The Onset of Apoptosis of Neurons Induced by Ischemia-Reperfusion Injury Is Delayed by Transient Period of Hypertension in Rats

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Summary
We investigated the potential neuroprotective effect of transient hypertension on neuronal cell death induced by ischemia-reperfusion. Recovery of neurons, terminally differentiated cells, is almost entirely dependent upon active transcription and repair of DNA damage. We focused on the histochemical detection of distribution of NOR (argyrophilic nucleolar proteins) reflecting nucleolar integrity, immunohistochemical detection of PARP-1 (poly(ADP-ribose) polymerase-1), MADD (mitogen-activated death domain), a protein accumulated in nucleoli upon stimulation by ischemia, the active form of caspase-3, a universal proteolytic enzyme of apoptosis. The terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick-end-labeling method (TUNEL) proved the presence of in situ DNA fragmentation. We used the model of transient focal cerebral ischemia in rats with occlusion of middle cerebral artery. In experimental group of rats, the transient hypertension was induced by constriction of the abdominal aorta. The period of ischemia lasted 15, 30, 60 and 120 min followed by 48 h of reperfusion. We examined the frontal lobe of the ipsilateral hemisphere for apoptosis of neurons and compared it with the intact brain tissue. In normotensive rats with transient focal cerebral ischemia, we found disintegrated nucleoli of cortical as well as subcortical neurons at all investigated periods of ischemia, whereas the neurons of intact animals showed compact nucleoli with a few satellites. Nuclear positivity for MADD and PARP-1 was apparent in the neocortex after 15 min and peaked after 30 min of ischemia. On the other hand, the subcortical neurons showed nuclear positivity after 60 and 120 min. The immunohistochemical reaction for active caspase 3 was apparent after 30 min onwards predominantly in the cortex. The TUNEL staining was distinct after 60 and 120 min. In hypertensive rats, we found disintegrated nucleoli of cortical as well as subcortical neurons at all investigated periods of ischemia, whereas the neurons of intact animals showed compact nucleoli with a few satellites. Nuclear positivity for MADD and PARP-1 and caspase 3 was apparent after 30 min of ischemia. On the other hand, the subcortical neurons showed nuclear positivity after 60 and 120 min. The immunohistochemical reaction for active caspase 3 was apparent after 30 min onwards predominantly in the cortex. The TUNEL staining was distinct after 60 and 120 min. In hypertensive rats, we found disintegrated nucleoli of cortical as well as subcortical neurons after 60 and 120 min. In summary, we detected delayed activation of neuronal apoptosis in transiently hypertensive rats with focal cerebral ischemia compared to normotensive animals. The apoptotic phenotype was confirmed by a panel of complementary methods showing rapid proteolysis-nucleolar segregation, MADD, PARP-1 and caspase-3 positivity as well as ultimate DNA fragmentation proved by the TUNEL assay.

Key words
Neuroprotection • Hypertension • MADD • PARP-1 • Caspase-3 • Apoptosis
**Introduction**

The precise mode of cell death after cerebral ischemia and reperfusion is still controversial. However, evidence has been mounting that neuronal death, including ischemia-induced death, occurs via apoptosis as well as necrosis (Longa et al. 1989). Apoptosis may represent a different mode of cell death in cerebral ischemia, which may have implications for stroke therapies. Necrosis predominates in more intense forms of ischemic damage, whereas apoptosis may occur in milder forms of ischemia. In addition, unlike the acute damage of necrosis, apoptotic injury may take time to develop. Therefore, demonstrating the presence of apoptosis as the result of ischemia-reperfusion injury may expand the therapeutic window for stroke by potentially making available delayed neuroprotective interventions aimed at blocking the apoptotic cascade.

Recent evidence demonstrates that apoptosis involves the activation of caspases, a growing family of cysteine proteases, which specifically target proteins possessing a characteristic tetrapeptide motive cleaving them precisely after aspartate residues. Procaspases can undergo autocatalysis or cleave other caspase zymogens, thus initiating an orchestrated cascade of events triggering several unique morphological changes such as chromatin condensation or nuclear and nucleolar fragmentation (Sahara et al. 1999, Horký et al. 2001). The nucleolus represents a highly dynamic nuclear compartment, easily accessible for light microscopic visualization. Nucleolar morphology can be evaluated histologically using the silver staining method for nucleolar organizer regions (NORs). NORs are loops of ribosomal DNA within the nucleus that transcribe ribosomal RNA and are usually tightly aggregated within the nucleoli in interphase cells. Currently, the morphological description of apoptosis is characterized by a segregation of the nucleolus (Reipert et al. 1999).

PARP-1 (poly(ADP-ribose) polymerase-1), an ubiquitous nuclear enzyme, localized in nucleolus, is a unique sensor of DNA breaks (reviewed by D’Amours et al. 1999). This property to detect even negligible DNA lesions is important for protection of cells from DNA damage and for the maintenance of genome integrity.

We visualized MADD (mitogen-activated death domain) translocation to the nucleolus upon ischemia in neurons. MADD was identified as a substrate for c-Jun N terminal kinase. The evidence for the direct involvement of JNK in apoptosis comes from several studies on PC12 cells as an overexpression of a constitutively activated JNK kinase potentiates apoptosis induced by nerve growth factor (NGF) deprivation. Conversely, microinjection of a c-Jun-dominant negative mutant into rat sympathetic neurons protects the cells against apoptosis (Zhang et al. 1998). Based on the relationship between JNK3 activation and the neuronal stresses of hypoxia/ischemia in the human central nervous system, we used the immunohistochemical detection of MADD as a marker of active neuronal cell death.

In spite of the fact that a variety of cell death assays is available, an accurate and a neuron-specific apoptotic marker has not yet been revealed. We took the advantage of the accessibility of nucleoli for light microscopic examination in the neurons. We focused on the distribution of argyrophilic nucleolar proteins and associated their disintegration with nuclear positivity for MADD, PARP-1 reflecting ischemic injury. The presence of an irreversible phase of apoptosis was detected by active caspase-3 and TUNEL assay. Our results suggest a link between nucleolar morphology of terminally differentiated neurons and the onset of active cell death thus enabling to assess an early stage of apoptosis. We analyzed a potential neuroprotective effect of transient hypertension based upon prevention of neuronal apoptosis.

**Methods**

Male Wistar rats (Charles River) weighing 290-350 g were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines. The rats fasted overnight before the day of the experiment, but were allowed free access to tap water. The rats, anesthetized with chloral hydrate (400 mg/kg i.p.), were subjected to minimum regional ischemia. In brief, the middle cerebral artery was occluded by an occluder filament (made of a nylon monofilament thread 0.28 mm in diameter). The right common, internal, and external carotid arteries were identified through a cervical midline incision. The external carotid artery and the occipital artery (one of the branches of the external carotic artery) were ligated by a 3-0 silk suture. The pterygopalatine artery was encircled with a suture and retracted to the left side of the animal to prevent incorrect insertion of the occluder. The internal carotid artery was then temporarily closed by a microvascular clip and the common carotid artery was closed by a suture in 3-mm
proximity to the carotid bifurcation. A small incision was
made in the common carotid artery 1 mm to the carotid
bifurcation and the middle cerebral artery occluding
device was inserted from the right common artery approx.
7 mm into the internal carotid artery. After removing the
microvascular clip, the occluder filament was advanced
11-12.5 mm depending on the animal weight to block the
origin of the MCA. For recirculation, the occluder
filament was pulled out from the sutured wound.

Immunohistochemistry for PARP-1 and MADD
was performed with human anti-PARP-1 (Alexis) and
anti-MADD. Sections were dewaxed and rehydrated, then
microwaved in 0.01 M sodium citrate buffer (pH 6.0), the
buffer being brought to boiling point twice over a 5 min
period. Non-specific peroxidase activity was blocked by
immersing the sections for 30 min into methanol
containing 3 % H2O2. They were then incubated first in
20 % normal serum and then overnight at room
temperature in either of the primary antibodies (anti-
PARP-1 diluted 1:1000 in PBS with 10 % BSA). Bound
antibody was visualized by incubation with horseradish
peroxidase conjugated anti-mouse/rabbit immunoglobulin
diluted 1:400 in PBS with 10 % BSA; Amersham) and
reaction with 0.01 % H2O2 and diaminobenzidine.
Negative controls comprised sections immunostained as
above apart from omission of the primary antibody.

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**Fig. 1.** The percentage of neurons (cortical, subcortical) showing disintegrated nucleoli (AgNOR), immunoreactivity for
MADD, PARP-1, caspase-3 and TUNEL positivity after transient period of ischemia followed by reperfusion. Two
hundreds randomly selected cells were evaluated in each ipsilateral hemisphere (4 rats in each group).

**Fig. 2.** The percentage of neurons (cortical, subcortical) showing disintegrated nucleoli (AgNOR), immunoreactivity for
MADD, PARP-1, caspase-3 and TUNEL positivity after transient period of ischemia followed by reperfusion in
hypertensive rats. Two hundreds randomly selected cells were evaluated in each ipsilateral hemisphere (4 rats in each
group).
Fig. 3. Progressive, irreversible damage to cortical neurons after 60 min ischemia demonstrated by caspase-3 positive cytoplasmic staining (brown) and shrinkage of cellular volume.

Fig. 4. Detailed view of a caspase-3 positive cortical neuron (brown cytoplasmic staining) after 30 min of ischemia.

Fig. 5. A group of PARP-1 positive nuclei (dark brown grains) of a neuron in the thalamus after 120 min of ischemia.
Results

As shown in Figure 1, disintegrated nucleoli were found in cortical as well as subcortical neurons (basal ganglia, thalamus) in all investigated periods of ischemia followed by reperfusion, whereas the neurons of intact animals showed compact nucleoli with a few satellites. Nuclear positivity for MADD and PARP-1 was apparent after 15 min and peaked after 30 min. On the contrary, subcortical neurons showed MADD positivity after 60 and 120 min of ischemia. The immunohistochemical reaction for active caspase-3 was distinct after 30 min onwards predominantly in the cortex. Similarly, the TUNEL staining revealed DNA fragmentation in neurons after 60 and 120 min of ischemia. In hypertensive rats (Fig. 2), we found nucleolar disintegration, positivity for MADD, PARP-1 and caspase-3 (Figs. 3-5) after 30 min cortically and subcortically followed by TUNEL positivity of cortical neurons after 60 and 120 min. On the contrary, all the apoptotic markers were negative in neurons of hypertensive rats after 15 min of ischemia.

Discussion

Our data demonstrated a rapid onset of active neuronal cell death induced by ischemia (15 min) and reperfusion (48 h) detected by a panel of complementary approaches. A transient period of hypertension postponed the onset of active neuronal cell death in the model of ischemia-reperfusion injury but did not prevent it.

Visualization of the nucleolus, a highly dynamic nuclear compartment, proved to be a sensitive technique enabling us to reveal discrete nuclear changes of chromatin in the damaged neurons. Nucleolar segregation in cortical and subcortical neurons coincided with nuclear and nucleolar accumulation of MADD, a stress-activated neuronal specific death domain. Death receptors (e.g. TNF-R1) are characterized by the presence of a death domain within the cytoplasmic region and have been shown to trigger apoptosis upon binding of their cognate ligands (Stegh et al. 1998). Immunohistochemical detection of a death domain translocation from cytoplasm to nucleolus provides a new insight into the early phase of active cell death of terminally differentiated neurons. It has been shown that the nucleolar translocation of death domain was accompanied by inhibition of rDNA transcription in vitro.

Poly (ADP-ribose) polymerase-1 (PARP-1), an ubiquitous nuclear enzyme, is a unique sensor of single-stranded DNA breaks. Immunostaining of several cell lines and tissue sections revealed that PARP-1 is not randomly distributed within the nucleus, but has been shown to be localized in three well-defined subnuclear compartments: the chromatin in the internucleosomal space, the nuclear matrix and the nucleolus (reviewed by D’Amours et al. 1999). Activated caspase-3 recognizing a DEVD tetrapeptide motif within the amino-terminal domain of PARP-1 generates two proteolytic products, a 29 kDa amino-terminal fragment encompassing DNA-binding domain and 89 kDa carboxy-terminus harboring the substrate binding pocket. The caspase-3-induced PARP-1 cleavage separates the DNA-binding domain essential for the stimulation of the enzyme and catalytic domain. A later onset of caspase 3 nuclear positivity in neurons (30 min cortically and 60 min subcortically) may be due to a slower accumulation of the active form of the enzyme (Chen et al. 1998). These results are in accordance with previous experiments showing that the rapid onset of caspase-3 activation plays a crucial role in the execution of active cell death in neurons (Pulera et al. 1998, Velier et al. 1999, Guegan and Sola 2000) and its inhibition represents an attractive target for pharmacotherapy of brain ischemia (Gillardon et al. 1997, Gillardon et al. 1999).

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