Angiotensin II-NADPH oxidase-derived superoxide mediates diabetes-attenuated cell excitability of aortic baroreceptor neurons

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Li YL, Zheng H. Angiotensin II-NADPH oxidase-derived superoxide mediates diabetes-attenuated cell excitability of aortic baroreceptor neurons. Am J Physiol Cell Physiol 301: C1368–C1377, 2011.—Over-activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels is involved in diabetes-depressed excitability of aortic baroreceptor neurons in nodose ganglia. This involvement links to the autonomic dysfunction associated with high morbidity and mortality in diabetic patients. The present study examined the effects of an angiotensin II type I receptor (AT 1R) antagonist (losartan), a NADPH oxidase inhibitor (apocynin), and a superoxide dismutase mimetic (tempol) on the enhanced HCN currents and attenuated cell excitability in diabetic nodose neurons. In sham and streptozotocin-induced type 1 diabetic rats, HCN currents and cell excitability of aortic baroreceptor neurons were recorded by the whole cell patch-clamp technique. The angiotensin II level in nodose ganglia from diabetic rats was higher than that from sham rats (101.6 ± 4.8 vs. 38.9 ± 4.2 pg/mg protein, P < 0.05). Single-cell RT-PCR, Western blot, immunofluorescence staining, and chemiluminescence data showed that mRNA and protein expression of AT 1R, protein expression of NADPH oxidase components, and superoxide production in nodose neurons were increased in diabetic rats compared with those from sham rats. HCN current density was higher and cell excitability was lower in aortic baroreceptor neurons from diabetic than from sham rats. Losartan (1 µM), apocynin (100 µM), and tempol (1 mM) normalized the enhanced HCN current density and increased the cell excitability in the aortic baroreceptor neurons of diabetic rats. These findings suggest that endogenous angiotensin II-NADPH oxidase-superoxide signaling contributes to the enhanced HCN currents and the depressed cell excitation in the aortic baroreceptor neurons of diabetic rats.

baroreflex; ion channels

CLINICAL TRIALS and animal models of type 1 diabetes have demonstrated pathophysiological alterations in the arterial baroreflex control of the cardiovascular function and blood pressure (3, 6, 8, 20, 31, 34, 47), contributing to the overall increased morbidity and mortality in diabetic patients (17, 44). The arterial baroreflex normally minimizes short-term oscillations in blood pressure and maintains a homeostatic state, acting on both the sympathetic and parasympathetic limbs of the autonomic nervous system (22, 33). Serving as a primary afferent limb of the baroreceptor reflex, baroreceptor neurons within the nodose and petrosal ganglia respond to elevation of blood pressure by increasing their discharge (excitation) and initiating decrease in heart rate via the baroreflex arc. Although any part of the baroreflex arc could be responsible for the baroreflex dysfunction in the diabetic state, recent studies have suggested that the afferent component of the baroreflex is involved in diabetes-related impairment of the baroreflex sensitivity (19, 27). Our recent studies have focused on the excitability of the aortic baroreceptor (AB) neuronal cells in the nodose ganglia (NG). We have found that diabetes-enhanced hyperpolarization-activated cyclic nucleotide-gated (HCN) channel function mediates the depressed AB neuron excitability, which may contribute to the blunted baroreflex sensitivity in diabetes mellitus (29, 45).

Angiotensin II (ANG II) is a physiologically active component of the renin-angiotensin system, which plays an important role in the maintenance of BP and fluid homeostasis (38). It is well documented that systemic and tissue ANG II levels are increased during diabetes in patients and animal models (21, 41, 43). Allen et al. (1) have demonstrated ANG II receptor binding in neuronal cell bodies of the NG. More importantly, our recent study has indicated that exogenous ANG II can activate the HCN channels and subsequently decrease the membrane excitability of rat AB neurons via AT 1R-NADPH oxidase-derived superoxide (50). Based on these studies, we hypothesized that endogenous ANG II-NADPH oxidase-superoxide signaling might be involved in diabetes-induced HCN channel hyperactivation and depression of the cell excitability in AB neurons. In the present study, therefore, we measured the endogenous ANG II concentration, mRNA, and protein expression of AT 1R, protein expression of NADPH oxidase components (p22 phox, p40 phox, p47 phox, p67 phox, and gp91 phox), and superoxide production in the NG from sham and diabetic rats. We also measured the effect of AT 1R antagonist (losartan), NADPH oxidase inhibitor (apocynin), and superoxide dismutase mimetic (tempol) on HCN currents and cell excitability in the AB neurons from diabetic rats. Preliminary results from this study have been published in an abstract form (49).

METHODS

Male Sprague-Dawley rats (200–220 g) were housed two per cage under controlled temperature and humidity and a 12:12-h dark-light cycle. Water and rat chow were provided ad libitum. Experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 85-23, Revised 1996) and the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

Materials. Losartan and L158,809 were obtained as a gift from Merck. Apocynin was purchased from CalBiochem. Tempol was purchased from Alexis Biochemicals. Other chemicals used in this study were obtained from Sigma-Aldrich Chemical.

Induction of diabetes. Rats were randomly assigned to sham (n = 59) and diabetic rats (n = 65). Diabetes was induced by a single
intraperitoneal injection of streptozotocin (65 mg/kg, Sigma) in a 2% solution of 0.1 M cold citrate buffer. Sham rats received a similar injection of vehicle. Diabetes was identified by polydipsia, polyuria, and blood glucose >250 mg/dl (Accu-check Aviva, Boehringer Mannheim). Rats receiving streptozotocin but with blood glucose ≤250 mg/dl were excluded (n = 7) from the study. Blood glucose and body weight in all rats were measured weekly. All experiments were taken at 6–8 wk after single dose injection of streptozotocin or vehicle. When each rat was anesthetized with a combination of urethane (800 mg/kg ip) and chloralose (40 mg/kg ip), both NGs in each rat were removed for varied measurements. The rat was then euthanized with an intraperitoneal injection of pentobarbital sodium (150 mg/kg).

**ANG II measurement in NGs.** ANG II concentration in NG was measured by ANG II 125I radioimmunooassay kit (Buhlmann Laboratories, Switzerland). The final ANG II concentration was counted by 1470 Automatic Gamma Counter (Perkin Elmer, Shelton, CT) and calculated with a standard curve generated for each experiment.

**Labeling of AB neurons.** AB neurons in the NG were selectively retrograde-labeled by a transported fluorescent dye Dil (red color, Molecular Probes, Eugene, OR) as described previously (29). Briefly, under sterile conditions, a thoracotomy was made at the third intercostal space, and Dil (2 μl) was injected into the adventitia of the aortic arch with a fine-tipped glass pipette. After application of the dye, the surgical incision was closed. The rat was allowed to recover for at least 1 wk to allow the dye to be transported in a retrograde direction to the AB neurons in the NG.

**Single-cell real-time PCR for AT1R.** Nodose neurons were isolated (see Isolation of nodose sensory neurons) and loaded in a chamber with regular extracellular solution (in mM): 137 NaCl, 5.4 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 glucose with pH 7.4. A patch-clamp pipette (1–3 MΩ resistance) was used to break the membrane of single AB (A-type or C-type) neuronal cell identified by Dil labeling (29). Under the suction condition, the cell and pipette’s content were moved in and expelled into a 0.2-ml PCR tube containing following reagents: 5 μl lRNA guard, 1 μl iScript Reverse Transcriptase (Bio-Rad), and 10 μl iQ Syber Green Supermix (Bio-Rad), 40 nM (in the first round) or 300 nM (in the second round) of primers of the first round and a new internal primer. PCR reaction was performed in a 50-μl volume containing 25 μl iQ SYBR Green Supermix (Bio-Rad), 40 nM (in the first round) or 300 nM (in the second round) of each primer. In the first round of amplification, a 2-μl aliquot of the RT product was used and then 5 μl of the first-round product was used in the second round of amplification. Negative control samples were taken from the aspiration buffer without cells. The cDNA was amplified by real-time quantitative PCR with the Bio-Rad iCycler iQ System. After 10 min of denaturation at 94°C, the amplification was performed with 30–35 thermal cycles of 94°C for 1 min, 56°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 5 min. For quantification, AT1R gene was normalized to the expressed housekeeping gene RPL19. The data were analyzed by the 2-ΔΔCT method (32).

Western blot analysis for AT1R and NADPH oxidase components. NADPH oxidase is a complex enzyme consisting of two membrane-bound components (gp91phox and p22phox) and three cytosolic components (p47phox, p40phox, and p67phox) (11). Therefore, we measured the protein expression for each NADPH oxidase component. NGs from sham and diabetic rats were rapidly removed and immediately frozen in liquid nitrogen and stored at −80°C until analyzed. The protein of NG homogenate was extracted with the lysing buffer (10 mM Tris, 1 mM EDTA, 1% SDS, pH 7.4) plus protease inhibitor cocktail (Sigma, 100 μl/ml). Total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce; Rockford, IL). Equal amounts of the protein samples were loaded and then separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins of these samples were electrophoretically transferred to PVDF membrane. The membrane was probed with goat anti-AT1R, p22phox, p47phox, p40phox, or gp91phox antibodies (Santa Cruz) and a peroxidase-conjugated rabbit anti-goat IgG (Pierce Chemical, Rockford, IL). The signal was detected using enhanced chemiluminescence substrate (Pierce Chemical), and the bands were analyzed using UVP bioimaging system. The target protein was controlled by probing the Western blot with mouse anti-GAPDH antibody (Santa Cruz) and normalizing target protein intensity to that of GAPDH.

**Immunofluorescence for AT1R.** Each rat was perfused with 50 ml heparinized saline followed by 150 ml of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). Both NGs in each rat were rapidly removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for 12 h at 4°C, followed by soaking the NG in 30% sucrose for 12 h at 4°C for cryostat preparation. The NG was cut into 10-μm thick sections and then mounted on precoated glass slides. The NG sections were incubated with 10% goat serum for 1 h followed by incubation with goat anti-AT1R (Santa Cruz, CA) and mouse anti-R1T7 antibodies [an A-type neuron marker (39), Abcam, Cambridge, MA] overnight at 4°C. Then the sections were washed with PBS and incubated with fluorescence-conjugated secondary antibody and Alexa Fluor 488 conjugated isoclectin-B4 [IB4, a C-type neuron marker (46), Invitrogen] for 60 min at room temperature. After three washes with PBS, the sections were mounted on precoated microscope slides. Slides were observed under a Leica fluorescent microscope with corresponding filters. Pictures were captured by a digital camera system. No staining was seen when PBS was used instead of the primary antibody in the above procedure. Fifteen slides from each rat were measured for AT1R staining.

**Measurement of superoxide production.** NG samples were homogenized in PBS solution at 4°C. Total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). Superoxide ion production was measured using lucigenin chemiluminescence method as described previously (26, 28, 50). The homogenate (0.3 ml) was placed in 0.5 ml microfuge containing dark-adapted lucigenin (5 μM), and then accumulative light emission was recorded for 5 min in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

**Isolation of nodose sensory neurons.** NGs were removed from each rat and placed in ice-cold modified Ringer solution (mM): 137 NaCl, 25 NaHCO3, 3 KCl, 1.25 NaH2PO4, 1.2 CaCl2, 1.2 MgSO4, and 10 glucose. The NGs were dissected free and incubated for 30 min at 37°C in an enzymatic modified Ringer solution containing 0.1% collagenase and 0.1% trypsin. The tissues were then transferred to a Ringer solution containing 0.2% collagenase and 0.5% bovine serum albumin for a 30 min incubation at 37°C. The isolated cells were cultured at 37°C in a humidified atmosphere of 95% air-5% CO2 for 4 to 24 h before the patch-clamp experiments because data from Kwong et al. (25) and our preliminary experiment showed that ion channel currents recorded in the acutely dissociated neurons were
RESULTS

Induction of diabetes. Diabetes was induced by single-dose injection of streptozotocin (65 mg/kg). After 6–8 wk of streptozotocin injection, the mean blood glucose was significantly higher (421 ± 10 mg/dl, n = 52) compared with that in sham rats (vehicle injection, 95 ± 10 mg/dl, P < 0.05, n = 53). The body weight in diabetic rats was lower than that in sham rats (234 ± 9 g vs. 368 ± 11 g, P < 0.05).

ANG II concentration and AT1R mRNA and protein expression in NG from sham and diabetic rats. ANG II concentration in the NG tissue from diabetic rats was significantly elevated (101.6 ± 4.8 pg/mg protein, n = 8 rats, P < 0.05) compared with that in sham rats (38.9 ± 4.2 pg/mg protein, n = 8 rats). Using single-cell, real-time PCR, we found that diabetes induced a significant increase of the AT1R mRNA in AB neurons compared with sham (Fig. 1). From Western blot data, we further confirmed that protein expression of AT1R was increased in NG from diabetic rats compared with sham rats (Fig. 2A). Similarly, immunofluorescence staining for AT1R protein (Fig. 2B) was increased in the A- and C-type neurons from diabetic rats (Fig. 2B).

Protein expression of NADPH oxidase components and superoxide production in NG from sham and diabetic rats. In the NG from diabetic rats, the protein expression of p22phox, p40phox, p47phox, p67phox, and gp91phox was elevated compared with that in sham rats (Fig. 3).

In addition, superoxide production was higher in NG from diabetic rats than that from sham rats. Losartan (AT1R antagonist), apocynin (NADPH oxidase inhibitor), and tempol (superoxide dismutase mimetic) each decreased superoxide production in NG from diabetic rats (Fig. 4).

Effect of losartan, apocynin, and tempol on HCN currents in AB neurons from sham and diabetic rats. Figure 5A illustrates typical HCN current recordings and I-V curves before and after treatment of losartan in A-type AB neurons from diabetic rats. From mean data shown in Fig. 5B, HCN current density was markedly enhanced in A-type and C-type AB neurons from...
diabetic rats compared with that from sham rats. Extracellular treatment of losartan (1 μM), apocynin (100 μM), and tempol (1 mM) each significantly reduced the HCN current density in the A-type and C-type AB neurons from diabetic rats. The combined benefit of losartan, apocynin, and tempol on the HCN current density was nonadditive (Fig. 5B). The intracellular administration of losartan (1 μM, added to the recording pipette solution) also decreased the HCN current density in A- (31.4 ± 4.5 pA/pF) and C-type (13.2 ± 2.7 pA/pF) AB neurons from diabetic rats (n = 6 cells in each group, P < 0.05). However, losartan, apocynin, and tempol did not induce any alteration of the HCN current density in the A-type and C-type AB neurons from sham rats (Fig. 5B, P > 0.05).

In addition, like losartan, apocynin, and tempol, L158,809 (1 μM, an AT1 receptor antagonist), phenylarsine oxide (2 μM, a NADPH oxidase inhibitor), and polyethylene glycol-superoxide dismutase (50 U/ml, a membrane-permeable superoxide dismutase) also markedly decreased the HCN currents in the AB neurons from diabetic rats (n = 7 cells in each group, P < 0.05), which further confirmed that ANG II-NADPH oxidase-derived superoxide modulated the alteration of HCN channels in diabetic AB neurons.

**Effect of losartan, apocynin, and tempol on action potentials in AB neurons from sham and diabetic rats.** Figure 6A shows the original action potential recording obtained from an A-type AB neuron of diabetic rat before and after treatment with losartan (1 μM). Losartan increased the number of action potentials induced by a 50-pA current injection and hyperpolarized the resting membrane potential.

In addition, action potentials were elicited in current clamp (1 s, 50 pA for A-type neurons and 250 pA for C-type neurons) (50). Diabetes significantly depolarized the resting membrane potential, reduced the input resistance, and decreased the number of action potentials in A- and C-type AB neurons compared with that in sham rats (Fig. 6, B, C, and D). Furthermore, losartan, apocynin, and tempol each markedly hyperpolarized the resting membrane potential, raised the input resistance, and increased the number of action potentials in the A-type and C-type AB neurons from the diabetic rats (Fig. 6, B, C, and D). However, these inhibitors did not show any effect on the action potentials in the sham rats.
potential in both A-type and C-type AB neurons from diabetic rats. These results indicate that endogenous ANG II-NADPH oxidase-superoxide signaling is involved in the blunted AB neuron excitability in the diabetic state.

ANG II, an endogenous peptide, has been thought to be a prime candidate in the regulation of the HCN channel function and cell excitability in the diabetic state. This is known because circulating and tissue ANG II concentrations are elevated in humans and animals with diabetes (21, 41, 43). Previous autoradiographic study has identified a high density of ANG II receptor binding sites over the NG neuronal cell bodies (1). Widdop et al. (48) provided evidence for the direct neuronal effects of ANG II on the vagal afferent neurons. Indeed, our previous study confirmed AT1R existed in the NG neuronal cells (50). We also found that exogenous ANG II enhanced the HCN currents and subsequently reduced cell excitability in normal rat AB neurons via NADPH oxidase-derived superoxide because apocynin and tempol blunted the effect of the exogenous ANG II on the HCN currents and action potentials (50). More importantly, in the present study, ANG II concentration and protein expression of AT1R were increased in the NG neuronal cells from diabetic rats. At the same time, using single-cell, real-time PCR, we also found that the mRNA expression of AT1R was increased in AB neuron cells from diabetic rats (Fig. 1). In addition, AT1R antagonist, NADPH oxidase inhibitor, and superoxide dismutase mimetic significantly attenuated the HCN currents and increased the cell excitability (including hyperpolarization of the resting membrane potential, rise of the input resistance, and increase of the action potential number) in the AB neurons (without exposure to circulating ANG II) from diabetic rats (Figs. 5 and 6). The HCN channels in the NG neurons generally exhibit neither pacemaker activity nor oscillatory potentials, unlike that in the cardiac pacemaker cells and central neurons (14, 15). The resting membrane potential of the NG neurons is about −50 to −60 mV at which voltage-dependent sodium, calcium, and potassium channels are almost inactivated (14). The inactivation of these voltage-dependent channels can be recovered to the activation state during the hyperpolarization of the resting membrane potential, which means the number of available

Fig. 3. Protein expression of NADPH oxidase components in nodose ganglia from sham and diabetic rats. The representative (A) and summary (B) results for protein expression of NADPH oxidase components. Data are means ± SE, n = 6 rats in each group. *P < 0.05 vs. sham rats.

Fig. 4. Superoxide production in homogenates of the nodose ganglia from sham and diabetic rats, as measured by lucigenin chemiluminescence. MLU, mean light units. Data are means ± SE, n = 6 rats in each group. *P < 0.05 vs. sham rats; #P < 0.05 vs. diabetes.

DISCUSSION

The present study showed that mRNA and protein expression of the AT1R and the local tissue ANG II concentration in the NG were markedly elevated in the diabetic rats. In addition, protein expression of NADPH oxidase components and superoxide production were increased in the NG from diabetic rats. Diabetes significantly enhanced the HCN currents and reduced cell excitability of the AB neurons, as reported in our previous study (29). AT1R antagonist (losartan and L158,809), NADPH oxidase inhibitor (apocynin and phenylarsine oxide), or superoxide dismutase mimetic (tempol) and analogue (polyethylene glycol-superoxide dismutase) normalized the HCN currents and increased the cell excitability in the A-type and C-type AB
voltage-dependent channels for activation is increased if the NG neurons receive the depolarizing stimulus (14). Inhibition of the HCN channels has been shown to hyperpolarize the NG neurons (increasing the resting membrane potential) and to reduce action potential threshold in response to a depolarizing current stimulation, thus rendering the NG neurons more excitable (15, 29). In addition, the increase in input resistance induced by blocking HCN channels would make the NG neurons more excitable with the same current injection (15).

As mentioned above, our present study showed that AT1R antagonist, NADPH oxidase inhibitor, and superoxide dismutase mimetic normalized the HCN currents, hyperpolarized the resting membrane potential, and increased the input resistance and the frequency of the action potentials in the diabetic AB neurons (Figs. 5 and 6). Therefore, these results clearly indicate that the endogenous ANG II-NADPH oxidase-superoxide signaling pathway primarily mediates the enhanced HCN channel activity and the blunted cell excitability in the AB neurons in diabetes.

Under physiological conditions, normal cellular metabolism can produce the superoxide (35). This low level of the superoxide is essential for proper cell function (18). Our previous study has shown that tempol does not induce any alteration of the HCN currents and cell excitability in the AB neurons from sham rats, although tempol decreases this basal superoxide production (50). Our present study also confirmed losartan, apocynin, and tempol did not change the HCN currents and cell excitability in the AB neurons from sham rats (Figs. 5 and 6). In addition, in the present study, there was no significant difference among losartan, apocynin, and tempol in their ability to reduce the HCN currents (Fig. 5) and to increase the cell excitability (Fig. 6) in the diabetic AB neurons even though losartan and apocynin partially decreased the superoxide production in the NG from diabetic rats compared with tempol (Fig. 4). It is possible that the superoxide can affect the HCN channels only when the superoxide production reaches a higher level. As we know, several reactive oxygen species (ROS) are formed in the oxygen reduction reaction such as superoxide,
hydrogen peroxide, and hydroxyl radical (36). It is very difficult to distinguish the signaling pathway of each ROS because superoxide is rapidly dismutated into hydrogen peroxide and then the latter quickly forms hydroxyl radical. Many studies have shown that ROS can modulate the electrophysiological kinetics of ion channels (16, 24, 42). However, our present study found tempol and polyethylene glycol-superoxide dismutase normalized the enhanced HCN currents (Fig. 5) and increased the cell excitability (Fig. 6) in the diabetic AB neurons. From the present data, therefore, we consider that high level superoxide production modulates the activation of HCN channels in diabetic AB neurons. Additionally, there is no report on the mechanisms by which superoxide modulates the HCN channels so far. The limitation of the whole cell patch-clamp recording used in the present study prevents measurement for the direct modulation of superoxide on the HCN channels. Therefore, single channel recording is needed in further study to explore how the HCN channels are modulated by superoxide.

Our results suggest that elevation of local tissue ANG II plays an important role on the enhanced HCN channel activity and the blunted cell excitability in the AB neurons in diabetes. However, it is unclear how ANG II and losartan within an isolated AB neuron from diabetic rat interacts with AT1R to affect the HCN channel activity and cell excitability. Classical viewpoint about the effects of ANG II binding with AT1R is that ANG II binds with AT1R at the cell membrane, and following the phosphorylation of the AT1R, ANG II induces...
intracellular responses via activation of intracellular downstream signal transduction. However, Zhuo et al. (51) have found that there is substantial intracellular accumulation of ANG II in renal cortical endosomes during ANG II-dependent hypertension via an AT$_1$R-mediated process. Recent studies have shown that intracellular administration of ANG II increases the peak inward calcium current density and decreases the junctional conductance via intracellular AT$_1$R in cardiac myocytes (9, 10). Intracellular treatment with losartan (a selective AT$_1$R antagonist) abolishes the effect of intracellular ANG II (1, 2). Based on these studies, we reason that diabetes-induced elevation of intracellular ANG II concentration in the NG contributes to the enhanced HCN channel activity and the blunted cell excitability in the AB neurons in diabetes. This viewpoint is confirmed by our observation that intracellular administration of losartan (added to the recording pipette solution) decreased the HCN current density and increased the cell excitability in the AB neurons from diabetic rats (see text in RESULTS). Therefore, it is possible there is an intracellular ANG II production system in the NG tissue. It would be optimal to measure intracellular ANG II concentration in the AB neurons, but there is no appropriate measurement for it so far due to insufficient cellular material of tiny NG. This issue needs to be confirmed by further study.

Our recent study has shown that 5 mM CsCl [HCN channel blocker in NGs (15)] similarly increases the action potential frequency in A-type AB neurons of sham and diabetic rats (29). In the present study, losartan, apocynin, and tempol normalized the diabetes-enhanced HCN currents as seen in sham rats (Fig. 5B). However, they significantly enhanced the action potential frequency in the AB neurons of diabetic rats, even over the normal level seen in sham rats (Fig. 6D, $P < 0.05$). Taking these results together, we speculate that the changes of other ion channels (such as calcium channels, potassium channels, etc.) modulated by ANG II-NADPH oxidase-superoxide signaling may also contribute to the alteration of cell excitability in the AB neurons of diabetic rats.

Although expression of AT$_1$R protein is increased in A-type and C-type NG neurons from diabetic rats (Fig. 2), we cannot clearly confirm increased expression of AT$_1$R protein in A-type and C-type AB neurons from diabetic rats due to the limitation of the method (DiI labeling was lost from cells during the immunofluorescent staining procedure and could not be used as a marker of AB neurons with immunofluorescent staining). However, it is reasonable to assume that AT$_1$R protein is overexpressed in AB neurons from diabetic rats because AB neurons are a component of the nodose neurons, and single cell real-time PCR data indicate that diabetes increases the expression of AT$_1$R mRNA in the AB neurons (Fig. 1).

Our present study indicates that elevated endogenous ANG II binding with AT$_1$R mediates the enhanced HCN channel activity and the blunted cell excitability in the AB neurons of the diabetic rats via NADPH oxidase-derived superoxide production. However, the process that occurs in the long-term development of diabetes that causes an upregulation of this local ANG II-AT$_1$R-NADPH oxidase-superoxide signaling in NG neuronal cells is not evident from the present study. In addition, we cannot investigate the effect of circulating ANG II on the isolated cell function in the present study. Therefore, further study is needed to explore these outstanding issues.
In summary, the present study demonstrates that the endogenous ANG II-NADPH oxidase-superoxide signaling is elevated in the NG from diabetic rats. Although we don’t know how diabetes increases the endogenous ANG II concentration and protein expression of AT₁R and NADPH oxidase, our present study indicates that the endogenous ANG II-NADPH oxidase-superoxide signaling contributes to the enhanced HCN channel activity and the blunted cell excitability in the AB neurons (A- and C-type neurons) from diabetic rats. The results of the present study would be helpful to understand the mechanisms of angiotensin-converting enzyme inhibitors, AT₁R antagonists, and antioxidants for the treatment of complications including baroreflex dysfunction in diabetes mellitus.

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