Ischemic stroke induces rapid renal oxidative stress and lipometabolic change

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Abstract

Objective: Stroke is associated with high risks for mortality. The bidirectional communication between ischemic brain and other organ dysfunctions is widely recognized. However, the mechanisms underlying cerebrorenal crosstalk remains unclear. Herein, we investigated the effect of ischemic stroke on the kidney using a middle cerebral artery occlusion (MCAO) model. Moreover, we explored the effects of overexpression of liver-type fatty acid binding protein (L-FABP), as antioxidant role in this disease.

Methods: MCAO was performed in wild-type (WT) and L-FABP transgenic (Tg) mice. At 24 h after this procedure, renal hypoxia, oxidative stress, and lipid metabolism were examined. Hypoxia was examined by pimonidazole staining and oxidative stress was examined by heme oxygenase-1 staining in MCAO WT mice. The biomarkers such as urinary 8-OHdG for oxidative stress and silent information regulator 2 mammalian homolog 1, peroxisome proliferator-activated receptor α, and 3-hydroxy-3-methylglutaryl-CoA reductase for renal lipid metabolism were compared between MCAO WT and Tg mice.

Results: MCAO enhanced renal hypoxia and oxidative stress, and the biomarkers for oxidative stress tended to increase in the MCAO WT mice than in

the sham-operated control mice, and these were diminished by L-FABP overexpression. The expression levels of renal lipid metabolism-related genes markedly changed. These alterations were blocked in the L-FABP Tg mice.

Conclusions: Ischemic stroke-mediated renal oxidative stress and the resulting alteration of lipid metabolism could partly account for cerebrorenal connection and the induction of renal L-FABP activity and could be a novel therapeutic target for the prevention of renal diseases in patients with stroke.

Introduction

The interdependence of cerebrovascular disease and chronic kidney disease (CKD) is based on cerebrorenal interactions[1]. CKD and stroke share both traditional vascular risk factors, such as hypertension, dyslipidemia, and diabetes. Moreover, both the kidney and brain are vulnerable to arteriosclerotic injury, given the anatomical and functional similarities in their microvasculatures[2]. In addition, CKD patients have other risk factors, such as hemodynamic and mineral metabolism alterations, anemia, increased oxidant stress and uremic toxins, neurohormonal overactivity, endothelial dysfunction, and chronic inflammation, all of which further lead to the development of vascular diseases[3]. On the other hand, about 25% of patients hospitalized for acute stroke experience acute kidney injury (AKI)[4, 5]. In stroke, the kidney can be directly affected by inducing hyperactivation of the renal sympathetic nervous system, which can alter renal blood flow and glomerular filtration; the increased release of vasopressin can lead to electrolyte imbalance, cerebral salt wasting, hemodynamic instability, hormonal disturbances, and an inflammatory cascade in the kidney secondary to heightened immunologic response[6]. Moreover, as a compensatory mechanism that preserves blood flow to the injured brain areas, systolic hypertension may

happen after stroke-induced sympathetic hyperactivation and increase in plasma catecholamine concentrations[7), 8)]. In addition, evidence collected in the past years suggested that compensatory neurohumoral hyperactivation may be involved in the pathogenesis of this complex disease[5)].

Indeed, chronic activation of the sympathetic nervous system, although initially as a compensatory mechanism for impaired cerebral blood flow, could enhance ischemia and oxidative stress, thereby contributing to damage in other organs[9)]. However, the involvement of acute stroke-induced sympathetic nerve activation and oxidative stress on renal injury remains to be elucidated. Therefore, we investigated whether ischemic stroke could enhance renal oxidative stress and related renal lipid metabolism using a middle cerebral artery occlusion (MCAO) mice model in the present study. To further determine the involvement of renal oxidative stress and metabolic alteration just after the stroke, we also explored the effects of overexpression of liver-type fatty acid binding protein (L-FABP), which has antioxidant properties, on ischemic stroke-mediated renal injury or metabolism.

Materials and Methods

1. Animals

The mice were housed in the animal facilities of the Juntendo University Faculty of Medicine and were maintained on a 12-h light/dark cycle, with free access to food and water, unless noted otherwise. In this study, 8-week-old female mice weighing 17.9-28.1g were used. The L-FABP Tg mice, which were genetically modified to highly express human *L-FABP* chromosome genes in the proximal renal tubules[10], and their wild-type (WT) littermates with C57/BL6 background were used in this study. The experimental protocol was approved by the Animal Care and Use Committee of the Juntendo University (No.1177).

2. Middle cerebral artery occlusion model

MCAO and sham operation were performed in both WT and Tg mice, and the sham operation group was used as a control. Cerebral ischemia in anesthetized mice was induced by occlusion of the left middle cerebral artery for 60 min, as described previously[11]. Sham-operated mice underwent only incision and closure, except for MCAO. During this procedure, the body temperature was kept

at 37.0 °C ± 0.5 °C using a heating pad. To eliminate the influence of variations in decreased food intake among the mice after MCAO, the control animals were fasted and were given access to only water for 24 h after the sham operation; these were used as a basal control group. Thereafter, the respective mice from the MCAO and sham groups with similar % changes in body weight (mean MCAO: before 21.8g after 18.7g, mean sham: before 21.8g after 18.7g) were selected for this study. Each of the WT and Tg groups was divided into two subgroups: 1) MCAO group (24 h; n = 3-5 in each group) and 2) the sham operation group as Sham Fast (SF; n = 3-5 in each group). Consistent with a previous report[12], in our preliminary studies, we did not find a clear increase in the serum creatinine level at 24 h (data not shown).

3. Histologic analysis

The mice were sacrificed at 24 h after the operation. Their kidneys were perfused with ice-cold normal saline; one kidney was fixed in 4% paraformaldehyde overnight, and the other kidney was snap frozen in liquid nitrogen. For immunopathological analyses, frozen renal sections were stained with rabbit

antimouse heme oxygenase-1 (HO-1) (Anti-Heme Oxigenase 1 antibody; Abcam, CA, USA). Tissue hypoxia was detected using the Pimonidazole Hydrochloride (Hypoxyprobe-1^{TM} Kit; Hypoxyprobe, Inc., MA, USA), according to the manufacturer's instructions. In brief, a dose of 60 mg/kg pimonidazole was injected in the tail vein 60 min before the sacrifice. Cryosections were incubated first with Hypoxyprobe-1 Mab1 conjugated with FITC (1:500) at room temperature for 60 min, followed by 1:50 dilution with the secondary antibody anti-FITC Mab conjugated with HRP after washing three times with PBS.

4. Measurement of urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG)

With the MCAO and sham groups in a metabolic cage (mouse metabolic cage; CLEA, Shizuoka, Japan), urinary samples were collected for 24 h. Urinary creatinine levels were measured by immunoassay (DCA 2000 System; Bayer Diagnostics, Elkhart, Ind., USA). Urinary 8-OHdG concentrations were measured using a highly sensitive ELISA kit (New 8-OHdG Check; JaICA Shizuoka, Japan), and the results were expressed as the urinary 8-OHdG/creatinine ratio.

5. Quantitative real-time reverse transcriptase polymerase chain reaction analysis

Because renal ischemia or lipid overloading to the kidney causes dramatic changes in renal lipid metabolism and can further worsen renal dysfunction[13), 14)], we investigated whether MCAO affected renal lipid metabolism. To investigate the state of renal lipid metabolism, the expression levels of the related genes at 24 h after MCAO were assessed. Using whole kidney samples, the expressions of the genes associated with lipid metabolism in general, including *silent information regulator 2 mammalian homolog 1* (*Sirt1*) and *peroxisome proliferator-activated receptor α* (*PPARα*), and the genes associated with cholesterol metabolism, such as *3-hydroxy-3-methylglutaryl-CoA reductase* (*HMGCR*), were evaluated by real-time polymerase chain reaction (RT-PCR). The respective expressions of the genes associated with lipid metabolism in the WT and L-FABP Tg mice were compared at 24 h after MCAO and at fasting after the sham operation (SF, control group).

Total RNA was extracted from the whole kidney homogenates from each group using Trizol reagent (TRIzol™ Reagent; Life Technologies Inc., Carlsbad, CA, USA), as per the manufacturer's instructions. Total RNA in 1-μg aliquots were

reverse-transcribed using random primers (Random Decamers; Ambion, Austin, TX, USA) and reverse transcriptase (M-MLV; Invitrogen life Technologies, Carlsbad, CA, USA). The products were then subjected to RT-PCR (Applied Biosystems 7500 Real-Time PCR System with SYBR Green Master Mix; Applied Biosystems, Tokyo, Japan) using specific primers. The gene expression of the target sequence was normalized to GAPDH. All data were calculated from triplicate reactions. The forward and reverse primers used for each molecule are indicated in Table 1.

6. Statistical analysis

The measured values were expressed as the mean value ± SD. The *t*-test (Stat View ver.[5)] was used for statistical analysis, and $p < 0.05$ was considered as statistically significant.

Results

1. MCAO enhanced renal hypoxia and oxidative stress

The area of renal hypoxia, i.e., pimonidazole staining, was significantly enhanced by MCAO in WT mice (Fig. 1A and 1B). Pimonidazole staining was marked around the corticomedullary junction in MCAO WT mice.

Consistent with these findings, immunohistochemistry revealed the upregulated expression level of HO-1, which is a downstream antioxidant molecule of hypoxia-inducible factor-1, in MCAO WT mice (Fig. 1C). In addition, a tendency for increased urinary 8-OHdG levels was seen in WT mice after MCAO and was blocked by L-FABP overexpression (Fig. 2A).

2. MCAO altered renal lipid metabolism

As shown in Figure 2, in the kidneys from WT mice, the mRNA expression levels of Sirt1 and PPARα were significantly decreased after MCAO (Figs. 2B ($p<0.05$) and 2C ($p<0.05$)), whereas HMGCR was significantly increased (Fig. 2D ($p<0.05$)). All of these alterations were inhibited after MCAO in the L-FABP Tg

mice (Figs. 2B ($p<0.05$), 2C ($p<0.05$) and 2D ($p<0.26$, n.s.)).

Discussion

The salient findings of the present study were (1) the enhancement of renal hypoxia and oxidative stress, (2) marked alterations in renal lipid metabolism in the mice model of acute ischemic stroke, and (3) normalization of ischemic stroke-mediated alterations of renal lipid metabolism after elimination of renal oxidative stress by L-FABP overexpression (Tg mice). These results suggested that the ischemic stroke-mediated renal oxidative stress induced by renal hypoxia and its consequent alteration of lipid metabolism, which partly accounted for the cerebrorenal connection and the enhancement of renal L-FABP activity, could be a novel therapeutic strategy to prevent renal comorbidity in patients with stroke.

In the present study, enhanced renal hypoxia and oxidative stress were observed in the MCAO model. Ischemic stroke can directly affect the kidney by inducing hyperactivation of the renal sympathetic nervous system, which, in turn, could be involved in renal damage[6]. Indeed, renal denervation (RD) was reported to prevent oxidative stress, endothelial dysfunction, and subsequent organ damage

in spontaneously hypertensive rats, which were loaded with high salt and were prone to stroke[15]. Moreover, accumulating evidence from clinical and basic research has indicated the effectiveness of RD not only in lowering blood pressure but also in decreasing the renin–angiotensin system (RAS) activity and vascular endothelial impairment, thereby protecting the organs from damage[16–18]. Although we did not measure blood pressure levels in this study, such lines of evidence strongly suggest that inadequate stroke-mediated sympathetic nerve activation may enhance RAS and oxidative stress and become involved in the development of renal injury. Therefore, we first decided to investigate the effects of RD on ischemic stroke-mediated renal injury. However, RD after MCAO tended to worsen mortality and infarct area in the preliminary experiments. Therefore, we considered that acute stroke tends to activate sympathetic nerves to compensate for the impaired cerebral blood flow. This was the reason why we decided to manipulate oxidative stress but not sympathetic nervous activation in this study.

L-FABP is a 14-kDa protein that is presented in the cytoplasm of human proximal tubules. After binding to L-FABP, fatty acids are transported to the mitochondria or peroxisomes, where they are β-oxidized; this reaction plays a role in fatty acid homeostasis. In addition, L-FABP has high affinity and capacity to bind to long-

chain fatty acid oxidation products, thereby acting as effective endogenous antioxidants[19]. Many experimental models have determined that the pathological significance of L-FABP was the possible contribution of oxidative stress to the progression of tubulointerstitial injury[19, 20]. In the present study, the increased renal HO-1 expression and urinary 8-OHdG excretion were observed in MCAO mice (Figs. 1C and 2A). In addition, tubular L-FABP overexpression ameliorated the increase in urinary 8-OHdG excretion (Fig. 2A). These results indicated that acute ischemic brain injury could be involved in the enhancement of renal oxidative stress, which can be ameliorated by tubular L-FABP; however, the mechanisms were not clarified in the present study. However, as previously mentioned, activation of the sympathetic nervous system enhances oxidative stress[9, 18]. Indeed various animal models with activated sympathetic nervous system showed that RD inhibited oxidative stress[15–17], suggesting that stroke-induced sympathetic nerve activation may account for renal oxidative stress via vasoconstriction of the afferent glomerular arteriole.

Mammalian SIRT1 couples protein deacetylation with NAD^+ hydrolysis and links cellular energy and redox state to multiple signaling and survival pathways[21]. Cellular SIRT activity is controlled dynamically to meet metabolic and

environmental changes. Expression of SIRT1 increases with calorie restriction during starvation and nutrient deprivation or when cells are acutely exposed to conditions that cause oxidative stress and DNA damage. Decreased expression of SIRT1 was reported to be associated with high-fat diet, insulin resistance, high glucose, and senescence[21]. In the present study, the renal expression level of SIRT1 was markedly reduced after MCAO in WT mice. Although we cannot clarify the roles of this downregulation on renal injury in this study, the health benefits of SIRT1 have been well established[22]. Calorie restriction-mediated SIRT upregulation was shown to be associated with less injury in age-related and diabetic nephropathy models[23]. Further, consistent with our observations, Xu S et al. reported that renal SIRT1 expression and activity were markedly decreased in a rat model of septic acute kidney injury (AKI)[22]. Moreover, they showed that treatment with resveratrol, which is a chemical SIRT1 activator, effectively restored SIRT activity and renal injury and prolonged the survival time in a septic AKI model[22]. Taken together, these observations strongly suggested the active involvement of SIRT1 in ischemic stroke-induced AKI. In addition to the SIRT1 alteration in our model, its downstream lipid metabolism-related genes, such as *PPARα* and *HMGCR*, were markedly changed after MCAO. Several basic and

clinical studies showed that ischemic or toxic AKI upregulated renal cortical HMGCR activity and cholesterol accumulation[13), 24), 25)]. Although the precious role of renal cholesterol loading remains incompletely defined, it is believed to be a kind of adaptive response, in which previously injured tubular cells become resistant to further ischemic or toxic attack[13)]. On the other hand, lipid nephrotoxicity has been recently rediscussed[26)]. In support of our findings, renal ischemia was reported to cause dramatic changes in renal lipid metabolism and further progression of renal dysfunction[13),14)]. In addition, a recent large-scale epidemiologic study revealed that the incidence of AKI after intensive care unit admission was 31% lower in preadmission statin users than in non-statin users[26)]. Furthermore, Negishi K et al. previously demonstrated that PPARα agonists could ameliorate renal injury in a cisplatin-induced AKI model through upregulation of L-FABP[28)]. These lines of evidence indicated that alterations in lipid metabolism could be involved in the initiation and/or development of AKI and that activation of L-FABP could be a novel therapeutic strategy to prevent renal comorbidity in patients with stroke.

There are some limitations in this study. Firstly, we could not measure the infarct size in this experiment. However, it has been reported that the severity of

ischemic stroke was significantly associated with the severity of AKI, suggesting that the larger the infarct size, the greater the oxidative stress on the kidney, which may have a greater effect on renal dysfunction[29]. Secondly, the evaluation of the relationship between the staining areas of renal pimonidazole, HO-1 and other markers of systemic oxidative stress and the area of cerebral infarction may help further clarify the pathophysiology of cerebrorenal connection. However, these could not be evaluated in this study. Further studies are needed to clarify these points.

In conclusion, this study suggested that ischemic stroke-mediated renal oxidative stress and the subsequent alteration of lipid metabolism could account for cerebrorenal connection.

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Authors' contributions

TK and KT carried out the experiments. TO, HN, JY and YK contributed to the implementation of the research and analysis of the results. TK wrote the manuscript with support from TS, TU and YT. YS suprevised the project. All authors read and approved the final manuscript.

Conflicting interest statement

The authors decleare that there is no confrict of interest.

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Tables

Table 1. Primers for real-time polymerase chain reaction

	Forward	Reverse
Sirt1	GCAACAGCATCTTGCCTGAT	GTGCTACTGGTCTCACTT
PPARα	CGATGCTGTCCTCCTTGATGA	CTCGCGTGTGATAAAGCCATT
HMGCR	AGCCGAAGCAGCACATGAT	CTTGTGGAATGCCTTGTGATTG
GAPGH	CATTGTGGAAGGGCTCATGA	TCTTCTGGGTGGCAGTGATG

SIRT1: silent information regulator 2 mammalian homolog 1,

PPARα: peroxisome proliferator-activated receptor α,

HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase,

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure Legends

Fig. 1. Histological staining shows renal ischemia and oxidative stress 24 h after middle cerebral artery occlusion in wild-type (WT) mice

(A) Pimonidazole staining is concentrated around the corticomedullary junction (×40).

(B) Pimonidazole staining in sham-operated WT mouse (×40).

(C) Heme oxygenase-1 (HO-1) staining is concentrated (×200).

Fig. 2. Urinary levels of oxidative stress and expression of renal lipid metabolism-related genes 24 h after middle cerebral artery occlusion in wild-type (WT) and Tg mice

(A) The urinary 8-OHdG level on ELISA tends to increase in WT- middle cerebral artery occlusion (MCAO) but not Tg-MCAO. In WT-MCAO, mRNA expression levels of Sirt1 and PPARα were significantly decreased after MCAO (B and C), whereas HMGCR was significantly increased (D). However, such decrease of mRNA expression of Sirt1 and PPARα was not found in Tg-MCAO (B and C).

Values are measured using real-time polymerase chain reaction. Each group included five animals.

Figures

Fig 1.

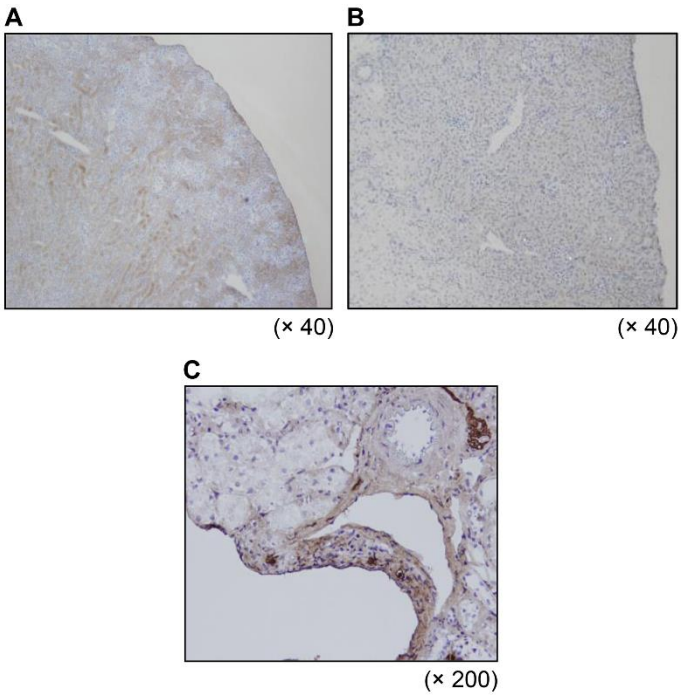
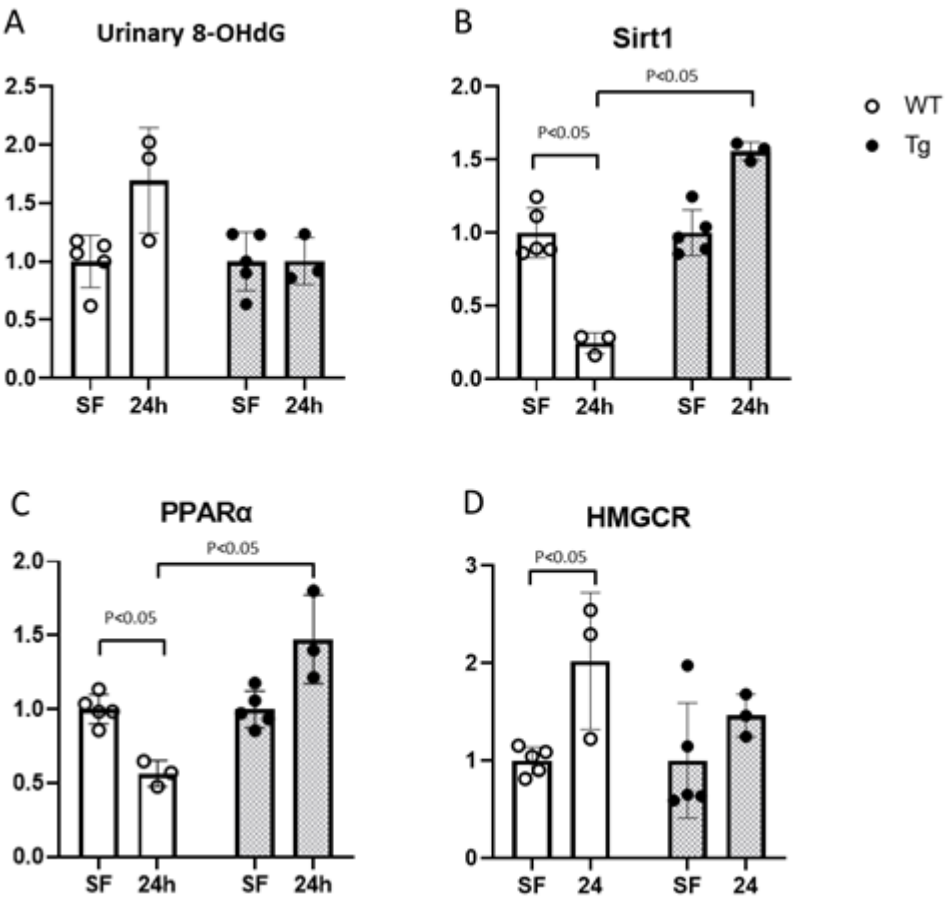


Fig 2.


A
Urinary 8-OHdG
2.5
2.0
1.5
1.0
0.5
0.0
SF
24h
SF
24h
B
Sirt1
P<0.05
P<0.05
2.0
1.5
1.0
0.5
0.0
SF
24h
SF
24h
WT
Tg
C
PPARα
P<0.05
P<0.05
2.0
1.5
1.0
0.5
0.0
SF
24h
SF
24h
D
HMGCR
P<0.05
3
2
1
0
SF
24
SF
24