Supplementation with N-acetylcysteine and taurine failed to restore glutathione content in liver of streptozotocin-induced diabetics rats but protected from oxidative stress

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Abstract

Rats were rendered diabetic with streptozotocin and supplemented or not with N-acetylcysteine (NAC) and taurine (TAU). The liver was examined for the quantity of glutathione (GSH), both total and oxidised (GSSG), by HPLC assay. Moreover, the liver expression of gamma-glutamyl-cysteine synthetase, cysteine dioxygenase and heme oxygenase 1 was evaluated. Streptozotocin-diabetic rats showed decreased levels of liver glutathione (GSH); dietary supplementation with the antioxidants NAC and TAU failed to restore liver GSH to the level of control rats. Gamma-glutamyl-cysteine synthetase expression was not reduced in the diabetic rats, so the low hepatic GSH level in the supplemented diabetic rats cannot be ascribed to decreased expression of the biosynthetic key enzyme. Moreover, the diabetic rats showed no evidence of increased expression of cysteine dioxygenase, which could have indicated that NAC-derived cysteine was consumed in metabolic pathways different from GSH synthesis. However, NAC+TAU treatment provided partial protection from glutathione oxidation in the liver of diabetic rats; moreover, the antioxidant treatment reduced the hepatic overexpression of heme oxygenase 1 (HO-1) mRNA which was detected in the diabetic rats. In conclusion, although NAC was not able to restore liver GSH levels, the antioxidant treatment restrained GSH oxidation and HO-1 overexpression, which are markers of cellular oxidative stress: diabetic rats probably exploit NAC as an antioxidant itself rather than as a GSH precursor.

1. Introduction

The role of oxidative stress in diabetes is currently under discussion. Several studies have underlined the participation of oxidative mechanisms in the generation of some Advanced Glycation End Products (AGE) [1], which are considered responsible for protein alterations leading to diabetic sequelae [2]. Moreover, signs of increased oxidative stress in diabetes have been recognised in plasma [3,4] and inside the cells [4,5]; studies on the content of antioxidant enzymes have yielded contrasting results [6–9], probably owing to differences in diabetes models, diabetes duration and organs analysed.

Abbreviations: GSH, glutathione; NAC, N-acetylcysteine; TAU, taurine; HO-1, heme oxygenase 1; AGE, Advanced Glycation End Products; UD, Untreated Diabetic rats; N+T, rats treated with NAC and TAU; CTR, control rats; GSSG, oxidised glutathione; PCA, perchoric acid; IAA, iodoacetate acid; FDNB, 1-Fluoro-2,4-dinitrobenzene; NEM, N-ethylmaleimide; gGCS-HS, heavy subunit of gamma-Glutamyl-Cysteine Synthetase; gGCS-LS, low subunit of gamma-Glutamyl-Cysteine Synthetase; CDO, Cysteine Dioxygenase; BSO, buthionine sulfoximine; MDA, malondialdehyde; TBA, thiobarbituric acid

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Glutathione (GSH) is the main cellular thiol participating in cellular redox reactions, and the liver is the main site of GSH synthesis [10]. Several studies have shown that GSH content is decreased in various organs of diabetic organisms [7,8,11]; this could be the cause and consequence of increased oxidative stress. In this report, we analysed whether dietary supplementation with N-acetylcysteine (NAC), a metabolic precursor of GSH possessing antioxidant properties in itself [12,13], and taurine (TAU), a betaineacid with detoxifying, antioxidative and antiglycative properties [14,15], were able to protect diabetic rats against diabetes-induced intracellular glutathione depletion, and if GSH metabolism was modulated by the treatment. The rationale to use this combined supplement was to combine the potential protective effects of the two substances, which have different properties.

2. Materials and methods

Eighteen male Sprague–Dawley rats aged 3 months were used. Six rats were used as controls (CTR group). Twelve rats were treated with streptozotocin (60 mg/kg body weight, i.p.) to render them diabetic; after the diabetes onset, six diabetic rats were left on a standard diet (Untreated Diabetic rats, UD group), while the other six diabetic rats were treated with NAC and TAU (0.12% and 0.8% in the drinking water, respectively) (N+T group) for the whole period of study (6 months); the estimated amount of antioxidant intake was 0.24–0.3 g NAC/die and 1.6–2 g TAU/die for each rat; the solution containing NAC and TAU given as drinking water to the rats was made fresh everyday.

All rats were maintained at 22–24 °C with a 12-h light/dark cycle; they had free access to food and water and were treated according to common procedures for good animal care. All rats were killed at 9 months of age while under anaesthesia with pentothal (65 mg/Kg).

Specimens of liver were immediately processed for GSH measurement. In order to calculate the oxidised to total glutathione ratio (GSSG/GSH) as precisely as possible, we implemented both Reed’s method [16], which enables total GSH to be evaluated, and Asensi’s method [17], which yields very reliable values for GSSG. Indeed, Reed’s method gives reliable results for total GSH, but it does not guarantee against in vitro casual GSH oxidation to GSSG, so that GSSG value can be artifically increased; on the other hand, Asensi’s method guarantees against GSH oxidation, but does not allow the evaluation of the reduced form of glutathione. Briefly, specimens for total GSH evaluation were homogenised and precipitated with perchloric acid (PCA) (10% final); thiol groups were blocked with iodoacetic acid (IAA) at alkaline pH; analytes were then converted to 2,4-dinitrophenyl derivatives with 1% 1-fluoro-2,4-dinitrobenzene (FDNB) at 4 °C in the dark overnight [16]. In the specimens used for GSSG evaluation, thiol groups were immediately blocked with N-ethylmaleimide (NEM) (20 mM) in PCA (6%); after precipitation and alkalisation, derivatization was performed with 1% FDNB [17].

Quantitative determination of derivatized analytes was performed in HPLC; the HPLC system was equipped with an NH2 Spherisorb column and a UV detector set at 365 nm; the flow rate was 1.5 ml/min. The mobile phase was maintained at 80% A (80% methanol) and 20% B (0.5 M sodium acetate in 64% methanol) for 5 min, followed by a 10-min linear gradient to 1% A and 99% B; the mobile phase was maintained at 99% B until GSSG eluted [16].

Total GSH content was evaluated in the chromatograms obtained with Reed’s method by adding GSH + 2GSSG, as suggested in [17], and expressed in GSH equivalents [10,18].

Expression of mRNA of Heme Oxygenase-1 (HO-1), of the heavy and low subunit of gamma-Glutamyl-Cysteine Synthetase (gGCS-HS and gGCS-LS, respectively) and of actin, as house-keeping gene, were evaluated in semi-quantitative RT-PCR. RNA was extracted with the RNeasy Midi Qiagen kit (Qiagen, S.p.A., Milan, Italy); 100 ng of RNA were reverse transcribed by MuLV Reverse Transcriptase (Perkin-Elmer) in the presence of polyadenylated oligonucleotides; aliquots of cDNA were then amplified with specific primers [19–21] which were synthesized by Tib Mol Biol (Genova, Italy) and Amplitaq DNA polymerase (Perkin-Elmer). Expression of Cysteine Dioxygenase (CDO) was similarly evaluated in RT-PCR; in this case, the primers were designed by reference to the sequence M35266 [22] of Cysteine Dioxygenase (Fw: 5′ CCT CTT GGT GCT GGT AAT GT 3′; Rv: 5′ CAG AAC TGG GAG CAA GG 3′).

The amplification products were run on 1.5% agarose gel, evidenced by ethidium bromide and analysed with the Gel Doc 2000 densitometer, (Milan, Italy) through the “Molecular Analyst” software (BioRad).

For Western blot (WB) analysis, liver tissue (150 mg) was homogenized in 1.5 ml cold phosphate buffer (5 mM, pH 7.4) containing 0.1% Triton X100 and protease inhibitor cocktail (Sigma-Aldrich, USA). The homogenate was clarified by centrifugation at 10,000 × g for 25 min at 4 °C. The supernatant was used for the determination of protein concentration (BCA, Pierce, USA) and for Western blotting for gGCS (heavy and light subunit), HO-1 and actin. Protein extracts were separated by SDS-PAGE in 6.5% (w/v) stacking and 10% (w/v) separation gels. Separated proteins were then transferred to a PVDF membrane (Amersham Biosciences, USA). After a 1-h blocking step with 5% milk in 20 mM TBS–0.05% Tween-20 (pH 7.5), the membranes were incubated for 1 h at room temperature with rabbit polyclonal antibodies (1:20,000) raised against rat gGCS heavy and light subunits, which were generously provided as a gift by Dr. T.J. Kavanagh (University of Washington, Seattle, USA) or with rabbit polyclonal antibodies (1:5000) raised against rat HO–1 (Stressgen Biotechnologies, USA). The membranes were washed three times, incubated for 1 h at room temperature with goat anti-rabbit IgG antibody conjugated
to horseradish peroxidase (Amersham Biosciences, USA) and again washed three times. Chemiluminescence was developed using ECL-plus substrate (Amersham Biosciences, USA) and quantification of chemiluminescence was performed with Gel Doc 2000 densitometer (BioRad, USA). To compare protein loading, membranes were reprobed with a mouse monoclonal anti-actin antibody (Sigma, Milan, Italy).

Malondialdehyde (MDA) was measured by a TBA (thiobarbituric acid)-test, performed on liver aliquots of 0.1 g according to [23]; the adduct was read in fluorescence at 530 nm ex/552 nm em. Protein carbonyl content in the liver was evaluated spectrophotometrically [24]. Plasma vitamin E was evaluated in HPLC [25]. Plasma sulfhydryl groups were assessed with Ellman’s reagent [26].

Statistical analysis included determination of mean, standard deviation and standard error of the mean (S.E.M.), ANOVA evaluation and Newman–Keuls Multiple Comparison post-Test. Data are expressed as mean ± S.E.M.; P values under 0.05 were considered significant.

3. Results

Rat growth was regular in the CTR group; at the end of the experimental period the weight of these rats was 632 ± 19 g. UD rats grew significantly less than CTR and reached at the end of the study a weight of 380 ± 19 g (P < 0.001 vs. CTR); NAC+TAU supplementation failed to protect from the diabetes-induced growth retardation: the rats of this group at the end of the study weighed 353 ± 24 g (P < 0.001 vs. CTR; not significant vs. UD group).

Total GSH content was 9.66 ± 0.77 microEquivalents/g of liver in control rats (CTR); untreated diabetic rats (UD) had a significantly lower content of total GSH (5.52 ± 0.29 microEquivalents/g of liver; P < 0.001 vs. CTR). Supplemented diabetic rats (N+T) had a total GSH content of 5.78 ± 0.53 microEquivalents/g of liver, which was not significantly different from the content of the UD, but was significantly lower than that seen in CTR (P < 0.001) (Fig. 1).

Liver GSSG content in CTR, UD and N+T rats was 52.2 ± 11.3, 129.5 ± 59.1, 60.6 ± 12.6 nmol GSSG/g of liver, respectively (Fig. 2); these data did not reach significance. Calculation of the GSSG/GSH percent ratio for each rat gave a similar pattern (Fig. 3).

Expression of HO-1 mRNA (Fig. 4) was significantly increased in UD compared to CTR (P < 0.01); N+T treatment induced a remarkable reduction of OH-1 mRNA expression (P < 0.05 vs. UD) up to a level not significantly different from CTR. The protein level of HO-1 did not differ significantly among groups (CTR: 34.3 ± 6.4; UD: 52.7 ± 8.9; N+T: 46.0 ± 5.4 Arbitrary Units), but showed a trend similar to the levels of the mRNA.

Expression of gGCS-HS mRNA did not differ among groups (Fig. 5A); on the contrary, expression of gGCS-LS mRNA was increased in UD in comparison with CTR (P < 0.05), and even more in N+T (P < 0.01); no significant difference existed between gGCS-LS mRNA expression in UD and N+T (Fig. 5B). The protein levels of gGCS-HS evaluated by WB did not differ among groups (Fig. 6A); the trend was anyway similar to that seen for the corresponding mRNA. The protein levels of gGCS-LS evaluated in WB was significantly increased in the N+T group in comparison with the other groups (Fig. 6B).
CDO mRNA expression was statistically decreased in both UD \((P<0.05)\) and N+T \((P<0.05)\) in comparison with CTR; no significant difference existed between CDO mRNA expression in UD and N+T (Fig. 7).

No significant differences among groups were detected as for MDA (CTR: 98.8±13.1; UD: 94.8±17.2; N+T: 104.9±13.7 pmol/mg protein) and protein carbonyl content (CTR: 1.69±0.16; UD: 1.37±0.15; N+T: 2.03±0.26 μmol/mg protein) in the liver.

No significant differences among groups were detected as for plasma vitamin E (CTR: 29.80±2.18; UD: 37.25±10.03; N+T: 26.0±1.89 nmol/ml) and plasma sulfhydryl...
groups (CTR: 517.2 ± 32.0; UD: 410.5 ± 113.5; N+T: 569.2 ± 113.9 \mu l).
cellular oxidative stress [39,40]; moreover, it is to be remembered that RT-PCR is more sensitive than WB. We suppose that the other oxidative indices tested (MDA, carboxyls, vitamin E, sulfhydryl groups) did not show difference among the groups because they may be markers less sensitive than HO-1 expression, or GSH content or oxidation.

We are not aware of any information on the combined effects of NAC+TAU on the levels or the oxidation balance of glutathione; however, it is conceivable that their association enhances the antioxidant defence of the organisms; in particular, it is unlikely that TAU renders NAC less available for GSH synthesis: on the contrary, since cysteine is a metabolic precursor of TAU [14], TAU supplementation should inhibit cysteine consumption for TAU synthesis and increase the availability of cysteine for GSH synthesis; this is consistent with the low CDO expression detected in N+T rats.

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