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Perindopril Attenuates Lipopolysaccharide-Induced Amyloidogenesis and Memory Impairment by Suppression of Oxidative Stress and RAGE Activation

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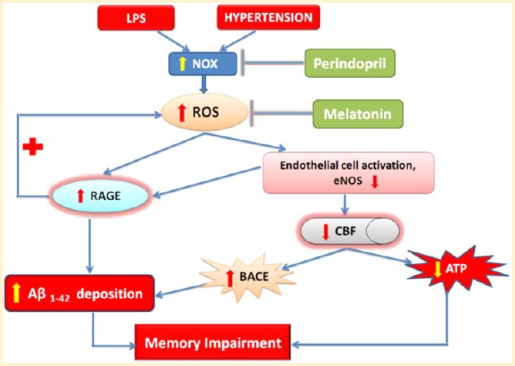
Supporting Information

ABSTRACT: Clinical and preclinical studies account hypertension as a risk factor for dementia. We reported earlier that angiotensin-converting enzyme (ACE) inhibition attenuated the increased vulnerability to neurodegeneration in hypertension and prevented lipopolysaccharide (LPS)-induced memory impairment in normotensive wistar rats (NWRs) and spontaneously hypertensive rats (SHRs). Recently, a receptor for advanced glycation end products (RAGE) has been reported to induce amyloid beta ($A\beta_{1-42}$) deposition and memory impairment in hypertensive animals. However, the involvement of ACE in RAGE activation and amyloidogenesis in the hypertensive state is still unexplored. Therefore, in this study, we investigated the role of ACE on RAGE activation and amyloidogenesis in memory-impaired NWRs and SHRs. Memory impairment was induced by repeated (on days 1, 4, 7, and 10) intracerebroventricular (ICV) injections of LPS in SHRs (25 μg) and NWRs (50 μg). Our data showed that SHRs exhibited increased oxidative stress (increased gp91-phox/NOX-2 expression and ROS generation), RAGE, and β-secretase (BACE) expression without $A\beta_{1-42}$ deposition. LPS (25 μg, ICV) further amplified oxidative stress, RAGE, and BACE activation, culminating in $A\beta_{1-42}$ deposition and memory impairment in SHRs. Similar changes were observed at the higher dose of LPS (50 μg, ICV) in NWRs. Further, LPS-induced oxidative stress was associated with endothelial dysfunction and reduction in cerebral blood flow (CBF), more prominently in SHRs than in NWRs. Finally, we showed that perindopril (0.1 mg/kg, 15 days) prevented memory impairment by reducing oxidative stress, RAGE activation, amyloidogenesis, and improved CBF in both SHRs and NWRs. These findings suggest that perindopril might be used as a therapeutic strategy for the early stage of dementia.



KEYWORDS: *Hypertension, oxidative stress, amyloidogenesis, memory impairment, angiotensin-converting enzyme, perindopril*

Evidence suggests that Alzheimer's disease (AD), the most common form of dementia (60−80%), apart from the amyloid beta ($A\beta_{1-42}$) deposition, is also characterized by sustained neuroinflammation and oxidative stress.[1,2] Recent studies have revealed that cardiovascular disease, such as hypertension, is also a risk factor for AD[3,4] and contributes to the cognitive deficit.[5] An increased amount of $A\beta_{1-42}$ deposition and neurofibrillary tangles has been reported in the brain of subjects with hypertension[6] and in mouse models of arterial hypertension.[7] Recently, it was shown that hypertension-induced $A\beta_{1-42}$ deposition was associated with increased expression of receptors for advanced glycation end products (RAGE).[8] RAGE is a multiligand receptor that, apart from binding with advanced glycation end products (main ligand of RAGE), mediates the transport of peripheral $A\beta_{1-42}$ into the brain.[9] Further, increasing evidence suggests that RAGE activation is also associated with increased oxidative stress[10,11] and progression of AD.[8]

Hypertension is a multifactorial disorder, but the renin angiotensin system (RAS) plays the major role in its regulation and development.[12,13] RAS not only regulates blood pressure but also plays an important role in regulation of cognitive functions.[14] The link between RAS and memory is further established when enhanced angiotensin-converting enzyme (ACE, an important component of RAS) activity has been reported in cerebro-spinal fluid (CSF) of AD brain[15] and ACE inhibition improved memory function in various rodent models of cognitive impairment.[16,17] Previously, we have reported that hypertension renders the brain more vulnerable to neurodegeneration and memory impairment because chronic inflammation induced by repeated (on days 1, 4, 7, and 10) intracerebroventricular (ICV) injections of lipopolysaccharide (LPS) at the dose of 25 μg impaired cognitive functions in spontaneously hypertensive rats (SHRs). However, this dose of LPS (25 μg, ICV) was unable to alter memory functions in normotensive wistar rats (NWRs), and a higher dose of LPS (50 μg, ICV) caused memory impairment in NWRs.[18] Additionally, chronic neuroinflammation was associated with increased activity and expression of ACE in the brain, and perindopril, an ACE inhibitor, prevented neurodegeneration and memory

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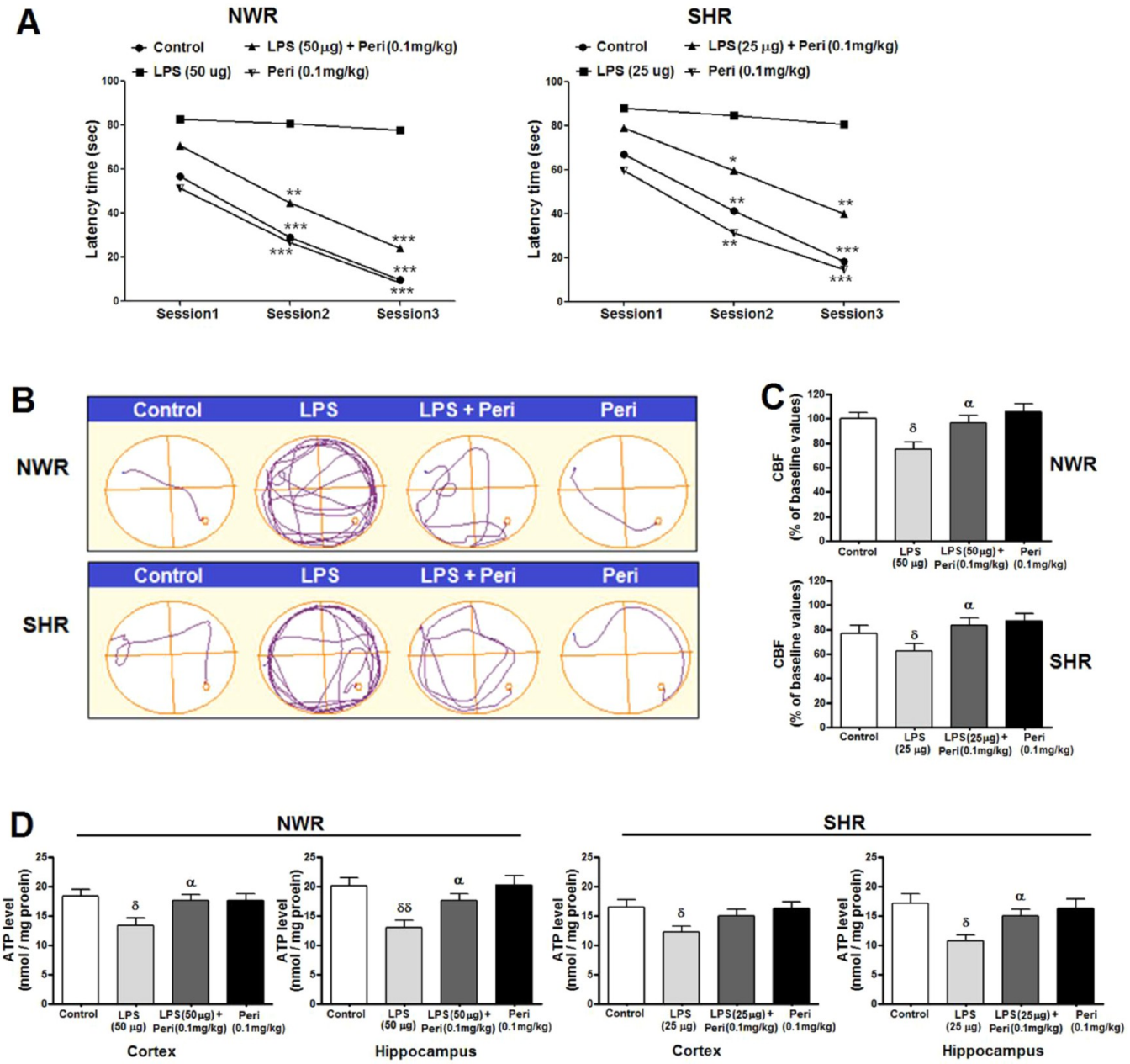

Figure 1. Effect of perindopril (0.1 mg/kg) on memory impairment, cerebral hypoperfusion, and brain energy metabolism following repeated administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Effect on latency time in the MWM test. Data are expressed as mean ± SEM. *Significant decrease (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) in comparison to session 1 of respective groups. (B) Representative MWM tracking of different groups of NWRs and SHRs during session 3. (C) Effect on cerebral blood flow (CBF). CBF was measured in arbitrary blood perfusion units and expressed as percentage of baseline blood perfusion unit ± SEM. (D) Effect on ATP level in cortex and hippocampus of both strains. Data values are expressed as mean ATP level (nmol/mg protein) ± SEM. $^{\delta}$Significant decrease ($^{\delta}p < 0.05$ and $^{\delta\delta}p < 0.01$) in comparison to respective control groups. $^{\alpha}$Significant increase ($^{\alpha}p < 0.05$) in comparison to respective LPS groups.

impairment in both SHRs and NWRs.[18] However, the involvement of ACE in modulation of RAGE activation and amyloidogenesis in a hypertensive state is still unexplored. Therefore, in the present study, the effect of ACE inhibition on RAGE expression and Aβ_{1-42} deposition was explored in SHRs and LPS (ICV)-induced memory-impaired SHRs and NWRs by employing perindopril at a non-antihypertensive dose.

RESULTS AND DISCUSSION

We have previously reported that hypertension renders the brain more vulnerable to dementia as repeated ICV injections of LPS at the dose of 25 μg resulted in memory impairment in SHRs but not in NWRs [a higher dose of LPS (50 μg) impaired memory functions], and ACE inhibition by perindopril prevented chronic neuroinflammation-induced memory impairment in both SHRs and NWRs.[18] However, the influence of ACE on RAGE expression and Aβ_{1-42} deposition in a hypertensive state is not well-explored, and the pathophysiological mechanistic link is also missing. In this study, we showed that control SHRs already exhibited increased RAGE and BACE expression as compared to control NWRs, but there was no evidence of Aβ_{1-42} deposition in control SHRs. Further, LPS (25 μg, ICV)-treated SHRs

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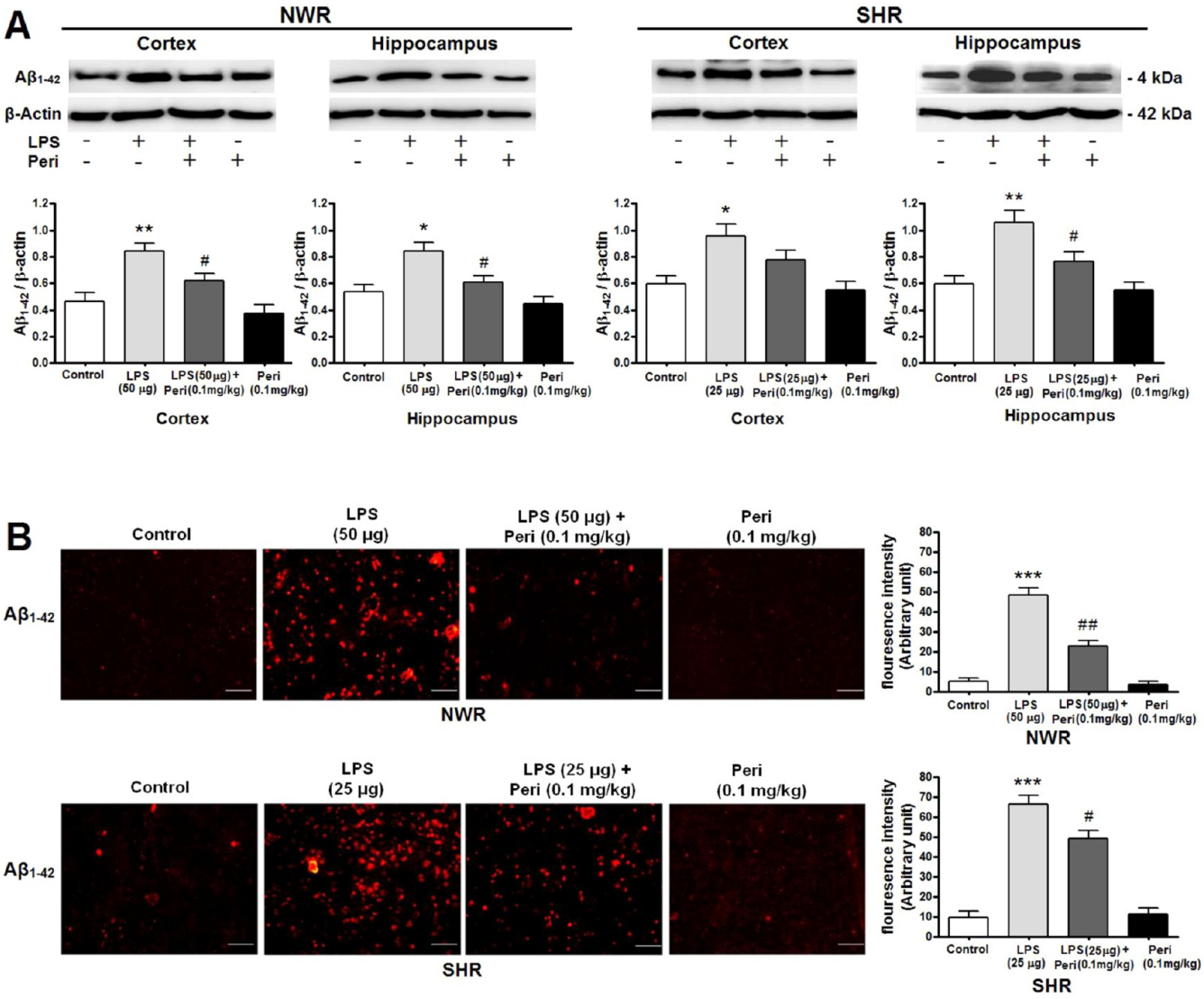

Figure 2. Effects of perindopril (0.1 mg/kg) pretreatment on Aβ_{1-42} deposition in cortex and hippocampus following chronic administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Representative Western blots with densitometric analysis of Aβ_{1-42} using β-actin as an internal control. (B) Representative photomicrographs with quantitative analysis of group data for Aβ_{1-42} deposition in the cortex region of NWRs and in SHRs. Data values are expressed as mean ± SEM. *Significant increase (*P < 0.05, **P < 0.01, and ***P < 0.001) in comparison to respective control groups, and #significant decrease (#P < 0.05 and ##P < 0.01) in comparison to respective LPS groups.

exhibited Aβ_{1-42} deposition as well as exaggerated RAGE and BACE expression, but similar effects were observed at 50 μg of LPS (ICV) in NWRs. Increased RAGE expression and amyloidogenesis were associated with increased oxidative stress and hypoperfusion following repeated administration of LPS. Further, we found that modulation of ACE by perindopril at a non-antihypertensive dose attenuated LPS-induced detrimental effects in both NWRs and SHRs.

In the present study, repeated administration of LPS (25 μg, ICV) on days 1, 4, 7, and 10 caused memory impairment in SHRs. However, memory impairment in NWRs was observed at a higher dose (50 μg, ICV) of LPS as evident from the lack of significant change in the latency period (Figure 1A) in the Morris water maze (MWM) test. The representative swim pattern of different groups of NWRs and SHRs during the third session is shown in Figure 1B. In this study, LPS (ICV) also resulted in amyloidogenesis, as evidenced by increased Aβ_{1-42} immunoblot (Figure 2A) and immunofluorescence (Figure 2B) in the cortex and hippocampus (data not shown for immunofluorescence staining) region at a lower dose in SHRs (25 μg, ICV) and at much higher dose in NWRs (50 μg, ICV). In agreement with this observation, previous studies have reported that LPS induces AD-like neuronal malfunction and deposition of Aβ_{1-42} fibrils.[19,20] Further, we evaluated the activity and expression of BACE, a rate-limiting step in the production of Aβ_{1-42}, and expression of APP (a substrate of BACE). Consistent with the increased Aβ_{1-42} deposition, LPS increased APP expression (Figure 3A) as well as BACE expression (Figure 3A) and activity (Figure 3B). Therefore, LPS-induced amyloidogenesis could be related with the change in expression of amyloidogenic proteins such as APP and BACE. Earlier studies also reported that pro-inflammatory cytokines (IL-1β and TNF-α) increased expression of APP[21] and Aβ_{1-42} deposition.[22] Interestingly, control SHRs displayed increased activity and expression of BACE without Aβ_{1-42} deposition as compared to control NWRs (Table 1), indicating that hypertension increases the susceptibility toward AD-like pathology.

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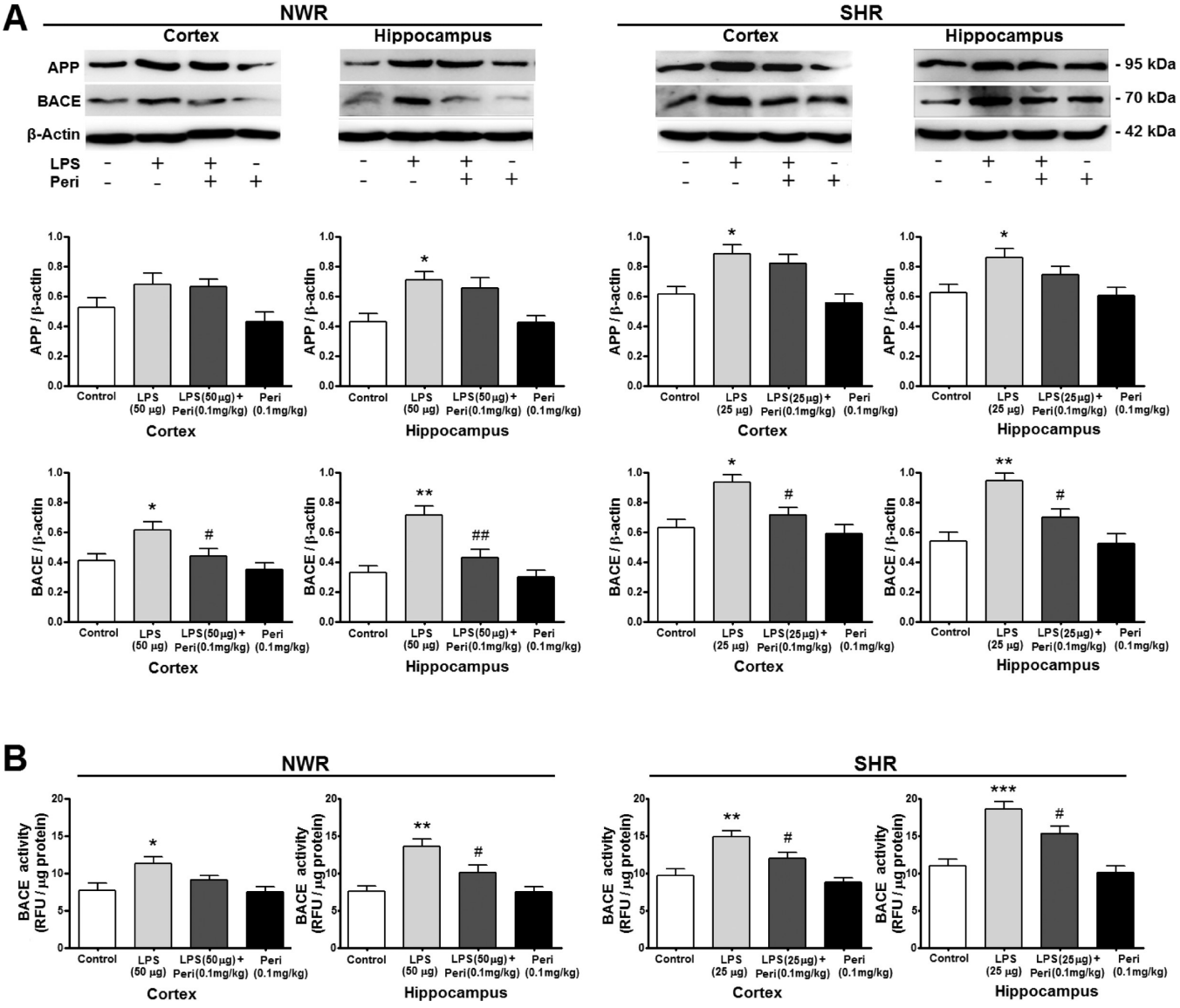

Figure 3. Effect of perindopril (0.1 mg/kg) pretreatment on APP expression and BACE expression and activity in the cortex and hippocampus following chronic administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Representative Western blots with densitometric analysis of the APP and BACE using β-actin as an internal control. (B) BACE activity by using enzyme assay kit. Data values are expressed as mean ± SEM. *Significant increase (*P < 0.05, **P < 0.01, and ***P < 0.001) in comparison to respective control groups, and $^{\#}$significant decrease ($^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01) in comparison to respective LPS groups.

Table 1. Basal Values of Different Parameters in Cortex and Hippocampus of NWRs and SHRs[a]

	cortex		hippocampus	
parameters	NWR	SHR	NWR	SHR
ROS level (fluorescence int.)	14.07 ± 3.78	26.73 ± 4.01[b]	10.07 ± 2.13	23.43 ± 4.92[b]
gp91-phox/β actin	0.453 ± 0.048	0.626 ± 0.064[b]	0.394 ± 0.054	0.591 ± 0.065[b]
p22-phox/β actin	0.707 ± 0.066	0.760 ± 0.057	0.617 ± 0.068	0.738 ± 0.081
RAGE/β-actin	0.302 ± 0.037	0.497 ± 0.053[b]	0.231 ± 0.043	0.555 ± 0.067[b]
$A\beta_{1-42}$/β-actin	0.466 ± 0.65	0.595 ± 0.62	0.534 ± 0.54	0.595 ± 0.058
$A\beta_{1-42}$ (fluorescence int.)	5.73 ± 1.45	7.66 ± 2.83	5.07 ± 1.37	6.82 ± 1.88
APP/β-actin	0.525 ± 0.068	0.617 ± 0.050	0.473 ± 0.53	0.602 ± 0.055
BACE/β-actin	0.41 ± 0.047	0.64 ± 0.052[b]	0.329 ± 0.048	0.542 ± 0.058[b]
BACE activity (RFU/μg protein)	7.749 ± 0.724	9.711 ± 0.729	7.585 ± 0.564	11.05 ± 0.654[b]

[a]Data are expressed as mean ± SEM. [b]Significant increase or decrease (*p < 0.05) in comparison to respective NWRs.

In our study, apart from the enhanced BACE expression, there was increased expression of RAGE; the major $A\beta_{1-42}$ receptor[23] was observed in control SHRs (Table 1), and LPS exacerbated RAGE expression in both the cortex and the hippocampus of

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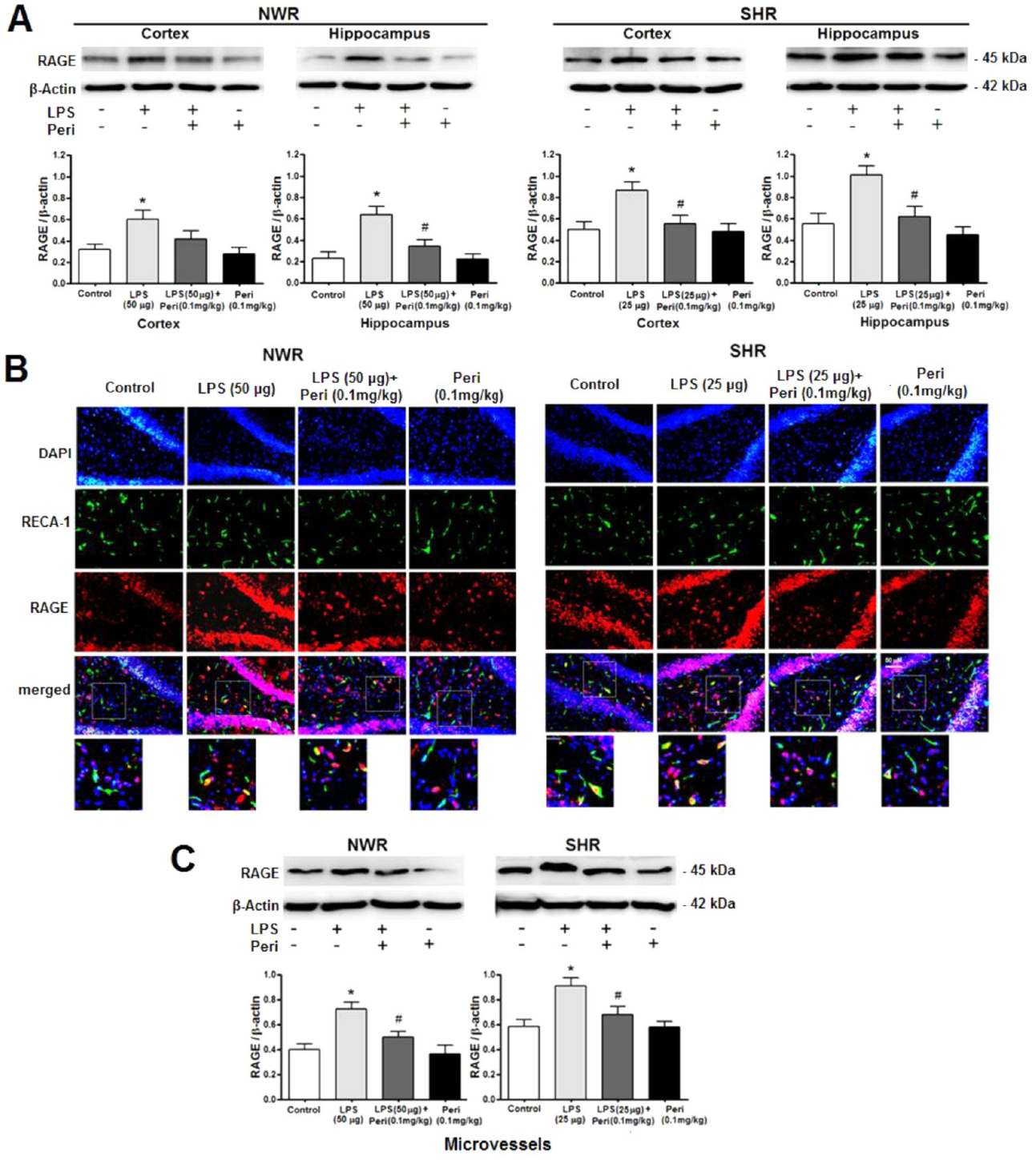

Figure 4. Effect of perindopril (0.1 mg/kg) pretreatment on RAGE protein expression following chronic administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Representative Western blots with densitometric analysis of the RAGE using β-actin as an internal control in the cortex and hippocampus. (B) Colocalization of RAGE and anti-endothelial cell antibody (RECA-1) double staining in the hippocampus. Scale bar = 50 μm. (C) Representative Western blots with densitometric analysis of the RAGE using β-actin as an internal control. Data values are expressed as mean ± SEM. *Significant increase (*$p < 0.05$) in comparison to respective control groups, and $^{\#}$significant decrease ($^{\#}p < 0.05$) in comparison to respective LPS-treated groups.

NWRs along with SHRs (Figure 4A). It is pertinent to mention here that activation of RAGE has a pivotal role in the brain Aβ_{1-42} deposition in both experimental[24] and clinical setups.[25] Therefore, overactivation of RAGE after LPS administration mediated transcytosis of Aβ_{1-42} and induced the AD-related pathology in both NWRs and SHRs. In the present study, inhibition of ACE by perindopril at a non-antihypertensive dose (0.1 mg/kg) improved memory functions (as evidenced by the significant decrease in latency times, Figure 1A) by preventing Aβ_{1-42} deposition in both NWRs and SHRs (Figure 2), indicating that ACE plays an important role in memory functions independent of blood pressure regulation. Perindopril prevented Aβ_{1-42}

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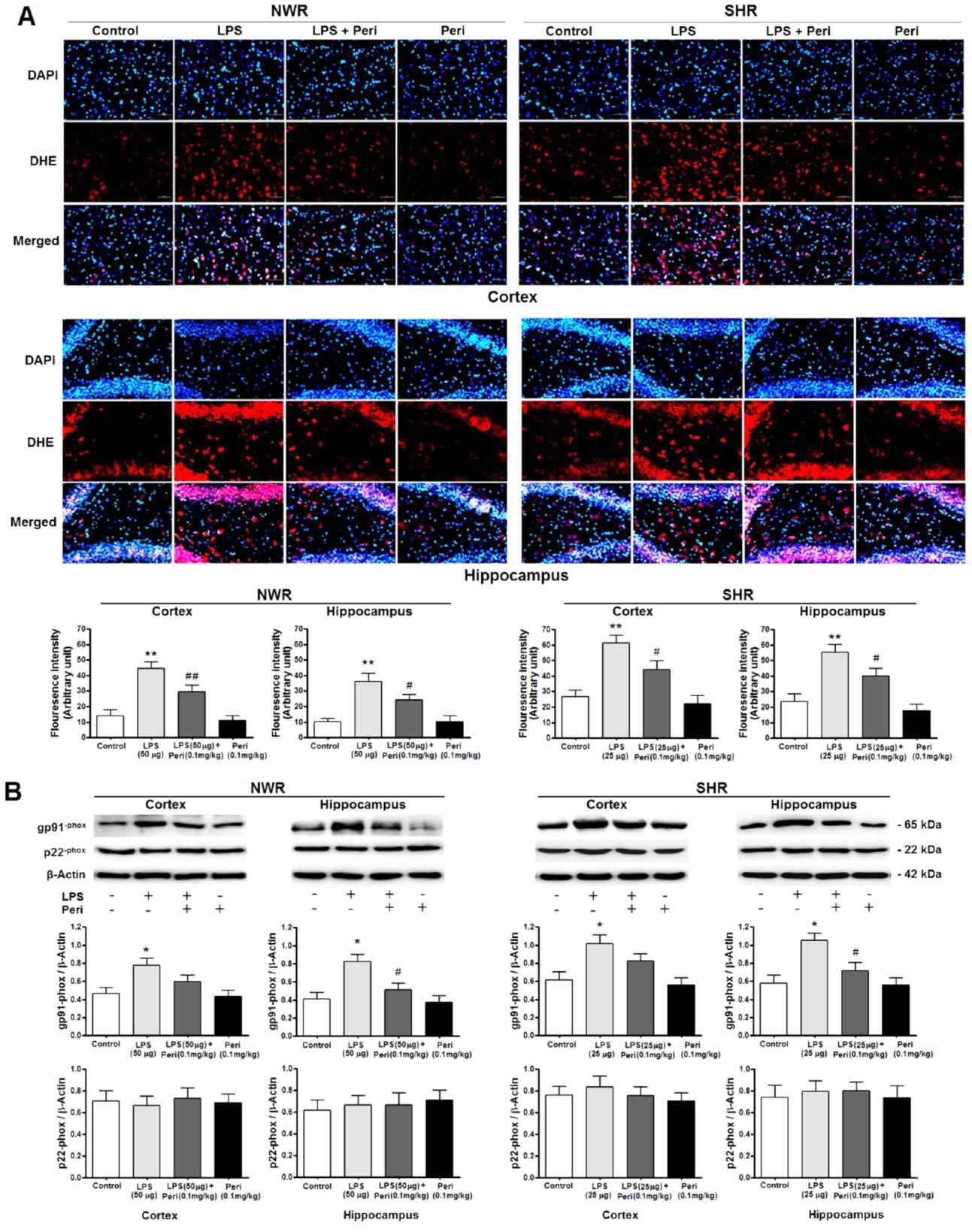

Figure 5. Effect of perindopril (0.1 mg/kg) on oxidative stress following chronic administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs) in cortex and hippocampus. (A) Representative photomicrographs and quantitative analysis of group data for ROS production in cortex and hippocampus regions of NWRs and SHRs assessed by DHE staining. Scale bar = 50 μm. (B) Representative Western blots with densitometric analysis of gp91-phox and p22-phox in cortex and hippocampus regions of NWRs and in SHRs using β-actin as an internal control. Data values are expressed as mean ± SEM. *Significant increase (*$p < 0.05$ and **$p < 0.01$) in comparison to respective control groups. $^{\#}$Significant decrease ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$) in comparison to respective LPS-treated groups.

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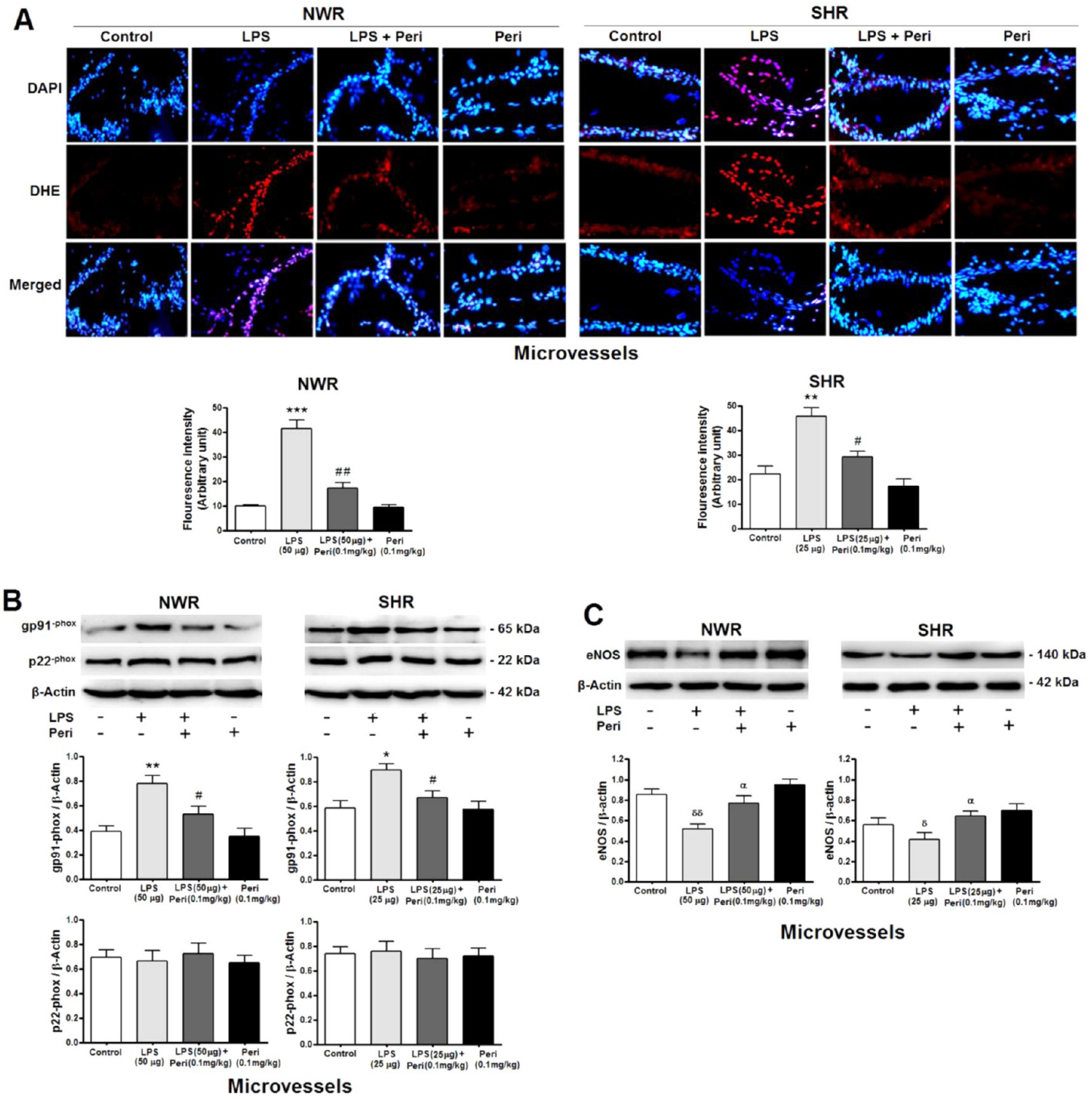

Figure 6. Effect of perindopril (0.1 mg/kg) on oxidative stress in brain microvessels upon repeated injections of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Representative photomicrographs and quantitative analysis of group data for ROS production in brain microvessels of NWRs and SHRs assessed by DHE staining. Scale bar = 50 μm. (B) Representative Western blots with densitometric analysis of the gp91-phox and p22-phox using β-actin as an internal control. (C) Representative Western blots with densitometric analysis of the eNOS using β-actin as an internal control. Data values are expressed as mean ± SEM. *Significant increase ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$), and $^{\delta}$significant decrease ($^{\delta}p < 0.05$ and $^{\delta\delta}p < 0.01$) in comparison to respective control groups. $^{\alpha}$Significant increase ($^{\alpha}p < 0.05$), and $^{\#}$significant decrease ($^{\#}p < 0.05$ and $^{\#\#}p < 0.01$) in comparison to respective LPS groups.

deposition by preventing increase in RAGE expression in both NWRs and SHRs (Figure 4A). Furthermore, rats pretreated with perindopril were also protected from LPS (ICV)-induced BACE expression and activity without affecting the expression level of APP (Figure 3). In line with this observation, earlier studies have demonstrated that ACE inhibitors assessable to the brain prevented memory impairment in rat[26] and mice[27,28] models of AD induced by Aβ_{1-42} injection. Recently, O'Caoimh et al.[29] also reported that centrally acting ACE inhibitors are associated with reduced rates of cognitive decline in AD patients.

Although the exact mechanism for the increased susceptibility for Aβ_{1-42} deposition in SHRs and decreased RAGE expression and Aβ_{1-42} deposition by perindopril is not known, oxidative stress[30] and endothelial dysfunction[31] seem to be common contributory factors found in both AD brains and in hypertensive condition. Further, several reports associate oxidative stress with

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RAGE activation.[11,32] Since RAGE is mainly expressed on the brain endothelium and mediates the transport of peripheral $A\beta_{1-42}$ into the brain,[9] in this study, oxidative stress and RAGE expression were evaluated on the brain endothelium. ROS production as evident from increased DHE immunostaining in the cortex and the hippocampus regions was significantly higher in control SHRs (Table 1) and LPS-treated SHRs and NWRs (Figure 5A). This increase in ROS generation was significantly abolished by the pretreatment with perindopril (Figure 5A). Further, similar results were observed in microvessels of control SHRs (Figure 6A). We have already shown that overactive central RAS (increased activity and expression of ACE and Ang II) exist in control SHRs, and ICV injections of LPS further amplified it in SHRs and in NWRs.[18] Thus, the oxidative stress in control SHRs and LPS-treated NWRs and SHRs might be mediated by the overactivation of ACE, resulting in increased Ang II production, triggering the oxidative stress, as Ang II is a well-known pro-oxidative molecule of RAS.[33] In addition, it has also been observed that NADPH oxidase-dependent production of ROS is an early and necessary step in the LPS-induced neuroinflammation,[34,35] strengthening the link between LPS administration and increased oxidative stress in both NWRs and SHRs. In this study, we have also analyzed the protein expression of both large and small subunits, that is, gp91-phox (NOX-2) and p22-phox, respectively, of cytochrome *b*558 (constituent of NADPH oxidase) in both NWRs and SHRs. Expression of gp91-phox was significantly increased in the cortex and hippocampus regions and in microvessels of control SHRs (Table 1 and Table 2) and LPS (ICV)-treated SHRs and NWRs (Figure 5B and Figure 6B). However, the expression level of p22-phox remained unaffected after LPS treatment in both NWRs and SHRs (Figure 5B and Figure 6B). Further, ACE inhibition by perindopril reduced the expression of gp91-phox in cortex and hippocampus brain regions (Figure 5B) as well as in the brain vessels (Figure 6B). Our data demonstrated that overactive central RAS in control SHRs and LPS-treated SHRs and NWRs induced oxidative stress through activation of gp91-phox. In agreement with this observation, a previous study by De Silva et al.[36] has demonstrated that RAS-induced oxidative stress in cerebral arteries is dependent on gp91-phox oxidase activity. Further, Girouard et al.[37] also demonstrated that the ROS signal was not observed after Ang II administration in mice lacking the gp91-phox subunit.

We further confirmed the RAGE activation by an immunofluorescence study in both endothelium and brain parenchyma. The double staining of RAGE and RECA-1 (rat endothelial cell antigen-1) clearly showed that RAGE was localized in brain vessels as well as in the brain parenchyma (Figure 4B). Immunoblot study also revealed the increased expression of RAGE in microvessels of control SHRs (Table 2) as well as in LPS-treated SHRs and NWRs (Figure 4C). In this study, control SHRs displayed oxidative stress and RAGE expression without $A\beta_{1-42}$ deposition. However, LPS treatment exaggerated oxidative stress and RAGE expression and induced amyloidogenesis. In support of this observation, oxidative damage has been observed in the AD brains[38,39] as well as in elderly patients without AD.[40] Finally, to confirm whether the RAGE activation and $A\beta_{1-42}$ deposition was driven by an oxidative stress-related mechanism, we chronically administered melatonin, an antioxidant, in both NWRs and SHRs. In our study, melatonin not only attenuated increased RAGE expression (Figure 7A) but also decreased $A\beta_{1-42}$ deposition in both NWRs and SHRs (Figure 7B). These results are backed by the study of Ko et al.,[41] who reported that free radicals are involved in AGE production and can promote the formation of $A\beta_{1-42}$. Further, RAGE is known to induce its own expression through the production of ROS in a feed-forward manner via activation of MAPK signaling like ERK1/2.[42] Here we also show that rats pretreated with perindopril were protected from LPS (ICV)-induced RAGE overactivation. This beneficial effect of the ACE inhibitor seems to be mediated by a reduction in activated gp91-phox-induced ROS generation in the brain vessels as well as in the brain parenchyma.

It the current study, control SHRs also exhibited impairment in cerebral blood flow (CBF) regulation (Table 2), although to a lesser extent than LPS-induced memory-impaired NWRs and SHRs (Figure 1C). These observations suggested that cerebral hypoperfusion, an early event in AD, already existed in control SHRs. Further, the decreased expression of eNOS, indicating endothelial dysfunction,[43] was observed in microvessels of control SHRs (Table 2) and LPS-treated NWRs and SHRs (Figure 6C), which might be responsible for the reduced CBF[44] observed in these rats. Reduction in the CBF might also be related to increased Ang II (a potent vasoconstrictor) in control SHRs and LPS-treated NWRs and SHRs. Further, this observation is supported by a study of Inaba et al.,[14] who demonstrated reduced CBF in renin/angiotensinogen transgenic mice due to overactivation of central RAS.

An earlier study also reported increased BACE and $A\beta_{1-42}$ levels in the brain during chronic cerebral hypoperfusion,[45] highlighting the role of RAS in AD-type dementia. In addition, we also observed a decreased ATP level in LPS-treated SHRs and NWRs (Figure 1D), demonstrating reduced brain energy metabolism during hypoperfusion. In support of this observation, a decreased ATP level has been reported in a rat model of chronic cerebral hypoperfusion.[46] ACE inhibition by perindopril improved endothelial functions and hypoperfusion, suggesting that the beneficial effects of an ACE inhibitor might be mediated by the reduction in the formation of Ang II.

In summary, the findings of the present study indicated that control SHRs exhibited increased RAGE and BACE expression without $A\beta_{1-42}$ deposition. Further, in the presence of a low dose of LPS (25 μg, ICV), activation of RAGE and BACE takes place largely via oxidative stress-dependent mechanisms and resulted in $A\beta_{1-42}$ deposition in the brain of SHRs. However, in NWRs, a much higher dose of LPS (50 μg, ICV) exhibited a similar response. Moreover, pretreatment with perindopril, even at a non-antihypertensive dose, prevented gp91-phox-induced oxidative stress (ROS generation) and associated RAGE activation, $A\beta_{1-42}$ deposition, and memory impairment in both NWRs and SHRs. These findings suggest the possibility that centrally active

Table 2. Basal Values of Different Parameters in Brain Microvessels of NWRs and SHRs[a]

	microvessels	
parameters	NWR	SHR
CBF (% of baseline values)	100.0 ± 5.978	76.891 ± 6.727[b]
eNOS/β-actin	0.853 ± 0.051	0.561 ± 0.063[b]
gp91-phox/β-actin	0.391 ± 0.044	0.588 ± 0.060[b]
p22-phox/β-actin	0.695 ± 0.058	0.740 ± 0.057
ROS level (fluorescence int.)	10.13 ± 0.296	22.20 ± 3.407[b]
RAGE/β-actin	0.401 ± 0.043	0.583 ± 0.058[b]

[a]Data are expressed as mean ± SEM. [b]Significant increase or decrease (*$p < 0.05$) in comparison to respective NWRs.

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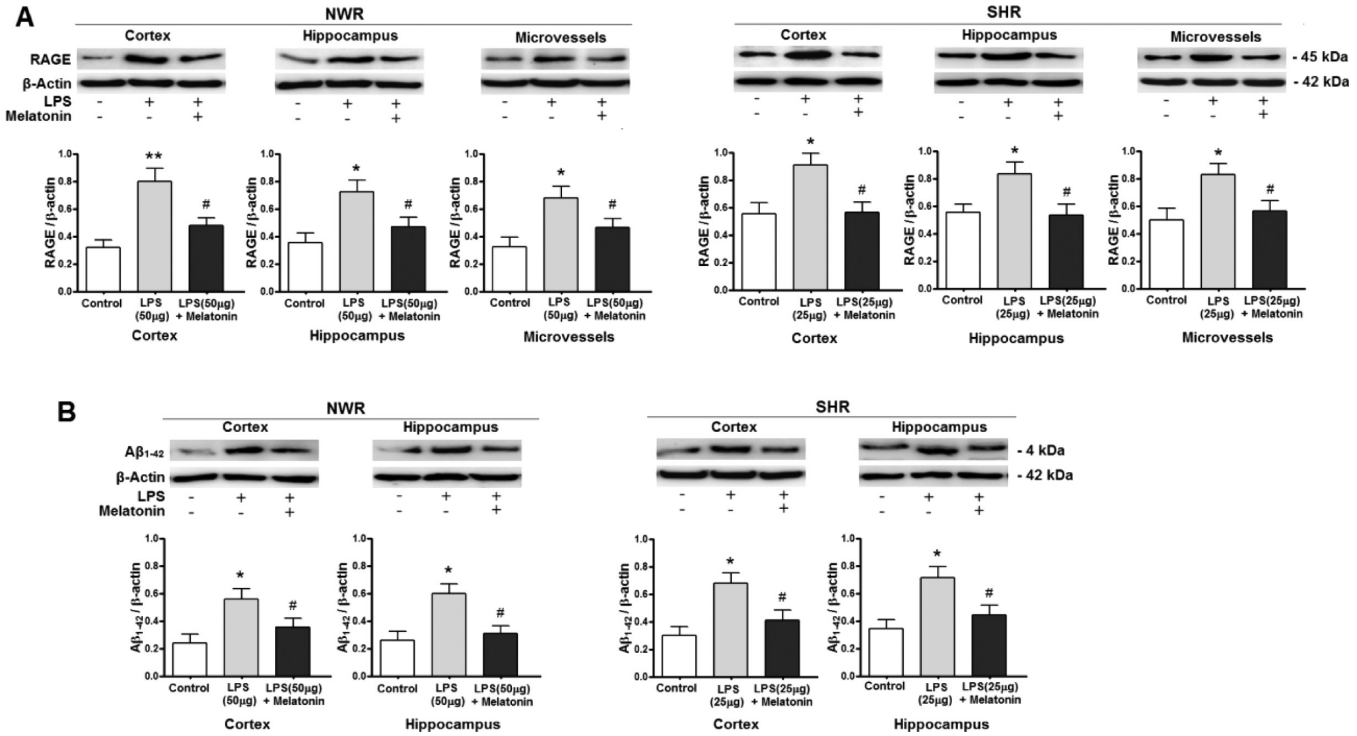

Figure 7. Effect of melatonin (10 mg/kg) on RAGE and Aβ_{1-42} protein expression following chronic administration of (ICV) LPS (50 μg in NWRs and 25 μg in SHRs). (A) Representative Western blots with densitometric analysis of the RAGE protein in brain microvessels and the cortex region using β-actin as an internal control. (B) Representative Western blots with densitometric analysis of the Aβ_{1-42} protein in cortex and hippocampus regions using β-actin as an internal control. *Significant increase ($^{*}p < 0.05$) in comparison to respective control groups, and $^{\#}$significant decrease ($^{\#}p < 0.05$) in comparison to respective LPS-treated groups.

ACE inhibitors could be a novel therapeutic strategy for the early stage of dementia in both normotensive and hypertensive subjects.

METHODS

Animals. The experiments were carried out with 8−9 week old male NWRs (systolic blood pressure = 105 ± 10 mmHg) and SHRs (systolic blood pressure = 165 ± 10 mmHg; Supporting Information Table 1) obtained from the Laboratory Animal Services Division of CSIR-Central Drug Research Institute, Lucknow, India. Experiments were performed according to internationally followed ethical standards and approved by the Institutional Animal Ethics Committee (IAEC no. IAEC/2012/2N dated 22.05.2014) of CSIR-CDRI and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Rats were maintained under standard housing conditions (room temperature 24−27 °C and humidity 60−65%) with a 12 h light and dark cycle. Food and water were available ad libitum.

Administration of LPS (ICV). ICV injections of LPS were given using the stereotaxic apparatus (Stoelting Co. USA) according to our previous report.[18] Rats were anaesthetized with chloral hydrate (300 mg/kg, i.p.). LPS (50 μg in NWRs and 25 μg in SHRs), dissolved in artificial CSF (147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$, and 2.2 mM dextrose), was administered slowly into each lateral cerebral ventricle (ICV) in a volume of 5 μL on days 1, 4, 7, and 10 using a Hamilton microsyringe. In the control group, artificial CSF was injected in the same volume.

Administration of Perindopril. To study the effect of modulation of ACE on RAGE activation and Aβ_{1-42} deposition, perindopril, an ACE inhibitor, was administered daily at a non-antihypertensive dose (0.1 mg/kg, p.o.) for 15 days in both SHRs and NWRs starting from the first dose of LPS.[17,18]

Administration of Melatonin. Melatonin, an antioxidant, was dissolved in ethanol and further diluted with saline. Melatonin (10 mg/kg) was administered intraperitoneally (i.p.) daily for up to 15 days starting from the first dose of LPS.[47]

Evaluation of Spatial Learning and Memory by Morris Water Maze Test. On the 13th day after first LPS injection, memory functions of NWRs and SHRs were tested in MWM.[17,18] The animals were given a daily session of five trials for three consecutive days. Data were acquired through a video camera, fixed above the center of the pool and connected to the ANY-maze video tracking software (ver. 4.73; Stoelting, USA). Time to reach the platform was recorded as latency time.

Estimation of CBF. CBF was measured by laser Doppler flowmetry (LDF 100, BIOPAC, USA) on the 15th day after the MWM test as described by Tota et al.[17] In brief, a microfiber laser Doppler probe was fixed on the skull (6 mm lateral and 1 mm posterior of bregma) of anesthetized NWRs and SHRs. CBF was monitored for 10 min, and values were recorded after each 30 s. Average values of CBF were calculated and expressed as the percentage of baseline values.

Isolation of Cerebral Microvessels. Brain was removed and rinsed with sucrose buffer (0.32 M sucrose, 3 mM HEPES, pH 7.4). After the cerebellum, pia mater, and choroids plexus were removed, the remaining brain was homogenized in three volumes of sucrose buffer followed by centrifugation at 1000g for 10 min at 4 °C. Sediments were resuspended in sucrose buffer and centrifuged twice at 100g for 30 s. The final pellet was again resuspended in the sucrose buffer, followed by centrifugation at 14 000g, and precipitate containing microvessels was stored at −80 °C until use.[48] The purity of the microvessels was evaluated by light microscopy and γ-glutamyl transpeptidase activity, a marker of brain microvessels.[48] Its activity was almost 9 times higher in microvessels than in the whole brain, with no significant differences between SHR and NWR rats as well as LPS-treated and/or perindopril-treated (results not shown).

Estimation of ROS by Fluorescence Microscopy. ROS was measured by dihydroethidium (DHE) microfluorography, as described previously by Munzel et al.[49] Briefly, isolated rat brain microvessels and coronal sections (15 μm thick) from fixed brains were incubated with 5

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μM DHE in PBS at 37 °C for 30 min in a dark humidified chamber. DAPI was used for counterstaining of nuclei. Fluorescent images were acquired using a Leica fluorescence microscope (Leica Microsystems, Germany), and intensities of the DHE fluorescent signals were quantified with ImageJ software (NIH, USA).

ATP Activity. ATP activity in the cortex and hippocampus of NWRs and SHRs was estimated using an ATP calorimetric/fluorometric assay kit as described previously.[17] The concentration of ATP was expressed in nanomoles per milligram protein.

Beta Site APP Cleaving Enzyme (BACE) Activity. The total activities of BACE or β-secretase present in the cortex and hippocampus regions were determined using a commercially available fluorometric BACE activity assay kit (Sigma-Aldrich Corporation, USA) according to the manufacturer's protocol. The level of BACE enzyme activity was expressed in relative fluorescence units per microgram of protein.

Estimation of Protein. Protein concentration was estimated by the method of Lowry et al.[50] in all of the brain tissue samples using bovine serum albumin (BSA) as standard.

Western Blot Analysis. Western blotting for different proteins was performed as described previously[18] using antibodies eNOS, APP, BACE, Aβ_{1-42}, RAGE (1:1000, Abcam, UK), gp91-phox, p22-phox (1:500, Santa Cruz Biotechnology, USA), and β-actin (1:10000, Sigma, St. Louis, MO, USA). After being incubated with respective secondary antibodies, blots were developed by the ECL chemiluminescence detection system (Millipore, USA). The band intensity was measured using spot densitometry analysis by MY IMAGE ANALYSIS software (Thermo Scientific).

Immunohistochemistry Analysis. For immunofluorescence, 15 μm thick coronal sections from fixed brains were cut on a cryostat and mounted to polylysine-coated slides. The sections were blocked using 1% BSA + 0.3% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 45 min at room temperature and incubated with primary antibodies, rabbit polyclonal antibodies against Aβ_{1-42} and RAGE (1:200, Abcam, UK), and mouse monoclonal antibody against RECA-1 (1:200, Abcam, UK) at 4 °C overnight. Sections were washed with PBS, followed by 2 h incubation with the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (1:400, Invitrogen, California), or Alexa Fluor 594 goat anti-rabbit IgG (1:400, Invitrogen, California) at room temperature and mounted with Prolong Gold antifade mounting medium with DAPI (Invitrogen, USA) to stain the nuclei. Images were acquired using a Leica fluorescence microscope (Leica Microsystems, Germany), and the intensities of the fluorescent signals were quantified with ImageJ software (NIH, USA).

Statistical Analysis. Statistical analysis was performed with Prism software version 5.0 (Graph Pad Software, San Diego, CA, USA). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Results were presented as mean ± SEM. A value of $p < 0.05$ was considered to be statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00274.

Blood pressure of animals (PDF)

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Author Contributions

R.G. performed most of the experiments, analyzed data, generated figures, and wrote the manuscript. S.A.B. helped in isolation of microvessels and in the fluorescence microscope imaging. K.H. and C.N. gave critical input on WB experiments, other crucial experiments, and critical reading of the manuscript. R.S. conceptualized, planned, and supervised the study and edited the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Aβ, amyloid beta; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; APP, amyloid beta precursor protein; BACE, beta site APP cleaving enzyme; CBF, cerebral blood flow; ICV, intracerebroventricular; LPS, lipopolysaccharide; NWR, normotensive wistar rats; RAGE, receptor for advanced glycation end products; RAS, renin angiotensin system; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats

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