Candesartan Augments Ischemia-Induced Proangiogenic State and Results in Sustained Improvement After Stroke

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Background and Purpose—We have shown that acute treatment with candesartan in an experimental model of stroke resulted in vascular protection and improved outcomes at 24 hours poststroke, but the mechanisms are unknown. We now examine effects of candesartan on proangiogenic factors and 7-day outcomes using the same treatment paradigm.

Methods—Male Wistar rats underwent 3 hours of middle cerebral artery occlusion followed by reperfusion. A single dose of 1 mg/kg candesartan intravenously was given at reperfusion. Animals received neurobehavioral testing before middle cerebral artery occlusion, at 24 hours after middle cerebral artery occlusion, and at 7 days. Blood pressure was measured by telemetry. Animals euthanized at 24 hours had brain tissue and cerebrospinal fluid collected for matrix metalloproteinase activity, vascular endothelial growth factor expression, and tube formation assay. Neurobehavioral testing included elevated body swing test, Bederson, beam walk, and paw grasp. Cerebrovascular density was quantified using immunohistochemistry at 24 hours and 7 days.

Results—Matrix metalloproteinase-2 activity and vascular endothelial growth factor expression were higher (P=0.035, P=0.042, respectively) and cerebrospinal fluid was significantly more proangiogenic (5× tube formation; P=0.002) in the candesartan group at 24 hours. Although no difference was seen in infarct size at 7 days, treatment improved Bederson scores (2.1 versus 2.9, P=0.0083), elevated body swing test (22.9 versus 39.4, P=0.021), and paw grasp (1.29 versus 2.88, P=0.0001) at 7 days. Candesartan treatment resulted in increased vascular density in the striatum at 7 days (P=0.037).

Conclusion—Candesartan after reperfusion augments ischemia-induced angiogenic state and provides long-term benefits. The beneficial effects may involve vascular protection and enhancement of early angiogenic remodeling. (Stroke. 2009; 40:1870-1876.)

Key Words: blood pressure ■ stroke ■ vascular protection

Manipulation of the renin–angiotensin system has emerged as an effective strategy to prevent stroke and other vascular events in patients at risk. It also appears that angiotensin II type 1 receptor blockade may provide particularly robust protection of the cerebral vasculature. We have shown that delayed acute treatment with candesartan, in an experimental model of stroke in rats, resulted in both neurovascular protection and improved function at 24 hours poststroke, which was beyond that of blood pressure-lowering alone. Although the mechanisms and long-term effects are unknown, it is possible that promotion of neovascularization with angiotensin type 1 receptor blockade, as has been reported by others, may be involved, enhancing long-term functional recovery. We now examine the effects of a single dose of candesartan on the proangiogenic state after stroke and 7-day behavioral and histological outcomes. We measured the activity of matrix metalloproteinases (MMPs), expression of vascular endothelial growth factor (VEGF), and ability of cerebrospinal fluid (CSF) from treated animals to induce tube formation in cultured brain microvascular endothelial cells. In addition, we quantified vessel density at 7 days after stroke.

Materials and Methods

The experimental protocol was approved by the Care of Experimental Animal Committee of the Medical College of Georgia/Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center. Forty adult male Wistar rats (Charles River Breeding Company, Wilmington, Mass), weighing between 270 and 300 g, were divided randomly into saline and candesartan treatment groups.

Experimental Cerebral Ischemia

Temporary (3-hour) middle cerebral artery occlusion (MCAO) was achieved using the intraluminal suture model under isoflurane anesthesia as we have previously reported. All animals were singly housed before and after surgery with free access to food and water.
At reperfusion, a single dose of 1 mg/kg candesartan or saline control was given intravenously through a tail vein at a volume of 1 mL/kg.

**Blood Pressure Telemetry**

For a subset of these animals (n = 7), blood pressure telemetry transmitters (Data Sciences International, St Paul, Minn) were implanted according to the manufacturer’s specifications as previously reported. Blood pressure measurements were obtained for 2 days before MCAO and for 7 days afterward.

**MMP Zymography**

Animals (n = 7) underwent MCAO and treatment with saline (n = 3) or candesartan (n = 4) and were euthanized at 24 hours for quantification of MMP-2 and MMP-9 activity. The animals were then pericardially perfused with ice-cold phosphate-buffered saline, euthanized, and the brains were extracted. The ischemic and nonischemic hemispheres were separated and stored at −80°C until homogenization. The samples were homogenized in buffer as described previously. The gelatinolytic activity of the samples was assessed by densitometric analysis (Gel-Pro v 3.1; Media Cybernetics, Carlsbad, Calif) and compared with a standard band of recombinant protein.

**VEGF Quantification**

VEGF was quantified by enzyme-linked immunosorbent assay kit (RayBiotech, Norcross, Ga) performed on the same brain homogenate supernatant used for the zymography as described previously. Briefly, antibody-coated wells were loaded with 100 μL of sample, incubated, washed, and incubated with secondary biotinylated antibody per manufacturer’s instructions. The secondary antibody binds a horseradish peroxidase–streptavidin conjugate, and TMB substrate is added. The resulting reaction is stopped and the spectrophotometric analysis is performed. Signal was read at 450 nm (BioTek, Winooski, Vt).

**Tube Formation Assay**

Growth factor-reduced Matrigel (BD Biosciences) was used according to the manufacturer’s protocol. Brain microvascular endothelial cells were counted and plated at 1 × 10^5 cells/mL. Once attached, cells were switched to serum-free medium and treated with 50 μL of CSF from either nonstroked or stroked candesartan (n = 3) or saline (n = 3)–treated animals and incubated for 24 hours. For inhibitor studies, 50 μL VEGF blocking antibody (1 μg/mL; R & D Systems, Minneapolis, Minn) was added 15 minutes before CSF treatment. Images of the tube-like structures were captured using a Zeiss Axiovert microscope. Analysis of the tube length was analyzed digitally using the Meta Morph imaging system.

**Neurobehavioral Tests**

Seventeen animals were given neurobehavioral testing before MCAO, at 24 hours after MCAO, and at 7 days. Tests that were used include elevated body swing test, Bederson, beam walk, and paw grasp performed in a blinded fashion.

**Infarct Size Determination**

Rats were deeply anesthetized with an 85% ketamine/15% xylazine combination and decapitated. For animals undergoing CSF collection, before decapitation, CSF was collected (approximately 150 μL) by inserting PE-50 tubing connected to a 26-gauge needle into the foramen magnum. For evaluation of infarct at 7 days (n = 10), rats were anesthetized and transcardially perfused with normal saline followed by 4% paraformaldehyde. Brains were quickly removed and fixed in 4% paraformaldehyde for 3 hours and then sliced into 2-mm coronal sections. These sections were further fixed in 4% paraformaldehyde for 24 hours and then transferred to 70% isopropl alcohol. Hematoxylin and eosin staining was performed on slide-mounted, paraffin-embedded 5-μm thick sections taken from the 2-mm sections. Grossly visible infarction areas were imaged and quantified by image analysis software (Zeiss-KS300, Oberkochen, Germany). Infarct areas were expressed as percentages of the contralateral hemisphere.

**Cavitation Measurements**

Cavitation was quantified as the difference in area between the hemispheres, expressed as a percentage of the contralateral hemisphere. These measurements were made on the hematoxylin and eosin-stained slices representing the state of the brain volume at 7 days, at which time edema had subsided and collapse of brain volume into the ischemic region had occurred.

**Endothelial Barrier Antigen and Laminin Analysis**

The immunohistochemical analyses were performed on additional slide-mounted, paraffin-embedded 5-μm thick sections collected at 7 days as described previously. Slides were deparaffinized, rehydrated in solution of 0.1% Triton-X-100 (Fisher Scientific), flooded with a solution of Proteinase K (20 μg/mL) and 0.05% Trypsin for antigen retrieval, and incubated for 30 minutes at 30°C. For endothelial barrier antigen analysis, slides were rinsed 3 times with phosphate-buffered saline and blocked with 2% normal calf serum solution (Sigma). A solution of primary mouse antiendothelial barrier antigen antibody (Sternberger Monoclonals SMI-71, Baltimore, Md) was flooded onto each slide and incubated at room temperature for 1 hour in a humidified chamber. Slides were washed 3 times in phosphate-buffered saline and a solution of secondary antibody, biotinylated horse antimouse (Vector BA-2001 #C0804, Burlingame, Calif) was applied, and incubated at room temperature for 30 minutes in a humidified chamber. Slides were then washed 3 times with phosphate-buffered saline and incubated in a solution of the fluorescent streptavidin conjugate SA-Cy3 (Jackson #016 to 160–084, West Grove, Pa) for 1 hour at room temperature in a humidified chamber. Slides were visualized by microscope (Zeiss-Observer.Z1) using 20× objective captured by a camera (Zeiss Axiocam HRc) in the regions of interest (area of image in millimeters squared) and densitometric measurements were made at 620 nm by Zeiss Axiosvision 4.6.3.0 software. Regions of interest were defined as cingulate cortex, lateral cortex, and striatum, in both hemispheres, with the cingulate cortex corresponding to the stroke penumbra, and the lateral cortex and striatum representing the core of the infarct. For the analysis of laminin, slides were prepared as described previously through the blocking step, then washed and fixed a solution of primary antibody (rabbit polyclonal; Novus Biologics, Littleton, Colo) for 1 hour at room temperature in a humidified chamber. The slides were washed 3 times with phosphate-buffered saline and flooded with a solution of biotinylated secondary antibody (Vector Labs #B1205) and incubated for 30 minutes at room temperature in a humidified chamber. Slides were then washed and incubated as described previously. Images were captured as described previously using the same regions of interest. In a blinded manner, 3 images per area per animal were analyzed and the number of vascular profiles averaged.

**Statistical Analyses**

Differences between candesartan and saline were determined by Student t test for average infarct size, edema, cavitation, MMP
activity, VEGF expression, and tube formation. A Wilcoxon rank sum test was used to assess the differences on the postperfusion values of the Bederson score. Differences from baseline behavior for paw grasp and beam walk were analyzed using *t* tests for 24-hour values and 7-day values. Because baseline values for both measures were 0 for all animals, no adjustment for baseline values was needed. An adjusted probability value of 0.025 was used to determine significance to account for the multiple tests. Differences from baseline for elevated body swing test at 7 days were analyzed for group difference adjusted for baseline by analysis of covariance. These analyses were performed using SAS 8.2 (SAS Institute Inc, Cary, NC).

**Results**

**Blood Pressure**

A single dose of 1 mg/kg candesartan administered at reperfusion lowered blood pressure for 4 days compared with saline controls (Figure 1). Baseline mean arterial pressure was approximately 95 mm Hg in both groups and demonstrated normal circadian variation for the 48 hours before MCAO. On MCAO, mean arterial pressure rose rapidly approximately 25 mm Hg in both groups. At reperfusion, the candesartan-treated animals returned to baseline after approximately 4 hours (which agrees with published data regarding time to onset of hypotensive effect for candesartan) and remained low for the follow-up period.

**Effect of Treatment on Neurobehavioral Testing and Weight**

Treatment improved Bederson scores at all time points, including Day 7 (2.1 versus 2.9 points, *P*=0.0083). Treatment also improved elevated body swing test performance, including Day 7 (22.9 versus 39.4% biased left turns, *P*=0.021), and improved paw grasp, including Day 7 (1.29 versus 2.88 points, *P*=0.0001). Beam walk was improved in the treatment group at 24 hours, but not at 7 days (Figure 2). Animals in the candesartan group had significantly higher weight at 7 days (82.8% versus 63.1% of preoperative weight, *P*=0.017).

**Effect of Treatment on Infarct Size and Cavitation**

Infarct size was not significantly different between the 2 groups (neither cortex or stratum) at 7 days (but cavitation was decreased slightly in the treated animals at 7 days; 4.3% versus 6.6% *P*=0.040) suggesting some beneficial preservation of brain tissue integrity in the treatment group (Figure 3).

**Effect of Treatment on MMP-2 and MMP-9 Activity, VEGF Expression, and Tube Formation**

MMP-2 activity was significantly increased in the stroke hemispheres of the treatment group (*P*=0.035) compared with stroke hemispheres of control animals. MMP-9 activity was not significantly different between the groups (Figure 4). VEGF expression was increased in the stroke hemispheres of the treatment group compared with controls (12.61 pg/mL versus 5.61 pg/mL, *P*=0.042).

Brain microvascular endothelial cells cultured in reduced growth factor Matrigel and incubated with CSF from candesartan-treated stroked animals exhibited 5× increased
tube formation \((P=0.002)\) at 24 hours compared with the saline group. Even without prior stroke, candesartan facilitated tube formation (Figure 5). Administration of a VEGF-blocking antibody before addition of the CSF significantly reduced the tube formation (data not shown).

**Effect of Treatment on Vascular Permeability at 24 Hours**

Despite the increase in VEGF and the resulting proangiogenic state, vascular permeability, as measured by the ratio of brain to plasma fluorescence after administration of fluorescein isothiocyanate-labeled albumin, was significantly decreased in both hemispheres of the candesartan-treated animals (Figure 6; \(*P<0.05\)). This confirms the uncoupling of the permeability and proangiogenic effects of VEGF by candesartan in this model.

**Effect of Treatment on Endothelial Barrier Antigen and Laminin Staining**

In both groups, endothelial barrier antigen and laminin staining were decreased in the ischemic hemisphere at 7 days and there was no difference between the treatments in any of the 3 areas for endothelial barrier antigen and for the lateral cortex and cingulate cortex in the laminin staining. In the candesartan-treated animals, however, laminin staining was significantly increased in the striatum at 7 days compared with saline-treated animals, indicating an increase in vascular density (Figure 7).

**Discussion**

A single dose of candesartan after reperfusion provided benefits at 7 days as measured by a variety of neurobehavioral tests. It also led to better recovery as measured by weight gain. This suggests that the status immediately after reperfusion is an important determinant of the ultimate damage due to the stroke. The concurrent elevation in 2 markers of extracellular matrix turnover and a proangiogenic state at 24 hours after stroke suggests the intriguing possibility that the observed beneficial effects of candesartan in our model may involve enhancement of early angiogenesis and remodeling rather than resulting merely from blood pressure-lowering. We have previously shown that blood pressure-lowering alone can be neurovascular protective, but we were unable to demonstrate improved functional outcome as a result. Interestingly, although elevated blood pressure has been implicated in increasing MMP activity, the elevation of MMP-2 activity seen in this experiment occurred in the context of...
normal and even slightly subnormal blood pressures, suggesting that the MMP-2 activity was independent of candesartan’s blood pressure-lowering effect. The mechanism by which candesartan might lead to increased MMP-2 activity and VEGF expression is unknown at this time, but it seems probable that it is mediated by one of its pleiotropic effects (e.g., antioxidant effect or angiotensin type 2 agonism).

It is becoming clear that it is the timing of MMP and VEGF expression that determines whether their actions will be positive (promoting angiogenesis and remodeling) or nega-
tive (promoting edema and hemorrhage). Specifically, in the experiments of Zhao et al, early inhibition of MMP-9 (within 24 hours) proved beneficial, whereas later inhibition was deleterious. In contrast, in our experiment, early elevation of MMP-2 and VEGF was associated with improved outcomes. It is possible that by blunting the typical acute hypertensive response after ischemic stroke, we allowed remodeling to get underway earlier (within 24 hours) with a decreased risk of hemorrhage and edema. Additionally, it has been shown in mice that angiotensin type 1 blockade can uncouple VEGF’s proangiogenic function from its permeability-inducing effects, reducing the hazards of VEGF elevation seen in our experiments. Even in control animals with no stroke, we were able to demonstrate a proangiogenic effect in the central nervous system.

Although the focus of our research is on the vasculoprotective effects of angiotensin antagonism, it is probable that the vascular and neuronal effects are inextricably linked and are mutually responsible for the recovery benefits seen in our model. In our experiment, there was increased vascularization in the striatum in the candesartan-treated animals at 7 days but no increase in blood–brain barrier staining or reduction in infarct size. This suggests that the benefits seen may be due to an enhancement of recovery due to increased neovascularization. Others have reported a transient and modest increase in early angiogenesis in the striatum after ischemia and reperfusion (6 days after stroke) associated with recovery. It is possible that candesartan augments this effect, leading to sustained benefits in neurological outcome.

The neurobehavioral benefit from candesartan treatment did not correspond to infarct size reductions in our experiment. Many animal models of stroke have reported little or no correlation between the observed neurobehavioral benefits and the morphological measures of stroke severity. In this experiment, the dose of candesartan lowered blood pressure below the baseline levels. Some experimental evidence suggests that overaggressive lowering of blood pressure with angiotensin receptor blockers can worsen infarct area. Given the blood pressures achieved, it would be expected that conditions were optimal for infarct extension. It is possible that this in fact occurred and is responsible for the lack of difference seen in infarct size at 7 days.

It is unknown whether a less aggressive dose would confer the same benefits, and research is ongoing to address this issue. Nonhypotensive doses of angiotensin receptor blockers have demonstrated benefits in a murine stroke model. Clinically, a more conservative approach is prudent; in the Acute Candesartan Cilexetil Therapy in Stroke Survivors (ACCESS) clinical trial, blood pressure-lowering with can-

Figure 6. Vascular permeability at 24 hours after MCAO. Candesartan-treated animals had significant protection in vascular permeability at 24 hours as measured by the brain to plasma fluorescein isothiocyanate ratio (mean±SEM) in both the stroke (S) and nonstroke (NS) hemispheres. *P<0.05.

Figure 7. Laminin staining in the saline (A) and candesartan (B)-treated animals. Shown are representative images from the ischemic hemisphere. Candesartan-treated animals had significantly more vascular density in the ischemic hemisphere (C; P<0.037).
desartan was targeted to achieve a 10% to 15% reduction over the first 24 hours poststroke and this resulted in improved long-term outcomes.

The use of candesartan as an acute stroke therapy has many exciting possible advantages. First, it is already an established medication approved for use in humans. Second, it can be administered without waiting for the stroke phenotype to be determined, because there is little risk of exacerbating hemorrhagic strokes. The additional attribute of possibly enhancing early angiogenesis and remodeling makes it a particularly promising intervention.

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