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Activation of NF-kB and ERK1/2 after permanent focal ischemia is abolished by simvastatin treatment

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We investigated the effects of simvastatin treatment on the expression of IL-1beta and MCP-1, the activity of NF-kB, and the signaling pathways related to NF-kB activation in a rat model of permanent middle cerebral artery occlusion (pMCAO). IL-1beta and MCP-1 expression, determined using RT-PCR, was enhanced by pMCAO; this effect was inhibited by the administration of simvastatin before ischemia. Pre-treatment with simvastatin abolished the ischemia-induced activation of NF-kB observed in vehicle-treated animals. The evaluation of signal transduction pathways, including extracellular signal-regulated kinase (ERK1/2), SAPK/JNK 46/54 and p38, indicated that only ERK1/2 phosphorylation was enhanced by ischemia, and this activation was prevented by simvastatin. ERK1/2-inhibitor, U0126, reduced brain ischemia but not cytokine induction. These results provide evidence that the HMG-CoA reductase inhibitor induces its effect in the protection of ischemic brain damage with a more complex mechanism which also involve anti-inflammatory properties rather than simple inhibition of ERK1/2 signaling pathway.

Keywords: Statin; Inflammation; Cerebral ischemia; Permanent middle cerebral artery occlusion; Transcription factor; MAP kinase; Rat

Introduction

Recent clinical trials have demonstrated that statins [3-hydroxy-3-methylglutaryl (HMG)-Co-A reductase inhibitors], the most widely used lipid-lowering drugs, reduce the incidence of stroke due to primary and secondary events by 25–30% (Amarenco et al., 2004). This putative neuroprotective activity of statins has been validated in animal studies by various investigators (Endres et al., 1998; Sironi et al., 2003; Amin-Hanjani et al., 2001; Laufs et al., 2000): the results obtained in adult rodents indicate that the prophylactic or post-ischemic administration of statins reduces the extent of the brain damage, an effect attributed to the drug's ability to increase the activity of endothelial nitric oxide synthase (eNOS), thus improving cerebral blood flow and reperfusion in the ischemic area (Endres et al., 1998; Sironi et al., 2003). However, it is unlikely that the induction of eNOS activity is the only molecular mechanism involved in neuroprotection because, in a neonatal model of hypoxia/ischemia, although statins improved behavioural and morphological parameters when administered before (but not after) the ischemic insult, they were unable to stimulate eNOS activity (Balduini et al., 2001). The role of inflammation in ischemic brain damage has been reported in humans and various animal models of stroke (Iadecola and Alexander, 2001), and its importance in stroke has been highlighted by observations that anti-inflammatory compounds or the deletion of proinflammatory genes are neuroprotective and by the fact that many mediators of the inflammatory process, such as cytokines and chemokines and their genes, are upregulated after an ischemic insult (Iadecola and Alexander, 2001; Barone and Feuerstein, 1999). Proinflammatory genes are mainly controlled by the transcription factor nuclear factor-kB (NF-kB), which is also upregulated in experimental stroke, although its role in neurodegeneration is still controversial (Nurmi et al., 2004; Schneider et al., 1999; Stephenson et al., 2000; Mattson and Camandola, 2001). One mechanism responsible for NF-kB induction involves the stimulation of the mitogen-activated protein kinase (MAPK) pathways, a family of MAPKs activated by focal cerebral ischemia (Mattson and Camandola, 2001). Prompted by the conflicting findings concerning eNOS activation and the different drug treatment schedules used, in the light of the recently demonstrated pleiotropic (Takemoto and Liao, 2001) or cholesterol-independent effects of statins (including anti-atherosclerotic, anti-inflammatory, anti-thrombotic, and neuroprotective actions), and considering the

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potential role of MAPK in ischemia and inflammation, we investigated the effect of simvastatin administration on the modulation of molecular signals of the inflammatory response, including IL-1beta, monocyte chemotactic protein-1 (MCP-1), NF-kB, and ERK1/2.

Materials and methods

Drug treatment

Simvastatin, kindly provided by Merck Sharp and Dôhme (Rahway, New Jersey), was chemically activated by means of alkaline hydrolysis before subcutaneous injection. The drug (20 mg/kg) was administered 1 h after middle cerebral artery occlusion (MCAO) (post-treatment) or three times (48, 24, and 2 h) before MCAO (pretreatment). U0126 (Sigma, St. Louis, MO) was dissolved in DMSO and injected intravenously 10 min after MCAO at 600 μg/kg (200 μl in 6% DMSO); control animals received vehicle.

Animals and surgery

Male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 180–200 g were allowed food and water ad libitum. The procedures involving the animals and their care at the Department of Pharmacological Sciences of the University of Milan respected the Institution's guidelines, which comply with national and international rules and policies. The rats underwent permanent MCAO as previously described (Sironi et al., 2003; Tamura et al., 1981). Sham-operated animals underwent the same surgical procedure as MCAO rats without electrocoagulation of middle cerebral artery.

Infarct size evaluation

The volume of the brain injury was determined at 2 and 24 h after MCAO by magnetic resonance imaging (MRI) using the trace of apparent diffusion coefficient maps, as previously described (Sironi et al., 2003). The variation in ischemic volume was expressed as a percentage in relation to the mean value in the 2-h group considered as 100%.

RT-PCR analysis

Total RNA was prepared by guanidium thiocyanate denaturation from ischemic cortical hemisphere, ipsilateral to artery's occlusion, collected 24 h after MCAO from vehicle (n = 5) and simvastatin pretreated rats (20 mg/kg/day administered 48, 24, and 2 h before MCAO; n = 5). The expression of MCP-1 and IL-1beta mRNA was determined by semi-quantitative RT-PCR as previously described (Balduini et al., 2003; Kim et al., 1995). The RT-PCR products were separated on 1.5% agarose gel, and the intensity of each band was quantified using NIH Image software and expressed in arbitrary units. The densities of the MCP-1 and IL-1beta bands were normalized using the corresponding GAPDH signal.

Preparation of nuclear extracts from rat cerebral cortex

Time-dependent nuclear factors binding activity was evaluated using ipsi- and contralateral cerebral cortex of rats sacrificed immediately (time 0; n = 6) or 3, 6, 16, and 24 h after artery occlusion (n = 6 each time point). In a separate set of experiments, the effects of pre (48, 24, and 2 h before MCAO)- and post-treatment (1 h after MCAO) with simvastatin (20 mg/kg) were determined 16 h after MCAO and compared with vehicle-treated animals. The nuclear extracts were prepared as described by Cercek et al. (1997). 2-Mercaptoethanol (5 mmol/l) and the protease inhibitors leupeptin (0.7 μg/ml), aprotinin (16.7 μg/ml), and PMSF (0.5 mmol/l), were added to all buffers just before use. The rat brains were minced in cold PBS and homogenized in ice-cold hypotonic lysis buffer (10 mmol/l Tris, pH 7.3, 10 mmol/l KCl, 1.5 mmol/l MgCl2, and 0.4% Nonidet P-40). After centrifugation at 9000 × g for 1 min, the pellet was washed in 20 mmol/l KCl buffer. Isolated nuclei were resuspended in 150 μl of 20 mmol/l KCl buffer, and 600 μl of 600 mmol/l KCl buffer was added. Nuclear proteins were extracted by incubation on ice for 30 min. After centrifugation at 9000 × g (4°C) for 15 min, the supernatant containing nuclear proteins was transferred to a precooled microcentrifuge tube. An aliquot of the extract was diluted 40 times with 484 mmol/l KCl buffer (mixture of 20 mmol/l KCl buffer and 600 mmol/l KCl buffer to give the same glycerol and salt concentrations as in the undiluted nuclear extracts) for the protein assay. The protein concentrations were determined spectrophotometrically.

Nuclear factors binding assays

NF-kB, c-Fos, c-Jun, NF-YA DNA binding activities were assessed using Trans-AM transcription factor assay kits (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. Five or ten micrograms of brain nuclear extracts was added to 96-well plates coated with an oligonucleotide containing the nuclear factors consensus site. The binding of NF-kB to DNA was visualized by means of anti-p65 antibody, which specifically recognizes activated NF-kB. Antibody binding was measured using a luminometer. The specificity of nuclear factors activation was determined by competition experiments using wild-type and mutant consensus oligonucleotides provided with the kit. ELISA was preferred to EMSA because it is more sensitive (Joussen et al., 2002; Kretz-Remy et al., 2001).

Signal transduction molecule immunoblotting

Immunoblotting was carried out on ipsi- and contralateral cerebral cortex collected immediately, 30 min, 1, 2, and 3 h after MCAO (n = 3 each h). The effects of pre- and post-treatment with simvastatin were determined 2 h after MCAO. The tissues were washed in cold PBS and homogenized in ice-cold buffer containing 10 mmol/l Tris, 10 mmol/l EDTA, 1 mmol/l EGTA, and protease inhibitors. The samples were briefly sonicated and centrifuged at 18,000 × g for 5 min at 4°C. The supernatants were collected and stored until use at −80°C. Equal amounts of protein were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Western blot analysis was performed using antibodies (1:1000 dilution in TBST containing 5% milk) against phospho-specific MAPK family members (anti-phospho-SAPK/JNK 46/54, anti phospho-p38 and anti-phospho-ERK1/2, from Cell Signaling Technology, Beverly, MA). The positive control for phospho-p38 was obtained by challenging human umbilical vein endothelial cells (HUVEC) for 5 min with TNFα 10 ng/ml. After incubation with horseradish peroxidase-conjugated secondary antibody, the