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Excitatory Amino Acid Release and Free Radical Formation May Cooperate in the Genesis of Ischemia-Induced Neuronal Damage

Domenico E. Pellegrini-Giampietro, Giovanna Cherici, Marina Alesiani, Vincenzo Carla, and Flavio Moroni

Dipartimento di Farmacologia Preclinica e Clinica "Mario Aiazzi Mancini" Universitá di Firenze, 50134 Firenze, Italy

Excessive stimulation of excitatory amino acid (EAA) receptors and abnormal production of oxygen-derived free radicals have repeatedly been implicated in the series of events linking brain hypoxia or ischemia to neuronal death. We report here that in rat hippocampal slices the KCl-stimulated output of labeled D-^{3}H aspartate or of endogenous aspartate and glutamate significantly increased under *in vitro* simulated hypoxic, hypoglycemic, or ischemic conditions. In particular, when the slices were incubated for 10 min at 32°C under "ischemic" conditions (namely, lack of oxygen and glucose), endogenous aspartate and glutamate in the supernatant increased by 10 and 20 times, respectively. Since radical scavengers (D-mannitol), drugs reducing free radical formation (indomethacin, corticosteroid), or enzymes able to metabolize them (catalase and superoxide dismutase) significantly reduced this output, it was supposed that free radicals caused EAA release. A direct demonstration of this concept was obtained by showing a significant release of EAA after incubation of hippocampal slices with enzymes and substrates known to cause the formation of free radicals, such as xanthine plus xanthine oxidase or arachidonic acid plus prostaglandin synthase. Neither ischemia nor the enzymatic reactions leading to free radical production increased the activity of the cytoplasmic enzyme lactate dehydrogenase in the incubation medium, thus ruling out a nonspecific cellular lysis. It appears therefore that during ischemic states, brain production of reactive molecules (free radicals) causes an increased output of EAA. This may trigger a series of events which could help to explain the delayed loss of neurons after a transient ischemic period.

Formation of large amounts of toxic oxygen-derived free radicals in hypoxic and ischemic brain has been proposed as an important step in the sequence of events that links cerebral blood flow reduction to neuronal death (Demopoulos et al., 1980; Siesjo, 1981; Raichle, 1983; Braughler and Hall, 1989; Hall and Braughler, 1989). Free radical formation has been demonstrated both during the acute ischemic attack (Demopoulos et al., 1980) and when blood and oxygen eventually return to the brain upon reperfusion (McCord, 1985). Certainly, free radical scavengers seem to be able to reduce anoxic or ischemic neuronal damage both *in vitro* and *in vivo* (Smith et al., 1980; Taylor et al., 1984, 1985; Itoh et al., 1986; Cao et al., 1988; Sasaki et al., 1988).

Another research line that has been thoroughly and independently investigated in order to understand the cause of neuronal damage induced by hypoxic or ischemic states deals with the excitatory amino acids (EAA) aspartate (Asp) and glutamate (Glu), synaptically released transmitters which are endowed with neurotoxic effects (Rothman and Olney, 1986, 1987; Choi, 1988a). An increased release of Glu and/or Asp has been observed both during anoxia *in vitro* (Bosley et al., 1983; Hauptman et al., 1984) and during cerebral ischemia *in vivo* (Benveniste et al., 1984; Erecinska et al., 1984; Hagberg et al., 1985; Globus et al., 1988). Furthermore, EAA receptor antagonists have been shown to offer protection against anoxic neuronal death *in vitro* (Rothman, 1984; Clark and Rothman, 1987; Goldberg et al., 1987) or ischemic brain damage *in vivo* (Simon et al., 1984; Gill et al., 1987; Andiné et al., 1988; Kochhar et al., 1988). Recently, a reduction in the number of EAA receptors following ischemia has also been reported (Westerberg et al., 1987; Leach et al., 1988).

Our working hypothesis was that these 2 metabolic events, namely, free radical formation and EAA release, are mutually related and cooperate in the series of events that link brain anoxia or ischemia to neuronal death. For this purpose we developed an *in vitro* model of simulated ischemia, and we studied the EAA release from the hippocampus, a brain region particularly sensitive to hypoxic or ischemic insults (Smith et al., 1984).

Materials and Methods

Materials. Allopurinol, corticosterone, indomethacin, kynurenic acid (KYN), D-mannitol, *o*-phthalaldehyde, and xanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Catalase (65,000 U/mg protein), lactate dehydrogenase (LDH; 550 U/mg protein), superoxide dismutase (SOD; 5000 U/mg lyophilisate), and xanthine oxidase (XOD; 1U/mg protein) were from Boehringer Mannheim GmbH (FRG). Arachidonic acid and prostaglandin synthase (PGS) were from Calbiochem Corporation (San Diego, CA). D-^{3}H-Asp (10–30 Ci/mmol) was from Amersham (Amity PG, Milan), ethylmercaptan from Fluka AG (Buchs, Switzerland), and 6,7,dinitroquinoxaline-2,3-dione (DNQX) from Tocris Neuramin (Buckhurst Hill, Essex, UK). All other reagents were from Merck (Darmstadt, FRG), analytical grade.

Preparation of rat hippocampal slices and in vitro *experimental models.* Male Wistar rats (Nossan strain, Milan), weighing 180–200 gm were used. After decapitation, their hippocampi were rapidly removed and plunged into ice-cold Krebs-bicarbonate buffer (in mM: NaCl, 122; KCl, 3.1; $MgSO_4$, 1.2; KH_2PO_4, 0.4; $CaCl_2$, 1.3; $NaHCO_3$, 25; and glucose, 10). Transverse slices (350 μm thick) were cut with a McIlwain tissue chopper from each hippocampus and then left to stand, dipped into Krebs-bicarbonate solution gassed with 95% O_2/5% CO_2 for 2 hr at room temperature in order to allow functional recovery. After this pe-

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Correspondence should be addressed to Prof. Flavio Moroni, Dipartimento di Farmacologia, Universitá di Firenze, Viale Morgagni, 65 50134 Florence, Italy.

Table 1. Excitatory amino acid release in various experimental models

Experimental condition	Release D-^{3}H-Asp	(%a)	(%b)	L-Asp	(%a)	(%b)	L-Glu	(%a)	(%b)
Control	14.0 ± 0.4	(100)		37.1 ± 8.6	(100)		72.6 ± 15	(100)	
50 mM KCl	27.7 ± 1.7[a]	(199)	(100)	272.5 ± 41[a]	(734)	(100)	1111 ± 168[a]	(1530)	(100)
"Hypoxia"	14.3 ± 1.6	(102)		38.2 ± 6.6	(103)		51.1 ± 9.7	(70)	
"Hypoxia" + 50 mM KCl	55.5 ± 5.3[b]		(200)	549.2 ± 41[b]		(201)	2835 ± 310[b]		(255)
"Hypoglycemia"	12.8 ± 1.4	(91)		45.3 ± 5.3	(122)		73.5 ± 10	(101)	
"Hypoglycemia" + 50 mM KCl	44.4 ± 3.4[b]		(160)	453.5 ± 54[b]		(166)	1622 ± 180[b]		(146)
"Ischemia"	24.5 ± 1.9[a]	(175)		395.2 ± 49[a]	(1065)		1465 ± 135[a]	(2017)	
"Ischemia" + 50 mM KCl	63.8 ± 6.5[b]		(230)	835.3 ± 71[b]		(306)	3832 ± 457[b]		(345)

D-^{3}H-Asp release was calculated as (cpm tritium in medium)/(cpm tritium left in the slices) × 100. Endogenous Asp and Glu release are expressed as pmol/mg protein/10 min. (%a) indicates percentage of basal control release; (%b) indicates percentage of stimulated release (KCl 50 mM). Values are means ± SEM of at least 5 experiments, all conducted in triplicate. See Materials and Methods for statistical procedures. "Hypoxia" was obtained by incubating rat hippocampal slices for 10 min in a medium bubbled with N_2 instead of O_2, "hypoglycemia" by incubation in a glucose-free medium, and "ischemia" by a combination of these 2 conditions.

[a] $p < 0.01$ vs control release.

[b] $p < 0.01$ vs KCl-induced release.

riod, viability was electrophysiologically assessed in some of the slices according to Corradetti et al. (1983), and they were then placed into test tubes (5 slices each) containing 650 μl of gassed solution at 32°C, with or without the drugs under study.

In order to simulate ischemic or hypoxic injury to the brain, viable hippocampal slices were incubated in test tubes for 10 min in glucose-free Krebs-bicarbonate solution saturated with 95% N_2/5% CO_2 ("ischemia"), while other slices were incubated with glucose-containing buffer bubbled with 95% N_2/5% CO_2 ("hypoxia") or with glucose-free solution gassed with 95%O_2/5% CO_2 ("hypoglycemia").

When the effects of enzymatically formed free radicals on hippocampal release were studied, the slices were incubated for 20 min at 32°C in Krebs-bicarbonate buffer gassed with 95% O_2/5% CO_2 and containing xanthine plus XOD or arachidonate plus PGS, which are excellent sources of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) (Kellogg and Fridovich, 1977).

In some experiments, a 50 mM KCl-containing buffer (with isomolar reduction of NaCl) was utilized, while in others $CaCl_2$ was omitted from the medium and replaced by 1 mM EGTA.

Studies on D-^{3}H-Asp release. For this set of experiments, hippocampal slices were previously incubated for 45 min at 32°C in oxygenated buffer containing D-^{3}H-Asp (final concentration, 50 nM), then washed for 30 min with fresh solution, and finally transferred into the test tubes (2 slices each) containing one of the different gassed solutions. The experimental reactions were carried out for 10 min and were then terminated by cooling the test tubes in an ice bath. The supernatant was separated from the slices by gentle filtration on Whatman GF/C filters, and the tritium content of both the supernatant and the solubilized slices, after the addition of an appropriate amount of scintillation fluid, was counted on a Packard (Tri Carb 1500) liquid scintillation analyzer. Labeled release was calculated as (cpm tritium present in the supernatant)/(cpm tritium left in the slices) × 100, and statistical differences were evaluated by performing analysis of variance on overall data and modified *t* statistics (Wallenstein et al., 1980) on individual comparisons.

Studies on endogenous Asp and Glu release. Hippocampal slices were prepared as described, and 5 of them were kept in each test tube for 10 min at 32°C in the required experimental conditions. After cooling the test tubes in an ice bath, aliquots (500 μl) of the supernatant were removed and stored at −20°C for subsequent analysis. Slices were used for protein determination (Lowry et al., 1951).

Endogenous Glu and Asp were measured by HPLC and fluorimetric detection after a precolumn derivatization procedure with *o*-phthalaldehyde and ethylmercaptan (Connick and Stone, 1988). Aliquots (200 μl) of each sample were rapidly mixed with 100 μl of the derivatizing solution before being injected in the HPLC apparatus. This reaction, which led to the formation of the 1-alkyl-thio-2-alkyl substituted isoindole derivatives of the amino acids, proved to be stable for at least 60 min, giving identical results at different times of injection within this period. Injections of 100 μl were made into an HPLC apparatus, consisting of a Perkin-Elmer ISS-100 autosampler, 2 Perkin-Elmer Series 10 pumps, a Perkin-Elmer Series 20 LC controller, and a Perkin-Elmer LS3 fluorescence spectrometer with a wavelength of 334 nm and emission cut-off filter at 426 nm. Separation was performed on a Perkin-Elmer reversed-phase C_{18} column (15 cm × 2.6 mm, 5 μm particle size) fitted with a C_{18} "guard pak" precolumn (Waters). Solvent A was 65% methanol in water, and solvent B was 50 mM sodium acetate and 50 mM NaH_2PO_4. The gradient program started from 20% of solvent A at the moment of injection and reached 80% in 12 min. Asp and Glu derivatives were identified by their retention times relative to reference injections of standard Asp and Glu, and their concentrations were quantified by comparing sample and reference peak heights. This procedure gave results that were comparable to those obtained with a gas chromatography-mass spectrometer method previously described (Moroni et al., 1981).

Glu and Asp release were expressed as pmol/mg protein/20 min, and statistical analysis was carried out as indicated for radiolabeled release.

LDH activity assay. Nonspecific lysis of neuronal cells was studied by detecting the activity of LDH, a typical cytoplasmic enzyme, in the supernatant of slices exposed for 10 min to the different described procedures, by means of a spectrophotometric assay (Vassault, 1983). A diagnostic kit (No. 228-UV) from Sigma was used, and a reference curve was constructed from LDH standards.

Results

EAA release under different in vitro *experimental conditions*

Table 1 shows that hippocampal slices preincubated with D-^{3}H-Asp released an increased amount of tritium when incubated for 10 min under ischemic conditions (no glucose, N_2 instead of O_2). This basal outflow was not affected when the slices were incubated under hypoxic or hypoglycemic conditions, and it almost doubled when slices were depolarized with 50 mM KCl; upon depolarization, "hypoxic," "hypoglycemic," and "ischemic" slices displayed a significantly higher efflux of label (Table 1).

Table 1 also shows that endogenous Asp and Glu release were qualitatively affected in the same manner by the different experimental challenges, the only difference being the more pronounced enhancement of basal and stimulated control outflows. Basal Asp and Glu release were increased by 10- and 20-fold, respectively, under ischemic conditions, while hypoxia and hypoglycemia alone were ineffective. In control slices (presence of glucose and normal oxygenation), 50 mM KCl induced a 7-fold potentiation of Asp output and a 15-fold potentiation of that of Glu. Hypoxia and hypoglycemia alone were capable of increasing Asp and Glu release, but, as in the case of D-^{3}H-Asp, the ischemic challenge yielded greater effects (Table 1).

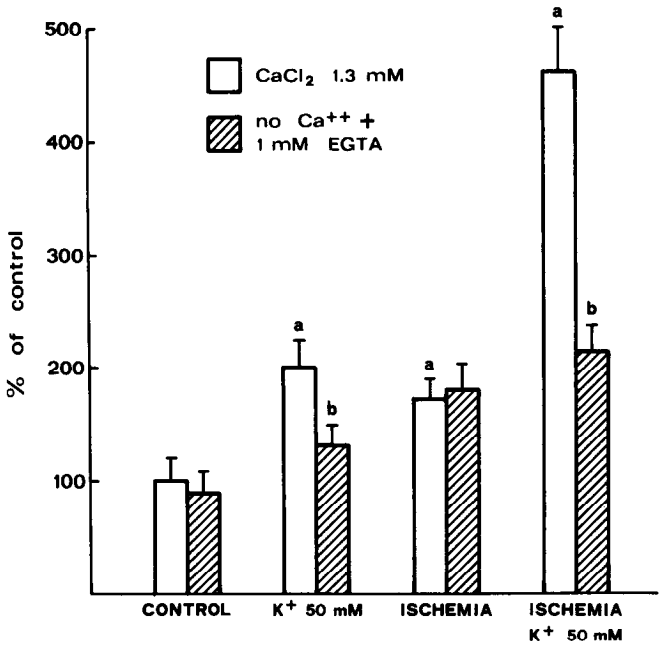

Figure 1. Ca^{2+} dependence of basal, stimulated (50 mM KCl), "ischemic" (incubation for 10 min in glucose- and O_2-free medium) and of stimulated "ischemic" release of D-^{3}H-aspartate from rat hippocampal slices. D-^{3}H-release is expressed as a percentage of control (see Table 1 for release values of different experimental conditions in the presence of Ca^{2+}). See Materials and Methods for statistical evaluation. Vertical bars are SEM of at least 5 experiments, all conducted in triplicate. $^a p < 0.01$ vs control release; $^b p < 0.01$ vs release of respective model in the presence of Ca^{2+}.

Ca^{2+}-dependence of EAA release induced by different *in vitro* conditions was studied by using Ca^{2+}-free buffers containing 1 mM EGTA. Radiolabeled and endogenous release induced by both KCl and ischemia plus KCl were partially dependent upon the presence of Ca^{2+} in the medium, while the ischemia-induced increase of basal release was not at all affected by the absence of Ca^{2+} (Figs. 1, 2).

In order to ascertain whether nonspecific lysis of the cell could be the cause of the observed outflow of labeled or endogenous amino acids, the activity of LDH, a specific cytoplasmic enzyme, was measured in the supernatant of slices treated under the experimental conditions described above. When slices were incubated alone for 20 min at 37°C, LDH activity in the incubation medium was 0.64 ± 0.05 U/liter (n = 4). After 10 min of hypoxia, the release was 0.70 ± 0.05, and in the presence of 0.5 mM xanthine plus 20 mU/ml XOD, it was 0.72 ± 0.06 U/liter (not significant). The addition of Triton X-100 (0.1%) to the incubation medium for 15 min increased LDH activity in the supernatant to 65.1 ± 0.5 U/liter.

Effects of free radical scavengers and enzyme inhibitors on ischemia-induced EAA release

Table 2 shows that D-mannitol (1 mM), a hydroxyl radical scavenger, was able to reduce (by 64%) the ischemia-induced increase in tritium outflow from hippocampal slices preloaded with D-^{3}H-Asp, when added to the incubation medium. The O_2^- scavenger system formed by SOD (50 μg/ml) plus catalase (50 μg/ml) was almost equally potent in this reducing effect (63%). Indomethacin (50 μM) and corticosterone (50 μM), which are inhibitors, at different stages, of enzymatic free radical production, partially reduced this increase (by 43 and 51%, respectively).

Table 2 also shows that the ischemia-induced release of endogenous Asp and Glu was reduced by the addition of different radical scavengers to the incubation medium. D-Mannitol (1 mM) was the most potent in this regard, inhibiting Asp release by 69% and Glu release by 60%. The scavenger system formed by SOD (50 μg/ml) plus catalase (50 μg/ml) reduced Asp and Glu outflow by 48 and 46%, respectively. The enzymatic inhibitors indomethacin (50 μM) and corticosterone (50 μM) caused

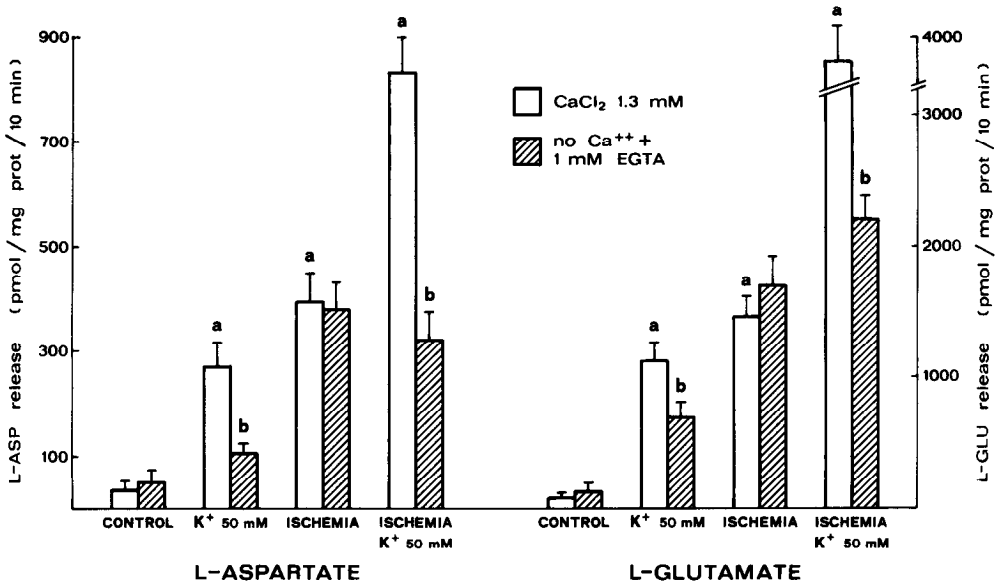

Figure 2. Ca^{2+} dependence of basal, stimulated, "ischemic," and stimulated "ischemic" release of endogenous Asp and Glu from hippocampal slices. See Figure 1 for experimental procedures. Values are expressed as pmol/mg protein/10 min and are means ± SEM of at least 5 experiments, all conducted in triplicate. See Materials and Methods for statistics. $^a p < 0.01$ vs control release; $^b p < 0.01$ vs release of respective model in the presence of Ca^{2+}.

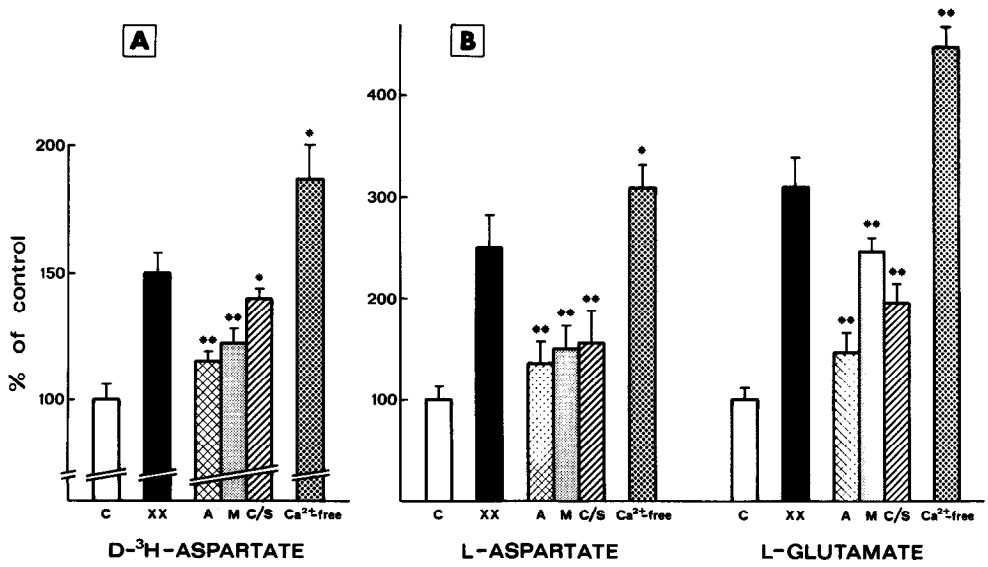

Figure 3. EAA release induced by xanthine (0.5 mM) plus XOD (20 mU/ml): Ca^{2+} dependence and effects of free radical scavengers (*C*, controls; *XX*, 0.5 mM xanthine plus 20 mU/ml xanthine oxidase; *A*, 10 μM allopurinol; *M*, 1 mM D-mannitol; *C/S*, 50 μg/ml catalase plus 50 μg/ml SOD). In Ca^{2+}-free medium, Ca^{2+} was omitted and 1 mM EGTA added. Results are given as a percentage of basal release (see text for control values) and are means ± SEM of at least 4 experiments, all conducted in triplicate. See Materials and Methods for statistics. *<0.05 vs xanthine plus XOD-induced release; **<0.01 vs xanthine plus XOD-induced release.

a less pronounced reduction (Table 2). None of the free radical scavengers or inhibitors tested modified the release of labeled or endogenous Asp and Glu induced by 50 mM KCl (data not shown).

Effects of xanthine plus XOD and of arachidonate plus PGS on EAA release

When slices preloaded with D-^{3}H-Asp were exposed to xanthine (0.5 mM) plus XOD (20 mU/ml) for 20 min, they released an increased amount of tritium (+50%, Fig. 3). This increase was further potentiated in the absence of Ca^{2+} in the medium. Allopurinol (10 μM), a specific inhibitor of XOD, was able to prevent almost completely (−70%) the xanthine plus XOD-induced release, which was also significantly reduced by the radical scavengers D-mannitol (1 mM) and SOD (50 μg/ml) plus catalase (50 μg/ml) (by 56 and 20% respectively; Fig. 3).

The xanthine plus XOD enzymatic reaction also led to a 2.5-fold Ca^{2+}-independent increase of endogenous Asp output and to a 3.2-fold increase of that of endogenous Glu (Fig. 3). This release was inhibited by 10 μM allopurinol (77% reduction for Asp and 81% for Glu), by 1 mM D-mannitol (58 and 34% reductions, respectively), and by SOD plus catalase (68 and 58%, respectively).

Similarly, the output of endogenous Asp and that of Glu increased from 35.9 ± 5.3 and 69.4 ± 14 pmol/mg protein, under basal conditions, to 61.4 ± 9.3 and 157.5 ± 18 pmol/mg protein, respectively, when the slices were incubated for 10 min in the presence of 100 μM arachidonate and 500 mU PGS ($n = 4$, $p < 0.01$).

LDH activity was not modified in the supernatant of slices exposed to these treatments.

Effects of EAA receptor antagonists on endogenous Glu and Asp release

Table 3 shows that KYN (100–300 μM), a nonselective EAA receptor antagonist (Stone and Burton, 1988) and DNQX (10–30 μM), a putative blocker of EAA receptors of the non-*N*-methyl-D-aspartate type (Honoré et al., 1988), when added to the "ischemic" incubation medium, were able to reduce endogenous Asp and Glu release from hippocampal slices. On the other hand, neither the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (AP5) nor the selective glycine antagonist 7-Cl-kynurenate (7-Cl-KYN) (Kemp et al., 1988) at fully active concentrations significantly reduced ischemia-induced EAA release.

Table 2. Effects of different free radical scavengers and inhibitors on simulated ischemia-induced EAA release *in vitro*

Condition	Release D-^{3}H-Asp	(%)	L-Asp	(%)	L-Glu	(%)
Control	14.0 ± 0.4	(100)	37.1 ± 8.6	(100)	72.6 ± 15	(100)
"Ischemia"	24.5 ± 1.9	(175)	395.2 ± 49	(1065)	1465 ± 135	(2017)
"Ischemia" + 1 mM D-mannitol	17.8 ± 1.8[b]	(127)	148.1 ± 17[b]	(399)	629.8 ± 73[b]	(867)
"Ischemia" + SOD (50 μg/ml) + catalase (50 μg/ml)	17.9 ± 2.0[b]	(128)	223.3 ± 25[b]	(602)	824.8 ± 94[b]	(1136)
"Ischemia" + 50 μM indomethacin	20.0 ± 3.0[a]	(143)	334.3 ± 31	(901)	1298 ± 131	(1788)
"Ischemia" + 50 μM corticosterone	19.2 ± 2.6[a]	(137)	312.9 ± 28[a]	(843)	1242 ± 125[a]	(1711)

"Ischemia" was obtained by incubating hippocampal slices in a glucose-free and O_2-substituted (with N_2) medium for 10 min. Values are means ± SEM of at least 5 experiments, all conducted in triplicate. See legend to Table 1 and Materials and Methods for experimental and statistical procedures.

[a] $p < 0.05$ vs ischemia alone.

[b] $p < 0.01$ vs ischemia alone.

Discussion

The aim of our study was to clarify the relationship between 2 widely studied pathogenetic events linking hypoxic or ischemic brain damage to the death of neurons: production of free radicals (Siesjo, 1981; Raichle, 1983; Braughler and Hall, 1989) and abnormal extracellular concentrations of EAA (Meldrum, 1985; Rothman and Olney, 1986; Choi, 1988a).

Our first step was to develop a satisfactory *in vitro* model of ischemia, in which EAA release could be detected. Similar models have been reported previously (Bosley et al., 1983; Hauptman et al., 1984; Drejer et al., 1985). Initial results showed that slices from rat hippocampi, not exposed to depolarizing solutions, released large amounts of D-^{3}H-Asp and of endogenous Asp and Glu only when incubated with a medium mimicking *in vivo* ischemic conditions (lack of both glucose and O_2). A much more pronounced outflow of EAA was observed when hypoxic, hypoglycemic, or ischemic conditions were tested in the presence of 50 mM KCl. However, we focused our attention on the non-depolarized ischemic approach because the simultaneous occurrence of both oxygen and glucose deprivation closely resembles what happens during stroke; because, in this case, the appearance of EAA in the medium was unaffected by the extracellular concentrations of Ca^{2+}; and because it was not associated with possible nonspecific cellular lysis, since the activity of the cytoplasmic enzyme LDH was not increased in the incubation medium. In our "ischemic" model, this efflux of EAA could not be ascribed simply to the rise in extracellular K^+ which has been shown during ischemia (Hansen, 1985), since the addition of KCl to the "ischemic" slices produced a further increase in EAA outflow, which was not lower than that induced by KCl in control slices. Furthermore, the KCl-induced EAA output was CA^{2+}-dependent, while the phenomenon we studied was not (see also Sànchez-Prieto and Gonzàlez, 1988; Ikeda et al., 1989).

The observation that the "ischemia"-induced appearance of EAA in the incubation medium could be significantly reduced by compounds such as D-mannitol, indomethacin, corticosterone, and the enzymes catalase and SOD, which have in common the ability to "scavenge" or reduce the formation of oxygen-derived free radicals, demonstrated that these active molecular species were involved in the elevation of EAA concentrations in the extracellular medium. A direct demonstration of this hypothesis was obtained by showing that the incubation of hippocampal slices with systems leading to free radical formation (xanthine plus XOD or arachidonate plus PGS) resulted in an increase of the release of both D-^{3}H-Asp and endogenous Asp and Glu. The possibility that free radical formation may release intracellular active components has been previously reported (Ohmori et al., 1980; Mannaioni and Masini, 1988).

The formation of free radicals during hypoxic or ischemic states is widely accepted (Braughler and Hall, 1989; Hall and Braughler, 1989). It has been proposed that they are formed mainly during the reperfusion of previously ischemic tissues,

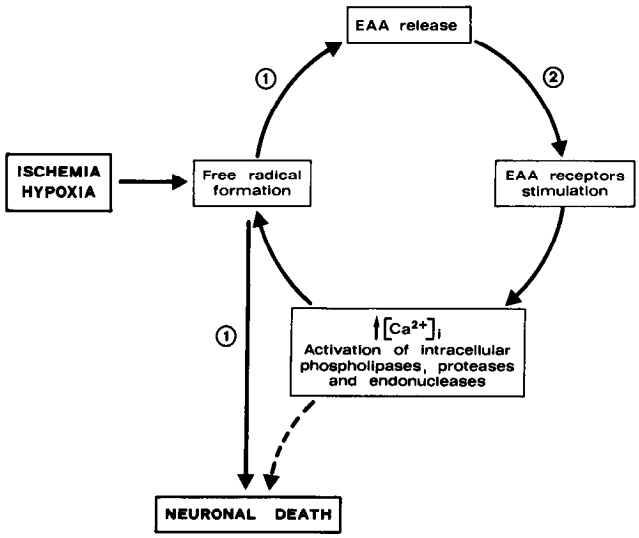

Figure 4. Hypothetical vicious circle illustrating the possible mutual cooperation between free radical formation and EAA release in the pathogenesis of neuronal damage induced by hypoxia and ischemia. Toxic free radicals, formed following the reduction in blood flow or oxygen supply to the brain promote the release of EAA, which in turn may exert their toxic effects by interacting with specific receptors. Stimulation of EAA receptors increases intracellular Ca^{2+} concentrations, resulting in both neuronal damage and further production of free radicals (Choi, 1988b; Siesjo and Bengtsson, 1989). This gives the possibility of starting another cycle. It is possible to interrupt this circle by using (*1*) free radical scavengers and enzyme inhibitors or (*2*) EAA receptor antagonists. See text for further discussion.

Table 3. Effects of different EAA antagonists on ischemia-induced EAA release

Condition	L-Asp	%	L-Glu	%
Control	38 ± 8	–	75 ± 15	–
"Ischemia"	392 ± 40	(100)	1500 ± 200	(100)
Ischemia + KYN 100 μM	350 ± 50	(89)	1380 ± 100	(92)
Ischemia + KYN 300 μM	290 ± 20[a]	(70)	1070 ± 110[a]	(71)
Ischemia + 7Cl-KYN 10 μM	350 ± 90	(92)	1520 ± 150	(101)
Ischemia + 7Cl-KYN 50 μM	330 ± 40	(87)	1490 ± 120	(99)
Ischemia+ AP5 50 μM	350 ± 35	(89)	1350 ± 130	(90)
Ischemia + AP5 100 μM	370 ± 40	(94)	1425 ± 140	(95)
Ischemia + DNQX 30 μM	270 ± 30[a]	(69)	1100 ± 80[a]	(73)
Ischemia + DNQX 100 μM	261 ± 15[a]	(66)	1080 ± 100[a]	(72)

Means ± SE of at least 4 experiments conducted in duplicate as picomol/mg protein/10 min and percentages of the "ischemia"-induced release are given. See Table 1 and Materials and Methods for experimental and statistical procedures.

[a] $p < 0.01$ vs ischemia alone.

by means of reactions involving XOD (McCord, 1985), PGS (Rehncrona et al., 1982; Kontos and Wei, 1986) or mitochondrial ubisemiquinone (Turrens et al., 1985; Patole et al., 1986). However, free radicals may also be formed during incomplete ischemia or even during complete ischemia or anoxia. In these cases, O_2 brought in by collateral vessels (Flamm et al., 1978), the "univalent leak" of O_2^- from mitochondria (Del Maestro, 1980), and the possibility of free radical formation even when tissues contain only 5% of normal O_2 levels (Demopoulos et al., 1980) may be of importance.

From these studies it is not possible to understand which might be the source of free radicals in our ischemic model. It is likely that those mechanisms which are operative during incomplete or complete ischemia are also important in our experimental conditions. In any case, our experiments utilizing an exogenous source of free radicals (xanthine plus XOD or arachidonic acid plus PGS) clearly demonstrate that they are indeed able to induce the outflow of EAA. Moreover, previous experiments from our group had shown that these enzymatic reactions were also able to release ^{3}H-noradrenaline from preloaded slices (Pellegrini-Giampietro et al., 1988). Altogether, these data suggest that free radicals, formed either under simulated ischemic conditions or by enzymatic reaction, lead to a nonspecific leakage of neurotransmitters.

It has been previously shown that EAA receptor agonists may promote enzymatic reactions leading to free radical production (Dumuis et al., 1988) and that excitotoxin-induced neuronal damage may be reduced by free radical scavengers (Dykens et al., 1987). This led us to hypothesize the existence of a vicious circle, in which free radical formation and EAA release could affect each other in the generation and propagation of ischemic neuronal death (Fig. 4). This proposed mechanism could account for the efficacy of free radical scavengers in preventing both EAA release (our data) and excitotoxin-mediated functional and morphological impairment (Lassmann et al., 1984; Baran et al., 1987; Dykens et al., 1987) (Fig. 4, point 1). In support of the existence of such a vicious circle stand also the experiments shown in Table 3 showing that EAA receptor antagonists such as KYN and DNQX reduced the "ischemia"-induced EAA output (Fig. 4, point 2). In the model here described, the EAA receptors involved appear to be of non-NMDA type, since AP5, a typical NMDA antagonist, or 7-Cl-KYN, a compound able to antagonize the strychnine-insensitive glycine site associated to the NMDA receptor ion channel complex, failed to reduce the EAA release in ischemic conditions.

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