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Immunohistochemical analysis of protein expression after middle cerebral artery occlusion in mice

Abstract The effect of transient focal cerebral ischemia on protein regulation was studied in mice using multiparametric immunohistochemistry. Injury was characterized by measurements of blood flow, regional protein synthesis and terminal transferase biotinylated-dUTP nick end labeling (TUNEL). The proteins studied were selected from a previously established list of differentially regulated proteins and included the GTPases dynamin, RhoB, CAS and Ran BP-1, the transcription factors Nurr1 and p-Stat 6, the protein kinase MAPK p49, the splicing factors SRPK1 and hPrp16, the cell cycle control proteins cyclin B1 and Nek2, the inflammatory proteins FKBP12 and Rag2, the cell adhesion protein paxillin and the folding protein TCP-1. Regulation patterns were diverse and comprised ipsi- and/or contralateral up- and down-regulation with or without topical association to impeding cell death. Some proteins (SRPK1, TCP-1 and Nurr1) also exhibited post-ischemic translocation from the nucleus to the cytosol. Our observations stress the importance of regional analysis for the interpretation of proteomic data, and contribute to the identification of new pathways that may be involved in the evolution of post-ischemic brain injury.

Keywords Immunohistochemistry · Protein expression · Gene regulation · Focal cerebral ischemia · Translocation

Introduction

Gene profiling methods are increasingly used for the detection of disease-relevant alterations of gene expression in neurological disorders, such as Alzheimer's disease [16], Parkinson's disease [11], aging [27], seizures [9, 40],

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spinal cord injury [5, 38], traumatic brain injury [29, 30] and cerebral ischemia [23, 25, 28, 40, 45]. The identification of novel modulators of neuronal death is thought to contribute to the understanding of injury mechanisms and the identification of targets for possible therapeutical interventions.

As regards brain ischemia, gene profiling by cDNA microarrays [25, 36] or serial analysis of gene expression (SAGE) [44] suggest that the number of genes that are up- or down-regulated is high. Even if only those genes are considered that are regulated by a factor of more than 10, close to 7% of the total genome is involved [43]. However, this does not mean that these changes are of relevance for the evolution of the disease process. Differential gene profiling is usually carried out by screening mRNA expression, but as brain ischemia is associated with a widespread and long-lasting inhibition of protein synthesis at the translational level, genes that code for short-lived proteins may be superinduced due to the release of feedback control [2, 8].

Many genes also respond to peri-infarct depolarizations, which spread over the entire hemisphere and which lead to the up-regulation of immediate-early genes in the intact, non-ischemic tissue [24]. Finally, the breakdown of the energy state in the core of the ischemic territory results in inhibition of both transcription and translation, which may be misinterpreted as active down-regulation. A more reliable approach for the evaluation of the role of selective gene regulation for injury evolution is, therefore, the analysis of protein translation in combination with independent markers of tissue injury.

Recently, we performed the first proteomic investigation of focal brain ischemia, using multi-Western blot analysis of hemispheric protein extracts [42]. Out of 400 proteins with a wide range of regulatory functions, up to 45% were up- or down-regulated at various time points after ischemia by a factor of 1.5 or more in both the ipsilateral and the contralateral hemisphere. To study the regional distribution of some of these proteins, we repeated the same experiment, and performed immunohistochemistry in combination with terminal transferase-biotinylated

dUTP nick-end labeling (TUNEL) and autoradiographic imaging of protein biosynthesis. This combination allows the precise topical association of the regulated proteins with the ischemic impact, which, in turn, can be differentiated into reversible and irreversible injury.

Our data revealed complex expression patterns, which frequently expanded beyond the ischemically damaged tissue and which have to be interpreted with care to avoid misconceptions on the pathogenetic role of regulated proteins for the evolution of tissue injury.

Materials and methods

Experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the local authorities. Animals were kept under standard diurnal lighting conditions and had free access to food and water until the day of the experiment. Anesthesia was induced by 1.5% halothane and maintained with 0.9% halothane in 70% N_2O and 30% O_2.

Induction of focal cerebral ischemia

Adult male C57BL/6J mice (Charles River, Germany) weighing 22–26 g were used. Three groups of animals ($n=4$ per group) were subjected to focal cerebral ischemia for 60 min, followed by 0, 3 and 12 h recirculation, respectively. Four animals were used as sham-operated controls.

Focal cerebral ischemia was produced by occluding the middle cerebral artery (MCA) with an intraluminal filament [14]. During the experiments blood flow was measured by laser Doppler flowmetry (LDF) using a 0.5-mm fiberoptic probe (Perimed, Stockholm, Sweden), which was attached to the intact skull overlaying the MCA territory (2 mm posterior, 6 mm laterally from bregma). LDF changes were monitored throughout the 1-h ischemia and up to 30 min after the onset of reperfusion. A midline neck incision was made and the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691; Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. An 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren; Heraus Kulzer, Dormagen, Germany) was introduced through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for occlusion of the MCA. The size of the thread (0.15–0.20 mm) was selected to match the body weight of the animals. One hour after the occlusion the thread was withdrawn to allow reperfusion of the MCA territory. Sham-operation was performed by inserting the thread into the common carotid artery, but without advancing it to the MCA.

Forty-five minutes before the animals were killed, L-[4,5-^{3}H]leucine (300 µCi/animal, specific activity 151 Ci/mmol; Amersham, Braunschweig, Germany) was administered intraperitoneally to evaluate cerebral protein synthesis (CPS) rates. After the predetermined reperfusion periods, experiments were terminated under halothane anesthesia by decapitation. The brains were removed, frozen with liquid nitrogen [32] and serially cut into 20-µm-thick coronal cryostat sections. Sections were mounted on poly-L-lysine-coated glass slides for immunohistochemistry, terminal transferase biotinylated-dUTP nick end labeling (TUNEL) and CPS autoradiography.

Temperature control

During surgery and up to 30 min after MCA occlusion, rectal temperature was kept at 37.0°C using a heating lamp and a heating pad connected to a thermistor. After recovery from the anesthesia, animals were maintained in an air-conditioned room at approximately 22°C.

Immunohistochemistry

Air-dried brain sections were fixed in ethanol/acetone (1:1 v/v) for 10 min. Thereafter, the sections were washed for 5 min in phosphate-buffered saline (PBS), treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity, and given two more 5-min rinses with PBS. For immunostaining, sections were incubated in PBS containing 5% normal goat serum or normal horse serum and 0.3% Triton X-100 for 30 min at room temperature. The slides were then incubated for 24 h at 4°C with the following primary antibodies: CAS goat polyclonal, cyclin B1 rabbit polyclonal, dynamin rabbit polyclonal, FKBP12 goat polyclonal, HIF-1α rabbit polyclonal, Nek2 goat polyclonal, Nurr1 rabbit polyclonal, p-Stat6 goat polyclonal, Rag2 rabbit polyclonal, Ran BP-1 goat polyclonal, RhoB rabbit polyclonal, SRPK1 goat polyclonal and TCP-1α goat polyclonal (all Santa Cruz Biotechnology), and with hPrp16 mouse monoclonal, MAPK p49 mouse monoclonal and paxillin mouse monoclonal (all BD Transduction Laboratories). Antibodies were diluted 1:100 in PBS containing 5% goat serum or normal horse serum and 0.3% Triton X-100, except RhoB antibody which was diluted 1:200. Thereafter, sections were washed three times in PBS and incubated with biotinylated secondary antibody (1:100; goat anti-rabbit antibody, goat anti-mouse antibody DAKO Diagnostics, Glostrup, Denmark, or horse anti-goat antibody Vector Co., Burlingame, CA) in PBS containing 5% normal goat serum or normal horse serum and 0.3% Triton X-100 at room temperature for 2 h. After washing in PBS three times for 5 min, slices were incubated with streptavidin-horseradish peroxidase (Vectastain Elite ABC; Vector) in PBS for 30 min at room temperature. Immunoreactivity was visualized via the detection of biotin-streptavidin-peroxidase complex by incubation with diaminobenzidine tetrahydrochloride (Sigma FAST DAB). Sections were dehydrated with increasing concentrations of ethanol and embedded. The specificity of the immunoreactivity was tested in selected experiments with the immunogene blocking peptide (Santa Cruz Biotechnology) before being used for immunohistochemistry according to the instructions of the manufacturer.

TUNEL staining

TUNEL was performed as described previously [47], with minor modifications. Briefly, coronal brain sections were fixed for 15 min in ice-cold 4% paraformaldehyde/PBS, pH 7.4. Subsequently, the sections were washed twice in 70% ethanol (1 min), once in PBS (3 min), and once in 0.3% hydrogen peroxide/PBS (5 min). After equilibration for 15 min in TDT buffer (100 mM potassium cacodylate, 2 mM cobalt chloride, 0.2 mM dithiothreitol), the buffer was quantitatively removed, sections were incubated in 50 ml TDT-Mix [10 pM biotin-16-dUTP (Boehringer, Mannheim, Germany) and 150 U/ml terminal deoxynucleotidyl transferase (Life Technologies, Eggenstein, Germany)] in TDT buffer, and covered with coverslips. After incubation for 60 min at 37°C, the reaction was terminated by washing the sections for 15 min in TB buffer (300 mM sodium chloride, 30 mM sodium citrate). Incorporated biotin was visualized using the avidin-biotin-peroxidase complex method (Vector), as recommended by the supplier. Finally, the sections were dehydrated and embedded in Eukitt (Kindler, Freiburg, Germany).

Measurement of cerebral protein synthesis

For CPS measurement, brain slices were incubated in 10% trichloroacetic acid to remove labeled free leucine and metabolites other than proteins. Subsequently, slices were exposed for 21 days with ^{3}H standards to tritium-sensitive x-ray film (Hyperfilm ^{3}H; Amersham) for autoradiography of ^{3}H-labeled proteins [32].

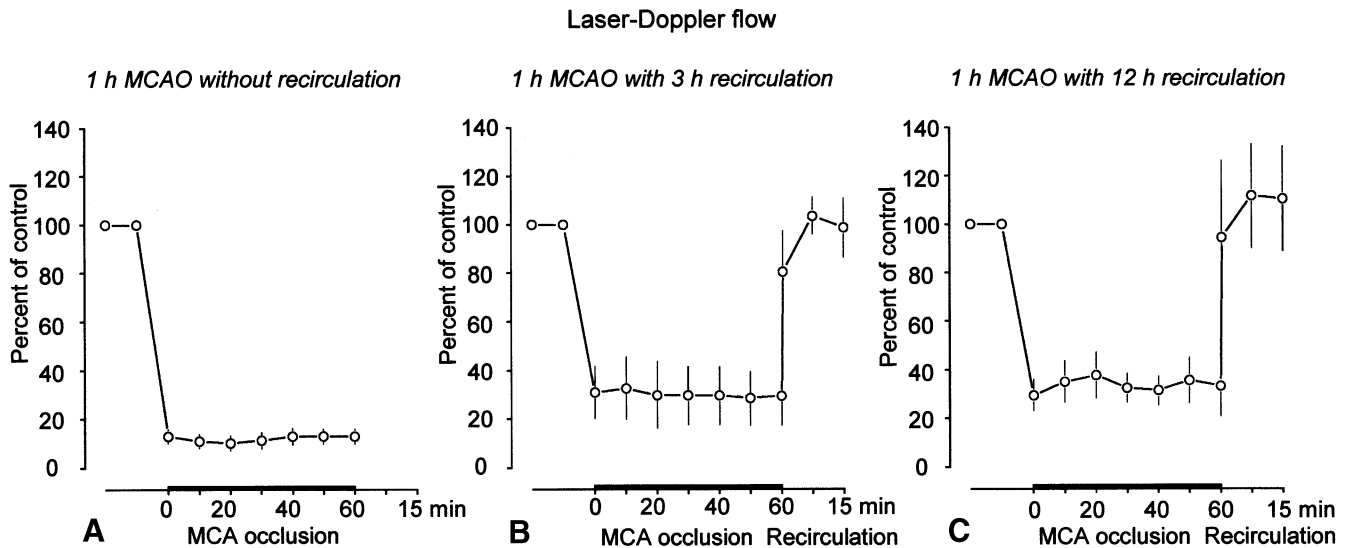## Laser-Doppler flow

Fig. 1 Measurement of blood flow in cerebral cortex of mice submitted to 60-min MCAO without (**A**) or with 3-h (**B**) and 12-h (**C**) recirculation. Blood flow was recorded by laser Doppler flowmetry in the territory of the occluded MCA and expressed as percentage of the pre-ischemic value (means ± SD; $n=4$ per group). Measurements were carried out during vascular occlusion and during the initial 15 min of reperfusion. Note abrupt decline of flow after vascular occlusion in **A–C**, and return of flow to control levels within 15 min of recanalization in **B** and **C** (*MCA* middle cerebral artery, *MCAO* MCA occlusion)

Incidence maps of regional alterations

To evaluate the regional reproducibility of immunostainings for TUNEL and CPS, regional incidence maps were constructed [14]. The areas of biochemical disturbances were outlined on representative coronal brain sections from each individual animal and superimposed at the level of caudate-putamen and in some cases also at the level of the hippocampus. Using the NIH image analysis software, the incidence of metabolic alterations was calculated for each pixel and expressed as a percentage of the number of animals per group.

Results

General physiological observations

The effects of intraluminal MCA thread occlusion on general physiological parameters have been described previously [16]. In this study the reduction of regional cerebral blood flow was measured by LDF. For the histochemical investigations, brains were selected according to the following criteria: (1) after MCA occlusion the LDF declined to below 35% of the basic level, (2) after withdrawal of the thread the LDF returned to the control level (100%) within 15 min. Fig. 1 shows the mean ischemic and post-ischemic LDF values in the three groups submitted to 60-min ischemia and 0, 3 and 12 h reperfusion, respectively. Statistical analysis did not reveal intergroup differences in the severity of ischemia or the efficacy of reperfusion.

Characterization of brain injury

Cerebral protein synthesis

Sixty minutes of intraluminal thread occlusion resulted in a suppression of global cerebral protein synthesis throughout the vascular territory of the MCA. During the recirculation, protein synthesis partly recovered in the cortex (12-h reperfusion), but not in the basal ganglia or in the thalamus (Figs. 2, 3).

TUNEL

After MCA occlusion, DNA fragmentations, as visualized by TUNEL, were clearly confined to neurons and appeared later than the inhibition of CPS (Figs. 2, 3). The number of TUNEL-positive neurons was counted at the level of caudate-putamen and hippocampus (data not shown), and incidence maps were calculated to illustrate the topical distribution of TUNEL positivity.

At the end of 1-h ischemia, neurons were TUNEL negative, but after 3 h of recirculation numerous TUNEL-positive neurons were detected both in caudate-putamen and hippocampus. TUNEL positivity was restricted to the central parts of the ischemic territory, where it clearly colocalized with the area of permanently suppressed protein synthesis. After 12 h, the number of TUNEL-positive neurons was lower, but the expansion of labeling was similar. These findings are in line with our earlier investigation of the evolution of ischemic cell death [14] and confirm that recirculation after 1 h of MCA occlusion prevents irreversible damage in the penumbra, but not in the core of the primary ischemic lesion.

Regional pattern of protein changes

Histochemical evaluation of selected proteins revealed six different patterns of regulation: early or late unilateral
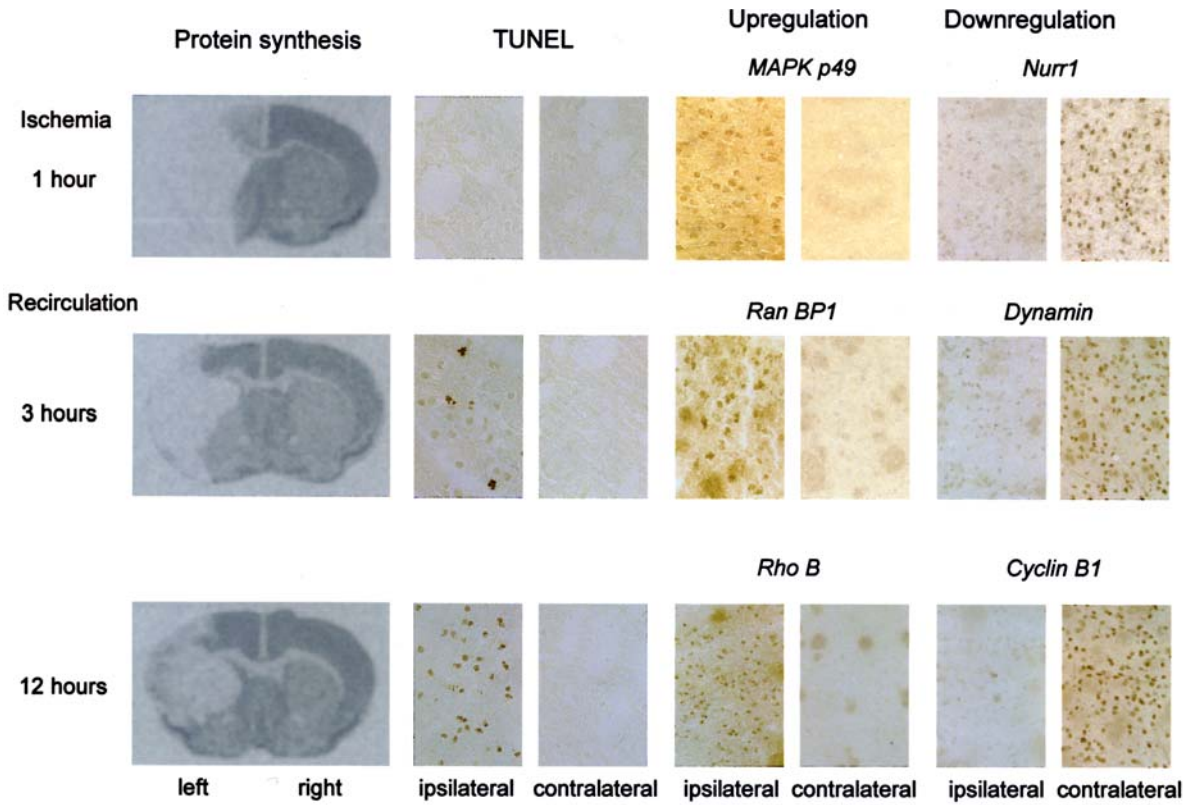
Fig. 2 Representative experiments showing inhibition of global CPS, TUNEL and various up- and down-regulated proteins at the end of 60-min MCAO and at 3 h and 12 h of post-ischemic recirculation, respectively. The topographical distribution of changes is summarized in the incidence maps of Figs. 3, 4, 5 (*CPS* cerebral protein synthesis, *TUNEL* terminal transferase biotinylated-dUTP nick end labeling)
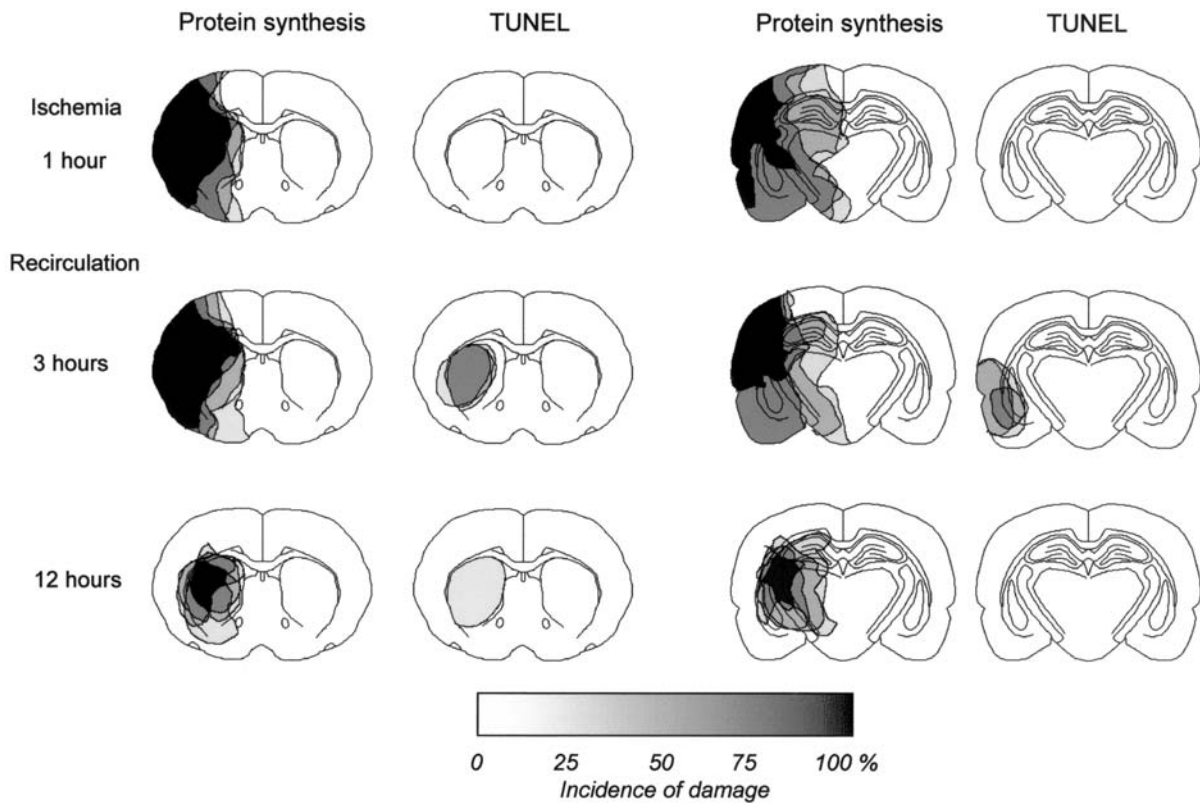

Fig. 3 Incidence maps of suppressed CPS and neurons positive for TUNEL on coronal sections of the mouse brains at various times after 60-min MCAO. Areas of disturbed metabolism were outlined at the level of caudate putamen (*left*) and dorsal hippocampus (*right*) and superposed to calculate the incidence of alterations as the percentage of the number of animals (*n*=4 per group)
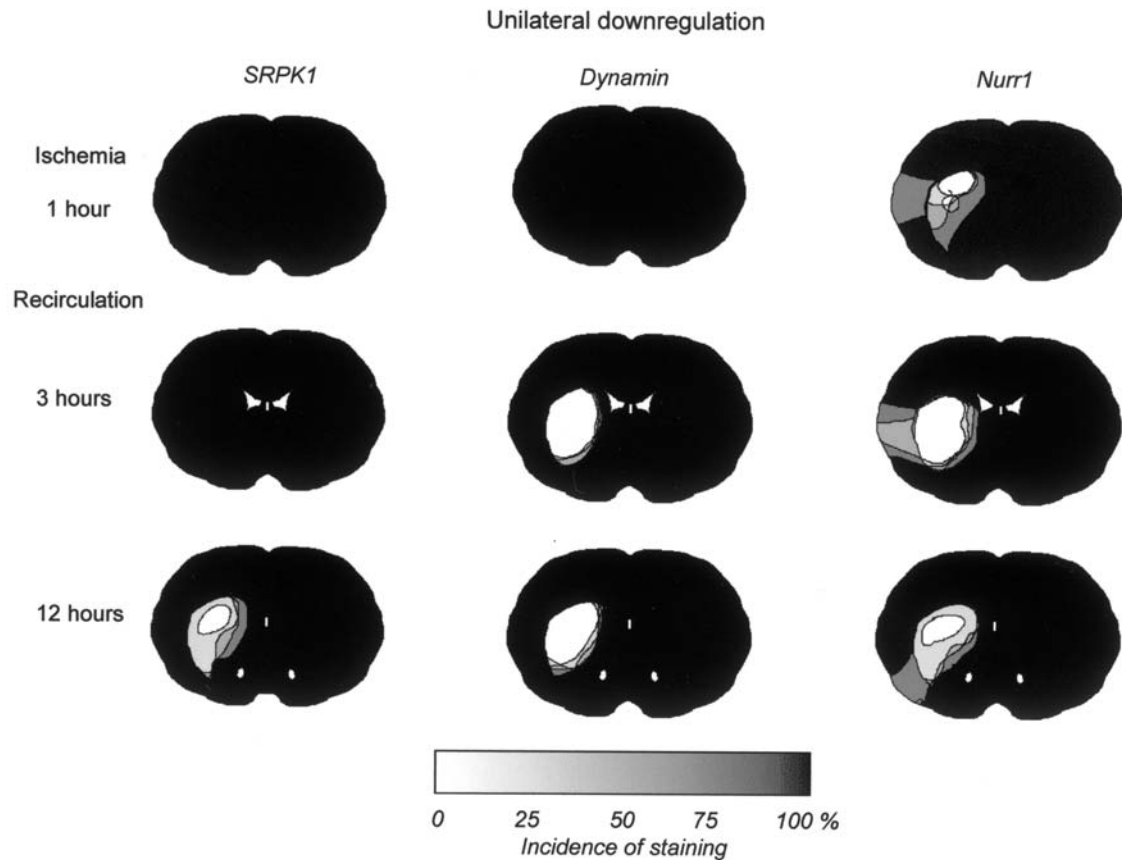
Fig. 4 Incidence maps of the topical representation of down-regulated proteins after 60-min MCA occlusion and various recirculation times (0, 3 and 12 h). The outlines of immunohistochemical stainings are projected on representative brain sections at the level of caudate putamen and expressed as percent group incidence for each protein and time point (*n*=4 per group). Note different dynamics of down-regulated proteins

down-regulation, mainly in the basal ganglia, early or late unilateral up-regulation both in the cortex and basal ganglia, and bilateral cortical up-regulation with or without unilateral up-regulation in basal ganglia (Figs. 4, 5). The functional characteristics of the regulated proteins are given in the Table 1.

The specificity of the immunohistochemical staining was confirmed in each experiment, using negative controls without application of the primary antibody, and in selected experiments by preabsorption of the primary antibody with the immunogene blocking peptide. Disease specificity was confirmed by comparison with sham-operated controls. Focal brain changes were evaluated by comparing the ipsilateral hemisphere with the non-ischemic contralateral side.

The down-regulated proteins (Nurr1, SRPK1, hPrp16, dynamin, cyclin B1 and Rag2) were constitutively expressed throughout the brain except in the inhibited area of caudate-putamen. The dynamics of the regulation showed different time patterns (Fig. 4). Sixty minutes of permanent focal ischemia suppressed the expression of the transcription factor Nurr1 mainly in the area of lateral basal

ganglia, but not that of the other proteins investigated. Following 3 h of recirculation, the expressions of four proteins (cyclin B1, dynamin, hPrp16 and Rag2) were inhibited and remained down-regulated at 12 h in the infarct core. Extent and incidence of down-regulation declined at 12 h of reperfusion in the case of Rag2 and Nurr1 in accordance with the partial recovery of CPS (Fig. 2).

SRPK1 was the only one of the 15 tested proteins to show a histochemically detectable decline only at the longer recirculation time. However, an intracellular translocation of the protein from the nucleus to the cytosol could already be observed after 3 h of recirculation (Fig. 6). The final loss of the protein in the ipsilateral side was accompanied by a stimulation of the expression in the contralateral, non-ischemic hemisphere (data not shown).

The up-regulated proteins showed infarct-dependent (MAPK p49, RhoB) or -independent (CAS, FKBP12, Nek2, paxillin, p-Stat6) distribution, or a combination of the two (TCP-1 and Ran BP-1). The MAPK p49 signal occurred after permanent occlusion and was localized to motor, sensory, insular and piriform cortex of the infarcted side. During recirculation, the area of MAPK p49 expression first decreased and later extended to the lateral caudate-putamen. RhoB immunostaining showed infarct specificity and appeared both in the cortex and the basal ganglia of the ischemic hemisphere during the recirculation.

Interestingly, Ran BP-1 and TCP-1 showed a constitutive expression profile bilaterally in the cortex, but after 3 h
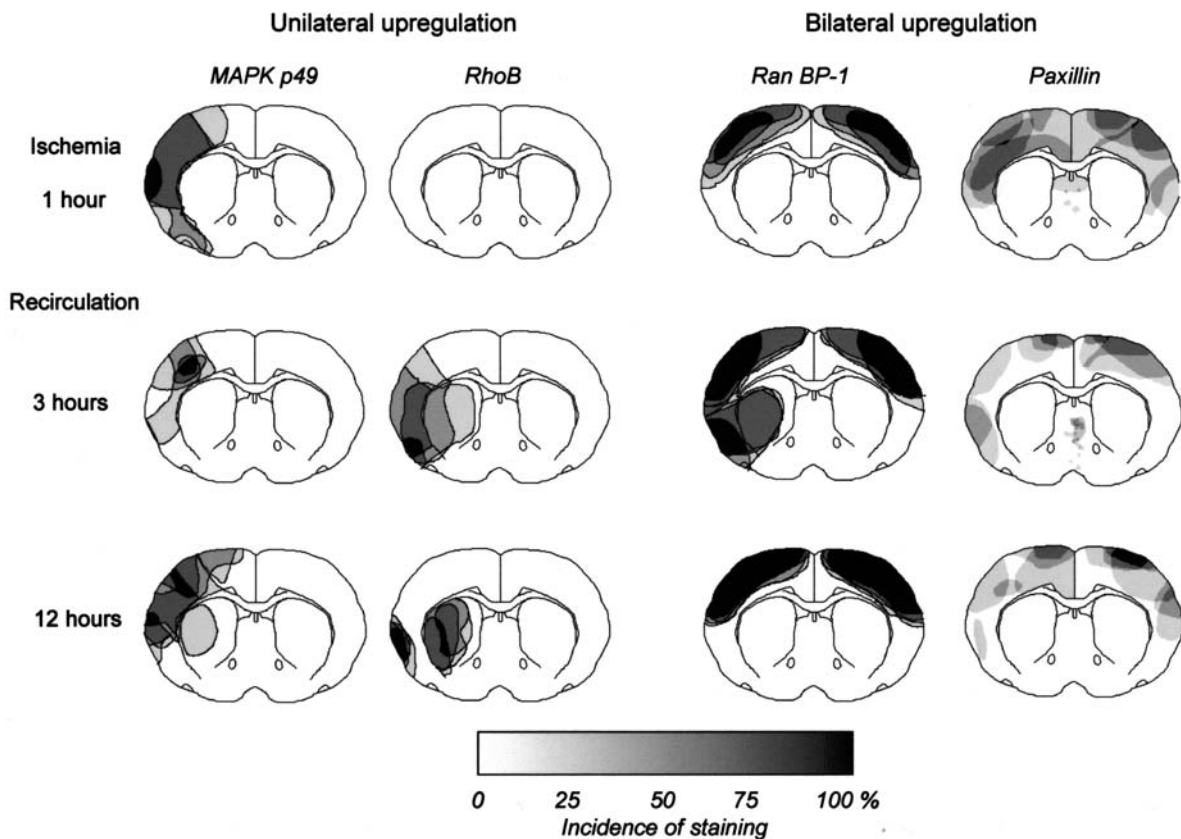
Fig. 5 Incidence maps of the topical representation of up-regulated proteins after 60-min MCAO and various recirculation times (0, 3 and 12 h). The outlines of immunohistochemical stainings are projected on representative brain sections at the level of caudate putamen and expressed as percent incidence for each protein and time point (n=4 per group). Note different patterns of uni- or bilaterally regulated proteins

of recirculation an ischemia-induced component of up-regulation was also observed in the piriform cortex and lateral caudate-putamen of the ipsilateral side.

Cellular association of regulated proteins

Cellular association was based on light microscopical appearance of histochemically positive cells. The majority of the proteins tested localized clearly in neurons. Only a few proteins showed immunoreactivity in cells with glial characteristics (Nek2, RanBP-1 in the cortex, FKBP12) but these associations require confirmation by cell-specific double labeling.

Table 1 Classification of selected proteins according to the distribution pattern after 1 h middle cerebral artery occlusion in mice

Regulation pattern	Protein	Function
Early unilateral down-regulation mainly in basal ganglia	Nurr1	Transcription factor
Late unilateral down-regulation mainly in basal ganglia	Cyclin B1	Cell cycle control, differentiation
	Dynamin	GTPase
	HPrp16	Splicing factor
	Rag2	Inflammatory protein, cell cycle control
	SRPK1	Splicing factor
Early unilateral up-regulation both in the cortex and basal ganglia	MAPK p49	Protein kinase
Late unilateral up-regulation both in the cortex and basal ganglia	RhoB	GTPase protein
Bilateral cortical up-regulation	CAS	GTPase protein, apoptosis, cytoskeleton
	FKBP12	Inflammatory protein, immunophillin
	Nek2	Apoptosis, cell cycle control, differentiation
	Paxillin	Cell adhesion protein
	p-Stat 6	Transcription factor
Bilateral cortical up-regulation with unilateral up-regulation in basal ganglia	Ran BP-1	GTPase
	TCP-1	Protein folding, neurodegeneration

Cytosolic translocation

SRPK1

| Ischemic hemisphere | Contralateral hemisphere |

TCP-1

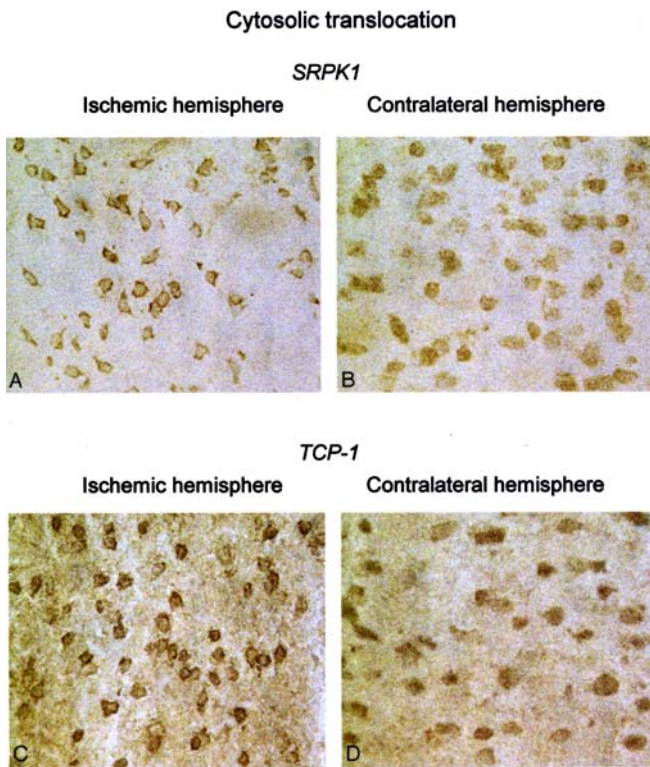| Ischemic hemisphere | Contralateral hemisphere |

Fig. 6 Translocation of proteins SRPK1 and TCP-1 from the nucleus into the cytoplasm after 60-min MCAO, followed by 3-h recirculation. Immunohistochemical stainings of striate nucleus of the ischemic hemisphere (**A**, **C**), and of the contralateral intact hemisphere (**B**, **D**). Note reduction of cell size in the ischemic hemisphere due to beginning pyknosis

Subcellular localization and translocation of regulated proteins

The subcellular localization was clearly cytosolic in the case of MAPK p49, RhoB, RanBP-1, FKBP12, Nek 2 and paxillin. Other proteins, like hPrp16, CAS, cyclin B1 and Rag2 were present in the nucleus. After ischemia SRPK1, Nurr1 and TCP-1 translocated from the nucleus to the cytoplasm (Fig. 6). Occasionally, such translocations were difficult to detect because the affected cells could undergo pyknosis (see Fig. 6 where the size of the TCP-1-positive pyknotic cells in the ischemic hemisphere is similar to that of the nuclei of the healthy non-ischemic side). In such cases, translocation preceded cell death in the core of the ischemic caudate-putamen.

Discussion

The present investigation stresses the complexity of protein changes after focal cerebral ischemia and describes several new proteins that previously have not been associated with the evolution of ischemic injury. Our model of 1-h transient middle cerebral artery occlusion represents an experimental paradigm that is particularly sensitive to molecular injury pathways. In fact, by selecting only ani-

mals in which recirculation was not impaired after the ischemic episode, tissue damage was mainly mediated by protracted, presumably gene expression-dependent molecular abnormalities.

This has been demonstrated by bioluminescence imaging of ATP, which upon recirculation revealed rapid recovery of energy metabolism, followed after a few hours by secondary energy failure [15]. The region of secondary breakdown of energy metabolism precisely matches that of ongoing inhibition of protein synthesis, indicating a close association with delayed cell death. Interestingly, primary recovery of protein synthesis, which is much slower than the initial recovery of energy metabolism, progresses from the peripheral to the central parts of the recirculated vascular territory, whereas secondary ATP failure progresses from the core to the periphery [15]. Depending on the speed of primary recovery of protein synthesis, on the one hand, and on the delay of secondary energy failure, on the other, the volume of delayed tissue injury may vary greatly [33]. As these variables depend both on the duration of ischemia and on the efficacy of post-ischemic recirculation, the dynamics of delayed tissue injury must be studied under strictly controlled experimental conditions. In the present study this was achieved not only by using a standardized MCA occlusion protocol, but also by selecting animals with the same recirculation profile, i.e., an initial blood reperfusion rate equal to or above 100% of control.

The different recovery profiles of energy metabolism and protein synthesis lead to the dissociation of post-ischemic gene regulation at the transcriptional and translational level. During ischemia, DNA transcription and RNA translation are equally suppressed because both pathways are energy dependent. With the recovery of energy metabolism, transcription is resumed, but the ongoing inhibition of protein synthesis prevents RNA translation [31]. In brain regions with secondary energy failure, transcription is again suppressed, whereas in those parts where protein synthesis is resumed both transcription and translation recover. This and the possibility of superinduction of DNA transcription in regions of disturbed translation [2, 8] create a complex pathobiochemical situation, the outcome of which depends on the individual recovery profile and, therefore, may vary under different experimental conditions.

The proteins studied in the present investigation were chosen from a list of selectively regulated genes that we previously established by multi-Western blot analysis of hemispheric protein extracts [42]. The GTPases RhoB, CAS, Ran BP-1 and dynamin are molecular switches that control a wide range of signal transduction pathways. They are mainly known for their role in regulating the actin cytoskeleton, but their ability to influence cell polarity, microtubule organization, membrane transport pathways and transcription factor activity is probably just as significant [7, 39].

The GTPase CAS was up-regulated bilaterally in the cerebral cortex, representing an expression pattern that apparently is unrelated to the evolution of focal brain ischemia. This differed from the other investigated GTPases,

which all exhibited focal abnormalities. The association of RhoB with impeding cell death has been described before [41] and is confirmed by the present study. Here we have demonstrated the inverse regulation of dynamin which is apparently down-regulated during infarct evolution. Dynamins are microtubule-associated proteins with putative function in endocytosis, actin cytoskeleton, synaptic transmission and neurogenesis [35].

Another new observation is the dual regulation of Ran BP-1, which exhibits both infarct-dependent and infarct-independent expression after focal ischemia. Beside a bilateral constitutive expression in the cortex, a Ran BP-1 signal appeared temporarily in the ipsilateral basal ganglia after 3 h of recirculation. This protein is localized in the cytoplasm and enhances the Ran GTPase activating protein (RanGAP) activity, which plays a role in the nucleocytoplasmic transport. Ran is implicated in diverse cellular processes including DNA replication, entry into and exit from mitosis and the transport of RNA and proteins through the nuclear pore complex. Active RanGTP and inactive RanGDP are tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins. Ran BP-1 binds specifically to the GTP-bound form of Ran and acts as an inhibitor of RanGEF function [4, 39]. Its up-regulation may reflect a temporary induction of ischemia-related transport mechanisms.

The regulation of these four GTPase proteins clearly follows different dynamics and shows different spatial distributions after the ischemia/reperfusion injury. Some studies have established a role of Rho GTPases in controlling the enzymatic activity of serine-threonine kinases known as mitogen-activated protein kinases (MAPKs) [41]. The new member of this enzyme family, MAPK p49, was tested in our study. It presented an early up-regulation in the preserved peri-infarct zone during and following ischemia, presumably taking part in the neuroprotection of the surviving neurons of the penumbra.

Another class of functional proteins examined in this study are the transcription factors Nurr1 and p-Stat6. The orphan nuclear receptor Nurr1 is encoded by an intermediate early gene that can be rapidly induced by different stress conditions. Nurr1 shows a distribution similar to that of dopamine-containing neurons [37] and is a potential regulator of gene expression of dopaminergic phenotypic markers. Gene coding for dopamine transporter and the dopamine biosynthetic enzyme tyrosine hydroxylase have been demonstrated to respond to Nurr1 [37]. The induction of Nurr1 mRNA following permanent focal ischemia [20] and global cerebral ischemia [19] has also been described. In our experiments the early down-regulation in the infarcted hemisphere is either due to post-translational protein modification or indirectly to the lack of energy supply. The translocation of Nurr1 in the hypoxia-damaged striatal cells from the nucleus to the cytosol is a novel finding that has not been reported before.

P-Stat6 was expressed in the cortex of both hemispheres with low intensity. The phosphorylation of Stat6 did not show an infarct-dependent profile in our experiments. Stat6 mediates signals for IL-4 and possibly IL-13,

and is thought to modulate the basal transcription machinery by binding to p300/CBP [21].

To the best of our knowledge, the ischemia-induced regulation of the cell cycle control proteins Rag2 and Nek2 has not been described before. However, the role of cyclin B1, another cell cycle regulator, in neuronal cell death following retinal ischemia/reperfusion [26] or transient MCA occlusion [17] has already been investigated. It was concluded that aberrant expression of cyclin B1 may play a role in necrotic cell death after retinal ischemia, but not in apoptosis associated with stroke. In our study, cyclin B1 and Rag2 were down-regulated in the injured basal ganglia during the reperfusion. In contrast, Nek2 was up-regulated bilaterally in the cortex during and after the MCA occlusion.

Cyclin B1 is mainly expressed in the G2/M phase of cell division which, in turn, is triggered by the cyclin B1-Cdc2 complex. In the M phase; cyclin B1 is phosphorylated in the cytoplasmic retention sequence, which is required for nuclear export. During the interphase, cyclin B1 shuttles between the nucleus and the cytoplasm because constitutive nuclear import is counteracted by rapid nuclear export [12]. Entry into mitosis requires not only the presence of cyclin-dependent proteins, but also a second, structurally distinct serine/threonine kinase known as NIMA. NIMA-related kinases like Nek2 investigated here are emerging as critical regulators of centrosome structure and function [10]. Similarly, the recombination activator gene Rag2, which is essential for immunoglobulin production [1], may provide new information on cell cycle-associated mechanisms of ischemic injury.

A novel observation is also the ischemia-dependent regulation of the splicing factors SRPK1 and hPrp16. Reversible phosphorylation plays an important role in pre-mRNA splicing in mammalian cells. SR-protein specific kinase (SRPK1) has been shown to phosphorylate the SR family of splicing factors [46]. According to our results, the inhibition of oxygen supply caused translocation of SRPK1 from the nucleus to the cytosol, blocking its nuclear function, and later led to its complete down-regulation in the focus of the infarcted area. HPrp16 is involved in the second step of splicing when the exons are fused with concomitant release of the intron lariat [49]. The post-ischemic down-regulation described here suggests disturbance in splicing activity, which is in line with our previous observations on the splicing regulating protein tra2-beta1 [6].

The behavior of the immunophilin FKBP12 (FK506 binding protein12) following focal cerebral ischemia has been investigated by Kato et al. [22]. They found that FKBP12 immunoreaction decreased rapidly in the ischemic core and increased in the penumbra after 4-h recirculation. We observed a peripheral up-regulation in both ischemic and healthy cortex. FKBP12 plays an important role for fate decisions between neuronal survival and death following cerebral ischemia and is involved in inflammatory reactions.

The post-ischemic up-regulation of TCP-1 (t-complex polypeptide) in the ipsilateral basal ganglia, is another interesting finding that may be linked to the recently dis-

covered role of endoplasmic reticulum (ER) dysfunction in the evolution of ischemic injury [34]. TCP-1 is a chaperone for the folding of tubulin [48], whose assembly into microtubules is critical to many cellular processes. As ER dysfunction results in disturbances of protein folding, up-regulation of TCP-1 may be a rescue response in analogy to the similarly up-regulated chaperone protein HSP70 [14].

Finally, the cell adhesion protein paxillin showed multifocal up-regulation in cerebral cortex, which may be of importance for controlling the recently documented long-distance migration of endogenous [3] or transplanted stem cells [18] to the ischemic lesion.

In summary, the combination of metabolic and molecular markers of ischemic injury with the immunohistochemical evaluation of biologically important regulatory proteins provides precise information on the association and disassociation between selective gene expression and disease evolution. Our observations stress the importance of regional analysis for the interpretation of genomic and proteomic data, and they contribute to the identification of new regulatory pathways that may be involved in the evolution of post-ischemic brain injury. Obviously, further analysis of these pathways will be necessary to specify their importance as new targets for neuroprotective drug design.

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