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Effect of Ischaemia on Protein Synthesis in Neuron and Glia-enriched Fractions from the Rabbit Spinal Cord

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Summary

The incorporation of ^{14}C-leucine into the post-mitochondrial supernatant and neuron, glia and myelin-enriched fractions isolated from the rabbit spinal cord was studied after ischaemia and subsequent recirculation. In the cell-free system, incorporation decreased to 55 % of the control value after 40 min ischaemia, but proteosynthesis returned to the pre-ischaemic value after 3 h recirculation and remained at this level during further recirculation. The incorporation of amino acids into proteins of neurons and neuroglia differed from the cell-free system and from each other. In the enriched neuronal fraction, protein synthesis fall after ischaemia and also during the first hours of recirculation, but during further recirculation it rose to 60 % above the control value. In the enriched glial fraction, specific radioactivity of proteins rose abruptly immediately after ischaemia and by the fourth day there was sixfold increase as compared with control values. The results indicate that the ischaemia-induced decrease in protein synthesis is only transient and that a significant increase occurs in the surviving cell populations, especially the neuroglia. The functional changes caused by spinal cord ischaemia are irreversible, however.

Key words:
Protein synthesis – Ischaemia – Neurons – Neuroglia

Protein synthesis in the nervous tissue is inhibited during ischaemia and the degree of inhibition depends on the intensity and duration of ischaemia and on age of the experimental animals (Dienel *et al.* 1980, Jílek *et al.* 1968). As distinct from the relatively rapid recovery of energy metabolism, the post-ischaemic restitution of cerebral protein synthesis is a slow process requiring a long recirculation period (Cooper *et al.* 1977). From the previous results it can be assumed that inhibition of the initiation of protein synthesis in the early phases of the post-ischaemic period may be due to the activation of a cytoplasmic factor blocking the function of the initiation factor eIF-2 (Nowak *et al.* 1985). The precise mechanism responsible for the decrease of protein synthesis during prolonged recirculation are still, however, unknown. The effect of ischaemia on protein synthesis, and especially on its recovery during recirculation, in neurons and neuroglia has so far not been studied adequately, although their sensitivity to ischaemia is known to differ. In an electron microscopic study, disaggregation of polyribosomes was found only in neurons,

whereas in the neuroglia, containing far fewer polysomes, there was no such change (Hartmann and Becker 1973). On the other hand, protein synthesis in the enriched glial and neuronal fraction was inhibited to approximately the same degree during anoxia-induced polysome disaggregation (Yanagihara 1976). In the present study we compared protein synthesis in a cell-free system from native spinal cord with that from enriched neuronal and glial fractions in spinal cord slices.

Material and Methods

Twenty-four Chinchilla rabbits of both sexes weighing 1–2 kg were used. Ischaemia of the lumbosacral cord was induced by ligature of the abdominal aorta just below the origin of the left (inferior) renal artery (Zivin and DeGirolami 1980). The rabbits were randomly divided into three experimental groups. The animals in the control group were anaesthetized with a barbiturate (Thiopental Spofa, 50 mg/kg i.v.) and the spinal cord from the L_2 to L_5 region was removed by extruding from the spinal canal with a teflon piston (Zivin and DeGirolami 1980). In the ischaemic group, the animals were anaesthetized and the abdominal aorta was ligated just below the origin of the left renal artery, also to prevent blood flow through a. radiculomedullaris (Adamkiewitzi); 40 min later, the spinal cord was removed as described above.

In the recirculation group, cross-clamping of the aorta was released after 40 min ischaemia and recirculation was allowed for 180 min, one or four days. Only animals with complete neurological failure (hind limb paralysis, loss of sensitivity, inability to retain urine and faeces) were studied further.

Incorporation of ^{14}C-leucine into proteins in postmitochondrial supernatants

A minimally modified form of the system elaborated by Cosgrove and Brown (1981) was used. Immediately after its removal, the spinal cord was washed in the ice-cold homogenization medium containing 0.32 mol/l sucrose, 4 mmol/l magnesium acetate and 7 mmol/l 2-mercaptoethanol and was homogenized in the same medium (1:4) in the glass-teflon homogenizer. The homogenate was centrifuged 10 min in a Janetzki K-24 centrifuge at 10 000 rpm and 4 °C. The resultant postmitochondrial supernatant (PMS) was used immediately for studying protein synthesis *in vitro*. The complete reaction mixture contained (per 100 l): PMS (about 400 g protein), 0.32 mol/l sucrose, 50 mmol/l Tris acetate at pH 7.55, 140 mmol/l potassium acetate, 4 mmol/l magnesium acetate, 7 mmol/l 2-mercaptoethanol, 2 mmol/l ATP, 0.4 mmol/l GTP, 0.5 mmol/l creatine phosphate, 5 μg creatine phosphokinase (EC 2.7.3.2.) and 24 kBq (0.65 Ci) ^{14}C-leucine (specific activity 8.880 MBq/mmol, Institute for Research, Development and Utilization of Radioisotopes, Prague). The samples, in quadruplicate, were incubated 15 min at 37 °C. The protein concentration was determined after Bradford (1976). Incubation was stopped by cooling the samples to 0–4 °C. TCA-insoluble material was measured according to Mans and Novelli (1961) on a Packard Tri-Carb C 2425 liquid scintillation counter.

Isolation of cytosols and ribosomes

The postmitochondrial supernatant (7 ml) was layered onto a discontinuous gradient containing 1 ml 1.3 mol/l and 1 ml 2.0 mol/l sucrose in the homogenization medium. The gradients were centrifuged for 5 h at 135 000 *x* g in a 8x11 ml rotor on a VAC 601 (Janetzki) ultracentrifuge. The upper part of the supernatant in 0.32 mol/l sucrose was used as the cytosol, the fraction in 2.0 mol/l sucrose as the free ribosomes and the fraction in 1.3 mol/l sucrose as the microsomes.

In the cell-free system, ^{14}C-leucine was incorporated into proteins in the same medium as for the PMS. The incubation mixture contained (in 100 μl) 500 μg cytosol protein, 100 μg microsome protein or 30 μg free ribosome protein. The samples were processed and their radioactivity was measured in the same manner as for the PMS.

Incorporation of ^{14}C-leucine into spinal cord slices

Incorporation was studied by the method of Blomstrand and Hamberger (1970). Immediately after its removal the spinal cord was washed in the incubation medium and slices 0.4 mm thick were cut with a McIlwain chopper (TD-02, Olomouc University). The slices were placed into an incubation flask

(100 ml) in medium (5 ml to 1 g spinal cord) containing 35 mmol/l Tris-HCl at pH 7.6, 5 mmol/l Na phosphate buffer at pH 7.6, 5 mmol/l KCl, 120 mmol/l NaCl, 2.5 mmol/l $MgCl_2$, 5 mmol/l glucose, 2.5 mmol/l ADP and 160 kBq/ml (4.3 μCi) ^{14}C-leucine. After 1 min aeration with 100 % O_2, the slices were incubated 1 h at 37 °C with gentle agitation. Incubation was stopped by cooling to 0–4 °C.

Isolation of neuronal and glial-enriched fractions

For this purpose the method of Farooq and Norton (1978) was employed. After incubation, the samples were washed three times in an isolation medium (F-2) containing 8 % (w/v) glucose, 5 % (w/v) fructose and 2 % (w/v) Ficoll (Pharmacia, Sweden) in 10 mmol/l phosphate buffer at pH 7.6. The tissue was disaggregated mechanically by mild vacuum suction of the slices through a capillary (i.d. 2.2 mm, length 3.5) connected to the side neck of a 100 ml flask containing 5 ml F-2 buffer. The resultant suspension was filtered through a sieve with 0.4 mm pores and the residue left on the filter was again drawn through the capillary into a flask containing the F-2 medium. Suction and filtration were repeated three times. The combined filtrates were left 15–20 min at 0–4 °C to settle. The supernatant was then carefully removed and the sediment, after brief stirring in a vortex mixer, was left to settle for another 15 min at 0–4 °C. The supernatants were combined and centrifuged for 15 min at 720 *x* g and 4 °C. The cellular sediment was used to measure radioactivity and for further fractionation in a Ficoll gradient. Samples containing the cellular sediment were suspended in an isolation medium containing 9 % Ficoll and applied to a discontinuous gradient composed, in equal parts (3 ml), of 34 %, 24 % and 12 % Ficoll. The gradient was centrifuged 10 min in a 6x14 ml swing-out rotor on a MSE Superspeed 65 ultracentrifuge at 8 500 *x* g and 4 °C. The pellet was used as the enriched neuronal fraction, the layer between 12 % and 24 % Ficoll as the enriched glial fraction and the layer floating on the surface of the gradient as myelin. The composition of the fractions was studied by light microscopy in phase contrast. The protein concentration and radioactivity were determined by the method of Bradford (1976) and Mans and Novelli (1961). The results were evaluated statistically by Student's t-test. Values of $p<0.05$ were regarded as statistically significant differences.

Results

The values of the incorporation of labelled leucine into the proteins of postmitochondrial supernatant (PMS) from rabbit spinal cord are illustrated in Fig. 1. It can be seen that significant changes occurred only in the phase of ischaemia manifested by a 44 % decrease in incorporation. After 3 h recirculation, ^{14}C-leucine incorporation returned to the control level.

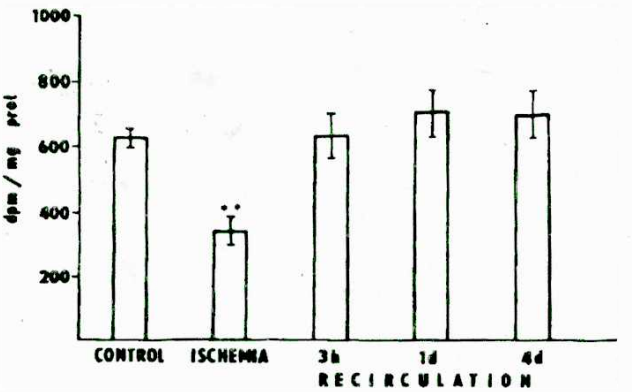

Fig.1

In vitro incorporation of ^{14}C-leucine into the proteins of the postmitochondrial supernatant of rabbit spinal cord after ligation of the abdominal aorta. The values are expressed in dpm/mg PMS protein ± S.E.M. ** = $p<0.01$

The activity of the individual components of the PMS after ischaemia is shown in Tab. 1. The ischaemia-induced decrease in ^{14}C-leucine incorporation into the PMS proteins is due largely to a reduced activity of ribosomes (especially the free ribosomes). The use of ischaemic cytosols together with control ribosomes (bound or free) was not manifested in any differences in formation of the polypeptide chain compared with the use of control cytosols. When ischaemic ribosomes were combined with control cytosols, however, incorporation fell significantly, the extent of inhibition being greater with free ribosomes ($p < 0.05$).

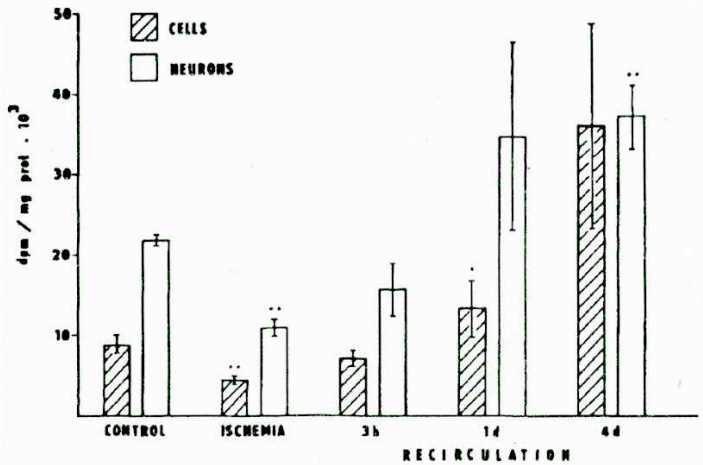

Fig.2
Incorporation of ^{14}C-leucine into the proteins of a cell suspension and the enriched neuronal fraction after the incubation of tissue slices from the spinal cord of rabbits subjected to ischaemia and recirculation. The values are expressed in dpm/mg protein ± S.E.M. * = $p < 0.05$, ** = $p < 0.01$

In the incubation of spinal cord slices, the post-ischaemic drop in protein specific activity in the unseparated cell suspension (Fig. 2) was even more pronounced (about 50 %, $p < 0.01$), with a correspondingly slower return to the control values. It can be seen that incorporation after 3 h recirculation was about 20 % lower ($p < 0.05$) than in the control. After longer recirculation intervals, however, there was a marked increase in protein specific radioactivity. After 24 h it amounted to 40 % ($p < 0.02$) and after 96 h it was actually 400 % of the control value. The pronounced increase of protein synthesis in the later recirculation phases was marked by considerable individual difference in the quality of recirculation, so that in some animals we found maximum activity on the first day and in others not until the fourth day of recirculation. The variance in the individual course of time changes is therefore manifested in a marked increase of the scatter, but the differences tended towards a higher statistical significance. Protein specific activity in the enriched cell fractions varied in the individual stages of the experiment.

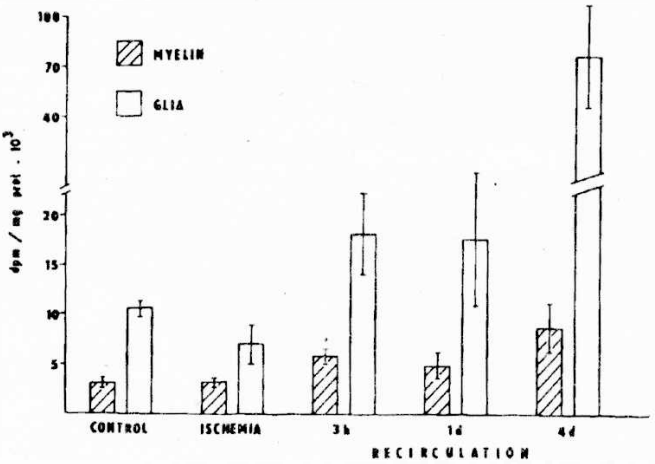

Fig.3
Incorporation of ^{14}C-leucine into the proteins of the enriched myelin and glial fractions after the incubation of tissue slices from the spinal cord of rabbits subjected to ischaemia and recirculation. The values are expressed in dpm/mg protein ± S.E.M. * = $p < 0.05$

The myelin fraction (Fig. 3) did not react directly to ischaemia, but in the glial (Fig. 3) and neuronal (Fig. 2) fractions there was a significant decrease in incorporation, which amounted to 34 % in the neuroglia and over to 50 % in the neurons ($p < 0.01$). Three hours' recirculation raised specific activity in the glial and myelin fractions well above the control value, whereas in the neurons it remained deeply (30 %) below the control level. After 24 h recirculation, specific activity in the glial and neuronal fractions was 50 % higher than in the controls; in the glial elements this meant a slight decrease compared with 3 h recirculation, but in the neurons a marked increase of specific activity.

In the last recirculation interval investigated, the amount of leucine incorporated into the neuronal fraction was 68 % higher than in the control ($p < 0.01$) – roughly the same as after one day recirculation. At the same time, there was an abrupt increase in the glial fraction and, in part, in the myelin fraction, in which specific activity attained six- and twofold values of the controls, respectively.

Discussion

Ligation of the abdominal aorta is an easily reproducible model of focal ischaemia in the spinal cord (DeGirolami and Zivin 1982). Over a period of 10–60 min the histopathological changes are proportional to the duration of ischaemia and are directly correlated to the loss of neurological functions. Continuous protein turnover in all cells and anatomical structures is indispensable for the maintenance of cell structure and function and depends on the regulatory mechanisms by which the organism adapts itself to various stimuli. Adaptability to ischaemic changes depends largely on the age of the experimental animals (Jílek *et al.* 1968). At the level of protein metabolism, the mechanisms include changes in protein synthesis and degradation rates. Cerebral ischaemia causes an abrupt decrease in protein synthesis *in vivo* (Kleihues and Hossmann 1971, Dienel *et al.* 1980), depending on the degree and duration of ischaemia. The primary factor causing inhibition or

arrest of protein synthesis during ischaemia is the exhaustion of energy substrates, in particular ATP, while the polyribosomal profile remains virtually intact. It can be seen from our results that 40 min ischaemia resulted in a roughly 45 % decrease of the *in vitro* incorporation of ^{14}C-leucine into proteins of the postmitochondrial supernatant compared with the control and that this decrease is due to inhibition of ribosomal activity. Morimoto *et al.* (1978), using the microsomal fractions, also reported reduced incorporation of ^{3}H-leucine after 60 min unilateral cerebral ischaemia. Conversely, when PMS was used, no difference was found after 5–20 min bilateral ischaemia of the brain in gerbils (Nowak *et al.* 1985) or after 15 min incomplete ischaemia of the rat brain (Cooper *et al.* 1977). Our earlier results also indicate that ischaemia alone does not reduce the capacity of either cytosols or ribosomes for incorporation (Burda *et al.* 1980). Morimoto and Yanagihara (1981) likewise found ribosomal activity to be unaltered after ischaemia. The causes of the given differences in the incorporation of labelled amino acids after ischaemia are to be found in differences in the degree of ischaemia and also in the technique used to prepare the components of the cell-free system. Where ribosomes were isolated by means of sodium deoxycholate, no changes were found in their activity after ischaemia (Burda *et al.* 1980, Morimoto and Yanagihara 1981), but if "microsomes" or the fractions of ribosomes isolated without detergents were used, their activity was inhibited. In our experience, gel filtration is unsuitable for removing the low molecular portion from the PMS (Cooper *et al.* 1977, Nowak *et al.* 1985). The effect of various amounts of endogenous amino acids in the control or ischaemic cytosol on incorporation is relatively small (Tab. 1), but gel filtration severely reduces the activity of ribosomes.

Table 1

Effect of ischaemia on in vitro incorporation of ^{14}C-leucine into proteins in a cell-free system

Cytosol	membrane-bound ribosomes	dpm/mg±S.E.M.	%
Control	Control	7 664±709	100.0
40 min ischaemia	Control	7 986±699	104.2
Control	40 min ischaemia	5 429±515*	70.8
Cytosol	**Free ribosomes**	**dpm/mg±S.E.M.**	**%**
Control	Control	50 975±9 634	100.0
40 min ischaemia	Control	42 564±8 800	83.5
Control	40 min ischaemia	18 050±1 650*	35.4

*The results are the mean values from groups of 4 animals. * = $p<0.05$*

This decrease is greater in intact ribosomes than in ischaemia-damaged ribosomes and it can seriously distort the true differences between the activity of control and ischaemic ribosomes. After removing the ligature, the incorporation of ^{14}C-leucine into the PMS rises and after 3 h of reperfusion reaches pre-ischaemic values. It also remains at this level at the subsequent intervals studied, i.e. 1 and 4 days later. This post-ischaemic recovery of activity is somewhat faster, for instance, than after 20 min bilateral cerebral ischaemia in gerbils, in which, after 4 h recirculation, incorporation amounted to only 60 % of the control values. Similarly to our results, however, the amount of newly synthesized protein reached the control levels after 24 h recirculation (Nowak *et al.* 1985). Complete recovery of the activity of the PMS might mean that 40 min ischaemia was not sufficient for the induction of irreversible changes in protein synthesis in the spinal cord. The results of morphological observations, however, show severe damage and necrosis of some parts of the grey matter of the rabbit spinal cord even after much shorter ligation of the abdominal aorta (about 15 min) (DeGirolami and Zivin 1982). Similarly, ATP production in some parts of the spinal cord remains low after 40 min ischaemia and 4 days survival (Danielisová *et al.* 1987). For the above reason, normalization of the incorporation of labelled amino acids into the PMS proteins during recirculation may be due to an increased synthetic capacity of the cell population which was not damaged by ischaemia, and/or to the proliferation of cells of non-neuronal origin. We attempted to resolve this problem by studying the incorporation of ^{14}C-leucine into spinal cord slices, followed by separation and the measurement of protein specific activity in the neuron and glia cell-enriched fractions. The ability of spinal cord slices to incorporate leucine after 40 min ischaemia is inhibited in approximately the same manner as in the case of the PMS. On the other hand, the initial slow increase of incorporation is afterwards succeeded by an abrupt increase in activity, mainly after 4 days survival, when it attains fourfold values of the controls.

The different degree of inhibition of protein synthesis during ischaemia and the more pronounced changes during recirculation testify unequivocally to lower sensitivity of glial cells to ischaemic and hypoxic injury (Blomstrand 1970, Albrecht and Šmialek 1975). Protein synthesis in the neuroglia, which is normally much lower than in the neurons (Blomstrand and Hamberger 1969, Tiplady and Rose 1971, Lisý and Lodin 1973, Albrecht and Šmialek 1975), alters during recirculation and exceeds that in neurons. At the same time, the recovery of inhibited incorporation into the glia cells can be seen to be significantly faster than into neurons. The increase of protein specific radioactivity values in the neuroglia can be ascribed, to some extent, to the postischaemic (hypoxic) excitation found *in vivo* (Albrecht and Šmialek 1975) and in morphological observations (Yap and Spector 1965). When evaluating this increase, the demonstrable death of some parts of the spinal cord (in particular the neurons) should be taken into consideration (DeGirolami and Zivin 1982). Under these conditions, changes presumably take place in the properties of the neurons which may alter the yield during isolation and thus seriously distort the results. Another possible factor could be a change which occurs in the non-radioactive precursor in the cell pools as a result of the changed functional, metabolic and physicochemical properties of the tissue. The fact remains that part

of the nerve cell population survived the chosen ischaemic treatment and that, despite this, the damage of tissue functions was irreversible.

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