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THE EFFECTS OF NORMOVOLEMIC HEMODILUTION ON PROTEIN SYNTHESIS RECOVERY FOLLOWING POSTISCHEMIC REPERFUSION IN THE RAT BRAIN

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INTRODUCTION

Hemodilution, improving brain perfusion, has been presented as an effective way of brain recovery after cerebral ischemia. A rationale for this possibility is based on the Hagen-Posseuille equation postulating that blood flow is inversely proportional to blood viscosity, when other blood parameters remain constant (11). Hematocrit values influence blood viscosity more than other rheological characteristics. When hematocrit is reduced by hemodilution, cerebral blood flow increases in both normal and ischemic brains (11). In a the therapeutic application, hemodilution decreases hematocrit rapidly down to 33% and raises cardiac output (26). As a result, erythrocytes reduce their density and improve their ability to squeeze through capillaries, thus enhancing cerebral blood flow (26).

An ischemic insult in the brain tissue causes biochemical, physiological and delayed morphological changes that may lead to cell death. However, the most prominent structural damage develops during the reperfusion period and not during the ischemic insult itself (28). Distinct neuronal populations within different brain areas have specific vulnerability to ischemia (named *selective vulnerability*) (6). For example, CA1 and CA4 layers of the hippocampus, the pars dorsolateralis of the corpus striatum and the cortical pyramidal cells in layers 2 and 5 belong to selective vulnerable brain areas (15).

Physiologically, working protein synthesis machinery is essential for cell survival and recovery after an ischemic insult. During early post-ischemic reperfusion, inhibition of protein synthesis accompanied by disaggregation of polysomes can be registered. Post-ischemic reperfusion causes protein synthesis alterations, relatively to the duration and severity of ischemia, intra-ischemic temperature, residual blood flow and age of the affected subject (8). Later, post-ischemic inhibition of protein synthesis persists despite ATP and energy renewal with blood circulation.

Protein synthesis inhibition is caused by impairment of two initiation factors, eIF-2 (3) and eIF-4G (9). In fact, oxygen radical overproduction, inactivation of detoxicant systems and consumption of antioxidants in the ischemic brain during reperfusion damage antioxidative defence mechanisms (7). Factor eIF-4G is therefore inac-

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tivated for a long time as a result of free radicals attack (9). A two or three times increase in phosphorylation of the eIF-2a subunit inhibits protein synthesis at 30 min after the ischemic episode (3).

We assumed that hemodilution could improve post-ischemic protein synthesis recovery by reducing the blood elements responsible for oxygen radical production. By using an *in vivo* model of cerebral ischemia with four-vessel occlusion (vertebral arteries, carotid arteries), this study demonstrated that the degree of phosphorylated eIF-2 (i.e. the magnitude of the reduction of eIF-2 activity) correlates with the inhibition of the ternary complex formation with GTP and Met-tRNA$_i$.

METHODS

Experimental animals and surgery

Male Wistar rats (250 - 350g) were used in this study. Animals were divided into three groups. The first group (n = 4) contained the control sham-operated animals. The rats in the second group (n = 16) underwent 30 minutes of forebrain ischemia followed by different times of reperfusion (30 minutes, 2, 4 and 6 hours). In the third group of animals (n = 16) ischemia and reperfusion timeframes were applied as in the second group, but hemodilution with hydroxyethyl starch (10% HES 200/0.5, osmolarity 308 mosm/l) was used.

Transient ischemia was induced by standard four-vessel occlusion (24), modified by Schmidt-Kastner (25). On the first day, the rats were anaesthetized by ketamine (100 mg/kg) and xylazine (15 mg/kg), and the vertebral arteries were cauterized by an electrocauter. The following day, rats underwent preparation to the reversible ischemia caused by the ligature of carotid arteries with polyethylene strips, and the vessel catheters were inserted into the caudal artery and the femoral vein. The atraumatic reversible compression of the carotid arteries with polyethylene-coated clips caused forebrain ischemia. During ischemia, blood was withdrawn and simultaneously replaced by the blood substitute solution – HES – for a total amount of 4 ml (2 ml x 2 times) through the vessel catheters. During ischemia, rats were placed on a heating thermo-pad to maintain a constant body temperature of 37 °C. The body temperature was controlled by a thermistor placed in the rat ear. Sham-operated animals were treated in the same way but the carotid arteries were not occluded. Rats were decapitated at the end of the recirculation period (30 minutes, 2 hours, 4 hours or 6 hours), brains were removed from skulls, dissected into the hippocampus, striatum and neocortex parts and immediately processed for biochemical analysis. Protein determination was carried out by Bradford's method (2).

Translation assays in vitro

Fresh brain tissue was homogenized 1:2 (w/v) with buffer (50 mM Hepes/KOH, pH 7.55, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiotreitol and 0.32 M sucrose) and centrifuged at 11000 g for 10 min at 4 °C to obtain postmitochondrial supernatant (PMS). The complete reaction system in a final volume of 50 ml contained 50 mM Hepes/KOH, pH 7.55, 140 mM potassium acetate, 4 mM magnesium acetate, 2.5 mM dithiotreitol, 0.32 M sucrose, 1 mM ATP, 0.75 mM GTP, 20 mM phosphocreatine, 150 ml/ml creatine kinase, 50 mM amino acids, 100 mg PMS proteins and 5 mCi of [4,5-^{3}H]leucine (25 mM; 66 Ci/mmol). The reactions were carried out at 30 °C for 45 min and then placed in 1 ml of distilled water. The samples were incubated for 20 min at 37 °C after addition of 0.5 ml of 1 M KOH containing 2 mg/ml leucine and the protein was precipitated with 25% trichloroacetic acid (TCA) containing 2 mg/ml leucine, and washed before filtration on a Whatman GF/C glass fibre filter. The filters were counted for radioactivity after agitation for 60 min with Normascint 22 scintillation liquid.

Factor eIF-2 assay

The GTP-dependent binding of eIF-2 factor to Met-tRNA$_i$ was measured in the postmitochondrial supernatant (PMS) as described by Martín et al. (17, 18), in the presence of Mg^{2+} (1.5 mM) and GTP-regenerating capacity (2.5 mM phosphoenolpyruvate and 2 U/ml of phosphopyruvate kinase). The results are expressed as pmoles of Met-tRNAi incorporated/mg of protein/10 min of incubation.

RESULTS

Figure 1 shows the development of the hematocrit values during the application of normovolemic hemodilution. The total blood volume (4 ml) was withdrawn in two separate withdrawals of 2 ml each and was replaced by HES immediately during ischemia. The observed protein synthesis results are expressed in the subsequent figures, divided according to the brain area studied: the cortex (Fig. 2), the striatum (Fig. 3) and the hippocampus (Fig. 4). Data show the activity of protein synthesis machinery as presented by *in vitro* incorporation of [4.5-^{3}H]leucine into the TCA insoluble polypeptides.

The ischemic insult caused a significant decline of protein synthesis in all the studied brain regions, as compared to the control values. This reduction, with protraction of the post-ischemic recirculation time interval, gradually increased and the highest values were obtained after 6 hours of post-ischemic reperfusion in all the studied areas. The cortex showed the best ability to improve its post-ischemic recov-

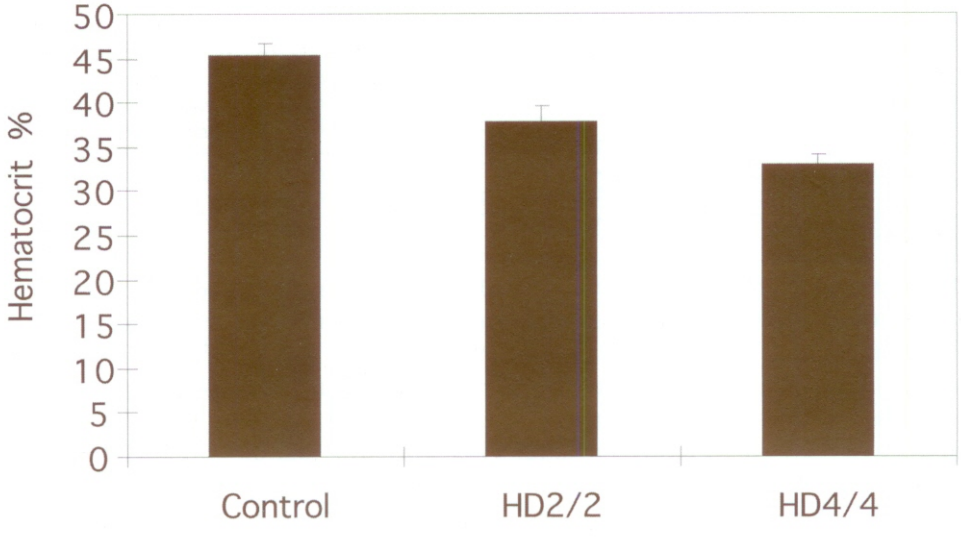

Fig. 1. - *Change of the hematocrit values (in %) during the application of normovolemic hemodilution.* The presented control values were taken from intact animals, before withdrawing and replacing any blood; HD 2/2 are the hematocrit values after withdrawing 2 ml of blood and replacing it with 2 ml of HES; HD 4/4 are the hematocrit values after withdrawing 4 ml of blood and replacing it with 4 ml of HES.

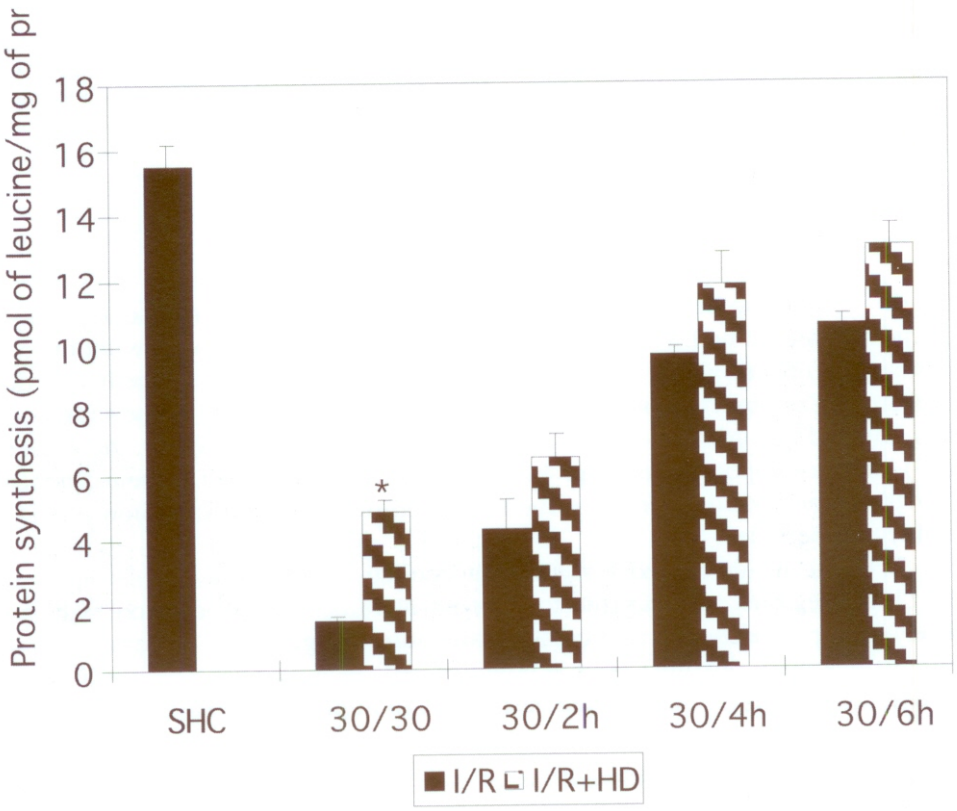

Fig. 2. - *The effects of post-ischemic recirculation on the activity of the protein synthesis machinery in the rat brain cortex with the application of normovolemic hemodilution (I/R + HD) or without (I/R), expressed as in vitro incorporation of [4,5-^{3}H]leucine into the TCA insoluble polypeptides.*
Values are means ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey-Kramer test. Significantly different results from comparison with and without the application of hemodilution are marked with * at the level $p < 0.01$. SHC means sham control, 30/30 means 30 min of ischemia and 30 min of post-ischemic reperfusion, 2 h means 2 hours, 4 h means 4 hours and 6h means 6 hours of post-ischemic reperfusion, respectively.

ery. The normovolemic hemodilution application caused improved recovery of the injured protein synthesis machinery and, in all brain areas studied, induced increased incorporation of *in vitro* labeled amino acid. The time course of this increase progressed with the extension of the reperfusion interval: the highest increase of [4.5-^{3}H]leucine incorporation was found in the cortex after 30 min ($p < 0.01$) of post-ischemic recirculation (Fig. 2). Prolonged hemodilution recirculation intervals led to slight increases in leucine incorporation, but without statistical significance. Similar results, even if not statistically significant, have been found in the striatum (Fig. 3) and the hippocampus (Fig. 4).

The eIF-2 activity in the neocortex was evaluated by measuring the formation of the ternary complex with GTP and Met-tRNA$_i$ in the presence of Mg^{2+} and GTP-regenerating capacity. As shown in Figure 5, the eIF-2 activity decreased signifi-

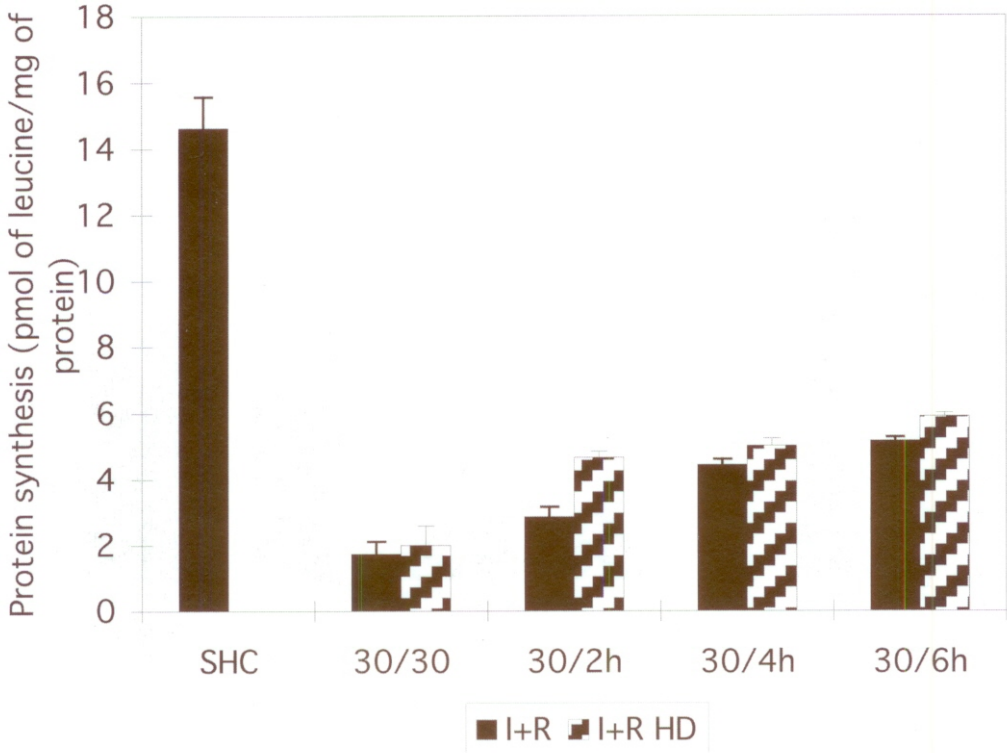

Fig. 3. - *The effects of post-ischemic recirculation on the activity of the protein synthesis machinery in the rat brain striatum with the application of normovolemic hemodilution (I/R + HD) or without (I/R), expressed as in vitro incorporation of [4,5-^{3}H]leucine into the TCA insoluble polypeptides.*
Values are means ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey-Kramer test. SHC means sham control, 30/30 means 30 min of ischemia and 30 min of post-ischemic reperfusion, 2 h means 2 hours, 4 h means 4 hours and 6h means 6 hours of post-ischemic reperfusion, respectively.

cantly in ischemic animals as compared to controls. A comparison between the post-ischemic recirculation results and the hemodilution results did not show significant changes, however, a small increase in reinitiating was found when normovolemic hemodilution was used.

DISCUSSION

Previous studies found out that post-ischemic reperfusion, with reintroduction of blood into the ischemically damaged tissue, causes, especially in the so-called *selective vulnerable* brain regions, deep and severe inhibition of protein synthesis thus disabling the protective protein synthesis or inactivating protein resynthesis during or after ischemia (15), and leading to cell death. The precise mechanism is not known, but our results indicate that changes in initiation factor complexes (eIF2/GEF or eIF4G) may be responsible for this inhibition. Increased Ca2+ con-

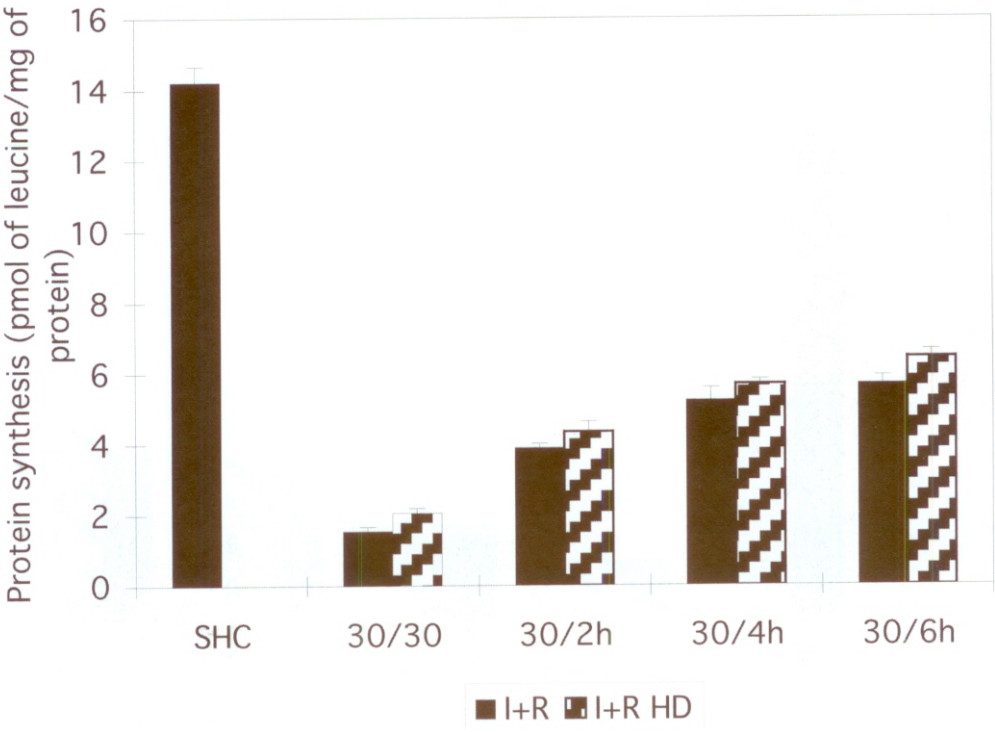

Fig. 4. - *The effects of post-ischemic recirculation on the activity of the protein synthesis machinery in the rat brain hippocampus with the application of normovolemic hemodilution (I/R + HD) or without (I/R), expressed as in vitro incorporation of [4,5-^{3}H]leucine into the TCA insoluble polypeptides.*
Values are means ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey-Kramer test. SHC means sham control, 30/30 means 30 min of ischemia and 30 min of post-ischemic reperfusion, 2 h means 2 hours, 4 h means 4 hours and 6h means 6 hours of post-ischemic reperfusion, respectively.

centration may also be involved in protein synthesis inhibition, as suggested by previous evidence in ischemic brain studies.

During post-ischemic reperfusion, reactive hyperaemia is a usual concomitant event (13, 20) with blood flow two- to three times higher than the control levels (5), and thus forming suitable conditions for oxygen radical formation. The swelling of capillary endothelial cells follows reactive hyperaemia, and thus brain hypoperfusion can occur. There is no evidence in literature that reduced blood flow after the global or focal ischemia has damaging effects. Decreased blood flow is consistent with reduced post-ischemic metabolism and increased blood flow in the post-ischemic period should not be protective (15). Cellular defenses are reduced, and the amount of the red blood cells and the PMN leucocytes, which are the main source of the reactive oxygen species, dramatically increases. Following growth of free radical production is obvious during the early reperfusion period (10, 22). A considerable increase of superoxide, coinciding with up-regulation of the enzyme COX-2 (19) – a probable source of free radicals –, was found 24h after a 10-min global

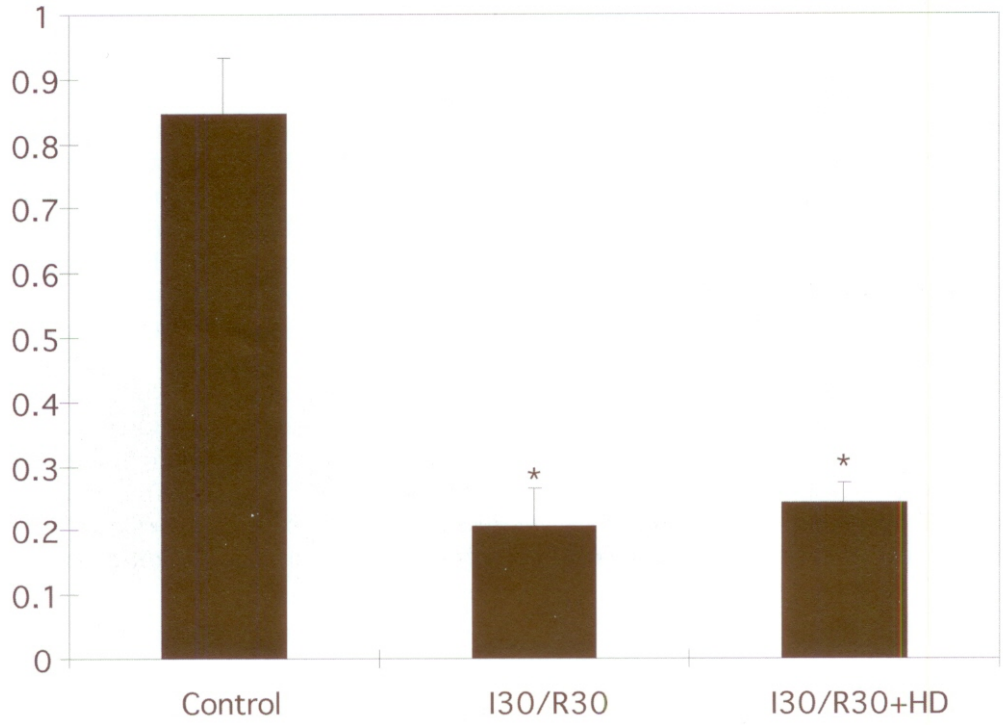

Fig. 5. - *The activity of eIF2 in the cortex measured as its capacity to form the ternary complex in the same subcellular fractions as described in Material and Methods.*
Values are means ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey-Kramer test (* means $p < 0.001$ versus control). Control means results from control animals, I30/R30 means 30 min of ischemia followed 30 min of post-ischemic reperfusion, I30/R30 + HD means the same procedure with hemodilution.

ischemia in the vulnerable CA1 pyramidal layer of the hippocampus. Generation of free radicals, according to previous studies, can be reduced or blocked by addition of superoxide dismutase, catalase, or allopurinol as inhibitors of xanthine oxidase or by some other antioxidants and free radical scavengers in the ischemic tissue (1, 12, 21, 27).

Other authors attempted to prevent the reactive oxygen product formation by surgical or various therapeutic processes, focused on cerebral blood flow decrease. Burda and co-workers (4) prevented free oxygen radical formation by short-term hypoperfusion with improved protein synthesis recovery in the rat brain cortex. Hypoperfusion can be compared to hemodilution of our study, since both processes decrease the formation of reactive oxygen species by reducing number of circulating blood elements in the rat brain. Our results showed improved protein synthesis in the rat neocortex after hemodilution. However, this effect remained only for a short time (30 min) during post-ischemic recirculation. Longer reperfusion did not show similar results, probably due to the gradual onset of other destructive processes affecting the post-ischemic protein synthesis machinery, like eIF-4G degradation

caused by activated calpain (9). Blocking the proteasome activity by use of PS-519 is extremely protective for the lesion core during focal ischemia (23). Very little is known about the rates of protein degradation after ischemia. A significant decrease in ubiquitin immunoreactivity was found in the CA1 region after global ischemia and this evidence suggests that a major degradation pathway is blocked (16).

We have achieved positive results in the rat brain cortex but not in selective-vulnerable regions, such as the hippocampus and the striatum. The neocortex belongs to the relatively resistant rat brain regions and the post-ischemic protein synthesis recovery shows better results in this area than in vulnerable regions. The rat brain cortex, as a whole, shows higher levels of the labeled amino acid incorporation *in vitro*, although some layers in the cortex are more vulnerable to ischemic insults. Hemodilution positively affects the activity of the protein synthesis machinery in this rat brain area after prolonged reperfusion too, though this effect is not so dominant as in the first half an hour of reperfusion immediately after ischemia. The selective vulnerable cells in the hippocampus or dorsolateral striatum show profound and permanent inhibition of protein synthesis after ischemia. Thus, we can conclude that, although hemodilution increased protein synthesis in selective vulnerable regions after ischemia, this improvement is not of significant importance.

SUMMARY

Normovolemic hemodilution is a possible way to improve the brain recovery after ischemia and reperfusion. Therefore we have decided to examine how this process may affect the post-ischemic protein synthesis machinery. We analysed rat brains after 4-vessel-occlusion and different time intervals of reperfusion using normovolemic hemodilution. We achieved an important increase of [4,5-^{3}H]leucine incorporation into polypeptides *in vitro* in the rat brain neocortex 30 minutes after ischemia, but concurrently there was no significant change in the hippocampus and striatum. By extending the time course of reperfusion we did not observe any important deviation of *in vitro* [4,5-^{3}H]leucine incorporation in the studied brain areas. Thus, although hemodilution increased protein synthesis in selective vulnerable regions after ischemia, this improvement is not of significant importance.

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