Original Article

Attenuation of Focal Brain Ischemia by Telmisartan, an Angiotensin II Type 1 Receptor Blocker, in Atherosclerotic Apolipoprotein E–Deficient Mice

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The effects of an angiotensin II (Ang II) type 1 (AT_1) receptor blocker (ARB) on focal brain ischemia and atherosclerotic lesions were explored in atherosclerotic apolipoprotein E–deficient (ApoEKO) mice treated with a high-cholesterol diet (HCD). The ischemic brain area and neurological deficit 24 h after middle cerebral artery (MCA) occlusion were significantly greater in ApoEKO mice treated with HCD for 10 weeks than in those with a normal standard diet. The reduction of cerebral surface blood flow in the penumbral region and the increase in superoxide production in the ischemic area were exaggerated in HCD-treated ApoEKO mice. Histological analysis showed atherosclerotic changes in the proximal aorta and deposition of lipid droplets in the arterial wall in the brain. Administration of an ARB, telmisartan (0.3 mg/kg/day), for the last 2 weeks after 8 weeks of HCD feeding attenuated the ischemic brain area, the neurological deficit, the superoxide production in the ischemic area, and the reduction of cerebral blood flow in the penumbra, without significantly changing blood pressure or serum cholesterol level. Telmisartan also decreased atherosclerotic lesion formation in the proximal aorta of HCD-treated ApoEKO mice, although it did not remarkably change lipid deposition in the cerebral arteries. These results suggest that the blockade of the AT_1 receptor attenuates ischemic brain damage induced in an atherosclerosis model. This inhibitory action is mediated through the attenuation of the reduction in cerebral blood flow and of oxidative stress in the brain; it also mediated through telmisartan's anti-atherosclerotic effect. (*Hypertens Res* 2008; 31: 161–168)

***Key Words*: cerebral ischemia, atherosclerosis, angiotensin, receptors, oxidative stress**

Introduction

Atherosclerosis is associated with various cardiovascular events, such as coronary heart disease and stroke. Recent clinical studies have indicated a close relationship between atherosclerosis of the carotid and coronary arteries and the risk of stroke (*1–3*). In these studies, regression of atherosclerosis seemed to lower the risk of stroke. However, the pathogenesis and effective prevention of ischemic brain damage in atherosclerosis patients have not been well studied. Previous studies also suggested that the renin-angiotensin system plays an important role in stroke. Angiotensin II was found to be significantly involved in ischemic brain damage in a study using a mouse model of brain infarction induced by middle cerebral artery (MCA) occlusion (*4–6*). Focal brain ischemia was reduced in angiotensin II type 1 (AT_1) receptor–deficient mice (*4*), whereas it was exaggerated in angiotensin II type 2 (AT_2) receptor–deficient mice (*5*). Peripheral administration of an AT_1 receptor blocker (ARB) significantly reduced the

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This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Cardiovascular Research Foundation, the Takeda Science Foundation, and the Mitsubishi Pharma Research Foundation.

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Received June 1, 2007; Accepted in revised form August 5, 2007.

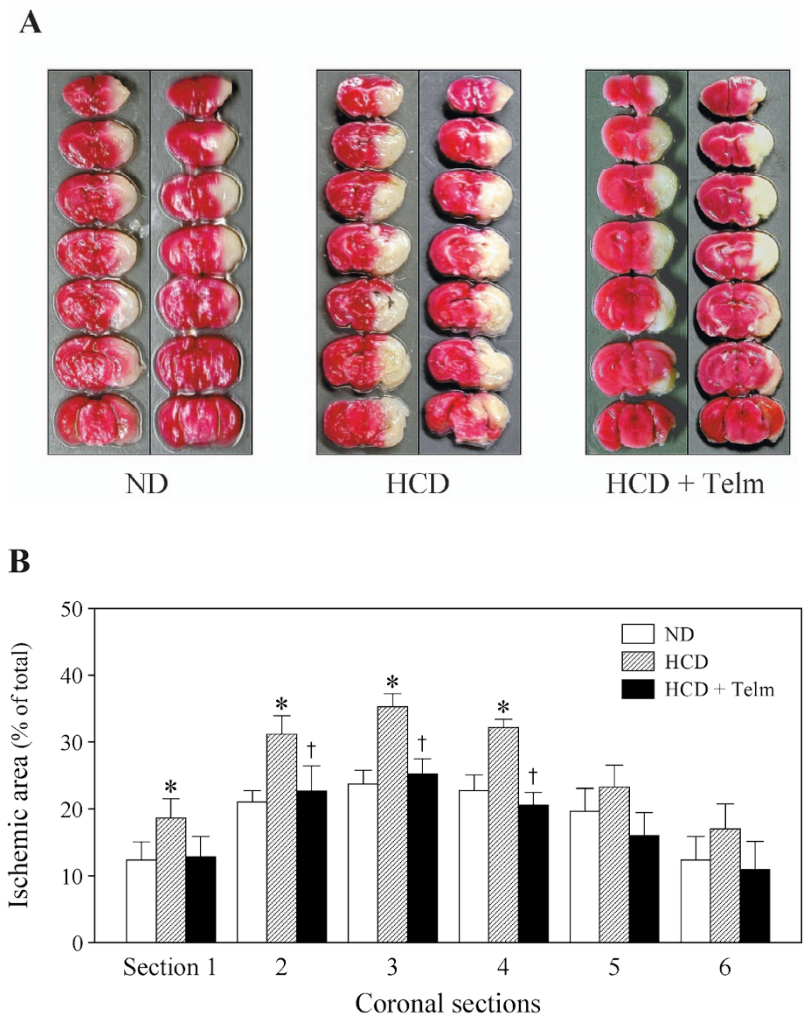

Fig. 1. *Focal brain ischemic area 24 h after MCA occlusion in apolipoprotein E–deficient (ApoEKO) mice treated with a high-cholesterol diet (HCD). ApoEKO mice were fed for 10 weeks with either a normal standard diet (ND) or a HCD containing 1.25% cholesterol. Coronal sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC). Brain sections were numbered from frontal (section 1) to caudal (section 6). Some mice were treated with telmisartan (Telm; 0.3 mg/kg/day) for 14 days using an osmotic minipump after 8 weeks of HCD feeding. A: TTC staining of brain sections from ApoEKO mice 24 h after MCA occlusion. B: Morphometry of ischemic area determined with TTC staining and expressed as a percentage of total area. n = 7–8 for each group. *$p < 0.05$ vs. ND. †$p < 0.05$ vs. HCD without telmisartan. Values are means ± SEM.*

ischemic brain area and neurological deficit after MCA occlusion (*5*, *7*). On the other hand, apolipoprotein E–deficient (ApoEKO) mice treated with a high-cholesterol diet (HCD) developed atherosclerotic changes. Blockade of the AT_1 receptor markedly attenuated atherosclerotic changes in ApoEKO mice treated with HCD (*8*). In addition, atherosclerotic lesion formation was lower in ApoE/AT_1a receptor–deficient mice than in ApoEKO mice (*9*), while it was higher in ApoE/AT_2 receptor–deficient mice (*10*). Moreover, previous reports suggested that suppression of oxidative stress is important in ARBs' inhibitory effects on ischemic brain damage as well as atherosclerosis (*6*, *8*, *10*).

The present study was designed to explore 1) the influence of atherosclerosis on ischemic brain damage using ApoEKO mice; 2) whether or not ARB inhibits ischemic brain damage in ApoEKO mice after treatment with HCD, in which atherosclerotic formation has been observed; and 3) whether or not the inhibition of ischemic brain damage is related to the inhibition of atherosclerosis.

Methods

Animals

Adult male ApoEKO mice (B6.129P2-*Apoe*^{tm1Unc}, The Jackson Laboratory, Bar Harbor, USA; 10× backcrossed) were

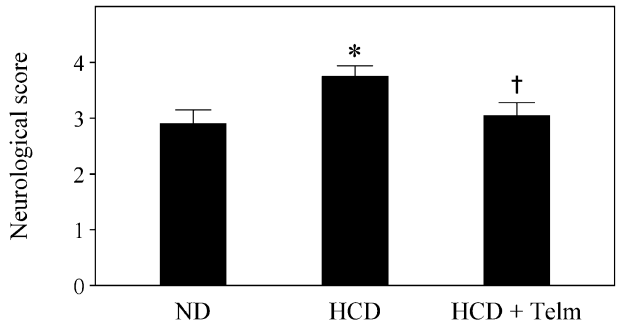

Fig. 2. *Neurological score after MCA occlusion in ApoEKO mice treated with HCD. MCA occlusion was performed, and the neurological score was used to evaluate neurological deficit 24 h after the operation. ND, normal standard diet; HCD, high-cholesterol diet; Telm, telmisartan. n = 7–8 for each group. *p < 0.05 vs. ND. †p < 0.05 vs. HCD without telmisartan. Values are means ± SEM.*

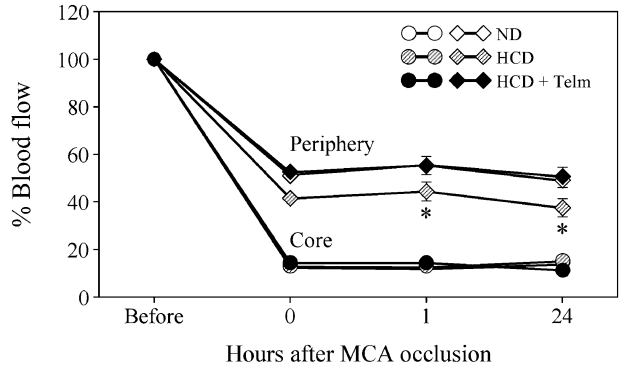

Fig. 3. *Change in cerebral blood flow after MCA occlusion in ApoEKO mice treated with HCD. Cerebral surface blood flow was determined immediately, 1 h after, and 24 h after MCA occlusion by laser-Doppler flowmetry in the core and periphery (penumbra). Change in blood flow was expressed as a percentage of basal flow rate. ND, normal standard diet; HCD, high-cholesterol diet; Telm, telmisartan. n = 6–7 for each group. *p < 0.05 vs. ND. Values are means ± SEM.*

housed in a room where lighting was controlled with a 12-h light/dark cycle and room temperature was kept at 25°C (*10*). They were given a standard diet (MF, Oriental Yeast, Tokyo, Japan) or HCD (1.25% cholesterol, 10% coconut oil in MF) for 10 weeks from 6 weeks of age and water ad libitum. Telmisartan (0.3 mg/kg/day; provided by Nippon Boehringer Ingelheim Co., Ltd., Kawanishi, Japan), an AT_1 receptor–selective ARB, was administered for the last 2 weeks after 8 weeks of HCD feeding, using an osmotic minipump (Alzet model 1002, Durect, Cupertino, USA) implanted intraperitoneally. Serum cholesterol level was measured by the cholesterol oxidase method (Cholesterol E-test, Wako Pure Chemical Industry, Tokyo, Japan). Blood pressure was measured by the indirect tail-cuff method with a blood pressure monitor (MK-1030, Muromachi Kikai, Tokyo, Japan). The experimental protocol was approved by the Animal Studies Committee of Ehime University.

MCA Occlusion

Focal cerebral ischemia was induced by occlusion of the MCA using an intraluminal filament technique according to the method previously described (*6*, *11*). Brain samples were obtained 24 h after MCA occlusion, and 1-mm-thick coronal sections were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) as previously described (*6*, *11*). Neurological deficit was evaluated 24 h after MCA occlusion using the neurological scores developed by Huang *et al*. (*12*).

Laser-Doppler Flowmetry

Cerebral surface blood flow was determined in the territory of the MCA by laser-Doppler flowmetry using a flexible fiberoptic extension to the master probe (Omegaflo FLO-C1, Omegawave, Tokyo, Japan). The tip of the probe was fixed to the intact skull over the territory supplied by the proximal part of the MCA (core; 2 mm caudal to bregma and 6 mm lateral to midline) and the peripheral penumbra of the MCA territory (periphery; 2 mm caudal to bregma and 3 mm lateral to midline) (*13*, *14*) using a tissue adhesive (Aron Alpha; Toa, Tokyo, Japan). Changes in cerebral surface blood flow after MCA occlusion were expressed as percentages of the baseline value.

In Situ Detection of Superoxide Anion in Brain Sections

Histological detection of superoxide anion was carried out as described previously (*15*). In brief, frozen, enzymatically intact, 10-μm-thick coronal sections were prepared from the mouse brain 24 h after MCA occlusion, then incubated with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 min at 37°C in a humidified chamber protected from light. Red fluorescence of ethidium on the specimen was detected using an Axioskop microscope (Axioskop 2 Plus with AxioCam, Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. The fluorescence intensity was analyzed and quantified using imaging software (Densitograph, Atto, Tokyo, Japan).

Determination of Atherosclerotic Lesion Size in Proximal Aorta

The mice were sacrificed at 16 weeks of age, and fresh-frozen sections of the proximal aorta were prepared as previously described (*10*). The area of atherosclerotic lesions in the proximal aorta and the area of lipid in the aortic wall were deter-

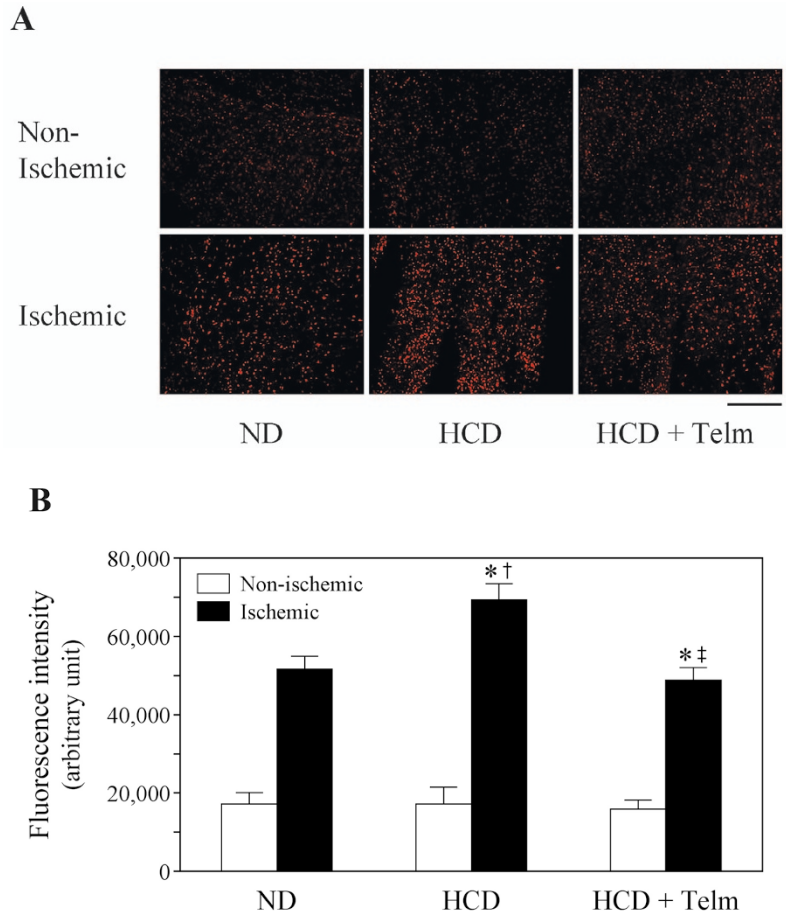

Fig. 4. *Detection of superoxide anion production in brain after MCA occlusion in ApoEKO mice treated with HCD. Fresh-frozen brain sections were prepared 24 h after MCA occlusion, and superoxide anion production was detected with dihydroethidium (DHE, 10 μmol/L). A: Photos show reproducible staining of brain sections (cortex) from non-ischemic and ischemic areas. Scale bar shows 100 μm. B: Intensity analysis of superoxide production stained with DHE. ND, normal standard diet; HCD, high-cholesterol diet; Telm, telmisartan. n = 6–7 for each group. *$p < 0.05$ vs. Non-ischemic. †$p < 0.05$ vs. ND. ‡$p < 0.05$ vs. HCD without telmisartan. Values are means ± SEM.*

mined in cross sections taken intermittently throughout a 2–3 mm length of the aortic arch with oil-red O staining and counterstaining with hematoxylin. Quantitative analysis was performed with Densitograph imaging software. The mean value of three sections was taken as the value for each animal. Fresh-frozen coronal sections of the brain including the MCA territory were also stained with oil-red O and counterstained with hematoxylin to determine lipid deposition in brain arteries in ApoEKO mice after HCD treatment.

Statistical Analysis

Values are expressed as mean±SEM in the text and figures. The effects of the different treatments on all data were evaluated by analysis of variance followed by Bonferroni's multiple range test. A value of $p<0.05$ was considered statistically significant.

Results

Ischemic Brain Damage after MCA Occlusion in ApoEKO Mice with HCD

In ApoEKO mice after 10 weeks of HCD treatment, the serum cholesterol level was markedly increased and systolic blood pressure tended also to be elevated. In these mice, the ischemic brain area in sections 1 to 4 after MCA occlusion was about 48% greater than in mice with normal standard diet (ND) ($p<0.05$, Fig. 1). Neurological deficit, determined by the neurological score, was significantly increased in the HCD group (Fig. 2). Mortality in the 24 h after MCA occlusion was also increased in the HCD group (41% in ND group *vs*. 59% in HCD group).

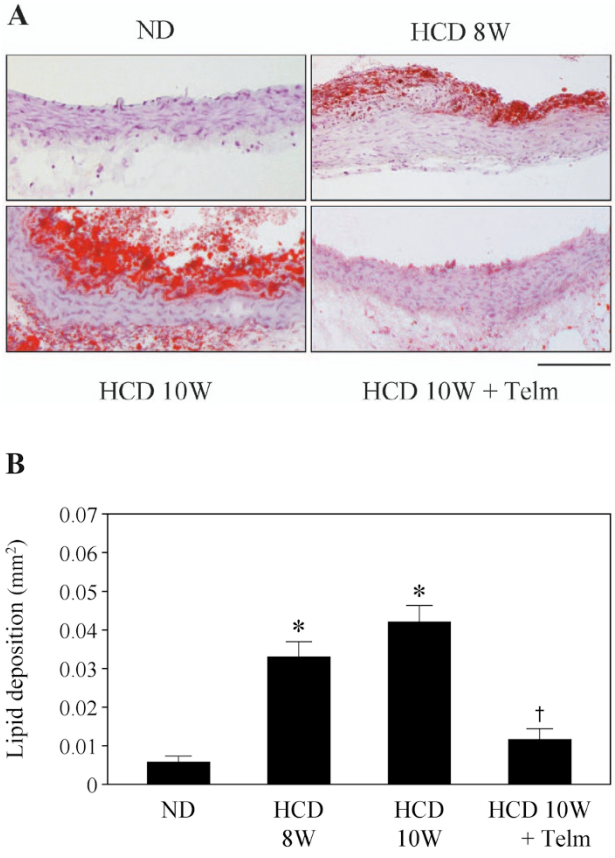

Fig. 5. *Atherosclerotic changes in ApoEKO mice treated with HCD for 10 weeks. Fresh-frozen sections of the proximal aorta were prepared and stained with oil red-O to determine lipid deposition and counter-stained with hematoxylin to measure the lesion area. A: Representative staining of proximal aorta with oil red-O in ApoEKO mice with or without treatment with telmisartan. Scale bar shows 100 μm. B: Morphological measurement of lipid deposition in cross sections of proximal aorta. n = 6–7 for each group. ND, normal standard diet; HCD, high-cholesterol diet; Telm, telmisartan. *p < 0.05 vs. ND. †p < 0.05 vs. HCD for 10 weeks without telmisartan. Values are means ± SEM.*

Change in Cerebral Surface Blood Flow after MCA Occlusion in ApoEKO Mice

Cerebral surface blood flow in the MCA territory was reduced after occlusion (Fig. 3). The reduction in the core region was similar in the ND and HCD groups. However, the flow reduction in the peripheral (penumbral) region was even greater in the HCD group (Fig. 3).

In Situ Detection of Superoxide Production in Brain after MCA Occlusion

To examine oxidative stress in the ischemic area, superoxide production was detected as fluorescence using dihydroethidium (Fig. 4). Treatment of ApoEKO mice with HCD did not significantly affect superoxide production in the cortex on the non-ischemic side. As previously reported, superoxide production was increased in the ischemic area after MCA occlusion. This increase was further enhanced in the HCD group compared to that in the ND group (Fig. 4).

Atherosclerotic Lesion Formation in Proximal Aorta and Lipid Deposition in Cerebral Arteries of ApoEKO Mice after HCD Treatment

ApoEKO mice treated with HCD for 10 weeks developed atherosclerotic changes accompanied by lipid accumulation in the proximal aorta (Fig. 5). Atherosclerotic lesions were already apparent after 8 weeks of HCD feeding (Fig. 5). Figure 6 shows oil-red O staining of cerebral arteries in the circle of Willis (Fig. 6A) and a branch of the MCA (Fig. 6B) at 24 h after MCA occlusion. After treatment with HCD for 10 weeks, lipid deposition was observed in the arterial wall of the brain in ApoEKO mice, such as in the circle of Willis, as well as in a branch of the MCA. On the other hand, only slight thickening of the arterial wall was observed in the circle of Willis, and it was not apparent in branches of the MCA in ApoEKO mice. Such lipid deposition and arterial thickening were not observed in the brains in the ND group.

Effect of Telmisartan on Ischemic Brain Damage in ApoEKO Mice with HCD

Telmisartan was administered for the last 2 weeks after 8 weeks of HCD feeding, at which time atherosclerotic changes were apparent in the proximal aorta. Telmisartan decreased the ischemic brain area and the neurological deficit after MCA occlusion (Figs. 1 and 2). Telmisartan also attenuated the exaggerated reduction of surface cerebral blood flow after MCA occlusion in HCD-treated ApoEKO mice (Fig. 3). In addition, telmisartan attenuated the increase in superoxide production in the ischemic area of the brain after MCA occlusion (Fig. 4).

Effect of Telmisartan on Atherosclerotic Changes in ApoEKO Mice with HCD

Administration of telmisartan for 2 weeks after 8 weeks of HCD feeding also markedly attenuated atherosclerosis formation in the proximal aorta (Fig. 5). On the other hand, telmisartan treatment for 2 weeks did not seem to significantly reduce lipid deposition in arteries or thickening of the arterial wall in the brain, although these changes tended to be attenuated (Fig. 6). This dose of telmisartan did not significantly affect systolic blood pressure or plasma cholesterol level in HCD-treated ApoEKO mice (Table 1).

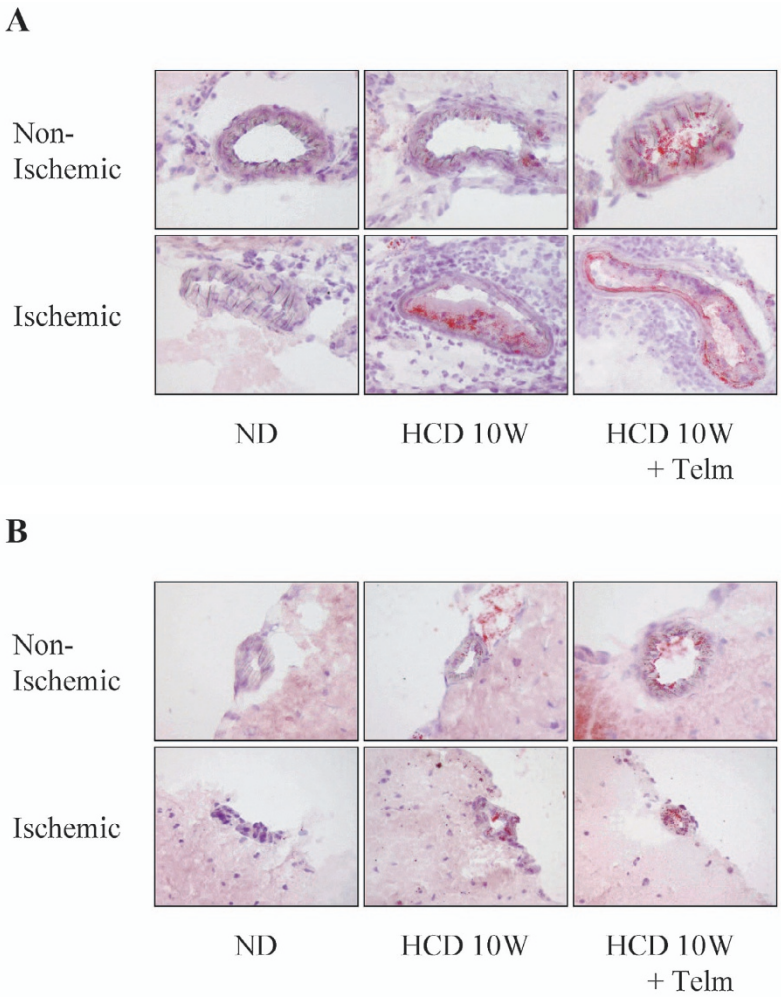

Fig. 6. *Lipid deposition in cerebral arteries of ApoEKO mice treated with HCD. Fresh-frozen coronal sections of the brain were prepared and stained with oil red-O. Photos show reproducible staining of cerebral arteries from non-ischemic and ischemic sides. A: Coronal section of artery of circle of Willis. B: Artery of brain surface in MCA territory. Magnification: ×200. ND, normal standard diet; HCD, high-cholesterol diet; Telm, telmisartan. n = 4–5 for each group.*

Discussion

The results of this study indicated that HCD treatment worsened ischemic brain damage induced by MCA occlusion in ApoEKO mice, and that an ARB, telmisartan, inhibited ischemic brain damage in ApoEKO mice after MCA occlusion. Telmisartan's inhibitory action was mediated by attenuation of oxidative stress in the ischemic region and of the blood flow reduction in the brain. Since telmisartan inhibited atherosclerotic changes in the proximal aorta in ApoEKO mice, it is possible that the attenuation of ischemic brain damage by telmisartan is due, at least in part, to the inhibition of atherosclerotic changes.

In our study, atherosclerosis was induced in ApoEKO mice by HCD feeding. ApoEKO mice treated with HCD for 8 to 10 weeks developed atherosclerotic plaques in the proximal aorta (Fig. 5). In these mice, the ischemic brain area and neurological deficit after MCA occlusion were significantly increased (Figs. 1 and 2). These results indicate that atherosclerotic change was involved in the worsening of ischemic brain damage after MCA occlusion. In fact, mild lipid deposition and slight thickening of vessel walls was observed in the cerebral arteries, although there was no apparent formation of atherosclerotic plaque (Fig. 6). Therefore, the increase in ischemic brain damage in ApoEKO mice may not be due directly to the structure of cerebral arteries by atherosclerotic plaque formation. Such slight changes in lipid deposition and arterial thickening may rather cause stiffness of the cerebral arteries. In our study, the reduction of cerebral surface blood flow after MCA occlusion was exaggerated in ApoEKO mice after HCD feeding (Fig. 3). Moreover, we have already

Table 1. Systolic Blood Pressure and Serum Cholesterol Concentration in ApoEKO Mice Treated with HCD

	SBP (mmHg)	Cholesterol (mg/dL)
ND	97.6±2.0	432±37
HCD	103.7±1.8	1,847±351*
HCD+telmisartan	98.4±2.1	1,811±156*

Animals were treated with HCD for 10 weeks with or without telmisartan (0.3 mg/kg/day for 14 days after 8 weeks of HCD feeding) as described in Methods. Values are mean±SEM. $n=8$–10 for each group. SBP, systolic blood pressure; ND, normal standard diet; HCD, high-cholesterol diet. *$p<0.05$ *vs*. ND.

reported that superoxide production is increased in the ischemic area of the brain after MCA occlusion (*6*). Such an increase in superoxide production in the ischemic area was further enhanced in HCD-treated ApoEKO mice compared with those with ND (Fig. 4). Since previous reports indicate that oxidative stress is involved in atherosclerotic lesion formation (*16–18*), the increase in superoxide production in the ischemic area may be due, at least in part, to changes in brain tissue, including neural and glial cells and vessels.

It has been suggested that the blockade of the renin-angiotensin system inhibits stroke in hypertensive and diabetic patients (*19–21*). ARBs appear to have beneficial effects in hypertensive patients to reduce the frequency of stroke and the decline in cognitive function after stroke. However, the detailed mechanism by which ARBs protect against ischemic brain damage remains to be elucidated. Recent studies have demonstrated the existence of all components of RAS, including angiotensin II receptors in the central nervous system (*22*, *23*) as well as its importance in the regulation of blood pressure and water intake (*24–26*). It was previously indicated that the ischemic region is smaller in AT_1a receptor–deficient mice (*5*). We have reported that pretreatment of animals with a non-hypotensive dose of an ARB, valsartan, attenuated the ischemic area and neurological deficit as well as the increase in superoxide production and the reduction of cerebral surface blood flow after MCA occlusion in non-atherosclerotic mice (*6*). These results suggest that ARBs inhibit ischemic brain damage through the regulation of brain blood flow and oxidative stress by the blockade of AT_1 receptor stimulation. There is also a possibility that the collateral circulation is changed by treatment with telmisartan. Although found no significant changes in the number or diameter of brain arteries after immunostaining of tissue sections with anti–α-smooth muscle actin antibodies (data not shown), it remained possible that telmisartan may affect vascular function and functional collateral circulation, thereby improving brain blood flow in the peripheral region (Fig. 3). Telmisartan may affect body temperature after MCA occlusion. However, telmisartan did not significantly change rectal temperature at 24 h after MCA occlusion (data not shown).

On the other hand, clinical and basic studies suggest ARB has beneficial effects on cardiovascular changes in atherosclerosis. Previous clinical and basic studies have shown antidiabetic and anti-atherosclerotic actions of telmisartan (*27–30*). Among recently developed ARBs, telmisartan has a unique property in addition to blocking the AT_1 receptor. Previous papers indicated that telmisartan acts as a selective modulator of peroxisome proliferator–activated receptor-γ (PPARγ) and increases its activity (*31*, *32*). Recently, Luo *et al*. reported that a PPARγ agonist, rosiglitazone, showed neuroprotective effects that were mediated at least partially by anti-inflammatory action (*33*). Therefore, it may be possible that telmisartan improves ischemic brain damage in the atherosclerotic model, not only *via* the blockade of AT_1 receptor stimulation but also by anti-inflammatory action and the regulation of carbohydrate and lipid metabolism *via* PPARγ activation. This possibility needs to be examined.

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