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# Ischemia and reoxygenation-induced amino acid release and tissue damage in the slices of rat corpus striatum

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**Summary.** Ischemic incubation significantly increased amino acid release from rat striatal slices. Reoxygenation (REO) of the ischemic slices, however, enhanced only taurine and citrulline levels in the medium. Ischemia-induced increases in glutamate, taurine and GABA outputs were accompanied with a similar amount of decline in their tissue levels. Tissue final aspartic acid level, however, was doubled by ischemia. Lactate dehydrogenase (LDH) leakage was not altered by ischemia, but enhanced during REO. Presence of tetrodotoxine (TTX) during ischemic period caused significant decline in ischemia-induced glutamate output, but not altered REO-induced LDH leakage. Although omission of extracellular calcium ions from the medium during ischemic period protected the slices against REO-induced LDH leakage, this treatment failed to alter ischemia-induced glutamate and GABA outputs. The release of other amino acids, however, declined 50% in calcium-free medium. Blockade of the glutamate uptake transporter by L-trans-PDC, on the other hand, doubled ischemia induced glutamate and aspartic acid outputs. These results indicate that more than one mechanisms probably support the ischemia-evoked accumulation of glutamate and other amino acids in the extracellular space. Although LDH leakage enhanced during REO, processes involved in this increment were found to be dependent on extracellular calcium ions during ischemia but not REO period.

**Keywords:** Ischemia – Reoxygenation – Glutamate release – Amino acids – LDH

## Introduction

Because the brain critically depends on its blood flow for a continuous supply of oxygen and glucose, a reduction in one or both of them causes a cascade of biochemical events and eventually leads to neuronal death. It has been repeatedly demonstrated that energy deprivation of the neuronal tissues rapidly enhances neurotransmitter release from their nerve endings and subsequent activation of postsynaptic receptors and/or receptor-coupling events are important processes in developing the neuronal damage. Although other neurotransmitters, such as dopamine, seem to be also implicated (Globus et al., 1987; Buisson et al., 1992), there is a considerable amount of evidence suggesting that extracellular accumulation of excitatory amino acids play a major role in ischemia-induced damaging processes (Martin et al., 1994; Szatkowski and Attwell, 1994).

Results derived from *in vivo* and *in vitro* studies indicate that, not only the excitatory or inhibitory amino acids, but also others accumulate in the extracellular medium during hypoxic/ischemic conditions (Rego et al., 1996; Larsen et al., 1998). Several distinct mechanisms of amino acid release from ishemic tissue have previously been proposed (Phillis and O'Regan, 2003). Since ischemia did not cause a significant LDH leakage from the brain slices (Tatsumi et al., 1998; De La Cruz et al., 2002), it is reasonable to consider that a damage of the neuronal membranes may not be involved in the release processes. Dependency of ischemia-induced amino acid release on extracellular calcium ions is also controversial. Although a calcium-independent mechanism, which probably occurs by reversal of the sodium dependent transmitter carriers, has been reported for glutamate output (Roettger and Lipton, 1996; Kulik et al., 2000; Rossi et al., 2000; Phillis et al., 2000), requirement of calcium ions, especially during early phase of the ischemia, seems to be existing (Pisani et al., 1998; Kulik et al., 2000; Nishizawa, 2001; Nelson et al., 2003).

Like excessive release of amino acids, mechanistic details of the tissue damage induced by ischemia are still controversial. It is generally accepted that intrasynaptic accumulation of glutamate during ischemia stimulates postsynaptic glutamate receptors and thus causes sodium

and calcium influx through glutamate-gated ion channels (Choi, 1990; Szatkowski and Attwell, 1994; Balestrino, 1995; Kristian and Siesjö, 1998; White et al., 2000). While resultant elevation of intracellular sodium concentration leads to subsequent influx of water and cell swelling, elevation of intracellular calcium concentration activates a variety of calcium-dependent enzymes, such as proteases, nitric oxide synthase, and finally causes neuronal damage. In agreement with this hypothesis, NMDA receptors antagonists (Newell et al., 1995; Kimura et al., 1998; Mathews et al., 2000; Nishizawa, 2001), blockers of the calcium channels (Kimura et al., 1998; Tatsumi et al., 1998; Oka et al., 2000a) or inhibition of nitric oxide synthase (Xu et al., 2000; Oka et al., 2000b) protect the neurons against ischemia-induced damage. It must be also noted that neuronal damage determined by the leakage of LDH was only present during REO of ischemic brain slices (Tatsumi et al., 1998; De La Cruz et al., 2002), indicating that processes involved in neuronal damage are activated during ischemia, nevertheless REO seems to be the critical period in resultant tissue injury.

The following study was designed to investigate both amino acid release and LDH leakage from slices of rat corpus striatum during ischemia and REO periods. The importance of reverse transport mechanism in ischemia-induced glutamate release and contribution of extracellular calcium ions and glutamate in ischemia-REO-induced LDH leakage were reinvestigated in the present study. By measuring the tissue initial and tissue final amino acid levels, it was also aimed to determine whether ischemia or ischemia-like conditions have any effect on amino acid metabolism in brain slice preparation.

## Materials and methods

### *Materials*

L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC), TTX, and amino acids were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). MK-801 was purchased from Research Biochemicals International (Natick, MA, U.S.A.). Other chemicals were pure analytical grade and obtained from Merck KGaA (Darmstadt, Germany) or from Sigma Chemical Co.

### *Preparation and incubation of striatal slices*

Male and female Wistar Albino rats (weighing 250–300 g and obtained from Experimental Animals Breeding and Research Center, Bursa, Turkey) were used. All experimental protocols were approved by the Uludağ University Medical Center Institutional Review Board for animal research, and all efforts were made to minimize the number of animals used and their suffering.

Rats were decapitated and brains were removed quickly and placed in cold oxygenated physiological medium with 95% $O_2$ and 5% $CO_2$ (in mmol/L: 120 NaCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 3.5 KCl, 25 $NaHCO_3$ and 10 glucose). When glucose-free or calcium-free medium was used, the constant osmolarity was maintained by increasing the NaCl concentration. Calcium-free medium was also containing EGTA (1 mM). After dissection of the corpus striatum, slices (0.3 mm of thickness) were prepared with a McIlwain tissue chopper (Brinkmann Instruments; Westbury, NY, U.S.A.). Slices were washed with physiological medium to remove the membrane debris and then transferred to 2-ml incubation tubes. Each tube was divided into four separate chambers and contained four slices that did not touch each other. The slices were incubated in a water bath at 37°C and the medium was changed every 10 min with fresh oxygenated medium. Following 90 min equilibration period, striatal slices were incubated in each condition-control (gassed with 95% $O_2$ and 5% $CO_2$), anoxic (first degassed under vacuum and then gassed with 95% $N_2$ and 5% $CO_2$), aglycemic (glucose excluded but gassed with 95% $O_2$ and 5% $CO_2$), or ischemic (glucose and oxygen-free medium) for 1 h at 37°C. During this period, incubation medium was changed at 10-min intervals with fresh medium and collected samples were acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for glutamate and aspartic acid. Because anoxia and aglycemia did not cause a significant increase in glutamate output, only ischemic medium was used for REO model. In these assays, striatal slices were incubated in control or ischemic medium for 1 h. At the end of this incubation, slices were transferred into the glucose and oxygen-containing medium and incubated for another 5 h (REO period), as indicated previously (Tatsumi et al., 1998). REO was terminated by transferring the slices into the 2 ml of 0.4 M $HClO_4$. Half of the incubation medium collected at the end of each incubation period was acidified with $HClO_4$ (final concentration 0.4 M) and than assayed for amino acids measurement. The remaining half was used for determination of the LDH leakage. Slices were homogenized in $HClO_4$ and homogenates were used for determination of tissue final amino acids and protein levels. For measurement of the tissue initial amino acid levels, incubation of slices was terminated at the end of equilibration period by transferring the slices in 2 ml of 0.4 M $HClO_4$. When tested, MK-801 was present in the medium during both ischemic and REO periods. TTX was added into the medium during only ischemic period.

### *Quantification of amino acids*

Amino acid levels in the medium or in the tissue were determined by a HPLC system (HP 1100 series, Hewlett-Packard, Palo Alto, CA, U.S.A.) coupled to a post-column derivatization unit (Pickering Laboratories, Mountain View, CA, U.S.A.). This system was combined with a quaternary pump (HP, G1311A), a fluorometric detector (HP, G1321A) and an autosampler (HP, G1329A). Amino acids separated on lithium exchange column (Pickering Labs., series number 5338) with Li280 and Li750 eluents (Pickering Labs.) were reacted with OPA in a post-column derivatization unit (both from Pickering Labs.). The flow rate of the quaternary pump and post column derivatization unit was 0.3 ml/min. Column and post-column reaction temperatures were adjusted to 40°C and 45°C, respectively. Other chromatographic conditions, such as the gradient program of the Li280, Li750 and lithium regenerant eluents, were similar with the conditions published elsewhere (Grunau and Swiader, 1992). OPA reactive compounds were detected at excitation 330 nm, emission 465 nm wavelengths, and chromatograms were analyzed with a software (HP Chemstation, revision A. 08. 03., 847).

Acidified samples or tissue homogenates were centrifuged for 5 min in a Beckman microfuge and pH of the samples were adjusted to 2–2.5 with 2 M of LiOH. A portion of the supernatant (20 $\mu$l) was than injected onto HPLC system without further purification. Amino acid levels were calculated by comparing peak heights of the samples with amino acid standards. Amino acid standards were prepared in physiological medium, acidified with $HClO_4$ and processed together with samples.

Tissue protein levels were measured in 50 $\mu$l of homogenate according to the procedure of Lowry et al. (1951). Protein standards were also prepared in 0.4 M $HClO_4$ and processed together with the tissue samples.

*LDH assay*

LDH activity in the incubation medium was assayed using a commercial kit from Merck-Biotrol Diagnostics (France). Namely, 100 $\mu$l of incubation medium was mixed with 0.6 ml of reactive mixture in a temperature controlled cuvette (30°C). Changes in the absorbance were read at 340 nm after 30 sec then every minute during 2 minutes and LDH activity was calculated according to averaged absorbance change and then corrected with the protein levels of the slices.

*Data analysis*

All results in the text are expressed as mean ± SEM. Differences between the results were tested by Tukey-Kramer multiple comparison test or Student's t test. A probability of $p < 0.05$ was considered significant.

## Results

### *Anoxia, aglycemia and ischemia-induced alterations in glutamate and aspartic acid release and tissue glutamate and aspartic acid levels*

Incubation of striatal slices in oxygen-free (anoxic condition) or in glucose-free (aglycemic condition) medium for 1 hour did not cause significant alterations in glutamate and aspartic acid release. In oxygen plus glucose-free medium (ischemia), however, a several fold increase in the release of both amino acids were observed (Fig. 1A and Fig. 2A). Although aglycemia failed to affect the glutamate and aspartic acid outputs, tissue final levels of these amino acids were found to be different from their controls; while tissue final level of aspartic acid was almost doubled, tissue glutamate level was declined by aglycemia. Like aglycemia, ischemic conditions caused similar changes in tissue glutamate and aspartic acid levels (Fig. 1B and Fig. 2B).

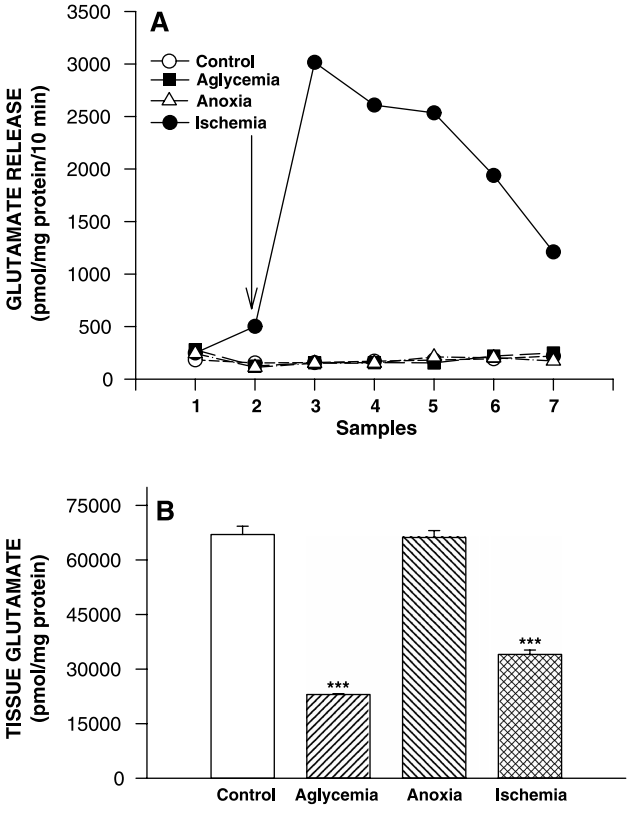


**Fig. 1.** Anoxia, aglycemia and ischemia-induced alterations in glutamate release (**A**) and tissue final glutamate levels (**B**) in rat striatal slices. After 90 min of equilibration period, striatal slices were incubated for another 10 min in normoxic conditions for determination of basal glutamate release (sample 1). Incubation of the slices was then continued for 60 min in control, aglycemic, anoxic or ischemic conditions. During this period, the incubation medium (2 ml) was changed at 10-min intervals to fresh medium. Collected samples were acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for glutamate levels. At the end of experiments, slices were removed from the incubation tubes and homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue final glutamate and protein levels. Data are mean ± SEM (bars) values, in pmol/mg protein/10 min. The number of determinations is 3 for each point. *** $p < 0.001$, significantly different from the value obtained in control condition

### *Ischemia and REO-induced alterations in amino acid release*

Since neither anoxia nor aglycemia alter the glutamate and aspartic acid outputs, only ischemic medium was used in the next series of experiments. As indicated in Table 1, not only the excitatory amino acids, but the levels of others (except glutamine) in the medium, were also enhanced by ischemia. The most striking increases were obtained in GABA (8900%), glutamate (1709%), aspartic acid (800%), taurine (400%) and glycine (350%) levels. Increases in the levels of other amino acids were quite similar to each other and were around 200% of control levels. Glutamine levels, on the other hand, reduced 50% in ischemic medium.

Doubling of citrulline level was the most evident observation during REO of the ischemic slices (Table 1). A similar but smaller increase was also detected in taurine level. The levels of other amino acids in the medium, however, remained unchanged or decreased slightly during the REO period.

### *Involvement of reverse transport mechanism in ischemia-induced glutamate release*

A possible involvement of reversed glutamate transporter in ischemia-induced amino acid release was tested with L-trans-PDC, a glutamate uptake inhibitor. Under control

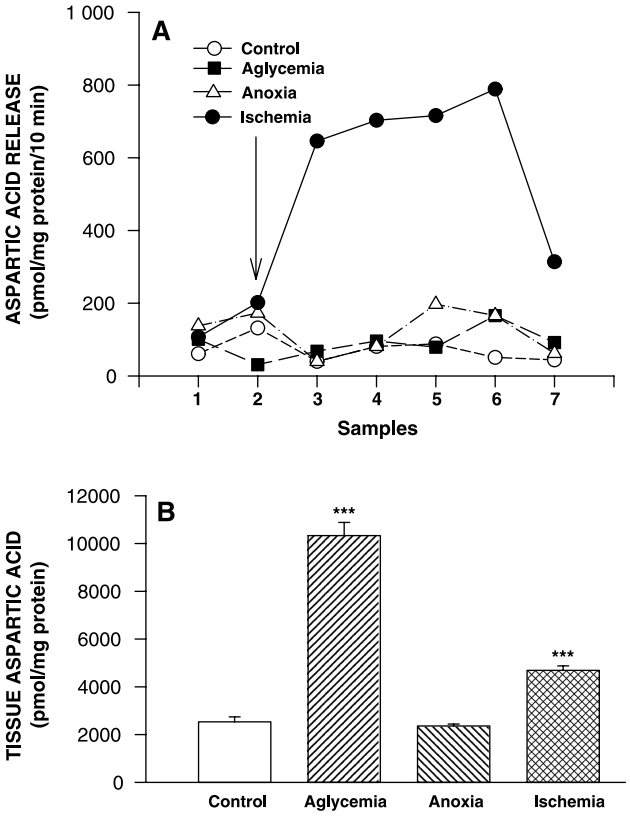


**Fig. 2.** Anoxia, aglycemia and ischemia-induced alterations in aspartic acid release (**A**) and tissue final aspartic acid levels (**B**) in rat striatal slices. After 90 min of equilibration period, striatal slices were incubated for another 10 min in normoxic conditions for determination of basal aspartic acid release (sample 1). Incubation of the slices was then continued for 60 min in control, aglycemic, anoxic or ischemic conditions. During this period, the incubation medium (2 ml) was changed at 10-min intervals to fresh medium. Collected samples were acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for aspartic acid levels. At the end of experiments, slices were removed from the incubation tubes and homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue final aspartic acid and protein levels. Data are mean ± SEM (bars) values, in pmol/mg protein/10 min. The number of determinations is 3 for each point. *** $p < 0.001$, significantly different from the value obtained in control condition

conditions (in the presence of both glucose and oxygen), striatal slices released $745 \pm 152$ pmol of glutamate/mg of protein and $295 \pm 32$ pmol of aspartic acid/mg of protein during 60 min of incubation period. When oxygen and glucose were omitted from the medium, glutamate and aspartic acid release were increased to $12309 \pm 923$ and $2479 \pm 77$ pmol/mg protein, respectively. Presence of L-trans-PDC (200 $\mu$M) in the medium almost doubled ischemia-induced glutamate output ($21754 \pm 2088$ pmol/mg protein). Similar to glutamate release, ischemia-induced aspartic acid release was also enhanced by L-trans-PDC ($3964 \pm 236$ pmol/mg protein, Fig. 3). Ischemia-induced release of other amino acids (except GABA and L-arginine, which were not determined in this part of the studies), however, was not altered by L-trans-PDC (data not shown).

### *Extracellular calcium dependency of amino acid release during ischemia and REO*

Omission of extracellular calcium from the medium (no calcium added, but containing 1 mM EGTA), did not alter ischemia-induced glutamate and GABA outputs (Table 2). Increments in the levels of other amino acids, however, were declined around 50% by lack of the extracellular calcium ions (Table 2). Removal of the calcium ions during REO period caused similar alterations in amino acid release.

### *LDH leakage from the slices during ischemia and REO*

One hour incubation of the slices under ischemic conditions failed to affect the LDH leakage ($125 \pm 15$ vs. $141 \pm 13\,\mu$ $\Delta$O.D/$\mu$g protein, $p > 0.05$). REO of the ischemic slices, on the other hand, increased LDH leakage to $511 \pm 34\,\mu$ $\Delta$O.D/$\mu$g protein from its control value $223 \pm 12\,\mu$ $\Delta$O.D/$\mu$g protein ($p < 0.001$, Fig. 4).

### *Effect of TTX on ischemia-induced amino acid release and LDH leakage*

Presence of the sodium channel inhibitor TTX (1 $\mu$M) in the medium caused significant decline in ischemia-induced glutamate ($11984 \pm 614$ vs. $6135 \pm 150$, $p < 0.05$) and aspartic acid ($1455 \pm 27$ vs. $914 \pm 200$, $p < 0.01$) outputs. Similar but a lesser decline was also obtained in taurine release ($3093 \pm 255$ vs. $2322 \pm 85$, $p < 0.05$). The release of other amino acids (except GABA and L-arginine, which were not determined in this part of the study) was not altered significantly by TTX. In contrast to the decreasing effect of TTX on ischemia-induced glutamate output, this toxin failed to inhibit the LDH leakage determined during REO period ($401 \pm 42$ vs. $358 \pm 16$, $p > 0.05$).

### *Involvement of glutamate and extracellular calcium ions in REO-induced LDH leakage*

Involvement of glutamate in REO-induced LDH leakage was tested by MK-801, a NMDA receptor antagonist. Presence of MK-801 (100 $\mu$M) in the medium during ischemia and REO periods caused a 30% decline in

**Table 1.** The amounts of amino acids released from rat striatal slices during ischemia and REO periods

| Amino acid | Ischemic period (pmol/mg protein/1 h) | | REO period (pmol/mg protein/5 h) | |
|---|---|---|---|---|
| | Control | Ischemia | Control | Reoxygenation |
| Glutamate | 849 ± 20 | 14512 ± 546*** | 219 ± 29 | 227 ± 23 |
| Aspartic acid | 416 ± 100 | 3218 ± 243*** | 505 ± 237 | 688 ± 67 |
| Taurine | 1627 ± 250 | 6618 ± 346*** | 3815 ± 93 | 5076 ± 218** |
| Glutamine | 6235 ± 740 | 2912 ± 230*** | 6287 ± 482 | 1981 ± 145*** |
| GABA | 97 ± 5 | 8674 ± 385*** | 1155 ± 177 | 533 ± 45 |
| Glycine | 948 ± 122 | 3252 ± 252*** | 2805 ± 167 | 2694 ± 74 |
| Citrulline | 159 ± 27 | 378 ± 62* | 471 ± 62 | 1031 ± 66*** |
| Arginine | 893 ± 56 | 1324 ± 63* | 2614 ± 35 | 2667 ± 134 |
| Alanine | 2020 ± 228 | 3790 ± 207*** | 6810 ± 394 | 4637 ± 158*** |
| Serine | 1374 ± 214 | 3335 ± 344*** | 3872 ± 262 | 3769 ± 136 |
| Leucine | 1735 ± 144 | 3650 ± 133*** | 5833 ± 258 | 4643 ± 127*** |
| Isoleucine | 1047 ± 123 | 2157 ± 96*** | 3423 ± 164 | 2707 ± 93*** |
| Tyrosine | 679 ± 89 | 1327 ± 96*** | 2033 ± 121 | 1940 ± 39 |
| Phenylalanine | 963 ± 105 | 1745 ± 96*** | 3118 ± 166 | 3295 ± 64 |
| Threonine | 1260 ± 134 | 2801 ± 120*** | 3326 ± 262 | 2952 ± 118 |
| Valine | 1446 ± 107 | 2868 ± 131*** | 4436 ± 226 | 3477 ± 66*** |
| Methionine | 587 ± 36 | 1541 ± 47*** | 2446 ± 119 | 2497 ± 75 |

After 90 min of equilibration period, striatal slices were incubated either in normal (control) or in ischemic conditions for 60 min (ischemic period) followed by 5 h of incubation in normal physiological medium (REO period). At the end of these periods, incubation medium was removed, acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for their amino acids. Data are expressed as mean ± SEM of 8 (control) or 10 (ischemic and REO groups) determinations, except GABA and arginine (number of determinations is 4 for these amino acids). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different from corresponding control values

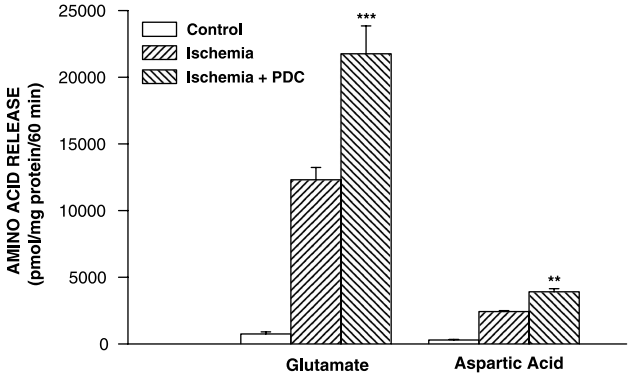


**Fig. 3.** Effect of glutamate transport inhibitor L-trans-PDC on ischemia-induced glutamate and aspartic acid outputs. After the equilibration period, striatal slices were incubated in ischemic medium for 60 min in the absence or presence of L-trans-PDC (200 $\mu$M). L-trans-PDC was added to the medium 10 min before ischemia and was present in the medium during the 60-min ischemic period. At the end of incubation, samples were collected, acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for amino acid levels. Slices were homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue final amino acid and protein levels. Data are mean ± SEM (bars) values, in pmol/mg protein/60 min. The number of determinations is 4, 7 and 6 for control, ischemia and ischemia plus L-trans-PDC groups, respectively. ** $p<0.01$, *** $p<0.001$, significantly different from the value obtained in ischemic conditions without L-trans-PDC

LDH leakage (448 ± 37 vs. 314 ± 30 $\mu$ $\Delta$O.D/$\mu$g protein, $p<0.05$), but this treatment failed to alter the ischemia-induced amino acid release (data not shown). REO-induced LDH leakage, on the other hand, was abolished almost completely by removal of the extracellular calcium during ischemic period. In contrast, enhanced LDH leakage was not altered when calcium ions were omitted from the medium during REO (Fig. 5). In agreement with these findings, removal of calcium during both ischemia and REO periods, did not cause a further decline in LDH leakage (Fig. 5).

### *Amino acid metabolism in control and ischemic striatal slices*

One hour ischemia followed by 5 hours REO caused nearly 50% depletion in tissue final glutamate, taurine and GABA levels. Tissue final aspartic acid level, on the other hand, was enhanced by ischemia (Table 3). The changes in the tissue final levels of other amino acids were lesser in extent. Ischemia-REO period, moreover, caused significant declines in total (released from plus remained in the slices) glutamate, glutamine and GABA levels (Table 3). Table 3 also indicates that the amounts of

**Table 2.** Effect of $Ca^{+2}$ removal on amino acid release during ischemia and REO periods

| Amino acid | Ischemic period (pmol/mg protein/1 h) | | REO period (pmol/mg protein/5 h) | |
|---|---|---|---|---|
| | $+Ca^{+2}$ | $-Ca^{+2}$ | $+Ca^{+2}$ | $-Ca^{+2}$ |
| Glutamate | $19082 \pm 668$ | $18928 \pm 903$ | $261 \pm 27$ | $208 \pm 10$ |
| Aspartic acid | $3594 \pm 175$ | $2663 \pm 108^{***}$ | $337 \pm 72$ | $101 \pm 60$ |
| Taurine | $8423 \pm 431$ | $4809 \pm 333^{***}$ | $4574 \pm 281$ | $3516 \pm 139$ |
| Glutamine | $1939 \pm 43$ | $1000 \pm 42^{***}$ | $1472 \pm 130$ | $712 \pm 60^{***}$ |
| GABA | $11865 \pm 692$ | $12071 \pm 511$ | $1226 \pm 138$ | $1157 \pm 45$ |
| Glycine | $3088 \pm 147$ | $2392 \pm 95^{***}$ | $2171 \pm 50$ | $1490 \pm 35^{**}$ |
| Citrulline | $416 \pm 37$ | $244 \pm 26^{*}$ | $1282 \pm 57$ | $560 \pm 42^{***}$ |
| Alanine | $4151 \pm 236$ | $2840 \pm 87^{***}$ | $3997 \pm 99$ | $3121 \pm 61^{*}$ |
| Serine | $3192 \pm 169$ | $2102 \pm 164^{***}$ | $3182 \pm 68$ | $1763 \pm 45^{***}$ |
| Leucine | $3806 \pm 69$ | $1462 \pm 32^{***}$ | $4577 \pm 125$ | $2674 \pm 46^{***}$ |
| Isoleucine | $1966 \pm 46$ | $786 \pm 19^{***}$ | $2489 \pm 77$ | $1376 \pm 29^{***}$ |
| Tyrosine | $1372 \pm 49$ | $652 \pm 53^{***}$ | $1771 \pm 62$ | $974 \pm 22^{***}$ |
| Phenylalanine | $1759 \pm 27$ | $819 \pm 27^{***}$ | $2838 \pm 64$ | $1649 \pm 22^{***}$ |
| Threonine | $2329 \pm 85$ | $1506 \pm 58^{***}$ | $2170 \pm 40$ | $1382 \pm 13^{***}$ |
| Valine | $2657 \pm 56$ | $1160 \pm 55^{***}$ | $3215 \pm 75$ | $1914 \pm 38^{***}$ |
| Methionine | $1309 \pm 100$ | $427 \pm 21^{***}$ | $2181 \pm 140$ | $1068 \pm 39^{***}$ |

After 90 min of equilibration period, striatal slices were incubated in $Ca^{+2}$-containing or $Ca^{+2}$-free ischemic medium for 60 min (ischemic period). Striatal slices in reoxygenation group were first incubated $Ca^{+2}$-containing ischemic conditions for 1 h and then incubated in normal physiological medium containing with or without $Ca^{+2}$ ions for 5 h (REO period). $Ca^{+2}$-free medium was prepared by replacing the $CaCl_2$ with equimolar NaCl and by adding 1 mM of EGTA in the medium. At the end experiments, incubation medium was removed, acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for their amino acids. Data are expressed as mean ± SEM of 6 (for ischemic group) or 4 (for REO groups) determinations. $^{*}\,p<0.05$, $^{**}\,p<0.01$, $^{***}\,p<0.001$, significantly different from corresponding control values

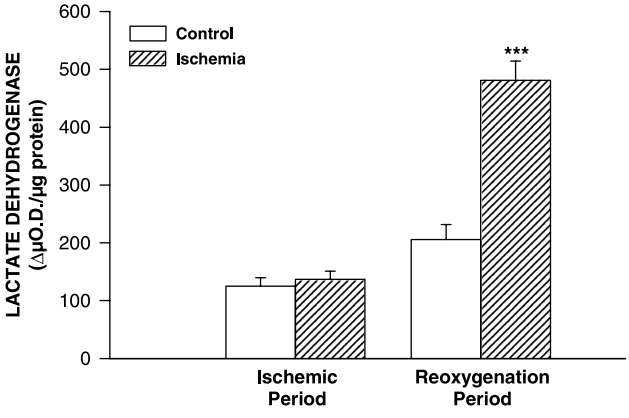


**Fig. 4.** LDH leakage from striatal slices during ischemia and REO periods. After the equilibration period, striatal slices were incubated either in control or ischemic conditions for 60 min, followed by 5 hours incubation in normoxic conditions. At the end of each period, half of the incubation medium (1 ml) was acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for amino acid levels. Another half of the medium was used for determination of LDH leakage as indicated in the text. Slices were homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue final amino acid and protein levels. Data are mean ± SEM (bars) values, in $\mu\Delta O.D/\mu g$ protein. The number of determinations is 8 for control, 14 for ischemia and 10 for REO periods. $^{***}\,p<0.001$, significantly different from the corresponding control value

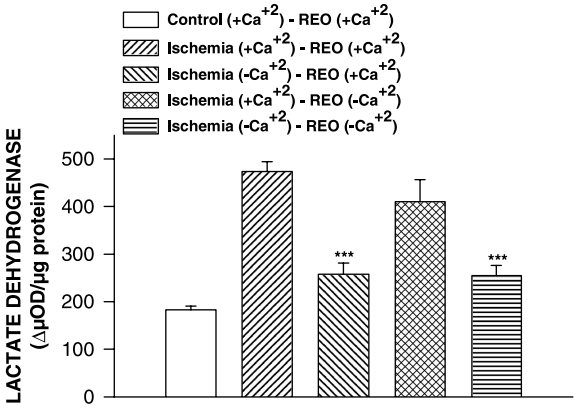


**Fig. 5.** Effect of $Ca^{+2}$ removal on ischemia-REO-induced LDH leakage from striatal slices. After the equilibration period, striatal slices were incubated either in control or ischemic conditions for 60 min, followed by 5 hours incubation in normoxic conditions. $Ca^{+2}$ was removed from the medium during ischemia and/or REO periods. At the end of each period, half of the incubation medium (1 ml) was acidified with $HClO_4$ (final concentration 0.4 M) and then assayed for amino acid levels. Another half of the medium was used for determination of LDH leakage as indicated in the text. Slices were homogenized in 2 ml of 0.4 M $HClO_4$, and homogenates were used for determination of both tissue final amino acid and protein levels. Data are mean ± SEM (bars) values, in $\mu$ $\Delta O.D/\mu g$ protein. The number of determinations is 6 for control, 12 for the ischemic groups which were contained $Ca^{+2}$ during REO period, and 8 for other two groups. $^{***}\,p<0.001$, significantly different from the value obtained in ischemia ($+Ca^{+2}$)-REO ($+Ca^{+2}$) group

**Table 3.** Tissue initial, tissue final and total amino acid levels in rat striatal slices

| | Tissue initial (pmol/mg protein) | Tissue final (pmol/mg protein) | | Total[c] (pmol/mg protein) | |
|---|---|---|---|---|---|
| | | Control | Ischemia | Control | Ischemia |
| Glutamate | 52465 ± 2012[a] | 42903 ± 1685 | 24176 ± 812*** | 43901 ± 1745 | 38934 ± 868* |
| Aspartic acid | 10862 ± 380[b] | 4352 ± 248 | 8522 ± 250*** | 5288 ± 429 | 12427 ± 391*** |
| Taurine | 18857 ± 704 | 13453 ± 735 | 7332 ± 335*** | 18895 ± 963 | 19026 ± 658 |
| Glutamine | 2288 ± 150[b] | 805 ± 34 | 691 ± 67 | 13327 ± 1205 | 5596 ± 391*** |
| GABA | 15568 ± 433[a] | 17090 ± 582 | 7010 ± 163*** | 18341 ± 561 | 16470 ± 553* |
| Glycine | 2051 ± 73[b] | 2144 ± 153 | 1808 ± 89 | 5897 ± 400 | 7754 ± 168*** |
| Citrulline | 133 ± 10[b] | 554 ± 96 | 525 ± 46 | 1184 ± 160 | 1935 ± 119** |
| Arginine | 640 ± 31[b] | 737 ± 23 | 698 ± 31 | 4244 ± 60 | 4750 ± 256 |
| Alanine | 1862 ± 159[b] | 1555 ± 79 | 1094 ± 84** | 10385 ± 666 | 9522 ± 209 |
| Serine | 1728 ± 41[b] | 1409 ± 138 | 1176 ± 115 | 6658 ± 515 | 8269 ± 283* |
| Leucine | 614 ± 37[b] | 989 ± 42 | 725 ± 57** | 8557 ± 403 | 9018 ± 142 |
| Isoleucine | 409 ± 11[b] | 675 ± 61 | 581 ± 51 | 5146 ± 290 | 5445 ± 126 |
| Tyrosine | 321 ± 21[b] | 474 ± 39 | 458 ± 50 | 3189 ± 231 | 3725 ± 67* |
| Phenylalanine | 361 ± 19[b] | 514 ± 28 | 521 ± 32 | 4598 ± 269 | 5466 ± 142* |
| Threonine | 1352 ± 145[b] | 1017 ± 79 | 885 ± 97 | 5591 ± 431 | 6668 ± 201* |
| Valine | 701 ± 35[b] | 915 ± 46 | 757 ± 58 | 6785 ± 349 | 7102 ± 84 |
| Methionine | 217 ± 22[b] | 289 ± 19 | 260 ± 23 | 3322 ± 148 | 4306 ± 103*** |

After 90 min of equilibration period, incubation on some slices were terminated for determination of tissue initial amino acid levels. Incubation of remained slices were continued either in normal (control) or in ischemic conditions for 60 min (ischemic period) followed by 5 h of incubation in normal physiological medium (REO period). Incubation was terminated by transferring the slices in 2 ml of 0.4 M $HClO_4$. After homogenization of the slices, samples were centrifuged and then assayed for their amino acids as indicated in the text. Data are expressed as mean ± SEM of 8 (control) or 10 (ischemic and REO groups) determinations, except GABA, arginine and tissue initial amino acids (n is 4 for these amino acids). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly differ from corresponding control values. [a] $p < 0.05$, [b] $p < 0.001$, significantly differ from total amino acid levels obtained in control slices. [c] Represents the sum of amino acids that were released from and remained in the slices

most amino acids that were released spontaneously from control slices significantly exceeds the quantity of their tissue initial levels. Although this enhancement was further extended by ischemia-REO, only the increases obtained in glycine, citrulline, serine, tyrosine, phenylalanine, threonine and methionine levels reached to significant levels.

## Discussion

### *Amino acid release during ischemia and REO periods*

Since glutamate is widely recognized to be a neurotoxic neurotransmitter in anoxic/ischemic conditions (Martin et al., 1994; Szatkowski and Attwell, 1994), much of the studies have been centered on the release of this amino acid. Results derived from these studies showed that several possible sources might be involved in glutamate efflux from the ischemic tissues (Phillis and O'Regan, 2003). One of the proposed sources is reversed operation of glutamate reuptake transporters. During ischemia, collapse of the membrane potential and ionic transmembrane gradients can cause reversed operation of glutamate reuptake transporters and thus contribute to glutamate accumulation in extracellular space. Indeed, studies on glutamate release during ischemic conditions have demonstrated involvement of a reversal of glutamate transport (Roettger and Lipton, 1996; Phillis et al., 2000; Rossi et al., 2000; Kulik et al., 2000), however, opposite results are also present (Heron et al., 1995; Obrenovitch et al., 1998). In the present study, inhibition of glutamate transporter by L-trans-PDC failed to decrease ischemia-induced glutamate output, but caused a further increment. Similar results were also obtained in terms of aspartic acid release. Although this finding suggests that reversed transport seems unlikely in ischemia-induced glutamate output from striatal slice preparation used in present study, L-trans-PDC has also an enhancing effect on glutamate release through heteroexchange (Volterra et al., 1996) or by a mechanism independent of inhibiting glutamate transporters (Ohta et al., 2002). It has been concluded that if the conditions prevailing during energy deprivation facilitate reverse glutamate transport, this process will also be associated with an increase in the release of glutamate by heteroexchange (Jabaudon et al., 2000). In support of this conclusion, L-trans-PDC, as demonstrated in the present paper, has been shown to increase glutamate

release from energy deprived rat organotypic hippocampal slice cultures (Jabaudon et al., 2000). Since a decrease or reversal of the electrochemical $Na^+$ gradient promotes reversed operation of the neurotransmitter transporters (Levi and Raiteri, 1993) and since ischemia collapses the transmembrane ionic gradients by inhibiting the $Na^+$,$K^+$-ATPase, it might be expected that blocking of the $Na^+$ channels may protect the neurons against the ischemia-induced glutamate output. In present study, TTX, a sodium channel blocker, caused significant decline in enhanced glutamate release. Although this finding in a good agreement with the previous observations (Calo et al., 1997; Djali and Dawson, 2001) and supports the hypothesis mentioned above, failure of the TTX on ischemia-induced glutamate efflux from rat cerebral cortex has been also reported (Nelson et al., 2003).

In a good agreement with the previous findings (Rego et al., 1996; Larsen et al., 1998), not only the excitatory amino acids, but the levels of others (except glutamine) in the medium also increased under ischemic conditions. At present, the mechanistic details of release process were not investigated, but involvement of the membrane distribution seems unlikely since no significant increase in LDH leakage was observed during 60 min of ischemia. As observed in glutamate release, TTX caused a partial decline in taurine and aspartic acid outputs, indicating that the release of these amino acids depends, at least partly, reversal of the transmembrane $Na^+$ gradient. With the exception of glutamate and GABA outputs, moreover, the release mechanism was found to be dependent on extracellular calcium ions in the medium. All results summarized above may suggest that probably more than one mechanisms involved in enhanced amino acid release under ischemic conditions. One important consequence of the cerebral ischemia is neuronal and especially astroglial swelling (Petito and Pulsinelli, 1984), that occurs as a result of anoxic depolarization leading to $Na^+$ and $Cl^-$ accumulation together with the water. As cells swell, they attempt to restore their normal volume by extruding osmotically active solutes including amino acids. Thus, this process known as ''regulatory volume decrease'' may contribute to ischemia-evoked amino acid release. The phenomenon of regulatory volume decrease has been extensively investigated in a variety of cell types and it has been established that swelling-induced release of certain amino acids such as glutamate and taurine, occur, at least in part, via diffusional efflux through swelling activated $Cl^-$ channels (see review Phillis and O'Regan, 2003). In addition to this mechanism, it has been reported that destabilization and deterioration of the plasma membrane as a consequence of phospholipid hydrolysis, would also allow the amino acids to diffuse across the plasma membrane, the rate of diffusion being dependent on concentration gradients (Song et al., 1998; Phillis and O'Regan, 2003). When the ratios of tissue initial amino acid concentrations (reflecting intracellular levels) were compared with their basal releases, it was noteworthy that amino acid neurotransmitters (glutamate, aspartic acid and GABA) and taurine which had the highest ratios, were preferentially released during ischemia. The release of other amino acids with the lower ratios, on the other hand, were quite similar and lesser in extent, supporting the conclusion that loss of the plasma membrane integrity as a result of the enhanced phospholipase activity would allow the amino acid efflux into the extracellular space, the rate of loss being dependent on concentration gradients (Phillis and O'Regan, 2003).

With the exception of amino acids with highest intracellular/extracellular concentration ratios, the amounts of others measured in the medium exceeded their tissue initial values (see Table 3). Similar findings have also been reported for tyrosine in rat brain slices incubated under normoxic conditions (Arneric et al., 1987; Büyükuysal and Mogol, 2000) and for other amino acids (except taurine) in isolated anoxic/reperfused rat heart (Song et al., 1998), implying that de novo synthesis or degradation of proteins or small peptides by proteolytic enzymes could be the main source for most of the amino acids (especially with lower intracellular/extracellular concentration ratios) released from the brain slices incubated under ischemic or even normoxic conditions.

The most striking observation during REO period was doubling of the citrulline level in the medium. Since citrulline is known as a co-product of NO synthesis from L-arginine (Garthwaite et al., 1989) and since enhanced formation of NO and resultant degradation of NO to cytotoxic free radicals, such as peroxynitrite or hydroxyradical have been involved in ischemia-REO-induced neuronal damage (Maiese et al., 1996; Eliasson et al., 1999; Oka et al., 2000b; Xu et al., 2000; Rodrigo et al., 2001), this alteration in citrulline level may indicate an increased synthesis of NO during REO. In addition to citrulline, taurine level in the medium also enhanced during REO period. Similar change in taurine release has been noted in isolated anoxic-reperfused rat heart (Song et al., 1998). Since taurine is known as an inhibitory amino acid (Saransaari and Oja, 2000), a rise in its level during ischemia and/or REO periods probably provide a protective mechanism against excitotoxicity.

*Ischemia-REO-induced tissue damage*

One of the most important finding related to ischemia-induced damage is that NMDA receptor antagonists can reduce the neuronal death even when used after ischemia (Gill et al., 1988; Hartley and Choi, 1989). This protection by late application of NMDA antagonists indicates that neuronal damage that becomes apparent after a delay is an active process, and because there is more time for giving the therapy, blocking the post-ischemic events should be a more promising strategy against ischemia-induced neuronal damage (Szatkowski and Attwell, 1994). In rat brain slice preparations, ischemia-induced tissue injury determined by the leakage of the LDH, has been observed only after a transient exposure to hypoxia/glucose deprivation followed by REO (Tatsumi et al., 1998; De La Cruz et al., 2002). In a good agreement with these observations, LDH leakage significantly increased when ischemic slices were transferred into oxygen and glucose containing medium (Fig. 4). Presence of MK-801 in the medium during both ischemia and REO periods attenuated the LDH leakage by 30%, but did not alter enhanced amino acid output. Although these data support the glutamate excitotoxicity in ischemia-REO-induced LDH leakage, limited effect of MK-801 even at high doses used in present or in other studies (Oka et al., 2000a) indicates that other glutamatergic receptors, such as AMPA and/or metabotropic receptors, or other cellular processes which do not directly related to glutamate might be also implicated (Newell et al., 1995; Rootwelt et al., 1998; Calabresi et al., 2000; Jarvis et al., 2001). It is must be noted that, neither blocking of kainat/AMPA receptors with CNQX (50 $\mu$M) nor metabotropic receptors with MCPG(±)- (50 $\mu$M) exerted a NMDA-like effect on REO-induced LDH leakage in ongoing studies of this laboratory. Since TTX caused 50% decline in ischemia-induced glutamate output without altering the REO-induced LDH leakage and since LDH leakage enhanced during REO, a period where the enhanced glutamate output has been returned to its control level, it seems likely that not only the glutamate release, but combination of the glutamate release with other factors that are activated during ischemia and/or REO periods could contribute the LDH leakage (Yamamoto et al., 1999).

The present study also demonstrated that removal of extracellular calcium from ischemic medium fully protected the slices against REO-induced LDH leakage, suggesting that under ischemic conditions, activation of cellular processes which are linked to neuronal damage (except glutamate output) needs extracellular calcium ions in the medium. In contrast, LDH leakage remained unchanged when calcium ions were removed from the medium during REO of the ischemic slices. This result may indicate that calcium ions present in the cells might be in a sufficient level for sustaining on the damaging processes which occur during REO period.

*Amino acid metabolism in ischemic slices*

Although removal of glucose from the medium failed to enhance both glutamate and aspartic acid outputs (Fig. 1A and Fig. 2A), tissue final levels of these amino acids significantly altered under aglycemic condition (Fig. 1B and 2B); while aspartic acid level was almost doubled, tissue level of glutamate was depleted under aglycemia, which may indicate an increased synthesis of aspartic acid from glutamate in glucose-free medium. In support of this conclusion, it has been shown that glutamate-aspartate conversion in cultured astrocytes (Bakken et al., 1998) or in rat hippocampal slices (Madl and Royer, 1999) is preferentially utilized when oxygen is available but glycolysis is impaired. Thus, when glucose is unavailable, glutamate probably serves as a substrate that produces ATP by an increased catabolism of glutamate through AATase, GDH and the TCA cycle (Madl and Royer, 1999). Like aglycemia, tissue final and total aspartic acid levels were also found higher in ischemia-REO group, suggesting that ischemia, like agylcemia, has a similar effect on the metabolism of this amino acid.

Results presented in this paper also indicate that ischemia, in addition to excitatory amino acids, may also affect GABA metabolism in rat striatal slices. As shown in Table 3, total GABA level of control slices was higher than its tissue initial value ($18341 \pm 561$ vs. $15568 \pm 433$ pmol/mg protein, $p < 0.05$), which probably suggests a continued synthesis of this amino acid during incubation period. In slices exposed to ischemia followed by REO, on the other hand, total GABA level declined significantly ($18341 \pm 561$ vs. $16470 \pm 553$, $p < 0.05$), indicating a decreased synthesis of GABA under these conditions. In support of this conclusion, it has been shown that a permanent ischemia induced by middle cerebral artery occlusion in mice (Green et al., 1992) or hypoglycemia in rat hippocampal slices (Madl and Royer, 2000) causes significant decline in GABA synthesis.

In summary, results presented here demonstrate that not only the excitatory and inhibitory amino acids, but the release of others was also enhanced by ischemia. Since LDH leakage was not altered during ischemia and since there was a clear difference between the dependency of amino acid release on extracellular calcium ions, neither distribution of neuronal membranes nor a common mechanism seems to be

involved in ischemia-induced amino acid release. In addition to amino acid release or LDH output, the present study also demonstrated that ischemia and/or ischemia-like conditions also alter amino acid metabolism which may directly be linked to neuronal damage and/or involved in sustaining the neuronal viability under these conditions.

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I dedicated this study to my dear niece Ayça Köker whose lost her life after brain damage.

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