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Calcium activity and post-ischemic suppression of protein synthesis

B. Djuricic, G. Röhn, W. Paschen* and K.-A. Hossmann

Max-Planck-Institute for Neurological Research, Department of Experimental Neurology, Gleuelerstraße 50, D-50931 Cologne (Germany)
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Abstract. Increase in intracellular calcium concentration is a prominent feature of ischemia and has been considered a major factor in the initiation of ischemic pathology, which involves inhibition of protein synthesis. A reduction of calcium ion activity during and immediately after in vitro ischemia did not prevent inhibition of protein synthesis in hippocampae slices. When slices were overloaded with calcium by NMDA receptor activation or by the calcium ionophore A23187, no significant inhibition of protein synthesis was observed. We conclude that calcium overload plays only a limited role in ischemic inhibition of protein synthesis.
Key words. Protein synthesis; CA^{2+} activity; ischemia; hippocampus; slice; in vitro.

The pathogenic mechanisms underlying the phenomenon of delayed neuronal death after ischemia are not entirely understood. Considerable attention has been directed towards the disturbance of ionic homeostasis, in particular that of calcium; this interest was increased when a link between the activation of glutamate-operated ion channels and calcium influx during ischemia was established[1]. Several drugs interfere with calcium influx[2], and if cellular Ca^{2+} overload is a causative factor of post-ischemic damage, these drugs would offer a range of therapeutic possibilities. In vivo studies of the effects of these agents are complicated by the multiplicity of the actions, however. These include vascular effects, which may alter the ischemic outcome and may not be related to any influence on the neuronal calcium homeostasis itself.

The present study examined the effect of a reduction of calcium ion activity during and immediately after ischemia on protein synthesis in an in vitro model of neuronal tissue ischemia, namely the hippocampal slice preparation. The hippocampus is very sensitive to ischemia, being damaged after short periods of ischemia that do not affect other brain regions[3]. As a biochemical marker of injury, the rate of protein synthesis was chosen. Protein synthesis has a higher ischemic threshold than energy metabolism in vivo[4], and it is a sensitive indicator of impending cell death. It never fully recovers in neurons destined to die[5,6], and interventions that improve survival of hippocampal cells after ischemia also restore protein synthesis[6].

* To whom correspondence should be addressed.

Materials and methods

Female CDF-344 rats aged 3 months were anesthetized with 4% halothane in 30% O_2 (remainder NO_2) and decapitated. Brains were removed and placed in chilled (0 °C) standard artificial cerebrospinal fluid (aCSF), which contained (in mM) NaCl, 120; $NaHCO_3$, 26; KCl, 3; KH_2PO_4, 1.4; $CaCl_2$, 2.4; $MgSO_4$, 1.3; glucose, 10; and HEPES, 5; pH was adjusted to 7.4. Artificial CSF was equilibrated with a gas mixture of O_2/CO_2 (95%/5%) for at least 45 min before the experiment. Hippocampi were dissected out and 400 µm slices were made using a tissue slicer. Slices were allowed to recover for 60 min at 33 °C in O_2/CO_2 atmosphere.

In vitro ischemia (15 min) was induced by placing slices in glucose- and HEPES-free aCSF under a gas mixture of N_2/CO_2 (95%/5%). Ca^{2+} availability in ischemic aCSF was reduced by removing Ca^{2+} from the medium and inclusion of 5 mM EGTA; in other experiments, reduction of ischemic increase in calcium activity was achieved by pre-loading slices with ruthenium red (20 µM), Fura2/AM (50 µM) or dantrolene (20 µM) before exposure to ischemia. Prior to ischemia slices were washed in Ca^{2+}-free medium, or incubated in the presence of pharmacological agent for 5 min (1 h for Fura2/AM loading, followed by 30 min incubation in standard aCSF to allow hydrolysis of the ester) under standard conditions (i.e., medium contained glucose, and atmosphere was 95% O_2/5% CO_2).

In a separate series of experiments 300 µm slices were used in order to establish whether slice thickness was a limiting factor regarding penetration of a pharmacological agent. In particular, Fura2 effects were tested. The effects of Fura2 loading and ischemia were undistinguishable in 400 µm thick slices and 300 µm thick slices,

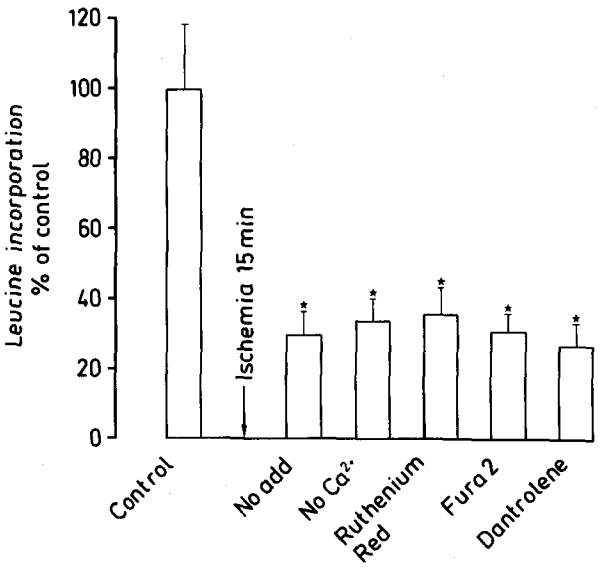

Figure. Protein synthesis in hippocampal slices after 15 min of in vitro ischemia. Ca^{2+} availability during ischemia was reduced by omitting Ca^{2+} from aCSF and adding 5 mM EGTA (no Ca^{2+}), or by including ruthenium red (20 μM), Fura2 (50 μM), or dantrolene (20 μM) in aCSF. Vertical bars indicate SD for 5–8 samples. No add, no additions, *, $p < 0.05$ compared to controls. Experiments with Fura2 and dantrolene were performed in nominally Ca^{2+}-free medium.

and in the present paper we report data on 400 μm slices, except when otherwise stated.

After 15 min incubation in modified aCSF, medium was exchanged for standard aCSF. Protein synthesis was measured by adding 4.5 μCi/ml of L-[1-^{14}C]leucine (specific activity 54 mCi/mMol) in aCSF after experimental treatment. Incubation in the presence of the radiolabeled leucine was carried out for 30 min. The experiment was terminated by transferring individual slices to 1 ml of 10% trichloroacetic acid (TCA). Slices were homogenized by sonification, centrifuged, the pellet washed once with 5% TCA, and centrifuged again. The pellet was resuspended in 1 M NaOH, and aliquots were taken for protein determination and liquid scintillation counting. In separate sets of experiments, ATP levels were measured in hippocampal slices.

Statistical analysis was performed by one-way ANOVA followed by Scheffe's group comparisons, $p < 0.05$ being significance limit.

Results

Following 15 min of in vitro ischemia, protein synthesis in hippocampal slices was reduced to 1/3 of controls during the first 30 min of recovery (fig.). The amount of radiolabeled leucine in slices that were ischemic was unchanged compared to controls (174 ± 54 nCi/mg slice protein in controls, and 186 ± 60 nCi/mg slice protein in slices exposed to ischemia; mean ± SD, n = 12), indicating that the radiolabeled leucine availability was not a factor in the observed reduction of the rate of protein synthesis. ATP levels returned to 80 ± 11% (n = 5) of controls after 30 min recovery and did not change during the next 4 h. Protein synthesis, on the other hand, remained severely depressed (50% of control at 4 h of recovery). This indicates that in vitro ischemia causes a similar dissociation between energy metabolism and protein synthesis as in vivo[7,8].

We tested whether preventing an increase in intracellular calcium activity lessens the effect of ischemia on protein synthesis. Increase in intracellular calcium ion activity was prevented by removing Ca^{2+} from aCSF, by blocking voltage-dependent calcium channels, by buffering increase in intracellular calcium, or by preventing release of calcium from intracellular stores. However, none of the treatment regimens prevented the suppression of protein synthesis (fig.).

In detail the following experiments were performed. Influx of extracellular calcium during ischemia was prevented by removing Ca^{2+} from the ischemic aCSF or by blocking voltage-dependent calcium channels by ruthenium red. The inorganic dye is an effective blocker of the voltage-activated influx of calcium, occurring for example during depolarization[9]. In addition, ruthenium red does not interact with Na^+ channels of cells as several organic and inorganic Ca^{2+} channel blockers do[10]. However, early post-ischemic inhibition of protein synthesis in treated groups was indistinguishable from that of the untreated groups (fig.). Blockade of receptor-operated calcium channels by MK-801, a non-competitive blocker of NMDA receptors, also failed to improve post-ischemic protein synthesis (data not shown). The effects of reducing intracellular calcium activity were assessed by incubating slices in Ca^{2+}-free medium during ischemia, combined with pre-loading slices with 20 μM dantrolene or 50 μM Fura2. Dantrolene blocks depolarization- and NMDA-induced release of calcium from ryanodine-sensitive intracellular stores in neurons in vitro[11], and was used both in vivo[12,13] and in vitro[14] for investigation of the effects of ischemia- or glutamate-induced calcium overflow on the nerve tissue. Fura2 was used to provide intracellular buffering of Ca^{2+}, similarly to the approach of von Tscharner[15]. The degree of the early post-ischemic inhibition of protein synthesis was not affected by this approach, and protein synthesis was as severely depressed in dantrolene and Fura2 groups as in the untreated groups (fig.). There were no differences between slices 400 μm thick and 300 μm thick (data not shown).

In experiments designed to study the effects of Fura2 we noted that the dye by itself causes a certain degree of inhibition of protein synthesis. For example, in 300 μm slices incubated in a Ca^{2+}-containing medium and loaded with 0.1 μM, 1.0 μM, and 10.0 μM Fura2/AM for 1 h, followed by 30 min hydrolysis period the rate of

protein synthesis was $60 \pm 17\%$, $55 \pm 24\%$, and $41 \pm 14\%$, respectively (means $\pm$ SD, $n = 5$; $p < 0.05$ compared to controls).

In separate experiments calcium was omitted or dantrolene was added during the post-ischemic period, but again the extent of inhibition of protein synthesis was not altered: protein synthesis was $22 \pm 2\%$ of control when Ca^{2+} was omitted from post-ischemic aCSF, and $19 \pm 2\%$ of control if dantrolene was present during recovery.

In order to investigate further the possible effects of Ca^{2+} on protein synthesis, hippocampal slices incubated under normoxic conditions were overloaded with Ca^{2+} by activation of NMDA-dependent Ca^{2+} channels (100 µM NMDA), or by exposing slices to calcium ionophore A23187 (10 µM) for 15 min. The protein synthesis rate was measured immediately after the treatment as described above. These treatments led to similar effects on the protein synthesis: in slices exposed to NMDA, protein synthesis was $76 \pm 6\%$ of the control, and in those exposed to A23187, $70 \pm 11\%$ of the control (mean $\pm$ SD, $n = 5-8$ in each group). It should be noted that these treatments may cause tissue swelling[16] and consequently reduce the amount of radiolabeled leucine in the tissue. For example, in our experiments the amount of radioactive leucine in slices exposed to NMDA or A23187 was reduced to respectively 84% and 79% of control. When the incorporated amino acid is expressed as percentage of the labeled amino acid present in the slice, the protein synthesis in treated slices was not significantly different from that in controls (not shown). Thus, the apparent inhibition of protein synthesis by calcium overload is a combination of two effects, a reduction of the amino acid content in the tissue and a marginal inhibition which may be attributed to direct calcium effects.

Discussion

A prominent pathophysiological feature of ischemia of the nervous system is the increase in intracellular calcium[17]. Given the number of functions of calcium in the nerve cell, local changes in the concentration of this ion can be assumed to participate in the development of ischemic neuronal damage[18]. The idea of Ca^{2+} overload as the causative factor in ischemic cell injury prompted investigations to block calcium entry into the neuron. However, in vivo application of calcium channel blockers (e.g. dihydropyridines) or NMDA antagonists, or of inhibitors of intracellular calcium release (e.g. dantrolene) did not reduce brain damage in global ischemia models[12,19,20]. Our observations in an in vitro model of neuronal tissue ischemia are also at variance with the calcium hypothesis of ischemic cell injury. In fact, neither inhibition of calcium influx nor the inhibition of intracellular calcium release was able to reverse the inhibition of protein synthesis which previously has been shown to precede the manifestation of ischemic cell damage. This observation is in line with a previous in vivo study of global brain ischemia which failed to detect a protective effect of calcium antagonists on post-ischemic inhibition of protein synthesis[21].

There may be concern regarding the effectiveness of penetration of drugs into the slice, particularly with those drugs with a high molecular weight such as Fura2/AM (MW 1001). Two lines of evidence showed that diffusion was not limiting. First, no differences were observed between 300 µm and 400 µm slices regarding Fura2 effect on postischemic inhibition of protein synthesis. Second, significant inhibition of protein synthesis was observed when intracellular calcium was chelated with Fura2 in both 300 µm and 400 µm slices; the degree of inhibition was similar, indicating that Fura2 penetrates the whole slice thickness during 1 h of loading (Djuricic, unpubl. data). The fact that intracellular calcium chelation inhibits protein synthesis indicates that protein synthesis requires Ca^{2+}, and that cautious interpretation of results is needed when changes in the ion activity are blocked. In addition, it is worth noting that Fura2 interferes with protein synthesis even if very low concentrations are used for loading.

An indirect argument against the importance of calcium for ischemic injury is the paucity of the metabolic disturbance when intracellular calcium was deliberately increased. Neither depolarization nor activation of receptor-operated Ca^{2+} channels had a marked effect on protein synthesis; apparent inhibition observed in slices overloaded with calcium is probably mostly due to the reduction in radiolabeled precursor availability because of the tissue swelling. This finding is fully in line with the previous observation that calcium overload is not a prerequisite for the induction of hypoxic cell death in vitro[22].

In contrast to these negative findings, calcium antagonists have been shown to reduce ischemic injury in animal models of focal ischemia[23,24]. It is not clear, though, to what extent the beneficial effect of calcium channel blockers such as nimodipine has to be attributed to the vasodilating effect of the drug[24]. It should also be noted that in models of reversible focal cerebral ischemia, Ca^{2+} continues to accumulate for at least 30 min after the onset of reperfusion[23,24], while intracellular calcium normalizes within a few minutes after the onset of recirculation following reversible global ischemia[1]. It is, therefore, possible that under certain conditions, as in the periphery of a focal ischemia, calcium antagonists are able to interfere with an ongoing pathological process that may lead to irreversible cellular damage. Our data, however, indicate that ischemia inhibits protein synthesis even under conditions when ischemic increase in calcium activity is prevented. We conclude that calcium overload

plays a limited role in the ischemic inhibition of protein synthesis.

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