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Intracellular sodium increase and susceptibility to ischaemia in hearts from type 2 diabetic *db/db* mice

Abstract *Aims/hypothesis:* An important determinant of sensitivity to ischaemia is altered ion homeostasis, especially disturbances in intracellular Na^+ (Na_i^+) handling. As no study has so far investigated this in type 2 diabetes, we examined susceptibility to ischaemia–reperfusion in isolated hearts from diabetic *db/db* and control *db/+* mice and determined whether and to what extent the amount of Na_i^+ increase during a transient period of ischaemia could contribute to functional alterations upon reperfusion. *Methods:* Isovolumic hearts were exposed to 30-min global ischaemia and then reperfused. ^{23}Na nuclear magnetic resonance (NMR) spectroscopy was used to monitor Na_i^+ and ^{31}P NMR spectroscopy to monitor intracellular pH (pH_i). *Results:* A higher duration of ventricular tachycardia and the degeneration of ventricular tachycardia into ventricular fibrillation were observed upon reperfusion in *db/db* hearts. The recovery of left ventricular developed pressure was reduced. The increase in Na_i^+ induced by ischaemia was higher in *db/db* hearts than in control hearts, and the rate of pH_i recovery was increased during reperfusion. The inhibition of Na^+/H^+ exchange by cariporide significantly reduced Na_i^+ gain at the end of ischaemia. This was associated with a lower incidence of ventricular tachycardia in both heart groups, and with an inhibition of the degeneration of ventricular tachycardia into ventricular fibrillation in *db/db* hearts. *Conclusions/interpretation:* These findings strongly support the hypothesis that increased Na_i^+ plays a causative role in the enhanced sensitivity to ischaemia observed in *db/db* diabetic hearts.

Keywords Cardiac ischaemia–reperfusion · *Db/db* mice · Intracellular sodium · Type 2 diabetes · Ventricular arrhythmias

Abbreviations I_{NaL}: slowly inactivating component of Na^+ current · LVDP: left ventricular developed pressure · LVEDP: left ventricular end diastolic pressure · Na_i^+: intracellular sodium · NHE: Na^+/H^+ exchange · NMR: nuclear magnetic resonance · pH_i: intracellular pH · $TmDOTP^{5-}$: thulium(III)-1,4,7,10-tetraazacyclododecane-N,N^I,N^{II},N^{III}-tetra-(methylene phosphonate) · VF: ventricular fibrillation · VT: ventricular tachycardia

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Introduction

Several studies suggest that type 2 diabetes mellitus has direct adverse effects on the heart, independently of obstructive coronary artery disease [1]. Both clinical [2] and experimental [3] investigations have indeed pointed to a cardiomyopathy in humans producing abnormalities in ventricular function. However, relatively few experimental studies on the possible mechanisms of ventricular dysfunction have been performed in animal models of type 2 diabetes [4–6]. Diabetic *db/db* mice provide an animal model of type 2 diabetes [7]. The natural progression of diabetes in *db/db* mice, with initial insulin resistance followed by an insulin-secretion defect [8], is similar to the pathogenesis of type 2 diabetes in humans [9]. Recent studies using perfused working hearts from diabetic *db/db* mice have demonstrated altered cardiac metabolism and reduced contractile performance [5, 6]. Reduced cardiac function was also assessed in vivo in 12-week-old diabetic *db/db* mice [10]. Impaired metabolism, i.e. increased fatty

acid oxidation and decreased glucose utilisation, appeared to be an important causative factor in the contractile dysfunction [5, 6]. To date, only a few investigators have reported results from ischaemia–reperfusion studies using *db*/*db* hearts [6]. At 6 weeks, *db*/*db* hearts showed normal recovery of contractile function following ischaemia, whereas at 12 weeks function was markedly reduced [6].

An important determinant of sensitivity to ischaemia is altered ion homeostasis, especially disturbances in intracellular Na^+ (Na_i^+) handling [11, 12]. As no study has so far investigated this in type 2 diabetes and altered Na^+ handling may indeed have functional as well as pro-arrhythmogenic consequences [13], we examined susceptibility to ischaemia–reperfusion in isolated hearts from 12- to 15-week-old *db*/*db* mice and from age-matched control *db*/+ mice, and determined whether and to what extent the amount of Na_i^+ increase, monitored through ^{23}Na nuclear magnetic resonance (NMR) spectroscopy during a transient period of ischaemia, could contribute to functional alterations upon reperfusion. ^{31}P NMR spectroscopy was also used to monitor intracellular pH (pH_i), since the accumulation of H^+, i.e. a decrease in pH_i during severe ischaemia, is a promoting factor for the imbalance of other cations, especially Na^+ [14–16]. In addition, since it is known that Na^+/H^+ exchange (NHE) contributes to Na^+ overload and that inhibitors of NHE exert substantial protection when present throughout ischaemia and reperfusion [16, 17], we compared the effects of NHE inhibition by cariporide in diabetic and control mouse hearts. A preliminary report on this study, in which Na_i^+ was non-invasively measured in the beating perfused mouse heart, has already been published [18].

Materials and methods

Experimental animals

Animals were cared for and used in accordance with the European convention for the protection of vertebrate animals used for experimental purposes, and institutional guidelines no. 86/609/CEE November 24, 1986. Male C57BL/KsJ-leprdb/leprdb diabetic (*db*/*db*) mice and their non-diabetic control littermates C57BL/KsJ-leprdb/lepr+ (*db*/+) were purchased from Janvier (Le Genest St. Isle, France). All animals used in this study were males between 12 and 15 weeks of age. The animals were housed in groups (five or six) and given free access to food and water.

Heart perfusions

Mice (fed dietary status) were anaesthetised with sodium pentobarbital (100 mg/kg) and heparinised (100 U) i.p. The heart was quickly removed and placed in ice-cold Krebs–Henseleit bicarbonate buffer. After removing extraneous

tissues, the aorta was cannulated with an 18-gauge steel cannula (in NMR study groups, with an 18-gauge plastic cannula). The heart underwent a Langendorff perfusion (80 mmHg perfusion pressure) with modified Krebs–Henseleit bicarbonate perfusate, consisting of (mmol/l): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.75, EDTA 0.5, glucose 11, gassed with 95% O_2/5% CO_2 (pH 7.4, 37°C) (20-min stabilisation period) and was then immersed in a set volume of warmed perfusate (37°C) so that coronary flow could be estimated by timed collection of coronary effluent. All perfusion solutions were filtered (0.8 μm; Millipore) prior to use. Isovolumic left ventricular developed pressure (LVDP) was monitored using a fluid-filled polyvinylchloride film balloon inserted into the left ventricle via the mitral valve, and was connected to a Statham 23 ID pressure transducer. The initial left ventricular end diastolic pressure (LVEDP) was adjusted to 8–10 mmHg. LVDP was calculated by subtracting LVEDP from systolic pressure. Pressure signals were recorded on a Gould 2,200 recorder (Gould Instruments, Ballainvilliers, France) and IOX data acquisition system (EMKA Technologies, Paris, France). For all experiments, hearts were allowed to contract spontaneously. For ^{23}Na NMR measurements, we used the shift agent thulium(III)-1,4,7, 10-tetraazacyclododecane-N,N^I,N^{II},N^{III}-tetra-(methylene phosphonate) ($TmDOTP^{5-}$) (Macrocyclics, Dallas, TX, USA), as previously described [11]. $TmDOTP^{5-}$ was dissolved in H_2O and subsequently added to the perfusate to reach a concentration of 3.5 mmol/l. Since $TmDOTP^{5-}$ is a sodium salt that binds Ca^{2+} to a significant extent, both Na^+ and Ca^{2+} were corrected. NaCl was adjusted to keep the total Na^+ content unchanged, and the total Ca^{2+} content was increased to 3.42 mmol/l, resulting in a free ionised Ca^{2+} of 0.85–0.95 mmol/l, measured by using a Ca^{2+} ion-selective electrode (Orion).

NMR spectroscopy

The ^{23}Na and ^{31}P spectra were recorded on a spectrometer (AM 400 WB; Bruker BioSpin, Ettlingen, Germany) at 105.84 and 161.98 MHz, respectively, using a multinuclear NMR probe. The spectrometer was equipped with a 9.4 T vertical magnet and the perfused heart was inserted into a multinuclear 10-mm diameter glass NMR tube, in which the temperature was maintained at 37°C. Magnetic field homogeneity was optimised using the ^1H resonance of H_2O. Each Na NMR spectrum (1-min time-resolved) was obtained by accumulation of 280 consecutive free induction decays using 90° pulses and a 205-ms interpulse delay with a 2,500-Hz spectral width and a 1-k data points time domain. ^{23}Na NMR spectra were processed for the quantitative analysis of the intracellular component Na_i^+ in two steps. First, removal of the overlapping spectral extracellular component was carried out by the HLSVD method [19]. Second, the Na_i^+ peaks were quantified with a time domain fitting routine (AMARES) [20]. The Na^+

resonance of a standard solution containing a fixed amount of Na^+ and $TmDOTP^{5-}$ in a glass capillary was used for absolute quantification. Eighteen pre-ischaemic spectra were obtained followed by spectra collected over 30 min of ischaemia (30 spectra). Na^+ spectra were not collected during reperfusion because we found there to be a negative effect of the presence of Na^+ shift reagent on coronary flow rate at reperfusion (effect on coronary flow has been previously noted by others [21]), which appeared to be deleterious at reflow after severe ischaemia in the mouse heart. ^{31}P spectra were obtained from 288 free induction decays following 75° pulses repeated every second using 4-k data points and a 6,000 Hz spectral width (5-min time-resolved spectra). To study changes in pH_i during the early phase of reflow following ischaemia with greater time resolution, 1-min time-resolved spectra were acquired. Values for pH_i were derived from the chemical shift of the inorganic phosphate resonance as previously described [11]. In some of the experiments we observed heterogeneity of the inorganic phosphate peaks during reperfusion. The averaged inorganic phosphate chemical shifts were therefore used to calculate pH_i.

Experimental protocol

The experimental protocol consisted of 15 min of control perfusion followed by 30 min of no-flow ischaemia and 40 min of reperfusion. In all study groups, except when otherwise mentioned, perfusate contained a similar free ionised Ca^{2+} concentration (0.9 mmol/l), adjusted by using 0.5 mmol/l EDTA when Na^+ shift agent was not used. In each group, diabetic or non-diabetic hearts either received or did not receive the NHE inhibitor cariporide (1 μmol/l; a gift from Aventis Pharma) [22]. When present, cariporide was added to the perfusion solution 10 min before inducing ischaemia and was present throughout.

Arrhythmia study

Unipolar ECG was recorded continuously on a Gould 2,200 recorder (Gould Instruments) from perfused hearts, using a reference electrode connected to the steel cannula and a silver electrode flanking the heart. Chart speed was set at 10 mm/s during control perfusion and ischaemia. A few seconds before reperfusion, it was set at 25 or 50 mm/s

Table 1 Characteristics of control (*db/+*) and diabetic *(db/db)* mice (*n*=9/group)

	Control *db/+*	Diabetic *db/db*
Body weight (g)	28.1±0.4	46.8±0.9*
Heart dry weight (mg)	25.8±0.7	25.6±0.2
Plasma glucose (mmol/l)	12.5±0.8	37.2±4.5*
Plasma triglycerides (mmol/l)	0.7±0.1	1.9±0.2*

Values are means±SEM. Plasma samples were collected from fed mice
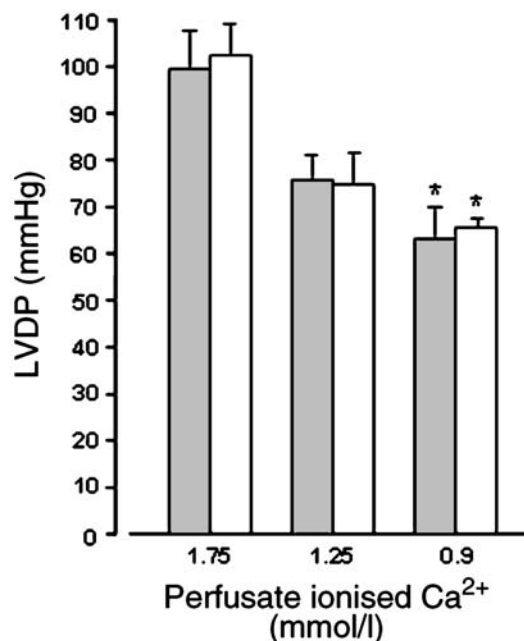*Significantly different from control

Fig. 1 Mean±SEM values of left ventricular developed pressure (LVDP) after 30 min of control perfusion with perfusate containing either 1.75 or 1.25 mmol/l (*n*=6 *db/db* and *db/+* hearts for each concentration) or 0.9 mmol/l (*n*=7 *db/db* and *db/+* hearts) ionised Ca^{2+}. *$p<0.05$ vs 1.75 mmol/l. Results from *db/db* hearts, *grey bars*; results from *db/+* hearts, *open bars*

so as to obtain a permanent high-speed recording [23]. The ECG was retrospectively analysed, in a blind manner, for the incidence, time to onset, and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) during reperfusion. All analyses were carried out in accordance with the Lambeth Conventions [24]. VT was defined as four or more consecutive premature beats of ventricular
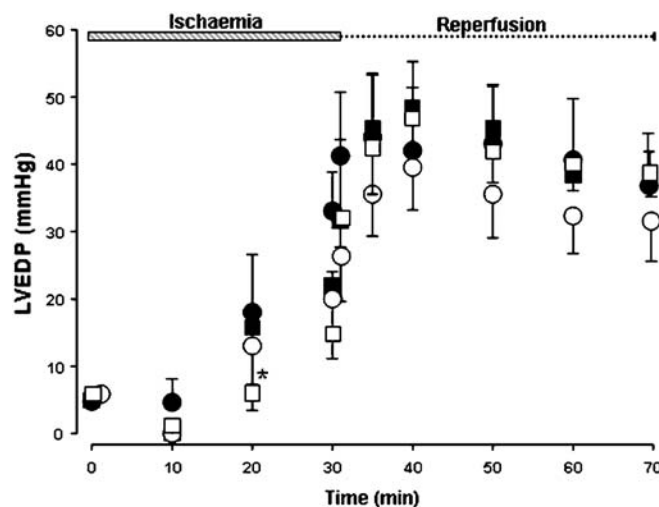

Fig. 2 Left ventricular end diastolic pressure (LVEDP) during ischaemia and reperfusion in isovolumic perfused hearts from non-diabetic *db/+* (*solid circles*) and diabetic *db/db* male mice (*solid boxes*), and in hearts from *db/+* (*open circles*) and *db/db* mice (*open boxes*) receiving cariporide. Means±SEM, *n*=5 hearts in each group. *$p<0.05$ for difference from *db/db* hearts without cariporide

origin, and VF was defined as a signal in which individual QRS deflections could no longer be distinguished from one another and for which the rate could not be determined.

Analysis of plasma metabolites

Blood samples (fed dietary status) were taken from the body cavity after excision of the heart. The blood was centrifuged in an Eppendorff centrifuge at 17,000 g and 4°C for 15 min. The resulting plasma sample was stored at −20°C before analysis. Plasma glucose and triglyceride levels were measured with kits from J2L Elitech (Labarthe-Inard, France).

Statistical analysis

Results are presented as means±SEM. The data were analysed using either Student's t test for unpaired data or ANOVA followed by the appropriate post hoc test to locate differences between groups. Binominally distributed variables, such as the incidence of VT or VF, were compared using the χ^2 test for a $2 \times n$ table, followed by a sequence of 2×2 χ^2 tests with Yates's correction. Significance was set at $p < 0.05$.

Results

Animal characteristics

At 12–15 weeks of age, diabetic db/db mice weighed significantly more than their control non-diabetic $db/+$ littermates (Table 1). This observation is in agreement with previous studies [5, 6]. Heart weight was similar in both groups of mice. As expected [5], db/db mice had significantly elevated plasma levels of glucose and triglyceride, reflecting their diabetic status with hyperglycaemia and hyperlipidaemia.

Ventricular function in hearts from db/db mice

We first tested, in pilot experiments, the response of the hearts to standard perfusate buffer Ca^{2+} concentrations. LVDP measured after 30-min perfusion with nominally
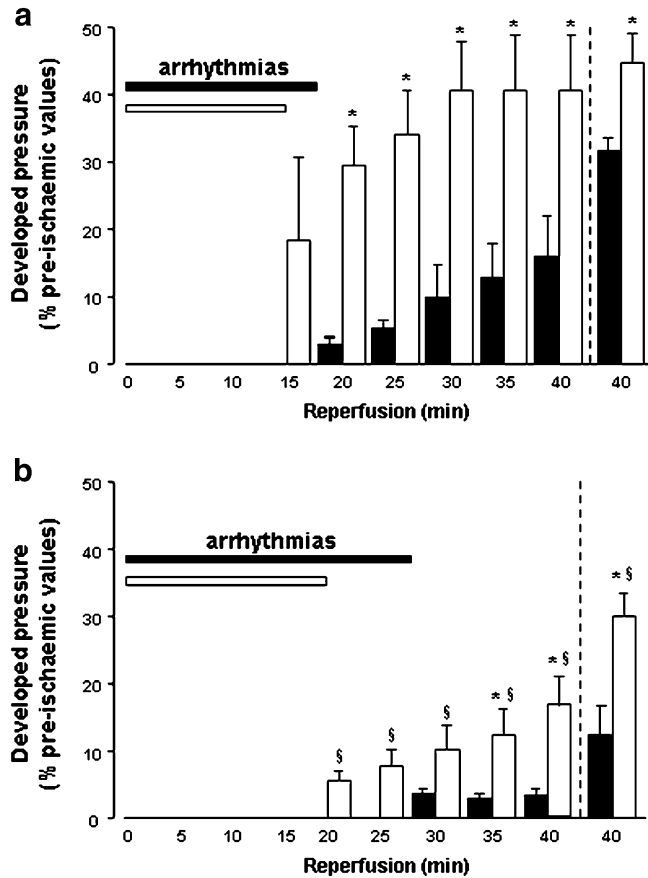

Fig. 3 LVDP during reperfusion following no-flow ischaemia. a Hearts from non-diabetic $db/+$ mice receiving (open columns) or not receiving (filled columns) cariporide. b Hearts from diabetic db/db mice receiving (open columns) or not receiving (filled columns) cariporide. The horizontal bars indicate the time-period of occurrence of ventricular arrhythmias for the groups of hearts receiving (empty bars) or not receiving (filled bars) cariporide. Means±SEM, $n=5$ hearts in each group. The columns right of the dotted line (a, b) show recovery of developed pressure for $db/+$ and db/db hearts that received perfusate containing 1.75 mmol/l ionised $Ca^{2+}(40_{1.75})$ *$p < 0.05$ for difference from hearts without cariporide. §$p < 0.05$ for difference from $db/+$ hearts with cariporide
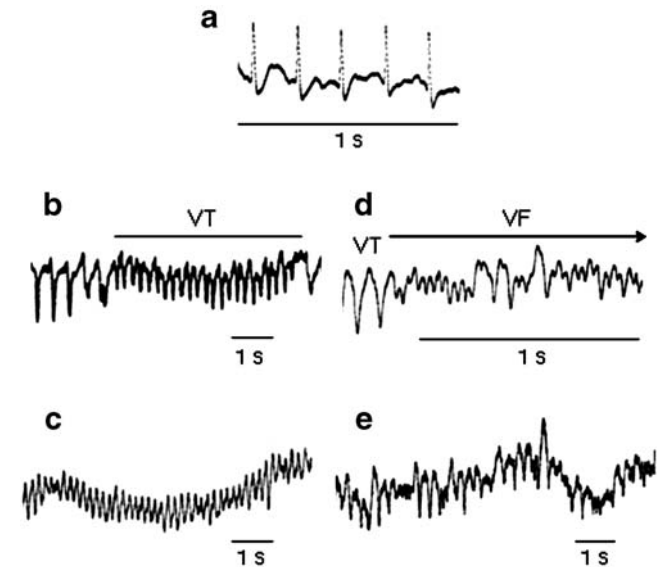

Fig. 4 Representative recordings of reperfusion-induced ventricular arrhythmias obtained from hearts of db/db mice. a Normal sinus rhythm; b non-sustained VT; c sustained VT; d degeneration of VT into VF; e VF

2.0 mmol/l Ca^{2+} (approximately 1.75 mmol/l ionised Ca^{2+}) was in the range of values previously reported for the mouse heart under similar conditions [25, 26], with no difference between control and diabetic hearts (Fig. 1). Decreasing perfusate calcium from 1.75 to 1.25 and then to 0.9 mmol/l ionised Ca^{2+} decreased LVDP, as previously described [25, 26], again with no significant difference in hearts from diabetic mice compared with hearts from non-diabetic mice (Fig. 1). Nor was there any significant difference in coronary flow rate (2.2±0.2, n=11, and 2.4±0.2 ml/min, n=12, in db/+ and db/db hearts, respectively) or heart rate (378±10, n=9, and 352±10 beats/min, n=11, in db/+ and db/db hearts, respectively) between hearts from the two groups.

During ischaemia, LVDP rapidly decreased to zero for all groups whereas LVEDP increased. LVEDP increase (Fig. 2) was not significantly different between groups over the entire period of ischaemia except for a slightly smaller increase in the db/db hearts in the presence of cariporide (only significant at 20 min of ischaemia, p<0.05). There were no significant differences in LVEDP during reperfusion between db/db and db/+ hearts and between hearts in each group receiving or not receiving

cariporide. Correct measurements of LVDP upon reperfusion were not possible at some time points over the first 15–20 min, depending on heart groups, due to the development of reperfusion-induced arrhythmias (Fig. 3a,b). Fig. 3a,b also shows that the percentage of LVDP recovery was significantly lower in db/db hearts that did not receive cariporide than in in db/+ hearts under similar perfusion conditions. Cariporide markedly improved the recovery of LVDP in db/+ hearts, from 15–20 min to the end of reperfusion. Although to a lesser extent, a significant improvement in LVDP recovery was also observed in hearts from diabetic db/db mice. We next examined whether the difference in functional recovery between cariporide-treated and untreated hearts would also be present at a more physiological calcium concentration. A group of hearts from both db/+ and db/db mice was perfused with buffer containing 1.75 mmol/l ionised Ca^{2+}. Under these conditions, ventricular function recovery was higher than in the presence of 0.9 mmol/l ionised Ca^{2+}. However, cariporide again significantly increased the recovery of LVDP in both diabetic and non-diabetic hearts, as shown in Fig. 3 at 40 min of reperfusion.

Fig. 5 Incidence (%) and duration in seconds (s) of VT (**a**) and of VF (**b**) in hearts from diabetic db/db and from non-diabetic db/+ mice receiving or not receiving cariporide. Means±SEM, n=5 hearts in each group. #p<0.05 vs db/+ hearts; *p<0.05 vs without cariporide. s, seconds; %, percentage of incidence
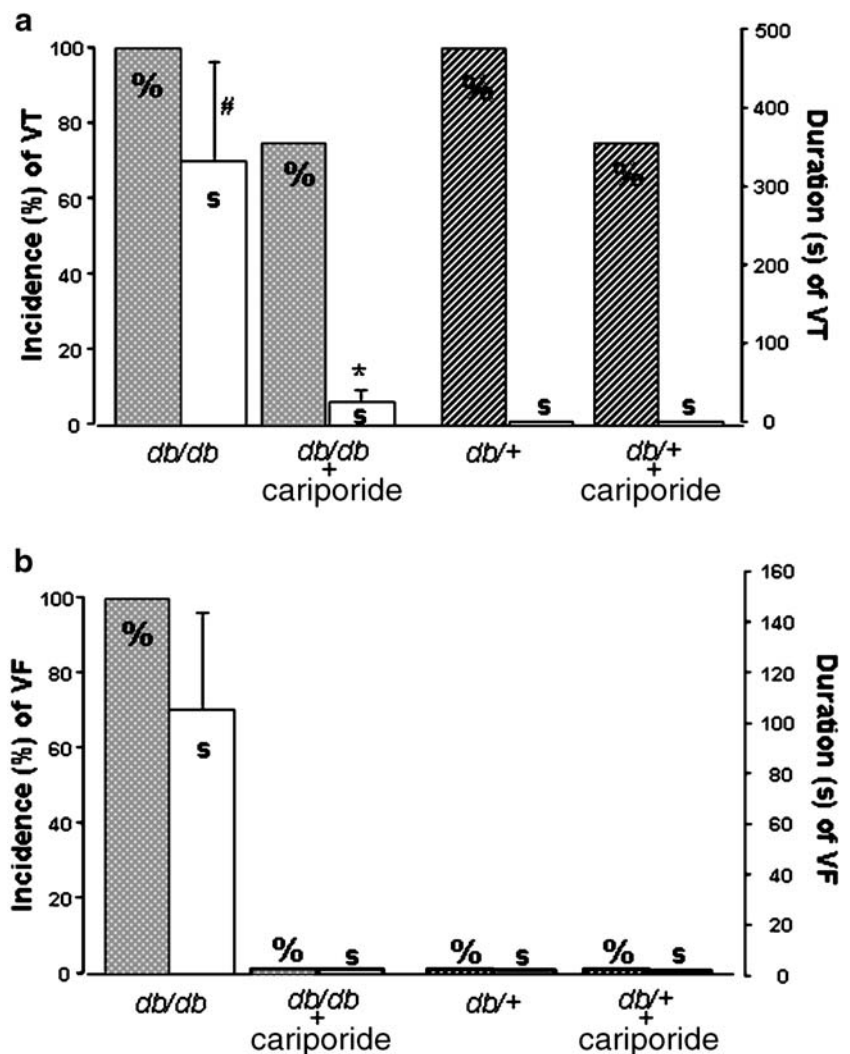
Reperfusion-induced arrhythmias

Reperfusion-induced arrhythmias (Fig. 4) occurred in all groups of hearts and all of them terminated spontaneously. The time-periods of occurrence of ventricular arrhythmias (from the earliest onset of arrhythmia to the latest end of arrhythmia in each group) were 15–1,635 s in *db/db* vs 20–1,121 s in *db/+* hearts without cariporide, whereas they were 30–1,116 s in *db/db* and 30–882 s in *db/+* hearts, respectively, in the presence of cariporide (Fig. 3). Although the incidence of VT (Fig. 5a) was 100% in both *db/db* and *db/+* hearts, duration of VT in *db/db* hearts was significantly greater than in *db/+* hearts. Cariporide similarly decreased the incidence of VT (to 75%) in both *db/db* and *db/+* hearts. Duration of VT was also significantly reduced in *db/db* hearts in the presence of cariporide, but the short duration of VT in *db/+* hearts remained unchanged.

In all *db/db* hearts, degeneration of VT into VF (Fig. 5b) was observed (100% incidence). This did not occur in *db/+* hearts. No occurrence of VF was observed in any of the two groups of hearts receiving cariporide.

## Changes in Na_i^+ in hearts from *db/db* mice

There were no differences in the mean values of Na_i^+ obtained from the ^{23}Na NMR spectra during control perfusion between *db/db* (13.9±1.5 mmol/l, n=8) and *db/+* hearts (14.2±0.7 mmol/l, n=8). These values were unchanged in hearts receiving cariporide (15.2±1.9 and 15.1±0.9 mmol/l for the *db/db* and *db/+* hearts, respectively, n=5

hearts in each group). As shown in Fig. 6, Na_i^+ increased more rapidly during no-flow ischaemia in hearts from diabetic *db/db* mice than in hearts from non-diabetic *db/+* mice, reaching 179.0±4.9% and 158.0±8.1% of control values at end ischaemia, respectively (*p*<0.05). Hearts receiving cariporide showed a similar time course of Na_i^+ increase to that observed in the absence of cariporide over the first 20 min of ischaemia. Although not significantly different, a tendency towards a lesser increase in Na_i^+ was observed in the group of *db/+* hearts receiving cariporide. However, in both *db/db* and *db/+* hearts, the presence of cariporide significantly reduced the rise in Na_i^+ at the end of ischaemia (157.6±9.4% vs 179.0±4.9% in *db/db* hearts and 126.2±10.9 vs 158.0±8.1% in *db/+* hearts).

Changes in pH_i in hearts from *db/db* mice

Since the presence of the NHE inhibitor cariporide reduced the rise in Na_i^+ at the end of ischaemia and since NHE contributes significantly to the integrated control of pH_i during ischaemia and reperfusion [15], we examined pH_i during ischaemia and reperfusion. As shown in Fig. 7, the two main observations from this pH_i investigation were: (1) cariporide significantly accentuated the decrease in pH_i during ischaemia in diabetic hearts but not in non-diabetic hearts; and (2) the rate of pH_i recovery was markedly increased over the first 25 min of reperfusion, particularly at the very beginning of reperfusion, in diabetic compared with non-diabetic hearts.

Fig. 6 Changes (mean percent of control values±SEM) in Na_i^+ during 30 min of no-flow ischaemia in hearts from non-diabetic *db/+* (*filled circles*) and from diabetic *db/db* mice (*filled boxes*) that did not receive cariporide, and in hearts from *db/+* (*open circles*) and *db/db* (*open boxes*) mice that received cariporide. n=8 hearts per group without cariporide and n=5 hearts per group with cariporide. *p*<0.05 vs without cariporide. *Inset:*^{23}Na NMR spectra of isolated perfused mouse heart showing Na_i^+ increase during ischaemia. Na_e is the extracellular peak shifted by TmDOTP^{5-}
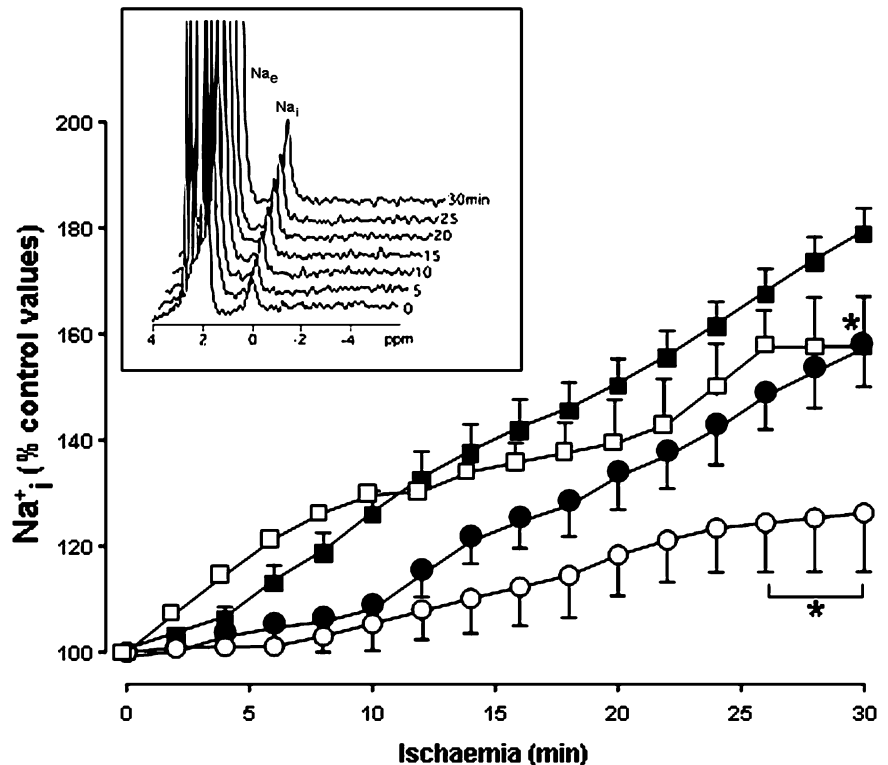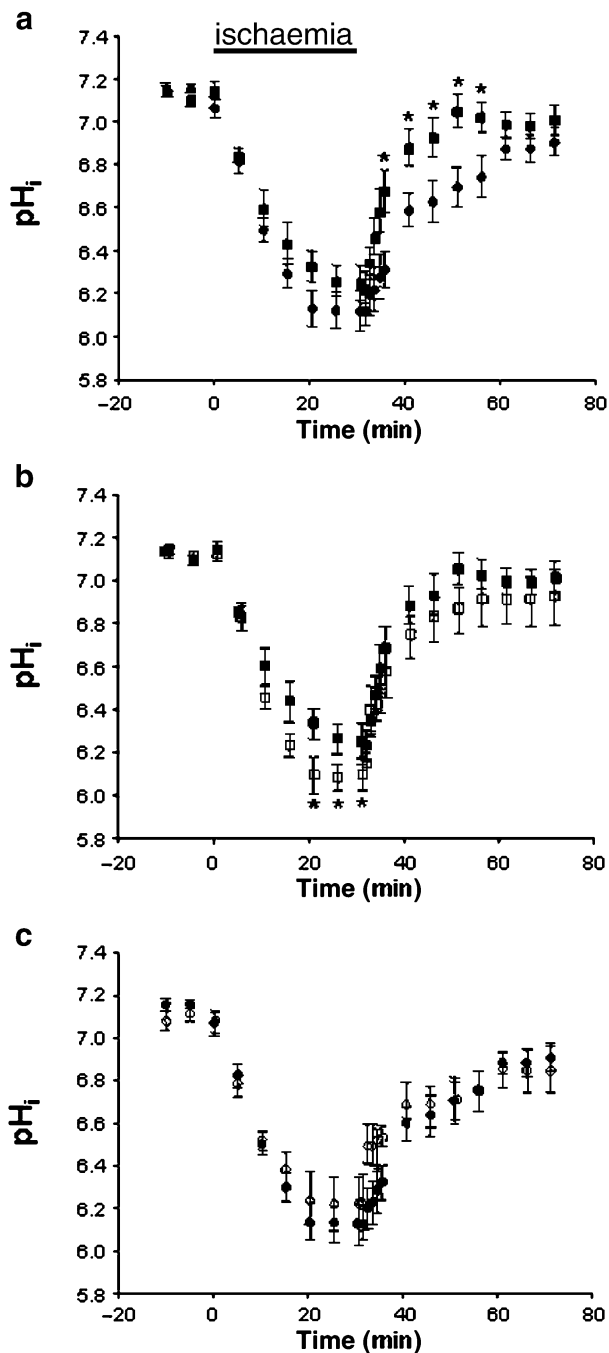
Fig. 7 Mean values±SEM of pH$_i$ during 30 min of no-flow ischaemia and reperfusion in **a** hearts from non-diabetic *db*/+ (*solid circles*) and from diabetic *db*/*db* mice (*solid boxes*) that did not receive cariporide, in **b** hearts from diabetic *db*/*db* mice that received cariporide (*open boxes*), and **c** in hearts from non-diabetic *db*/+ mice that received cariporide (*open circles*). *$p < 0.05$ vs *db*/+ (**a**); *$p < 0.05$ vs *db*/*db* without cariporide (**b**). $n = 7$ *db*/*db* with or without cariporide, $n = 6$ *db*/+ with or without cariporide

Discussion

The main feature of this study was that increased susceptibility to ischaemia–reperfusion in *db*/*db* hearts mainly consisted of a higher duration of VT and the degeneration of VT into VF upon reperfusion after global ischaemia.

This was associated with delayed and lower levels of recovery of developed pressure. It could be argued that the relatively low level of ionised Ca^{2+} used in the present work may have affected the functional response of the hearts to ischaemia and reperfusion injury, as well as the response to cariporide treatment. LVDP recovery was indeed higher in hearts perfused with a more physiological Ca^{2+} concentration, i.e. 1.75 mmol/l vs 0.9 mmol/l. However, the comparison of LVDP recovery after 40 min of reperfusion in the presence and absence of cariporide in each of the two groups of mouse hearts clearly showed a beneficial effect of cariporide (30.0±3.4% of the pre-ischaemic level vs 12.1±4.3% in the absence of cariporide for the *db*/*db*, $p = 0.012$, and 44.6±4.3 vs 31.6±1.8%, $p = 0.023$, for the *db*/+ hearts). In the only previous report on ischaemic sensitivity in hearts from *db*/*db* mice, perfused working hearts demonstrated decreased post-ischaemic recovery of coronary flow compared with control hearts, and because coronary flow is an important determinant of cardiac function the decreased post-ischaemic function could also reflect impaired flow as a result of vascular or endothelial dysfunction in addition to myocyte dysfunction. No such difference was observed in the present work, since coronary flow recovery after 5 min of reperfusion averaged 71% (1.57±0.15 ml/min, $n = 11$) and 73.8% (1.77±0.26 ml/min, $n = 12$) in *db*/+ and *db*/*db* hearts, respectively. In the above mentioned study, treatment aimed at normalising metabolic disturbances (with an activator of peroxisome proliferator-activated receptor-α) failed to improve mechanical recovery after ischaemia–reperfusion, even though carbohydrate oxidation was stimulated and palmitate oxidation was decreased. This finding suggested that in the severe type 2 diabetic *db*/*db* mice, at least not all the adverse effects of diabetes on the recovery of contractile function after ischaemia–reperfusion were due to altered metabolism [6].

An important determinant of sensitivity to ischaemia–reperfusion is altered ion homeostasis, especially disturbances in Na_i^+ handling [11, 12]. This study is the first to demonstrate that myocardial Na_i^+ increased substantially in isolated mouse hearts during ischaemia and increased significantly more in hearts from diabetic *db*/*db* hearts than in hearts from control *db*/+ mice. In this context, the present pH$_i$ data are of particular interest. Indeed, Fig. 7a shows a trend towards a lesser pH$_i$ decrease during the last 10 min of ischaemia in *db*/*db* hearts, although the differences observed between pH$_i$ values of these hearts and those of *db*/+ mice over the same period were not significant. The accentuated pH$_i$ decrease in cariporide-treated *db*/*db* hearts over this same period of ischaemia (Fig. 7b) seems to indicate that NHE was not totally inhibited in diabetic hearts. No information is available to date concerning NHE activity in type 2 diabetic *db*/*db* mouse hearts. In streptozotocin-induced diabetic rats, we previously reported a ~50% increase in levels of Na_i^+ at baseline [27], these results being consistent with a decrease in NHE activity in this model [28]. No such increase was found here in *db*/*db* hearts. We can therefore infer from these data and from the

pH$_i$ data that NHE activity would at least not be decreased in type 2 diabetic *db*/*db* mouse hearts. Consistent with the pH$_i$ data in *db*/*db* hearts, this study also shows that the NHE inhibitor cariporide significantly reduced the rise in Na$_i^+$ at end ischaemia. However, and surprisingly, this effect appeared less pronounced in diabetic (−21%) than in control (−31%) hearts. This apparent contradistinction could be explained if Na$^+$ influx during ischaemia also occurs via other routes than NHE, which may be Na$^+$/HCO$_3^-$ cotransport [29] and the voltage-gated Na$^+$ channel, especially a slowly inactivating component of Na$^+$ current (I_{NaL}) [17, 21, 30, 31]. We have previously shown that such a current is partially inhibited by known NHE blockers and also reduced in diabetic rat hearts [30]. Since the *db*/*db* hearts show an increased Na$_i^+$ accumulation, this must therefore result from NHE- and/or Na$^+$/HCO$_3^-$ cotransport-mediated Na$^+$ influx. More Na$^+$ influx means more acid-equivalent efflux. Because pH$_i$ at the end of ischaemia shows no significant difference between *db*/*db* and *db*/+ hearts, we can envisage two possibilities: (1) there is a higher ischaemic proton production in the *db*/*db* hearts; and/or (2) the intracellular H$^+$ buffering capacity is decreased. The cariporide-sensitive Na$^+$ influx during ischaemia in control hearts may have resulted not only from inhibition of NHE, but also from some degree of cariporide-induced inhibition of I_{NaL}, the latter being reduced in diabetic hearts. Nevertheless, cariporide treatment was associated with a similar lower incidence of VT in both groups of hearts but with a markedly shorter duration of VT in diabetic *db*/*db* hearts. Moreover, the most prominent effect of cariporide was that it totally inhibited the degeneration of VT into VF in *db*/*db* hearts, the incidence of which was 100% without cariporide, whereas none of the *db*/+ hearts exhibited VF. In this regard, Na$_i^+$ accumulation has been shown by others to be a strong predictor of VF [32]. Of particular interest is our observation of a high incidence of VF in diabetic *db*/*db* hearts with exacerbated ischaemia-induced Na$_i^+$ increase. It is known that increases in Na$_i^+$ in ischaemic cardiomyocytes generate Ca^{2+} loading via reverse Na$^+$/Ca^{2+} exchange, which mediates much of the damage incurred upon reperfusion [33, 34]. Since diabetic *db*/*db* mouse hearts have a deficiency in cardiomyocyte Ca^{2+} handling, in particular an increased Ca^{2+} leakage from the sarcoplasmic reticulum, as recently shown by Belke et al. [35], this may in turn precipitate the occurrence of ventricular arrhythmias [36]. Our data indicate that reducing accumulation of Na$_i^+$ to a modest extent may indeed have marked beneficial effects on the occurrence of VF and on ventricular function.

It has also previously been shown that one factor promoting degeneration of VT into VF during early reperfusion is the rate at which extracellular pH is restored [23] and extracellular pH restoration influences pH$_i$ [37]. We report here a markedly higher recovery rate of pH$_i$ in *db*/*db* hearts during reperfusion, with an abrupt slope over the first 5 min, compared with *db*/+ hearts. However, since this pH$_i$ recovery rate on reperfusion was not influenced by

cariporide in either *db*/+ or *db*/*db* hearts, this seems to exclude the possibility of pH$_i$ recovery rate playing a significant role in the development of VF, which was highly cariporide-sensitive in *db*/*db* hearts. Therefore, the rapid rise of pH$_i$ on reperfusion, which has been shown to be mediated principally by metabolite washout (lactate and CO$_2$) over the first 2–3 min and also by Na$^+$/HCO$_3^-$ cotransport [38–40], may suggest changes in the activity of these mechanisms in *db*/*db* hearts.

Conclusion

In this study we found that hearts from *db*/*db* mice had enhanced susceptibility to cardiac ischaemia, associated with a higher rise in Na$_i^+$. The data also suggest a higher sensitivity of *db*/*db* hearts to the protective effect of cariporide, as assessed particularly by the marked inhibition of the degeneration of VT into VF upon reperfusion. It must be acknowledged that data obtained with the use of a leptin-signalling-deficient model to represent type 2 diabetes should be interpreted with caution. In view of emerging evidence for cardiac effects of leptin and that leptin may represent an autocrine/paracrine regulator of cardiac function [41], future work will have to examine the possible interplay between leptin-signalling pathway(s) and Na$_i^+$-regulating mechanisms, especially NHE.

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