Prophylactic angiotensin type 1 receptor antagonism confers neuroprotection in an aged rat model of postoperative cognitive dysfunction

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Abstract
Postoperative cognitive dysfunction (POCD) is a common geriatric complication, although its exact neuropathogenesis remains elusive. Blockers of the renin–angiotensin system (RAS) ameliorate cognitive deficits in inflammatory brain disorders, with its effects on POCD not yet fully elucidated. The aim of the present study was to investigate regulation of the brain RAS and the effect of angiotensin II receptor type 1 (AT1) inhibition on surgery-induced cognitive impairment in a well-established rat POCD model. We observed upregulation of angiotensin II protein expression and AT1 subtype B transcript levels in the hippocampus after laparotomy, suggesting surgical stress activates the hippocampal RAS in aged rats. Chronic pretreatment with 0.1 mg/kg/day candesartan, an AT1 antagonist, significantly attenuated surgery-induced cognitive deficits in the Morris water maze task without altering blood pressure. Candesartan also decreased hippocampal blood–brain barrier (BBB) permeability. Concomitant with these functional benefits, we observed significant inhibition of hippocampal neuroinflammation, evidenced by decreased glial reactivity and phosphorylation of the NF-κB p65 subunit, as well as marked reductions in interleukin-1β, tumor necrosis factor-α, and cyclooxygenase-2. Our results are the first to show that activation of the brain RAS after surgery contributes to POCD in aged rats. Chronic treatment with low doses of candesartan may elicit blood pressure-independent neuroprotective effects in POCD by improving BBB function and promoting resolution of neuroinflammation.

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1. Introduction

With the increase in the aging population and improvements in anesthesia and surgery, it is likely that postoperative cognitive dysfunction or decline (POCD) will become an ever-growing concern in senior patients [1]. POCD delays rehabilitation and is directly associated with impairments in daily functioning, and increased morbidity and mortality [1]. However, the exact neuropathogenesis of POCD remains largely to be determined.

POCD etiology may include patient-related (e.g., advanced age, genetic susceptibility), anesthetic, and surgical factors, although major surgical intervention appears to be the principle culprit when compared with anesthesia [2]. POCD animal models show postoperative hippocampal neuroinflammation marked by blood–brain barrier (BBB) disruption, glial response, and activation of inflammation/NF-κB signaling [3,4]. Unfortunately, there is currently no established preventative or therapeutic strategy for POCD because of its elusive pathogenic mechanisms.

For years, the renin–angiotensin system (RAS) has been well known as a circulating humoral system involved in blood pressure regulation and water homeostasis. The existence of an indepen-
dient intrinsic cerebral RAS with all its components, has also been well established [5]. In addition, more recent studies have involved the brain RAS in inflammatory brain disorders, including neurodegenerative diseases [6]. More importantly, accumulating preclinical evidence indicates that augmentation of brain RAS activity contributes to cognitive impairment progression in a variety of animal models including Alzheimer’s disease (AD) [7], hypertension [8], type 2 diabetes mellitus [9], and traumatic brain injury [10]. There is also conclusive clinical evidence that treatment with angiotensin receptor blockers significantly attenuates the incidence, progression, and pathology of AD [11,12].

In the brain, as in the periphery, it is accepted that most of the physiological and pathological effects of RAS activation are the consequence of angiotensin II (Ang II) stimulation, dependent on Ang II type 1 receptors (AT1) [6]. So far, the exact role played by Ang II and its receptors in POCID development has not been determined. Therefore, using an established POCD model, we investigated the regulation of the brain RAS and the influence of AT1 inhibition on neurobehavioral outcome, BBB permeability, and brain inflammation after surgery.

2. Materials and methods

2.1. Subjects

Aged male Sprague-Dawley rats (20-month-old; weight, 550–650 g) were used for all experiments. Animals were purchased from the Dongchuan Laboratory Animal Center (Changsha, Hunan, China) and bred under standardized housing conditions with food and water ad libitum. All rats were allowed to adapt to their new environment for at least 7 days before beginning experiments. The experimental protocol was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. LA2014113).

2.2. Experiment protocol

2.2.1. Assessment of brain and circulating RAS regulation after surgery

To study the effects of peripheral surgical trauma on RAS activity, rats were randomly assigned to surgery (n = 20) or control (n = 4) groups, and received laparotomy or sham operations, respectively. RAS components in the hippocampus and plasma were dynamically determined at 3, 6, 12, 24, and 72 h after surgery using radioimmunoassay and real-time reverse transcription-PCR (qRT-PCR) (n = 4 per time point).

2.2.2. Effect of AT1 blockade on spatial learning and memory

To evaluate the role of the AT1 in surgery-induced cognitive decline, the AT1 antagonist, candesartan (Takeda Pharma, Osaka, Japan), was used to block AT1 receptors. Rats were randomly assigned to vehicle + sham, vehicle + surgery, CAND + surgery, and CAND + sham groups (n = 9 each). Rats in the CAND + surgery and CAND + sham groups were intraperitoneally administered with candesartan at a non-hypotensive dose of 0.1 mg/kg daily for 14 consecutive days pre-treatment [13]. This dosing protocol has been shown to effectively protect against streptozotocin-induced memory deficits [13], and recall impairment caused by repeated stress in rats [14]. Candesartan was dissolved in a vehicle solution (1 mg in 950 L of phosphate buffered saline and 50 L 1 M Na2CO3). Rats in the other two groups received an identical volume of vehicle solution. Following the pretreatment phase, the animals either underwent laparotomy or sham surgery under isoflurane anesthesia. Spatial learning and memory were then tested on the Morris water maze (MWM) task.

2.2.3. Effect of AT1 blockade on physiological parameters in aged rats

To determine the systemic effect of the candesartan dose used, another 10 aged rats were treated with candesartan or vehicle as described above (n = 5 each group). Blood pressure and heart rate measurements were performed using the CODA rat tail-cuff system (Kent Scientific, Torrington, CT, USA) on the 3rd, 7th, and 14th day of treatment. Two hours after the last injection, 0.5 mL blood samples were collected for arterial blood gas (ABG) analysis [15].

2.2.4. Effect of AT1 blockade on BBB permeability and neuroinflammation

To further determine if AT1 blockade could be of therapeutic benefit, separate animal cohorts were randomly assigned to vehicle or candesartan treatment for 2 weeks, followed by laparotomy or sham surgery. Hippocampal expression of inflammatory markers, including phosphorylated Nk-xB p65, interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2), were assessed on postoperative day 1 (n = 4). In addition, two glial cell activation markers, cluster of differentiation 11b (CD11b) and glial fibrillary acidic protein (GFAP), were also examined at postoperative days 1 and 7 (n = 4). BBB integrity was determined on postoperative days 1 and 3 (n = 4–6).

2.3. Anesthesia and surgery

Brief anesthesia (1.5% isoflurane for 5 min) was performed in an anesthetic chamber [15]. Animals were then removed, endotracheally intubated, and mechanically ventilated with 1–2% isoflurane in 100% oxygen. Laparotomy was aseptically performed using a previously described method developed as a model of POCID in aged rats [16]. Briefly, the abdominal region was shaved and sterilized. A 3-cm vertical incision was made approximately 0.5 cm below the lower right rib. The viscera were vigorously manipulated by inserting an index finger up to the second knuckle into the opening. Similarly, the incised muscle was also manipulated by inserting about 1 cm of the index finger. Next, approximately 10 cm of the small intestine was exteriorized and vigorously rubbed with the thumb and index finger for 30 s. The laparotomy duration was 20–25 min. Animals in the sham operation group were treated in an identical manner for the same duration, except for the laparotomy.

2.4. Radioimmunoassay

Hippocampal and blood samples were obtained immediately after rats were sacrificed. Ang II content and renin activity in plasma and tissue were measured by radioimmunoassay using commercially available kits (North Institute of Biological Technology, Beijing, China), following the manufacturer’s instructions. Protein concentrations of hippocampal samples were determined using a BCA protein assay kit (Applygen Technologies Inc., Beijing, China).

2.5. qRT-PCR

Transcript levels of AT1 subtypes, AT1A and AT1B, were analyzed by qRT-PCR. Briefly, total RNA was extracted using Eastep Universal RNA Extraction Kit (Promega, Madison, WI, USA). Total RNA (2 μg) was reverse transcribed. The primer sequences used were: AT1A-forward, 5'-CTCAAGCGTTCTCTCCAAAATGAG-3'; reverse, 5'-TAGATCTGAGCCAGGGTGAAAATG-3'; AT1B-forward, 5'-CTTCCTCAGC-3'; β-Actin was used as an internal control with the following forward and reverse primers: 5'-AGAGCTATGAGCTGCC-3' and 5'-AATGAATGATGTACTCCATGATG-3', respectively. Data were analyzed by the 2-ΔΔCt method.
2.6. MWM test

MWM tests were started on day 2 postsurgery (allowing for incision healing after surgery), and performed by investigators blinded to the group conditions, as previously described [15]. Swimming was video tracked (Sunny Instruments Co. Ltd., Beijing, China). Latency, path length, swim speed, time, and distance to first platform crossing, and time spent in the previous platform quadrant were analyzed.

2.7. Measurement of BBB permeability

Sodium fluorescein (NaF, MW 376 Da, Sigma–Aldrich, St. Louis, MO, USA), a marker of paracellular flux, and Evans blue (EB, Sigma), a tracer for transendothelial transport that strongly binds to serum albumin producing a high-molecular weight complex (EBA, MW 68,500 Da), were used according to the protocols of Hawkins et al., with minor modifications [17]. Briefly, a solution of both dyes (2%, 5 mL/kg) was administered intravenously and allowed to circulate for 30 min. Intravascular dyes were removed by transcardial perfusion with isotonic saline. Hippocampal samples were homogenized in 3.0 mL of cold 7.5% trichloroacetic acid (Sigma) and centrifuged at 10,000×g for 10 min. EBA (absorbance, 620 nm) and NaF (excitation, 485 nm; emission, 535 nm) concentrations in supernatants were analyzed using a fluorescent microplate reader. Dyes were expressed as μg/mg of brain tissue against standard curves.

2.8. Western blot

Western blots were performed using the following primary antibodies: anti-CD11b (1:500; Millipore, Billerica, MA, USA); anti-GFAP and anti-phospho-p65 NF-κB (1:1000; CST, Danvers, MA, USA); anti-IL-1β (1:1000; Abcam, San Diego, CA, USA); anti-TNF-α (1:1000; Abcam); and COX-2 (1:1000; Abcam). Fluorescently labeled secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) were used.

Fig. 1. Surgery effects on RAS activity in aged rats. Kinetics of surgery-induced changes in renin activity (A and B) and angiotensin II (C and D) in blood plasma and hippocampal tissues, as well as transcript levels of AT1 subtype A (E) and B (F) in the hippocampus are shown. Values are mean ± SEM, n = 4. # p < 0.05, vs. control.
2.9. Statistical analysis

All data are expressed as mean ± SEM. Values of escape latency in the MWM studies, and blood pressure and heart rate were analyzed using two-way analysis of variance (ANOVA) for repeated measurements. ABG data were analyzed using t-tests. All other data were analyzed using one-way ANOVAs followed by least square difference multiple comparison tests. All statistics were performed using SPSS (version 14; SPSS Inc., Chicago, IL; USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Surgery activates the brain RAS

Compared with controls, plasma renin activity increased during the 72-h period observed, reaching a maximum value 6 h postsurgery (Fig. 1A; $p < 0.05$). Hippocampal renin activity increased within 3–6 h postsurgery, and decreased within 12–24 h before a second peak at 72 h (Fig. 1B; $p < 0.05$). Plasma Ang II levels were not altered following surgery. Conversely, hippocampal Ang II expression increased at 12 h, and reached a peak level at 24 h (Fig. 1C and D; $p < 0.05$). Surgery also caused downregulation of AT1A mRNA expression over time, with a minimum at 72 h (51.6 ± 8.7% control, $p < 0.05$), whereas AT1B increased with a peak at 12 h (249.4 ± 61.7% control) (Fig. 1E and F).

3.2. Low candesartan dose does not significantly affect blood pressure and physiological homeostasis

No significant differences in mean arterial pressure, systolic pressure, diastolic pressure, and heart rate were detected between vehicle- and candesartan-treated rats during the 2-week pretreatment phase (Supplementary Fig. 1). There was no significant difference in any measured variable for ABG values at the end of treatment (Supplementary Fig. 2). These findings indicate that low-dose, chronic, peripheral candesartan administration does not significantly affect systemic blood pressure and acid-base homeostasis.

3.3. Candesartan treatment improves learning and memory outcome after surgery

All rats appeared to swim normally and no differences were observed in swimming speeds among groups (data not shown). Animals in the vehicle + surgery group showed longer escape latencies compared with the vehicle + sham group (Fig. 2A; $p < 0.05$). Rats in the CAND + surgery group took less time to find the platform than those in the vehicle + surgery group ($p < 0.05$). There was no significant difference in latencies between the vehicle + sham and CAND + sham groups. In the probe test, the time spent in the target quadrant by rats in the vehicle + surgery group was much shorter than those in the vehicle + sham group (Fig. 2B). However, the amount of time and distance to achieve their first platform crossing were both longer (Fig. 2C and D), confirming the presence of memory impairments after surgery. All these
changes were significantly alleviated by candesartan pretreatment, indicating that candesartan has a cognition-improving effect.

3.4. Candesartan markedly restores BBB disruption after surgery in aged rats

On postoperative day 1, there was a marked increase in both hippocampal NaF (Fig. 3A; *p < 0.01) and EB (Fig. 3B; #p < 0.05) content in operated rats compared with sham-operated vehicle-treated rats, showing that surgery led to BBB dysfunction. These increases were significantly alleviated by candesartan pretreatment (p < 0.05). On postoperative day 3, significant NaF levels between vehicle + sham and vehicle + surgery groups were still present (p < 0.05), although EB content between the two groups was no longer significant (p > 0.05). These results show that surgery-induced BBB dysfunction lasts for at least 3 days, and candesartan markedly restores BBB disruption.

3.5. Candesartan treatment reduces glial activation and neuroinflammation

To assess the effect of candesartan on glial reactivity in aged rats after surgery, we examined hippocampal expression of CD11b, an indicator of reactive microglia, and GFAP, an astrocyte marker. Marked increases in hippocampal CD11b levels on postoperative days 1 and 7 (Fig. 4A and B; *p < 0.05) were attenuated by candesartan pretreatment (p < 0.01). There was no significant difference in GFAP content in the hippocampus of rats under different experimental conditions on postoperative day 1 (p > 0.05). Nevertheless, GFAP was upregulated on day 7 and also inhibited by candesartan (p < 0.01). In addition, candesartan pretreatment significantly lowered surgery-induced increases in hippocampal protein expression of multiple inflammatory markers on postoperative day 1 (p < 0.05), including phosphorylated NF-κB p65 (representing NF-κB signaling activation), early pro-inflammatory cytokines IL-1β and TNF-α, and inflammation-related enzyme, COX-2.

4. Discussion

The major focus of our study was to determine the role of brain RAS signaling in mediating POCD, and then to explore the therapeutic potential of AT1 inhibition in vivo. Here, we demonstrate that surgical stress induces hippocampal RAS activation and cognitive impairment in aged rats. Chronic administration of candesartan, an AT1 antagonist, at a non-hypotensive dose before surgery, confers significant protection from surgery-induced spatial learning and memory deficits. Moreover, candesartan provides significant protection from increased hippocampal BBB permeability, accompanied by marked reductions in hippocampal NF-κB activation, inflammatory molecules, and glial reactivity.

To the best of our knowledge, this study is the first to examine the role of Ang II in POCD in aged rats. A previous randomized, placebo-controlled, prospective study showed that candesartan does not affect postoperative cognition in elderly patients recovering from cardiac surgery [18]. Nevertheless, it is worth noting that...
there were no differences in relevant cognitive function parameters after surgery compared with the preoperative status, potentially the main reason for no beneficial effect of candesartan. To address this issue, we used a well-established animal POCD model [16] to examine the protective effects of candesartan. To date, there is not yet a definite animal POCD model and the most widely used surgical models are abdominal operations, including exploratory laparotomy [16,19], partial hepatectomy [20], splenectomy [4], and nephrectomy [21]. The spleen is essential for proper immune responses, and the liver and kidney have been shown to be involved in metabolic regulation of the AD-related peptide, amyloid-β [22], therefore we performed laparotomies, a necessary part of most abdominal operations in humans.

Ang II is a stress hormone and the most important effector peptide of the RAS. Consistent with the response of the brain RAS to brain trauma [10], following laparotomy we observed Ang II upregulation within 12–24 h, increased AT1B gene expression at 12 h, and a two-phase change in hippocampal renin activity, indicating activation of the brain RAS after surgery. In accordance with this, surgery also caused increased inflammatory factor production in the hippocampus on postoperative day 1. These changes occurred independently of circulating Ang II levels, which remained relatively stable after surgery, highlighting the major role of the local cerebrovascular Ang II system.

Neuroinflammation characterized by increases in cytokines and glial reactivity, is thought to modulate POCD progression. Here, we observed a significant increase in hippocampal levels of CD11b, but not GFAP, on postoperative day 1. Interestingly, by day 7, both were increased. One explanation for this pattern of change may be that microglia cell priming mainly occurs in the initial phase

Fig. 4. Candesartan pretreatment reduces surgery-induced upregulation of glial reactivity and protein expression of inflammatory markers in the hippocampus of aged rats. Representative western blotting images and statistical analysis of CD11b, GFAP (A and B), phosphorylated NF-κB p65, IL-1β (C), TNF-α, and COX-2 (D) are shown. Values are mean ± SEM, n = 4. *p < 0.05, vs. vehicle + sham; #p < 0.05, vs. vehicle + surgery.

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whereas astrocyte activation may persist into the chronic periods after surgery [27]. Furthermore, the aging process may serve as a “priming” stimulus for microglia [24], and upon secondary stimulation with surgical trauma, these primed glial cell release excessive quantities of proinflammatory cytokines, yielding postoperative behavioral changes. Importantly, candesartan not only effectively modulates glial cell activation, but also prevents acute cytokine increases on day 1. Subsequent functional improvement in MWM performance is concomitant with this anti-inflammato-
ry action.

Previous studies by our laboratory and others have suggested that the NF-κB pathway is involved in neuroinflammation and cognitive dysfunction in aged animals [3,15,25]. Moreover, in normo-
tensive rats, systemic candesartan administration reduces lipopolysaccharide-induced activation of transcription factors regulating expression of multiple inflammatory genes, including acti-
vating protein-1 and NF-κB [26]. In agreement with this, we observed a marked increase in phosphorylation of p65, a critical NF-κB component, on postoperative day 1 that was mitigated by candesartan. As NF-κB downstream target genes (IL-1p, TNF-α, and COX-2) also showed corresponding changes, it suggests candesartan might preserve cognitive function by inhibiting surgery-
induced neuroinflammation via NF-κB signaling.

Cerebral endothelial cells forming the BBB provide an appropriate environment to allow proper neural function. Recently, BBB disruption has been identified in POC(3,4,25). In the present study, increased paracellular and transcellular BBB permeabilities were accompanied by cognitive deficits after surgery in aged rats. Furi-
themore, candesartan treatment attenuated augmented BBB permea-
ibilities and cognitive impairment. Our results are consistent with previous studies, in which RAS inhibition attenuates cognitive impairment by reducing BBB permeability in both diabetic [27] and hypertensive rats [8]. Although the precise mechanisms responsible for the surgery-induced increase in BBB permeability are not clear, one possible mechanism is that surgery inappropriately increases hippocampal Ang II thereby directly modulating BBB permeability via activation of AT1 highly expressed in cerebrovascular endothelial cells [28]. Further research on this issue may be warranted.

Altogether, our study indicates that surgery-induced cognitive decline in aged rats is accompanied by a homeostatic dysfunction in brain RAS activity. Chronic treatment with low candesartan doses may elicit blood pressure-independent protective effects on cognitive impairment by restoring BBB disruption and reducing neuroinflammation. The ready availability of clinical therapy with RAS inhibitors in inflammatory conditions makes AT1 a promising therapeutic target to prevent surgery-induced cognitive decline.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.04.153.

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