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Glutamate-evoked redox state alterations are involved in tissue transglutaminase upregulation in primary astrocyte cultures

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Abstract The aim of this study was to evaluate the involvement of oxidative stress in glutamate-evoked transglutaminase (TGase) upregulation in astrocyte cultures (14 DIV). A 24 h exposure to glutamate caused a dose-dependent depletion of glutathione intracellular content and increased the ROS production in cell cultures. These effects were receptor-mediated, as demonstrated by inhibition with GYKI 52466. The pre-incubation with glutathione ethyl ester or cysteamine recovered oxidative status and was effective in significantly reducing glutamate-increased tissue TGase. These data suggest that tissue TGase upregulation may be part of a biochemical response to oxidative stress induced by a prolonged exposure of astrocyte cultures to glutamate.

Keywords: Glutamate; ROS production; Primary astrocyte culture; Tissue transglutaminase; Glutathione; Antioxidant

1. Introduction

Transglutaminases (TGases) are a family of calcium-dependent enzymes that catalyze an acyl transfer reaction between peptide-bound glutamine residues and primary amines. This in turn can result in the ε-(γ-glutamyl) lysine cross-linking of proteins or their poly-amination, with consequent protein conformational changes. Several findings have demonstrated that the upregulation of tissue type of TGases (tTG) was involved in different pathological conditions associated with neurodegenerative diseases [1–3]. However, a possible role for the high levels of enzyme activity in cell damage, or the proposed implication in the tissue response to external injury, is not yet clear. Although it has been reported that the transamidating activity of tTG is regulated by several factors, including Ca^{2+}, GTP, retinoids, nitric oxide, and sphingosylphosphocholine [4–7], the molecular mechanism underlying neuronal cell damage has not been completely understood.

Previously, we demonstrated that glutamate receptor-mediated events upregulated tTG in cerebellar granule cells as well as in cultured astrocytes [8,9]. Elevated extracellular glutamate levels have been shown to affect neuronal functions, mainly by activation of specific ionotropic and metabotropic receptors, and have been demonstrated to be involved in the neuronal loss associated with many neurological diseases [10,11]. Additionally, increasing evidence suggests a gliotoxic action of extracellular glutamate for astroglial cells. In particular, it has been reported that, in differentiated astrocytes, a brief exposure to glutamate causes cell swelling, whereas a prolonged incubation induces cell damage, resulting from calcium overload and reactive oxygen species (ROS) production [12–14]. The mechanisms of this toxicity involve alterations in glutamate transport, glutathione depletion, and macromolecular synthesis [13]. Consequently, several calcium-dependent enzymes may be activated, causing mitochondria impairment, decrease in ATP levels, and subsequent neuronal cell death [12,15]. Recently, it has been demonstrated, in Swiss 3T3 fibroblasts, that intracellular ROS were essential for the activation of tTG by both lysophosphatidic acid and TGF-β, and this effect was inhibited by the ROS scavengers [16]. Conversely, there is substantial evidence that a number of agents, scavenging ROS and improving mitochondrial function, ameliorate cell death in various models of neuronal damage [17–19]. The present study was therefore aimed to investigate the involvement of oxidative stress in the expression of glutamate-evoked tTG in primary astrocyte cultures.

2. Materials and methods

2.1. Materials

Cell culture medium and sera, TRIzol for RNA extraction and Thermoscript RT-PCR system for cDNA synthesis, were from Invitrogen (Milano, Italy). Aprotinin, leupeptin, pepstatin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), glutathione reduced (GSH) ethyl ester, cysteamine–HCl, and other chemicals of analytical grade were obtained from Sigma (Milano, Italy). 1-[4-Aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) was from Research Biochemicals Inc. (Natick, MA). RT-PCR specific primers for different rat TGase isoforms were synthesized by

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Abbreviations: GSH/GSSG, reduced/oxidised glutathione; GYKI 52466, 1-[4-aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; TGase, transglutaminase; tTG, tissue transglutaminase

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Primm (Milano, Italy). Monoclonal tTG antibody CUB 7402 was from LabVision Corp. (Fremont, CA). Horseradish peroxidase (HRP)-conjugated secondary antibody was from Amersham Pharmacia Biotech (Milano, Italy).

2.2. Cell cultures

Primary cultures of astrocytes were prepared from newborn albino rat brains (1–2-day-old Wistar strain) and checked for purity as previously described [9]. Cerebral tissues, after dissection and careful removal of the meninges, were mechanically dissociated through sterile meshes of 82-μm pore size (Nitex). Isolated cells were suspended in Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml), and plated at a density of 3×10^6 cells/100-mm dish and of 0.5×10^5 cells/chamber of multichambered slides. Cells were maintained at 37 °C in a 5% CO_2/95% air humidified atmosphere for two weeks and medium exchanged every three days. All efforts were made to minimize both the suffering and number of animals used. All experiments conformed to the guidelines of the Ethical Committee of the University of Messina, Italy.

2.3. Glutamate treatment

Astrocytes at 14 days in vitro (DIV) were treated with glutamate (10–500 μM) for 24 h as previously described [9]. In a subset of experiments to assess the inhibition of glutamate effects, cell cultures were pre-incubated with either the specific AMPA/KA receptor antagonist, GYKI 52466 (100 μM), or GSH ethyl ester (50–100 μM), or cysteamine–HCl (100–500 μM), which were added 30 min before glutamate exposure. Four replicates were carried out for each sample.

2.4. Cytotoxicity assessment

Astrocyte survival analysis was performed by MTT reduction assay, evaluating mitochondrial dehydrogenase activity. In brief, the culture medium was removed and replaced with MTT solution. After incubation at 37 °C for 30 min, the MTT solution was removed and blue purple formazan crystals dissolved in dimethylsulfoxide for colorimetric quantification at 570 nm by using a plate reader (Sunrise, Tecan Italia, Cologno Monzese, Italy).

Lactate dehydrogenase (LDH) release was also tested to assess necrotic cell death as described by Chen et al. [13].

2.5. Glutathione measurement

Cells were scraped off and lysed in 50 μM sodium phosphate buffer, pH 7.4. The protein concentration in cell extracts was determined by Bradford assay (Bio-Rad, Richmond, CA). Then, total glutathione intracellular content (GSH + GSSG) was chemically determined according to Chen et al. [13].

2.6. Evaluation of intracellular peroxide concentrations

To evaluate production of ROS, astrocyte cultures were treated with 2′,7′-dichlorofluorescein diacetate for 20 min, rinsed with serum-free medium, and intracellular peroxide levels were measured for individual cells in multiple cultures under fluorescein optics, as previously described [16].

2.7. RT-PCR

Total RNA was isolated using TRIzol and reverse-transcribed using the Thermoscript RT-PCR kit according to the manufacturer's instructions. cDNA was amplified for 35 cycles with specific primers for rat TGase 1, tTG, TGase 3, and β-actin (as reaction internal control), using a Hybaid PCR Sprint thermocycler (Celbio; Milano, Italy). RT-PCR products were separated on 2% agarose gel and quantified by densitometric analysis with an AlphaImager 1200 System (Alpha Innotech, San Leandro, CA). Amplified cDNAs were directly sequenced and sequences matched with known cDNA sequences of rat TGases.

2.8. Western blotting

Analysis of tTG expression in glutamate-exposed astrocytes, in the presence or absence of externally added GSH ethyl ester (50–100 μM) or cysteamine–HCl (100–500 μM), was performed with specific tTG mouse antibody, CUB 7402, as reported by Ientile et al. [8]. Immunoblots were scanned and quantified by densitometric analysis with AlphaImager 1200 System.

2.9. Statistical analysis

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

3. Results

First, we evaluated the glutamate-evoked oxidative stress by measuring the depletion of intracellular GSH levels. Glutamate (100–500 μM) produced a dose-dependent decrease in the intracellular GSH levels below control levels. In particular, the average GSH content was 13.8 ± 2.2 nmol/mg of protein in control cultures. The GSH dropped to 73.6% and 65.6% of the control, after a 24 h exposure to 100 μM glutamate and 500 μM glutamate, respectively (Fig. 1A). Consistent with previous observations [9], the 24 h exposure of 14 DIV cultured astroglial cells to glutamate (100–500 μM) did not significantly affect cell functions and viability, as revealed by the MTT reduction assay and LDH release measurement (data not shown).

The pre-incubation with GYKI 52466, the selective inhibitor of KA/AMPA receptors, diminished glutamate effects, indicating the involvement of receptor-linked events in glutathione decrease (Fig. 1B). Oxidative stress levels produced by

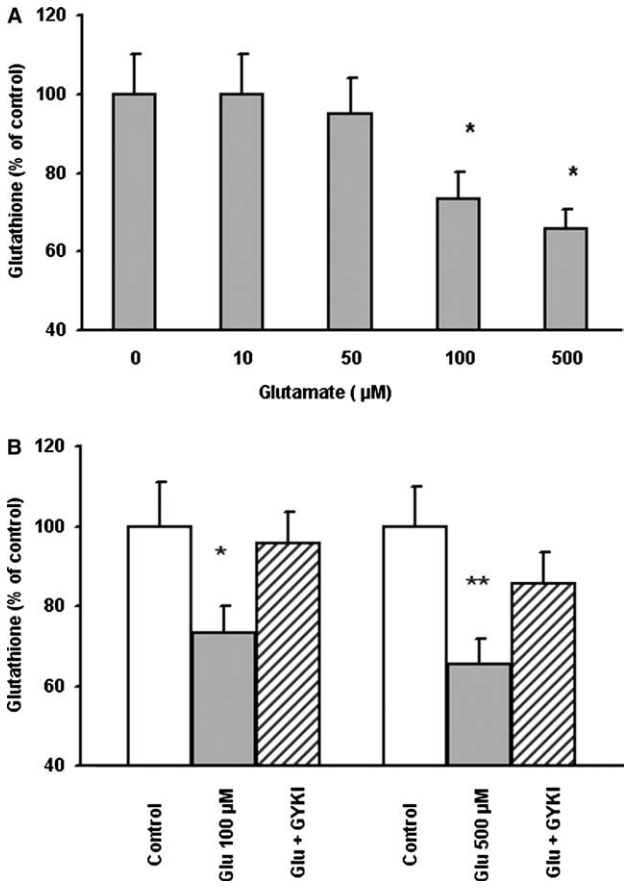

Fig. 1. Evaluation of intracellular GSH content in primary cultures of astrocytes (14 DIV) exposed to glutamate. Cell cultures were incubated for 24 h with glutamate (10–500 μM) (A); in presence or absence of AMPA/KA receptor antagonist, GYKI 52466 (100 μM) (B); then, GSH intracellular content was measured by a colorimetric assay as reported in Section 2. * $P < 0.05$, ** $P < 0.01$ significant differences compared with the control.

glutamate exposure were also evaluated by measuring intracellular ROS production. Glutamate produced a significant increase (33%) of intracellular ROS in comparison with untreated control cultures (Fig. 2).

To directly assess the involvement of oxidative stress in the expression of different TGase isoforms in astroglial cells, we evaluated the effects of the most effective concentration of glutamate (500 μM), in the absence or presence of either GSH ethyl ester or cysteamine–HCl, the latter able to increase GSH content. Under our experimental conditions, tTG mRNA transcript levels were strongly increased by 500 μM glutamate in comparison to controls. Conversely, TGase 1 mRNA levels were low in control cultures and were not significantly affected in glutamate-treated astrocytes (Fig. 3). No evidence was obtained for the presence of TGase 3 transcript.

The addition of GSH ethyl ester (50–100 μM) or cysteamine–HCl (100–500 μM), 30 min before glutamate exposure, was able to reduce the increase in tTG expression induced by glutamate. In particular, the most significant effects were observed in the presence of 100 μM GSH ethyl ester or 500 μM cysteamine–HCl. A partial inhibition of glutamate-induced tTG upregulation was also obtained by pre-incubating astrocyte cultures with GYKI 52466 (100 μM), the selective antagonist of AMPA/KA receptors (Fig. 3).

Western blot analysis of tTG expression confirmed that this was upregulated by glutamate injury, and that GSH ethyl ester, in a concentration range 50–100 μM, reduced glutamate-evoked increase in tTG. Analogous effect was obtained by pre-incubation with cysteamine–HCl, which, at 500 μM concentration, was able to counteract glutamate-dependent increase in tTG (Fig. 4).

The pre-incubation of primary astrocyte cultures either with the membrane permeant GSH delivery agent, GSH ethyl ester, or with cysteamine–HCl, was able to counteract glutamate effects on ROS production and intracellular GSH depletion. In particular, both compounds significantly reduced glutamate-dependent ROS increase, but only GSH ethyl ester (100 μM) was able to restore ROS basal levels (Fig. 5A). However, GSH ethyl ester (100 μM) or cysteamine–HCl (500 μM), in a similar manner, almost completely prevented glutamate-induced GSH depletion (Fig. 5B).

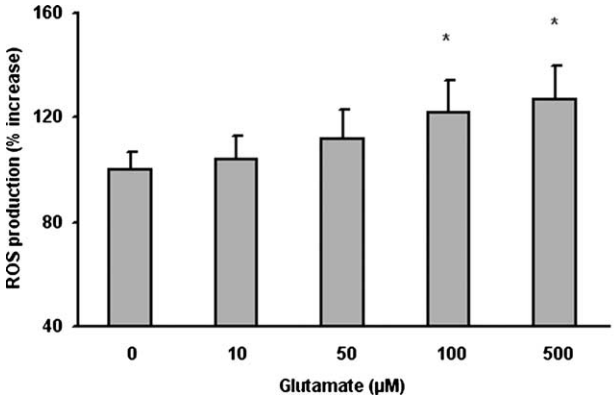

Fig. 2. Evaluation of ROS production in primary cultures of astrocytes (14 DIV) exposed to glutamate. Cell cultures were incubated for 24 h with glutamate (10–500 μM); then, changes in ROS production were evaluated by flow cytometric analysis, after treatment with DCFD. * $P < 0.05$ significant differences compared with the cultures in the absence of glutamate.

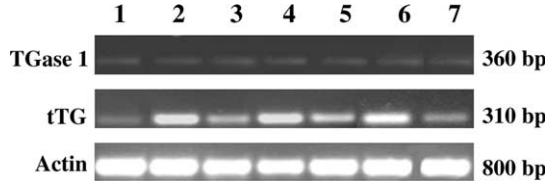

Fig. 3. Analysis of glutamate effects on TGase expression in astrocytes. Cell cultures (14 DIV) were exposed to 500 μM glutamate for 24 h, in the presence or absence of either glutamate receptor inhibitor, or antioxidants. RT-PCR analysis of TGase 1, tTG and TGase 3 expression was carried out. After 35 cycles of amplification with specific primers, mRNA transcripts were found only for tTG and, in lower amounts, for TGase 1. *Lane 1*, control; *lane 2*, glutamate; *lane 3*, glutamate + GYKI 52466 (100 μM); *lane 4*, glutamate + cysteamine (100 μM); *lane 5*, glutamate + cysteamine (500 μM); *lane 6*, glutamate + GSH ethyl ester (50 μM); *lane 7*, glutamate + GSH ethyl ester (100 μM).

4. Discussion

The present data indicate that stimulation of cultured astrocytes with high concentrations of glutamate results in oxidative stress, as indicated by the reduction of GSH levels and overproduction of intracellular ROS. However, GSH depletion was not necessarily associated with alterations in cell viability, considering that glutamate at indicated doses, after 24 h exposure, did not produce cell damage. Indeed, the altered redox status is consistent with a transporter-mediated toxicity evoked by glutamate [12]. In accordance with other reports, it is possible to suggest that glutamate uptake-induced impairment of cystine/glutamate antiporter leads to depletion of GSH content and biochemical alterations, resulting in the delayed toxic effect for primary astrocyte cultures [13,20].

The present results also extend previous observations on TGase expression in primary astrocytes treated with glutamate. In fact, we provided evidence that, under our conditions, different TGase isoforms are expressed, although a prolonged exposure to 500 μM glutamate was able to significantly affect only tTG protein expression. Indeed, the occurrence of a significant increase in tTG mRNA levels, more abundant than TGase 1, is in agreement with previous observations demonstrating that tTG upregulation was associated with in vitro as well as in vivo models of cell stress response [16,21]. The causative effect of oxidative stress on the rises in tTG expression in astrocyte cultures was confirmed by both inhibition of ROS production and intracellular GSH depletion through

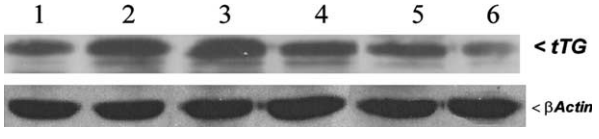

Fig. 4. Western blot analysis of tTG expression. Immunoblotting of tTG antigen was performed with specific monoclonal antibody. *Lane 1*, control; *lane 2*, glutamate (500 μM); *lane 3*, glutamate + cysteamine (100 μM); *lane 4*, glutamate + cysteamine (500 μM); *lane 5*, glutamate + GSH ethyl ester (50 μM); *lane 6*, glutamate + GSH ethyl ester (100 μM).

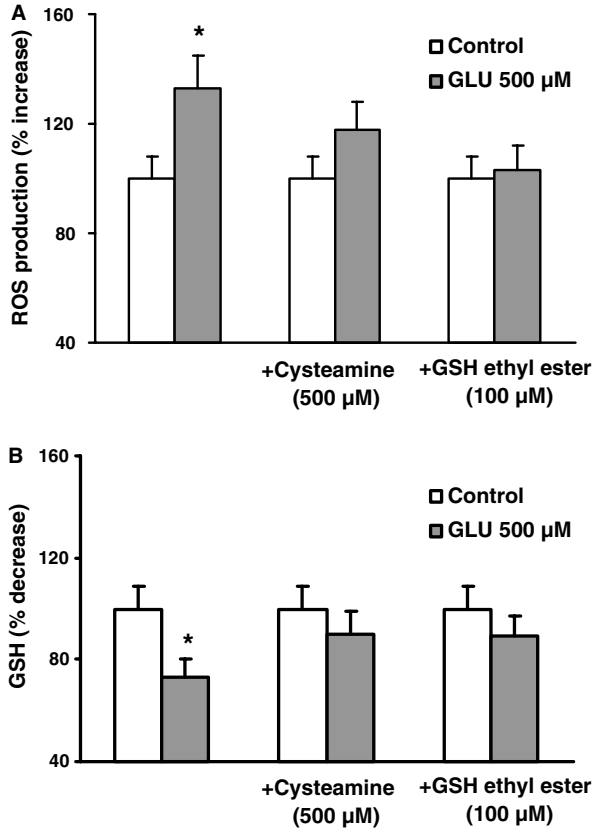

Fig. 5. (A) Evaluation of ROS production in primary cultures of astrocytes (14 DIV), exposed to glutamate in the presence or absence of externally added GSH ethyl ester or cysteamine–HCl. (B) Evaluation of intracellular GSH content in primary cultures of astrocytes (14 DIV), exposed to glutamate in the presence or absence of cysteamine–HCl or GSH ethyl ester. * $P < 0.05$ significant differences compared with the control.

GSH ethyl ester or cysteamine–HCl addition. In fact, the recovery of redox status, achieved by these agents, was accompanied by a concomitant, dose-dependent, reduction of glutamate-induced tTG upregulation. In this regard, the greater effect observed in the presence of GSH ethyl ester could reside in its more powerful action, than cysteamine–HCl, in restoring ROS control levels. It has been reported, in Swiss 3T3 fibroblasts, that ROS are involved in the in situ activation of TGase, and that intracellular Ca^{2+} may play an important role to mediate this effect [16]. Indeed, in astrocytes, alterations of glutamate transport, induced by prolonged exposure to glutamate, trigger increases in ROS production followed by increases in calcium-dependent enzymes. Further, these effects can be reverted by antioxidants, such as GSH ethyl ester that increases glutamate uptake and inhibits glutamate toxicity lessening cell damage. Considering the influence of GSH depletion or supplementation in the regulation of tTG expression, it is possible to propose that TGase reactions mediate early changes in astrocyte cultures induced by a prolonged exposure to glutamate. Indeed, several findings have demonstrated that GSH levels in the brain were lowered in cerebral ischemia [22,23] and that GSH depletion caused an enlargement of mitochondria in the brain [24]. Furthermore, an increasing body of evidence suggests that mitochondria alterations may be a key regulator of cell death [25] and mitochondria swelling has been observed in early brain damage [24,26]. In relation to this, it has been reported that alteration in mitochondrial functions significantly increases tTG activity [27], suggesting that tTG-dependent hyperpolarization of mitochondria can be considered as an important early event in the biochemical changes evoked by oxidative stress [28]. However, further experiments must be carried out to provide information on the regulatory mechanisms of TGase gene transcription evoked by oxidative stress.

A remarkable bulk of experimental data has clearly indicated that several neurodegenerative injuries are associated with the excessive activation of glutamate receptors [11,29,30], suggesting that pathways, other than excitotoxicity, may contribute to cell vulnerability through altered glutamate transport especially regarding astrocytes [31]. In various models of brain wounds to adult rats, the astrocyte response was characterized by increases in protein expression such as glial fibrillary acid protein and vimentin, and these alterations were typical features in brain tissue from patients with neurodegenerative diseases [32–34]. In this context, in several neuropathological conditions, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, TGase increases and the presence of cross-linked products may be directly related to cell damage and neuronal loss [2,35]. Considering that excitotoxicity and oxidative stress are interdependent phenomena, it is possible to hypothesize that TGase upregulation may be a component of biochemical response to oxidative stress triggered by glutamate receptor activation in brain tissue.

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