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Amlodipine and Atorvastatin Exert Protective and Additive Effects Via Antiapoptotic and Antiautophagic Mechanisms After Transient Middle Cerebral Artery Occlusion in Zucker Metabolic Syndrome Rats

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We examined the neuroprotective effects amlodipine and/or atorvastatin in metabolic syndrome (MetS) Zucker fatty rats against transient (90 min) middle cerebral artery occlusion (MCAO). The rats were pretreated with vehicle, amlodipine, atorvastatin, or amlodipine plus atorvastatin for 28 days, and 24 hr after transient MCAO the infarct size was assessed via hematoxylin and eosin staining, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling (TUNEL) and microtubule-associated protein 1 light chain 3 (LC3) expression were examined by immunohistochemistry to evaluate apoptosis and autophagy, respectively. Compared with the vehicle group, rats treated with amlodipine or atorvastatin alone showed a significant decrease in infarct volume ($P < 0.01$), which was further decreased in the amlodipine plus atorvastatin group ($P < 0.001$). Compared with the vehicle group, the numbers of TUNEL- and LC3-positive cells were markedly reduced by amlodipine or atorvastatin alone ($P < 0.01$) and further decreased by amlodipine plus atorvastatin ($P < 0.001$). The number of apoptotic TUNEL/autophagic LC3 double-positive cells was also significantly decreased with amlodipine or atorvastatin alone compared with vehicle ($P < 0.01$) and was further decreased by amlodipine plus atorvastatin ($P < 0.001$). These data suggest additive neuroprotective effects of combination amlodipine and atorvastatin treatment after acute ischemic stroke in MetS model Zucker rats. These effects are mediated, at least in part, via antiapoptotic and antiautophagic mechanisms. Further studies are now needed to expand these preliminary results to understand fully the mechanisms involved in the protective effects of amlodipine and atorvastatin against ischemic stroke.

Key words: cerebral ischemia; amlodipine; atorvastatin; apoptosis; autophagy

Ischemic stroke is the third leading cause of death in many countries, exceeded only by heart disease and cancer (Dirnagl et al., 1999). The main risk factors for stroke include hypertension and hyperlipidemia, which are primary and adjustable factors.

Apoptosis is a form of programmed cell death and is the main pathway for cell death (Mattson et al., 2001). Autophagy is the second type of programmed cell death and is highly conserved during evolution (Mortimore and Poso, 1987). Homologous genes involved in autophagy have been found in yeast, *Drosophila*, and vertebrates (Klionsky and Emr, 2000). There is also evidence that autophagic function is strengthened by apoptotic stimulation, suggesting a potential synergy between apoptosis and autophagy (Ohsawa et al., 1998; Bursch et al., 2000). In addition, certain signals that induce apoptosis can also lead to autophagy (Schwartz et al., 1993).

Amlodipine is a dihydropyridine calcium channel blocker that lowers blood pressure and can protect the brain from ischemic damage (Annoura et al., 1999;

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Lukic-Panin et al., 2007). Amlodipine is highly lipophilic and long-acting and easily crosses the blood–brain barrier (Nagahiro et al., 1998), exhibits antioxidative effects (Chen et al., 1997), and inhibits neuronal apoptosis (Mason et al., 1999). Atorvastatin is a new generation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors with pleiotropic effects, including lipid-lowering (Bell and Yellon, 2003), antiatherosclerosis (Raal et al., 1999), antioxidative (Nagotani et al., 2005), and antiinflammatory effects (Chiang et al., 2009). Atorvastatin can also reduce the occurrence of cardiogenic cerebral embolism (Amarenco et al., 2004).

The Zucker fatty rat has a deficit in the leptin receptor gene that results in hyperphagia (Ionescu et al., 1985). These animals are a good model of obesity and metabolic syndrome (MetS; Vaziri et al., 2005), including insulin resistance, hyperlipidemia, and hypertension (Arenillas et al., 2009). MetS is also associated with an increased risk for cardiovascular disease and ischemic stroke (Malik et al., 2004; Arenillas et al., 2009). To simulate the pathological process of clinical stroke, we performed middle cerebral artery occlusion (MCAO) in Zucker fatty rats, and examined the neuroprotective effect of amlodipine and atorvastatin alone or in combination. Although both drugs have been extensively evaluated individually to prevent ischemic stroke, the neuroprotective effects of both drugs in combination have not been examined.

MATERIALS AND METHODS

Experimental Model

Experiments were performed with Zucker fatty rats (Disease Model Cooperative Research Association, Kyoto, Japan). Eight-week-old rats weighing 250–280 g were maintained in a temperature-regulated room (21–23°C) with a 12-hr light/dark cycle. Rats were randomly divided into four groups (n = 5/group) and treated with 1) vehicle (0.5% methyl cellulose in saline), 2) amlodipine (3 mg/kg/day; Yamanaka et al., 1991) 3) atorvastatin (10 mg/kg/day; Birnbaum et al., 2006), or 4) amlodipine (3 mg/kg day) plus atorvastatin (10 mg/kg/day). Amlodipine and atorvastatin were provided by Pfizer (Tokyo, Japan). The allocated drugs were administered from the age of 8 weeks by oral gavage every evening for 28 days. Physiological parameters of the rats were tested and are presented in more detail elsewhere (Kawai et al., 2010). There were no differences among the four experimental groups in terms of physiological parameters, including regional cerebral blood flow. All experimental procedures were approved by the Animal Committee of the Graduate School of Medicine and Dentistry, Okayama University.

MCAO and Reperfusion

At 12 weeks of age, the rats were lightly anesthetized by inhalation of a 69%/30% (v/v) mixture of nitrous oxide/oxygen and 1% halothane using a facemask. A midline neck incision was made and the right common carotid artery exposed, and then inhalation of anesthetics was stopped. When the rat began to regain consciousness, the right MCA was occluded by inserting 4-0 silicon-coated surgical nylon thread through the common carotid artery (Nagasawa and Kogure, 1989; Abe et al., 1992). With this technique, the tip of the thread occludes the origin of the right MCA. The reliability of successfully inducing stroke is almost complete in this model (Koizumi et al., 1986). All of the animals in our study showed neurological evidence of stroke. The body temperature was monitored throughout the procedures with a rectal probe and was maintained at 37°C ± 0.3°C with a heating pad. The surgical incision was then closed, and the animals were allowed to recover at room temperature. After 90 min of MCAO, cerebral blood flow was restored by removing the nylon thread. Twenty-four hours after reperfusion, the animals were euthanized. Frozen brain sections were prepared as previously described (Zhang et al., 2010) and were assayed by hematoxylin and eosin (H&E) and immunohistochemical staining.

Brain Preparation and Quantitative Analysis of Infarct Volume

Animals were sacrificed under deep anesthesia with pentobarbital (10 mg/250 g rat). Rats were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Coronal brain sections (20 μm thick) were prepared with a cryostat and mounted on silane-coated glass slides. For quantitative analysis of infarct volume, the H&E-stained sections were examined by light microscopy (BX-51; Olympus, Tokyo, Japan). The area of the infarct was measured in five sections by pixel counting in Photoshop 7.0, and the volume was calculated (Shang et al., 2010).

TUNEL Staining

Cell apoptosis was assessed by using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling (TUNEL) in situ cell death detection kit (Roche, Nonnenwald, Germany) in accordance with the manufacturer's instructions. Briefly, the brain sections were fixed with 4% paraformaldehyde and rinsed three times in PBS, and endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. The sections were then incubated in sodium citrate buffer with 0.1% Triton X-100 for 20 min at 4°C, followed by the TUNEL reaction mixture for 60 min at 37°C.

Single Immunohistochemical Analysis

We performed immunohistochemistry for caspase 3 and microtubule-associated protein 1 light chain 3 (LC3). Brain sections were first fixed with 4% paraformaldehyde, rinsed three times in PBS (pH 7.4), and incubated in 0.3% H_2O_2/methanol for 30 min to block endogenous peroxidase activity. The sections were then blocked with 3% bovine serum albumin in PBS with 0.25% Triton X-100 for 3 hr and then incubated with primary antibody for caspase 3 antibody (1:200; Cell Signaling Technology, Beverly, MA; No. 9661) or rabbit anti-LC3 polyclonal antibody (1:200 dilution; MBL; No. PM046) overnight at 4°C. The sections were washed with PBS and incubated for 2 hr with relevant biotinylated secondary antibodies (1:500; Vector Laboratories, Burlingame, CA), followed by incubation with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 min. After

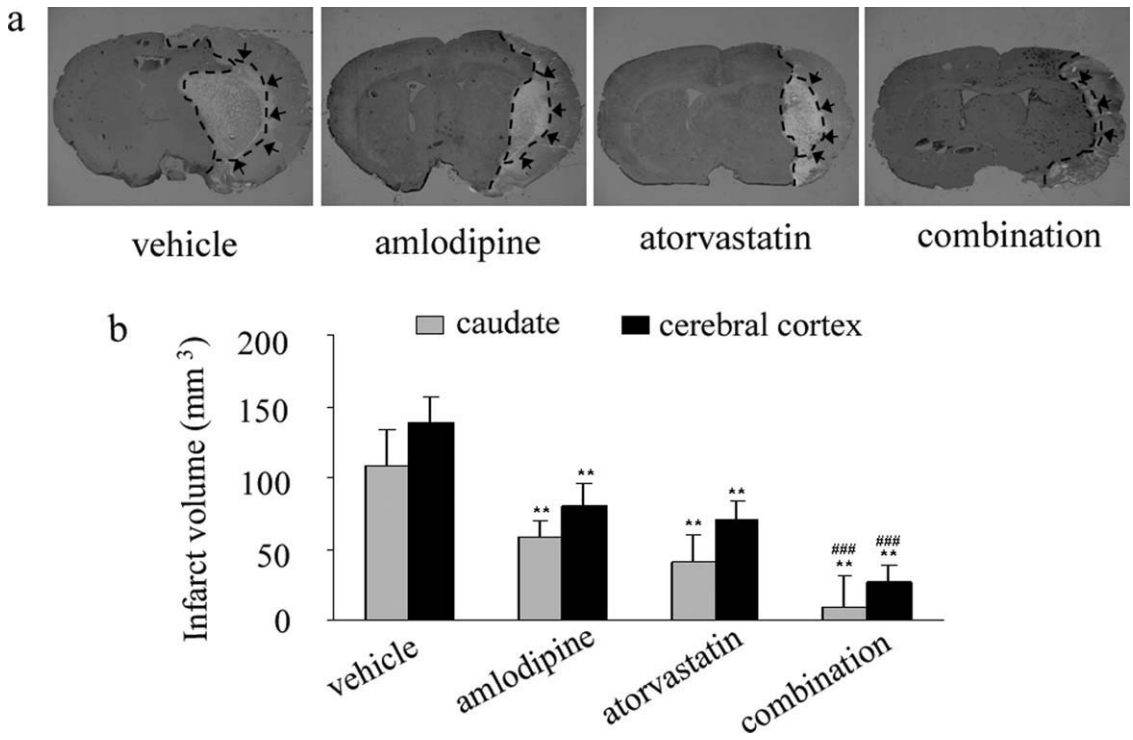

Fig. 1. Hematoxylin and eosin staining (**a**) and quantitative analysis of infarct volume (**b**) at 24 hr after transient middle cerebral artery occlusion. $^{**}P < 0.01$ vs. vehicle; $^{\#\#\#}P < 0.001$ vs. amlodipine or atorvastatin alone.

washing with PBS, diaminobenzidine tetrahydrochloride was used as a color substrate for chromogenic reactions.

Double-Immunofluorescence Analysis of TUNEL and LC3

Double-immunofluorescence studies were performed for TUNEL and LC3. Brain sections were first fixed with 4% paraformaldehyde and then rinsed three times in PBS. Sections were incubated in sodium citrate buffer with 0.1% Triton X-100 for 20 min at 4°C, rinsed three times in PBS, and blocked with 10% normal goat serum in PBS with 0.25% Triton X-100 for 1 hr. The sections were then incubated with rabbit anti-LC3 (1:200) for 3 hr at room temperature, rinsed in PBS, followed by incubation with the secondary antibody Texas red-labeled goat anti-rabbit IgG antibody (1:250 dilution; Invitrogen, Carlsbad, CA) plus the TUNEL enzyme and label or FITC avidin D (1:9 dilution; Roche, Indianapolis, IN). The double-labeled immunofluorescence sections were scanned with a confocal microscope equipped with an argon and HeNe1 laser (LSM-510; Zeiss, Jena, Germany).

Quantitative Analysis

To estimate the number of TUNEL-, caspase 3-, and LC3-positive cells and double TUNEL/LC3-positive cells per square millimeter, five sections at the level of the anterior commissure were chosen from each animal (2, 4, 6, 8, and 10 mm caudal from the frontal pole). Five randomly chosen, nonoverlapping, high-power fields (×400) in the cortex of the periinfarct area were examined in each section (n = 3 per group). Colocalization of TUNEL with LC3 was determined as cells in which the nucleus was stained with TUNEL and the nucleus plus cytoplasm was stained with LC3. The number of TUNEL and LC3 double-labeled cells was calculated.

Statistical Analysis

Data are presented as means ± standard deviation. Statistical analysis was performed by one-way analysis of variance followed by Student's *t*-test for post hoc analysis. $P < 0.01$ was considered statistically significant.

RESULTS

Quantitative Analysis of Infarct Volume

At 24 hr after the 90-min MCAO, large infarcts were observed in the lateral cortex and the underlying caudoputamen on H&E-stained brain sections (Fig. 1). The amlodipine, atorvastatin, and combination groups showed a significant decrease in infarct volumes compared with the vehicle group (all $P < 0.01$). Furthermore, the infarct volume in the combination group was significantly decreased compared with amlodipine or atorvastatin groups (both $P < 0.001$; Fig. 1B).

Effects of Amlodipine and Atorvastatin on Apoptosis

TUNEL- and caspase 3-positive cells were widely distributed in the cerebral cortex and the dorsal caudate

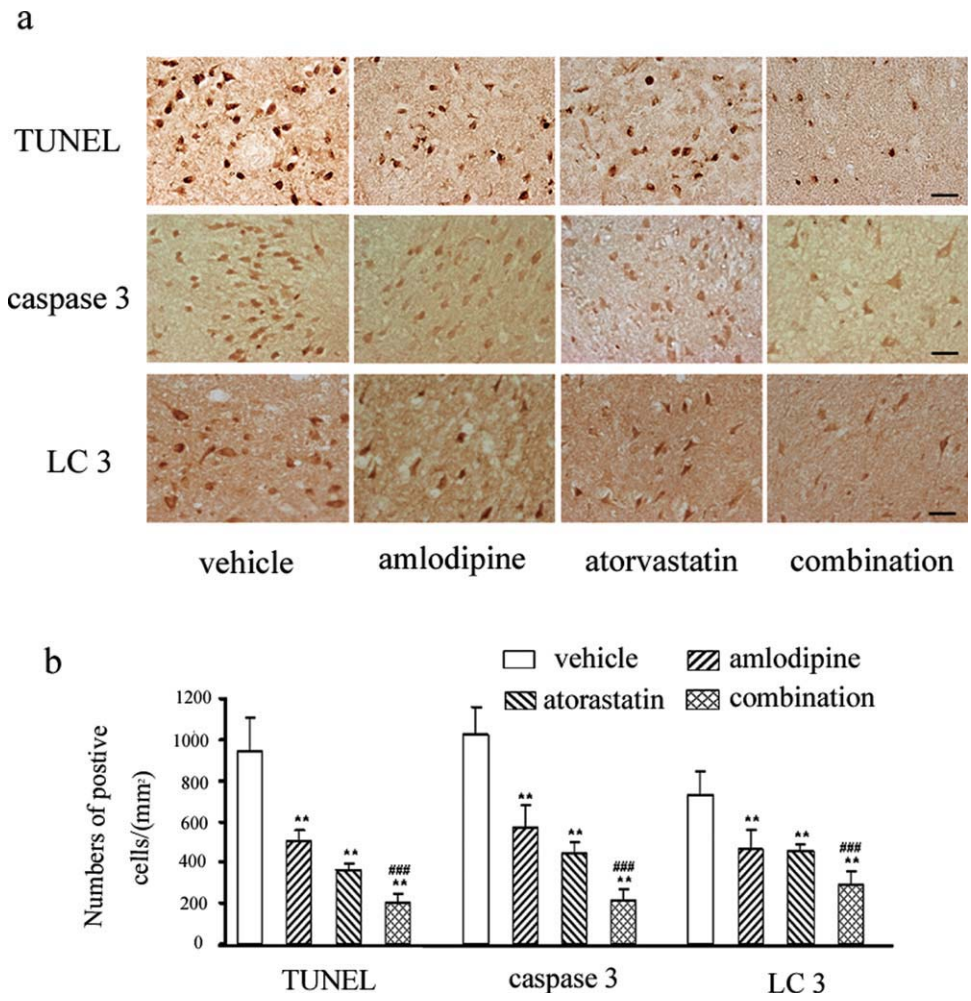

Fig. 2. **a**: Representative photomicrographs of TUNEL (top panels)-, caspase 3 (middle panels)-, and LC3 (bottom panels)-positive cells at 24 hr after transient middle cerebral artery occlusion. **b**: Quantitative analysis. $^{**}P < 0.01$ vs. vehicle; $^{\#\#\#}P < 0.001$ vs. amlodipine or atorvastatin alone. Scale bars = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

supplied by the MCA but were not found in other areas of the ipsilateral hemisphere or the contralateral side. TUNEL and caspase 3 staining was present in the vehicle group (947.2 ± 86.6 and 907.1 ± 63.8 cells/mm^2) but was greatly reduced by amlodipine (506.9 ± 53.4 and 501.6 ± 56.5 cells/mm^2), atorvastatin (362.9 ± 40.4 and 394.9 ± 43.1 cells/mm^2), and combination therapy (202.8 ± 32.2 and 192.1 ± 26.5 cells/mm^2; all $P < 0.01$; Fig. 2A,B). The numbers of TUNEL- and caspase 3-positive cells were significantly lower in the combination therapy group than with amlodipine or atorvastatin alone ($P < 0.001$).

Effects of Amlodipine and Atorvastatin on Autophagy

LC3-positive stained cells were distributed throughout the cerebral cortex and dorsal caudate supplied by the MCA. Cytoplasmic LC3 staining was present in neurons in the vehicle group (522.4 ± 62.7 cells/mm^2) but decreased in the amlodipine, atorvastatin, and combination therapy groups (330.8 ± 51.3, 325.5 ± 43.9, and 209.1 ± 35.8 cells/mm^2, respectively; all $P < 0.01$; Fig. 2A,B). Furthermore, the number of LC3-positive cells was significantly lower in the combination therapy group than with amlodipine or atorvastatin alone ($P < 0.001$).

Analysis of TUNEL and LC3 Double-Immunofluorescence Staining

Double-immunofluorescence staining analysis showed that neurons stained for both TUNEL and LC3 were expressed mainly in the cerebral cortex and dorsal caudate of the MCA territory. The number of double-positive cells was 369.3 ± 39.8 cells/mm^2 in the vehicle-treated group, which decreased significantly to 116.9 ± 27.3, 104.6 ± 25.7, and 46.2 ± 15.1 cells/mm^2 in

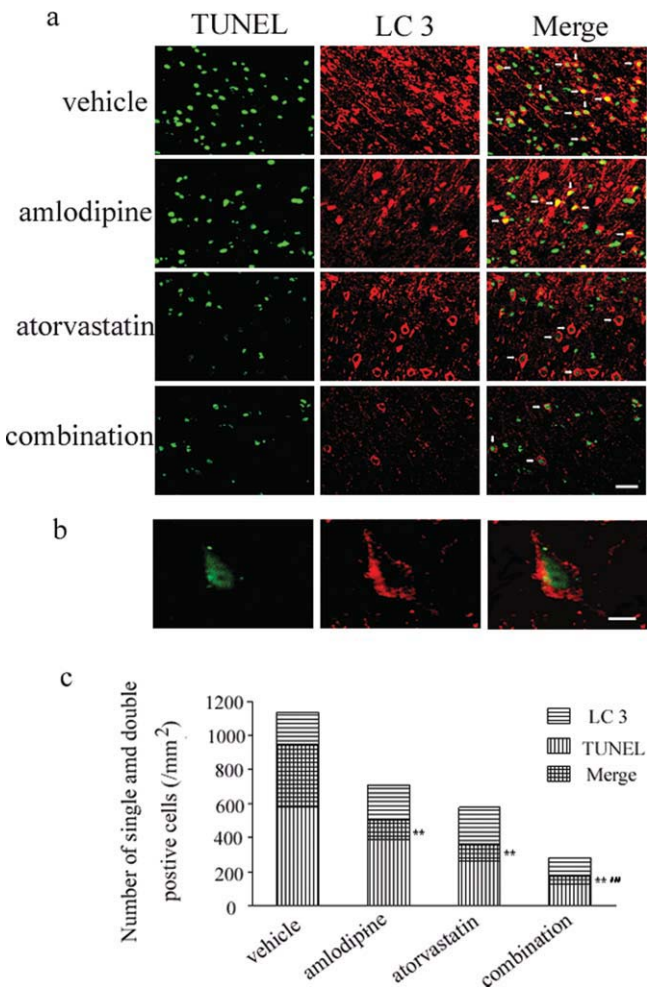

Fig. 3. **a,b**: Representative photomicrographs of apoptotic TUNEL/autophagic LC3 double-stained cells at low (a) and high (b) magnification. **c**: Quantitative analysis. $^{**}P < 0.01$ vs. vehicle; $^{###}P < 0.001$ vs. amlodipine or atorvastatin alone. Scale bars = 20 μm in a; 5 μm in b. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the amlodipine, atorvastatin, and combination therapy groups, respectively ($P < 0.01$). The number of double-positive cells was also significantly lower in the combination therapy group compared with amlodipine or atorvastatin alone ($P < 0.001$; Fig. 3A,B).

DISCUSSION

Necrosis (passive) and apoptosis and autophagy (active) are the three established types of cell death following cerebral ischemia (Shang et al., 2010). The primary mechanisms involved in these processes include calcium overload and the release of oxygen free radicals, excitatory amino acids, and inflammatory cytokines. Apoptosis is considered to be responsible for the delayed neuronal death following ischemia (Lipton, 1999). However, recent studies have also reported the activation of autophagy following ischemia (Shang et al., 2010), although the contribution of autophagy to neuronal death/survival remains largely unknown (Adhami et al., 2007).

Amlodipine binds primarily to L-type Ca^{2+} channels and thereby limits the influx of calcium into smooth muscle cells, lowers intracellular calcium, increases cell cyclic adenosine monophosphate (cAMP), and relaxes blood vessels (Nagahiro et al., 1998; Kikuya et al., 2000). Amlodipine was also shown to exert antioxidative effects in vitro and in vivo by inhibiting the oxidizability of the cell membrane and low-density lipoproteins (Chen et al., 1997; Mason, 2002). Amlodipine can inhibit lipid peroxide formation at concentrations as low as 10.0 nmol/liter, an effect attributed to its high lipophilic properties and its chemical structure, by quenching free radical reactions. Furthermore, amlodipine was reported to inhibit platelet activation by increasing endothelial nitric oxide bioavailability (Loke et al., 2000; Berkels et al., 2004) and to protect rat cerebellar granule cells against apoptosis (Mason et al., 1999).

Statins (HMG-CoA reductase inhibitors) are widely used clinically to lower blood lipid levels (Bell and Yellon, 2003). Statins also exhibit pleiotropic effects against atherosclerosis, inflammation, and the formation of thrombi and can promote angiogenesis (Kureishi et al., 2000; Tsuchiya et al., 2007). We previously reported that atorvastatin and pitavastatin had antioxidative effects and reduced infarct volume (Nagotani et al., 2005) and that statins also prevented and reduced atherosclerotic changes in the common carotid artery of stroke-prone spontaneously hypertensive rats (Tsuchiya et al., 2007). A study on normocholesterolemic mice also revealed that prophylactic treatment with statins augmented cerebral blood flow, reduced cerebral infarct size by 30%, and improved neurological function (Endres et al., 1998). Atorvastatin shows several powerful and well-tolerated effects, including lipid-lowering, antiatherosclerosis, antioxidative, and antiinflammatory effects. As such, atorvastatin is widely used to treat hyperlipidemia. Interestingly, the results presented here were independent of changes in regional blood flow; we found no differences in cerebral blood flow among the four groups prior to MCAO (Kawai et al., 2010).

Apoptosis is the major cause of cell death in the ischemic penumbra (Sharp et al., 2000), and the severity of the brain damage may depend on how many apoptotic cells can be reversed (Li et al., 1998). The caspases are a family of cysteine proteases that are critical mediators of programmed cell death. Activation of caspase-3 appears to be a key event in execution of the apoptotic cascade in central nervous system diseases (Awasthi et al., 2005). Autophagy is another mechanism of cell death, and LC3, a mammalian gene homolog of yeast ATG8 (Aut7/Apg8; Kabeya et al., 2000), is localized to autophagic vacuoles and the cell membrane of autophagic vacuoles (Lockshin and Zakeri, 2004). Rami et al. (2008) reported that expression of the autophagic proteins Beclin-1 and LC3 was significantly increased in the cerebral ischemic penumbra of rats after focal cerebral ischemia. These two phenomena are also found in many

neurodegenerative diseases, including Parkinson's disease (Anglade et al., 1997), schizophrenia (Margolis et al., 1994), and ischemic stroke (Shang et al., 2010).

The present study has shown that treatment with amlodipine or atorvastatin alone decreased the infarct volume in the cerebral cortex and caudoputamen and that combination therapy further decreased infarct volume relative to either drug alone. Meanwhile, treatment with amlodipine or atorvastatin alone significantly reduced the numbers of TUNEL-positive and LC3-positive cells in the cerebral ischemic penumbra, and combination therapy elicited a greater effect than either drug alone. The number of TUNEL/LC3 double-stained cells was also decreased by amlodipine or atorvastatin alone and was further decreased by combination therapy. Overall, these data suggest that amlodipine and atorvastatin in combination act in an additive or synergistic manner to protect against ischemic neuronal damage by inhibiting both apoptosis and autophagy. Similarly to our previous report showing antiapoptotic and antiautophagic effects of glial cell line-derived neurotrophic factor (GDNF) and hepatocyte growth factor (HGF; Shang et al., 2010), the proportional reduction of apoptotic/autophagic double-positive cells by amlodipine, atorvastatin, and combination therapy suggests that these antihypertensive (amlodipine) and antihyperlipidemic (atorvastatin) drugs activate mechanisms upstream of these neuroprotective proteins (GDNF and HGF).

In summary, we have demonstrated that pretreatment with amlodipine or atorvastatin inhibits apoptosis and autophagy of ischemic neuronal cells induced by MCAO and that this effect is greater with combination therapy. Because apoptosis and autophagy are important processes in ischemic neuronal cell death, combination therapy that targets both pathways may be more beneficial than either drug individually to reduce acute ischemic brain damage and prevent recurrent stroke at a chronic stage. Further studies are now needed to expand the preliminary findings reported here, to understand how amlodipine and atorvastatin regulate the apoptotic and autophagic pathways and thus protect against ischemic stress and whether initiating treatment immediately after ischemia is as effective as pretreatment.

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