

## Original Article



# Insulin sensitization causes accelerated sinus nodal dysfunction through autophagic dysregulation in hypertensive mice

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## ABSTRACT

Insulin sensitizers, while effective in glucose-lowering for diabetes control, are linked to an increased risk of heart disease through mechanisms that are not well understood. In this study, we investigated the molecular mechanisms underlying the effects of insulin sensitization on cardiac sinus node dysfunction. We used pharmacologic or genetic approaches to enhance insulin sensitivity, by treating with pioglitazone or rosiglitazone, or through phosphatase and tensin homolog (*PTEN*) deletion in cardiomyocytes respectively. We employed an angiotensin II (Ang II)-induced hypertensive animal model which causes sinus node dysfunction and accumulation of oxidized calcium/calmodulin-dependent protein kinase II (CaMKII), which also serves as a biomarker for this defect. While neither *PTEN* deficiency nor insulin sensitizers caused sinus node dysfunction in normotensive mice, both accelerated the onset of sinus node dysfunction and CaMKII oxidation in hypertensive mice. These abnormalities were accompanied by a significant defect in autophagy as revealed by unc-51 like autophagy activating kinase 1 (ULK1) signaling. Indeed, mice deficient in *ulk1* in cardiomyocytes and the sinus node also showed early onset of slow atrial impulse conduction with frequent sinus pauses and upregulated CaMKII oxidation following Ang II infusion similar to that seen with *PTEN* deficiency, or treatment with insulin sensitizers. To further elucidate the role of autophagy in sinus node dysfunction, we treated mice with a peptide D-Tat-beclin1 that enhanced autophagy, which significantly abrogated the frequent sinus pauses and accumulation of oxidized CaMKII induced by insulin sensitizers treatment, or *PTEN* deficiency in hypertensive animals. Together, these findings provide clear evidence of the detrimental cardiac effects of insulin sensitization that occurs through failure of autophagy-mediated proteolytic clearance.

**Keywords:** Insulin Sensitizer; Autophagy; ULK1; Hypertension; Heart

## INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterized by multiple pathophysiologic abnormalities and is associated with a 2- to 3-fold increased risk in cardiovascular disease compared to non-diabetic individuals [1,2]. Therefore, when selecting medications to treat individuals with T2DM, it is important that the agent does not have cardiovascular morbidity and mortality.

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Although rosiglitazone (RSG), a peroxisome proliferator-activated receptor (PPAR)- $\gamma$  agonist, is effective in lowering blood glucose through increased insulin sensitivity, it was reported that it significantly increases the risk of heart failure and cardiovascular death by recent studies [3-5]. However, the precise mechanisms through which the insulin sensitizer causes these adverse events are not clear. Here we focused on the cardiac sinoatrial node to better understand the potentially detrimental effects of insulin sensitization.

The Sinoatrial node is the nascent pacemaker of the heart and is responsible for the initiation of the heartbeat [6,7]. It spontaneously generates an electrical impulse that propagates through the heart, causing cardiac muscle contraction [6]. Throughout life, this small number of cells is required to generate continuous electrical impulses [6,8]. With aging, oxidative stress can be overcome by the removal of oxidized proteins and maladaptive organelles [8,9]. These self-preserving processes are collectively referred to as autophagy [9]. During autophagy, a preautophagosome engulfs cytosolic components and forms an autophagosome, which subsequently fuses with a lysosome, leading to the proteolytic degradation of internal components [9]. To date, more than 36 autophagy (Atg)-related proteins have been identified [10]. Among these, Atg1 shows strong homology with *Caenorhabditis elegans* uncoordinated-51 (UNC-51), which has a mammalian homolog known as ULK1 [11].

Stimulation of insulin signaling through deletion of PTEN, a potent negative regulator of this pathway, or by PPAR- $\gamma$  activation, promotes mammalian target of rapamycin (mTOR) activity, a negative regulator of autophagy that suppresses ULK1 [12,13]. We, therefore, hypothesized that treatment with PIO or RSG, or PTEN deficiency increases the accumulation of oxidized proteins by impeding autophagy in sinus nodal cells. Sinus node dysfunction commonly occurs in the setting of hypertension, a condition characterized by excessive activation of renin-angiotensin II (Ang II) signaling [14,15]. Recently, Ang II-induced calmodulin kinase II (CaMKII) oxidation has been shown to provoke sinus node dysfunction in humans and animals [14,16]. However, the specific role of autophagy in CaMKII oxidation in the sinus node remains unclear.

The present study aimed to investigate the effects of insulin sensitization on sinus node function and its regulation on autophagy in hypertensive animals. Our results show that treatment with PIO or RSG, or PTEN deficiency accelerates sinus node dysfunction through attenuating autophagic clearance of oxidized CaMKII in hypertensive mice. As sinus node dysfunction plays a central role in the initiation and progression of heart failure, mechanisms that clarify our understanding by which insulin sensitization links to cardiovascular disease will enhance the discovery of cardioprotective drugs.

## METHODS

### Mice

In the current study, *ulk1<sup>fl/fl</sup>* and *Myh6-cre* mice were purchased from Jackson Lab. *PTEN<sup>fl/fl</sup>* mice were kindly provided by Dr. Tak Mak. All mice were in C57BL/6 background and corresponding wild-type littermate controls were used. All animal experiments were approved and performed by the NIH guidelines (Guide for the care and use of laboratory animals) and the Ewha Womans University Animal Care Committee. *PTEN<sup>fl/fl</sup>* or *ulk1<sup>fl/fl</sup>* mice were mated with *Myh6-cre* (cre transgene under the control of the cardiac-specific mouse alpha myosin heavy chain promoter) to produce *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* or *Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>*.

Only male mice 6–10 weeks old were used in this study, and all mice were housed in specific pathogen-free conditions. Mice were genotyped by polymerase chain reaction using the following primers; *PTEN* wild type allele (5'-CTCCTCTACTCCATTCTTCCC-3' and 5'-ACTCCCACCAATGAACAAAC-3', generating a 200-bp fragment) or the *PTEN* floxed allele (5'-CTCCTCTACTCCATTCTTCCC-3' and 5'-ACTCCCACCAATGAACAAAC-3', generating a 300-bp fragment). All samples were subjected to the following conditions: 94°C 5 min, (94°C 60 s, 62°C 60 s, 72°C 60 s; 10 cycles) and 72°C 10 min; *ulk1* wild type allele (5'-CAGTTAGGTTCACTGCAGACTTG-3' and 5'-TTTATCCGTCTTCTGCTATTGG-3', generating a 291-bp fragment) or the *ulk1* floxed allele (5'-CTTGGGTGGAGAGGCTATTC-3', 5'-AGGTGAGATGACAGGAGATC-3', generating a 280-bp fragment). All samples were subjected to the following conditions: 94°C 2 min, (94°C 20 s, 65°C 15 s, 68°C 10 s; 10 cycles), (94°C 15 s, 50°C 15 s, 72°C 10 s; 28 cycles) and 72°C 2 min; *Myh6-cre* allele (5'-ATGACAGACAGATCCCTCCTATCTCC-3', 5'-CTCATCACTCGTTGCATCATCGAC-3', generating a 300-bp fragment). All samples were subjected to the following conditions: 94°C 3 min, (94°C 30 s, 58°C 60 s, 72°C 60 s; 35 cycles) and 72°C 2 min. Mice received Ang II (800 ng/min per kg body weight), PIO (13.8 µg/min per kg body weight), RSG (20 µg/min per kg body weight), D-Tat-beclin1 (4 µg/min per kg body weight) or Saline (0.9% NaCl) infusions via a subcutaneously implanted osmotic minipump (Alzet, model 1002) for 4 weeks. Blood pressure was measured by tail cuff in unanesthetized mice using a BP2000 (Visitech Systems, Inc., USA). Systolic and diastolic blood pressure were obtained as the average of 7–12 measurements that showed stable readings over 10–15 min. Mice were anesthetized with 375 mg/kg 2,2,2-tribromoethanol (Avertin, Sigma Chemical Co.) by intraperitoneal injection.

### Echocardiographic assessment

For echocardiography, Vevo 2100 was used at Cardiovascular Research Center in Seoul. Mice were anesthetized with 2% isoflurane and maintained with 1.5% isoflurane followed by application of depilatory cream to the chest and wiped clean to remove all hair in the area of interest. The scanning probe (20 MHz) was used to obtain 2D images of the parasternal long axis. These 2D images were converted to M-mode.

### Isolated Langendorff heart and ex vivo electrocardiogram (ECG) recording

Hearts of anesthetized mice were carefully excised and aorta cannulated. Hearts were secured by tying below the innominate artery and perfused retrogradely by the nonrecirculating Langendorff technique with Krebs-Henseleit buffer containing 10 mmol/L glucose (pH 7.4). Perfusion fluid was controlled by a peristaltic pump continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. ECGs were continuously recorded using a Biopac system-ECG100 (Biopac system, Inc., USA).

### Isolation of adult cardiomyocytes and sinus nodal cells from heart

Right atria were digested with collagenase II (0.1 g/1 mL, Worthington Inc., USA) for 2 h. Cells were plated on laminin-coated six-well culture plates with Leibovitz Media (to a density of 200,000 cells/well). Cells were maintained using Leibovitz Media to select for atrial cardiomyocytes or using 20% FBS-DMEM to select for sinus nodal cells at 37°C under an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub> for 3 days. Subsequently, the protein was extracted and measured for T-Box 3 (TBX3; a sinus node marker) and modifier of locomotor activity 2 (MLCA2; an atrial cardiomyocyte marker) using Western blotting.

### Glucose uptake in primary cultures of cardiomyocytes

The isolated sinus nodal cells were treated with 0.1 µM insulin for 30 min and washed twice with 2 ml of PBS buffer to remove serum and glucose. The cells were then incubated for

30 min in 1 mL of fresh KR buffer and glucose uptake assay was done over 30 min after the addition of 0.5  $\mu\text{Ci/mL}$  2-deoxyglucose. The assay was terminated by rapid washes with 1 mL of ice-cold PBS buffer. Cells were disrupted with 1 mL of 0.5 M NaOH for 60 min at 37°C, and cell-associated radioactivity was determined by scintillation counting. Glucose uptake was expressed as a percentage of basal uptake.

### Western blotting

Hearts or cells were isolated and lysed, extracts were quantified for protein content and boiled with loading dye, and 200  $\mu\text{g}$  was used in gel electrophoresis. After blotting, membranes were incubated with antisera directed against LC3 I & II (1:2,000), ox-CaMKII (1:2,000), total-CaMKII (1:2,000), TBX3 (1:1,000), MLCA2 (1:1,000), beta-actin (1:3,000), phospho-ULK1 (1:1,000), total-ULK1 (1:1,000) antibodies, and then with secondary antibodies (mouse-specific HRP-conjugated antibody or rabbit-specific HRP-conjugated antibody). Bands were visualized using an ECL detection kit and quantified by densitometry.

### Statistical analysis

Values are means  $\pm$  SE. The significance of differences was determined by a two-way analysis of variance (ANOVA), or a one-way ANOVA followed by a Bonferroni post-hoc analysis in GraphPad Prism 9. Differences were considered significant when  $p < 0.05$ .

### Materials

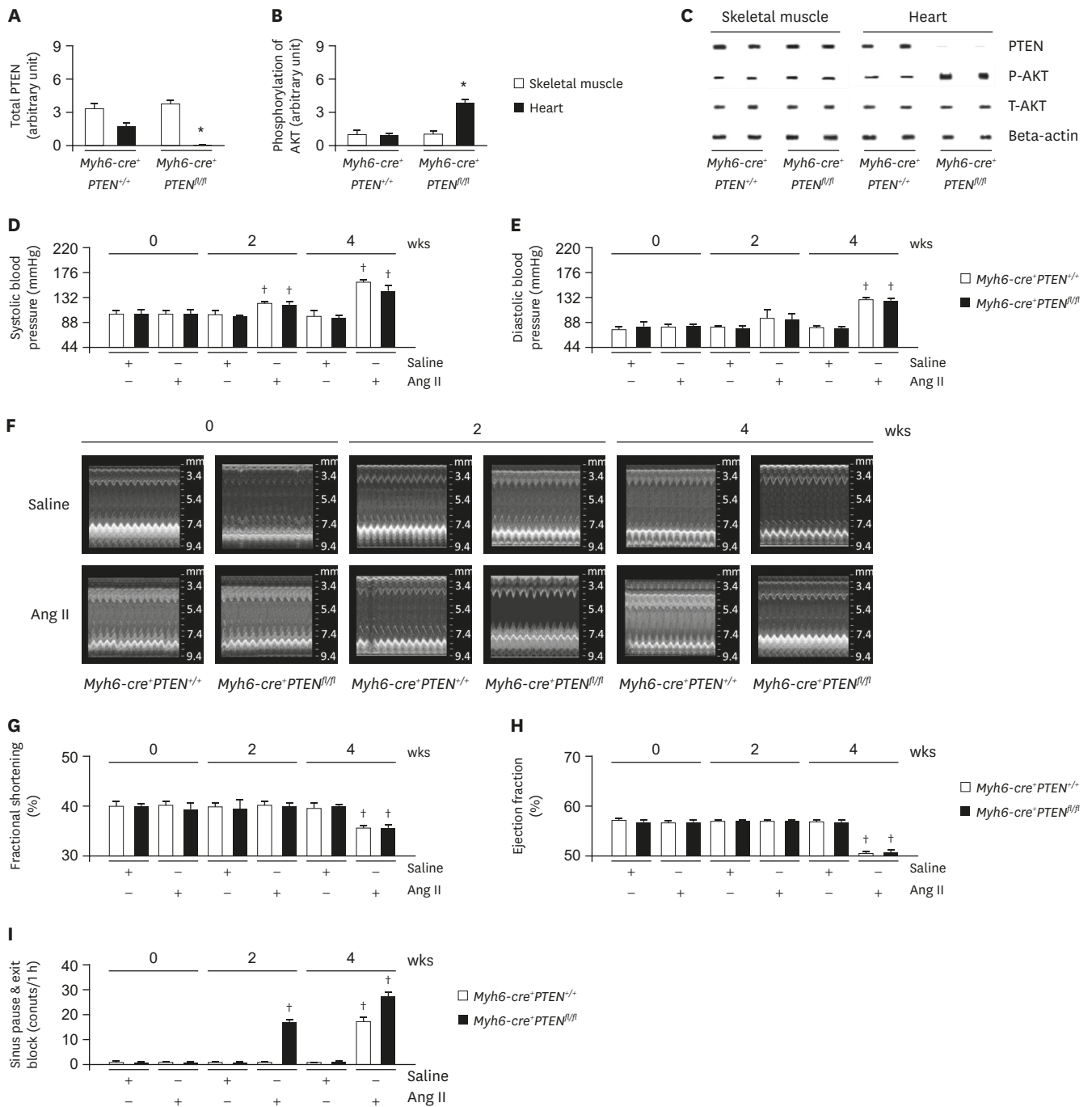
Primers for genotyping were obtained from Integrated DNA Technologies. Total-CaMKII (ab22609), TBX3 (ab154828), and MLCA2 (MYL-7; ab68086) antibodies were obtained from Abcam. Ox-CaMKII (GTX36254) was purchased from GeneTex. Beta-actin (#8457), LC3 I & II (#12741), phospho-ULK1 (#12753), and total-ULK1 (#6439) were purchased from Cell Signaling Technology. D-Tat-Bec1-1 was obtained from Calbiochem. Pioglitazone (#E6910) and Angiotensin II (#A9525) were purchased from Sigma Aldrich. The enhanced chemiluminescence detection kit was purchased from Advansta.

## RESULTS

### PTEN deficiency accelerates Ang II-induced sinus nodal dysfunction

To study the effects of enhanced insulin sensitivity on cardiac dysfunction, we generated cardiac-specific *PTEN* knockout (KO; *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>*) mice. Western blotting showed a significant reduction in the amount of PTEN protein in the heart, but not in skeletal muscle, of *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice (**Fig. 1A and C**). In support of enhanced insulin signaling, AKT phosphorylation was up-regulated in the hearts of *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice compared to those of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice (**Fig. 1B and C**). Mice were infused with Ang II or saline for 4 weeks at which time both systolic and diastolic blood pressure increased (**Fig. 1D and E**), and fractional shortening and ejection fraction decreased in the Ang II group. PTEN deficiency did not result in any changes in the contractile function (**Fig. 1F-H**). To determine whether our model of Ang II infusion promoted sinus node dysfunction, I obtained ECG in Langendorff-perfused hearts of mice (**Fig. 1I and J**). In saline-infused normotensive mice, hearts of *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice did not show any changes in sinus node function compared to those of saline-treated *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice. On the other hand, hearts of Ang II-infused *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice exhibited sinus pauses indicative of sinus node dysfunction starting at 2 weeks, at a similar time to when they developed hypertension, much earlier than control *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice that started showing sinus pauses only at 4 weeks of Ang II infusion. At this time, *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice

Autophagy regulation of sinus node function

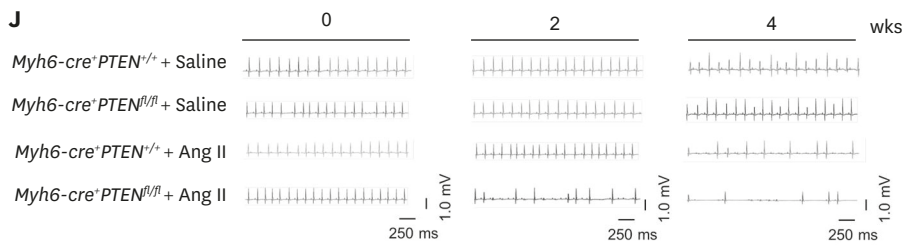


**Figure 1.** PTEN deficiency promotes sinus node dysfunction in hypertensive animals. (A-C) Western blot analysis for total AKT, phosphorylation of AKT, or total PTEN from cardiac ventricle or skeletal muscle of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice. (D-E) Systolic and diastolic blood pressure in *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice infused with Ang II (800 ng/min per kg body weight) or Saline (0.9% NaCl) for 4 weeks. (F) M-mode echocardiographic images from *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice infused with Ang II (800 ng/min per kg body weight) or saline (0.9% NaCl) for 4 weeks. (G-H) Fractional shortening and ejection fraction were determined from the M-mode images. (I) Sinus pauses and exit blocks were counted from ECG recordings from hearts isolated from mice infused with Ang II (800 ng/min per kg body weight) or Saline (0.9% NaCl) for 4 weeks. (J) Representative ECG recordings from Langendorff-perfused hearts. Results are the means  $\pm$  SE of 5 experiments in each group. Ang II, angiotensin II; ECG, electrocardiogram; wks, weeks.

\*Significantly different from *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* hearts,  $P < 0.05$ . †Significantly different from Saline-infused *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice,  $p < 0.05$ .

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**Figure 1.** (Continued) PTEN deficiency promotes sinus node dysfunction in hypertensive animals. (A-C) Western blot analysis for total AKT, phosphorylation of AKT, or total PTEN from cardiac ventricle or skeletal muscle of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice. (D-E) Systolic and diastolic blood pressure in *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice infused with Ang II (800 ng/min per kg body weight) or Saline (0.9% NaCl) for 4 weeks. (F) M-mode echocardiographic images from *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice infused with Ang II (800 ng/min per kg body weight) or saline (0.9% NaCl) for 4 weeks. (G-H) Fractional shortening and ejection fraction were determined from the M-mode images. (I) Sinus pauses and exit blocks were counted from ECG recordings from hearts isolated from mice infused with Ang II (800 ng/min per kg body weight) or Saline (0.9% NaCl) for 4 weeks. (J) Representative ECG recordings from Langendorff-perfused hearts. Results are the means  $\pm$  SE of 5 experiments in each group. Ang II, angiotensin II; ECG, electrocardiogram; wks, weeks.

\*Significantly different from *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* hearts,  $P < 0.05$ . †Significantly different from Saline-infused *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice,  $p < 0.05$ .

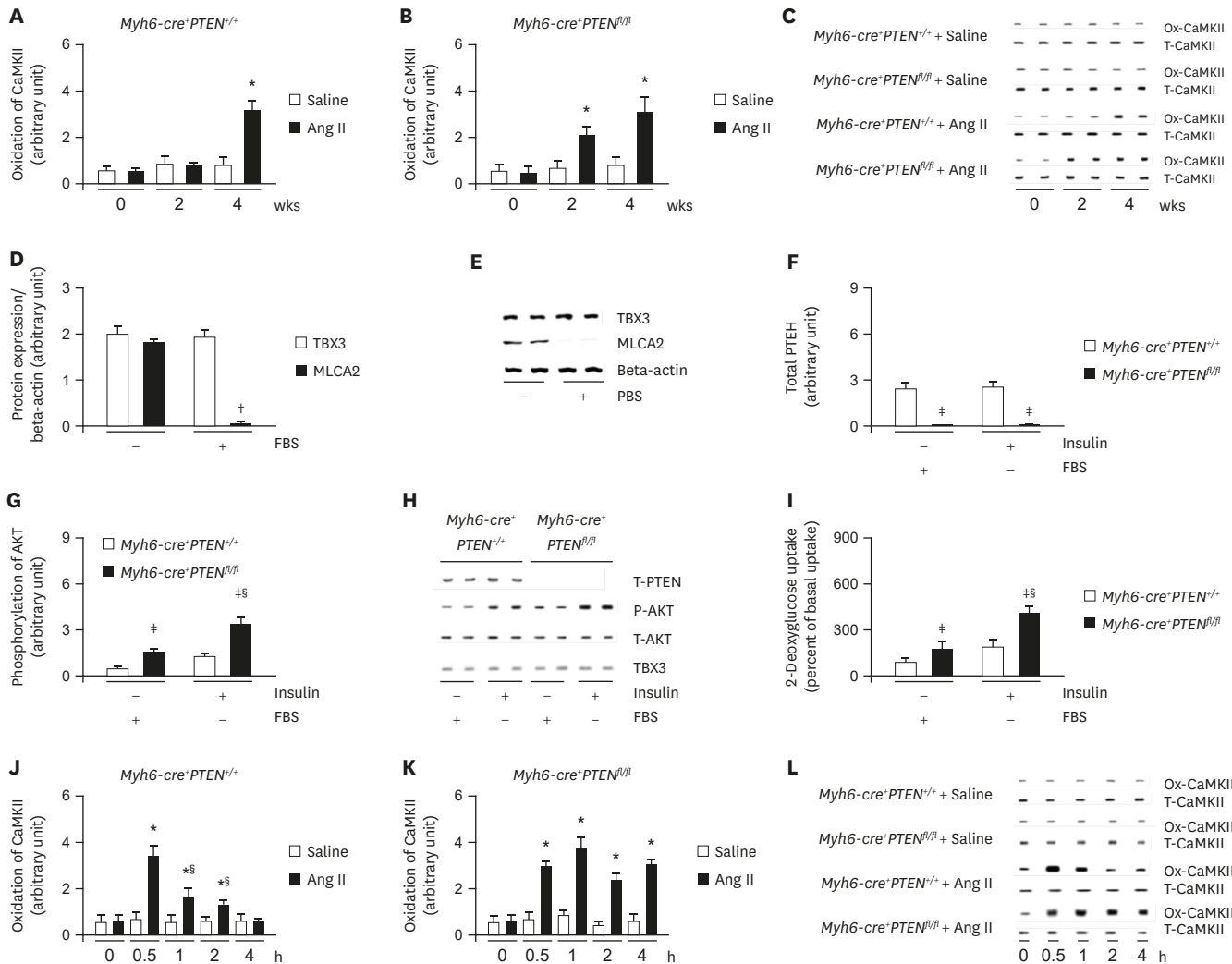
showed more frequent and longer sinus pauses compared to the control group. Overall, PTEN deficiency in Ang II-infused group resulted in the accelerated onset of frequent sinus pauses.

### Cardiac PTEN deletion accelerates CaMKII oxidation in Ang II-treated sinus nodal cells

Previous studies have reported that oxidized CaMKII promotes sinus dysfunction [14,16]. Indeed, I observed CaMKII oxidation in the right atrium of our hypertensive animals after 4 weeks of Ang II infusion. While PTEN deficiency did not change CaMKII oxidation in normotensive mice, it accelerated CaMKII oxidation in the early stages of hypertensive mice at 2 weeks of Ang II infusion (**Fig. 2A-C**). To confirm that this occurred in the sinus nodal cells, we prepared primary cell cultures of the sinus node from the right atrium. Initially, the isolated cells of the right atria contained cells expressing TBX3 (a sinus node marker) and MLC2A (a marker for atrial cardiomyocytes) (**Fig. 2D and E**). Following FBS incubation for 3 days, only TBX3-expressing cells remained and were confirmed using Western blot (**Fig. 2E**). TBX3-expressing cells were incubated with insulin or PBS for 30 min. Insulin treatment did not alter the level of PTEN in TBX3-expressing cells (**Fig. 2F**). In PBS-treated PTEN KO cells, basal AKT phosphorylation levels were increased compared to PBS-treated control cells. Moreover, in response to insulin, there was a two-fold increase in AKT phosphorylation and over a two-fold increase in glucose uptake compared to PBS-treated control cells (**Fig. 2G-I**). TBX3-expressing cells were also incubated with Ang II or saline for 4 h. Following Ang II exposure, an approximately three-fold induction of CaMKII oxidation was observed after 30 min, after which it declined and reached basal levels at about 4 h in wild-type cells. In PTEN KO cells, saline did not increase CaMKII oxidation during the 4 h period. However, when incubated with Ang II, PTEN deficiency led to a sustained increase in CaMKII oxidation, peaking at 60 min, and remained high during the 4 h period (**Fig. 2J-L**).

### PTEN deficiency substantially decreases autophagic flux in sinus nodal cells

It has been reported that autophagy is constitutively active in sinus nodal cells [8]. In the present study, we measured amounts of LC3-II that indicate autophagy in these cells. As LC3-II is continuously consumed by autolysosome, it is not sufficient to monitor static levels of LC3-II [17]. Therefore, to measure autophagic flux, cells were treated with bafilomycin A1, an autophagosome-lysosome fusion inhibitor [17]. Following autolysosome inhibition, saline or Ang II treatment led to an elevated amount of LC3-II indicating basal autophagic action in the cells. However, LC3-II accumulation did not occur in PTEN deficient cells indicating



**Figure 2.** PTEN deletion promotes CaMKII oxidation with hypertension.

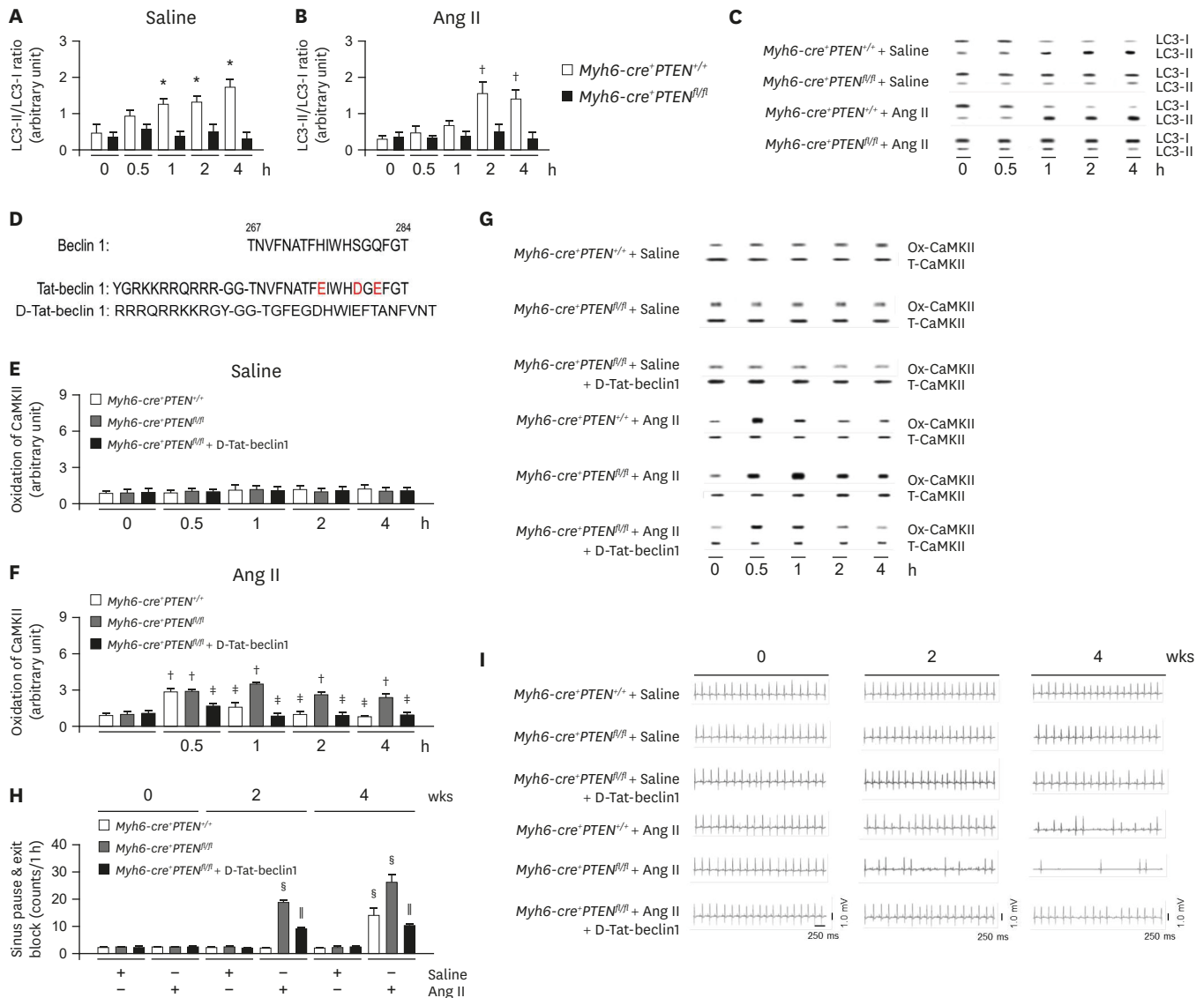
(A-C) Following infusion with Ang II (800 ng/min per kg body weight) or saline (0.9% NaCl) for 4 weeks, right atria of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice were isolated to evaluate CaMKII oxidation using Western blotting and densitometry. (D-E) Cells were isolated from right atria of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>fl/fl</sup>* mice using collagenase II and incubated with serum free or 20% FBS-DMEM media for 3 days. Protein was extracted to determine TBX3 (sinus node marker) and MLCA2 (cardiomyocyte marker) using Western blotting. Cells with dominant TBX3 expression were mainly in 20% FBS-DMEM. (F-I) Following serum starvation for 3 h, insulin (0.1 μM) was added to TBX3 expressing cells for 30 min. Medium was replaced with PBS buffer and glucose uptake was evaluated. Protein was extracted to measure total AKT, phosphorylation of AKT, or total PTEN using Western blotting. (J-L) Following the indicated times, Ang II (20 μM) or Saline (0.9% NaCl) was added to the TBX3 expressing cells. Protein was extracted to evaluate oxidized and total CaMKII. Results are the means ± SE of 5 experiments in each group.

Ang II, angiotensin II; wks, weeks; Ox-CaMKII, oxidized CaMKII; h, hour; T, total.

\*Significantly different from saline-treated groups,  $p < 0.05$ . †Significantly different from cells with serum free,  $p < 0.05$ . ‡Significantly different from cells of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice,  $p < 0.05$ . §Significantly different from insulin treated cells of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice,  $p < 0.05$ . ¶Significantly different from Ang II-treated cells for 30 min,  $p < 0.05$ .

a defect in basal autophagic flux (Fig. 3A-C). We, therefore, hypothesized that PTEN deficiency prevents autophagic flux in sinus nodal cells. In addition, since oxidized proteins are known to be degraded via autophagy, we measured CaMKII oxidation after treatment with D-Tat-beclin1, an autophagy inducer. The D-Tat-beclin1 fusion protein is composed of a cell-permeable Tat domain that is attached to an 18 amino acid peptide comprising of the active domain (amino acids 267-284) of beclin1 with retro-inverso-sequenced D-amino acids which renders them more resistant to proteolytic degradation (Fig. 3D) [18]. Neither D-Tat-beclin1 nor PTEN deficiency affected CaMKII oxidation in sinus nodal cells cultured

with saline. However, when exposed to Ang II, PTEN deficiency led to high CaMKII oxidation levels which were sustained during the 4 h period. However, the accumulation of CaMKII oxidation that occurred with PTEN deficiency was abolished when pre-incubated with D-Tat-beclin1 (Fig. 3E-G). To assess whether sinus node dysfunction occurred due to a defect in autophagy, animals were infused with D-Tat-beclin1 for 4 weeks. Indeed, D-Tat-beclin1 led to a significant recovery of sinus nodal function in Ang II-infused PTEN KO mice (Fig. 3H and I).



**Figure 3.** Autophagy flux in PTEN deleted sinus nodal cells.

Sinus nodal cells were isolated from right atria of *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* or *Myh6-cre<sup>+</sup>PTEN<sup>0/0</sup>* mice and incubated 20% FBS-DMEM for 3 days. (A-C) To prevent fusion of autophagosome-lysosome, cells were pretreated with bafilomycin A1 (100 nM) for 30 min. Following the indicated times, Ang II (20 μM) was added to the culture medium with saline (0.9% NaCl). All samples were then subjected to Western blot for evaluating LC3-II and LC3-I. (D) Sequences of cell-permeable Tat protein and beclin1 amino acids 267-284. Red letters indicate amino acid substitutions to enhance hydrophilicity. Retro-inverso sequenced D-amino acids (D-Tat-beclin1) with more stability due to resistance against *in vivo* proteolysis was used. (E-G) To assess the relationship between autophagy and CaMKII oxidation, sinus nodal cells were pretreated with D-Tat-beclin1 for 1 h. Following indicated time, Ang II (20 μM) or saline (0.9% NaCl) was added to the culture medium. Samples were determined using Western blot for oxidized CaMKII. (H-I) Using Langendorff-perfused hearts, sinus pauses and exit blocks were counted from ECG recordings. Results are the means ± SE of 5 experiments in each group.

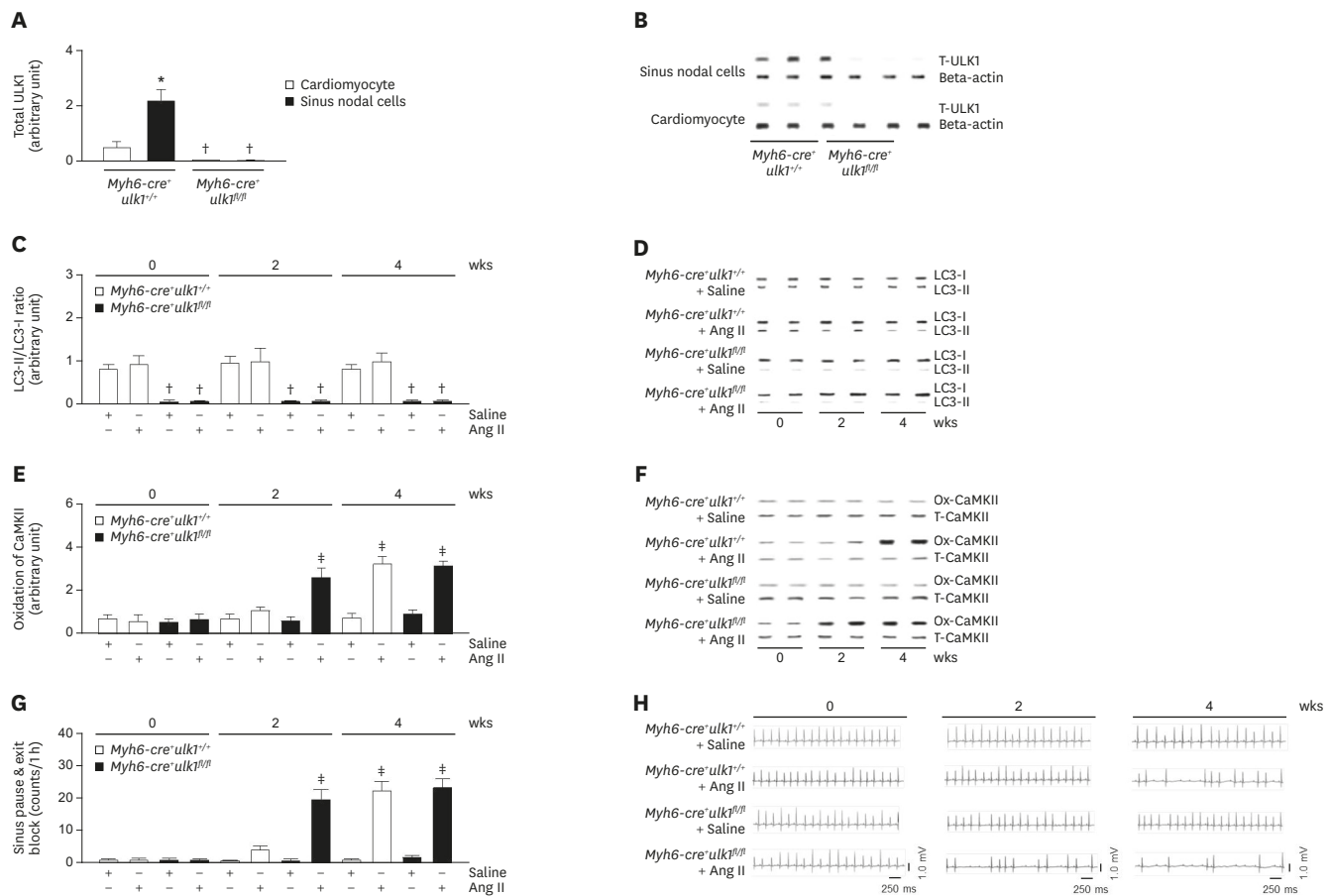
Ang II, angiotensin II; LC3, microtubule-associated protein1 light chain 3; Ox-CaMKII, oxidized CaMKII; h, hour; T, total.

\*Significantly different from saline-treated cells for 0 min,  $p < 0.05$ . †Significantly different from Ang II-treated cells for 0 min,  $p < 0.05$ . ‡Significantly different from Ang II-treated cells for 30 min,  $p < 0.05$ . §Significantly different from saline-infused *Myh6-cre<sup>+</sup>PTEN<sup>+/+</sup>* mice,  $P < 0.05$ . ¶Significantly different from Ang II-infused *Myh6-cre<sup>+</sup>PTEN<sup>0/0</sup>* mice,  $p < 0.05$ .



**ULK1 deficiency accelerates Ang II-induced sinus nodal dysfunction**

The above results suggest that PTEN deficiency induces sinus node dysfunction through defective ULK1/beclin1-mediated autophagy in the sinus node. To test this hypothesis in vivo, we generated cardiac-specific *ulk1* KO (*Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>*) mice. ULK1 protein levels in these mice were deficient not only in cardiomyocytes but importantly also in sinus nodal cells. Efficient deletion of *ulk1* was confirmed by Western blotting of lysates of cardiomyocytes and sinus nodal cells from *Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>* mice (Fig. 4A and B). In support of ULK1's essential role in autophagy, autophagy flux was abrogated as assessed by low levels of LC3-II in bafilomycin A1-treated sinus nodal cells of *Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>* mice compared to *Myh6-cre<sup>+</sup>ulk1<sup>+/+</sup>* controls (Fig. 4C and D). Notably, the lack of ULK1 did not influence sinus node function or CaMKII oxidation under saline-treated normotensive conditions (Fig. 4E and F). However, Ang II-infused *Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>* mice demonstrated the earlier appearance of more frequent sinus pauses and elevated CaMKII oxidation in the right atrium compared to *Myh6-cre<sup>+</sup>ulk1<sup>+/+</sup>* mice, similar to PTEN deficient mice (Fig. 4G and H).



**Figure 4.** ULK1 deletion elevates CaMKII oxidation and sinus pauses with hypertension.

*Myh6-cre<sup>+</sup>ulk1* and *Myh6-cre<sup>+/β</sup>ulk1* mice were generated from *Myh6-cre<sup>+</sup>ulk1<sup>fl/fl</sup>* heterozygous intercrosses. (A-B) Sinus nodal cells and cardiomyocytes were isolated from hearts using collagenase II. Samples were extracted and expression levels of total ULK1 were measured using Western blotting. (C-F) Following infusion with Ang II (800 ng/min per kg body weight) or saline (0.9% NaCl) for 4 weeks, sinus nodal cells were isolated and homogenized from *Myh6-cre<sup>+/+</sup>ulk1* or *Myh6-cre<sup>fl/fl</sup>ulk1* mice. All samples were then subjected to Western blotting for evaluating LC3, oxidized CaMKII, and total CaMKII. (G-H) From same groups, ECG was recorded from Langendorff-perfused hearts. Sinus pauses and exit blocks were counted from ECG recordings. Results are the means ± SE of 5 experiments in each group.

Ox-CaMKII, oxidized CaMKII; T, total; Ang II, angiotensin II; LC3, microtubule-associated protein1 light chain 3; wks, weeks.

\*Significantly different on isolated cardiomyocytes from *Myh6-cre<sup>+/+</sup>ulk1* mice,  $p < 0.05$ . †Significantly different from *Myh6-cre<sup>+/+</sup>ulk1* mice,  $p < 0.05$ . ‡Significantly different from saline-infused *Myh6-cre<sup>+/+</sup>ulk1* mice,  $p < 0.05$ .

### Thiazolidinediones accelerate sinus nodal dysfunction through aberrant autophagy in hypertensive animals

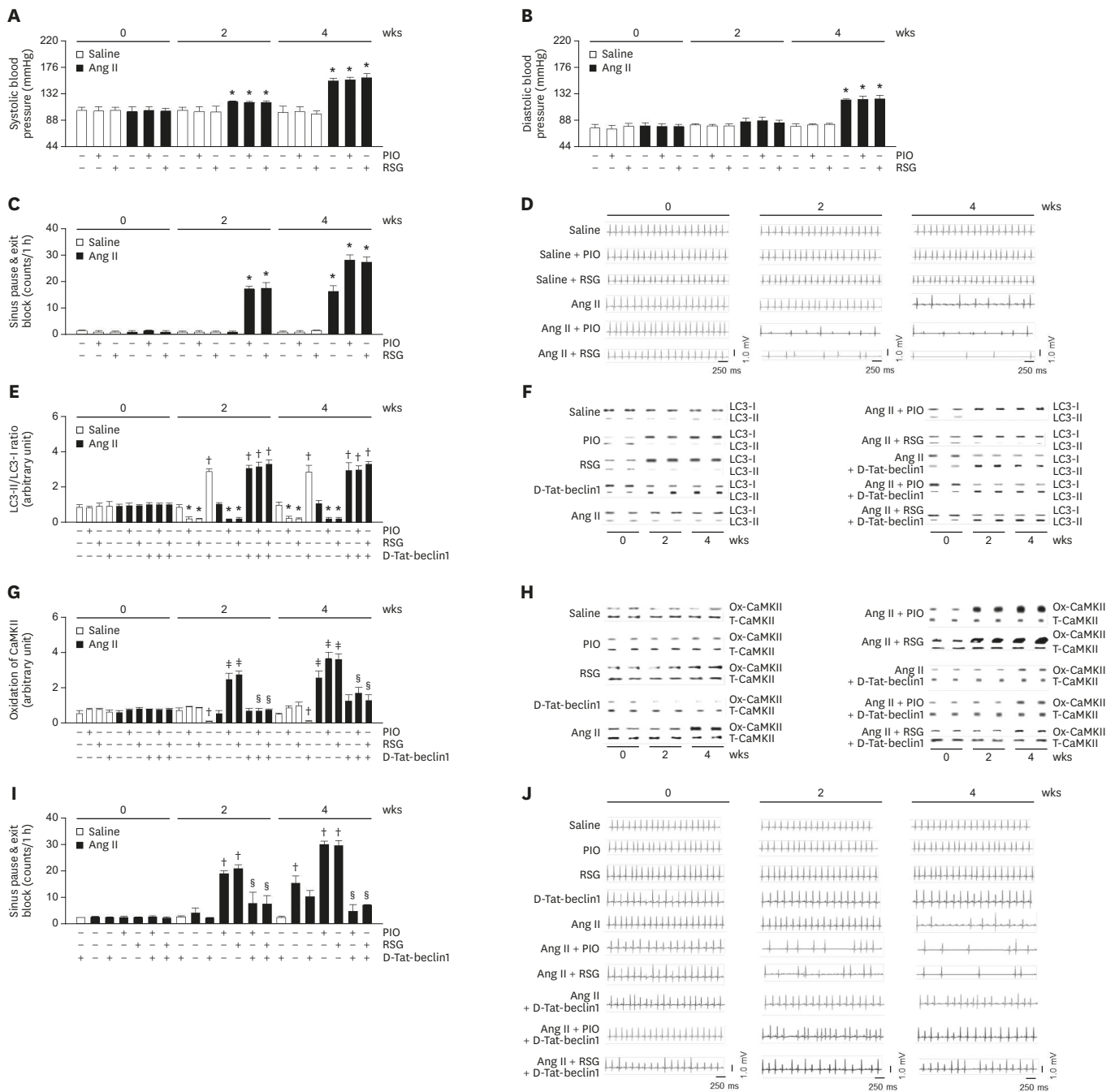
In addition to a genetic model of enhanced insulin sensitivity, we also used a pharmacologic approach by treating insulin sensitizers, pioglitazone (PIO) or rosiglitazone (RSG). To study the effects of these thiazolidinediones (TZDs) on hypertension-induced sinus nodal dysfunction, these treatments were given to wild type mice for the duration of Ang II or saline infusion of 4 weeks, at which time both systolic and diastolic blood pressure were increased in Ang II-infused group (**Fig. 5A and B**). In saline-infused normotensive mice, neither PIO nor RSG treatment exerted any influence on sinus node function compared to the saline-treated control group. On the other hand, both groups treated with either PIO or RSG in Ang II-infused hearts exhibited sinus pauses indicative of sinus node dysfunction starting at an earlier time of 2 weeks, similar to the time of onset of systolic hypertension. At 4 weeks of Ang II infusion, TZDs caused more frequent and longer sinus pauses (**Fig. 5C and D**). We next assessed the effects of TZDs on autophagy. Interestingly, autophagy flux was defective as assessed by low levels of LC3-II in the sinus node of both normotensive and hypertensive mice (**Fig. 5E and F**). To definitively show the essential role of autophagy in TZD-induced sinus node dysfunction, animals were infused with D-Tat-beclin1 for the 4 weeks duration along with TZDs. Saline-infused normotensive or Ang II-infused hypertensive mice were treated with bafilomycin A1 to assess for autophagy flux *in vivo* and observed that D-Tat-beclin1 infusion was able to rescue the autophagy defect that was caused by TZDs and led to increased conversion of LC3-I to LC3-II indicating enhanced autophagic flux (**Fig. 5E and F**). D-Tat-beclin1 alone did not influence sinus node function in normotensive or hypertensive animals and interestingly, CaMKII oxidation was attenuated even in normotensive along with hypertensive animals (**Fig. 5G, H and Fig. 6**). Importantly, D-Tat-beclin1 showed recovery of sinus nodal function and a substantial decrease in CaMKII oxidation caused by TZDs in hypertensive animals (**Fig. 5I, J and Fig. 6**).

## DISCUSSION

Insulin resistance is considered a core defect in the pathogenesis of type 2 diabetes. Indeed alleviating insulin resistance in multiple metabolic tissues has been shown to protect against type 2 diabetes, whether through genetic or pharmacologic means [19,20]. PPAR $\gamma$  agonists in particular, through their insulin-sensitizing effects, have been effective in the treatment of hyperglycemia. However, despite the successful use of PPAR $\gamma$  agonists for glycemic control, its continued use has been dampened by long-term studies which showed potentially harmful effects of TZDs on the cardiovascular system [5].

Although insulin signaling has been implicated in cytoprotection and promoting cell survival in multiple tissues, cardiomyocyte/muscle-specific PTEN KO mice have been reported to provoke a dramatic decrease in cardiac contractility [21]. Importantly, while the role of insulin signaling in myocardial contractile function is extensively studied, its role in sinus nodal function is less clear. Here we study the effects of insulin sensitization on the sinus nodal function by generating mice with PTEN deficient in cardiomyocytes and the sinus node. Given the common occurrence of atrial fibrillation that is associated with heart failure [22], we next assessed sinus nodal function that is critical in spontaneously generating electrical impulses to initiate the heartbeat. Ang II is well known to induce defective electrical signals from the sinus node [14]. In our experiments, sinus nodal dysfunction occurred after 4 weeks of Ang II infusion. Interestingly, cardiac PTEN deficiency led to accelerated sinus nodal dysfunction appearing as

Autophagy regulation of sinus node function



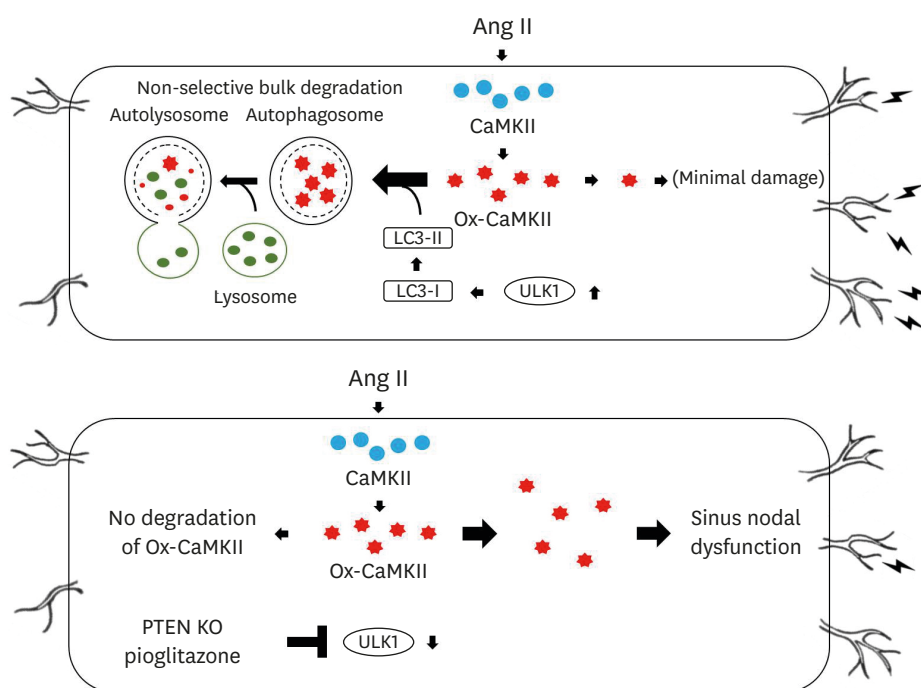
**Figure 5.** Sinus node function in PIO or RSG treated hypertensive animals.

Wild type mice were infused with saline (0.9% NaCl), Ang II (800 ng/min per kg body weight), PIO (13.8 µg/min per kg body weight), RSG (20 µg/min per kg body weight) or D-Tat-beclin1 (4 µg/min per kg body weight) for 4 weeks. Some groups were treated bafilomycin A1 (10 ng/min per kg body weight) to evaluate LC3 level by preventing fusion of autophagosome-lysosome. (A-B) Systolic and diastolic blood pressure in WT mice infused with Ang II (800 ng/min per kg body weight), PIO (13.8 µg/min per kg body weight), RSG (20 µg/min per kg body weight) or Saline (0.9% NaCl) for 4 weeks. (C-D and I-J) Following Langendorff-perfusion of the hearts, sinus pauses and exit blocks were counted from ECG recordings. (E-H) Sinus nodal cells were isolated from all right atria and then subjected to Western blotting for evaluating LC3, oxidized CaMKII, and total CaMKII. Results are the means ± SE of 5 experiments in each group. Ox-CaMKII, oxidized CaMKII; T, total; Ang II, angiotensin II; LC3, microtubule-associated protein1 light chain 3; wks, weeks.

\*Significantly different from saline-infused WT mice,  $p < 0.05$ . †Significantly different from D-Tat-beclin 1-infused normotensive animals for 0 week,  $p < 0.05$ .

‡Significantly different from Ang II-infused WT animals for 0 week,  $p < 0.05$ . §Significantly different from PIO-infused hypertensive animals for 2 weeks,  $p < 0.05$ .

early as 2 weeks of Ang II infusion. Importantly, cardiac PTEN deficiency alone did not affect sinus node function in saline-infused normotensive animals.



**Figure 6.** Autophagy is essential in attenuating Ang II-induced sinus node dysfunction exacerbated by TZDs or PTEN deficiency.

Ang II-induced CaMKII oxidation is degraded by non-selective bulk degradation pathway in normal hearts. Following treatment with PIO or RSG, or with PTEN deletion, ox-CaMKII accumulates due to a defect in autophagy flux. This increase in ox-CaMKII is associated with significant sinus node dysfunction in early hypertension. D-Tat-beclin1 restores autophagy and attenuates sinus nodal dysfunction.

Ox-CaMKII, oxidized CaMKII; Ang II, angiotensin II; LC3, microtubule-associated protein1 light chain 3.

As CaMKII oxidation has recently been reported to contribute to sinus nodal dysfunction in Ang II-induced hypertensive mice [14], we determined CaMKII oxidation in sinus nodal cells *in vitro*. Although Ang II exposure led to induction of CaMKII oxidation within 30 min, it diminished over time in culture. This suggests that sinus nodal cells have the ability to eliminate oxidized CaMKII. However, PTEN deficiency led to sustained Ang II-induced CaMKII oxidation over time in these cells. Collectively, our experiments show that PTEN deficiency attenuates the ability of sinus nodal cells to remove oxidized CaMKII.

As fatty acids are the main energy source of cardiomyocytes, substantial oxidative stress exists in the heart [23]. Given the effects of oxidized proteins on damaging cellular organelles and causing cellular aging, leading to dysfunction or death, it is imperative for cardiac cells to continuously eliminate oxidized molecules [8]. This general process is referred to as autophagy [9]. Although autophagy is expected to be considerably active in cardiomyocytes, the ultrastructural analysis revealed intriguingly that the sinus nodal cells, rather than cardiomyocytes, contain autophagic vacuoles in various stages of degradation by lysosomal digestion [8]. In addition, previous studies have reported that insulin inhibits autophagy via the suppression of ULK1. Based on this information, we hypothesized that PTEN deficiency leads to the accumulation of oxidized CaMKII by disrupting autophagic flux in sinus nodal cells exposed to Ang II. To test this hypothesis, we first assessed the essential role of ULK1 in sinus nodal dysfunction *in vivo* by generating cardiac-specific ULK1 KO mice driven by Myh 6 promoter, which is distributed in the sinus node and cardiomyocytes [24]. Indeed, ULK1 was efficiently disrupted in the sinus node with substantial decreases in autophagic flux. Interestingly, no significant defects in the sinus nodal function were observed under

basal normotensive conditions. However, Ang II infusion led to the accumulation of oxidized CaMKII, and furthermore, sinus nodal function was disrupted in the ULK1 cardiac-specific KO animals similar to that seen with cardiac PTEN deficiency. Importantly, D-Tat-Beclin1 was able to completely eliminate the accumulation of oxidized CaMKII and protect against Ang II-induced sinus nodal dysfunction in these mice.

Insulin sensitizers, TZDs, were widely used as monotherapy or in combination with other glucose-lowering agents to treat type 2 diabetes mellitus [20,25,26]. However, recent studies showed RSG, a TZD, to be associated with an increase in cardiovascular mortality particularly through its association with increased heart failure in clinical trials [4,5]. While insulin sensitizers can exert beneficial effects on cardiac metabolism, HDL, and vasculature, their potentially detrimental effects on the heart have not yet been fully elucidated [27-30]. To this end, we examined the effects of insulin sensitizers on sinus nodal function in normotensive and hypertensive mice. Interestingly, similar to PTEN deficient heart, both TZDs, PIO or RSG, also led to accelerated oxidized CaMKII and sinus nodal dysfunction appearing early after 2 weeks of Ang II infusion compared to 4 weeks at which time control mice displayed sinus nodal dysfunction. Neither, on the other hand, affected sinus node function in normotensive animals.

In summary, our experiments show that treatment with TZDs or PTEN deficiency can exacerbate sinus nodal dysfunction in the early stages of hypertension. Given that Ang II concentrations are elevated in obesity [31], further research needs to be conducted to investigate the relationship between sinus nodal function and Ang II in models of obesity. The present study shows the critical role of autophagy with D-Tat-Beclin1 abrogating sinus nodal dysfunction through activation of autophagy. These results suggest that pharmacologic strategies to promote autophagy as supplemental agents for diabetes therapy may attenuate detrimental effects of TZDs or other insulin-sensitizing drugs. Together, our results broaden our understanding of the detrimental cardiac effects of insulin sensitizers and highlight a novel important role of autophagy in the protection against sinus nodal dysfunction.

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