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Blockade of AT1 Receptors Protects the Blood–Brain Barrier and Improves Cognition in Dahl Salt-Sensitive Hypertensive Rats

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Abstract

BACKGROUND—The present study tested the hypothesis that inappropriate activation of the brain renin–angiotensin system (RAS) contributes to the pathogenesis of blood–brain barrier (BBB) disruption and cognitive impairment during development of salt-dependent hypertension. Effects of an angiotensin II (AngII) type-1 receptor blocker (ARB), at a dose that did not reduce blood pressure, were also examined.

METHODS—Dahl salt-sensitive (DSS) rats at 6 weeks of age were assigned to three groups: low-salt diet (DSS/L; 0.3% NaCl), high-salt diet (DSS/H; 8% NaCl), and high-salt diet treated with ARB, olmesartan at 1 mg/kg.

RESULTS—DSS/H rats exhibited hypertension, leakage from brain microvessels in the hippocampus, and impaired cognitive functions, which were associated with increased brain AngII levels, as well as decreased mRNA levels of tight junctions (TJs) and collagen-IV in the hippocampus. In DSS/H rats, olmesartan treatment, at a dose that did not alter blood pressure, restored the cognitive decline, and ameliorated leakage from brain microvessels. Olmesartan also decreased brain AngII levels and restored mRNA expression of TJs and collagen-IV in DSS/H rats.

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CONCLUSIONS—These results suggest that during development of salt-dependent hypertension, activation of the brain RAS contributes to BBB disruption and cognitive impairment. Treatment with an ARB could elicit neuroprotective effects in cognitive disorders by preventing BBB permeability, which is independent of blood pressure changes.

Keywords

blood–brain barrier; blood pressure; cognitive impairment; hypertension; receptors; vascular cognitive impairment

Although increased blood–brain barrier (BBB) permeability is observed as a result of cerebral ischemia,[1,2] disruption of the BBB has also been identified in other pathological conditions, such as multiple sclerosis.[3] Furthermore, cerebrovascular abnormalities have been observed in cognitive disorders.[4] BBB permeability increases with normal aging, and further increases in patients with vascular dementia,[5] suggesting that BBB dysfunction is present from an early stage in patients exhibiting mild symptoms of cognitive impairment and minor cerebral microvascular diseases, such as lacunar stroke.

Accumulating evidence has suggested a potential relationship between hypertension and the risk of cognitive impairment.[6–8] For instance, the Honolulu-Asia Aging Study and other clinical studies have shown that high midlife systolic blood pressure is a significant predictor of reduced cognitive function in later life, as well as a risk factor for vascular dementia.[6–8] Additionally, a strong relationship between average dietary sodium intake and mortality due to stroke and cardiovascular diseases risk has been determined.[9,10] In spontaneously hypertensive rats and stroke-prone spontaneously hypertensive rats, a disturbed fence function of tight junctions (TJs) has been demonstrated in BBB endothelial cells.[11,12] However, it remains unclear whether BBB disruption and cognitive impairment occur during development of salt-dependent hypertension.

The brain renin–angiotensin system (RAS) plays an important role in the pathogenesis of cognitive impairment. Increased angiotensin II (AngII) levels result in impaired cognitive function in renin/angiotensinogen transgenic mice.[13] Furthermore, treatment with an angiotensin receptor blocker (ARB) ameliorates the cognitive impairment in mice fed a high-salt and cholesterol diet, or type-2 diabetic mice.[14,15] In addition, ARB treatment decreases BBB permeability in diabetic rats,[16] which suggests that activation of the brain RAS is involved in the pathogenesis of cognitive impairment and BBB permeability in certain pathophysiological conditions. However, Hirawa *et al*.[17] reported that long-term inhibition of RAS improves memory function in aged, low-salt treated, normotensive, Dahl salt-sensitive (DSS) rats. However, it has not been determined whether increased BBB permeability induced by inappropriate activation of brain RAS contributes to cognitive deficits observed during development of salt-dependent hypertension.

Therefore, the present study tested the hypothesis that augmentation of BBB permeability and cognitive impairment are associated with inappropriate activation of the brain RAS during development of salt-dependent hypertension. To test this, BBB permeability was analyzed, as well as TJ expression, cognitive function, and the effects of an ARB at a subpressor dose, in DSS hypertensive rats.

METHODS

Animals

All experimental procedures were performed according to guidelines for the care and use of animals established by the Kagawa University Medical School (Kagawa, Japan). Adult,

male, 5-week-old, DSS rats (average weight 190 g) were purchased from Seak-Yoshitomi, Fukuoka, Japan. All animals were housed in a room with controlled lighting and temperature (25 °C). After a 1-week adjustment period, the 6-week-old rats were assigned to three groups: low-salt diet (DSS/L; 0.3% NaCl; Oriental Yeast, Osaka, Japan, $n = 16$), high-salt diet (DSS/H; 8% NaCl; Oriental Yeast, $n = 16$), and high-salt diet treated with the ARB, olmesartan (Daiichi-Sankyo, Tokyo, Japan, $n = 16$). The olmesartan dose was 1 mg/kg, once a day, by oral gavage for 4 weeks, which was determined on the basis of previous rat studies.[18,19] Furthermore, our preliminary experiments showed that 1 mg olmesartan/kg body weight/day did not alter blood pressure in DSS/H rats (data not shown). Systolic blood pressure was monitored in conscious rats using tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) each week. For BBB permeability detection, eight animals per group were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal) and perfused with a fixative solution composed of 2.5% glutaraldehyde and 2% paraformaldehyde. In the remaining animals, brain tissues were collected following decapitation to measure AngII and gene expression, as well as for immunohistochemical analyses ($n = 8$ for each).

BBB permeability

As previously described,[12] microvessel permeability was analyzed in the hippocampus and corpus callosum through the use of horseradish peroxidase. Following perfusion with 2.5% glutaraldehyde/2% paraformaldehyde, the brains were separated and maintained in another fixative solution composed of 1.25% glutaraldehyde/1% paraformaldehyde, for 24 h. For light microscope observation, sections were incubated in 0.01 mol/l acetate buffer (pH 3.3), followed by tetramethylbenzidine and hydrogen peroxide.

Passive avoidance test

To analyze cognitive functions, a shuttle avoidance cage (64 × 35 × 33 cm; Melquest, Toyama, Japan) and an isolation cabinet (72 × 67 × 63 cm; Melquest) were used, as previously described.[20] Briefly, the rats were individually placed in a chamber and administered 20 inescapable, electric shocks (0.4 mA) for 5 s each. A tone signal was presented during the first 5 s of each trial. If there was no avoidance response within that period, the tone signal remained on and the shock was delivered through the grid floor. In the case of a no-escape response during this period, both tone and shock were automatically terminated. Cognitive function was determined by avoidance rate percentage.

Immunohistochemistry

Occludin colocalization with PECAM-1-positive endothelial cells was assessed in hippocampal microvessels. Brain tissue was immersed in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and stored at −80 °C. The tissue was then cut into 10-μm thick frozen sections. Following immersion in methanol and phosphate-buffered saline wash steps, the sections were blocked with serum-free protein block (Dako Cytomation, Glostrup, Denmark) for 15 min. Subsequently, the primary antibodies were diluted with Canget signal immunostain (Toyobo, Tokyo, Japan) and incubated overnight at 4 °C. The antibodies included goat polyclonal anti-mouse platelet endothelial adhesion molecule (PECAM-1; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-occludin (1:200; Santa Cruz Biotechnology). The sections were then incubated at room temperature for 1 h with fluorescein-conjugated secondary antibody in Canget signal immunostain (Toyobo; 1:800, donkey anti-goat and donkey anti-rabbit; Molecular Probes, Eugene, OR), mounted with Vectashield (Vector, Burlingame, CA), and examined under a fluorescence microscope (IX71SIF-2; Olympus, Tokyo, Japan).

Gene expression

Occludin, *claudin-5*, *collagen-IV*, and *metalloproteinase* (*MMP*)-*2* and *9* were analyzed by laser-capture microdissection in the hippocampus and corpus callosum of frozen brain samples. First, tissue blocks were sectioned at 10-μm thickness using a cryostat microtome (Leica CM1950; Leica Microsystems, Tokyo, Japan). The sections were incubating in increasing concentrations of ethanol, followed by xylene. For RNA isolation from laser-capture microdissection samples, a Micro Scale RNA Isolation Kit was used (RNAqueous-Micro; Ambion, Austin, TX). All primers for real-time PCR are shown in Table 1.

AngII measurements

After brain tissues were quickly removed, part of the cerebral cortex and hippocampus were immediately separated and frozen. For plasma sample collection, a proteolytic enzyme inhibitor cocktail (EDTA-2Na 500 mmol/l; Captopril 10 nmol/l; Pepstatin 1 mmol/l + 1,10-phenanthroline 125 mmol/l) was added to each sample. Brain and plasma AngII were measured using a previously described radioimmunoassay method.[21,22]

Statistical analyses

All values were expressed as mean ± s.d. Differences in blood pressure and passive avoidance test results among groups were determined using repeated measurements of analysis of variance. Differences in AngII protein concentrations and mRNA levels were examined using one-way analysis of variance. When overall analysis of variance *P* values were <0.01, Bonferroni's correction for multiple comparisons was used to assess individual group differences.

RESULTS

Systolic blood pressure and body weight

Low-salt diet treatment for 4 weeks did not alter systolic blood pressure in the DSS rats (123 ± 4 mm Hg, Figure 1a), whereas DSS/H rats exhibited increased systolic blood pressure (176 ± 5 mm Hg) at 4 weeks. Olmesartan did not alter systolic blood pressure in DSS/H rats (174 ± 4 mm Hg) at 4 weeks. Changes in body weight throughout the experimental period were similar between groups (data not shown).

Cognitive functions

Figure 1b shows cognitive function as determined by avoidance rate percentage. DSS/L rats exhibited gradual improvement in cognitive functions, whereas cognitive functions in the DSS/H rats were significantly decreased. Olmesartan treatment significantly ameliorated the cognitive deficits in the DSS/H rats.

BBB microvessel leakage

As previously mentioned, BBB permeability was evaluated by measuring percentage leakage of horseradish peroxidase-tetramethylbenzidine. [12] DSS/L rats exhibited very little leakage (7 ± 1%) from microvessels in the hippocampus and corpus callosum (Figure 2). In contrast, substantial leakage took place in the ventral hippocampus and corpus callosum of DSS/H rats (34 ± 3%). Olmesartan markedly reduced BBB microvessels permeability in DSS/H rats (16 ± 7%).

Gene expression in the hippocampus and corpus callosum

mRNA expressions of *occludin*, *claudin-5*, and *collagen-IV* in the hippocampus and corpus callosum were less in the DSS/H rats compared with the DSS/L rats. In addition, DSS/H rats

showed to increase the genes expression of *MMP-9*. Olmesartan treatment restored expression of genes encoding *occludin*, *claudin-5*, *collagen-IV*, and *MMP-9* in DSS/H rats. *MMP-2* mRNA expression remained similar between groups (Figures 3 and 4).

Immunohistochemistry

To determine TJ expression, occludin colocalization in PECAM-1-positive endothelial cells was analyzed in microvessels of the hippocampus and corpus callosum by immunohistochemistry. PECAM-1-positive endothelial cells were present in all groups (Figure 5). Occludin expression was observed around microvessels of the hippocampus and corpus callosum in the DSS/L rats. However, very low occludin expression was observed in hippocampal microvessels of the DSS/H rats, but olmesartan treatment significantly restored expression.

Plasma and brain AngII levels

Plasma AngII levels were decreased in DSS/H rats, compared with DSS/L rats (Figure 6a). In contrast, DSS/H rats exhibited approximately twofold greater AngII levels in the hippocampus, compared with DSS/L rats (Figure 6b). However, cerebral cortical AngII levels were similar between DSS/L and DSS/H rats (data not shown). In the DSS/H rats, olmesartan treatment significantly decreased hippocampal AngII concentrations to levels similar to the DSS/L rats (Figure 6b). Treatment with olmesartan tended to increase plasma AngII levels, but these changes were not statistically significant (Figure 6a).

DISCUSSION

Small-vessel disease is a major risk factor for vascular dementia,[23,24] and brain vessel remodeling is a key contributor to cerebrovascular dysfunction in several diseases.[25] The present study demonstrated, for the first time, that cognitive impairment is associated with increased hippocampal AngII levels and BBB permeability during hypertension development in DSS/H rats. Furthermore, treatment with the ARB, olmesartan, at a dose that did not alter blood pressure, markedly restored BBB disruption and reduced cognitive decline in DSS/H hypertensive rats. These data were consistent with the hypothesis that inappropriate activation of the brain RAS contributes to the pathogenesis of BBB injury and cognitive impairment during the development of salt-dependent hypertension. ARB treatment could elicit beneficial effects for cognitive impairment by protecting against BBB injury in patients with salt-dependent hypertension, independently of blood pressure changes.

The present study showed that RAS inhibition with an ARB protected cognitive deficits in DSS/H hypertensive rats in a blood pressure-independent manner. These results were consistent with previous studies showing that long-term treatment with an ARB or angiotensin-converting enzyme inhibitor ameliorated impaired cognitive function in aged, low salt-treated, normotensive, DSS rats.[17] Additionally, a series of studies reported disruption of passive avoidance retention by AngII infusion in the central nervous system.[26,27] Interestingly, AngII administration to the hippocampal area resulted in induction of long-term potentiation in the hippocampus.[28] The present study showed that cognitive impairment was associated with increased hippocampal AngII levels in DSS/H hypertensive rats. These data were consistent with the hypothesis that augmentation of brain RAS activity contributes to the progression of cognitive decline.[13–17,28] It should be noted that AngII levels were measured using the direct radioimmunoassay method,[21,22] without separation of Ang peptides by high-performance liquid chromatography. Therefore, AngII values should represent actual concentrations of AngII + AngIII.

The present study also demonstrated that cognitive deficits were associated with augmented BBB permeability in the DSS rats. The BBB neurovascular unit acts as an obstacle for substance delivery to the central nervous system. In particular, under physiological conditions, TJs and collagen-IV are key components in the regulation of BBB function.[2] Impaired BBB function has been shown to contribute to the pathophysiology of several brain diseases, including cognitive disorders.[29] In the present study, cognitive deficits and increased BBB permeability were accompanied by reduced expression of *TJs* and *collagen-IV* and increased *MMP-9* gene expression in the hippocampus and corpus callosum of DSS/H hypertensive rats. Furthermore, ARB treatment attenuated BBB injury and the reduction in *TJ* and *collagen-IV* expression in the DSS/H rats. Collectively, these results were consistent with the hypothesis that AngII-induced reductions in neurovascular unit components of the BBB, such as TJs and collagen-IV, contribute to altered BBB permeability and associated cognitive impairments.

Although the precise mechanisms responsible for the deleterious effect of AngII on BBB injury are not clear, one possible mechanism is that AngII stimulates the production of proinflammatory cytokines and activates MMPs, which further mediate TJ disruption and BBB permeability and ultimately lead to cognitive dysfunction.[30–32] In aged, low-salt-treated, normotensive, DSS rats, improved cognitive function mediated by RAS inhibitors was associated with preservation of neuronal capillary densities in the hippocampal area.[17] Therefore, it is possible that some of the neuroprotective effects of ARBs on BBB permeability are accompanied by restoration of TJ and collagen-IV expression in the BBB. In the present study, MMP-9 expression was significantly increased in hippocampal tissues of DSS/H rats. Although *in situ* zymography was not performed to measure MMP activity due to the small amount of tissue, these results suggest a potential role for MMP-9 in BBB injury in DSS rats. Olmesartan treatment also resulted in significantly decreased hippocampal MMP-9 expression in the DSS/H rats. Previous studies have shown that cognitive deficits and BBB permeability are associated with increased oxidative stress.[13,33] Therefore, it can be speculated that ARB effects on MMP-9 expression are mediated by their antioxidative stress effects. However, further studies are needed to address these issues.

The present study showed that a high-salt diet inappropriately increased AngII contents in the hippocampus of DSS rats, whereas plasma AngII levels were significantly decreased. These results were consistent with previous studies showing that a high-salt diet increased AngII levels and AT1 receptor density in tissues.[34,35] Systemic olmesartan treatment decreased AngII levels in hippocampal tissues of DSS/H rats, suggesting that at least some neuroprotective effects of ARB could be explained by reduced brain AngII levels. However, the precise mechanisms responsible for high salt-induced activation of the brain RAS, as well as effects of olmesartan on brain AngII levels, remain poorly understood. Nevertheless, we recently demonstrated that systemic treatment for 2 weeks with the ARB candesartan decreases brain AngII levels, as well as angiotensin-converting enzyme and angiotensinogen gene expression, in AngII-infused hypertensive rats, although candesartan concentrations in brain tissues were below detectable limits.[36] In contrast, other ARBs, such as losartan, partially penetrate the BBB and selectively inhibit central AT1 receptors.[37] These results suggest that ARBs penetrate the BBB at very low concentrations, but these levels could be sufficient to regulate the brain RAS.

In conclusion, the present study supported the hypothesis that RAS activation in the hippocampus plays a key role in the pathogenesis of reduced neurovascular TJs and BBB disruption, which leads to cognitive deficits in DSS hypertensive rats. Furthermore, in the DSS/H hypertensive rats, RAS inhibition attenuated these changes without altering blood pressure. These data supported the recently introduced clinical concept that the neurovascular unit is an important therapeutic target for brain protection.[38] However, the

precise mechanisms underlying the neurovascular protective effects of RAS inhibitors, as well as the pathophysiological relevance in preventing cognitive decline, need to be further investigated to better understand the role of local RAS in the pathogenesis of cognitive disorders. Furthermore, DSS rats do not emulate exactly the pathophysiology of salt-sensitive hypertensive patients. Therefore, future clinical studies will be needed.

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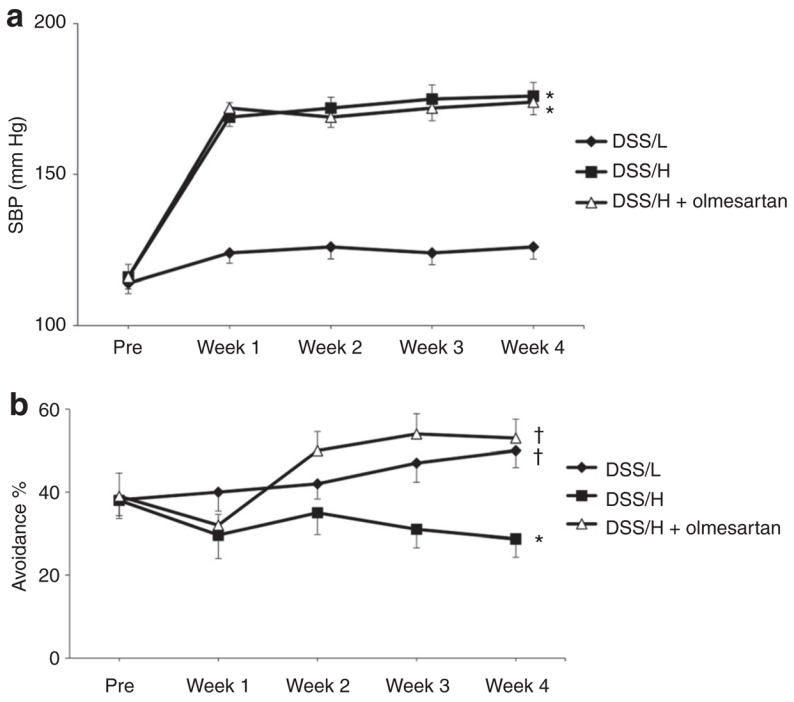

Figure 1.

Blood pressure and cognitive functions. (**a**) Systolic blood pressure. High-salt diet intake elevates systolic blood pressure in DSS/H rats. Treatment with olmesartan (1 mg/kg body weight/day) does not significantly decrease systolic blood pressure in DSS/H rats. **P* < 0.01 vs. DSS/L. (**b**) Cognitive functions. DSS/L rats exhibit gradual improvement in cognitive functions, with regard to percentages in avoidance tests, which were assessed once per week for 4 weeks. In contrast, at week 4, DSS/H rats exhibit learning and memory deficits. Olmesartan treatment restores cognitive decline and improves cognitive ability to levels similar to the DSS/L rats. **P* < 0.01 vs. DSS/L. †*P* < 0.01: vs. DSS/H. DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet; SBP, systolic blood pressure.

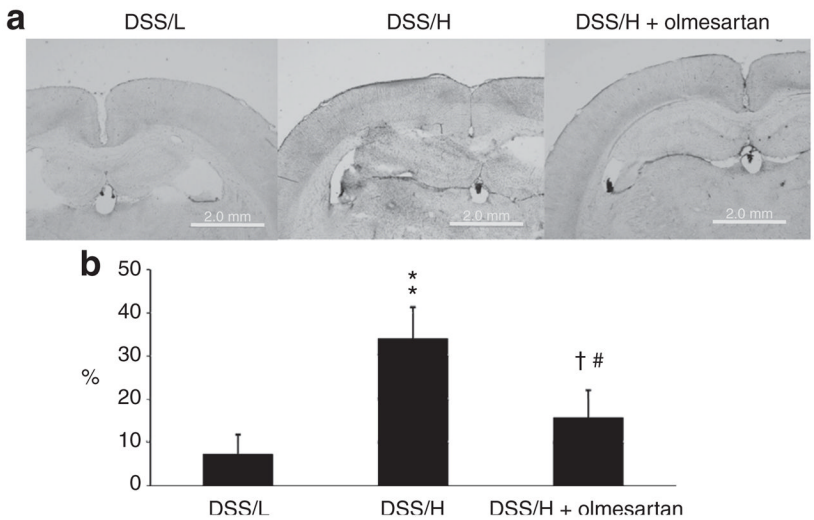

Figure 2.

BBB permeability. (**a**) DSS/H rats exhibit increased microvessels permeability in the hippocampus and corpus callosum compared with DSS/L rats, as indicated by HRP-TMB reaction (violet stain). Olmesartan treatment reduces microvessels permeability in the hippocampus and corpus callosum of DSS/H rats. (**b**) Quantitative analysis of BBB permeability in terms of the percentage stained area of hippocampus and corpus callosum. **P* < 0.01 vs. DSS/L. †*P* < 0.01 vs. DSS/H. BBB, blood–brain barrier; DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet; HRP, horseradish peroxidase; TMB, tetramethylbenzidine.

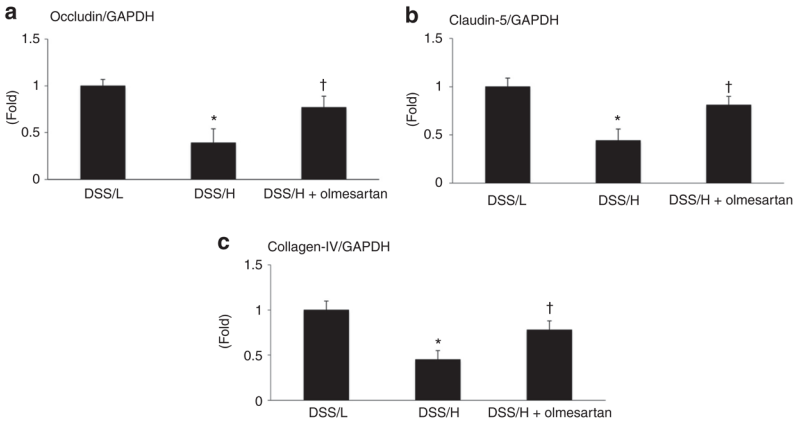

Figure 3.

mRNA expression of *TJ* genes and *collagen-IV*. *Occludin*, *claudin-5*, and *collagen-IV* mRNA levels in the hippocampus are less in DSS/H rats than in DSS/L rats. High-salt diet decreases mRNA levels of *occludin* and *claudin-5*, as well as *collagen-IV*, compared with levels in the DSS/L rats. Olmesartan treatment in DSS/H rats restores expression of genes encoding *TJ* proteins and *collagen-IV*. *$P < 0.01$ vs. DSS/L. †$P < 0.01$ vs. DSS/H. DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet; TJ, tight junction.

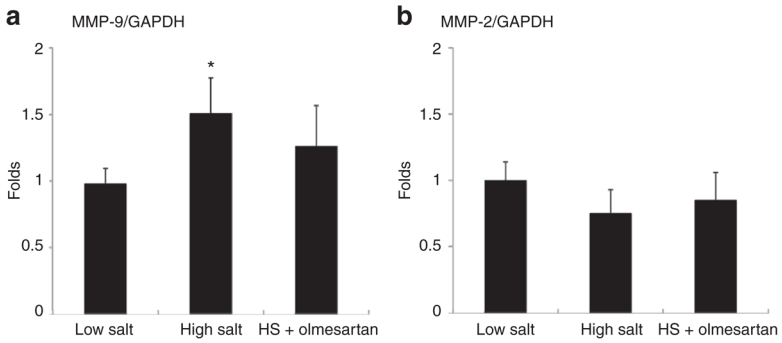

Figure 4.

mRNA expression of *MMP-2* and *MMP-9*. (**a**) *MMP-9* gene expression is increased in the hippocampus of DSS/H rats compared with control rats. In addition, there are no differences between the DSS/H^+ olmesartan and DSS/L groups. (**b**) No changes are observed in *MMP-2* gene expression between the groups. **P* < 0.01 vs. DSS/L. DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet; HS, high salt; MMP, metalloproteinase.

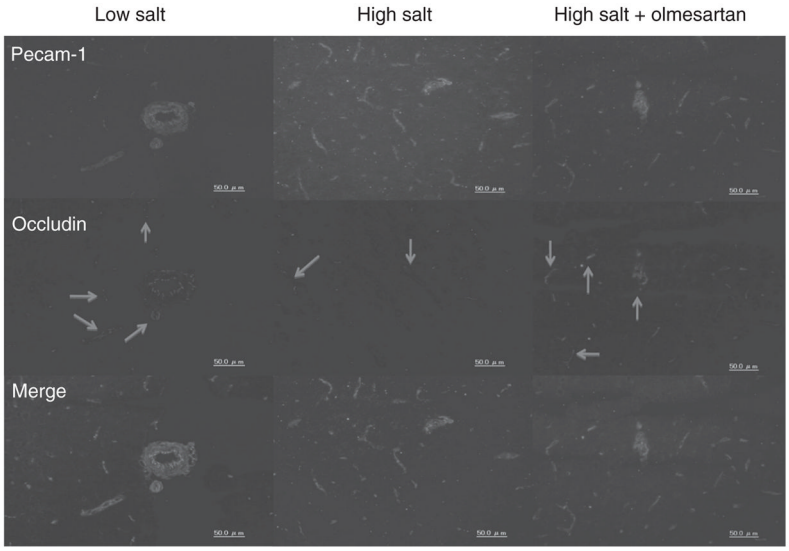

Figure 5.

Immunostaining of microvessels. PECAM-1-positive endothelial cells are observed in all groups. Occludin is expressed in microvessels of the hippocampus and corpus callosum in DSS/L rats. However, corresponding levels are low in DSS/H rats. Olmesartan treatment restores occludin expression in microvessels of the hippocampus and corpus callosum in DSS/H rats. DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet.

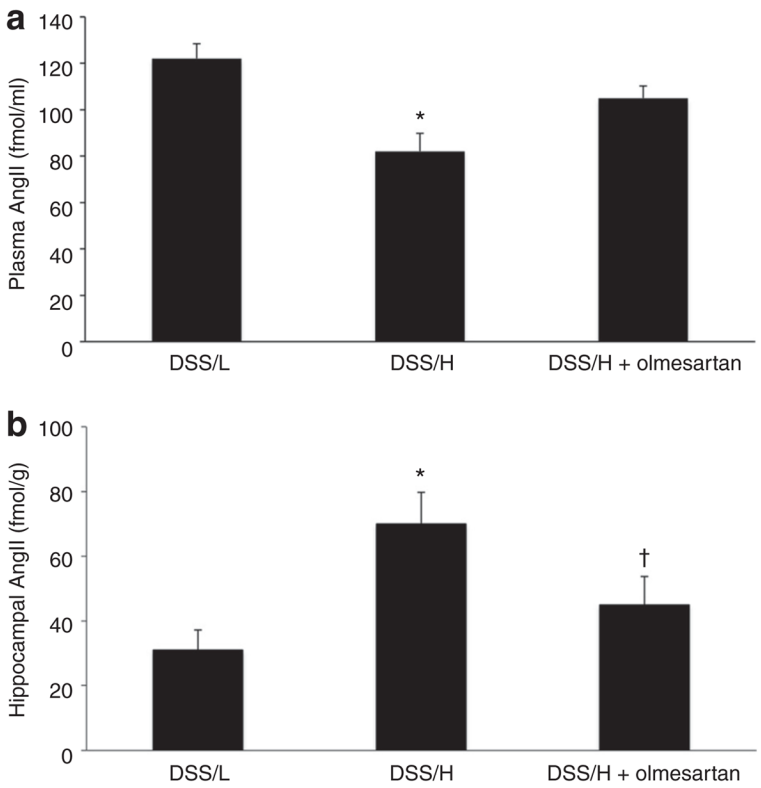

Figure 6.

Plasma and brain AngII levels. (**a**) Plasma AngII expression is decreased in DSS/H rats compared with DSS/L rats. **P* < 0.01 vs. DSS/L. (**b**) High-salt diet increases AngII levels in the hippocampus of DSS/H rats. Olmesartan treatment decreases AngII levels in the hippocampus of DSS/H rats. **P* < 0.01 vs. DSS/L. †*P* < 0.01 vs. DSS/H. AngII, angiotensin II; DSS/H, Dahl salt-sensitive/high-salt diet; DSS/L, Dahl salt-sensitive/low-salt diet.

Table 1

Primers used for real-time Pcr

	Occludin	Claudin-5	Collagen-IV	MMP-2	MMP-9	GAPDH
F	5′-ATGATGAACAG CCCCCTAATGT-3′	5′-TGTGAGGACT TGACCGACCTT-3′	5′-GAGGGTGCTG GACAAGCTCTT-3′	5′-GGTGGTGGTCA CAGCTATTTCTT-3′	5′-GGATCCCCC AACCTTTACCA-3′	5′-TGAACGGGA AGCTCACTGG-3′
R	5′-TGTCGACTCTT TCCGCATAGTC-3′	5′-CGGCAGTTTG GTGGCTACTT-3′	5′-TAAACGGACTG GGCTCGGAATTC-3′	5′-GCCAGTCCGAT TTGATGCTT-3′	5′-AAGGTCAGAAC CGACCCTACAA-3′	5′-TCCACCACCC TGTTGCTGTA-3′

Data were normalized against GAPDH expression and were expressed as fold-differences between control and treated groups.

MMP, metalloproteinase.