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Reversible inhibition of mammalian glutamine synthetase by tyrosine nitration

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Abstract The effect of tyrosine nitration on mammalian GS activity and stability was studied in vitro. Peroxynitrite at a concentration of 5 μmol/l produced tyrosine nitration and inactivation of GS, whereas 50 μmol/l peroxynitrite additionally increased *S*-nitrosylation and carbonylation and degradation of GS by the 20S proteasome. (−)Epicatechin completely prevented both, tyrosine nitration and inactivation of GS by peroxynitrite (5 μmol/l). Further, a putative "denitrase" activity restored the activity of peroxynitrite (5 μmol/l)-treated GS. The data point to a potential regulation of GS activity by a reversible tyrosine nitration. High levels of oxidative stress may irreversibly damage and predispose the enzyme to proteasomal degradation.

Keywords: Ammonia; Cerebral cortex; Liver; Nitrosylation; Oxidative stress; Proteasome; Tyrosine nitration; Proteolysis

1. Introduction

Glutamine synthetase (GS; glutamate ammonia ligase, EC 6.3.1.2) catalyzes the conversion of glutamate and ammonia to glutamine and plays a major role in ammonia detoxification, interorgan nitrogen flux, acid–base regulation, cell proliferation and protection from apoptotic stimuli [1].

GS activity is highly susceptible to reactive nitrogen and oxygen species. For instance, inhibition of nitric oxide (NO) synthase (NOS) increased GS activity in rat brain and cultured rat astrocytes [2–4], suggesting that a NOS-dependent generation of reactive nitrogen species accounts for tonic GS inhibition in the brain. Further, NOS inhibition protected GS from *N*-methyl-D-aspartate (NMDA)-induced inactivation in vital brain slices [5]. NO may recombine with the superoxide anion to generate peroxynitrite, which potently nitrates protein tyrosine residues [6]. Indeed, GS tyrosine nitration and inactivation was found in brains of different rat models related to hyperammonemia and hepatic encephalopathy, in cultured astrocytes exposed to ammonia, and in livers of septic rats [7–10]. Recently mass spectrometric analysis of peptides derived from sheep GS inactivated by peroxynitrite in vitro identified a nitrated tyrosine residue corresponding to Tyr^{335} of rat GS, which is highly conserved among eukaryotic GS [9,11]. This tyrosine residue may directly stack with the purine system of GS-bound ADP/ATP, and in addition might contribute to the interface with the neighbouring subunit. Hence it seems plausible that GS activity is sensitive to tyrosine nitration. However, a potential contribution of oxidative modifications different from tyrosine nitration to GS inactivation has not yet been excluded. Further, the impact of tyrosine nitration and oxidation on the stability of mammalian GS is unknown.

The present study by an in vitro approach examined the contribution of tyrosine nitration and other oxidative modifications to GS inactivation and degradation by the 20S proteasome.

2. Materials and methods

2.1. Materials and reagents

A commercial sheep brain GS preparation (G-6632, Sigma, Taufkirchen, Germany) was used in this study. Following SDS gel electrophoresis and Coomassie brilliant blue staining the GS presents as a double band at about 45 kDa (Fig. 1A). Mass spectrometric analysis confirmed that both bands relate to GS (Dr. Sabine Metzger, Biological Medical Research Center, Heinrich-Heine-University, Düsseldorf, Germany).

L-Glutamine, hydroxylamine-HCl, adenosine diphosphate, $MnCl_2$, imidazol-HCl, (−)epicatechin and the polyclonal antibodies against GS and *S*-nitrosocystein were from Sigma (Taufkirchen, Germany). The monoclonal anti-GS antibodies were from Transduction-Laboratories (San Diego, CA). The monoclonal and polyclonal antibodies against 3′-nitrotyrosine were purchased by Upstate Biotechnology (Lake Placid, NY). The horseradish peroxidase (HRPOD)-coupled anti-mouse-IgG antibody was from BioRad (Hercules, USA) and the HRPOD-coupled antibody recognizing rabbit IgG was from DAKO (CA, USA). The protein A/G agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxynitrite and the purified 20S proteasome were from Calbiochem-Novabiochem (Schwalbach, Germany). Succinyl-leucine-leucine-valine-tyrosine-4-methylcoumarin-7-amide (Suc-LLVY-MCA) was from Bachem (Weil am Rhein, Germany).

2.2. Peroxynitrite treatment of GS

Sheep GS (500 ng) was treated with peroxynitrite at concentrations of 0–50 μmol/l in KH_2PO_4 buffer (pH 7.0) under continuous vortexing in the presence or absence of 30 μmol/l (−)epicatechin in a total volume of 100 μl. The addition of peroxynitrite to the buffer system did not lead to a pH change of the sample. Aliquots were taken for dot blot analysis of covalent modifications and determination of enzyme activity.

2.3. Preparation of spleen proteins from lipopolysaccharide-treated rats

The experiments were approved by the responsible local authorities. Lipopolysaccharide (LPS, dissolved in 0.9% saline) was injected to rats

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Abbreviation: GS, glutamine synthetase

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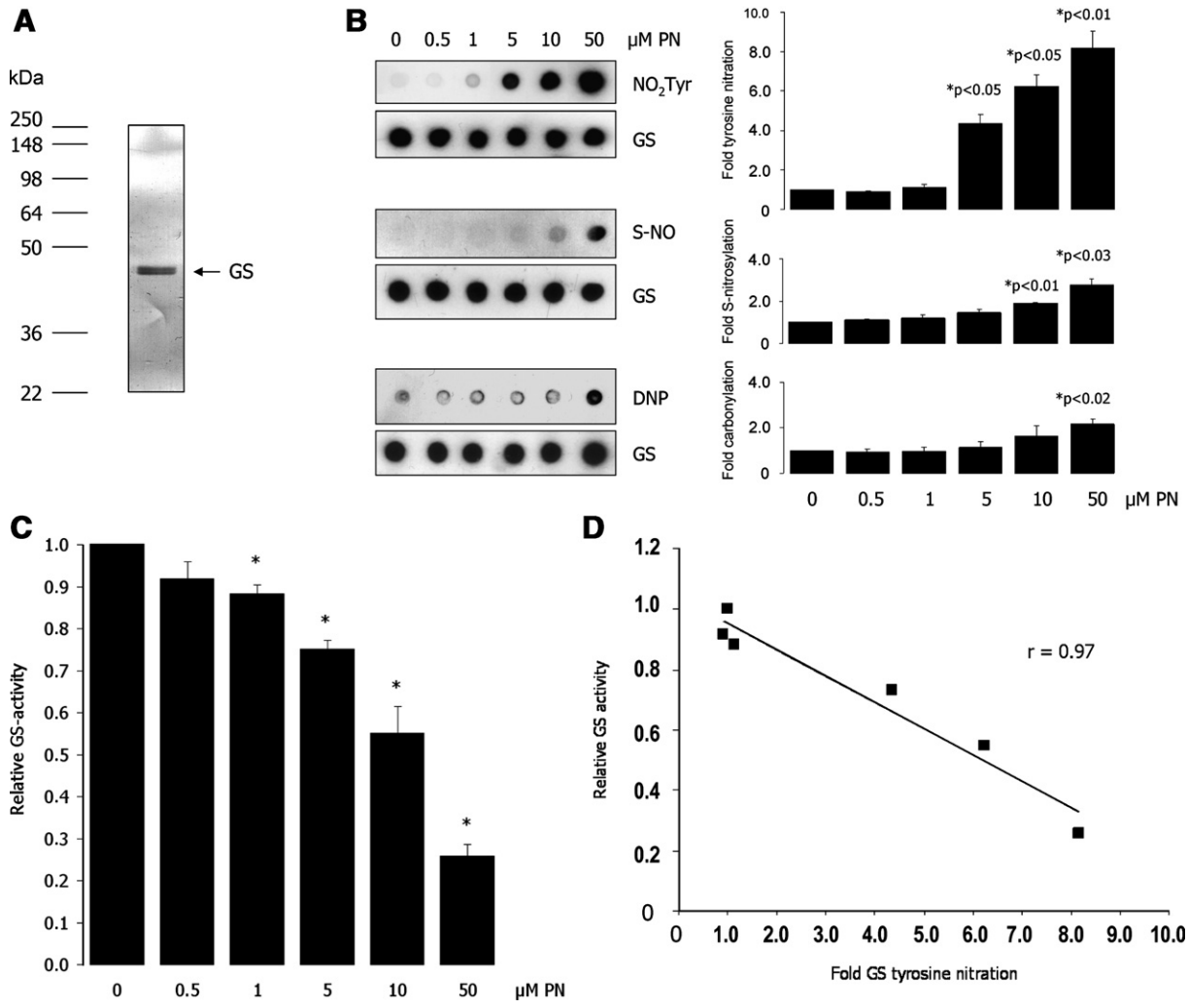

Fig. 1. Peroxynitrite-induced covalent modifications and inactivation of GS. (A) Representative Coomassie brilliant blue staining of the used commercial sheep brain GS preparation. (B,C) GS reacted with 0–50 μmol/l peroxynitrite. Samples were analyzed for the indicated modification or GS activity, respectively. (B) Peroxynitrite-induced tyrosine nitration, *S*-nitrosylation and carbonylation of GS. Representative dot blots of 3–4 independent experiments are shown. Changes in tyrosine nitration, *S*-nitrosylation and carbonylation were quantified by densitometry and were normalized to overall GS immunoreactivity. The level of modified GS at the zero time point was set to one. (C) Peroxynitrite-induced inactivation of GS. GS activity was normalized to that found in absence of peroxynitrite. (D) Regression analysis of a linear relationship between GS tyrosine nitration and relative GS activity.

intraperitoneally at a dose of 4 mg/kg body wt and animals were housed individually. Spleen samples were homogenized with an ultra-turrax using 10 mmol/l Tris–HCl buffer (pH 7.4) containing 1% Triton X-100, 150 mmol/l NaCl, 10 mmol/l $Na_4P_2O_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mmol/l NaF, 1 mmol/l sodium vanadate, 20 mmol/l β-glycerophosphate, and protease inhibitor cocktail (Boehringer Mannheim, Germany). Lysates were centrifuged at 20 000 × *g* at 4 °C and protein concentration of the supernatant was estimated by the Bradford assay (BioRad, Munic, Germany). Likewise, protein extracts from liver and cerebral cortices of non LPS-treated rats were prepared.

2.4. Incubation of peroxynitrite-treated GS with spleen protein from LPS-treated rats

Peroxynitrite-treated GS (50 ng) was incubated with 40 μg of spleen protein from LPS-treated rats supplemented with 2 mmol/l $MgCl_2$ and 2 mmol/l $CaCl_2$ in a total volume of 40 μl. For control the putative "denitrase" activity of the spleen extract was inhibited by heating to 95 °C for 2 min [12] before incubation with the peroxynitrite-treated GS. Similar incubations were performed with protein preparations from rat liver and cerebral cortex. Removal of 3′-nitrotyrosine immunoreactivity was followed by Western- and dot blot analysis.

2.5. GS activity assay

The glutamine synthase-catalyzed formation of γ-glutamylhydroxamate from glutamine and hydroxylamine was measured as described [9].

2.6. Detection of GS carbonylation

To monitor protein carbonylation the OxyBlot-protein oxidation detection kit (Chemicon) was used. 25 ng GS was denaturated by addition of SDS (final concentration: 6%), followed by derivatization of carbonyl groups with dinitrophenylhydrazine according to the manufacturers protocol. Aliquots were dotted to nitrocellulose and the 2,4-dinitrophenyl (DNP) moiety was detected by using a 1:5000 dilution of a polyclonal antibody specifically recognizing DNP. No unspecific binding of the anti-DNP antibody to the negative control samples (absence of 2,4-dinitrophenylhydrazine) was observed.

2.7. Immunoblotting

GS was analyzed by Western Blotting using 10% SDS–polyacrylamide gels and a semi-dry transfer apparatus (Biometra, Fastblot B33, Göttingen, Germany). For dot blot analysis equal amounts of GS (10 ng) in a volume of 2 μl were applied to a nitrocellulose