Direct renin inhibition prevents cardiac dysfunction in a diabetic mouse model: comparison with an angiotensin receptor antagonist and angiotensin-converting enzyme inhibitor

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Abstract
Hyperglycaemia up-regulates intracellular AngII (angiotensin II) production in cardiac myocytes, effects of which are blocked more effectively by renin inhibition than ARBs (angiotensin receptor blockers) or ACEIs (angiotensin-converting enzyme inhibitors). In the present study, we determined whether renin inhibition is more effective at preventing diabetic cardiomyopathy than an ARB or ACEI. Diabetes was induced in adult mice for 10 weeks by STZ (streptozotocin). Diabetic mice were treated with insulin, aliskiren (a renin inhibitor), benazeprilat (an ACEi) or valsartan (an ARB) via subcutaneous mini-pumps. Significant impairment in diastolic and systolic cardiac functions was observed in diabetic mice, which was completely prevented by all three RAS (renin–angiotensin system) inhibitors. Hyperglycaemia significantly increased cardiac oxidative stress and circulating inflammatory cytokines, which were blocked by aliskiren and benazeprilat, whereas valsartan was partially effective. Diabetes increased cardiac PRR (prorenin receptor) expression and nuclear translocation of PLZF (promyelocytic zinc finger protein), which was completely prevented by aliskiren and valsartan, and partially by benazeprilat. Renin inhibition provided similar protection of cardiac function to ARBs and ACEis. Activation of PLZF by PRR represented a novel mechanism in diabetic cardiomyopathy. Differential effects of the three agents on oxidative stress, cytokines and PRR expression suggested subtle differences in their mechanisms of action.

Key words: angiotensin-converting enzyme, angiotensin receptor blocker, diabetic cardiomyopathy, intracrine, prorenin receptor, renin–angiotensin system (RAS)

INTRODUCTION
The RAS (renin–angiotensin system) has been significantly associated with diabetes-induced organ damage, including diabetic cardiomyopathy. The RAS consists of two major subcomponents, the circulating and the local tissue system. Recently, we presented evidence that the tissue RAS can be further divided into extracellular and intracellular systems [1,2]. The intracellular RAS is distinguished from the local extracellular RAS in that the synthesis of AngII (angiotensin II) occurs inside the cells and AngII actions are not mediated by plasma membrane AT1 and AT2 (angiotensin type 1 and angiotensin type 2) receptors [3,4]. The local RAS has been demonstrated to have a role in hypertrophy, fibrosis, inflammation, oxidative stress and thrombosis, independent of systemic AngII [5]. Local AngII levels in the heart are increased in pathological conditions such as myocardial

Abbreviations: A, peak velocity of late filling wave; ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; AngII, angiotensin II; ARB, angiotensin receptor blocker; AT1, angiotensin type 1; AT2, angiotensin type 2; BP, blood pressure; CO, cardiac output; CVD, cardiovascular disease; DHE, dihydroethidium; E, peak velocity of early filling wave; EF, ejection fraction; FS, fractional shortening; HR, heart rate; IFNγ, interferon γ; IL-1β, interleukin-1β; IVRT, isovolumic relaxation time; LVEF, left ventricular ejection fraction; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; PI3K, phosphoinositide 3-kinase; PLZF, promyelocytic zinc finger protein; PPARγ, peroxisome proliferator-activated receptor γ; PRR, prorenin receptor; RAS, renin-angiotensin system; ROS, reactive oxygen species; STZ, streptozotocin; STZ-Alsk, STZ + aliskiren; STZ-Benz, STZ + benazeprilat; STZ-Vals, STZ + valsartan; STZ-Veh, STZ + saline; TNFα, tumour necrosis factor α.

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infarction and diabetes [5,6]. Recently, our laboratory demonstrated that the intracellular RAS constituted the major part of the local RAS in hyperglycaemic conditions [1,7–11]. We reported a several fold increase in intracellular AngII levels in cultured cardiac myocytes, when grown in high-glucose medium or from the hearts of diabetic rats. The observation of increased cardiac intracellular AngII levels had previously been described in diabetic patients [12]. We reported that the intracellular AngII was biologically active and produced cardiac hypertrophy in mice [4]. Significantly, growth effects of intracellular AngII in cultured cardiac myocytes and in the heart were not prevented by AT1 receptor antagonists. Further, high glucose-stimulated cardiac myocyte production of AngII was chymase-dependent, in contrast to ACE (angiotensin-converting enzyme)-dependent conversion in cardiac fibroblasts [13,14]. These observations suggested that treatment with an ACEi (ACE inhibitor) or ARB (angiotensin receptor blocker) may be only partially protective in diabetic cardiomyopathy, since the former would inhibit AngII production only by cardiac fibroblasts and the latter would block actions of only extracellular AngII, without affecting intracellular AngII production and actions in cardiac myocytes. Accordingly, we showed that renin inhibition proved more effective than an ARB or ACEi, in preventing cardiomyocyte superoxide production and fibrosis after one week of diabetes [5].

Several studies have been performed to compare the relative efficacy of aliskiren with ACEis or ARBs in hypertensive CVDs (cardiovascular diseases), mainly renal function [15–17]. These studies have shown a similar therapeutic profile of the three classes of drugs. A comparative effect of all three RAS blockers on cardiac function in diabetes has not been reported. The latter is important, due to the changes in the characteristics of the cardiac RAS in diabetes, i.e. from an extracellular to an intracellular system, ACE-dependent to largely chymase-dependent system, and possibly an AT1 receptor-dependent to an AT1 receptor-independent system [11]. Diabetic patients remain at an increased risk of cardiovascular events compared with non-diabetics, despite the use of ACEis and ARBs, suggesting insufficient RAS inhibition as one of the possible explanations, in addition to other mechanisms [16,18]. In this context, a renin inhibitor might provide more complete inhibition of the RAS in diabetes. The objective of the present study was to determine whether direct renin inhibition, which blocks both the intracellular and extracellular RAS, is more effective in preventing diabetic cardiomyopathy in a mouse model of Type 1 diabetes, than an ARB or an ACEi, which block only extracellular AngII.

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MATERIALS AND METHODS

Animals

All protocols were approved by the Institutional Animal Care and Use Committee and conformed to the NIH guidelines. Male C57Bl/6J mice were purchased from The Jackson Laboratory and fed ad libitum.

At 12 weeks of age, animals were randomized into six groups (n = 10): (i) control, (ii) STZ (streptozotocin), (iii) STZ + saline (STZ-Veh), (4) STZ + aliskiren (20 mg/kg of body weight; Novartis; STZ-Alsk), (5) STZ + valsartan (2 mg/kg of body weight; Novartis; STZ-Val), (6) STZ + benazepril (10 mg/kg of body weight; Novartis; STZ-Benz). STZ (50 mg/kg of body weight per day; Zanosar) was injected intraperitoneally for 5 consecutive days. Doses of the RAS inhibitors were based on results of a preliminary study described in the Supplementary online material (at http://www.clinsci.org/cs/124/cs1240529add.htm). Control groups received 0.1 M sodium citrate buffer (pH 4.5). After 2 weeks, all STZ-injected mice reached a blood glucose value of ≥250 mg/dl and were considered diabetic. At this point, diabetic mice in treatment groups were implanted with osmotic mini-pumps (ALZET 1004; 0.11 μl/h), containing one of the aforementioned agents, for 10 weeks (Figure 1). Mini-pumps were replaced every 4 weeks. An insulin group was included to verify that the cardiac effects observed in the diabetic group were due to hyperglycaemia. The insulin group received 0.7 unit of insulin/day (Humulin N; Eli Lilly) by subcutaneous injection during the 10 weeks of the treatment period.

Echocardiographic measurements

Transthoracic echocardiography was performed on anaesthetized mice, using a VisualSonic Vevo 2100 and a 35-MHz probe, before injection with STZ (Before STZ) and 0, 4, 6, 8 and 10 weeks after establishment of diabetes. Briefly, mice were anaesthetized with 3–5% isoflurane that was reduced to 1.5% to maintain the HR (heart rate) between 400 and 500 beats/min. The heart was imaged in the two-dimensional, short-axis and four-chamber view. LV (left ventricular) FS (fractional shortening), EF (ejection fraction), CO (cardiac output), HR, LVIDd and LVIDs (LV internal dimension at end-diastole and LV internal dimension at end-systole respectively), IVRT (isovolumic relaxation time), peak velocity of early and late filling waves (E and A respectively) and mitral deceleration time were measured.

MAP (mean arterial pressure)

Arterial pressure was measured at the end of the study (10 weeks of diabetes) in anaesthetized animals. The right carotid artery was cannulated and arterial pressure was measured with a BP (blood pressure) analyser (BPA 400; Micro-Med). MAP was calculated as follows: 1/3 systolic pressure + 2/3 diastolic pressure.

Isolation of cardiac myocytes

Adult mouse cardiac myocytes were isolated using Langendorff’s perfusion system, as described previously [19]. Briefly, hearts were perfused retrograde for 5 min, followed by digestion with 0.1% collagenase II ( Worthington Biochemical) for 10 min at a rate of 3 ml/min. After stopping digestion with a calcium-containing buffer, cells were washed with PBS and collected by centrifugation (180 g, 1 min). Myocytes were stored in RNA later solution for RNA analysis.
Renin inhibition in diabetic cardiomyopathy

Male c57b16 mice at 10–12 weeks of age were injected with either 0.1 M citrate buffer (pH 4.5) or STZ (50 mg/kg of body weight) for 5 days. After 2 weeks, animals with blood glucose levels of $\geq 250$ mg/dl were considered diabetic and osmotic mini-pumps containing one of the drugs were implanted subcutaneously. Echocardiographic measurements were taken before STZ injections (Before STZ) and at two-week intervals, beginning at 4 weeks after mini-pump installation. After conclusion of the study at 10 weeks, MAP was measured and tissues were collected.

ROS (reactive oxygen species) staining
Hearts were fixed in 4% PFA (paraformaldehyde) and frozen in OCT compound (Tissue-Tek). Frozen sections (20 $\mu$m) were incubated with 10 $\mu$M DHE (dihydroethidium; Sigma–Aldrich), at 37 $^\circ$C, for 30 min in a humidified chamber protected from light. Fluorescence images ($\times 60$) were obtained with a Leica TCS SP5X and analysed using ImageJ. Mean DHE fluorescence was calculated by subtracting integrated density of the background signal from the integrated density of the fluorescence staining for ten fields/heart, five hearts/group and normalized to control.

Histological analysis
Heart sections (5 $\mu$m) were fixed with 4% formaldehyde and permeabilized using 0.1% Triton X-100. After blocking, the sections were incubated with anti-PRR (prorenin receptor) (1:30 dilution; Everest Biotech) and anti-PLZF (promyelocytic zinc finger protein; 1:50 dilution; Santa Cruz Biotechnology) antibodies, followed by incubation with respective secondary antibodies (1:500 dilution). Specificity of staining was determined by using secondary antibody alone. The sections were co-stained with phalloidin (1:100 dilution). Images ($\times 60$) were acquired with a confocal fluorescence microscope (Leica TCS SP5X). Fluorescence intensities were determined using ImageJ after subtracting background fluorescence and normalizing to control.

Real-time PCR
Gene expression of PRR was determined using a TaqMan assay (Applied Biosystems). RNA was extracted using an RNeasy Fibrous Tissue Kit (Qiagen). cDNA was made using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed in 20 $\mu$l reaction mixture containing cDNA, Taqman Universal PCR master Mix, and 20X specific gene expression assay mix (Applied Biosystems). Data were normalized to 18S mRNA. Each sample was run in duplicate, and the threshold cycle, $\Delta C_t$, was calculated as $C_t$ (target gene) $- C_t$ (18S). The relative changes in target gene in different treatment groups were determined by the formula $2^{-\Delta \Delta C_t}$, where $\Delta \Delta C_t = \Delta C_t$ (control) $- \Delta C_t$ (treatment group).

Plasma cytokines
Blood was collected in tubes containing EDTA and centrifuged at 1600 g for 15 min at 4 $^\circ$C. Plasma was collected and stored at $-20$ $^\circ$C. Milliplex Mouse Cytokine/Chemokine kit was used to determine plasma levels of IL-1$\beta$, TNF$\alpha$, IFN$\gamma$, and MCP-1.

Fibrosis
For characterization of fibrosis in the ventricular tissue, Picrosirius Red, which specifically binds to collagen, and Fast Green, which stains non-collagen proteins, were used. Briefly, paraffin embedded sections were de-paraffinized with xylene (60 $^\circ$C for 15 min), rehydrated through graded concentrations of ethanol [100, 95, 85, 70% (v/v)], washed, and stained with 0.1% Picrosirius Red/0.1% Fast Green overnight. Stained slides were washed, rapidly dehydrated through graded ethanol concentrations, and mounted with Permount. For quantification of fibrosis, sections were air dried for 5 min following staining, as described by others [20]. One ml dye extraction solution (0.1 M NaOH/methanol, 1:1) was used to extract dyes from the stained section, which was read at 356 and 605 nm (Sirius red and Fast Green) by spectrophotometer. The absorbance ratio (356/605 nm) determined the amount of collagen to total protein ratio.
Statistical analysis
All data were expressed as the means ± S.E.M. One-way ANOVA with Tukey’s post-hoc test or multiple comparisons using two-way ANOVA with the Bonferroni post-hoc test were used for statistical analysis where appropriate (GraphPad). *P < 0.05 was considered statistically significant.

RESULTS
We have reported previously that 1 week of STZ-induced hyperglycaemia in rats significantly up-regulated intracellular AngII levels in cardiac myocytes and was associated with increased oxidative stress, cardiac myocyte apoptosis and fibrosis [5]. The latter conditions were corrected more efficiently by a renin inhibitor, which completely reduced intracellular AngII levels, in comparison with an ARB or ACEi. In the present study, we evaluated the comparative efficacy of the three RAS blocking agents on diabetes-induced cardiac dysfunction after 10 weeks.

STZ-injected mice have a similar degree of hyperglycaemia and MAP, regardless of treatment
Body weight and blood glucose were monitored on a bi-weekly basis to determine whether aliskiren, benazeprilat or valsartan have an effect on these parameters. As shown in Figure 2(A), mice in STZ, STZ-Alsk, STZ-Benz and STZ-Vals groups had weights similar to the control group after 10 weeks in the study. Mice injected with STZ had an increase in blood glucose 2 weeks after injection, which remained elevated throughout the study, regardless of treatment (Figure 2B). Heart weight to tibia length ratio was not significantly different between the groups (Figure 2C). Similarly, no significant change in the MAP was observed as a result of hyperglycaemia or treatments (Figure 2D).

These observations suggested that the effect of the three RAS blockers on cardiac function were not modified by the degree of hyperglycaemia or BP.

Hyperglycaemia-induced diastolic dysfunction is prevented by aliskiren, benazeprilat and valsartan
Diabetic cardiomyopathy is correlated with early diastolic dysfunction independent of changes in MAP [21]. We used echocardiography to non-invasively monitor diastolic function in control mice, diabetic mice and diabetic mice treated with an ARB, ACEi or renin inhibitor. Diastolic function was evaluated by measuring the $E/A$ ratio, IVRT and mitral valve deceleration time (Figure 3). Representative echocardiograms of diastolic parameters from control and STZ-Veh mice are presented in Figures 3(A) and
Figure 3 Measurement of diastolic function by echocardiography
Representative pulsed-wave Doppler images of Mitral valve flow of control and STZ-Veh mice at 10 weeks of diabetes (A and B, respectively). Mitral valve flow velocity (E/A) in control, STZ-injected (STZ), STZ-injected with saline mini-pumps (STZ-Veh), and STZ-injected mice treated with insulin (STZ-Insulin), before STZ injection and 4, 6, 8 and 10 weeks after becoming diabetic (C). Mitral valve flow velocity (D), IVRT (E), and deceleration time of early mitral inflow (F) of control, STZ-Veh, and those treated with aliskiren (STZ-Alsk), benazeprilat (STZ-Benz) and valsartan (STZ-Vals), before STZ treatment and at 10 weeks after becoming diabetic. Values are expressed as the means ± S.E.M. *P < 0.05 against control.

3(B), respectively. The STZ-Veh group, which received saline-containing osmotic mini-pumps, was included to control for the impact of surgery on the study parameters. Hyperglycaemia caused a progressive decrease in the E/A ratio, which reached statistical significance following 6 weeks of diabetes and continued to decrease through 10 weeks (Figure 3C). A similar decline in cardiac function was observed in the STZ and STZ-Veh groups; therefore data from only the STZ-Veh group have been shown in subsequent Figures. Insulin reversed all parameters of the STZ-induced decrease in cardiac function (Supplementary Figure S2 at http://www.clinsci.org/es/124/csl1240529add.htm; only the E/A ratio shown in Figure 3A), indicating that the cardiac effects observed in the diabetic group were due to hyperglycaemia. Compared to before STZ, hyperglycaemia led to a significant decrease in the E/A ratio at 10 weeks, which was completely prevented by aliskiren, benazeprilat and valsartan (Figure 3D). IVRT was significantly increased in the STZ-Veh group after 10 weeks, which was prevented by aliskiren and benazeprilat. Valsartan partially prevented the increase in IVRT by hyperglycaemia (Figure 3E). Mitral valve deceleration time was significantly increased in the 10 week STZ-Veh group and was prevented by all treatments (Figure 3F).
hyperglycaemia-induced oxidative stress in the heart

Oxidative stress is a well-documented mechanism that contributes to cardiac remodelling and diabetic cardiomyopathy [23,24]. Microscopic analysis of frozen heart sections stained with DHE was used to assess oxidative stress. Diabetic mice had a significant increase in DHE staining, compared with control mice, which was completely prevented by aliskiren and benazeprilat, whereas valsartan was only partially protective (Figure 5). A quantitative analysis of the oxidative stress is presented in Figure 5(F).

Fibrosis is not significantly increased in STZ-injected mice after 10 weeks of diabetes

Figures 6(A)–6(E) show the representative images of heart sections from different groups stained with Picrosirius Red and Fast Green. There was no significant increase in collagen content in STZ-Veh mice compared with controls. Similarly, there was no change in collagen staining in aliskiren, benazeprilat or valsartan treated mice. A semi-quantitative analysis of fibrosis is presented as the absorbance ratio (356/605 nm), which represents the ratio of the amount of collagen to total protein, and confirmed similar collagen content in all groups of animals (Figure 6F).

Hyperglycaemia-induced increase in plasma cytokines is prevented by aliskiren and benazeprilat, but only partially attenuated by valsartan

AngII mediates cytokine production observed in diabetes [25,26]. STZ-Veh mice had significantly elevated plasma levels of TNFα, IL-1β, IFNγ and MCP-1. Treatment with aliskiren and benazeprilat completely prevented the observed increases in these inflammatory cytokines. However, valsartan only partially prevented the increase in all four of these cytokines, of which TNFα, IL-1β levels remained significantly elevated, as compared with control (Figure 7).

PRR expression and nuclear translocation of PLZF is significantly increased by hyperglycaemia

PRR expression has been shown to be increased in high glucose conditions [27]. In the present study, the gene expression of PRR was increased in isolated cardiac myocytes of STZ-Veh mice (Figure 8A). This increase was prevented by aliskiren, benazeprilat and valsartan. Figure 8(D) shows representative images of PRR immunostaining in frozen heart sections, which was significantly increased in the STZ-Veh group, compared with control. Aliskiren and valsartan completely prevented the increase, whereas the benazeprilat effect was non-significant. A quantification of PRR from immunostained sections is presented in Figure 8(B). PLZF, a transcription factor that directly binds to and mediates the effects of the PRR [28], showed increased translocation to the nucleus (Figure 8C), suggesting enhanced transcriptional activity of PLZF. Treatment with all three RAS blockers prevented the increased expression of PRR, PLZF and translocation of the latter to the nucleus.
**DISCUSSION**

The results of the present study provide, for the first time, a direct comparison between a renin inhibitor, an ARB and an ACEi in the context of diabetic cardiomyopathy. We observed that renin inhibition completely prevented diastolic and systolic cardiac dysfunction in diabetic animals. An ARB and ACEi were similarly effective. However, there were differences in the effect of these drugs on oxidative stress, plasma pro-inflammatory cytokines, and PRR expression. Furthermore, we demonstrated increased nuclear localization of PLZF, in association with increased PRR expression, which represented a novel cellular mechanism operating in diabetic cardiomyopathy.

Diabetic cardiomyopathy has been described as ventricular dysfunction, including early diastolic dysfunction, in diabetic patients, without coronary artery disease or hypertension [21,29]. We demonstrated that diabetic mice had impaired diastolic function, as well as a modest decrease in systolic function, determined by echocardiography, after 10 weeks of hyperglycaemia. These effects were independent of BP, as STZ-induced diabetes in C57BL6 mice is a normotensive model [30]. Previously, we had shown that the intracellular AngII levels were significantly increased in cardiac myocytes in diabetes [13]. A renin inhibitor was more effective than an ACEi in reducing AngII levels and associated cardiac fibrosis, oxidative stress and apoptosis in rats, independent of AT1 [5,13]. In another study on
The cardioprotective effects of the three different classes of drugs in SHR (spontaneously hypertensive rats), aliskiren improved coronary endothelial function and cardiac hypertrophy to the same degree as an ACEi and ARB; however, it provided better long-term cardiac AngII suppression [31]. It is noteworthy that aliskiren accumulates in tissues, including cardiac myocytes [5,32,33], whereas there are no data regarding ARBs. On the basis of these previous findings, we anticipated that renin inhibition would more completely block the cardiac RAS, thus preventing diastolic impairment more effectively than an ARB or ACEi, which block only cardiac fibroblast-generated extracellular AngII [14]. Consistent with this hypothesis, the latter agents have been beneficial in improving several physiological parameters in human diabetic nephropathy, retinopathy and cardiomyopathy; however, long-term morbidity and mortality in diabetic patients remain high compared with non-diabetics [15,16]. In our study, all three inhibitors of the RAS were effective at preventing alterations in LV function associated with diabetic cardiomyopathy to a similar extent. However, there were significant differences in protection provided against oxidative stress, inflammatory status, and PRR expression. Renin inhibition was completely effective in all three parameters, while the other two agents were partially effective in one or two parameters studied. Although these differences did not impact the heart function at 10 weeks of hyperglycaemia, their long-term effect is not clear.

Oxidative stress is a major mechanism which contributes to impaired heart function in diabetes and the RAS has a significant role in the generation of oxidative stress [22,24,34]. Consistent with this, a renin inhibitor, which inhibits both intra- and extra-cellular AngII formation, completely protected the diabetic heart from oxidative stress. Treatment with an ARB, which did not block intracellular AngII actions in cardiac myocytes and in diabetic hearts [4,5], was only partially effective at preventing oxidative stress. The incomplete protection was probably due to the ARB only inhibiting extracellular AngII-dependent ROS generation and limited cellular permeability to inhibit intracellular AT1 receptors. These observations corroborated our previously reported observations in rat heart after 1 week of diabetes and suggest involvement of the intracellular RAS in cardiac oxidative stress [5].

Figure 6 Detection of cardiac fibrosis by Picrosirius Red staining
Representative images from control and STZ-Veh mice (A), and diabetic mice treated with vehicle (B), aliskiren (C), benazeprilat (D) or valsartan (E). Semi-quantitative calculation of Picrosirius Red staining represented as an absorbance ratio of 356 nm (Picrosirius Red)/605 nm (Fast Green), after normalization to control (F).
in intracellular AngII synthesis in cardiac myocytes. Intracellular AngII would generate ROS likely through intracellular AT1-receptor-dependent and non-AT1-receptor-dependent mechanisms. In support of the former mechanism, AngII was shown to bind to nuclear AT1 receptors and increase ROS generation in kidney cells and cardiac myocytes [35–37]. Recently, both AT1 and AT2 receptors were described in the inner mitochondrial membrane and were associated with the age-related increase in mitochondrial oxidative stress [38]. In support of an AT1-receptor-independent mechanism, increased oxidative stress was observed in kidneys of transgenic mice expressing intracellular AngII through direct interaction of AngII with proteins of the mitochondrial respiratory chain [39,40]. These studies supported, and provided the likely mechanism of, intracellular AngII-mediated oxidative stress in diabetes.

Elevated levels of pro-inflammatory cytokines, such as TNFα, IL-1β, IFNγ and IL-6 have been reported in diabetic animals, as well as in patients [41]. In the present study, we observed that TNFα, IL-1β, IFNγ and MCP-1 levels were significantly increased in diabetic mice, compared with controls. Renin and ACEi completely prevented the increase in all four cytokines; but, the ARB was only partially effective. Similar to our findings, an ARB and ACEi were ineffective at preventing the increased TNFα or IL-1β in diabetic rats in another study [42]. In addition to AngII, PRR-mediated renal and retinal inflammation has been described in diabetes [43,44]. In another study, the glucose-induced increase in IL-1β expression in mesangial cells was blocked by PRR siRNA (small interfering RNA), but only partially by valsartan [45]. We observed increased PRR expression in diabetic hearts (Figure 8); however, whether PRR contributed to inflammation and aliskiren had a role in preventing such PRR-mediated inflammation is not clear from our studies.

Despite our previous reports of the inability of ACEi and ARBs to inhibit the intracellular RAS [4,5,13] a complete preservation of cardiac function by these agents was observed in the present study. One possibility for the observed difference was that even partial effects of these drugs were sufficient for the heart to maintain function in the short term. Another explanation was that the non-RAS related effects of ARBs and ACEis, such as those on PPARγ (peroxisome-proliferator-activated receptor γ) and bradykinin, might have contributed to the cardioprotective effects of these drugs [46,47]. Additionally, it was reported that valsartan suppressed LPS (lipopolysaccharide)-induced macrophage activation and improved insulin resistance, independent of AT1 receptor or PPARγ, through an unknown mechanism [48]. ACEis enhanced not only kinin levels, but kinin B1 and B2 receptor function, via allosteric mechanisms that involved ACE and B2 receptor heterodimerization and direct binding to B1 receptor [49]. Blockade of B2 receptor reduced the renal protective effects of ramipril, an ACEi, in diabetic nephropathy [50]. Although ARBs and ACEis have pleiotropic mechanisms of cardiac protection, aliskiren is known as a specific inhibitor of renin. The latter observation and an overall better protection profile of aliskiren have suggested, but not confirmed, a role of intracellular AngII in diabetic cardiomyopathy. Studies utilizing genetically modified animal models, such as AT1a-receptor-knockout mice in which experimental effects of PRR in diabetes have been described [44,52–54]. In the heart, PRR, which was primarily co-localized with the cardiac RyR2 (ryanodine receptor 2) in the Z-disc, was significantly elevated in diabetic TGR(mRen2)-27 rats [27]. Previously direct intracellular interaction of PRR with the transcription factor PLZF was demonstrated in several cell types in vitro

* Figure 7 Measurement of plasma cytokine levels

TNFα (A), IL-1β (B), IFNγ (C) and MCP-1 (D) in control, STZ-Veh, STZ-Alsk, STZ-Benz and STZ-Vals, after 10 weeks of diabetes and normalization to control. Values are expressed as the means ± S.E.M. *P < 0.05 against control, #P < 0.05 against STZ-Veh.
This interaction caused nuclear translocation of PLZF resulting in negative regulation on PRR and positive regulation of PI3K (phosphoinositide 3-kinase)-p85 expression [28]. Studies in the heart reported direct interaction of PLZF with the AT2 receptor, which resulted in AngII-induced nuclear localization of PLZF and enhanced expression of PI3K-p85 and GATA4 [55,56]. In other systems, PLZF targets included smooth muscle α-actin in chicken embryonic fibroblasts, Redd1 in spermatogonial progenitor cells and c-myc in several cell lines [57,58]. If the latter targets of PLZF are validated in cardiac myocytes, these would imply a significant role of this transcription factor in heart contraction and growth. We observed that the hyperglycaemia increased prorenin [5] and PRR gene expression in cardiac myocytes, which was associated with increased nuclear translocation of PLZF (Figure 8). Aliskiren, benazeprilat and valsartan reduced cardiac myocyte PRR density and nuclear translocation of

Figure 8  Cardiomyocyte expression of PRR and nuclear translocation of PLZF
PRR gene expression in cardiac myocytes of control, STZ-Veh, STZ-Alisk, STZ-Benz and STZ-Vals, after 10 weeks and normalization to control (A). Quantification of PRR immunostaining after normalization to actin staining (B). Quantification of nuclear localization of PLZF, represented as a percentage of nuclei showing nuclear PLZF staining (C). Representative images of PRR immunostaining in the heart (D). Representative images of PLZF immunostaining in the heart (E). Grey, actin filaments; blue, nuclei; red, PRR; green, PLZF. Values are expressed as the means ± S.E.M. *P < 0.05 against control, #P < 0.05 against STZ-Veh (A–C).
PLZF in diabetic mice. The effect of aliskiren on PRR expression corroborates earlier findings in TGR(mRen2)-27 rats [27]. The mechanism by which these three agents regulate the PRR–PLZF pathway and a precise role of PLZF in diabetic cardiomyopathy remain to be determined. Association of PRR with PLZF in diabetic cardiomyopathy and prevention of nuclear co-localization of the latter by all three drugs, represent significant novel findings.

Conclusions
Both ARBs and ACEis have been shown to be cardioprotective in various pathological states. A renin inhibitor is expected to have a similar profile. However, no single agent among ARBs and ACEis has been sufficient to reduce morbidity and mortality to the desired levels in patients with CVDs. The latter observations have suggested the existence of residual risk and the potential benefits of dual therapy [17]. Unexpectedly, a combination of ACEis and ARBs produced a worse outcome than any single RAS blocker, the reasons for which are not clear [59]. Similarly, addition of aliskiren to an ARB and ACEi has not provided a favourable outcome [16]. Interestingly, head-to-head comparison of the three classes of drugs has not been performed in the context of diabetes. The intracellular RAS, which is strongly activated by hyperglycaemia, might be the residual risk that is not mitigated by ARBs and ACEis. The intracellular RAS is inhibited completely by a renin inhibitor, suggesting that a renin inhibitor might have an advantage in diabetes. In the present study, direct renin inhibition provided similar protection from cardiac dysfunction in the short term, but better protection from oxidative stress and inflammation, as compared with an ARB in diabetic cardiomyopathy. However, whether the latter superior effects of renin inhibition would translate into a better outcome in long-term treatment in diabetes remains to be investigated.

CLINICAL PERSPECTIVES

- In view of the intracellular cardiac RAS activation by hyperglycaemia, we studied the comparative efficacy of a renin inhibitor, an ACEi and an ARB in preventing diabetes-induced cardiac dysfunction.
- We observed that all three classes of RAS inhibitors prevented diastolic and systolic dysfunction in an STZ-induced mouse model of diabetes. However, these drugs had differential effects on oxidative stress and inflammation in the heart, which might influence their long-term efficacy. We also described the activation of a novel PRR/PLZF signalling pathway in the diabetic heart.
- These studies suggest that a renin inhibitor, the newest among the RAS inhibitors, is as effective as an ACEi or ARB in maintaining cardiac function in diabetes.

AUTHOR CONTRIBUTION
Candice Thomas researched data and wrote the paper. Qian Chen Yong researched data and reviewed the paper prior to submission. Rachid Seqqat and Niketa Chandel researched data. David Feldman assisted in research design, provided drugs and reviewed the paper prior to submission. Kenneth Baker reviewed and edited the paper prior to submission and contributed to discussion, and Rajesh Kumar designed and supervised the research and wrote the paper. Kenneth M. Baker and Rajesh Kumar are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Renin inhibition in diabetic cardiomyopathy


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SUPPLEMENTARY ONLINE DATA

Direct renin inhibition prevents cardiac dysfunction in a diabetic mouse model: comparison with an angiotensin receptor antagonist and angiotensin-converting enzyme inhibitor

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MATERIAL AND METHODS

Preliminary dose-determination study
A preliminary dose-determination experiment was performed to choose the dosage of aliskiren, benazeprilat and valsartan for the present study. There is no standard measure of the degree of RAS inhibition to compare different agents [1]. Anti-hypertensive and renal/plasma renin-elevating effects of RAS blockers are generally used by investigators as a measure of the degree of RAS inhibition; however, these methods may give different results when compared side-by-side [2,3]. The dose selection requires consideration of disease conditions and experimental models being investigated. In this manuscript, we studied cardiac dysfunction caused by the local RAS, not the renal or circulatory RAS, in a diabetic model that did not develop hypertension. Furthermore, AngII synthesis in diabetes is significantly chymase-mediated; thus, ACEIs might not cause a similar reactive rise in renin, as a renin inhibitor or an ARB. Therefore anti-hypertensive or renin elevating effects were not considered appropriate for dose selection in the present study. Our hypothesis was that a renin inhibitor would be more cardioprotective than an ACEi or ARB, as the renin inhibitor blocks intracellular AngII synthesis more than the ACEi in diabetes and ARBs do not prevent effects on intracellular AngII [4]. Therefore we determined the effects of the three agents on intracellular AngII levels in cardiomyocytes of diabetic mice after 1 week of treatment. We tested a range of doses (1.85, 7.4 and 18.5 mg/kg of body weight per day) of the three RAS blockers, which have been shown to have protective effects in various models of CVDs [5–7].

Intracellular AngII measurement
A total of 33 adult male C57BL/6j mice were divided into 11 groups. One group of animals served as control, whereas others were made diabetic by STZ treatment (50 mg/kg of body weight per day, intraperitoneally) for 5 days, as described in the main text. Animals in all diabetic groups, except one, received valsartan, benazeprilat or aliskiren at a dose of 1.85, 7.4 and 18.5 mg/kg of body weight per day, respectively, by subcutaneous minipumps. After 1 week, the animals were killed, hearts perfused by the Langendorff method and cardiac myocytes isolated. Cardiac myocytes were immediately processed for AngII extraction. AngII was measured by a competitive quantitative ELISA [4]. The latter method had previously been validated by MS [8]. AngII levels presented in Table S1 are similar to those we reported in rat cardiomyocytes [9]; however, these are significantly higher than described by other groups [10]. The differences are probably due to the fact that we measured AngII in purified cardiomyocytes, which are the major AngII producing cells in the heart, as opposed to whole heart preparations used by other groups. Heart fluids and matrix would add to the weight of the heart, thereby generating lower AngII concentrations per unit weight. Additionally, we represented the AngII concentration as per mg of protein, not total cell weight.

As shown in Table S1, aliskiren completely prevented the diabetes-induced intracellular AngII levels at a dose of 18.5 mg/kg of body weight per day. Benazeprilat showed a plateau effect above 7.4 mg/kg of body weight and valsartan at a dose above 1.85 mg/kg of body weight. Incomplete inhibition of the increase in AngII levels by benazeprilat further confirmed our
previous observations of chymase-mediated intracellular AngII generation in cardiomyocytes in diabetes [8,9]. Inhibition by valsartan was probably due to prevention of AT1-mediated uptake of extracellular AngII by cardiomyocytes, which largely came from ACE-mediated synthesis in cardiac fibroblasts [11]. We chose doses of 2, 10 and 20 mg/kg of body weight for valsartan, benazeprilat and aliskiren, respectively, for the present study, which represented the lowest maximally effective doses on intracellular AngII levels. Importantly, similar doses of these RAS blockers have been reported to have protective effects in various models of CVDs [5–7]. Particularly, the dose of valsartan (2 mg/kg of body weight) might appear low; however, even smaller doses of 0.3 and 1 mg/kg of body weight were sufficient to provide end-organ protection from malignant hypertension, inflammation-induced vascular injury and improved adipocyte differentiation in atherosclerotic and diabetic models [6,12–14].

**Plasma and renal renin**

Furthermore, we determined plasma renin levels and renal renin mRNA expression at the end of the main study at 10 weeks. As shown in Figure S1, all three agents significantly increased plasma renin levels, as measured by Western blot analysis. Similarly, renin mRNA expression in kidneys was increased significantly by all three drugs. On the basis of the above measures of the efficacy of these drugs, we believe that any differential effect of the drugs in the heart were unlikely due to the doses used in the present study.

**Insulin treatment prevented the STZ-induced decrease in cardiac function**

Figure S2 shows that STZ-induced hyperglycaemia for 10 weeks resulted in decreased cardiac function, as determined by echocardiographic measurement of E/A ratio, isovolumic relaxation time, mitral valve deceleration time, EF and FS. Insulin treatment (0.7 unit/day; Humulin N) completely prevented deterioration of the heart function.

<table>
<thead>
<tr>
<th>Group</th>
<th>AngII (fmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.0 ± 12.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>100.0 ± 10</td>
</tr>
<tr>
<td>Aliskiren (mg/kg of body weight per day)</td>
<td></td>
</tr>
<tr>
<td>1.85</td>
<td>80.7 ± 5.1</td>
</tr>
<tr>
<td>7.4</td>
<td>50.0 ± 5.8</td>
</tr>
<tr>
<td>18.5</td>
<td>32.6 ± 9.5</td>
</tr>
<tr>
<td>Benazeprilat (mg/kg of body weight per day)</td>
<td></td>
</tr>
<tr>
<td>1.85</td>
<td>120.5 ± 14.8</td>
</tr>
<tr>
<td>7.4</td>
<td>79.6 ± 9.9</td>
</tr>
<tr>
<td>18.5</td>
<td>77.5 ± 4.9</td>
</tr>
<tr>
<td>Valsartan (mg/kg of body weight per day)</td>
<td></td>
</tr>
<tr>
<td>1.85</td>
<td>78.3 ± 15.5</td>
</tr>
<tr>
<td>7.4</td>
<td>83.2 ± 13.8</td>
</tr>
<tr>
<td>18.5</td>
<td>63.6 ± 13.5</td>
</tr>
</tbody>
</table>

**Figure S1**  **Analysis of renin following treatment with the three RAS inhibitors**

Left-hand panel, plasma prorenin and renin levels were determined by Western blot analysis and quantified by densitometry. Values were combined to obtain total renin values. Data are presented as fold change compared with the control group. Right-hand panel, renin mRNA expression was determined in kidneys by real-time reverse transcription–PCR and normalized to 18S mRNA. Data are presented as fold change in expression compared with the control group. n = 4, *P < 0.05 against control.
Figure S2  Measurement of diastolic and systolic function by echocardiography in control, STZ and STZ-insulin mice

Data shown are before STZ treatment and at 10 weeks after becoming diabetic; except that 8 weeks was used for MV (mitral valve) deceleration time in the STZ group, due to technical difficulty separating E and A waves at 10 weeks. Values are expressed as the means ± S.E.M. *P < 0.05 against before STZ.
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