Research Report

Synergistic benefit of combined amlodipine plus atorvastatin on neuronal damage after stroke in Zucker metabolic rat

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ABSTRACT

Stroke is a major neurologic disorder and a leading cause of death in the world. We compared neuroprotective effects of single or combination therapy of amlodipine (AM) and atorvastatin (AT) in such a metabolic syndrome model Zucker rat after 90 min of transient middle cerebral artery occlusion (tMCAO). The animals were pretreated with vehicle, AM, AT, or the combination of AM plus AT for 28 days, and at 24 h of tMCAO, infarct volume and immunohistochemical analyses were performed. The combination of AM plus AT treatment decreased the infarct volume stronger than each single treatment with AM or AT. The numbers of positive cells of oxidative stress markers such as 8-hydroxy-2′-deoxyguanosin (8-OHdG), 4-hydroxy-2-nonenal (4-HNE), and advanced end glycation products (AGE) and inflammation markers such as tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) decreased dramatically in the combination-treated group compared with single AM- or AT-treated group. The present study showed that single AM or AT treatment showed neuroprotective effects both with antioxidative and anti-inflammatory mechanisms, but combination therapy of AM plus AT presented a further synergistic benefit in acute ischemic neural damages.

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1. Introduction

Stroke is a major neurologic disorder and a leading cause of death in the world. Oxidative stress is strongly related to the pathophysiology of stroke (Abe et al., 1995; Hayashi et al., 1999), and inflammatory response is one of the first immune processes after injury relating after oxidative stress. Therefore, antioxidative and anti-inflammatory actions could be an important strategy in decreasing ischemic brain damage (Sun et al., 2002; Villegas et al., 2004).

The calcium channel blocker (CCB) AM is most commonly used for hypertensive patients in the world and does not only lower blood pressure but also directly protect neurons under ischemic damage (Opie and Schall, 2002; Lukic-Panin et al., 2007). Statin (3-hydroxy-3-methylglitaryl coenzyme A, HMG-CoA reductase inhibitor) is also widely used for lowering serum

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Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosin; 4-HNE, 4-hydroxy-2-nonenal; AGE, advanced end glycation products; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; MCA, middle cerebral artery; AM, amlodipine; AT, atorvastatin

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cholesterol level in the world (Farmer, 1998), among which AT has strong pleiotropic effects that decreased the risk of stroke and other cardio vascular events in patients with hyperlipidemia (Amarenco et al., 2006).

Zucker-fatty rat is a good model of obesity and metabolic syndrome with deficient leptin receptor (Vaziri et al., 2005), showing serum characters of insulin resistance, hypertriglyceridemia, and hyperlipidemia (HL) (Zucker, 1965; Zucker and Antoniades, 1972). Metabolic syndrome is the major risk factor for cardiovascular and ischemic stroke, needing single therapy not only for such as HT or HL but also for both diseases (Malik et al., 2004; Arenillas et al., 2009). Although there have been several reports of single treatment with AM (Yamagata et al., 2004; Lukic-Panin et al., 2007) or AT (Hayashi et al., 2005; Nagotani et al., 2005; Lee et al., 2008; Cui et al., 2010), their combination has not been investigated with respect to neuroprotection focusing on antioxidative and anti-inflammatory effects. In the present study, therefore, we compared neuroprotective effects of single or combination therapy of AM and AT in such a metabolic syndrome model Zucker rat after transient middle cerebral artery occlusion (tMCAO).

2. Results

2.1. Infarct volume

Compared with vehicle-treated controls (190.0±50.6 mm$^3$, n=5), quantitative analysis showed that AM-treated (146.7±27.8 mm$^3$, n=5, *p<0.05) and AT-treated (124.1±13.6 mm$^3$, n=5, *p<0.05) groups significantly reduced the infarct volume determined by CV staining (Fig. 1, upper pictures). Moreover, the combination of AM plus AT-treated group greatly decreased the infarct area (82.3±19.0 mm$^3$, n=5 **p<0.01 vs vehicle group, *p<0.05 vs AM and AT groups), (Fig. 1, lower illustration).

2.2. Physiological parameters in Zucker rat

We monitored physiologic parameters, including regional cerebral blood flow during the experiments, and found no significant differences among the four experimental groups. Blood pressure was decreased in the amloidipine-treated group compared with other groups (p<0.01).

2.3. Peroxidative markers

Immunohistochemical analysis showed that no staining for 8-OHdG, 4-HNE, and AGE in the sham control brain (Fig. 2, sham) but that tMCAO induced strong staining of 8-OHdG, 4-HNE, AGE in nerve cells of the ischemic core (core) and the boundary penumbral (penumbra) zones (Fig. 2, vehicle). Compared with this vehicle group, single treatment with AM or AT significantly reduced the number of positively stained cells for these oxidative injury markers (*p<0.05, **p<0.01) in the ischemic core (Fig. 2, upper pictures) and the ischemic penumbra (Fig. 2, lower illustrations). Among the single treatment, treatment with AT decreased the number of positively stained cells compared with AM treatment both in the ischemic core and the ischemic penumbra (*p<0.05, **p<0.01 vs AM group). Combination of AM plus AT treatment showed a further reduction of the number of positively stained cells of 8-OHdG both in the ischemic core and the ischemic penumbra compared with AM or AT single treatment group (*p<0.05,
Such a combination treatment also showed a further reduction of the number of 4-HNE and AGE positive cells, but the difference was significant with vehicle and AM groups and not with AT group (Fig. 2, lower illustration). The number of positively stained cells was generally greater in the ischemic core than those in the penumbra in all 3 peroxidative markers, while the difference was not significant (Fig. 2, lower illustrations). In terms of intracellular localization, both the nuclei and cytoplasm were strongly stained in 8-OHdG, but preferential cytoplasmic staining was found in 4-HNE and AGE in the vehicle treatment group (Fig. 2, upper panels, insets). Treatment of AM or AT reduced the staining strength in all 3 peroxidative markers, and greatly reduced the nuclear staining of 8-OHdG (Fig. 2, upper panels, insets). Combination of AM plus AT treatment showed a further reduction of the staining in the nuclei and cytoplasm. The sections without first antibody did not show any positive staining in each peroxidative marker.

### 2.4. Inflammatory proteins

Immunohistochemical analysis showed that no staining for TNF-α and MCP-1 in the sham control brain but that tMCAO induced strong staining of TNF-α and MCP-1 in nerve cells of the ischemic core and the penumbra (Fig. 3, Vehicle). Staining property of these 2 inflammatory proteins was not so strong compared with that of the 3 peroxidative markers (Fig. 2) at 24 h.
after tMCAO. Compared with the vehicle group, single treatment with AM reduced the number of positively stained cells for these inflammation markers in the ischemic core and the ischemic penumbra, but the difference did not reach statistical significance except for the ischemic penumbra of MCP-1 ($p < 0.05$).

Single treatment with AT significantly reduced the number of positively stained cells for these inflammation markers ($* p < 0.05$, $** p < 0.01$ vs vehicle group) in the ischemic core (Fig. 3, upper pictures) and the ischemic penumbra (Fig. 3, lower illustrations). Among single treatment, the effect of AT was much stronger than that of AM in the ischemic core and the ischemic penumbra ($* p < 0.05$, $** p < 0.01$ vs AM group). Combination of AM plus AT treatment showed a further reduction of the number of positively stained cells of both in the ischemic core and the ischemic penumbra compared with the vehicle and AM groups ($* p < 0.01$ vs AM group) but did not with the AT group (Fig. 3, lower illustrations).

The number of positively stained cells was generally greater in the ischemic core than those in the penumbra in both 2 inflammation markers at 24 h after tMCAO, while the difference was not significant (Fig. 3, lower illustrations). In terms of intracellular localization, both the nuclei and cytoplasm were strongly stained at vehicle treatment (Fig. 3, upper pictures, inlets). Treatment of AM or AT reduced the staining strength both of the nuclei and cytoplasm, with a further reduction in the combination of AM plus AT treatment. The sections without first antibody did not show any positive staining in each inflammation marker.

### 3. Discussion

In this study, we first discovered that a combination of AM plus AT showed a stronger reduction in infarct volume (Fig. 1), oxidative stress (Fig. 2), and inflammation (Fig. 3) in the ischemic brain compared with the vehicle group and the single treatment with AM or AT.
The calcium channel blocker (CCB) AM is a long-acting dihydropyridine, which not only lowers the blood pressure but also protects brain from ischemic damage (Rami et al., 2008; Annoura et al., 1999). AM primarily binds L-type Ca²⁺ channel which can catch free radical by aromatic ring. Our previous report showed that AM reduced infarct volume and brain edema through antioxidative mechanism (Lukic-Panin et al., 2007). Another group reported that AM decreased myocardial oxygen consumption of normal Syrian hamster (Loke et al., 2000). With respect to the above previous reports and our present data, AM possesses direct neuroprotective effect not only by antioxidative but also by anti-inflammatory mechanisms, which can also slow atherosclerosis.

Statin is primarily the blood lipid-lowering drug, but AT shows various pleiotropic effects such as lipid-lowering, preventing atherosclerosis, antioxidative, and anti-inflammation. Reactive oxygen species (ROS) lead to harmful effects by direct damage of cellular protein, lipid, and DNA in the brain during and after ischemia (Zhang et al., 2001; Schaller and Graf, 2004). Statins have antioxidant properties such as scavenging ROS (Franzoni et al., 2003), inhibiting ROS-induced DNA breakage, and restraining superoxide generation in blood vessel. We have demonstrated that AT and simvastatin reduced an infarct volume with strong antioxidative effect (Nagotani et al., 2005), that AT, simvastatin, and pitavastatin showed strong neuroprotective effects (Hayashi et al., 2005) and that simvastatin also inhibited atherosclerotic changes of common carotid artery in stroke-prone spontaneously hypertensive rats (SHR-SP) (Tsuchiya et al., 2007). Statins also exert an activation of induced endothelial nitric oxide synthase (eNOS) (Endres et al., 1998), an inhibition of Rho kinase (Zhou and Liao, 2009), a reduction of TNF-α and SOD levels (Hajipour et al., 2009), and an inhibition of MCP-1 (Shiraya et al., 2009). All of the above pleiotropic mechanisms may contribute to the strong protective effect of AT found in the present study (Figs. 1–3).

Our data clearly revealed that either single treatment with AM or AT significantly decreased infarct volume comparing with the vehicle group. However, the combination of AM plus AT treatment decreased the infarct volume stronger than each single treatment (Fig. 1). Amlodipine treatment decreased the blood pressure (Table 1), but this should not contribute to the reduction of infarction volume (Fig. 1) because the degree of blood pressure decrease and that of infarction volume reduction was not correlated. The numbers of positive cells for oxidative (Fig. 2) and inflammation (Fig. 3) markers decreased dramatically in the combination-treated group compared with single AM- or AT-treated group. These results suggest that the combination of AM plus AT treatment shows a synergistic effect for protecting ischemic brain after tMCAO. The present study also showed that single AT was more protective than single AM both in the antioxidative and anti-inflammatory effects (Figs. 2 and 3). Furthermore, both single AM or AT treatment and AM plus AT combination treatment were more effective to reduce oxidative damage than inflammatory damage (Fig. 2 vs 3). Statin may be more effective in antioxidation than anti-inflammation at this time point of 24 h after tMCAO.

In summary, the present study showed that a single treatment with AM or AT have neuroprotective effects with antioxidative and anti-inflammatory mechanisms, but combination therapy of AM plus AT showed a further benefit in acute ischemic neural damages (Figs. 1–3). A previous report showed a beneficial effect of AT in acute ischemic stroke patients to ameliorate clinical severity and with reducing their infarct size (Rodriguez-Yanez et al., 2008). However, the present study suggests a strong potential of this synergistic effect of AM plus AT combination as promising clinical therapeutic strategies for ischemic stroke.

4. Experimental procedures

4.1. Animals

Male Zucker-fatty rats (supplied from Disease Model Cooperative Research Association, Kyoto, Japan) at the age of 8 weeks (body weight 250–280 g) were used in this study. All experimental procedures were approved by the Animals Committee of the Okayama University Graduate School of Medicine. The animals were divided into 5 experimental groups such as the sham control group, the vehicle (0.5% methyl cellulose in physiological saline)-treated control group, the amlodipine (AM, 3 mg/kg per day)-treated group, the atorvastatin (AT, 10 mg/kg per day)-treated group, and the combination of AM plus AT (3+10 mg/kg per day)-treated group (n=5 in each group).

4.2. Drug treatment and focal cerebral ischemia

From the age of 8 weeks, drugs were administered by oral gavage every morning for 28 days. On the day before middle cerebral artery occlusion (MCAO) at 12 weeks of age, rats were anesthetized with a pentobarbital (5 mg/250 g rat, i.p.), and burr hole (diameter 2 mm) was made with 3 mm dorsal and 5 mm lateral to the right from the Bregma, which is located in the upper part of the MCA territory. After the burr hole was made, the rats were allowed to recover overnight at ambient condition. On the day of

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* p<0.01 for comparison with the amlodipine-treated group.
  b p<0.01 for comparison with the sham control group.
MCAO, the animals were anesthetized with a nitrous oxide/oxygen/isoflurane mixture (69%/30%/1%) during surgery with the use of an inhalation mask. The right MCA was occluded by an insertion of a 4-0 surgical nylon thread with silicon coating through the common carotid artery as described previously (Kawai et al., 2010). Body temperature was maintained at 37±0.3 °C using a heating pad during the surgical procedure. After 90 min of transient occlusion (tMCAO), the filament was gently removed to restore blood flow of MCA territory, and the incision was closed. Sham control animal were incised in the same region, and the incision was closed without thread insertion. Regional cerebral blood flow (rCBF) of the right frontoparietal cortical region was measured before, during, and after tMCAO through the burr hole using a laser blood flow meter (flo-C1; Omegawave, Tokyo, Japan). At 24 h after the reperfusion or sham operation, the Zucker rats (vehicle-treated control, the AM-treated, the AT-treated, and the combination of AM plus AT-treated groups) were sacrificed under deep anesthesia with pentobarbital (10 mg/250 g rat, i.p.). The rats were then transexally perfused with heparinized saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2). The whole brain was removed and immersed in the same fixation for 12 h at 4 °C. After washing with PBS, the tissues were transferred into graded sucrose of 10%, 20%, and 30% subsequently, then embedded in OCT in powdered dry ice, and stored at –80 °C until use. Twenty micrometer-thick coronal brain sections were cut in a cryostat at –18 °C and mounted on a silane-coated glass.

4.3. Histology and immunohistochemistry

For evaluation of the infarct volume, the brain sections were stained with cresyl violet (CV), which were then examined with a microscope (BX-51; Olympus Optical, Tokyo, Japan). The sections were measured in five sections by pixel counting using a computer program for Photoshop 7.0, and the volume was calculated.

For immunohistochemistry, the following primary antibodies were used: mouse anti-8-hydroxy-2′-deoxyguanosine (8-OhdG) antibody (1:50; JaICA, Shizuoka, Japan), mouse anti-4-hydroxy-2-nonenal (4-HNE) antibody (1:50; JaICA, Shizuoka, Japan), and mouse anti-advanced end glycation products (AGE) antibody (1:200; Transgenic, Kobe, Japan) for detections of oxidative DNA damage (8-OHdG), lipid peroxidation (4-HNE), and protein peroxidation (AGE), respectively. First, antibodies for tumor necrosis factor alpha (TNF-α) (1:50; R&D Systems, Abingdon, UK) and rabbit monocline chemotactant protein-1 (MCP-1) (1:100; abcam Cambridge, UK) were used as inflammation markers. To estimate the expression of each signal, the brain sections were incubated in 30% methanol and 0.3% hydrogen peroxide in PBS in order to quench endogenous peroxidase activity. After blocking non-specific reaction by normal house serum, the sections were incubated with the respective first antibody for 4 °C overnight; on the next day, the brain sections were washed twice with PBS and reacted with secondary antibody (1:500) at room temperature for 2.5 h. After incubation with ABC Elite complex (Vector Laboratories, Burlingame, CA, USA), the signal was visualized with diaminobenzidine tetrahydrochloride. In each study, a set of sections was stained in a similar way without the first antibody as a negative control. The sections were captured and examined by a microscope (BX-51; Olympus Optical, Tokyo, Japan).

4.4. Quantitative analysis

For the semiquantitative evaluation of immunohistochemical stainings such as 8-OhdG, 4-HNE, AGE, TNF-α, and MCP-1, the positively stained nerve cells were counted in the cerebral cortex at the ischemic core zone and the ischemic boundary zone (penumbra) in 15 sections per rat brain. Values are expressed as number of positive nerve cells/0.5 mm² in means±S.D. Statistical analysis was performed by non-repeated measures analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test. In all statistical analyses, significance was accepted at *p<0.05.

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