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Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion

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Abstract

We have shown that reduction of blood pressure (BP) immediately after the onset of reperfusion reduced neurovascular damage and improved functional outcome after experimental cerebral ischemia and candesartan is particularly effective in improving long-term functional outcome. In this study, we sought to determine if early BP lowering with candesartan, in the presence of an occluded cerebral artery, will reduce injury and improve outcome after experimental stroke. Male Wistar rats underwent 24 h or 7 days of middle cerebral artery occlusion (MCAO). A single dose of 1 mg/kg candesartan was administered intravenously at 3 h after MCAO. Animals received neurobehavioral testing at 3 h, 24 h, and 7 days, and blood pressure was measured by telemetry. Animals had brain tissue collected for infarct size (24 h and 7 days), hemoglobin content, matrix metalloproteinase (MMP) activity, and vascular endothelial growth factor (VEGF) expression (24

h only). Candesartan significantly decreased blood pressure, infarct size (-20% ; $p=0.021$), hemoglobin excess (-50% ; $p=0.0013$), and edema (-35% ; $p=0.0005$) at 24 h after MCAO. This resulted in a reduced cerebral perfusion deficit ($p=0.034$) in the ischemic hemisphere compared with saline and significantly improved Bederson scores and paw grasp. MMP-2, MMP-9, and VEGF were significantly increased by MCAO, but there were no differences between candesartan- and saline-treated animals. There were no significant differences in behavioral outcome at day 7. BP lowering with candesartan reduces early brain injury after experimental stroke even when the artery remains occluded. The early benefits were not sustained at 7 days, as seen in reperfused animals, however. The neuroprotection and neurorestorative properties of candesartan may occur by separate distinct mechanisms.

Keywords

Candesartan; Blood pressure; Stroke; Reperfusion

Introduction

Blood pressure (BP) lowering in the acute stroke period has been controversial for decades but is enjoying a resurgence of interest as a strategy to reduce disability and attenuate hemorrhage growth [1] in patients. There remains concern, however, that certain individuals may experience untoward reductions in cerebral blood flow and neurologic worsening when blood pressure is reduced aggressively [2]. Reduction of BP immediately after the onset of reperfusion has been shown to be neurovascular protective and improve functional outcome after experimental cerebral ischemia [3–5]. The angiotensin II type 1 receptor antagonist (ARB), candesartan, is particularly effective in improving longer-term functional outcome [6, 7]. We have shown that this tactic increases the expression and activity of endogenous protective proteins (vascular endothelial growth factor (VEGF)) when administered after reperfusion [6]. Most patients do not experience complete recanalization and early reperfusion, however, even if they receive intravenous tissue plasminogen activator, so it is important to determine the outcomes of BP lowering when the artery remains occluded [8].

In the present study, we sought to determine if early BP lowering with candesartan, in the presence of an occluded cerebral artery, will reduce injury and improve outcome after experimental stroke.

Materials and Methods

The Institutional Animal care and Use Committee (IACUC) of the Charlie Norwood VA Medical Center approved the protocol. Male Wistar rats ($n=127$), from the Charles River Breeding Company (Wilmington, MA, USA) within a narrow range of body weight (270–305) were used.

Experimental Cerebral Ischemia

Anesthesia was performed by using 2% isoflurane via inhalation. Cerebral ischemia was induced using the intraluminal suture middle cerebral artery occlusion (MCAO) model [9]. Nineteen to 21 mm 3–0 surgical nylon filament was introduced from the external carotid artery lumen into the internal carotid artery to block the origin of the right MCA. The animals were kept under anesthesia for only 15 min for the surgical procedure. Temperature was maintained at $36.5\text{--}37.5^{\circ}\text{C}$ using a controlled heating system. After 3 h of occlusion, the animals received either candesartan (Astra-Zeneca) 1 mg/kg or an equal volume of saline (1 mL/kg) intravenously, and the animals were returned to their cages. The selected

candesartan dose was previously shown to be neurovascular protective [5, 6]. Animals were euthanized at either 24 h or 7 days after stroke.

Physiological Monitoring

In a subset of animals ($n=16$), cerebral perfusion was measured using the Periscan PIM 3 System (Stockholm, Sweden). A skin incision was performed, and the skull was exposed and cleaned. Whole brain scan was performed using the PIM3 to measure cerebral perfusion in both hemispheres at baseline, 3 h, 24 h, and 7 days after MCAO (at 5 min, 21 h, and 7 days after candesartan or saline treatment) ($n=10$). An additional group of animals was included to evaluate perfusion at 3 and 6 h after treatment ($n=6$).

Blood Pressure Telemetry

In a set of 30 rats, telemetry transmitters (Data Sciences, Inc.) were implanted according to manufacturer's specifications under general anesthesia with ketamine and xylazine. A midline incision was used to expose the abdominal aorta that was briefly occluded to allow insertion of the transmitter catheter into the abdominal aorta. The catheter was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed using nonabsorbable suture (3–0). Rats were allowed to recover from surgery for 10 days and returned to housing for data acquisition until undergoing MCAO. The individual rat cages were placed on top of the telemetry receivers, and arterial pressure waveforms were continuously recorded throughout the study. Data were recorded every 10 min for 48 h before the stroke and until sacrifice at 24 h after the onset of stroke. Use of inhaled anesthetics during stroke surgery, with rapid recovery of consciousness, allowed frequent collection of arterial pressure data in conscious animals close to the time of onset of ischemia. A separate set of sham animals, where the artery was exposed and anesthesia was administered, were also included for comparison.

Infarct Size/Hemorrhage Determination

At 24 h after the onset of MCAO, animals were anesthetized with a cocktail of ketamine (45 mg/kg) and xylazine (15 mg/kg) via intramuscular injection then perfused with saline and euthanized, and their brains were removed. The brain tissue was sliced into seven 2-mm-thick slices in the coronal plane and stained with a 2% solution of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, Missouri, USA) for 15–20 min. Images of the stained sections were taken. Using image analysis software (ImageJ, NIH), infarction zones were measured and infarct volume was calculated. The ischemic and non-ischemic hemispheres of the slices for the enzyme-linked immunosorbent assay (ELISA) were separated and processed, using the non-ischemic side as a control. After homogenizing the slices in the core of the infarct and taking the supernatants, ELISA was performed to measure the hemoglobin in the brain tissue [10].

For evaluation of infarct at 7 days ($n=15$), rats were anesthetized and transcardially perfused with normal saline, followed by 4% paraformaldehyde (PFA). Brains were quickly removed and fixed in 4% PFA for 3 h, then sliced into 2-mm coronal sections. These sections were further fixed in 4% PFA for 24 h and then transferred to 70% isopropyl alcohol. Hematoxylin and eosin (H&E) staining was performed on slide-mounted, paraffin-embedded, 5- μ m-thick sections taken from the 2-mm sections. Grossly visible infarction areas were imaged and quantified by image analysis software (ImageJ) [11]. Infarct areas were expressed as percentages of the contralateral hemisphere.

Edema/Cavitation Measurements

Edema or 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 H&E stained slices, representing the state of the brain volume at either 24 h (edema) or 7 days (cavitation), at which time edema had subsided and collapse of brain volume into the ischemic region had occurred.

MMP Zymography

A separate set of animals underwent MCAO as above and treatment with either saline ($n=9$) or candesartan ($n=8$) and euthanized at 24 h for quantification of MMP-2 and MMP-9 activity. The animals were anesthetized with a cocktail of ketamine (45 mg/kg) and xylazine (15 mg/kg) via intramuscular injection. The animals were then pericardially perfused with ice-cold PBS, and the brains were extracted. The ischemic and non-ischemic hemispheres were separated and stored at -80°C until homogenization. The samples were homogenized in buffer as described previously [12]. The gelatinolytic activity of the samples was assessed by densitometric analysis (Gel-Pro v 3.1, Media Cybernetics, Carlsbad, CA, USA).

VEGF Quantification

Brain lysates were subjected to heparin beads (Sigma, St. Louis, MO, USA) as described before [13]. Briefly, the beads were pelleted at $5,000\times g$ for 1 min, washed in 400 mM NaCl and 20 mM Tris, and loaded onto a 4–20% gradient Tris glycine precast gel (BioRad, Hercules, Gibbstown, NJ, USA). After blocking, the membrane was incubated with VEGF primary antibody (Calbiochem, Gibbstown, NJ, USA), and detection was accomplished using a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (GE Healthcare). The films were subsequently scanned, and band intensity was quantified using densitometry software (Alpha Innotech, San Leandro, CA, USA).

Neurobehavioral Tests

All animals underwent neurobehavioral testing before MCAO, at 24 h after MCAO, and at 7 days. Tests that were used included Bederson score, beam walk, and paw grasp, performed in a blinded fashion [14, 15].

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Differences between candesartan and saline were determined by Student t test for average infarct size, edema, cavitation, matrix metalloproteinase (MMP) activity, and VEGF expression. A Wilcoxon rank sum test was used to assess the differences on the post-perfusion values of the Bederson score. Differences from baseline behavior for paw grasp and beam walk were analyzed using t tests for 24-h 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. These analyses were performed using SAS 8.2 (SAS Institute Inc, Cary, NC, USA).

Results

BP Lowering with Candesartan

In all animals, baseline mean arterial pressure (MAP) was between 95 and 97 mmHg and was elevated by 30–35 mmHg upon MCAO. As is seen in Fig. 1, candesartan significantly

lowered BP within a few hours of administration, to an ultimate value that was previously reported when the artery was reopened at 3 h [5, 6].

Infarct, Hemoglobin, and Edema

In animals euthanized at 24 h after the onset of MCAO, candesartan treatment significantly lowered infarct size ($50.9 \pm 12.4\%$ vs $41.9 \pm 9.08\%$, $p=0.021$), hemoglobin excess (2.92 ± 1.22 vs 1.6 ± 0.6 , $p=0.0013$), and edema ($19.7 \pm 5.42\%$ vs $13 \pm 4.83\%$, $p=0.0005$). However, in the animals that survived 7 days, the differences were no longer significant (Fig. 2).

Behavioral Outcomes

At 24 h after the onset of PMCAO, animals treated with candesartan scored better on the Bederson ($p=0.0002$) and the assessment of paw grasp ($p=0.0022$) and were not significantly better on the beam walk ($p=0.095$) (Fig. 3). At 7 days, there were no significant differences in behavioral measures.

Cerebral Perfusion

When cerebral perfusion in the ischemic hemisphere was expressed as a percentage of the contralateral hemisphere (Fig. 4), there was a dramatic drop at 10 min after MCAO and this was maintained for 3 h. Candesartan treatment resulted in a significant improvement in cerebral perfusion at 24 h ($p=0.034$). In a subset of animals ($n=6$) where perfusion was measured at 6 and 9 h after MCAO (3 and 6 h after candesartan or saline treatment), the relative deficit in the ischemic hemisphere remained similar to that measured immediately after treatment (-20% to -40% , not shown). At 7 days after MCAO, hemispheric perfusion had returned to baseline in both groups (candesartan or saline).

MMPs and VEGF

MMP-2 activity and VEGF expression, but not MMP-9 activity, were significantly increased in the stroke hemispheres of both treatment groups. Candesartan treatment did not result in a further significant increase in either MMP-9, MMP-2, or in VEGF at 24 h (Figs. 5 and 6).

Discussion

It is feared that lowering BP will reduce cerebral blood flow and worsen the outcome [16]. In this study, we demonstrated that lowering the BP acutely with candesartan in animals with an occluded cerebral artery was associated with an actual reduction in tissue injury (infarct, hemorrhage, edema) and better function at 24 h after the onset of ischemia. This early improvement is identical to what we reported in a model of temporary MCAO [5]. Although we demonstrated an apparent improvement in cerebral perfusion at 24 h in the treated animals, this is unlikely to be the mechanism of the reduction in neurovascular injury, but a response to it. The improvement in the perfusion deficit in the ischemic hemisphere that we demonstrated at 24 h was not present at 3 and 6 h after candesartan administration, making it less likely that early reperfusion of the ischemic hemisphere resulted in less tissue damage. It is more likely that vascular protection by BP lowering and candesartan resulted in neuroprotection through preservation of matrix-trophic factor coupling [17].

Unlike our previous report, where early candesartan administration was associated with an increase in VEGF and matrix proteins and 7-day functional improvement [6], the early protection in this study was lost at 7 days when the artery remained occluded. Whether repeated dosing of candesartan throughout the acute stroke period (to 7 days) would have allowed sustained benefit remains to be determined. It is also possible that early reperfusion is necessary for the neurorestorative effects of candesartan on VEGF, vascular remodeling,

and longer-term recovery. Previous reports from our group and others have shown the importance of reactive oxygen species in neovascularization and VEGF signaling, and optimal conditions might only be achieved in a reperfused state [18, 19].

The clinical relevance of our study lies in the fact that less than 10% of ischemic stroke patients receive reperfusion therapy [20], and BP lowering with candesartan is likely to be safe and provide early benefits to the vast majority of patients not treated with thrombolysis. In patients, where spontaneous recanalization may occur, the early benefit may be sustained, as was seen a clinical trial of candesartan in acute stroke patients [21]. Future studies of candesartan in acute ischemic stroke (Scandinavian Candesartan Acute Stroke Trial (SCAST)) will determine whether this therapy will grant sustained benefits to stroke survivors.

In conclusion, BP lowering with candesartan, administered 3 h after permanent MCAO, reduced tissue damage and resulted in an increase in perfusion in the ischemic hemisphere at 24 h. The failure to induce the formation of trophic factors as is seen in reperfused animals [6] may explain the lack of benefit we reported at 7 days.

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Fig. 1. Changes of MAP (mmHg) of PMCAO rats before and after injection (arrow) with saline or candesartan at 1 mg/kg. Sham animals received the same anesthesia and surgical preparation to expose the artery. *Black horizontal bars* indicate nighttime in the 12:12-h light/dark cycles

Fig. 2. Infarct size, edema, and hemoglobin content. Treatment significantly lowered infarct size (**a**, $*p<0.05$), edema (**b**, $**p<0.01$), and hemoglobin excess(**c**, $**p<0.01$) at 24 h after MCAO. No difference in infarct size or cavitation was seen at day 7(**d**, **e**)

Fig. 3. Neurobehavioral tests. Treatment improved Bederson scores (**a**, $**p < 0.01$) and paw grasp (**c**, $**p < 0.01$) at 24 h after MCAO but not at day 7. However, treatment did not improve beam walk (**b**) at either time point after MCAO

Fig. 4. Candesartan treatment significantly improved cerebral perfusion at 24 h after MCAO (**a**, $p < 0.05$ vs saline). Laser Doppler scan images (PIM 3) of orientation and tissue perfusion (**b**)

Fig. 5. MMP-9 (a) and MMP-2 (b) activity was increased as compared with the non-ischemic control hemisphere in saline and candesartan treated animals at 24 h after MCAO. Candesartan treatment did not significantly alter MMP-2 or MMP-9 (a, b)

Fig. 6. Candesartan treatment did not result in a significant increase in VEGF-121 (**a**), VEGF-165 (**b**), or VEGF-189 (**c**) at 24 h after MCAO