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Original article

Diabetes-induced stimulation of the renin-angiotensin system in the rat brain cortex

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ABSTRACT

Cerebrovascular disease is a threat to people with diabetes and hypertension. Diabetes can damage the brain by stimulating the renin-angiotensin system (RAS), leading to neurological deficits and brain strokes. Diabetes-induced components of the RAS, including angiotensin-converting enzyme (ACE), angiotensin-II (Ang-II), and angiotensin type 1 receptor (AT1R), have been linked to various neurological disorders in the brain. In this study, we investigated how diabetes and high blood pressure affected the regulation of these major RAS components in the frontal cortex of the rat brain. We dissected, homogenized, and processed the brain cortex tissues of control, streptozotocin-induced diabetic, spontaneously hypertensive (SHR), and streptozotocin-induced SHR rats for biochemical and Western blot analyses. We found that systolic blood pressure was elevated in SHR rats, but there was no significant difference between SHR and diabetic-SHR rats. In contrast to SHR rats, the heartbeat of diabetic SHR rats was low. Western blot analysis showed that the frontal cortexes of the brain expressed angiotensinogen, AT1R, and MAS receptor. There were no significant differences in angiotensinogen levels across the rat groups. However, the AT1R level was increased in diabetic and hypertensive rats compared to controls, whereas the MAS receptor was downregulated ($p < 0.05$). These findings suggest that RAS overactivation caused by diabetes may have negative consequences for the brain's cortex, leading to neurodegeneration and cognitive impairment.

1. Introduction

Worldwide, hypertension and diabetes are the most prevalent diseases associated with a higher risk of macrovascular and microvascular complications (Perreault et al., 2017). Compared to people with diabetes with normotension, those with hypertension have an earlier onset of heart and vascular disease and stroke. These harmful impacts of hypertension extend beyond the major arteries, as it has been shown to hasten the development of microvascular problems such as nephropathy, retinopathy, and neuropathy in individuals with diabetes (UK Prospective Diabetes Study Group, 1998). Compared to those without diabetes, hypertensive individuals with diabetes are more likely to encounter intracerebral hemorrhage and have worse outcomes (Gerstein et al. 2008). Numerous researches show that genetic and environmental factors contribute to diabetes and hypertension-induced pathophysiological complications (Govindarajan et al. 2006). Furthermore, vascular and metabolic changes brought on by hypertension, including arterial stiffness, astrogliosis, microglial activation, and stroke, have been linked to brain damage (Dennis et al. 1989; Ishida et al. 2006; Shi et al. 2010; Wei et al. 2011; Anwer et al. 2011; Carvalho, and Moreira 2018;) Diabetes is also a significant independent risk factor that might hasten dementia, cerebral infarction, and cognitive dysfunction (Allen et al. 2004).

In the cerebral cortex, the pathogenesis of diabetes and hypertension-induced stroke, cerebral hemorrhage, and neurovascular injury is poorly understood. However, one of the potentially

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harmful variables that may result in neurological impairments and damage in the brain is the activation of the local renin-angiotensin system (RAS) brought on by diabetes. It is now widely acknowledged that the brain expresses most of the RAS's components and that the central nervous system (CNS) has its own local RAS (Yang et al. 1999; Agassandian et al. 2017; Sapouckey et al. 2017). However, still, the existence of the brain RAS has been disputed by certain researchers (van Thiel et al. 2017). Furthermore, diabetes-induced components of the RAS system consisting of angiotensin-converting enzyme (ACE), angiotensin-II (Ang-II), and angiotensin type 1 receptor (AT1R) are found to be linked to many neurological disorders, both within the retina and brain (Govindarajan et al. 2006; Mehta and Griendling 2007; Saavedra 2012; Tian et al. 2012; Ola et al. 2013; Ola et al. 2017; Abiodun and Ola 2020).

The profound effects of the intrinsic Ang-II generated within the brain are mediated by AT1R. The RAS has a counter-regulatory arm called the ACE2/Ang-(1–7)/Mas receptor axis, which works to counteract the effects of Ang-II. The Mas receptor's ligand, Ang-(1–7), is produced via the proteolytic cleavage of Ang-II by ACE2 (Santos et al. 2003). The Mas-R has been found in various brain regions, including the cortex and hippocampus, and diverse brain regions related to cardiovascular regulation (Young et al. 1988; Freund et al. 2012). Some pathophysiological processes associated with brain RAS-induced diabetes encephalopathy include higher levels of oxidative stress, inflammation, apoptosis, and endothelial dysfunction (Goossens, 2012; Muris et al., 2013; Wong, 2013). However, little is known about how diabetes affects and regulates the major constituents of the renin-angiotensin system in the brain cortex and how this affects neurodegeneration.

It is crucial to comprehend how the brain's RAS is controlled in both normal and pathological conditions. To better understand how the major RAS components are expressed and activated in the brain cortex, we investigated the expression and activation of the major components of RAS; angiotensinogen, AT1R, and MAS receptors in the brain cortex of normal, diabetic, and spontaneous hypertensive transgenic rats (SHR). In addition, we measured the systolic pressure and heart rate in hypertensive and diabetic-hypertensive rats. This study establishes the role of localized RAS in the brain, allowing Ang-II to affect neuronal activity in paracrine. Additionally, in the case of diabetic-hypertensive situations, the overactivation of the Ang-II-AT1R axis while the lowered level of the protective axis through the MAS receptor may cause neuronal injury in the brain cortex. We speculate that these results could improve our understanding of the neuronal damage in diabetic and hypertensive brains and point to a possible treatment target for preventing stroke and other cerebrovascular diseases.

2. Materials and methods

2.1. Chemicals and kits

The streptozotocin used was provided by Sigma Aldrich. Both primary and secondary antibodies were procured from Santa Cruz, USA. Analytical-grade chemicals and reagents were utilized for everything else.

2.2. Experimental animals

Ten-week-old Wistar Kyoto rats weighing 250–280 g were used in this investigation. The animals came from King Saud University's College of Medicine's Experimental Animal Care Center in Riyadh, Saudi Arabia. In addition, Harlan Laboratories in Indianapolis, USA, provided ten-week-old transgenic hypertensive and normotensive Lewis rats. The rats were housed in a normal temperature and humidity-controlled environment with free access to the standard laboratory rat food and water. The rats were sedated using ketamine. The Wake Forest University animal care and use committees authorized the treatment of all rats in accordance with the guidelines of the NIH. The experimental animal protocol has been approved by the Experimental Animal Care Committee (approval number KSU-SE-21–04), King Saud University, Riyadh, Saudi Arabia.

2.3. Induction of diabetes mellitus

Both the hypertensive and normal rats were split into two groups. A single IP injection of streptozotocin (65 mg/kg), freshly produced in a 50 mM citrate buffered solution (pH 4.5), induced diabetes in one group of rats from each. Three days after the injection, blood glucose levels and body weight were assessed. To confirm diabetes, fasting blood glucose levels were measured using an ACCU-CHEK glucometer from tail vein blood samples. Animals were considered diabetic and included in the study if their blood glucose level was more than 250 mg/dl. The control, diabetic, hypertensive, and diabetic-hypertensive animal groups, each consisting of seven rats, were used for experiments after four weeks of streptozotocin induction.

2.4. Blood pressure and heartbeats measurements

Tail-cuff plethysmography measured systolic blood pressure and heart rate in the hypertensive and diabetic-hypertensive groups (Hatteras Instruments, Cary, NC). Tail-cuff plethysmography is a non-invasive method of measuring blood pressure in small animals, such as rats and mice. It involves placing a cuff around the animal's tail and inflating the cuff to a pressure that occludes the blood flow. These changes can be used to calculate the systolic blood pressure and heart rate.

2.5. Tissue harvesting and brain homogenate preparation

Under anesthesia, blood was collected from the rats, and they were then all given at least 100 ml of saline perfusion before having their brains dissected. After isolating the frontal brain cortex, it was promptly cleaned with ice-cold saline, put into labeled Eppendorf tubes, and maintained at −70 °C until analysis. Brain homogenates were made by first applying a brief burst of ultrasonication in a 10 mM HEPES buffer (pH7.4) that contained 100 mM NaCl, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1 mM PMSF, 1% Triton X-100 and 0.2% SDS and a protease inhibitor. After that, samples were centrifuged at 15,000 g for 15 min in a cooled centrifuge, and the supernatants were collected. Finally, each brain sample's protein concentrations were calculated using the Lowry method.

2.6. Determination of blood glucose

The Randox Glucose Assay kit (Randox, UK) was used to measure the glucose level in accordance with the manufacturer's instructions. In brief, 0.1 ml of the sample was combined with 1.0 ml of the reagent (GOD-PAP), which is composed of glucose oxidase (GOD), peroxide, and 4-aminophenazone (POD), in one test tube. In another test tube, 1.0 ml of the reagent was combined with 0.1 ml of the prepared standard glucose. After being well combined, the mixtures were incubated for 25 min at 25 °C. At a wavelength of 500 nm, the absorbance of the standard and the samples was measured compared to the reagent blank. In the samples, the glucose concentration was expressed as mg/dL.

2.7. Western blot analysis

Using the Western blotting method, the protein expression in the brains of control, diabetic, hypertensive, and diabetic-hypertensive rats was examined. Cortex-specific protein samples were extracted and transferred to PVDF membranes after being separated on SDS-polyacrylamide gels (10–15%). In addition, 5% non-fat milk in Tris-buffer saline-tween-20 (TBST) was used to block the membranes. Anti-angiotensinogen, anti-AT1R, anti-MAS1, and anti-actin primary antibodies (Santa Cruz Biotechnology, Dallas, USA) were incubated with the membranes overnight at 4 °C. For internal control, a mouse monoclonal β-actin antibody was utilized. The membranes were treated with their corresponding secondary horseradish peroxidase-conjugated antibodies (1:5000) after being incubated with the primary antibodies overnight. t. After the membranes had been washed four times with TBS-T for five minutes each, bands were seen on an LI-COR C-DiGit Blot Scanner from Biosciences, USA. Densitometry analysis and Image Studio Acquisition Software were used to measure protein bands (Image-Lab software). Band intensities were measured by delineating a square or circle around the protein's bands. Calculations were made of the band intensities ratios of the protein of interest to β -actin. The band intensity ratio of the control protein sample to Beta-actin in each experiment is taken to be 100%. The band intensities ratios of other protein samples to β-actin were compared with the control ratios, as was done in our lab previously (Al-Dosari et al. 2017).

2.8. Statistical analysis

A two-way repeated-measure ANOVA was used to analyze the data. Values are shown as means ± SEM. Differences were deemed significant for all studies at $P < 0.05$. SPSS version 18.0 was used to conduct all statistical analyses.

3. Results

3.1. Blood glucose analyses in normal, diabetic, and hypertensive rats

Rats were divided into four groups: diabetic, SHR control (SHR-C), control, and diabetic SHR (SHR-D). Rats were made diabetic by injecting streptozotocin. Our analyses of the blood glucose level showed that streptozotocin caused a significantly higher blood glucose level in these rats compared to the control rats (350 ± 23 mg/dL in diabetic vs. 115 ± 6 mg/dL control; $p < 0.01$) (Fig. 1). The blood glucose level was also significantly higher in the STZ-induced SHR rats compared to the SHR control (470 ± 26 mg/dL (SHR-D) greater than 126 ± 3 mg/dL (SHR-C); $p < 0.01$).

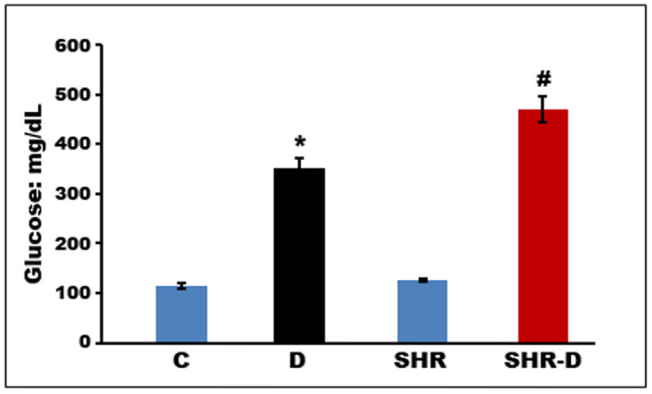

Fig. 1. Serum blood glucose measurements in normal and spontaneously diabetic hypertensive (SHR-D) rats. Serum glucose levels were compared with control, diabetic, and hypertensive rats (SHR-C). Data provided as means ± SEM; $^{*,\#}p < 0.01$ in comparison to controls and SHR controls.

3.2. Blood pressure and heart rate analysis in SHR-C and SHR-D rats

We measured the STZ-diabetic SHR rats' and SHR rats' systolic pressures and heart rates (Fig. 2). As predicted, the data show that SHR rats had high blood pressure, but there was no appreciable difference in blood pressure between the STZ-diabetic and the SHR rats. However, heart rate was significantly lower in the SHR-diabetic rats compared to the SHR rats. Under diabetes, cardiac output decreased with heart rate from 460 beats per minute to 350 beats per minute. The STZ-SHR heart rate reduction may contribute to the onset of hypertension. Therefore, the reduced heart rate in STZ-SHR may have a role in the development of hypertension.

3.3. AGT expression in the normal, diabetic, hypertensive, and diabetic-hypertensive rat cortex

By using Western blot analysis, the amounts of angiotensinogen protein expression in the brain of control, diabetic, hypertensive, and diabetic-hypertensive rats were determined (Fig. 3). In comparison to the controls, the diabetic and SHR rats' levels of angiotensinogen increased somewhat, but not significantly, according to the protein band densitometry study. Additionally, there was no apparent difference in the expression of AGT in the brain between hypertension and hypertensive-diabetic rats.

3.4. AT1R expression in the rat brain cortex

The levels of AT1R protein in the cortexes of control, diabetic, hypertensive, and diabetic-hypertensive rats were determined by Western blotting (Fig. 4). Densitometry analysis of the protein bands showed that in the diabetic, SHR-C, and SHR-D brain cortexes, the AT1R level increased by 40%, 50%, and 90%, respectively, compared to the controls. No significant change in the expression of AT1R in the brain cortex was observed between diabetic and hypertensive rats. However, compared to other groups, the diabetic hypertensive had noticeably higher AT1R expression in the cerebral cortex.

3.5. MAS receptor protein expression in the rat brain cortexes

Western blotting was used to measure the levels of protein expression of the ang (1–7) receptor (MAS receptor) in the brain cortexes of control, diabetic, hypertensive, and diabetic-hypertensive rats (Fig. 5). Densitometry analysis of the protein bands revealed that the MAS receptor was reduced by 65% in the brain cortex of SHR diabetic rats ($P < 0.05$ vs. control rats). The MAS receptor was decreased by 45% in the cortex in control diabetic rats. Our results show MAS receptor had considerably less expression in the cerebral cortex of diabetic, SHR, and SHR-diabetic rats than in controls ($P < 0.01$). However, the level of MAS receptors was not significantly different between diabetic and SHR rats. Notably, the MAS receptor was reduced considerably in SHR rats with diabetes than in controls.

4. Discussion

One of the potentially harmful elements that may cause neurological impairments in the brain is diabetes-related activation of the RAS. The ACE/Ang-II/AT1R axis, which are diabetes-induced components of the RAS system, have been related to many neural

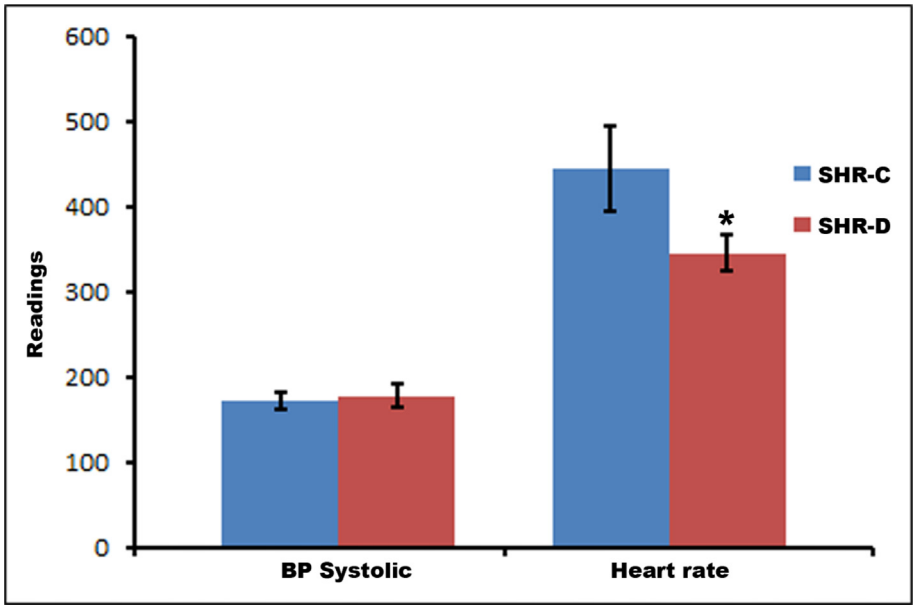

Fig. 2. Systolic blood pressure and heart rate measurements in spontaneously diabetic-hypertensive (SHR-D) and hypertensive rats (SHR-C). Data presented as means ± SEM; *p < 0.05 compared to controls and SHR controls.

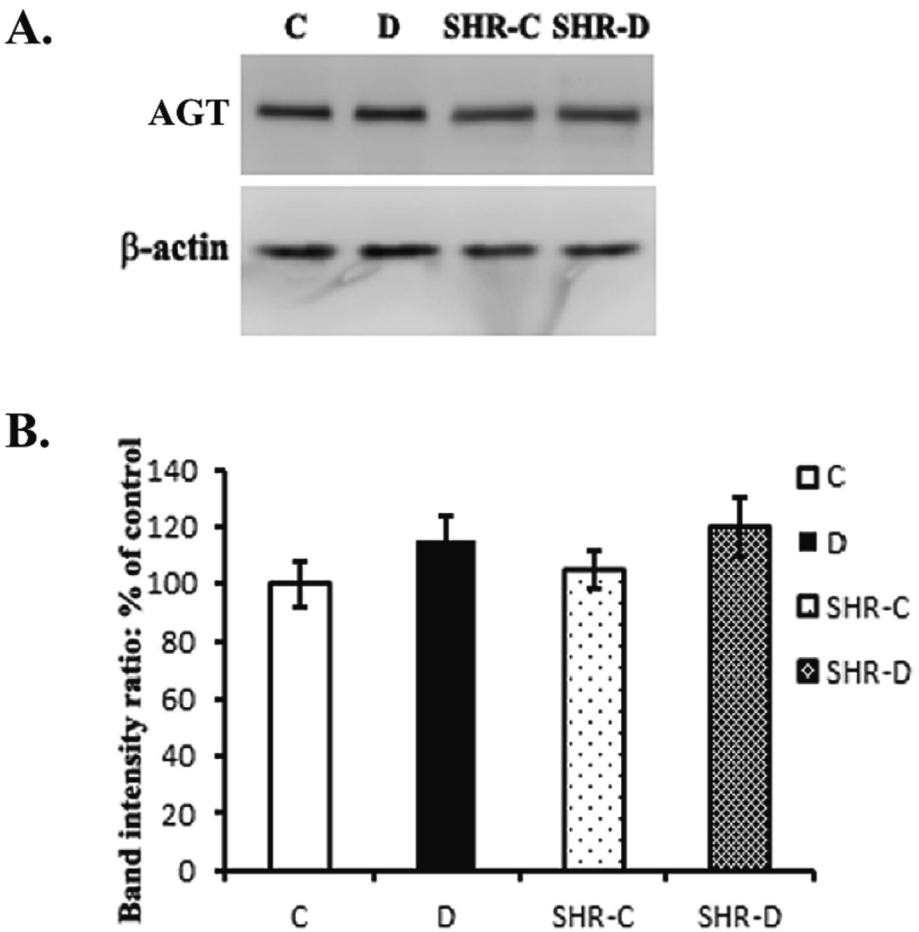

Fig. 3. Angiotensinogen expression in rat brain cortexes from control, diabetic, hypertensive, and diabetic-hypertensive rats was analyzed using a Western blot. By using densitometry, the band's intensities were measured. Immunoblots of angiotensinogen and β-actin bands are shown in the top panel (Fig. 3A). AGT stands for angiotensinogen, C (control), D (diabetes), SHR-C (spontaneously hypertensive rats-control), and SHR-D (spontaneously diabetic-hypertensive rats). The data are shown as means ± SEM (Fig. 3B).

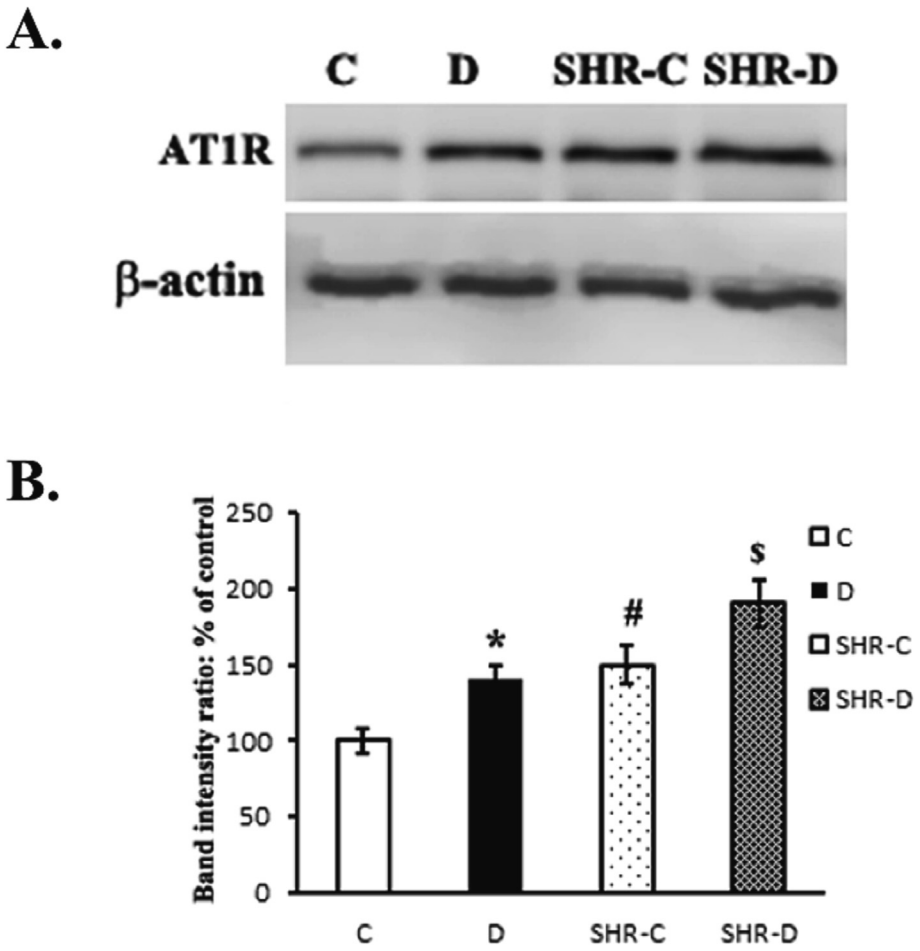

Fig. 4. Angiotensin II receptor (ATR1) expression in rat brain cortexes from control, diabetic, hypertensive, and diabetic-hypertensive rats was analyzed using a Western blot. By using densitometry, the band's intensities were measured. AT1R and β-actin bands are shown in the top panel (Fig. 4A) immunoblots. Angiotensin type 1 receptor (ATR1). Data provided as means ± SEM; *,#,$p < 0.05 compared to controls (Fig. 4B).

diseases in the retina and brain (Govindarajan et al. 2006; Mehta and Griendling 2007; Saavedra 2012; Tian et al. 2012; Ola et al. 2013; Ola et al. 2017). In this study, first, we measured the systolic pressure and heart rates of the SHR and STZ-diabetic SHR rats. Secondly, we analyzed the protein expression of different components of RAS; angiotensinogen, AT1R, and Ang 1–7 receptor (MAS receptor) in the frontal brain cortexes in the four groups of rats: normal, diabetic, SHR, and SHR diabetic. It also analyzed how their expression was regulated in the cerebral cortex of rats with hypertension and diabetes.

As anticipated, the SHR rats had high blood pressure that bordered on hypertension, but there was no significant change in blood pressure between the STZ-diabetic and the SHR rats. The primary step in the development of hypertension in SHR rats is thought to be an elevated heart rate (Lundin and Hallback-Nordlander 1980; Korner and Swales 1991). However, the heart rate of SHR-diabetic rats was substantially lower than that of SHR rats. Our findings of heart rate impairment in STZ-induced diabetes align with earlier findings in rats with diabetes and diabetes with hypertension (Cavaliere et al., 1980; Widdop et al., 1990). Furthermore, the studies prove that having both hypertension and diabetes mellitus together worsens myocardial and cerebral vascular dysfunction more than having either condition by itself (Gerstein et al. 2008).

Numerous investigations conducted in the past have shown that specific groups of astrocytes and neurons express components of RAS proteins, including angiotensinogen (AGT) (Stornetta et al. 1988; Intebi et al. 1990; Milsted et al. 1990; Yang et al. 1999; Agassandian et al. 2017; Sapouckey et al. 2017). A few studies, though, have disputed the presence of the brain RAS (van Thiel et al. 2017). Immunoblotting results from this study also reveal that AGT was expressed in the frontal cortex of the rat brain. However, the physiological function of AGT is still not well understood. The expression of AGT in the cerebral cortex did not differ significantly between diabetic, hypertensive, or diabetic-hypertensive rats. Although there is little information regarding the regulation of AGT in the diabetes brain, a few studies suggested that high glucose circumstances greatly enhance the expression level of AGT in diabetic kidney cells and cultured podocytes (Yoo et al. 2007; Paeng et al. 2017).

Previously, it was discovered that the Ang-II receptors, including the AT1R, are expressed in several brain areas, including the hypothalamus (Moellenhoff et al. 2001; Lazartigues et al. 2002; Chen et al. 2004). However, our results of expression of AT1R indicated that the receptor is highly expressed in the brain cortex. Furthermore, the level increased in the diabetic and hypertensive rats, consistent with the finding of Cai et al. 2021, who reported an increased level of both mRNA and protein levels of AT1R in diabetic brains (Arrick et al. 2008; Cai et al. 2021). Additionally, compared to other groups, the diabetic-hypertensive group showed much higher AT1R expression. Accordingly, diabetes may result in AT1R activation, which causes most of Ang-II's detrimental effects by promoting oxidative stress, apoptosis, and neurodegenerative consequences (Kusaka et al. 2004; Ola et al. 2013).

The Mas receptor is regarded as the RAS's counter-regulatory arm and is frequently thought to have physiological effects that

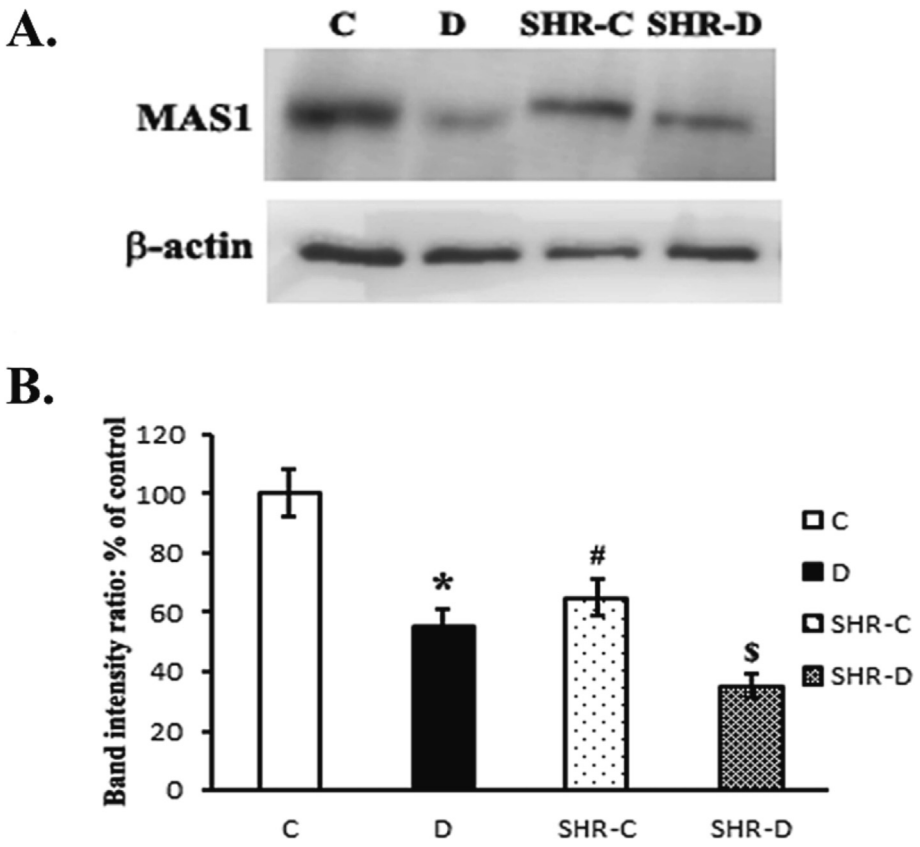

Fig. 5. MAS receptor expression was analyzed using a Western blot in the rat brain cortexes of control, diabetic, hypertensive, and diabetic-hypertensive rats. By using densitometry, the band's intensities were measured. Immunoblots of the MAS1 and β-actin bands are displayed in the top panel (Fig. 5A). Data provided as means ± SEM; *,#, $p < 0.05 compared to controls (Fig. 5B).

counteract the activity of Ang-II. According to many previous studies, insufficient endogenous Ang-(1–7) in the brain caused reduced tonic baroreceptor reflex and diabetes-induced alterations in hypertensive and diabetic rats (Young et al. 1988; Senanayake et al. 1994; Renno et al. 2012). In the present work, diabetes-induced SHR rats and diabetic animals appeared to have compromised Mas receptor pathways in the cerebral cortex. These findings align with a study by Li et al. (2016) that found a malfunction in the MAS receptor pathway in the brain's cortex under hypertensive and diabetic hypertension circumstances (Li et al. 2016). A growing body of research indicates that Ang II is crucial in developing cerebrovascular injury, although the function of other RAS components in neurodegeneration is not yet known. Through the AT1R, Ang II stimulates NADPH oxidase, an enzyme present in various brain areas, to produce reactive oxygen species (Fernando et al., 2005; Wright and Harding, 2011; Solleiro-Villavicencio and Rivas-Arancibia, 2018). Additionally, research has demonstrated that oxidative stress and chronic inflammation brought on by RAS in the brain may cause apoptosis, which in turn causes neuronal dysfunction (Zhao et al. 2015; Kim et al. 2017; de Morais et al. 2018; Carvalho and Moreira 2018).

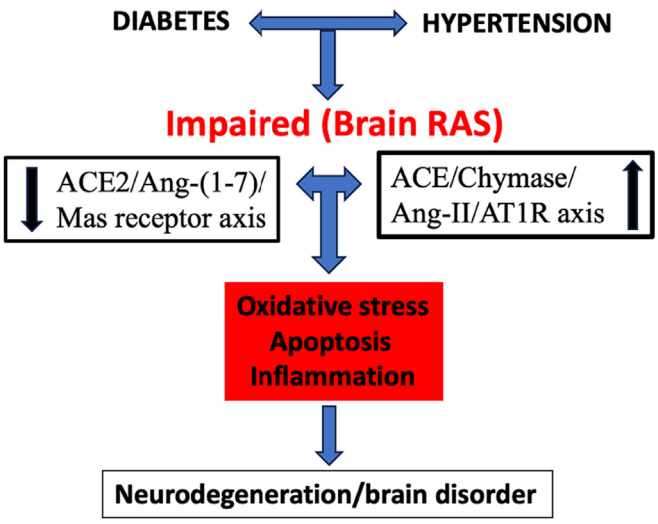

Fig. 6. Flow diagram of potential links between diabetes and hypertension and their influence on the dysregulation of the brain renin-angiotensin system, thereby influencing neurodegeneration.

5. Conclusions

Our investigation demonstrated that the RAS system is expressed and regulated in the cerebral cortex under diabetes and hypertension settings, which may influence neurodegeneration in the brain cortex, as depicted in the flow diagram of Fig. 6. A better knowledge of the RAS activation and Ang-II receptor signaling within the brain will make it easier to develop novel strategies to treat hypertension-mediated neuronal dysfunction within the brain.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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