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Ischemia-induced modifications of protein components of rat brain postsynaptic densities

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Abstract

Considering that postsynaptic densities (PSD) are a functionally active zone involved in excitatory synaptic transmission we evaluated the influence of global, postdecapitative cerebral ischemia of 15 min duration on characteristic protein constituents of PSD in rats. Ischemia induced changes in the assembly and function of calcium, calmodulin-dependent kinase II (CaMKII), calpains and a novel, 85 kDa/RING3 kinase but to different extents.

CaMKII is translocated toward the PSD very rapidly and extensively after the first seconds of ischemia. Concomitantly, the total phosphorylating potency of this kinase with endogenous, as well as exogenous, substrates was elevated but to a lower extent than suggested by the increased protein content.

Of the two brain-specific isoforms of calpain (μ and m), only recently recognized in PSD, the proteolytically activated, 76 kDa subunit of μ-calpain was significantly down-regulated after 15 min of brain ischemia. However, this effect is coupled with the decline of fodrin, the only calpain substrate that has been demonstrated to be a calpain target in vivo. Together, these findings may suggest that calpains, primarily activated by calcium in ischemic PSD, are subsequently degraded.

A new observation is the relatively high phosphorylating activity of a novel, 85 kDa/RING3 kinase in the PSD which independently of other kinase systems, was greatly enhanced after ischemia.

These data provide evidence that the signal transduction processes could be rapidly altered by short-term (15 min) brain ischemia due to changes in the assembly and function of PSD connected proteins.

Keywords: PSD; CaMKII; Calpain; Protein phosphorylation; Brain ischemia

1. Introduction

Synapses are highly specialized sites of cell-cell contact mediating the transmission of impulses between neurons. Electron microscopy has revealed the presence of a dense submembraneous cytomatrix associated with the synaptic junction, called the postsynaptic density—PSD (Kennedy, 1993). The postsynaptic plasma membrane, in particular at excitatory synapses, has a pronounced PSD thought to anchor and cluster a variety of ion channels and receptors. Classical PSD preparations contain a complex of more than 30 proteins, amongst them components of the cytoskeleton, several receptors, signal-transducing enzymes and their substrates, as well as a set of proteins containing a novel, protein-interacting module named the PDZ domain (Garner et al., 1993; Sheng, 1996).

One of the mechanisms which govern the functions of enzymes, especially those with a broad spectrum of substrate specificity, is targeting to selected locations in the cell using high- affinity interactions between the enzyme and a subcellular compartment. The enzyme could be constitutively present in the targeted compartment or recruited there after the cell receives a stimulus. Considering postsynaptic densities as a func-

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tionally active zone intimately connected with excitatory synaptic transmission, we have asked the question if global cerebral ischemia generates signals which can significantly modify the protein composition and function of the classical PSD preparation. Special attention was paid to the enzymes previously reported to be involved in brain ischemic pathology. Namely, a calcium calmodulin-dependent protein kinase-CaMKII (Goldenring et al., 1984; Aronowski and Grotta, 1996; Zalewska and Domańska-Janik, 1996a,b), a neutral calcium-activated proteinases- calpain (Bartus et al., 1998, Zalewska et al., 1998) and an inducible, mitogen-activated phosphokinase p85/RING3 protein (Ostrowski et al., 1998).

2. Material and methods

2.1. Animals and sampling technique

Three-month-old Wistar rats under light ether anesthesia were used for all experiments. The brains were collected using three different protocols:

1. the brains were frozen in situ in liquid nitrogen and dissected by the method of Tzigaret et al. (1993)—this group is referred to as the in situ control group—C_{is}.
2. conventional decapitation and immediate dissection of the brain in ice-cold PBS—referred to as decapitated control group—C_d.
3. the heads were kept at 37°C for 15 min before further preparation—referred to as postdecapitative ischemic group—I.

In separate experiments, and prior to routine PSD preparations, tissue slices from the forebrains of four rats were prepared using McIlwain tissue chopper and incubated in Krebs-Ringer-bicarbonate buffer for 30 min at 34°C with or without calcium.

2.2. Tissue fractionation

The preparations of PSD were performed separately for all three experimental groups by following the procedure of Cohen et al. (1977). Briefly: 10 rats from each experimental group were employed. Two additional preparations from preincubated brain slices were analyzed independently. The tissue was homogenized in a motor-driven teflon/glass homogenizer in ice-cold buffer A containing 0.32 M sucrose, 1 mM $NaHCO_3$ pH 7.5, 1 mM $MgCl_2$, 0.5 mM $CaCO_3$ and 100 μM leupeptin. After the removal of nuclei and undisrupted cells, the supernatant was washed two times in buffer A and centrifuged at 17,000 g for 10 min. The pellet (crude P2 fraction) was suspended in buffer B containing 0.32 M sucrose, 1 mM $NaHCO_3$ pH 7.5 and subjected to a discontinuous sucrose density gradient by centrifugation over 0.85/1.0/1.2 M sucrose for 2 h at 100,000 g. The interface between 1.0 and 1.2 M sucrose was collected (synaptoneurosomal SN1 fraction), resuspended in 4 × vol. of buffer B, centrifuged at 48,000 g for 20 min (SN2 fraction), then dissolved in 6 mM Tris/HCl pH 8.1 to a protein concentration 4 mg/ml, mixed (1:1 v/v) with 1% Triton X-100 in 6 mM Tris/HCl pH 8.1 and incubated at 40°C for 15 min. This fraction was centrifuged at 48,000 g for 20 min. The pellet (synaptic membranes SM) was resuspended in buffer B and subjected to overnight centrifugation at 275,000 g over a 1.0/1.5/2.0 M sucrose gradient. The interface between the 1.5/2.0 M sucrose layers contained PSD structures. This fraction was collected and treated with 12 ml H_2O and spun down at 275,000 g for 1 h. The final PSD was resuspended in 100 μl 20 mM Tris/HCl, pH 7.5 containing 20% glycerol, 1.0 mM EDTA, 1.0 mM EGTA and kept frozen at −80°C. All steps of the PSD purification were performed at 4°C.

2.3. PAGE and Western blotting

Fractions containing 5–10 μg of protein were resolved on 10% SDS-PAGE according to Laemmli (1970). The proteins were electrotransferred onto nitrocellulose Hybond C Extra at 680 mA for 3 h. The membranes were probed with specific antiprotein primary antibodies: a monoclonal antibody to the α (50 kDa) subunit of CaMKII (Boehringer, Mannheim), a monoclonal antibody 1D10A7, recognizing the large subunit of both μ and m-calpain, (with an approximately 7 times higher affinity for m-calpain) (Kasai et al., 1986), a rabbit polyclonal antibody C30-5 against autolysed μ-calpain and two antibodies specific for fodrin, generously provided by Dr Saido (Saido et al., 1993) and Dr Nixon (antibody XQ¤3-3.1). The secondary, peroxidase-linked antibodies were detected by the ECL system (Amersham) according to the provided protocol.

2.4. CaMKII-dependent phosphorylation of PSD protein

The phosphorylation medium (25 μl) contained 50 mM HEPES (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 0.05 mM ATP and 5 μCi [γ-^{32}P]ATP (3000 cpm/pmol), 1 mg/ml BSA and 5 μM PKI-tide (Sigma). To measure the total activity the assay medium contained 2 mM $CaCl_2$ and 3 μM calmodulin. To determine the Ca^{2+}-independent phosphorylation, 2 mM EDTA was added to the reaction mixture (devoid of calcium and calmodulin) with, or without calmodulin inhibitor W-7 (100 μM). The reaction was started by the addition of the PSD fraction (15 μg/sample). After incubation at

0°C for 1 min the reaction was stopped by the ddition of sample buffer (5X). Electrophoresis was erformed by the method of Laemmli. Gels were ried and exposed to X-ray film (Hyperfilm®-Mp- mersham) at −80°C for 24 h.

CaMKII specific activity was measured in the PSD raction as previously described for brain homogenates Zalewska and Domańska-Janik, 1996a), using syntide- as exogenous substrate.

.5. Phosphorylation of renaturated PSD proteins on VDF membranes

A previously described procedure was used (Rachie t al., 1993), 20–100 μg of homogenate or PSD pro- eins were boiled in 2 × sample buffer (1:1 v/v) and eparated on 10% SDS-PAGE gels. After bathing the el in transfer buffer for 30 min, the proteins were elec- roblotted onto a PVDF membrane at 100 v for 60 min n transfer buffer (25 mM Tris/HCl pH 8.2; 192 mM lycine, 20% methanol and 0.05% SDS). The blotted roteins were denatured for 30 min at room tempera- ure by incubating the membranes with continuous low agitation in 10 ml of denaturing buffer (7 M gua- idine/HCl, 50 mM Tris, 3 mM DTT, 3 mM EDTA H 8.3). The blotted proteins were then allowed to enature by incubating the membrane in renaturation uffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.05% Tween 20) for 12–14 h at °C. Following renaturation, the membranes were then ncubated in phosphorylation buffer (50 mM Tris H 7.5, 5 mM $MgCl_2$, 5 mM DTT, 50 μCi of γ-^{32}]ATP) for 30 min at room temperature, with con- tant agitation. Phosphorylation was terminated by washing the membranes four times in 50 mM Tris H 7.5 and 0.05% Tween 20 and then once with 0 mM Tris pH 7.5. After the last wash the membrane as briefly submerged in 100% methanol, air-dried nd autoradiographed.

2.6. Competition between protein phosphorylation on PVDF membranes and the 'classical' phosphokinase ystems

Control (Cis) PSD samples suspended in 20 mM Tris/HCl and 5 mM $MgCl_2$ buffer pH 7.5 with a final oncentration of 20 μg protein/10 μl were incubated with or without 25 μM of unlabelled ATP and the fol- owing kinase activators:

1. 100 μM cAMP for PKA activation
2. 0.5 μM PDBu and 5 mM $CaCl_2$ for PKC activation
3. 5 μM calmodulin and 5 mM $CaCl_2$ for CaMKII activation

After 5 min, the samples were boiled with 2 × sampling buffer and resolved by SDS PAGE for further phosphorylation of proteins with labeled ATP as described above.

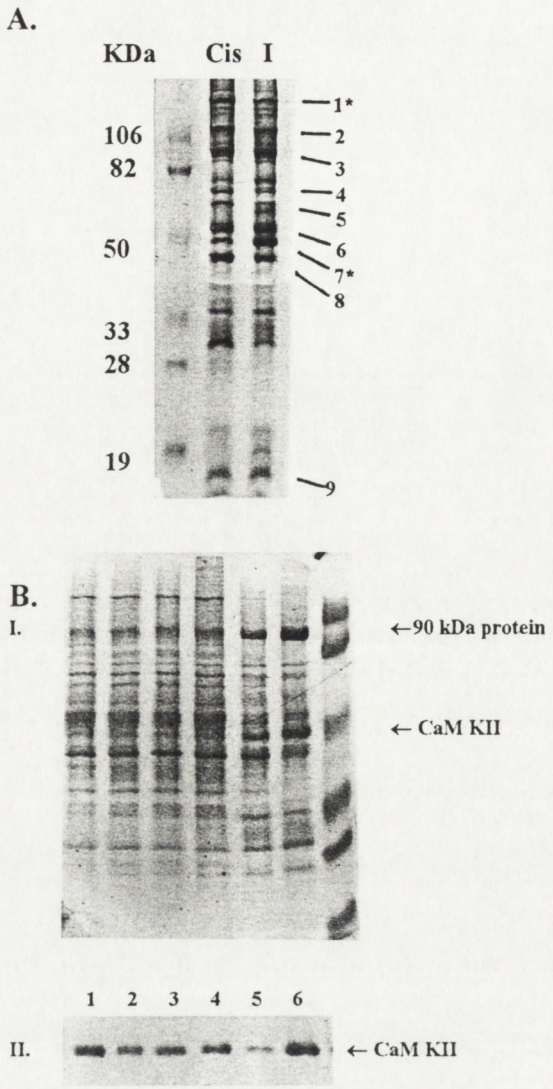

Fig. 1. Characteristic of the PSD fraction prepared from control in situ (C_{is}) and ischemic (I) brains. (A) SDS-PAGE was performed as described in Material and methods. Lanes loaded with 5 μg of proteins were stained with Coomassie Brilliant Blue. The mobilities of some known PSD proteins are shown on right: 1. Fodrin, 2. NF-M, 3. 90 kDa protein region, 4. Synapsin I, 5. NF-L, 6. α and β Tubulin, 7. CaMKII, 8. Actin, 9. Calmodulin; (B) SDS-PAGE was performed for fractions from stages of the PSD purification procedure and stained for proteins (upper) or Western-blotted for CaMKII (lower). Gels were loaded with 5 μg of proteins per lane in the following pattern; 1. homogenate, 2. P2-particulate fraction, 3. crude synaptoneurosomes, 4. purified synaptoneurosomes, 5. synaptic membranes, 6. Post- synaptic densities.

2.7. Densitometry

Relative optical densities were measured using the image analysis system Gel Scan XL. Statistical analyses were performed by using the ANOVA program.

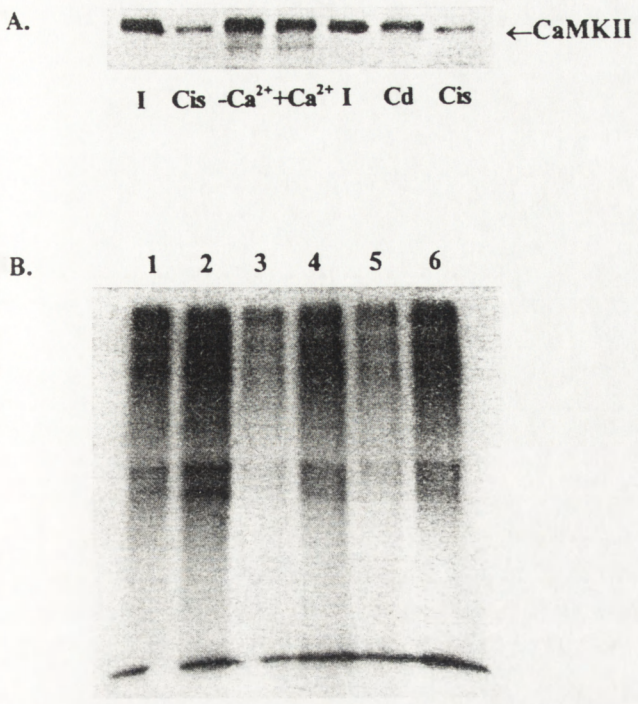

Fig. 2. Effect of ischemia on CaMKII protein content and endogenous kinase activity. (A) Western-blot of PSD proteins isolated from in situ frozen (C_{is}), conventionally decapitated (C_d) or postischemic (I) brains. In addition, two PSD probes isolated from the brain slices preincubated in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of calcium are presented. The gels were loaded with 10 μg of proteins lane then blotted and probed with α-subunit-specific CaMKII antibody; (B) Autoradiogram of SDS-PAGE of the PSD fractions isolated from control (rows 1, 3, 5) or ischemic (2, 4, 6) brains. Proteins were phosphorylated with ^{32}P-γATP as described in Material and methods. The phosphorylation was performed in the presence of all CaMKII activators (1, 2), with omission of calcium (3, 4) and with calmodulin inhibitor W7 (5, 6). The gels were loaded with 15 μg of proteins per lane.

Proteins concentrations were estimated by the method of Bradford (1976).

3. Results

The electrophoretic pattern of control PSD (Fig. 1A) was found to be similar to that previously described for the classical preparation of this fraction (Carlin et al., 1980). Ischemia only resulted in subtle alterations—the attenuation or enhancement of certain protein bands. In particular, a decrease in fodrin (band 1) and an increase in CaMKII protein (band 7) was suspected after visual inspection of the electrophoretograms. For further evaluation of these changes, Western blotting was employed.

The calcium/calmodulin-dependent protein kinase II is known as one of the major constituents of the PSD fraction and can be used as a purification marker. Accordingly, we found that the α-subunit of this protein accumulates in the PSD during the purification procedure (Fig. 1B). In particular, the preferential postsynaptic localization (line 6) of this protein was evident compared with presynaptic membranes (line 5). The amount of α-CaMKII in PSD, but also in other membrane fractions, markedly increased after 15 min of postdecapitative ischemia (Fig 2A). The PSD immediately prepared from decapitated rats (Cd) and from brain slices incubated for 30 min with or without calcium in the medium ($+Ca^{2+}$ and $-Ca^{2+}$) displayed, similar to as 15 min ischemia (I), a significant enrichment of CaMKII content compared to brains frozen in situ (Cis).

Table 1
Specific activity of calcium, calmodulin dependent kinase II (CaMKII) in the rat brain PSD fraction after ischemia

	Specific CaMKII activity with exogenous substrate nmol/min/mg protein[b]		Endogenous CaMKII activity Absorbance units[a]	
	Control	Ischemia	Control	Ischemia
Total	4.77±0.27	7.55±0.18	0.475	1.1372
Ca^{2+}-independent	0.975±0.13	1.14±0.15		

[a] densitometry of the representative autoradiogram presented in Fig. 2B.
[b] mean values from four experiments±S.D.

The endogenous phosphorylating activity of PSD-associated CaMKII was evaluated by SDS-PAGE and autoradiography after incubation of the samples with labeled ATP in the presence of specific kinase activators and inhibitors (Fig. 2B). As demonstrated in Table 1, ischemia in the presence of CaMKII activators, resulted in a significant enhancement of all phosphorylated protein bands in the PSD. However, compared with the densitometry of Western blots, this increase is lower than expected on the basis of the observed 5-fold enrichment of the amount of enzyme protein (Fig. 2A). In addition, the specific CaMKII activity has been measured using an exogenous, well defined synthetic substrate. In this experiment the specific activities measured in the PSD fraction were also significantly enhanced after ischemia (Table 1).

In contrast to a well established PSD component like CaMKII, the association of the calcium-activated neutral proteinase (calpain) with this subcellular compartment has only recently been recognized. Generally, two major isoforms of calpain have been described in mammalian brain. One form requires micromolar concentrations of calcium for activation in vitro (μ-calpain) while the other needs millimolar concentrations (m-calpain) (for review see Zalewska, 1996). Under ischemia, m-calpain, the dominating isoform in brain homogenate, is translocated from the cytosol to plasma membranes (Fig. 3B). Densitometry revealed an increase of the membrane- bound calpain fraction

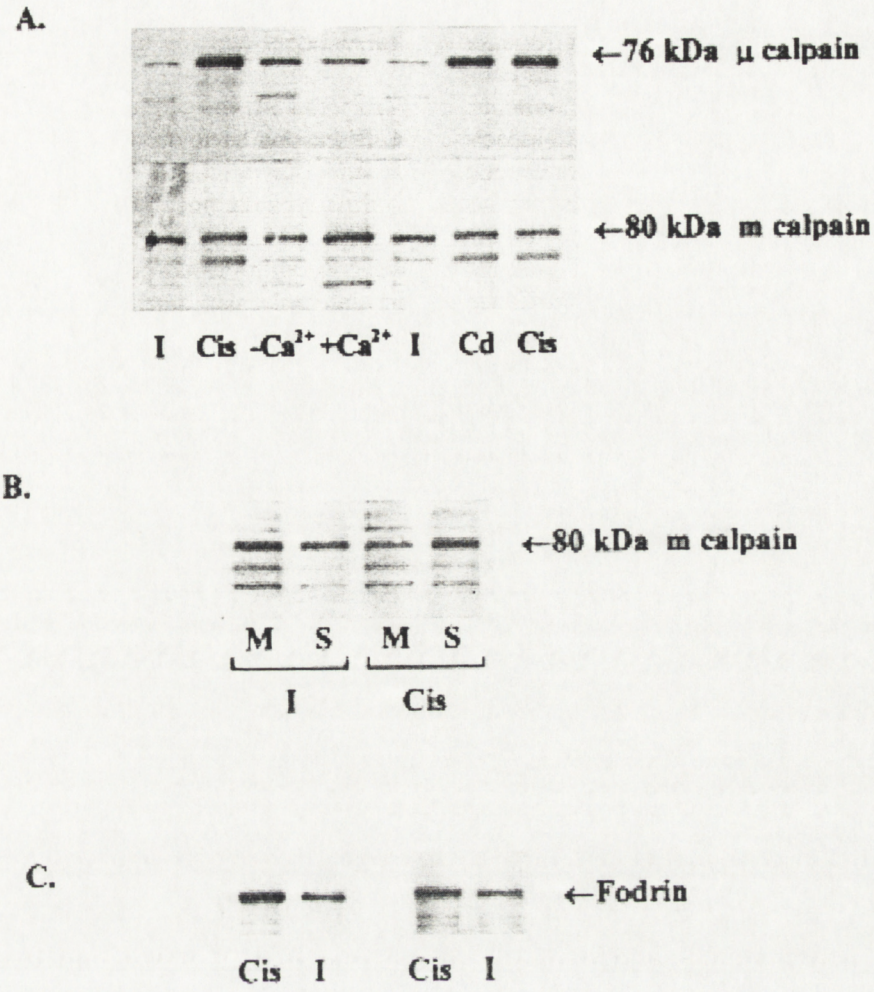

Fig. 3. Effect of ischemia on calpain content and activity in PSDs and brain homogenates. (A) ~~and (B)~~ The PSD samples are designed as in legend to Fig. 2A. The gels containing 10 μg protein lines were blotted and probed with antibody C30-5, recognizing autolyzed form of μ calpain (~~A~~) and with antibody 1D10A7 preferentially recognizing the large subunit of m-kalpain (~~B~~). (C) The soluble (S) and total membrane (M) fractions of brain homogenate (10 μg protein line) were probed with anti-calpain antibody 1D10A7. Control—C_{is}, Ischemia I: (~~D~~) The PSD from control (C_{is}) and ischemic (I) brain were probed with two anti-fodrin antibodies as described in Material and methods.

from a basal 30% to 60% of total after ischemia. Both isoforms of calpain (μ and m) have been detected in PSD preparations on Western blots (Fig. 3A ~~and B~~). However, in this fraction a considerable lowering of the 76 kDa (active) subunit of μ calpain resulted from ischemia (upper panel). This coincides with a relatively stable content of the m isoform (80 kDa band on the lower panel). Furthermore, the in vitro incubation of brain slices (Fig. 3A, ~~B~~, rows designeted + Ca^{2+} and $-Ca^{2+}$) also resulted, as in the case of CaMKII, in a response similar to that found in ischemia with a visible decline of the 76 kDa μ-calpain levels.

Since the functional meaning of the above changes in calpains after ischemia was unclear, we measured its physiological substrate, fodrin, in the post-ischemic

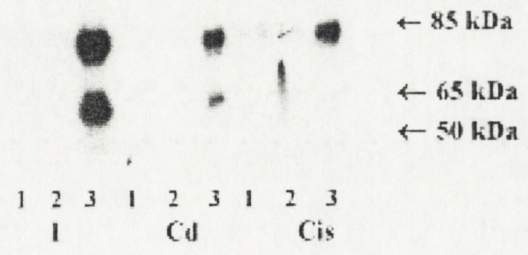

Fig. 4. Autoradiogram of homogenates (lines 1 containing 50 μg prot. lane), P2 particulate fractions (lines 2 with 50 μg prot./lane) or PSDs (lines 3–20 μg prot./lane) blotted on Immobilon membrane and phosphorylated after denaturation/renaturation cycle as described in Material and methods. Experimental groups as in legend to Fig 2A.

PSD (Fig. 3D). Densitometry of fodrin-specific Western blots (Fig. 3D) revealed a significant decrease of this proteins content to about 60% ± 3.5 of the control value.

In spite of the 'classical' signal transduction-associated kinases activated by specific second messengers, post-translational protein modifications may also depend on other phosphorylating systems. We used a denaturation/renaturation method of blotted proteins to identify the intrinsic, PSD-connected phosphokinase activity and its response to ischemic stress. The results of this experiment revealed a strongly phosphorylated major band of approximately 85 kDa. Another, substantially weaker, band was noticed at 60 kDa. These renaturable kinases, particularly those of higher molecular weight, were more active in the PSD than in any other fraction (Fig. 4). Moreover, the phosphokinase activity considerably increased under ischemia as well as after conventional decapitation. We were unable to influence the phosphorylation of these proteins by activating the endogenous, PSD-connected kinase systems like PKA, PKC or CaMKII (Fig. 5). Moreover, the extent of 85 kDa protein phosphorylation in brain homogenate was, in contrast to PSD, not influenced by ischemia. Furthermore, only the in situ control, but not the other preparations, were affected by phosphatase inhibitors such as 30 mM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, or 10 mM β-glycerophosphate (Fig. 5). This means that the differences in phosphorylating activity between either of the controls and the ischemic PSD did not result from their ex vivo dephosphorylation but is rather due to kinase translocation or activation under ischemia.

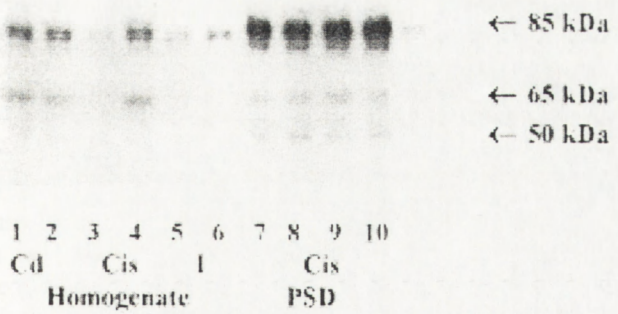

Fig. 5. Lanes 1–6: autoradiograms of tissue homogenized in standard buffer (lanes 1, 3, 5) or in antiphosphatase cocktail (2, 4, 6). Gels were loaded with 100 μg protein lane and phosphorylated after denaturation renaturation cycle as described. Lanes 7–10: The PSD samples from control (C_{is}) rats containing 20 μg of protein were preincubated in 20 mM Tris HCl pH 7.5, 5 mM $MgCl_2$ (lane 7) or with the above medium containing: 100 μM cAMP (lane 8), 5 mM $CaCl_2$ and 0.5 μM PDBu (lane 9) or 5 mM $CaCl_2$ and 5 μM calmodulin (lane 10). After addition of ATP to 25 μM in final volume of 10 μl, the samples were incubated for 5 min then boiled with 2 × SB. After resolution on SDS-PAGE, blotting and denaturation renaturation cycle, the Immobilon membranes were probed with ^{32}Pi-γATP and autoradiographed.

4. Discussion

Post-synaptic membranes forming the PSD are a highly specialized site of contact between neuronal cells. Recently, it has become clear that plastic changes in the molecular assembly of the PSD can regulate function, modulate downstream signals and determine the final response of a neuron to stimulus (Sheng, 1996). Ischemia provides a great metabolic stress with well recognized pathological consequences but its influence on the PSD structure and function still remain to be elucidated. From previous studies it may be concluded that among various processes occurring in ischemic neurons, an early activation of calcium dependent enzymes can trigger a cascade of secondary events leading to neuronal degeneration (Siesjo, 1993). In this paper we provide evidence that the PSD structure, due to a rapid remodeling of its protein composition, may be the first target of ischemia-induced changes in signal transduction.

In agreement with the previously observed translocation of CaMKII to the bulk of cerebral plasma membranes (Zalewska and Domańska-Janik, 1996a,b), brain ischemia also resulted in a rapid translocation of CaMKII to the PSD. However, this translocation is comparatively more extensive, resulting in a 5-fold increase of CaMKII protein, and is nearly complete during the interval between decapitation and homogenization (less than 1 min). As demonstrated, only the in situ tissue freezing method was able to preserve the constitutive, relatively low CaMKII level in this fraction. This is in agreement with data reported by Hu and Wieloch (1995) who also used an in situ freezing procedure and demonstrated a CaMKII redistribution to the PSD under ischemia in vivo. Aronowski and Grotta (1996) succeeded in demonstrating a 3-fold increase of CaMKII in the PSD fraction after brain ischemia employing conventional decapitation for collecting the tissue. A similar enrichment was also reported by Suzuki et al. (1994).

But most importantly, and in sharp contrast to changes in other regions of cerebral membranes, the rapid and massive translocation of CaMKII protein toward the PSD was able to completely compenste for the inhibition of phosphorylating enzyme activity which generally occurs during ischemia. In contrast, the specific activities of CaMKII in PSD, estimated using endogenous as well as exogenous substrates, revealed an increase of protein phosphorylation after ischemia. In similar experiments, the specific CaMKII activity in bulk cerebral membranes dropped to about 20% of control (Zalewska and Domańska-Janik,

1996a). This observation indicates the privileged position of the PSD structure in an efficient targeting of CaMKII.

Calpains (μ and m isoforms) represent the other calcium dependent system deeply involved in ischemic pathology (review Zalewska, 1996). The direct connection of calpains with the PSD structure could theoretically have a great impact on cytoskeletal network remodeling as well as on the function of the protein triggered by the calcium signal. Such a calcium-dependent activation of a PSD-connected, constitutive calpain system is probably responsible for the decrease of the calpain substrate, fodrin, observed immediately after ischemia in PSD (Fig. 3C) as well as in the other brain structures (Zalewska et al., 1998). However, in contrast to this strong indication of calpain activation under ischemia, its targeting to the membrane compartment was found only in the bulk of isolated membranes of brain homogenate (Fig. 3C) and not in connection with the PSD (Fig. 3A,B). The ischemic response in PSD consisted rather in the down-regulation of an active, 76 kDa subunit of μ calpain. This observation corresponds to a previously reported decrease of μ-calpain proteolytic activity in rat brain homogenate after 15 min of postdecapitative ischemia. However, in this case it was accompanied by a shift of the remaining activity toward membranes (Zalewska et al., 1998). In the light of the data presented herein, it seems that postsynaptic densities, containing constitutive calpains which can be activated by calcium under ischemia, also possess the ability to down-regulate calpain to protect cell membranes from further overdegradation. An underlying mechanism, based on the autoproteolytic activity of membrane-bound μ calpain itself was recently proposed by Inomata and Kawashima (1995).

In a separate set of experiments ($+Ca^{2+}$ and $-Ca^{2+}$ groups) we have used brain slices as an in vitro model for studying PSD function. However, after 30 min of incubation of the slices with or without calcium in the medium, the protein composition of the subsequently isolated PSD was similar to that found in the ischemic brain. This observation indicates that brain tissue, when studied in vitro, is severely compromised. As reported previously, the ATP level in the slices is abnormally low, the intracellular concentration of Ca^{2+} is elevated and the NMDA receptor/channel is stimulated (Feig and Lipton, 1990). All of this suggests that the damaging process in incubated brain slices might be similar to that mediating the anoxic/ischemic injury in vivo.

Several proteins connected with signal transduction have been localized in the PSD. There are at least two, in addition to CaMKII, well defined protein kinases identified so far in this structure: PKC and PKA (Kelly et al., 1979; Goldenring et al., 1984; Wolf et al., 1986). From our back-phosphorylation experiment in vitro (Fig. 5), these kinases are probably not directly involved in the intrinsic phosphorylating activity of the denatured/renaturated proteins observed only recently in the PSD fraction. If this were the case the phosphorylation should be blocked by pre-stimulation of the responsive kinase systems with their specific second messengers in the presence of non-radioactive ATP. In contrast, the PSD-specific phosphorylation system described here did not respond to such treatments. We were unable to determine the proteins involved in the observed phosphorylations, mainly because of the extremely low quantities of isolated PSD (about 1 mg protein per 10 g of cerebral tissue). However, using SDS-PAGE and a membrane autophosphorylation assay identical to that used in this paper, the 85 kDa cytokine-inducible serine/threonine kinase has been identified in other cellular systems (Rachie et al., 1993), with its activity induced in cell cultures in response to treatment with IL-1, LPS and IFN-γ. The 85 kDa kinase is also activated in the lung, kidney, brain, liver and heart after systemic administration of IL-1β, EGF and PMA in mice (Ostrowski et al., 1998). Its size and properties are very similar to the RING 3 kinase (Denis and Green, 1996). Although the role of 85 kDa RING 3 kinase is still unclear, previously published studies indicating its activation by growth factors and cytokines may reflect the involvement of this enzyme in specific signal-transduction pathways. In this study we found that the 85 kDa kinase is obviously much more active in PSD than in parallel samples of the homogenate and P2 fraction (Fig. 4) and is further activated by 15 min of global brain ischemia. These observations extend the possible regulatory role of this enzyme on signal transduction processes in the brain which are greatly altered by ischemia.

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Research report

Isoforms of protein kinase C in postsynaptic densities after cerebral ischemia

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Abstract

Relatively mild ischemic insult can lead to delayed neuronal cell death in vulnerable brain regions. We provide evidence that the protein composition of the postsynaptic densities (PSD) undergoes rapid modification after 15 min postdecapitative as well as 5 min transient global ischemia. We observed a significant increase in cPKC and nPKC protein content in the postischemic PSD. Of the calcium-regulated PKC isoforms, the α and β subtypes increase in PSD over ten times above the control values whereas γPKC, an isoform most abundant in the native PSD structure, shows relatively smaller changes under ischemic conditions. For the first time, the PSD membrane translocation of Ca^{2+}-independent isoforms δ and ε is shown. The yield of the PSD protein preparation from the postischemic cortex was two times higher compared with control. This correlated with an abundant increase in electron density and changes in ultrastructure of PSD isolated from postischemic cortex. Also sections from CA1 gerbils hippocampus after transient ischemia showed persistent enlargement of postsynaptic densities up to 24 h of reperfusion. This was accompanied by elevation of the PSD/cytoskeleton-associated α, β PKC immunoreactivity and other changes in neuronal and glial cell morphology typical of the early postischemic degeneration. Sustained changes in PKC composition and organization of postsynaptic membranes during and after ischemia may cause persistent alteration in synaptic transmission and subsequently contribute to delayed neuronal injury.

Theme: Excitable membranes and synaptic transmission

Topic: Postsynaptic mechanisms

Keywords: Postsynaptic density; Isoforms of protein kinase C; Brain ischemia; Synapse morphology

1. Introduction

Nerve cells are connected via synaptic junctions. The efficiency of signal transmission in chemical synapses depends on the coordinated action of neurotransmitters released from the presynaptic terminals, activation of their receptors gathered in the postsynaptic membrane and the propagation of the impulse in the postsynaptic cell [8,11,14].

Brief, transient cerebral ischemia causes changes in synaptic transmission and consequently in neuronal function. Ischemia-vulnerable brain regions undergo drastic changes, manifested in delayed neuronal cell death, mostly in CA1 area of hippocampus. Enhanced release of excitatory neurotransmitters influences a marked accumulation of proteins and the protein makeup of the postsynaptic densities (PSD), which is directly involved in the transduction of the ischemic signal [5,9,10]. Modulations of receptor function and down stream pathways both involve post-translational modifications of proteins, amongst

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others, phosphorylation and dephosphorylation reactions. There are several families of protein kinases and phosphatases implicated in the modulation of synaptic transmission. The members of the protein kinase C family are key enzymes in the mediation of ischemic signals in the nerve cells. The activation/translocation of Ca^{2+}-dependent PKC to particulate fractions with subsequent down-regulation triggered by transient brain ischemia was reported in rats and gerbils [3,6]. The classical PKC isoforms, whose activities depend on the cytosolic concentration of Ca^{2+} (α, β, γ), are involved in generation of second messengers and glutamate excitotoxicity. Simultaneously, the Ca^{2+}-independent forms of PKC can be activated, but their role in the ischemic signal transduction remains to be elucidated. On the postsynaptic site, intracellular signaling molecules are coupled to the membrane and are organized into different pathways. Proteins belonging to the PSD 95/103 family are the main molecules that are known to assemble this specific membrane bound formation [21]. The molecular structure of these proteins makes them well suited for the formation, upon stimulus, of functional complexes of proteins recruited from the cytosol or gathered to exert their activity in this special location. Suzuki at al. have characterized Ca^{2+}-dependent PKC activities in PSD from different regions of rat brain, pointing out the extremely low contents of PKC molecules in PSD [24].

Detailed studies were performed to elucidate if the PKC isoforms are targeted to the postsynaptic density upon ischemic insult. We employed antibodies to specific PKC isoforms to monitor their existence in the PSD and the influence of ischemia on their cellular distribution. Post-embedding immunogold detection method was used to study the effect of transient ischemia on the cellular localisation of Ca^{2+}-dependent isoforms in brain sections. Changes in the morphology and immunolabelling of CA1 region of hippocampus were examined on the EM level to reveal the changes in synaptic junction structure.

The results of the present study indicate that 15 min global postdecapitative ischemia in rats induces a marked increase in the content of conventional PKC and also Ca^{2+}-independent isoforms δ and ε in the PSD structure. These changes are accompanied by an increase in the yield of PSD preparation from the ischemic cortex. In the model of 5 min ischemia in gerbil followed by reperfusion, the early elevation in size and density of PSD proceded up to 24 h postischemia and was accompanied by an increased content of α, β PKC isoforms in this structure, possibly reflecting their role in the transduction of the ischemic signal. The sustained molecular changes, which are already seen in the PKC composition and organization of postsynaptic densities during ischemia are likely to contribute to persistent alteration of synaptic transmission that might be detrimental to the neurons in ischemia vulnerable brain regions leading to their death during reperfusion after transient insult.

2. Material and methods

2.1. Postdecapitative ischemia and preparation of subcellular fractions

Male Wistar rats (200–300 g) were used for the PSD preparation. The experimental procedure was approved by the institute's committee on animal studies. Brains were collected under light ether anaesthesia. Postdecapitative ischemia was produced by incubation of the heads at 37°C for 15 min. After that, the heads were cooled down and the brains dissected on ice. Brains for control preparations were obtained by freezing in situ with liquid nitrogen and dissected by method of Tzigaret et al. [27]. Each experimental group contained ten brains. Isolation of PSDs was performed according to the procedure of Cohen et al. [2] with minor modifications [5].

The tissue (10 g) was homogenized in isoosmotic buffer containing 0.32 M sucrose, 1 mM NaHCO3, 0,5 $CaCl_2$, 1 mM $MgCl_2$ supplemented with 100 μM leupeptine and spun for 10 min at 17 300 *g*. After the wash of the pellet and sedimentation in the same conditions the P2 fraction contained organelles and fragments of plasma membranes. P2 fraction was separated by 0.85/1.0/1.2 M sucrose density gradient. The synaptoneurosomal fraction (SN) was collected from the interface between 1.0/1.2 M sucrose. Synaptic membranes (SM) were obtained after 15 min incubation at 4°C in buffer containing 0.5% Triton X-100. Further, the SM pellet was subjected to 1.0/1.5/2.0 M sucrose density gradient overnight centrifugation at 275 000 *g*. The PSD fraction was collected form interface 1.5/2.0 M sucrose, diluted with 12 ml of water and centrifuged 275 000 *g* for 1 h.

To improve the purity of the fraction, the final PSD was additionally diluted with 0,5% Triton X-100 with 75 mM KCl, kept in the cold for 1 h and respun for 1 h at 275 000 *g*. A portion of the PSD was dissolved in 1% SDS and the protein concentration were determined by the method of Lowry [15].

2.2. Transient ischemia model

The adult Mongolian gerbils (50–70 g, both sexes) were subjected to brain ischemia by 5 min bilateral occlusion of the common carotid arteries under halotane/N_2O anaesthesia in strictly controlled normothermic conditions. Animals subjected to ischemia were allowed to recover for 6 or 24 h after the insult. Sham operated animals served as controls. Before removal, the brains were fixed by a transcardial perfusion with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PBS), followed by a brief rinse with 0.9% saline.

2.2.1. Electron microscopy studies

Pellets of PSD fractions were fixed by immersing them first in 4% glutaraldehyde in 0.2 M cacodylate buffer pH

7.4 and than in 1% osmium tetroxide resuspended in the same buffer. The pellets were dehydrated by standard procedures and embedded in Epon. Thin sections were cut on a LKB microtome and stained sequentially with 4.7% uranyl acetate and lead citrate. The sections were examined and photographed in a JEOL 1200EX electron microscope.

Blocks of tissue were cut from stratum pyramidale of the CA1 sector of hippocampus, incubated overnight in PBS, treated with 1% osmium tetroxide for 1 h, dehydrated in the gradient of ethanol, and finally embedded in Epon. Ultrathin sections were treated according to postembedding immunogold procedures. The primary monoclonal antibody to α, β PKC (Amersham) was diluted in PBS 1:10. After pre-treatment with 10% hydrogen peroxide the antiserum was applied to the sections mounted on the formvar-coated golden grids and incubated for 2 h. After washing, the sections were exposed for 30 min to secondary antibody coupled with 10 nm gold particles, diluted 1:20 in PBS (Janssen, Beerse, Belgium). The control staining was performed, where primary antibody was replaced by normal rat serum diluted 1:20 in PBS. Sections were stained with 4.7% uranyl acetate for 15 min and with lead citrate for 2 min. Photographs were taken from stratum pyramidal of the CA1 area of the hippocampus of the control and experimental animals.

2.2.2. SDS–PAGE electrophoresis and immunoblot analysis

The samples of homogenate, P2, synaptoneurosoms, synaptic membranes and PSD fraction containing 5 μg of protein were separated by 7.5% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose Hybond C Extra membrane (Amersham). The proteins were analysed by the Western technique. Antibodies against isoforms of protein kinase C (Transduction Laboratories) were used as primary antibodies; horseradish peroxidase-conjugated anti-mouse antibody was used as the secondary antibody. Immunoblots were developed with an ECL system (Amersham) and quantified using a densitometer UltroScan XL (Pharmacia-LKB).

3. Results

The PSD structure from control and ischemic rat brains was purified to study the contribution of protein kinase C in the ischemic signal transduction. We applied an isolation procedure described by Cohen et al. [2] as the PSD fraction obtained by this method is well characterised morphologically and its protein pattern is established electrophoreticaly. The yield of the control preparations was 135±29 μg/1 g of tissue (n=5). In the case of ischemic PSD, the yield was significantly enhanced to 260±11 μg/1 g of tissue (n=5), what account for 193±4% of the control value (Fig. 1). No significant differences were observed between the control and is-

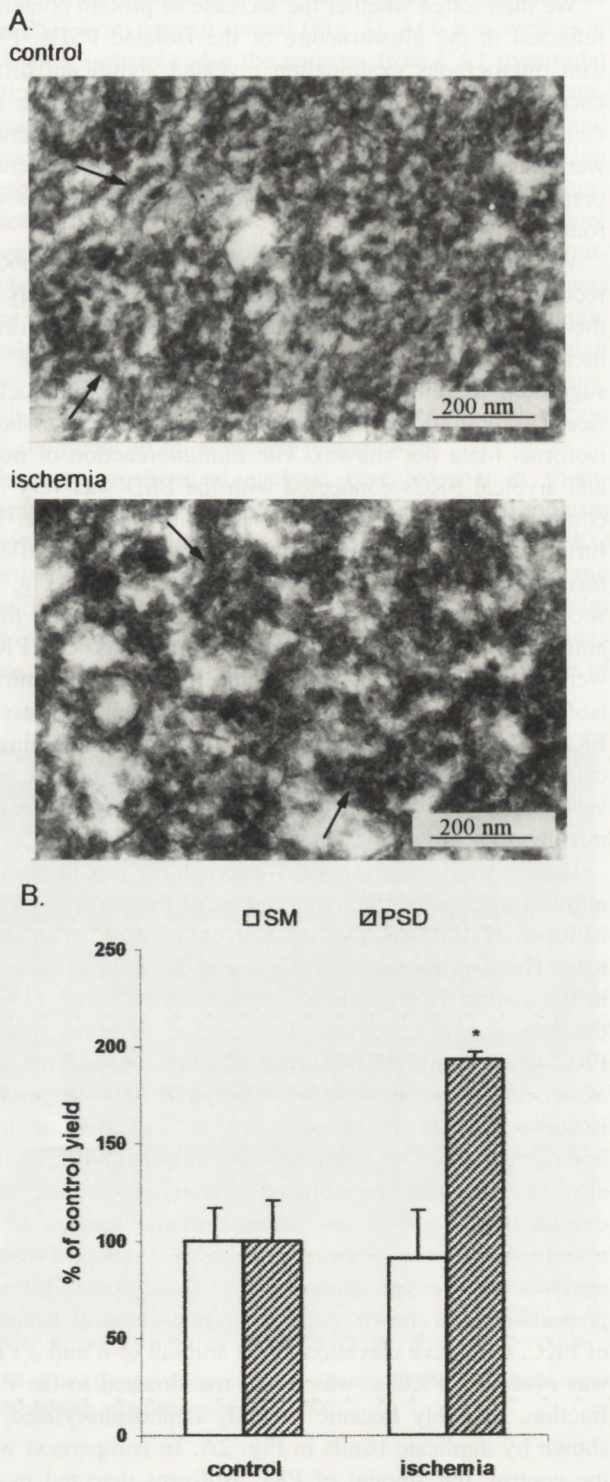


Fig. 1. **A**. Electron micrographs of isolated cortical PSDs from control and ischemic rats. Densely stained structures of PSD are present in control and ischemic preparations (arrows). Note the difference in the size of PSDs between control and ischemic structures. **B**. Yield of postsynaptic densities (PSDs) and synaptic membranes (SM) from control and ischemic rat brains. Data were expressed as mean±SD percentage of control (n=5). A significant difference in protein yield (*) was observed between the two groups (P<0.05, Student's t-test).

chemic animals in the protein yield of P2 or synaptic membrane fractions (Fig. 1).

We then tested whether the increase of protein content is reflected in the ultrastructure of the isolated PSDs. Electron micrographs examination revealed significant differences in the shape and size between postischemic and control PSDs (Fig. 1A). In controls, most of the structures were curved in shape and thin. In contrast, PSD structures prepared from ischemic brains looked much thicker and round.

We analysed the whole spectrum of specific antibodies recognising isoenzymes belonging to the PKC family. In the control PSD, besides the classical α, β and γ isoforms, there were also ε, δ (Fig. 2) the isoforms that are suggested to phosphorylate receptors for growth factors (see Discussion) as well as small amounts of τ and λ isoforms (data not shown). The immunoreaction of novel and atypical PKCs connected with the PSD was very low compared with their recognition in the homogenate. For further detailed examination we chose the classical isoforms and two novel PKC isoforms (ε and δ). Fig. 2A shows a protein kinase C immunoblots probed with these antibodies. In the control preparations, classical PKCs were detected in all of the isolated fractions. In contrast, isoforms δ and ε, which belong to a novel subclass of PKC, were mostly observed in fractions containing a cytosolic compartment (homogenate, P2), whereas only very weak reaction was seen in the detergent treated membranous structures, including PSD.

Densitometric analysis of Western blots run in parallel allowed to establish the contribution of the particular PKC isoforms in PSD relative to their expression in homogenate. The data showed that the α and β isoforms detected in the control PSD fraction accounted for 13 and 14% of the homogenate reactivity. In the case of brain specific PKC-γ, in control PSD almost 40% of the enzyme was associated with this fraction. The calcium independent isoforms, δ and ε, showed only 5 and 3% of total homogenate reactivity, respectively. In ischemic PSDs, but also in the other membranous fractions isolated after decapitation, a substantial increase in the amount of all tested isoforms was observed. In Fig. 2C the densitometric analyses of Western blots from at least three different preparations are shown. Among the non-classical isoforms of PKC, a massive elevation in the amount of δ and ε PKC was observed. PKC-ε, which was translocated to the PSD fraction, probably became partially dephosphorylated, as shown by duplicate bands in Fig. 2A. In comparison with the control, the amount of PKC isoforms detected in the postischemic PSD structure increased almost ten times in the case of α and β, and 12 times in the case of ε PKC. Surprisingly, the elevation in the amount of PKC-γ was not as large as the translocation of the other classical isoforms. However, it should be stressed again that, in control PSD, the amount of γ type of PKC was the highest among all the isoforms tested.

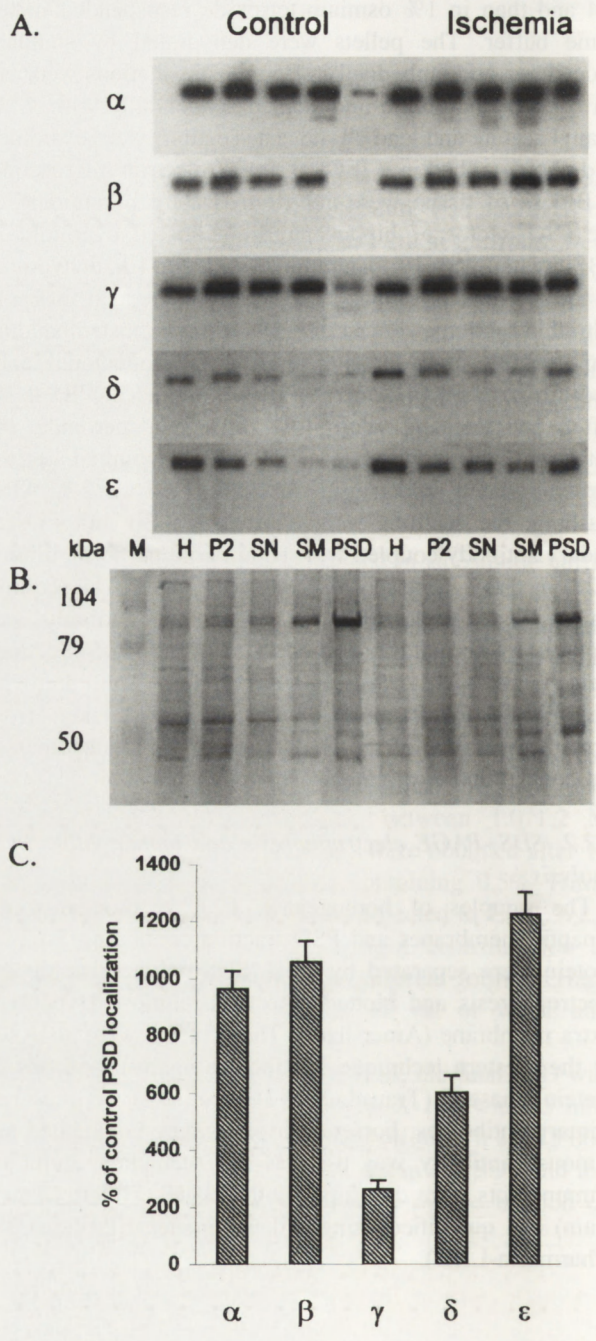
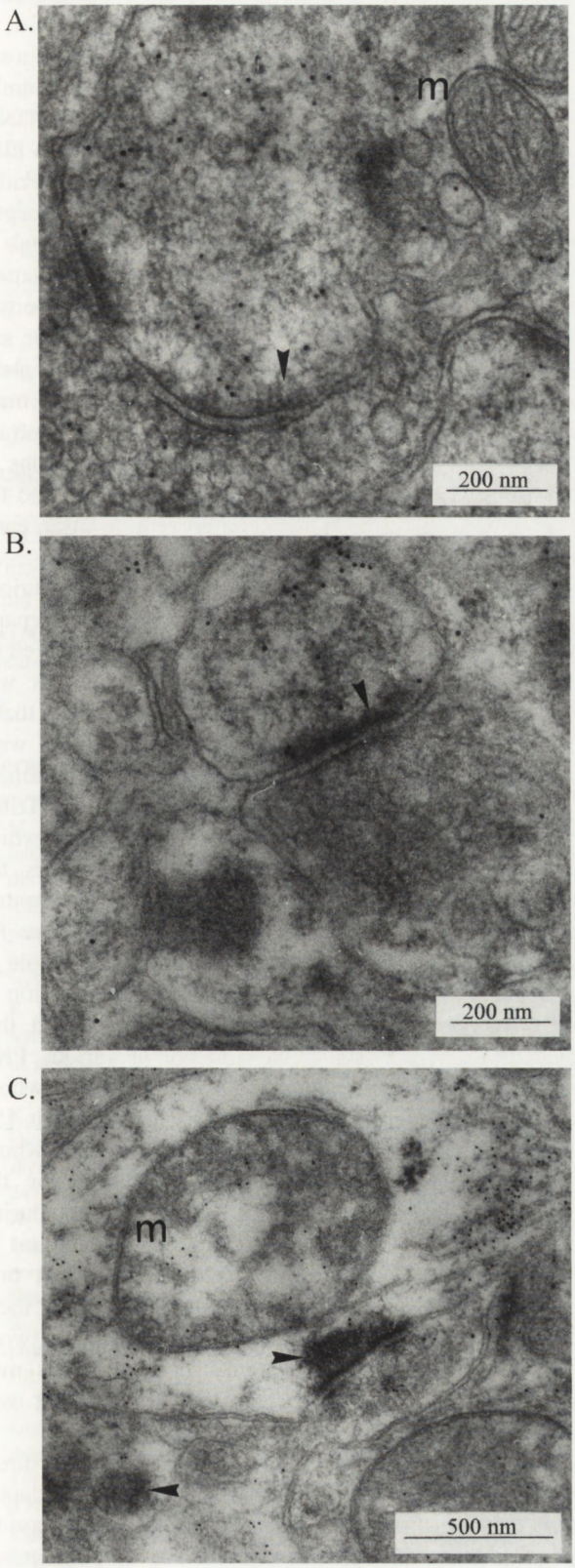


Fig. 2. Western blot analysis of protein kinese C isoenzymes in the subcellular fractions from cortex of control and 15 min postdecapitative ischemic rat brains. **A**. An immunoblot of PKC isoforms in 5 μg of protein samples from homogenate (H), P2, synaptoneurosoms (SN), synaptic membranes (SM) and PSD. The blots were labelled with antibodies against α, β, γ, δ, ε PKC and visualised with an ECL system. Samples were separated by 7.5% SDS–PAGE and blotted to a nitrocellulose membrane (NC). **B**. NC stained for protein profile, M represents molecular weight standards. **C**. The optical density of the PKC isoform bands from ischemic PSD was calculated and presented as % of the parallel run control PSD reactivity. The values are means±S.D. (n=5).

For further investigation of alterations in PSD appearance and composition, EM analysis of postischemic gerbil brain sections was performed. Earlier light microscopic

studies showed no obvious changes in hippocampal morphology up to 24 h of reperfusion after transient ischemic insults in gerbils [12]. By contrast at later times, selective neuronal degeneration occurred in CA1 region of hippocampus. At the ultrastructural level, transient 5 min cerebral ischemia affected directly the postsynaptic densities, which appeared bigger and denser in comparison with PSD from control animal (Fig. 3). At 6 h as well as at 24 h after ischemia also the neurons from CA1 pyramidal section showed appearance different relative to sham-operated animal. As it is shown in Fig. 3, neurons and astrocyts were swollen, with clear cytoplasm, lacking organelles. Numerous mitochondria showed altered ultrastructure: their membranes were discontinuous, crists were shortened and matrix swollen. Neuron terminals contributing to the formation of symmetric and asymmetric contacts were also affected by transient ischemia. Postsynaptic densities were thicker and fluffier than control ones, which were more compact and strictly attached to the membrane. Such an appearance of cells in postischemic CA1 region was dominant in all screened preparations.

Immunocytochemical analysis revealed that in sham-operated animals α and β PKC was located in cytoplasm of neurons and glial cells. At 24 h after ischemia PKC reactivity was translocated to neurotubules of axons and dendrites and was associated with pericarion over Golgi and mitochondria. Densely immunoreactive PSD structures were also seen. Fig. 4 shows high power magnification of



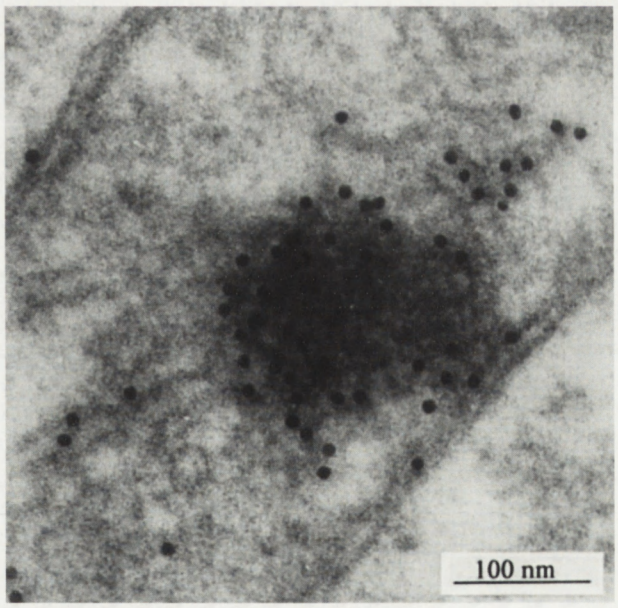


Fig. 4. The high power magnification of postischemic PSD shows dense gold labelling connected with α, β PKC.

Fig. 3. Electron micrographs of synapses in CA1 region of hippocampus from control gerbil (**A**) and animal subjected to 5 min ischemia followed by 6 and 24 h of reperfusion (**B** and **C**). Arrows show intact PSD structure in **A** and dense, fluffed PSD in **B** and **C**. Note the difference in mitochondria (m) appearance in control and postischemic slices. In comparison to control synapse, where immunostaining (black dots) is mostly diffuse in cytoplasm, in postischemic synapse anti-PKC staining is connected with PSD and neurotubules of dendrites and axons.

characteristic postischemic PSD. This structure was fluffed and thickly decorated with gold particles associated with anti-PKC antibody.

4. Discussion

The data presented in this paper demonstrate that ischemia induces in rat brain rapid translocation of protein kinase C isoforms to the postsynaptic densities. Simultaneously, the amount of total protein in the PSD is almost doubled. The microscopic differences in the appearance of PSD from control and ischemic preparations support the view of the possible role of this structure in the processes going on in postsynaptic neurons during ischemia. Electron microscopic observation of the purified PSD samples find the reflection in rapid and sustained remodeling of postsynaptic densities in the CA1 hippocampal sections after 5-min transient ischemic insult in gerbils.

An earlier light microscopic examination revealed only very scarce changes in the morphology of neurons and surrounding glial cells at 24 h after the ischemic insult [12]. By contrast at the EM level, changes in morphology of neuronal cells, especially those of PSD and mitochondria were evident. Our data are in agreement with the recent study of Martone et al. [16], who showed profound changes in the three-dimensional ultrastructure of PSD in various hippocampal sections after transient cerebral ischemia in rats.

The association of PKC with the membranes has served as a hallmark for its in vivo activation [18]. Although such an association is taking place during ischemia, activation is not observed when the ATP level is severely reduced, the amount of protein phosphorylation diminishes rapidly [28]. However, during reperfusion, the concentration of ATP is restored and protein phosphorylation, due to specific localization of actually translocated kinases, might be accelerated. The presence of PKC in the postsynaptic density structure might thus modify cellular signaling immediately after restoration of blood recirculation.

In general, the differences in the actual make-up of enzyme isoforms may confer functional specificity to the site of their location and this holds to the PSD compartment as well. It is well documented that changes in cPKCs properties are associated with ischemia-related brain pathology [1,3,6]. Initial activation followed by a subsequent loss in the amount of membrane calcium-dependent PKC isoforms during and after ischemia primarily depends on the elevation of intracellular Ca^{2+} concentrations and a concomitant activation of calcium-dependent proteases–calpains [4]. These responses are mainly initiated by glutaminergic stimulation of NMDA receptors (NR) [7]. The receptors, located in postsynaptic membranes, are gathered by specific proteins, which belong to the PSD-95 protein family [8]. Recently, it has been shown that the NR1 subunit of NMDA receptor is physically and functionally connected with active PKC-γ through noncovalent interaction [23]. The activation of the NMDA receptor/channel by glutamate allows calcium entering the cell to activate associated PKC-γ which in turn enhances synaptic transmission due to the rapid phosphorylation of the NR1 receptor subunit promoting open channel conformation [23].

The other isoforms that belong to a novel PKC group are activated in response to growth factor receptor stimulation [19]. The brain-derived neurotrophic factor (BDNF) elicits a rapid increase in PSD NR1 phosphorylation [22]. Additionally, the tropomyosin receptor kinase (Trk B) protein coimmunoprecipitates with NR1 NMDA receptor subunits. Therefore, novel, calcium independent forms of PKC may mediate the response of NR1 to trophic factors. Elevation of the amount of TrkB in the PSD structure was observed at 4 h of recovery after 15 min of ischemia in rats [9], suggesting that prolonged NMDA receptor phosphorylation and activation in a calcium-independent manner may have occurred under these conditions. In contrast, it was recently reported that BDNF protects neurons in vitro from excitotoxic injury caused by glutamate and the underlying mechanism involves the prevention of an early loss of membrane PKC activity [25].

The data presented here confirm the existence of various PKC isoforms in the PSD structure. In control preparations, the Ca^{2+}-dependent PKC isoforms were observed on Western blots in all tested subcellular fractions. It was calculated, based on the optical density of the bands, that a relatively large portion of PKC α, β and γ isotypes were recovered in the particulate fractions. Sequential treatment of the membranes with hypoosmotic conditions and Triton X-100 renders a detergent insoluble PSD fraction, which broadly corresponds to cytoskeletal proteins. Perhaps, the high amount of PKC γ associated with PSD in control brains is a reflection of this isoenzyme preference for neuronal postsynaptic localization and its putative role in the regulation of synaptic plasticity via phosphorylation of NMDA receptors. In our earlier study we showed that beside the enrichment of PSD structure in various PKC isoenzymes there were a number of other proteins whose content in PSD was changed after ischemic insult [5]. Among them was calcium/calmodulin kinase II whose 5-fold increase in content could compensate for the inhibition of this enzyme activity, which occurs during ischemia [5]. There also was decrease in the amount of fodrin which is a substrate for calcium-dependent proteases–calpains, and subsequent down-regulation of these enzymes, an event likely to protect cytoskeletal network from overdegradation. On the other hand, calpains may contribute to the down-regulation of PKCs, what was observed under the ischemic conditions (1, 3, 6).

In addition to the occurrence of PKCs in the direct proximity to its potential substrates, a number of adaptor proteins have been detected that can bind protein kinase C. Among them are RACKs (receptor for activated C kinase),

AKAP 79 (a kinase-anchoring protein), cytoskeletal proteins and actin [13,17,20]. More recently a new protein targeting PKC, named InaD, was discovered in the photoreceptor neurons of *Drosophila* [26]. InaD belongs to the family of proteins possessing PDZ domains that are involved in the formation of a specially organised architecture of signalling domains at the synaptic junctions. In view of recent data and our findings, PKC appears to be one of the enzymes dynamically involved in building the signalling domains in postsynaptic cells upon ischemic insult. The changes in the ultrastructure of PSD strongly confirm this concept. These findings support and extend the view of the possible regulatory role of protein kinases on signal transduction processes, which are altered by ischemia. Clearly, more detailed immunocytochemical and functional studies are needed to bridge the EM observations with biochemical data and describing the postischemic fate of CA1 hippocampal neurons.

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Research report

Opposite reaction of ERK and JNK in ischemia vulnerable and resistant regions of hippocampus: involvement of mitochondria

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Abstract

Delayed ischemic death of neurones is observed selectively in CA1 region of hippocampus at 3–4 days of reperfusion. Signals generated immediately during and after ischemia are further propagated by a variety of kinases, proteases and phosphatases. Tissue samples from dorsal (vulnerable) and abdominal (resistant) parts of gerbil hippocampi were collected to determine the activation state of key signaling molecules: Akt, Raf-1, JNK, ERK1/2 in the course of reperfusion after 5 min of global cerebral ischemia. Western blot analysis of phosphorylated forms of the kinases revealed persistent activation of JNK, being limited mostly to vulnerable CA1 region. On the contrary, activation of ERK, although observed transiently in both parts, was enhanced for a longer time in the abdominal hippocampus. The levels of the active/phosphorylated Akt and Raf-1 kinases did not change significantly during the recovery period. No significant correlation between postischemic JNK activation and c-Jun phosphorylation or its contribution to AP1-like complex formation was found. In contrast, the amount of active JNK linked with mitochondrial membranes was significantly increased and preceded neuronal death in CA1. In the same period of time the AP1 complex, augmented in CA1 region, did not appear to contain a classical c-Fos protein. These results are consistent with the theory that either long-lasting activation of JNK and/or contrasting ERK and JNK activities in critical time of reperfusion, contribute to selective apoptosis of CA1 neurons. This, in connection with the translocation of activated JNK to mitochondria and time/regional differences in AP1 binding protein complexes can affect final postischemic outcome.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Brain ischemia; Neurodegeneration; ERK/JNK protein kinases; AP1 transcription factor; JNK translocation

1. Introduction

Moderate, transient ischemia triggers a cohort of potentially devastating cellular processes, although the morphological signs of neuronal disintegration are only seen in specific brain areas at 3–4 days of initially successful reperfusion. Among others, the members of MAP kinase pathway, such as Raf-1, ERK1/2 and JNK are supposed to take part in the enzymatic amplification and regional specification of post-ischemic signaling [10]. The general phenomenon of different spatial or cellular sensitivity to moderate cerebral ischemia can be easily observed in hippocampus. In this structure tissue reaction to ischemic challenge, differs from massive apoptosis-like neuronal death in CA1 sector [25,28] to full functional and morphological recovery in the adjacent parts, including CA2, CA3 and the dentate gyrus (DG). Spatial discrepancy in the activation of survival-connected extracellular-response kinase, ERK1/2 [32] and stress activated/c-Jun N-terminal protein kinase, JNK [18], has been suggested to influence the balance between cell death or survival in these two parts of hippocampus [22,31]. Also, the activity of Raf-1 kinase, located up-stream of ERK and JNK in MAP kinase pathway, may influence cell survival. Raf-1 is the main protein recruited by GTP-bound Ras in the sequential MAPK-phosphorylation cascade [1]. On the other hand, conventional PKCs are powerful additional modulators of Raf-1 activation/phosphorylation [30]. Transient activation

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and subsequent down-regulation of PKC has been reported by our and other groups in similar ischemic models [13,7]. It has been demonstrated that Raf-1, co-activated by PKC, can be directed to the mitochondria membrane and then become engaged in deactivation (phosphorylation) of pro-apoptotic Bad [4]. In addition to its pro-survival function Raf-1 is involved in down-stream regulation of MAPK/ERK modules and their further neuroprotective effects, i.e., through direct GSK-3β [20] and/or RSK phosphorylations, or alternatively, by transcriptional regulation of pro-survival gene expression [3].

In contrast to many known ERK effectors, the only process in which a target for JNK has been directly defined is c-Jun phosphorylation [2]. JNK-dependent c-Jun phosphorylation is critical for formation of the activation protein-1 (AP1) complex. We have previously shown that ischemia induces elevation of AP1-like protein binding ability in hippocampal homogenates [35]. Moreover, composition of AP1 may depend on the time of its appearance after the insult [11]. c-Jun phosphorylation and expression of AP1 proteins have been mutually suggested to contribute to the genetic mechanism underlying vulnerability and/or resistance of CA1 to excitotoxicity and ischemia [16,17,33]. In this work we explore this possibility by comparing the influence of transient ischemia on canonical AP1 cFos/cJun heterodimer formation in both, ischemia—vulnerable and ischemia—resistant, hippocampal regions.

One other player in controlling the balance between survival and apoptosis is Akt/PKB kinase, a key protein in the growth factor stimulated pathway involving PI3 kinase [6]. The Akt promotes cell survival through its ability to phosphorylate and inactivate several apoptosis-inducting targets including Bad [8,4], forkhead transcription factors [5] and caspase 9 [29]. According to the recent reports, Akt activity increases transiently during postischemic reperfusion of hippocampus [34].

In order to asses how the activity of Akt, Raf-1, ERK1/2 and JNK kinases correlate with transduction of the lethal ischemic signal, we have quantified of active forms of these enzymes in cell lysates from dorsal, containing vulnerable CA1 region, and abdominal, ischemia-resistant, parts of hippocampus. An attempt has been made to find the major determinants of the different postischemic outcome between these two regions of hippocampus. After having preestablished on increase in the active P-JNK level in postischemic CA1 we have further evaluated c-Jun phosphorylation and its participation in the formation of AP1 complex in this region. In addition, we have explored the connection of P-JNK with mitochondrial fraction in the course of postischemic reperfusion.

2. Materials and methods

Ischemic model and sample preparation

Male Mongolian gerbils, weighing 50–70 g, were used to perform brain ischemia by 5 min bilateral ligation of the common carotid arteries under halotan/N_2O anesthesia in strictly controlled normothermic conditions as previously described [35]. Animals after ischemia were allowed to recover up to 4 days. Sham operated animals served as controls. The experimental procedure was approved by the Local Commission for Ethics of Experiments on Animals.

Tissue samples for Western blot analysis were obtained by freezing the brains in situ with liquid nitrogen to prevent protein dephosphorylation during the procedure of decapitation and dissection [36,37]. Thawing and dissection of the brain was carried out at 0 °C. Manual 'unfolding' of dorsal hippocampus, containing CA1 sector, was completed under binocular with a fissura hippocampalis taken as a starting orientation point. The accuracy of the dissection was standardized under microscope on cross-sectioned hippocampal slices. The remaining tissue (the sectors CA2 to CA4 plus gyrus dentatus—DG) was designed as an abdominal part (AbP). The tissue was homogenized using teflon pistol in Eppendorf tubs (15 strokes) in 250 μl of homogenization buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF. After homogenisation samples were sonicated four times for 5 s. and centrifuged 15000 *g* for 10 min at 4 °C.

Protein–DNA binding activity

Tissue samples for electrophoretic mobility shift assay were obtained by decapitation under anesthesia. Preparation of protein extracts for DNA binding activity was carried out as described previously [35]. A 10-μg amount of nuclear proteins was incubated with ^{32}P end-labeled double-stranded oligonucleotide probe containing AP1 consensus binding motif (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The EMSA-supershift of AP1 components was performed with antibody against phospho-c-Jun (ser-73) and c-Fos (Santa Cruz Biotechnology) as described earlier [11].

Mitochondria preparation

Tissue samples for Western blot analysis of mitochondria were obtained by decapitation under anesthesia. The hippocampi were immediately isolated and homogenized 10–12 strokes in Dounce homogenizer in 10 volumes of ice cold buffer. We did not divide hippocampi into CA1 and dorsal regions because time-consuming procedure may have caused mitochondria damage. The homogenization buffer contained 15 mM Tris–HCl, pH 7.6; 0.25 M sucrose, 1 mM $MgCl_2$; 1 mM EGTA; 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and the mixture of phosphatase inhibitors (Sigma) and protease inhibitors (Sigma). The homogenates were centrifuged at 1000 *g* at 4 °C for 10 min. The pellets were

liscarded, and the supernatants were centrifuged at 17000 g at 4 °C for 20 min to get cytosolic and the crud nitochondrial fractions. The pellets were further diluted with homogenization buffer and mixed with four volumes of 12% Ficoll in Krebs–Ringer buffer prior to centrifugation 99000 g at 4 °C for 30 min. The pellets containing nitochondria were collected in homogenization buffer.

The protein content was determined by the method of Lowry et al. [27] using the Bio-Rad DC Protein Assay Kit.

Western blot analysis

Western blot analysis was carried out on 10% sodium lodecyl sulfate–polyacylamide gel electrophoresis (SDS–PAGE) for P-Raf-1 and Raf-1 immunodetection according o the method of Laemmli [26]. Samples of 50 mg of protein were loaded to each lane. After electrophoresis, proteins were electrotransfered to a Hybond-C extra membrane (Amersham). The membranes were incubated over night with primary antibody against Raf, P-Raf, in the dilution 1:500 in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) with 0.05% Tween X-100. Polyclonal antibody against P-Raf-1 and monoclonal anti Raf-1 were purchased from Santa Cruz Biotechnology. After incubation with secondary antibody conjugated with horseradish peroxidase the blots were developed with ECL detection system (Amersham). The density of the bands were evaluated densitometricaly using UltroScan XL LKB). The membranes for Western blots of mitochondria were stripped after probed with P-JNK antibodies and the second immunoreaction were done with anti HSP-60, the mitochondrial matrix protein to assess the gel loading.

Western blot analysis for detection of P-Akt, Akt, P-ERK, ERK, P-JNK and JNK was carried out on 10% SDS–PAGE. Antibodies were purchased in New England BioLabs (Beverly, MA, USA). Western blot analysis of P-c-Jun (ser-73) and c-Fos was performed on nuclear fractions prepared for EMSA using the same antibodies as for supershift EMSA.

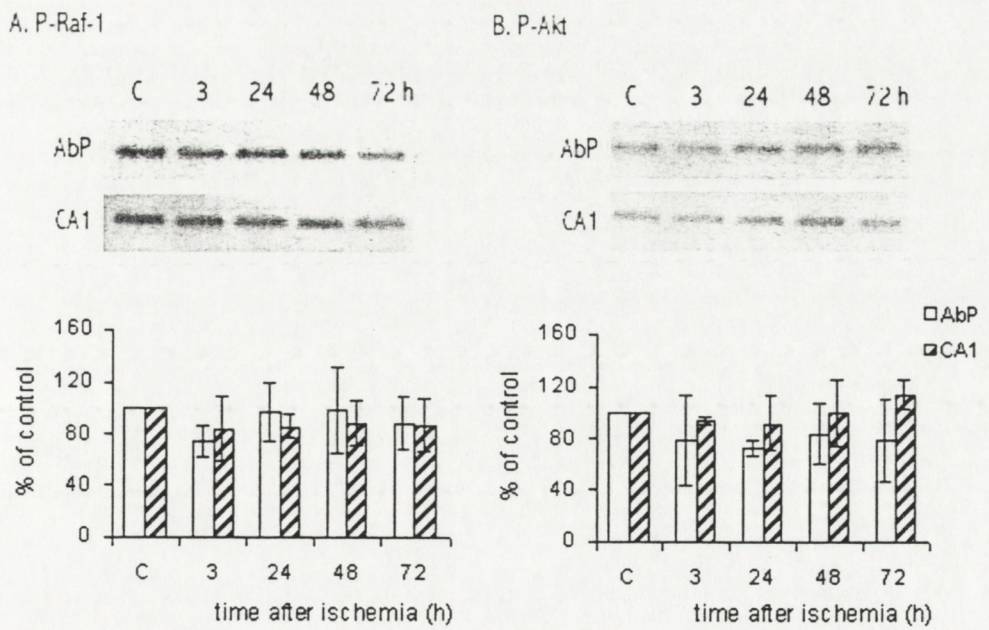
Statistical analysis

Data are presented as mean values±standard deviation. Number of experiments are given in legends to figures. Differences between mean values were tested for significance by analysis of variance and the *t*-test.

3. Results

Looking at the differences between signalling proteins being activated after ischemia in dorsal (vulnerable) and abdominal (resistant) parts of hippocampus we demonstrate rather their quantitative than a qualitative character. In contrast, a parallel study revealed that almost all neurons (94%) in the CA1 sector degenerated during 7 days of postischemic survival whereas in the remaining parts of hippocampus they still appeared morphologically intact (unpublished).

The main findings during 4 days of postischemic recovery can be described as follows.



ig. 1. Western blots and densitometric analysis of phospho-Raf-1 (A) and phospho-Akt (B) in the CA1 and abdominal (AbP) regions of hippocampi after schemia. Results shown are from sham-operated control (C) and from gerbils subjected to 5 min of ischemia followed by 3, 24, 48, 72 h of reperfusion. The lanes representing one time point in (A) and (B) contain samples (50 μg of protein) taken from CA1 and AbP of the same animal. The Western blots were evaluated densitometrically and expressed as a percentage of control (mean±S.D., $n=4$).

Raf-1 and Akt kinases

Protein extracts obtained from the vulnerable (CA1) and the resistant abdominal part (AbP) of hippocampus were examined by western blot for the content of the total and phosphorylated/active forms of these proteins. Fig. 1A and B refer to temporal profiles of Raf-1 and Akt activation in the above regions. No significant changes in the phosphorylation state of these two kinases in comparison to the control, sham-operated animals, were found in the early (3 h) postischemic period as well as in the case of longer reperfusion up to 72 h. The only exception was a transient tendency towards decreasing of Raf-1 protein content at 3 h.

Mitogen activated kinases: ERK and JNK activity

ERK: Fig. 2A shows representative immunoblots probed with antibody specific to total ERK protein as well as to P-ERK1/2, together with the densitometric analysis of this and parallel blots ($n=5$). In the resistant areas of abdominal hippocampus the amounts of P-ERK increased in all tested time points, with a peak at 24 h after ischemia. In CA1 this increase was significantly less pronounced and readily down-regulated to the control level at 48 and 72 h, a period when neuronal death was well advanced. In contrast, in the abdominal part, more than five-time elevated P-ERK levels were observed up to the third day of reperfusion.

JNK: The phosphorylated form of JNK accumulated exclusively in CA1 region (Fig. 2B). Initial increase to about 250% of control values at 3 h of reperfusion was followed by further enhancement reaching 350% of content after 3 days. Concomitantly, in the abdominal part of hippocampus, P-JNK levels remain almost unaffected despite the total amount of JNK protein revealed rather a tendency to moderate elevation at 24 h of reperfusion.

The 63 or 73 serine residues of c-Jun are the only unquestionable substrates for JNK. Therefore we have measured postischemic accumulation of phosphorylated c-Jun in both investigated hippocampal regions (Fig. 3C). The amount of P-c-Jun did not change significantly in the abdominal part in comparison to control level whereas in the dorsal part (CA1) it sharply increased, but only at 3 h of recovery.

AP1 transcription factor

In view of the progressive activation of JNK kinase in CA1 (Fig. 2B), and having in mind previously reported biphasic increase of AP1-like protein in the whole hippocampus [11], here we have explored this transcriptional factor binding ability and its composition in hippocampal sub-regions during reperfusion. Fig. 3A shows initial elevation appeared at 3 h postischemia in both regions, and was little more accentuated in CA1. The second, delayed

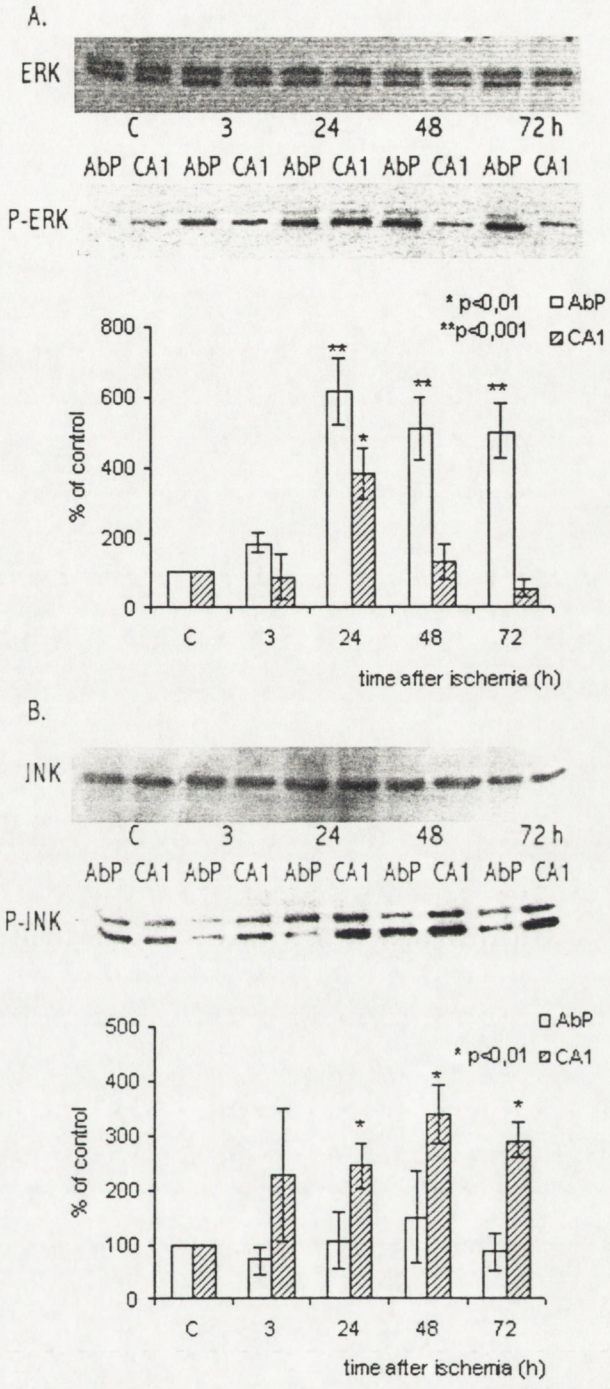


Fig. 2. Western blots and densitometric analysis of total ERK plus phospho-ERK (A) and total JNK plus phospho-JNK (B) in the CA1 and abdominal (AbP) regions of hippokampi after ischemia. Results shown are from sham-operated control (C) and from gerbils subjected to 5 min of ischemia followed by 3, 24, 48, 72 h of reperfusion. The one time point in (A) and (B) contain samples from the same animal. Equal amounts of protein (50 μg) were run in parallel for CA1 and AbP regions then, after blotting, evaluated densitometrically. Data are expressed as a percentage of control (mean±S.D., $n=4$).

phase of AP1 expression, was not significantly different in these two hippocampal regions. Super-shift EMSA with antibody recognizing proteins which contribute the canonical AP1: c-Fos/c-Jun heterodimer, revealed a lack of

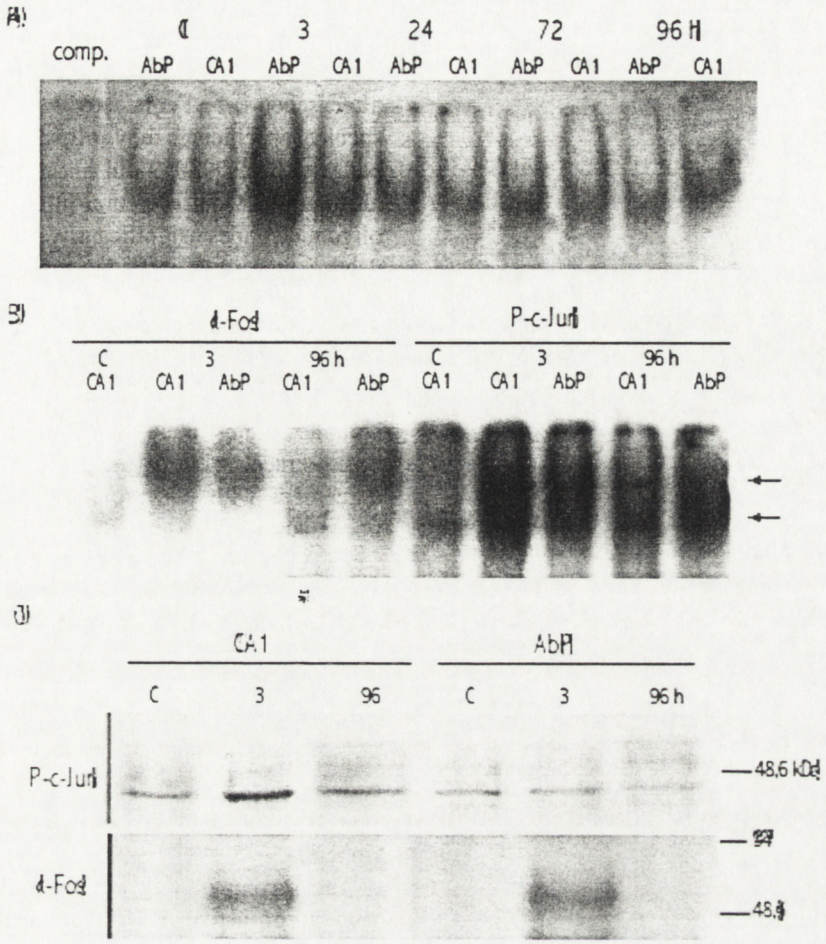


Fig. 3. Representative autoradiograms of AP1 EMSA (A), supershifts of AP1 binding by anti-c-Fos and anti-P-c-Jun (B) and Western blot of P-c-Jun and c-Fos proteins (C) in nuclear extracts from CA1 and AbP regions after ischemia. The lanes for given time point contain samples from the same animal. Samples of hippocampal regions nuclear extracts were obtained from sham operated control (C) or gerbils subjected to 5 min ischemia followed by 3, 24, 72 and 96 h of reperfusion. Aliquots of extracts were incubated with radiolabelled AP1 probe under routine conditions (A) or with addition of antibody against c-Fos or P-c-Jun (ser-73) (B) then followed by gel electrophoresis. Arrows in (B) indicate the positions of AP1-oligonucleotide complexes and these shifted by antibodies, asterisk indicate the CA1 96 h band depleted with c-Fos super shift. In C equal amounts of nuclear proteins (30 μg) were subjected to SDS–PAGE and Western with P-c-Jun (ser-73) or c-Fos antibody.

reaction with c-Fos antibody in the AP1 complex extracted from CA1 at 96 h recovery. This was evident both when compared with samples at 3 h postischemia and with parallel, time-matched AbP samples (Fig. 3B). The amount P-c-Jun increased almost in parallel to the total AP1 content, being more pronounced in the super-shifts from CA1 (Fig. 3B).

Western blot analysis of c-Fos protein content in the nuclear fractions showed a significant increase in both parts of hippocampus but only at 3 h after ischemia. In contrast, P-c-Jun was enhanced exclusively in CA1 region along the course of postischemic reperfusion (Fig. 3C).

Activated JNK binds to the mitochondrial fraction

Mitochondrial fraction isolated from hippocampus during postischemic recovery was evidently enriched in P-JNK protein. This was seen at 24, 48 and 72 h after ischemia as compared either with a non-ischemic or with an early (3 h) postischemic samples. Representative immunoblots, normalized to total proteins as well as to a mitochondria matrix marker—HSP-60, are shown on Fig. 4. As can be judged from HSP-60 blots increased P-JNK binding to mitochondrial fraction was transiently induced at about 24–48 h, then down-regulated at 72 and 96 h, preceding clear signs of neuronal degeneration.

4. Discussion

This report demonstrates that the relations between ERK and JNK pathways differ substantially in ischemia vulnerable and resistant regions of hippocampus. This corresponds with the earlier data reported by Sugino et al. [31] and Gu et al. [18]. Our data reveal that forebrain ischemia influence activation of P-ERK1/2 and P-JNK, but not P-Raf-1 and P-Akt. This last result is in contradiction to a recent report by Ouyang et al. who demonstrated substan-

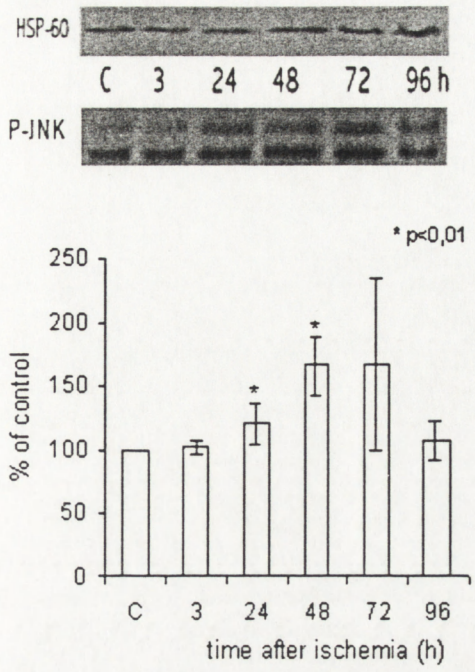


Fig. 4. Western blots and densitometric analysis of P-JNK present in mitochondrial fraction obtained from hippocampi of sham operated control (C) or gerbils subjected to 5 min ischemia followed by 3, 24, 48, 72 and 96 h of reperfusion. The mitochondrial fraction (30 μg) were immunoblotted with anti-P-JNK and anti-HSP60. Densities of P-JNK bands were evaluated and data expressed as a percentage of control (mean±S.D., $n=4$).

tial elevation of phosphorylated AKT up to 1 day of recovery [34]. However, the ischemia employed in their study was more severe, compared to present model (15 vs. 5 min duration). In addition, the effect on AKT activation described by Quyang et al. was not region-specific, with the same magnitude of P-Akt elevation in either CA1 or dentate gyrus dentatus—thus, it did not correlate with preferential vulnerability of the CA1 structure to ischemic damage. In our work, the lack of noticeable P-Akt activation after much shorter, but still devastating for CA1 neurons ischemic insult implicates that even if it is induced by more severe ischemic stress, this response would be not decisive for selective cell death or survival in hippocampus.

This is in contrast to the unique spatio-temporal change observed in the pattern of ERK/JNK. While the transient elevation of P-ERK1/2 in the vulnerable CA1 sector has a tendency to normalization or even down-regulation after 24 h, JNK phosphorylation/activation is steadily increased during entire investigated recovery period. At the same time, in the abdominal hippocampus, P-JNK remains stably at the control level, whereas P-ERK increases more than five times. Our data fit to the assumption derived from most of the in vitro studies performed on neuronal cultures that the ERK module of MAPK pathway is antiapoptotic [21] whereas the JNK promotes cell death [32]. A similar conclusion was reached by Gu et al. [17] on global cerebral ischemia in rats' four-vessel occlusion model. Despite of some differences with our data concerning mainly the time and evolution of ERK/JNK activation after ischemia and connected most probably with model or species specificity, they proved that inhibition of ERK pathway by PD98059 did not affect CA1 neuronal survival. In contrast, intracerebral infusion of JNK 1/2 antisense oligonucleotides significantly attenuated CA1 neuronal death in the same ischemic model [16].

Two intriguing observations gathered during this study deserve a note. Firstly, AP1 complex formed at the late phase of postischemic recovery in degenerating CA1 does not appear to contain a classical c-Fos protein. The AP-1 complex is thus likely to be composed of P-c-Jun homo- or heterodimers with partners other than c-Fos. This is in agreement with our previous data from the whole hippocampus [11], extended here by showing its specificity for CA1 region. Since c-Jun:Fos heterodimers are more stable then c-Jun homodimers and bind more efficiently to the AP1 site, the complex may influence different gene expression in analyzed regions of hippocampus. In addition, as several members of this inducible transcription factor family have been shown to possess intrinsic transrepressive domains (for a review, see Ref. [19]), further experiments will be required to explore this attractive possibility of functional modulation of AP-1 complex after ischemia.

The second finding is that substantially increasing the amount of active P-JNK can be associated with mitochondrial fraction isolated from ischemia-injured hippocampus. This implicates role for postischemic enhancement of JNK activity other then classical N-terminus c-Jun phosphorylation. There are several in vitro data suggesting translocation and involvement of this kinase in mitochondrial Bcl2/Bcl_{xl} protein phosphorylation [14,23,24]. Thus, it seems plausible to suggest a role of JNK translocation in the delayed postischemic cell death. In cellular signaling, the mitochondria translocation of JNK would predate cytochrome c release and caspase 3 activation in the Bcl-dependent apoptotic pathway. Recent studies indicate that JNK activation can be driven directly by an oxidizing stress with subsequent opening of mitochondrial transition pore (MTP) and adenine-nucleotide translocase (ANT) activation [9]. In correspondence, transient MTP opening has been reported after a global brain ischemia in hippocampus [15]. Our recent experiments confirm an early, transient appearance of cytoplasmic cytochrome c in hippocampus subjected to ischemia (unpublished). Moreover, Cassarino et al. report NFκB activation to be one other effect connected with JNK activation [9]. Similarly, we have demonstrated induction of NFκB in CA1 hippocampus as early as 3 h after ischemia [12] Taken together, all these responses (activation/translocation of JNK, NFκB and cytochrome *c* release) appear to be triggered by an early, ischemia-induced mitochondrial dysfunction in hippocampal CA1 sector. Continuing this

line of evidences, in further experiments we would like to assess a broad repertoire of functional proteins in mitochondrial fraction isolated from hippocampus and their possible changes during the course of postischemic recovery.

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Erratum

Erratum to "Opposite reaction of ERK and JNK in ischemia vulnerable and resistant regions of hippocampus: involvement of mitochondria" [Molecular Brain Research, 110 (2003) 245–252]☆

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Due to a mistake the name of the second author was misspelled in the original article. The correct name should be Joanna Dlużniewska.

The Publisher apologizes for the inconvenience caused.

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Research report

Neuroprotection by cyclosporin A following transient brain ischemia correlates with the inhibition of the early efflux of cytochrome *C* to cytoplasm

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Abstract

The efflux of mitochondrial protein cytochrome *C* to cytoplasm is one of the key events of mitochondrial dysfunction observed in post-ischemic pathology. We investigated the effect of intra-carotid infusion of 5–10 mg/kg of cyclosporin A (CsA) on the neuronal survival in CA1 sector of hippocampus and on the subcellular localization of cytochrome *C* in the model of 5 min gerbil brain ischemia. To discriminate between the immunosuppressive and the mitochondria protecting component of CsA action, we compared the effect of CsA with one other immunosuppressant FK506. Almost 75% of neurons in ischemia-affected brain area were saved after CsA but not after FK506 treatment. This protective effect was only observed when the drug was infused immediately upon reperfusion. Early CsA treatment was able to block an initial phase of cytochrome *C* release, occurring transiently at 30 min post-ischemia, an effect never observed after FK506 administration. We assessed the neuroprotective potency of CsA vs. FK506 in rat cortical primary culture treated with compounds that mimic destructive signals induced by brain ischemia. In all cases, neuronal death and cytochrome *C* release were evidently suppressed by CsA applied not later than 30 min after the initial insult. Thus, early treatment with CsA in vitro and after bolus intra-carotid injection in vivo can save neurons by inhibition of cytochrome *C* efflux to cytoplasm.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Brain ischemia; Mitochondria; Cytochrome *C* translocation; Neuroprotection; Route of drug administration

1. Introduction

Cyclosporin A (CsA) is a compound, which beside its well known immunosuppressive activity, can stabilize mitochondrial membrane and block MPT (mitochondrial permeability transition) both in vitro and in vivo [9]. However, the problem still existing is a relatively poor and/or variable penetration of this compound into brain due to active ATP-dependent, P-glycoprotein-mediated outward transport system at the blood–brain barrier (BBB) [20]. Hence, the route and model of administration will limit the effectiveness of this drug applied to protect neurons in various brain pathologies. Recently, it was reported that pharmacokinetic parameters after intra-carotid injection of CsA do not differ significantly between normal, ischemic and reperfused rat brain. All these conditions resulted in efficient drug penetration into brain tissue with brain–blood ratio reaching the levels from 0.5 to 1 [25].

To discriminate which of the mentioned two mechanisms of action of CsA: calcineurin inhibition directly responsible for immunosuppression and/or stabilization of mitochondria membrane is involved; another immunosuppressive drug, FK 506, can be used for comparison. Both compounds, CsA and FK 506 (Tacrolimus), are specific inhibitors (through cyclophilin A and FKBP ligation, respectively) of a type 2B Ser/Thr phosphatase-calcineurin, the enzyme abundant in CNS. Beside of the role of this phosphatase in immunosuppression also other, potentially destructive signalling path-

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ways affected by ischemia can be modified by calcineurin and are taken for consideration in anti-ischemic effect of the inhibitors [24]. On the other hand, only CsA but not FK 506 can interact and inhibit D-type cyclophilin, a protein directly involved in MPT opening [3] and responsible for mitochondria membrane stabilization.

One of the best studied models of forebrain global ischemia is that induced by transient (5 min) common carotid artery occlusion in gerbils [12]. It results in delayed, well-defined lesion in a selected brain area, i.e. CA1 sector of hippocampus. The model has been standardized and used for years for testing the effect of various neuroprotective treatments in our laboratory [4,6].

Here, we used this model to evaluate the neuroprotection offered by intra-carotid bolus injection of CsA with a special focus on its mitochondria stabilizing activity. This route of drug administration should help to overcome the potential problem of sufficient penetration into brain under conditions other than ischemia-induced, acute energy deficit and related blood–brain barrier disruption.

The presented data obtained in experiments in vivo and in vitro performed directly on primary cortical culture suggest that factors other then barrier permeability are responsible for the time limits of effectiveness of CNS protection by CsA after ischemia. In particular, they emphasize the key role of its inhibitory effect on the permeability of outer mitochondria membrane to cytochrome *C*, which is observed transiently in a very early phase of ischemic reperfusion. Transient appearance of cytochrome *C* in cytoplasm of a tissue recovering after ischemia would be decisive for initiation of the pathological signalling cascade leading to neuronal death. In injured animals treated with CsA immediately at the beginning of reperfusion, MPT is not initiated, mitochondrial integrity and cytochrome *C* localization well preserved and destructive signals are not induced.

2. Material and methods

2.1. Experimental models

2.1.1. Global, transient cerebral ischemia in gerbils

All animal experiments were approved by the ethical committee of Polish Academy of Sciences. Male Mongolian gerbils weighing 50–70 g were used. The ischemic insult was performed by 5 min ligation of the common carotid arteries under halotane in N_2O/O_2 (70:30) anaesthesia in strictly controlled normothermic conditions as previously described [5]. A group of animals received CsA (Sandimune–Novartis) injection in the dose of 1.25, 2.5, 5.0 and 10.0 mg/kg directly to the left carotid artery at various time of post-ischemic reperfusion as precisely indicated in Section 3. FK 506 (Tacrolimus, Fujisawa Ph.) in the dose of 2 mg/kg was applied i.p. in the single injection 2 h before ischemic insult or 3 h after ischemia. Sham operated animals were injected with FK 506 (2 mg/kg) or 5 or 10 mg/kg of CsA.

2.1.2. Induction of apoptosis in rat primary cortical cells in vitro

Mixed primary cultures were prepared from brain cortex of 18–19-day-old rat embryos (Wistar) under sterile conditions. Dissected tissue was placed in Ca^{2+}- and Mg^{2+}-free HBSS (Hanks' balanced salt solution, Gibco BRL), dispersed mechanically (10–12 pipette strokes) and then chemically dissociated by 15 min incubation in 0.2% trypsin (Gibco BRL). After centrifugation at 1000 rpm for 3 min, the pellets were resuspended in Dulbecco's modified Eagle medium (D-MEM, Gibco BRL) supplemented with 10% foetal bovine serum (FBS) under antibiotic and antimycotic protection (AAS, Sigma, 1:100). After triturating, the debris were removed by filtration through Millipore cell strainers (45 μm in diameter). Viable cells were plated at a density of 5×10^4 cells/cm^2 on poly-L-lysine 24-well tissue culture plates in 500 μl D-MEM supplemented with 10% FBS and AAS. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and allowed to grow for 7 days before apoptosis induction. At this time, the medium was changed and cells were incubated for additional 6 h either in D-MEM containing 2% FBS and 0.1% dimethyl-sulfoxide (DMSO) or in this medium containing separately 25 nM staurosporine (STS), 500 μM sodium glutamate (Glu) or 25 μM hydrogen peroxide (H_2O_2). In parallel experiments, these compounds were applied together with 0.5 μM CsA (dissolved 100 × in 10% DMSO) or 0.5 μM FK 506. In the separate set of experiments, CsA was added with a delay in respect to the STS, Glu or H_2O_2 treatment.

2.2. Histological and cytological estimation of cell death

2.2.1. Hippocampus

Animals were subjected to 5 min of transient ischemia and allowed recovery period of one week. The animals were perfused with ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) under pentobarbital anaesthesia. The histological evaluation was performed on paraffin-embedded and fixed, 10-μm-sections stained by hematoxylin/eosin or immunohistochemically by double-labelling with anti-GFAP/ MAP2 (glial fibrillary acidic protein and microtubule-associated protein 2, respectively) antibodies as recommended by the manufacturer (Dako/ Sigma). Extend of cell damage of CA1 hippocampal region was quantified under Zeiss Axioscop 2 as the mean number of the persisted, intact neurons at the coronal sections. At least three defined 300 μm fields of CA1 were saved by camera MC 10095 (Carl Zeiss Jena) and counted in a computer-assisted image analysis system (KS 300, Carl Zeiss Jena).

2.2.2. Cell culture

The assessment of apoptosis separately in neurons and astrocytes was performed by the triple-staining method on paraformaldehyde (4%)-fixed cultures. The cells were

washed in PBS and blocked for 30 min by 50% sheep serum, 10% FBS in PBS. All antibodies were diluted in PBS/gelatin with 0.2% Triton X-100. The monoclonal antibody Tuj1 (IgG2a), a neuronal marker, directed against β-tubulin, isoform III (gift from Dr. Frankfurter) was diluted 1:2000 and applied overnight in 4 °C. After washing, the cells were incubated with an goat anti-mouse IgG-FITC (Jackson ImmunoResearch), diluted 1:100. Next, the same cultures were incubated for 1 h with rabbit, anti-cow GFAP, the marker of astrocytes, diluted 1:100 and with goat anti-rabbit IgG-Cy3 (Jackson ImmunoResearch) for 1 h in dark. After washing in PBS, cells were additionally incubated in 5 μM Hoechst 33258 (Sigma) for 20 min in order to visualize nuclei. The cells from at least three independent experiments were counted under Axioskop 2 fluorescence Microscope (Zeiss). Obtained data were statistically evaluated in groups not smaller than 300 cells each.

2.3. Preparation of tissue and cytochrome C immunoblotting

Gerbils in a separated series were subjected to 5 min ischemia and 0.5, 1, 3, 24, 48, 72 and 96 h of reperfusion. Tissue samples for Western blot analysis of cytosolic fraction were obtained by decapitation under anesthesia. The hippocampi were immediately isolated and homogenized 10–12 strokes in Dounce homogenizer in 10 volumes of ice cold buffer. We did not separate CA1 from abdominal hippocampal region because time-consuming procedure may have caused mitochondria damage. The homogenization buffer contained 15 mM Tris–HCl pH 7.6, 0.25 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA (ethylenediaminetetraacetic acid), 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM dithiothreitol (DTT) and the mixture of phosphatase inhibitors (Sigma) and protease inhibitors (Sigma). The homogenates were centrifuged at 1000 × *g* at 4 °C for 10 min. The pellets were discarded and the supernatants were centrifuged at 17,000 × *g* at 4 °C for 20 min to get cytosolic and the crud mitochondrial fractions. The protein content was determined using the BioRad DC Protein Assay Kit.

Cytosolic fractions were separated by 0.1% sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. Immunoblotting was performed according to standard procedure with monoclonal anti-cytochrome *C* antibody purchased from BD Pharmingen and visualized using horseradish peroxides (HRP)-conjugated anti-mouse antibody and enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, UK). The membranes were stripped and the immunoreactions were done with anti β-actin, the cytosol marker protein to assess the gel loading and anti cytochrome oxidase subunit IV (Cox IV), the marker of mitochondria to verify the purity of the fractions.

2.4. Confocal microscopy

Cells grown on cover slips in 24-well plates were fixed with 4% paraformaldehyde diluted in PBS for 30 min. Unless indicated otherwise, washes with PBS were per-

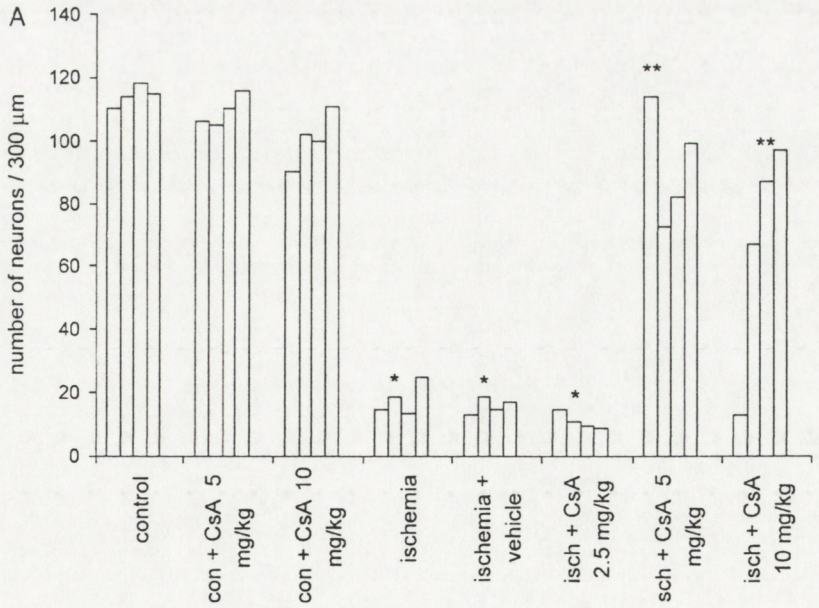

Fig. 1. Neuroprotective effect of CsA in global cerebral ischemia. (A) Histological outcome in the CA1 sector of the hippocampus after 5 min of ischemia followed by 7 days recovery. Quantification of survived neurons was performed as described in Section 2. Each bar represents one animal. $*p < 0.05$ vs. control, $**p < 0.05$ vs. ischemia. (B) Examples of histological images of coronal sections of CA1 of the hippocampus from control, ischemic and CsA-treated ischemic animals, 7 days after the insult. Sections were double stained with MAP2 and GFAP antibodies to show neurons and astrocytes. Note that, in hippocampus of CsA treated animals, even ischemia-injure regions (centre of the picture) contain reduced number of microglia (blue-stained small cells) and reactive astrocytes (magenta) commonly observed at unprotected ischemic outcome.

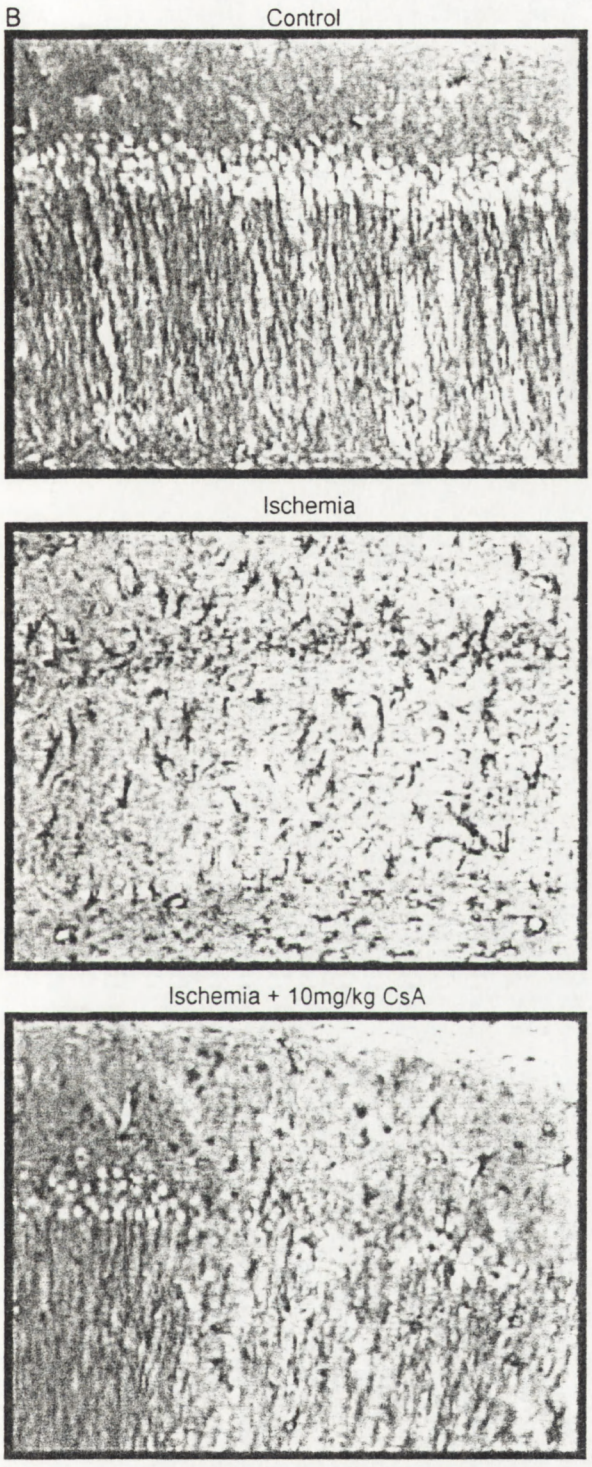

Fig. 1 (*continued*).

formed between each step and all primary and secondary antibodies were diluted in PBS containing 0.1% Triton X-100. To minimize non-specific binding of all secondary antibodies raised in goats and to enhance penetration of antisera, preincubation was performed for 20 min with PBS containing 1% Triton X-100. Then cells were blocked in PBS containing 10% goat serum and 0.1% Triton X-100 (1 h). In the first primary incubation, cells were incubated with mouse monoclonal antibody against cytochrome *C* (diluted 1:500, overnight at 4 °C, BD Pharmingen). This primary antibody was detected with a FITC-conjugated goat anti-mouse IgG1 (diluted 1:500, 1 h at room temperature, Southern Biotechnology Associates). The cytochrome *C*-labelled cells were incubated with a second mouse monoclonal antibody raised against β-tubulin III (diluted 1:500, overnight at 4 °C, Sigma), followed by Texas Red-conjugated goat anti-mouse IgG2b (diluted 1:500, 1 h at room temperature, Southern Biotechnology Associates). As a control for immunocytochemistry (in order to exclude non-specific background staining), first antibodies were omitted during procedure. After a final wash, cells on the cover slips were mounted in Fluoromount-G (Southern Biotechnology Associates).

To obtain detailed images of the cells, a confocal laser scanning microscope (Zeiss LSM 510) was used. An argon laser (488 nm) and helium-neon laser (543 nm) were utilized for the excitation of FITC and Texas Red, respectively. Following acquisition, images were processed using the Zeiss LSM 510 software package v. 2.8 and Corel Draw v. 9.0.

2.5. Statistics

Statistical significance of the differences between the results was computed using one-way ANOVA test followed by Dunnett's test, from GraphPad Prism 3.02.

3. Results

Gerbils subjected to 5 min forebrain ischemia followed by 7 days recovery under standard laboratory conditions displayed typical neuronal losses, restricted mainly to CA1 region of hippocampus. In control, the mean number of morphologically intact neurons per 300 μm length scored in CA1 was 114.25 ± 11.5. When sham-operated animals were treated with CsA in its effective doses (5 and 10 mg/kg), the average number of neurons in CA1 decreased insignificantly to 105 ± 7.9 per 300 μm length. This lowering can be linked rather with a side effect of surgical procedure than with a direct toxic effect of CsA on hippocampal neurons as it was independent on the applied drug concentration. In the group of four animals with 5 min ischemia and 7 days recovery, almost all neurons in CA1 disappeared, leaving in average only 18.25 ± 5 per 300 μm of intact neurons in CA1 area (equal to 14.9% of the untreated control; Fig. 1). Gerbils subjected to 5 min ischemia and treated with CsA immediately after recirculation were effectively protected against CA1 neuronal death. A single dose of 5 or 10 mg/kg body weight gave a similar protection with average survivals increasing up to 75% of cells found in CsA-treated, sham-operated animals. When drug injection was delayed up to 6

or 24 h, the protective effect of CsA completely disappeared (not shown). Also the animals receiving 2.5 mg/kg of CsA did not respond positively to the treatment (Fig. 1A).

The time-limited effectiveness of CsA for protection of neurons against apoptosis was verified using cell culture paradigm. The extend of cell death was determined after treatment with either glutamate (500 μM), H_20_2 (25 μM) or staurosporine (25 nM)—the well known inductors of apoptosis mirroring certain aspects of post-ischemic brain injury. Reactive oxygen species, excitotoxicity and inhibition of protein kinases signalling can facilitate MPT, seemingly by increasing pore sensitivity or intracellular calcium ions concentration [23]. Cells selected by morphological criteria as apoptotic possessed highly condensed nuclei, reacted strongly with Hoechst 33258 and were frequently fragmented to apoptotic bodies. In our experiments at 6 h post-treatment, 30–40% nuclei of the cells identified immunochemically with anti-β-tubulin III as neurons showed typical apoptotic

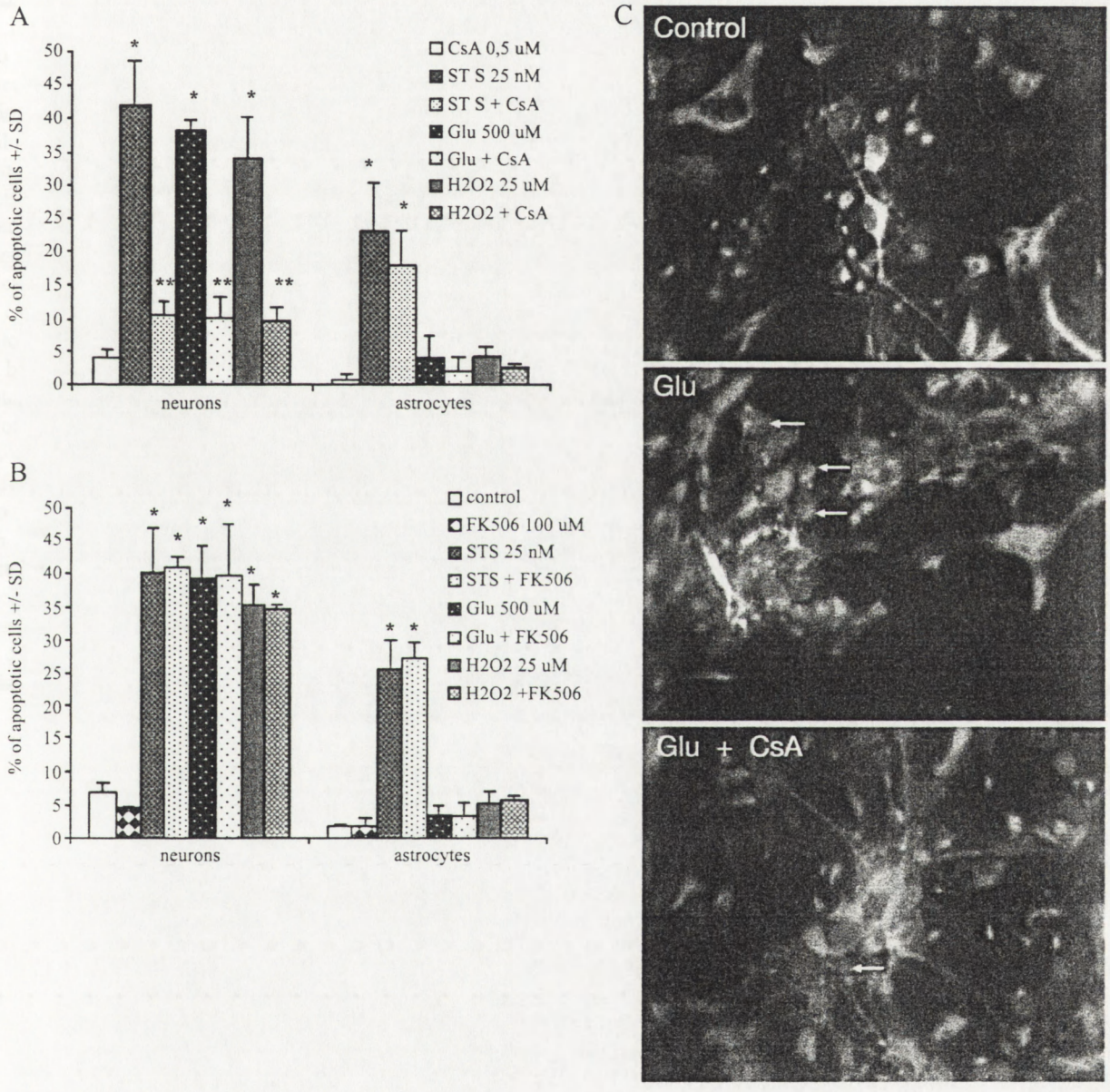

Fig. 2. Cyclosporin A but not FK506 protects neurons from apoptotic death in the primary culture of rat cortex. Cell death was induced by adding of 25 nM STS or 500 μM glutamate or 25 μM H_2O_2 to the culture medium for 6 h. CsA (0.5 μM) (A, C) or 0,5 μM FK506 (B) were added simultaneously with the apoptogens. Number of cells with nuclei displaying apoptotic feature and neurons or astrocytes morphology were evaluated after Hoechst 33258 staining and immunolabelling with Tuj1 and GFAP antibodies, respectively, as described in Section 2. (A, B) The results are expressed as the mean ± S.D. of cells number from five independent cultures. *$p < 0.05$ vs. control, **$p < 0.05$ vs. STS or glutamate or H_2O_2-treated cultures. (C) Culture neurons are stained with antibody against Tuj1/IgG FITC (green), astrocytes with anti GFAP/IgG-Cy3 (red) and nuclei with Hoechst 33258 (blue). Morphology of nuclei with typical apoptotic fragmentation in neurons are indicated by arrows (40× magnification).

characteristic. In contrast, astrocytes (immunoreactive with anti-GFAP antibody) were relatively resistant to injury except of week reaction to staurosporine treatment (Fig. 2).

Non-toxic doses of cyclosporin A were established in control cultures by growing cells by 24 h at different drug concentrations. Direct cytotoxicity toward the whole cell population was found in concentrations of 5 μM of CsA. In contrast, 10 times lower, 0.5 μM CsA applied concomitantly with the apoptogens protected cell cultures significantly by decreasing frequency of neuronal death from 30–40% to about 10%. The apoptotic reaction of astrocytes after staurosporine treatment was less sensitive to CsA protection (Fig. 2A).

To verify possible contribution of the alternative to MPT-connected mechanisms of CsA protection involving calcineurin inhibition, we tested influence of FK506 in the parallel experiments. However, neither in vivo (not shown) nor in vitro (Fig. 2B) we were able to notice any protection by this drug when applied before or shortly after ischemia or apoptogenic treatments in a dose of 2 mg/kg or 0.5 μM concentration, respectively.

Next we have verified the time-course of neuroprotection offered by CsA in vitro. The cultures treated with apoptogens were supplemented with cyclosporin A (0.5 μM) but with a delay of 0.5–3 h in respect to the initial, apoptosis-inducing treatments (Fig. 3). The ability of CsA to protect cells in vitro, similarly as previously observed in vivo, successively decreased with time and was non-significant after 3 h.

To confirm mitochondria-connected mechanism of cell death in vitro, we used immunocytochemical approach to detect possible cytochrome *C* appearance in cytoplasm of the cells treated with neurotoxic compounds and modulation of this translocation from mitochondria by CsA treatment. The disperse cytochrome *C* staining (red) in neurons (marked green by anti-β-tubulin III co-staining) were observed as early as 30 min after initiating treatments (Fig. 4). Cytoplasmatic immunoreactivity of released cytochrome *C* continued to increase up to the time when massive neuronal death had occurred. Moreover, simultaneous addition of CsA to the medium was able to block this reaction in majority of neurones. These cells, injured with selected apoptogens in the presence of CsA preserved, similarly as cells in control cultures, mitochondrial distribution of cytochrome *C* which appeared as fine and punctuate staining during the whole observation period.

Then, we decided to look precisely on time-course of cytochrome *C* appearance in cytosol fraction obtained from ischemia-injured hippocampus in vivo. Using the Western blot method, we found significant increase of cytosolic cytochrome *C* level at 30 min of reperfusion (Fig. 5). This early reaction disappeared at longer recovery time (1 and 3 h) then reoccurred again at 24–48 h, the time being expected for the cell-death-execution phase in this ischemic model [7].

Cyclosporin A applied in bolus inter-carotid injection at early reperfusion completely block cytochrome *C* translocation at 30 min after ischemia (Fig. 5B). Of course, application of the drug in similar experimental setting inhibited also the later phase of cytochrome *C* translocation in accordance with its previously observed effect on cell death inhibition (not shown).

4. Discussion

The following major novel observations are reported in the present study:

- Single inter-carotid injection of 5–10 mg/kg of cyclosporin A immediately after reperfusion in global cerebral ischemia can effectively protect neurons against delayed death in CA1 hippocampus.

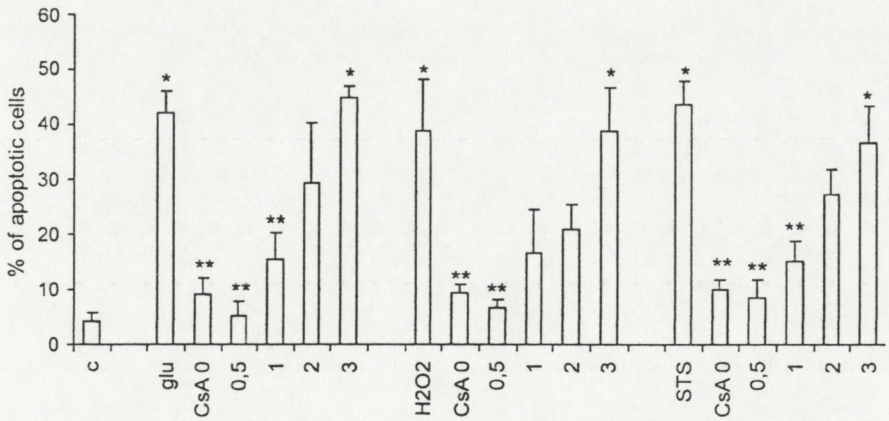

Fig. 3. Early CsA application protects neurons from apoptotic death in rat cortex primary culture. Cyclosporin A (0.5 μM) was added to the culture medium together with apoptogens (25 nM STS or 500 μM glutamate or 25 μM H_2O_2 mM) or with the delay of 0.5 or 1 or 2 or 3 h with respect to the time of the initiation of apoptosis. The number of cells with apoptotic nuclei were estimated in cultures stained with Hoechst 33258 and immunolabelled with Tuj1 and GFAP antibodies, as described in Section 2. The results are expressed as the mean ± S.D. of cell number from three independent cultures. *$p < 0.05$ vs. control, **$p < 0.05$ vs. STS or glutamate or H_2O_2-treated cultures.

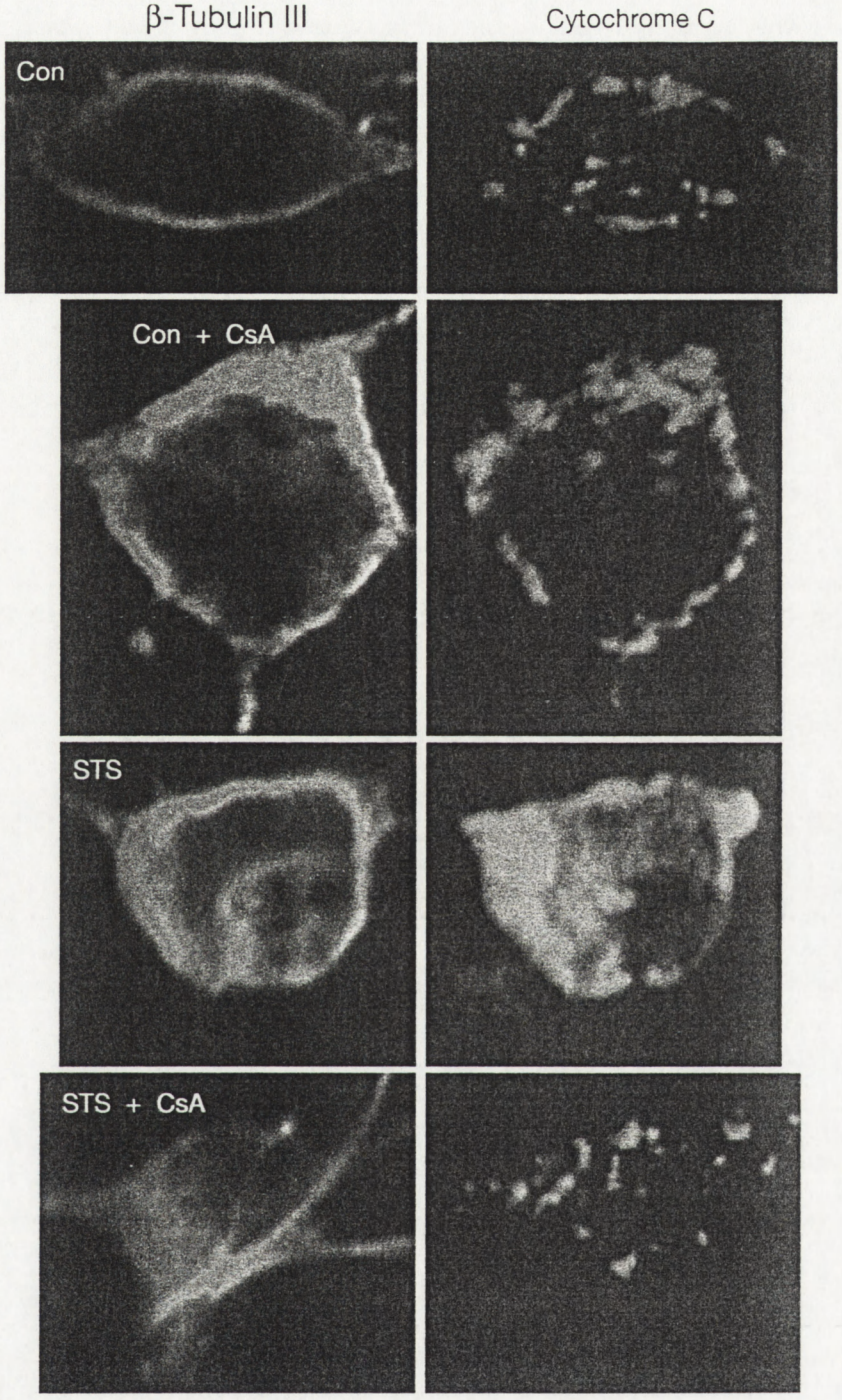

Fig. 4. Cyclosporin A inhibits early cytochrome *C* appearance in affected neurones in vitro. The representative confocal images of neurons from primary rat cortical cultures double immunostained for β-tubulin III (green FITC fluorescence, left column) and cytochrome *C* (red TXRD fluorescence, right column). Cells were cultured 7 days in condition described in Section 2. In control and control + CsA cultures cytochrome *C* localized in mitochondria is shown as a bright red dots and areas. In experimental cultures, cells were treated with 25 nM STS for 30 min. Cytosolic appearance of cytochrome *C* is observed as disperse and diffuse red fluorescence throughout the cell. Cells treated 30 min with 25 nM STS and 0.5 μM CsA look similar as controls with mitochondrially localized bright fluorescence. Magnification of each single cell was increased 2.5-fold with 60× objective using the zoom function of confocal software.

- Such an early CsA treatment can inhibit initial transient appearance of cytochrome *C* in cytoplasm, which in untreated animals become apparent at 30 min after ischemic insult.
- The time-limit of effectiveness of CsA to prevent lethal neuronal injury and early cytochrome *C* release were confirmed directly in primary cortical culture in vitro treated with staurosporine or glutamate or H_2O_2.

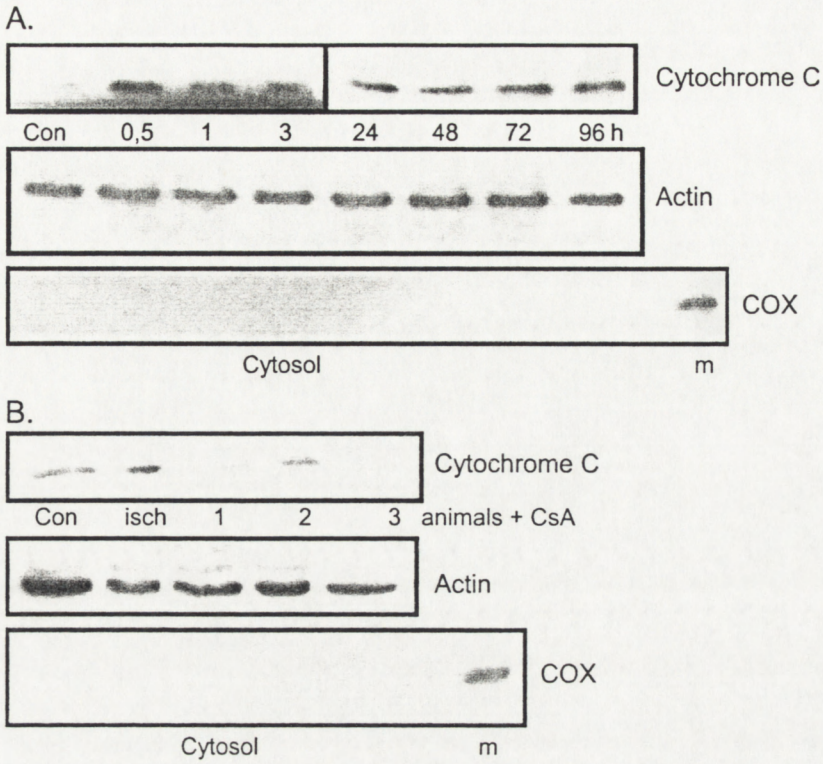

Fig. 5. Cyclosporin A inhibits early cytochrome *C* appearance in cytoplasm after transient cerebral ischemia in gerbils. (A) Western blot analysis of cytochrome *C* in cytosolic fraction (50 μg of protein) from hippocampi of control (con) and gerbils subjected to 5 min of ischemia followed by the reperfusion of 0.5, 1, 3, 24, 48, 72 and 94 h. (B) Western blot analysis of cytochrome *C* in cytosolic fraction (50 μg of protein) from hippocampi of control (con) and gerbils subjected to 5 min ischemia (isch) and ischemic animals treated with 10 mg/kg CsA immediately after the insult (1–3). All animals recovered 0.5 h. After treatment, the tissue was processed as described in Section 2. β-Actin and Cox IV immunoreactivities are shown to assess the gel loading and to verify the purity of cytosolic fraction, respectively. m—mitochondria.

Of note, FK506 given in recommended by other investigators effective doses of 2 mg/kg in vivo or 0.5 μM in vitro failed to give similar protection to neurons.

The above results support the hypothesis that the primary and decisive effect of CsA on neuronal fate consists in its ability to inhibit the early phase of MPT opening and cytochrome *C* release after ischemia. Nevertheless, the data do not allow to exclude completely additional routes of neuroprotection, which could be offered by CsA due to its immunosuppressive properties [15]. However, according to the data provided by the drug suppliers (Novartis and Fujisawa Ph.), cyclosporin A and FK506 should maintain their therapeutic levels at least by 24 h. Thus, this mechanism, which should be shared by the both tested compounds, is either of less importance or located downstream of MPT inhibition. Indeed, in gerbils, the classical neuroinflammatory response to ischemia initiates not earlier than 24 h after the insult [7]. On the other hand, the lack of protection in animals treated with CsA after 6 h or longer period of reperfusion seems to exclude the primary pathogenic importance of neuroinflammation and immunosuppression on neuronal survival in gerbil ischemic model. Nevertheless, neuronal calcineurin inhibition may participate in a variable degree to the other route of cyclosporin A protection in later period after ischemia by preventing the Bad protein dephosphorylation as suggested recently by Uchino et al. [24]. The phosphorylation-dependent sequestration and functional inactivation of this proapoptotic protein in cytoplasm is regulated at least by two phosphokinases, AKT/PKB (P-Ser 136) and Raf-1 (P-Ser 112) [2] as well as by phosphatase-calcineurin [26]. However, according to our data, the AKT and Raf-1 activity does not change significantly in/after ischemia in gerbils [30]. It is in contrast to observations in similar ischemic model in rats where increases [18] or decreases [24] of AKT phosphorylation has been reported. Moreover, according to available data, activation of calcineurin is relatively late post-ischemic event [24], which is not covered by the drug administration design here employed. Taking these facts into consideration, we can assume that Bad phosphorylation/dephosphorylation balance is not of primary importance in death/survival decision that is taken in the first hours of recovery and effectively blocked by CsA in gerbils.

Addressing to the positive results showing partial effectiveness of FK 506 in protection of neurones after ischemia [24] or axonal transsection [10] also the other mechanisms than calcineurin inhibition can be proposed. It has been reported that a new synthetic FKBP (12 or 52) ligands, which in contrast to FK506 do not inhibit calcineurin phosphatase activity, also display neuroprotective properties

[10]. It was suggested that this FK506 effect can be achieved simple by stabilizing the FKBPs to degradation by the classical mechanism involving enzymatic substrate protection. This property would not be shared by CsA, the ligand interacting with cyclophilins (A and D) but not with FKBP. Neither our results nor these reported by others [27] revealed any significant effect of FK506 given in the single dose directly after ischemia, being the window of opportunity for CsA treatment. Summarizing, after global cerebral ischemia in gerbils contribution of mechanism(s) triggered by FK 506 may be either insignificant or combinatorial [21] and/or down-stream to the protection offered exclusively by CsA on the early MPT blockade.

The above mechanistic assumptions were successfully verified in in vitro experiments. CsA was effective in blocking cells death in a time-limited manner. In primary cortical cells subjected to proapoptotic conditions the CsA at 0.5 μM concentration, which is likely to occur in brain parenchyma in vivo [24], effectively prevented cytochrome *C* release and neuronal death (Fig. 4). Our supposition and experimental data are in agreement with MPT inhibition by CsA in isolated brain mitochondria [11]. The half-maximal dose of CsA was estimated in vitro at approximately 23 nM.

High consistence of the results on the time-course of CsA protection obtained in vivo and in vitro seems to rule out the role of drug bio-availability to brain tissue in the in vivo model. Recent data strongly suggest that pharmacokinetic parameters after intra-carotid injection of CsA do not differ significantly between normal, ischemic and reperfused rat brain: all these conditions resulted in a high level of drug penetration into brain tissue with a brain–blood ratio from 0.5 to 1 [25].

Other routes of CsA delivery appear to be less reproducible and effective, which may explain variable neuroprotective effects of the drug obtained in the different models. For example, Yoshimoto and Siesjö [29] did not observe protection following intravenous administration of CsA (10 mg/kg), unless the BBB was first disrupted by needle perforation. Higher intravenous doses (50 mg/kg) tested by these authors were associated with increased systemic toxicity [29]. In contrast, Friberg et al. [8] reported a good penetration of CsA after i.v. injection without needle insertion with blood/brain ratio about 0.3. However, more recently the same group reported ineffectiveness of i.v. CsA administration in an experimental stroke model, while considerable protection with intra-peritoneal drug delivery was achieved [16]. Additionally, Matsumoto et al. [16] found relatively narrow concentration range (at about 20 mg/kg) of the therapeutic window with increased toxicity at higher CsA doses. Thus, we can agree with the authors who insist on a very strict and precise requirement for further use of CsA as a therapeutic agent.

The mechanism(s) of CsA transport to brain is not clear. There are doubts if P-glycoprotein, a secretory transporter located on brain capillary endothelium, is indeed responsible for the reported variability of CsA penetration. For example, when verapamil, a competitive P-glycoprotein function inhibitor, was injected in mice concomitantly with CsA, no significant increase of CsA concentration could be detected in brain [22]. Other possible mechanisms of limited CsA availability include a barrier imposed by tight junctions and physicochemical properties of CsA itself. Newly synthesized CsA analogues that would readily penetrate the BBB are urgently needed since inconsistent bioavailability and potential toxicity of CsA delay its promising therapeutic application. The available clinical data are rare and rather incidental, however positive. A successful CsA trial on patients anaesthetized for early clipping of a aneurysm and treated with CsA at 12 h before operation and then for the following 14 days was reported [19]; neurological outcome in all patients treated with cyclosporin A was significantly better than in patients without treatment.

In line with the majority of recent reports, our finding supports the hypothesis that the neuroprotective effect of CsA is mediated mainly through inhibition of the mitochondrial permeability transition. Here, we demonstrated that post-ischemic MPT could rapidly and transiently induce cytochrome *C* release from mitochondria. This would explain the already reported early activation of two proteolytic systems, i.e., caspases and calpains in the model of hyperglycaemic rats subjected to ischemia [14], as well as in rat and gerbil subjected to forebrain transient ischemia [17,31]. The role of activation of these two proteases by cytosolic cytochrome *C* is well documented in an in vitro model of neuronal apoptosis [1]. Furthermore, treatment with calpain inhibitor is able to block cells death in gerbil ischemic model in vivo [13,28].

5. Conclusions

Our results confirm the therapeutic potential of CsA in global cerebral ischemia after its bolus intra-carotid injection in in vivo as well as in several models of neuronal apoptosis in vitro. The underlying mechanism would consist of the inhibition of early cytochrome *C* efflux to cytoplasm.

Acknowledgements

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Research report

Transient cerebral ischemia induces delayed proapoptotic Bad translocation to mitochondria in CA1 sector of hippocampus

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Abstract

Delayed ischemic brain damage is associated with mitochondrial dysfunction, but the underlying mechanisms are not known in detail. Recent data suggest that the process is associated with multidirectional changes in the activities of various proteins located in mitochondria. Of these, the stress-activated kinase JNK is delay-activated postischemia. We induced 5 min cerebral ischemia in gerbils followed by 3, 24, 48, 72 and 96 h of reperfusion. Here we show the postischemic translocation of proapoptotic protein Bad to mitochondria. Immunoelectron microscopic examination revealed the co-appearance of Bad and Bcl-2 proteins in postischemic mitochondria in ischemia-vulnerable CA1 sector of hippocampus as opposed to the ischemia-resistant DG region. Mitochondrial increase of Bad protein is coincident with a transient decrease of the active, phosphorylated form of prosurvival kinase, Raf-1, under conditions of long reperfusion. The above demonstrated sequence of events is likely to play a role in delayed postischemic nerve cell death.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Brain ischemia; Neurodegeneration; Mitochondria; Bad translocation; Raf-1 kinase

1. Introduction

Various experimental models have been used to study the mechanisms underlying the delayed neuronal cell death after transient brain ischemia. Research data accumulated during the last decade extended the list of data pointing to disturbed mitochondrial function as a key player in the pathogenesis of postischemic neurodegeneration [6,15,23]. In this pathology, permeabilization of mitochondrial membranes is a critical event that results in release (from inter-membrane space) of various molecules that are crucial for apoptosis. Recently, it was shown that ischemic insult induces early cytosolic translocation of cytochrome *c* from mitochondria and activation of caspases [8,11,23]. Concomitant activation of calpains and protein kinases were observed in various models of ischemia [4,8,24,33], leading to delayed death of vulnerable and to survival of resistant neurons. Recently, we showed delayed, postischemic accumulation of phosphorylated stress-activated kinase (P-JNK) in mitochondria [31]. This event may contribute to activation of terminal effectors of apoptosis, that is, proteins belonging to the Bcl-2 family which take part in the controlling of the integrity of the outer mitochondrial membrane [1,3].

There are experimental data showing that Raf-1 kinase maintains cell survival by at least two separate pathways: one connected with MAP kinase cascade in the cytoplasm, second, which become apparent when the kinase is associated with mitochondria [7,32]. In mitochondria, Raf-1 can suppress apoptosis in vitro [2], inactivate proapoptotic

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Bad protein [18] or negatively regulate VDAC channels [10]. Interestingly, P-Raf-1 when targeted to mitochondrial membranes via the connection with Bcl-2 protein [28] can serve as a heterodimeric partner for Bad, increasing the probability to release this protein out of mitochondria and thus to inactivate its proapoptotic action [28,29].

In this study, we have aimed to evaluate the effect of ischemia on the amount of mitochondrially located proapoptotic Bad protein and prosurvival Raf-1 kinase. We show a sustained elevation of mitochondrially localized Bad protein. The amount of Bad and Bcl2 proteins revealed by immunocytochemical electron microscopy study is increased in ischemia-vulnerable CA1 region of hippocampus. The amount of active Raf-1 kinase is transiently decreased in the mitochondrial fraction. We conclude that the delayed postischemic elevation in the amount of mitochondrially located Bad and the decrease in P-Raf-1 kinase might be the additional causative factors of delayed ischemia-induced metabolism, leading to neurons degradation.

2. Materials and methods

2.1. Ischemic model

Male Mongolian gerbils, weighing 50–70 g, were used to perform brain ischemia by 5 min bilateral ligation of the common carotid arteries under halotan/N_2O anesthesia in strictly controlled normothermic conditions as previously described [30]. Animals after ischemia were allowed to recover up to 4 days. Sham-operated animals served as controls. The experimental procedure was approved by the Local Commission for Ethics of Experiments on Animals.

2.2. Cell fractions preparation

Mitochondria were obtained from hippocampi of control and ischemic animals after 3, 24, 48, 72 and 96 h of reperfusion.

Tissue samples for Western blot analysis of cytosolic fraction and mitochondria were obtained by decapitation under anesthesia. The hippocampi were immediately isolated and homogenized 10–12 strokes in Dounce homogenizer in 10 volumes of ice-cold buffer as described previously [31]. The homogenization buffer contained 15 mM Tris–HCl, pH 7.6, 0.25 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT and the mixture of phosphatase and protease inhibitors (Sigma). The homogenates were centrifuged at 1000×*g* at 4 °C for 10 min. The pellets were discarded and the supernatants were centrifuged at 17,000×*g* at 4 °C for 20 min to get cytosolic and the crud mitochondrial fractions. The pellets were further diluted with homogenization buffer and mixed with 4 volumes of 12% Ficoll in Krebs–Ringer buffer prior to centrifugation 99,000×*g* at 4 °C for 30 min. The pellets containing mitochondria were collected in homogenization buffer.

The protein content was determined using the BioRad DC Protein Assay Kit.

2.3. SDS-PAGE

Mitochondrial fractions (30 μg) were loaded on 10% gels for P-Raf-1 kinase and on 12% polyacrylamide gels to look for Bad protein. The following antibodies were used for Western blots: anti-P-Raf-1 and anti-Bad from Santa Cruz Biotechnology (cat. no. sc-227, sc-942, respectively). The accuracy of gel loading was evaluated by Western blots with anti-HSP-60 (Sigma) or cytochrome oxidase subunit IV (Cox IV) (Molecular Probes) for mitochondrial preparation. A contamination of mitochondria with the synaptic membranes was checked with anti-dystrofin antibody (Novo Castra cat. no. NCL-DYS2), which recognized Dp 71 form of postsynaptic density-specific dystrofin. For visualization, the ECL from Amersham was used.

Soluble, cytosolic fractions (50 μg) were loaded on 12% polyacrylamide gels to look for Bad and P-Bad proteins. Western blots were done with the following antibodies: anti-Bad from Santa Cruz Biotechnology (cat. no. sc-942) and anti-P-Bad which recognize phosphorylated ser 112 from New England BioLabs (cat. no. 9291). The accuracy of gel loading was evaluated by Western blots with anti β-actin antibody (Sigma).

2.4. Immunocytochemical electron microscopy studies

Before removal, the control and postischemic brains were fixed by a transcardial perfusion with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PBS), followed by a brief rinse with 0.9% saline. Blocks of tissue were cut from stratum pyramidale of the CA1 and dentate gyrus (DG) sectors of hippocampus, washed overnight in PBS, treated with 1% osmium tetroxide for 1 h, dehydrated in the gradient of ethanol, and finally embedded in Epon. Ultrathin sections were treated according to post-embedding immunogold procedures. The primary polyclonal antibodies to Bcl-2 and Bad (Santa Cruz Biotechnology sc-783; sc-942-G) were diluted in PBS 1:10. After pretreatment with 10% hydrogen peroxide, the antisera were applied to the sections mounted on the formvar-coated nickel grids and incubated for 2 h. After washing, the sections were exposed for 30 min to secondary antibody coupled with 12 nm (Bcl-2) and 20 nm (Bad) gold particles, diluted 1:20 in PBS (Janssen, Beerse, Belgium). The control staining was performed, where primary antibody was replaced by normal rat serum diluted 1:20 in PBS. Sections were stained with 4.7% uranyl acetate for 15 min and with lead citrate for 2 min. The sections were examined and photographed in a JEOL 1200EX electron microscope.

2.5. Statistics

Statistical significance of the differences between the results was computed using one-way ANOVA test followed by Dunnett's test, from GraphPad Prism 3.02.

3. Results

3.1. P-Raf-1 detached from the mitochondrial fraction

Mitochondria isolated from hippocampus during postischemic recovery were evidently depleted of P-Raf-1 protein. The time points examined (3, 24, 48, 72 and 96 h, n=4–5 animals per time point) were chosen on the basis of our previous observation that application of CsA later than an hour after ischemia in vivo or 2 h after proapoptotic stimulation in vitro failed to protect neurons against death [5]. Decreased amount of P-Raf-1 kinase connected with mitochondria was seen at 24 and especially 48 h after ischemia as compared either with a non-ischemic or with a 3-h postischemic sample. Representative immunoblot, normalized to total proteins as well as to a mitochondria matrix marker, HSP-60, is shown in Fig. 1.

3.2. Bad protein binds to postischemic mitochondrial fraction

In the time course of recovery, mitochondrial fraction was enriched in Bad protein (Fig. 2A) tested in the same samples as were analyzed for the P-Raf-1. The control band was very weak, and the elevated amount of Bad was seen 24 h after ischemia and was sustained throughout the duration of the experiment. At 3 h of reperfusion, mitochondrial Bad content displayed control value, possibly reflecting control level of P-Raf-1 kinase. We did not find phosphorylated Bad in the mitochondrial fraction. Neither did the antibody recognizing P-ser 112, which is thought to be phosphorylated by Raf-1 kinase nor the antibody against P-ser 136 probably phosphorylated by Akt kinase, gave positive bands on Western blots. The amount of Bad and P-Bad find that cytosolic fraction was not markedly decreased after ischemia (Fig. 2B), in parallel with its increase in mitochondria. We explain this observation as being the result of (i) the use of samples of whole hippocampi for Western blots, a procedure chosen to

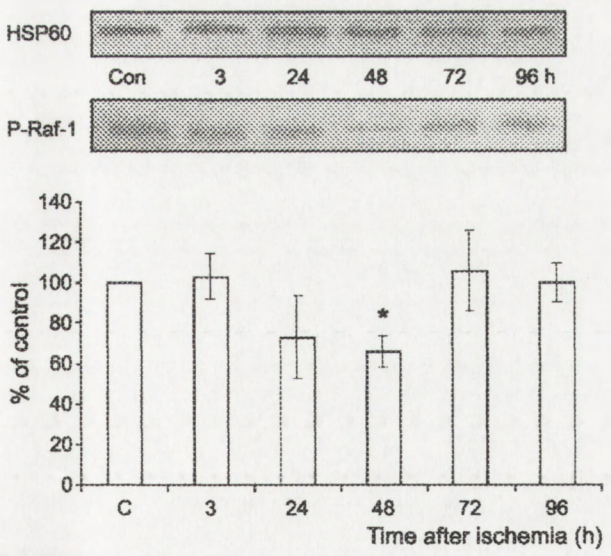

Fig. 1. Postischemic changes in the amount of P-Raf-1 kinase associated with mitochondria obtained from hippocampi of sham-operated controls (C) or gerbils subjected to 5 min ischemia followed by 3, 24, 48, 72 and 96 h of reperfusion. The mitochondrial fractions (30 μg) were immunoblotted with anti-P-Raf-1 and anti-HSP60 to assess the gel loading. Densities of the P-Raf-1 bands were evaluated and data expressed as a percentage of control (mean±S.D., n=4). *p<0.05 vs. control.

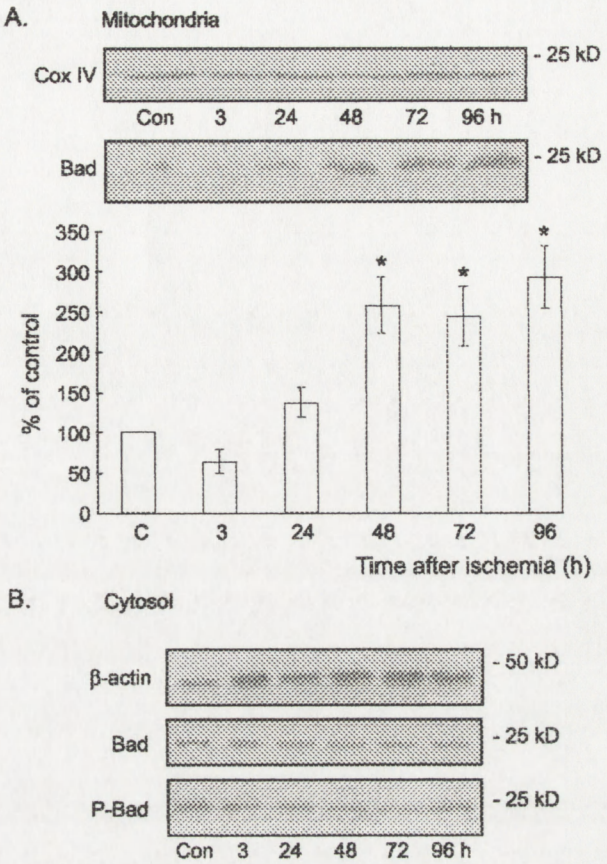

Fig. 2. Representative immunoblots demonstrate that transient brain ischemia results in the translocation of Bad to mitochondria. (A) Postischemic changes in the amount of Bad protein associated with mitochondria obtained from hippocampi of sham-operated controls (C) or gerbils subjected to 5 min ischemia followed by 3, 24, 48, 72 and 96 h of reperfusion. The mitochondrial fractions (30 μg) were immunoblotted with anti-Bad and anti-Cox IV antibodies to assess the gel loading. Densities of the bands were evaluated and expressed as a percentage of control (mean±S.D., n=4). *p<0.05 vs. control. (B) Transient brain ischemia causes slight decrease in the amount of cytosolic Bad and P-Bad. The soluble fractions (50 μg) obtained from whole hippocampi were immunoblotted with anti-Bad, anti-P-Bad (ser 112) and anti-β-actin antibodies to assess the gel loading.

maximally reduce the time of preparation of mitochondria; (ii) a low Bad content in control mitochondria, which in conjunction with the abundance of Bad in the soluble fraction renders the difference in the cytosol between control and 48–96 times statistically insignificant.

The translocation of Bad protein to mitochondria has been further documented by EM immunolocalization of Bad protein in ischemia-vulnerable (CA1) and ischemia-resistant dentate gyrus (DG) sectors separately.

3.3. Bad and Bcl-2 coexist in postischemic mitochondria

The brain sections were double-labeled with anti-Bad and anti-Bcl-2 antibody and visualized with gold particles

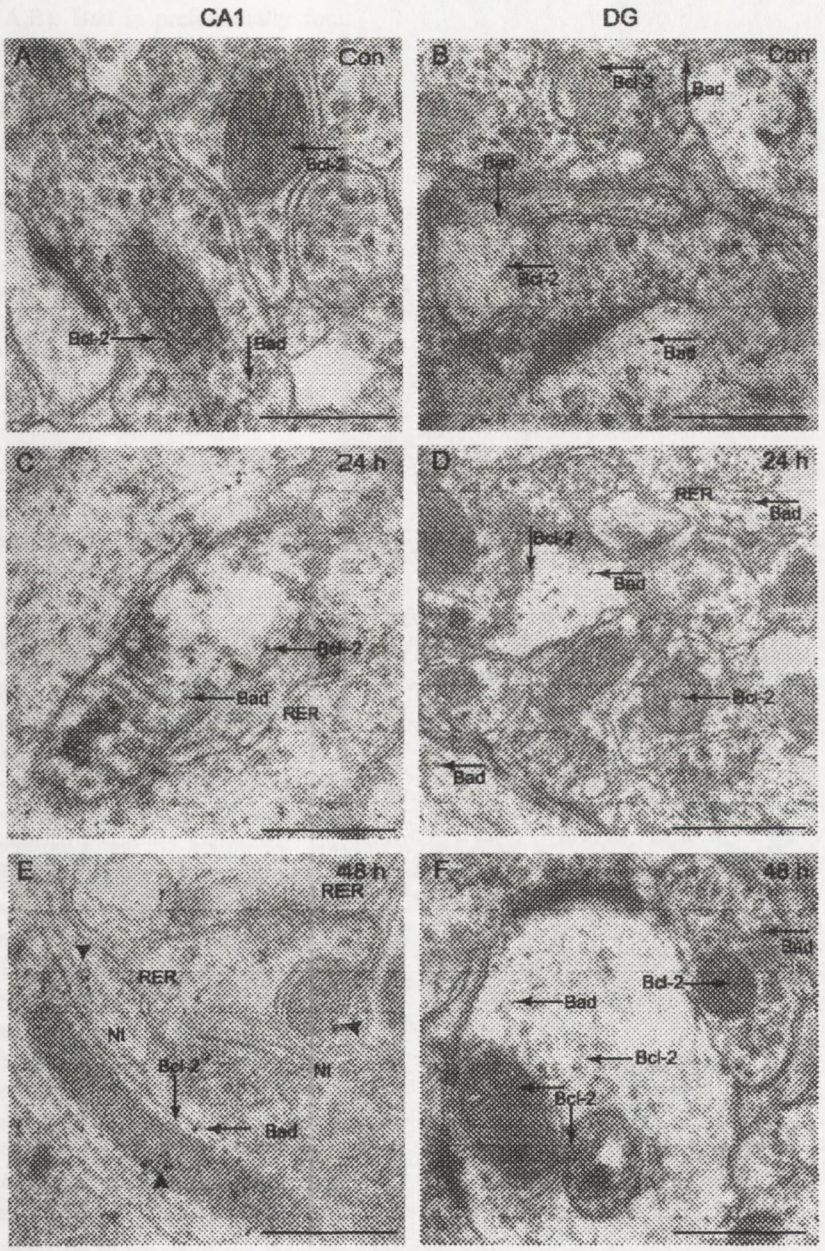

Fig. 3. Electron micrographs of CA1 (left column) and *dentate gyrus* DG (right column) regions of hippocampus from control gerbil (Con) and animals subjected to 5 min ischemia followed by 24 and 48 h of reperfusion. Arrows show immunosignal for Bad and Bcl-2 represented by colloidal gold particles of 20 and 12 nm, respectively. Bar=500 nm. In CA1 and DG, in neuropil of control gerbil the immunosignals for Bcl-2 are located mainly on mitochondria, whereas immunosignals for Bad are located on synaptic vesicles or in synaptoplasm (A, B). In comparison to control slices, in postischemic CA1 regions, the colocalization of Bad and Bcl-2 is observed (C, E). Small aggregates of gold particles (arrowheads) connected with Bad (20 nm) and Bcl-2 (12 nm) are located mostly on mitochondria or in close proximity. They are also associated with rough endoplasmic reticulum (RER) and with neurotubules (Nt). Note the colocalization of Bad and Bcl-2 in injured mitochondria at 24 h after ischemia observed in the region undergoing delayed degeneration (C). In contrast, in ischemia-resistant DG, there are no changes in the morphology of mitochondria and in the cellular distribution of Bad and Bcl-2 at 24 and 48 h after the insult (D, F).

of 20 and 12 nm diameters, respectively. Fig. 3 shows the representative micrographs from CA1 (left panel) and DG (right panel) regions of hippocampus, illustrating cellular localization of Bad and Bcl-2. At the ultrastructure level, in control, in both brain regions, analyzed proteins are found in synaptic terminals and in neurons body, at endoplasmic reticulum and on synaptic vesicles (Fig. 3A,B). Bcl-2 is often found in the connection with mitochondria (Fig. 3A,B). Bad is preferentially found in cytoplasm. Ischemia causes evident translocation of Bad protein to changed mitochondria observed in CA1 region. The dense Bad labeling in mitochondria is seen after 24 and 48 h of reperfusion (Fig. 3C,E). Additionally, some mitochondria, especially in the cell soma, were swollen and the outer membrane was disrupted (Fig. 3C). In contrast, transient ischemic insult did not influence the integrity of mitochondria in DG, as it is shown in Fig 3D and F. Moreover, there were no changes in the localization of Bad and Bcl-2 proteins in the observed substructures.

4. Discussion

Transient brain ischemia causes delayed neuronal death in CA1 region of hippocampus. Brief ischemia/reperfusion (IR) insult induces changes in the signal transduction pathways which lead to the degeneration of vulnerable cells [8,24]. Mitochondria play a crucial role in the regulation of apoptotic proteolysis and the intact mitochondrial membranes are necessary to maintain the cell viability. There is a complex mechanism preventing permeabilization of the outer mitochondrial membrane to the proapoptotic proteins: cytochrome *c*, Smac/DIABLO and AIF. Release of cytochrome *c* to cytoplasm causes activation of caspases cascade in the so-called execution phase of apoptosis. Ischemic insult induces also the early efflux of cytochrome *c* from mitochondria at the very beginning of reperfusion [5]. It is suggested that mitochondrial permeability transition (MPT) is responsible for this efflux. MPT is regulated by Bcl-2 family proteins. The proapoptotic Bad is a heterodimeric partner for Bcl-xl and Bcl-2 [29] and its phosphorylation regulates its availability for heterodimerization with Bcl-xl and Bcl-2 on the mitochondrial membranes [25]. Bad and other BH3-only proteins influence the antiapoptotic properties of Bcl-2 and Bcl-xl proteins. Here we show significant postischemic elevation in the mitochondrial content of Bad protein in ischemia-vulnerable CA1 sector of hippocampus with a parallel slight decrease in the cytosolic content of its phosphorylated form. Transient brain ischemia induces Bad translocation to mitochondria as shown by Western blot of mitochondrial fraction and by double immunolabeling procedure followed by EM study in the fields of ischemia-vulnerable (CA1) and ischemia-resistant (DG) separately in the control and postischemic animals. Parallel occurrence of proapoptotic Bad and antiapoptotic Bcl-2 was found on postischemic mitochondria in CA1 region of hippocampus, as opposed to control samples where these proteins were mostly localized separately. Mitochondria in both cell soma and in the synaptic area were labelled with anti-Bad and anti-Bcl2 antibody (Fig. 3C,E). Electron microscopic observations revealed also the presence of ultrastructurally changed mitochondria in the CA1 sector. Our data appear to demonstrate a significant aspect of the mechanism underlying neuronal cell death after transient ischemia. In contrast, in ischemia-resistant *dentate gyrus* region, ischemic insult did not influence the pattern of Bad and Bcl-2 localization. The mitochondria have well-preserved structure and are labelled only with anti-Bcl-2 antibody.

Bad dephosphorylation by calcineurin has been reported to correlate with cell death in hippocampi of rats subjected to transient global ischemia [26] and after traumatic spinal cord injury [21]. In our model of transient brain ischemia, it seems that calcineurin-mediated Bad dephosphorylation might play a role in the delayed amplification of ischemic signal [5], in accordance with the here observed elevation of this protein in mitochondrial compartment after longtime reperfusion. In this case, it cannot be counterbalanced by immediate re-phosphorylation because of the concomitant reduction of Raf-1 kinase in mitochondria, as shown here. In the previous study [31], we documented the absence of significant postischemic changes in the total level of active Akt and P-Raf-1 kinases in the ischemia-vulnerable (CA1), and ischemia-resistant (CA2, CA3, DG) parts of hippocampus. Based on this observation, we suggest that the intracellular localization of active Raf-1 kinase is of importance for the cell fate. Here we show that in postischemic mitochondria, P-Raf-1 content is transiently decreased. This might promote stabilization of dephosphorylated Bad in postischemic mitochondria. In vitro experiments have shown that Bad can be phosphorylated by Akt and/or Raf-1 kinases [9,20]. It is also suggested that Akt promotes survival by inducing the activity of mitochondrial Raf-1 in a Ras-independent manner [13]. Raf-1 can phosphorylate Bad, which upon phosphorylation is sequestrated in the cytosol in a complex with 14-3-3 protein [19,28]. Moreover, Raf-1 kinase is found in the complex with 14-3-3 [22]. Raf-1/14-3-3 complexes in cytoplasm might be regulated by external signals. The insulin-like growth factor receptor type 1 reacts with 14-3-3 which results in Raf-1 activation and translocation to mitochondria to protect a cell against proapoptotic agents [17]. There is another indication from the experiments in vitro that Raf-1 targeted to mitochondria protects N2a cells against apoptosis induced by the inhibition of PKC [2]. The protective effect of Raf -1 was confirmed by the finding of negative regulation of mitochondrial VDAC channels by this kinase [10]. In mitochondria, Raf-1 is found in protein complexes with Bcl-2 [28] as well as with other protein adaptors (Grb10, BAG-1) [14,27], reflecting a variety of pathways and stimuli which might be involved in its action. Here we

show a postischemic increase of Bad, and transient decrease of Raf-1 kinase proteins content in mitochondria, and the two phenomena are presumably of importance for the nerve cell fate. Additionally, our observations correlate with the increased activity of calcineurin in cytoplasm after the ischemic insult [16]. Such relatively late (>24 h) postischemic changes can be suggested as a possible target for the reported neuroprotective action of calcineurin inhibitors [12,26].

It should be pointed out that this study addresses the delayed reaction of ischemia-vulnerable neurons to the transient ischemic insult, when the execution phase of cell death occurs. We believe that the description of late postischemic events might be helpful in understanding the whole process and in finding new targets and tools to prevent or diminish postischemic degeneration.

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Komentarz

Wstęp

Udar mózgu występuje u 1 na 250 osób i jest trzecią w krajach rozwiniętych a czwartą w Polsce przyczyną zgonów. Szansa wystąpienia udaru rośnie wraz z wiekiem i u osób po 65 roku życia dotyka około 5% tej grupy wiekowej. Już kilkuminutowe przerwanie krążenia krwi powoduje uszkodzenia mózgu wymagające rehabilitacji zmierzającej do przywrócenia choć części utraconych funkcji. Warunkiem znalezienia skutecznych metod zapobiegania występowaniu i rozwijaniu się poniedokrwiennej śmierci neuronów jest badanie mechanizmów tego procesu.

Całkowite i uogólnione niedokrwienie mózgu wywołuje nagłe załamanie homeostazy tkanki oraz charakterystyczny zespół ciężkich zaburzeń neurologicznych. W zależności od czasu trwania niedokrwienia jak i towarzyszących mu zaburzeń systemowych, ostre zmiany metaboliczne mogą cofnąć się całkowicie lub spowodować szybką śmierć organizmu. Pomiędzy tymi skrajnymi reakcjami na niedokrwienie znajduje się całe spektrum trwałych uszkodzeń strukturalno - funkcjonalnych, znanych pod nazwą zespołu encefalopatii poniedokrwiennej. Bezpośrednie skutki ostrego niedoboru tlenu i glukozy występujące w czasie zatrzymania krążenia mózgowego, prowadzą w ciągu kilkunastu minut do ustania funkcji mózgu, nieodwracalnych zmian strukturalnych i śmierci organizmu. Wydaje się, że mechanizm powstawania opóźnionych uszkodzeń poniedokrwiennych, rozwijających się selektywnie tylko w niektórych, wyjątkowo wrażliwych strukturach mózgu takich jak hipokamp, jest wysoce specyficzny i znacznie odbiegający od tego, co obserwuje się w ostrej fazie patologii niedokrwiennej. Uszkodzenia te powstają prawdopodobnie w wyniku rozwoju określonej sekwencji wtórnych zaburzeń biochemicznych i molekularnych indukowanych stresem niedokrwiennym. Nasze ostatnie badania jak i liczne doniesienia z piśmiennictwa wskazują, że szczególnie istotne w przekazywaniu sygnału prowadzącego do opóźnionej śmierci neuronów w ischemii mózgu są dynamicznie zmieniające się kompleksy białkowe w

gęstościach postsynaptycznych (PSD) i w mitochondriach. Translokacje białek sygnałowych do wyżej wspomnianych struktur prowadzą do i) rozprzestrzeniania bodźca w komórce, co może skutkować jej przeżyciem lub eliminacją, oraz ii) do końcowych etapów śmierci neuronów we wrażliwych na krótkie niedokrwienie rejonach mózgu, którym towarzyszy wtórne załamanie energetyczne (Siesjo i wsp., 1999) oraz aktywacja szlaków sygnałowych charakterystycznych dla programu apoptozy (Brenner i Kroemer, 2000).

Mechanizm(y) i związki pomiędzy zdarzeniami, indukującymi bądź towarzyszącymi poischemicznej, opóźnionej śmierci neuronów, nie są w pełni poznane i stanowiły bezpośredni cel podejmowanych przeze mnie badań. Nadrzędnym celem prowadzonych prac jest stworzenie uporządkowanego opisu przekazywania sygnału w niedokrwionych komórkach, który może pomóc w znalezieniu nowych strategii leczniczych przeciwdziałających uszkodzeniom neuronów w ischemii mózgu.

Model badawczy

Przerwanie dopływu krwi do mózgu powoduje poważne zaburzenia metaboliczne, obserwowane w trakcie niedokrwienia i po przywróceniu krążenia. W modelu przejściowego, 5 minutowego niedokrwienia mózgu gerbila mongolskiego (*Meriones unguiculatus*) morfologiczne oznaki ubytku neuronów obserwowane są w sektorze CA1 hipokampów po 2-3 dniach reperfuzji (Gadamski i Mossakowski, 1992; Kirino, 1982). Podobny skutek czasowego niedokrwienia mózgu obserwuje się również w innych modelach ischemii u szczura (Krajewski i wsp., 1995; Pahlmark i wsp., 1993). Zanik neuronów we wrażliwych rejonach mózgu ma wiele cech charakterystycznych dla programowanej śmierci komórek – apoptozy (Domańska-Janik i wsp. 1999). Zarówno w modelu ischemii u szczura jak i u gerbila, po upływie 7 dni od niedokrwienia większość (ponad 80 %) neuronów wrażliwej

okolicy CA1 hipokampa ginie, a złożony, wieloczynnikowy proces prowadzący do śmierci opisywany jest jako opóźniona, selektywna poniedokrwienna śmierć neuronów (Wieloch i Kamme, 1998).

Sygnał ischemiczny

Już w trakcie pierwszych minut trwania ischemii obserwowane jest gwałtowne zahamowanie aktywności elektrycznej mózgu (Williams i Grossman, 1970) i postępujące wyczerpywanie się związków wysokoenergetycznych (Domańska-Janik i wsp., 1985; Levy i Kelly, 1997). Dochodzi do spadku potencjału błonowego mitochondriów oraz uwalniania białek pro-apoptotycznych. Zaburzona zostaje homeostaza jonowa zarówno w komórkach nerwowych jak i glejowych. Jednocześnie obserwowany jest nadmierny w stosunku do fizjologicznego wyrzut do przestrzeni międzykomórkowych pobudzających aminokwasów, głównie kwasu glutaminowego (Obrenovitch i Richards, 1995). Kwas glutaminowy uwalniany jest z pęcherzyków synaptycznych z zakończeń nerwowych oraz z tzw. puli metabolicznej aminokwasów. Komórki glejowe, na skutek wyczerpania się związków wysokoenergetycznych i odwrócenia działania transporterów aminokwasów (Attwell i wsp., 1993; Phillis i O'Regan, 2003) stają się dodatkowym źródłem m.in. pobudzających neurotransmiterów i zwiększają tym samym pobudzenie komórek nerwowych. Aktywacja receptorów kwasu glutaminowego i związany z tym znaczny wzrost stężenia jonów wapnia w cytoplaźmie neuronów powodują aktywację wielu enzymów regulowanych jonami wapnia (Zipfel i wsp., 2000), co w konsekwencji prowadzi do uruchomienia szlaków sygnałowych prowadzących do przezwyciężenia patologicznego bodźca lub do eliminacji komórek w rejonach szczególnie wrażliwych. Jeśli niedokrwienie trwa stosunkowo krótko (do około 15 min) wówczas po przywróceniu krążenia następuje odbudowa ATP, jednak niewydolny

łańcuch oddechowy jest źródłem wolnych rodników, które utleniają białka, lipidy i kwasy nukleinowe przyczyniając się do wtórnego uszkodzenia komórek.

PSD i białka przekazujące sygnał wapniowy

Receptory neurotransmiterów umieszczone w błonie postsynaptycznej skupiają wokół siebie szereg białek przekazujących sygnały do wnętrza komórki. Podbłonowa, elektronogęsta struktura wielobiałkowa nosi nazwę „gęstości postsynaptyczne" (PSD) (Kennedy, 2000). Znajdujemy tu białka receptorowe dla kwasu glutaminowego (typu NMDA i mGluR), szkieletowe z rodzin MAGUK, GKAP, Shank i Homer, kinazy białkowe, neurofilamenty, mikrotubule, aktyny i wiele innych (Peng i wsp., 2004). Dotychczasowe badania PSD po niedokrwieniu mózgu wykazały znaczny przyrost ilości białka związanego z tą strukturą, który odzwierciedlał przyrost objętości PSD obserwowany w mikroskopie elektronowym (Hu i wsp. 1998, Martone i wsp. 1999, **Domańska-Janik i wsp. 1999b**, **Zabłocka i wsp. 2001**). Mając na uwadze złożone oddziaływania składników PSD wydaje się, że przebudowa gęstości postsynaptycznych jest jednym z pierwszych etapów szlaków wewnątrzkomórkowego przekazywania sygnału. Kaskada zdarzeń zapoczątkowanych niedokrwieniem rozprzestrzenia się od miejsc kontaktu synaptycznego na wszystkie przedziały komórkowe i oddziałuje na funkcję struktur wewnątrzkomórkowych (mitochondriów, siateczki śródplazmatycznej, jądra komórkowego, cytoszkieletu) jak również obejmuje białka macierzy zewnątrzkomórkowej.

W badaniach prowadzonych na modelu krótkotrwałego niedokrwienia mózgu gerbila oraz w przedłużonej do 15 minut ischemii mózgu szczura zaobserwowano wzrost ilości białek związanych z PSD zarówno w obrazach ultrastrukturalnych jak i posługując się metodą western blot z zastosowaniem przeciwciał rozpoznających aktywne formy kinaz białkowych (**Domańska-Janik i wsp., 1999b; Zabłocka i wsp., 2001**). Jednym z białek

charakterystycznych dla PSD jest kinaza białkowa II, której aktywność zależy od jonów Ca^{2+} i kalmoduliny (CamKII). CamKII jest translokowana do PSD już w pierwszych sekundach ischemii mózgu. Jednocześnie aktywność tej kinazy mierzona wobec egzogennego jak i endogennego substratu jest większa niż w mózgu kontrolnym jednak wzrost aktywności jest mniejszy niż można by wnioskować na podstawie przyrostu ilości białka. Nasze badania **(Domańska-Janik i wsp,. 1999b)** jak i doniesienia innych (Aronowski i Grotta, 1996; Hu i Wieloch, 1995) sugerują, że masowy przyrost ilości CamKII w czasie niedokrwienia kompensuje hamowanie aktywności fosforylacyjnej, które występuje w czasie załamania energetycznego w niedokrwionych neuronach. Ponadto obniża się aktywność CamKII w innych frakcjach błonowych. Wyniki te wskazują na istotną rolę CamKII w reakcji ischemicznej gęstości postsynaptycznych. Równie istotny wydaje się udział kinaz białkowych C (PKC).

PKC obejmuje rodzinę 11 serynowo-treoninowych kinaz białkowych, których aktywność zależy od fosfolipidów i w zalezności od podgrupy od Ca^{2+} i diacyloglicerolu (Nishizuka, 1988; Nishizuka, 1992). PKC pełni kluczową rolę w niemal wszystkich procesach komórkowych włączając w to podziały, różnicowanie czy homeostazę jonową. Izoformom PKC przypisuje się też regulacyjną rolę w procesie apoptozy i regeneracji (Metzger i Kapfhammer, 2003). Z prac wielu badaczy oraz naszych obserwacji na modelu niedokrwienia mózgu gerbila wiemy o szybkiej aktywacji izoform PKC regulowanych jonami wapnia Ca^{2+} i diacyloglicerolem (cPKC) jak również izoform, których aktywność nie zależy od jonów Ca^{2+} (nPKC) (Cardell i wsp., 1990; Domańska-Janik i Zabłocka, 1993; Saluja i wsp., 1999). PKC obecnej w PSD przypisuje się rolę jednego z regulatorów aktywności receptora NMDA (Jimenez i Tapia, 2004) oraz wpływ na aktywność szlaku MAPK poprzez oddziaływanie z kinazą Raf-1 (Ueda i wsp., 1996).

Kinazy białkowe należące do rodziny PKC reagują na stres ischemiczny szybką zmianą aktywności obserwowaną już w pierwszym okresie po przywróceniu krążenia (Domańska-Janik i Zabłocka, 1993). W PSD po niedokrwieniu następuje 4-18 krotny przyrost ilości białka różnych izoform PKC w porównaniu z ich ilością u zwierząt kontrolnych (**Zabłocka i wsp., 2001**). Pośród cPKC obserwujemy 10 krotny przyrost powszechnie występujących izoform α i β, podczas gdy poziom izoformy γ, specyficznej dla neuronów podwyższa się dwukrotnie. Prawdopodobnie jest to spowodowane najwyższym kontrolnym poziomem tej izoformy w PSD. Ponadto, nasze badania po raz pierwszy pokazały spowodowane ischemią przemieszczenie do PSD izoform δ i ε, których kontrolny poziom w tej strukturze jest niemal niewykrywalny. Badania na izolowanej, oczyszczonej frakcji PSD zostały potwierdzone badaniami immunohistochemicznymi w mikroskopie elektronowym, które pokazują utrzymujący się co najmniej do 24 godzin po przywróceniu krążenia znaczny przyrost wielkości gęstości postsynaptycznych i ilości cPKC, głownie w części CA1 hipokampów.

W badaniach wykorzystujących izolowaną frakcję PSD pochodzącą z mózgów zwierząt poddanych ischemii lub ze skrawków hipokampa w warunkach symulujących tę patologię wykazano, ze poza wspomnianymi kinazami białkowymi enzymy proteolityczne – kalpainy są elementem struktury PSD (Domańska-Janik i wsp., 1999a; **Domańska-Janik i wsp., 1999b**). Dwie izoformy kalpainy: μ i m mają różne wymagania dotyczące stężenia jonów Ca^{2+} koniecznych do ich aktywacji. Stwierdzono, że ischemia obniża ilość białka μ-kalpainy w PSD, podczas gdy poziom m-kalpainy nie zmienia się. Jednocześnie obserwowano obniżenie ilości fodryny, która jest białkiem cytoszkieletowym obecnym w PSD, i substratem kalpain. Wydaje się, ze obniżenie ilości enzymu proteolitycznego aktywowanego już w mikromolarnym stężeniu Ca^{2+} ma znaczenie ochronne i zabezpiecza komórki przed rozległą degradacją cytoszkieletu.

W wielobiałkowym kompleksie PSD kinazy białkowe CamKII i PKC wydają się mieć wiele funkcji. Po przebytym epizodzie niedokrwiennym mogą one aktywować różnorodne ścieżki sygnałowe, często o przeciwstawnej funkcji, miedzy innymi mogą modulować aktywność kinazy białkowej Raf-1. Jej ilość w strukturze PSD także rośnie po niedokrwieniu co w konsekwencji może zmieniać aktywność szlaków kinaz białkowych, w tym MAPK. Jednocześnie pewna pula kinazy Raf-1 może być związana z mitochondriami, gdzie ma swoje specyficzne substraty, różne od tych które może fosforylować gdy występuje przy błonie plazmatycznej (Bronisz i wsp., 2002; Wang i Reed, 1998).

Ischemia i szlaki kinaz MAP i JNK

Jednym z lepiej poznanych wieloetapowych szlaków kontrolujących ekspresję genów jest szlak MAPK. W zależności od bodźca pobudzającego lub modyfikacji białek towarzyszących w tworzeniu sieci sygnalizacyjnej, kinazy te wykazują plejotropowe działania. Obecnie rodzina kinaz MAP dzielona jest na trzy grupy, w zależności od końcowej kinazy szlaku: ERK1,2; p38/SAPK oraz JNK/SAPK (Johnson i Lapadat, 2002).

Szlak prowadzący do aktywacji ERK1,2 zaangażowany jest głównie w procesy podziału, wzrostu i funkcjonowania komórek w odpowiedzi na czynniki wzrostowe, ale także na niespecyficzne bodźce stresowe (Góźdź i wsp., 2003). Wewnątrzkomórkowy wzrost stężenia Ca^{2+} i depolaryzacja są także czynnikami aktywującymi kinazy ERK1 i 2 prawdopodobnie w wyniku stymulacji białek syn-GAP i Ras (Rosen i wsp., 1994). Białko syn-GAP jest charakterystyczne dla gęstości postsynaptycznych i moduluje aktywność białka Ras o aktywności GTP-azowej, które z kolei kontroluje aktywność kinazy Raf-1, wpisanej jako jedno z pierwszych białek kaskady MAPK (Komiyama i wsp., 2002). Białko synGAP podlega negatywnej regulacji poprzez fosforylację przez kinazę CamKII (Chen i wsp., 1998),

co w efekcie wpływa na szybkość całego szlaku. Istnieje także możliwość modulacji ERK1,2 przez kinazę białkową C (PKC) poprzez jej oddziaływanie z kinazą Raf-1.

Ścieżka p38/SAPK łączona jest z reakcjami komórek na różnorodny stres, który prowadzi do aktywacji szlaku apoptozy. Z reakcją stresową komórek wiąże się również aktywacja szlaku JNK/SAPK, jednak jak pokazują badania mutantów pozbawionych JNK1 i 2, szlak ten jest niezbędny w procesie apoptozy rozwojowej i prawidłowego formowania układu nerwowego (Chang i Karin, 2001). Kinaza JNK aktywowana jest w wyniku przekazywania sygnału poprzez co najmniej dwa różne kompleksy białkowe. Pierwszy szlak obejmuje kompleks tworzony przez białko JIP1 z kinazami MLK i MKK7, która aktywuje JNK (Yasuda i wsp. 1999). Lokalizacja kompleksu przy powierzchni komórki sugeruje powiązanie z receptorami odbierającymi bodźce zewnątrzkomórkowe. Na podstawie badań *in vitro* istnieją dowody, ze białko PSD-95, które jest typowe dla gęstości postsynaptycznych, odgrywa kluczową rolę w aktywacji JNK poprzez bezpośrednie oddziaływanie z kinazą MLK (Savinainen i wsp., 2001). JNK jest również aktywowana przez inną kinazę szlaku MAPK - MKK4, która nie łączy się z białkiem JIP, ale nie można wykluczyć obecności innych białek kompleksujących. Jednym z podstawowych substratów JNK jest jedno z białek czynnika transkrypcyjnego AP1 - białko c-Jun. Wydaje się, że zależna od fosforylacji aktywność c-Jun jest istotna w wyborze szlaku do przeżycia lub śmierci. Ostatnio coraz więcej doniesień łączy aktywność kinazy JNK z mitochondriami, sugerując, ze taka lokalizacja komórkowa odgrywa istotną rolę w procesie apoptozy (Deng i wsp., 2001; Ito i wsp., 2001; Kharbanda i wsp., 2000).

W wyniku krótkotrwałej ischemii mózgu obserwowana jest aktywacja zarówno pro-życiowych ERK 1,2 jak i JNK (**Zabłocka i wsp., 2003**). Jednak równowaga pomiędzy aktywnością tych dwóch ścieżek sygnałowych jest różna w różnych rejonach hipokampa. I

tak w rejonach, które nie wykazują cech uszkodzenia po niedokrwieniu (CA2, 3, DG) już w pierwszych godzinach po przywróceniu krążenia ilość aktywnych form ERK1,2 (P-ERK) wzrasta dwukrotnie, a po 24 godzinach aż 6-krotnie w stosunku do kontroli, i na tak wysokim poziomie utrzymuje się przez kolejne dni. We wrażliwej na niedokrwienie części CA1 przyrost ilości P-ERK1,2 nie jest istotny statystycznie i występuje przejściowo po 24 i 48 godzinach po ischemii. Jednocześnie w rejonie CA1 ilość aktywnej formy JNK (P-JNK) wzrasta w stosunku do kontroli już po 3 godzinach po niedokrwieniu i pozostaje na wysokim poziomie przez kolejne dni. W pozostałych, niewrażliwych na krótkotrwałe niedokrwienie rejonach hipokampa, wzrost ilości P-JNK występuje przejściowo, 24 godziny po przywróceniu krążenia i jest znacznie mniejszy niż ilość aktywnych form ERK1,2. Wydaje się zatem, że stres ischemiczny powoduje aktywację obu szlaków MAPK, przy czym zmieniona zostaje równowaga pomiędzy ścieżką ERK i JNK (**Zabłocka i wsp., 2003**). Na podstawie obrazu morfologicznego hipokampa w 7 dni po niedokrwieniu można przypuszczać, ze dominacja szlaku ERK nad ścieżką JNK w rejonach CA2, 3 i w zakręcie zębatym skutkuje „przezwyciężeniem" stresu ischemicznego i promuje przeżycie.

W poszukiwaniu kolejnych etapów kaskady przykazywania sygnału ischemicznego w rejonie CA1 badano fosforylację podstawowego substratu JNK jakim jest białko c-Jun czynnika transkrypcyjnego AP1 (**Zabłocka i wsp., 2003**). Wydaje się, że wzrost fosforylacji tego białka odgrywa istotną rolę w pierwszych godzinach po przywróceniu krążenia, natomiast w dłuższym czasie poziom P-c-Jun powraca do wartości kontrolnych. Mając w pamięci długotrwały wzrost ilości aktywnej formy JNK kolejne badania udziału tego białka w poischemicznej śmierci neuronów dotyczyły innych przypuszczalnych substratów i translokacji aktywnej kinazy do mitochondriów.

Ischemia, mitochondria i apoptoza

Najnowsze badania wydają się potwierdzać dawno powstałą hipotezę o kluczowej roli mitochondriów w uszkodzeniu poischemicznym (Fiskum, 1983; Fiskum i wsp. 1999). Mitochondria oprócz swej powszechnie znanej funkcji tworzenia związków wysokoenergetycznych biorą czynny udział w regulacji wewnątrzkomórkowego stężenia jonów wapnia oraz procesu apoptozy. W badaniach *in vitro* i *in vivo* wykazano, że cytochrom c może, oprócz swojej funkcji w łańcuchu oddechowym, pełnić rolę aktywatora kompleksu białkowego (apoptosomu), którego składnikami są proenzymy proteolityczne – kaspazy 9 i 3. Aktywacja apoptosomu prowadzi do aktywacji kaspaz i degradacji komórki (Orrenius, 2004). Istnieje hipoteza zakładająca udział magakanału (MPT) występującego w błonach mitochondrialnych, w wypływie do cytoplazmy białek o niskich masach cząsteczkowych, w tym również cytochromu c i innych białek proapoptotycznych (AIF, endonukleaza G, Smac/Diablo) (Saelens i wsp., 2004). Jednak z doświadczeń z zastosowaniem związków zamykających kanał MPT wynika, ze nie jest to jedyny mechanizm uwalniania białek z mitochondriów. W procesie tym podnoszona jest rola białek z rodziny Bcl, z których niektóre mają właściwości ochronne dla komórek (Bcl_2, Bcl_{xl}) a inne sprzyjają uruchomieniu procesu apoptozy (Bax, Bid, Bad, Bik) (Cory i Adams, 2002; Krajewski i wsp., 1995). Wydaje się, że białka Bcl2 i Bclxl mogą modulować otwarcie kanału MPT. Równowaga pomiędzy pro-i antyapoptotycznymi białkami z rodziny Bcl związanymi z mitochondriami może odgrywać istotną rolę w uruchamianiu kaskady proteaz. Dodatkowo, na funkcje białek z rodziny Bcl mogą wpływać modyfikacje posttranslacyjne, głównie fosforylacje przez kinazy Raf-1 i JNK. Z badań *in vitro* pochodzą przesłanki, że enzymy te mogą modulować przepuszczalność błony mitochondrialnej poprzez oddziaływanie z pro- i anty-apoptotycznymi białkami z rodziny Bcl (Bae i wsp., 2001; Ito i wsp., 2001; Tournier i wsp., 2000). A zatem, podobnie

jak balans pomiędzy białkami z rodziny Bcl tak i równowaga pomiędzy związanymi z błonami mitochondrialnymi aktywnymi kinazami Raf-1 i JNK może wpływać na integralność zewnętrznej błony mitochondrialnej.

W modelu opóźnionej poniedokrwiennej śmierci neuronów w hipokampie gerbila wykazano przejściową, występującą w krótkim czasie reperfuzji (0,5 – 1 godzina) obecność cytochromu c w cytoplaźmie **(Domańska-Janik i wsp., 2004)**. Ponownie cytochromu c w cytoplazmie wykrywany jest po 24 aż do około 72 godzin po przywróceniu krążenia, równocześnie z pierwszymi morfologicznymi symptomami śmierci neuronów. Można sugerować, że wypływ cytochromu c z mitochondriów jest czułym markerem ich uszkodzenia. Wykazano, że wczesne zastosowanie 0,5 μM cyklospory A (CsA) ma działanie ochronne dla komórek. Cyklosporyna A jest lekiem, który prócz swojej dobrze znanej aktywności immunosupresyjnej może stabilizować błonę mitochondrialną i blokować wypływ niskocząsteczkowych białek, w tym cytochromu c. Z naszych badań *in vitro* i *in vivo* wynika, że CsA hamuje wczesną fazę wypływu cytochromu c z mitochondriów i zabezpiecza neurony rejonu CA1 hipokampa przed uszkodzeniem **(Domańska-Janik i wsp., 2004)**. Natomiast zastosowanie CsA później niż 1 godzinę po przywróceniu krążenia nie chroni komórek przez śmiercią.

Postawiono zatem pytanie czy brak neuroprotekcji przy opóźnionym podaniu CsA wiąże się z przebudową błony mitochondrialnej, tak że staje się ona niewrażliwa na ten lek?

Określono poziom związanych z mitochondriami kompleksów białkowych składających się z pro-apoptotycznego białka Bad i anty-apoptotycznego Bcl2 oraz

przypuszczalnie fosforylujących je aktywnych form kinaz białkowych Raf-1 i JNK w różnym czasie po ischemii **(Zabłocka i wsp., 2004; Dłużniewska i wsp., 2004).** O ile ochronne dla komórek białko Bcl2 fizjologicznie lokalizuje się głównie w mitochondriach oraz w innych błonowych strukturach wewnątrzkomórkowych, to pro-apoptotyczne białko Bad jest fosforylowane i pozostaje nieaktywne w cytoplaźmie w kompleksie z białkiem 14-3-3 (Berg i wsp., 2003). W przebiegu reperfuzji obserwowano trwały przyrost ilości białka Bad związanego z mitochondriami, z jednoczesnym, przejściowo obniżonym poziomem fosforylującej go kinazy Raf-1. Równolegle w cytoplaźmie obniżała się ilość ufosforylowanej, nieaktywnej pro-apoptotycznie formy Bad **(Dłużniewska i wsp., 2004)**. Występowanie kinazy Raf-1 w mitochondriach zostało dodatkowo potwierdzone znalezieniem kompleksu białek Bad i P-Raf-1 otrzymanych metodą koimmunoprecypitacji z preparatów oczyszczonych mitochondriów izolowanych z hipokampów gerbila. W tym samym, późnym okresie po niedokrwieniu wykazano we frakcji mitochondrialnej przyrost ilości aktywnej formy kinazy JNK **(Zabłocka i wsp., 2003)**, która może fosforylować/dezaktywować anty-apoptotyczne białka Bcl2 i Bcl_{xl}. Translokację białka Bad do mitochondriów potwierdziły obserwacje w mikroskopie elektronowym. Badano komórkową lokalizację białek Bad i Bcl2 metodą immunocytochemiczną z wykorzystaniem specyficznych przeciwciał znakowanych cząsteczkami złota. Wykazano poniedokrwienne przemieszczenie białka Bad do mitochondriów jedynie w rejonie CA1 hipokampa, natomiast obrazy rejonu zakrętu zębatego (DG) nie wykazywały zmian lokalizacji badanych białek w przebiegu reperfuzji **(Dłużniewska i wsp. 2004)**. Wydaje się zatem, że odległe w czasie od epizodu niedokrwiennego tworzenie indukowanych bodźcem ischemicznym kompleksów Bad-Bcl2 oraz zmieniona lokalizacja komórkowa kinaz białkowych Raf-1 i JNK są elementami przebudowy błon mitochondrialnych, które stają się przepuszczalne dla cytochromu c, niezależnie od obecności cyklosporyny A. Poniedokrwienne zachwianie

równowagi pomiędzy ilością ochronnej P-Raf-1 i aktywowanej stresem P-JNK może wpływać na skierowanie komórki na ścieżkę prowadzącą do śmierci poprzez uwolnienie z mitochondriów białek aktywujących kompleks apoptosomu (w tym cytochromu c) i w efekcie kaspazę 3.

Podsumowanie

Przejściowe, krótkotrwałe niedokrwienie mózgu inicjuje cały szereg reakcji, które doprowadzają do przezwyciężenia stresu lub do eliminacji wrażliwych komórek nerwowych. Trzeba podkreślić, ze szczególnie wrażliwe na ischemię są neurony rejonu CA1 hipokampa, którym przypisuje się kluczową rolę w procesie uczenia się. Dlatego niezwykle ważne jest opisanie procesów inicjowanych niedokrwieniem zarówno w komórkach przeżywających ten epizod jak również umierających, aby można było znaleźć skuteczne sposoby ochrony tych tak istotnych dla każdego organizmu komórek. Zmiany lokalizacji wewnątrzkomórkowej białek sygnałowych (kinazy białkowe) oraz uczestniczących w procesie apoptozy (pro- i anty-apoptotyczne białka z rodziny Bcl) wpisują się w coraz bardziej skomplikowany obraz reakcji poniedokrwiennej tkanki nerwowej. Sekwencja zdarzeń opisywanych w niedokrwionych komórkach wskazuje na wiele równolegle aktywowanych szlaków metabolicznych, które pozostają w ściśle określonych relacjach, i oddziałują nawzajem na siebie. Próby stworzenia mapy szlaków komplikuje fakt, że ten sam enzym/białko może występować w wielu regionach komórki, gdzie pełni rożne funkcje. Jednym z takich białek sygnałowych jest kinaza białkowa Raf-1; jedna z pierwszych w kaskadzie MAPK, której aktywacja zależy od białka Ras i regulacji jego aktywności. Pierwsze elementy kompleksu zlokalizowane są w gęstościach postsynaptycznych (PSD). Do niedawna uważano, ze jest to jedyne miejsce działania Raf-1. Obecnie wiadomo, ze aktywna kinaza białkowa Raf-1 może być związana z mitochondriami, gdzie pełni różne funkcje; np. fosforyluje pro-apoptotyczne białko Bad

(Scheid i wsp., 1999) oraz co ciekawsze, tworzy kompleks z białkiem VDAC, które wchodzi w skład megakanału, i moduluje przepuszczalność kanału. Wydaje się, że dzieje się to poprzez zmiany konformacji białek kompleksu, a nie poprzez fosforylację któregoś z jego elementów (Le Mellay i wsp., 2002). Z przeglądu piśmiennictwa wyłania się spójny obraz, że zlokalizowana mitochondrialnie kinaza Raf-1 chroni komórki przed apoptozą.

Na podstawie obserwacji własnych oraz danych z literatury postulujemy, że tworzenie się nowych, wywołanych przez krótką ischemię patologicznych kompleksów białkowych, zlokalizowanych w miejscach kontaktu synaptycznego (PSD) oraz w mitochondriach może wpływać istotnie na pro-apoptotyczne ukierunkowanie sygnału ischemicznego w okresie reperfuzji we wrażliwym rejonie CA1 hipokampa.

Gęstości postsynaptyczne reagują na niedokrwienie co objawia się bardzo szybkim, znaczącym przyrostem ilości białek i objętości struktury. Białka szkieletowe z rodziny PSD 95 stanowią „kotwicę" dla wielu kinaz białkowych (między innymi izoform PKC), które wpływają na aktywność receptorów dla neurotransmiterów z jednej strony i zmiany aktywności kaskad przekazywania sygnału do wnętrza komórek z drugiej. Istotnym elementem translokacji kinaz białkowych do PSD są jony wapnia, których stężenie w cytoplaźmie zwiększa się w czasie niedokrwienia. Zmienione w wyniku przejściowego niedokrwienia kompleksy białkowe w PSD wydają się wpływać na aktywność szlaków kinaz białkowych MAPK i JNK. Wydaje się, że w komórkach CA1 hipokampa przewaga aktywności szlaku JNK stanowi istotny element procesu prowadzącego do eliminacji tych neuronów.

Równocześnie ze zmianami składu białkowego PSD, w badanym przez nas modelu niedokrwienia mózgu gerbila obserwujemy w czasie reperfuzji dwufazowy wypływ cytochromu c z mitochondriów. Wczesny, przejściowy wypływ prawdopodobnie pełni funkcję amplifikującą sygnał prowadzący do śmieci neuronów. Długotrwały wypływ

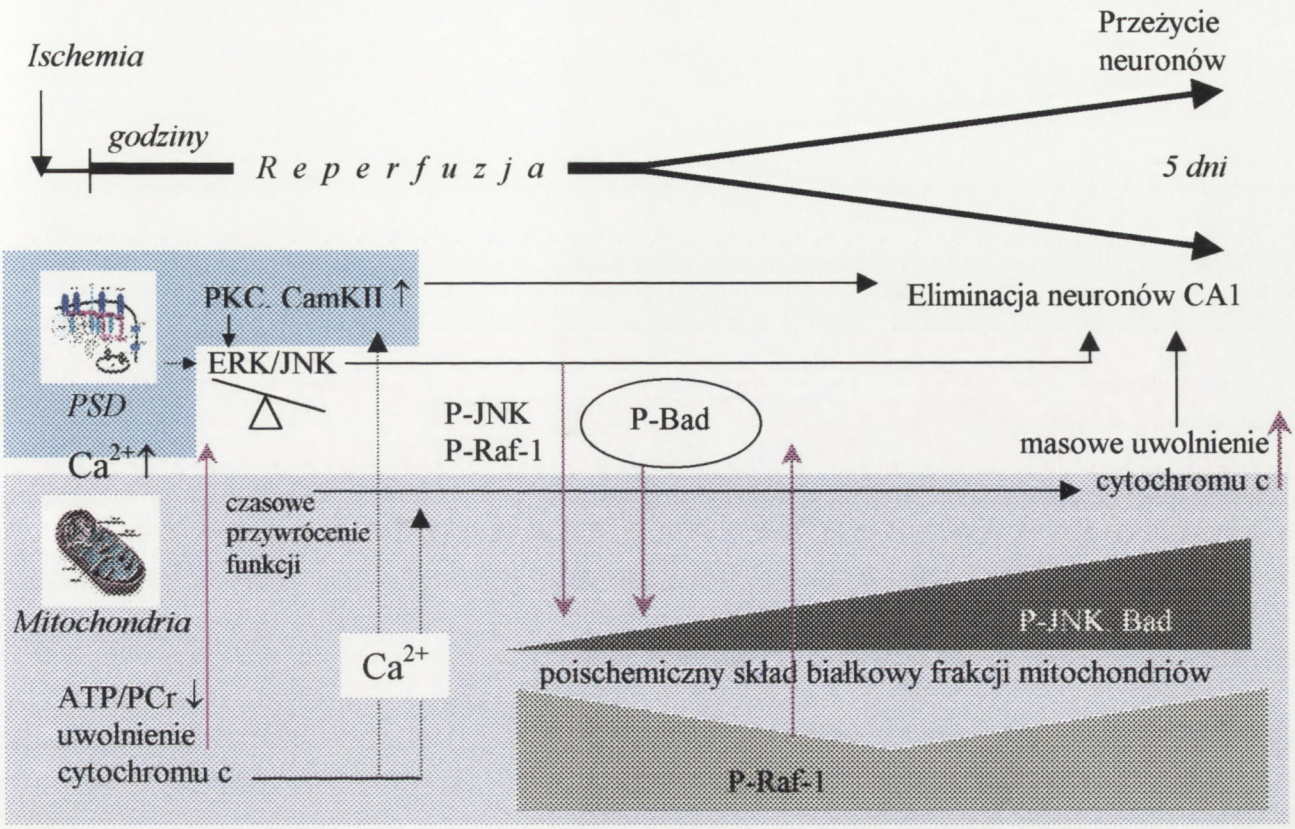
cytochromu c, obserwowany od drugiej doby po przywróceniu krążenia wydaje się być związany z fazą masowej aktywacji proteaz. Sugerujemy, że zmienione na skutek krótkotrwałego niedokrwienia kompleksy sygnałowych kinaz białkowych z zewnętrzną błoną mitochondrialną przyczyniają się zarówno do wczesnego jak i późnego wypływu cytochromu c z mitochondriów a tym samym do uruchomienia kaskady kaspaz.

Ostatnio ukazała się praca wskazująca na nową, kolejną rolę cytochromu c w przedłużaniu i rozprzestrzenianiu sygnału wzbudzonego stresem (Boehning i wsp., 2003). W badaniach *in vitro* wykazano, że cytochrom c łączy się z receptorami IP3 zlokalizowanymi w siateczce śródplazmatycznej, powoduje trwałe otwarcie kanałów błonowych i wypływ jonów wapnia do cytoplazmy. Te z kolei mogą powodować wtórne uszkodzenie mitochondriów. Koncepcja ta jest zgodna z wynikami naszych badań i wnioskami sformułowanymi na podstawie obserwacji cytochromu c niezwiązanego z mitochondriami w pierwszym okresie reperfuzji po przejściowym niedokrwieniu mózgu **(Domańska-Janik i wsp., 2004)**. Ponadto, z naszych ostatnich, wstępnych doświadczeń *in vivo* wynika, że w krótkim czasie po przywróceniu krążenia (30 minut) po niedokrwieniu, cytochrom c związany jest z frakcją mikrosomów. Tym samym potwierdza się hipoteza o udziale cytochromu c w wewnątrzkomórkowym przekazywaniu sygnału wywołanego ischemią mózgu.

Zmiany składu białkowego gęstości postsynaptycznych oraz translokacja białek do oraz z mitochondriów wydają się być istotnym elementem procesu opóźnionej poischemicznej śmierci neuronów.

Schemat uproszczonej sekwencji przemieszczania białek sygnałowych w PSD i mitochondriach w procesie poniedokrwiennej, opóźnionej śmierci neuronów w sektorze CA1 hipokampa przedstawia Figura 1.

↑ - wzrost zawartości, ↓ - obniżenie zawartości, ➔ - translokacja,



Na podstawie przedstawionych wyników badań można pokusić się o wysnucie kilku bardziej ogólnych wniosków, które odnoszą się zarówno do procesu poischemicznej śmierci neuronów jaki i mogą przyczynić się do lepszego zrozumienia procesów wewnątrzkomórkowych.

1. Przejściowa ischemia mózgu inicjuje w niedokrwionych komórkach szybkie zmiany w wewnątrzkomórkowej lokalizacji a tym samym funkcji białek sygnałowych.
2. Lokalizacja wewnątrzkomórkowa białka może określać jego aktywność i udział w odmiennych szlakach metabolicznych.
3. Wielobiałkowe kompleksy, tworząc dynamiczne struktury funkcjonalne wyższego rzędu, mogą istotnie wpływać na los komórki w odpowiedzi na stres niedokrwienny.

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