Inhibition of glutamate release by delaying ATP fall accounts for neuroprotective effects of antioxidants in experimental stroke

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ABSTRACT

Excitotoxic neuronal injury related to excessive glutamate release is believed to play a key role in the pathogenesis of focal cerebral ischemia. Reversal of neuronal glutamate transporters caused by ATP fall and subsequent imbalance of membrane ionic gradients accounts for most glutamate release after cerebral ischemia. ATP synthesis from oxidative phosphorylation derives from the coupled functioning of the mitochondrial respiratory chain (MRC) and the ATP synthase; interestingly, the MRC is one of the main sites of cellular reactive oxygen species (ROS) generation even in physiological circumstances. Hence, we have studied the effect of the antioxidants glutathione, superoxide dismutase, and α-tocopherol on infarct outcome, brain ATP, and glutamate levels after permanent middle cerebral artery occlusion (MCAO) in Fischer rats; we have also characterized the actions of antioxidants on MRC complexes. Our results show that intraperitoneal administration of antioxidants 2 h before MCAO enhances ATP synthesis and causes a neuroprotective effect concomitant to inhibition of ischemia-induced increase in brain glutamate. Antioxidants also increased mitochondrial ATP and MRC complex I–III activity and respiration, suggesting that these actions are due to removal of the inhibition caused by endogenous ROS on MRC. These findings may possess important therapeutic repercussions in the management of ischemic stroke.

Key words: cerebral infarct • MCAO • reactive oxygen species

Although the management of stroke has improved remarkably over the past decade due mainly to the advent of thrombolysis, most neuroprotective agents—although successful in animal studies—have failed in humans (reviewed in 1, 2). This emphasizes the need for new strategies with neuroprotective effects.

Glutamate, a major excitatory neurotransmitter, has long been recognized to play key roles in the pathophysiology of ischemia, due to its excessive accumulation in the extracellular space and the subsequent activation of its receptors, mainly the N-methyl-D-aspartate (NMDA) type of glutamate receptor (3–5). Indeed, most efforts to reduce ischemia-induced brain injury have
focused primarily on attenuating this excitotoxicity with several neuroprotective drugs that block glutamate receptors or inhibit glutamate release induced by brain ischemia (6). However, although some trials are still ongoing, the results from several completed trials have been disappointing (6, 7).

Regarding glutamate release as a possible target for neuroprotection, it has recently been shown that the reversed operation of neuronal glutamate transporters accounts for most glutamate release induced by severe ischemia (8, 9). This reversal occurs when ATP levels fall (10) after blood-flow cessation, an effect that brings about the alteration of membrane ionic gradients and the subsequent misfunction of glutamate transporters. In this context, using both in vitro and in vivo models of cerebral ischemia, we and others have found that strategies that prevent ATP fall are neuroprotective (11–19). Most ATP synthesis derives from mitochondrial oxidative phosphorylation: mitochondria use oxidizable substrates to produce NADH/FADH$_2$ that is used by the MRC to produce an electrochemical potential of protons; this potential is used to produce ATP from ADP and P$_i$ by the ATP synthase. Interestingly, the MRC is a major site of cellular free radical generation (20) and is therefore particularly susceptible to oxidative damage (21).

The present study was therefore designed to determine whether antioxidants may be neuroprotective by increasing ATP levels and, subsequently, by decreasing ischemia-induced glutamate release in a rat model of permanent middle cerebral artery occlusion (MCAO).

METHODS

Animals

Adult male Fischer rats weighing 250–275 g were used. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (following DC 86/609/EU). Rats were housed individually under standard conditions of temperature and humidity and a 12 h light/dark cycle (lights on at 08:00) with free access to food and water.

Experimental groups

Several groups were used for determinations of infarct size and neurological assessment, brain ATP, and brain glutamate concentrations: MCAO 2 h after an intraperitoneal (i.p.) injection of saline (MCAO; $n$=6) and MCAO 2 h after an i.p. injection of three different antioxidants, reduced glutathione 2 g/Kg (MCAO + GSH; $n$=4–6), the superoxide dismutase mimetic Mn (III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) 10 mg/Kg (MCAO + MnTBAP; $n$=4–6) and α-Tocopherol 100 mg/Kg (MCAO + tocopherol; $n$=4–6). Additional groups consisted of sham-operated animals 2 h after an i.p. injection of saline (SHAM; $n$=4–6) or antioxidants (SHAM + GSH, MnTBAP, or tocopherol; $n$=4–6). Injection volume was 1 ml/100 g body weight.

Middle cerebral artery occlusion (MCAO)

Permanent focal cerebral ischemia was induced by ligature of the left common carotid artery (CCA) and occlusion of the ipsilateral distal middle cerebral artery (MCA) as described previously (18). Rats in which the MCA was exposed but not occluded served as sham-operated controls (SHAM). Following surgery, subjects were returned to their cages and were allowed
free access to water and food. The body temperature of animals was monitored throughout the experiment and was maintained at 37.5 ± 0.5°C by using a heating pad.

**Brain extracellular glutamate levels**

Brain glutamate levels were determined by microdialysis as described (18). Briefly, rats were anesthetized with 2.5% halothane in a mixture of 70% nitrogen/30% oxygen and secured in a Kopf stereotaxic frame with the tooth bar at –3.3 mm below the interaural zero. Microdialysis guide cannula (CMA guide, Stockholm, Sweden) was implanted in the left striatum according to the following coordinates: +1.0 mm anteroposterior and +3.0 mm mediolateral to the bregma and –8.0 mm dorsoventral from the surface of the brain (22). The correct placement of the probes was verified by dye perfusion on test animals prior to proceeding with experimental groups. The microdialysis probe (CMA 12, Stockholm, Sweden) was lowered through the guide cannula. The day after cannulae implantation, probes were perfused with artificial CSF (KCl: 2.5 mM; NaCl: 125 mM; MgCl₂·6H₂O: 1.18 mM; CaCl₂·2H₂O: 1.26 mM; NaH₂PO₄·H₂O: 0.5 mM; Na₂HPO₄·2H₂O: 5 mM) at a rate of 1 µl/min. After a 60-min resting period, samples were collected every 30 min. Three basal samples were taken prior to MCAO to achieve steady baseline concentration of glutamate. These samples were averaged, and all subsequent values were expressed as a percentage of these basal pre-ischemic levels. The in vitro probe recovery of a solution containing 1 ng/µl of glutamate was 20.5 ± 1.5% (n=6). Dialysate samples were collected at the times indicated during 24 h and stored at –40°C until glutamate determination by HPLC as described (19).

**Infarct size**

The brains were removed 48 h after MCAO, and a series of 2 mm of coronal brain slices were obtained (Brain Matrix, WPI, UK) and stained in 1% TTC (2,3,5-triphenyl-tetrazolium chloride, Merck) in 0.1 M phosphate buffer. The infarcted area of each slice, which is not stained, was quantified by image analysis (Scion Image for Windows 2000, Scion Corporation, Frederick, MD) and expressed as total infarct volume.

**Neurological characterization after MCAO**

Prior to killing, a neurological test was made by two unaware independent observers as described previously (23). According to the test, animals were scored by the following: 0 points—no deficit; 1 point—failure to extend right forepaw fully; 2 points—decreased grip of right forelimb while tail pulled; 3 points—spontaneous circling or walking to contralateral side; 4 points—walks only when stimulated with depressed level of consciousness; 5 points—unresponsive to stimulation.

**Brain ATP levels**

For determination of ATP levels, ipsilateral side of the forebrains was collected before or 15 min after MCAO. In both cases, cortices were dissected and homogenized as described previously (18). The homogenate was centrifuged at 10,000 g for 3 min at 4°C, and the supernatant was mixed 1:1 with Tris-acetate buffer solution (pH 7.75). Finally, luciferin-luciferase was added
(final concentration 1.5 mg/mL) and ATP production was measured in a Fluoroskan Ascent FL microplate reader (Labsystems, Helsinki, Finland).

**Isolation of rat brain mitochondria and submitochondrial particles (SMP)**

Rat brain mitochondria and submitochondrial particles were prepared as described (24). Briefly, the brain mitochondria were prepared by homogenization and differential centrifugation followed by a Ficoll gradient. A suspension of freshly prepared brain mitochondria was exposed to three cycles of freeze-thaw to obtain submitochondrial particles.

**Determination of ATP levels in isolated mitochondria**

Isolated mitochondria were resuspended in a buffer containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 µM EGTA, and 5 mM potassium phosphate, pH 7.4. Mitochondria (final concentration of 1.5 mg prot/mL) were incubated with glutamate/malate (final concentration of 2.5 mM) plus ADP (180 µM) in the absence or presence of GSH (100 µM), SOD plus catalase (SOD/CAT; 100 U/ml each), and α-tocopherol (200 µM) for 10 min. SOD was used in combination with catalase, in order to remove H₂O₂ formed from superoxide anion dismutation. At the end of this period, ATP levels were determined as described above.

**Specific activities of MRC complexes in rat brain SMP**

All enzyme assays were performed at 37°C in a final volume of 1 ml, using a Beckman DU-7500 spectrophotometer (Beckman, Fullerton, CA). The activity of complex I–III (NADH-CoQ₁ reductase), complex II–III (succinate-cytochrome c reductase), and complex IV (cytochrome c oxidase) were measured as described (25–27) in SMP samples with 1 mg/ml of mitochondrial protein, in the absence or presence of reduced glutathione (GSH; 100 µM), oxidized glutathione (GSSG; 100 µM), superoxide dismutase plus catalase (SOD/CAT; 100 U/mL each), and α-tocopherol (200 µM).

**Oxygen consumption by brain MRC complex I-III in SMP**

Incubation of SMP (0.5 mg of protein/ml) was performed at 37°C with continuous stirring in 2 ml of buffer (50 mM potassium phosphate, 100 µM EGTA pH 7.2). NADH (50 µM) was used to quantify complex I–III dependent respiration, in the absence or presence of antioxidants at the concentrations above indicated. SMP respiration was measured polarographically by using a Clark-type micro oxygen electrode (Digital Model 10 Rank Brothers Ltd, Cambridge, UK).

In a different set of experiments, hydrogen peroxide (H₂O₂; 20–40 µM) was used as an oxidant agent, able to cause subsequent inhibition of MRC complex I. To test the effect of antioxidants on H₂O₂-induced inhibition of MRC complex I–III oxygen consumption by SMP, antioxidants were added, at the concentration above indicated, either before or after H₂O₂ addition to SMP and oxygen consumption was then measured.

**Protein determination**

The protein content was determined by using bicinchoninic acid (28).
Chemicals and statistical analyses

Unless otherwise stated, chemicals were from Sigma (Madrid, Spain). Results are expressed as mean ± SEM of the indicated number of experiments; statistical analysis involved one-way ANOVA (ANOVA, or the Kruskal-Wallis test when the data were not normally distributed) followed by individual comparisons of means (Student-Newman-Keuls, or Dunn’s method when the data were not normally distributed). P<0.05 was considered statistically significant.

RESULTS

Effect of the antioxidants GSH, MnTBAP, and tocopherol on infarct outcome

Infarct volume measured 48 h after permanent MCAO showed a reduction in the groups treated with GSH, MnTBAP and tocopherol when compared with the vehicle-treated group (P<0.05, n=4–5, Fig. 1A).

MCAO induced an increase in neurological damage scores measured 48 h after MCAO (1.8 ± 0.2 points, n=6), consistent with previous observations (23). Treatment with GSH, MnTBAP, and tocopherol before MCAO partly prevented neurological deficit (0.8 ± 0.3, 0.5 ± 0.3, and 1.1 ± 0.1 points, respectively, n=6, P<0.05).

Effect of the antioxidants GSH, MnTBAP, and tocopherol on brain glutamate concentrations after MCAO

Permanent MCAO caused an increase in brain glutamate concentration (basal value: 2.4 ± 0.2 µM, n=12; Fig. 1B). The onset of glutamate increase began immediately after occlusion and was decreased after 24 h (Fig. 1B). MCAO-induced increase in brain glutamate levels was decreased by previous treatment with GSH, MnTBAP, or tocopherol (Fig. 1B).

Effect of the antioxidants GSH, MnTBAP, and tocopherol on brain ATP levels before and after MCAO

Sham-operated animals showed a reduction in brain ATP levels when compared with control values (Fig. 2A; control values: 14.1 ± 1.8 µmol/g prot, n=8, 100%). Reduced glutathione, MnTBAP, and tocopherol increased basal brain ATP levels in sham-operated animals as shown when measured 2 h after their administration (Fig. 2A). Occlusion of MCA caused a reduction of ATP levels when compared with the sham group (Fig. 2A). Previous treatment with GSH, MnTBAP, or tocopherol prevented ATP reduction induced by the ischemic insult (Fig. 2A). Brain ATP levels were also increased by antioxidants in control animals (137 ± 19, 186 ± 14, and 189 ± 23% in GSH-, MnTBAP, and tocopherol-treated animals, respectively, P<0.05, n=4).

Effect of GSH, SOD/CAT, and tocopherol on ATP levels from isolated mitochondria

The production of ATP by isolated rat mitochondria incubated with glutamate/malate/ADP (complex I-, III-, and IV-dependent respiration; control value: 11.8 ± 1.0 µmol/g of mitochondrial protein, n=4) was increased by GSH, SOD/CAT, and tocopherol (Fig. 2B).
**Effect of GSH, SOD/CAT, and tocopherol on mitochondrial respiratory chain complexes in brain SMP**

Reduced glutathione, SOD/CAT, and tocopherol all increased MRC complex I–III activity (Fig. 3A) and respiration (Fig. 3B). Oxidized glutathione failed to increase complex I–III activity (Fig. 3A).

In addition, the antioxidants tested did not affect either complex II–III (92 ± 5, 91 ± 7, and 102 ± 8% of control activity in GSH-, SOD/CAT, or tocopherol-treated SMP, respectively; n=6, P>0.05) or complex IV activities (103 ± 5, 101 ± 8, and 108 ± 5% of control activity in GSH-, SOD/CAT- or tocopherol-treated SMP, respectively; n=6, P>0.05).

**Characterization of the antioxidant effect of GSH, SOD/CAT, and tocopherol**

The effect of prior or subsequent addition of antioxidants on oxygen consumption elicited by complex I–III substrate NADH was studied after exposure of SMP to H₂O₂ (40 µM). H₂O₂ caused a decrease in complex I–III oxygen consumption, which was not affected by subsequent addition of either GSH, SOD/CAT, or tocopherol (Fig. 4). However, SOD/CAT and tocopherol prevented H₂O₂-induced inhibition of complex I–III respiration when added prior to H₂O₂ (Fig. 4).

**DISCUSSION**

We and others have provided evidence showing that strategies able to prevent ischemia-induced ATP fall are neuroprotective in both in vitro and in vivo models of brain ischemia (11–19). The MRC, which is coupled to the synthesis of ATP for the oxidative phosphorylation, is a major source of reactive oxygen species and has been shown to be vulnerable to oxidative stress (21). We have therefore postulated that antioxidants may be neuroprotectants by increasing ATP levels and thus preventing ATP fall induced by ischemia. To test this hypothesis, we have studied the effect of several antioxidants on infarct outcome, brain ATP, and brain glutamate levels after permanent MCAO in rats and we have characterized the actions of these compounds on MRC complexes. Our results show that antioxidants exhibit a remarkable and specific protection, which occurs concomitant to an inhibition of both ischemia-induced increase in glutamate release and decrease in brain ATP levels.

We first tested the effect of several antioxidants on infarct outcome, all of them known as potent protective compounds against oxidants in biological systems (29). In in vivo experiments, SOD was substituted by its molecular weight analog MnTBAP. Our results show that intraperitoneal administration of the antioxidants GSH, MnTBAP, or tocopherol prior to MCAO was effective in improving stroke outcome, as demonstrated by a reduction in infarct volume and an improvement of neurological scores.

As it is well known, glutamate plays a predominant role in the pathogenesis of ischemic brain injury: this excitatory amino acid is released in high concentrations in the core of the cerebral infarction and in the penumbral tissue, leading to a massive influx of calcium that activates a
variety of catabolic processes that subsequently produce cell death (3–5). Therefore, we tested the correlation between the neuroprotective effect of antioxidants and brain glutamate levels; indeed, these compounds reduced MCAO-induced increase in brain glutamate concentrations. Glutamate release induced by severe ischemia is largely due to reversed operation of neuronal glutamate transporters (8, 9). This reversal takes place when ATP levels are severely depleted after ischemia (10, 18, 30). The next step was therefore to test the effects of antioxidants on brain ATP levels in control, sham, and MCAO-exposed animals. In agreement with the literature, MCAO induced a remarkable decrease in brain ATP levels, which was reversed by antioxidants treatment up to the levels of sham-operated rats, therefore explaining the inhibition of MCAO-induced glutamate release by these compounds. Moreover, our results show that sham operation causes a decrease in brain ATP levels. This might be explained by our previous data demonstrating that psychological stress in rats induced by exposure to acute immobilization stress causes a decrease in brain ATP levels (31), however, not sufficiently severe to provoke an increase in brain extracellular glutamate concentrations. Interestingly, we have found that the antioxidant compounds used in this report cause an increase in brain ATP levels in all groups. The observation that brain ATP levels are increased in non-ischemic animals by antioxidants indicates that these compounds target a physiological component of ATP synthesis. Our results showing that reduced glutathione, SOD/CAT, and tocopherol all increase ATP production in isolated brain mitochondria stimulated by complex I–III substrates indicate that mitochondrial oxidative phosphorylation is the source of the increased brain ATP levels after administration of antioxidants.

To investigate the action of antioxidants on mitochondrial ATP synthesis, we tested the effects of antioxidants on MRC complexes oxygen consumption and activity in rat brain SMP. We have found that GSH, SOD/CAT, and tocopherol all increased complex I–III activity and respiration. In in vitro experiments, SOD was used in combination with catalase, in order to remove H2O2 formed from superoxide anion dismutation. Oxidized glutathione did not affect complex I–III activity or respiration, suggesting that anti-oxidation is the mechanism responsible for the actions of the reduced compounds. It has been shown that the anion peroxynitrite (ONOO−), which results from the reaction between nitric oxide (NO) and superoxide, inhibits complex I–III respiration (24, 32, 33); however, an NO synthase inhibitor, L-NAME, did not increase complex I–III dependent respiration (data not shown), suggesting that NO formed from a mitochondrial NO synthase is not responsible for the endogenous inhibition, at least at this in vitro level. This result, however, does not discard inhibitory actions of NO on mitochondria in the whole cell. On the other hand, GSH, SOD/CAT, and tocopherol did not affect complex II–III or IV activity. These results demonstrate that antioxidants target mitochondrial respiratory chain complex I–III, resulting in an increased oxygen consumption and ATP production that delays ischemia-induced ATP loss and subsequent reversal of glutamate transporters. Then, these data indicate that this action of antioxidants occurs only in those cells with sufficient oxygen pressure for oxidative phosphorylation, thus implying that a neuroprotective effect of antioxidants should be expected either in a preventive protocol or after an early therapeutic administration that can reach neurones with sufficient oxygen pressure, as those in the ischemic penumbra or after recanalization. We recently showed that aspirin and salicylates are neuroprotective by increasing ATP levels and decreasing glutamate release (17–19); the present study suggests that such actions are likely to be due to the well-known antioxidant properties of salicylates (31, 34–37).
We have also characterized the anti-oxidative mechanism of these compounds by studying their effects on MRC complex I–III inhibition of respiration caused by a pro-oxidative agent, H₂O₂. Our results show that these compounds can prevent H₂O₂-induced inhibition of complex I–III respiration when added before but not after the addition of H₂O₂, indicating that they can scavenge the formation of reactive oxygen species (ROS) but fail to reverse established oxidative damage, at least at this level. Only GSH failed to cause this effect; this is possibly due to a lower anti-oxidative capacity of this compound, which is more evident after a highly oxidative situation, such as the one that occurs after a bolus addition of H₂O₂.

ROS have been implicated in the pathophysiology of many neurologic disorders and brain dysfunctions (38–40). In the context of cerebral ischemia, mitochondrial dysfunction associated with ischemia (41, 42), its reversal by antioxidant approaches (43, 44), and generation of ROS by mitochondria after brain ischemia have been reported in the literature (45). But ROS generation does not only occur in ischemic situations: It has been demonstrated that ~2–5% of the electron flow in healthy isolated brain mitochondria produces superoxide anion and H₂O₂ (20, 46). It is plausible that this basal ROS production constitutes a physiological “brake” of ATP synthesis from oxidative phosphorylation; its removal by antioxidants may be beneficial in situations in which cellular energetic levels are compromised, such as brain ischemia.

To our knowledge, this is the first report of this mechanism of action of antioxidants, with very important implications not only as a mechanism to prevent glutamate release caused by cerebral ischemia, but also in other pathophysiological conditions that arise from a depletion of cellular energetic stores, such as ischemic heart disease. Our data are in agreement with prior evidences, indicating that intra-ischemic glutamate release in the penumbral cortex correlates positively with enhanced free radical activity in experimental models of brain ischemia (47–50). In addition, this work may be involved in previously reported neuroprotective actions of antioxidants in cerebral ischemia (51), including endogenous such as melatonin (52), vitamin C (53), or uric acid (54), among others. According to our results, maximal efficacy of antioxidants should be expected if the antioxidant is given 1) either before stroke onset or 2) as soon as possible after the beginning of the ischemia, in order to decrease the risk of early neurological worsening, which is associated with high concentrations of glutamate in blood and cerebrospinal fluid (55, 56).

In summary, our results show that antioxidants exhibit a remarkable and specific protection that occurs concomitantly with an inhibition of both induced-induced increase in glutamate and decrease in brain ATP levels. These results may possess important therapeutic implications, either in the management of patients at risk of ischemic events, or for early treatment of stroke patients to avoid early neurological deterioration. Hopefully, future clinical studies will confirm this neuroprotective effect.

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Figure 1. Effect of the antioxidants glutathione (GSH), the low-molecular-weight superoxide dismutase analog MnTBAP and tocopherol on infarct volume (A) and brain glutamate concentrations (B) after permanent middle cerebral artery occlusion (MCAO). Pre-ischemic glutamate concentration was 2.4 ± 0.2 µM, n=6. Data are mean ± SEM, n = 4–6, * P<0.05 vs. sham, #P<0.05 vs. MCAO. (See Materials and Methods for details).
Figure 2. Effect of antioxidants on ATP levels. A) Effect of glutathione (GSH), the superoxide dismutase (SOD) analog MnTBAP and tocopherol on brain ATP levels in sham-operated and MCAO animals (control value: 14.1±1.8 µmol/g prot, n=8, 100%). Data are mean ± SEM, n=6, *P<0.05 vs. Sham, # P<0.05 vs. MCAO. B) Effect of GSH, SOD plus catalase (SOD/CAT) and tocopherol on ATP production by glutamate/malate-dependent respiration (control value: 11.8±1.0 µmol/g of mitochondrial protein) in isolated brain mitochondria. Data are mean ± SEM, n=4, *P<0.05 vs. control (See Materials and Methods for details).
Figure 3. Characterization of the effect of antioxidants on mitochondrial respiratory chain activities and respiration. 

A) Effect of the antioxidants glutathione (GSH), SOD/CAT, and tocopherol on complex I–III specific activity. Effect of oxidized glutathione (GSSG).

B) Effect of antioxidants on complex I–III dependent respiration (control value: 11.2±0.9 μM O2/min) by submitochondrial particles (SMP). Data are mean ± SEM, n = 6–10, *P<0.05 vs. control (See Materials and Methods for details).
Figure 4. Characterization of the antioxidant action of GSH, SOD/CAT, and tocopherol. Effect of the addition of GSH, SOD/CAT, or tocopherol before (gray columns) or after (white columns) H₂O₂ on the inhibition of complex I–III dependent respiration by H₂O₂ (40 µM) (black column). Data are mean ± SEM, n = 8–10, *P<0.05 vs. control (See Materials and Methods for details).