



Research Paper

Vitamin D receptor activation regulates microglia polarization and oxidative stress in spontaneously hypertensive rats and angiotensin II-exposed microglial cells: Role of renin-angiotensin system

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ABSTRACT

Hypertension is one of the major predisposing factors for neurodegenerative disease characterized with activated renin-angiotensin system (RAS) in both periphery and brain. Vitamin D (VitD) is recently recognized as a pleiotropic hormone with strong neuroprotective properties. While multiple lines of evidence suggest that VitD can act on RAS, the evidence concerning the crosstalk between VitD and RAS in the brain is limited. Therefore, this study aims to evaluate whether VitD can modulate brain RAS to trigger neuroprotective actions in the brain of spontaneously hypertensive rats (SHR). Our data showed that calcitriol treatment induced VDR expression and inhibited neural death in the prefrontal cortex of SHR. Sustained calcitriol administration also inhibited microglia M1 polarization, but enhanced M2 polarization, accompanied with decreased expression of proinflammatory cytokines. We then further explored the potential mechanisms and showed that SHR exhibited overactivated classical RAS with increased expression of angiotensin II (Ang II) receptor type 1 (AT1), angiotensin converting enzyme (ACE) and Ang II production, whereas the counteracting arm of traditional RAS, ACE2/Ang(1–7)/MasR, was impaired in the SHR brain. Calcitriol nonsignificantly suppressed AT1 and ACE but markedly reduced Ang II formation. Intriguingly, calcitriol exerted pronouncedly impact on ACE2/Ang(1–7)/MasR axis with enhanced expression of ACE2, MasR and Ang(1–7) generation. Meanwhile, calcitriol ameliorated the overactivation of NADPH-oxidase (Nox), the downstream of RAS, in SHR, and also mitigated oxidative stress. In microglial (BV2) cells, we further found that calcitriol induced ACE2 and MasR with no significant impact on ACE and AT1. In accordance, calcitriol also attenuated Ang II-induced Nox activation and ROS production, and shifted the microglia polarization from M1 to M2 phenotype. However, co-treatment with A779, a specific MasR antagonist, abrogated the antioxidant and neuroimmune modulating actions of VitD. These findings strongly indicate the involvement of ACE2/Ang(1–7)/MasR pathway in the neuroprotective mechanisms of VitD in the hypertensive brain.

1. Introduction

Hypertension is tightly related to neuropathy and is one of the major predisposing factors for neurodegenerative disorders, such as Alzheimer's disease, which is associated with neuroinflammation and oxidative damage [1]. Although the mechanisms remain elusive, it has

been recently demonstrated that renin-angiotensin system (RAS), originally acknowledged for its role in the regulation of blood pressure, now is generally accepted that brain has its intrinsic RAS with the major components, including angiotensin converting enzyme (ACE) and angiotensin II (Ang II) receptor type 1 (AT1), widely distributed in the central nervous system [2]. By binding with Ang II, AT1 stimulation

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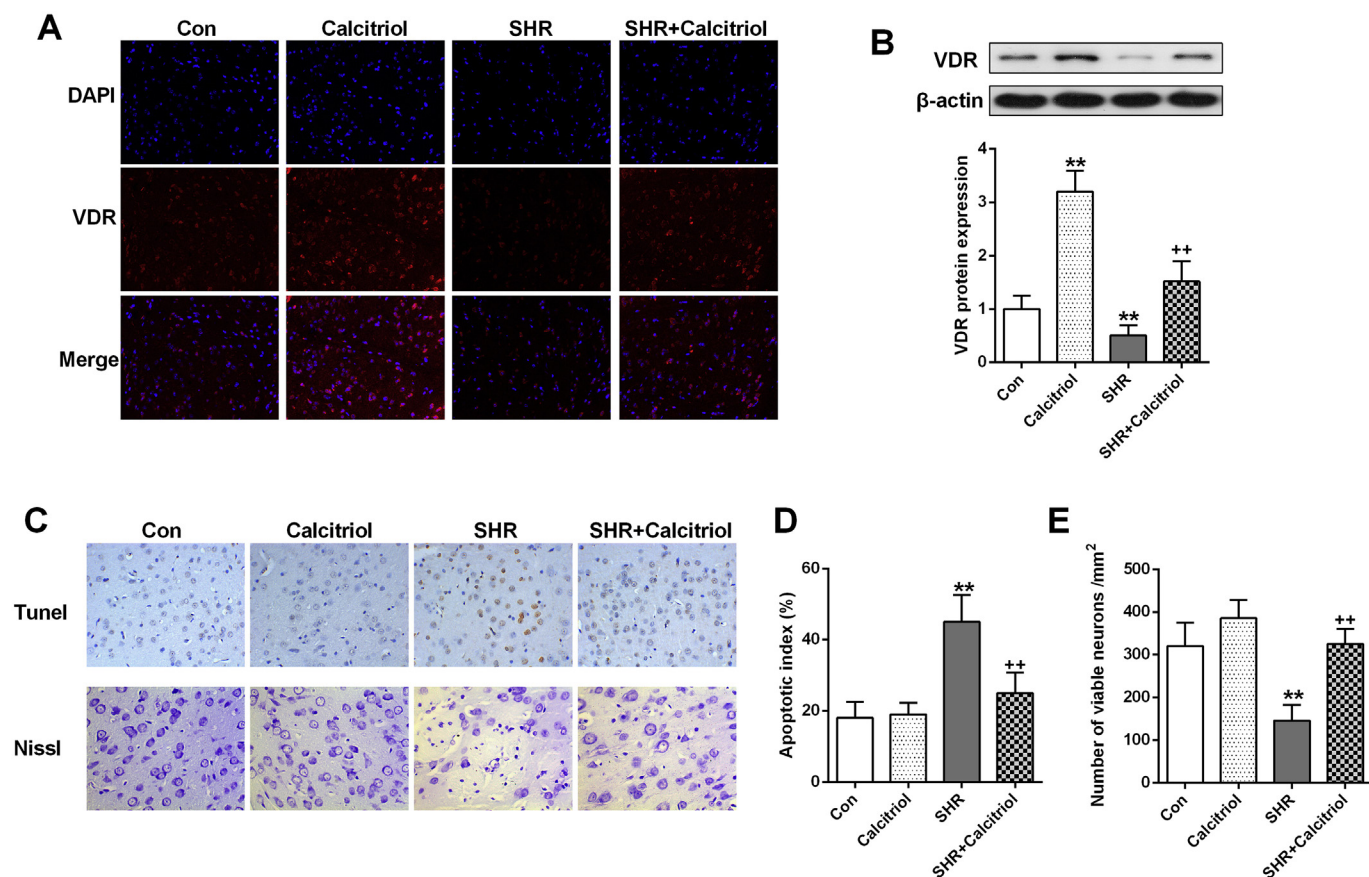


Fig. 1. Neuroprotective effects of calcitriol on hypertensive brain. Representative images of immunofluorescence assays of VDR (A) and VDR Western blot analysis (B) in the prefrontal cortex. (C) Representative images of TUNEL and Nissl staining. Statistical graphs of TUNEL (D) and Nissl (E) staining. 100 ng/kg calcitriol was administrated daily for 6 weeks in both control (Con) and SHR animals. Data are means \pm SD (n = 7). ** p < 0.01 compared to control group. ++ p < 0.01 compared to SHR group.

triggers NADPH-oxidase (Nox) activation, leading to reactive oxygen species (ROS) generation and shifting the microglia from immunoregulatory M2 phenotype to proinflammatory M1 phenotype [3]. However, as a newly identified heptapeptide of RAS, angiotensin-(1–7) (Ang-(1–7)), catalyzed by ACE2 from Ang II, is protective against inflammation and oxidative damage through binding with MasR, counteracting the deleterious effects of ACE/Ang II/AT1 pathway [4]. The spontaneously hypertensive rat (SHR), a widely used animal model of hypertension, exhibits neurological dysfunction, neuroimmune activation and oxidative brain damage [5–7]. The overactivated ACE/AngII/AT1 signaling but weakened ACE2/Ang(1–7)/MasR signaling also has been observed in the brain tissues of SHR, raising the possibility that rebalancing the RAS system might be neuroprotective [8].

Vitamin D (VitD), a recently recognized pleiotropic hormone, contains anti-inflammatory, antioxidant and neuroprotective properties in addition to its classical function in calcium-phosphate homeostasis [9,10]. Recently, VitD is recognized as a neuroactive steroid and its receptor, vitamin D receptor (VDR) is widely distributed in the central nervous system [11]. To start its bio-action, 1,25-dihydroxyvitamin D₃ (calcitriol), the active form of VitD, binds with VDR, which subsequently acts on VitD-response elements (VDREs) to exert transcriptional control over a batch of genes [12]. Our previous study showed that 1,25-dihydroxyvitamin D₃ (calcitriol), the active form of VitD, is protective against neurological deficits and attenuates Nox2-induced oxidative stress in a rat model of traumatic brain injury [13]. Interestingly, VitD is well-known for its action in cardiovascular system and its deficiency is directly related to the progression of hypertension, heart failure and myocardial infarction [14]. The underlying mechanisms are closely linked to its modulating effect on RAS system. Although

multiple lines of evidence indicate the regulatory effect of VitD on RAS in the periphery [15,16], whether VitD is involved in the maintenance of local RAS homeostasis in the brain remains unknown.

Therefore, the aim present study is two-fold, to firstly evaluate the neuroprotective effects of VitD in the hypertensive brain and to systematically explore the impact of VitD on the two RAS axis in both central nervous system and in microglial (BV2) cells.

2. Materials and methods

2.1. Animals and drug treatment

Male, six-month-old, Wistar-Kyoto (WKY) rats and SHR rats (200–230 g) were housed separately under standard conditions of temperature (23 ± 2 °C) and light (12:12 h light/dark cycle), with free access to rodent chow and water. All animal use procedures were carried out in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China, with the approval of the Animal Ethics Committee of our University. Systolic blood pressure (SBP) and heart rate (HR) were measured at the end of the experiment in conscious rats by the volume–pressure recording tail-cuff method (Softron, China).

Rats were randomly divided into four groups: WKY, WKY + Calcitriol, SHR and SHR + Calcitriol. The animals in different groups received daily gavage of vehicle or 100 ng/kg calcitriol between 8:00 a.m. and 9:00 a.m. for 6 weeks. Calcitriol (Roche, Mannheim, Germany) was suspended in saline containing 0.5% Tween 80. The treatment protocol was in accordance with our previous research

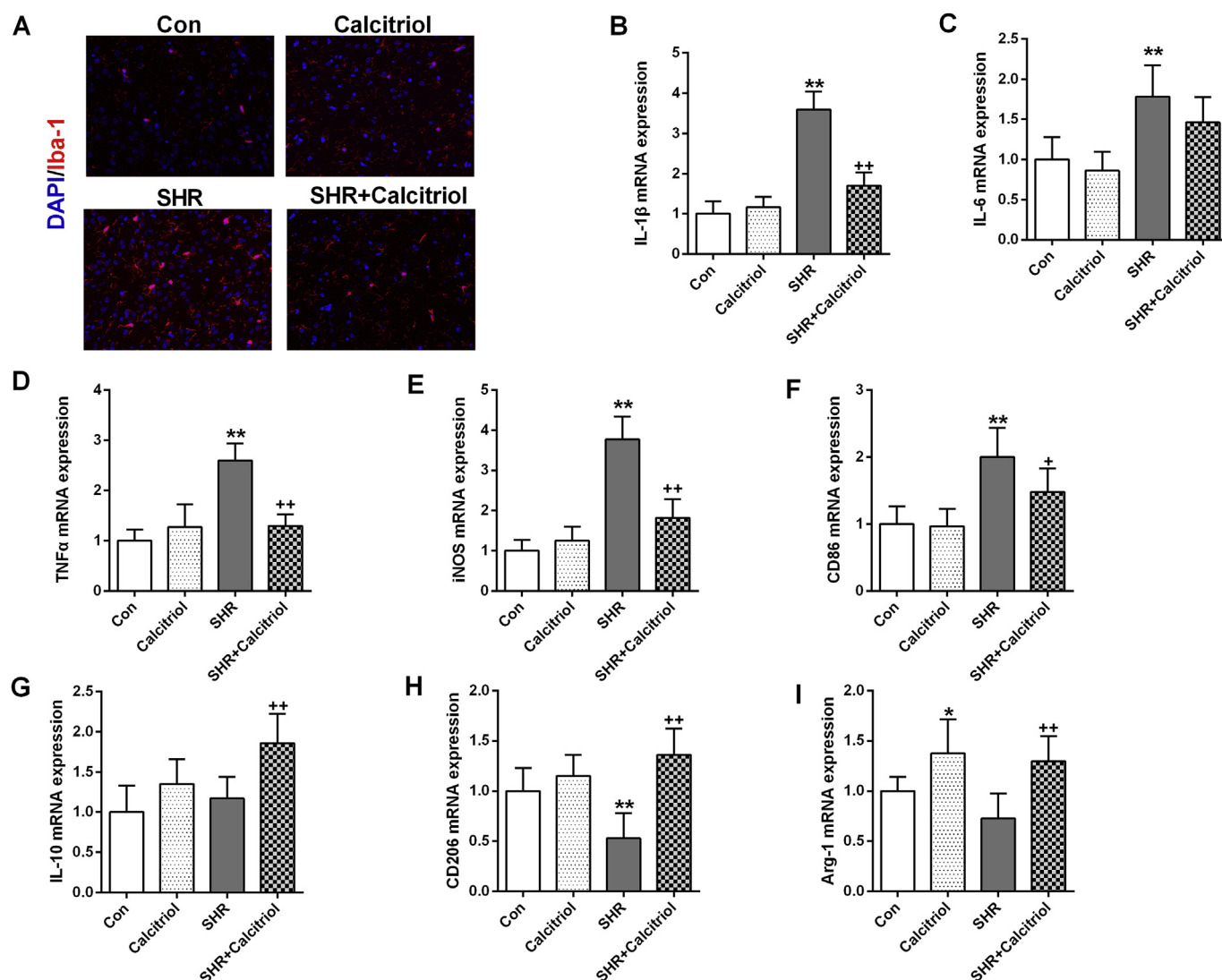


Fig. 2. Anti-inflammatory effects of calcitriol on hypertensive brain. Representative images of immunofluorescence assays of Iba-1 (A). Expression of proinflammatory M1 mediators, IL-1 β (B), IL-6 (C), TNF α (D), iNOS (E) and CD86 (F), and immunoregulatory M2 mediators, IL-10 (G), CD206 (H) and Arg-1 (I). 100 ng/kg calcitriol was administered daily for 6 weeks in both control (Con) and SHR animals. Data are means \pm SD (n = 7). *p < 0.05, **p < 0.01 compared to control group. +p < 0.05, ++p < 0.01 compared to SHR group.

showing that 100 ng/kg calcitriol can effectively activate VDR without disturbing calcium-phosphate homeostasis [17].

2.2. Cell culture and treatment

BV2 murine microglial cells were cultured at humidified atmosphere of 5% CO₂ in DMEM/F12 medium with 10% heat-inactivated fetal calf serum at 37 °C. Prior to experiments, BV2 cells were seeded into 24-well and 6-well plates at a density of 3×10^5 and 1×10^6 cells per well, respectively. Following one day incubation, serum free medium (SFM) was added and incubated in SFM for an additional 4 h. For drug treatment, cells were pre-treated for 30min with 1 μ M calcitriol before exposure to 100 nM Ang II for 24 h. The dose of calcitriol was chosen according to our preliminary data and previous research showing the immune-modulating effectiveness of calcitriol in inflammatory conditions [18]. The concentration of Ang II was selected based on previous findings to activate AT1 and downstream inflammatory signaling [19]. 1 μ M A779 was added simultaneously with Ang II to block MasR as previously reported [20].

2.3. Real-time PCR analysis

Total RNA was extracted by using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Quantitative PCR was performed on Bio-rad Cx96 Detection System (Bio-rad, USA) using SYBR green PCR kit (Applied Biosystems, USA) and gene-specific primers (Supplementary Table 1 and Table 2). Each cDNA was tested in triplicate. Thermo profile conditions were: 50 °C for 2min, 95 °C for 10min, 40 cycles of amplification at 95 °C for 15s and 60 °C for 1min. Relative quantitation for PCR product was normalized to β -actin as an internal standard.

2.4. Western blot analysis

For western blotting analysis, total protein was prepared and the concentrations were analyzed using Bradford method. Samples were loaded on precast 12% SDS-PAGE gels with approximately 50 μ g protein in each lane. The following antibodies and concentrations were used overnight at 4 °C; anti-VDR (Santa Cruz, sc-13133, 1:300), anti-AT1 (Proteintech, 25343-1-AP, 1:1000), anti-ACE (Abcam, ab254222, 1:1000), anti-ACE2 (Proteintech, 21115-1-AP, 1:1000), anti-MasR

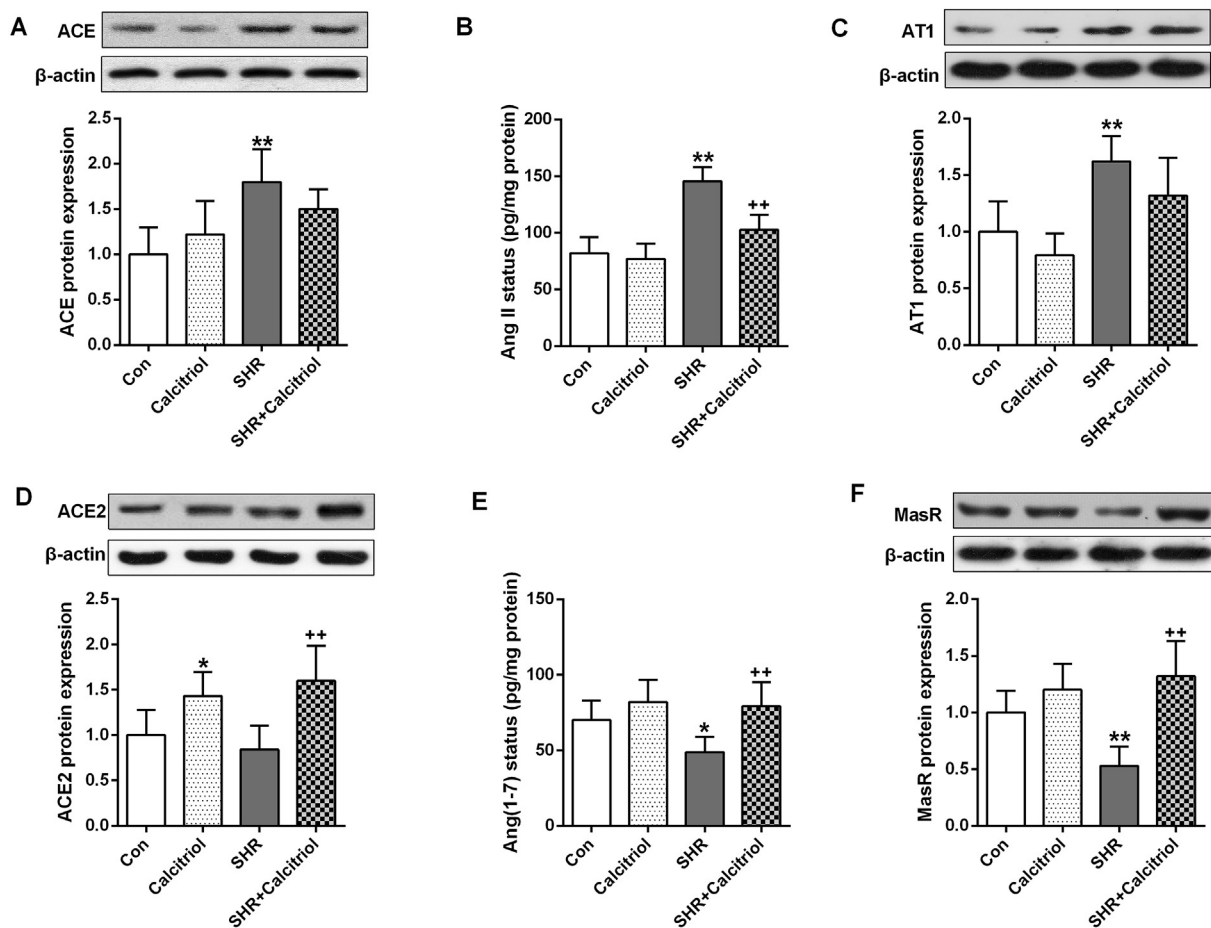


Fig. 3. The impacts of calcitriol on RAS in the hypertensive brain. Protein expression or concentrations of ACE (A), Ang II (B), AT1 (C), ACE2 (D), Ang (1–7) (E) and MasR (F). 100 ng/kg calcitriol was administrated daily for 6 weeks in both control (Con) and SHR animals. Data are means \pm SD (n = 7). * p < 0.05, ** p < 0.01 compared to control group. ++ p < 0.01 compared to SHR group.

(Novus, NBP1-78444, 1:1000), anti-Nox2 (Santa Cruz, sc-130549, 1:800), anti-phospho-p47phox (Ser370) (Invitrogen, PA5-36863, 1:500), anti-p47phox (Santa Cruz, sc-17844, 1:200), Nox4 (Proteintech, 14347-1-AP, 1:1000). It was then probed with HRP-conjugated secondary antibody for 40min. The film signals were digitally scanned and then quantified using Image J software. The signals were normalized to β -actin as an internal standard and the relative protein expression was presented as fold change over control group.

2.5. Histopathological staining

The tissues were fixed in 10% phosphate-buffered paraformaldehyde and then embedded in paraffin, prepared for histopathological examination and immunohistochemical staining. For nissl staining, brain paraffin tissues were sectioned with 20 μ m thickness, which were immersed in cresyl violet (0.1%), rinsed in distilled water, and differentiated in 95% ethyl alcohol. The presence of apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated FITC-dUTP nick end labeling (Tunel) method, which detects fragmentation of DNA in the nucleus during apoptotic cell death *in situ* [21]. The apoptosis detection assay was performed using a commercially available kit following the manufacturer's protocol (Keygen Biotech, Nanjing, China).

2.6. Immunostaining

For the immunofluorescent analysis, paraffin-embedded coronal sections of the tissues (6 μ m thickness) were dewaxed in xylol, rehydrated, and rinsed in PBS. Antigen retrieval was performed by boiling

the sections in a citric acid buffer (0.01 mol/L, pH 6.0), followed by incubation with blocking 5% goat serum for 1 h at room temperature. For immunocytochemistry, cells grown on coverslips were fixed with ice-cold 4% paraformaldehyde in PBS for 10 min. Cells were blocked using 1% BSA + 0.3% (v/v) Triton X-100 + 0.3 M glycine (1% BSA) in PBS for 45 min at room temperature. The sections or cells were then incubated with the following primary antibodies: anti-AT1 (Proteintech, 25343-1-AP, 1:200), anti-MasR (Novus, NBP1-78444, 1:30), anti-VDR (Santa Cruz, sc-13133, 1:200), anti-Iba-1 (Abcam, ab178847, 1:200), anti-CD206 (Proteintech, 18704-1-AP, 1:500) or anti-CD86 (Invitrogen, 13-0862-82, 1:1000). The sections were washed with PBS three times and stained with DAPI (Beyotime Biotechnology, China) to stain the cell nuclei. Immunofluorescent images were taken with an inverted fluorescence microscope (Olympus, Japan) and analyzed by using the Image J software to obtain the mean fluorescence density of each visual field.

2.7. Ang II and Ang (1–7) analysis

The brain tissues were homogenized and centrifuged at 9500 rpm for 20 min at 4 $^{\circ}$ C. The supernatant was used for the measurement of Ang II and Ang (1–7) by using the commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits (Cusabio, China).

2.8. NADPH oxidase activity assay

NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay as we previously described [13]. The

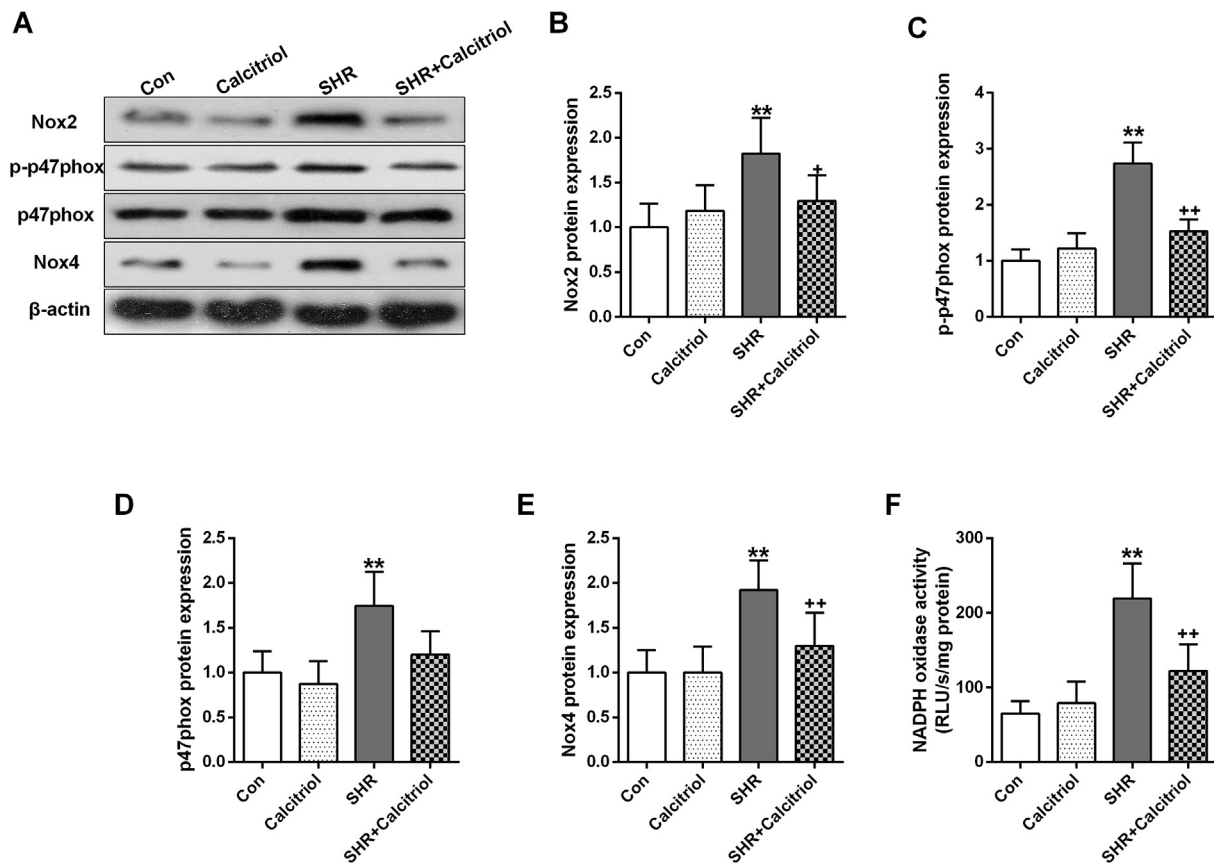


Fig. 4. The impacts of calcitriol on protein expression and activity of Nox in the hypertensive brain. Representative Western blot (A) and statistical graphs of protein expression of Nox2 (B), p-p47phox (C), p47phox (D) and Nox4 (E), and total Nox activity (F). 100 ng/kg calcitriol was administrated daily for 6 weeks in both control (Con) and SHR animals. Data are means \pm SD (n = 7). **p < 0.01 compared to control group. *p < 0.05, **p < 0.01 compared to SHR group.

homogenized brain tissues or BV2 cells were used for assaying NOX enzymatic activity. Relative light units (RLU) were measured every minute continuously for 5 min via a standard luminometer (Thermo Fisher, USA). The results of NOX activity were calculated as RLU/s/mg protein.

2.9. Oxidative parameters detection

Generation of reactive oxygen species (ROS) was determined by fluorescent-labeled dihydroethidium (DHE). Frozen slices (15 μ m thick) were incubated in DHE for 30 min at 37 $^{\circ}$ C in a dark humidified chamber. The sections were rinsed three times in PBS and were observed by using an inverted fluorescence microscope (Olympus, Japan). Cellular superoxide generation was determined with dichlorodihydrofluorescein diacetate (DCFH-DA), which can be oxidized by ROS to form DCF, a fluorescence probe with 525 nm wavelength emission under the excitation of 488 nm wavelength. 5 μ M DCFH-DA was added to cell medium and cultured for 45 min. Then, the fluorescence emission was measured on a FLUOstar plate reader (BMG LABTECH, Germany).

Malondialdehyde (MDA) levels were measured using the thiobarbituric acid reactive substances (TBARS) method. The activities of superoxide dismutase (SOD) and catalase (CAT) were determined using the SOD and CAT assay kits, respectively (Nanjing Jiancheng Bioengineering Institute, China).

2.10. Data analysis

Results from the experiment were expressed as means \pm SD and analyzed using SPSS. For body weight, repeated measures analysis of

variance (ANOVA) with factors of treatment and week were conducted. Differences of other parameters were determined by one-way or two-way ANOVA test, followed by Tukey's test for post hoc comparisons. The prior level of significance was established at p < 0.05.

3. Results

3.1. Neuroprotective effects of VitD in SHR

As revealed in [Supplementary Fig. 1.](#), calcitriol had no significant effect on body weight growth or SBP in both WKY and SHR animals, whereas it markedly reduced the HR in SHR, indicating potential cardioprotective actions. Moreover, in the prefrontal cortex, we found that sustained calcitriol treatment induced VDR expression in both WKY and SHR ([Fig. 1A](#) and [B](#)). Since calcitriol can stimulate VDR expression via the activation of its gene expression and the stabilization of the receptor protein [10], our results indicate that calcitriol can effectively cross the blood-brain barrier and act on the brain. In addition, SHR displayed decreased VDR expression and translocation compared with WKY rats, suggesting impaired VitD signaling in the hypertensive brain. The neuroprotective effects of VitD were evaluated by using TUNEL method and Nissl staining ([Fig. 1C](#)). TUNEL-positive cells were stained deep brown in the cortex. Compared with WKY group, TUNEL-positive cells were much more abundant in the cortex of SHR, which was restored by calcitriol administration ([Fig. 1D](#)). In Nissl staining, the neurons were significantly shrunken, irregularly arranged, and weakly stained in SHR group, indicating the loss of Nissl bodies, whereas calcitriol treatment increased the number of viable neurons, exhibiting regularly arranged, deeply stained and normal forms ([Fig. 1E](#)).

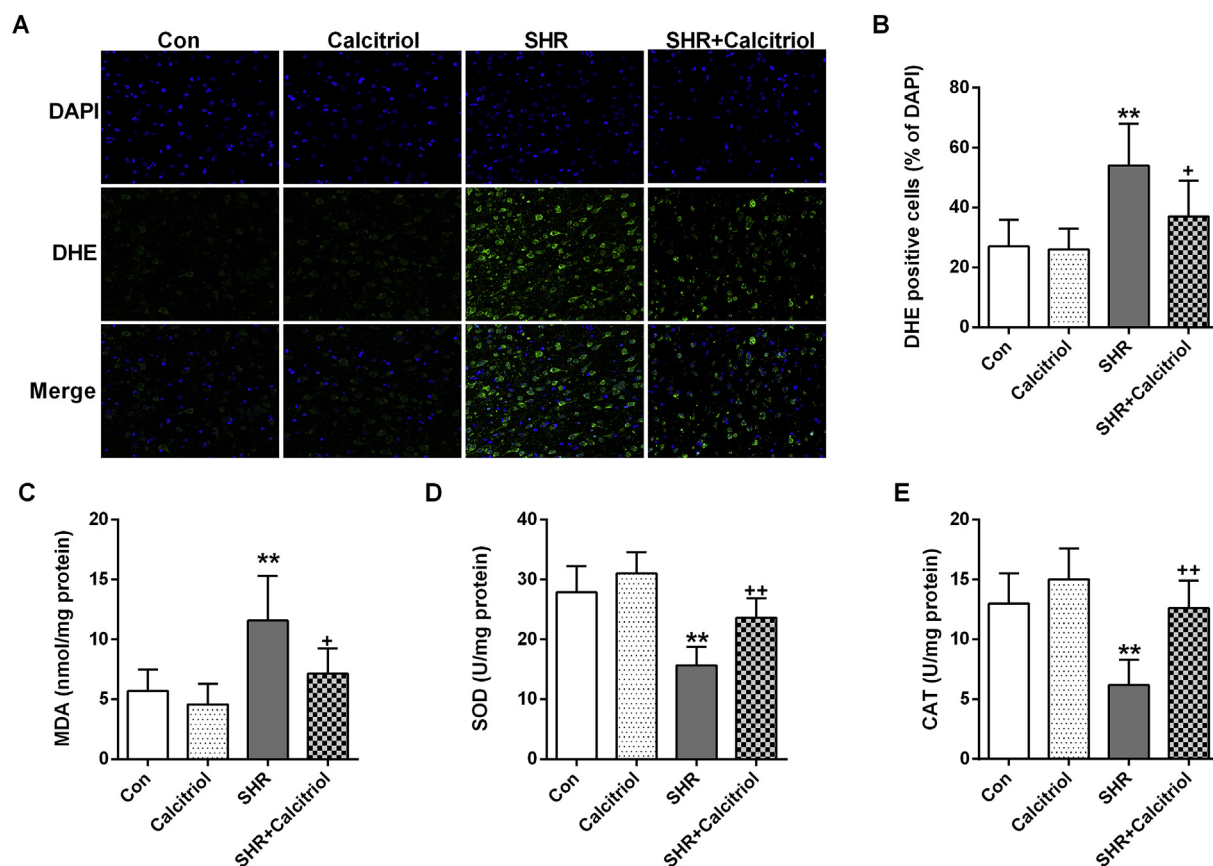


Fig. 5. Anti-oxidative effects of calcitriol in the hypertensive brain. Representative images (A) and statistical graph (B) of immunofluorescence assays of DHE. Concentrations of lipid peroxidation product, MDA (C) and activity of the antioxidant enzymes, SOD (D) and CAT (E). 100 ng/kg calcitriol was administered daily for 6 weeks in both control (Con) and SHR animals. Data are means \pm SD (n = 7). ** p < 0.01 compared to control group. + p < 0.05, ++ p < 0.01 compared to SHR group.

3.2. Anti-inflammatory effects of VitD in SHR

We then explored the neuroimmune modulating effects VitD in SHR. As shown in Fig. 2A., calcitriol attenuated the exaggerated abundance of Iba-1 (biomarker of microglia activation) and mitigated the over-expression of proinflammatory cytokines, including IL-1 β and TNF α in SHR (Fig. 2B and D). We further analyzed M1 and M2 polarization state and the data showed that the markers of M1 phenotype (iNOS and CD86) were induced but the expression of M2 marker, CD206, was inhibited in SHR, indicating that the microglia was prone to M1 polarization (Fig. 2E–I). Interestingly, calcitriol shifted microglia polarization from M1 to M2 phenotype, decreasing iNOS and CD86 expression but increasing IL-10, CD206 and Arg-1 expression in SHR + Calcitriol group compared with vehicle treated SHR.

3.3. Effects of VitD on RAS in SHR

RAS and downstream Nox activation play an essential role in microglial polarization [3]. To further clarify the underlying neuroprotective mechanisms of VitD, we firstly assessed the co-expression of VDR and AT1 or MasR in the brain of WKY animals. As shown in Supplementary Fig. 2., Double immunofluorescent staining showed that VDR was co-localized with AT1 and MasR in the cortex, respectively. Then, the major components of brain RAS and Nox signaling were analyzed. As previously reported, SHR rats were characterized with increased status of ACE/Ang II/AT1 but decreased MasR expression and Ang(1–7) concentration [8], suggesting the activated ACE/Ang II/AT1 arm and compromised ACE2/Ang(1–7)/MasR arm in the hypertensive brain (Fig. 3). Chronic calcitriol treatment induced ACE2 expression in

both WKY and SHR group, the enzyme functioning to catabolize the excessive Ang II into Ang (1–7), thereby accompanied with decreased Ang II but increased Ang (1–7) status in SHR + Calcitriol group compared with vehicle treated SHR. In addition, calcitriol administration also ameliorated the suppressed MasR expression in SHR.

3.4. Effects of VitD on Nox signaling in SHR

The downstream of RAS, Nox pathway, and neural oxidative stress were further examined. The Nox enzyme complex is composed of membrane subunits (Nox family) and cytosolic subunits (p47phox, p67phox, p40phox, and Rac2) [22]. The phosphorylation of p47phox in stressful conditions is an essential mediator of Nox activation [23]. We found that the major components of Nox, including Nox2, Nox4, p-p47phox and p47phox, and total Nox activity were all synchronously increased in SHR, which were partly restored by calcitriol treatment (Fig. 4). In accordance, as shown in Fig. 5., calcitriol also markedly alleviated ROS production (Fig. 5A and B) and lipid peroxidation products (MDA) (Fig. 5C) and enhanced the activity of anti-oxidant enzymes (SOD and CAT) (Fig. 5D and E).

3.5. Effects of VitD and Ang II on RAS in BV2 cells

BV2 murine microglial cells were used to further confirm the effects of VitD on RAS. In line with the findings in vivo, calcitriol induced VDR expression in BV2 cells. However, VDR expression was also induced by Ang II exposure and was further exaggerated by calcitriol co-treatment (Fig. 6A and B), indicating an acute compensatory mechanism. As previously reported in the other cell types [24], Ang II caused elevated

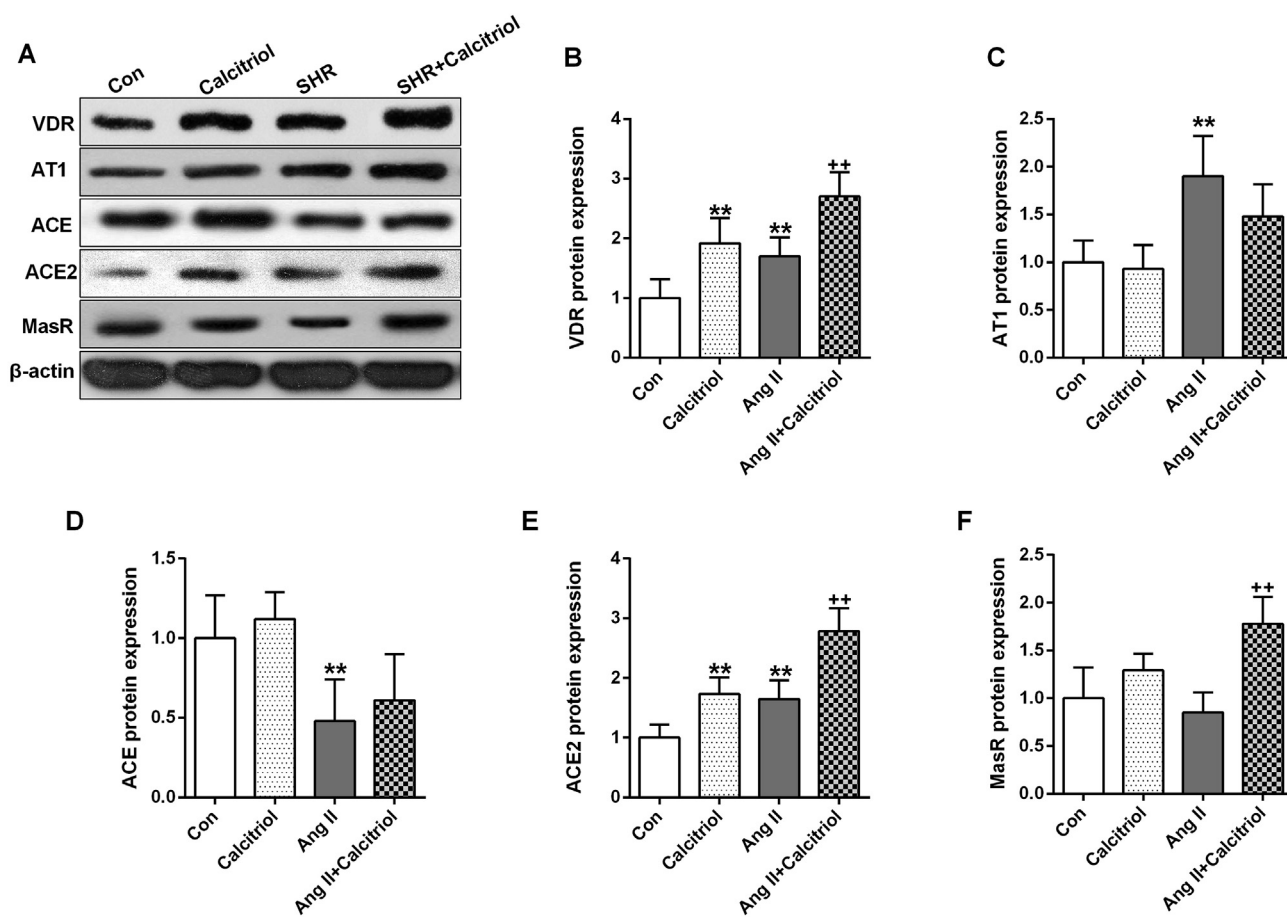


Fig. 6. The impacts of calcitriol treatment on VDR activation and RAS in microglia BV2 cells. Representative western blots (A) and statistical graphs of protein expression of VDR (B), AT1 (C), ACE (D), ACE2 (E) and MasR (F). 1 μ M calcitriol was pre-treated for 30min before exposure to 100 nM Ang II for 24 h. Data are means \pm SD (n = 6). ***p* < 0.01 compared to control group. ++*p* < 0.01 compared to SHR group.

AT1 and ACE2 expression but inhibited ACE expression through potential negative feedback response. While calcitriol had no significant impact on AT1 and ACE expression, it induced ACE2 expression (Fig. 6C–E). When co-treated with Ang II, MasR was also induced by calcitriol (Fig. 6F). These findings are in line with the *in vivo* results, further strengthening the modulating effect of calcitriol on ACE2/Ang (1–7)/MasR pathway.

3.6. Anti-oxidant and anti-inflammatory effects of VitD via MasR

As the robust effect of VitD on ACE2/Ang(1–7)/MasR signaling, we then used A779, a selective MasR antagonist [25], to confirm the anti-oxidant and anti-inflammatory mechanisms of VitD. As expected, Ang II triggered Nox activation with increased expression the major components of Nox and enhanced Nox activity, whereas VitD partly restored Ang II-induced altered Nox signaling by decreasing Nox2 protein levels, p47phox phosphorylation and NADPH activity, thereby attenuating the Ang II-induced ROS accumulation (Fig. 7). However, in the presence of A779, the modulating effect of VitD on Nox signaling was abrogated (Fig. 7). Similarly, Ang II also induced the expression of biomarkers or cytokines secreted by the proinflammatory microglia M1 phenotype but decreased the expression of Arg-1 and CD206, the biomarkers of immunoregulatory M2 phenotype (Fig. 8). VDR activation by calcitriol pretreatment effectively inhibited M1 polarization and promoted M2 polarization in the presence of Ang II, which was also compromised by A779 co-treatment. These data strongly indicated the involvement of ACE2/Ang(1–7)/MasR pathway in the neuroprotective mechanisms of VitD.

4. Discussion

Hypertension greatly increases the vulnerability to neurodegeneration and brain aging, and SHR animals, found in this study and previous researches, are featured with enhanced neural apoptosis and neurological deficits [26,27]. However, the pathological mechanisms are still elusive and the neuroprotective strategies for the hypertensive brain remain limited.

In the present study, we showed for the first time that VDR activation was neuroprotective in the hypertensive brain with anti-apoptosis, anti-inflammatory and anti-oxidant activities. VitD is recently appealing growing attention and is now becoming a neuroactive steroid involved in the maintenance of brain homeostasis with strong activities in neurotransmission, neurotrophic signaling and neuroimmune function [28]. The beneficial effects of VitD on central nervous system have been observed in animal models or clinical patients with neuropsychiatric disease, including Alzheimer's disease, Parkinson's syndrome, cerebral ischemia and multiple sclerosis [11,29,30]. In our recent researches [13,21], we also found that calcitriol, the active metabolite of VitD, is effective in the prevention of oxidative stress and apoptosis progression induced by traumatic brain injury. These results are in accordance with our findings, further lending weight to the hypothesis that VitD is likely to preserve neurological function in the context of hypertension.

Aside from neuroactivities, VitD is also well-known for its actions in the cardiovascular system. VitD deficiency is prone to increase the susceptibility to hypertension through its actions on RAS system [31]. VDR activation by calcitriol or its analogues can directly inhibit AT1

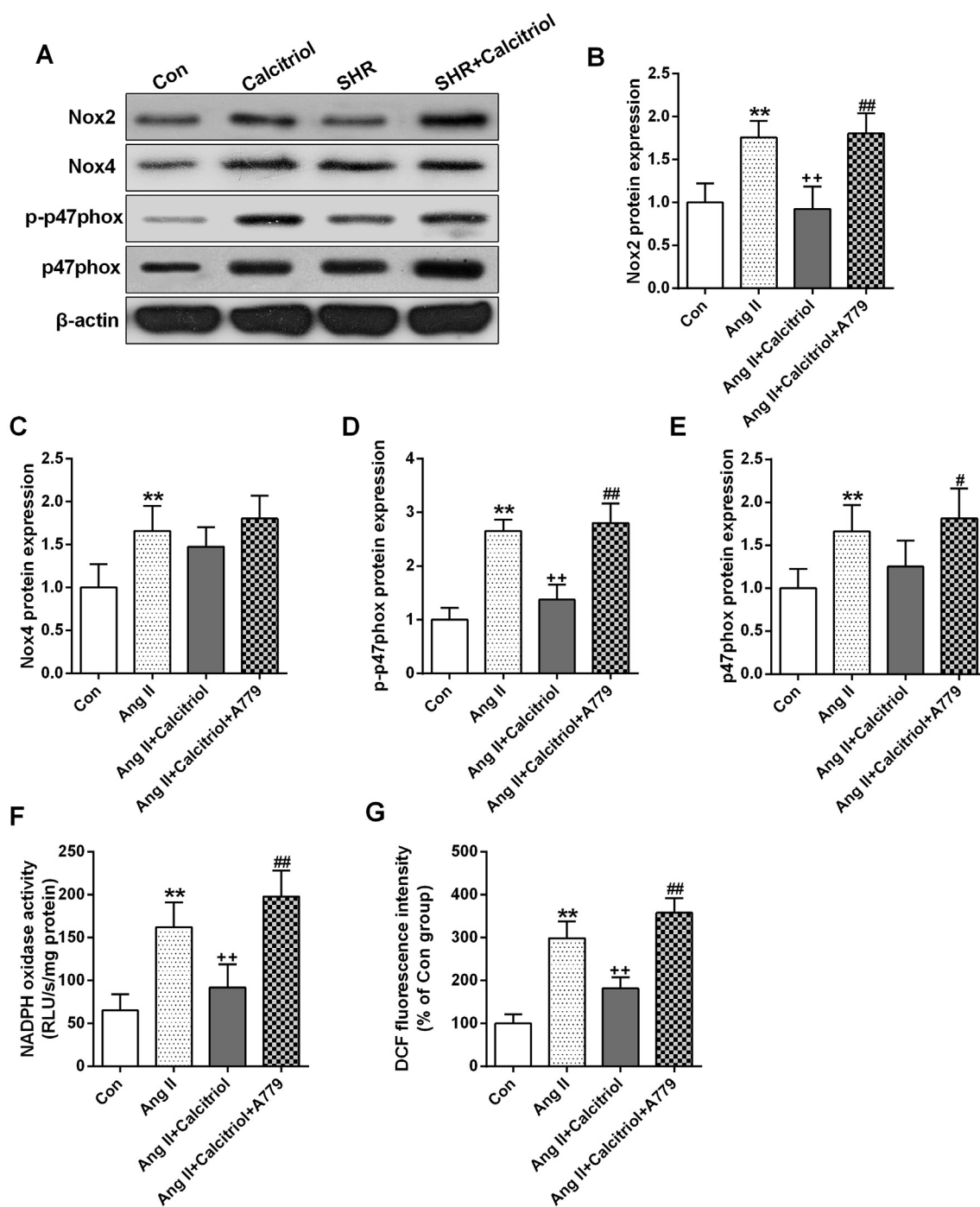


Fig. 7. The impacts of calcitriol treatment on Nox signaling and cellular superoxide generation in microglia BV2 cells. Representative western blots (A) and statistical graphs of protein expression of Nox2 (B), Nox4 (C), p-p47phox (D) and p47phox (E). NADPH oxidase activity (F) and ROS generation (DCF fluorescence intensity) (G). 1 μ M calcitriol was pre-treated for 30min before exposure to 100 nM Ang II for 24 h. 1 μ M A779 was added simultaneously with Ang II to block MasR. Data are means \pm SD (n = 6). ** p < 0.01 compared to control group. ++ p < 0.01 compared to Ang II treated group. ### p < 0.05, ## p < 0.01 compared to Ang II + Calcitriol treated group.

expression and local Ang II generation in myocardium, renal arteries or kidney tissues [32–34]. However, the direct anti-hypertensive effect of VitD activation is controversial and the results are inconsistent according to the different VitD analogues, various doses and treatment duration used in different studies [32,35,36]. As previous researches [32,35,37], the present study also didn't observe a significant decline of blood pressure in SHR following 6-week treatment with 100 ng/kg calcitriol, suggesting that the neuroprotective efficacy of calcitriol is blood pressure-independent. Considering the pro-inflammatory and

pro-oxidant actions of ACE/Ang II/AT1 pathway and the modulating effect of VitD on RAS, it raises the possibility that RAS is very likely to be involved in both pathological progression of neurodegeneration and neuroprotective mechanism of VitD in the hypertensive brain.

Our research showed that ACE/Ang II/AT1 signaling was robustly activated in SHR brain, whereas calcitriol pronouncedly decreased local Ang II formation. However, the deduction of AT1 and ACE status unexpectedly didn't meet statistical difference. It should be noted that ACE2 is the key enzyme responsible for catabolizing Ang II into Ang

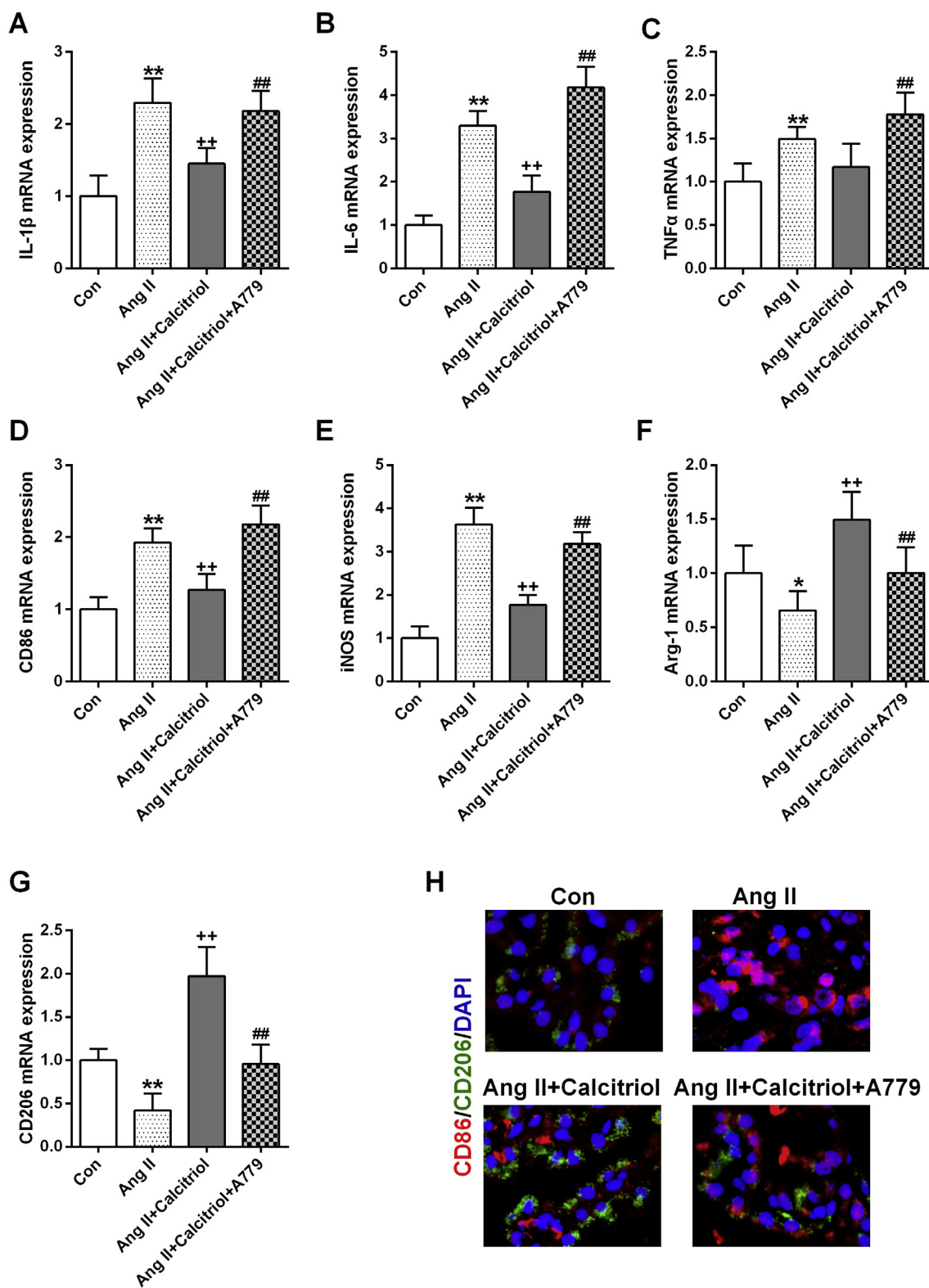


Fig. 8. The impacts of calcitriol treatment on microglial polarization in BV2 cells. Expression of proinflammatory M1 mediators, IL-1 β (A), IL-6 (B), TNF α (C), CD86 (D) and iNOS (E), and immunoregulatory M2 mediators, Arg-1 (F) and CD206 (G). Representative images of immunofluorescence assays of CD86 (red) and CD206 (green) (H). 1 μ M calcitriol was pre-treated for 30min before exposure to 100 nM Ang II for 24 h. 1 μ M A779 was added simultaneously with Ang II to block MasR. Data are means \pm SD (n = 6). * p < 0.05, ** p < 0.01 compared to control group. ++ p < 0.01 compared to Ang II treated group. ## p < 0.01 compared to Ang II + Calcitriol treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(1–7), which binds to MasR to counteract deleterious actions of Ang II [38]. Therefore, we further firstly explored the impacts of VitD on the protective arm of RAS, ACE2/Ang(1–7)/MasR pathway. Interestingly, we found that calcitriol induced ACE2 expression in both WKY and SHR rats, which may cleave Ang II into Ang(1–7), providing an explanation for the fact that Ang II was decreased but ACE was unchanged following calcitriol treatment. In support, Ang(1–7) generation was enhanced in SHR + calcitriol group, revealing enhanced conversion of Ang II to Ang(1–7). In addition to the normalized Ang(1–7) status, the compromised MasR signaling in SHR animals was also restored by calcitriol treatment, implying that calcitriol shifted the balance between ACE/Ang II/AT1 and ACE2/Ang(1–7)/MasR axis to the protective side. Of note, Ang(1–7) and Ang II can also bind with angiotensin type 2 receptor (AT2), triggering anti-inflammatory actions and counteracting AT1 overstimulation [39]. It has been demonstrated that the binding affinity of Ang II to AT2 is much higher than Ang(1–7) [40] and our data showed elevated Ang II but reduced Ang(1–7) concentrations in the hypertensive brain, making it difficult to evaluate the condition of AT2 signaling. Therefore, although we can't exclude the possibility that AT2 might also be involved in the underlying mechanism, which is a major limitation of the present study, this research mainly focused on the protective ACE2/Ang(1–7)/MasR axis in the following experiment.

By binding to AT1, Ang II triggers immune system, shifting the microglia from immunoregulatory M2 phenotype to proinflammatory M1 phenotype, whereas Ang(1–7) contains the opposing effects via evoking M2 polarization and suppressing the M1 inflammatory response [3]. Although the interaction between RAS and macrophage polarization is well acknowledged, there is only one study demonstrating the tendency to M1 polarization in the SHR liver [41]. We firstly explored the microglial polarization in the hypertensive brain and showed the shift of M2 toward M1 phenotype with increased expression of pro-inflammatory cytokines or mediators secreted by M1 phenotype and decreased expression of M2 biomarkers. Nox, the most predominant source of ROS generation and downstream of RAS, promotes pro-inflammatory activation of microglia [3]. In line with previous findings [19], Nox was activated and ROS was accumulated, resulting in elevated oxidative damage and exhausted anti-oxidative activity in the brain of SHR, which may further exaggerate the inflammatory situation. As expected, VDR activation favored the polarization of microglia toward the anti-inflammatory M2 phenotype. Repeated calcitriol administration inhibited microglial activation and the expression of pro-inflammatory cytokines and promoted the expression of M2 biomarkers in the SHR brain. Moreover, calcitriol suppressed Nox overactivation with decreased expression of major components of Nox and reduced total Nox activity, leading to the neuroprotective actions against oxidative stress. These findings are in accordance with our previous research showing the beneficial effects of VitD in the animal model of traumatic brain injury through regulating Nox-derived oxidative damage [13].

To further explore the role RAS in hypertension-induced brain damage and the neuroprotective mechanisms of VitD, microglial BV2 cells were treated with Ang II to stimulate inflammation. The modulating effect of VitD on RAS was supported by the in-vitro findings that calcitriol enhanced ACE2 expression in BV2 cells and further amplified Ang II-induced ACE2 expression via a potential adaptive response. Likewise, calcitriol pretreatment also induced MasR expression in cells exposed to Ang II but didn't affect ACE and AT1 expression. These data corroborated with each other and all pointed out the importance of the opposing arm of traditional RAS, ACE2/Ang(1–7)/MasR, in the neuroactivities of VitD. Therefore, A779, the antagonist of MasR, was used to block the ACE2/Ang(1–7)/MasR axis. In correspondence with the in-vivo findings, calcitriol pretreatment mitigated Ang II-induced Nox activation by decreasing the expression of Nox complex and suppressing total Nox activity, and ameliorated Ang II-induced ROS accumulation in BV2 cells, whereas co-treatment with A779 abrogated the modulating effect of calcitriol on Nox and compromised the anti-

oxidative activity. In parallel, Ang II exposure also potentiated the tendency of polarization to cytotoxic pro-inflammatory M1 phenotype and triggers inflammatory activation, which was attenuated by calcitriol pretreatment. A779 exposure also reduced the capacity of neuroimmune-modulation of VitD and prevented the calcitriol-induced immunoregulatory M2 polarization.

Collectively, the present study demonstrated that the altered balance between ACE/Ang II/AT1 and ACE2/Ang(1–7)/MasR axis in the hypertensive brain may contribute to the phenotypic transition of microglia from immunoregulatory M2 toward proinflammatory M1 polarization, providing an interesting target given that SHR is not only a neurodegenerative model but also a widely used rodent model of attention-deficit/hyperactivity disorder [42]. In addition, we also firstly showed that VitD is neuroprotective in the hypertensive brain by modulating brain ACE2/Ang(1–7)/MasR axis, shedding novel insight into the neuroactivities of VitD. Intriguingly, it has been reported that VitD also preserves structural and functional integrity of the kidney and cardiovascular system in SHR animals [31,32]. Considering the protective effects of VitD have been observed in various organs, it would be interesting for future studies to explore the potential beneficial effects of VitD in hypertension-induced other tissue damage, such as retinopathy and pancreatic dysfunction.

Conflicts of interest

All authors have no financial disclosures and no conflict of interest.

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Appendix A. Supplementary data

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