Research Report

Protection against ischemic stroke damage by synergistic treatment with amlodipine plus atorvastatin in Zucker metabolic rat

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ABSTRACT

Ischemic stroke is a major neurologic disorder and a leading cause of disability and 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. The animals were pretreated with vehicle, AM, AT, or the combination of AM plus AT for 28 days, and physical and serum parameters were analyzed, then 90 min of transient middle cerebral artery occlusion (tMCAO), was performed followed by immunohistochemical analyses at 24 h. Without affecting serum levels of lipids, adiponectin, and leptin, the combination therapy of AM plus AT ameliorated the post-ischemic brain weight increase. The single treatment with AM or AT itself exerted neuroprotective effects with reducing inductions of MMP-9 and AT2R, as well as with preserving collagen IV, and the combination therapy of AM plus AT showed a further synergistic benefit against acute ischemic neural damages. Single AT was more protective on these 3 molecules than single AM at this time point of 24 h after tMCAO. Thus, the combination therapy with AM plus AT extended the neuroprotective effect of single treatment with AM or AT on a part of neurovascular unit and a hypertension-related receptor.

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1. Introduction

Ischemic stroke is a major neurologic disorder and a leading cause of disability and death in the world. For prevention of ischemic stroke, anti-hypertensive drugs and statins (3-hydroxy-3-methylglutaric coenzyme A, HMG-CoA reductase inhibitor) are commonly used for hypertensive or hyperlipidemic patients, respectively. Amlodipine (AM) is a calcium channel blocker (CCB), and the most commonly used for hypertensive patients. AM is the longest-acting dihydropyridine, which does not only lower the blood pressure but also protects brain from ischemic damage (Rami and Krieglstein, 1994; Annoura et al., 1999). AM principally binds L-type Ca²⁺ channel which can catch free radical by electron-rich aromatic...
showed that AT and simvastatin reduced an infarct volume (Malik et al., 2004; Arenillas et al., 2009). Our previous report only single therapy for such as HT or HL but also both diseases factor for cardiovascular and ischemic stroke, needing not 1965; Zucker and Antoniades, 1972). Obesity is the major risk 1998; Asahi et al., 2001) and neurovascular unit including collagen IV (Guo et al., 2008; Yamashita et al., 2009). Angiotensin 2 (AT2) is a vasoconstrictor peptide which induces ROS (Wassmann et al., 2002), and AT2 receptor (AT2R) blockers (ARB) reduced infarct volume in the ischemic rat (Nishimura et al., 2000; Kobayashi et al., 2009).

Zucker-fatty rat is a well-understood model of obesity and metabolic syndrome with deficient leptin receptor (Vaziri et al., 2005), showing metabolic characters of insulin resistance, hypertriglyceridemia, and hyperlipidemia (HL) (Zucker, 1965; Zucker and Antoniades, 1972). Obesity is the major risk factor for cardiovascular and ischemic stroke, needing not only single therapy for such as HT or HL but also both diseases (Malik et al., 2004; Arenillas et al., 2009). Our previous report showed that AT and simvastatin reduced an infarct volume with drastic antioxidative effect (Nagotani et al., 2005); that AT, simvastatin and pitavastatin reduced drastic 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).

Although there have been several reports of single treatment with AM (Lukic-Panin et al., 2007; Nishida et al., 2008) or AT (Wassmann et al., 2002; Nagotani et al., 2005; Hayashi et al., 2005; Liu et al., 2006), their combination has not been investigated with respect to focusing protective effect on MMP-9, collagen IV, and AT2R. In the present study, therefore, we examined and compared such a single treatment with AM or AT and the combined therapy with AM plus AT on such molecular markers.

2. Results

2.1. Physical data and serum chemicals

The data of body weight, brain weight, serum levels of TG, T-cho, HDL-cho, LDL-cho, adiponectin, and leptin are shown in Table 1. Body weight of Zucker-lean rats was 258.3±8.8 g, and that of Zucker-fatty rats was 285.6±19.5 g in any of 5 groups significantly higher than the Zucker-lean rats (p<0.01). Brain weight of sham group was 1.3±0.1 g, which became heavier to 1.5±0.0 g (p<0.01) after tMCAO in the vehicle groups. Treatment of AT and combination of AM plus AT groups ameliorated such a brain weight increase after tMCAO to 1.4±0.1 and 1.4±0.0 g (p<0.01) than vehicle, respectively.

Serum TG level of Zucker-lean rat was 41.4±6.4 mg/dL, and that of Zucker-fatty rats was 218.0±74.4 mg/dL in which any of 5 groups significantly higher than the Zucker-lean rats (p<0.01). T-cho level of Zucker-lean rat was 71.0±3.1 mg/dL, and that of Zucker-fatty rats was 107.0±117.4 mg/dL in any of 5 groups significantly higher than Zucker-lean rats (p<0.01). HDL-cho level of Zucker-lean rat was 30.4±1.5 mg/dL, and that of Zucker-fatty rats was 47.4±50.2 mg/dL in which any of 5 groups significantly higher than the Zucker-lean rats (p<0.01). LDL-cho level of Zucker-lean rat was 10.2±1.1 mg/dL, and that of Zucker-fatty rats was 11.0±13.6 mg/dL in among 5 groups, both of AM and combination of AM plus AT groups significantly higher than the Zucker-lean rats (p<0.01). Adiponectin level of Zucker-lean rat was 5.6±1.5 mg/L, and that of Zucker-fatty rats was 9.0±10.2 mg/L in which any of 5 groups significantly higher than the Zucker-lean rats (p<0.01). Leptin level of Zucker-lean rat was 0.6±0.1 ng/mL, and that of Zucker-fatty rats was 9.4±11.6 ng/mL in which any of 5 groups significantly higher than the Zucker-lean rats (p<0.01).

Table 1. Physical data and serum chemicals.

<table>
<thead>
<tr>
<th>Zucker-lean rats (n=5)</th>
<th>Zucker-fatty rat</th>
<th>tMCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Sham (n=5)</td>
<td>Vehicle (n=5)</td>
</tr>
<tr>
<td>258.3±8.8</td>
<td>385.6±19.5</td>
<td>400.8±15.0</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1.5±0.0</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>41.4±6.4</td>
<td>218.0±142.3</td>
</tr>
<tr>
<td>T-cho (mg/dL)</td>
<td>71.0±3.1</td>
<td>107.3±7.6</td>
</tr>
<tr>
<td>HDL-cho (mg/dL)</td>
<td>30.4±1.5</td>
<td>49.0±2.6</td>
</tr>
<tr>
<td>LDL-cho (mg/dL)</td>
<td>10.2±1.1</td>
<td>11.0±1.0</td>
</tr>
<tr>
<td>Adiponectin (mg/L)</td>
<td>5.6±1.5</td>
<td>9.6±2.0</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>0.6±0.1</td>
<td>10.3±2.1</td>
</tr>
</tbody>
</table>

*p<0.01 for comparison with the lean group.

Table 1. Physical data and serum chemicals.
2.2. Immunohistochemistry of MMP-9 and collagen IV

Immunohistochemical analysis of Zucker-fatty rat showed that almost no staining for MMP-9 in the sham control brain (data not shown), but that tMCAO induced strong staining of MMP-9 in the ischemic core (core) and the boundary penumbral (penumbra) zones at 24 h after the reperfusion (Fig. 1, vehicle group). The MMP-9 expression was observed in nerve cells and blood vessels in both ischemic core and penumbra. Compared with this vehicle group, single treatment with AM significantly reduced the intensity of MMP-9 staining in the ischemic core (*p<0.05 vs. vehicle group) (Fig. 1, upper pictures) and the ischemic penumbra (**p<0.01 vs. vehicle group) (Fig. 1, lower illustrations). Single treatment with AT further reduced the intensity of MMP-9 staining in both of the ischemic core and penumbra (**p<0.01 vs. vehicle group). Among single treatments, the effect of AT was much stronger in decreasing the intensity of MMP-9 staining than AM treatment both in the ischemic core (*p<0.05 vs. AM group) and the ischemic penumbra (**p<0.01 vs. AM group). Combination of AM plus AT treatment also showed a reduction of the intensity of MMP-9 staining both in the ischemic core (**p<0.01 vs. vehicle group) and the penumbra (**p<0.01 vs. vehicle group), but did not show a further reduction than single AT treatment.

Immunohistochemical analysis of Zucker-fatty rat showed a degradation of collagen IV staining in the ischemic core and penumbra (Fig. 1, vehicle group) at 24 h after tMCAO, compared with sham control group (data not shown). Collagen IV was observed as peri-vascular staining of cerebrovascular endothelium (Fig. 1). Single treatment with AM improved the intensity of collagen IV staining in the ischemic core (Fig. 1, upper pictures) and the ischemic penumbra (Fig. 1, lower illustrations), but difference was not

![Fig. 1](attachment:image.png)
significant. Single treatment with AT further improved the intensity of collagen IV staining in both of the ischemic core (**\( p < 0.01 \) vs. vehicle group, \( \ast \ast \ p < 0.05 \) vs. AM group) but still not in the ischemic penumbra. However, combination of AM plus AT treatment showed a significant improvement of the collagen IV staining both in the ischemic core (**\( p < 0.01 \) vs. vehicle group) and the penumbra (\( \ast \ast \ast \ p < 0.01 \) vs. AM group). The sections without first antibody did not show any positive staining in both of MMP-9 and collagen IV markers.

### 2.3. Immunohistochemistry for angiotensin 2 type 1 receptor

Immunohistochemical analysis showed that AT2R is expressed in nerve cells of sham control brain of Zucker-fatty rats (Fig. 2, Sham) and that tMCAO induced strong staining of AT2R in nerve cells of the ischemic core and the penumbra at 24 h of reperfusion (Fig. 2, vehicle, \( \# \# \ p < 0.01 \) vs. sham group). Single treatment with AM reduced the number of such AT2R-positive cells (**\( p < 0.01 \) vs. vehicle group) in the ischemic core (Fig. 2, upper picture) and the penumbra (Fig. 3, lower illustrations), compared with vehicle group. Single treatment with AT also significantly reduced the number of such AT2R-positive cells (**\( p < 0.01 \) vs. vehicle group) both in the ischemic core (Fig. 2, upper picture) and the penumbra (Fig. 3, lower illustrations). Combination of AM plus AT treatment showed a similar effect with single AT treatment and did not show a further reduction than single AT treatment both in the ischemic core and penumbra. The sections without first antibody did not show any positive staining in AT2R.

### 3. Discussion

In the present study, the body weight was heavier in Zucker-fatty rats than Zucker-lean rats (Table 1). The brain weight was increased in Zucker-fatty rats at 24 h after tMCAO from 1.3±0.1 to 1.5±0.0 g probably due to brain edema (Table 1). However, AT and combination of AM plus AT groups decreased such a change of the brain weight to 1.4 g (Table 1), probably due to reduction of brain edema after tMCAO (Wang et al., 2010). Serum levels of TG, T-cho, HDL-cho, LDL-cho, adiponectin, and leptin were higher in Zucker-fatty rats than Zucker-lean rats (Table 1). Otherwise, any serum levels did not show significant difference within 5 Zucker-fatty rats groups, as our previous reports showing that the statin treatment did not decrease the cholesterol levels in the rat (Hayashi et al., 2005; Nagotani et al., 2005).

Adiponectin, a hormone secreted by adipocytes, possesses anti-atherogenic, anti-inflammatory, and anti-diabetic property (Okamoto et al., 2006). Similar to a previous report (Honnma et al., 2010), serum adiponectin level in Zucker-fatty rats was 2 times higher than in Zucker-lean rats in the present study (Table 1). Although treatment with statin slightly elevated serum adiponectin levels in human patients and mice (Krysiai et al., 2009; Ohashi et al., 2010; Ishihara et al., 2010), our present study did not reach a significant effect of statin on such a serum adiponectin level as another report (Chen et al., 2007). Zucker-fatty rat congenitally lacks the leptin receptor gene, which causes hyperleptinemia and hyperphagia (Ionescu et al., 1985). Leptin production was significantly enhanced in enlarged adipose cells of the Zucker-fatty rats (Turban et al., 2002), and the serum level of leptin is related to detrimental metabolic consequences (Bodkin et al., 1996). Similar to previous reports (Chen et al., 2007; Barbu et al., 2009), serum level of leptin was 16–19 times higher in Zucker-fatty rats than Zucker-lean rats, and the serum levels were not different among 5 Zucker-fatty rat groups in the present study (Table 1).

Ischemia and reperfusion cause deterioration of cerebral microvascular basal lamina, especially collagen IV with activating MMP-9 by ROS (Maier et al., 2006; Yamashita et al., 2009; Hu et al., 2010). Atorvastatin significantly ameliorated MMP-9 activation in the ischemic region (Liu et al., 2006), and pravastatin reduced the collagen IV damage following transient ischemia (Trinkl et al., 2006). Our present data revealed that the activation of MMP-9 (Fig. 1) was markedly improved in AM, AT, and AM plus AT combination groups, and the reduction of collagen IV was also markedly recovered in AM.

![Fig. 2](image-url) - (A) Photomicrographs for AT2R immunostaining of the ischemic core of Zucker rat brain treated with vehicle, AM, AT, or AM plus AT combination, and (B) quantitative analysis of the positive cells. Note tMCAO induced strong staining of AT2R in nerve cells of the vehicle group, which were greatly reduced in AM, AT, and AM plus AT groups (A). Scale bar=100 μm (**\( p < 0.01 \) vs. sham control group, \( \ast \ast \ p < 0.01 \) vs. vehicle group, \( \ast \ p < 0.05 \) vs. AM group).
plus AT combination group [Fig. 1]. We have reported that AM reduced an infarct volume and brain edema with antioxidative effects on neuronal protein, lipid, and DNA (Lukic-Panin et al., 2007); that AT, simvastatin and pitavastatin reduced an infarct volume with a strong antioxidative effect (Nagotani et al., 2008; Hayashi et al., 2005); and that simvastatin inhibited atherosclerotic changes of common carotid artery in stroke-prone spontaneously hypertensive rats (SHR-SH) (Tsuchiya et al., 2007). AT2 is known to induce ROS, which were significantly reduced in rat vascular endothelium treated with AT (Wassmann et al., 2002), and an induction of AT2 protein was inhibited by AM treatment in brain of SHR-SP (Nishida et al., 2008). Our present data clearly revealed that AT2R was upregulated in neuronal cells at 24 h after tMCAO, and that such as activation of AT2R was markedly improved in AM, AT, and AM plus AT combination groups (Fig. 2).

We previously found that a combination of AM plus AT showed stronger reductions of infarct volume, oxidative stress, and inflammation in the ischemic brain compared with vehicle group and single treatment with AM or AT (Kawai et al., 2010). These results demonstrated a synergistic effect of the combination of AM plus AT treatment for protecting ischemic brain after tMCAO. The present study extends such a synergistic protective effect of AM plus AT on MMP-9, collagen IV and AT2R, and also shows that single AT was more synergistic effect against acute ischemic neural damages (Figs. 1 and 2). From the results of this study, we are confident that this synergistic effect of AM plus AT combination is a promising clinical preventive therapeutic strategy for ischemic stroke.

4. Experimental procedures

4.1. Animals

Adult (250 to 280 g, 8 weeks old) male Zucker-lean and Zucker-fatty rats (supplied from Disease Model Cooperative Research Association, Kyoto, Japan) were used in this study. All experimental procedures were approved by the Animals Committee of the Okayama University Graduate School of Medicine. We studied 5 experimental groups with Zucker-fatty rats including 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 mg/kg+10 mg/kg per day)-treated group (n=5 in each group). Zucker-lean rats were analyzed just for physical parameters and analysis of serum chemicals.

4.2. Drug treatment and focal cerebral ischemia

Drugs were administered orally gavage every evening for 28 days starting from age 8 weeks. On the 3 days before middle cerebral artery occlusion (MCAO) at 12 weeks of age, rats were anesthetized with a nitrous oxide/oxygen/isoflurane mixture (69/30/1%), and their blood samples collection were performed. On the day before the MCAO, rats were anesthetized with 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.

On the next day, 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 insertion of a 4–0 surgical nylon thread with silicon coat through the common carotid artery, according to our previous report (Kawai et al., 2010). Body temperature was monitored and kept 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 animals were incised same region, and the incision was closed without thread insertion. Regional cerebral blood flow (rCBF) of the right frontoparietal cortex 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 transcardially 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. Coronal brain sections with 20 μm thickness were prepared using cryostat at −18 °C and mounted on a silane-coated glass.

4.3. Serum analysis

To assess serum levels, blood was drawn from the jugular vein 3 days before a sham or MCAO operation. Serum level of triglyceride (TG), total cholesterol (T-cho), high-density lipoprotein-cholesterol (HDL-cho), and low-density lipoprotein-cholesterol (LDL-cho) were measured by standard biochemical method in SRL, Inc. (Tokyo, Japan). The serum concentration of adiponectin was measured with a rat adiponectin ELISA kit (Otsuka, Tokyo, Japan) and leptin was measured with a leptin measurement kit (Morinaga, Yokohama, Japan) according to the manufacturer’s specifications.

4.4. Immunohistochemistry

For the immunohistochemical analysis, the brain sections (20 μm thickness) 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 at 4 °C overnight for rabbit anti-matrix metalloproteinase-9 (MMP-9) (1:150; Millipore, Billerica, MA, USA), rabbit anti-collagen IV (collagen IV) (1:100; Novotec, Lyon, France), or rabbit ant-angiotensin 2 type 1 receptor
(AT2R) (1:500; Abcam Cambridge, UK). On the next day, the brain sections were washed twice with PBS, and reacted with the same anti-rabbit biotin-labeled secondary antibody (1:500; Jackson ImmunoResearch Laboratories, Inc., PA, USA) 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).

4.5. Quantitative analysis

For the semiquantitative evaluation of immunohistochemical staining, such as for MMP-9, collagen IV, and angiotensin 2 type 1 receptor (AT2R1). The intensity of MMP-9 and collagen IV staining and AT2R1 positively stained nerve cells were evaluated 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 intensity and number of positive nerve cells/0.5 mm² in means±SD. The staining intensity was measured using Scion Image software (Scion Corporation). Statistical analysis was performed by nonrepeated 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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