ONLINE APPENDIX – Supplemental Data and Materials.

RESEARCH DESIGN AND METHODS

Adenoviral infection of cultured ARVC

Cardiomyocytes were infected with adenoviral vectors containing a dominant negative mutant Rac1 (Ad-RacN17, Vector Biolabs) or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection (MOI) of 100 PFU/cell. Adenovirus-mediated gene transfer was implemented as we previously described (1).

Gp91^{phox} and Nox4 knockdown using small interfering siRNA

To knock down gp91^{phox} (Nox2) and Nox4 expression, a small interfering RNA (siRNA) against rat Nox2 or Nox4 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA) and a scrambled siRNA was employed as a control. Transfection was performed using TransMessenger Transfection Reagent (Qiagen) according to manufacturer's protocol as described previously (1-5).

NADPH oxidase activity

NADPH-oxidase activity was assessed in tissue or cell lysates by lucigenin-enhanced chemiluminescence (50 µg of protein, 100 µM NADPH, 5 µM lucigenin) with a multilabel counter (Victor3 Wallac) (4-6). Some experiments were performed in the presence of diphenyleneiodonium, an inhibitor of NADPH oxidase. The light signal was monitored for 5 seconds, and counts per second (CPS) were presented as NADPH oxidase activity that was diphenyleneiodonium inhibitable.

Intracellular ROS measurement

The production of H_2O_2 was measured by using 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA, Invitrogen), as an indicator, as described in our recent reports (4-6). Briefly, heart tissues were homogenized in assay buffer. The homogenates were incubated with DCF-DA at 37ºC for 3 hours. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in fluorescence were expressed as arbitrary unit.

To evaluate tissue superoxide production, fresh frozen ventricular myocardium (10 µm slices) was incubated for 1 h at 37°C with dihydroethidium (DHE, 2 µM) as described (7). Superoxide production in cultured cardiomyocytes was measured with DHE as described previously (8).

Measurement of superoxide generation in isolated mitochondria

Mitochondria were isolated from the freshly harvested heart as described in our recent report (1). Mitochondrial superoxide generation was measured on addition of pyruvate/malate by using DHE as an indicator (9).

Measurement of thioredoxin reductase activity

Thioredoxin reductase activity in heart tissue lysates was determined by using a commercial kit (Cayman Chemical) according to manufacturer's instructions.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

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Total RNA was extracted from heart tissues using the Trizol Reagent (Gibco-BRL) following the manufacturer's instruction. Real-time RT-PCR was performed to analyze mRNA expression for beta-myosin heavy chain (β-MHC), atrial natriuretic pepetide (ANP), Collagen I and III (Col I and III), transforming growth factor-beta1 (TGF-β1), osteopontin, alpha-smooth muscle actin (α-SMA), tumor necrosis factor-alpha (TNF-α), Rac1, gp91^{phox}, p47^{phox}, GRP78, C/EBP homologous protein (CHOP), X-box protein 1 (XBP1) and GAPDH as previously described (2; 3; 6). The primers for TNF-α, Rac1, gp91^{phox}, p47^{phox} and GAPDH were described in previous reports (2; 3; 6) and the primers for β-MHC, ANP, Col I, Col III, TGF-β1, α-SMA, GRP78, XBP1 and CHOP were shown in Supplemental Table-1.

Western blot analysis

Rac1, gp91^{phox}, p67^{phox}, GRP78, ATF-6, GAPDH and phosphorylation of PERK were determined by western blot analysis using respective specific antibodies.

Assessment of cardiac function

Mouse hearts were isolated and perfused on a Langendorff-system. Myocardial function was then determined as described in our previous study (1; 3). Maximal and minimal first derivatives of force (+dF/dt_{max} and –dF/dt_{min}) as the rate of contraction and relaxation were analyzed by PowerLab Chart program (ADInstruments).

REFERENCES

1. Shen E, Li Y, Shan L, Zhu H, Feng Q, Arnold JM, Peng T: Rac1 is required for cardiomyocyte apoptosis during hyperglycemia. *Diabetes* 58:2386-2395, 2009

2. Shen E, Fan J, Chen R, Yee SP, Peng T: Phospholipase Cgamma1 signalling regulates lipopolysaccharide-induced cyclooxygenase-2 expression in cardiomyocytes. *J Mol Cell Cardiol* 43:308-318, 2007

3. Peng T, Shen E, Fan J, Zhang Y, Arnold JM, Feng Q: Disruption of phospholipase Cgamma1 signalling attenuates cardiac tumor necrosis factor-alpha expression and improves myocardial function during endotoxemia. *Cardiovasc Res* 78:90-97, 2008

4. Li Y, Feng Q, Arnold M, Peng T: Calpain activation contributes to hyperglycaemia-induced apoptosis in cardiomyocytes. *Cardiovasc Res* 84:100-110, 2009

5. Li Y, Arnold JM, Pampillo M, Babwah AV, Peng T: Taurine prevents cardiomyocyte death by inhibiting NADPH oxidase-mediated calpain activation. *Free Radic Biol Med* 46:51-61, 2009

6. Zhu H, Shan L, Peng T: Rac1 mediates sex difference in cardiac tumor necrosis factor-alpha expression via NADPH oxidase-ERK1/2/p38 MAPK pathway in endotoxemia. *J Mol Cell Cardiol* 47:264-274, 2009

7. Zanetti M, d'Uscio LV, Peterson TE, Katusic ZS, O'Brien T: Analysis of superoxide anion production in tissue. *Methods Mol Med* 108:65-72, 2005

8. Peshavariya HM, Dusting GJ, Selemidis S: Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. *Free Radic Res* 41:699-712, 2007

9. Hool LC, Di Maria CA, Viola HM, Arthur PG: Role of NAD(P)H oxidase in the regulation of cardiac L-type Ca2+ channel function during acute hypoxia. *Cardiovasc Res* 67:624-635, 2005

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Supplemental Table 1. Primers and their sequences

Gene		Primer sequences
ANP		
	Forward	5’ CTGCTAGACCACCTGGAGGA 3’
	Reverse	5’AAGCTGTTGCAGCCTAGTCC 3’
β-MHC		
	Forward	5’ TGCAAAGGCTCCAGGTCTGAGGGC 3’
	Reverse	5’GCCAACACCAACCTGTCCAAGTTC 3’
Col I		
	Forward	5’ ACGGCTGCACGAGTCACAC 3’
	Reverse	5’ GGCAGGCGGGAGGTCTT 3’
Col III		
	Forward	5’ GTTCTAGAGGATGGCTGTACTAAACACA3’
	Reverse	5’ TTGCCTTGCGTGTTTGATATTC 3’
α-SMA		
	Forward	5’ GTCCCAGACATCAGGGAGTAA 3’
	Reverse	5’ TCGGATACTTCAGCGTCAGGA 3’
CHOP		
	Forward	5’ GCATGAAGGAGAAGGAGCAG 3’
	Reverse	5’ CTTCCGGAGAGACAGACAGG 3’
GRP78		
	Forward	5’ TCATCGGACGCACTTGGAA 3’
	Reverse	5’ CAACCACCTTGAATGGCAAGA 3’
XBP1		
	Forward	5’ CCTGAGCCCGGAGGAGAA 3’
	Reverse	5’ CTCG AGCAGTCTGCGCTG 3’

Supplemental Table 2. Characteristic symptoms of diabetes in Rac1-ko and wild-type mice
(* $P < 0.05$ versus non-diabetes)

Group	Blood glucose (mM)	Food up-take (g/mouse/day)	Water up-take (ml/mouse/day)
Non-diabetes			
WT	<12	3.13 ± 0.18	9.50 ± 0.71
Rac1-ko	<12	2.98 ± 0.14	8.50 ± 0.71
Vehicle	<12	3.29 ± 0.44	8.50 ± 0.71
Apocynin	<12	2.84 ± 0.06	9.00 ± 1.41
Diabetes			
WT	30.20 ± 4.07*	6.42 ± 0.50*	48.50 ± 2.12*
Rac1-ko	25.87 ± 6.94*	6.43 ± 0.61*	47.50 ± 3.54*
Vehicle	24.44 ± 4.96*	6.43 ± 0.08*	45.50 ± 2.12*
Apocynin	23.76 ± 4.20*	6.24 ± 0.65*	43.00 ± 2.83*

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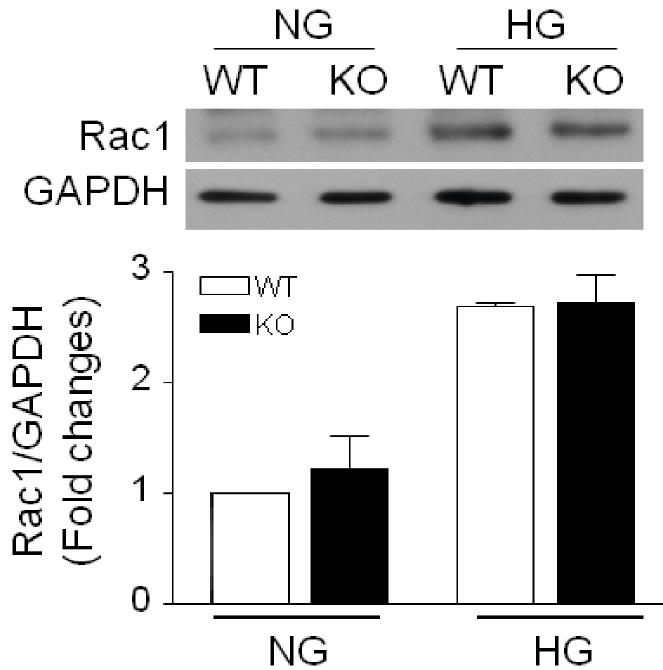
Supplemental Figure 1. Rac1 protein expression in cardiac fibroblasts from wild-type (WT) and Rac1 knockout mice (KO). Cardiac fibroblasts were isolated from adult WT and KO mice and cultured. The second generations of cardiac fibroblasts were incubated with normal (NG, 5.5 mM) and high glucose (HG, 33 mM) for 24 hours. The protein levels of Rac1 were determined by western blot analysis. Top panel is the representative blot for Rac1 and GAPDH proteins from 3 different cultures and lower panel is the quantification of Rac1 relative to GAPDH. Data are MEAN ± SD from 3 different experiments.



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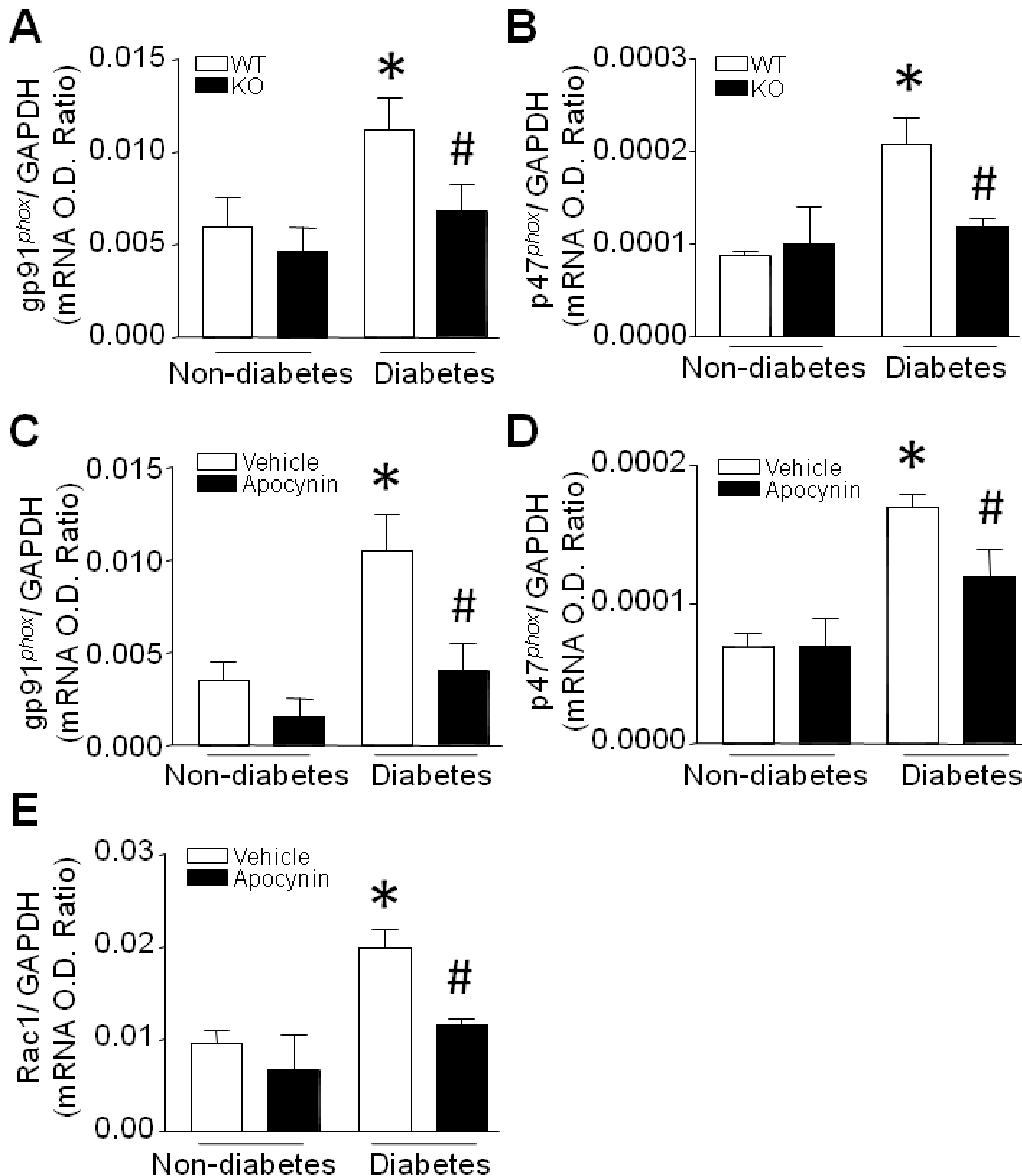
Supplemental Figure 2. Effects of Rac1 knockout and apcynin on NADPH oxidase subunits' expression. A & B: Rac1-ko mice and their wild-type littermates were injected with STZ. Two month later, myocardial gp91^{phox} (A) and p47^{phox} (B) expressions were determined by real-time RT-PCR. C-E: wild-type mice were rendered diabetic by STZ injection and apocynin was administrated in the drinking water for 2 months. Myocardial gp91^{phox} (C), p47^{phox} (D) and Rac1 (E) expressions were measured. Data are MEAN ± SD, n = 6 – 8. * $P < 0.05$ *versus* non-diabetes in WT or vehicle; # $P < 0.05$ *versus* diabetes in WT or vehicle.



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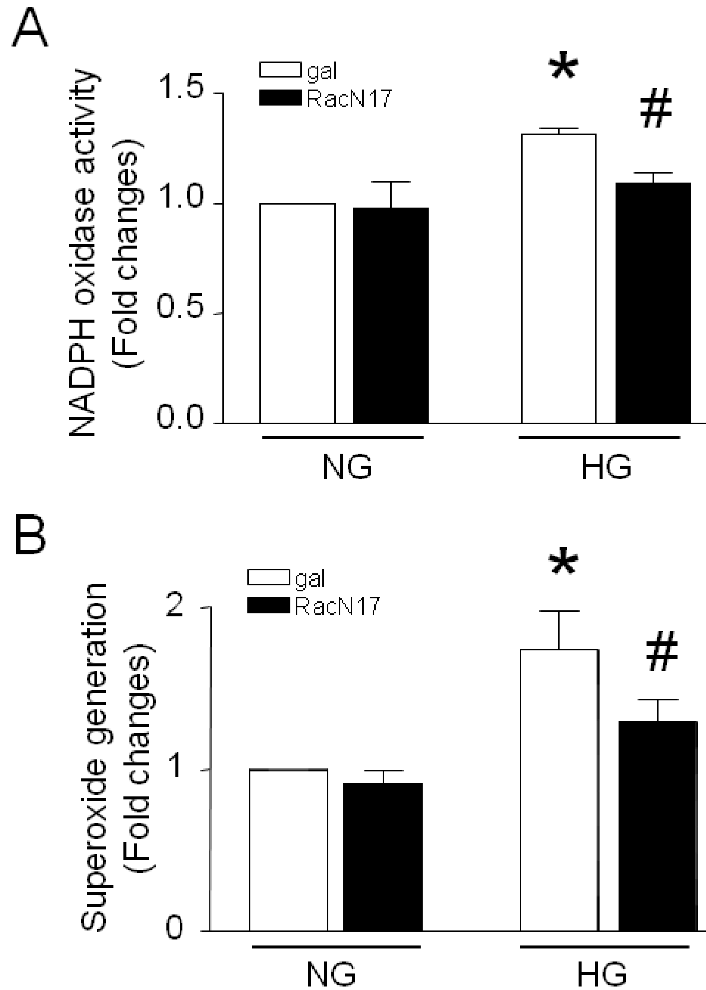
Supplemental Figure 3. Effects of dominant negative mutant Rac1 on NADPH oxidase activity and superoxide production in cardiomyocytes. Isolated adult rat cardiomyocytes were infected with Adenoviral viruses containing a dominant negative mutant Rac1 (Ad-RacN17 or RacN17) or β-gal (Ad-gal or gal) as a control for 24 hours, and then incubated with normal (NG, 5.5 mM) or high glucose HG, 33 mM) for another 24 hours. NADPH oxidase activity (A) and superoxide production (B) were measured by lucigenin-enhanced chemiluminescence and using DHE as an indicator, respectively. Data are MEAN ± SD, n = 3 – 4. * $P < 0.05$ *versus* gal in NG; # $P < 0.05$ *versus* gal in HG.



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