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The new role of LOX-1 in hypertension induced neuronal apoptosis

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ABSTRACT

Lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) was originally identified as a receptor for oxLDL predominantly expressed in endothelial cells. Recently up-regulation of LOX-1 has been implicated in oxidative stress and cell apoptosis in many cell types. However, LOX-1 expression in neurons or regulation of neuronal apoptosis by LOX-1 has not been reported. To investigate the possible roles of LOX-1 in hypertension induced brain damage, we examined the distribution of LOX-1 in cortex and hippocampus and compared its expression in 32-week-old SHR and WKY rats. Immunofluorescence revealed that LOX-1 positive cells were located principally at the cortex involved in sensory information processing and were mainly expressed in neurons. We also found up-regulated mRNA expression of LOX-1, Bax and caspase-3 and down-regulated mRNA expression of Bcl-2 in SHR group. Compared with WKY group, SHR group showed increased LOX-1 positive cells and TUNEL positive cells. Furthermore, double-labeling method indicated that LOX-1 expression was colocalized with TUNEL positive cells, which means that LOX-1 expression was involved in hypertension related cell apoptosis. These findings indicated that LOX-1 expression was up-regulated in the cortex of SHR and its expression has implication in neuronal apoptosis. Elevated Bax/Bcl-2 ratio may be involved under this event.

1. Introduction

LOX-1 is a type II membrane protein receptor for oxLDL. It has diverse roles in the host-defense system and inflammatory responses, and is involved in the pathogenesis of various diseases such as hypertension, atherosclerosis and cardiovascular diseases [1]. Recent studies showed that LOX-1 is a multiple ligand receptor induced by reactive oxygen species (ROS) and oxidative stress, and its expression can further exacerbate oxidative stress [2], thus plays an important role in oxidative stress induced cell injury [3]. It has been demonstrated that LOX-1 expressed in endothelial cells [4], smooth muscle cells [5] and monocytes/macrophages [6] are all involved in cell apoptosis. Microglia is termed as "macrophages in the central nervous system (CNS)" and it has been suggested that LOX-1 may be expressed in microglia other than vascular endothelial cells in stroke [7].

Spontaneously hypertensive rats (SHR), which are normotensive at birth and develop sustained hypertension between 3 and 6 months of age, are the most extensively investigated model for evaluating hypertensive brain damage and its treatment [8]. The time-dependent rise of arterial blood pressure (BP), occurrence of brain atrophy and glial reaction, and loss of nerve cells are shared to some extent with what occurs in human hypertensive brain [9]. SHR, therefore, can represent a reasonable model of hypertension related brain damage. Oxidative stress is believed to play an important role in the development of hypertension and hypertension related organ damage [10]. Increased production of superoxide anion and hydrogen peroxide has been demonstrated in experimental hypertensive models [11] and human hypertension [12] based on increased levels of biomarkers of lipid peroxidation and oxidative stress.

However, there has been no exact evidence about LOX-1 expression in neurons or regulation of neuronal apoptosis by LOX-1 in SHR rats. This study was designed to explore the expression and distribution of LOX-1 in SHR and age-matched WKY rats and evaluate its role on hypertension induced neuronal apoptosis and its possible molecular mechanisms.

2. Materials and methods

2.1. Animals

Thirty-two-week-old male SHR ($n = 8$) and WKY rats ($n = 8$) were provided by Shanghai Slac Laboratory Animal Center. All procedures were conducted according to guidelines established by the Institutional Animal Care and Use Committee and The National

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Institutes of Health. The rats were housed under humidity-, temperature-, and light cycle-controlled conditions, with free access to food and water. BP values were measured by an indirect tail-cuff method. Before killing, rats were anesthetized with chloral hydrate (10%). One half of the animals in each group were sacrificed by decapitation. The brains were kept in the sample protectors (TaKaRa) in liquid nitrogen for realtime RT-PCR. The other animals were perfused intraventricularly with saline and 4% paraformaldehyde (PFA). Then brains were removed and postfixed in 4% PFA overnight. Targeted brain pieces were chosen, dehydrated, embedded and cut into 10-μm-thick sections. Three sections containing both the cortex and hippocampus were chosen from each rat for immunohistochemical analysis.

2.2. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cortex with the use of Trizol reagent (Invitrogen) and the RNA samples were transcribed to cDNA with the PrimeScript RT Master Mix kit (TaKaRa) according to the manufacturer's instructions. Real-time RT-PCR was performed with SYBR ExScript RT-PCR Kit (TaKaRa) on an iQ Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA). The primers for rat LOX-1(forward: 5′-CTGATCAGAAGTCATGTGGCAAGAA-3′, reverse: 5′-GGATGGCCAGAGTCACAGCA-3′; Caspase-3 (forward: 5′-GAGACAGACAGTGGAACTGACGATG-3′, reverse: 5′-GGCGCAAAGTGACTGGATGA-3′); Bax (forward: 5′-GCGTCCACCAAGAAGCTGA-3′, reverse: 5′-ACCACCCTGGTCTTGGATCC-3′); Bcl-2 (forward: 5′-GACTGAGTACCTGAACCGGCATC-3′, reverse: 5′-CTGAGCAGCGTCTTCAGAGACA-3′) and B-actin (forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′, reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′) were designed and synthesized by TaKaRa Biotechnology. Amplification was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard edition optical system software (Bio-Rad). Relative quantification by the comparative method ($2^{-\triangle\triangle Ct}$) was used and data was presented as the mean ± SD of three separate experiments done in triplicate.

2.3. Immunohistochemistry

Sections were deparaffinized with xylene and rehydrated according to standard protocols. Then they were blocked with 10% goat serum for 1 h, followed by incubation with rabbit anti-LOX-1 antibody (1:100, ABcam) at 4 °C overnight. After being washed in PBS, the sections were incubated with FITC-labeled goat anti-rabbit IgG (1:100, Invitrogen) and counterstained with 4′,6′-diamino-2-phenylindole (DAPI, 1:1000, Sigma–Aldrich). Fluorescence microscopy was carried out using an Olympus BX51 microscope equipped with a mercury lamp power supply. For negative controls, primary antibody was replaced with PBS.

Double immunostaining was performed to identify the cell type that LOX-1 is expressed. Briefly, the sections were treated with 0.3% Triton for 30 min, followed by incubation with 10% goat serum for 1 h. Then they were incubated overnight at 4 °C with the mixture of mouse anti-NeuN (or goat anti-IBA-1) (1:100, ABcam) and rabbit anti-LOX-1 primary antibodies. After being washed, sections were incubated with CY3-labeled goat anti-mouse IgG (or CY3-labeled mouse anti-goat IgG) (1:100, Boster) and FITC-labeled goat anti-rabbit IgG (1:100, Invitrogen) for 2 h, respectively. Cell nuclei were counterstained by DAPI.

2.4. Terminal dUTP nick end-labeling (TUNEL) and immunofluorescence double-labeling assay

To identify whether LOX-1 expressing cells were undergoing apoptosis, a double-labeling assay for detecting apoptotic cells, using TUNEL assay and antigens of LOX-1, using immunofluorescence, was performed [13]. Briefly, sections were blocked with 10% goat serum, and then incubated with rabbit anti-LOX-1 antibody at 4 °C overnight. After washes, sections were incubated with TRITC-labeled goat anti-rabbit IgG (1:100, invitrogen) for 2 h. Following washing and rinsing, the sections were treated with 20 μg/ml proteinase K solution for 15 min, followed by incubation with equilibration buffer for 30 min and TdT reaction mixture for 2 h, respectively. The slides were then immersed in 2 × SSC to stop the reaction and DAPI was added on the sections and mounted.

TUNEL assay (promega) was performed to analyze the apoptotic ratio of cells in the secondary auditory cortex of SHR and WKY rats. The procedure was conducted according to the manufacturer's instructions as described above. The number of LOX-1 positive neurons and TUNEL positive cells was counted restricted to III, IV and V layers of the secondary auditory cortex in which region LOX-1 was mainly expressed. TUNEL positive cells were normalized to DAPI stained cells. Immunoreactive cells from 9 random fields (3 samples per group, 3 fields per sample) were counted using a 20 objective lens by an observer blind to the treatment groups.

2.5. Statistical analysis

The quantitative data were represented as mean ± SD and analyzed using SPSS 16.0 software. Statistical comparisons between groups were carried out using Student's *t* test and one-way ANOVA. $P < 0.05$ was considered to be significant.

3. Results

3.1. SHR group showed higher systolic blood pressure (SBP)

We measured the SBP level in two groups. SBP values were 103.5 ± 3.6 mm Hg in WKY group and 184.4 ± 8.1 mm Hg in SHR group ($p < 0.01$). The results indicated that SHR group has significantly higher SBP than age-matched WKY group.

3.2. Distribution of LOX-1 expression in SHR and WKY rats

To verify whether LOX-1 protein was expressed in CNS, we performed immunofluorescence with anti-LOX-1 antibody. Since LOX-1 was a membrane glycoprotein with a C-type lectin-like extracellular domain and a short cytoplasmic tail [4], it was mainly located in the membrane of cells. In this study, we focused on the cortex and hippocampus (Fig. 1A) and found that a clear anti-LOX-1 labeling was obvious in some selected brain regions of cortex, such as the primary and secondary auditory cortex, ectorhinal cortex, perirhinal cortex, lateral entorhinal cortex, piriform cortex and posterodorsal and posteromedial cortical amygdaloid nuclei (Fig. 1B–H). However, only a non-specific staining was observed in the hippocampus (Fig. 1I). We also found that LOX-1 positive cells were distributed in different layers of cerebral cortex depending on the anatomical regions. It was mainly expressed in the II and III layers of lateral entorhinal cortex, piriform cortex and cortical amygdaloid nucleus, and in the III, IV and V layers of the auditory cortex, ectorhinal cortex and perirhinal coretx.

3.3. LOX-1 expressed in neuron in the CNS

We performed double immunostaining using anti-LOX-1 antibody and anti-NeuN antibody (or anti-IBA-1 antibody) to examine whether LOX-1 expressing cells were neurons or microglias. Neuron nuclear antigen (NeuN) is a neuronal nuclear antigen which marks mature neurons and ionized calcium binding adaptor molecule 1 (IBA-1) was specifically expressed in activated microglia

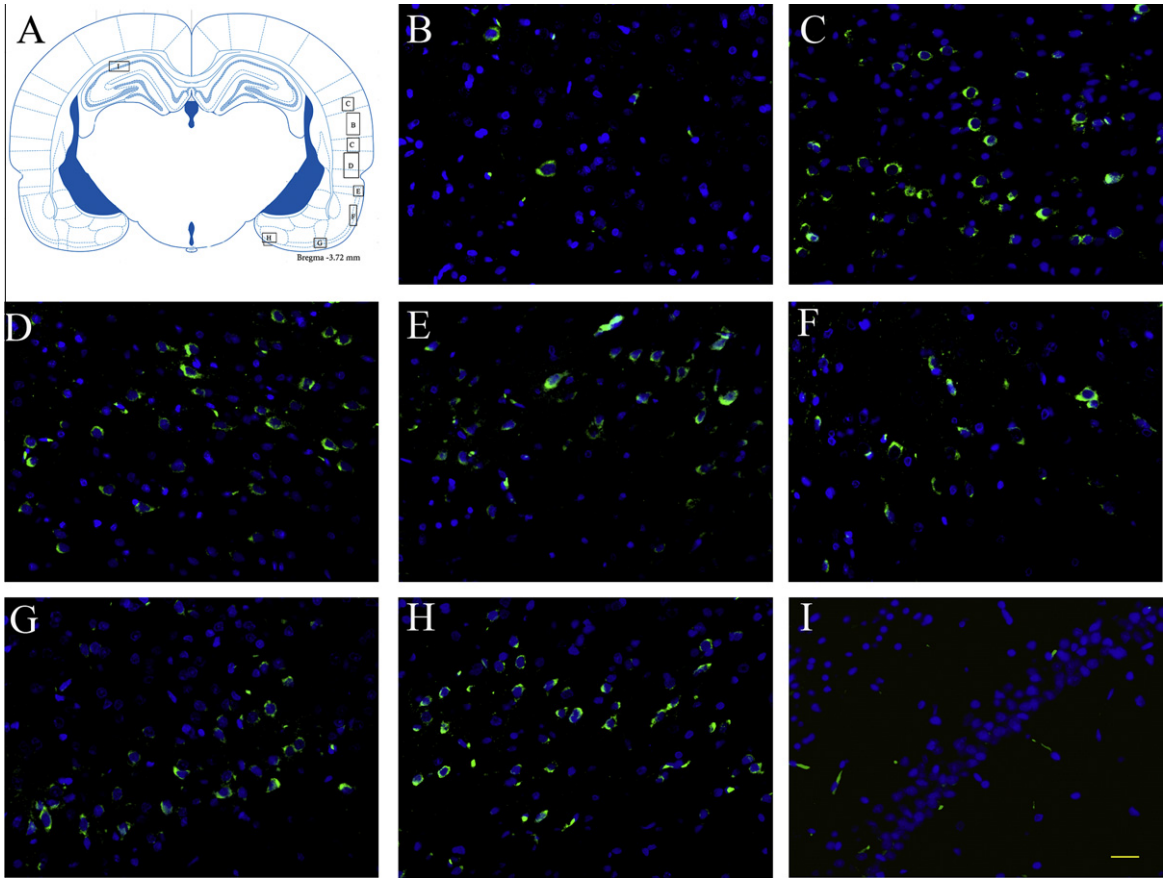

Fig. 1. Immunostaining of anti-LOX-1 antibody in cortex and hippocampus of SHR. Brain sections were stained with rabbit anti-LOX-1 antibody and DAPI. The anatomy level and positive region were shown in sketch map (A). LOX-1 (green color) was expressed in primary (B) and secondary (C) auditory cortex, Ectorhinal cortex and perirhinal cortex (D), lateral entorhinal cortex (E), piriform cortex (F), posterodorsal cortical amygdaloid nucleus (G) and posteromedial cortical amygdaloid nucleus (H), but not in hippocampus (I) (bar = 25 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

among brain cells. As shown in Fig. 2, IBA-1 positive cells were triangle or amoebae shape with a smaller nuclear, which means microglia was under active state in hypertension, while NeuN positive cells showed round or ellipse shape with a larger nuclear. By double immunostaining, we found that LOX-1 positive cells were all NeuN positive cells (neurons) rather than IBA-1 positive cells (microglias). Thus, LOX-1 was exclusively expressed in neurons.

3.4. SHR group showed increased expression of LOX-1 and apoptotic cells

To determine whether LOX-1 plays an important role in the cell apoptosis process in hypertension. We performed real-time RT-PCR to analyze the change of LOX-1 and caspase-3 mRNA levels in SHR and WKY group. We also compared the number of LOX-1 positive neurons and TUNEL/DAPI positive cell ratios in the secondary auditory cortex in two groups. The results indicated that LOX-1 and caspase-3 mRNA were both elevated in SHR group (Figs. 3B and 4B, $p < 0.05$, respectively). Compared with age-matched WKY group, LOX-1 positive neurons and the proportion of apoptotic nuclei were both increased significantly in the secondary auditory cortex in SHR group (Figs. 3A and 4A, $p < 0.01$, respectively). These results revealed that hypertension up-regulated LOX-1 expression and promoted cell apoptosis in cortex.

3.5. Role of LOX-1 expression in cell apoptosis

To further detect the possible effect of LOX-1 expression on cell apoptosis, double immunostaining using anti-LOX-1 antibody and TUNEL method was performed in cortex of SHR. As demonstrated in Fig. S1, LOX-1 positive cells were all TUNEL positive cells, which indicated that LOX-1 expression was colocalized with apoptotic cells. Thus, LOX-1 expression in neuron may be related to neuronal apoptosis.

3.6. Bax and Bcl-2 mRNA expression in the two groups

To explore the possible mechanism under LOX-1 related neuronal apoptosis, we assessed Bax and Bcl-2 mRNA expression in cortex in the two groups. As shown in Fig. S2, SHR group showed up-regulated mRNA expression of proapoptotic factor Bax (Fig. S2 A, $p \leqslant 0.01$) and down-regulated mRNA expression of antiapoptotic factor Bcl-2 (Fig. S2 B, $p \leqslant 0.05$). Therefore, hypertension elevated Bax/Bcl-2 ratio of mRNA expression significantly (Fig. S2 C, $p \leqslant 0.01$).

4. Discussion

In this research, we found that LOX-1 expression was increased in SHR group and was mainly distributed in the cortex involved in sensory information processing. Instead of microglia, LOX-1 was expressed in neuron and its expression was highly colocalized with the apoptotic cells. Elevated Bax/Bcl-2 expression ratio may be involved under this event. To our knowledge, this is the first evidence to clearly show LOX-1 expression in neurons and verify the relationship between LOX-1 and neuronal apoptosis. It would be of interest to identify the signal pathway underline this phenomenon and this issue should be extensively investigated.

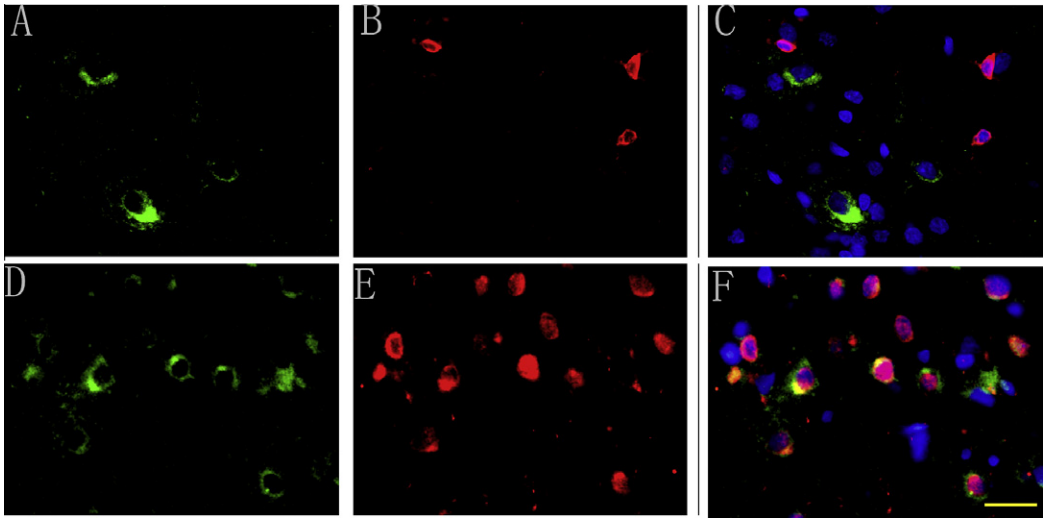

Fig. 2. Double immunostaining of anti-LOX-1 and anti-NeuN antibody or anti-IBA-1 antibody in cortex of SHR. LOX-1 was expressed in cell membrane (A, D, green color). IBA-1 was expressed in cell plasma (B, red color) and NeuN was expressed in cell nuclei (E, red color). All LOX-1 expressing cells were NeuN positive cells (C, E, yellow color) (bar = 25 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

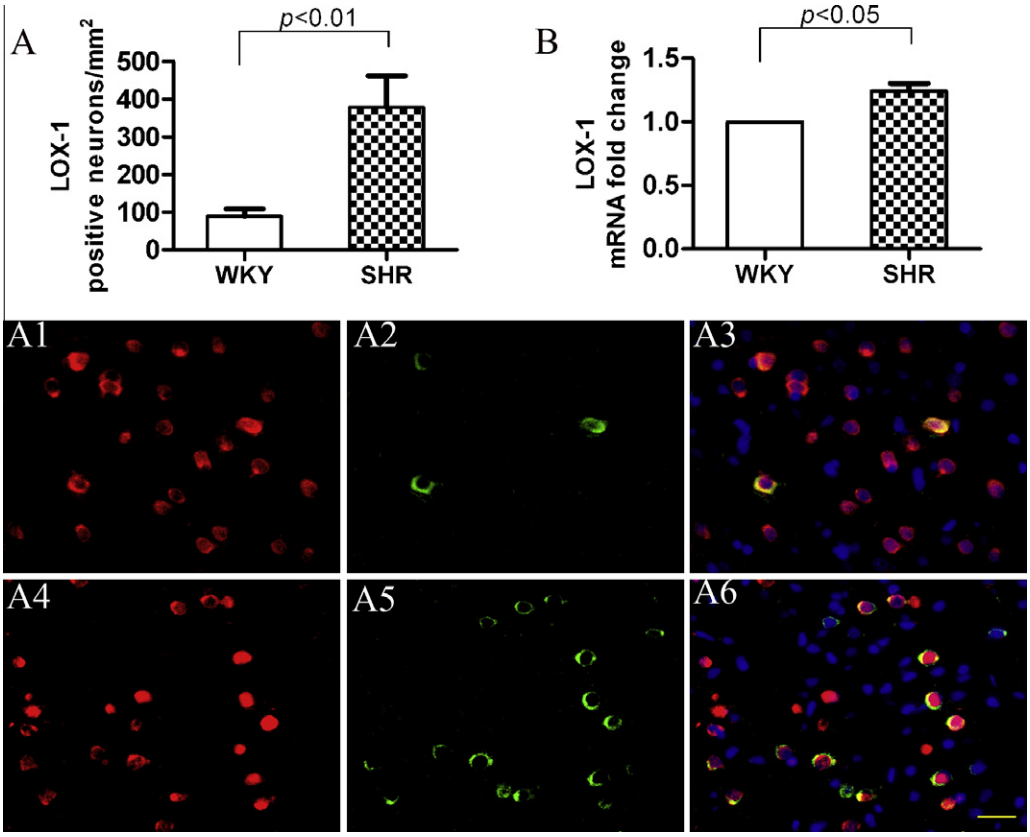

Fig. 3. Effects of hypertension on LOX-1 expression. A1–A3 and, A4 and A5 show LOX-1 positive neurons in WKY and SHR group, respectively (A1, A4, neuron, red color; A2, A5, LOX-1, green color; A3, A6, LOX-1 positive neuron, yellow color). (A) Shows LOX-1 positive neurons in the secondary auditory cortex of the two groups, and (B) shows the expression of LOX-1 mRNA levels. Hypertension increased the LOX-1 positive neurons in the secondary auditory cortex (bar = 25 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LOX-1 was originally identified to be a novel ox-LDL receptor in vascular bovine aortic endothelial cells [14]. Since discovered, it has been extensively discussed in the initiation and process of atherosclerosis and atherosclerosis related disorders [15]. Recently, LOX-1 was considered to be participated in hypertensive organ damage [2,16,17]. But little is known about whether LOX-1 is involved in hypertension related brain damage.

Hypertension is associated with increased oxidative stress. Although there is a still hot debate on whether oxidative stress is the cause or the result of hypertension [10], accumulating studies have generally supported that increased BP is associated with increased oxidative stress [10,18]. So far, oxidative stress has been demonstrated in hypertensive patients [19,20] and experimental hypertensive models [11,21–23]. Therefore, oxidative stress is a

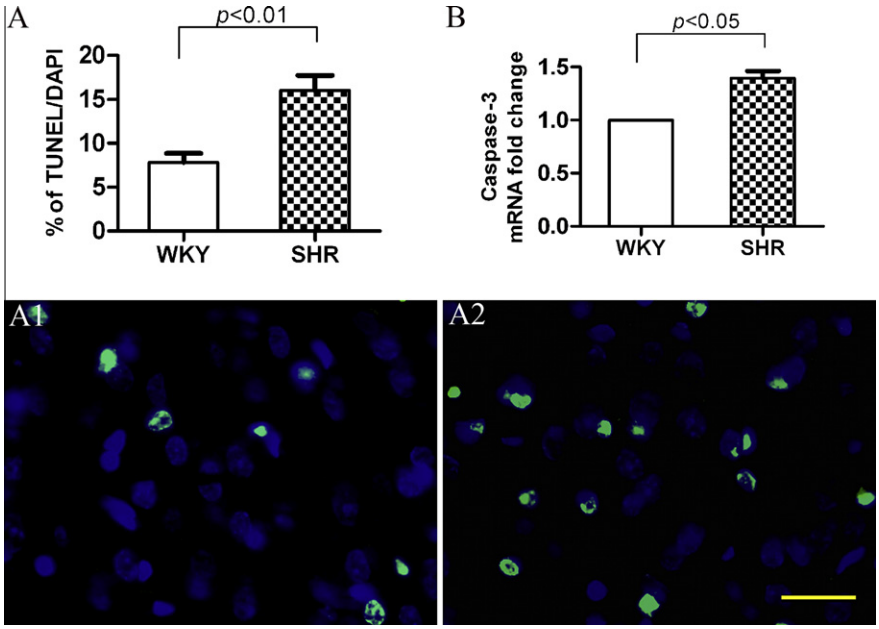

Fig. 4. Effects of hypertension on cell apoptosis by the TUNEL method. A1 and A2 show TUNEL positive cells in WKY and SHR group, respectively. (A) Shows the statistical percentage of apoptotic cells in the two groups, and (B) shows the expression of caspase-3 mRNA levels in two groups. Hypertension increased the apoptotic cell ratio in the secondary auditory cortex. (bar = 25 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

common trait of hypertensive conditions [24]. As the most active organ with greatest oxygen consumption and rich in fatty acids, brain is more susceptible to oxidative stress injury than other organs, thus suggesting that oxidative stress may play an important role in the hypertension related brain damage [25]. LOX-1 has intimate relationship with oxidative stress. The receptor can be induced or up-regulated by ROS and oxidative stress. Binding of LOX-1 to its multiple ligand can generate ROS and exacerbate oxidative stress [2]. This vicious cycle may exacerbate hypertension and its vascular damage. Hence, we inferred that LOX-1 may play part in hypertensive brain damage.

Though LOX-1 up-regulation has been extensively investigated in vessel and organ damage such as heart, kidney and lung, it has been confirmed only in brain abscess [26] and stroke [7] in CNS. Studies assessing LOX-1 expression in hypertensive brain are limited. We found hypertension up-regulated LOX-1 expression in 32-week-old SHR, compared with WKY group. To ascertain the effect of hypertension on LOX-1 expression, we also examined LOX-1 positive cells in 8-week-old SHR and WKY rats in preliminary test. No difference was found in SBP between the two groups (SHR group averaged 105.46 ± 4.44 mm Hg, while WKY group averaged 102.22 ± 3.33 mm Hg, $p > 0.05$). Moreover, we did not found any LOX-1 positive cells in the two groups. It indicates that LOX-1 expression is induced by hypertension or hypertension related pathological changes. But since LOX-1 expression can also be induced in other disease, we do not think LOX-1-related phenomenon is specific to hypertension-induced injury.

Schwarz et al. [7] examined LOX-1 mRNA expression in stroke and found it was mainly up-regulated in cortex and striatum other than hippocampus, which was consistent with our observations. They also found that LOX-1 was not expressed in vascular endothelial cells, but rather in a non-neuronal cell type. Thus, they inferred that LOX-1 expressed in microglia. Microglia is termed as "macrophages in the CNS" and plays an important role in neuro-inflammation injury. In addition, LOX-1 has been demonstrated to be expressed in macrophages [6]. Thus, we initially inferred that LOX-1 may be expressed in microglia and exert its effect on hypertension related neuro-inflammation. However, the results turned out that LOX-1 was expressed in neurons indicating that LOX-1 expression may have other effects other than neuro-inflammation. We also found LOX-1 expression was mainly distributed in cortex involved in sensory information processing, such as auditory cortex, perirhinal cortex, entorhinal cortex, piriform cortex and amygdaloid nuclei. The auditory cortex is the most highly organized processing unit of sound. We found, compared with the primary auditory cortex, LOX-1 was mainly expressed in the secondary part of auditory cortex. The perirhinal and ectorhinal cortex were involved in visual perception and memory. The entorhinal cortex plays a crucial role in spatial cognition. The function of piriform cortex and cortical amygdaloid nuclei are all related to the sense of smell and pheromone-processing. Thus, the cortices that LOX-1 expressed were all involved in sensory information processing in SHR, which indicates some interesting implications.

Since LOX-1 has been found to be involved in apoptosis of different cell types [4–6,27–29] and anti-LOX-1 therapy can provide protection on cell apoptosis [30], LOX-1 expression may be related to neuronal apoptosis. To verify our point of view, we performed Realtime RT-PCR to investigate the expression of LOX-1 and caspase-3 mRNA in two groups and found that LOX-1 and caspase-3 mRNA were both elevated in SHR group. Since LOX-1 expression was more obvious in the secondary auditory cortex, we also compared LOX-1 positive neurons and TUNEL/DAPI cell ratios in this region in two groups. We found that SHR group showed significantly increased LOX-1 positive neurons and elevated apoptotic cell ratio compared with age-matched WKY group. To further prove our point, we performed the double-labeling assay using TUNEL and immunofluorescence, which was a powerful tool to correlate apoptosis with specific antigen expression to identify whether factors were involved in the induction and execution of the apoptotic pathway [13]. In our study, apoptotic cells were consistently observed to be expressed in the cortex of SHR which suggests that cell apoptosis may be an important process in hypertension related brain damage. In addition, we found the expression of LOX-1 was colocalized with apoptotic cells, thus clearly verified that LOX-1 was involved in cell apoptosis. This is in line with the report that LOX-1 was colocalized with apoptotic

cells in atherosclerotic lesions [31]. Thus, we suggest that under hypertension, LOX-1 plays an important role in neuronal apoptosis. We also observed that not all TUNEL-positive cells were LOX-1 positive cells, which means LOX-1 related phenomenon is not a generic feature of neuronal apoptosis. LOX-1 pathway may be one of the mechanisms under hypertension-induced neuronal apoptosis. To explore the underlying mechanism, we further assessed mRNA expression of Bax and Bcl-2 in the two groups and found SHR group showed increased mRNA expression of Bax and decreased mRNA expression of Bcl-2. It indicates that the elevated Bax/Bcl-2 ratio, which is a widely used parameter to determine the cell susceptibility of apoptosis [32] may be involved under the event. LOX-1 has been demonstrated to increase Bax/Bcl-2 ratio or through caspase-3 and caspase-9 pathway to promote apoptosis in VSMC and endothelial cells [5,15,33]. Therefore, LOX-1 related Bax/Bcl-2 expression ratio elevation may be involved in LOX-1 induced neuronal apoptosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.143.

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