Zinc Inhibits Astrocyte Glutamate Uptake by Activation of Poly(ADP-ribose) Polymerase-1

Sang Won Suh, Koji Aoyama, Conrad C Alano, Christopher M Anderson, Aaron M Hamby, and Raymond A Swanson

Department of Neurology, University of California, San Francisco, and Veterans Affairs Medical Center, San Francisco, California, USA

Several processes by which astrocytes protect neurons during ischemia are now well established. However, less is known about how neurons themselves may influence these processes. Neurons release zinc (Zn^{2+}) from presynaptic terminals during ischemia, seizure, head trauma, and hypoglycemia, and modulate postsynaptic neuronal function. Peak extracellular zinc may reach concentrations as high as 400 μM. Excessive levels of free, ionic zinc can initiate DNA damage and the subsequent activation of poly(ADP-ribose) polymerase 1 (PARP-1), which in turn lead to NAD^+ and ATP depletion when DNA damage is extensive. In this study, cultured cortical astrocytes were used to explore the effects of zinc on astrocyte glutamate uptake, an energy-dependent process that is critical for neuron survival. Astrocytes incubated with 100 or 400 μM of zinc for 30 min showed significant decreases in ATP levels and glutamate uptake capacity. These changes were prevented by the PARP inhibitors benzamide or DPQ (3,4-dihydro-5-(4-(1-piperidinyl)butoxyl)-1(2*H*)-isoquinolinone) or PARP-1 gene deletion (PARP-1 KO). These findings suggest that release of Zn^{2+} from neurons during brain insults could induce PARP-1 activation in astrocytes, leading to impaired glutamate uptake and exacerbation of neuronal injury.

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INTRODUCTION

Astrocytes perform several functions that are essential for normal neuron activity, including glutamate uptake, K^+ and H^+ buffering, water transport, and metabolite exchange (1,2). These astrocyte functions can also influence neuronal survival during ischemia and other brain insults (2). Glutamate homeostasis is a key aspect of astrocyte-neuron interaction during ischemia because of the sensitivity of many neurons to glutamate excitotoxicity (3). Microdialysis studies indicate that extracellular glutamate is maintained at low concentrations in the (viable) ischemic penumbra (4), and failure of astrocyte glutamate uptake may trigger the conversion of ischemic but viable tissue to infarction (5,6). Astrocyte glutamate uptake is accomplished by Na^+-dependent transporters (7,8). These transporters move glutamate into astrocytes against a steep concentration gradient by coupling glutamate translocation to the transmembrane Na^+, K^+, and voltage gradients. These gradients are in turn maintained by membrane Na^+/K^+ ATPase activity, such that glutamate uptake is ultimately ATP dependent.

While the importance of glutamate uptake and other astrocyte processes is well established, little is known about how neurons themselves may affect this process. The present study explores a mechanism by which neurons may activate astrocyte poly(ADP-ribose) polymerase 1 (PARP-1), thus impairing energy metabolism and glutamate uptake in these cells. PARP activity is shared by a family of enzymes, of which PARP-1 is the most abundant and well characterized (9,10). When activated by DNA strand breaks or kinks, PARP-1 consumes NAD^+ to form poly(ADP-ribose) polymers on several acceptor proteins, including histones, DNA polymerase, and DNA ligases (9,11,12). This process appears to facilitate DNA repair; however, excessive PARP-1 activation results in NAD^+ and ATP depletion when DNA damage is extensive (10,13,14). Because glutamate uptake requires ATP, it follows that extensive PARP-1 activation in astrocytes could impair glutamate uptake.

Zinc (as Zn^{2+}) leads to PARP-1 activation, probably as a result of increased production of reactive oxygen species in the presence of elevated Zn^{2+} (15–19). Neurons release Zn^{2+} from presynaptic terminals during pathological conditions such as ischemia, seizure, brain trauma, and hypoglycemia (20-27). In those conditions extracellular Zn^{2+} concentration may reach 100–400 μM (28–32), although direct measurements of brain extracellular Zn^{2+} have not confirmed elevations to this level (33).

The present study examines the effect of Zn^{2+} elevations on ATP levels and glutamate uptake in cultured astrocytes. We show that Zn^{2+} can inhibit glutamate uptake in mouse cortical astrocytes by PARP-1 activation and subsequent ATP depletion, and that this effect is attenuated by the PARP inhibitors benzamide or DPQ (3,4-dihydro-5-[4-(1-piperidinyl) butoxyl]-1(2*H*)-isoquinolinone) and by PARP-1 gene deficiency.

Address correspondence and reprint requests to *Sang Won Suh, Department of Neurology (127), VAMC 4150 Clement Street, San Francisco, CA, 94121. Phone: (415) 221-4810 (2480); Fax: (415) 750-2273; E-mail: sang.suh@ucsf.edu.*
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METHODS AND MATERIALS

The studies were performed in accordance with protocols approved by the animal studies committee of the San Francisco Veterans Affairs Medical Center. Reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA) except where noted.

Cell cultures

Wild-type astrocyte cultures were prepared from cortices of one-day-old Swiss-Webster mice (Simonsen, Gilroy, CA, USA) as described previously (34,35) and plated into 24-well Falcon culture plates. The PARP-$1^{-/-}$ mice were the 129S *Adprt1*^{tm1Zqw} strain obtained from Jackson Laboratory (Bar Harbor, ME, USA) and originally developed by Wang et al. (36). The wild-type and PARP-$1^{-/-}$ cortices were harvested, freed of meninges, dissociated with papain digestion (with DNase) and subsequent trituration, and plated on 24-well Falcon culture plates or glass coverslips. Cells were treated for 48 h with 20 μM cytosine arabinoside at confluence (12–15 d in vitro) to prevent microglial proliferation. This medium was replaced with Eagle's minimal essential medium containing 5 mM glucose supplemented with 5% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM glutamine, 100 nM sodium selenate, and 200 nM α-tocopherol. The astrocyte cultures were used, when confluent, at 20 to 30 d in vitro (37).

Experimental procedures

Experiments were initiated by replacing the culture medium with artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM) KCl, 3.1; NaCl, 134; $CaCl_2$, 1.2; $MgSO_4$, 1.2; KH_2PO_4, 0.25; $NaHCO_3$, 15.7; and glucose, 2. The pH was adjusted to 7.2 while the solution was equilibrated with 5% CO_2 at 37 °C. Osmolarity was verified at 290–310 mOsm with a Wescor vapor pressure osmometer (Logan, UT, USA). Zinc and PARP inhibitors were added from concentrated stocks prepared in ACSF immediately before use and adjusted to pH 7.2 when necessary. An exposure to Zn^{2+} ($ZnCl_2$) was performed at 37 °C in a 5% CO_2 atmosphere. Thirty minutes of exposure to 100 or 400 μM Zn^{2+} (as $ZnCl_2$) was performed in ACSF. After the exposure, cultures were washed two times with ACSF and were placed back into the incubator. When used, 1 mM of benzamide or 25 μM of DPQ was added to the medium one hour before and during the Zn^{2+} exposure. Cultures were then maintained in ACSF until biochemical studies were performed.

ATP assay

For ATP assays, cells were lysed in boiling buffer containing 100 mM Tris and 4 mM EDTA, pH 7.75. Fifty milliliters of cell lysates were mixed with 50-mL aliquots of luciferase/luciferin mixture provided with an ATP bioluminescence assay kit (Roche Diagnostics GmbH, Mannheim, Germany), and photon emission was detected with a luminometer. ATP concentrations were calibrated against ATP standards and expressed as nanomoles per milligram of protein.

Glutamate uptake

Glutamate uptake was measured as described previously (38) with minor modifications. Assays were initiated by replacing the culture medium with ACSF. After a 20-min preincubation in this medium, each culture well received 1.67 μCi/mL L-[^{14}C(U)]glutamate plus unlabeled glutamate to achieve a total glutamate concentration of 100 μM. Uptake was terminated after a 7-min incubation at 37 °C by two washes in ice-cold Hank's balanced salt solution, followed immediately by cell lysis in 0.1N NaOH. Aliquots were divided for scintillation counting and protein determinations.

Cell death determinations

Astrocyte death was quantified by measuring lactate dehydrogenase (LDH) activity in cell lysates harvested 24 h after Zn^{2+} exposures. Percentage cell death was calculated by normalizing the LDH values to LDH activity measured in lysates from control (wash only) culture wells (38,39).

Statistical analyses

All data are presented as means ± SEM and were assessed by analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test, as post-hoc comparison, for differences between selected pairs of groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

Zn^{2+} Inhibits Uptake of Glutamate into Cultured Mouse Astrocytes

The basal rate of L-[^{14}C]glutamate uptake in wild-type primary mouse cortical astrocyte cultures was 8.08 ± 0.35 nmol/min/mg protein. Astrocytes incubated with Zn^{2+} (100–400 μM) for 30 min exhibited delayed and dose-dependent reductions in glutamate uptake that became more pronounced with progressive time after zinc exposure and washout (Figure 1). Studies performed with 10 μM Zn^{2+} added to the medium showed no effect on glutamate uptake (not shown).

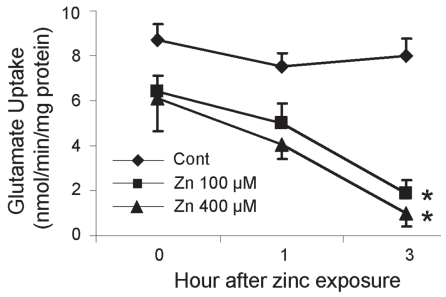

Figure 1. Zn^{2+} impairs astrocyte glutamate uptake in a dose- and time-dependent manner. Astrocytes were exposed for 30 min to Zn^{2+} at the designated concentrations, and glutamate uptake was assessed immediately (zero hours), one hour, or three hours after zinc removal. Data points are means of three to four independent experiments (*n* = 3-4), each with measurements from two to three culture wells. **P* < 0.05 versus control at the designated time point.

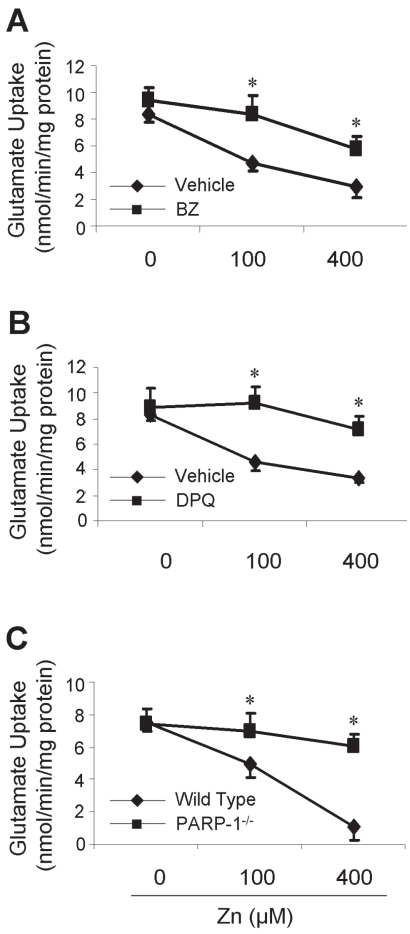

Figure 2. PARP inhibitors and PARP-1 gene deletion prevent Zn^{2+}-induced inhibition of glutamate uptake. Astrocytes were treated for 30 min with Zn^{2+} alone or with (A) 1 mM benzamide (BZ) or (B) 25 μM DPQ. (C) Comparison of Zn^{2+} effects on glutamate uptake in wild-type and PARP-1 deficient (PARP-$1^{-/-}$) astrocytes. Glutamate uptake was assessed one hour after washout of Zn^{2+}. Data points are means of three to four independent experiments (*n* = 3-4), each with measurements from two to three culture wells. **P* < 0.05 compared with vehicle (A, B) or control (C) at the designated zinc concentration.

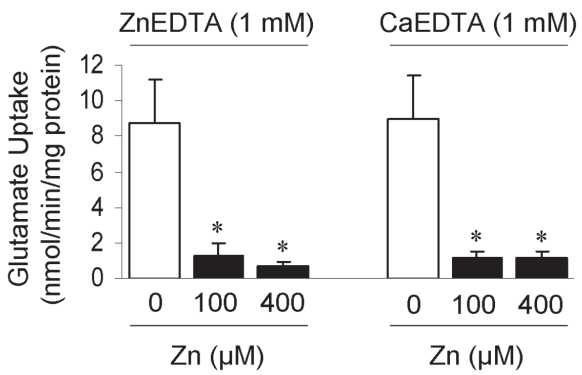

Figure 3. The addition of a zinc chelator, CaEDTA, did not reverse the effect of zinc of glutamate uptake. Astrocytes were exposed for 30 min to Zn^{2+} at the designated concentrations, and glutamate uptake was measured three hours later. The cultures were also treated with 1 mM of the zinc chelator CaEDTA or, as a control, ZnEDTA, for 5 min prior to initiation of the glutamate uptake assay. Data points are means of three independent experiments (*n* = 3), each with measurements from two to three culture wells. **P* < 0.05 compared with zero (0) zinc-exposed control.

PARP Inhibitors and PARP-1 Gene Deletion Prevent Zn^{2+}-Induced Astrocyte Glutamate Uptake Inhibition

As shown in Figure 2A and 2B, astrocytes incubated with Zn^{2+} showed reduced glutamate uptake at one hour after Zn^{2+} washout, and this reduction was significantly prevented in cultures treated with benzamide (1 mM) or DPQ (25 μM). Zinc-induced glutamate uptake inhibition was also attenuated in astrocytes prepared from PARP-$1^{-/-}$ mice, further suggesting that the effect of Zn^{2+} on glutamate uptake is mediated by PARP-1 activation (Figure 2C).

Zinc-Induced Glutamate Uptake Inhibition is Not Reversed by Subsequent Addition of a Zinc Chelator

Prior studies have shown that Zn^{2+} can inhibit glutamate uptake into the cells by a direct effect on glutamate transporters (30,40). This is an unlikely explanation for the present observations because astrocyte glutamate uptake capacity continued to fall long after washout of zinc; however, it is possible that Zn^{2+} could remain bound to cell membranes long after Zn^{2+} washout from the medium. To evaluate this possibility, an additional study was performed in which astrocytes were incubated with 1 mM of CaEDTA (a zinc chelator) (41) or 1 mM of ZnEDTA (control) for 5 min at three hours after Zn^{2+} exposure. These concentrations are in substantial excess of the original Zn^{2+} added to the culture wells. The inhibition of astrocyte glutamate uptake by Zn^{2+} was not reversed by incubation with the zinc chelator CaEDTA (Figure 3), thus arguing against an effect of retained zinc bound at the cell membranes.

Zn^{2+} Reduces Both Astrocyte ATP Levels and ATP/ADP Ratios

We proposed that zinc reduced glutamate uptake as a result of PARP-1 activation and resultant energy failure. To further test this idea, we measured the effect of Zn^{2+} on astrocyte ATP concentrations and ATP/ADP ratios. The basal ATP concentration was 20.3 ± 0.8 nmol/mg protein and the basal ATP/ADP ratio was 3.1. The ATP concentrations were decreased after Zn^{2+} (100-400 (μM) exposure in a time-dependent manner at both concentrations (Figure 4). The ATP/ADP ratio was also decreased by Zn^{2+}, indicating an impairment in high-energy phosphorylation. The reductions in ATP concentrations and ATP/ADP ratios were both attenuated in astrocytes derived from PARP-$1^{-/-}$ mice (Figure 4).

Zinc-induced inhibition of glutamate uptake is not due to membrane disruption

These results support the idea that zinc-induced PARP-1 activation causes energy failure, which in turn leads to impaired glutamate uptake. However, an alternative possibility is that zinc-in-

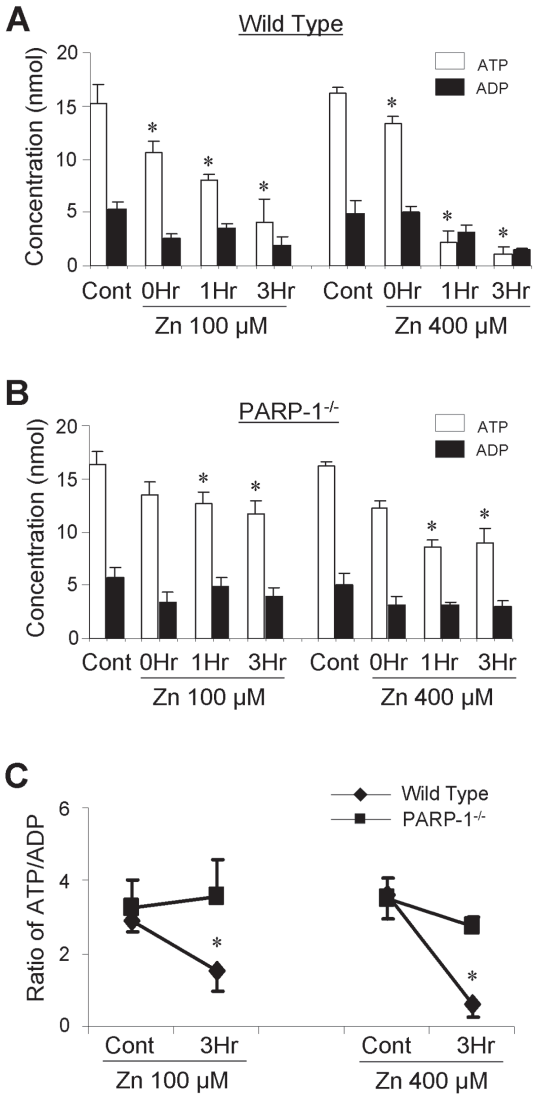

Figure 4. Zn^{2+} affects astrocyte energy status in a PARP-1-dependent manner. (A) Astrocytes were exposed for 30 min to Zn^{2+} at the designated concentrations, and the levels of ATP and ADP were assessed immediately (zero hours), one hour, or three hours after zinc removal. Data points are means of three independent experiments (n = 3), each with measurements from two to three culture wells. *P < 0.05 versus controls. (B) Wild-type and PARP-1-deficient (PARP-$1^{-/-}$) astrocytes were exposed for 30 min to Zn^{2+} at the designated concentrations and the levels of ATP and ADP were assessed immediately (zero hours), one hour, or three hours after zinc removal. Data points are means of four independent experiments (n = 4), each with measurements from two culture wells. *P < 0.05 versus wild-type astrocyte. (C) Zinc-induced changes in ATP / ADP ratio were also blocked in the PARP-$1^{-/-}$ cells. Data points are means of three independent experiments (n = 3), each with measurements from two culture wells. *P < 0.05 versus wild-type astrocyte.

duced PARP-1 activation causes disruption of the cell membrane, and this is the proximate cause of ATP depletion and glutamate uptake failure. To evaluate this possibility we measured LDH activity retained the in the cultures at serial time points after zinc exposure. As shown in Figure 5, LDH activity in the intracellular compartment did not decrease until time points long after ATP depletion and glutamate uptake failure had occurred.

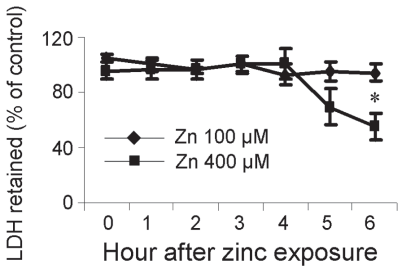

Figure 5. Zn^{2+} did not acutely cause cell membrane disruption. Astrocyte membrane integrity was evaluated by measuring the lactate dehydrogenase (LDH) retained in the cultures at serial time points after 30 min incubation with Zn^{2+}. Data points are means of four independent experiments (n = 4), each with measurements from two culture wells. *P < 0.05 versus immediately (zero hours) after zinc removal.

DISCUSSION

Zn^{2+} contributes to neuronal death in several conditions, including stroke, epilepsy, head trauma, and hypoglycemia, and intracerebroventricular administration of the zinc chelator CaEDTA reduces neuronal death (20–27). It is generally assumed that zinc-induced neurotoxicity in these settings results from direct effects of zinc on the neurons. The present studies suggest an additional or alternative mechanism: zinc released from neurons may induce failure of astrocyte glutamate uptake through a mechanism involving PARP-1 activation and energy failure. The resulting increase in extracellular glutamate levels would be expected to contribute to neuronal demise, and activation of neuronal AMPA/kainate receptors could in turn stimulate additional Zn^{2+} release (18).

Astrocytes express both EAAT1 and EAAT2 glutamate transporter subtypes, with EAAT2 being responsible for the vast majority of glutamate uptake in most brain structures (8). Zn^{2+} has been previously reported to modulate glutamate uptake through direct effects on EAAT1 but not EAAT2 (30,42). Several lines of evidence argue that the inhibitory effect of Zn^{2+} on glutamate uptake observed in the present studies

cannot be attributed to a direct effect of Zn^{2+} on glutamate transporters. First, the effect of Zn^{2+} incubations was delayed in onset and reached maximal effect 3 hours after washout of Zn^{2+} from the culture medium. Moreover, the uptake inhibition was not reversed by the addition of the very high affinity Zn^{2+} chelator, CaEDTA, thereby excluding an effect mediated by residual Zn^{2+} bound to glutamate transporters or other cell membrane components. By contrast, the effect of Zn^{2+} on glutamate uptake was markedly attenuated both by pharmacological PARP inhibitors and by PARP-1 gene deletion.

The roughly parallel time course of glutamate uptake failure (see Figure 1) and ATP depletion (see Figure 4) further suggest a causative role for PARP-1 activation in the zinc-induced glutamate uptake failure. ATP-dependent processes are sensitive both to absolute ATP concentrations and the ATP/ADP ratio. Here, both of these measures declined after Zn^{2+} exposure, and these declines were blocked by PARP-1 gene deficiency. Consumption of cytosolic NAD^+ by PARP-1 leads to a fall in the ATP/ADP ratio by impairing glycolysis and, indirectly, by impairing respiration (37,43,44). In addition, de novo synthesis of NAD^+ is stimulated by NAD^+ consumption and leads to depletion of the total ATP and adenylate pools (45). Thus reductions in both the ATP/ADP ratio and total ATP levels occur with extensive PARP-1 activation, and both may contribute to impaired glutamate uptake.

An unresolved question is which events may link the increased intracellular Zn^{2+} levels to increased PARP-1 activity. Possible candidate mediators are reactive oxygen species (ROS) such as superoxide ion, nitric oxide (NO), and peroxynitrite. In fact, several studies have previously reported that Zn^{2+} overload results in elevated intracellular levels of ROS in a PKC- and NADPH oxidase-dependent manner (16,46), suggesting that ROS generation may contribute to Zn^{2+}-induced PARP-1 activation. NO, produced by NO synthases (NOS), is a well-established mediator of neuronal cell death in a variety of models of neuronal injury (47) in which Zn^{2+}-induced cell death plays a prominent role (20,21,23,25).

Zn^{2+}, released by neurons into the synapse and surrounding extracellular space during ischemia and other insults, may result in free Zn^{2+} concentrations of up to 100-400 μM in some brain regions (30–32). These concentrations are comparable to the levels of Zn^{2+} observed in the present studies to inhibit glutamate uptake. It should be noted, however, that there are several uncertainties regarding the extrapolation of these cell culture findings to the in vivo setting. First, protein binding of Zn^{2+} in vivo may lower true free Zn^{2+} activity (48). Second, a recent study using fluorescent indicators of free Zn^{2+} showed the extracellular levels achieved during ischemia to be much lower than the estimated maximal values (33). Third, it is possible that Zn^{2+} could have direct effects on neurons at concentrations well below those that cause impaired astrocyte glutamate uptake. On the other hand, Zn^{2+} released from intracellular protein binding sites may also contribute to Zn^{2+}-mediated cell injury (49,50). Given these considerations, the present results indicate a potential mechanism by which neuronal Zn^{2+} release could lead to impaired astrocyte function and further neuronal injury, but additional studies will be required to confirm this process in vivo.

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