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ORIGINAL ARTICLE

Homocysteine-induced toxicity increases TG2 expression in Neuro2a cells

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Abstract High levels of homocysteine promote cell damage mainly through induction of oxidative stress, endoplasmic reticulum (ER) stress, and activation of pro-inflammatory factors. The effects of homocysteine were here examined in the continuously dividing neuroblastoma cell line Neuro2a. Cell treatment with homocysteine (100–500 μM) for 4 h increased ROS production while reducing cell viability in a dose-dependent manner. Cell exposure to 250 μM homocysteine was able to induce transglutaminase 2 up-regulation and increased in situ transglutaminase activity. These effects were prevented by the incubation with the transglutaminase activity inhibitor cystamine. Homocysteine also induced NF-κB activation that seemed associated with transglutaminase 2 up-regulation since the specific NF-κB inhibition by SN50 was able to reduce transglutaminase expression and activity levels. In the light of these observations, it may be postulated that TG2 up-regulation is involved in cell response to homocysteine-induced stress, in which NF-κB activation plays also a pivotal role.

Keywords Homocysteine · Transglutaminases · NF-κB · Neuro2a cells · ROS

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Introduction

Homocysteine (Hcy) is a non-protein sulfur containing amino acid derived from methionine metabolism in humans. High levels of Hcy contribute to neuronal cell damage in different neuropathological conditions, including Alzheimer's disease, age-related dementia, and impaired cognitive function (Mattson and Shea 2003; Boldyrev and Johnson 2007). Underlying mechanisms by which elevated Hcy promotes cell damage are oxidative stress, endoplasmic reticulum (ER) stress and activation of pro-inflammatory factors (Dickhout et al. 2007; Hernanz et al. 2007). Particularly, the effects on ER stress can be relevant to the relationship between excitotoxin-mediated mechanisms and protein alterations. The physiological roles of the ER include regulation of protein synthesis, folding and targeting, and maintenance of cellular calcium homeostasis. Thus, Hcy-induced misfolding or malfunctioning of numerous intracellular proteins are increasingly important and attract much attention because mechanisms underlying Hcy-evoked ER stress can explain many processes of cell injury.

Biological protein assembly is mediated not only by non-covalent interactions but also by enzyme-catalyzed covalent cross-linking. The most important group of cross-linking enzymes is the transglutaminase (TG) family. TGs are calcium dependent acyl-transferases that catalyze the reaction between the γ-carboxamide group of glutamine residues and the ε-amino group of lysine residues or polyamines. Transamidation increases the stability of the protein substrates, since the cross-linked protein polymers are resistant to mechanical stress and proteolytic degradation (Griffin et al. 2002).

The transglutaminase-catalyzed binding has been shown to be associated with intracellular inclusions of several

neurodegenerative disease-related proteins, suggesting that protein cross-linking is a general mechanism or common pathway involved in the pathophysiology of Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Muma 2007). Notably, increased TG2 expression and activity have been shown in all of these diseases (Ruan and Johnson 2007).

Using primary cultures of neuronal and astroglial cells, we previously demonstrated that increases in TG activity and TG2 expression may be associated to excitotoxic cell damage induced by glutamate exposure as well as excitotoxicity-related oxidative stress (Ientile et al. 2002; Campisi et al. 2004).

To ascertain the effects of Hcy on TG2 expression and activity we used in this study a continuously dividing cell line, such as the neuroblastoma Neuro2a that has been widely employed as a model to study neurotoxic effects. We also examined the occurrence of an activation of NF-κB pathway as a downstream Hcy effect, and evaluated its possible role in the regulation of TG2 expression.

Materials and methods

Materials

The mouse neuroblastoma cell line, Neuro2a (ATCC-CCL 131), was obtained from American Type Culture Collections (ATCC) (Rockville, MD, USA). Eagle's Minimum Essential Medium (MEM), cystamine, glutamine, and other chemicals of analytical grade were from Sigma (Milan, Italy). Foetal bovine serum (FBS) was from Invitrogen Life Technologies (Milan, Italy). 5-(biotinamido)pentylamine was purchased from Pierce (Celbio, Milan, Italy), while FITC-conjugated streptavidin was from Amersham Pharmacia Biotech (Milan, Italy). The NF-κB inhibitor SN50 was from Santacruz (DBA, Milan, Italy).

Cell culture and treatment

Neuro2a cells were grown in MEM, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin and 50 U/ml penicillin, at 37°C in a 5% CO_2/95% air humidified atmosphere. Cells were maintained for at least 2 weeks, with medium renewal every 2 days.

Subconfluent cells were first treated for 4 h with different concentrations of Hcy (10–500 μM) in order to test the effects on cell viability. Changes in cell viability were evaluated by the MTT reduction assay.

Subsequently, cell cultures were exposed to 250 μM Hcy for 4 h, in the presence or absence of a well known inhibitor of TG activity, cystamine, used at a concentration of 500 μM which does not significantly affect cell viability. In a subset of experiments cell cultures were also incubated with the NF-κB inhibitor SN50 (50 μg/ml), that was added to the culture medium 30 min prior to stimulation with Hcy.

Evaluation of reactive oxygen species production

Reactive oxygen species (ROS) production was measured by the evaluation of dichlorofuorescein (DCF) released after addition of 2′, 7′-dichlorodihydrofluorescein-diacetate (H2-DCFDA) to untreated and treated cell cultures as described by Campisi et al. (2004).

Analysis of TG expression

Semi-quantitative RT-PCR analysis of different TG isotypes, namely TG1, TG2 and TG3, and beta-actin, as well as Western blotting of TG2 and beta-actin were carried out as reported by Ientile et al. (2004).

In situ transglutaminase activity

Determination of in situ TG activity was performed by spectrophotometrical quantitation of 5-(biotinamido)pentylamine (BAPA) incorporation into cell proteins as described by Zhang et al. (1998).

The subcellular distribution of TG activity was analysed by confocal laser scanning microscopy after 2 mM BAPA addition to cell cultures 1 h prior to the end of Hcy treatment. Then, cell cultures were washed three times with PBS and incubated for 2 h with FITC-conjugated streptavidin (1:1000 in PBS).

Electrophoretic mobility shift assay (EMSA)

The presence of DNA binding activity by NF-κB in nuclear extracts of untreated and treated Neuro2a cells was evaluated by EMSA and supershift assay as described by Caccamo et al. (2005a).

Statistical analysis

All values are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA, followed by Newman–Keuls post hoc test.

Results

To evaluate Hcy-induced cell redox status alteration, Neuro2a cell cultures were exposed to different Hcy doses (10–500 μM). Hcy did not significantly alter ROS levels in Neuro2a cells when used in a concentration range of

10–100 μM. Higher Hcy concentrations, i.e. 250–500 μM, produced a two–threefold increase in ROS production, respectively, in comparison to untreated cells (Table 1). The MTT assay demonstrated that Hcy (100–500 μM) also reduced cell viability in a dose-dependent manner. The treatment with 250 μM Hcy decreased the number of viable cells by around 10% compared with controls, while 500 μM Hcy drastically reduced cell viability by 40% (Table 1). Thus, the subsequent experiments were carried out using 250 μM Hcy.

To determine which TG isoenzymes were expressed under these experimental conditions a qualitative RT-PCR analysis was performed, showing a very limited amount of TG2 in the control Neuro2a cells compared with Hcy-treated ones. The presence of mRNA transcripts for TG1 and TG3 was not detected. Notably, a significant TG2 up-regulation was observed in Hcy-exposed Neuro2a cells in comparison to controls (Fig. 1a). TG2 expression in Neuro2a cells was also evaluated by Western blotting. The densitometric analysis of immunoblots clearly indicated that Hcy treatment significantly increased TG2 protein levels compared with those observed in untreated cells (Fig. 1b).

Then, the effect of Hcy-induced stress on the in situ TG activity in Neuro2a cells was also evaluated. Consistent with the observed increases in TG2 expression, significant changes of in situ TG activity occurred in cell cultures treated with Hcy (250 μM) (Fig. 2). To further confirm these results, the in situ enzyme activity was determined in the presence of cystamine, a well known inhibitor of TG activity. The treatment with cystamine significantly reduced the

Table 1 Hcy effects on cell viability (MTT assay) and ROS production in neuroblastoma Neuro2a cells

	MTT assay (arbitrary units)	ROS production (relative DCF fluorescence)
Controls	100	100
Hcy 10 μM	115 ± 12	105 ± 11
100 μM	95 ± 10	155 ± 18
250 μM	90 ± 9	257 ± 28*
500 μM	60 ± 7*	300 ± 19*

Cell viability was assessed by the estimation of uptake and reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). Cell cultures were incubated with MTT (0.5 mg/ml in MEM) for 4 h after Hcy treatment. Then, blue formazan crystals, deriving from MTT reduction by mitochondrial succinate dehydrogenase, were solubilized in 100 μl SDS, 10% in HCl 0.01 N, at 37°C overnight. Absorbance was determined at 550 nm after background subtraction at 650 nm

ROS production was measured by DCF formation from H2-DCFDA as reported by Caccamo et al. (2005a). Fluorescence intensities were then measured (ex 488 nm, em 525 nm). Data represent the mean ± SE of values from three separate experiments. *$P < 0.01$ in comparison to controls

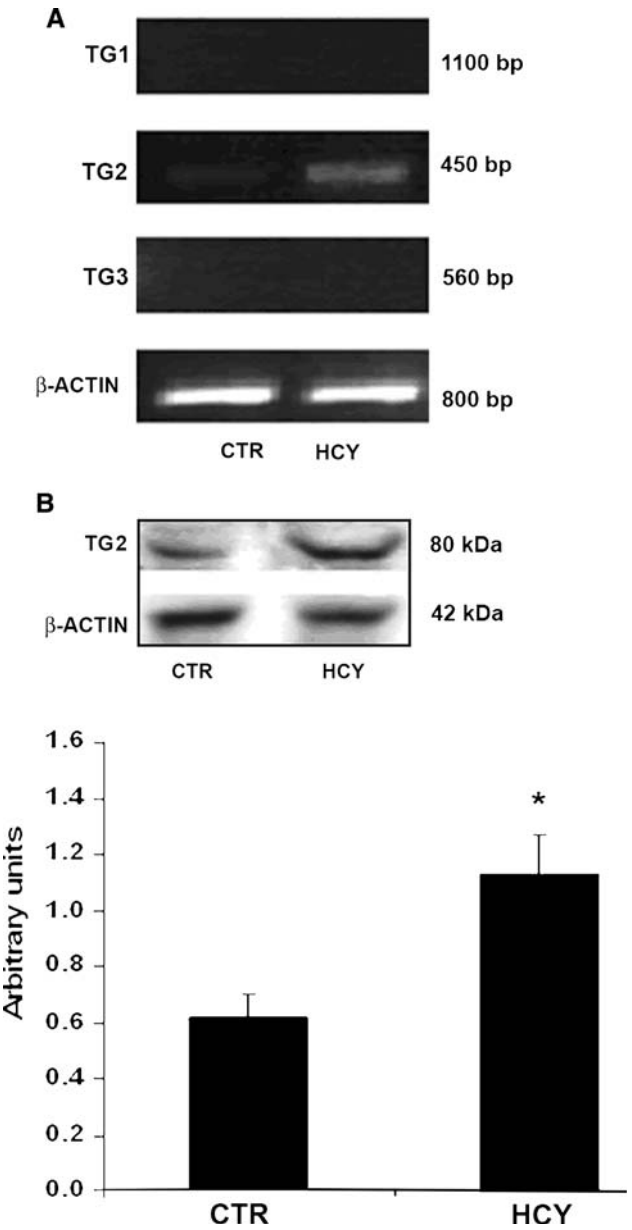

Fig. 1 Analysis of TG expression in untreated and Hcy-treated neuroblastoma Neuro2a cells. **a** Semi-quantitative RT-PCR analysis of mRNA transcripts for TG1, TG2, TG3, and beta-actin. Results of densitometric analysis, after normalisation against beta-actin levels, are mean ± SEM from three to five experiments. **b** Immunoblots of TG2 and beta-actin. Results of densitometric analysis, after normalisation against beta-actin levels, are mean ± SEM from three experiments. *$P < 0.05$ significant differences in comparison to control cells

Hcy-induced increase in TG activity (Fig. 2). The changes observed in living cells, likely due to increased intracellular calcium concentrations, indicate that activation of TG2 was dependent on Hcy-induced stress in Neuro2a cells.

We also demonstrated that increases in TG2 expression were associated to an increase in DNA binding activity by p50/p65 heterodimer NF-κB complexes in nuclear extracts

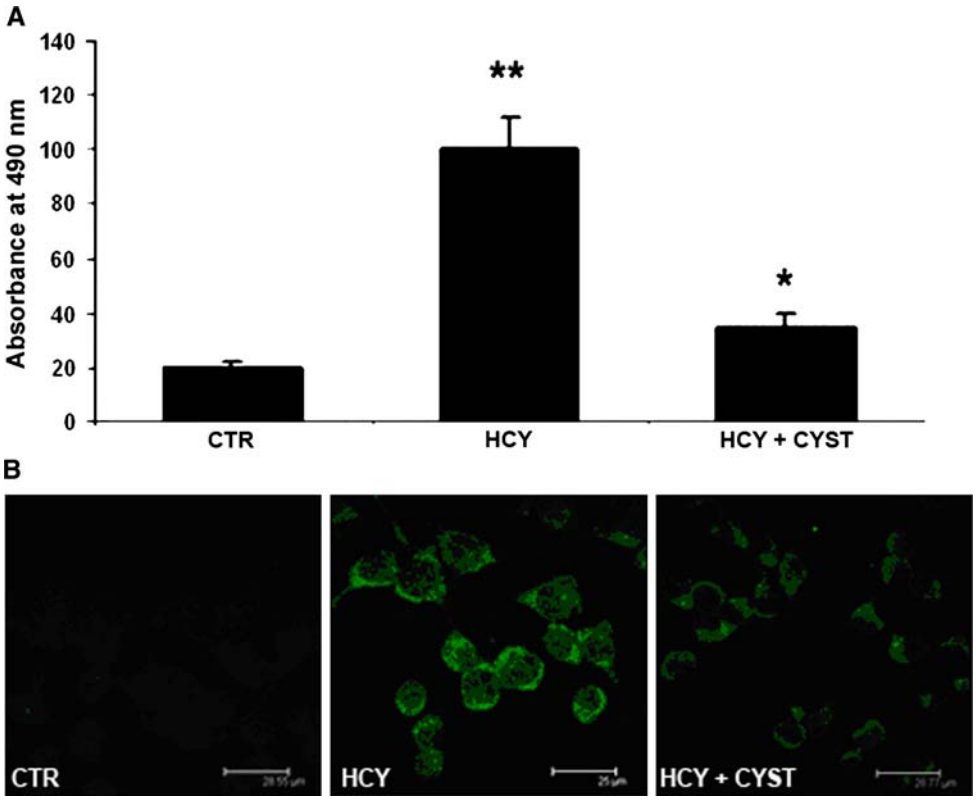

Fig. 2 Analysis of in situ TG activity in untreated and Hcy-treated Neuro2a cells in the presence or absence of the TG activity inhibitor cystamine. **a** Spectrophotometrical determination of BAPA incorporation into cell proteins. BAPA (2 mM) was added to cell cultures 1 h prior to the end of Hcy treatment. Then, BAPA fluorescence was quantitated by absorbance reading at 490 nm as described by Zhang et al. (1998). Results are mean ± SEM from three to five experiments. $*P < 0.05$ and $**P < 0.01$ significant differences in comparison to control cells. **b** Confocal laser scanning microscopy analysis of in situ TG activity distribution in untreated and Hcy-treated cells in the absence or presence of 500 μM cystamine. In situ TG activity was evaluated on the basis of 2 mM BAPA incorporation into protein substrates

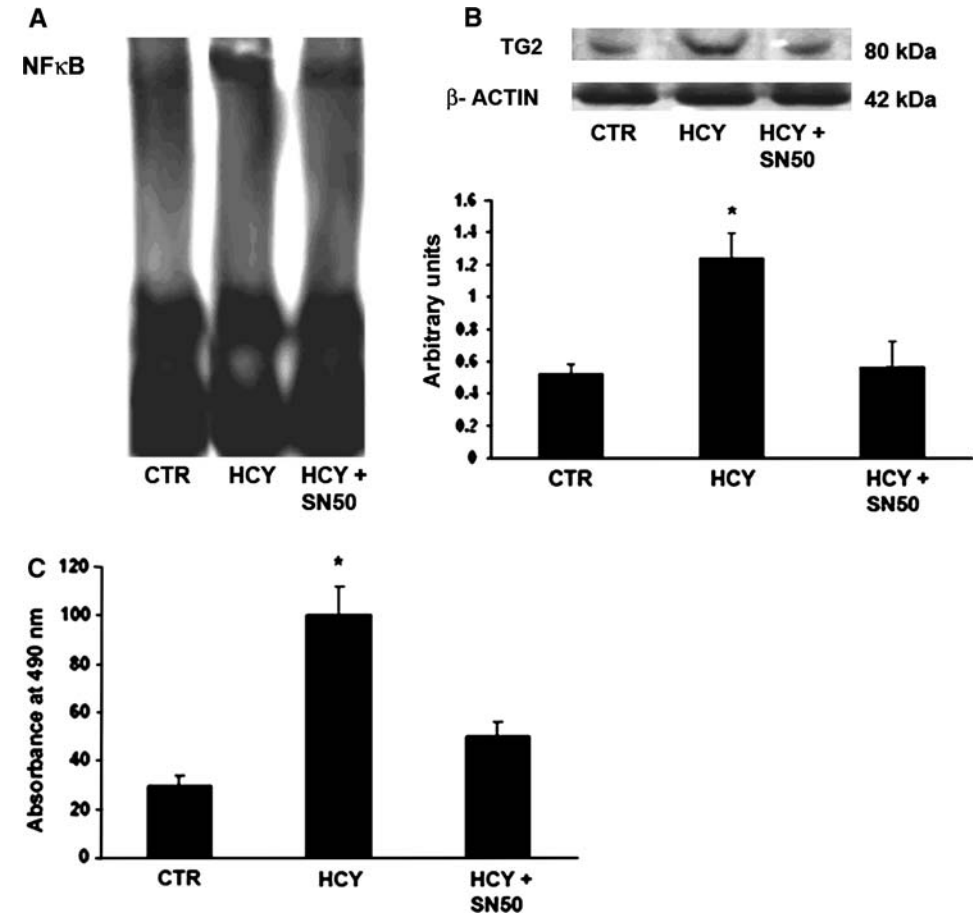

Fig. 3 NF-κB activation is associated with Hcy-induced TG2 up-regulation. **a** Analysis of NF-κB activation by EMSA. The presence of NF-κB DNA binding activity in Neuro2a cells exposed to 250 μM Hcy for 4 h in the presence or absence of the specific NF-κB inhibitor SN50, was evaluated by incubation of nuclear proteins (10 μg) with an oligonucleotide probe (1 pmol) containing the NF-κB recognition motif present in TGM2 promoter. SN50 was added to cell cultures 30 min prior to Hcy exposure. **b** Western blot analysis of TG2 expression in untreated and Hcy-treated cells in the presence or absence of SN50 (50 μg/ml). Results of densitometric analysis were normalised against beta-actin levels and are shown as means ± SEM from three experiments. **c** Reduction of in situ TG activity as an indirect effect of NF-κB inhibition by SN50. Results are mean ± SEM from three separate experiments. $*P < 0.05$ significant difference in comparison to control cells

of Hcy-treated neuroblastoma cells in comparison with untreated ones, where lower levels of active NF-κB complexes were found (Fig. 3a). Notably, when Neuro2a cells were incubated with the NF-κB inhibitor SN50 prior to Hcy exposure, a significant reduction of TG2 expression was observed (Fig. 3b). Further, when the cells were treated with SN50 prior to Hcy exposure a reduction in the TG activity was observed in comparison to untreated cells (Fig. 3c). These data suggest a direct relationship between Hcy-induced NF-κB activation and TG2 expression.

Discussion

Accumulating data demonstrate that Hcy may promote cell injury, thus contributing to development of neurodegenerative diseases. In vitro Hcy treatment induced ER stress, mitochondrial alterations and neuronal cell damage (Zieminska et al. 2006; Lenz et al. 2006).

In this study, we found that micromolar concentrations of Hcy produced an early increase in ROS production in neuroblastoma Neuro2a cells. Since the effects occurred in undifferentiated cells, Hcy-induced alterations appear to be not dependent on glutamate receptor activation. Under these conditions, Hcy exposure promoted TG2 up-regulation and increased the in situ TG activity. Moreover, these effects were associated with the activation of NF-κB, since the specific inhibition of NF-κB nuclear translocation by SN50 was able to reduce TG increases.

Recently, much attention has been focused on the potential role(s) played by TG2 in oxidative stress-induced cell death both under physiological and pathological conditions (Muma 2007). This is particularly important given the controversial functions ascribed to TG2 including a role in the apoptosis or necrosis (Nicholas et al. 2003; Piacentini et al. 2005) as well as its proposed role as intracellular survival protein (Fésus and Szondy 2005). However, the activation of NF-κB, one of the most studied transcription factors, has long been known to be involved in oxidative stress in numerous cell types (Gloire et al. 2006). Further, we previously demonstrated that excitoxicity-related oxidative stress activated the NF-κB pathway in primary cultures of astrocytes, and that increases in NF-κB levels were significantly reduced by the pre-incubation with different antioxidants (Caccamo et al. 2005a, b). Then, the concomitant NF-κB nuclear translocation and TG2 up-regulation may be part of biochemical mechanisms involved in the cell response to stress triggered by Hcy treatment.

Further, in this study the pre-incubation with the specific NF-κB inhibitor SN50 blocked NF-κB nuclear translocation and reduced Hcy-induced increases in TG2 expression in Neuro2a cells. On the basis of these results, one can suggest that the NF-κB activation plays a pivotal role in the molecular pathways leading to Hcy-evoked TG2 up-regulation.

In this context, it may be postulated that TG2 up-regulation is involved in the mechanisms and changes associated with Hcy-induced stress response. We suggest that TG-mediated cross-linking might be involved in the unfolded protein response associated with changes in Ca2+ levels and ER-altered functions following Hcy exposure. Nevertheless, despite the relevance of numerous experimental data suggesting a role for TG2 in several neurodegenerative diseases, further studies are needed to characterize the relationship between TG activity and multiprotein-aggregates leading to cytotoxic mechanisms. Research efforts towards the identification of proteins modified by TG2 under stress conditions will provide further information for the understanding of intracellular TG2 up-regulation.

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