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POST-ISCHEMIC DELIVERY OF THE 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE INHIBITOR ROSUVASTATIN PROTECTS AGAINST FOCAL CEREBRAL ISCHEMIA IN MICE VIA INHIBITION OF EXTRACELLULAR-REGULATED KINASE-1/-2

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Abstract—After recent clinical trials, statins have gained increasing significance in secondary stroke prevention. From experimental studies, it is well established that statins have beneficial action when delivered prophylactically prior to a stroke. Conversely, much less is known about the effects of statins on injury development when delivered after ischemia. We here examined the effects of a post-ischemic delivery of rosuvastatin (0.5, 5 or 20 mg/kg, administered i.p. immediately after reperfusion onset), a potent 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, on brain injury and cell signaling after focal cerebral ischemia, induced by 90 min of intraluminal middle cerebral artery occlusion in mice. In animals receiving normal saline, 0.5 or 5 mg/kg rosuvastatin, middle cerebral artery occlusions resulted in reproducible brain infarcts at 24 h after reperfusion onset, which did not differ in size. However, rosuvastatin, administered at higher doses (20 mg/kg), reduced infarct volume at 24 and 48 h after ischemia (by 34±16% and 18±3%, respectively, $P<0.05$). Western blots revealed that rosuvastatin decreased phosphorylated extracellular-regulated kinase-1/-2 and reduced activated caspase-3 levels in ischemic brain areas, while endothelial NO synthase expression, p38 and Jun kinase phosphorylation were not influenced by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Rosuvastatin also significantly diminished expression levels of inducible NO synthase in the ischemic brain. Our results indicate that rosuvastatin may have utility not only as stroke prophylaxis but also as acute therapy inhibiting executive cell death pathways.

Key words: statin, neuroprotection, signal transduction.

Clinical trials in the last decade have shown the safety and efficacy of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (so-called statins) in reducing the risk for coronary heart disease and ischemic stroke (Scandinavian Simvastatin Survival Study Group, 1994; Plehn et al., 1999; White et al., 2000). While initial studies focused on patients with elevated plasma cholesterol levels (Scandinavian Simvastatin Survival Study Group, 1994), it has been demonstrated more recently that patients with normal or low cholesterol levels similarly profit from statin therapy (Heart Protection Study Collaborative Group, 2002). The latter observations considerably expanded the use of statins in secondary stroke prevention.

In ischemic stroke, statins exert their beneficial action not only via reduction of plasma cholesterol levels. Experimental studies have shown that statins ameliorate endothelial dysfunction, reflected by an upregulation of endothelial NO synthase (eNOS) (Endres et al., 1998; Laufs et al., 1998; Hernandez-Perera et al., 1998; Kaesemeyer et al., 1999) and, in addition, might also have direct neuroprotective actions (Zacco et al., 2003). In focal cerebral ischemia, the efficacy of statins, including rosuvastatin, has repeatedly been shown (e.g. Endres et al., 1998; Laufs et al., 2002). Yet, in all latter studies, statins were delivered prophylactically over several days *prior* to stroke.

To date, only one study has examined the effects of an acute *post-ischemic delivery* of statins. In that study, actions of simvastatin were evaluated in a model of permanent cortical ischemia, induced by transcranial electrocoagulation of the middle cerebral artery (MCA) in rats (Sironi et al., 2003). Simvastatin significantly reduced infarct size, both when applied at low (0.2 mg/kg) and higher (2–20 mg/kg) doses (Sironi et al., 2003). This finding is very interesting and it may be of clinical relevance, since statins are well tolerated in humans and may safely be administered in acute stroke patients. Unfortunately, the mechanisms mediating the acute neuroprotective effects of statins remained insufficiently understood.

To elucidate these mechanisms, we here examined the effects of the synthetic HMG-CoA reductase inhibitor rosuvastatin on ischemic injury following transient focal ischemia induced by intraluminal MCA occlusions. Rosuvastatin is a very potent statin, which has recently become available (McKenney et al., 2003; Blasetto et al., 2003; Jones et al., 2003). Two reasons encourage usage of rosuvastatin in stroke therapy: First, rosuvastatin, in contrast to most other statins (except for pravastatin, which has low potency), does not require hepatic metabolism for activation (McTaggart et al., 2001; Martin et al., 2003). Thus, rosuvastatin has very rapid bio-availability, which is crucial in a quickly developing pathology like stroke. Secondly, rosuvastatin appears to have particularly prominent neuroprotective actions, which may be superior to other

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Abbreviations: ECL, enhanced chemoluminescence; eNOS, endothelial NO synthase; ERK, extracellular-regulated kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; iNOS, inducible NO synthase; JNK, Jun kinase; LDF, laser Doppler flow; MAP, mitogen-activated protein; MCA, middle cerebral artery; TBS, Tris-buffered saline.

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statins, based on recent *in vitro* findings (Zacco et al., 2003).

To elucidate the actions of rosuvastatin, we made use of immunohistochemical techniques, analyzing expression and activation patterns of the mitogen-activated protein (MAP) kinases extracellular-regulated kinase (ERK)-1/-2, p38 and Jun kinase (JNK), of the executioner caspase-3, of eNOS and inducible NO synthase (iNOS).

EXPERIMENTAL PROCEDURES

Induction of MCA thread occlusions

Experiments were carried out according to Nation Institutes of Health guidelines for the care and use of laboratory animals and approved by local government authorities. Efforts were done in order to minimize animal suffering. Adult male C57BL/6 mice (20–25 g) were anesthetized with 1% halothane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. Focal cerebral ischemia was induced using an intraluminal filament technique (Hata et al., 2000; Hermann et al., 2001a; Kilic et al., 2002; Wang et al., 2005). Briefly, the left 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 into the common carotid artery and advanced 9 mm distal to the carotid bifurcation, thereby interrupting MCA blood flow (diameter of thread: 180–200 μm).

Ninety minutes after occlusion, reperfusion was initiated by thread withdrawal. Immediately thereafter, 0.5 ml of normal saline or 0.5 ml of normal saline containing rosuvastatin (0.5, 5 or 20 mg/kg b.w.) was injected intraperitoneally. During the experiments, cerebral blood flow was monitored by laser Doppler flow (LDF) measurements using a flexible 0.5 mm fiberoptic probe (Perimed, Stockholm, Sweden), which was attached to the intact skull overlying the MCA territory (2 mm posterior/6 mm lateral from bregma). LDF changes were recorded during ischemia and up to 30 min after reperfusion onset. At that time, anesthesia was discontinued and animals were placed back into their cages.

After 24 or 48 h of reperfusion, mice were re-anesthetized with halothane and decapitated. Brains were removed and immediately frozen with dry ice. Brains were cut into 18 μm cryostat sections. From additional animals killed at 24 h after ischemia, tissue samples were retrieved both from the ischemic and contralateral non-ischemic parietal cortex for Western blots. In animals subjected to 90 min ischemia followed by 24 h of reperfusion, few animal dropouts were seen (less than 10% of animals spontaneously died). In animals killed 48 h after ischemia, however, some animal deaths were noted in the normal saline-treated group (three of nine control mice died, all animals exhibiting severe brain edema; in contrast: only one of seven dead animals in the rosuvastatin-treated group).

Cresyl violet staining and analysis of histological injury

Coronal brain sections from five equidistant rostrocaudal brain levels, 2 mm apart, were submitted to Cresyl Violet stainings using a standard histological protocol (n=5–7 animals/group). On the digitized sections, the border between infarcted and non-infarcted tissue was outlined using an image analysis system, and the infarct volume and brain swelling were measured, as previously described (Kilic et al., 2002; Wang et al., 2005).

Measurement of plasma rosuvastatin levels

Further adult male C57BL/6 mice (20–25 g) were anesthetized with 1% halothane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. Rosuvastatin (0.5, 5 or 20 mg/kg) was injected intraperitoneally (n=4 animals/dose). Thirty minutes later, mice were decapitated and blood was taken from the animals' trunks. Blood samples were supplemented with EDTA and centrifuged. To the plasma, equal amounts of 0.1 M sodium acetate (pH 4.0) were added. These samples were analyzed, as previously described (Hull et al., 2002).

Western blots

Tissue samples obtained from the ischemic and contralateral non-ischemic parietal cortex 24 h after reperfusion onset were dissected, complemented with lysis buffer, homogenized and centrifuged (for protocol see also Kilic et al., 2005). Supernatants were used for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples from animals belonging to the same treatment group were pooled (n=4 animals/group). Equal amounts of protein were diluted in 6× sample buffer, boiled and loaded on polyacrylamide gels. Following separation, proteins were transferred onto PVDF membranes. Membranes were dried overnight, incubated in blocking solution and immersed with monoclonal mouse anti-eNOS (610296; BD Biosciences, Basel, Switzerland), mouse anti-phosphorylated ERK-1/-2 (M8159; Sigma, St. Louis, MO, USA) and mouse anti-diphospho-p38 (M8177; Sigma) as well as polyclonal rabbit anti-diphospho-JNK-1/-2 (JNK-2, sc-572; Santa Cruz, Heidelberg, Germany) and rabbit anti-activated caspase-3 (CM1; BD Biosciences) antibodies, each diluted 1:500 in 0.1% Tween 20/0.1 M Tris-buffered saline (TBS). As a loading control, a monoclonal mouse anti-β-actin (A5316; Sigma) antibody was used. Membranes were rinsed, incubated in peroxidase-coupled secondary antibodies (Santa Cruz), diluted 1:2000 in 0.1% Tween 20/0.1 M TBS, washed, immersed in enhanced chemoluminescence (ECL) solution and exposed to ECL-Hyperfilm (Amersham, Braunschweig, Germany). Blots were carried out repeatedly in order to ensure reproducibility of the data.

Immunohistochemistry for iNOS

Brain sections were fixed in 4% paraformaldehyde/0.1 M PBS, washed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton (PBS-T)/10% normal goat serum (NGS). Sections were incubated overnight at 4 °C with polyclonal rabbit anti-iNOS (NOS-2, sc-650; Santa Cruz) antibody, diluted 1:100 in PBS-T. After washing, sections were stained with a Cy-3 labeled secondary antibody, counterstained with DAPI and coverslipped. Sections were evaluated by one of the investigators (Ü.K.) blinded for the experimental condition by counting iNOS (+) cells in a total of six regions of the non-ischemic and ischemic MCA territory. Thus, rectangular fields measuring 62,500 μm^2 were analyzed (see also Hermann et al., 2001b). Subsequently mean values were calculated for all regions.

Statistics

For statistical analyses, a standard software package (SPSS for Windows 10.1, Microsoft) was used. Differences between groups were compared by using one-way ANOVA followed by Scheffé tests (comparisons between ≥3 groups) or two-tailed t-tests (comparisons between two groups). Values are given as means±S.D., n values indicating the number of different samples analyzed. P values less than 0.05 were considered significant.