ONLINE SUPPLEMENT

Role of Phosphoinositide 3-Kinase α, PKC and L-type Ca^{2+} Channels in Mediating the Complex Actions of Angiotensin II on Mouse Cardiac Contractility

Wenbin Liang[1,2], Gavin Y. Oudit[3,4], Mikin M. Patel[1,2], Ajay M. Shah[5], James R. Woodgett[6], Robert G.Tsushima[7], Michael E. Ward[8,9] and Peter H. Backx[1,2]

[1]Departments of Physiology and Medicine, [2]Division of Cardiology, University Health Network, University of Toronto, Toronto, Canada; [3]Division of Cardiology, Department of Medicine, [4]Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada; [5]Cardiovascular Division, King's College London BHF Centre of Excellence, London, UK; [6]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada; [7]Department of Biology, York University, Toronto, Canada; [8]Division of Respirology, [9]the Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Toronto, Canada

Running Head: AngII has complex inotropic effects.

Address Correspondence to: Dr. Peter H. Backx
Room 68, Fitzgerald Building
150 College Street, Toronto, Ontario, Canada, M5S 3E2
Phone: 416-946-8112; Fax: 419-946-8380
Email: p.backx@utoronto.ca

Extended Materials and Methods

Animals

C57BL/6 mice (male, 8-12 weeks) were obtained from Charles River Laboratories (Montreal, Canada). Mice lacking PI3Kα activity in the myocardium (DN–PI3Kα),[1] and mice lacking PI3Kγ activity (PI3Kγ-/-)[2] or NOX2-NADPH oxidase activity ($p47^{phox}$-/-)[3, 4] have been described and were bred in our animal facility. $GSK3\beta^{flox/flox}$ mice were generated as previously described.[5] Mice expressing the tamoxifen-inducible Cre recombinase protein fused to mutated mouse estrogen receptor (Mer) under the cardiac-specific α–myosin heavy chain promoter (α-myosin MerCreMer)[6] were obtained from Jackson Laboratories. The $GSK3\beta^{flox/flox}$ mice were crossed with α-myosin MerCreMer mice. α-myosin MerCreMer/$GSK3\beta^{flox/flox}$ mice (12 weeks of age) were treated with 20 mg/kg tamoxifen citrate (i.p.; Calbiochem) once a day for 4 consecutive days to generate conditional cardiac-specific GSK3β knockout mice (GSK3β cKO). Treated mice were used 5 days later following the final injection of tamoxifen. GSK3β protein levels were reduced by >90% in GSK3β cKO hearts as assessed by western blotting analysis (data not shown). Only littermate mice were used as controls for the studies using DN–PI3Kα, PI3Kγ-/-, $p47^{phox}$-/- and GSK3β cKO mice. A total of 90 mice were used in Langendorff studies. The care and use of animals conformed to the standards of the Canadian Council on Animal Care.

Isolated Langendorff heart study

Hearts were extracted from mice after intraperitoneal (IP) heparin injection (10 IU/g body weight) followed 5 min later by IP injection of ketamine (1 mg/g) plus xylazine (0.2 mg/g) and mounted in a Langendorff perfusion system. Hearts were perfused via the aorta with a modified Krebs solution containing (in mmol/L): 118 NaCl, 23 $NaHCO_3$, 3.2 KCl, 1.2 KH_2PO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, 0.5 Na_2-EDTA, 11 glucose, and 2 Na-pyruvate, which was bubbled with 95% O_2–5% CO_2 (pH=7.40, free Ca^{2+}=1.5 mmol/L) and kept at 37°C. An inflatable water-filled balloon was inserted into the left ventricle (LV) via a small incision in the left atrium for LV pressure measurement (BIOPAC Systems Inc., Goleta, CA).

After mounting, hearts were equilibrated for 20 minutes at a perfusion pressure of 80 mmHg and an end-diastolic pressure of 5-10 mmHg. In studies with wild-type C57BL/6 mice, only hearts with a sinus heart rate of ≥ 300 beats/min and a LV systolic pressure of ≥ 80 mmHg at the end of the equilibration period were included in the studies. In some studies, coronary flow rate was controlled with a peristaltic pump and set to a fixed value, determined by the requirement to achieve a coronary perfusion pressure of 80 mmHg during the equilibration period. In some studies with a constant flow rate, hearts were pretreated (before AngII infusion) with a vasodilator (P1075, 100 nmol/L) after the equilibrium period, which caused a small decrease in both coronary perfusion pressure and baseline $+dP/dt_{max}$. Heart rate and the rate of LV pressure changes ($+dP/dt_{max}$ and $-dP/dt_{min}$), as well as LV relaxation time constant (Tau),[7] were estimated from LV pressure recordings using Acqknowledge program (BIOPAC Systems Inc., Goleta, CA).

Angiotensin II (AngII, Sigma-Aldrich, St. Louis, MO) stock solution (1.5 mmol/L) was prepared in Milli-Q water. P1075 (Tocris Bioscience, Ellisville, MO) stock was prepared in ethanol at 10 mmol/L. GF 109203X (Calbiochem, San Diego, CA) stock were prepared in

DMSO at 3.3 mmol/L. On the day of experiments, drug stock solutions were diluted in Krebs solution and administered by slow parallel injection into the perfusion line. Final concentrations of vehicles (ethanol and DMSO) in perfusate were ≤ 0.03%. The delay from the start of the drug injection to the arrival of drugs at the heart was 2-3 min.

Ventricular myocyte isolation

As previously described,[8] hearts were isolated and retrogradely perfused for 5 min with nominally Ca^{2+}-free Tyrode solution containing (in mmol/L): 136 NaCl, 5.4 KCl, 0.5 Na_2HPO_4, 10 HEPES, 1 $MgCl_2$, and 10 glucose (pH=7.40 with NaOH). Following perfusion with a Ca^{2+}-free Tyrode solution containing collagenase (0.15 mg/ml, Yalkult, Japan) and protease (0.028 mg/ml, type XIV, Sigma) for ~10 min, the left ventricular free wall was dissected, cut into small pieces and gently triturated to release single myocytes. Isolated myocytes were stored in KB solution, containing (in mmol/L): 100 K-glutamate, 10 K-aspartate, 2.5 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 5 HEPES, 20 glucose, 20 taurine, 5 creatine, 0.5 EGTA, and 0.1% albumin (pH=7.2 with NaOH), and used for study within 8 h of isolation.

Myocyte cell shortening

Cell shortening were measured as previously described.[9] Myoctes were placed in a chamber (volume=0.5mL) on a microscope stage (IX50, Olympus Inc., Center Valley, PA) and bathed in Tyrode's solution as above with 1.2 mmol/L $CaCl_2$ added. Myocytes were field-stimulated at 1 Hz using square pulses (5 ms × 6 V, S44 Grass Stimulator，Grass Instrument Co., Quincy, MA) during cell length recording with a video edge detector coupled to a high frequency (240 Hz) charge-coupled camera (Crescent Electronics Inc., Sandy, UT) sampled at 1 kHz. Cell shortening was measured in myocytes without treatment or after 5 min and 15 min of AngII treatment. In some studies, myocytes were pretreated with irbersartan (10 μmol/L) for 10 min before AngII treatment. Cell shortening was expressed as percent shortening relative to the resting diastolic length.

Recording of L-type Ca^{2+} currents

L-type Ca^{2+} currents were recorded at 22°C using amphotericin B-perforated patch-clamp technique (Axopatch 200B, Axon Instruments, Foster City, CA). The external recording solution contained (in mmol/L) 137 NaCl, 5.4 CsCl, 1 $MgCl_2$, 1.2 $CaCl_2$, 10 HEPES, and 10 glucose (pH=7.35 with NaOH). The pipettes had a resistance of 2.0-2.5 MΩ when filled with a solution containing (in mmol/L) 130 CsCl, 10 NaCl, 0.5 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES, as well as 200 μg/ml amphotericin B (pH=7.2 with CsOH). Series resistance was compensated by 80%-90%. Myocytes were held at –85 mV and Na^+ currents were inactivated by applying an 800-mS voltage ramp from –70 mV to –40 mV before stepping to 0 mV for 200 mS to activate L-type Ca^{2+} currents. Currents were recorded every 10 seconds during AngII perfusion.

Statistics

Differences between two means were assessed using paired or unpaired Student's *t*-tests. Differences among multiple means were assessed by one-way analysis of variance (ANOVA)

and evaluated post hoc by Bonferroni's test. A P value<0.05 was considered significant. Group data are expressed as mean±S.E.M.

References

1. Shioi T, Kang P, Douglas P, Hampe J, Yballe C, Lawitts J, Cantley L, Izumo S. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J.* 2000;19:2537-2548.

2. Sasaki T, Irie-Sasaki J, Horie Y, Bachmaier K, Fata J, Li M, Suzuki A, Bouchard D, Ho A, Redston M, Gallinger S, Khokha R, Mak T, Hawkins P, Stephens L, Scherer S, Tsao M, Penninger J. Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)Kγ. *Nature.* 2000;406:897-902.

3. Tkalcevic J, Novelli M, Phylactides M, Iredale JP, Segal AW, Roes J. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity.* 2000;12:201-210.

4. Li J-M, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM. Essential role of the NADPH oxidase subunit $p47^{phox}$ in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-α. *Circ Res.* 2002;90:143-150.

5. Patel S, Doble BW, MacAulay K, Sinclair EM, Drucker DJ, Woodgett JR. Tissue-specific role of glycogen synthase kinase 3β in glucose homeostasis and insulin action. *Mol Cell Biol.* 2008;28:6314-6328.

6. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res.* 2001;89:20-25.

7. Weiss JL, Frederiksen JW, Weisfeldt ML. Hemodynamic determinants of the time-course of fall in canine left ventricular pressure. *J Clin Invest.* 1976;58:751-760.

8. Sun H, Kerfant B-G, Zhao D, Trivieri MG, Oudit GY, Penninger JM, Backx PH. Insulin-like growth factor-1 and PTEN deletion enhance cardiac L-type Ca^{2+} currents via increased PI3Kα/PKB signaling. *Circ Res.* 2006;98:1390-1397.

9. Sun H, Oudit GY, Ramirez RJ, Costantini D, Backx PH. The phosphoinositide 3-kinase inhibitor LY294002 enhances cardiac myocyte contractility via a direct inhibition of $I_{k,slow}$ currents. *Cardiovasc Res.* 2004;62:509-520.

Heart function changes	[AngII]		
	3 nmol/L (n=5)	**30 nmol/L** (n=5)	**300 nmol/L** (n=4)
Changes in $+dP/dt_{max}$			
Peak decrease (%)	12.3±1.7	13.2±2.9	14.0±3.0
Peak increase (%)	13.4±3.1	21.3±3.1	20.4±3.4
Time of peak increase (min)	11.4±2.2	6.0±0.1*	6.0±0.1*
Changes in $-dP/dt_{min}$			
Peak decrease (%)	16.7±1.2	23.1±1.8	21.7±5.1
Peak increase (%)	10.7±2.9	15.5±3.9	15.4±4.3
Time of peak increase (min)	11.2±1.7	7.0±0.5*	6.6±0.6*
Changes in heart rate			
Peak increase (beats/min)	71.5±20.6	53.9±19.9	55.4±25

Table S1. Effects of AngII (3, 30, and 300 nmol/L) on cardiac function in isolated wild-type C57BL/6 mouse hearts. Hearts were Langendorff perfused at a constant coronary flow rate in the presence of a vasodilator (P1075, 100 nmol/L). Peak changes in dP/dt were expressed as percent changes relative to baseline values. * $p<0.05$ vs. values in 3 nmol/L AngII group by ANOVA.

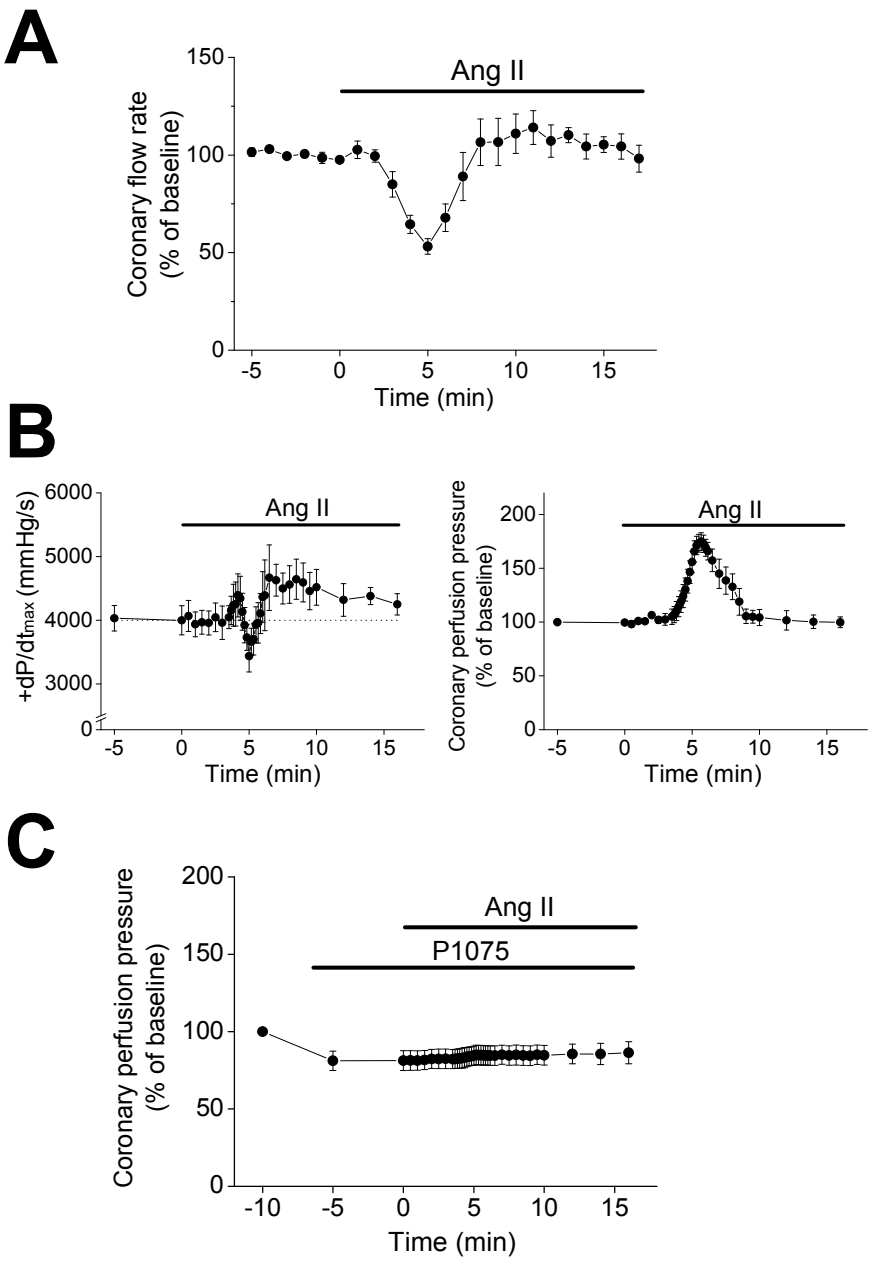

Figure S1. A. Time course of coronary artery flow rate of isolated hearts (n=4) during infusion of AngII (3 nmol/L). Hearts were perfused at a constant coronary perfusion pressure. **B.** Time courses of $+dP/dt_{max}$ (left panel) and coronary perfusion pressure (right panel) of isolated mouse hearts (n=5) during AngII infusion (3 nmol/L). Hearts were perfused at a constant coronary flow rate. **C.** Time course of coronary artery perfusion pressure of isolated mouse hearts (n=4) during AngII infusion (3 nmol/L). Hearts were perfused at a constant flow rate in the presence of a vasodilator (P1075, 100 nmol/L).

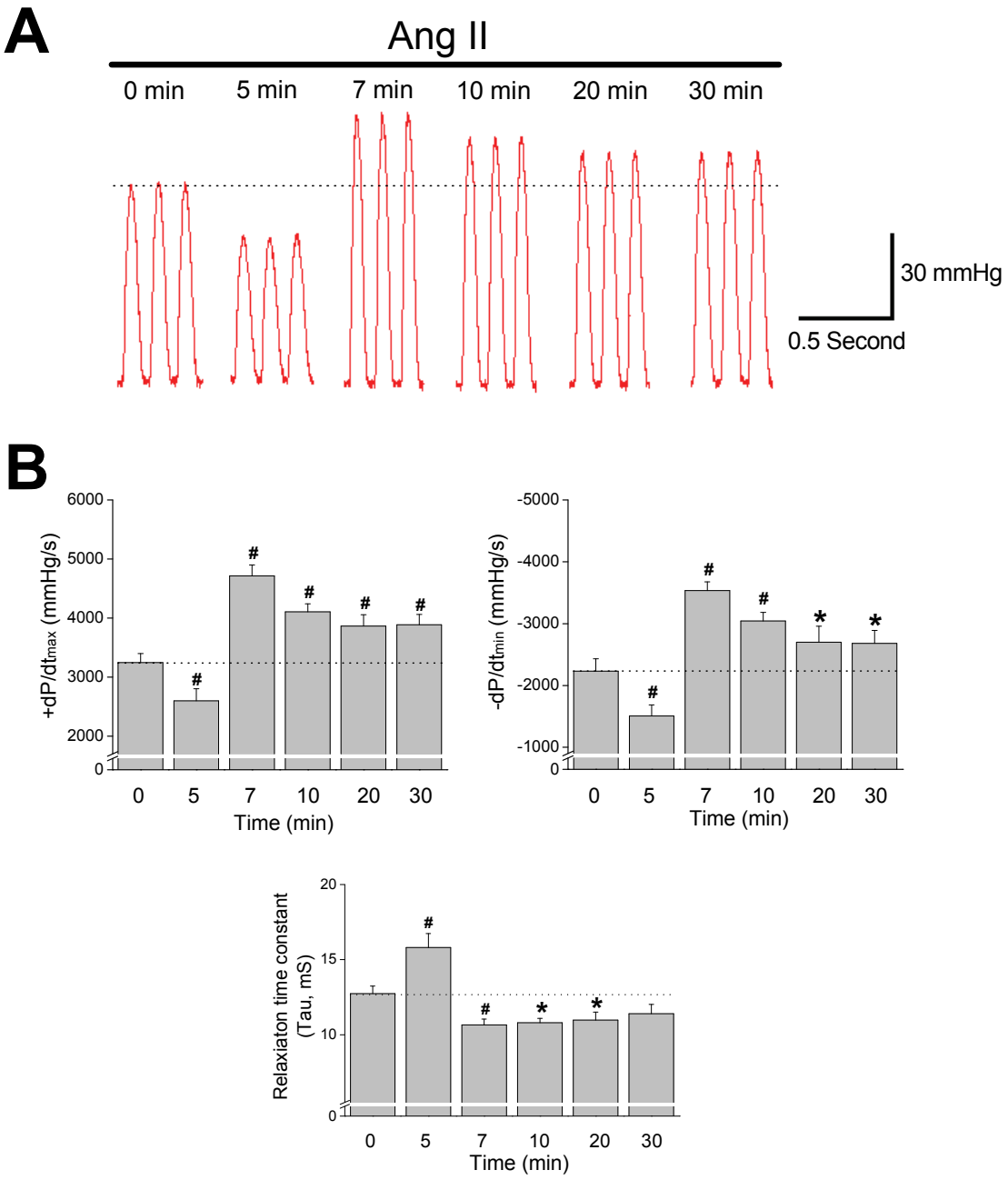

Figure S2. A. Representative left ventricle (LV) pressure traces in isolated Langendorff hearts at the indicated time points after infusion of AngII (30 nmol/L). Hearts were perfused at a constant coronary flow rate in the presence of a vasodilator (P1075, 100 nmol/L). **B.** Summary of $+dP/dt_{max}$ (top left panel), $-dP/dt_{min}$ (top right panel), and LV relaxation time constant (Tau, lower panel) at indicated time points after AngII infusion. n=6 for all three panels; #p<0.01, *p<0.05 vs. corresponding values at 0 min, by one-way repeated measures ANOVA.

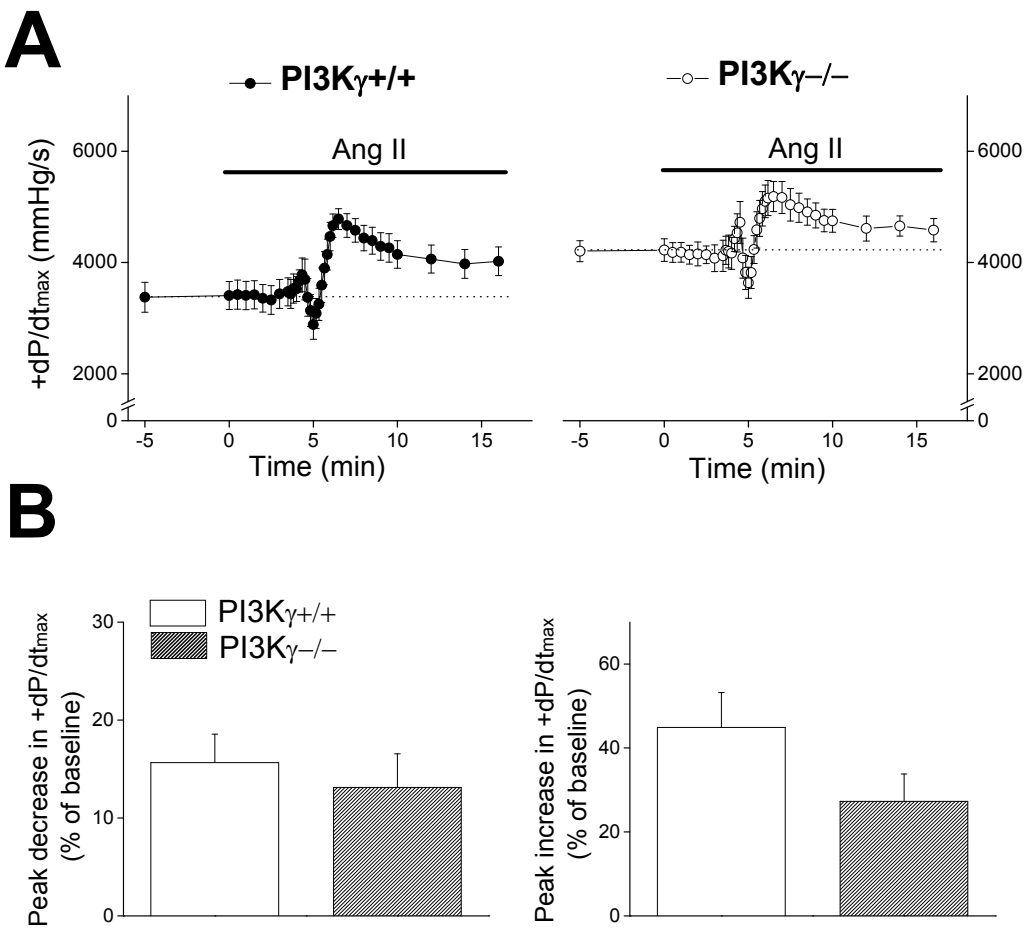

Figure S3. A. Time courses of $+dP/dt_{max}$ during AngII (30 nmol/L) infusion in PI3Kγ+/+ (left, n=7) and PI3Kγ-/- hearts (right, n=5). Mouse hearts were perfused at a constant flow rate in the presence of a vasodilator (P1075, 100 nmol/L). **B.** Summary of peak decreases (left) and peak increases (right) of $+dP/dt_{max}$ during AngII infusion in PI3Kγ+/+ (blank bars) and PI3Kγ-/- hearts (filled bars).

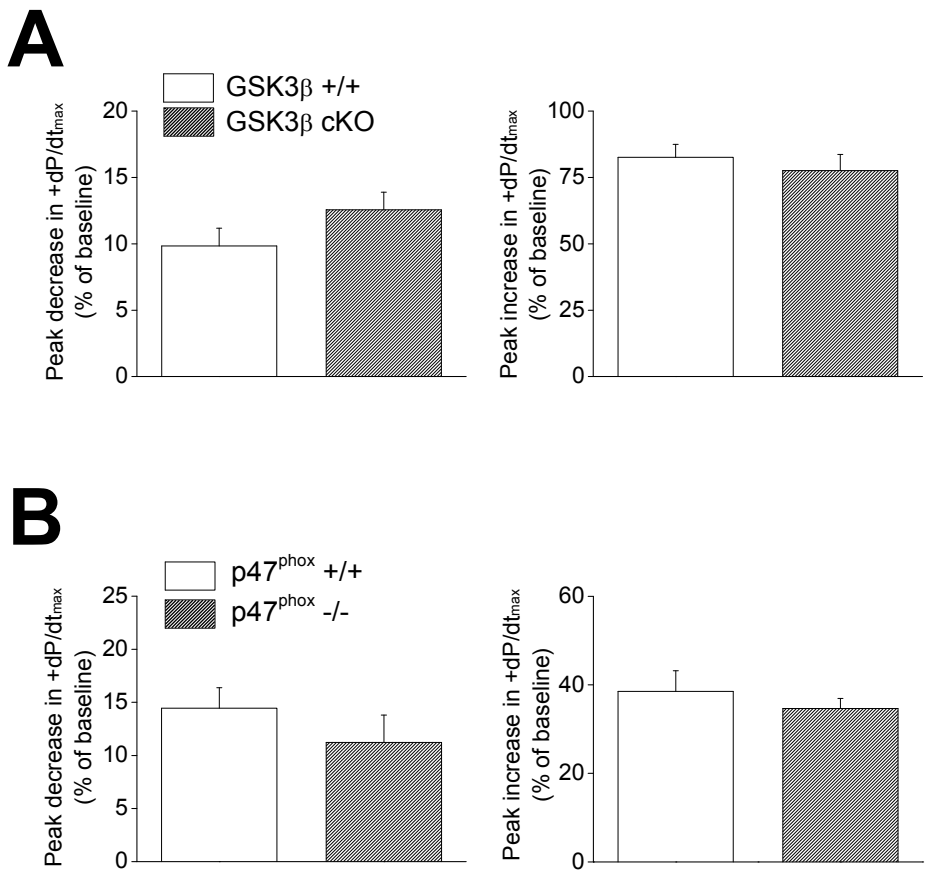

Figure S4. A. Summary of peak decreases (left) and peak increases (right) of $+dP/dt_{max}$ during AngII (30 nmol/L) infusion in wild-type littermate hearts (GSK3β+/+, blank bars, n=5) and hearts with conditional knockout of GSK3β (GSK3β cKO, filled bars, n=5). **B.** Summary of peak decreases (left) and peak increases (right) of $+dP/dt_{max}$ during AngII (30 nmol/L) infusion in $p47^{phox}$+/+ (blank bars, n=5) and $p47^{phox}$-/- hearts (filled bars, n=5). Hearts were Langendorff perfused at a constant flow rate in the presence of a vasodilator (P1075, 100 nmol/L).