Neuropsychopharmacology (2012) 37, 2817–2829

www.neuropsychopharmacology.org

Candesartan, an Angiotensin II AT_1-Receptor Blocker and PPAR-γ Agonist, Reduces Lesion Volume and Improves Motor and Memory Function After Traumatic Brain Injury in Mice

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Traumatic brain injury (TBI) results in complex pathological reactions, the initial lesion worsened by secondary inflammation and edema. Angiotensin II (Ang II) is produced in the brain and Ang II receptor type 1 (AT_1R) overstimulation produces vasoconstriction and inflammation. Ang II receptor blockers (ARBs) are neuroprotective in models of stroke but little is known of their effect when administered in TBI models. We therefore performed controlled cortical impact (CCI) injury on mice to investigate whether the ARB candesartan would mitigate any effects of TBI. We administered candesartan or vehicle to mice 5 h before CCI injury. Candesartan treatment reduced the lesion volume after CCI injury by approximately 50%, decreased the number of dying neurons, lessened the number of activated microglial cells, protected cerebral blood flow (CBF), and reduced the expression of the cytokine TGFβ1 while increasing expression of TGFβ3. Candesartan-treated mice also showed better motor skills on the rotarod 3 days after injury, and improved performance in the Morris water maze 4 weeks after injury. These results indicate that candesartan is neuroprotective, reducing neuronal injury, decreasing lesion volume and microglial activation, protecting CBF and improving functional behavior in a mouse model of TBI. Co-treatment with a peroxisome proliferator-activated receptor-gamma (PPARγ) antagonist significantly reduced some of the beneficial effects of candesartan after CCI, suggesting that PPARγ activation may contribute to part or to all of the neuroprotective effect of candesartan. Overall, our data suggest that ARBs with dual AT_1R-blocking and PPARγ activation properties may have therapeutic value in treating TBI.
Neuropsychopharmacology (2012) **37,** 2817–2829; doi:10.1038/npp.2012.152; published online 15 August 2012

Keywords: traumatic brain injury; candesartan; TGFβ; inflammation; neuroprotection; PPARγ

INTRODUCTION

Traumatic brain injury (TBI) results in complex pathophysiological reactions. The initial direct injury is considerably worsened by secondary cascades that activate many different signaling pathways. These cascades result in blood–brain barrier dysfunction, edema formation, an enhanced inflammatory response, increased cell death, gliosis, and cerebral cavity formation (O'Connor *et al*, 2011). TBI also often results in motor and learning impairment leading to long-term disability (Hamm, 2001; O'Connor *et al*, 2011; Santos *et al*, 2005). These secondary cascades, which occur minutes to days following injury, provide a therapeutic window to intervene, to prevent, or reduce the extent of the secondary damage. As a result of the multiple mechanisms initiated by brain injury that lead to neuronal dysfunction, it would seem that a drug that had multimodal action to combat the harmful secondary cascades would be advantageous to treat recovery from TBI. Drugs that block the angiotensin II (Ang II) receptors (Ang II receptor blockers, ARBs) are neuroprotective, anti-inflammatory, and vasodilatory attacking three potentially devastating sequelae of TBI (Benigni *et al*, 2010). In addition, ARBs are already widely used FDA-approved drugs with limited side effects that have significant benefit in animal models of stroke (Thone-Reineke *et al*, 2006). However, little is known of the involvement of Ang II in the response to TBI, or the ability of ARBs to have a beneficial therapeutic effect. The probable beneficial properties of

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Received 27 February 2012; revised 18 July 2012; accepted 20 July 2012

ARBs together with their known efficacy in treating stroke made a strong case for investigating their potential for treating TBI.

Ang II is a multifunctional effector hormone that contributes to the regulation of blood pressure, vascular tone, and fluid volume (Davisson *et al*, 2000). Ang II is synthesized from the precursor angiotensinogen, in most tissues including the brain (Paul *et al*, 2006; Saavedra, 1992). In the brain, Ang II is involved in the regulation of cerebral blood flow (CBF), the autonomic and hormone systems, and stress response (Benicky *et al*, 2011; Saavedra *et al*, 2011). Ang II type 1 receptors (AT_1R) are responsible for most of the well-characterized peripheral and central actions of Ang II (Benicky *et al*, 2011). However, excessive AT_1R stimulation leads to inflammation, increasing oxidative stress, and endothelial dysfunction both in the periphery (Savoia and Schiffrin, 2007) and in the brain (Ozacmak *et al*, 2007). In the brain, excessive AT_1R stimulation has been linked to cerebrovascular remodeling and inflammation leading to neuronal injury and vulnerability (Ando *et al*, 2004a; Nishimura *et al*, 2000; Yamakawa *et al*, 2003; Zhou *et al*, 2005). In addition, reduced Ang II formation offers protection from stroke, because the core lesion area after middle cerebral artery occlusion is significantly reduced in angiotensinogen-knockout mice (Maeda *et al*, 2009).

The neuroprotective effect of ARBs has been demonstrated in animal models and clinical studies. Peripherally administered ARBs cross the blood – brain barrier and have direct CNS effects (Nishimura *et al*, 2000), reducing both cerebrovascular remodeling and inflammation (Ando *et al*, 2004b; Yamakawa *et al*, 2003; Zhou *et al*, 2005). ARBs ameliorate stroke by protecting the cerebrovascular flow (Engelhorn *et al*, 2004; Ito *et al*, 2001; Nishimura *et al*, 2000); lessen the cognitive impairment seen after whole brain irradiation in rats (Robbins *et al*, 2010) and reduce brain inflammation protecting the rats from behavior associated with sickness and depression (Benicky *et al*, 2011).

Several large clinical studies have demonstrated that ARBs prevent the cognitive dysfunction produced by stroke in patients with and without hypertension (Fogari and Zoppi, 2004; Hansson *et al*, 1999; Igase *et al*, 2012; Meredith *et al*, 2004; Poon, 2008; Van Mieghem *et al*, 2010; Zanchetti and Elmfeldt, 2006). Furthermore, ARBs reduce the incidence and progression of Alzheimer's disease and dementia in hypertensive patients more effectively than other antihypertensive medications (Davies *et al*, 2011).

We hypothesized that treatment with candesartan may improve the behavioral and neurological outcome after TBI, in a manner similar to that observed in rodent models and in humans affected by stroke (Awad, 2011; Guan *et al*, 2011; Ito *et al*, 2001; Liu *et al*, 2008; Stenman and Edvinsson, 2004). We therefore investigated the effect of administering candesartan to mice just before they received a controlled cortical impact (CCI) injury to determine whether short- or long-term inhibition of central AT_1Rs would reduce the cortical and hippocampal damage and hence protect against cognitive impairment. While we were completing these data, a report was published demonstrating that administration of low doses of candesartan to mice within 4 h of TBI prevented secondary brain damage and reduced cerebral inflammation at 24 h post-injury (Timaru-Kast *et al*, 2012). Our studies confirm and extend these observations. We show that administration of candesartan can reduce cell death, inflammation and lesion volume, reduce TGFβ1 expression post-injury, and improve functional outcome up to 4 weeks after injury.

Some ARBs, including candesartan, are peroxisome proliferator-activated receptor-gamma (PPARγ) agonists (An *et al*, 2010; Benson *et al*, 2004; Erbe *et al*, 2006). PPARγ is a nuclear hormone receptor whose activation leads to beneficial effects in the regulation of multiple pathways. PPARγ activation mitigates some of the major factors influencing TBI outcome, such as excessive inflammation, reduction of oxidative stress, and inhibition of apoptosis in various tissues, including the brain (Gillespie *et al*, 2011; Ricote *et al*, 1998; Rotman and Wahli, 2010). PPARγ agonists have been investigated as potential therapeutics for treating TBI (Gillespie *et al*, 2011). We asked whether the beneficial effects of candesartan was a consequence of both AT_1R blockade and PPARγ agonist activity. We show that candesartan's actions may indeed be mediated by the dual activities of AT_1R blockade and PPARγ activation. Therefore, our studies show that candesartan exerts broad and long-term beneficial effects, and thus may be a potentially valuable therapeutic for treating TBI.

MATERIALS AND METHODS

Animals and CCI Injury

All animal studies were approved by the USUHS Institutional Animal Care and Use Committee and were conducted in accordance with the NRC guide to the Care and Use of Laboratory Animals. Nine-week-old male C57BL/6 mice (NCI, MD), weighing 22–28 g, were kept under 12 : 12 light and dark cycle with access to food and water *ad libitum*. Typically, surgery was done after 1 week of recovery from transportation-related stress. Mice were anaesthetized with isoflurane (3% induction : 1.5% maintained) and placed in a stereotaxic frame. Body temperature was kept constant using an isothermal heating pad (Stoelting, IL) throughout surgery. The skull was fixed in a stereotactic frame and a craniotomy was performed above the left parietal cortex. We performed moderate CCI injury (coordinates; 2 mm lateral, −2 mm posterior to Bregma) at an impact depth of 2 mm, with a 2 mm diameter round impact tip (speed 3.6 m/s, dwell time 100 ms) and 12° angle of dura mater, using an electromagnetically driven CCI injury device (Impact One stereotaxic impactor CCI, Leica Microsystems Gmbh, Wetzlar, Germany). The bone flap was replaced but not sealed, the skin was sutured, and the mice were allowed to recover fully from anesthesia before transfer to their cages. Sham-injured animals received the same craniotomy without the impact injury.

Drug Treatment

Osmotic minipumps were employed to dispense candesartan (1 mg/kg/day) continuously until killing at 3 or 28 days post-injury (dpi). Minipumps (ALZET, Cupertino, CA: model 1007; delivering 0.5 μl/h for 7 days; or model 1004; delivering 0.11 μl/h for 28 days) were filled with candesartan

(CV-11974, Astra-Zeneca, Sweden) dissolved in 0.1 N Na_2CO_3, pH = 7.4 (2 mg/ml for model 1007; 9.2 mg/ml for model 1004), or vehicle (0.1% saline and 0.1 N Na_2CO_3 at pH = 7.4) the day before implantation and primed at 37 °C overnight. At 5 h before CCI injury, animals were anesthetized with isoflurane and the loaded minipumps were implanted at the back of the neck of each mouse so that the drug was delivered subcutaneously. For some experiments (presented in Figures 3 and 6) mice were administered drug or vehicle by i.p. injections with either candesartan (1 mg/kg/day), vehicle, and/or the PPARγ antagonist, (T0070907, 1.5 mg/kg, Sigma-Aldrich). At the time of killing (either 1, 3 or 28 dpi), mice were anesthetized with ketamine/xylazine and killed by decapitation.

Determination of CBF and Blood Pressure

Regional CBF (rCBF) was measured around the cortical impact area using a laser-Doppler flowmeter (PeriFlux System 5000 LDPM, Perimed). Changes in rCBF were measured in the impact area using a flexible fiber optic extension to the LDPM probe tip 404 as described previously (Villapol *et al*, 2011). Changes in rCBF were expressed as the percentage of the baseline value recorded before CCI injury. Animals were anesthetized with isoflurane for 1–2 min while rCBF was measured. The rCBF values were taken at basal levels before, 2 min, 2 h, and 18 h after cortical impact. Tail blood pressure was obtained using the CODA mouse tail-cuff system, an indirect blood pressure method that utilizes volume pressure recording sensor, coupled to a PC-based data acquisition system (Kent Scientific, CT). A minimum of 10 measurements were taken from each mouse.

Behavioral Testing

To examine the effects of the candesartan on neurological outcome after CCI injury, two investigators who were blinded to the treatment status of the mice performed behavioral tests. A rotarod test was used to evaluate motor coordination. Mice were trained on the rotarod (Ugo Basile, Collegeville, PA, USA) for 2 days before injury, and tested at specific time points after injury. Each day, mice performed at least two trials, separated by a 15-min rest period. Mice were allowed to stand for 15 s on the rod before the rotarod started to rotate with speed linearly increasing from 4 to 40 r.p.m. in 2 min. The average latency before animals fell off the rod was recorded.

Morris Water Maze Test Used to Evaluate Spatial Learning and Memory Talks

The Morris water maze (MWM) was performed as previously described (Hamm, 2001) from 24 to 28 dpi. The 4 ft diameter tank (Stoelting Morris) was filled with tap water (23–25 °C) to a depth of 25 cm and a clear plastic platform (4 × 12 inch) was placed in the northwest quadrant of the tank 1 cm beneath the surface of the water. Highly visible black cues were placed on the walls of the room. Initial trial: on day 1 (24 dpi), each mouse was placed on the platform in the tank and kept there for 15 s. Follow-up trials: on days 2–5, each mouse underwent four trials, each separated by 2 min. Mice were randomly placed into one of the four quadrants and allowed 60 s to swim to the platform. If a mouse did not reach the platform in 60 s, they were gently guided to the platform by the investigator and allowed to remain there for 15 s. A video tracking system recorded all parameters including swim speed and the latency to find the platform. Final probe trial: on day 5 (28 dpi), 1 h after the last training trial, a probe trial was performed with mice placed in the tank without the platform. The time spent in the quadrant where the platform had been located was recorded and compared with time spent in the other three quadrants. All data were analyzed with ANY-maze 4.50 software (Stoelting, Wood Dale, IL).

Tissue Preparation and Histology

Animals were randomized into two groups; one group was used for histology and binding analyses and the second group for qPCR. For histology and binding analyses, mice were killed at 3 dpi, brains were dissected, fresh frozen in cold isopentane on dry ice, and stored at − 80 °C. Fresh frozen brains were cut to 16 μm thick coronal sections on a cryostat, mounted on gelatin-coated glass slides and stored at − 80 °C until use. To quantify AT_1R mRNA expression with qPCR, mice were killed at 1 dpi, brains dissected, and sliced into 300 μm thick sections with a cryostat. Punches were taken from the pericontusional cortex (5 punches per section) using a 1 mm microdissection punch (Harris Uni-core needles, Electron Microscopy Sciences, Hatfield, PA). To measure mRNA levels of PPARγ brains were dissected at 3 dpi and a single punch was taken from the cortical lesion region using a 5 mm punch cannula (Zivic Instruments, Pittsburgh, PA).

Cell Death Assay

Sections were processed for DNA strand breaks (TUNEL assay, labeling of fragmented DNA) using the fluorescence *In Situ* Cell Death Detection Kit (Roche, IL), according to the manufacturer's instructions. TUNEL-positive nuclei were counted in cortical and hippocampal regions in 3 to 5 coronal sections for each animal, with five animals per group.

RNA Isolation and qPCR of AT_1R and PPARγ

The extracted punches were immediately placed in TRIzol reagent (Invitrogen, CA) and homogenized by trituration with a pipette, then stored at − 80 °C until use. RNA was isolated by adding 0.2 volumes of chloroform, centrifuging at 16 000 *g* for 15 min, and extracting the aqueous layer. RNA was further purified using the RNeasy Lipid Tissue Mini kit (Qiagen, MD) and treated with RNase-Free DNase according to the manufacturer's instructions. RNA was quantified and RNA integrity checked by agarose gel electrophoresis. qPCR was performed using SYBR Green qPCR MasterMix (Qiagen) with primers specific for murine AT_{1A} type receptor (forward (5′–3′): AGCCTGCGTCTTGTT TTGAG, reverse (5′–3′): GCTGCCCTGGCTTCTGTC), AT_1 total receptor (forward (5′–3′): TGTTCCTGCTGCTCACGT GTCTC, reverse (5′–3′): CATCAGCCAGATGATGATGC), and PPARγ (forward (5′–3′): CACAATGCCATCAGGTTTGG, reverse (5′–3′): GTGATTTGTCCGTTGTCTTTCC). The amplification

conditions consisted of denaturation and enzyme activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s (CFX96, Bio-Rad). mRNA expression levels were normalized to the housekeeping gene cyclophilin A (reverse (5′–3′): GCACTGGAGAGAAAGGATTTGG, forward (5′–3′): CCAGTGCCATTATGGCGTGT). Quantification of relative changes in mRNA levels between samples taken from animals after surgery with those from naive mice were calculated using the delta delta threshold cycle (ΔΔCt) method (Susarla *et al*, 2011).

Immunohistochemistry

Fresh frozen 16 μm thick sections were post-fixed in 4% paraformaldehyde for 1 h. Sections were blocked with 10% normal goat serum in PBS and 0.1% Triton X-100 (TX) for 1 h. The following primary antibodies were incubated at 4 °C overnight anti-GFAP, mouse monoclonal (1 : 200, Millipore, CA); anti-NeuN, mouse monoclonal (1 : 200, Chemicon, Temecula, CA) for mature neurons; and anti-Iba-1, rabbit polyclonal (1 : 100, Wako,VA) for microglia; anti-TGFβ1 rabbit polyclonal (IDFR-B, 1 : 1000, kind gift of Dr Flanders, NIH) and anti-TGFβ3 (1 : 1000, sc-83, Santa Cruz Biotechnology, CA). This novel TGFβ1 antiserum does not stain sections taken from TGFβ1 knockout mice (manuscript in preparation). Sections were washed in PBS and 0.1% Triton X-100 (PBS-T) three times and incubated with the corresponding Alexa Fluor 568-conjugated IgG secondary antibodies (all 1 : 1000; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. Sections were rinsed with PBS and distilled water and coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen, Chicago, IL). Images were acquired on an Olympus BX61 with attached qImaging Retiga EXi Aqua CCD camera, and iVision software (BioVision Technologies, Exton, PA).

Ang II Receptor Autoradiography

For receptor autoradiography, brains were removed from mice that were either treated or not treated with candesartan for 3 days, snap frozen, and 16 μm thick sections were cut in a cryostat at −15 °C. Sections were incubated with [^{125}I]-sarcosine1Sar1-ANG II ([^{125I}]-Sar^{1}-ANG II; ARC, St Louis, MO) as described previously (Tsutsumi and Saavedra, 1991). Optical densities of autoradiograms were analyzed by computerized densitometry using Scion Image 4.0.2 (Scion, Frederick, MD) based on the NIH Image program.

Quantification and Image Analysis

The lesion area was determined in every 16th section throughout the entire lesion (a total of 9 sections at 256 μm intervals). The area of each of the corresponding ipsilateral hemispheres was similarly determined. Lesion volume was obtained by multiplication of the sum of the lesion areas by the distance between sections. Percent lesion volume was calculated by dividing the lesion volume by the total ipsilateral hemisphere volume (similarly obtained by multiplying the sum of the areas of the ipsilateral hemispheres by the distance between sections). To obtain cell counts of specific labeled cells, each labeled cell was counted with the ×40 objective in five fields per section, at least three sections per animal, $n = 5$–12. Quantitative image analysis of the immunoreactive areas for TGFβ1, TGFβ3, and Iba-1 were performed on 15 cortical and hippocampal sections through the level of impact site (AP −2.0 mm) taken with the ×20 objective and using the same densitometric analysis method as described previously (Villapol *et al*, 2011). Immunofluorescence intensity was calculated using the threshold method and defined as the number of pixels, divided by the total area (mm^2) in the imaged field with the average background subtracted. All images were captured and analyzed using iVision.

Statistical Analysis

All data in this study are expressed as mean ± SEM, except for lesion volume presented as mean ± SD. $^{*}p < 0.05$ or less was considered statistically significant. Intergroup differences were evaluated by one-way ANOVA followed by the Newman–Keuls Multiple Comparison test. CBF and blood pressure data were analyzed by two-way ANOVA with Bonferroni post-tests. All statistics were performed with Prism 5.03 software (GraphPad Software, San Diego, CA).

RESULTS

Candesartan Treatment Reduces Lesion Volume and Cell Death after CCI

We implanted an osmotic minipump dispensing candesartan (1 mg/kg/day) starting 5 h before the injury and continuously until the time of killing (Figure 1a). Examination of cresyl-violet stained sections of brains taken from either candesartan- or vehicle-treated mice showed that candesartan reduced the mean lesion volume at 3 and 28 dpi. At 3 dpi, mice treated with candesartan had a 43% decrease in lesion volume compared with vehicle-treated mice ($^{***}p < 0.005$, $n = 7$–8) (Figure 1b). This difference was maintained at 28 dpi, where treatment with candesartan reduced the lesion cavity by approximately 31% ($^{*}p < 0.05$, $n = 11$–12) (Figure 1b). To determine whether the beneficial effect of candesartan was due in part to a reduction in cell death, we performed a TUNEL assay on sections taken from candesartan- or vehicle-treated mice at 3 dpi. Candesartan-treated injured mice (CCI-CD) had a significantly lower number of TUNEL-positive cells compared with vehicle-treated injured (CCI-VH) in the cortex ($^{*}p < 0.05$), (Figures 2a–c). Double staining with TUNEL and NeuN showed that approximately 80% of TUNEL-positive cells corresponded with neurons (data not shown), indicating that candesartan prevented significant neuronal cell death in the perilesional area. Furthermore, there were significantly fewer TUNEL-positive cells in the CA1, CA3, and dentate gyrus regions of the ipsilateral hippocampus in candesartan-treated mice after CCI (Figures 2d-f). Thus, candesartan reduced the amount of cell death and specifically neuronal cell death after CCI. To ensure that candesartan was acting as an antagonist at AT$_1$R in the brain, we analyzed radiolabeled ligand binding to the AT$_1$R. There was an approximately 45% reduction in binding to the AT$_1$R in both the

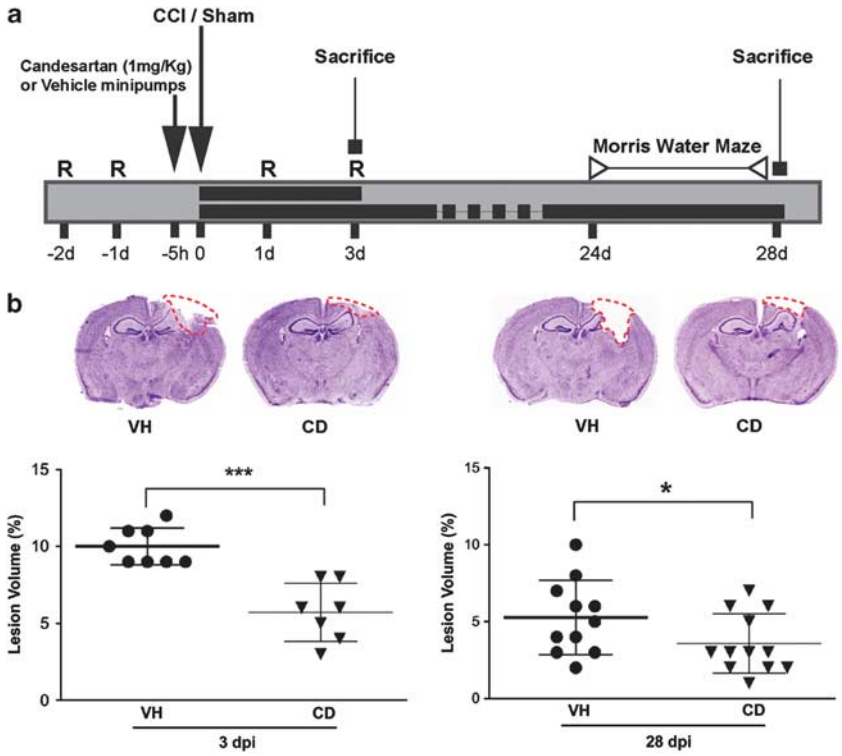


Figure 1 Neuroprotective effect of candesartan treatment on lesion volume following CCI injury in mice. (a) Experimental design. Osmotic pumps containing candesartan (CD, 1 mg/kg/day) or vehicle (VH) were implanted subcutaneously 5 h before craniotomy (sham) or CCI injury. Mice were pre-trained in the rotarod task (R), at 1 and 2 days before CCI and tested at 1 and 3 days post-injury (dpi). MWM testing began at 24 dpi, and continued daily until 28 dpi, when the mice were killed. (b) Representative sections of injured brains at 3 dpi stained with cresyl-violet (top). The dotted line indicates the lesion area composed of the cavity and edematous area. Candesartan treatment significantly reduced the mean lesion volume by 43% compared with vehicle-treated mice at 3 dpi (mean ± SD $n = 7$–8, ***$p < 0.001$) and by 31% at 28 dpi (mean ± SD, $n = 11$–12, *$p < 0.05$).

paraventricular nucleus (PVN) and the subfornical organ (SFO), studied in sections taken from mice treated with candesartan for 3 days, in comparison with binding to sections taken from mice treated with vehicle only (Supplementary Figure 2). To determine whether expression or function of the AT_1R was altered after injury, we examined the expression of AT_1R mRNA together with ligand-specific binding in different brain regions after TBI. In rodents, there are two isoforms of AT_1Rs; AT_{1a} and AT_{1b} receptors (Burson *et al*, 1994; Johren and Saavedra, 1996). qPCR analysis indicated no change in expression in either total AT_1R ($AT_{1a} + AT_{1b}$) mRNA or $AT_{1a}R$-specific mRNA at 1 dpi, in comparison with expression in sham mice (Supplementary Figure 3). Autoradiography also showed no alteration in ligand-specific binding to the PVN or SFO after injury. AT_1R density in the cortex was not sufficient to detect by autoradiography. Taken together, our data show no gross difference in expression of the AT_1R after CCI injury.

Candesartan Treatment Reduces Microglial Activation

To determine whether candesartan altered the activation or survival of glial cells, we examined staining for glial-specific markers in sections taken from candesartan- or vehicle-treated mice at 3 dpi. Candesartan treatment significantly decreased the number of Iba-1-positive cells and the amount of Iba-1 immunoreactivity in the perilesional area at 3 dpi (Figures 2g-j) (*$P < 0.05$). Candesartan treatment also led to a change in morphology of microglia, with reduced numbers of microglia showing hypertrophy or ameboid morphology (Figures 2g and h with inset g′ and h, respectively). Candesartan treatment did not alter the number of GFAP-positive astrocytes, or mature oligodendrocytes (APC-positive cells) in the perilesional area at 3 dpi (data not shown). Thus, the glial-specific effects of candesartan are restricted to reducing microglial activation.

Blockade of AT_1 Receptors Increases CBF but Does not Significantly Affect Blood Pressure after CCI Injury

Laser-Doppler analysis showed that CBF decreased immediately after CCI to approximately 50% of the pre-injury level in both candesartan-treated and -untreated mice (Figure 3a). At 2 and 18 h after CCI, CBF recovered more quickly in candesartan-treated mice than in mice treated with vehicle. Indeed at 18 h after injury in mice treated with candesartan, CBF had rebounded to just above pre-injury levels (Figure 3a). To determine whether the dose of candesartan used in these experiments altered blood pressure, we recorded blood pressure using the indirect tail-cuff system to obtain baseline levels before injury and at 2 and 18 h post-injury. In vehicle- and candesartan-treated

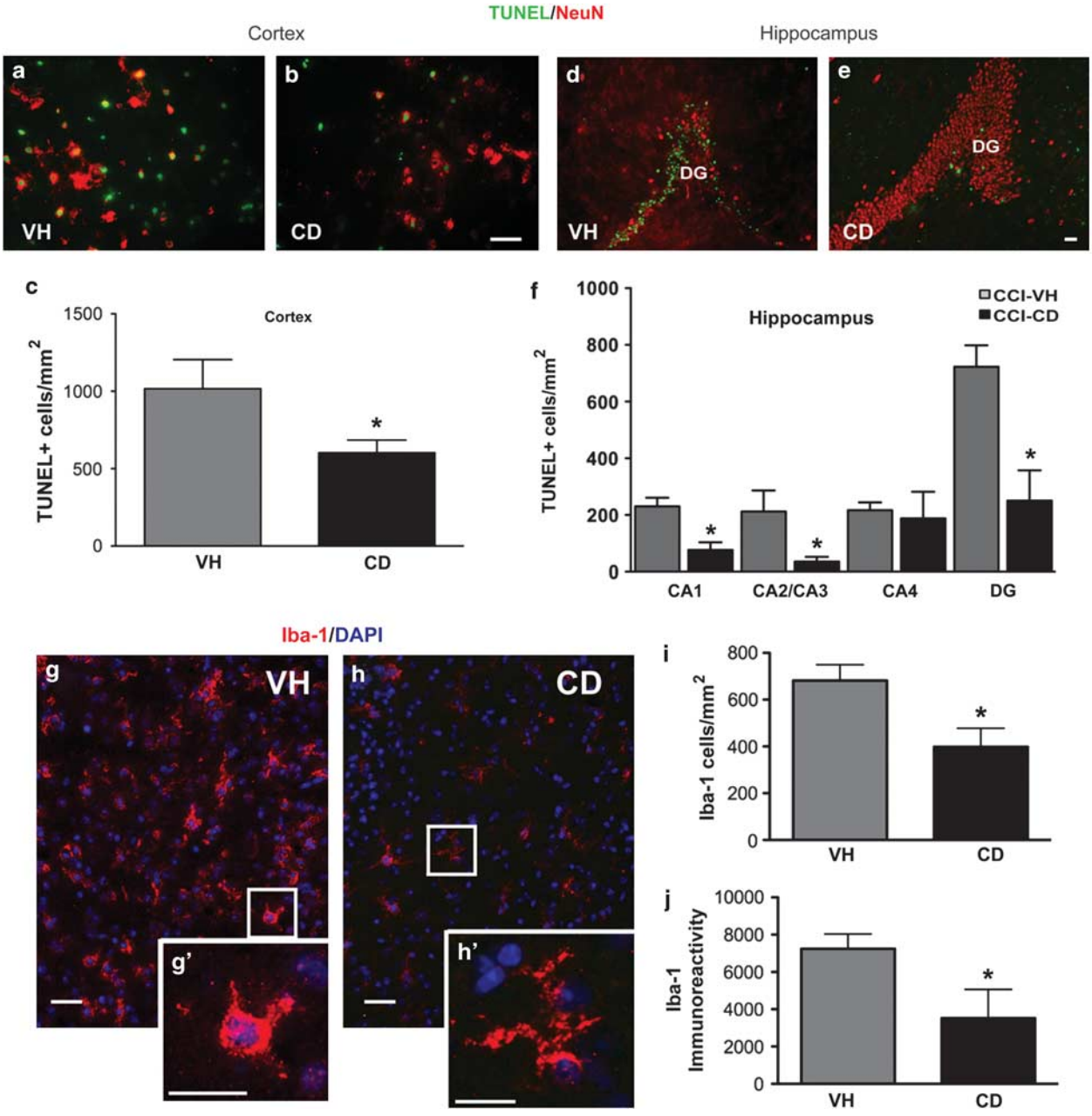
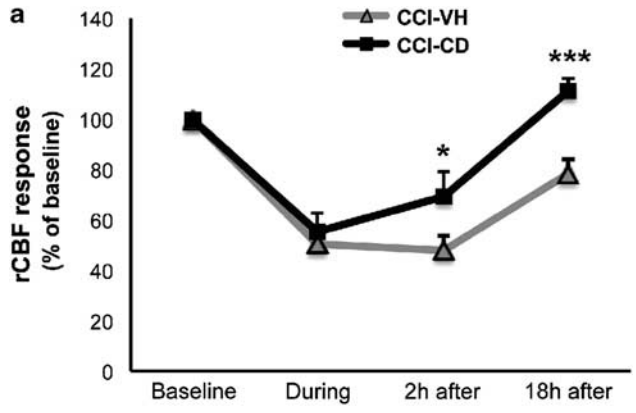


Figure 2 Candesartan treatment reduces the number of dying cells and activated microglial cells after CCI injury. Sections of brains from cortex (a, b) or hippocampus (d, e) taken from mice treated with candesartan (CD) or vehicle (VH) and killed at 3 dpi, showing dying cells, labeled by TUNEL (green) and the neuronal marker NeuN (red) (c, f). Graphs show the number of TUNEL-positive cells at 3 dpi was significantly reduced after injury in CD-treated mice (CCI-CD) compared with those treated with VH (CCI-VH) in the cortex and in the CA1, CA2/3, and dentate gyrus (DG) of the hippocampus (mean ± SEM, $n = 4$, $^*p < 0.05$). (g, h) At 3 dpi, increased numbers of Iba-1-positive microglia (red) were found in brain sections taken from VH-treated mice in comparison with CD-treated mice. In the VH-treated mice, the microglia had the ameboid and hypertrophy morphology characteristic of activated microglia (g′), in comparison with the more ramified morphology in CD-treated animals (h′). (i, j) Quantitative analysis of Iba-1-positive cells in injured cortex showed a reduction after CD treatment in the number of Iba-1-positive cells and in the fluorescence intensity of Iba-1 staining in the perilesional area (mean ± SEM, $n = 4$, $^*p < 0.05$). Scale bars represent 50 μm (a–e, g, and h) and 25 μm (g′, h′).

mice groups, mean baseline blood pressure (before drug administration) was between 114 and 117 mm Hg. No significant differences in blood pressure were detected between groups after CCI (Figure 3b). We also weighed the mice at 3 and 28 dpi to show that there were no differences between candesartan- or vehicle-treated animals after injury (Supplementary Figure 1).

Effect of Candesartan Treatment Reduces Expression of TGFβ1, but Increases TGFβ3 Expression

As ARBs can reduce the amount of TGFβ expressed in certain tissues (Sun *et al*, 1998), we sought to determine whether candesartan treatment reduced TGFβ expression. TGFβ1 and TGFβ3 expression was upregulated at 3 dpi, with



b

Blood Pressure (mm Hg)

	CCI-VH	CCI-CD
Basal	114±9.1	117.4±7.3
2 h after CCI	101.2±11.7	91.8±4.9
24 h after CCI	105.6±3.7	97.85±5.2

Figure 3 Changes in the CBF and blood pressure after CCI injury in mice treated with vehicle or candesartan. Mice were administered candesartan or vehicle by daily injection for 3 days, starting 5 h before injury. (a) Regional CBF (rCBF) was measured before injury, during, 2 and 18 h after injury, and was expressed as % baseline values (arbitrary units) in the ipsilateral hemisphere. At 2 and 18 h post-injury, candesartan treatment increased CBF values compared with vehicle. (mean ± SEM, $n = 12–17$, $^*p < 0.05$, $^{***}p < 0.001$). (b) Blood pressure (BP) in CCI mice with vehicle (CCI-VH) or candesartan-treated (CCI-CD) measured before, and 2 and 24 h after injury, (mean ± SEM, $n = 6$).

both cytokines colocalized mainly in reactive astrocytes in the ipsilateral hemisphere (data not shown). Candesartan treatment significantly reduced (by 50%) astroglial TGFβ1 expression in the ipsilateral cortex (Figure 4e) and hippocampus, relative to TGFβ1 expression in vehicle-treated mice (Figures 4a-f). Surprisingly, candesartan treatment led to an upregulation of astroglial TGFβ3 expression (approximately 150–200%) in the ipsilateral cortex (Figure 4k) and hippocampus compared with TGFβ3 expression in vehicle-treated mice (Figures 4g-l).

Candesartan Treatment Improves Motor Function, Spatial Learning and Memory Outcome after TBI

To determine whether candesartan treatment benefited the recovery from injury, we subjected mice to behavioral tests to determine the extent of their functional recovery. Mice were pre-trained on the rotarod for 2 days before CCI injury. All groups of mice behaved similarly during training. Candesartan or vehicle was administered by minipump, implanted 5 h before injury, as with the rest of the experiments. After CCI injury, mice showed a reduced ability to perform this test, as judged by their shorter latency to fall. At 1 and 3 dpi, candesartan-treated mice were able to stay on the rotarod for significantly longer time periods relative to vehicle-treated mice suggesting that candesartan treatment enhances motor performance after TBI, and had a similar effect on the 'sham' operated mice, enhancing their ability to stay longer on the rotarod (Figure 5a). We found no effect of candesartan treatment on the performance of naive mice in this test (Figure 5a). To determine the effect of candesartan treatment on cognitive ability at a later time point, we evaluated spatial learning and memory by the MWM test (Figure 5b). Mice were trained for 4 trials per day from days 24 through 28 dpi. On day 28, with the platform removed, candesartan-treated mice after CCI spent significantly more time in the correct quadrant, than vehicle-treated mice after CCI, showing that candesartan treatment led to a greater ability to learn and remember ($n = 7–12$, $^*p < 0.05$). There was no observable difference between candesartan- and vehicle-treated mice after sham injury (Figure 5b). These results suggest that candesartan treatment significantly improved spatial learning and memory 4 weeks after CCI injury.

PPARγ Activation may Contribute to the Neuroprotective Effects of Candesartan Following Brain Injury

We wished to determine whether the PPARγ agonist activity of candesartan was contributing to its efficacy in promoting recovery after CCI. We first examined whether short-term candesartan would elevate PPARγ mRNA expression. No significant differences were observed in PPARγ mRNA expression in the cortex when comparing naive mice to injured mice, nor between those treated with candesartan compared with mice treated with vehicle at 3 dpi (Figure 6a). We then treated mice with candesartan, the PPARγ antagonist, T0070907, or a combination of candesartan with T0070907, injecting once daily, starting 5 h before injury as previously. At 3 dpi, the beneficial effects of candesartan treatment alone were maintained—with reduced lesion volume, decreased number of Iba-1-positive microglial cells, and an enhanced ability to stay on the rotarod as compared with vehicle-treated mice (Figure 6). However, candesartan's protective effects were reduced or eliminated when given together with T0070907. Mice treated with T0070907 alone showed no significant difference from vehicle-treated mice after CCI. Mice treated with T0070907 together with candesartan showed significantly more microglial activation than mice treated with candesartan alone and were not significantly different from vehicle-treated mice (Figure 6c), However, mice co-treated with candesartan and T0070907 developed a lesion volume and showed recovery of motor function that was neither significantly different from vehicle-treated nor candesartan-treated mice (Figures 6b and d). Thus, PPARγ antagonism diminishes or abolishes the beneficial effects of candesartan treatment on recovery from CCI injury.

DISCUSSION

The search for an effective therapy to treat TBI patients has been ongoing for many years. After over 30 failed clinical trials for TBI it is becoming increasingly recognized that the sequelae of events after TBI is so complex that a multifunctional approach is more likely to be effective (Loane and Faden, 2010; Marklund and Hillered, 2011). ARBs are known to have a multifaceted action in the brain. They reduce brain inflammation, protect from stroke, and are

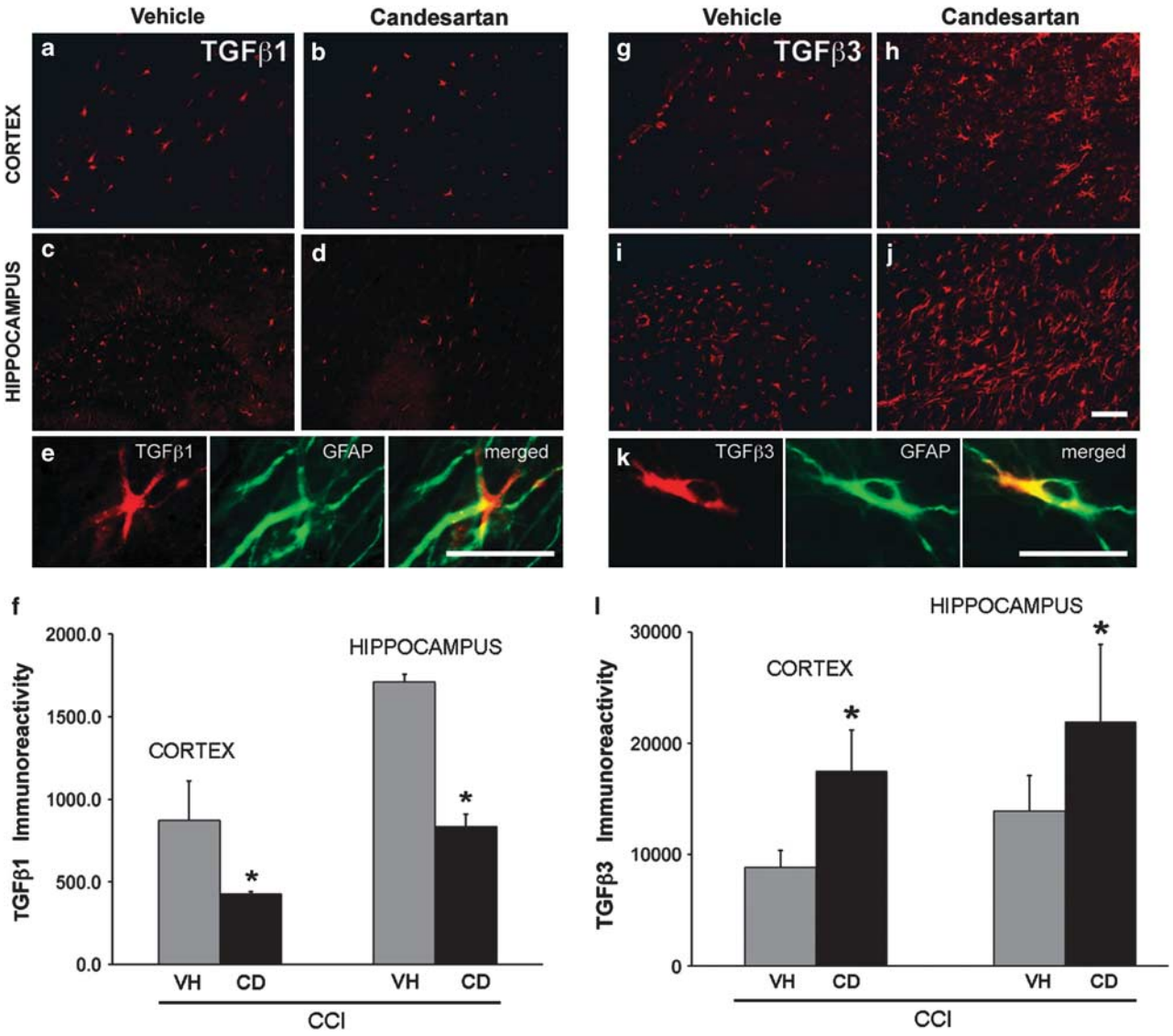


Figure 4 Candesartan treatment modulates TGFβ1 and TGFβ3 expression after CCI. TGFβ1 immunoreactivity (red) was decreased by candesartan treatment in the ipsilateral cortex (b) and hippocampus (d) in comparison with that in vehicle-treated mice (a, c, respectively). (f) Quantitative analysis of TGFβ1 immunoreactivity showed that candesartan treatment led to a reduction of TGFβ1 immunoreactivity of 47% in the cortex and 49% in the hippocampus compared with vehicle-treated groups (mean ± SEM, $n = 4$, $^*p < 0.05$). Conversely, candesartan treatment increased TGFβ3 immunoreactivity in the cortex (h) and hippocampus (j) in comparison with immunoreactivity in vehicle mice (g, i, respectively). (l) Quantitative analysis of TGFβ3 immunoreactivity showed that candesartan treatment led to an increase in TGFβ3 immunoreactivity of 50% in the cortex and 36% in the hippocampus compared with vehicle-treated groups (mean ± SEM, $n = 4$, $^*p < 0.05$). Higher magnification images show TGFβ1 and TGFβ3 colocalization with the astroglial marker, GFAP (e, k, respectively). Scale bars represent 50 μm (a–d and g–j) and 25 μm (e, k).

directly neuroprotective (Benicky *et al*, 2011; Kasahara *et al*, 2010; Rodriguez-Pallares *et al*, 2008). In this study, we show that administration of the ARB candesartan resulted in a significant reduction in lesion volume, neuronal cell death, and activated microglial cells after CCI injury in the mouse. The improved pathology resulted in better motor and cognitive recovery up to 4 weeks after the injury. We also show that some of the beneficial effects of candesartan may be due to PPARγ activation. Thus, the ARBs may have potential therapeutic value for treating TBI because of their dual action on the AT_1R and PPARγ receptors.

TBI initially causes an acute decrease in the CBF, often with periodic episodes of cerebral vasospasm in the days to weeks following injury (Barkhoudarian *et al*, 2011). These alterations in CBF contribute to the detrimental effects on structure and function resultant from TBI. Studies after ischemia indicate that candesartan exerts an important beneficial effect. Blockade of AT_1R in the cerebral vasculature decreases vasoconstriction and this diminishes the CBF reduction provoked by ischemia (Ariza *et al*, 2006; Baranov and Armstead, 2003; Engelhorn *et al*, 2004; Ito *et al*, 2002). The mechanisms of CBF protection by ARBs include increased microcirculation, partially through the formation of new collaterals resulting in an increase in vascular flow (Li *et al*, 2008), increased expression of endothelial nitric oxide synthase in the cerebral vasculature (Yamakawa *et al*, 2003) and direct reduction of cerebral vasoconstriction resulting from AT_1R blockade (Ito *et al*,

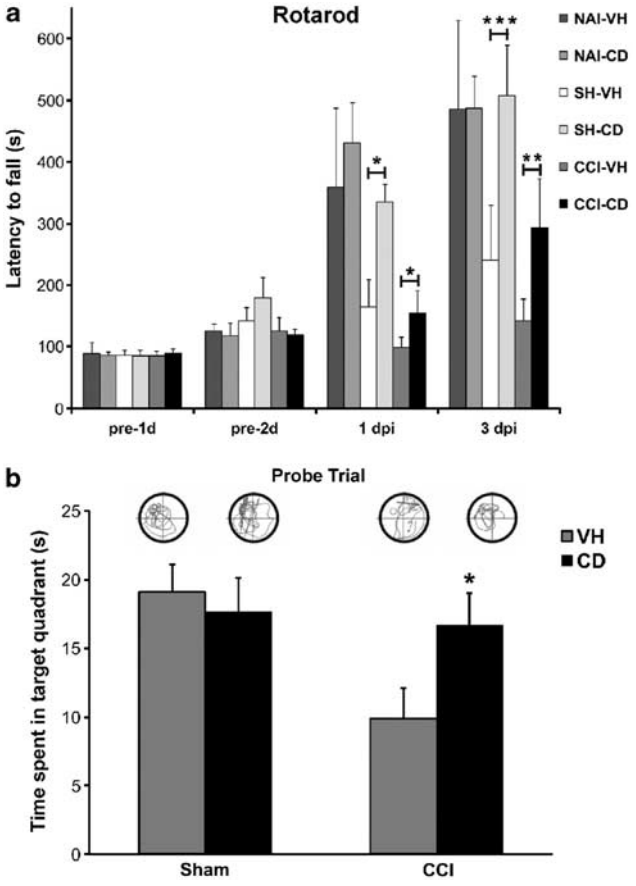


Figure 5 Candesartan treatment improves motor and cognitive function in mice after CCI. (a) Time (seconds) that mice were able to remain on the rotarod in pre-training and at 1 and 3 days post-injury (dpi). Candesartan treatment (CD) enhanced the ability of mice to stay on the rotarod after either sham (SH) surgery or CCI mice compared with vehicle (VH)-treated mice but did not alter the ability of naive (NAI) mice to perform this test (mean ± SEM, $n = 8$–12, ***$p < 0.008$, **$p < 0.005$, *$p < 0.05$) at 1 and 3 dpi. (b) MWM testing showed that after CCI injury mice treated with CD spent more time in the northwest (NW) quadrant from where the platform was removed in the probe trial, compared with mice receiving VH at 28 dpi. Thus, CD treatment led to a greater ability to learn and remember the location of a hidden platform (mean ± SEM, $n = 5$ SH-VH, $n = 3$ SH-CD, $n = 7$ CCI-VH, $n = 8$ CCI-CD, *$p < 0.05$).

2002; Nishimura *et al*, 2000; Zhou *et al*, 2006). When measured 18 h after CCI, we found that in mice treated with candesartan the reduction in CBF returned to pre-injury levels, in comparison with the still depressed CBF in vehicle-treated mice after CCI (Figure 3). Our results concur with other reports showing that candesartan partially restored diminished CBF following fluid percussion brain injury in a neonatal pig model (Baranov and Armstead, 2003). Demonstrated effects of PPARγ on the vasculature seem restricted to a long-term protective effect that may even be mediated via inhibition of the renin – angiotensin system, mediated by AT_1R (Sugawara *et al*, 2011). Thus, the ability of candesartan to protect CBF after TBI demonstrates the important role of cerebrovascular AT_1R blockade (Zhou *et al*, 2006).

TGFβ1 is an important cytokine that is rapidly induced following TBI (Wang *et al*, 2007). It has many functions in the nervous system, which are both beneficial and detrimental to the recovery from TBI including promoting glial scar formation (Chodobski *et al*, 2003; Vivien and Ali, 2006; Wang *et al*, 2007). Ang II can induce the expression or activation of TGFβ1 in many different tissues (Harasawa *et al*, 2010; Jiao *et al*, 2011; Sun *et al*, 1998; Yu *et al*, 2001). Indeed, TGFβ1 mediates Ang II dependent functions in the heart (Rosenkranz, 2004). In the spinal cord, Lanz *et al.* (2010) show that Ang II-dependent neuroinflammation in an experimental autoimmune encephalomyelitis model is mediated through upregulation of TGFβ, and that candesartan ameliorated this neuroinflammation acting through AT_1Rs in astrocytes and microglia. We found that candesartan potently reduces TGFβ1 expression in cortical and hippocampal astrocytes following CCI injury. Therefore, our results support the concept that TGFβ1 acts as a downstream mediator of Ang II in the nervous system. Conversely and somewhat surprisingly, we found that candesartan treatment led to an upregulation of TGFβ3 expression in cortical and hippocampal astrocytes. TGFβ3, despite its similarity to TGFβ1, favors repair processes, and has been used in clinical trials for scar reduction in the skin (Occleston *et al*, 2008). Thus, candesartan differentially regulates two different TGFβ family members through unknown mechanisms. The significance of the candesartan-mediated induction of TGFβ3 levels is not known.

Increased neuronal cell death after TBI has many potential mechanisms including reduced blood flow, cerebral vasoconstriction, blood – brain barrier breakdown, excitotoxicity, oxidative stress, and pro-inflammatory processes (Barkhoudarian *et al*, 2011). Previous studies have demonstrated that candesartan reduces superoxide production and preserves antioxidant capacity after global cerebral ischemia (Sugawara *et al*, 2005) and after intracerebral hemorrhage (Jung *et al*, 2007). As AT_1R expression in the cortex is low, it is probable that the reduction in lesion volume and neuroprotection mediated by candesartan is a result of a more global reduction in inflammation and oxidative stress, mediated by the actions of candesartan on other cell types, including the cerebral vasculature (Zhou *et al*, 2006). Following peripheral administration of bacterial endotoxin, candesartan decreases inflammation throughout the brain preventing microglia activation (Benicky *et al*, 2011). Candesartan also directly reduces markers of inflammation in neuronal, microglial, and cerebrovascular endothelial cell cultures (Benicky *et al*, 2011) and in human circulating monocytes (Larrayoz *et al*, 2009). Indeed, we found that candesartan treatment reduces the activation of microglial cells in the injured cortex after TBI (Figure 2). As microglial expression of AT_1R is very low (data not shown), we postulate that effects beyond AT_1R blockade may be at least partially responsible for the therapeutic effects of candesartan.

One major additional effect of candesartan is PPARγ activation. The nuclear receptor PPARγ is expressed in most if not all cells in the CNS, including astrocytes, oligodendrocytes, microglia, and neurons (Bernardo and Minghetti, 2006). PPARγ activation curtails inflammation through decreasing the expression and release of pro-inflammatory cytokines, and reducing the activation of microglial activation (Gillespie *et al*, 2011). PPARγ agonists have beneficial effects in a variety of animal models including spinal cord

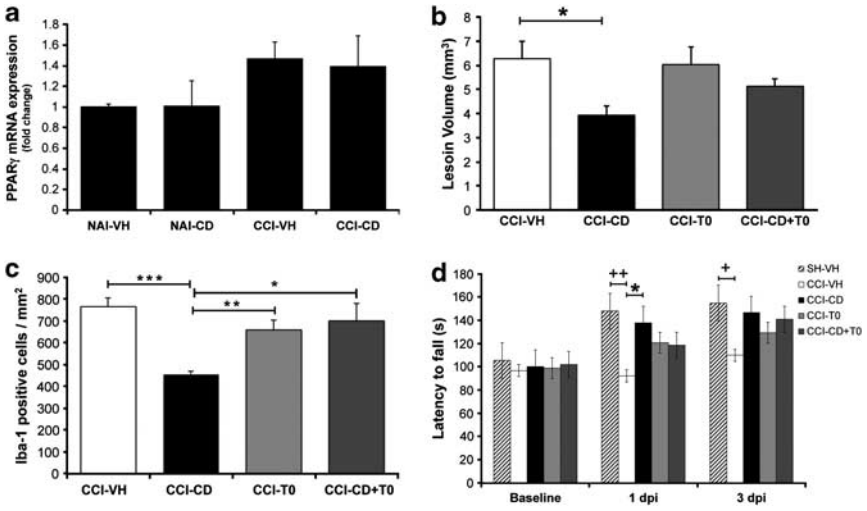


Figure 6 The influence of PPARγ antagonist on the neuroprotective effects of candesartan following brain injury. Mice were administered vehicle, candesartan (CD) and/or the PPARγ antagonist, T0070907 by daily injection for 3 days, starting 5 h before injury. (a) Effects on PPARγ mRNA expression. PPARγ mRNA expression was not significantly altered after injury (CCI) and/or after CD treatment in the perilesional cortex as compared with vehicle-treated naive (NAI) mice at 3 dpi (mean ± SEM, $n = 4$). (b) Effects on lesion volume. At 3 dpi, CD significantly reduced the lesion volume (*$p < 0.05$, CCI-VH *vs* CCI-CD). T0070907 administration alone (CCI-T0) or together with CD (CCI-CD + T0) did not alter the lesion volume compared with the vehicle group (NS, $p > 0.05$), nor was it significantly different than in mice treated with CD alone (NS, $p > 0.05$, CCI-VH *vs* CCI-CD + T0) (mean ± SEM, $n = 7$–8). (c) Effects on Iba-1-positive microglial cells. CD significantly reduced the number of Iba-1-positive cells in the injured cortex (***$p < 0.001$, **$p < 0.005$, CCI-VH *vs* CCI-CD). This effect was abolished by co-treatment with T0070907 (*$p < 0.05$, CCI-CD *vs* CCI-CD + T0). Treatment with T0070907 alone (CCI-T0) did not change the number of Iba-1-positive cells (NS, $p > 0.05$, CCIVH *vs* CCI-T0) (mean ± SEM, $n = 4$–7). (d) Effects on the ability of mice to remain on the rotarod. CCI significantly reduced the ability of mice to remain on the rotarod ($^{++}p < 0.01$, SHVH *vs* CCI-VH). CD treatment alone significantly enhanced the ability of CCI injured mice to remain on the rotarod at 1 dpi (*$p < 0.05$, CCI-VH *vs* CCI-CD). The protective effect of CD at 1 dpi was no longer significant after co-administration of T0070907 (NS, $p > 0.05$, CCI-VH *vs* CCI-CD-T0) (mean ± SEM, $n = 7$; data were analyzed by two-way ANOVA with Bonferroni post-tests). VH, vehicle; CD, candesartan; T0, T0070907; NS, not significant.

injury, stroke and TBI (Gillespie *et al*, 2011; McTigue *et al*, 2007; Zuhayra *et al*, 2011). The anti-inflammatory properties of PPARγ suggest that PPARγ activation may contribute to the anti-inflammatory effects of ARBs (Malchiodi-Albedi *et al*, 2008). Telmisartan is the ARB with the greatest PPARγ agonist activity (Benson *et al*, 2004). However, candesartan can also activate PPARγ-mediated gene expression (Erbe *et al*, 2006; Zorad *et al*, 2006). Indeed, the significant reduction in the protective effects of candesartan by co-administration of a PPARγ antagonist (Figure 6) suggests that PPARγ agonist activity forms a significant if not the major component of the beneficial, anti-inflammatory actions of candesartan. Candesartan did not appear to induce expression of PPARγ mRNA at 3 dpi (Figure 6). Instead, candesartan may bind directly to PPARγ as part of the heterodimer with retinoid X receptor causing dissociation of co-repressor molecules and recruitment of transcriptional coactivators to induce transcription (Erbe *et al*, 2006).

The relative role of AT_1R blockade and PPARγ activation in the therapeutic effects of ARBs is still under investigation. The protection of CBF, decreased vasoconstriction, regulation of TGFβ expression, and direct neuroprotective effects, are probably attributable to blockade of the AT_1R. However, it appears that the contribution of AT_1R blockade and PPARγ activation is dependent on cell type. For example, candesartan protects human circulating monocytes expressing very few AT_1Rs by activating PPARγ (Larrayoz *et al*, 2009). Conversely, the neuroprotective effect of another ARB, telmisartan in neuronal cultures expressing AT_1Rs, is independent of PPARγ activation (Pang *et al*, 2012).

In our study, PPARγ inhibition abolished the significant protective effects of candesartan on microglial activation (Figure 6c). However, PPARγ inhibition reduced, but did not eliminate the beneficial effect of candesartan on lesion volume or motor function, as treatment with T0070907 together with candesartan was not significantly different from treatment with either vehicle or candesartan alone (Figures 6b and d). The PPARγ agonist activity of some ARBs, including candesartan, may not be independent of their AT_1R-blocking properties. There is cross-talk between AT_1R and PPARγ activation (Xiao *et al*, 2009); PPARγ agonists reduce AT_1R-mediated inflammation and hypertension *in vivo* (Ji *et al*, 2009), and downregulates AT_1R expression (Zhao *et al*, 2008), whereas Ang II, by stimulating the AT_1R, downregulates PPARγ activity (Tham *et al*, 2002). Further studies are necessary to elucidate the relative role of AT_1R inhibition and PPARγ activation in neuroprotection after TBI. Nevertheless, use of the ARBs as treatment for TBI may have more efficacy because of the dual anti-inflammatory action of inhibition of AT_1R and activation of PPARγ (Yi *et al*, 2008).

While we were completing the studies presented here, Timaru-Kast *et al* (2012) published their study showing that a 0.1 mg/kg dose of candesartan, but not a 1 mg/kg dose, administered by subcutaneous injection up to 4 h after injury, reduced the lesion size after CCI injury in mice and improved the neurologic severity score. We found efficacy of candesartan at 1 mg/kg/day, administered by osmotic

minipump, starting 5 h before injury. The difference in the efficacy of 1 mg/kg/day between our data and that of Timaru-Kast *et al* (2012) may be explained by different mechanisms of administration. There is a significant drop in blood pressure following TBI that could be detrimental (Guan *et al*, 2011; Sookplung *et al*, 2011). Thus, medication that lowers blood pressure further may be problematic in treating TBI. Candesartan can lower blood pressure at high doses (Omura-Matsuoka *et al*, 2009). Injection of 1 mg/kg candesartan immediately following injury may be more detrimental than injecting it 5 h before injury. Indeed, Timaru-Kast *et al*, (2012) found that 1 mg/kg significantly lowered blood pressure. With the use of indirect tail cuff measurements, we found only a slight nonsignificant drop in blood pressure (Figure 3). It is probable that the neuroprotective effects of candesartan are not dependent on its hypotensive action, and thus the dose dependence of neuroprotection and blood pressure reduction can be separated. Indeed, beneficial effects of candesartan after stroke have been shown for doses that are not hypotensive (Omura-Matsuoka *et al*, 2009). The results of Timaru-Kast *et al* (2012), show that post-injury administration of candesartan up to 4 h after injury are beneficial to recovery when assessed at 24 h after injury. We have shown that the beneficial cognitive effects of candesartan can be detected up to 28 days after injury. Ultimately, for ARBs to be pursued as therapeutics, we need to determine the maximum time window after injury for candesartan administration to retain its efficacy in improving functional recovery 1 or 2 months after injury.

Together our data show that treatment with ARBs in mice attenuated the TBI-induced neuronal death, in part through reducing the amount of inflammatory response. This improvement in pathology bestowed improved cognitive and motor functional outcomes after injury. The reduced lesion size, and enhanced cell survival translated into longer-term improvement in cognitive memory, as shown by the improved function in the MWM. To our knowledge, this is the first study that shows that candesartan can enhance recognition and spatial memory 4 weeks after TBI. Thus, candesartan, and therefore potentially other ARBs that cross the blood – brain barrier, could be promising therapeutics for TBI. The ability of these drugs to address several different mechanisms simultaneously makes them particularly attractive treatments for TBI.

ACKNOWLEDGEMENTS

This study was supported by a pilot award from the Center for Neuroscience and Regenerative Medicine (CNRM) grant (AJS). SV is supported by a CNRM postdoctoral fellowship. JMS and ESL are supported by the Division of Intramural Research Programs, National Institute of Mental Health, National Institutes of Health. We thank Laura Tucker, Oz Malkesman, and Amanda Fu from the CNRM pre-clinical core facility for expert advice, Dr Tim ÓNeil for his assistance with blood pressure measurements. We are grateful to members of the Symes and Saavedra laboratories for their helpful comments and suggestions. The opinions and assertions contained herein are the private opinions of the authors and are not to be construed as reflecting the views of the Uniformed Services University of the Health Sciences or the US Department of Defense.

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)