Peroxy nitrite Inhibits Glutamate Transporter Subtypes*

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The reuptake of glutamate in neurons and astrocytes terminates excitatory signals and prevents the persistence of excitotoxic levels of glutamate in the synaptic cleft. This process is inhibited by oxygen radicals and hydrogen peroxide (H_2O_2). Here we show that another biological oxidant, peroxynitrite (ONOO^−), formed by combination of superoxide (O_2^−) and nitric oxide (NO), potently inhibits glutamate reuptake by purified or reconstituted high affinity glutamate transporters reconstituted in liposomes. ONOO^− reduces selectively the V_max of transport; its action is fast (reaching 90% within 20 s), dose-dependent (50% inhibition at 50 μM), persistent upon ONOO^− (or by product) removal, and insensitive to the presence of the lipid antioxidant vitamin E in the liposomal membranes. Therefore, it likely depends on direct interaction of ONOO^− with the glutamate transporters. Three distinct recombinant glutamate transporters from the rat brain, GLT1, GLAST, and EAAC1, exhibit identical sensitivity to ONOO^−. H_2O_2 also inhibits reconstituted transport, and its action matches that of ONOO^− on all respects; however, this is observed only with 5–10 mM H_2O_2 and after prolonged exposure (10 min) in highly oxygenated buffer. NO, released from NO donors (up to 10 μM), does not modify reconstituted glutamate uptake, although in parallel conditions it promotes cGMP formation in synaptosomal cytosolic fraction. Overall, our results suggest that the glutamate transporters contain conserved sites in their structures conferring vulnerability to ONOO^− and other oxidants.

EXPERIMENTAL PROCEDURES

Glutamate uptake in neurons and astrocytes is essential to maintain resting extracellular glutamate concentration below levels inducing significant activation of excitatory amino acid (EAA)^2 receptors (1). Thereby it provides a high signal to noise ratio for excitatory neurotransmission and prevents harmful receptor overstimulation. Altered transport function has been associated with neuronal damage in ischemia/reperfusion injury (2) and amyotrophic lateral sclerosis (ALS) (3, 4). The uptake process is mediated by glycoproteins located in the plasma membrane of neuronal and glial cells. At least four different transporters are now cloned, i.e. GLAST (5), GLT1 (6), EAAC1 (7), and EAAT4 (8), constituting a gene family with specialized brain distributions (9–11). There is increasing evidence that glutamate transport is regulated, e.g. via protein kinase C-mediated phosphorylation (12) and arachidonic acid (13, 14). Oxygen radicals and H_2O_2 induce persistent inhibition of glial glutamate uptake, probably via direct interaction with the transport process (15). Sodium nitroprusside, a NO generator, decreases uptake into synaptosomes (16). When generated simultaneously, ‘O_2^− and NO react together at a diffusion-limited rate to form the strong oxidant ONOO^− (17). Several biological or toxic effects originally attributed to either NO or ‘O_2^− are now thought to be mediated by ONOO^− (18–22). In the present study, we address the possibility that ONOO^− affects glutamate uptake by direct interaction with the glutamate transporters.

Purification and Reconstitution of Glutamate Transporters into Liposomes—This was done as described previously (23). Briefly, crude rat brain plasma membranes were solubilized with CHAPS and centrifuged. The supernatant (CHAPS extract) was passed through a wheat germ agglutinin-agarose column, and the glycoproteins eluted with N-acetyl-glucosamine (partially purified transporters). For reconstitution, this fraction (20 mω CHAPS and 0.1–0.2 mg of protein/ml) was mixed with 1.5 volumes of a reconstitution mixture consisting of phospholipid, cholate, and salt, incubated on ice, and gel-filtered on spin columns (13) equilibrated with KCl to remove detergent and sodium ions. The liposomes form spontaneously during this gel filtration, and KP, becomes their internal medium. This preparation (10, 24)^2 contains immunoreactivity to the 3 rat brain glutamate transporters cloned to date: GLT1, GLAST, and EAAC1. In some experiments, vitamin E (1.00 or 1.25 w/w) was added to the reconstitution mixture. To test membrane integrity, liposomes were formed in the presence of ^25mRb, exposed to ONOO^− or H_2O_2, and blotted on Millipore filters (0.45-μm pores). Radioactivity retained was compared to control.

Cloning and Sequencing of the Glutamate Transporters—Rat GLT1 and EAAC1 clones were the same as published previously (6, 24). For GLAST; a cDNA library (in the pCD2 vector) from the cerebral cortex

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5 The abbreviations used are: EAA, excitatory amino acid; ALS, amyotrophic lateral sclerosis; DEA, diethylamine; MAHMA, N,N'-di- methyl-1,6-hexanediamine; SNAP, 5(1S)-N-sitron-o-N-acetylpenicillamine; DOTAP, N-[1,2-dioleoyloxy]propyl]-N,N,trimethylammonium methyl-sulfate, KP, potassium phosphate buffer; NaP, sodium phosphate buffer; PBS, phosphate-buffered saline; CHAPS, 3(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate.

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was screened with a 160-nucleotide probe from the N-terminal (5). The probe was obtained by polymerase chain reaction-amplification from cDNA of rat brain using synthetic primers. Positive clones were isolated, and plasmid DNA was prepared from 500 ml of Luria-Bertani broth cultures using the Wizard Maxiprep DNA isolation system (Promega Biotech Inc.). The cDNA fragment was subcloned into the pBluescript vector. The cDNA fragment was subcloned into the pBluebroth cultures using the Wizard Maxiprep DNA isolation system (Promega, Madison, WI) and plasmid DNA was isolated from 500 ml of Luria-Bertani strain JM103. The plasmid DNA was isolated as described above. DNA sequencing was performed by the deoxyxylan chain termination method (26) using Sequenase (U. S. Biochemical Corp.) and primers derived from the GLAST cDNA sequence as well as from the pBluescript T7 and T3 promoters. The sequences were analyzed with the DNA analysis software (Genetigene, Lillestroem, Norway) and found to be identical to GLAST.

Transfection of Glutamate Transporter Plasmid cDNAs in HeLa Cells and Reconstitution into Liposomes—HeLa cells infected with VTF-7 recombinant vaccinia virus (6, 25, 26) were transfected with pT7-GLT1, pT7-EAAC1, or pT7-GLAST plasmids (1.5 μg/ml) by electroporation (27). The sequences were analyzed with the DNA analysis software (Genetigene, Lillestroem, Norway) and found to be identical to GLAST. Glutamate Transporter Assay—Glutamate transport in liposomes was measured using an inwardly directed Na+ gradient in the presence of a negative membrane potential, as described (13, 23). Briefly, liposomes were diluted 1:10 or 1:100 with PBSG, and the uptake assay was run for 2 min, unless otherwise specified, 2 min (NO releasers), or 10 min (H2O2) and gel-filtered to remove the compounds and their by products. 80% of liposomes were isolated as described above. DNA was isolated as described above. DNA sequencing was performed by the dideoxy chain termination method (26) using Sequenase (U. S. Biochemical Corp.) and primers derived from the GLAST cDNA sequence as well as from the pBluescript T7 and T3 promoters. The sequences were analyzed with the DNA analysis software (Genetigene, Lillestroem, Norway) and found to be identical to GLAST. Glutamate Transporter Plasmid cDNAs in HeLa Cells and Reconstitution into Liposomes—HeLa cells infected with VTF-7 recombinant vaccinia virus (6, 25, 26) were transfected with pT7-GLT1, pT7-EAAC1, or pT7-GLAST plasmids (1.5 μg/ml) by electroporation (27). The sequences were analyzed with the DNA analysis software (Genetigene, Lillestroem, Norway) and found to be identical to GLAST. Glutamate Transporter Assay—Glutamate transport in liposomes was measured using an inwardly directed Na+ gradient in the presence of a negative membrane potential, as described (13, 23). Briefly, liposomes were diluted 1:3 with 130 mM NaCl, 20 mM NaP, (pH 7.4), 1% glycerol (PBSG) plus ONOO− or NO releasers (buffer a) or with Krebs/HCO3−, pH 7.4 (oxygen-saturated) plus H2O2 (buffer b), incubated at room temperature for 70 s (ONOO−), unless otherwise specified, 2 min (NO releasers), or 10 min (H2O2) and gel-filtered to remove the compounds and their by products. 80% of liposomes were isolated from spin columns were diluted 1:5.5 volumes with PBSG, and the uptake assay was started by adding 1.4 μCi of [3H]glutamate plus 2.8 μM valinomycin and incubated for 70 s later. In the kinetic experiments, uptake assay was run for 2 min, unless otherwise specified (13). Then, liposomes were collected on filters and counted for radioactivity. Statistical analysis was done by Student’s t test (2-tailed).

Lipid Peroxidation—Lipid peroxidation was measured on lipid suspensions treated with ONOO− or H2O2 as in the glutamate uptake assay, using an LPO-586 kit from Bioxtech S.A.

Monitoring of NO Release and ONOO− Accumulation—Stock solutions of DEA/NO and MAHMA/NO in 10 mM NaOH were added to NaP, (0.1 M, pH 7.4) in a standard UV cell to give final concentrations of 0.1, 1, and 10 mM. The rate of NO release was determined by monitoring the disappearance of the characteristic UV absorption (A250 = 250-252 nm) exhibited by the NO adducts (27). Nitrite accumulation was detected following the method of Ignarro et al. (28).

Cyclosporin G Formation in Cortical Crude Synaptosomal Cytosol—Synaptosomal cytosol was prepared according to Knowles et al. (29) without the final chromatographic step. 150 μl of cytosol were added to 50 μl of PBS buffer + (in mM) 5 MgCl2, 5 GTP, 1 isobutylmethylxanthine (final concentration). Incubation at room temperature was started by addition of 0.1 M DCE (control) or DEA/NO and terminated 2 min later with 20 μl of HCO3−. After centrifugation (12000 × g, 1 min), the cytosol GMP formed was determined by radioimmunoassay using the 125I-CMP kit (RPA 525, Amersham) on supernatant aliquots diluted 1:100 or 1:1000 with PBSG + 4 mM EDTA. The cyclic GMP content, expressed as nanomoles/sample, was calculated by extrapolation from a standard curve constructed following the kit manual.

RESULTS

The possible direct interaction of ONOO− with glutamate uptake was first studied on a preparation of partially purified brain glutamate transporters reconstituted in liposomes. Liposomes were exposed to ONOO− or to decayed ONOO− as control (see legend to Fig. 1). The agents were then removed by gel filtration and the uptake assay run. ONOO− (but not decayed ONOO−) inhibited uptake dose dependently (Fig. 1A). A 70-s exposure to 50 μM ONOO− reduced uptake by 50 ± 6%. Threshold inhibition (−10 ± 6%) was seen with 5 μM ONOO−, while nearly complete inhibition (−85 ± 5.1%) required 250 μM ONOO−. The ONOO− effect developed almost immediately, reaching ≥90% within 20 s and then increasing slightly in the next 2-3 min (Fig. 1B). Conversely, 50 μM ONOO− preincubated for 20 s at pH 7.4 before addition to liposomes was totally devoid of effect. Characterization of uptake kinetics without ONOO− gave a Km value of 11 ± 0.8 μM and a Vmax of 5.7 ± 1 nmol/min/mg of protein. ONOO− (50 μM, 70 s) reduced Vmax by 50% without affecting Km (Fig. 1C). Different from ONOO−, 3 fast NO donors, failed to significantly modify reconstituted glutamate uptake. In a few cases, MAHMA/NO (1 mM) or SNAP (1 mM) were used, while in most experiments we utilized DEA/NO (0.1-10 mM) (15). Uptake assay was run after preincubation (2 min) of NO and removal of DEA/NO or in its presence. In either case, the compound produced weak inhibition (~10%) that was similar at 0.1, 1, and 10 mM (Fig. 2A). However, in the same condition, NO release from DEA/NO, observed spectrophotometrically (see “Experimental Procedures”), was found to proceed dose dependently, with a t1/2 of 2 min. NO2− accumulated accordingly. Finally, we confirmed that active NO was indeed released from DEA/NO. Thus, the compound-induced
dose-dependent accumulation of cGMP in synaptosomal soluble fraction from rat cortex cGMP was 0.1 ± 0.03 nmol/sample in controls (2 min, PBSG), 0.08 ± 0.03 in 1 mM DEA, 0.17 ± 0.02 in 0.1 mM DEA/NO (+117%), and 0.33 ± 0.05 in 1 mM DEA/NO (+312%) (n = 3 in duplicate). Different from NO and similar to ONOO\(^-\), H\(_2\)O\(_2\) is another biological oxidant, inhibited reconstituted glutamate transport. Like ONOO\(^-\), H\(_2\)O\(_2\) selectively reduced uptake \(V_{\text{max}}\) however, it induced a comparable level of inhibition only at mM concentrations (38.9 ± 8.2% at 10 mM), after long exposure (10 min) and in highly oxygenated buffer (Fig. 2B).

Glutamate uptake inhibition with ONOO\(^-\) or H\(_2\)O\(_2\) could be due to peroxidation of the liposome membranes resulting either in loss of the ion gradients fueling the uptake process or changes in the lipidic environment of the transporter proteins. To exclude the first possibility, liposomes were preloaded with \(^{86}\)Rb and exposed to ONOO\(^-\) (5–50 \(\mu\)M). No release of radioactivity was observed at times parallel to uptake inhibition (Fig. 1D, left). To address the second possibility, we prepared liposomes containing the lipophilic antioxidant vitamin E among the lipid constituents (1:100 or 1:25 w/w). The effect of ONOO\(^-\) (50 \(\mu\)M, 70 s) was compared in liposomes with and without vitamin E, finding the same extent of uptake inhibition in all types of liposomes, independent of their vitamin E content (Fig. 1D, right). Moreover, by use of a standard assay, we failed to detect any significant signal of lipid peroxidation in conjunction with uptake inhibition. H\(_2\)O\(_2\) (10 mM, 10 min) behaved identically to ONOO\(^-\) in the above experiments. We then tested the effect of ONOO\(^-\) and H\(_2\)O\(_2\) on uptake by recombinant transporter subtypes. HEK293 cells were transfected with cDNAs encoding the glutamate transporters GLT1, GLAST, or EAAC1 and their cell membranes reconstituted into liposomes. Uptake capacity was found about 100- (GLT1), 20- (GLAST), and 50-fold (EAAC1) higher with respect to mock-transfected cells similarly reconstituted. As shown in Table I, \(\mu\)M ONOO\(^-\) inhibited uptake by any transporter subtype dose-dependently and with equivalent potency. Again, mM H\(_2\)O\(_2\) behaved comparably.

**DISCUSSION**

ONOO\(^-\), a biological oxidant and the combination product of O\(_2\) and NO, potently inhibits purified and recombinant glutamate transporters reconstituted in liposomes. NO alone appears unable to directly modify glutamate transport. Thus, 3 fast NO releasers, MAHMA/NO (1 mM), SNAP (1 mM), and DEA/NO (0.1, 1, and 10 mM), failed to inhibit reconstituted transport. To be sure that active NO indeed was released, we checked the dose- and time-dependent NO disappearance from the DEA/NO adduct paralleled NO\(_2\) accumulation and that DEA/NO dose-dependently enhanced cGMP levels in synaptosomal soluble fraction.

We reported previously that oxygen radicals and H\(_2\)O\(_2\) induce long-lasting decreases of glutamate transport in astrocytic cultures, probably due to protein oxidation (15). In agreement, here we find that H\(_2\)O\(_2\) directly affects reconstituted glutamate transport. Its mode of action is superimposable to that of ONOO\(^-\). Thus, both agents selectively reduce uptake \(V_{\text{max}}\). This effect involves some persistent modification of proteoliposomes, because it is observed after removal of the compounds and their by products via gel filtration. However, it unlikely depends on oxidation of the lipid component. Thus, (a) ONOO\(^-\) or H\(_2\)O\(_2\) inhibition is not attenuated in liposomes containing vitamin E among the membrane constituents (up to \(\sim\)1:10 molar ratio with phospholipids) and (b) a standard lipid per-oxidation assay does not reveal detectable levels of malonaldehyde or 4-hydroxyalkenals paralleling uptake changes. Generalized membrane damage is also ruled out because no radioactive leakage is observed from \(^{86}\)Rb-filled liposomes exposed to 5–50 \(\mu\)M ONOO\(^-\) or to 10 mM H\(_2\)O\(_2\). Therefore, uptake inhibition likely depends on direct interaction of ONOO\(^-\) and H\(_2\)O\(_2\) with the glutamate transporter proteins resulting in covalent modification of their structure. Recombinants of the 3 major cloned rat brain subtypes, GLT1, GLAST (both glial), and EAAC1 (neuronal) are all similarly inhibited by ONOO\(^-\) (or H\(_2\)O\(_2\)). Lack of differential sensitivity suggests that one or more "oxidant-vulnerable site(s)" are present in conserved regions of these proteins.

Although similar in the mode of action, ONOO\(^-\) is significantly more potent and rapid than H\(_2\)O\(_2\) in inhibiting reconstituted glutamate transport. Thus, while H\(_2\)O\(_2\) effect is seen at mM concentrations and after several minutes, ONOO\(^-\) acts in the \(\mu\)M range and within seconds (\(\sim\)90% of inhibition at 20 s, paralleled by disappearance of the active species), in line with its reported half-life and rate of decomposition at pH 7.4, resulting in the formation of potent oxidant intermediates with reactivity of hydroxyl radical (OH) and other reactive species such as nitronium ion and nitrogen dioxide (31). Moreover, ONOO\(^-\) is effective in normal air-equilibrated buffer, while H\(_2\)O\(_2\) only in a hyperoxegenated buffer, suggesting that its transformation into OH\(^-\) via ‘O\(_2\)’ driven Fenton reaction may be required (32). A common primary target for ONOO\(^-\), H\(_2\)O\(_2\) or downstream products such as ‘OH is oxidation of cysteine sulf-hydryl groups. Thiol oxidation by ONOO\(^-\) proceeds 103-fold faster than with H\(_2\)O\(_2\) (32). In addition, ONOO\(^-\) could induce nitrosylation and/or nitration of aromatic amino acids. Targeting of transporter SH groups by H\(_2\)O\(_2\) and ONOO\(^-\) would be consistent with our previous observation that glial uptake, inhibited by oxygen radicals, is significantly restored with thioredoxin, a disulfide-reducing agent (15).
Inhibition of glutamate uptake by ONOO$^-\$ may be of pathological significance, contributing to the build up of excitotoxic extracellular glutamate. The conditions for local formation of ONOO$^-\$ at glutamate synapses exist. Thus, both O$_2^-$ and NO can be generated as a result of activation of EAA receptors (33, 34). If formed in conjunction, O$_2^-$ and NO react together to give ONOO$^-\$ at a diffusion-limited rate (17). Several pathological situations would favor this process, e.g. because NO levels are enhanced (via activation of inducible NO synthase (35, 36)), because O$_2^-$ catabolism is reduced, or superoxide dismutase (SOD) activity is altered, as proposed for mutant SOD1 in familial ALS (37, 38). Indeed, enhanced protein tyrosine nitration, a marker for ONOO$^-\$ formation, was recently reported in mutant SOD1 transgenic mice (39) as well as in other animal models of neurodegenerative diseases thought to involve excitotoxicity (40, 41). The case of ALS is intriguing, as this pathology has been associated with defect of glutamate transport (2). μM ONOO$^-\$ is highly neurotoxic to cultured neurons (21). Due to its half-life, ONOO$^-\$ can travel quite a distance from the site of production to damage critical neuronal constituents, such as the neurofilament proteins (37, 38) or the mitochondrial enzyme aconitase (19, 20). The present study indicates that the glutamate transporters could be other important targets of ONOO$^-\$ toxicity.

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