synthetase (γ-GCS), which, together with γ-glutamyl transpeptidase (γ-GT), is involved in de novo cell synthesis of GSH in the γ-glutamyl cycle (Meister and Anderson, 1983; Lapenna et al., 2004; Lu, 2009); thus such enzymes control cell glutathione metabolic status. There is experimental evidence that glutathione levels decline with age, thus resulting in reduced cell antioxidant capacity; as shown in nonvascular tissues, such age-dependent glutathione decrease may be related to altered γ-GCS expression leading to impaired enzymatic activity and tripeptide biosynthesis (Liu and Choi, 2000). However, to date little is known about glutathione metabolic status of the aged mammalian arterial tissue and its association with vascular oxidative stress and function. Thus we have here investigated glutathione metabolic status together with vascular oxidative stress and function in the aged rabbit aorta.

2. Materials and methods

2.1. Animals

Two groups of New Zealand White (NZW) rabbits were used in the study, which was approved by the local institutional animal care and use ethics committee: Group A, comprising 9 young adult control rabbits, about 4 months of age, and Group B, comprising 9 aged rabbits, about 4.5 years old (the oldest commercially available rabbits). In this regard, it is of note that commercial NZW rabbits have a life-span of 5–7 years, so that the age of 4.5 years corresponds to the aging of rabbits and, when extrapolated to the human context, to the aging of humans. Moreover, NZW rabbits are sexually mature at age of about 4 months, possibly corresponding to the status of young adult subjects in the

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* Corresponding author at: U.O.C. Attività di Medicina per Intensità di Cure ad Indirizzo Geriatrico, Ospedale Clinicizzato "Santissima Annunziata", Via dei Vestini, 66100 Chieti, Italy.
E-mail address: dlapenna@unich.it (D. Lapenna).

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human context; thus rabbits about 4 months of age were used as controls for the aged rabbits.

2.2. Aortic biochemical analyses

Reagents of the highest purity were from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Before sacrifice, rabbits were given heparin (500 U/kg) intravenously through the marginal ear vein. The excised aortas were placed in a buffer (buffer A) with antioxidant characteristics, namely argon-purged ice-cold 0.1 M Tris-HCl buffer, pH 7.6, plus 2 mM EDTA and 0.2 mM butylated hydroxytoluene (BHT), and cleaned of surrounding tissue. Aortic rings about 3-mm width were obtained from thoracic aorta for vascular function assessment (see below). The aortas above and below the rings were opened with longitudinal cuts and any possible blood material was removed; longitudinal aortic portions were rapidly snap-frozen in liquid nitrogen and stored at −80 °C until biochemical analysis. A longitudinal portion of the aortic wall was directly homogenized in ice-cold 5% sulfosalicylic acid plus 5 mM EDTA to assay total glutathione, GSSG and GSH, and another in ice-cold buffer A for the other biochemical assays, except for hydrogen peroxide (H_2O_2), which was assessed in another aortic portion homogenized in ice-cold 40 mM Tris-HCl buffer, pH 7.6, in the absence of EDTA, which interferes in the H_2O_2 assay. The homogenization media were argon-purged, and dark amber vials were generally used under minimal light exposure. It is worth noting that Tris has per se scavenging-antioxidant properties (Hicks and Gebicki, 1986), which are maximized by addition of the antioxidants BHT and EDTA and removal of oxygen by argon purging (according also to our experience, EDTA, but not the lipophilic antioxidant compound BHT, is useful as metal-chelating antioxidant at higher concentration in glutathione assay). Thus, considering the experimental conditions used and the biochemical analyses performed, sample auto-oxidation is substantially prevented. The homogenate obtained in sulfosalicylic acid plus EDTA was centrifuged at 10,500 ×*g* for 20 min, and the resulting supernatant used to assess total glutathione, GSSG and GSH. The homogenate obtained in buffer A was subjected to a first centrifugation at 1500 ×*g* for 15 min, and the resulting supernatant used to assay γ-GT activity, lipid peroxidation, namely fluorescent damage products of lipid peroxidation (FDPL) and thiobarbituric acid reactive substances (TBARS), and protein carbonyls; the activity of γ-GCS was measured on the supernatant resulting from a further centrifugation at 8500 ×*g* for 20 min, while that of GSSG-Red on the cytosolic fraction obtained after ultracentrifugation at 105,000 ×*g* for 60 min. The homogenate obtained in Tris-HCl buffer without EDTA was centrifuged at 20,000 ×*g* for 20 min, and the supernatant heated at 95 °C for 3 min to allow enzymatic proteins inactivation, followed by further centrifugation at 6000 ×*g* for 10 min; the resulting supernatant was used for H_2O_2 assay.

Total glutathione (GSH + 2GSSG) was determined by the Tietze's GSSG-Red enzyme recycling method (Tietze, 1969) basically as previously reported (Lopez-Torres et al., 1994; Lapenna et al., 2004), and its concentrations calculated as nmol/mg protein (Lapenna et al., 2004). GSSG was assessed by the same recycling method after derivatization of the aortic homogenate GSH with 2-vinylpyridine to block and mask GSH thiol group. GSH was calculated from the difference between total glutathione and GSSG values, considering that reduction of one molecule of GSSG results in two molecules of GSH; as for total glutathione, GSH and GSSH concentrations are expressed as nmol/mg protein. The glutathione redox ratio was calculated based on the concentrations of GSSG and total glutathione, namely GSH + 2GSSG, as: [GSSG] / [GSH + 2GSSG] × 100.

GSSG-Red activity was assayed by monitoring spectrophotometrically the oxidative disappearance of NADPH at 340 nm as previously reported (Lapenna et al., 1996; Lapenna et al., 2004). Results were calculated as nmol NADPH oxidized/min (mU)/mg protein, using a molar extinction coefficient for NADPH of 6220 at 340 nm (Lapenna et al., 1996; Lapenna et al., 2004).

The activity of γ-GCS was assayed basically according to the method of Sekura and Meister (1977), as we previously reported (Lapenna et al., 1996; Lapenna et al., 2004). The inorganic phosphate (P_i) formed by the specific enzymatic activity was detected spectrophotometrically at 405 nm using a commercial kit. Results are expressed as μg P_i/min/mg protein.

The activity of γ-GT was measured using a commercially available kit based on the use of glycylglycine and L-γ-glutamyl-3-carboxy-4-nitroanilide (GCNA) as the donor substrate, after 4 h incubation of the relative homogenate supernatant at 4 °C in 0.1 M Tris/HCl buffer, pH 8.2, plus 5 mM EDTA and 1% sodium deoxycholate (Lapenna et al., 1996; Lapenna et al., 2004). The release of 5-amino-2-nitrobenzoate (ANB) from GCNA by γ-GT was determined spectrophotometrically at 405 nm. Results are expressed as nmol ANB released/min (mU)/mg protein.

FDPL, which result from the interaction of lipoperoxidation aldehydes such as 4-hydroxynonenal with biomolecular primary amino groups, were assayed after extraction with BHT-containing chloroform/methanol (2:1 v/v), followed by addition of 0.05 M KCl solution and centrifugation at 1300 ×*g* to separate aqueous and chloroform phases. The organic layer was dried under a stream of argon, resuspended in chloroform/methanol (10:1 v/v), and read spectrofluorometrically at 430 nm emission with excitation at 360 nm (Lapenna et al., 2004). Results are expressed as units of relative fluorescence (URF)/mg protein.

TBARS assay, which detects aldehydic products of lipid peroxidation such as malondialdehyde, was performed basically according to the method by Ohkawa et al. (1979) using BHT and EDTA as antioxidants during the assay heating phase to prevent sample auto-oxidation. Results were calculated as nmol TBARS/mg protein, using a molar extinction coefficient of 154,000.

Protein carbonyls, expression of protein oxidation, were determined spectrophotometrically at 370 nm by the 2,4-dinitrophenylhydrazine assay basically as previously reported (Oliver et al., 1987; Qu et al., 2000) after homogenate supernatant treatment with streptomycin sulfate (1% final concentration) to remove DNA. Results were calculated as nmol protein carbonyls/mg protein, using 21,000 as the molar extinction coefficient of carbonyls (Oliver et al., 1987).

H_2O_2 was determined spectrophotometrically at 560 nm by the aqueous ferrous oxidation in xylenol orange (FOX-1) assay in the presence of 100 mM sorbitol as reaction amplifier basically as previously reported (Slezak et al., 1995; Lapenna et al., 2014); the assay was rendered absolutely specific for H_2O_2 using catalase (1.2 mg/ml) to valid the H_2O_2 signal (Lapenna et al., 2014). Results were calculated as nmol H_2O_2/mg protein, using a molar extinction coefficient for H_2O_2 of 267,000 at 560 nm (Lapenna et al., 2014).

Protein concentrations were assayed by Bradford's method as we previously reported (Lapenna et al., 1996; Lapenna et al., 2004).

2.3. Ex vivo vascular function assessment

Aortic rings were suspended in an organ bath kept at 37 °C containing Krebs-Henseleit bicarbonate buffer composed by (in mM) 119 NaCl, 4.75 KCl, 1.19 $MgSO_4$, 2.54 $CaCl_2$, 1.19 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose, pH 7.4; such buffer was continuously gassed with 95% O_2 and 5% CO_2. Aortic rings were equilibrated for 90 min with a resting tension of 2 g. Isometric tension was recorded with a Grass FT03 force-displacement transducer and recorded on a multichannel SensorMedics Dynograph Recorder R611. After the equilibration period, rings were contracted with 0.4 μM phenylephrine; acetylcholine (1 μM) was then added to the bath to assess the endothelium integrity. After acetylcholine testing, the rings were re-equilibrated for 60 min and again contracted as before with phenylephrine; endothelium-dependent vasodilation was then assessed with 1 μM acetylcholine.