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Glutamate Uptake Is Inhibited by Arachidonic Acid and Oxygen Radicals via Two Distinct and Additive Mechanisms

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SUMMARY

Reuptake of glutamate in astrocytes, a critical mechanism involved in the maintenance of physiological excitatory amino acid neurotransmission, is inhibited by both arachidonic acid (AA) and reactive oxygen species (ROS), via incompletely defined molecular mechanisms. Because ROS are generated during AA metabolism and AA can be released as a result of ROS-mediated phospholipase A_2 activation, it seems likely that their effects on uptake are mediated by a common mechanism. However, here we show that rapid (10-min) uptake inhibitions by AA or by ROS generated by the xanthine plus xanthine oxidase (XO) reaction are selectively abolished by distinct agents; bovine serum albumin (BSA) acts only on AA, whereas the scavenger enzymes superoxide dismutase (SOD) and catalase (CAT) and the disulfide-reducing agent dithiothreitol (DTT) act only on ROS. Moreover, when added together, xanthine/XO and AA decrease uptake in a fully additive manner. In particular, the effect of xanthine/XO is seen also in the presence of maximal AA inhibition. No major signs of cell damage or chemical reaction between AA and radicals accompany their cumulative effects on uptake. Finally, uptake inhibition elicited by AA and xanthine/XO together is attenuated but not blocked by either BSA, DTT, or SOD/CAT individually, whereas it is fully blocked and substantially reversed by a combination of SOD/CAT and BSA or SOD/CAT, DTT, and BSA. Together, these data indicate that AA and ROS act on glial glutamate transport via distinct noninteracting mechanisms. Therefore, they could independently and additively contribute to the impairment of reuptake function, a phenomenon observed in pathological conditions such as ischemia/reperfusion injury.

L-Glutamate and related EAAs play an essential role in fast synaptic transmission and synaptic plasticity in the central nervous system (1, 2). However, dysfunction of the EAA system leads to neuronal death by "excitotoxicity," a mechanism thought to take place in several acute and long term neurodegenerative brain diseases (3). Rapid development of neuronal injuries is often observed in response to enhanced extracellular glutamate concentration and overstimulation of EAA receptors, particularly of the NMDA type. Ca^{2+} entry through NMDA receptor channels triggers several metabolic events, including release of AA (4) and formation of ROS (5). Both AA and ROS have been found to inhibit high affinity Na^+-dependent glutamate reuptake (6, 7) and to enhance extracellular glutamate levels (8, 9), thereby possibly starting a dangerous cycle. These agents likely affect glutamate transport directly, because both AA (6) and ROS (7) specifically reduce electrogenic uptake current in voltage-clamped astrocytes without important effects on membrane holding current. However, at present it is not clear whether AA and ROS act via common or separate molecular mechanisms and whether their inhibitory effects are additive.

AA induces functional modulation of several receptor or channel proteins. Its effects depend in some cases on a direct action of the fatty acid on the target protein or its lipidic microenvironment (10, 11) and in others on the formation of downstream mediators such as eicosanoid metabolites (12) or ROS intermediates (13). Thus, ROS may be formed during cycloxygenase or lipoxygenase metabolism (14). NMDA-dependent superoxide anion production would be secondary to AA release (5). Moreover, AA administration to cortical slices (15) or cultured astrocytes (16) results in progressive ROS accumulation. In turn, ROS have been shown to enhance free AA levels by increasing PLA_2 activity (17) and inducing phospholipid hydrolysis (18). Therefore, based on these data, one could hypothesize that AA and ROS stimulate each other and converge to affect glutamate uptake via a common inhibitory pathway. However, in our previous studies characterizing the effects of AA (19) and ROS (7) individually, we have observed some important differences; noticeably, AA selectively reduces the V_{max} of uptake, whereas ROS both decrease V_{max} and slightly enhance K_m. Moreover, whereas the AA effect rapidly

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ABBREVIATIONS: EAA, excitatory amino acid; AA, arachidonic acid; ROS, reactive oxygen species; DTNB, 5,5′-dithio-bis(nitrobenzoic acid); SOD, superoxide dismutase; XO, xanthine oxidase; CAT, catalase; BSA, bovine serum albumin; DTT, dithiothreitol; NMDA, *N*-methyl-D-aspartate; LDH, lactate dehydrogenase; SH, sulfhydryl; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; PLA_2, phospholipase A_2.

reaches a plateau and is reversible, the ROS effect increases progressively and is persistent. Here we present direct evidence that AA and ROS affect glutamate transport via two distinct, noninteracting, and additive mechanisms.

Materials and Methods

Astrocytic cell cultures. Astroglial cells were obtained from the cerebral cortices of newborn rats by mechanical dissociation, plated, and grown *in vitro* for 2 weeks as described previously (19). The primary cultures were then enriched (>95%) in type 1 astrocytes according to the procedure of McCarthy and de Vellis (20), replated, and kept for 4–5 days until reaching monolayer confluence.

Na^+-dependent glutamate uptake assay in astrocytes. Cortical astrocytic cultures (≈200 μg of protein/35-mm Petri dish) were incubated for 10 min at 25°, under circular shaking conditions, in the presence of different agents in oxygenated Krebs/bicarbonate buffer (124 mM NaCl, 4.6 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2 \cdot 6H_2O$, 0.416 mM $KH_2PO_4 \cdot 2H_2O$, 26.75 mM $NaHCO_3$, 10 mM glucose, adjusted to pH 7.4) containing 40 μM [^{3}H]glutamate (63.5 Ci/mmol; isotopic dilution, 1/25,000; NEN, Dreieich, Germany). The Na^+-independent component of uptake, determined in samples where Na^+ was replaced by choline, was determined to be minor and was subtracted from total [^{3}H]glutamate uptake. Uptake was stopped by the addition of ice-cold buffer with 100-fold excess unlabeled glutamate (to displace [^{3}H]glutamate bound to cells but not transported inside), and cells were removed from the culture dishes with 0.2 N NaOH and counted by liquid scintillography for incorporated radioactivity. Comparable results were obtained by substituting choline for Na^+ in the stop solution to block transport function during washes. Protein content was measured in aliquots of solubilized cells (21). The [^{3}H]glutamate concentration utilized in this study is close to the previously reported K_m value for Na^+-dependent high affinity glutamate uptake in rat cortical astrocytes (19, 22). Absolute uptake values ranged between 48 and 65 nmol/mg of protein/10 min (see also Ref. 7) and have been expressed as percentage of control, to facilitate comparisons among different experiments. In some of the experiments different protocols were used (for details, see figure legends).

LDH activity assay. LDH activity, as a marker of cell membrane disruption (23), was measured in samples (0.15 ml) taken from the supernatant medium of astrocytic cultures exposed to different agents for the same time as in the uptake assay (10 min). A diagnostic LDH kit from Merck (catalogue number 3399) was used. Data are expressed as units/liter/minute/milligram of protein, following the calculation protocol of the kit manual.

SH group oxidation by ROS. The different capacity of oxygen radicals to oxidize SH groups in the absence or presence of AA was measured with a spectrophotometric assay. DTT at a fixed concentration (50 μM) was used as the SH group donor. ROS were generated by the xanthine (500 μM)/XO (50 milliunits/ml) system. AA (15–500 μM in 1% DMSO) was added in some samples and in the respective controls (together with DTT and xanthine). The reaction was started by addition of XO to 1 ml of Krebs/O_2 buffer containing xanthine plus DTT (with or without AA) and was allowed to proceed for 10 min. Then, 1 ml of 1 mM DTNB was added. DTNB immediately reacted with residual nonoxidized DTT and was reduced to thio-bis(nitrobenzoic acid), resulting in a color change (to yellow) that was monitored at 432 nm with a spectrophotometer. Comparison with a calibration curve of thio-bis(nitrobenzoic acid) levels obtained by reaction of different DTT concentrations (1–50 μM) with 1 mM DTNB allowed the calculation of the amount of DTT not oxidized by ROS. From this, the amount of DTT oxidized was then calculated and expressed as percentage of initial DTT.

HPLC analysis of AA transformation in the presence of oxygen radicals. [^{3}H]AA (1 μCi, 60–100 mCi/mmol; NEN), with or without 50 μM unlabeled AA, was added to astrocytic cultures with xanthine (500 μM), with or without XO (50 milliunits/ml), and incubation was carried out under the same conditions (time, temperature, and buffer) as used for the glutamate uptake assay (see above). The reaction was terminated by addition of formic acid and cooling on ice. [^{14}C]Oleic acid (0.1 μCi, 60 Ci/mmol) was added as an internal standard, and samples were extracted with ethyl acetate (1:1, v/v) and injected onto a Jasco 880 PU HPLC coupled to a Flo-one/β series A100 Radiomatic (Canberra-Packard) radiodetector. Chromatographic analysis using a reverse phase C-18 column (250 × 4.6 mm, 5-μm particle size; Bio-Rad) was performed following the method of Powell (24) with slight modification, allowing for simultaneous detection of both AA and its more polar derivatives with prostanoid, hydroxyeicosanoid, hydroperoxyeicosanoid, and epoxyeicosanoid structures. Protein content was assessed in the aqueous phase of the extraction mixture, which contained the cell homogenate (21). Data were calculated by normalizing the cpm of chromatographic peaks for internal standard recovery and sample protein content.

Materials. XO (0.4–1.1 units/mg) was obtained from Fluka. The purity of the enzyme was checked by gel electrophoresis, which yielded a single major band of ≈130 kDa. The enzymes SOD (3250–3570 units/mg) and CAT (48,700 units/mg) and the compounds AA, xanthine, DL-DTT, and α-tocopherol (free and acetate ester) were purchased from Sigma, DTNB was from Aldrich, and fatty acid-free BSA was from Calbiochem. All reagents were prepared fresh for each experiment. AA, made in stock aliquots (10^{-2} M) in DMSO and stored at −80°, was applied to experimental preparations after rapid sonication, at a final DMSO concentration of 1% (v/v). Coadministration of AA and xanthine/XO was performed by rapid dilution of a small volume of AA (100×) to its final concentration in the culture dish just after addition of xanthine/XO.

Results

Pharmacological distinction of AA- and ROS-induced glutamate uptake inhibition. In a first set of experiments, we tested whether the effects of AA and ROS on glutamate uptake were sensitive to the same pharmacological blockers (Fig. 1). As reported previously, inhibition by AA (50 μM) was prevented by BSA (1 mg/ml), whereas inhibition by the oxygen radical-generating system xanthine (500 μM) plus XO (50 milliunits/ml) was abolished by either the scavenger mixture SOD (90 units/ml) plus CAT (3000 units/ml) or the disulfide-reducing agent DTT (2 mM). However, when the opposite treatments were performed, the effect of xanthine/XO was nearly unaffected by BSA and that of AA was totally insensitive to SOD plus CAT or DTT. Basal uptake was unaffected by SOD plus CAT, whereas BSA as well as DTT slightly enhanced transport, in agreement with our previous observations (7, 19). Nonetheless, these treatments failed to significantly reduce inhibition by xanthine/XO and AA, respectively. Finally, AA as well as xanthine/XO inhibition was similarly unaffected by the lipophilic antioxidant α-tocopherol (20–200 μM, in 2% DMSO) (data not shown).

Additive effects of AA and ROS on glutamate uptake with either simultaneous or sequential administration. In a second set of experiments we applied AA and xanthine/XO simultaneously to astrocytes, to see whether their inhibitory effects on glutamate uptake would be additive or occlusive. A fixed amount of xanthine/XO (500 μM/50 milliunits/ml) was selected, whereas AA was added either at a concentration close to the IC_{50} for uptake inhibition (15 μM) or at two concentrations reported to be at a plateau of the effect (50 and 100 μM) (6, 19). As shown in Fig. 2, xanthine/XO and AA at 15 μM, 50 μM, and 100 μM, when individually incubated, in this set of experiments reduced uptake by 26 ± 3.17%, 18.5 ± 2.18%, 36.7

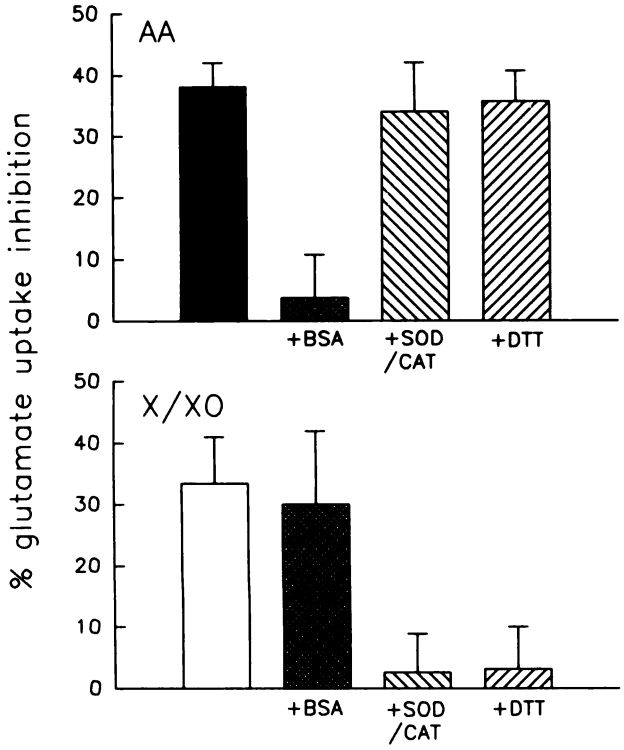

Fig. 1. Pharmacological distinction between AA- and ROS-induced glutamate uptake inhibition. *Upper*, uptake inhibition induced by AA (50 μM, in 1% DMSO) in the absence (■) or presence of different agents; *lower*, effect of the mixture of xanthine (500 μM) plus XO (50 milliunits/ml) without (□) or with the same agents as above, i.e., BSA (1 mg/ml), SOD (90 units/ml) plus CAT (3000 units/ml), or DTT (2 mM). In all experiments agents were added together with AA or xanthine/XO and uptake assays were performed for 10 min in their presence and compared with appropriate controls (with or without DMSO). The value for each group is the mean ± standard error of six different experiments. Only the AA plus BSA group was statistically different from AA alone, whereas the xanthine/XO plus SOD/CAT and xanthine/XO plus DTT groups were homogeneously different from xanthine/XO alone (one-way analysis of variance followed by the Tukey method of multiple comparisons).

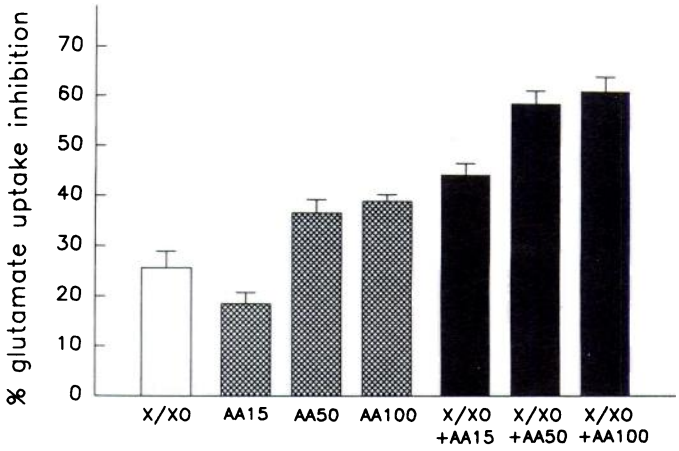

Fig. 2. Coadministration of AA and ROS, resulting in additive inhibition of glutamate uptake. Glutamate uptake assays were performed for 10 min in the presence of xanthine/XO (500 μM/50 milliunits/ml) (□), AA (15, 50, or 100 μM) (■), or a combination of them (■) and were compared with controls. For the coadministration procedure, see Materials and Methods. The value for each group is the mean ± standard deviation of nine different experiments. Groups with AA plus xanthine/XO are all significantly different from groups with either AA (at the corresponding concentration) or xanthine/XO ($p < 0.001$, two-tailed Student's *t* test).

TABLE 1

Extracellular LDH activity in astrocytic cultures after exposure to AA and ROS

Astrocytic cultures were exposed for 10 min (as in the uptake assay) to different agents. Each determination was performed in duplicate, and the results of at least four experiments were pooled. One-way analysis of variance reveals that none of the treatments with AA, Xanthine/XO, or their combination resulted in extracellular LDH activity significantly different from controls. Values are mean ± standard deviation.

Treatment	LDH activity
	units/liter/min/ mg of protein
Control	22.9 ± 8.6
Xanthine/XO	25.9 ± 6.6
AA, 15 μM	23.1 ± 7.2
Xanthine/XO + AA, 15 μM	24 ± 2.9
AA, 50 μM	23.3 ± 5.7
Xanthine/XO + AA, 50 μM	28.9 ± 7.4
Triton X-100, 0.1%	1156 ± 62

± 2.66%, and 38.9 ± 1.36%, respectively. Because 50 and 100 μM AA decreased uptake to the same extent, we confirmed that, indeed, the fatty acid effect is saturated at these concentrations. Therefore, we went on to test xanthine/XO efficacy in the presence of either submaximal or maximal AA inhibition. Coadministration of xanthine/XO and 15 μM AA led to uptake inhibition of 44.4 ± 2.25%, whereas xanthine/XO plus 50 μM AA decreased uptake by 58.8 ± 2.55% and xanthine/XO plus 100 μM AA decreased uptake by 61.4 ± 3.01%. Therefore, inhibition by xanthine/XO plus 15 μM AA reached a level identical to the sum of their individual effects, whereas xanthine/XO plus 50 μM AA and xanthine/XO plus 100 μM AA inhibited uptake by 94% and 95% of the sums, respectively.

Although this finding suggests that AA and ROS might act via independent mechanisms, it is insufficient, by itself, to demonstrate this hypothesis. A trivial possibility is that AA and ROS, although not individually toxic (7, 19), when added together affect the cell membrane integrity, disrupt the ion gradients that drive uptake, and thereby result in an apparent enhancement of inhibition. To clarify this point we measured the extracellular release of LDH, a marker of membrane leakage (23), under the same experimental conditions as in the uptake assays described above. As shown in Table 1, neither AA, xanthine/XO, nor AA plus xanthine/XO caused significant increase of extracellular LDH, compared with controls. Another possibility is that ROS formed by the xanthine/XO reaction chemically interact with AA during coadministration, generating new and more potent inhibitory species (e.g., AA peroxides). If this is the case, then enhanced uptake inhibition should occur when AA and xanthine/XO are added together but not when they are applied in a sequential nonoverlapping manner. Taking advantage of the fact that uptake inhibition by ROS is persistent and outlasts their presence (7), we tested this possibility. The effect of AA (15 μM) was measured in cells that had been previously treated for 10 min with xanthine/XO, which showed reduced transport capacity (−35.5 ± 1.9% of the uptake observed in cells that had been pre-exposed to control buffer) (Fig. 3), and after ROS had been inactivated by reaction with SOD plus CAT. Under these conditions, AA further reduced uptake to −58.6 ± 4.5% of control, with a net 23.1% increment of inhibition. Interestingly, 15 μM AA caused 23.4 ± 2.2% inhibition in parallel experiments in cells that had been pretreated with control buffer. In one experiment with 100 μM AA,

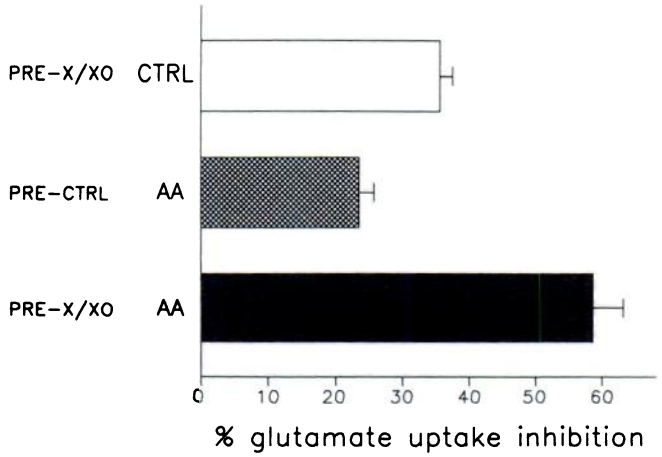

Fig. 3. Sequential administration of ROS and AA, resulting in summation of their inhibitory effects. [^{3}H]Glutamate uptake assays were performed for 10 min in the absence (*CTRL*) or presence (*AA*) of 15 μM AA, after a 10-min pre-exposure to xanthine/XO (*PRE-X/XO*) or to control conditions (*PRE-CTRL*). In all groups preincubation was terminated by substitution of the cell supernatant with a solution containing a cocktail of ROS scavengers (90 units/ml SOD plus 3000 units/ml CAT). Then, after three washes, [^{3}H]glutamate in Krebs buffer was added to start the uptake assay. Data are expressed as percentage inhibition versus control pretreatment/control treatment. The value for each group is the mean ± standard deviation of six different experiments. The xanthine/XO pretreatment/AA treatment group is statistically different from both xanthine/XO pretreatment/control treatment and control pretreatment/AA treatment groups ($p < 0.001$, two-tailed Student's *t* test).

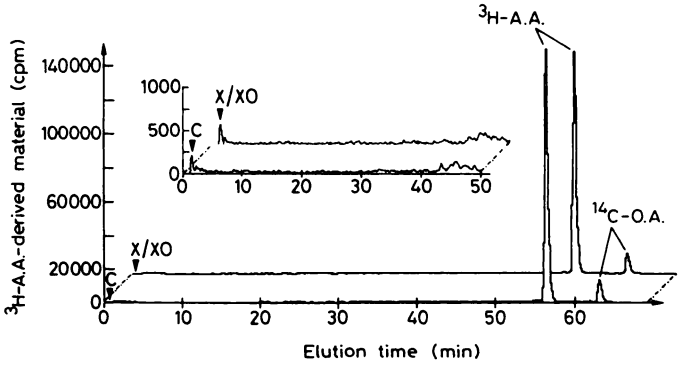

Fig. 4. Chromatographic profile of [^{3}H]AA-derived material after exposure to ROS. Representative reverse phase HPLC chromatograms of [^{3}H]AA-derived material obtained after a 10-min incubation of [^{3}H]AA in control buffer plus xanthine (*C*) or buffer plus xanthine/XO (*X/XO*) bathing intact astrocytic cultures are shown. In some experiments 50 μM unlabeled AA was added to catalyze AA conversion, but this resulted in a comparable chromatographic profile. For details, see Materials and Methods. *Inset*, same chromatograms at ≈60-fold magnification, with expanded time scale between 0 and 50 min, corresponding to the elution area expected for a wide variety of AA oxidation products. The main peaks have the same retention time as the authentic [^{3}H]AA standard. Smaller peaks ([^{14}C-*O.A.*) correspond to the internal standard ([^{14}C]oleic acid) and indicate comparable recovery for control buffer plus xanthine and xanthine/XO groups after the extraction procedure.

uptake was found to be reduced by 76% in xanthine/XO-pretreated cells but by only 42% in control cells. Therefore, very similarly to coadministration, sequential application of xanthine/XO and AA resulted in additive inhibition of glutamate uptake.

Evaluation of the cross-interference of AA and ROS with the respective putative mechanisms of uptake inhibition. The precise mechanism by which AA inhibits glutamate uptake is not clear. However, blockade or reversal of its effect by BSA (19, 25) and insensitivity to metabolic blockers (6) and to radical scavengers and antioxidants (see Fig. 1) suggest that the presence of the free fatty acid form of the molecule is required. As a consequence, to retain its inhibitory potency in the presence of oxygen radicals AA should not be significantly consumed by reaction with them. We tested this point by adding AA (1 μCi of [^{3}H]AA, with or without 50 μM unlabeled AA) to astrocytic cultures, with or without the ROS-generating system xanthine/XO, under our standard glutamate uptake assay conditions. After the incubation period, the amounts of residual free [^{3}H]AA and of possible oxidation products were evaluated by a reverse phase HPLC method and compared for the two experimental conditions. As seen in Fig. 4, chromatograms of [^{3}H]AA-derived material under control conditions and in the presence of xanthine/XO were almost identical and showed a single major peak, with the retention time of authentic [^{3}H]AA. No [^{3}H]AA enzymatic products were detected, in agreement with a previous study (25). As a result of six different observations, the amount of [^{3}H]AA recovered in the group incubated with xanthine/XO was determined to be 93.3 ± 18.8% (not significant, paired Student's *t* test) of that in the group without xanthine/XO. No other defined peaks with areas of >0.3% of the [^{3}H]AA peak area were detected in either experimental group, and even the background radioactivity measured at retention times attributable to derivatives more polar than [^{3}H]AA (between 0 and 50 min) (Fig. 4, *inset*) was comparable in samples incubated with and without xanthine/XO.

TABLE 2

Oxidation of SH groups by ROS and interaction with AA

For experimental details, see Materials and Methods. Note that DTT was present in excess, so that the SH groups would not be fully oxidized by Xanthine/XO in 10 min. Oxidation with Xanthine/XO, with or without AA, was calculated with respect to the corresponding control containing Xanthine, with or without AA. Data are the average ± standard deviation of three different experiments performed in duplicate.

Treatment	SH group oxidation
	%
Xanthine/XO	82.7 ± 2.1
Xanthine/XO + AA, 15 μM	83 ± 1
Xanthine/XO + AA, 50 μM	78.7 ± 4.7
Xanthine/XO + AA, 100 μM	69.3 ± 2.1[a]
Xanthine/XO + AA, 250 μM	44 ± 3[b]
Xanthine/XO + AA, 500 μM	29.3 ± 1.5[c]

[a] Paired Student's *t* test with two-tailed analysis indicates significant reduction of SH oxidation by Xanthine/XO in the presence of AA at ≥100 μM, compared with corresponding controls, $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

Inhibition of glutamate uptake by ROS might depend on oxidation of relevant protein thiol groups (7). To test whether AA would interfere with such processes, e.g., by acting as a competitive substrate for ROS, we measured thiol group oxidation using DTT (50 μM) as an SH group donor. Table 2 shows that oxygen radicals generated by the xanthine plus XO reaction (500 μM plus 50 milliunits/ml) in 10 min oxidized SH groups by ≥80%. Addition of AA at increasing concentrations (15–500 μM) to xanthine/XO caused a progressive decrease of SH oxidation. However, AA influenced oxidation only when present at concentrations of ≥100 μM, whereas at 15 or 50 μM (i.e., at the concentrations primarily used in the present studies on glutamate uptake) it did not modify the xanthine/XO effects.

Restoration, by a combination of blockers of both effects, of glutamate uptake function inhibited by coadministration of AA and ROS. In this final set of experiments we analyzed the effects of blockers of either AA or ROS mechanisms (Fig. 1), or of combinations of these blockers, on uptake decline induced by coapplication of AA and ROS. To better evaluate whether these agents would attenuate, block, or even reverse the inhibitory process, we added them some time after AA plus xanthine/XO, when uptake was already partially impaired. As shown in Fig. 5, the rate of uptake decreased soon after application of xanthine/XO (500 μM/50 milliunits/ml) plus AA (15 μM), and within 20 min it was reduced to ≈40% of its control value. With 50 μM AA the same level of inhibition was reached within 10 min (data not shown). Inhibition was particularly fast in the first minutes, and ≈60% of the effect seen at 20 min was already present after 5 min. Inhibition then continued to increase but at a progressively lower rate. Based on these observations, pharmacological blockers were added 5 min after AA plus xanthine/XO and overall inhibition was measured at 20 min. Application of either a ROS blocker (90 units/ml SOD plus 3000 units/ml CAT or 2 mM DTT) or an AA blocker (1 mg/ml BSA) gave similar effects, partially reducing the rate of uptake decline without stopping the process.

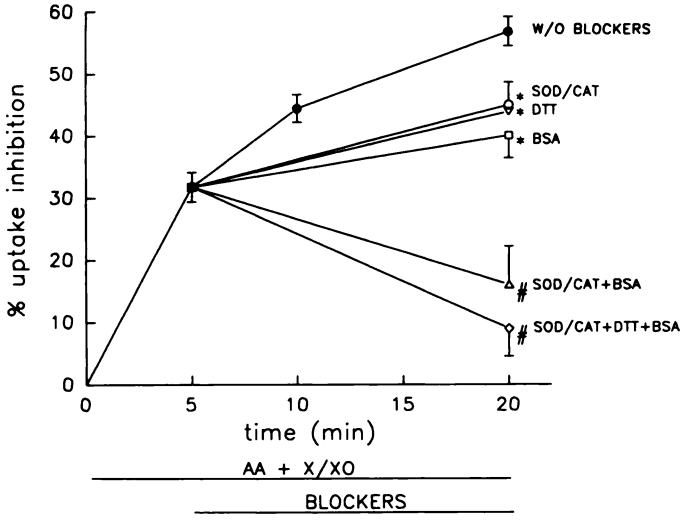

Fig. 5. Glutamate uptake inhibition by conjunct action of AA and ROS and restoration by pharmacological blockers. ●, Time course of uptake inhibition by the combination of 15 μM AA plus xanthine/XO (500 μM/50 milliunits/ml). *Open symbols*, levels of uptake inhibition by AA plus xanthine/XO in the presence of different AA or ROS blockers or different combinations of them. Concentrations of various agents were as in Fig. 1. AA and xanthine/XO were coadministered to astrocytes and [^{3}H] glutamate uptake assays were performed in parallel. Inhibition was calculated at 5 and 20 min by comparison with corresponding controls. Blockers were added at 5 min and uptake values were measured at 20 min. The value for each group represents the mean ± standard error of six different experiments. Statistical comparison between experimental groups was performed by one-way analysis of variance followed by the Tukey method. *, SOD/CAT, DTT, and BSA groups are all significantly different from the AA plus xanthine/XO group (*W/O BLOCKERS*) ($p < 0.01$) and homogeneous among themselves; #, SOD/CAT plus BSA and SOD/CAT plus DTT plus BSA groups are different from both the AA plus xanthine/XO group and the aforementioned groups ($p < 0.01$) and homogeneous among themselves. Inhibition by AA plus xanthine/XO in the presence of SOD/CAT, DTT, or BSA is significantly enhanced with respect to that observed at 5 min, whereas inhibition in the presence of SOD/CAT plus BSA or SOD/CAT plus DTT plus BSA is significantly reduced.

In contrast, a combination of AA and ROS blockers not only produced full blockade but also induced significant uptake recovery. In particular, addition of DTT, BSA, and SOD plus CAT produced, at 20 min, an overall inhibition of ≈9%, representing 15% of the level expected in the absence of these agents. Taking into account the fact that when the agents were applied uptake inhibition was already ≈33%, the agents were able to induce >70% uptake recovery.

Discussion

Enhanced levels of free fatty acids such as AA, as well as formation of ROS, have been reported, often conjunctly, in several acute and long term neurodegenerative diseases and have been suggested to take part in the development of neurotoxicity (26, 27). Oxidation products, including reactive intermediates, eicosanoids, and lipid peroxides, are thought to be the common mediators of the toxicity produced by fatty acids and free radicals (28). However, a comprehensive understanding of the mode of action of these species and of their interrelations is still lacking. An effect exerted by both AA and ROS is inhibition of glutamate uptake, which leads to enhanced extracellular levels of this potentially toxic transmitter (3). We have previously shown that AA and ROS affect uptake kinetics differently and that the onset, duration, and saturability of their effects are also different (compare Refs. 7 and 19). Here we present more direct evidence that they reduce glutamate transport via two distinct noninteracting mechanisms. Thus, (a) agents able to abolish AA effects fail to protect against ROS, and vice versa; (b) xanthine/XO and AA administered together to astrocytes inhibit uptake in an additive manner and, in particular, xanthine/XO retains its full effect in the presence of maximal AA inhibition; and (c) uptake inhibition by AA plus xanthine/XO is only partially reduced by any AA or ROS blocker, whereas it is fully antagonized by a combination of blockers.

Previous studies from our laboratory, as well as from other laboratories, indicate that AA does not act on glutamate uptake through mechanisms mediated by eicosanoid formation or protein kinase C activation or indirectly, e.g., via Na^+/K^+-ATPase inhibition (6, 19, 25). The present data also argue against a mechanism involving ROS intermediates (14). Based on the observation that BSA, a protein that binds free but not phospholipid-esterified fatty acids, relieves inhibition by either AA or the PLA_2 activator melittin (19, 25), we proposed that the uptake rate is decreased as a result of interaction of the free fatty acid molecule with the glutamate transporter or its lipidic microenvironment (19). Indeed, we now have direct evidence that AA interferes with high affinity Na^+-dependent glutamate transport directly. Thus, it reversibly inhibits transport by a purified glial glutamate transporter (29) reconstituted in artificial liposomes.[1] On the other hand, ROS also likely interact with glutamate transport directly, because they specifically inhibit Na^+-dependent electrogenic uptake current in voltage-clamped astrocytes (7). Other indirect mechanisms (e.g., impairment of Na^+/K^+-ATPase) might provide an additional minor component of inhibition (7). However, as suggested by selective reversal with DTT, the molecular mode of action of ROS differs from that of AA and includes long-lasting oxidation of functionally relevant thiol groups (7) (see also Ref. 30).

[1] D. Trotti, manuscript in preparation.

Mechanisms of cross-induction, such as PLA_2 stimulation by oxidant species or, conversely, free radical formation in response to AA, although documented in central nervous system preparations (5, 15–17), are unlikely to play an important role in the inhibition of glutamate transport by AA and ROS. If they did play a role, common effectors should mediate the actions of these agents and, as a consequence, common blockers should antagonize their effects. On the contrary, we find that agents such as BSA and DTT display full selectivity against one of the treatments. Moreover, depression of uptake rates takes place within seconds of either AA or ROS application and reaches a considerable level after 10 min (6, 7, 19) (see also Fig. 1), whereas, according to the literature, no clear evidence exists that ROS are produced in response to AA or that phospholipids are degraded by radicals with a similarly rapid time course. Also, the AA concentrations reported to induce detectable O_2^- accumulation in astrocytes (≥100 μM) are well above those required to inhibit glutamate uptake (16).

Astrocytes exposed to xanthine/XO and AA together take up glutamate at a significantly lower rate than do those exposed to either agent alone. This phenomenon is not related to the induction of major disruptive events leading to LDH leakage through the membranes and more likely depends on the convergence of the specific and distinct inhibitory actions of AA and ROS on glutamate transport. Thus, under all tested conditions, the level of uptake inhibition after incubation with AA plus xanthine/XO was identical to the sum of their individual effects. Noticeably, the xanthine/XO contribution was additive with the effect induced by 15 μM AA, a submaximally inhibitory concentration, but also with that induced by 50 or 100 μM AA, which are maximally inhibitory concentrations (Fig. 2). Moreover, AA (15 or 100 μM) added to cells that had been pretreated with xanthine/XO, which thus displayed reduced uptake capacity, further decreased uptake to an extent identical to its effect in cells that had not been pre-exposed to radicals. Therefore, these data, showing no occlusion or synergism between AA and ROS effects, support the idea that these agents affect glutamate transport through distinct mechanisms, as well as suggesting that their actions involve independent sites.

Chemical interaction between AA and oxygen radicals during coadministration to astrocytes either is not relevant for the observed effect on uptake or does not take place at all under our experimental conditions. Thus, comparable uptake inhibition was obtained with a different protocol, in which these agents were applied sequentially without being present together in the incubation medium. Furthermore, when we directly tested for chemical reactivity between them, we failed to detect significant [^{3}H]AA degradation upon incubation with xanthine/XO. Metabolic transformation to eicosanoids was also minimal, in agreement with our observation that intact astrocytic cultures metabolize exogenously added [^{3}H]AA at a significantly lower rate than do the corresponding astrocytic homogenates (25).[2] On the other hand, oxidation of thiol groups by xanthine/XO was not affected by 15–50 μM AA, whereas it was reduced by high concentrations of the fatty acid (>100 μM). These data indicate that, at least under our conditions, ROS react preferentially with SH groups rather than with free AA double bonds. In turn, AA is not immediately consumed by our cell preparation via enzymatic and/or nonenzymatic oxidation. Therefore, in the case of conjunct production *in vivo*, AA and oxygen radicals might exist, at least within a certain time frame, as separate entities exerting distinct biological actions. In the case of glutamate uptake, these might converge to modulate the same molecular event.

In situations where both AA and ROS act at the same time, the rate of glutamate uptake in astrocytes rapidly decreases within the first 5 min and then shows a slower progressive decline with time. Such progress can be stopped by the addition, 5 min after AA plus xanthine/XO treatment, of a combination of BSA and SOD/CAT but not either agent individually. Interestingly, BSA plus SOD/CAT (and, even more, BSA plus SOD/CAT plus DTT) also significantly reverses the inhibition already established. Therefore, these agents not only effectively neutralize both AA and ROS effects but also force uptake to proceed faster than its normal rate, to recover from pre-existing inhibition. This effect is probably due to the additional ability of DTT and BSA to enhance the uptake rate under basal conditions (7, 19). The pharmacological implications of the aforementioned result seem important; uptake function is not irreversibly compromised at early stages of exposure to free fatty acids and ROS, and even significantly inhibited transport can be mostly restored by appropriate intervention. This requires agents capable of removing both AA and ROS effects. In this specific case, lipophilic antioxidants would probably not be useful, because lipid peroxidation reactions seem not to be involved in the primary action of AA and ROS on glutamate transport (see also Ref. 7).

Efficient reuptake of glutamate in neuronal terminals and astrocytes is essential for maintaining physiological EAA neurotransmission (31). Administration of pharmacological uptake inhibitors results in rapid potentiation of cell responses to EAA (32) and may lead to delayed excitotoxicity (33). Direct evidence exists that glutamate uptake function is altered in at least two distinct types of neurodegenerative diseases, namely ischemia/reperfusion injury (34, 35) and amyotrophic lateral sclerosis (36). In the case of amyotrophic lateral sclerosis, oxidative stress (37) and excitotoxicity secondary to impaired uptake function (38) have been proposed as possible pathogenic mechanisms, but a link between them has not yet been demonstrated. On the other hand, in transient brain ischemia, massive liberation of AA (39) and burst formation of oxygen radicals (40) are well established phenomena. They take place at times partially overlapping and consistent with the observed reduction of glutamate transport capacity (35). Therefore, in view of the present data, it is possible that AA and oxygen radicals, independently and even additively at some times, converge to affect glutamate reuptake and thereby importantly participate in the elevation of extracellular EAA concentrations toward neurotoxic levels.

[2] A. Volterra and D. Trotti, unpublished observations.

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