Targeting γ-secretases protects against angiotensin II-induced cardiac hypertrophy

Short title: γ-secretase inhibition prevents LVH.

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ABSTRACT

Objective: The Notch pathway has been linked to pulmonary hypertension, but its role in systemic hypertension and, in particular in left ventricular hypertrophy (LVH), remains poorly understood. The main objective of this work was to analyze the effect of inhibiting the Notch pathway on the establishment and maintenance of angiotensin II (Ang-II)-induced arterial hypertension and LVH in adult mice with inducible genetic deletion of γ-secretase, and to test preclinically the therapeutic efficacy of γ-secretase inhibitors (GSIs).

Basic Methods: We analyzed Ang-II responses in primary cultures of vascular smooth muscle cells obtained from a novel mouse model with inducible genetic deletion of the γ-secretase complex, and the effects of GSI treatment on a mouse cardiac cell line. We also investigated Ang-II-induced hypertension and LVH in our novel mouse strain lacking the γ-secretase complex and in GSI-treated wild-type mice. Moreover, we analyzed vascular tissue from hypertensive patients with and without LVH.

Main Results: Vascular smooth muscle cells activate the Notch pathway in response to Ang-II both "in vitro" and "in vivo". Genetic deletion of γ-secretase in adult mice prevented Ang-II-induced hypertension and LVH without causing major adverse effects. Treatment with GSI reduced Ang-II-induced hypertrophy of a cardiac cell line "in vitro" and LVH in wild-type mice challenged with Ang-II. We also report elevated expression of the Notch target *HES5* in vascular tissue from hypertensive patients with LVH compared with those without LVH.

Conclusions: The Notch pathway is activated in the vasculature of mice with hypertension and LVH, and its inhibition via inducible genetic γ-secretase deletion protects against both conditions. Preliminary observations in hypertensive patients with LVH support the translational potential of these findings. Moreover, GSI treatment protects wild-type mice from Ang-II-induced LVH without affecting blood pressure. Our results unveil the potential use of GSIs in the treatment of hypertensive patients with LVH.

KEY WORDS: Hypertension, Notch, γ-secretase inhibitors, left ventricular hypertrophy, angiotensin-II, vascular smooth muscle cell and cardiomyocyte.

CONDENSED ABSTRACT

Here, we generated a mouse model for inducible genetic deletion of the γ-secretase complex. Our results demonstrate that targeting these proteases in adulthood protects against hypertension and associated cardiac hypertrophy without causing major adverse effects. Detection of elevated expression of the Notch target *HES5* in vascular tissue from hypertensive patients with left ventricular hypertrophy (LVH) supports the translational potential of these findings. Moreover, treatment of wild-type mice with γ-secretase inhibitors (GSIs) protects them from Ang-II-induced LVH without affecting blood pressure. Our results unveil the potential use of GSIs in the treatment of hypertensive patients with LVH.

ABBREVIATIONS DEFINITION LIST

VSMCs - vascular smooth muscle cells
N3ICD - notch 3 intracellular domain
Ang-II - angiotensin II
4-OHT - 4-hydroxytamoxifen
LVH - left ventricular hypertrophy
Saline - sodium chloride 0.9%
GSI-gamma-secretase inhibitors

INTRODUCTION

Notch signaling plays key roles in vascular smooth muscle cells (VSMCs) during development and in vascular diseases such as pulmonary hypertension [1, 2]. Five Notch activating ligands and four receptors have been identified. Active Notch intracellular domains are generated by sequential proteolytic processing of the ligand-bound receptors in a process ultimately mediated by the γ-secretase complex.

Mice constitutively deficient for *Notch3* receptor show a below-normal increase in blood pressure upon treatment with angiotensin II (Ang-II). However, these mice have a high mortality rate (65%) attributed to heart failure, thus casting doubt on the potential of anti-hypertension therapies based on Notch inhibition [3]. Here, we generated a mouse model for inducible genetic deletion of the γ-secretase complex. Our results demonstrate that targeting these proteases in adulthood protects against Ang-II-induced hypertension and cardiac hypertrophy without causing major adverse effects. Detection of elevated expression of the Notch target *HES5* in vascular tissue from hypertensive patients with left ventricular hypertrophy (LVH) supports the translational potential of these findings. Importantly, treatment with γ-secretase inhibitor (GSI) protects against LVH in wild-type mice infused with Ang-II and prevents the development of Ang-II-induced hypertrophy in mouse cardiomyocytes. Taking together, our findings unveil a protective role of targeting the γ-secretases in Ang-II-promoted LVH.

METHODS

Mice. This study used *Cre*$^{ERT2/\,ERT2}$ mice, in which transgenic tamoxifen-inducible Cre is systemically expressed under the RNA polymerase II promoter [4], and *Psen1*$^{f/f}$*;Psen2*$^{-/-}$ mice, in which PS1 is flanked by *loxP* sites excisable by Cre recombinase, and are defective for *PS2* [5]. Both mouse lines were in the C57BL/6NCrl (obtained from Charles River) pure background and were crossed at the CNIO Animal Facility to generate *Psen1*$^{f/f}$*;Psen2*$^{-/-}$*;Cre*^{ERT2} mice, and male mice were used for experimental procedures at the CNIC. Age-matched littermates were injected intraperitoneally (i.p) either with 1 mg 4-hydroxytamoxifen (4-OHT) (Sigma Aldrich) in corn oil (Sigma Aldrich) or with corn oil alone (control group). The timing of injections is indicated in the figures. All animal procedures were approved by the CNIC-Instituto de Salud Carlos III (CNIC-ISCIII) Ethics Committee for Research and Animal Welfare.

Angiotensin II-induced hypertension and γ-secretase inhibitor (GSI) treatment in mice. Mice were infused with angiotensin II (Ang-II) as described [6]. Briefly, 8-week-old male mice were anesthetized with sevoflurane and a small incision was made in the interscapular area. Osmotic minipumps (#2004 at Alzet Corp) loaded with Ang-II (Sigma Aldrich) were implanted subcutaneously (s.c.) into the mid-scapular incision, which was then stapled. Ang-II was infused at 1.4 μg/kg/min for 4 weeks. Control animals received minipumps filled with 0.9% saline. For treatment with the GSI diaminobenzidine (DBZ from Synkom), we adapted a previous protocol [7]. Briefly, animals were i.p. injected with 3.3 mg/kg/day during 4 consecutive days and allowed to rest for another 3 days. This procedure was repeated for 5 weeks.

Cell-culture assays. Primary cultures of mouse vascular smooth muscle cells (VSMCs) were isolated and cultured as described [8]. Briefly, the thoracic aortas of 8-week-old mice were micro-dissected and digested with 2 mg/mL collagenase type IV (Worthington Biochemical Corporation) at 37ºC for 15 min in a 5% CO_2 incubator, to remove the adventitial and endothelial layers. VSMCs were released by a second collagenase digestion for 90 min at 37 ºC with constant agitation. Cells were then washed and suspended in minimal essential medium, supplemented with 1mM L-glutamine, 100 IU/ml penicillin, 100 μg streptomycin and 10% (v/v) fetal bovine serum and grown at 37 ºC in a humidified atmosphere at 5% CO_2. Cells were grown to confluence and treated for 5 days with vehicle (0.1% DMSO, Sigma Aldrich) or 4-OHT (600 nmol/L, Sigma Aldrich) as indicated. For Ang-II stimulation, cells were first rendered quiescent by serum deprivation for 48 h and then stimulated with 1 μmol/L Ang-II for the indicated times. Experiments were performed with cells between passages 4 and 9 (1:3 splitting after trypsinization). Cultures of NkL-TAg cells, a mouse cardiac cell line, were maintained as described [9]. Briefly, cells were kept in DMEM/F12 supplemented with 10% FBS and plated into petri dishes coated with 12.5 μg/ml of fibronectin in 0.1 % gelatin/PBS. To keep the cardiac phenotype only two first passages after thawing cells were used. The GSI DBZ was added to the culture medium at 250 nmol/L and kept for 48 hours before the addition of Ang-II. For Ang-II stimulation, cells were first rendered quiescent by serum deprivation for 48 h (with or without DBZ) and then stimulated with 1μmol/L Ang-II for the indicated times. For the hypertrophic assay of the cardiomyocytes we follow the protocol described before [10]. Briefly cells were maintained in the described culture medium, supplemented with solvent carrier in control or with 1 μmol/L Ang-II and with or without DBZ. 24 hours later, hypertrophy was assayed by measurement of the cell's surface area using ImageJ software. Results represent the average of 85-100 cells from 7 pictures from different areas in each group.

Protein analysis

Cell extracts were prepared by incubating cells on ice for 10 min in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, NP-40 0.5%, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 1 mg/ml pepstatin) followed by removal of cellular debris by centrifugation at 12000 xg for 10 min. Protein concentration was measured with the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories).

For immunoblots, 30 µg of protein were resolved on 4-20% SDS-PAGE gels, wet-transferred to nitrocellulose (Bio-Rad Laboratories) and immunoblotted. The following antibodies were used: anti-NOTCH3 (rabbit polyclonal sc-5593, Santa Cruz Biotechnology), anti-presenilin1 (rabbit polyclonal 529592, Merck), anti-γ-tubulin (mouse monoclonal GTU-88, Sigma Aldrich) and anti-GAPDH (mouse monoclonal MAB374, Chemicon International). Horseradish peroxidase-linked secondary antibodies were from DAKO. Immunocomplexes were visualized by chemiluminescence using the ECL detection system (GE Healthcare).

Quantitative real-time RT-PCR

Total RNA was purified from VSMCs and snap-frozen adventitia- and endothelium-free mouse aorta with Trizol (Life Technologies). cDNA was generated using Ready-to-Go (GE Healthcare). Quantitative real-time PCR was performed in an ABI PRISM 7700 thermal cycler (Life Technologies), using DNA Master SYBR Green I mix (Life Technologies).
The human primers used were:

# *HES1*	HES1-F:	5'-CAA GCT GGA GAA GGC GGA C-3'
	HES1-R:	5'-CAC ACT TGG GTC TGT GCT CA-3'
# *HEY1*:	HEY1-F:	5'-CCA GTA CAA GAG CAT CCC TGT-3'
	HEY1-R:	5'-AGT GGA CAA ACA CCC TTC CTC-3'
# *HES5*:	HES5-F:	5'-CTC AGC CCC AAA GAG AAA AAC-3'
	HES5-R:	5'-CAC GAG TAG CCT TCG CTG TAG-3'
# *β-ACTIN*:	ACT-F:	5'-CAA GGC CAA CCG CGA GAA GAT-3'
	ACT-R:	5'-CCA GAG GCG TAC AGG GAT AGC AC-3'

The mouse primers used were:

# *Hey1*:	Hey1-F:	5'-CAC CTG AAA ATG CTG CAC AC-3'
	Hey1-R:	5'-GGG TCC TGC TGT ATG TTG AAA-3'
# *Hes5*:	Hes5-F:	5'-GAG ATG CTC AGT CCC AAG-3'
	Hes5-R:	5'-GCT GCT CTA TGC TGC TGT TG-3'
# *Hes1*:	Hes1-F:	5'-GCG AAG GGC AAG AAT AAA TG -3'
	Hes1-R:	5'-TGT CTG CCT TCT CTA GCT TGG-3'
# *Nppb*:	Nppb-F:	5'-GAG GTC ACT CCT ATC CTC TGG-3'
	Nppb-R:	5'-GCC ATT TCC TCC GAC TTT TCT C-3'
# *Myh7*:	Myh7-F:	5'-GGA AGA CAG GAA GAA CCT ACT GC-3'
	Myh7-R:	5'-AAC TTG GAC AGG TTG GTG TTG-3'
# *Tgfb1*:	Tgfb1-F:	5'-CCG AAG CGG ACT ACT AT-3'
	Tgfb1-R:	5'-GTA ACG CCA GGA ATT GT-3'

# *18S rRNA*:	18S-F:	5'- CGG ACA GGA TTG ACA GAT TG -3'
	18S-R:	5'- CAA ATC GCT CCA CCA ACT AA -3'
# *β-Actin*:	Act-F:	5'-GGC ACC ACA CCT TCT ACA ATG-3'
	Act-R:	5'-GTG GTG GTG AAG CTG TAG CC-3'

The difference in PCR cycles with respect to housekeeping gene (*18S rRNA* in Supplementary Figure 4, or β-actin for the rest of Figures) for a given experimental sample (ΔCt) was subtracted from the corresponding ΔCt of the reference sample (such as wild-type) (ΔΔCt). Values of ΔΔCt were converted into fold expression ($2^{-\Delta\Delta Ct}$).

Histopathology and immunohistochemistry. Kidneys were harvested from euthanized mice and immediately fixed in 10%-buffered formalin (Sigma-Aldrich). Paraffin-embedded cross sections (5 µm-thick) were stained with hematoxylin and eosin (H&E) or were subjected to antigen retrieval using citrate buffer for immunohistochemistry with the following antibodies: rabbit polyclonal anti-NOTCH3 (1:25 dilution, sc-5593, Santa Cruz Biotechnology), rabbit polyclonal anti-smooth muscle actin (1:150 dilution, RB-9010-P0, Fisher Scientific). Horseradish peroxidase-linked secondary antibodies were from DAKO. Immunocomplexes were detected with diaminobenzidine (DAB+, Dako). An average of 200 VSMCs per animal were scored for NOTCH3 nuclear staining.

Transthoracic echocardiography in mice. Mice were anesthetized by inhalation of 1.5-2% isoflurane in a mixture with oxygen (0.8-1%) through a facial mask. Sufficient anesthesia was determined by negative paw pinch test. Fur was removed from the ventral surface of supine-placed mice by application of a topical depilatory agent and animals were warmed to maintain body temperature. Measurements were made with a Vevo 2100 system (VisualSonic) equipped with a 30-MHz linear transducer probe, as described [11]. Limb leads were attached for electrocardiogram monitoring. The heart was measured in M-mode with guided B-mode short axis recordings at the mid-ventricular level. LV end-diastolic (LVIDd) diameter, LV end-systolic diameter (LVIDs), end-diastolic interventricular septum (IVSd) and end-diastolic LV posterior wall (LVPWd) thicknesses were measured. LV fractional shortening (FS), LV ejection fraction (EF) and LV mass corrected were calculated following calculation definitions described in Visual Sonics Vevo 2100 Imaging System operator manual.

Blood pressure measurements in mice. Blood pressure (BP) was measured non-invasively with a tail-cuff device (Visitech System BP2000, NC) in trained conscious mice [12]. All measurements were taken at the same time in the morning. To increase accuracy the first 10 measurements were discarded. Mean values for individual mice were used for analysis.

Human arterial samples. Carotid artery tissue was obtained from 10 males aged over 60 years who underwent revascularization via endarterectomy due to internal carotid stenosis >75%. All patients had a previous diagnosis of essential hypertension and, despite being under chronic treatment with antihypertensive medications (including angiotensin converting enzyme inhibitors or angiotensin receptor blockers), systolic and diastolic blood pressure were maintained above 140 and 90 mm Hg respectively. Left ventricular mass (LVM) was estimated from measurements obtained by 2D-echocardiography. Patients were grouped into those with left ventricular hypertrophy (LVH; indexed LVM>131g/m^2; N=5) and those without (indexed LVM<131g/m^2; N=5) in accordance with the criteria reported by *Lang et al* [13]. When atherosclerotic plaque was observed, it was immediately removed and the remaining tissue samples frozen in liquid nitrogen. The study protocol was approved by the Research Ethics Committee of the University Clinic of Navarra and written informed consent was obtained from all participants.

Statistical Analysis

Values for each parameter within a group are expressed as mean ± SEM. For comparisons between groups, statistical significance was assessed by unpaired 2-tailed Student's *t* test. For within group comparisons, a paired 2-tailed Student's *t* test was used. In figures 2B and 3A, a repeated measures two-way ANOVA (mixed model) followed by Bonferroni post hoc test was performed. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Ang-II activates Notch signaling in VSMCs.

Notch signaling is essential for proper vascular development in mammals [2]. To mimic pharmacological intervention in adulthood while avoiding possible effects of Notch inactivation during embryonic development, we generated the inducible mouse line *Psen1$^{f/f}$;Psen2$^{-/-}$;Cre$^{ERT2/ERT2}$*, in which the γ-secretase complex is ubiquitously targeted after treatment with 4-hydroxytamoxifen (4-OHT). Using VSMCs isolated from *Psen1$^{f/f}$;Psen2$^{-/-}$;Cre$^{ERT2/ERT2}$* mice, we investigated the ability of Ang-II to activate the Notch pathway by monitoring the activation of NOTCH3, which is highly expressed by VSMCs [1, 14]. As expected, treatment of VSMCs with 4-OHT sharply reduced protein expression of PSEN1 **(Figure 1A)** and prevented the strong Ang-II-induced activation of NOTCH3 (measured as the NOTCH3 intracellular domain; N3ICD) that rapidly occurred in vehicle-treated cells (**Figure 1A**). Analysis of Notch downstream effectors by qPCR showed that while Ang-II had no effect on *Hes1*, the best-known Notch pathway effector, it upregulated *Hey1* and *Hes5,* two essential NOTCH3 effectors in VSMCs [1, 14] (**Figure 1B**). 4-OHT significantly reduced *Hes1* expression in control and Ang-II-treated VSMCs and blunted Ang-II-dependent *Hey1* and *Hes5* upregulation **(Figure 1B).**

Next, we infused wild-type mice with Ang-II or 0.9% saline buffer using mini-pumps [6]. Ang-II infusion increased NOTCH3 activity, measured as nuclear NOTCH3 in VSMCs from the media of renal arteries **(Figure 1C).** Accordingly, we also found higher expression of *Hes5* in aortic tunica media cells from the same animals (**Figure 1D**). However, aortic tunica media expression of *Hes1* (data not shown) and *Hey1* (**Figure 1D**) was similar in saline- and Ang-II-infused mice.

Targeting γ-secretases inhibit Ang-II-induced hypertension and LVH.

We next examined the effect of γ-secretase genetic deletion on the development of hypertension. As expected, VSMCs from 4-OHT-treated *Psen1$^{f/f}$;Psen2$^{-/-}$;Cre$^{ERT2/ERT2}$* mice showed a markedly reduced *Hey1* and *Hes5* mRNA expression **(Figure 2A)** indicating that the Notch pathway was affected. Neither 4-OHT nor vehicle treatments affected systolic blood pressure over a period of 6 days (**Figure 2B**), indicating that Notch signaling is not involved in the regulation of blood pressure at homeostasis. In contrast, Ang-II-promoted hypertensive systolic blood pressure was significantly blunted in 4-OHT-treated mice compared with controls **(Figure 2B)**.

To analyze whether γ-secretase deletion protects against Ang-II induced LVH we performed echocardiographic longitudinal studies. Neither 4-OHT nor Ang-II affected ejection fraction or fractional shortening **(Supplementary Figure 1)**. After Ang-II infusion in vehicle-treated mice, corrected left ventricular mass (LVMc) showed the expected progressive increase during follow-up (35% at 15 days after Ang-II infusion, $p<0.001$ versus baseline). Importantly, this pathological response was blunted in 4-OHT-treated mice (no significant differences versus baseline and $p<0.01$ when compared to vehicle-treated mice) (**Figure 2 C, Supplementary Figure 2 and Supplementary Videos**).

The γ-secretase complex is needed to maintain Ang-II-induced hypertension and LVH.

We next analyzed whether targeting γ-secretases could be also beneficial in mice with established hypertension. *Psen1$^{f/f}$;Psen2$^{-/-}$;Cre$^{ERT2/ERT2}$* mice were infused with Ang-II and, once systolic blood pressure reached >150 mmHg, were randomized for treatment with vehicle or 4-OHT. In vehicle-treated mice systolic blood pressure continued to increase >170 mmHg, whereas 4-OHT-treated mice maintained the baseline systolic value of ≈150 mmHg (**Figure 3A**). Similarly, whereas vehicle-treated mice significantly increased LVMc during follow-up ($p<0.05$ versus baseline at 24 days), LVMc in 4-OHT-treated mice showed no statistical differences between baseline and follow-up (**Figure 3B**).

Hypertensive patients with LVH have elevated vascular *HES5* expression.

In order to test the relevance of these findings in human patients, we analyzed the activation of Notch pathway in a small cohort of 10 hypertensive patients. Despite being treated with the same pharmacological regime (**Supplementary table 1**), half of patients display LVH while the other half do not. Thus we sought to investigate whether worst hypertensive condition correlates with an increase in the Notch pathway activity. Expression analysis of Notch effectors by qPCR demonstrated a markedly higher *HES5* expression in patients with LVH ($p<0.05$) compared to those without LVH, whereas both groups showed similar expression in *HES1* and *HEY1* **(Figure 3C)**.

The γ-secretase inhibitor diaminobenzidine protects against LVH *in vivo* and cardiomyocyte hypertrophy *in vitro* induced by Ang-II.

To reinforce the translational capability of the results obtained in mice with genetic ablation of γ-secretase complex, we performed a preclinical analysis in Ang-II infused mice in which the Notch pathway was inhibited during 5 weeks of treatment with the GSI diaminobenzidine (DBZ) starting 7 days after pumps implantation. Interestingly, DBZ did not prevent blood pressure elevation (**Supplementary Figure 3**), suggesting that secretase complex inhibition was less efficient after DBZ administration compared to genetic ablation likely due to inability for DBZ to reach small arteries. Since Ang-II promotes cardiac hypertrophy and remodeling in the absence of increased blood pressure [15], we tested by transthoracic echocardiography the effect of DBZ administration on LVH. Measurements at 18 and 38 days after Ang-II infusion (for a scheme of the experiment see **Supplementary Figure 3**) revealed a clear reduction in the LVH in DBZ-treated animals compared with vehicle-treated mice (**Figure 4A)**.

Prompted by this result we analyzed the effect of DBZ in the mouse cardiac cell line NkL-TAg [9]. Treatment of NkL-TAg cells with Ang-II upregulated NOTCH3 activity in a time-dependent manner, and DBZ abrogated this response (**Figure 4B**). This result reinforces our data using VSMCs (**Figure 1A**). We next analyzed the expression of natriuretic peptide B (*Nppb*), beta isoform of myosin heavy chain (*Myh7*) and tumor growth factor beta 1 (*Tgfb1*), three bona fide markers of cardiac hypertrophy [16]. As expected, treatment of NkL-TAg cells with Ang-II during 24h induced the expression of *Nppb*, *Myh7* and *Tgfb1* (**Supplementary Figure 4**) and augmented cell surface area. Importantly, DBZ prevented Ang-II-induced expression of hypertrophy markers **(Supplementary Figure 4)** and abrogated hypertrophy of NkL-TAg cells (**Figure 4C)**.

DISCUSSION

Herein we report that Ang-II treatment activates NOTCH3 *in vitro* in murine VSMCs and NkL-TAg cardiac cells and *in vivo* in VSMCs. These findings are consistent with a previous report showing that Ang-II activates the Notch pathway through γ-secretase-dependent cleavage in HEK293 cells ectopically expressing the Ang-II type 1 receptor [17]. We tested the relevance of these findings *in vivo* by generating a mouse model in which the γ-secretase complex is genetically targeted through the inducible ubiquitous elimination of *Psen1/Psen2* genes in adults. This approach mimics a pharmacological intervention and thus more closely resembles the clinical situation than germline knockout approaches. We show that deletion of the γ-secretase complex limits the development of Ang-II-induced systolic hypertension and LVH, an important pathological feature associated with hypertension. Moreover, targeting genetically γ-secretases in mice after hypertension is established prevents any further increase in systolic blood pressure and protects against LVH. Notably, the Notch effector *HES5* was strongly upregulated in carotid endarterectomy specimens obtained from patients with hypertension and LVH despite receiving antihypertensive treatment, supporting a translational potential of the results obtained in mice. To challenge this possibility we have treated hypertensive mice with DBZ. This potent cell permeable GSI prevented the development of Ang-II-induced LVH without affecting blood pressure. This, together with our observation that Ang-II-induced hypertrophy in NkL-TAg cells is inhibited by DBZ, suggest that the Notch pathway is important in the Ang-II response of both VSMCs and cardiomyocytes. Further studies are required to assess whether the reduction in LVH observed in 4-OHT-treated $Psen1^{f/f};Psen2^{-/-};Cre^{ERT2/ERT2}$ was due, at least partly, to a synergistic effect between decreased blood pressure and a direct inhibition in the hypertrophy by lack of Notch activity.

Constitutive *Notch3* deletion in the mouse has been reported to protect against Ang-II-induced hypertension, but was associated with a high mortality rate soon after Ang-II infusion, which was attributed to heart failure [3]. We reasoned that the mortality associated with constitutive *Notch3* ablation might be the result of developmental defects that manifest only upon challenge of the cardiovascular system with Ang-II. This is supported by our finding here that Notch inhibition in adult mice protects against Ang-II-induced LVH, with no increase in mortality or cardiac dysfunction. This protective outcome suggests that GSIs are a possible treatment for hypertension. Previously, Ozasa et al demonstrated that systemic GSI administration reduces Ang-II-induced vascular remodeling in mouse aorta without reporting adverse effects [17], but information regarding LVH was not provided. In apparent conflict with our results, NOTCH1 was reported to have a cardioprotective role in LVH [18]; but in this study the *Notch1* gene was eliminated specifically in the ventricles during fetal development, and developmental and non-systemic effects might therefore explain the discrepancy with our results.

Dysregulated Notch signaling has been linked to cancer, pulmonary hypertension, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and metabolic disorders, and preclinical studies in mouse models have identified Notch inhibition as a promising therapeutic intervention [1, 19, 20]. Our results suggest that GSIs, which are under evaluation in clinical trials for several diseases, might be an effective therapy for patients with hypertension, especially those whose LVH is refractory to antihypertensive therapy, and who are therefore at increased risk of heart failure [21].

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Figure

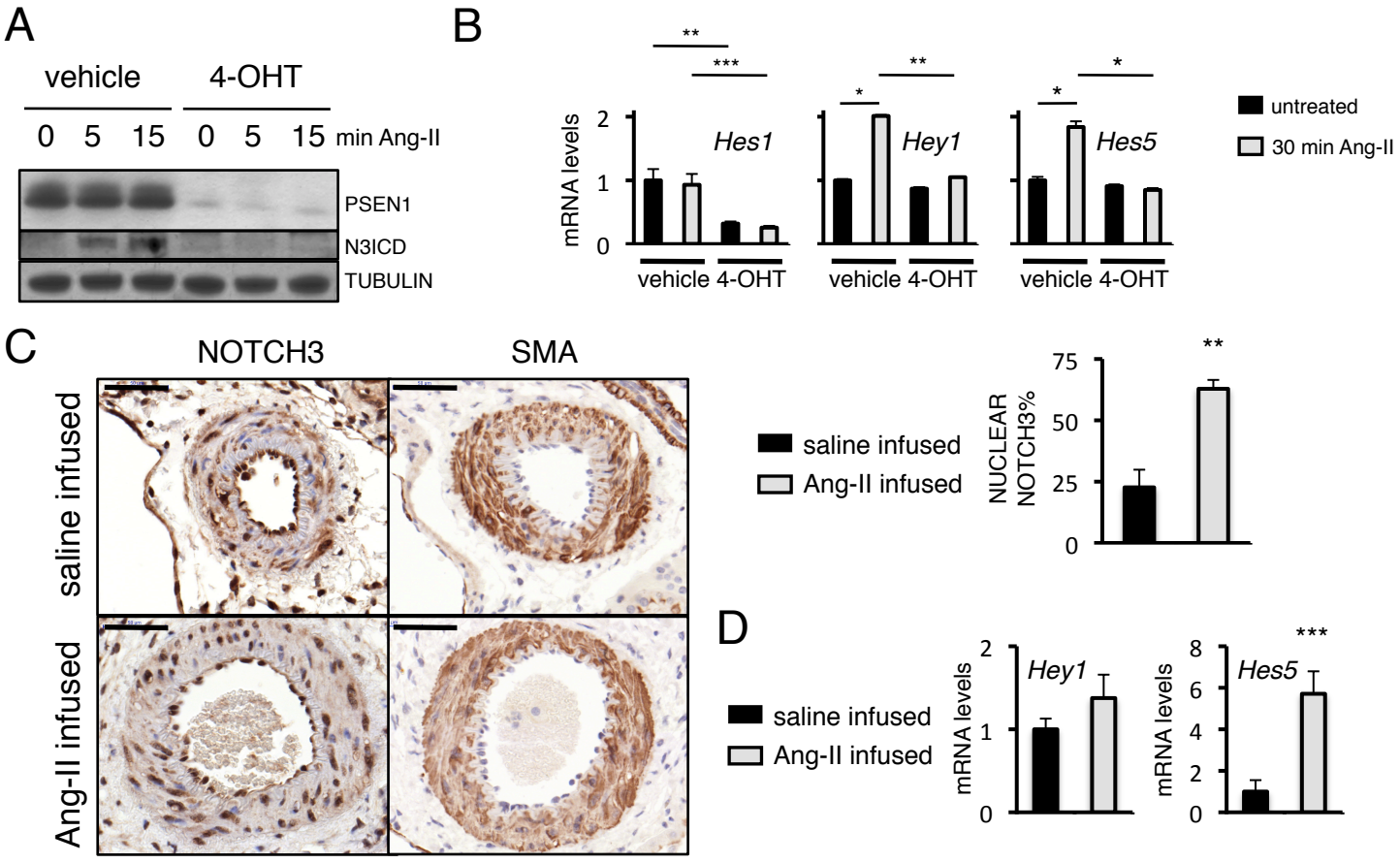

Figure 1. Ang-II activates Notch signaling in VSMCs.
(A, B) Primary VSMCs from *Psen1*$^{f/f}$;*Psen2*$^{-/-}$;*Cre*$^{ERT2/ERT2}$ mice were treated with vehicle or 4-OHT and activated with Ang-II for the indicated times. Cells were analyzed by western blot **(A)** and qPCR **(B)**. **(C)** Representative images of immunostaining for NOTCH3 and α-smooth muscle actin (SMA) in cross-sections of paraffin-embedded kidney from saline- or Ang-II-infused wild-type mice (n=5 mice per group). Bar, 50μm. The chart shows quantification of nuclear NOTCH3. **(D)** qPCR analysis of the indicated genes in adventitia- and endothelium-free aorta from saline- or Ang-II-infused *Psen1*$^{f/f}$;*Psen2*$^{-/-}$;*Cre*$^{ERT2/ERT2}$ mice (n=5).
*** p<0.001; **p<0.01; *p<0.05.

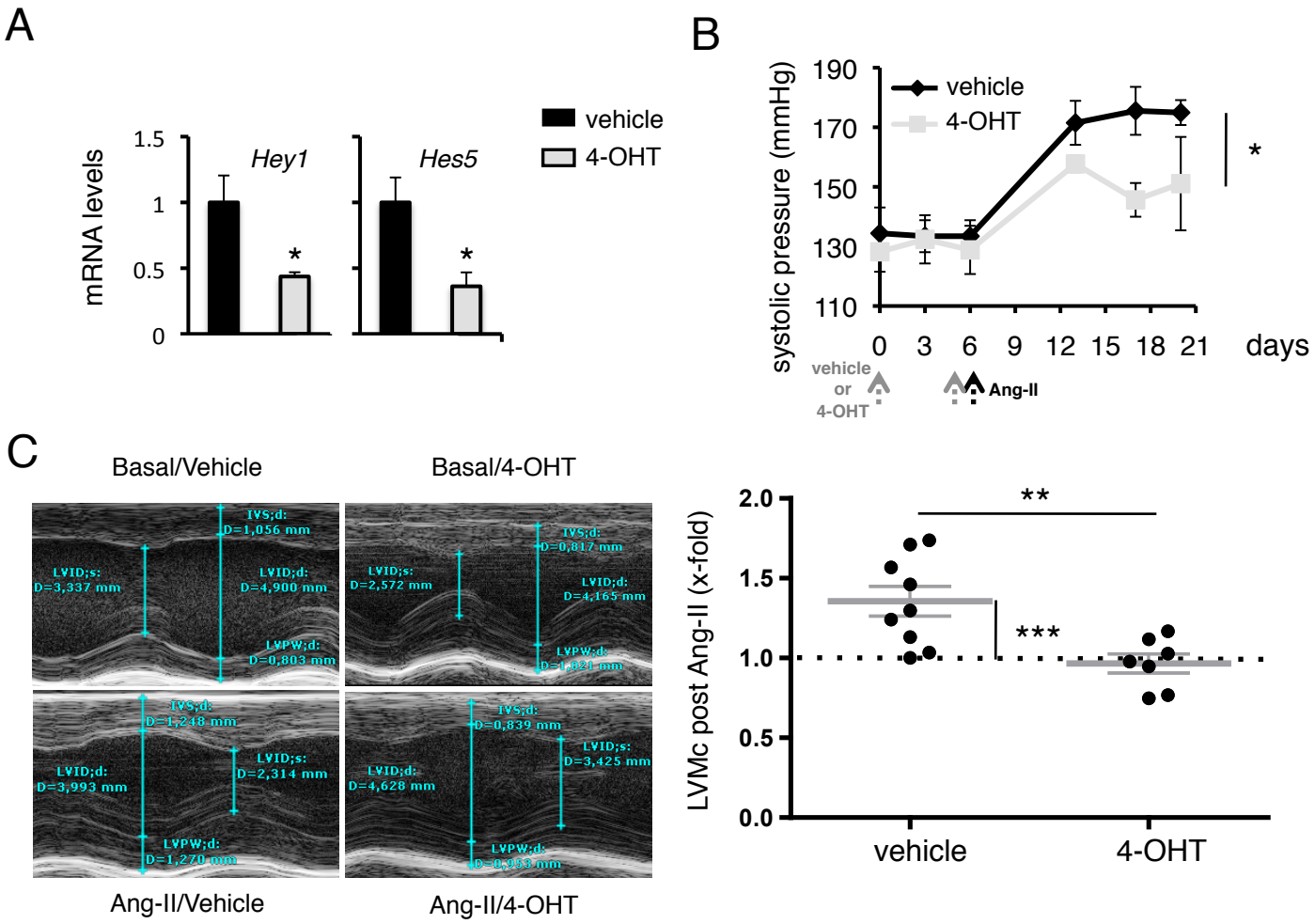

Figure 2. Targeting γ-secretases inhibit Ang-II-induced hypertension and LVH. *Psen1*$^{f/f}$;*Psen2*$^{-/-}$;*Cre*$^{ERT2/ERT2}$ mice were treated with vehicle or 4-OHT as indicated. **(A)** qPCR analysis in primary VSMCs from vehicle- (n=5) or 4-OHT-treated mice (n=4). **(B)** Systolic blood pressure in vehicle- (n=9) or 4-OHT-treated (n=7) mice. Dotted grey arrows indicate the time of injections with 4-OHT or vehicle; the dotted black arrow indicates the time of Ang-II pump implantation. **(C)** Representative echocardiograms from vehicle- or 4-OHT-treated mice before (basal, day 5 in 2B) and after 15 days of Ang-II infusion (day 21 in 2B). The parameters used for quantification of post-Ang-II infusion corrected left ventricular mass (LVMc=[0.8xLVM]+0.6) are indicated. Values on the chart correspond to the relative change between day 5 (dotted line) and day 21 after pump implantation.
*** p<0.001; ** p<0.01; * p<0.05.

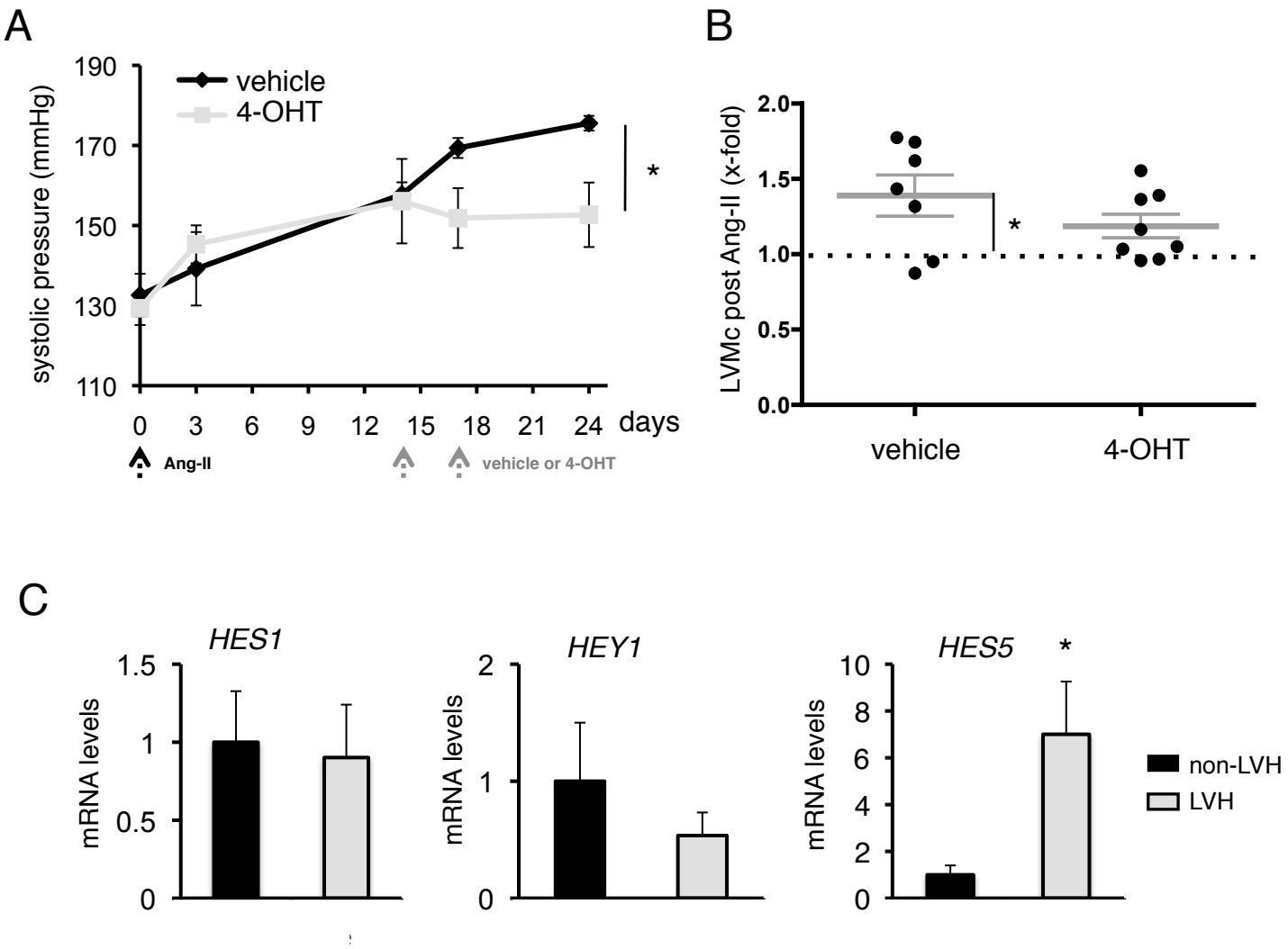

Figure 3. The γ-secretase complex is needed to maintain Ang-II-induced hypertension and LVH. *Psen1*$^{f/f}$;*Psen2*$^{-/-}$;*Cre*$^{ERT2/ERT2}$ mice were treated with vehicle or 4-OHT as indicated. **(A)** Systolic blood pressure in mice exposed to Ang-II (dotted black arrow) and subsequently treated (dotted gray arrows) with vehicle (n=8) or 4-OHT (n=9). **(B)** Quantification of LVMc measured by echocardiography 24 days after Ang-II pump implantation and treatment with vehicle (n=8) or 4-OHT (n=9) as in **(A)**. Values correspond to the relative change between day 1 (dotted line) and day 24 after pump implantation.. **(C)** qPCR analysis of NOTCH effectors in carotid endarterectomy samples from patients with hypertension with or without LVH (n=5 each group).
* $p<0.05$).

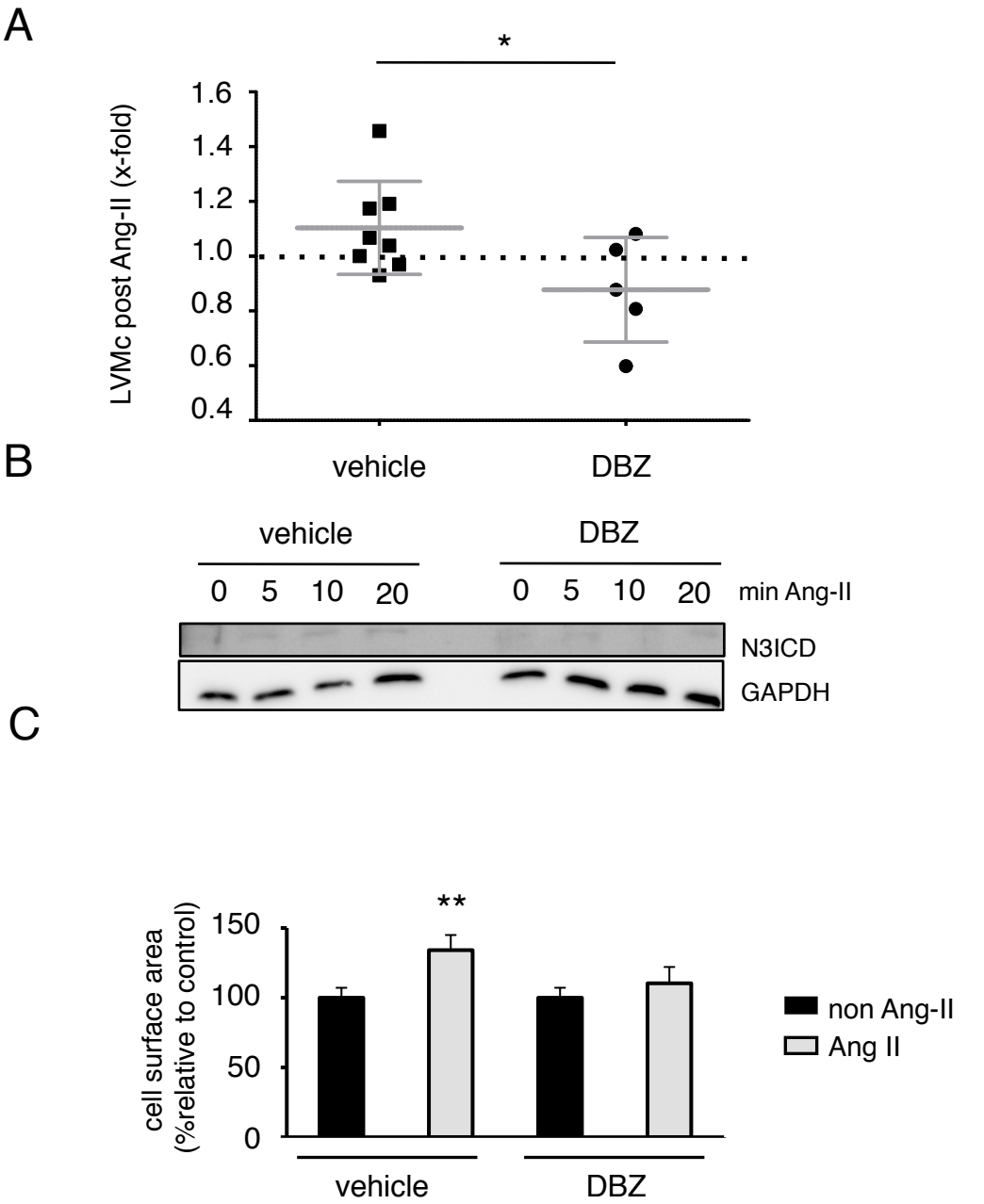

Figure 4. γ-secretase inhibitors protects against LVH *in vivo* and cardiomyocyte hypertrophy *in vitro* induced by Ang-II. (A) Quantification of LVMc between days 18 and 38 days after Ang-II pump implantation and vehicle- (n=8) or DBZ-treated (n=5) mice measured by echocardiography. **(B)** Mouse ventricular cells NkL-Tag were treated with vehicle or DBZ and activated with Ang-II for the indicated times. Cells were analyzed by western blot. **(C)** Mouse ventricular cells NkL-Tag were treated with vehicle or DBZ and activated or not with Ang-II for 24h and the area of cells was measured. Cell surface area (% relative to control) = Surface area (Ang-II)/Surface area (non-AngII) × 100. ** $p<0.01$).