

# Age-related changes in hypertensive brain damage in the hippocampi of spontaneously hypertensive rats

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**Abstract.** The aim of the present study was to investigate the age-related alterations in hypertensive brain damage in the hippocampi of spontaneously hypertensive rats (SHR) and the underlying mechanisms. Aging resulted in a significant increase in the number of activated astrocytes and apoptotic cells in the SHR group, which was accompanied by increased expression of oxidative stress markers (iNOS and gp47<sup>phox</sup>) and apoptotic regulatory proteins (Bax and caspase-3). In addition, the expression of PPAR- $\gamma$  and Bcl-2 were progressively reduced with increasing age in the SHR group. The 32 and 64-week-old SHRs exhibited significantly increased numbers of apoptotic cells, oxidative stress markers and pro-apoptotic proteins compared with age-matched WKY rats, which was accompanied by reduced expression of PPAR- $\gamma$ . Compared with the 16 and 32-week-old WKY group, the 64-week-old WKY rats exhibited increased oxidative stress and pro-apoptotic markers, and increased levels apoptotic cells. In conclusion, the present study indicated that both aging and hypertension enhanced brain damage and oxidative stress injury in the hippocampi of SHRs, indicated by an increased presence of apoptotic cells and astrocytes. In addition, reduced expression of PPAR- $\gamma$  may contribute to the age-related brain damage in SHRs.

## Introduction

Hypertension is a common disease that may result in pathological changes in important organs including the brain, heart and the kidneys. Hypertension-associated brain damage

can induce cerebral dysfunction, which may manifest as cognitive decline (1,2). Although it is generally believed that hypertension-associated vascular events are the main causes of dementia, many patients experience cognitive decline prior to a stroke (3). In addition, there is a delay between the onset of hypertension and cerebrovascular disease. During this period, a series of pathological alterations occur in the brain, including oxidative stress and cellular apoptosis (4). Furthermore, hypertension is known to enhance the levels of oxidative stress in the brain and vascular tissues (4). Additionally, increased oxidative stress can lower the level of neuronal activity and more importantly, result in the massive entrance of Ca<sup>2+</sup> into neurons, thus activating proapoptotic mechanisms and leading to neuronal loss (5). However, few studies have assessed the age-related changes of hypertensive brain damage in spontaneously hypertensive rats (SHR). Studies investigating the pathophysiological mechanisms involved in hypertension-associated cellular apoptosis and oxidative stress injury may provide new drug targets to prevent the progression of the pathological changes observed in hypertension.

SHRs are the model most extensively used for the evaluation of hypertensive brain damage and the potential treatments. The time-dependent rise in arterial blood pressure and the occurrence of brain atrophy, loss of nerve cells and glial reaction share, to an extent, similarities with the process occurring in human hypertensive brains (6). SHRs, therefore, represent a reasonable model of hypertension-associated brain damage. The hippocampus is a brain region involved in learning and memory (7), and is particularly sensitive to ischemia and represents a predictive site for assessing brain damage (8). However, the majority of investigations on the sensitivity of the hippocampus to hypertension and/or ischemia have been performed in acute conditions or following abrupt elevation of blood pressure (9). Only few studies have evaluated the influence of chronic hypertension or ischemia on the structure of the hippocampus (6). Furthermore, the SHR models used in previous studies were ~6-26-week-old, which is not representative of the advanced hypertensive period (3,10,11). Peroxisome proliferator-activated receptor (PPAR)- $\gamma$  is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily, and is involved in the oxidative stress response (12). Oxidative stress is able to attenuate

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PPAR- $\gamma$  expression and activity in vascular endothelial cells through the suppression of PPAR- $\gamma$  transcription (13). In addition, the PPAR- $\gamma$  agonist rosiglitazone has been shown to protect QZG cells against oxidative stress injury through modulating the PPAR- $\gamma$  pathway (14). Thus, PPAR- $\gamma$  may participate in oxidative stress-related injury.

The present study assessed whether hypertension is able to induce apoptosis and/or necrosis in the hippocampus of SHRs, and the associated age-related alterations. In addition, the oxidative stress pathway was investigated as to whether it was involved in this process and its potential underlying mechanisms.

## Materials and methods

**Animals.** 16, 32 and 64-week-old male normotensive Wistar-Kyoto (WKY) rats and SHRs were purchased from Shanghai Laboratory Animal Center ( $n=6$  per age group; Shanghai, China). They were housed under humidity (50–60%), temperature (21–23°C) and light cycle (12 h light/dark) controlled conditions, with free access to food and water. All procedures were conducted according to the guidelines established by the Institutional Animal Care and Use Committee and the National Institutes of Health (Bethesda, MD, USA). The study was approved by the ethics committee of The Second Affiliated Hospital or Xi'an Jiaotong University (Xi'an, China).

**Blood pressure measurements.** Systolic blood pressure (SBP) was measured by an indirect tail-cuff method (BP-2000; Visitech Systems, Inc., Apex, NC, USA). Briefly, unanesthetized rats were placed in a holding device mounted on a thermostatically controlled warming plate to make the pulsations of the tail artery detectable. Tail cuffs were fixed on the animals, which were allowed to acclimate to the cuffs for 10 min prior to each pressure recording session.

**Brain tissue preparation.** Rats were anesthetized with 10% chloral hydrate. For western blot analysis, rats ( $n=3$ ) were perfused transcardially with 0.9% saline (pH 7.4), following which the brains were rapidly removed and stored in sample protectors (Takara Biotechnology, Inc., Dalian, China) until use. For immunohistochemistry and the terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) assay, rats ( $n=3$ ) were perfused intraventricularly with 0.9% saline (pH 7.4) and 4% formaldehyde. The brains were subsequently removed and postfixed in 4% formaldehyde overnight. Targeted brain pieces were chosen, dehydrated, embedded in paraffin (Beyotime Institute of Biotechnology, Haimen, China) and cut into 10- $\mu$ m-thick sections. A total of 3 sections containing both the cortex and hippocampus were selected from each rat for immunohistochemical and TUNEL analysis.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the hippocampus with the use of TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the RNA samples were transcribed to cDNA using a PrimeScript RT Master Mix kit (Takara Biotechnology, Inc.) according

to the manufacturer's instructions. RT-qPCR was performed with SYBR ExScript RT-PCR Kit (Takara Biotechnology, Inc.) on an iQ Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers used were as follows: PPAR- $\gamma$ , forward 5'-GGAGCCTAAGTTTGAGTTTGCTGTG-3' and reverse 5'-TGCAGCAGGTTGTCTTGGATG-3'; caspase-3, forward 5'-GAGACAGACAGTGGAACTGACGATG-3' and reverse 5'-GGCGCAAGTGACTGGATGA-3'; Bax, forward 5'-GCGTCCACCAGAAGCTGA-3' and reverse 5'-ACCACCCTGGTCTTGATCC-3'; Bcl-2, forward 5'-GACTGAGTACCTGAACCGGCATC-3' and reverse 5'-CTGAGCAGCGTCTTCAGAGACA-3'; and  $\beta$ -actin, forward 5'-GGAGATTACTGCCCTGGCTCCTA-3' and reverse 5'-GACTCATCGTACTCCTGCTTGCTG-3'. The primers were designed and synthesized by Takara Biotechnology, Inc. Amplification was performed at 95°C for 30 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad Laboratories, Inc.). Relative quantification was performed using the comparative method ( $2^{-\Delta\Delta C_q}$ ) (15) and data was presented as the mean  $\pm$  standard deviation of three separate experiments conducted in triplicate.

**Immunohistochemistry.** In brief, sections were deparaffinized and rehydrated according to standard protocols. Subsequently, sections were treated with 3%  $H_2O_2$  for 20 min, followed by incubation with 0.3% Triton X-100 for 30 min and 10% goat serum for 1 h at room temperature. The slides were then incubated overnight at 4°C with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:2,000; ab16997; Abcam, Cambridge, UK). Following washing in phosphate-buffered saline (PBS), the sections were incubated with biotinylated goat anti-rabbit IgG (1:100; A10547; Invitrogen; Thermo Fisher Scientific, Inc.). The immuno-complexes were detected using 3,3'-diaminobenzidine. The series of sections obtained from the different groups were processed in the same way to avoid alterations in the intensity of the staining occurring as a result of different incubation conditions. For the negative control, the primary antibody was replaced with PBS. Activated astrocytes express high levels of GFAP and have altered morphology (larger cell bodies and thick branches). Using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) to count activated astrocytes, a threshold was set up for positive cell area, and within these area limits, each nucleated cell exhibiting GFAP labeling was counted. Labeled astrocytes were investigated in the right and left hippocampus in 5–6 sections per animal, using three animals per group.

**Western blot analysis.** For western blot analysis, the brain tissues were homogenized and the total proteins were extracted using radioimmunoprecipitation assay lysis buffer. The protein concentrations were determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). A total of 20  $\mu$ g of each sample was separated on 12% sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene fluoride membranes (both from Beyotime Institute of Biotechnology), and blocked in 10% non-fat milk at room temperature for 2 h. Membranes were incubated overnight at 4°C with rabbit polyclonal antibodies against PPAR- $\gamma$

(1:400; ab66343; Abcam), rabbit polyclonal antibodies against gp47<sup>phox</sup> (bs3261), Bax (bs2538), Bcl-2 (bs1511), caspase-3 (bs7004; all 1:800; Bio-World, Dublin, OH, USA) and a mouse monoclonal antibody against inducible nitric oxide synthase (iNOS; 1:500; sc-7271; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The blots were then washed with Tris-buffered saline-Tween-20 (0.1%) 3 times, and incubated with secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (1:5,000; BS13278; Bio-World) and HRP-conjugated goat anti-mouse immunoglobulin G (1:5,000; BS12478; Bio-World) for 2 h at room temperature. Following washing, protein bands were detected with chemiluminescent HRP substrate (SuperSignal West Pico; Thermo Fisher Scientific, Inc.) for 5 min at room temperature in the dark and exposed to X-ray film (Fujifilm Corp, Tokyo, Japan). The band intensities were analyzed using Quantity One software 4.6.2 (Bio-Rad Laboratories, Inc.) and normalized to the  $\beta$ -actin loading control.

**TUNEL assay.** For the TUNEL assay, a cell death detection kit was used (Promega Corp, Madison, WI, USA). Briefly, the sections were deparaffinized with xylene and rehydrated according to standard protocols, and were then incubated with proteinase-K for 20 min at room temperature followed by 3 washes in PBS. Subsequently, the sections were covered with equilibration buffer for 10 min at room temperature, followed by incubation with the rTdT incubation buffer (50  $\mu$ l) in the dark for 1 h at 37°C. The slides were then immersed in 2X SSC buffer for 15 min and washed with PBS. Sections were counterstained with 4',6'-diamino-2-phenylindole (DAPI; 1:1,000; Sigma-Aldrich, St. Louis, MO, USA), washed in PBS and mounted. Fluorescence microscopy was conducted using an Olympus BX51 microscope equipped with a mercury lamp. TUNEL-positive cells were normalized to the DAPI stained cells. The immunoreactive cells from 9 random fields (3 fields per sample, 3 samples per group) were counted using a 20x objective by an observer blinded to the treatment groups.

**Statistical analysis.** The data were presented as the mean  $\pm$  standard deviations and analyzed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). A two-way analysis of variance was used to determine the significant differences between strain and age followed by a Tukey-Kramer post-hoc test to evaluate the statistical significance between the hypertensive state and age groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Blood pressure.** The SBP levels in the 16, 32 and 64-week-old SHR groups averaged  $127.1 \pm 6.9$ ,  $184.4 \pm 8.1$  and  $193.9 \pm 11.3$  mmHg, respectively. In aged-matched WKY rats, the SBP levels were significantly lower ( $102.9 \pm 4.7$ ,  $103.5 \pm 3.6$  and  $110.0 \pm 9.8$  mmHg, respectively;  $P < 0.01$ ). A progressive augmentation of SBP was also observed in the SHR group with increasing age. Compared with the 16-week-old group, the 32 and 64-week-old groups showed significantly upregulated SBP levels ( $P < 0.01$ ). However, there was no significant difference between the two groups. Systolic pressure values were similar in the WKY groups of different ages.

**Quantitative analysis of activated astrocytes.** Activated astrocytes are markers of brain damage. The sections processed for GFAP immunohistochemistry exhibited thin, shallow, dark-brown astrocytes with slender branches in the WKY group (Fig. 1D). The SHR group exhibited increased occurrence of activated astrocytes, with large cell bodies and thick branches (Fig. 1C). Although the number of astrocytes was similar in the SHR and WKY groups of different ages, the ratio of activated astrocytes was increased progressively with age in the CA1 subfield in the SHR and WKY groups. The WKY group had 8.8, 12.9 and 21.8% activated astrocytes in the 16, 32 and 64-week-old groups, respectively (Fig. 1D-F). Compared with the age-matched WKY group, the SHRs exhibited a significant increase in the proportion of activated astrocytes (9.1, 25.3 and 53.1%, respectively; Fig. 1A-C). Apart from the 16-week-old group, the 32 and 64-week-old SHR groups were significantly different compared with the age-matched WKY groups ( $P < 0.01$ ). In addition, there were significant differences between the three SHR groups ( $P < 0.01$ ). The parameters investigated were similar in the 16 and 32-week-old WKY rats. The 64-week-old WKY group was significantly different compared with the 16 and 32-week-old WKY rats ( $P < 0.05$ ; Fig. 1G).

**Quantitative analysis of TUNEL-positive cells.** Sections processed for TUNEL staining revealed a few profiles of TUNEL-positive nuclei in the hippocampus of the 16 week SHR and WKY rats (13.7 and 13.4%, respectively; Fig. 2A and D). No statistically significant alteration was observed between the 16 week SHR and WKY rats. The apoptotic ratio of cells was reduced in the SHR group in comparison with the age-matched WKY rats. In the hippocampus of the 32-week-old SHRs, 24.9% of nuclei were TUNEL-positive (Fig. 2B). This number was reduced to 14.5% in the 32-week-old WKY group (Fig. 2E). A total of 46.8% TUNEL-positive nuclei were observed in the CA1 subfield of the 64-week-old SHRs (Fig. 2C), while the 64-week-old WKY group exhibited 22.2% TUNEL-positive nuclei (Fig. 2F). Apart from the 16-week-old group, the 32 and 64-week-old SHR groups were significantly different compared with the age-matched WKY groups ( $P < 0.01$ ). In addition, there were significant differences between the three SHR groups ( $P < 0.01$ ; Fig. 2G).

**RT-qPCR assay.** The expression of PPAR- $\gamma$  mRNA in the SHR group reduced progressively with increasing age ( $P < 0.01$ , compared within SHR groups). Compared with the age-matched WKY group, the 32 and 64-week-old SHR groups demonstrated a significantly reduced level of PPAR- $\gamma$  mRNA expression ( $P < 0.01$ ). In addition, aging resulted in an age-dependent reduction of PPAR- $\gamma$  mRNA expression levels in the WKY group. The 64-week-old WKY group were observed to be significantly different compared with the 16-week-old WKY group ( $P < 0.05$ ; Fig. 3A).

The expression levels of Bax and caspase-3 mRNA increased progressively with increasing age ( $P < 0.01$ , compared within SHR groups). The 32 and 64-week-old SHR groups were significantly different compared with the age-matched WKY groups ( $P < 0.01$ ; Fig. 3B and C). Compared with the 16-week-old group, the 64-week-old group exhibited



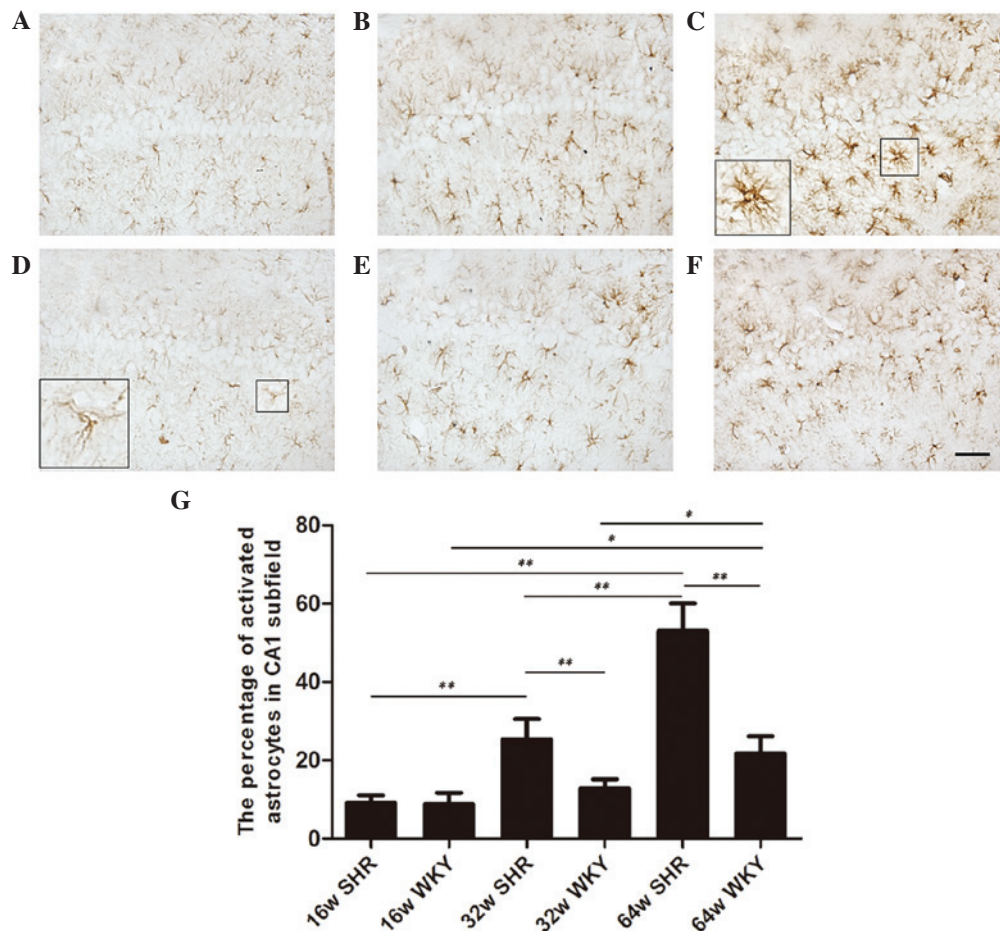


Figure 1. GFAP expression in the CA1 subfield of the hippocampi of SHRs and WKY rats aged 16, 32 and 64 weeks. The ratio of activated astrocytes increased progressively with increasing age in 16, 32 and 64-week-old (A-C) SHRs and (D-F) WKY rats. (D) The WKY group exhibited thin, shallow, dark-brown astrocytes with slender branches, while (C) the SHR group exhibited activated astrocytes with large cell bodies and thick branches. (G) The percentage of active astrocytes in the CA1 subfield of the hippocampus. The results are presented as the ratio of the number of activated astrocytes vs. the total number astrocytes in the CA1 subfield. The values are presented as the mean  $\pm$  standard error. \* $P < 0.05$ ; \*\* $P < 0.01$ . GFAP, glial fibrillary protein; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

a significant difference in caspase-3 mRNA expression levels ( $P < 0.01$ ; Fig. 3B). Aging resulted in an age-dependent reduction of Bcl-2 mRNA expression levels in the SHR group ( $P < 0.01$ , 16 vs. 32 and 64 weeks). Compared with the age-matched WKY groups, the Bcl-2 mRNA expression levels were observed to be significantly different in the 32 and 64-week-old SHR groups ( $P < 0.01$ ; Fig. 3D).

**Western blot analysis.** As shown in Fig. 4, the protein expression levels of PPAR- $\gamma$  were progressively reduced with increasing age in the SHR groups ( $P < 0.01$ , 16 vs. 32 week;  $P < 0.05$ , 32 vs. 64 week). Apart from the 16-week-old group, the 32 and 64-week-old SHR groups showed a significant difference compared with the age-matched WKY groups ( $P < 0.01$ ). In addition, aging resulted in an age-dependent reduction of PPAR- $\gamma$  protein expression levels in the WKY groups. However, only the 64-week-old WKY group was significantly different compared with the 16-week-old WKY group ( $P < 0.05$ ).

iNOS and gp47<sup>phox</sup> are markers used for assessing oxidative stress in brain tissues. As shown in Fig. 5, aging resulted in a significant upregulation of the protein expression levels of iNOS ( $P < 0.01$ , 16 vs. 32 week;  $P < 0.05$ , 32 vs. 64 week) and

gp47<sup>phox</sup> ( $P < 0.01$ , compared within SHR groups) in the SHR groups. Compared with the age-matched WKY group, the 32 and 64-week-old SHR groups exhibited significant differences in iNOS ( $P < 0.05$  and  $P < 0.01$ , respectively) and gp47<sup>phox</sup> ( $P < 0.01$ ) expression levels. In addition, the 64-week-old WKY group showed significantly increased expression of gp47<sup>phox</sup> and iNOS protein compared with the 16-week-old WKY group ( $P < 0.05$ ).

**The expression of Bax, Bcl-2 and caspase-3.** As shown in Fig. 6, the protein expression levels of Bax, caspase-3 and the ratio of Bax/Bcl-2 were progressively increased with increasing age in SHR group ( $P < 0.01$ ). Apart from the 16-week-old group, the levels in the 32 and 64-week-old SHR groups were all significantly different compared with the age-matched WKY groups ( $P < 0.01$ ). In addition, the expression levels of Bax and caspase-3 protein in the 64-week-old WKY group were significantly increased compared with the 16-week-old WKY group ( $P < 0.05$ ,  $P < 0.01$ , respectively). However, the expression of Bcl-2 protein was progressively reduced with increasing age in the SHR groups ( $P < 0.05$ , 16 vs. 32 week;  $P < 0.01$ , 16 vs. 64 week), but there was not a significant difference between the 32 and 64-week-old

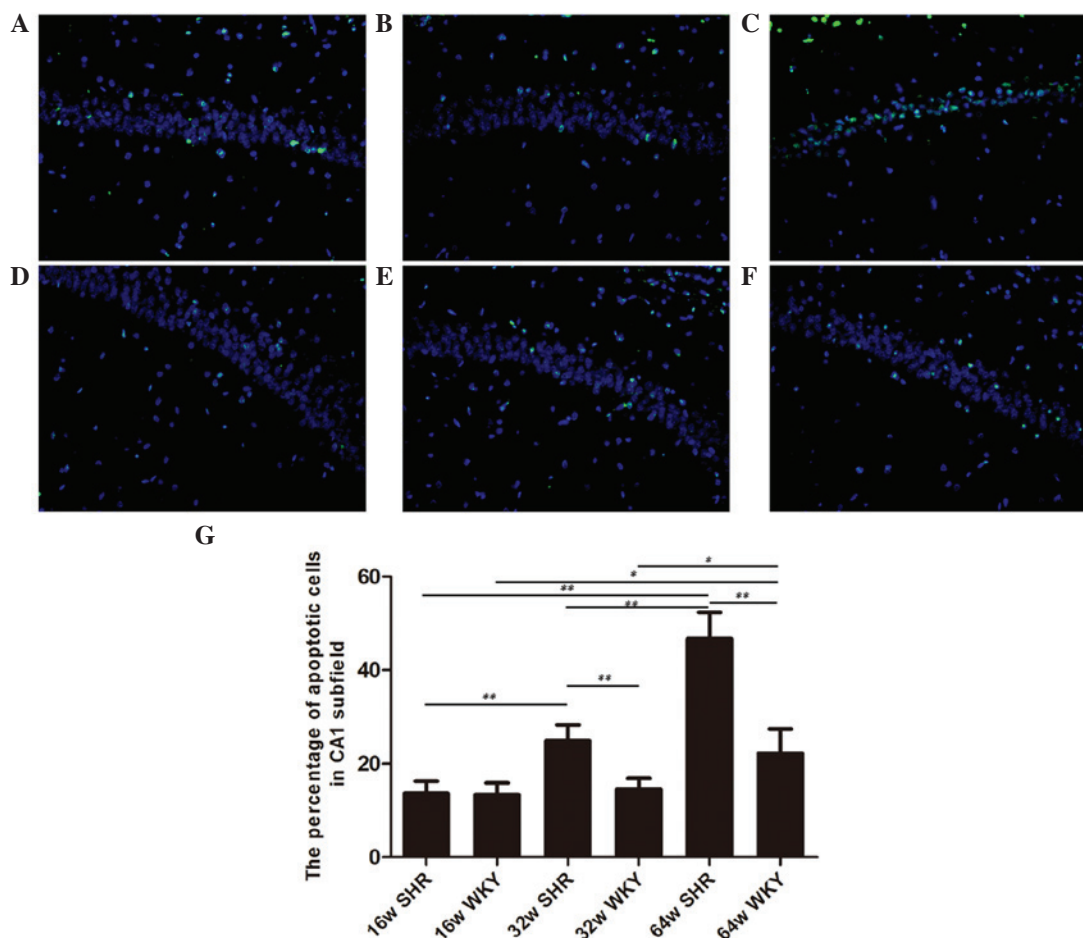


Figure 2. TUNEL assay in the CA1 subfield of the hippocampi of SHR and WKY rats aged 16, 32 and 64 weeks. The ratio of apoptotic cells increased progressively with increasing age in 16, 32 and 64-week-old (A-C) SHRs and (D-F) WKY rats. (G) The percentage of apoptotic cells in the CA1 subfield of the hippocampus. The results are expressed as the ratio of TUNEL-positive cells vs. total cells in the CA1 subfield. The data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ ; \*\* $P < 0.01$ . TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP end-labeling; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

SHR groups. Compared with the age-matched WKY group, the 32 and 64-week-old SHR groups exhibited significantly reduced Bcl-2 protein expression ( $P < 0.05$ ,  $P < 0.01$ , respectively).

## Discussion

The main findings of the current study was that SHRs exhibited an age-dependent increase in apoptotic cells in the hippocampus and that this was accompanied by increased levels of oxidative stress and reduced expression of PPAR- $\gamma$ . In addition, the degree of hypertensive brain damage, as indicated by activated astrocytes, was increased with increasing age, and the abnormally elevated Bax/Bcl-2 ratio may be involved in this process. To the best of our knowledge, the majority of previous studies assessing age-related oxidative stress levels and damage have been conducted in young/adult SHR models, ranging from 6-26 weeks of age (3,10,11). Few studies have examined the degree of age-related brain damage in the hippocampi of 32 and 64-week-old SHRs. The current study indicates that both aging and hypertension are able to exacerbate brain damage in SHRs, which was shown by the increased proportion of apoptotic cells and the expression of

oxidative stress markers. The reduced expression of PPAR- $\gamma$  may contribute to the age-related brain damage observed in SHRs.

SHRs represent a model of chronic hypertension, sharing several similarities with human hypertension and exhibiting behavioral and brain morphological alterations when compared with age-matched normotensive animals (16). Previous studies have observed significant reductions in the volume of grey matter, and a reduction in the number of neurons in the CA1 subfield of hippocampi in 6-7 month old SHRs, indicating that the volume reduction in grey matter potentially reflects neuronal loss occurring in certain hippocampal regions in SHRs (17,18). These previous studies suggest that depressed neuronal activity in SHRs occurs around 6 months of age (6,19). In the present study, no significant difference was observed in the ratio of TUNEL-positive cells between 16-week-old SHR and WKY groups, while the 32 and 64-week-old SHR groups showed a significant increase in the proportion of TUNEL-positive cells in the CA1 subfield of the hippocampus. The number of neurons in a given cerebral area is associated with the capability of the nervous system to receive, analyze and store information (20). The observation of increased levels of apoptotic cells in the hippocampus of SHR and aged WKY

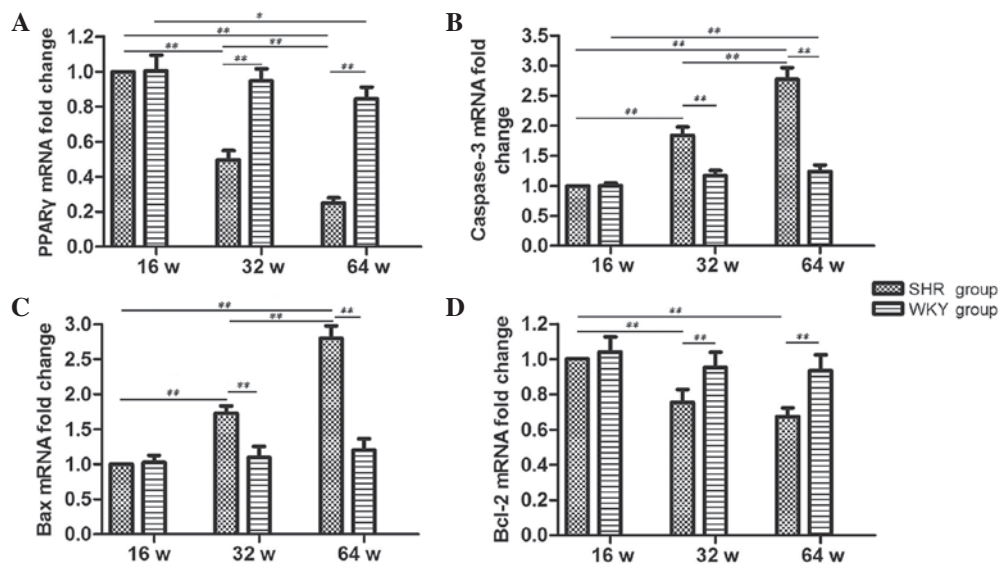


Figure 3. mRNA expression of (A) PPAR- $\gamma$ , (B) caspase-3, (C) Bax and (D) Bcl-2 in the hippocampi of SHRs and WKY rats aged 16, 32 and 64 weeks. Aging resulted in an age-dependent increase of Bax and caspase-3 mRNA expression and a reduction of PPAR- $\gamma$  and Bcl-2 expression in the SHR groups. Compared with the age-matched WKY groups, the 32 and 64-week-old SHR groups exhibited significantly elevated Bax mRNA expression and reduced Bcl-2 mRNA expression. The data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ ; \*\* $P < 0.01$ . PPAR- $\gamma$ , peroxisome proliferator-activated receptor; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

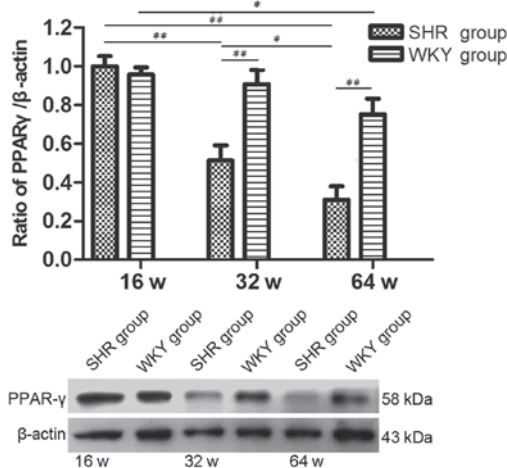


Figure 4. The protein expression levels of PPAR- $\gamma$  in the hippocampi of SHRs and WKY rats aged 16, 32 and 64 weeks. The data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ ; \*\* $P < 0.01$ . PPAR- $\gamma$ , peroxisome proliferator-activated receptor; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

rats suggests that brain function may undergo a progressive impairment.

The specificity of the TUNEL technique for apoptosis has been questioned, as it may detect both apoptotic and necrotic nuclei (21). Although based on the TUNEL data it is not possible to conclude whether the CA1 neurons were undergoing apoptosis and/or necrosis, the demonstration of TUNEL-positive nuclei in the current study suggests the occurrence of cell death in the hippocampi of SHRs (22). This observation is consistent with a previous report which showed that excessive reactive oxygen species (ROS) induced by hypertension damaged neuronal components and lead to neuronal loss via apoptosis or necrosis (23). The demonstration of GFAP-immunoreactive

astrocytes in the present study strengthens the suggestion of neuronal damage in the hippocampus of SHRs. GFAP is a cell-specific marker which distinguishes astrocytes from other glial cells. Increased expression of GFAP is considered to be an indicator of brain injury (24). The increased number of activated astrocytes in 32 and 64-week-old SHRs potentially indicates the detrimental effects of hypertension on the brain, and the attempt of astrocytes to protect the neuronal microenvironment.

Oxidative stress is suggested to be an important factor in aging and numerous age-related diseases (25), including essential hypertension (10). This is based on the presence of increased production of ROS, reduced NO synthesis and the reduced bioavailability of antioxidants, which have been verified in experimental and human hypertension (10,26). To date, oxidative stress has been demonstrated in patients with hypertension (27,28) and experimental hypertensive models (29-32). As the most active organ with the greatest oxygen consumption and richness of fatty acids, the brain is more susceptible to oxidative stress injury than other organs, thus suggesting that oxidative stress may serve an important role in hypertension-associated brain damage (33). This is supported by evidence that the activation of cerebral nicotinamide adenine dinucleotide phosphate (NADPH) oxidase preceded cerebral inflammation and cellular apoptosis (34). gp47<sup>phox</sup> is an important subunit of the NADPH oxidase complex, and functions in the spatial organization of the various subunits of the enzyme (11,35). It has been observed that the enhancement of NADPH oxidase activity in SHRs was associated with an increase in gp47<sup>phox</sup> expression in vessels (11), which indicates that gp47<sup>phox</sup> is an important marker for assessing oxidative stress injury in SHRs. In addition, iNOS has been considered to be a marker of oxidative stress due to its ability to generate toxic levels of nitric oxide, which subsequently lead to cellular apoptosis or necrosis in the brain (36,37). In



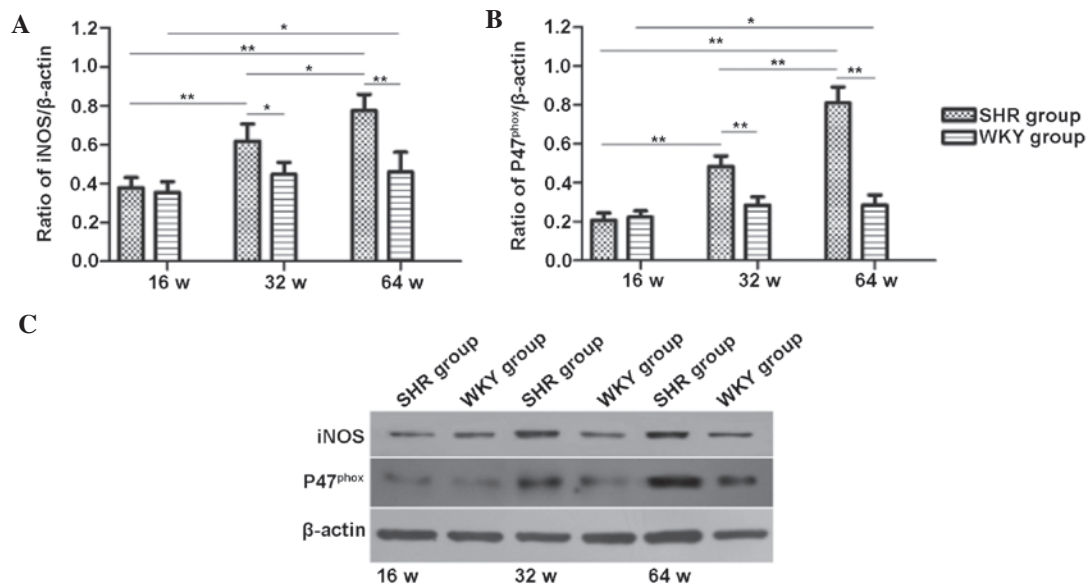


Figure 5. Quantification of (A) iNOS and (B) gp47<sup>phox</sup> protein expression, and (C) the corresponding western blot analysis, in hippocampi of SHRs and WKY rats aged 16, 32 and 64 weeks. The data are presented as the mean ± standard error. \*P<0.05; \*\*P<0.01. iNOS, inducible nitric oxide synthase; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

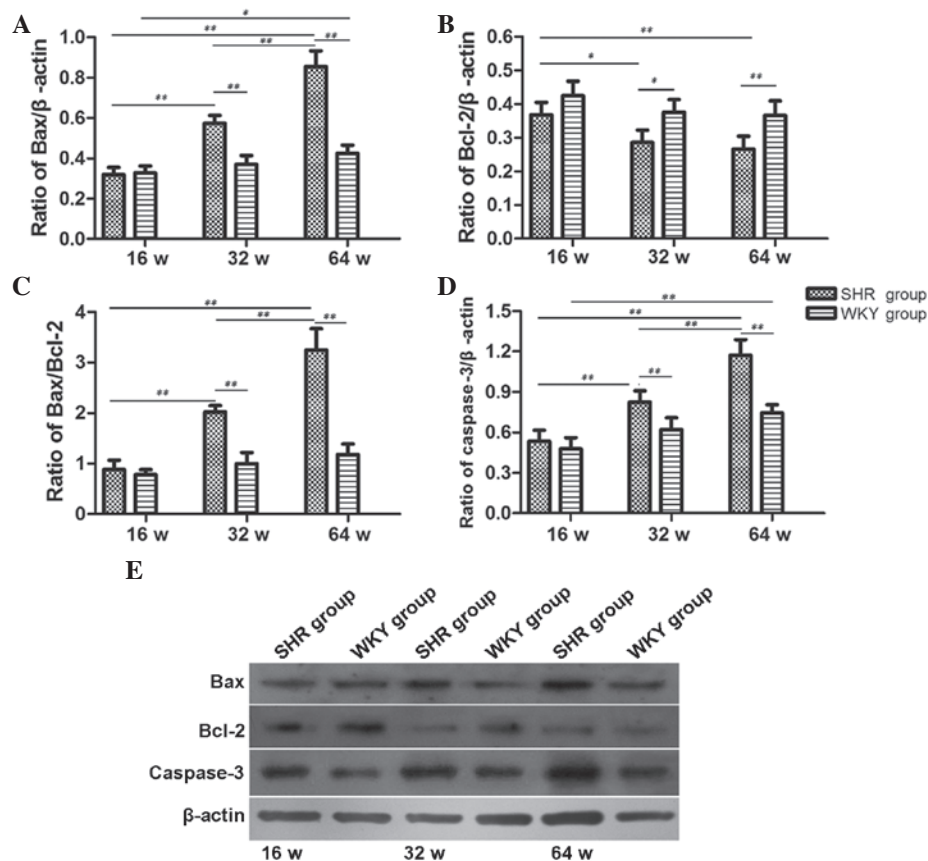


Figure 6. Protein expression levels of (A) Bax, (B) Bcl-2, (C) Bax/Bcl-2 and (D) caspase-3, and (E) the corresponding western blot analysis, in the hippocampi of SHRs and WKY rats aged 16, 32 and 64 weeks. Aging resulted in progressively increased expression of (A) Bax and (D) caspase-3 protein and (B) reduced Bcl-2 protein expression in the SHR groups. Compared with the age-matched WKY groups, the 32 and 64-week-old SHR groups exhibited significantly increased Bax and caspase-3 protein expression levels and reduced Bcl-2 protein expression. The ratio of Bax/Bcl-2 was increased with increasing age in the SHR groups. (C) The 32 and 64 week SHR groups were significantly different compared with the age-matched WKY groups. The data are presented as the mean ± standard error. \*P<0.05; \*\*P<0.01. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; w, weeks.

the current study, the increase in the protein expression levels of gp47<sup>phox</sup> and iNOS provides additional support for increased

ROS production and oxidative stress in the brains of SHRs and aged WKY rats.

PPAR- $\gamma$  is a ligand-activated transcription factor and serves beneficial roles in inhibiting inflammation and tissue injury (38). Recently, PPAR- $\gamma$  has been suggested to be involved in the oxidative stress response. Oxidative stress can attenuate PPAR- $\gamma$  expression and activity through the suppression of PPAR- $\gamma$  transcription (13). A previous study reported that once activated, the PPAR- $\gamma$  agonist was able to protect QZG cells against oxidative stress injury (14). Therefore, PPAR- $\gamma$  may participate in oxidative stress-related injury and exert beneficial effects. To the best of our knowledge, a systematic analysis of the expression profile of PPAR- $\gamma$  protein in SHRs of different ages has not been conducted. Diep *et al* (39) reported that PPAR- $\gamma$  expression increases with age during the development of hypertension. Wu *et al* (40) observed that the basal protein expression levels of PPAR- $\gamma$  in vascular tissues did not differ between the prehypertensive stage (5 weeks) or the evolving hypertensive state (13 weeks) of SHRs compared with age-matched WKY rats, while the protein levels of PPAR- $\gamma$  were lower in 21-week-old SHRs compared with age-matched WKY rats (40). In the present study, it was observed that PPAR- $\gamma$  expression was reduced with increasing age in the hippocampi of SHRs. Whilst this conflicts with the previous reports that PPAR- $\gamma$  expression is elevated in the brains of SHRs (41), the difference may be due to the increased age of the SHRs used in the present study. The current study demonstrated that SHRs exhibit an age-dependent reduction in PPAR- $\gamma$  expression in the hippocampus, which may be involved in the hypertension-associated oxidative stress injury.

Bax is a member of the Bcl-2 family, and promotes apoptosis, while Bcl-2 blocks cell death (42). In a previous study, 32-week-old SHRs exhibited increased mRNA expression of Bax and reduced mRNA expression of Bcl-2 (43). The Bax/Bcl-2 ratio is a widely used parameter to determine cell susceptibility to apoptosis, and in the present study, an age-dependent increase in the Bax/Bcl-2 ratio was observed in the 32 and 64-week-old SHRs. The increase in oxidative stress markers and apoptosis regulatory proteins observed in the hippocampi of SHRs may be, at least in part, responsible for the increased proportion of apoptotic cells in these rats. However, the present study also observed that the 64-week-old WKY rats exhibited significantly increased active astrocytes and TUNEL-positive cells compared with 16 and 32-week-old WKY rats, which was accompanied by increased levels of oxidative stress markers and proapoptotic proteins. We hypothesized that the results reported in the present study may arise for two reasons: i) aging is an important factor in inducing cellular apoptosis; and ii) normotensive WKY rats may develop obesity with as they age. Common mechanisms leading to oxidative stress may underlie hypertension and obesity (44).

In conclusion, the present study demonstrated that SHRs and aged WKY rats exhibited increased apoptotic cells in the hippocampus, with this accompanied by increased levels of oxidative stress, suggesting the possible role of oxidative stress in hypertension-associated brain damage. Aging resulted in an age-dependent increase in oxidative stress and apoptotic cells in the hippocampi of SHRs. Reduced mRNA and protein expression of PPAR- $\gamma$  in the hippocampus may be involved in hypertension-associated oxidative stress injury. Further studies should be undertaken to further dissect the mechanisms and signaling pathways involved.

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