Impaired sonic hedgehog pathway contributes to cardiac dysfunction in type 1 diabetic mice with myocardial infarction

Qing Xiao1,2†, Ning Hou1,2†, Yan-Ping Wang3, Li-Shan He1, Yan-Hua He1, Gui-Ping Zhang1, Quan Yi1, Shi-Ming Liu2, Min-Sheng Chen2, and Jian-Dong Luo1,2*

1Department of Pharmacology, Guangzhou Medical University, Guangzhou 510182, PR China; 2Guangzhou Institute of Cardiovascular Disease, Guangzhou key laboratory of Cardiovascular Disease, and the Second Affiliated Hospital, Guangzhou Medical University, Guangzhou 510260, PR China; and 3Department of Pathophysiology, Jinan University, Guangzhou 510182, PR China

Received 10 March 2012; revised 23 June 2012; accepted 25 June 2012; online publish-ahead-of-print 28 June 2012

Aims

The incidence and mortality of myocardial infarction (MI) in diabetic patients are higher than in non-diabetic patients; however, the mechanisms by which diabetes results in cardiac dysfunction are poorly understood. The present study tested the hypothesis that an impaired sonic hedgehog (Shh) pathway contributes to cardiac dysfunction in type 1 diabetic mice with MI.

Methods and results

Adult male C57/B6 mice and streptozotocin-induced type 1 diabetic mice were used. Myocardial proteins of Shh, Patched-1 (Ptc1), and glioma-associated oncogene-1 (Gli1) were significantly decreased in type 1 diabetic mice at 10 weeks, and this was accompanied by cardiac dysfunction. Although myocardial proteins of Shh, Ptc1, and Gli1 were significantly increased 7 days after MI compared with the sham group in control mice, these proteins were markedly decreased in streptozotocin-induced diabetic mice. Treatment with Shh pathway agonist for 21 days significantly increased Ptc1 and Gli1 proteins, enhanced capillary density, reduced the percentage myocardial infarct, and then improved cardiac function in diabetic mice with MI compared with those with no drug treatment. This treatment had no effects in control mice with MI. Conversely, treatment with Shh pathway antagonist for 21 days significantly decreased Ptc1 and Gli1 proteins, reduced capillary density, enlarged the percentage myocardial infarct, and then exacerbated cardiac dysfunction in control mice with MI compared with those with no drug treatment.

Conclusions

These findings indicate that in type 1 diabetic mice the myocardial Shh pathway is impaired and that the impaired Shh pathway contributes to cardiac dysfunction. Strategies that are aimed at augmenting the Shh pathway may offer useful means for improving diabetic cardiac dysfunction.

Keywords

Diabetes • Myocardial infarction • Sonic hedgehog pathway • Cardiac dysfunction

1. Introduction

Compared with non-diabetics, the incidence of myocardial infarction (MI) in diabetic patients is higher and recovery is slower, resulting in higher mortality rates.1 The aetiology of impaired myocardial healing in diabetes is multifaceted. Recent studies have shown that diabetes-associated MI is associated with a down-regulation of thioredoxin-1, haem oxygenase-1, hypoxia-inducible factor-1α, and vascular endothelial growth factor levels, as well as extracellular signal-regulated kinase-5 transcriptional activity. It also presents an up-regulation of nitrotyrosine, ubiquitin–proteasome activity, nuclear factor κB, tumour necrosis factor-α, and the receptor for advanced glycation end-products compared with MI that is not associated diabetes2–7; however, the cellular mechanisms underlying the exacerbated cardiac dysfunction observed in diabetes-associated MI are incompletely understood.

A morphogen is a substance governing the pattern of tissue development and the positions of the various specialized cell types within a

† These authors contributed equally to this work.

* Corresponding author: Tel: +8620 8134 0203; Fax: +8620 8134 0137. Email: jiandongluo@hotmail.com

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2012. For permissions please email: journals.permissions@oup.com.
tissue. The hedgehog proteins are well-known morphogens that play a critical role in several tissues during embryonic and postnatal development and adult life.\(^8\) The hedgehog protein family comprises sonic hedgehog (Shh), Indian hedgehog, and desert hedgehog; of these, Shh is the most widely expressed and thoroughly investigated. The Shh pathway has been studied and characterized extensively during embryogenesis concerning its role in the regulation of epithelial-mesenchymal interactions crucial to limb, lung, gut, hair follicle, bone formation, etc., and it is also required for the regulation of multiple key cellular events, in homeostatic conditions, and during tissue regeneration and repair after severe injury, in a wide range of adult tissues and organs, including bone marrow, central nervous system, peripheral nerves, cardiovascular system, and epithelial tissues.\(^11\) – \(^19\)

There is growing evidence that the hedgehog pathway is down-regulated in diabetic neuropathy and vasculopathy, and the delivery of exogenous hedgehog proteins has therapeutic potential for repairing diabetic tissues such as nerve, vasa nervorum, and penis.\(^20\) – \(^23\) Our previous studies have also reported that the Shh pathway is impaired in the skin tissue of type 1 diabetic mice, and the application of exogenous Shh accelerated diabetic wound healing by enhancing cutaneous NO function.\(^24\)

More evidence reveals that there is a close relationship between the hedgehog pathway and cardiovascular disease. Recent reports demonstrated that the hedgehog pathway is up-regulated in both acute and chronic MI, and left ventricular function is preserved after the hedgehog pathway and cardiovascular disease. Recent reports have indicated that the hedgehog pathway is down-regulated in diabetic tissues such as nerve, vasa nervorum, and penis. This finding suggests that the hedgehog pathway plays a critical role in several tissues during embryonic and postnatal development and adult life.\(^8\) The hedgehog protein family comprises sonic hedgehog (Shh), Indian hedgehog, and desert hedgehog; of these, Shh is the most widely expressed and thoroughly investigated. The Shh pathway has been studied and characterized extensively during embryogenesis concerning its role in the regulation of epithelial-mesenchymal interactions crucial to limb, lung, gut, hair follicle, bone formation, etc., and it is also required for the regulation of multiple key cellular events, in homeostatic conditions, and during tissue regeneration and repair after severe injury, in a wide range of adult tissues and organs, including bone marrow, central nervous system, peripheral nerves, cardiovascular system, and epithelial tissues.\(^11\) – \(^19\)

This study aimed to test the hypothesis that an impaired Shh pathway contributes to cardiac dysfunction in type 1 diabetic mice with MI. The blood glucose and the body weight were measured to confirm the diabetes model. The protein expression of Shh, Ptc1, and Gli1 measured by western blot analysis. Twenty-one days after MI, echocardiography was performed to assess ventricular function, and then the hearts were harvested in order to evaluate capillary density by immunohistochemical analysis and assess the percentage myocardial infarct by Masson’s trichrome staining.

This study was carried out in type 1 diabetic mice with and without MI, the blood glucose and the body weight were measured to confirm the diabetes model. The protein expression of Shh, Ptc1, and Gli1 measured by western blot analysis. Twenty-one days after MI, echocardiography was performed to assess ventricular function, and then the hearts were harvested in order to evaluate capillary density by immunohistochemical analysis and assess the percentage myocardial infarct by Masson’s trichrome staining.

### 2. Methods

#### 2.1 Animals and experimental design

Adult male C57/B6 mice were handled in accordance with 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 protocol was approved by the Animal Care and Use Committee of Guangzhou City, and approval reference number was 2010-14.

Adult male C57/B6 mice (body weights of approximately 20 g and with ages ranging between 6 and 7 weeks, from Guangdong Medical Laboratory Animal Center) were divided into the following nine groups: (i) control (CON, \(n = 25\)); (ii) control sham (CS, \(n = 15\)); (iii) control plus MI (CMI, \(n = 15\)); (iv) CMI plus Shh pathway receptor agonist SAG1.3 (CMI-SAG1.3, \(n = 15\)); (v) CMI plus Shh pathway receptor antagonist SANT-1 (CMI-SANT-1, \(n = 15\)); (vi) diabetes (DM, \(n = 25\)); (vii) diabetic sham (DS, \(n = 15\)); (viii) diabetes plus MI (DMI, \(n = 15\)); and (ix) DMI plus SAG1.3 (DMI-SAG1.3, \(n = 15\)).

The study was carried out in type 1 diabetic mice with and without MI, and 155 mice were used in all procedures. For mice without MI (CON, and DM), 20 mice were used for analysis of body weight and blood glucose, and then 16 of them for echocardiography; 30 mice were used for western blot analysis. For mice with MI or sham operation (CS, CMI, CMI-SAG1.3, CMI-SANT-1, DS, DMI, and DMI-SAG1.3), 75 mice were used for MI, and 30 mice for sham operation; 35 mice for echocardiography, and then for Masson’s trichrome staining; 35 mice for western blot analysis; and 35 mice for analysis of capillary density.

The surgery to induce MI was performed 7 weeks after induction of diabetes. The Shh pathway receptor agonist SAG1.3 (5 mg/kg/day; Merck, Darmstadt, Germany) or Shh pathway receptor antagonist SANT-1 (3.3 mg/kg/day; Merck, Darmstadt, Germany) were administered intraperitoneally 10 min after induction of MI, and the dose was given once a day and continued for 21 days. SAG1.3 was injected into the DMI and the CMI group, whereas SANT-1 was exclusively injected into the CMI group. For molecular analysis, left ventricular tissue sections from the border zone/risk area surrounding the infarct were harvested, flash frozen in liquid nitrogen and stored at \(-80\) °C. For histochemical analysis, the whole heart was sectioned horizontally between the point of ligation and the apex, and fixed overnight in 10% buffered formalin for paraffin sections or directly embedded in optimum cutting temperature (OCT) medium for frozen sectioning.

The heart tissue was harvested on day 7 after MI for detecting the expression of the Shh pathway by western blot analysis. Twenty-one days after MI, echocardiography was performed to assess ventricular function, and then the hearts were harvested in order to evaluate capillary density by immunohistochemical analysis and assess the percentage myocardial infarct by Masson’s trichrome staining.

### 2.2 Induction of type 1 diabetes

Adult male C57/B6 mice between 6 and 7 weeks of age were injected intraperitoneally with streptozotocin (STZ; Sigma Chemical, St Louis, MO, USA) dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5, 45 mg/kg). Either STZ or citrate buffer (control) was administered for 5 days consecutively during the first week of the study. Blood samples were collected from the vena caudalis. Whole blood glucose levels were measured using a glucose analyser [One Touch Ultra Mini Blood Glucose Monitoring System; Johnson & Johnson (China) Co.], and mice with a blood glucose level >16.7 mmol/L were considered diabetic and were used for MI experiments.\(^26\) Twenty mice were used for analysis of body weight and blood glucose.

### 2.3 Induction of MI

Adult male C57/B6 mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and artificially ventilated with a respirator. To provide analgesia, buprenorphine (0.1 mg/kg) was injected subcutaneously immediately before the operation, and subsequently administered every 8 h for the next 48 h. After administration of the anaesthetic, about 7–10 min were allowed for it to take effect. The depth of general anaesthesia was assessed by pinching the toe, tail or ear of the animal. Any reaction from the mouse indicated that the anaesthestic was too light and that additional anaesthetic agent should be given. Mice were subjected to either ligation of the left anterior descending coronary artery or to sham operation as previously described.\(^26\)\(^29\) The sham-operated animals contained an untied left anterior descending artery. (Suture material was in place, but the ligation was not tightened.) Seventy-five mice were used for MI, and 30 mice for sham operation.
2.4 Echocardiography

Transthoracic echocardiography was performed with a VisualSonics (Vevo 2100; VisualSonics Inc., Toronto, Ontario, Canada) equipped with a 25 MHz imaging transducer. Mice were then anaesthetized with 2% isoflurane gas with an inflow rate of 0.5–1.5 mL/min during the echocardiographic examination. The left ventricle (LV) was analysed through the parasternal long- and short-axis views, with Doppler images for LV systolic function, LV cavity diameter, wall thickness, diastolic function, and LV end-systolic and end-diastolic volume determination. Throughout the procedure, ECG, respiratory rate (RR), and heart rate (HR) were monitored. An increased rate (RR and HR) was a sign that the anaesthesia was too light, whereas a decreased or irregular rate (RR and HR) was indicative of anaesthesia that was too deep. The isoflurane gas volume was therefore regulated according to the rate in order to ensure an adequate depth of anaesthesia.5,23,30 Forty-one mice were used for echocardiography.

2.5 Assessment of percentage myocardial infarct (Masson’s trichrome staining)

On day 21 after induction of MI, adult male C57/B6 mice were anaesthetized with sodium pentobarbital (100 mg/kg) and assessed to be fully anaesthetized according to the methods described in Section 2.3. The hearts were perfusion-fixed with 10% buffered formalin and horizontally sectioned between the point of ligation and the apex, and then embedded in paraffin. The percentage myocardial infarct was obtained on Masson’s trichrome-stained tissue sections using Image Pro-Plus software (Media Cybernetics, Inc. Bethesda, MD, USA). The fibrosis and total LV area were measured and expressed as a final percentage myocardial infarct.5,31 Thirty-five mice were used for Masson’s trichrome staining.

2.6 Immunohistochemistry for capillary density

On day 21 after induction of MI, adult male C57/B6 mice were anaesthetized with sodium pentobarbital (100 mg/kg) and assessed to be adequately anaesthetized according to the methods described in Section 2.3. The hearts were horizontally sectioned between the point of ligation and the apex, and then embedded in OCT. Briefly, the OCT sections were stained for capillary density using rat polyclonal anti-CD31/PECAM-1 (1:100 dilution in phosphate-buffered saline; BD Pharmingen, San Jose, California, USA) followed by incubation with secondary anti-rat reagent according to the manufacturer’s specifications (DAKO). The antigen–antibody interaction was visualized using 3′,3′-diaminobenzidine (DAB) substrate using a DAB Substrate Kit (DAKO). The images were acquired digitally at ×200 magnification using an Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope and were used for CD31 counting. Counts of capillary density per square millimetre of the border zone of infarcted myocardium (MI group) or the free wall of the left ventricle (Sham group) were carried out using Image Pro-Plus software. Thirty-five mice were used for analysis of capillary density.

2.7 Western blot analysis

Western blot analysis was performed for the proteins Shh, Ptc1, and Gli1 as described.5,18,22,24,30 Briefly, the infarcted hearts were separated into two parts consisting of the ischemic region and the viable region. Equal amounts of protein (60 μg) were separated on SDS–polyacrylamide gels (10%) and electrotransferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with Shh (1:500 dilution; Millipore), Ptc1 (1:1000; Millipore), Gli1 (1:1000 dilution; Genetex), or β-actin (1:1000 dilution; Santa Cruz Biotechnology), and incubated with either goat anti-rabbit or rabbit anti-mouse second antibody (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed using a supersignal west pico chemiluminescent substrate (Pierce Manufacturing), and molecular band intensity was determined by densitometry (Bio-Rad image software). Sixty-five mice were used for western blot analysis.

2.8 Statistical analyses

All data were analysed with the statistical software GraphPad Prism 5.0 (GraphPad Software, Inc. Avenida de la Playa La Jolla, CA, USA), and all values are expressed as means ± SEM. The differences between two groups were analysed using Student’s unpaired t-test, and differences between three or more groups were evaluated via one-way ANOVA with Bonferroni correction. A probability value of ≤0.05 was considered significant.

3. Results

3.1 Cardiac dysfunction in type 1 diabetic mice

A 5 day low-dose STZ injection regimen was used to ensure 10 weeks of sustained hyperglycaemia and body weight loss throughout the study (Figure 1A). There was no significant difference in the ratio of heart to body weight (Figure 1B). Next, an echocardiographic assessment of cardiac function was performed at 7 and 10 weeks after STZ injection. The transmirtal filling pattern showed a reduction in E-wave velocity (MVE, 23.14%) and the ratio of the E- to A-wave (E/A, 22.38%), with a prolongation of deceleration time in the E-wave (DT, 23.81%). The pattern continued to present a significant reduction in the ratio of the E-wave deceleration rate (E/WDR, 30.17%), and a marked up-regulation of isovolumetric relaxation time (IVRT, 37.66%) and ejection time (ET, 27.04%) in diabetic mice compared with control mice at 10 weeks (Figure 1C and D). M-Mode images showed that LV contractility had no marked difference between diabetic mice and control mice at 7 and 10 week time points. Parameters reflecting LV contractility include LV end-diastolic dimension (LVIDd), LV end-systolic dimension (LVIDs), ejection fraction (EF), fractional shortening (FS), stroke volume (SV), and cardiac output (CO; see Supplementary material online, Figure S1).

3.2 Impaired myocardial Shh pathway in type 1 diabetic mice

Myocardial Shh, Ptc1, and Gli1 proteins were quantified by western blot analysis in control and diabetic mice at 4, 7, and 10 week time points. Shh and Ptc1 were decreased (by 48.26, 64.21 and 24.17%, respectively) in DMI (diabetes plus MI) compared with DS (control sham). Conversely, such proteins were significantly increased (by 30.64, 45 and 28.61%, respectively) in CMI (control plus MI) compared with CS (control sham). More importantly,
Figure 1  Cardiac dysfunction in type 1 diabetic mice. (A) Whole blood glucose concentration (left panel) and body weight (right panel) in mice with diabetes induced by administration of streptozotocin (STZ; 45 mg/kg/day for 5 days) and in control mice (citrate buffer; n = 10). (B) Haematoxylin-and eosin-stained four-chamber views of CON and DM hearts at 10 weeks, and quantitative analysis of the ratio of heart weight to body weight (n = 4). (C) Representative transmitral Doppler flow profile of CON and DM groups at 10 weeks. (D) Quantitative analysis of HR, MVE, MVA, E/A, DT, EWDR, IVRT, and ET of CON and DM groups at 7 and 10 week time points (n = 8). Values are presented as means ± SEM. *P < 0.05. Abbreviations: CON, control group; DM, diabetic group; DT, deceleration time of E-wave; E/A, the ratio of E-wave velocity to A-wave velocity; ET, ejection time; EWDR, rate of E-wave deceleration rate; HR, heart rate; IVRT, isovolumetric relaxation time; MVA, A-wave velocity; MVE, E-wave velocity.

Figure 2  Impaired myocardial Shh pathway in type 1 diabetic mice. Western blot analysis of myocardial Shh, Ptc1, and Gli1 was performed at 4, 7 and 10 week time points after STZ injection (n = 5). Values are presented as means ± SEM. *P < 0.05. CON indicates control group; DM, diabetic group.
protein levels of Shh, Ptc1, and Gli1 were significantly decreased (by 68.7, 81.32 and 45.79%, respectively) in DMI compared with CMI (Figure 3).

3.4 Lower capillary density and larger percentage myocardial infarct in type 1 diabetic mice with MI than in control mice with MI

Myocardial capillary density and percentage myocardial infarct were examined 21 days after MI. Compared with CS and DS, respectively, CMI and DMI showed reduced capillary density and obvious percentage myocardial infarct. Notably, capillary density was significantly decreased by 33.8%, and percentage myocardial infarct was significantly enlarged by 55.91% in DMI compared with CMI (Figure 4A and B; C and D).

3.5 Worse cardiac dysfunction in type 1 diabetic mice with MI than in control mice with MI

Cardiac function was examined by echocardiography 21 days after MI. MI resulted in cardiac function impairment in diabetic and control mice (Figure 4E and F). Compared with CS and DS, respectively, CMI and DMI showed significantly enlarged LV end-systolic and LV end-diastolic dimension, and reduced fractional shortening and ejection fraction. Notably, LV end-systolic and LV end-diastolic dimension were significantly increased (by 19.4 and 10.07%, respectively), and fractional shortening and ejection fraction were significantly reduced (by 58.33 and 46.24%, respectively) in DMI compared with CMI.
3.6 Shh pathway receptor agonist enhanced myocardial Ptc1 and Gli1 protein expression in type 1 diabetic mice with MI

The Shh pathway receptor agonist SAG1.3 (5 mg/kg/day) or the Shh pathway receptor antagonist SANT-1 (3.3 mg/kg/day) was injected intraperitoneally 10 min immediately after MI, and treatment was repeated daily for 7 days. SAG1.3 was injected into DMI and CMI, and SANT-1 was exclusively injected into CMI mice. On day 7 after treatment, myocardial Ptc1 and Gli1 protein levels were verified by western blot analysis in CMI and DMI. SAG1.3 had no effects on myocardial Ptc1 and Gli1 protein levels, but SANT-1 significantly inhibited expression of these proteins (decreased by 52.54 and 39.57%, respectively) in CMI. However, SAG1.3 markedly increased Ptc1 and Gli1 protein expression by 76.04 and 58.19%, respectively, in DMI (Figure 5).

3.7 Shh pathway receptor agonist enhanced capillary density and reduced percentage myocardial infarct in type 1 diabetic mice with MI

The Shh pathway receptor agonist SAG1.3 (5 mg/kg/day) or the Shh pathway receptor antagonist SANT-1 (3.3 mg/kg/day) was injected intraperitoneally 10 min immediately after MI and this treatment repeated for 21 days. SAG1.3 was injected into DMI and CMI, and SANT-1 was exclusively injected into CMI. On day 21 after treatment, the Shh pathway receptor agonist SAG1.3 significantly increased capillary density by 30.9% and reduced the percentage myocardial infarct by 19.91% in DMI. The treatment with SAG1.3 had no effects on these parameters in CMI. Conversely, treatment with the Shh pathway receptor antagonist SANT-1 significantly decreased capillary density by 31.15% and enlarged percentage myocardial infarct by 45.58% in CMI (Figure 6).

4. Discussion

The present study demonstrates, for the first time, that: (i) the myocardial Shh pathway is impaired; (ii) activation of the myocardial Shh pathway induced by MI is markedly weakened; and (iii) administration of a Shh pathway receptor agonist significantly increases the expression of Shh pathway proteins and ameliorates the cardiac dysfunction induced by MI in type 1 diabetic mice. These findings suggest that an impaired myocardial Shh pathway contributes to cardiac dysfunction in type 1 diabetic mice.

4.1 Shh pathway and cardiovascular system

The Shh pathway is critical for the cardiovascular system during embryonic and postnatal development and adult life. The Shh pathway is required for the development of the cardiac outflow tract, coronary artery, coronary vein, and ventricular septum in the embryo; it is also responsible for maintenance of the adult coronary vasculature, and is activated immediately for repair once there is ischemic injury in the adult heart. Shh signalling occurs through interaction of the Shh protein with its receptor, Ptc1, and then removes the inhibition of another Shh pathway receptor named smoothened. (In the absence of Shh protein, the Shh pathway receptor Ptc1 inhibits the activity of smoothened). This leads to the activation of a transcription factor, Gli, which induces the expression of downstream target genes, including Ptc1 and Gli. Thus, the
Present study, similar to the findings of previous reports,\textsuperscript{18,30} examined the expression of Shh, Ptc1, and Gli1 to represent the activation of the Shh signalling pathway in response to MI. Results from the present study showed that MI-induced expression of Shh, Ptc1, and Gli1 were all significantly up-regulated in control mice, suggesting that this signalling is intact and up-regulated in the CMI group.

Although the Shh pathway plays a pivotal role in embryonic heart development and adult cardiac maintenance, we wanted to know what would happen in diabetic mice with MI. Is the Shh pathway impaired in the diabetic heart and is this related to diabetic cardiovascular diseases?\textsuperscript{4}

4.2 Shh pathway in diabetes

Diabetes is characterized by chronic hyperglycaemia and is associated with significant cardiovascular pathology; however, the detailed cellular mechanisms underlying diabetic cardiomyopathy and diabetes-associated MI are not completely understood. Recent studies demonstrated that the hedgehog signalling pathway was impaired in nerve,
penis, vessels, and even monocytes of diabetic rats, which proved to be associated with diabetic complications such as diabetic neuropathy, vasculopathy, diabetic erectile dysfunction, diabetic wound healing, and diabetic monocyte dysfunction.\textsuperscript{20–24,42} To elucidate whether there was an abnormal Shh pathway in the myocardium of type 1 diabetic mice, Shh, Ptc1, and Gli1 were examined and cardiac function was tested. The data revealed that the Shh pathway was significantly down-regulated at 7 and 10 weeks in diabetic mice. Echocardiographic analysis showed that 10 weeks of sustained diabetic status led to diastolic dysfunction (e.g. MVE, DT, and IVRT) in the presence of normal systolic function (e.g. FS and EF). These results suggested that the Shh pathway was impaired in diabetes, which was accompanied by the cardiac dysfunction in the diabetic setting. It is worth noting that the body weight, heart mass, and surface areas of the diabetic mice were much less than those of the control mice, which should be taken into account when data such as LVIDs, LVIDd, SV, CO, MVE, and MVA are analysed statistically [all these parameters were divided by the body surface area (in square centimetres) in the present study].

4.3 Shh pathway in diabetic MI

Emerging evidence indicates that the hedgehog pathway is up-regulated in both acute and chronic MI, and both Shh and its target protein Ptc1 were found to be up-regulated in murine myocardium following the occlusion of the left carotid artery. The injection of an Shh-expressing plasmid into the myocardium was able to induce a Ptc1 response and to preserve left ventricular function after Shh gene transfer, suggesting that the Shh pathway is important in postnatal tissue repair.\textsuperscript{18,30,38} Therefore, a model of myocardial ischaemia was constructed to induce cardiac injury at 7 weeks after STZ treatment, the time at which a down-regulation of the Shh signalling pathway could be detected. We then further investigated the relationship between the Shh pathway and the diabetic heart.

4.3.1 Impaired Shh pathway and cardiac dysfunction in diabetic MI

Data from the present study suggest that the Shh pathway in diabetic mice with MI is significantly down-regulated compared with control mice with MI, followed by lower capillary density, a larger percentage myocardial infarct, and then worse cardiac dysfunction.

In order to investigate the relationship between the impaired Shh pathway and cardiac dysfunction, SAG1.3 was delivered to up-regulate the suppressed Shh pathway in DMI, as well as supplement to the self-activated Shh pathway in CMI, whereas SANT-1 was exclusively delivered to inhibit the activated Shh pathway in CMI, and then we investigated the protein expression of the Shh pathway and the cardiac function. The intraperitoneal injection of SAG1.3 led to a significant increase in the expression of downstream Ptc1 and Gli1, as well as enhanced capillary density, reduced percentage myocardial infarct and then improved cardiac function in diabetic mice with MI. These phenomena suggest that the impaired Shh pathway is a strong inducer of the exacerbated cardiac dysfunction after injury in diabetic mice. Although the mechanism underlying the impairment of the Shh pathway was not disclosed in the present study, the results could be explained not only by the already impaired Shh pathway in the basal state (diabetes) in the present study, which was not susceptible to activation by the upstream relative factors when combined with the stress of injury, but also by the pre-existing evidence that ischaemia-derived stress upon a hyperglycaemic background rather than in the presence of normal blood glucose levels limits some transcriptional processes upstream of Shh expression within the myocardial tissue. For example, diabetic hyperglycaemia-induced oxidative stress limited the ischaemia-induced expression of hypoxia-inducible factor-1α, and hyperglycaemia itself inhibited its functional activity, compared with non-diabetic conditions.\textsuperscript{7,43} Hypoxia-inducible factor-1α is a transcriptional activator of the Shh gene in response to cellular hypoxia and stress.\textsuperscript{44} On another note, hyperglycaemia led to high production of reactive oxygen species, and then reduced nitric oxide availability via peroxynitrite formation in the heart.\textsuperscript{45} In normal conditions, nitric oxide derived from inducible nitric oxide synthase enhances Shh expression in ischaemic tissues;\textsuperscript{46} therefore, the large amount of peroxynitrite formed in the heart might have impaired Shh expression.

Moreover, diabetic mice with MI had a significant down-regulation of the Shh pathway compared with the diabetic sham-operated animals, indicating that the diabetic Shh pathway was not only obstructed in self-up-regulation (the Shh pathway in normal mice was activated after induction of MI), but also further impaired in the stressed state (diabetes plus MI) compared with that in the baseline conditions (diabetes). This was slightly different in comparison to our previous study,\textsuperscript{24} which reported that activation of the Shh pathway was impaired only in wounds in diabetic mice and that there was no significant difference from the diabetic sham-operated animals. The difference may have resulted from different kinds of organs and different types of injuries, or it might be explained by oxidative stress induced by MI, which further impaired the Shh pathway upon the baseline (diabetes), as in one previous study, which reported that immediately after MI the administration of Ad.Trx1, an important factor maintaining the redox environment, rescued the reduction of two angiogenic growth factors similar to Shh in both normal and diabetic rats.\textsuperscript{5}

4.3.2 Shh pathway receptor antagonist SANT-1 and cardiac dysfunction in MI

There is growing evidence that the Shh pathway plays a pivotal role in the maintenance of cardiac homeostasis in embryos, neonatal, and adult animals. Mouse embryos homozygous null for Shh are known to have pharyngeal arch and cardiac defects that impede the development of the cardiac outflow tract, coronary artery, coronary vein, ventricular septum, etc.\textsuperscript{50–52} Mice, ablated the target gene Shh pathway (Myocardium-specific ablation of Shh signaling in the adult mice heart was accomplished by conditionally removing a homozygous floxed allele of Smoothened, an essential transducer of Shh signaling), showed loss of coronary blood vessels, tissue hypoxia, cardiomyocyte cell death, myocardial fibrosis, heart failure, and subsequent death.\textsuperscript{25} In the present study, the Shh pathway antagonist SANT-1 injected into control mice with MI significantly retarded the activation of the Shh pathway, reduced the capillary density, enlarged the percentage myocardial infarct, and then exacerbated cardiac dysfunction. These actions proved from a different viewpoint that the endogenous Shh pathway plays a protective role in MI to maintain cardiac homeostasis. This was similar to the diabetic state in the present study, in that once the normal activating capability of the Shh pathway was impaired, cardiac dysfunction would be strongly exacerbated.

A previous study\textsuperscript{53} examined the effects of cyclopamine (another antagonist of the Shh pathway receptor) on myocardial ischaemia–reperfusion-induced injury, and reported that cyclopamine prevented myocardial fibrosis and preserved cardiac dysfunction, with an
increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-positive nuclei, and had no effect on the number of α-smooth muscle actin-positive vessels. The results from Biljsma et al.13 conflicted with our discovered ration- ale. The reason for this discrepancy is unclear, but the authors men- tioned that their model was made by ligating the descending coronary artery for 30 min, and then it was reperfused for only 1 week, which was a short time for them to investigate the effect of cycloamine. Also, the model was different from the one used in the present study, and may have induced other signalling pathways that influenced the final result.

4.4 Mechanism of impaired Shh pathway in diabetic status
Diabetes-induced oxidative stress is well documented in the pathophysiology of diabetic complications.47 Suppression of the Shh pathway resulting from oxidative stress, which has been reported in bone marrow stromal cells in vitro,48,49 was confirmed in the myocardium in our laboratories (data not shown). Data from our laboratories suggested that antioxidant improves the impaired Shh pathway and cardiac function in diabetic mice. Moreover, it also up-regulated the Shh pathway, and then enhanced capillary density, reduced the percentage myocardial infarct, and eventually improved cardiac function in diabetic mice with MI. Furthermore, the in vitro experiments also showed that the Shh pathway was directly inhibited by oxidative stress in neonatal cardiomyocytes. These results demonstrated that oxidative stress contributes to Shh pathway inhibition in the myocardium of diabetic mice and diabetic mice with MI, and that antioxidant improved the impaired Shh pathway and ameliorated cardiac dysfunction in diabetic mice and diabetic mice with MI. In summary, the present study demonstrates that the Shh signalling pathway, which is up-regulated in normal mice following MI, is impaired and contributes to exacerbate cardiac dysfunction in diabetic mice following MI. Delivery of Shh pathway receptor agonists may contribute to improve diabetic cardiac dysfunction. Strategies aimed at augmenting the endogenous Shh pathway may provide an effective means for ameliorating diabetic cardiac dysfunction.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
We thank Yan Xiong, Gen-Shui Zhang, Ying-Hua Liu, Xiao-Qian Wu, Wen-Liang Chen, Wei-Wen Zhang, and Yuan Qin for valuable guidance in experiments and at every stage of the writing.

Conflict of interest: none declared.

Funding
This work was supported by the National Natural Science Foundation of China (no. 30873069 and no. 81173062 to Dr J-D. Luo) and by the Education Administration Research Foundation of Guangzhou City (no. 61098 to Dr J-D. Luo).

References