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Proteomics of experimental stroke in mice

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Abstract. Multi-Western blots of more than 400 proteins were performed from brain extracts of mice submitted to transient focal ischemia induced by 1 h middle cerebral artery (MCA) thread occlusion. Measurements were carried out in groups of six animals in sham-operated controls, at the end of 1 h ischemia, and after 3 and 12 h recirculation. After MCA occlusion up to 45% of proteins were up- or downregulated in the ipsilateral hemisphere by a factor of 1.5 or more, as compared to sham-operated controls. The temporal regulation of several proteins in the ischemia-affected hemisphere after 1 h MCA thread occlusion is described. In the non-ischemic hemisphere the number of regulated proteins was close to 50%, indicating a hitherto unrecognized involvement of the opposite side. The proteomic approach of brain injury analysis goes beyond previous screenings of gene expression at the transcriptional level and although our study provides further evidence for the complexity of multiinjury pathways in the evolution of ischemic brain damage it may help to identify key mediators of ischemic injury.

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INTRODUCTION

Brain ischemia produces tissue injury by two basically different mechanisms, which are broadly referred to as hemodynamic and molecular, respectively (Dirnagl et al. 1999, Lipton 1999). Hemodynamic injury results from the decline of blood flow below the threshold of energy failure, which in rodents amounts to approximately 20% of control flow and is histologically manifested as primary necrosis (Hossmann 1994). Molecular injury evolves in the absence of impaired energy metabolism, e.g. in the penumbra range of reduced blood flow during permanent vascular occlusion (Siesjö et al. 1995), or in areas in which energy metabolism recovers after transient vascular occlusion. Examples of molecular injury are delayed neuronal death in the selectively vulnerable areas of the brain after brief global ischemia (Kirino et al. 1985), or delayed brain infarction after longer than 30 minutes of transient focal ischemia (Du et al. 1996). Histologically, this type of injury may be manifested as necrosis, apoptosis or a combination of both (Asahni et al. 1997, Martin et al. 1998, Nicotera et al. 1999, Zeng and Xu 2000).

Intensive research into the mechanisms of molecular injury has led to the identification of a great number of injury pathways, which in specially sensitized experimental models can be alleviated by a multitude of pharmalogical interventions (Dirnagl et al. 1999, Kuroda and Siesjö 1997, White et al. 2000). However, all of these drugs failed to improve ischemic brain damage under clinical conditions, indicating that the key mediator of this type of injury remains elusive (Grotta 1995, Zoppo 1995).

Searching for hitherto unknown molecular injury pathways, differential gene profiling techniques are increasingly used (Jin et al. 2001, Read et al. 2001, Wang H et al. 2001, Wang XK et al. 1998). cDNA microarray studies from various laboratories suggest that the number of genes up- or downregulated after ischemia at the transcriptional level is very high, and even if only those genes are considered that are regulated by a factor of more than 10, still close to 7% of the genome are involved. However, a large part of these changes is only indirectly linked to the disease process. As molecular ischemic injury is heralded by an inhibition of global protein synthesis, many genes are superinduced due to the release of feedback control (Altus et al. 1987, Franco et al. 1990). Others respond to peri-infarct depolarization which leads to the sharp upregulation of various immediate-early genes (Kiessling and Gass 1994). Finally, the breakdown of the energy state in the core of the ischemic territory results in inhibition of transcription which may be misinterpreted as active downregulation. A more reliable association between molecular changes and injury evolution can, therefore, be expected when changes of gene products are analyzed at the translational level.

With the development of multi-Western blot techniques, such investigations have become feasible with high throughput. Here, we describe a study in which we take advantage of this new technology for the analysis of injury evolution after one-hour transient focal brain ischemia in the mouse.

METHODS

Animal preparation

Focal cerebral ischemia was induced in C57Black/6J mice by occluding the MCA with the intraluminal filament technique (Hata et al. 1998). Under light halothane anesthesia, a midline neck incision was made and the common and external carotid arteries were isolated and ligated. An 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren, Bayer Dental, Osaka, Japan) was introduced through a small incision into the common carotid artery and advanced about 9 mm distal to the carotid bifurcation for occlusion of the MCA. The diameter of the silicon tip was matched to the body weight of the animal (Hata et al. 1998), and the correct position of the filament was identified by recording Laser-Doppler Flow (LDF) from the ipsilateral parietal cortex. Recirculation was initiated at 1 h after MCA occlusion by removal of the intraluminal thread. Only those experiments were included for further analysis in which LDF declined to below 20% of control during thread insertion and promptly returned to above 80% of control after thread withdrawal. During surgery and up to the beginning of recirculation, rectal temperature was kept constant at 37°C, using a feedback controlled heating lamp. For longer survival times, animals were returned to the cages with water and food *ad libitum*.

The animal experiments were done in accordance with the animal protection guidelines approved by the governmental authorities within approved animal experiment proposals.

Multi-Western blots

Measurements were carried out in sham-operated controls, 1 h after MCA occlusion and at 3 h and 12 h reperfusion following 1 h MCA occlusion. Animals were sacrificed under anesthesia by decapitation. Brains were immediately removed, and ipsilateral and contralateral hemispheres were homogenized in lysis buffer (10 mM Tris, pH 7.4; 1 mM sodium ortho-vanadate, 1% SDS). After adding an equal volume of electrophoresis buffer (125 mM Tris, pH 6.8; 4% SDS; 10% glycerol; 0.006% bromophenol blue; 2% b-mercaptoethanol) samples were heated at 95°C for 3 minutes. Electrophoresis was performed by BD PharMingen (Heidelberg) in triplicate on pooled samples from 6 animals in 5–15% gradient SDS-polyacrylamide gels (0.1 × 16 × 16 cm). By using such a gradient system a wide range of proteins can be detected on the same gel (Fig. 1). Four-hundred micrograms of protein was loaded in one big well across the entire width of the gel. This translates into ~15 μg per lane on a standard 25 well gel. Gels were run overnight at room temperature and constant current in the mA range. Applying a wet electrophoretic transfer apparatus (TE Series, Hoefer) gels were then transferred to Immobilion-P nylon membrane (Millipore) for 1 hour at 1 A. After transfer, membranes were blocked for one hour with 5% milk powder. Next, membranes were clamped with a Western blotting manifold that isolates 45 channels across the membrane. In each channel, a complex antibody cocktail (BD Biosciences, Heidelberg, Germany) is added and allowed to hybridize for one hour. Blots were removed from the manifold, washed and hybridized for 30 minutes with secondary goat anti-mouse horseradish peroxidase. Membranes were washed and bands were visualized by chemiluminescence. Data analysis includes raw and normalized digital data from each blot.

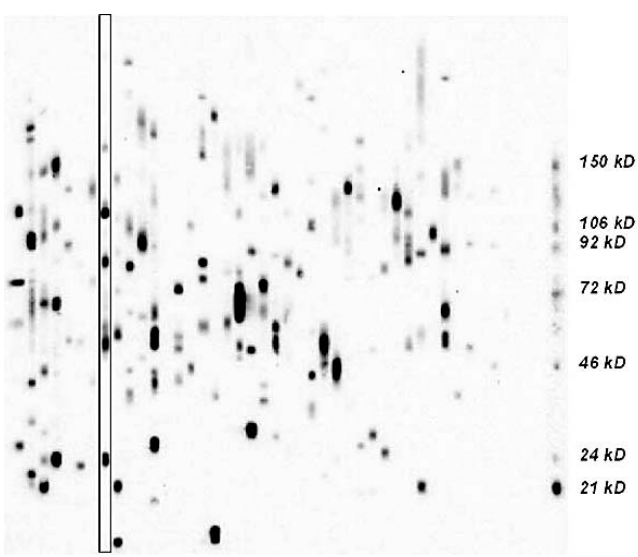

Fig. 1. Multi-Western blot of proteins extracted from mouse brain after 1 h MCA occlusion. Proteins are arranged in 45 lanes per template, using up to 6 antibodies per lane for simultaneous detection of proteins with widely differing molecular weights. This results in simultaneous detection of up to 270 proteins per template. Proteins detected in one lane are exemplarily shown for lane 9 by framing.

RESULTS

The evolution of ischemic injury after one hour MCA thread occlusion in C57Black/6J mice has been described before (Hata et al. 2000, Trapp et al. 2001). At the end of ischemia, both energy metabolism and protein biosynthesis are severely suppressed in the territory of the occluded MCA, the area of the cerebral protein synthesis (CPS) disturbance at the coronal level of caudate/putamen being about 50% larger than that of energy failure. After restoration of blood flow energy metabolism promptly recovers. CPS returns more slowly after recirculation times of more than six hours and is restored only in the most peripheral parts of the MCA territory, but never in the core region. In the area of persisting CPS, ATP secondarily fails, starting at three hours in the core of the MCA territory and then gradually expanding towards the more peripheral parts.

Secondary post-ischemic energy failure is accompanied by DNA fragmentations, as demonstrated by *in situ* nick translation (ISNT) for single and terminal-deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) for double-strand breaks. Single-strand breaks may become visible already at one hour after the beginning of recirculation, occasionally preceding energy failure (Mies et al. 2001, Trapp et al. 2001). In contrast, double-strand breaks either accompany or follow the secondary depletion of ATP (Hata et al. 2000).

Protein extracts were prepared from the ipsilateral (ischemic) and the contralateral (non-ischemic) hemisphere at the end of one hour MCA thread occlusion, and after 3 and 12 hours recirculation, respectively. Multi-Western blots (Fig. 1) were obtained from over

400 proteins, covering a wide spectrum of gene products associated with different physiological and pathological cell functions.

Proteins were considered to be regulated during or after ischemia when the change – compared to sham-operated controls – exceeded a factor of at least 1.5 in at least two of the three runs. Using this criterion, up to 45% of proteins were regulated in the ipsilateral and up to 49% in the contralateral hemisphere (Fig. 2). At the end of one-hour ischemia, the percentage of up- and downregulated proteins was 16% and 20% in the ischemic, and 19% and 15% in the non-ischemic hemisphere, respectively. During recirculation (12 h), both the number of upregulated proteins (29%) and downregulated proteins (20%) increased in the non-ischemic hemisphere, but in the ischemic hemisphere the up- and downregulated proteins shifted inversely in accordance with the evolution of secondary injury (3 h recirculation: 13% and 24%, 12 h recirculation: 12% and 33% for up- and downregulation, respectively). The temporal dynamics of regulation differed greatly for different proteins, with all combinations of early or late up- or downregulation being possible. In the following we refer to downregulation if at one or more time points protein concentration decreased as compared to the sham-operated controls (excluding upregulations at other times) and to upregulation if at one or more time points protein concentration increased (including downregulations at other times).

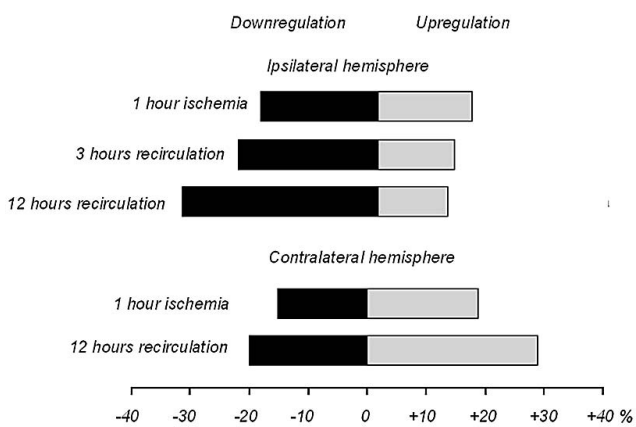

Fig. 2. Diagramatic representation of protein regulation after 1 h MCA occlusion, followed by 3 and 12 h recirculation in the ischemic hemisphere and 12 h recirculation in the non-ischemic hemisphere. Regulation was defined as a change by the factor of at least 1.5 as compared to sham-operated controls in at least two of the triplicate runs. Note inverse shift of up- and downregulated genes in the ischemic hemisphere during recirculation, and the high number of both, up- and downregulated genes in the contralateral hemisphere.

Regulated proteins could be grouped into functionally related classes. Not surprisingly, both up- and downregulations were present in most protein classes, but a few groups deviated from this rule: in the ischemic hemisphere all proteins classified as protein phosphatases were upregulated whereas the majority of protein kinases and associated proteins were downregulated. There was also a remarkably high incidence of downregulation among proteins associated with cell adhesion, extracellular matrix and synaptic transmission whereas all proteins related to neurodegeneration and all but one protein involved in cell splicing were upregulated.

In Fig. 3 the temporal regulation profile of several proteins in the ischemia-affected hemisphere after 1 h

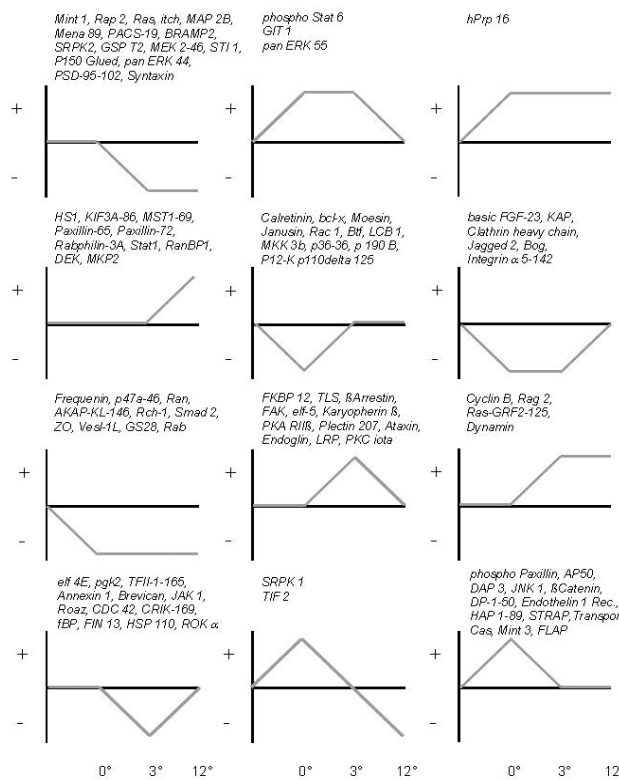

Fig. 3. The temporal regulation of selected proteins in the ischemia-affected hemisphere after 1 h MCA thread occlusion and 0, 3, and 12 hours of reperfusion is described. Proteins that show the same regulation are grouped and were considered to be regulated when the change – compared to sham-operated controls – exceeded a factor of at least 1.5 in at least two of the three runs. Upregulation of immunoreactivity of a given protein is represented by plus and downregulation by minus. The nomenclature of the proteins is according to the used antibodies (BD Biosciences, Heidelberg, Germany) that in some cases detect post-translational alterations (e.g. phospho Stat 6).

MCA thread occlusion is described. Proteins that show the same regulation at the end of cerebral ischemia and after 3 and 12 hours of recirculation are grouped.

DISCUSSION

The reproducibility and reliability of the multi-Western blot approach was tested by triple repetition and by comparing the multi-blot measurements of selected proteins with conventional Western blots. Although the sensitivity of measurements varied in each of the three runs, only few of the triplicate recordings exhibited incoherent results, as compared to the sham-operated controls, demonstrating that the sign of expression – up- or downregulation – could be determined with high reproducibility. The reliability of measurements was also supported by the conventional Western blots which were in full agreement with multi-blot data.

The present results also agree well with previously published data. Although few of the investigated proteins have been studied under similar experimental conditions before, most of these were regulated in a similar way. Examples of concordant alterations are the upregulation of hypoxia-inducible factor-1 (HIF-1) (Bergeron et al. 1999), phosphotyrosine (Bolay et al. 2002), cyclin (Campagne and Gill 1998) or the signal transducer and activator of transcription STAT3 (Planas et al. 1996). For some proteins such as heat shock protein Hsp70 (Massa et al. 1995), ubiquitin (Ubc) (Noga and Hayashi 1996) or synapsin (Bolay et al. 2002) either the present or the earlier measurements failed to document significant changes.

An unexpected result was the large number of proteins that were up- or downregulated in the opposite, non-ischemic hemisphere. It has been established that acute focal ischemia evokes depolarizations which spread into the peri-infarct tissue and which lead to the upregulation of immediate early genes that function as transcription factors for a great number of other genes (Hata et al. 2000, Kiessling and Gass 1994). However, this response is strictly confined to the ipsilateral hemisphere and never spreads to the opposite side. The pronounced contralateral response must, therefore, be due to another mechanism, the kind of which remains to be clarified. It would be tempting to speculate that this phenomenon is associated with brain plasticity and/or transhemispheric diachisis but other – possibly unspecific – reactions are equally possible.

CONCLUSIONS

At the present state of the analysis, it is difficult to predict if this approach leads to the expected identification of a key mediator of ischemic injury. However, the diversity of the changes observed clearly demonstrates, that high-throughput screening methods are the only way to explore the full spectrum of pathophysiological changes and, therefore, are mandatory for approaching this goal. The investigation of the spatio-temporal regulation of selected proteins by immunohistochemistry has to clarify which of the proteins that show an interesting regulation pattern in our study are really associated with neuronal death or survival.

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