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Original Contribution

Sepsis inhibits recycling and glutamate-stimulated export of ascorbate by astrocytes

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Abstract

Sepsis causes brain dysfunction. Because neurotransmission requires high ascorbate and low dehydroascorbic acid (DHAA) concentrations in brain extracellular fluid, the effect of septic insult on ascorbate recycling (i.e., uptake and reduction of DHAA) and export was investigated in primary rat and mouse astrocytes. DHAA raised intracellular ascorbate to physiological levels but extracellular ascorbate only slightly. Septic insult by lipopolysaccharide and interferon-γ increased ascorbate recycling in astrocytes permeabilized with saponin but decreased it in those with intact plasma membrane. The decrease was due to inhibition of the glucose transporter (GLUT1) that translocates DHAA because septic insult slowed uptake of the nonmetabolizable GLUT1 substrate 3-*O*-methylglucose. Septic insult also abolished stimulation by glutamate of ascorbate export. Specific nitric oxide synthase (NOS) inhibitors and nNOS and iNOS deficiency failed to alter the effects of septic insult. Inhibitors of NADPH oxidase generally did not protect against septic insult, because only one of those tested (diphenylene iodonium) increased GLUT1 activity and ascorbate recycling. We conclude that astrocytes take up DHAA and use it to synthesize ascorbate that is exported in response to glutamate. This mechanism may provide the antioxidant on demand to neurons under normal conditions, but it is attenuated after septic insult.
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Keywords: Ascorbate; Astrocytes; Brain; Dehydroascorbic acid; Glucose transporter; Glutamate; Interferon-γ; Lipopolysaccharide; NADPH oxidase; Nitric oxide synthase; Sepsis; Vitamin C; Free radicals

Most hospital patients with bacteremia show neurologic symptoms ranging from lethargy to coma [1]. This sepsis-associated encephalopathy is characterized by cytokine induction, nitric oxide synthase (NOS) activation, glutamate excitotoxicity, and ascorbate depletion in the brain [1–4]. Early in sepsis, bacterial toxins (e.g., lipopolysaccharide (LPS)) and inflammatory cytokines (e.g., interferon-γ (IFN-γ)) increase the expression of NADPH oxidase and NOS isoforms 1 and 2 (i.e., nNOS and iNOS) in the brain [1,3,5]. Whereas brain neurons express nNOS constitutively and microglia express iNOS during sepsis, astrocytes in vivo also express nNOS and are induced by brain injury to express iNOS [6,7].

Neurotransmission in brain requires high ascorbate and low dehydroascorbic acid (DHAA) concentrations [8]. The critical importance of extracellular ascorbate for excitatory neurotransmission has been shown by infusing ascorbate oxidase into rat striatum and observing that the enzyme's conversion of extracellular ascorbate to DHAA leads to a rapid decline in behavioral activation, such that a 50% decrease in extracellular ascorbate concentration is associated with nearly complete inhibition of motor, social, and approach

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BSA, bovine serum albumin; DHAA, dehydroascorbic acid; DPI, diphenylene iodonium; IFN-γ, interferon-γ; L-NAME, *NG*-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MEM, minimum essential medium; 7NI, 7-nitroindazole; NOS, nitric oxide synthase; 1400W, *N*-(3-amino-methyl)benzylacetamidine hydrochloride.

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behavior [9]. Although exogenous DHAA decreases brain injury in experimental models of cerebral ischemia [10,11], the neuroprotection is likely due to ascorbate generated by cellular uptake and reduction of DHAA (i.e., ascorbate recycling) [8]. Normally DHAA is generated in the brain by oxidation of ascorbate and it may also enter from the blood [12]. Cultured astrocytes are not injured by exposure to high levels of DHAA, unlike neuronal cells [13–17]. Astrocytes take up DHAA through a 45-kDa isoform of the glucose transporter GLUT1 [18] and express enzymes capable of reducing intracellular DHAA to ascorbate, namely, NADPH-dependent thioredoxin reductase, glutathione-dependent protein disulfide isomerase, and DHAA reductase [19–22].

Glutamate elevates extracellular ascorbate concentration in the brain because high-affinity glutamate uptake triggers ascorbate release from astrocytes [23–27]. In the brain, astrocyte endfeet are in close contact with neuronal synapses and astrocytes respond with ionic fluxes to the glutamate released from synapses [28]. Neurons cannot synthesize ascorbate de novo from glucose but instead take up ascorbate from the extracellular fluid [29,30]. Thus a demand-driven component of ascorbate export from astrocytes may occur just in time and in the right place to reach active neurons that are releasing glutamate. It is possible that the decrease in extracellular ascorbate concentration in septic brain results from failure of this mechanism. Therefore the present study evaluated the hypothesis that astrocytes produce extracellular ascorbate from DHAA through mechanisms sensitive to glutamate and sepsis.

Materials and methods

Materials

Antibiotic solution (penicillin G sodium 10,000 units/ml; streptomycin 10,000 μg/ml) was purchased from Gibco Laboratories (Burlington, ON, Canada). Anti-glial fibrillary acidic protein antibody was purchased from Dakopatts (Glostrup, Denmark). Anti-iNOS antibody (monoclonal) was from Transduction Laboratories (Lexington, KY, USA). Secondary antibodies, avidin, and biotin were obtained from Vector Laboratories (Burlington, ON, Canada). DHAA was from Fluka (Oakville, ON, Canada). 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), apocynin, L-ascorbic acid, diphenylene iodonium (DPI), L-glutamate, DL-homocysteine, *Escherichia coli* LPS, *NG*-nitro-D-arginine methyl ester (D-NAME), *NG*-nitro-L-arginine methyl ester (L-NAME), and 3-*O*-methylglucose were from Sigma Chemical Co. (St. Louis, MO, USA). IFN-γ was from Life Technologies, Inc. (Gaithersburg, MD, USA). *N*-(3-Aminomethyl)benzylacetamidine hydrochloride (1400W), 7-nitroindazole (7NI), and *NG*-propyl-L-arginine were from Calbiochem (San Diego, CA, USA). 3-*O*-Methyl-D-[1-^{3}H]glucose (5 Ci/mmol) was from Dupont Canada (Lachine, QC, Canada).

Cell cultures

The University of Western Ontario Council on Animal Care approved the experimental procedures. Primary astrocyte cultures were prepared from the neopallium of 1-day-old Wistar rats and CD1 mice and grown to confluence in 10% horse serum-supplemented minimum essential medium (MEM), according to our published procedure [31]. The same procedure was used to make astrocyte cultures from 1-day-old mice with targeted disruption of the nNOS and iNOS genes, as well as matched wild-type controls, which were produced on a C57BL/6 background. The nNOS- and iNOS-deficient mice were backcrossed to C57BL/6 for more than eight generations. Immunohistochemical analysis after 2 weeks in culture showed that approximately 99% of the cells expressed abundantly the astrocyte marker glial fibrillary acidic protein and conformed to the morphology of astrocytes. The sera and MEM that were used to culture cells did not contain detectable ascorbate (<1 μM). Brain cells cannot synthesize ascorbate de novo from glucose and thus these cultures became depleted of the vitamin.

Experimental procedures

The culture medium was replaced 1 day before the experiments. Temperature was maintained at 37°C for all incubations except where otherwise noted. Mouse astrocyte cultures were subjected to septic insult by incubation for 24 h with LPS (25 ng/ml) and IFN-γ (100 U/ml) in MEM supplemented with 10% fetal bovine serum. Nonseptic control cultures were incubated with vehicle (aqueous solution of bovine serum albumin (BSA)). Animal experiments have shown that systemic injection of LPS is an insult sufficient to cause motor impairment typical of sepsis-associated encephalopathy [32]. Injection of LPS also increases the concentration of IFN-γ in the brain [33]. But because primary astrocyte cultures lack the immune cells that are the main source of IFN-γ in septic brain, LPS does not induce IFN-γ expression in these cultures [34]. Furthermore, the combination of LPS and IFN-γ stimulates inflammatory responses (e.g., nitric oxide production) in astrocyte cultures more than either LPS or IFN-γ alone [35,36]. Therefore the combination of LPS and IFN-γ was used to model the effects of septic insult on astrocytes in vitro.

Incubation of astrocytes with LPS and IFN-γ causes rapid activation of NADPH oxidase and subsequent induction of iNOS [37,38]. Immunohistochemical assessment of iNOS expression was carried out in representative cultures using the immunoperoxidase staining procedure described previously [38]. After 24 h exposure to the LPS + IFN-γ or vehicle (BSA), other astrocyte cultures were incubated for 30 min in serum-free MEM with DHAA (400 μM) and either glutamate (1 mM) or vehicle (MEM). The rationale for exposing cells to 1 mM glutamate is that, in the brain, this excitatory neurotransmitter is released from nerve

endings into synaptic clefts, where its average concentration peaks in the range 1–5 mM [39]. For experiments using inhibitors of NADPH oxidase and NOS in mouse astrocyte cultures, these drugs or their vehicles (dimethyl sulfoxide and MEM) were added at the same time as the LPS + IFN-γ and were present for the entire 24.5-h incubation period. The acute effects of NOS inhibitors on ascorbate recycling were also examined in rat astrocyte cultures by omitting the preincubation step and applying the inhibitors at the same time as DHAA.

A cell permeabilization technique was used to allow measurement of intracellular DHAA reduction independent of plasma membrane transporters. Astrocyte cultures, after incubation with LPS + IFN-γ or vehicle for 24 h, were incubated for an additional 1 min in serum-free MEM containing the permeabilizing agent saponin (0.6 mg/ml MEM). Staining of the astrocytes by trypan blue was monitored by phase-contrast light microscopy to assess membrane permeability. In parallel experiments that studied DHAA reduction, 400 μM DHAA was added at the same time as saponin (absent trypan blue) and incubated for 1 min.

Ascorbate concentration was assayed by acidic extraction and HPLC with electrochemical detection, according to a published procedure [31]. Assay sensitivity was 2 pmol. When freshly prepared, 400 μM DHAA solution was found to contain 0.34 μM ascorbate and, to account for this contamination, the latter value was subtracted from the ascorbate concentrations measured in DHAA incubates. Intracellular ascorbate concentrations were normalized to 1 mg cell protein measured by the Lowry procedure.

3-*O*-Methylglucose uptake was measured to assess glucose transporter activity according to a published procedure [18]. Briefly, astrocytes were incubated with 3-*O*-methyl[^{3}H]glucose (60 μM; specific activity was adjusted with unlabeled 3-*O*-methylglucose to 6.7 mCi/mmol) in glucose-free transport medium. This medium consisted of (in mM) 134 NaCl, 5.2 KC1, 1.8 $CaCl_2$, 0.8 $MgSO_4$, and 20 Hepes, adjusted to pH 7.3 with NaOH. The final concentration of Na^+ was 138 mM as measured by flame photometry. Uptake was measured at 23°C to increase the accuracy with which the initial rate was quantified. Uptake was terminated by washing cultures with ice-cold isosmotic Tris–sucrose solution and then the cells were harvested by osmotic lysis (1 ml water/dish) and mechanical scraping. An aliquot of the cell harvest was used for protein measurement and the remainder was combined with scintillation cocktail. The radioactive contents of the media and cells were measured by liquid scintillation counting. Astrocyte uptake of 3-*O*-methylglucose proceeded as a linear function of time for at least 40 s. Therefore, the initial rate of uptake was measured using 30-s incubation periods. Uptake rate was computed based on the specific activity of radiolabeled 3-*O*-methylglucose in the media and expressed as pmol 3-*O*-methylglucose/mg cell protein/min.

Statistics

Data are presented as mean ± SEM values for n independent experiments that were performed on separate days. Comparisons between means based on a single level of treatment were evaluated using the two-tailed, paired t test. For two or more treatments, analysis of variance and the Tukey multiple comparison tests evaluated differences between means. $p < 0.05$ was considered significant.

Results

Ascorbate was not detectable in the cells or media of astrocyte cultures grown under standard conditions. Incubation of astrocyte cultures with DHAA (400 μM) increased intracellular ascorbate to physiological levels and raised extracellular ascorbate concentration slightly, reflecting rapid ascorbate recycling and slow export under basal conditions (Fig. 1). The presence of cells was necessary because cell-free incubations of DHAA in MEM failed to

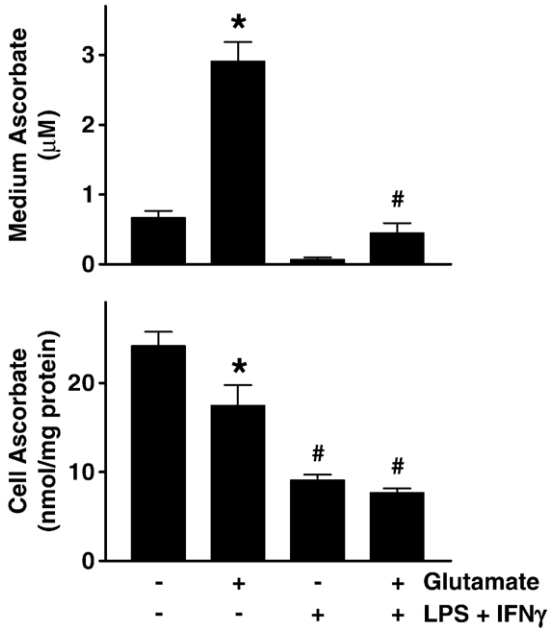

Fig. 1. Effects of glutamate and septic insult on the concentrations of ascorbate produced from DHAA by astrocytes. CD1 mouse astrocyte cultures were incubated with the combination of LPS (25 ng/ml) and IFN-γ (100 U/ml) or else with vehicle (BSA) for 24 h and then were incubated in serum-free MEM with DHAA (400 μM) for an additional 30 min. Either glutamate (1 mM) or its vehicle (MEM) was added simultaneously with DHAA. Subsequently the ascorbate concentrations in the medium (top) and cells (bottom) were measured by HPLC-based electrochemical assay. Plotted are the mean ± SEM values for $n = 9$ independent experiments. $^*p < 0.05$ for the effect of glutamate because, in nonseptic cultures, glutamate increased medium ascorbate and decreased cell ascorbate concentrations compared to its own vehicle. $^{\#}p < 0.05$ for the effect of LPS + IFN-γ because this septic insult abolished the increase in medium ascorbate caused by glutamate in nonseptic cultures and also lowered cell ascorbate concentrations compared to nonseptic cultures that were nominally glutamate-free or glutamate-treated.

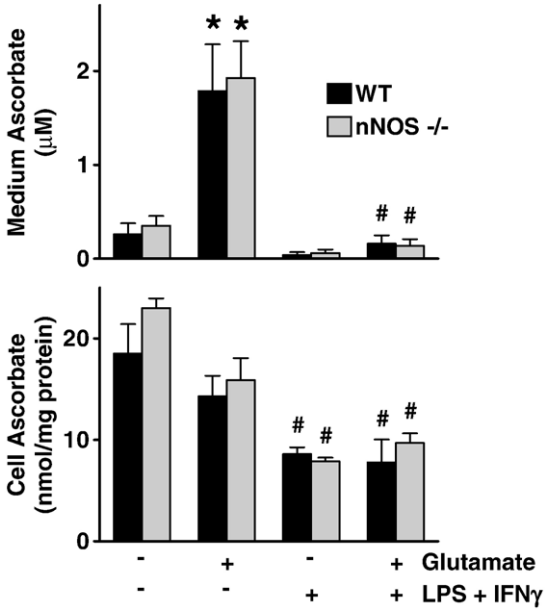

Fig. 2. Effects of glutamate and septic insult in nNOS-deficient astrocytes. nNOS$^{-/-}$ and wild-type C57BL/6 mouse astrocytes were incubated according to the conditions described in the Fig. 1 legend. Plotted are mean ± SEM values for ascorbate concentration in the medium (top) and cells (bottom) for $n = 4$ independent experiments. The responses of nNOS$^{-/-}$ and wild type did not differ ($p > 0.05$). *$p < 0.05$ for the effect of glutamate because, in nonseptic cultures, glutamate increased medium ascorbate compared to its own vehicle. $^{\#}p < 0.05$ for the effect of LPS + IFN-γ because this septic insult abolished the increase in medium ascorbate caused by glutamate in nonseptic cultures and also lowered cell ascorbate concentrations compared to nonseptic cultures that were nominally glutamate-free or glutamate-treated.

produce detectable ascorbate (data not shown). Glutamate (1 mM added simultaneous with DHAA) increased the concentration of ascorbate in the medium of the astrocyte cultures while causing a corresponding decrease in the cells, but did not change the total amount of ascorbate produced from DHAA. Because the cells were the only source of ascorbate increase in the medium, we designated this change as ascorbate export. Glutamate did not alter the total protein content of cell-attached astrocytes (data not shown). Septic insult (LPS + IFN-γ for 24 h) decreased ascorbate recycling and glutamate-stimulated ascorbate export (Fig. 1).

Next, the roles of nNOS, iNOS, and NADPH oxidase were investigated because septic insult increases the activities of these enzymes. We observed in CD1 mouse astrocytes that 24.5 h incubation with the nNOS inhibitor 7NI (100 μM [40]) and the iNOS inhibitors *NG*-propyl-L-arginine (50 μM [41]) and 1400W (100 μM [42]) did not prevent attenuation of ascorbate recycling by LPS + IFN-γ (data not shown). Subsequent experiments used astrocytes derived from transgenic mice and wild-type (C57BL/6) controls. LPS + IFN-γ (24 h) induced immunoreactive iNOS in wild-type but not iNOS-deficient (iNOS$^{-/-}$) mouse astrocyte cultures (this result is shown in Fig. 4 of Ref. [43]). The septic insult caused similar inhibition of ascorbate recycling and export in wild-type, nNOS-deficient (nNOS$^{-/-}$, Fig. 2) and iNOS$^{-/-}$ astrocytes (Fig. 3), as well as in iNOS$^{-/-}$ astrocytes treated with the nNOS inhibitor 7NI (Fig. 3). Taken together, these results indicate that nNOS and iNOS are not required for the novel actions of LPS + IFN-γ that we found.

The acute effect of L-NAME was studied in rat astrocytes because the nonspecific NOS inhibitor had been shown previously to increase ascorbate recycling in this species after 24 h [38]. We observed that 30 min incubation with L-NAME and D-NAME (an analog that does not inhibit NOS) increased ascorbate export and D-NAME also enhanced ascorbate recycling, but 7NI, *NG*-propyl-L-arginine, and 1400W did neither (Fig. 4). L-NAME did not affect ascorbate stability because when 10 μM ascorbate was incubated in MEM without cells, the concentrations of ascorbate remaining after 30 min were similar in the presence of 1 mM L-NAME (7.0 ± 0.4 μM) and vehicle control (7.3 ± 0.4 μM). Thus L-NAME increases extracellular ascorbate concentration in astrocyte cultures by a mechanism that requires cells but not NOS activity.

NADPH oxidase inhibitors had disparate effects on ascorbate recycling. One of them, DPI (0.1 μM [44]), increased the ascorbate recycling by nonseptic and septic

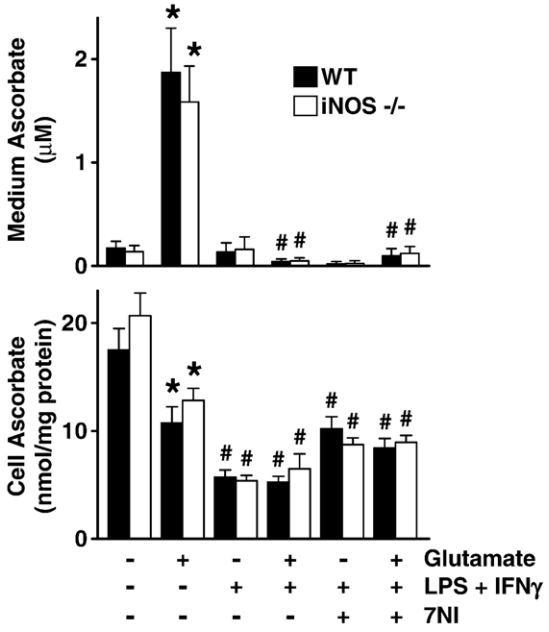

Fig. 3. iNOS deficiency and nNOS inhibition do not prevent septic inhibition of ascorbate recycling and export. iNOS$^{-/-}$ and wild-type C57BL/6 mouse astrocytes were incubated according to the conditions described in the Fig. 1 legend. Where indicated, the nNOS inhibitor 7NI (100 μM) or its vehicle (DMSO) was added at the same time as LPS + IFN-γ and present for the entire experiment (24.5 h). Plotted are mean ± SEM values for ascorbate concentration in the medium (top) and cells (bottom) for $n = 5$ independent experiments. iNOS deficiency, alone or in combination with the nNOS inhibitor, did not alter ascorbate levels under any conditions ($p > 0.05$). *$p < 0.05$ for the effect of glutamate compared to its own vehicle. $^{\#}p < 0.05$ for the effect of LPS + IFN-γ compared to its own vehicle.

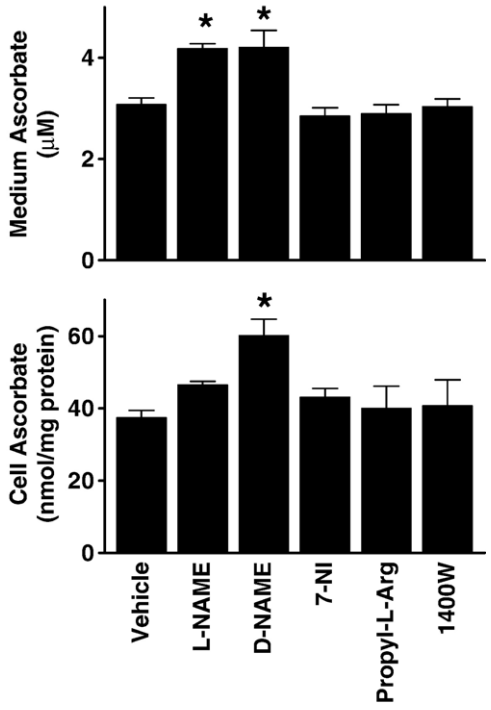

Fig. 4. Effects of L-NAME and D-NAME on ascorbate recycling. Rat astrocyte cultures were incubated for 30 min with DHAA (400 μM) and one of the following: L-NAME (1 mM), D-NAME (1 mM), 7NI (100 μM), *NG*-propyl-L-arginine (50 μM), 1400W (100 μM), or vehicle. Plotted are mean ± SEM values for ascorbate concentration in the medium (top) and cells (bottom) for $n = 3$ independent experiments. *$p < 0.05$ compared to vehicle control.

CD1 mouse astrocytes and the glutamate-stimulated ascorbate export by the septic cells (Fig. 5). Higher DPI concentrations (0.5–5 μM) induced cell death. DPI neither reduced DHAA (400 μM) to ascorbate nor increased ascorbate (10 μM) stability in cell-free incubations (30 min; data not shown). Whereas these results seemed to support a role for NADPH oxidase, two other inhibitors of the enzyme failed to alter ascorbate recycling or export by astrocytes. Apocynin (1 mM [44]) had no effect on these parameters in the same experiments as DPI (Fig. 5) and, similarly, AEBSF (20 μM [45]) was ineffective in subsequent experiments (data not shown). The results with apocynin and AEBSF indicate that enhancement by DPI of ascorbate recycling and export does not require NADPH oxidase.

The intracellular and extracellular ascorbate concentrations produced by astrocytes were higher if the 30 min incubation with DHAA was in glucose-free rather than glucose-replete (7.5 mM) medium (Fig. 6), a finding that is consistent with DHAA uptake through GLUT1 [18]. However, septic insult lessened the sensitivity to glucose of ascorbate export by astrocytes incubated with DHAA. Therefore the initial rate of uptake of the GLUT1 substrate 3-*O*-methylglucose was measured to assess changes in the activity of this transporter. We found that 3-*O*-methylglucose uptake was inhibited after LPS + IFN-γ exposure and stimulated after DPI treatment (Fig. 7). Thus, these treatments caused similar changes in GLUT1 activity (Fig. 7) and ascorbate recycling (Fig. 5).

To assess if septic insult alters the reduction of intracellular DHAA, astrocytes were incubated for 24 h with LPS + IFN-γ or vehicle and then were incubated for 1 min with saponin and either trypan blue or DHAA (400 μM). Saponin rapidly (within a few seconds) caused extensive cell staining with trypan blue, indicating permeabilization of the plasma membrane. The concentration of ascorbate derived from DHAA after cell permeabilization was higher in septic (2.4 ± 0.1 nmol ascorbate/mg protein) than in nonseptic cultures (1.7 ± 0.2 nmol ascorbate/mg protein; $n = 4$ experiments performed in triplicate; $p < 0.05$).

Discussion

The principal findings of this study are that, first, astrocytes replace extracellular DHAA with ascorbate and second, septic insult inhibits GLUT1 activity, ascorbate recycling, and glutamate-stimulated ascorbate export. Previous studies generated DHAA from ascorbate and ascorbate oxidase in the culture medium and observed that astrocytes convert DHAA to intracellular ascorbate, but the

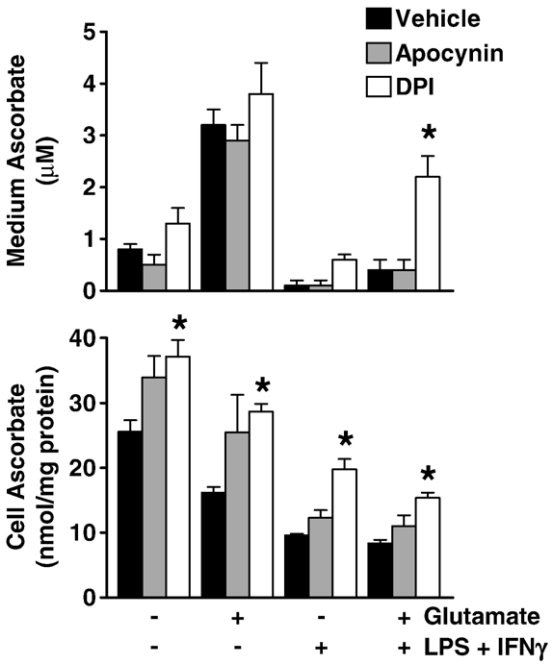

Fig. 5. Effects of the NADPH oxidase inhibitors DPI and apocynin on the concentrations of ascorbate produced from DHAA. CD1 mouse astrocytes were incubated according to the conditions described in the Fig. 1 legend. Where indicated, DPI (0.1 μM), apocynin (1 mM), or vehicle was added at the same time as LPS + IFN-γ and present for the entire experiment (24.5 h). Plotted are mean ± SEM values for ascorbate concentration in the medium (top) and cells (bottom) for $n = 5$ independent experiments. The effects of glutamate and LPS + IFN-γ had the same significance ($p < 0.05$) as in Fig. 1. Apocynin had no effect ($p > 0.05$) compared to its own vehicle. *$p < 0.05$ for the effect of DPI compared to its own vehicle.

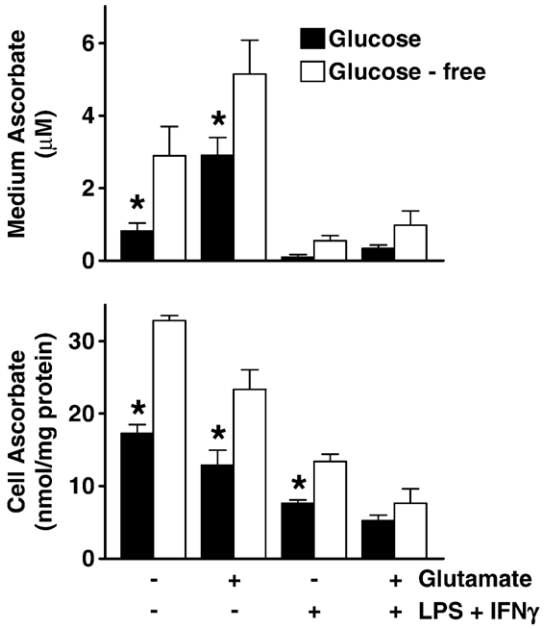

Fig. 6. Effect of glucose on the concentrations of ascorbate produced from DHAA. CD1 mouse astrocyte cultures were treated as described in the Fig. 1 legend except that the serum-free MEM, used for the final 30 min incubation with DHAA and glutamate, either contained 7.5 mM glucose or was nominally glucose-free. Plotted are mean ± SEM values for ascorbate concentration in the medium (top) and cells (bottom) for $n = 4$ independent experiments. The effects of glutamate and LPS + IFN-γ had the same significance ($p < 0.05$) as in Fig. 1. *$p < 0.05$ for the effect of glucose.

cells may have become hypoxic because ascorbate oxidase converts molecular oxygen to water [14,38]. The present experiments used synthetic DHAA instead, and the absence of ascorbate oxidase allowed measurement of extracellular ascorbate. Because the volume of intracellular water (approximately 2 μl) was much less than that of the medium (1.5 ml), the ascorbate released from astrocytes in culture was diluted much more than in brain, where the volume of intracellular fluid is fivefold greater than that of extracellular fluid [46]. Incubation with DHAA also raised extracellular ascorbate concentration in cultures of HepG2 cells [47] and luteal cells [48], showing that the role DHAA plays in ascorbate export is not restricted to astrocytes. Physiologically important levels of DHAA may occur in brain extracellular fluid because microdialysis in the striatum of freely moving mice observed that the contents of ascorbate and DHAA in the microdialysate were similar [49]. Moreover, the tissue concentration of DHAA in striatum is approximately one-tenth that of ascorbate [50,51].

The present experiments used an HPLC-based electrochemical assay to determine that authentic ascorbate is released by glutamate from astrocytes simultaneously exposed to DHAA. We reported previously that incubation with high concentrations of glutamate (EC_{50} = 200 μM) accelerates the uptake of [^{14}C]vitamin C by ascorbate-free astrocytes and stimulates the release of radioactivity from [^{14}C]ascorbate-loaded astrocytes through a mechanism that requires the swelling of endfeet and is blocked by inhibitors of volume-regulated anion channels [27]. A high rate of glutamate uptake also causes redistribution of ascorbate from cells to extracellular fluid in vivo [23,26]. Glutamate released from neurons is taken up by Na^+-dependent, high-affinity glutamate transporters in astrocyte endfeet and swells these cell processes [52], leading to activation of volume-regulated anion channels that are permeant to glutamate [53] and ascorbate [27]. The EC_{50} of glutamate for triggering ascorbate efflux is much greater than the affinity constants (K_m) of glutamatergic receptors but corresponds to the affinity constants of astrocytic Na^+–glutamate cotransporters [27]. Furthermore, blockers of these cotransporters attenuate the glutamate-induced increase in extracellular ascorbate concentration [23].

DHAA reduction in astrocytes maintains a DHAA gradient that favors continued uptake across the plasma membrane, and the consequent accumulation of intracellular ascorbate also contributes to an ascorbate gradient that drives ascorbate export. The drop in intracellular ascorbate concentration caused by glutamate in primary astrocyte cultures was approximately 30%, which was insufficient to reverse the ascorbate concentration gradient across the plasma membrane. In these neuron-free cultures some of the exported ascorbate would have been reabsorbed into astrocytes, which express the Na^+–ascorbate cotransporter SVCT2 [8], and this reabsorption would have mitigated the loss of intracellular ascorbate. But in brain the ascorbate that astrocytes put into the extracellular fluid would be taken up by neurons that express SVCT2 [8,13] and thus glutamate may elicit larger decreases in the ascorbate concentration within astrocytes in vivo.

Astrocyte endfeet are in close contact with synapses and arterioles [28]. Glutamate acts on neurons to trigger the production of oxidants [54] but stimulates astrocytes to export ascorbate that may function as a counter-balancing antioxidant. Further, because ascorbate potentiates vaso-

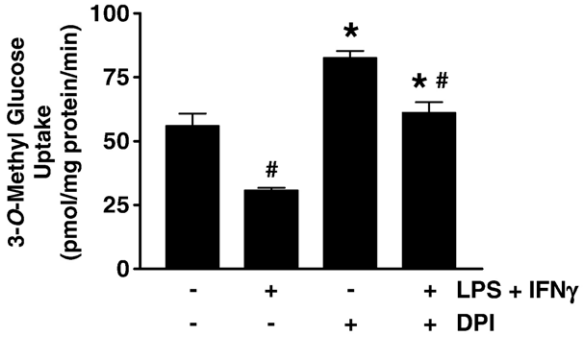

Fig. 7. Effects of septic insult and DPI on glucose transporter activity. CD1 mouse astrocytes were incubated with LPS + IFN-γ or vehicle (BSA) for 24 h and then glucose transporter activity was assessed by measuring the initial rate of uptake of 3-*O*-methyl[^{3}H]glucose (60 μM). Plotted are the mean ± SEM values for $n = 4$ independent experiments. $^{\#}p < 0.05$ for the effect of LPS + IFN-γ compared to its own vehicle. *$p < 0.05$ for the effect of DPI compared to its own vehicle.

dilation by nitric oxide [55], the exported ascorbate may decrease the resistance of cerebral arterioles. Thus, ascorbate recycling and export by astrocytes may functionally link neurons to blood vessels, contributing to a tight coupling between neuronal activity and blood-flow distribution within the brain. This astrocytic mechanism may explain how injection of DHAA into mice before focal cerebral ischemia causes dose-dependent increases in cerebral blood flow during reperfusion [11].

Septic insult inhibited the stimulation by glutamate of ascorbate export, so that the ascorbate synthesized from DHAA inside astrocytes was not released. Our observation that septic insult abolishes glutamate stimulation of ascorbate export may be explained by impairment of glutamate transporters and anion channels. On the one hand, high-affinity uptake by Na^+–glutamate cotransporters stimulates export of ascorbate [23,26,27] and LPS + IFN-γ inhibits this uptake [38]. On the other hand, septic insult lowers intracellular ATP concentration [36] to levels that may prevent activation of ATP-dependent anion channels [57] through which ascorbate may exit astrocytes [27,58].

Just as glucose diminished ascorbate recycling by astrocytes under the conditions of the present study, the elevated concentrations of glucose associated with sepsis [56] may impair GLUT1-mediated DHAA uptake and ascorbate recycling in vivo. The underlying mechanism is competition between glucose and DHAA for transport by GLUT1 [8]. But we also found that septic insult decreases ascorbate recycling and export even under glucose-free conditions. Septic inhibition of ascorbate recycling does not require nNOS or iNOS because it occurs in $nNOS^{-/-}$ and $iNOS^{-/-}$ astrocytes and is unaffected by specific inhibitors of these enzymes.

LPS induces glucose-6-phosphate dehydrogenase, the key enzyme of the pentose phosphate pathway, and thereby increases NADPH content in astrocytes [59]. The capacity of astrocytes to reduce DHAA to ascorbate could increase because NADPH is a cofactor for enzymes that catalyze this reaction [22]. Indeed, we observed that pretreatment with LPS + IFN-γ did increase reduction of DHAA to ascorbate by astrocytes that were permeabilized with saponin. However, septic insult decreased ascorbate recycling by non-permeabilized astrocytes with intact plasma membrane. The decrease was due to septic inhibition of the GLUT1 transporters that translocate DHAA into astrocytes [18], because we found that the septic insult slowed uptake of the nonmetabolizable GLUT1 substrate 3-*O*-methylglucose.

DPI increased GLUT1 activity and ascorbate recycling by nonseptic and septic astrocytes, whereas other inhibitors of NADPH oxidase did not. Independent of its action on NADPH oxidase, DPI also inhibits glucose-6-phosphate dehydrogenase [60] and may thereby elicit a compensatory increase in GLUT1 activity that accelerates DHAA uptake and so enhances ascorbate recycling. How DPI increased the ascorbate export response to glutamate is uncertain, but a partial explanation is that DPI's acceleration of ascorbate recycling raises the intracellular ascorbate concentration and therefore provides a steeper gradient favoring diffusion of ascorbate out of the cells through ion channels. The mechanism by which high concentrations of DPI (0.5–5 μM) induced cell death in our astrocyte experiments is unknown. In other cell types, however, DPI induces apoptosis through cyclin B1 down-regulation and cell cycle arrest [61].

In conclusion, astrocytes create a favorable microenvironment for neurons by clearing DHAA and exporting ascorbate. The high glutamate concentrations at synapses may stimulate astrocytes to export ascorbate at the time and place appropriate to reach active neurons. After infection, impaired astrocytic regulation of extracellular concentrations of neurotoxic DHAA [17] and neuroprotective ascorbate [9,62,63] may contribute to the clinical problem of sepsis-associated encephalopathy. Because septic insult diminishes ascorbate recycling and glutamate-stimulated export, it may attenuate the rise in extracellular ascorbate concentration that is required for activation of behavior [9]. Therefore, interventions that restore the ability of astrocytes to replace extracellular DHAA with ascorbate should be investigated as adjunctive treatments to the current antimicrobial and supportive therapy of sepsis. Finally, because the present study used primary astrocyte cultures as an in vitro model of astrocyte function, in vivo experiments will be required to establish the clinical importance of these mechanisms.

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