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Reduction in IL-33 expression exaggerates ischaemia/reperfusion-induced myocardial injury in mice with diabetes mellitus

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Aims The underlying mechanism(s) of vulnerability of the diabetic myocardium to ischaemia/reperfusion (I/R)-induced injury is not fully understood. Interleukin-33 (IL-33) has been reported showing the beneficial effect to the myocardium on I/R injury. The aims of this study were to test whether diabetes mellitus (DM) affects myocardial levels of IL-33 and to examine whether reduction in IL-33 is responsible for exaggerated I/R injury in the diabetic myocardium.

Methods and results DM hearts were challenged with I/R *in vivo*, whereas while isolated cardiomyocytes *in vitro* were conditioned with high glucose (HG) followed by an anoxia/reoxygenation (A/R) challenge. Myocardial levels of IL-33 were decreased in mice with DM which was associated with increased protein kinase C βII (PKCβII) activation. Exogenous IL-33 prevented the DM-induced PKCβII activation and attenuated I/R injuries (myocardial infarction size and apoptosis). HG-conditioned myocytes incurred exaggerated apoptosis when compared with naïve myocytes after A/R which was attenuated by IL-33. HG activated PKCβII in cardiomyocytes, which was further enhanced by A/R. IL-33 prevented the PKCβII activation in myocytes with HG or HG and A/R. Inhibition of PKCβII prevented the beneficial effect of IL-33. Finally, IL-33 up-regulated diacylglycerol kinase zeta (DGK-zeta) in cardiomyocytes and reversed the down-regulation of myocardial DGK-zeta in mice with DM.

Conclusion Our results indicate that decreased levels of IL-33 are responsible for the increased sensitivity of the myocardium to I/R in DM. Reduction in IL-33 results in a chronic activation of PKCβII. I/R further enhances PKCβII activation in the diabetic myocardium which results in exaggeration of myocardial injury.

Keywords Ischaemia/reperfusion injury • IL-33 • PKCβII • Diabetes mellitus

1. Introduction

Patients with diabetes mellitus (DM) have an increased risk of coronary heart disease, and cardiovascular complications are responsible for the majority of diabetes-related deaths.[1] In acute coronary syndromes, the presence of DM has a negative impact on the outcomes of the disease.[2] Due to the exposure to abnormal substrate and cytokines, it appears that the myocardium of diabetic patients is more prone to the ischaemia/reperfusion (I/R)-induced injury than those of individuals without DM.[3,4] However, the exact mechanism(s) by which the diabetic myocardium is more vulnerable to I/R injury remains unknown.

Interleukin-33 (IL-33) is a 30 kDa protein and is a new member of the IL-1 cytokine family.[5,6] It is broadly expressed in many tissues including the heart. Previous studies have demonstrated that IL-33 has beneficial actions on various cardiovascular pathologies.[5] By interacting with its receptor ST-2L, IL-33 can (i) prevent cardiomyocyte apoptosis induced by hypoxia *in vitro* and (ii) attenuate myocardial infarction and improve cardiac function and survival after I/R *in vivo*.[7] Furthermore, it has been demonstrated that IL-33 can inhibit NFκB activation and attenuate myocardial fibrosis and myocyte hypertrophy.[8] The above studies suggested that changing in myocardial levels of IL-33 may contribute to various myocardial pathologies including I/R injury in the diabetic myocardium.

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Protein kinase C (PKC) is a serine/threonine protein kinase family which has least 11 isoforms that function in different biological systems. PKCβ is one of conventional (c-PKC) isoforms. The PKCβ activity is dependent on both Ca^{2+} and phosphatidylserine and is greatly enhanced by diacylglycerol (DAG). The clearance of the DAG is attributed to diacylglycerol kinase (DGK). Thus, an increase in cellular levels of DGK could lead to a decrease in PKCβ activity.[9,10]

The myocardial PKCβ activity is increased in diabetic rodents.[11] It has been reported that chronic activation of PKC by hyperglycaemia in DM leads to an increase in vascular permeability, extracellular matrix synthesis, and myocardial dysfunction.[12] Thus, there is a possibility that increased PKC activity causes the vulnerability of the myocardium to I/R-induced injury. Whether the increased PKC activity is related to myocardial IL-33 level remains to be determined.

The present study aimed to test the hypothesis that the diabetic myocardium is more vulnerable to I/R-induced injury than those of mice without DM due to decreased myocardial levels of IL-33. The association of IL-33 expression, PKCβ activation, and myocardial I/R injury in DM was addressed. The study shows that DM results in a decreased myocardial IL-33 expression which is associated with an increased myocardial infarction size and enhanced apoptosis after I/R. Treatment of high glucose (HG) conditioned cardiomyocytes or diabetic mice with exogenous IL-33 attenuated anoxia/reoxygenation (A/R) or I/R-induced injury. Further, treatment of cardiac myocytes with IL-33 results in an increase in DGK-zeta expression and prevents the HG-induced activation of PKCβ. Thus, the present study demonstrates a novel mechanism underlying the sensitization of the myocardium of DM to I/R injury.

2. Methods

2.1 Mice

C57BL/6 mice were obtained from Charles River Canada (St Constant, Quebec, Canada) and were housed in Victoria Research Labs Vivarium Service with a 12 h light/dark cycle and free access to rodent chow and tap water. The mice were used for *in vivo* experiments, as well as a source for cardiac myocytes for *in vitro* experiments. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the University of Western Ontario Animal Care and Use Committee (Protocol No. 2009-043).

2.2 *In vivo* STZ model of DM

Type I DM was induced in 8-week-old male, C57BL/6 wild-type mice by intraperitoneal (ip) injection with streptozotocin [STZ in citrate buffer; 50 mg/kg, daily for 3 consecutive days].[13] These sex-matched litter mates injected with vehicle were served as a control. Blood glucose was determined 5 days after the final injection. DM was considered to be induced once hyperglycaemia became evident (blood glucose levels >22 mM). Eight weeks after the induction of DM, mice were subjected to a myocardial I/R challenge as described below.

2.3 I/R model

The mouse model of myocardial I/R involved ligation of the left anterior descending artery (LAD) as described previously.[14,15] Briefly, mice were anaesthetized with ketamine (150 mg/kg) and xylazine (5 mg/kg, ip). When adequacy of anaesthesia was reached as indicated by the disappearance of pedal withdrawal reflex, the tracheal intubation was performed and the mice were artificially ventilated with a MiniVent (Type 845). Post-thoracotomy, the LAD was occluded using a 6-0 suture with a piece of tubing interposed between the artery and suture and the thorax closed. Thirty minutes later, the thorax was reopened, the tubing removed, and the suture cut. The thorax was closed again and the heart allowed to reperfusion for 24 h. As a control, sham-operated mice underwent the same surgical procedure except arterial occlusion. To determine the role of IL-33 on I/R injury in mice with DM, diabetic mice were given either IL-33 (20 mg/kg, ip) or saline 24 and 2 h prior to I/R.

2.4 Myocardial infarct size

Myocardial infarct size was measured as reported previously.[15] Briefly, 24 h after reperfusion, the mouse was anaesthetized, artificially ventilated, and the chest opened again. Immediately following re-occlusion of the LAD at original ligation site, the heart was isolated and perfused (retrograde) with 4.0% Evans Blue through the aorta to stain non-ischaemic myocardial tissue. The heart was then cut into four transverse slices. The slices were stained with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) to determine the infarct area (IFA) and photographed under a dissecting microscope. Left ventricular (LV) area, area at risk (AAR), and IFA were determined with SigmaScan software. The area of the myocardium without TTC staining was considered as IFA. Infarct size was expressed as the percentage of the IFA in AAR.

2.5 Myocardial apoptosis

Myocardium apoptosis was assessed with an *in situ* cell death detection kit (Roche) as described previously.[14] Briefly, 24 h after I/R, the hearts were harvested and fixed with 10% formalin for 24 h. The specimens were processed for paraffin embedding. After deparaffinization, rehydration, and antigen retrieval with sodium citrate (pH 6.0), the tissue sections were treated with proteinase K (10–20 μg/mL in Tris/HCl). Subsequently, the sections were incubated with TUNEL reaction mixture at 37°C for 60 min and myocardial apoptosis was assessed by immunofluorescence microscopy (Zeiss Axiovert 200M). To localize positive apoptotic signal within the myocardium, the myocardial sections were also counterstained for troponin T with Texas red (myocytes), and Hoechst for (nuclei).

Cardiomyocyte apoptosis was assessed by determination of caspase 3 activity, fragmented DNA, and apoptotic cells attaining.[14] For measuring the caspase 3 activity, the myocytes were washed with PBS and lysed with Cell Lysis Buffer (BioVision). Subsequently, the cell lysates were centrifugated at 10 000 *g* for 10 min at 4°C. The supernatants were incubated with a caspase 3 fluorometric substrate DEVA-AFC (BioVision). The caspase 3 activity was determined by measuring the fluorescence intensity with a Victor 3 multilabel counter (Perkin Elmer).

Apoptotic myocyte death was determined with a quantitative Cell Death Detection ELISA Kit (Roche) to detect fragmented DNA according to the manufacturer's instructions.[14] Briefly, cardiomyocytes were washed with PBS, lysed with cell lysis buffer in the kit, and harvested in an Eppendrof tube. After centrifugation at 20 000 *g* for 10 min at 4°C, the supernatants were collected for detection of histone-associated DNA fragments with ELISA.

For direct detection of apoptotic cells in myocytes, the cells were seeded on cover slips and cultured with or without HG followed by challenged with a normoxia/reoxygenation (N/R) or A/R. Subsequently, the cells were fixed with 4% paraformaldehyde and apoptotic cells were detected with TUNEL staining as described above for the myocardium apoptosis.

2.6 Myocardial function

Mouse cardiac function was evaluated with a pressure–volume loop analysis system as described previously.[16] Briefly, mice were anaesthetized with ketamine (150 mg/kg) and xylazine (5 mg/kg) and the adequacy of anesthesia was monitored for the disappearance of pedal withdrawal reflex. Subsequently, a Millar tip transducer catheter (Model SPR-893, 1.4 Fr) was advanced into the LV via the right carotid artery. After recording of the basic haemodynamic parameters, LV pressure–volume loops were generated by occlusion of the inferior caval vein using a PowerLab system (AD

Instruments, USA) connected to the Millar catheter.[17] LV end-systolic pressure–volume relation was calculated and used as an index of myocardial contractile function.

2.7 Cardiac myocytes culture

Cardiac myocytes were isolated from neonatal mouse hearts.[14] In brief, the harvested hearts were minced, digested, washed, and the cells suspended in M199 with 10% foetal calf serum (FCS). The myocytes were enriched by a preplating approach to remove contaminating cells (fibroblasts and endothelial cells readily adhere, while myocytes do not). The non-adherent cells were removed and cultured in M199 supplemented with 10% FCS. After 48 h in culture, the cells had formed a confluent monolayer consisting of 95% myocytes beating in synchrony.

2.8 *In vitro* model of hyperglycaemia

Cardiac myocytes were cultured with M199 containing 30 mM glucose for up to 72 h to simulate the hyperglycaemia of DM.[18] As a control, the cells were incubated in M199 with 30 mM mannitol.

2.9 A/R model

As *in vitro* correlates to I/R *in vivo*, cardiomyocytes were exposed to an A/R as described previously.[14] Briefly, the myocyte monolayer were exposed to anoxia for 30 min and then reoxygenated for up to 24 h. The control cardiac myocytes were exposed to normoxia (N/R). In some experiments, recombinant IL-33 (0.2–5 ng/mL) was given to myocytes while they were challenged with A/R.

2.10 Protein expression

Myocardial tissue and cardiac myocytes protein expression was assessed with western blot as described previously.[16] Briefly, tissue or cell lysates were resolved on SDS–polyacrylamide gels (PAGE) and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were probed with primary antibodies against one of the following proteins: IL-33, DGK-zeta, β-actin, GAPDH, and P-Cadhrin. After incubated with related secondary antibodies, the specific bands were visualized with an ECL detection system according to the manufacturer's instructions.

2.11 PKCβII activation

Myocardial tissue PKCβII activation was assessed by detection of membrane-bonded PKCβII as described previously.[19] Briefly, cell membrane fraction of the myocardium was separate with a membrane/cytoplasm fractionation kit (BioVision). Membrane-bonded PKCβII and cytosolic PKCβII were determined with western blot. The ratio of membrane bonded to cytosolic PKCβII was used as an index of tissue PKCβII activation. PKCβII activation in cardiac myocytes was determined by detecting the phosphorylation status of intracellular PKCβII by western blot.[11]

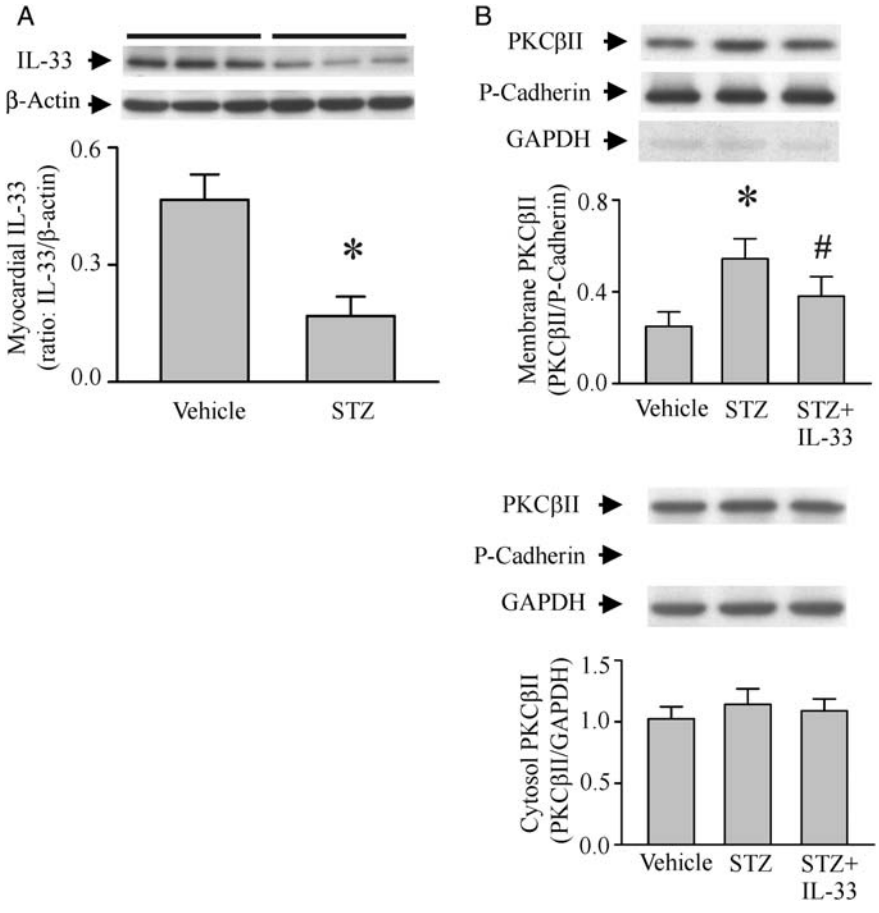

Figure 1 DM decreases myocardial levels of IL-33 and activates PKCβII. (*A*) Myocardial IL-33 expression was decreased in STZ-treated mice when compared with those of vehicle-treated mice. Upper panel: actual blots; lower panel: densitometry analysis data. $n = 3$; $^{*}P < 0.05$ compared with vehicle. (*B*) Exogenous IL-33 attenuated the activation of myocardial PKCβII by DM. Myocardial PKCβII activation was assessed 24 h after the administration of IL-33 by measuring membrane-bonded PKCβII with western blot. $n = 3$; $^{*}P < 0.05$ compared with vehicle; $^{\#}P < 0.05$ compared with STZ.

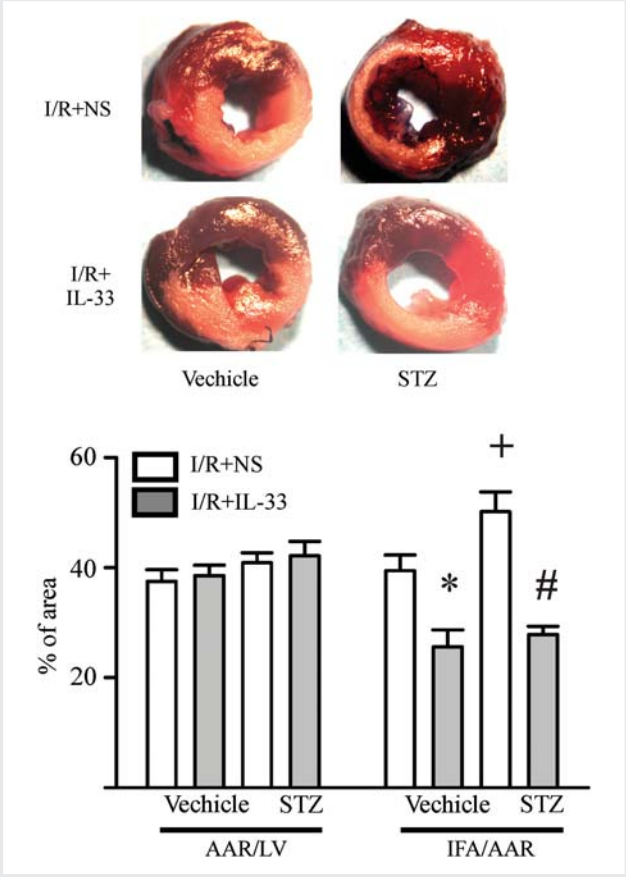

Figure 2 Myocardial infarction size was measured. Upper panels are actual images and lower panels are quantitative data. $n = 5$; $^{*}P < 0.05$ vs. vehicle mice/I/R; $^{+}P < 0.05$ compared with vehicle mice/I/R; $^{\#}P < 0.05$ vs. STZ mice I/R.

2.12 PKCβII small-interference RNA transfection

Small-interference RNA (siRNA) specific for PKCβII was purchased from Santa Cruz Biotechnology. Cardiac myocytes were transfected the siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.[20] Transfection efficiency was ~70% as determined with western blot (see Supplementary material online, *Figure S1*) and the cardiac myocytes were used in experiments 48 h after the siRNA transfection.

2.13 Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by a Bonferroni correction for multiple comparisons. GraphPad Software program was used for statistical analysis. A *P*-value of <0.05 is considered to be statistically significant.

3. Results

3.1 Myocardial IL-33 expression, PKCβII activation, and I/R-induced myocardial injury in mice with DM

Myocardial IL-33 expression is decreased in mice with DM (*Figure 1A*), which is associated with an increased activation of PKCβII (*Figure 1B*). In addition, an I/R challenge to the myocardium resulted in an increased myocardial infarction size (*Figure 2*), and exaggerated myocardial apoptosis and dysfunction in mice with DM compared with mice without DM (*Figure 3*). To determine whether the deficiency of myocardial IL-33 in diabetic mice causes activation of PKCβII and sensitization of the myocardium to I/R-induced injury, DM mice were administrated with IL-33. Subsequently, the PKCβII activation, I/R injury, and myocardial function were assessed. As shown in *Figure 1B*, exogenous IL-33 abolished the PKCβII activation, attenuated I/R injury (myocardial infarction size and apoptosis), and improved myocardial function in both mice with and without DM. However, the protective effect was more prominent in mice with DM than those without DM (*Figures 2* and *3*).

3.2 Role of IL-33 on A/R-induced apoptosis in cardiomyocytes with HG

To study the mechanism(s) by which the diabetic myocardium is protected by exogenous IL-33, the cardiomyocytes were exposed to HG to mimic hyperglycaemia *in vivo*. Subsequently, the HG-treated cardiomyocytes were challenged with an A/R with or without IL-33. As shown in *Figure 4A* and *B*, HG-treated cardiomyocytes incurred exaggerated apoptosis after the A/R challenge compared with those of myocytes without HG. Treatment of cardiomyocytes with IL-33 (1–5 ng/mL) attenuated myocyte apoptosis after A/R, while 0.2 ng/mL IL-33 showed no effect (*Figure 4C*). We further evaluated the effect of IL-33 on A/R-induced apoptosis in HG-conditioned myocytes. As shown in *Figure 4D*, exogenous IL-33 (5 ng/mL) attenuated the A/R-induced apoptosis in HG-conditioned cardiomyocytes.

3.3 Effect of IL-33 on HG-induced activation of PKCβII in cardiomyocytes and on cardiomyocyte DGK-zeta expression

Treatment of cardiac myocytes with HG resulted in an increase in PKCβII phosphorylation (*Figure 5A*) which is an indication of PKCβII activation.[11] IL-33 prevented the increase in PKCβII phosphorylation (*Figure 5B*). Further, HG-induced activation of PKCβII was dramatically enhanced when the HG-treated cardiomyocytes were challenged with A/R. Again, the IL-33 significantly attenuated the A/R-induced activation of PKCβII in HG-treated cardiomyocytes (*Figure 5C*). To further demonstrate whether PKCβII activation is associated with the A/R-induced myocyte apoptosis, cardiac myocytes were pretreated with either a PKCβII inhibitor [3-(1-(3-Imidazol-1-ylpropyl)-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione] or an siRNA specific to PKCβII prior to being challenged with A/R. As shown in *Figure 6A* and *B*, blockade of PKCβII by either the inhibitor or the siRNA prevented the A/R-induced myocyte apoptosis. Furthermore, as shown in *Figure 6B*, both exogenous IL-33 and siRNA showed protection to cardiomyocytes; however, exogenous IL-33 to siRNA-treated myocytes did not show additional protection. These results indicate that IL-33 protects cardiomyocytes through inhibition of PKCβII.

PKCβII activation is a complex event including a series of serine/threonine transphosphorylation and autophosphorylation followed by relocation of activated PKCβII to plasma membrane. The PKCβII activity is greatly affected by the availability of DAG, an intermediate product synthesized from glucose.[12] DGKs are intracellular lipid kinases which can regulate PKCβ activity by altering the availability of DAG.[10] In the heart, DGK-zeta has been reported being related

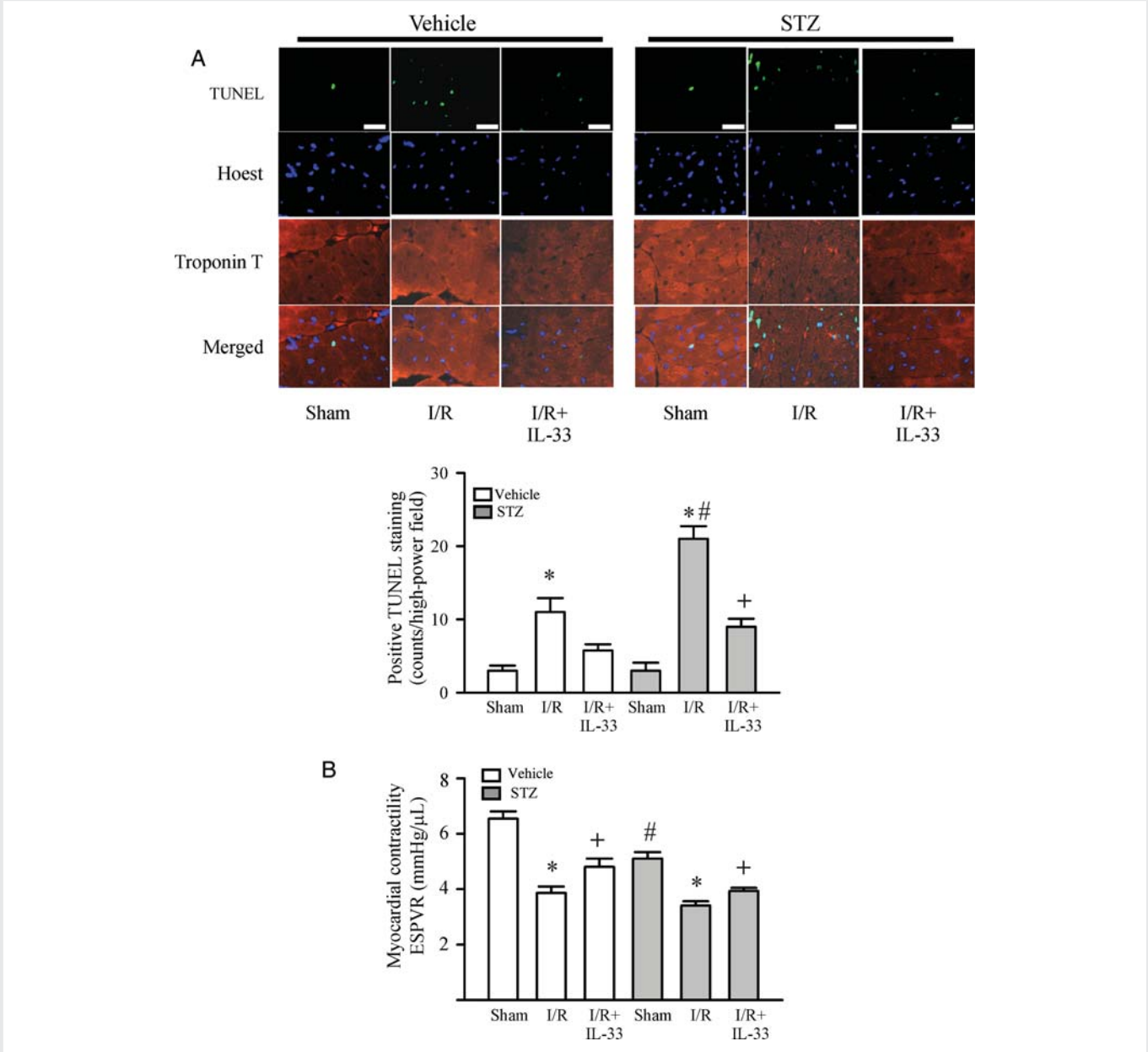

Figure 3 I/R results in an exaggerated myocardial apoptosis and dysfunction in mice with DM which was attenuated by exogenous IL-33. (*A*) Myocardial apoptosis assessed with TUNEL staining. The upper panel is an actual imagine of TUNEL immunofluorescence staining. Scale bar = 20 μm; lower panel: quantitative analysis data. $n = 5$; $^{*}P < 0.05$ compared with respective sham mice; $^{\#}P < 0.05$ compared with I/R/vehicle mice; $^{+}P < 0.05$ compared with respective I/R. (*B*) IL-33 improved cardiac contractility after I/R in mice with or without DM. $n = 7$; $^{*}P < 0.05$ compared with respective sham mice; $^{\#}P < 0.05$ compared with vehicle/sham mice; $^{+}P < 0.05$ compared with respective I/R.

to I/R injury.[21] Thus, we assessed the effect of IL-33 on DGK-zeta expression in cardiomyocytes and myocardium of diabetic mice. As shown in *Figure 6C*, treatment of cardiomyocytes with IL-33 (1–5 ng/mL) increased myocyte DGK-zeta expression. Furthermore, DM resulted in decreased levels of myocardial DGK-zeta which was prevented by exogenous IL-33 (*Figure 6D*).

4. Discussion

After a myocardial infarction, the prognosis of patients with DM is much worse than that of patients without DM.[2] Experimental studies have demonstrated that an I/R challenge to the myocardium of diabetic animals resulted in a larger infarction size than those of animals without DM.[4] However, the underlying mechanism(s) by which the diabetic myocardium is more vulnerable to I/R-induced injury remains poorly understood.

Previous reports indicate that aldose reductase, an NADPH-dependent enzyme, is activated in DM and is responsible for diabetes-related vascular and neurological complications.[22,23] In the heart, it has been demonstrated that the I/R challenge results in an increase in myocardial aldose reductase activity. Inhibition of the aldose reductase attenuates the I/R-induced myocardial injury.[24] These studies

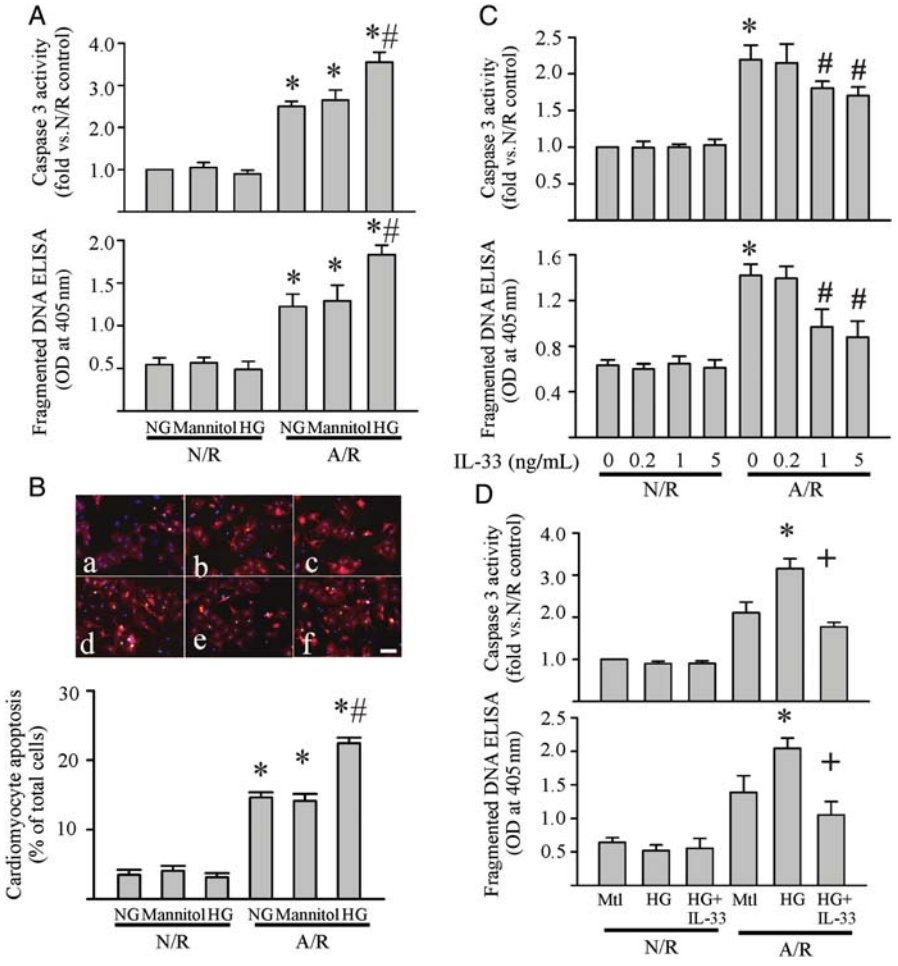

Figure 4 A/R-induced apoptosis was exaggerated in cardiomyocytes with HG which was attenuated by IL-33. (*A*) Cardiomyocyte caspase 3 activity and fragmented DNA were measured. $n = 3$; $^{*}P < 0.05$ compared with respective N/R; $^{\#}P < 0.05$ compared with normal glucose (NG)/A/R or mannitol/A/R. (*B*) Cardiomyocytes were grown on cover slips and challenged with HG followed by A/R. Apoptosis assessed with TUNEL staining. Representative staining for TUNEL (green signal), troponin T (red signal), and Hoechst 33342 (blue signal). Upper panel—actual images; N/R: a, NG; b, mannitol; c, HG; A/R: d, NG; e, mannitol; f, HG. Scale bar = 20 μm; lower panel—quantitative data. $n = 3$; $^{*}P < 0.05$ compared with respective N/R; $^{\#}P < 0.05$ compared with M199/A/R or mannitol/A/R. (*C*) Cardiac myocytes were challenged with an N/R or A/R with or without IL-33 (0.2–5 ng/mL) and apoptosis assessed. $n = 3$; $^{*}P < 0.05$ compared with N/R; $^{\#}P < 0.05$ compared with A/R without IL-33. (*D*) Cardiac myocytes were cultured with mannitol or HG for 48 h followed by A/R with or without IL-33 (5 ng/mL) and myocyte apoptosis assessed. $n = 4$; $^{*}P < 0.05$ compared with mannitol/A/R; $^{+}P < 0.05$ compared with HG/A/R.

imply that the aldose reductase plays key roles in the I/R injury in the diabetic myocardium. In addition, (i) chronic hyperglycaemia leads to a decreased microvasculature density in the myocardium[25] and (ii) either hyperglycaemia or I/R can result in a robust free radical species production.[9] The oxidant stress to microvascular cells can cause further damage to the diabetic myocardium. Thus, others believe that microcirculation dysfunction of the diabetic myocardium causes the vulnerability to I/R injury.

Our study provides a novel insight to I/R injury in the diabetic myocardium. Using a mouse model of type I DM and I/R challenge to the diabetic mice, we found that DM decreases myocardial levels of IL-33 which is associated with increased myocardial PKCβII activation and exaggerated I/R injury. Administration of IL-33 to diabetic mice or cardiomyocytes attenuates I/R- and A/R-induced apoptosis. In addition, we demonstrated that A/R challenge enhances HG-induced PKCβII activation which is associated with the exaggerated myocyte apoptosis, and IL-33 attenuates A/R or I/R injury by inhibition of PKCβII activation. Finally, we provided evidence indicating that exogenous IL-33 increased DGK-zeta expression in cardiomyocytes and myocardium of mice with DM.

It is generally believed that cytokines play important roles in the I/R-induced myocardial injury[14] and myocardial dysfunction in DM.[26] Previous studies have demonstrated that increased expression of TNFα, IL-6, MCP-1, and recently HMGB1 in both settings is detrimental and showed cytotoxic effects to cardiomyocytes.[14,27,28] IL-33 is a newly identified cytokine which belongs to IL-1 family. Interestingly, in contrast to those cytotoxic cytokines, IL-33 has been reported showing beneficial effects to the cardiovascular system including preventing the development of arthrosclerosis and attenuating I/R-induced myocardial injury and fibrosis.[7,8] In the present study,

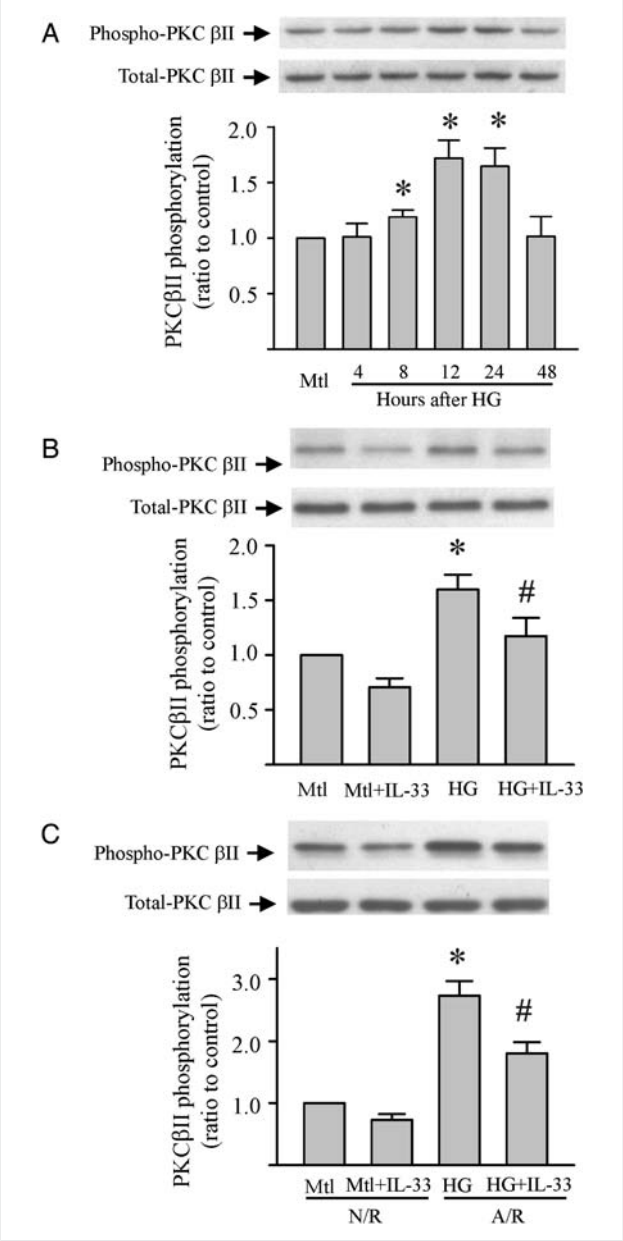

Figure 5 IL-33 prevented the A/R-challenge enhanced PKCβII activation in cardiomyocytes with HG. (*A*) Cardiomyocyte PKCβII phosphorylation increased after HG. $n = 3$; $^{*}P < 0.05$ compared with control. (*B*) IL-33 attenuated HG-induced PKCβII phosphorylation. $n = 3$; $^{*}P < 0.05$ compared with mannitol; $^{\#}P < 0.05$ compared with HG. (*C*) A/R enhanced HG-induced PKCβII phosphorylation which was attenuated by IL-33. $n = 3$; $^{*}P < 0.05$ compared with HG/N/R; $^{\#}P < 0.05$ compared with HG/A/R.

we discovered for the first time that myocardial levels of IL-33 are decreased in diabetic mice. The reduction in myocardial IL-33 results in the diabetic myocardium is more sensitive to I/R. However, the mechanism which causes a reduction in myocardial IL-33 in diabetes is not clear. One potential mechanism is the induction of microRNA (miR) in the diabetic heart. By microarray assay, we recently found that several miRs are elevated in the diabetic myocardium (unpublished observation). Of which miR-375, miR-380-5p are directly targeting the 3′-UTR of the IL-33 based on the bio-information tools (microrna.org, targetscan.org). Thus, the increase in myocardial miRNA expression could lead to a reduction in myocardial IL-33 in diabetes.

PKCβ is an important kinase that contributes to myocardial dysfunction in various pathologies.[12] In I/R myocardial injury, an increase in the PKCβ level resulted in myocyte death. Genetic and pharmacological inhibition of PKCβ resulted in diminished phosphorylation of c-Jun NH_2-terminal mitogen-activated protein kinase and attenuated I/R-induced myocardial injury and apoptosis.[29] These results indicate that PKCβ is a pivotal kinase in I/R-induced myocyte apoptosis. Our results agree with the role of PKCβ in I/R-induced myocardial injury.

Chronic activation of PKCβ has been demonstrated in DM and is believed to contribute to DM-related complications.[12] In addition, it has been reported that I/R can activate PKCβ and contribute to myocardial injury.[29] Thus, we postulate that enhanced myocardial injury after I/R in diabetic mice is attributed to exaggerated activation of PKCβ. Indeed, our data support the contention. We found that treatment of cardiomyocytes with HG activated PKCβII, which was further enhanced when the HG treated cardiomyocytes were further challenged with an A/R. IL-33 prevented the exaggerated PKCβII activation and attenuated the A/R-induced apoptosis in myocytes with HG.

Our results indicate that myocardial PKCβII was increased in diabetic mice. However, myocardial IL-33 was decreased and exogenous IL-33 attenuated the activation of PKCβII. Therefore, we believe that decreased myocardial IL-33 contributes to chronic activation of PKCβII in diabetes. As a c-PKC isoform, the PKCβII activation and activity is dependent on the DAG level which is regulated by DGK.[12] Therefore, a change in the DGK level could affect the PKCβII activation. In this study, we demonstrated for the first time that treatment of the cardiomyocyte with IL-33 increased the DGK-zeta expression; furthermore, we found that myocardial DGK-zeta was decreased in diabetic mice, which was reversed by exogenous IL-33.

It has been demonstrated that an increase in myocardial aldose reductase activity results in cytosolic NADH/NAD^+ elevation.[23,30] The resultant of the above changes is the increase in PKCα/β activity and myocardial injury.[30] However, our data support that a reduction in IL-33 leads to a decrease in the DGK level which further activates PKCβII. It is not clear whether a reduction in myocardial IL-33 could further activate aldose reductase. To this end, we measured the aldose reductase activity, lactate/pyruvate ratio (a measure for cytosolic NADH/NAD^+), and intracellular levels of ATP in cardiomyocyte with HG. As shown in Supplementary material online, *Figure S2*, after HG treatment for 48 h, myocyte aldose reductase activity and lactate/pyruvate ratio were elevated. The metabolic changes were exaggerated after A/R. The IL-33 treatment attenuated the HG or HG with an A/R-induced increase in aldose reductase activity and lactate/pyruvate ratio. Based on the above data, it seems that HG-induced activation of aldose reductase is related to the down-regulation of IL-33. However, the exact pathway by which to link the IL-33 and activation of the aldose reductase remains to be determined.

One limitation of the present study is to use normal cardiomyocytes treated with HG *in vitro* to mimic diabetic myocardium *in vivo*. However, based on the aldose reductase activity and lactate/pyruvate ratios, it seems that cardiomyocytes show similar abnormalities as those of the diabetic myocardium (see Supplementary material online, *Figure S2*). The metabolic difference between HG-treated myo-

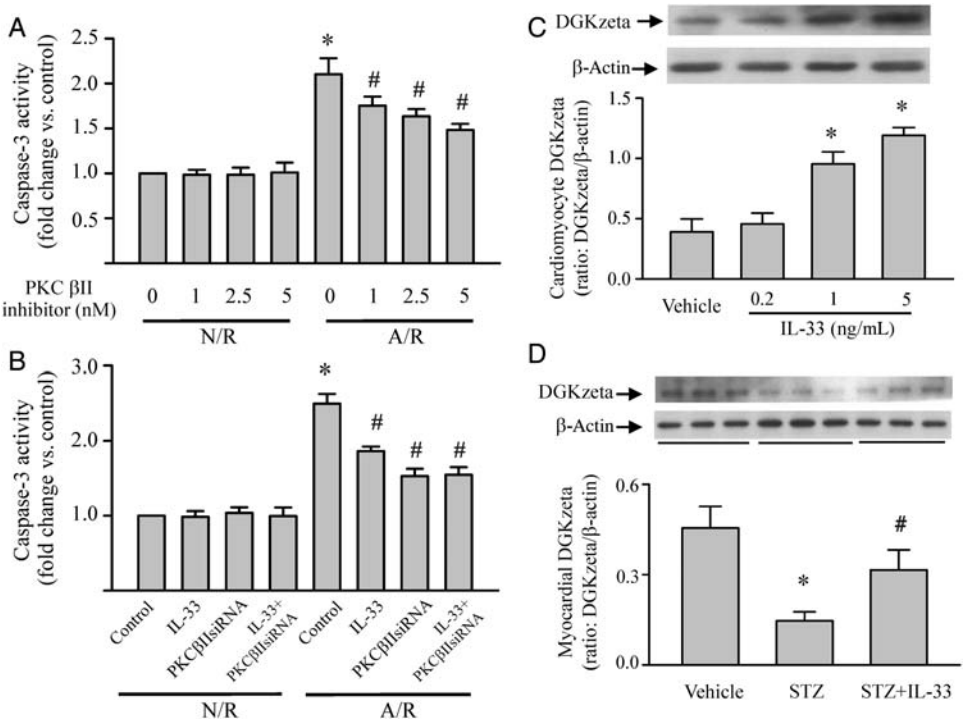

Figure 6 (*A*) Cardiomyocytes were pretreated with a PKCβII inhibitor (1–5 nM) or vehicle followed by an A/R and myocyte caspase 3 activity assessed. $n = 3$; $^{*}P < 0.05$ compared with N/R; $^{\#}P < 0.05$ compared with A/R. (*B*) Cardiomyocytes were pretreated with either IL-33 or transfected with PKCβII siRNA or the combination of the treatment; the control cells were treated with vehicle. Myocyte caspase 3 activity assessed after N/R or A/R. $n = 3$; $^{*}P < 0.05$ compared with control N/R; $^{\#}P < 0.05$ compared with control A/R. (*C*) Cardiomyocytes were treated with IL-33 (0.2–5 ng/mL) for 24 h and DGK-zeta protein expression evaluated with western blot. Upper panel: representative blots; lower panel: densitometry analysis data. $n = 3$; $^{*}P < 0.05$ compared with vehicle. (*D*) DM mice were given saline or saline-containing IL-33 (20 μg/kg), control mice were given saline, and myocardial DGK-zeta expression was assessed. The upper panel is actual western blots and the lower panel is densitometric data. $n = 3$; $^{*}P < 0.05$ compared with vehicle; $^{\#}P < 0.05$ compared with STZ.

cytes and the diabetic myocardium is that HG slightly increases intracellular ATP (see Supplementary material online, *Figure S2C*), while the diabetic myocardium incurs decreased intracellular ATP.[31] However, intracellular ATP was dramatically decreased after A/R in HG-treated myocytes (see Supplementary material online, *Figure S2C*) which is similar to ATP levels in the diabetic myocardium after I/R.[32] Thus, we believe that our *in vitro* setting mimics the metabolic abnormalities of the diabetic myocardium *in vivo*. Another limitation of the study is to use the STZ mouse model of diabetes. The blood glucose levels of the diabetic mice were not controlled with insulin. They are rather severe diabetic mice challenged with I/R. Thus, the results should be interpreted within the limits of the experimental setting.

In conclusion, our study demonstrated for the first time that DM decreases myocardial IL-33 expression. The resultant of decreased IL-33 is down-regulation of DGK-zeta and chronic activation of PKCβII. The enhanced activation of PKCβII after I/R in the diabetic myocardium results in exaggeration of myocardial injury.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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