Role of AT₁ receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury

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Submitted 16 December 2003; accepted in final form 12 February 2004

Kusaka, Ikuyo, Gen Kusaka, Changman Zhou, Mami Ishikawa, Anil Nanda, D. Neil Granger, John H. Zhang, and Jiping Tang. Role of AT₁ receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. Am J Physiol Heart Circ Physiol 286: H2442–H2451, 2004; 10.1152/ajpheart.01169.2003.—The objective of the present study was to examine the role of the angiotensin II type 1 receptor (AT₁-R) in the diabetes-aggravated oxidative stress and brain injury observed in a rat model of combined diabetes and focal cerebral ischemia. Diabetes was induced by an injection of streptozotocin (STZ; 55 mg/kg iv) at 8 wk of age. Two weeks after the induction of diabetes, some animals received continuous subcutaneous infusion of the AT₁-R antagonist candesartan (0.5 mg·kg⁻¹·day⁻¹) for 14 days. Focal cerebral ischemia, induced by middle cerebral artery occlusion/reperfusion (MCAO), was conducted at 4 wk after STZ injection. Male Sprague-Dawley rats (n = 189) were divided into five groups: normal control, diabetes, MCAO, diabetes + MCAO, and diabetes + MCAO + candesartan. The major observations were that 1) MCAO produced typical cerebral infarction and neurological deficits at 24 h that were accompanied by elevation of NAD(P)H oxidase gp91phox and p22phox mRNAs, and lipid hydroperoxide production in the ipsilateral hemisphere; 2) diabetes enhanced NAD(P)H oxidase gp91phox and p22phox mRNA expression, potentiated lipid peroxidation, aggravated neurological deficits, and enlarged cerebral infarction; and 3) candesartan reduced the expression of gp91phox and p22phox, decreased lipid peroxidation, lessened cerebral infarction, and improved the neurological outcome. We conclude that diabetes exaggerates the oxidative stress, NAD(P)H oxidase induction, and brain injury induced by focal cerebral ischemia. The diabetes-aggravated brain injury involves AT₁-Rs. We have shown for the first time that candesartan reduces brain injury in a combined model of diabetes and cerebral ischemia.

angiotensin type 1 receptor antagonist

It has been established that diabetes is a risk factor for cerebral ischemia, and the relative risk of cerebral ischemia in diabetic patients is approximately twice as much as in patients without diabetes (6, 15, 28). In addition, diabetes is strongly related to early brain injury and to the poor outcome after cerebral ischemia (10, 28, 54). Clinical studies on diabetic patients showed that hyperglycemia augments brain lesions associated with cerebral ischemia (29, 47). In animal models of cerebral ischemia, hyperglycemic animals suffered greater neurological deficit with extensive brain damage and widespread necrosis than nonhyperglycemic animals (17, 53). One of the mechanisms of diabetes-enhanced brain injury is oxidative stress caused by hyperglycemia (58).

Reactive oxygen species-mediated oxidative stress is believed to produce tissue injury in wide variety of diseases, including diabetes (58). Several enzymes, especially NAD(P)H oxidase, are recognized as being potentially able to produce reactive oxygen species during diabetes (31). NAD(P)H oxidase consists of five major subunits: a plasma membrane spanning cytochrome b₅₅₈ composed of the large subunit gp91phox, the smaller p22phox subunit, and three cytosolic compounds (p47phox, p67phox, and p40phox) (19, 30). When cells are stimulated, the cytosolic component p47phox becomes heavily phosphorylated, and the entire cytosolic complex migrates to the membrane, where it associates with cytochrome b₅₅₈ to assemble the active oxidase (4). The active oxidase can transfer electrons from NAD(P)H to oxygen, forming reactive oxygen species (4, 30).

Several recent studies indicate that the increased angiotensin II levels during diabetes enhanced the production of reactive oxygen species by activating NAD(P)H oxidase (20, 21). Angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists, which have been used in the management of diabetic complications, inhibit NAD(P)H oxidase (21, 41). In addition, angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists have been used in cerebral vascular disorders, including cerebral ischemia (2, 14, 51, 56). For example, candesartan blocked brain angiotensin II type 1 receptors (AT₁-Rs) (37) and protected brain tissues from cerebral ischemia (24, 26, 38). In the present study, we addressed the following hypotheses: 1) diabetes aggravates NAD(P)H oxidase expression and lipid peroxidation after cerebral ischemia; 2) diabetes enhances brain injury and neurological deficit after cerebral ischemia; and 3) the AT₁-R antagonist candesartan reduces NAD(P)H oxidase and lipid peroxidation, decreases brain injury, and improves neurological functions in the presence of diabetes and cerebral ischemia. We tested the effect of candesartan in a combined rat model of diabetes and cerebral ischemia.

MATERIALS AND METHODS

Male Sprague-Dawley rats (n = 189), weighing 300–350 g, were randomly divided into five groups: 1) normal control, 2) cerebral ischemia by middle cerebral artery occlusion/reperfusion (MCAO), 3) diabetes, 4) diabetes + MCAO, and 5) diabetes + MCAO + candesartan. All animals were killed at 4 wk after the induction of streptozotocin (STZ)-induced diabetes and 24 h after cerebral ischemia, respectively. All procedures were performed according to an institu-
tionally approved protocol and were in accordance with the guidelines provided by the American Academy of Accreditation of Laboratory Animal Care. Animals were housed under identical 12 h cycling-controlled light conditions and fed standard rat laboratory diet with free access to water.

Diabetes Animal Model

The detailed method for STZ-induced diabetes rat model has been described in our previous publications (33, 43). The animals were anesthetized with 1,2,4-trichloroanisole (40 mg/kg ip) and urethane (400 mg/kg ip). Diabetes was induced by a single injection of STZ at a dose of 55 mg/kg (Sigma Chemical; St. Louis, MO) via the tail vein. Control and cerebral ischemia groups received injections of equal amounts of saline (vehicle). Body weights and blood glucose levels (tail vein samples, Glucometer, Beyer) were measured at the time of injection (day 0) and on days 7, 14, 21, and 28.

Candesartan Treatment

Under anesthesia as described above, an osmotic minipump was implanted subcutaneously under the dorsal skin (2-cm cut) as described previously (44). In the candesartan treatment group, rats were treated with continuous subcutaneous infusion of candesartan (0.5 mg kg⁻¹ day⁻¹ from the osmotic minipump) from day 14 after the STZ injection to day 28 before death. No side effects of minipump placement were noticed.

MCAO Model

The experimental MCAO rat model was conducted as described previously (5, 57). General anesthesia was induced and maintained with 1,2,4-trichloroanisole (40 mg/kg ip) and urethane (400 mg/kg ip). Rats were placed in the supine position on a heated operating table, with body temperature maintained around 37 ± 0.5°C. Throughout the experiment, frequent checks were made to ensure that the animals were adequately anesthetized. This was done by applying a painful stimulus to a paw and observing blood pressure responses.

Under an operating microscope, the left femoral artery was dissected and cannulated using polyethylene-50 tubing to allow continuous monitoring for mean blood pressure and sampling for analysis of blood gases. The left common carotid artery, including its bifurcation, was exposed and dissected. All branches of external carotid artery were isolated, coagulated, and transected. The external carotid artery was divided, leaving a stump of ~3-4 mm. The internal carotid artery was then isolated, and the pterygopalatine artery was ligated close to its origin. The internal carotid artery was then clamped with a small vascular clip. The common carotid artery was also clamped with a small 5-mm aneurysm clip. The stump of the external carotid artery was reopened, and a 4.0 monofilament nylon suture with a slightly enlarged and round tip was inserted up through the internal carotid artery. When a small resistance was felt, insertion was stopped. The distance from bifurcation of the common carotid artery to the tip of the suture was ~20 mm in all rats. After occlusion for 2 h, the suture was withdrawn through the internal carotid artery into the external carotid artery, allowing reflux. The skin was sutured and the rats were allowed to wake up. To complete the surgery, the operator applied 0.1% lidocaine locally to the wound and allowed the rat to recover. A successful occlusion of the left middle cerebral artery is achieved when the right forelimb is paretic after filament introduction (49). All rats were killed at 24 h after cerebral ischemia.

Neurological Evaluation

Before death, each rat was neurologically scored for focal deficits using of a six-point neurological scoring system (7, 34) as described previously (5, 57) grade 0, no apparent deficits; grade 1, contralateral forelimb flexion; grade 2, decreased grip of contralateral forelimb while the rat’s tail is pulled; grade 3, spontaneous movement in all directions, contralateral circling only if the rat’s tail is pulled; grade 4, spontaneous contralateral circling; and grade 5, death.

Scoring was performed blindly (by G. Kusaka) on individual animals and averaged in groups.

Measurement of Infarct Size

Rats were anesthetized as described above and decapitated 24 h after MCAO. The brains were immediately removed and placed in ice-cold PBS for 15 min. Coronal sections of the brain were cut into 2-mm slices by a brain slicer (Harvard Apparatus; South Natick, MA) as described previously (5, 57). Brain slices were immersed in 2% 3,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution (in PBS, pH 7.4) at 37°C for 15 min, followed by 10% formaldehyde solution. The infarct area and hemisphere area of each section were traced and quantitated by an image analysis system (Scion Image, Scion; Frederick, MD) (5). The possible interference of brain edema to infarct volume was corrected by standard methods (contralateral hemisphere volume – volume of non ischemic ipsilateral hemisphere), with infarcted volume expressed as a percentage of the contralateral hemisphere (8).

Brain Water Content

Rats were anesthetized as described above and decapitated at 24 h after MCAO. The brain was dissected into the cortex, basal ganglia area, and cerebellum. The brain samples were weighed immediately after dissection (wet weight) and then dried at 105°C for 24 h. The percent water content was calculated as [(wet weight – dry weight)/ dry weight] × 100% as described previously (9).

Determination of Oxidative Stress

The level of lipid peroxidation products [malondialdehyde (MDA)] was measured using a LPO-586 kit (OxisResearch; Portland, OR) in brain samples at 24 h after MCAO. Brains were homogenized in 20 mM phosphate buffer (pH 7.4), and 0.5 M butylated hydroxytoluene in acetonitrile was added to prevent sample oxidation. The homogenates were centrifuged at 3,000 g for 10 min at 4°C to remove large particles. Equal amounts of proteins in each sample were allowed to react with a chromogenic reagent at 45°C for 60 min. The samples were centrifuged at 15,000 g for 10 min at 4°C, and supernatants were measured at 586 nm. The level of MDA was calculated with the standard curve according to the manufacturer’s instructions.

RNA Isolation and RT-PCR

The mRNA expression of NAD(P)H oxidase in brain samples collected at 24 h after MCAO was examined by semiquantitative RT-PCR (22). The gene descriptions, accession numbers, and primers used for RT-PCR are listed in Table 1. In brief, total RNA was isolated from the brain with RNA STAT-60 (TEL-TEST CmbH; Friendswood, TX). All samples were treated with RQ1 RNase-Free DNase (Promega; Madison, WI) before RT-PCR. cDNA was prepared from 1 μg of total RNA using the SuperScript First-Strand Synthesis System for the RT-PCR kit (Invitrogen; Carlsbad, CA). The thermal cycle profile for PCR amplification of 25–40 cycles was 1) denaturing for 1 min at 94°C; 2) annealing primers for 1 min at 55°C; and 3) extending the primers for 1.5 min at 72°C, using specific primers for p22phox, gp91phox, and p47phox (Invitrogen). A portion of 10 μl of the PCR products was electrophoresed in 2% agarose gel in Tris-borate-EDTA buffer. For the quantitative analysis of RT-PCR products, the density of bands for mRNA was determined by a densitometer. Intensities of the bands were normalized and expressed relative to the intensity of the band of NAD(P)H as described by us previously (11). Because cerebral ischemia was produced in the left hemisphere and diabetes affects both hemispheres, we collected and separated both hemispheres from each animal and compared the left hemisphere from control animals with left hemispheres from other groups. Similarly,
the right hemisphere from the control group was compared with right hemispheres from other groups. All bands were normalized to the density of the appropriate GAPDH band before comparison.

**Immunohistochemical Analysis**

Rats were anesthetized as described above and perfused transcardially [0.01 mol/l PBS (200 ml) at pH 7.4 and then 4% formaldehyde solution (400 ml)] at 24 h after MCAO. The brains were quickly removed, postfixed in 4% formaldehyde solution at 4°C overnight, and immersed in 30% sucrose until they sank. The brains were then embedded, and axial sections (10 μm thick) were cut with the use of a cryostat as described by us previously (57). After treatment with 1% H2O2 for 10 min and 3% normal goat serum in PBS for 30 min, sections were incubated with a 1:100 dilution of p22 phox, gp91 phox, and p47 phox goat polyclonal antibody (Santa Cruz Biotechnology; control (n/H11005). The sections were then rinsed in water, –3). The MCAO model was conducted at 27 days after STZ injection.

**Blood gases.** Blood gases evaluated on day 27 after STZ injection, before the MCAO surgical procedure, are summarized in Table 2. Blood pH values in the diabetes + MCAO and diabetes + MCAO + candesartan groups were decreased markedly (P < 0.0001 by ANOVA) compared with the MCAO group. The value of blood pH in the MCAO group was in the normal range and was similar to values obtained from normal control rats (not shown). Consistently, PCO2 and PO2 values were increased in the diabetes + MCAO and diabetes + MCAO + candesartan groups compared with controls, even though no statistical significances were obtained (P < 0.05 by ANOVA).

**Infarct size.** Representative samples of TTC-stained brain sections are shown in Fig. 4. The white-colored areas represent the infarcted regions in these sections. For the summary shown in Fig. 5, the data are the sum of five animals in each group by ANOVA. Additional animals were used to induce MCAO without diabetes (n = 27, including the 12 animals in the normal control group listed in the Figs. 1–3). The MCAO model was conducted at 27 days after STZ injection.

**Cerebral Ischemia in the Presence of Diabetes**

MCAO was induced in the above-mentioned diabetes (n = 30) and diabetes + candesartan (n = 27) groups. Additional animals were used to induce MCAO without diabetes (n = 27, including the 12 animals in the normal control group listed in the Figs. 1–3). The MCAO model was conducted at 27 days after STZ injection.

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**Statistical Analysis and Agents**

The differences among groups were compared by Student’s t-test or one-way ANOVA. All values are expressed as means ± SE. The level of significance was set at P < 0.05.

Chloralose, urethane, and lidocaine were all purchased from Sigma. The minipump for candesartan injection was purchased from Alzet Pharmaceuticals (Palo Alto, CA). Candesartan was purchased from Takeda Chemical (Tokyo, Japan). candesartan (Can)-treated rats. Candesartan was applied 14 days after streptozotocin (STZ) injection and was applied daily until day 28 (blood glucose measured on day 27). n, no. of rats/group. *P < 0.0001 vs. the control group by ANOVA.

**RESULTS**

**Diabetes Before Cerebral Ischemia**

Three groups of animals were randomly assigned to normal control (n = 12), diabetes (n = 30), and diabetes treated with candesartan (n = 27) groups to characterize the effect of candesartan in diabetic rats.

**Blood glucose.** Diabetes was confirmed by hyperglycemia after STZ injection. Plasma glucose concentrations of rats with diabetes were approximately fivefold greater than those of time-matched normal controls from day 7 to day 27 after STZ injection (P < 0.05 vs. control by ANOVA; Fig. 1). Candesartan, chronically applied for 2 wk (from days 14 to 27), did not affect the level of blood glucose in diabetic rats (P > 0.05 vs. diabetes and P < 0.001 vs. control by ANOVA; Fig. 1).

**Body weight.** Body weights in the diabetes and diabetes + candesartan groups were significantly (P < 0.05 vs. control by ANOVA) reduced with time (growth retardation, 18% and 21% reduction at 27 days, respectively) compared with controls (Fig. 2). Candesartan did not improve the body weight loss after diabetes.

**Blood pressure.** Blood pressure was measured on day 27 after STZ injection. There was no difference in blood pressure between diabetic and control groups (115 ± 2 and 113 ± 1 mmHg, respectively. P > 0.05 by ANOVA). Candesartan, however, significantly decreased blood pressure (100 ± 2 mmHg) in diabetic rats compared with control and diabetic groups (P < 0.0001 by ANOVA; Fig. 3).

**Table 1. Oligonucleotide primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Oligonucleotide primers used for RT-PCR</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td><strong>p22phox</strong></td>
<td>5′-GTTTGTGTGGGCTGCTGGAGCT-3′</td>
<td>5′-TGCGCGGGTCGCTGGATGCT-3′</td>
</tr>
<tr>
<td><strong>gp91phox</strong></td>
<td>5′-ACGTAGTGGGACGACATTG-3′</td>
<td>5′-TTCGACGTTGGGCGCTGATA-3′</td>
</tr>
<tr>
<td><strong>p47phox</strong></td>
<td>5′-CCACCGAACATGTGTTGAG-3′</td>
<td>5′-CCGCTGATGTCCTTCCTGCT-3′</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>5′-ACCAAGTGATGCTGGCATCAC-3′</td>
<td>5′-TCGACCAGCTGTGTTGCTGTA-3′</td>
</tr>
</tbody>
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**Fig. 1. Time course of blood glucose levels for normal control, diabetic, and candesartan (Can)-treated rats.** Blood glucose measured on day 27 after STZ injection. Additional animals were used to induce MCAO without diabetes (n = 27, including the 12 animals in the normal control group listed in the Figs. 1–3). The MCAO model was conducted at 27 days after STZ injection.
(32.3 ± 6.3% vs. 13.2 ± 3.1%, respectively, P < 0.05 by ANOVA; Fig. 5). The infarct size in the diabetes + MCAO + candesartan group was similar to that of MCAO alone (P > 0.05) and markedly smaller than the diabetes + MCAO group (9.7 ± 2.5%, P < 0.05 vs. diabetes + MCAO).

Neurological evaluation. The neurological scores at 24 h after MCAO are shown in Fig. 6. Neurological dysfunction in the diabetes + MCAO group (n = 30) was significantly increased compared with the MCAO-alone (n = 27) group (3.2 ± 0.3 vs. 2.1 ± 0.4, respectively, P < 0.05 by ANOVA) . Improved neurological function was observed in the diabetes + MCAO + candesartan group (n = 27) (1.6 ± 0.3, P < 0.0001 vs. diabetes + MCAO).

Mortality. Higher mortality was seen in the diabetes + MCAO group compared with the MCAO-alone group (22.2% vs. 11.1%, respectively). Candesartan treatment decreased mortality in diabetes + MCAO animals to 11.1%.

Brain Edema in Different Groups

Another five groups of animals (n = 5 in each group) were assigned for studies of brain water content: 1) normal control, 2) MCAO, 3) diabetes, 4) diabetes + MCAO, and 5) diabetes + MCAO + candesartan.

There were no differences in water content in the contralateral cortex, contralateral basal ganglia, and cerebellum among groups (Fig. 7). In the MCAO group, the ipsilateral cortical and basal ganglia water contents were significantly higher than those of normal controls (81.6 ± 0.1% vs. 80.1 ± 0.1% in the cortex, P < 0.001, 78.3 ± 0.2% vs. 77.4 ± 0.1% in basal ganglia, P < 0.01, respectively). The water content in the ipsilateral cortex in the diabetes + MCAO group was significantly increased compared with that in the MCAO group (82.9 ± 0.3% in the cortex, P < 0.01). The water content in the ipsilateral basal ganglia in the diabetes + MCAO group tended to be increased but not significantly compared with the MCAO group (78.8 ± 0.2%, P > 0.05). In the candesartan treatment group, the water content was markedly lower in the ipsilateral cortex (80.9 ± 0.4%, P < 0.01) and relatively lower in the ipsilateral basal ganglia (78.1 ± 0.3, P > 0.05) than in the diabetes + MCAO group.

Lipid Peroxidation in Different Groups

Another five groups of animals were assigned for studies of brain lipid peroxidation: 1) normal control (n = 5), 2) MCAO (n = 5), 3) diabetes (n = 4), 4) diabetes + MCAO (n = 5), and 5) diabetes + MCAO + candesartan (n = 5).

The levels of MDA, or lipid peroxidation products, in the ipsilateral hemisphere in the MCAO group and in both hemispheres in diabetes groups were significantly increased compared with those in the control group (P < 0.01 by ANOVA; Fig. 8). The diabetes + MCAO group had tremendously enhanced MDA levels, especially in the ipsilateral hemisphere, compared with MCAO or diabetes groups (P < 0.05). Candesartan decreased MDA levels to almost normal control levels (P < 0.01 vs. diabetes + MCAO).

NAD(P)H Expression

Another five groups of animals (n = 5 in each group) were assigned for studies of NAD(P)H oxidase mRNA expression: 1) normal control, 2) MCAO, 3) diabetes, 4) diabetes + MCAO, and 5) diabetes + MCAO + candesartan.

The primer sequences used in RT-PCR are summarized in Table 1. Figure 9 shows the original results of RT-PCR in the three subunits of NAD(P)H oxidase. The signals were normalized with the housekeeping gene GAPDH for comparison. The mRNA levels of p22phox and gp91phox were significantly higher in the ipsilateral hemisphere in the MCAO group and in both hemispheres in the diabetes group than in normal controls (P < 0.05 by ANOVA, Figs. 10 and 11). The diabetes + MCAO group had markedly enhanced expression of gp91phox in the
ipsilateral hemisphere, significantly \((P < 0.001)\) higher than that in the control, MCAO, and diabetes groups (Figs. 10 and 11). The mRNA level of \(p22^{\text{phox}}\) in the ipsilateral hemisphere in the diabetes + MCAO group was significantly higher than that in the control, diabetes, and MCAO groups \((P < 0.05\) by ANOVA). Candesartan treatment abolished \((P < 0.05\) vs. diabetes + MCAO) the elevated expressions of \(p22^{\text{phox}}\) and \(gp91^{\text{phox}}\).

On the contrary, the mRNA expression of \(p47^{\text{phox}}\) in the diabetes, MCAO, diabetes + MCAO groups showed a limited tendency to rise but failed to reach statistical significance \((P > 0.05)\) by ANOVA; Fig. 12).

**Immunohistochemistry of NAD(P)H Oxidase**

Another five groups of animals were assigned for studies of NAD(P)H oxidase protein distribution by immunohistochemistry: 1) normal control \((n = 3)\), 2) MCAO \((n = 4)\), 3) diabetes \((n = 4)\), 4) diabetes + MCAO \((n = 4)\), and 5) diabetes + MCAO + candesartan \((n = 4)\).

Figures 13 and 14 show the immunohistochemistry staining of \(p22^{\text{phox}}\) and \(gp91^{\text{phox}}\) in different groups. Figures 13A and 14A show brain slides from rats of the diabetes + MCAO group. The dashed lined semicircle indicates the infarct area after MCAO. Other slides show samples from the normal cortex \((B)\), MCAO \((C)\), diabetes \((D)\), diabetes + MCAO \((E)\), and diabetes + MCAO + candesartan \((F)\). Slides of MCAO, diabetes + MCAO, and diabetes + MCAO + candesartan are all from ipsilateral hemisphere. MCAO increased the expression of \(p22^{\text{phox}}\) and \(gp91^{\text{phox}}\) in the ipsilateral cortex (Figs. 13C and 14C), and diabetes markedly enhanced these expressions (Figs. 13E and 14E). The insets in Figs. 13E and 14E demonstrated the positive staining in cortex neuronal cells in the membrane and cytoplasm. The immunohistochemistry of \(p47^{\text{phox}}\) was not conducted because its mRNA level was not increased statistically.

**DISCUSSION**

Three major observations were made in the present study. First, diabetes enhanced the brain edema, infarction volume, mortality, and neurological dysfunction that results from cerebral ischemia. Second, diabetes enhanced the mRNA expression of NAD(P)H oxidase and lipid peroxidation in both hemispheres in rats. Focal cerebral ischemia produced an increase in ipsilateral mRNA expression of NAD(P)H oxidase and lipid peroxidation. After cerebral ischemia and reperfusion, diabetes further potentiated the expression of NAD(P)H oxidase and lipid peroxidation in the injured brain tissues (ipsilateral hemisphere). Third, the AT1-R antagonist candesartan reduced NAD(P)H oxidase expression and lipid peroxidation in the injured brain tissues. Our results are consistent with those of others, showing that an elevated level of angiotensin II in diabetes augmented NAD(P)H oxidase expression, increased reactive oxygen species and lipid peroxidation (42), and enhanced brain injury after cerebral ischemia in patients (6, 29). Our data are also consistent with published studies showing that angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists (such as candesartan) can be used to manage diabetic complications (41). Because candesartan reduced brain injury after focal cerebral ischemia in the presence of diabetes as shown in this study, we suggest that candesartan should be considered for diabetes-aggravated brain injuries after stroke.
The mechanisms of diabetes aggravated brain injury after focal cerebral ischemia is related to angiotensin II-NAD(P)H oxidase pathways for oxidative stress, as reported in other tissues (20, 41). Although xanthine oxidase, cyclooxygenases, and NAD(P)H oxidase are well known as potential sources of reactive oxygen species, NAD(P)H oxidase is considered the most important source of reactive oxygen species in diabetes (55). Diabetes, which was represented by hyperglycemia (Fig. 1) and body weight loss (Fig. 2) in the present study, slightly but significantly enhanced the expression of NAD(P)H oxidase mRNAs of two subunits (Figs. 9–14): p22phox and gp91phox. Diabetes was also associated with significant enhancement of lipid peroxidation and effects on NADPH oxidase resulted in the enhancement of lipid peroxidation. The effect of diabetes on NAD(P)H oxidase resulted in a slight but significant enhancement of lipid peroxidation (Fig. 8). However, it was not sufficient to produce observable brain injuries because diabetes alone did not produce brain edema (Fig. 7) or brain infarction (not shown). Once focal cerebral ischemia was induced in diabetic rats, a much more pronounced elevation of NAD(P)H oxidase mRNAs, especially the two subunits p22phox and gp91phox, occurred (Figs. 9–14) in the ipsilateral hemisphere in parallel with a striking enhancement of lipid peroxidation (Fig. 8). These strong biochemical and molecular biological changes were accompanied by enhanced brain edema, enlarged brain infarction, deteriorated neurological function (Figs. 6–8), and higher mortality. Because angiotensin II activates NAD(P)H oxidase during diabetes (21), it is not surprising that the AT1-R antagonist candesartan reduced NAD(P)H oxidase, decreased lipid peroxidation, protected brain tissues, and improved neurological function as shown in the present study.

The major effects of angiotensin II in the brain include vasoconstriction, reactive oxygen species production, cellular hypertrophy, and apoptosis (40). Two subtypes of G protein-coupled receptors, AT1-R and AT2-R, are involved in the renin-angiotension system. Most of the effects of angiotensin II in the brain are mediated by AT1-R, which is expressed in the blood-brain barrier (40). Several studies have demonstrated that an AT1-R antagonist prevented brain injury, especially in focal cerebral ischemia (14, 24, 25, 27). The protective effect of AT1-R blockade is not directly correlated with blood pressure reduction but is related to the improvement of cerebral
blood flow and brain edema (2, 24, 38). Candesartan is a nonpeptide angiotensin II receptor antagonist with high selectivity and an affinity for AT₁-R (36). Candesartan differs from other AT₁-R antagonists, such as irbesartan, losartan, or its active metabolite EXP 3174, because of its insurmountable and long-lasting inhibitory action at the AT₁-R (48). Administered peripherally, candesartan effectively inhibits responses mediated by AT₁-Rs localized inside the blood-brain-barrier (18). Candesartan has been used to reduce brain injury caused by cerebral ischemia in animals (26, 37) but not in a combined animal model of cerebral ischemia and diabetes. Therefore, we chose candesartan for treatment in our study and used peripheral route of application.

Activation of NAD(P)H oxidase by angiotensin II generates reactive oxygen species that cause or aggravate tissue injuries, especially in the cerebral tissues because the levels of endogenous antioxidants are low in the brain (12). Consistently, cerebral ischemia produces less brain injury in mice lacking functional NAD(P)H oxidase in the central nervous system (50) and in mice with AT₁-R knockout (51). During diabetes, hyperglycemia activates NAD(P)H oxidase and generates radical oxygen species, especially in vascular cells or leukocytes (23, 35). Engagement of the receptor for advanced glycation end products (RAGE) by products of nonenzymatic glycation/oxidation triggers the generation of reactive oxygen species. Activation of NAD(P)H oxidase contributed, at least in part, to enhancing oxidant stress via RAGE (52). In addition, S100B-RAGE interaction triggered intracellular generation of reactive oxygen species via activation of phospholipase D (46). Similar observations were made in diabetic rats created by STZ injection (45), the same rat model used in the present study. Several studies examined the expression of subunits of NAD(P)H oxidase in diabetes more specifically. For example, hyperglycemia induced expression of the p22phox subunit of NAD(P)H oxidase in human endothelial cells (13). The expression of the monocyte NAD(P)H oxidase subunit p22phox was increased in Type 2 diabetic patients (3). In addition, renal expression of the p47phox component of NAD(P)H oxidase was increased during diabetic nephropathy (39). Diabetes aggravates brain injury by intracellular acidosis, accumulation of extracellular glutamate, brain edema formation, blood-brain barrier disruption, and tendency for hemorrhagic transformation (1, 16, 32). However, no literature is available on the possible involvement of NAD(P)H oxidase subunits in brain injury after focal cerebral ischemia in the presence of diabetes. This study used a com-
Fig. 13. Immunohistochemistry of p22phox is shown. A: sample of a STZ + MCAO rat brain. The dashed line indicates the infarct area (bar = 1 mm). B–F: normal cortex (B), MCAO (C), STZ (D), STZ + MCAO (E), and STZ + MCAO + Can (F) at higher magnification (bar = 100 μm). Strong staining was observed in the ipsilateral cortex in MCAO, especially in STZ + MCAO samples. The inset in E (bar = 20 μm) shows p22phox staining in the membrane and cytoplasm.

Fig. 14. Immunohistochemistry of gp91phox. A: sample of a STZ + MCAO rat brain. The dashed line indicates the infarct area (bar = 1 mm). B–F: normal cortex (B), MCAO (C), STZ (D), STZ + MCAO (E), and STZ + MCAO + Can (F) at higher magnification (bar = 100 μm). Strong staining was observed in the ipsilateral cortex in MCAO, especially in STZ + MCAO samples. The inset in E (bar = 20 μm) shows gp91phox staining in the membrane and cytoplasm.
bined rat model of cerebral ischemia and diabetes and demonstrated that the AT1R antagonist candesartan has the potential to prevent brain injury aggravated by diabetes, which increases angiotensin II and activates NAD(P)H oxidase, after cerebral ischemia.

GRANTS

This study was partially supported by an American Heart Association Bugher Foundation Award for Stroke Research (to J. H. Zhang) and National Institutes of Health Grants NS054694, HD-45120, and HD-15338 (to J. H. Zhang) and HL-26441 (to D. N. Granger).

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