Aged garlic extract and S-allylcysteine increase the GLUT3 and GCLC expression levels in cerebral ischemia

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Abstract

Background. During cerebral ischemia, energy restoration through the regulation of glucose transporters and antioxidant defense mechanisms is essential to maintain cell viability. Antioxidant therapy has been considered effective to attenuate brain damage; moreover, the regulation of transcription factors that positively regulate the expression of glucose transporters is associated with this therapy. Recently, it has been reported that the use of antioxidants such as S-allylcysteine (SAC), a component of aged garlic extract (AGE), improves survival in experimental models of cerebral ischemia.

Objectives. The aim of this study was to determine the effect of AGE and SAC on the level of mRNA expression of the main neuronal glucose transporter (GLUT3) and the glutamate cysteine ligase catalytic subunit (GCLC) in rats with transient focal cerebral ischemia.

Material and methods. Cerebral ischemia was induced in male Wistar rats by middle cerebral artery occlusion (MCAO) for 2 h. The animals were sacrificed after different reperfusion times (0–48 h). Animals injected with AGE (360 mg/kg, intraperitoneally (i.p.)) and SAC (300 mg/kg, i.p.) at the beginning of reperfusion were sacrificed after 2 h. The mRNA expression level was analyzed in the fronto-parietal cortex using quantitative polymerase chain reaction (qPCR).

Results. Two major increases in GLUT3 expression at 1 h and 24 h of reperfusion were found. Both treatments increased GLUT3 and GCLC mRNA levels in control and under ischemic/reperfusion injury animals.

Conclusions. This data suggests that SAC and AGE might induce neuroprotection, while controlling reactive oxygen species (ROS) levels, as indicated by the increase in GCLC expression, and regulating the energy content of the cell by increasing glucose transport mediated by GLUT3.

Key words: antioxidants, cerebral ischemia, glucose transporters

Damage induced by cerebral ischemia and restoration of blood flow (reperfusion) has been linked to mitochondrial dysfunction and the concomitant production of noxious levels of reactive oxygen species (ROS).[1] Reactive oxygen species are released during mitochondrial respiration, causing tissue injury and cellular dysfunction.[2,3] Therefore, the reduction of ROS by antioxidants has been considered as a potential therapeutic method that, through the regulation of the cellular redox state, might ameliorate ischemic injury.[4–6]

Interestingly, antioxidant agents activate the hypoxia inducible factor-1α (HIF-1α) in cultured brain endothelial cells, astrocytes and neurons subjected to normoxia, hypoxia or ischemia.[7–9] This factor increases anaerobic glycolysis through the upregulation of glucose transporters (GLUTs), which possess hypoxia response elements (HRE) in their promoters,[10,11] and this upregulation facilitates cell survival by maintaining adenosine triphosphate (ATP) production.[9,12] Glucose transporters are essential for an adequate supply of energy in neurons, principally under conditions where the energy demand is high as occurs in hypoxia and brain ischemia.[13] The glucose transporter 3 (GLUT3) is the main transporter in neurons with the highest affinity for glucose (K_m 1.4 mmol/L),[10,11] its expression increases at the onset of cerebral ischemia, and its subsequent decline is followed by neuronal death.[14] On the other hand, treatment with antioxidant agents also increases expression levels of proteins involved in cellular redox regulation.[9,15] The glutamate cysteine ligase catalytic subunit (GCLC) is an enzyme that participates in the production of glutathione, an important endogenous antioxidant peptide.[16] The glutamate cysteine ligase catalytic subunit is increased in brain slices subjected to oxygen-glucose deprivation,[17] and in vitro models of chemical hypoxia-induced neurotoxicity.[18] Therefore, regulation of GLUT and GCLC are 2 potential mechanisms by which antioxidants might prevent the deleterious effects of ROS in cerebral ischemia.

In line, some antioxidant agents, such as aged garlic extract (AGE), and its main active component, S-allylcysteine (SAC), have been shown to prevent brain cell death and to improve neurologic deficit induced in experimental models of cerebral ischemia/reperfusion.[5,6,19,20] The effects of AGE and SAC in brain ischemia have been associated with their high antioxidant potential; nevertheless, their complete mechanism of action is not well understood. We assume that these antioxidants act in association with the regulation of GLUT3 expression to increase the entrance of glucose to the cell to augment ATP concentration, but are also associated with the regulation of GCLC mRNA to achieve a reduced cellular redox state, which is characterized by a high glutathione concentration. Therefore, we have determined the temporal expression patterns of GLUT3 induced by cerebral ischemia/reperfusion and the possible changes following reperfusion in the presence of AGE and SAC. We also have evaluated the effect of these antioxidants on GCLC expression in our model of cerebral ischemia/reperfusion. Our purpose was to identify a possible mechanism by which antioxidants participate in neuroprotection in the middle cerebral artery occlusion (MCAO) model of ischemia/reperfusion.

Material and methods

Reagents

Aged garlic extract Kyolic® was obtained from Wakunaga of America Co., Ltd. (Mission Viejo, USA), and SAC was synthesized by the reaction of L-cysteine with allylbromide and purified by recrystallization from ethanol-water, according to a previous report.[21] TRIzol Reagent, SuperScript® III First Strand Synthesis SuperMix and ramdom hexamer primers were obtained from Invitrogen Life Technologies (Carlsbad, USA); commercial predesigned TaqMan Probes system was performed in a 7500 Real-time PCR System (Applied Biosystems, Foster City, USA) using specific assay GLUT3 (Rn00567331_M1) and GCLC (Rn00563101_M1) (Applied Biosystem). 18S ribosomal RNA (18S rRNA, assay ID: Hs99999901_s1, Applied Biosystems). All other reagents were obtained from known commercial sources.

Experimental animals

Adult male Wistar rats weighing 280–320 g were included in this study. The mRNA expression encoding for GLUT3 and GCLC was studied in 3–8 animals per group. Nine animal groups were subjected to transient focal cerebral ischemia induced by MCAO during 2 h,[22] and then were sacrificed by decapitation after different times (0, 1, 2, 3, 4, 6, 10, 24, and 48 h) of reperfusion (n = 4). Briefly, animals were anaesthetized with isoflurane (2.5–3.0%). The left common carotid artery was exposed at the level of the external and internal carotid artery bifurcation. A 3-0 nylon monofilament was inserted into the external carotid artery and advanced into the internal carotid artery at a depth of about 17 mm to block the origin of the middle cerebral artery (MCA). Body temperature was kept at 37 ±0.5°C during the procedure. Two hours after the induction of ischemia, the filament was removed to allow reperfusion. The animals were returned to their cages and monitored until they recovered from anesthesia. Neurological deficit was determined 30 min before reperfusion and was scored on a 2-point scale: failure to extend right paw fully = 1; circling to right (more than 5 turns over a period of 30 s) = 1. Animals that presented neurological deficits <2 were exclude from the study.[5,22] An animal control group was subjected to surgery without MCAO. Another 3 control groups were injected intraperitoneally (i.p.) with a single dose of the antioxidant agents, AGE (360 mg/kg), SAC (300 mg/kg) or with their vehicles consisting in 20% ethanol in sterile water (control group). Finally, the last 3 animal groups were subjected to MCAO,

and then received a single i.p. injection of one of the above antioxidant agents at the beginning of reperfusion. All animal groups administered with the antioxidant agents were sacrificed by decapitation 2 h after the treatment injection. Experimental procedures were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, and with the ethical guidelines established by the National Institute of Neurology and Neurosurgery "Manuel Velasco Suárez" in Mexico City, Mexico (approved project No. 20/11 (624)).

Quantitative polymerase chain reaction

Total RNA extraction was performed on the whole fronto-parietal cortex, using TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions, and the amount and purity were determined using a spectrophotometer. cDNA was synthesized from 5 µg total RNA, using the kit SuperScript® III First Strand Synthesis SuperMix (Invitrogen Life Technologies). Quantitative polymerase chain reaction (qPCR) was performed in a 7500 Real-time PCR System (Applied Biosystems), using specific assay GLUT3 (Rn00567331_M1) and GCLC (Rn00563101_M1) (Applied Biosystem). 18S ribosomal RNA (18S rRNA, assay ID: Hs99999901_s1, Applied Biosystems) was used as a control to normalize the relative mRNA amount of the amplified genes. Reactions were done in triplicate and consisted of a denaturation cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and of annealing/extension at 60°C for 1 min. Values of cycle threshold (Ct) were determined through automated threshold analysis using SDS v. 1.3.1. software (Applied Biosystems, Waltham, USA). This software uses the comparative Ct method of relative quantification to determine relative gene expression levels.

Statistical analysis

Data analysis was performed using SPSS v. 13.0 (SPSS Inc., Chicago, USA) statistical software package. The results are presented as mean and standard deviation (mean ±SD). The statistical significance of differences between groups was analyzed using analysis of variance (ANOVA) and post hoc Tukey analysis was used for statistical evaluations. A value of $p < 0.05$ was considered significant.

Results

Effects of cerebral ischemia/reperfusion on GLUT3 mRNA expression levels in fronto-parietal cortex

Considering that major alterations in cerebral glucose concentration during ischemic injury have been reported,[23–25] temporal expression of glucose transporters induced by cerebral ischemia/reperfusion in the fronto-parietal cortex was evaluated. Figure 1 shows that focal cerebral ischemia (MCAO group) increased the GLUT3 mRNA expression level by 224% compared with control rats (1.41 ±0.24-fold; $p < 0.0001$). In the case of animals subjected to 2 h of MCAO followed by different times of reperfusion, 2 major increases in the GLUT3 mRNA expression were observed. After 1 h of reperfusion, the GLUT3 mRNA expression level remained elevated and reached 31% (0.8 ±0.08-fold) more than in the MCAO group ($p < 0.0001$). After 2, 3 and 4 h of reperfusion, the GLUT3 expression level was reduced to 52.4%, 67.7%, and 50%, respectively (2 h reperfusion = –1.21 ±0.08-fold; 3 h of reperfusion = –0.82 ±0.17-fold; 4 h of reperfusion = –1.27 ±0.17-fold; reperfusion group vs MCAO, $p < 0.0001$) reaching the baseline levels of expression (control group). After 6 and 10 h of reperfusion, the levels of GLUT3 were even lower that baseline reaching 22.1% and 14.6% (6 h of reperfusion = –1.98 ±0.01-fold; 10 h of reperfusion = –2.17 ±0.05-fold; reperfusion group vs MCAO, $p < 0.0001$). A more pronounced increase was found after 24 h of reperfusion, when the GLUT3 mRNA expression level was increased to 163% (1.62 ±0.26, 24 h of reperfusion vs MCAO $p < 0.0001$). Finally, after 48 h of reperfusion, GLUT3 mRNA decreased again 33% compared to the MCAO group (–1.68 ±0.14, $p < 0.0001$).

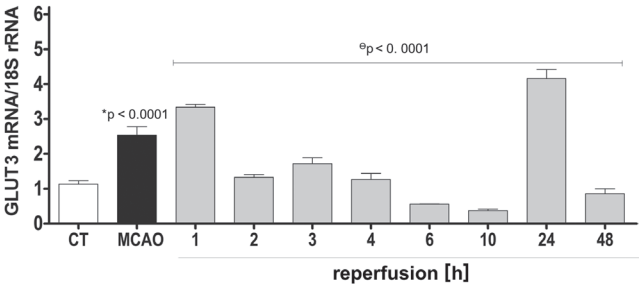

Fig. 1. Effect of cerebral ischemia/reperfusion on GLUT3 mRNA expression level in fronto-parietal cortex. Rats were subjected to middle cerebral artery occlusion (MCAO) for 2 h, and were, thereafter, sacrificed at different times of reperfusion: 0, 1, 2, 3, 4, 6, 10, 24, and 48 h. cDNA was synthesized using 5 µg of total RNA. The qPCR was performed to determine the expression level of GLUT3 mRNA, and ΔCt method was used to quantify the relative expression level. The changes in GLUT3 mRNA expression level (dark bars) are expressed as relative GLUT3 expression normalized to 18S ribosomal RNA. Results are the mean ±SD values of 4 animals included in each group. Statistical differences were calculated using ANOVA and post hoc Tukey analysis. Differences are indicated above each bar. *$p < 0.0001$ vs CT; $^{\theta}p < 0.0001$ vs MCAO

Effects of AGE and SAC on GLUT3 mRNA expression levels in cerebral ischemia/reperfusion

Recent evidence suggests a key role of GLUT3 as a potential therapeutic target in the treatment of cerebral ischemia/reperfusion.[26,27] Therefore, a possible effect of the AGE and its principal component SAC on GLUT3

Table 1. Single and combined effects of cerebral ischemia/reperfusion and antioxidant agents on GLUT-3 mRNA levels in brain cortex

Experimental condition	§ GLUT-3 levels	p-value between the indicated experimental conditions	
Control (a)	1.13 ±0.10		
MCAO/2R (b)	1.33 ±0.08	(b) vs (a) NS	
Aged garlic extract (c)	6.38 ±0.36	(c) vs (a) 0.00002	
Aged garlic extract + MCAO/2R (d)	4.43 ±0.38	(d) vs (a) 0.0002	(d) vs (b) 0.0002
SAC (e)	4.82 ±0.85	(e) vs (a) 0.002	
SAC + MCAO/2R (f)	3.51 ±0.59	(f) vs (a) 0.005	(f) vs (b) 0.008

§ main neuronal glucose transporter (GLUT3) levels are expressed in arbitrary units of GLUT-3 mRNA/18S rRNA
Results are the mean ±SD (n = 3–8).
Middle cerebral artery occlusion (MCAO)/2R – animals subjected to middle cerebral artery occlusion were sacrificed after 2 h of reperfusion.
NS – not statistically significant difference between the indicated experimental conditions.
Aged garlic extract (AGE) and S-allylcysteine (SAC) doses were 360 mg/kg and 300 mg/kg body wt, respectively.
AGE and SAC were administered at the beginning of reperfusion and animals were sacrificed after 2 h of reperfusion.

Table 2. Single and combined effects of cerebral ischemia/reperfusion and antioxidant agents on GCLC mRNA levels in brain cortex

Experimental condition	§ GCLC levels	p-value between the indicated experimental conditions	
Control (a)	1.00 ±0.03		
MCAO/2R (b)	1.09 ±0.13	(b) vs (a) NS	
Aged garlic extract (c)	3.06 ±0.38	(c) vs (a) 0.006	
Aged garlic extract + MCAO/2R (d)	2.92 ±0.45	(d) vs (a) 0.01	(d) vs (b) 0.02
SAC (e)	2.92 ±0.16	(e) vs (a) 0.001	
SAC + MCAO/2R (f)	2.25 ±0.22	(f) vs (a) 0.005	(f) vs (b) 0.02

§ glutamate cysteine ligase catalytic subunit (GCLC) levels are expressed in arbitrary units of GCLC mRNA/18S rRNA.
Results are the mean ±SD (n = 3–8).
Middle cerebral artery occlusion (MCAO)/2R – animals subjected to middle cerebral artery occlusion were sacrificed after 2 h of reperfusion.
NS – not statistically significant difference between the indicated experimental conditions.
Aged garlic extract (AGE) and S-allylcysteine (SAC) doses were 360 mg/kg and 300 mg/kg body wt, respectively.
AGE and SAC were administered at the beginning of reperfusion and animals were sacrificed after 2 h of reperfusion.

mRNA expression level in cerebral ischemia/reperfusion was tested here. Table 1 shows that both treatments increased the baseline expression of the mRNA encoding for GLUT3 (AGE and SAC groups). In the control rats, AGE increased GLUT3 mRNA expression level by 564.6% over basal conditions ($p < 0.0001$), while SAC increased the GLUT3 mRNA expression level by 426.5% ($p = 0.002$) (Table 1, row c and e vs a). On the other hand, AGE and SAC administration to the animals subjected to 2 h of ischemia and 2 h of reperfusion increased the GLUT3 mRNA expression level by 333% ($p = 0.0002$) in the MCAO/2R+AGE group and 263.9% in the MCAO/2R+SAC group (Table 1, row d and f vs b; $p = 0.008$).

Effects of AGE and SAC on GCLC mRNA expression levels in cerebral ischemia/reperfusion

Considering that oxidative stress generated during the ischemia/reperfusion process aggravates the damage, we evaluated the effect of AGE and SAC on GCLC mRNA expression level to determine a possible mechanism by which antioxidant molecules function as regulators in neuroprotection. Table 2 shows that both AGE and SAC administration induced a significant increase in the expression level of GCLC mRNA over control values in physiological conditions. The AGE treatment increased GCLC mRNA expression level by 306% ($p = 0.006$) in the control group + AGE, and by 292% ($p = 0.001$) in the control group + SAC (Table 2, row c and e vs a). In another observation, the increase induced by AGE and SAC on GCLC expression level was still present in animals subjected to brain ischemia, in the MCAO/2R+AGE group, the GLUT3 mRNA increased expression level 268% ($p = 0.02$), and 206% ($p = 0.02$) in the MCAO/2R+SAC group vs the MCAO/2R group (Table 2, row d and f vs b)

Discussion

The present study shows that in the fronto-parietal cortex dissected from animals exposed to transitory focal cerebral ischemia, the expression level of GLUT3 mRNA is increased after 2 h of injury and continued until 1 h of reperfusion period. We found that early transient increase in GLUT3 mRNA expression is presumably an acute response to brain ischemia, which could be explained by the activation of the HIF-1α, a potent inducer of GLUTs expression under hypoxia/ischemia situations. The HIF-1α mediates transcriptional regulation of glycolytic genes that possess hypoxia-response elements (HRE) in their promoters, including GLUT3.[11] During ischemia, deficiency of energy substrates induces the activation of cAMP response element-binding protein (CREB), which is also an important transcriptional factor of the GLUT3 gene.[10,27,28] Coupled to this, CREB is activated by the PI3K/Akt pathway,[29] a mechanism of neuroprotection in cerebral ischemia/reperfusion injury.[30]

In addition to neurons, it is possible that astrocytes contribute to the increase in GLUT3 expression. It has been demonstrated that in cultured astrocytes, under ischemic/reperfusion conditions, the ischemic stress increased

the expression levels of GLUT3, in order to enhance intracellular glucose storage during reperfusion, apparently as a protective mechanism against lethal ischemic stress.[27–31] Possibly, this transcriptional activation generates a prolonged protective effect. Interestingly, from 2 h to 10 h of reperfusion, the level GLUT3 decreased, and of 2 h to 4 h had normalized relative to the control, possibly through HIF inactivation, since HIF-1α under normoxic conditions is rapidly hydroxylated by prolyl hydroxylase and degraded.[32] Furthermore, during early reperfusion, the sudden increase in glucose might induce a decrease in GLUT expression, as a compensatory mechanism due to the excess supply of glucose that occurs with blood flow recovery.[27]

The second increase in GLUT3 mRNA expression observed at 24 h of reperfusion may be associated with the need for glucose to maintain the energy demands for cell repair, since during reperfusion there is an increase of ROS that induces damage and neuronal necrosis.[5] It has been shown that the increase in GLUT3 expression is related to increased glucose utilization.[10,14,25,27]

This data also shows that antioxidant agents such as SAC and possibly other components that have been identified in AGE are effective in enhancing GLUT3 and GCLC mRNA expression under ischemic and 2 h reperfusion. Previous studies have reported that AGE and SAC delays the appearance of neuronal damage and prevents cognitive impairments in some models of cerebral ischemia/reperfusion, possibly associated with their antioxidant potential.[4–6,19] Supporting this hypothesis in cultures of brain cells, some antioxidant agents increased the expression of GLUTs associated with a reduced redox state.[7–9] The increase of GLUT3 mRNA induced by AGE and SAC may be explained by the ability of some antioxidants to regulate signaling pathways. Antioxidants such as resveratrol induce Akt phosphorylation; this protein kinase is also involved in the activation of CREB.[33] Thus, it is likely that GLUT3 plays a key neuroprotective role in brain ischemia as an endogenous mechanism and as antioxidant's mechanism of action.

Comparison of the effect exerted by AGE and SAC on the mRNA baseline levels of GLUT3 shows that AGE is the most effective. The fact that AGE possesses a major effect suggests that components of the extract besides SAC are contributing to the outcome. Previously, we showed that AGE scavenges $O2^{\cdot-}$, $ONOO^{-}$, $OH^{\cdot}$, and $ROO^{\cdot}$.[34] Although several studies have suggested that SAC, the major compound in AGE, is the principal compound responsible for the inhibition of oxidative damage in the ischemic brain,[35] a wide variety of other potential antioxidant compounds present on AGE, such as sulfur compounds,[36] may be responsible for the upregulation of GLUT3 observed in our study.

Brian ischemia is known to induce oxidative stress that ultimately may lead to brain cell death. One strategy to slow the progression of brain damage is to prevent the formation and action of free radicals. To this end, maintenance of the reduced glutathione pool, which is the main intracellular antioxidant, is critical to cell survival during brain ischemic injury.[37] Interestingly, we found that AGE and SAC showed a positive inductive effect on the expression of the GCLC in the fronto-parietal cortex during focal cerebral ischemia and 2 h of reperfusion. This result could be explained by the activation of Nrf2, since it has been shown that some antioxidants increase the activity of the Nrf2 pathway in animal models of stroke and induce protection against ischemia injury.[2] Nrf2 activates a number of Nrf2-dependent genes encode antioxidant proteins, as GCLC and GCLM (glutamate-cysteine ligase), both enzymes involved in the synthesis of glutathione.[2,16,38]

Therefore, the present findings show the temporal course of GLUT3 mRNA expression in brain cortex during cerebral ischemia/reperfusion. This study also determined that the treatment with AGE and SAC increased the baseline expression of GLUT3, which may significantly account for their protective effect during brain ischemia. Furthermore, our results suggest that, in addition to the intrinsic antioxidant capability of AGE and SAC, the mechanism by which these antioxidants exert their neuroprotective effect may be by enhancing cellular antioxidant systems expression and facilitating the GLUT3 activity after an ischemic/reperfusion.

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