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Neurobiology of Disease

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Neurobiology of Disease 22 (2006) 445 – 451

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

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Received 5 August 2005; revised 5 December 2005; accepted 15 December 2005
Available online 9 February 2006

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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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.nbd.2005.12.004

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

blot was developed using the Amersham ECL System. Band intensities were quantified by densitometric scanning using a system incorporating a video camera and a computer analysis package (NIH Image). The results are expressed in arbitrary optical density units.

Statistical analysis

The data are expressed as mean values ± SE. Statistical differences were evaluated by means of the StatView program, using analysis of variance (ANOVA) followed by Fisher's or Scheffé's test. A P value of <0.05 was considered significant.

Results

To evaluate whether the previously described beneficial effect of statin treatment on cerebral ischemia can be at least partially attributed to its anti-inflammatory action, we examined the activity of simvastatin on modulators of the inflammatory response after pMCAO. Fig. 1 shows the expression of IL-1beta and MCP-1 24 h after MCAO (when both have reached peak expression) (Kim et al., 1995; Berti et al., 2002) in the ipsilateral cerebral cortex of animals treated three times (48, 24, and 2 h) with either vehicle or simvastatin before the artery's occlusion. In agreement with other studies, the expression of these biochemical markers of the inflammatory response increased several fold in the injured hemisphere of the vehicle-treated animals as compared with undetectable amount found in the contralateral side. However, the administration of simvastatin for 3 days before pMCAO markedly attenuated the ischemia-induced mRNA expression of IL-1beta (−62.5%, $P < 0.05$) and MCP-1 (−62.0%, $P < 0.05$) (Fig. 1). As some of these molecular modulators of inflammation are controlled by transcription factor NF-kB, we determined the effect of simvastatin treatment on NF-kB activation in ischemic animals.

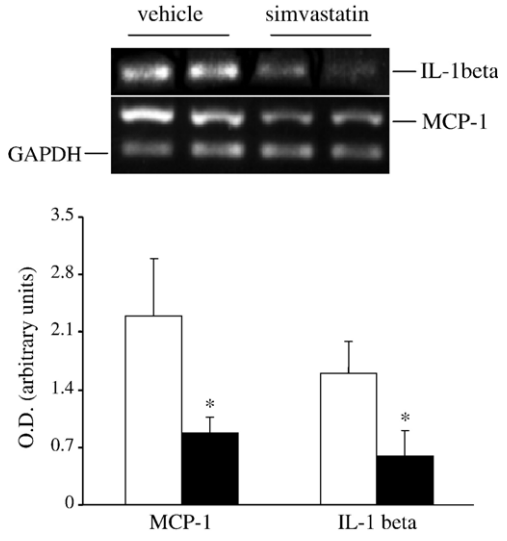

Fig. 1. Representative RT-PCR of IL-1beta and MCP-1 mRNA in the ischemic cerebral cortex of animals treated with either vehicle (white column) or simvastatin (black column) before MCAO. The bar graph shows the results of the densitometric analysis of the IL-1beta and MCP-1 bands from five individual experiments, normalized with the corresponding GAPDH (*$P < 0.05$ vs. vehicle).

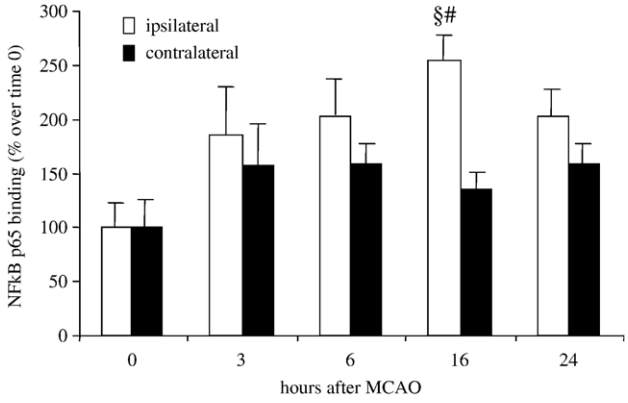

Fig. 2. Time course of NF-kB activation in rat cortical homogenates. The nuclear extracts were prepared from rat cerebral cortex, ipsi- and contralateral to artery's occlusion, at different times after MCAO. NF-kB p65 DNA binding activity was assessed using a TransAm transcription factor assay kit. The data represent the average ± SE of three separate binding assays and are expressed as percent of activation over the contralateral side at time 0. #$P < 0.05$ vs. contralateral brain cortex after 16 h of MCAO; §$P < 0.05$ vs. time 0.

As a first approach, we investigated the ability of the NF-kB p65 subunit to bind to DNA at different times after pMCAO. The data in Fig. 2 show that the binding activity of the NF-kB p65 subunit in the cerebral cortex of the side contralateral to the lesion remained unchanged over time, whereas the ischemic side ipsilateral to the occlusion showed a trend toward increased binding activity which reached its peak and statistical significance 16 h after MCAO and declined as early as after 24 h. In order to determine whether simvastatin treatment can interfere with the ischemia-induced activation of the transcription factor, 16 h after artery occlusion, we evaluated the binding of the NF-kB p65 subunit to DNA in the injured ipsilateral cerebral cortex of animals treated with either vehicle or simvastatin before or after MCAO. In animals receiving vehicle before MCAO (vehicle group), the binding of the NF-kB p65 subunit was significantly increased compared with sham-operated rats (Fig. 3). In contrast, the administration of simvastatin 20 mg/kg for three times before artery occlusion (pretreated group)

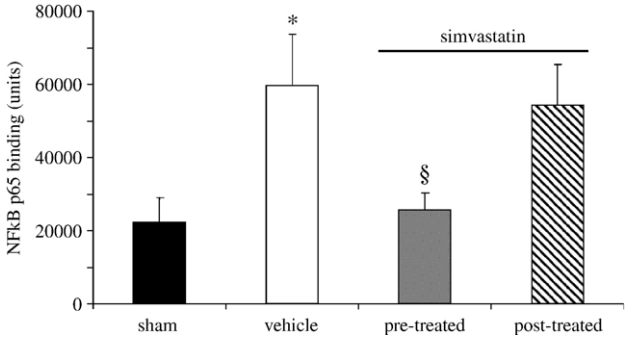

Fig. 3. Effect of statin administration on NF-kB activation. The nuclear extracts were prepared from the cerebral cortex ipsilateral to the occlusion 16 h after a sham operation or MCAO. The sham group did not receive any treatment, whereas the "vehicle" and "pretreated" groups received vehicle or simvastatin 20 mg/kg/day 48, 24, and 2 h before MCAO. The "post-treated" group received a single administration of simvastatin 1 h after MCAO. The data represent the average ± SE of six separate binding assays and are expressed as units of NF-kB activation. *$P < 0.05$ vs. sham; §$P <$ 0.05 vs. vehicle.

abolished the NF-kB activation observed in the vehicle-treated animals. However, this inhibitory effect was no longer observed if the drug was given as a single injection 1 h after the induction of the injury (Fig. 3; post-treated group). To investigate whether the effect of simvastatin is specific for NF-kB, we evaluated the activity of other inducible (c-Fos and c-Jun) or constitutive (NF-YA) transcription factors. The activity of NF-YA, c-Jun, and c-Fos was not significantly affected by either ischemia or treatment (data not shown). The modulation of MAPK signal transduction pathways by ischemia was also examined with the aim of better elucidating the molecular mechanism(s) underlying the protective effect of simvastatin. To this end, we evaluated the phosphorylation state of ERK1/2, ERK5, SAPK/JNK 46/54, p38, Akt, FAK, JAK2, src, and PLC between 30 min and 3 h after pMCAO. As shown in Fig. 4, the phospho-p38 signal was undetectable under our experimental conditions, whereas the levels of phospho-SAPK/JNK 46/54 were not affected by permanent MCA occlusion in the short time period considered. On the contrary, the levels of phospho-ERK1/2 were enhanced in the ischemic hemisphere and reached their highest expression 2 h after injury. Starting from this observation, we investigated the effects of pre- and post-MCAO simvastatin administration on phosphorylated signaling proteins. Under our experimental conditions, the levels of phospho-ERK5, phospho-Akt, phospho-FAK, phospho-JAK2, phospho-src, and phospho-PLC were not induced by MCAO or modulated by statin treatment (data not shown). However, the results shown in Fig. 5

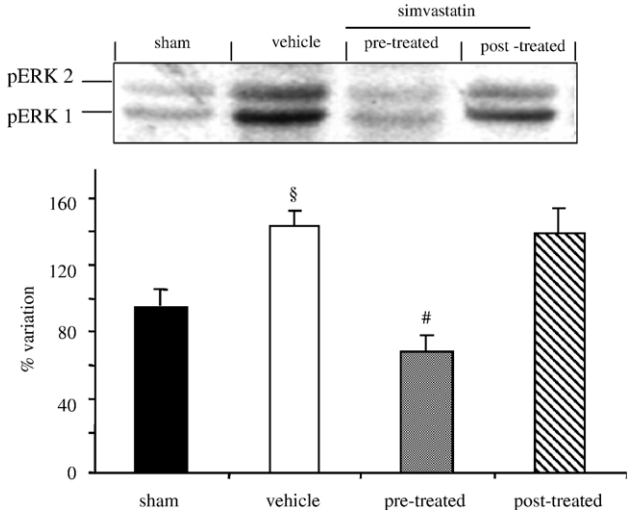

Fig. 5. Effect of statin administration on ERK1/2 activation. The brain homogenates, obtained from cerebral cortex 2 h after MCAO, were fractionated on a 12% SDS-polyacrylamide gel followed by immunoblotting with anti-phospho-ERK1/2 antibody. The blots are representative of three separate experiments. The quantitative analysis was based on densitometric evaluations in three different experiments after normalization with total ERK1/2. §$P < 0.05$ vs. sham operated rats; #$P < 0.05$ vs. vehicle.

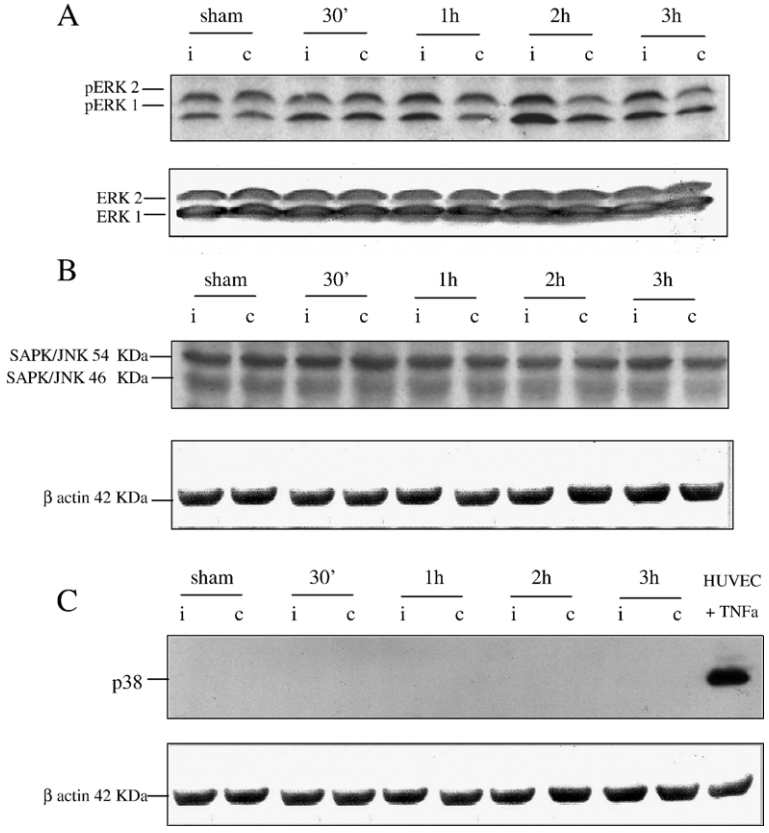

Fig. 4. Time course of MAP kinase activation in rat cortical homogenates. The brain homogenates were fractionated on a 12% SDS-polyacrylamide gel followed by immunoblotting with anti-phospho-ERK1/2 antibody (A), anti-phospho-SAPK/JNK (B), and anti-phospho-p38 (C). Total ERK1/2 (A) and β actin (B and C) were used to normalize data. The blots are representative of three separate experiments. i—ipsilateral side; c—contralateral side.

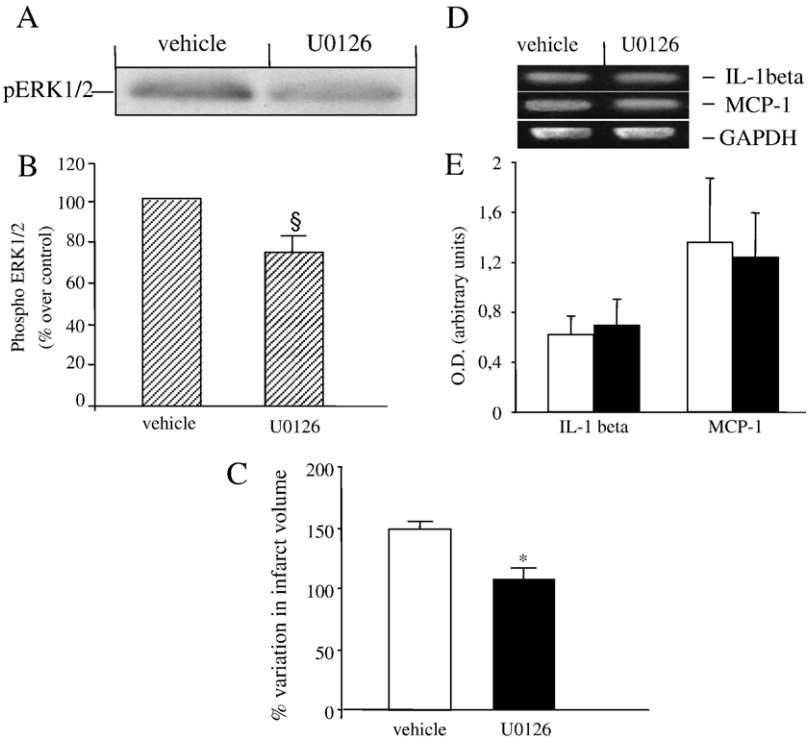

Fig. 6. Effect of U0126 administration on ERK1/2 activation, infarct size, and inflammatory markers. The brain homogenates were fractionated on a 12% SDS-polyacrylamide gel followed by immunoblotting with anti-phospho-ERK1/2 antibody (A); densitometric evaluations of three different immunoblotting experiments after normalization with total ERK1/2 (B). Changes in infarct volume at 24 h after MCAO compared to damage visualized by MRI 2 h after the ischemic insult (n = 6, C). Panel D shows RT-PCR of IL-1beta and MCP-1 mRNA in the cerebral cortex of vehicle- or simvastatin-treated animals before MCAO; densitometric analysis of the IL-1beta and MCP-1 bands from five individual experiments, normalized with the corresponding GAPDH (E; white column = vehicle, black column = U0126).

clearly demonstrate that the ischemia-induced increase in the levels of phospho-ERK1/2 is prevented only when simvastatin is administered before MCAO. In order to further investigate the role of ERK1/2 in the effects mediated by statin, animals were treated, after MCAO, with U0126 a selective inhibitor of MEK/ERK pathway. U0126 reduced by 30% the early ischemia-induced phosphorylation of ERK1/2 (Figs. 6A and B) and prevented the evolution of the infarct size (Fig. 6C). U0126, however, did not show any significant effect on the expression of IL-1beta and MCP-1 (Figs. 6D and E).

Discussion

Several lines of evidence demonstrate that cerebral ischemia is associated with the infiltration of inflammatory cells into the ischemic territory, which is preceded by increased expression of proinflammatory mediators such as cytokines, chemokines, and adhesion molecules (Iadecola and Alexander, 2001; Barone and Feuerstein, 1999). The importance of these molecular signals of inflammation in ischemic injury is demonstrated by experimental data showing that interventions aimed at inhibiting cytokine activation, blocking cytokine receptors, or interfering with adhesion molecules reduce infarct volume and improve functional outcome (Iadecola and Alexander, 2001; Barone and Feuerstein, 1999). Statins, the most widely used therapeutic agents for the control of plasma cholesterol, have a number of lipid-independent actions, such as anti-atherosclerotic, anti-inflammatory, anti-thrombotic, and neuroprotective activities (Takemoto and Liao, 2001). The anti-inflammatory properties of statins are well established (Takemoto and Liao, 2001), although the mechanism of action involved is not yet completely understood. We here show for the first time that the expression of the proinflammatory mediators IL-1beta and MCP-1, which are known to be activated by ischemic injury, is inhibited in animals treated with simvastatin for 3 days before MCAO. This biochemical effect of simvastatin preadministration correlates with the reduction in the extent of ischemic damage previously reported in rat and mouse models of focal ischemia, thus suggesting that the inhibition of post-ischemic inflammation may have beneficial effects on the evolution of brain injury (Endres et al., 1998; Sironi et al., 2003). The transcription factor NF-kB, whose dual role in apoptosis and inflammation is still debated (Mattson and Camandola, 2001), is crucial in cerebral inflammation, as demonstrated by the observations that both transient and permanent MCAO enhances NF-kB activity, thus contributing to infarction, and that NF-kB knockout mice develop less ischemic damage after a focal insult (Nurmi et al., 2004; Stephenson et al., 2000). Consistent with its anti-inflammatory action, and in line with previous observations in human blood monocytes (Hilgendorff et al., 2003), we found that the administration of simvastatin for 3 days before MCAO prevented the ischemia-induced activation of NF-kB. However, the ability of simvastatin to inhibit the induction of the transcription factor was lost if it was given after MCAO. This lack of effect may be explained by the fact that the animals were sacrificed at the time

of peak NF-kB activation (16 h after the ischemic insult) and therefore received a single dose of simvastatin 1 h after MCAO, which may not be high enough to inhibit the ischemia-induced activation of NF-kB. The biological activity of many extracellular signals, including proinflammatory mediators, is transduced to the nucleus via the activation of the MAP kinase cascade, a group of serine and threonine kinases that regulate gene expression by modulating transcription factors such as NF-kB (Liu et al., 2001). Three members of the MAPK family, ERK1/2, SAPK/JNK, and p38, are all activated early after focal cerebral ischemia, and inhibition of this pathway reduces the expression of proinflammatory cytokines and confers protection against the insult (Wu et al., 2000; Irving and Bamford, 2002; Ferrer et al., 2003; Wang et al., 2004). In this study, we found that simvastatin pretreatment abolished MCAO-induced ERK1/2 phosphorylation and that, in analogy with the data reported for NF-kB, the administration of the drug following the injury failed to affect the ischemia-induced enhanced activation of the kinase. As it has been demonstrated that statins can directly interfere with intracellular signaling pathways (Davignon and Mabile, 2001), the most likely mechanism responsible for the inhibitory effect of simvastatin pretreatment involves the inhibition of small GTPases prenylation via reduced farnesylation and geranylgeranylation because in vivo studies have demonstrated that these statin-related effects on intracellular signaling mechanisms can be overcome by adding mevalonate or farnesol (Guijarro et al., 1998). However, we cannot exclude the possibility that simvastatin may interfere with the activity of other signal transduction molecules. The proposed hypothesis of a direct link between activation of ERK1/2 and induction of inflammatory markers, inferred by data obtained from simvastatin pretreatment, was not validated by investigations carried out with the MEK/ERK pathway inhibitor U0126. Indeed, we found, in agreement with previous data (Namura et al., 2001) that intravenous administration of U0126 after MCAO reduced the levels of phospho-ERK1/2 and decreased the infarct size. U0126, however, did not affect significantly the expression of IL-1beta and MCP-1. At least in our experimental conditions, the inhibition of phospho-ERK-1/2 is not related with the anti-inflammatory effect of simvastatin, thus indicating that the drug may exert its neuroprotective effect through several mechanisms besides ERK1/2 signaling pathway. In this study, we failed to show the known ischemia-induced modulation of SAPK/JNK, and we were unable to detect the levels of phospho-p38 in either intact or ischemic tissue. Although a strong positive signal in stimulated endothelial cells was observed. The lack of expression and ischemia-induced modulation of these activated kinases may be explained by the different degree of severity of the injury, the different experimental conditions and/or the different animal model. In a mouse model of cerebral ischemia, increased phospho-p38 expression was observed early after injury and lasted for only 10–30 min after MCAO (Wu et al., 2000), whereas the time course examined in our experiments ranged from 30 min to 3 h when the signal may no longer be visible. In conclusion, we have demonstrated that the administration of simvastatin before MCAO can inhibit the ischemia-induced activity and expression of IL-1beta, MCP-1, NF-kB, and ERK1/2, thus suggesting that the prevention of post-ischemic inflammation by statins may be one of the mechanisms involved in their neuroprotective effect in stroke. However, a direct link between anti-inflammatory effects of simvastatin and its inhibitory activity on ERK1/2 could not be established in the model of permanent MCAO used, and further studies will be needed to disentangle this issue.

Acknowledgment

This study was supported by a grant from Ministry of Education, University and Research (FIRB, #RBNE01BNFK).

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