High Glucose Alters Cardiomyocyte Contacts and Inhibits Myofibrillar Formation

Daniela Dyntar, Pavel Sergeev, Jelena Klisic, Patrice Ambühl, Marcus C. Schaub, and Marc Y. Donath

Context: The frequency of diabetes-related heart failure along with the prevalence of diabetes is increasing. Diabetic cardiomyopathy is considered to be a distinct disease in the absence of discernible coronary artery and other defined heart disease. Previously we have shown that glucose and palmitic acid induce degeneration of myofibrils and modulate apoptosis in cultivated cardiomyocytes.

Objective: Here we studied the mechanisms of diabetic cardiomyopathy in more detail.

Results: Streptozotocin-induced diabetes led to a significant increase in cardiac cell apoptosis. Furthermore, cardiomyocyte contacts were reduced. In vitro, prolonged exposure of cultured adult cardiomyocytes to high glucose concentrations drastically reduced myofibrillar formation. In particular, sarcomeric myosin heavy chains and cardiac α-actin were reduced, whereas the nonsarcomeric smooth muscle α-actin remained unaffected. The deleterious effects of glucose on myofibrillar formation were prevented by antioxidative regimens.

Conclusions: Thus, a diabetic milieu leads to multiple structural alterations of the heart including apoptosis, loss of intercellular contacts, and malformation of contractile structures.

Materials and Methods

Diabetic animals

Ethical approval for rat studies was granted by the Zurich Cantonal Animal Experimentation Committee. Diabetes was induced in female rats (−175 g, corresponding to an age of 6–7 wk) by a single iv injection of STZ (50 mg/kg, freshly prepared in 0.1 M citrate buffer (pH 4.5); Sigma Chemicals, St. Louis, MO) in the tail vein. Control rats were injected with buffer only. The diabetic and control rats were housed under similar conditions at 22 °C with a 12:12 h light, 12:12 h dark cycle (lights on at 0700 h) and allowed free access to water and chow. Blood samples were obtained from tail-tip bleedings, and blood glucose concentration was measured with a glucometer (Freestyle; Disetronic Medical Systems, Burgdorf, Switzerland).

Ventricular myocyte morphometry

To assess myocyte dimensions, formalin-fixed myocardial tissue was digested according to the method developed by Gerdes et al. (14), allowing the evaluation of isolated individual cells. Then hearts were quickly removed, trimmed of excess tissue, and placed in PBS for approximately 5 min followed by a cold 10% formaldehyde solution in PBS. After several days of fixation the tissue was cut into small pieces (approximately 1 × 1 × 1 mm) and placed into a 12.5 mol/liter KOH solution. Samples were shaken gently at room temperature for approximately 20 h at a slow speed. The tissue was then placed into PBS and vortexed vigor-
ouly for approximately 3 min and ultrasonicated for 10 min. After centrifugation, cells were suspended in 10% formaldehyde PBS until analysis by light microscopy (microscope Axioslab; Zeiss, Jena, Germany).

**Cell culture**

Ventricular cardiac muscle cells of adult female rats (Sprague Dawley-Ivanovas, 2 months old) were isolated as described (11, 15). After perfusion, the heart tissue was minced and incubated at 37°C for another 10 min in Kraft Bruhe medium (16) containing collagenase. Cells were cultured in dishes coated with 0.1% gelatin in M-199 supplemented with 20% fetal calf serum (FCS) (Sigma), 1% penicillin/streptomycin, and 20 μM creatine. For inhibiting growth of contaminating cells, 10 μM/liter 1-b-D-arabinofuranosyl-cytosine were added throughout the culture period (17). The 20% FCS medium was changed to medium containing 10% FCS after 2 and 7 d. In some experiments, basic medium (5.5 mM glucose was supplemented with 27.8 mM glucose, 50 μg/ml (+)-α-tocopherol (vitamin E; Sigma) and 1 mM N-acetyl-L-cysteine (NAC) (Sigma). In the present study, in control incubations osmolality was not corrected with a metabolically inactive molecule.

**Immunocytochemistry**

Heart left ventricles were rapidly removed, tissue samples placed in optimal cutting temperature medium (Tissue-Tek; Sakura-Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at −70°C. Cryosections (5–5 μm) were prepared, collected on gelatin precoated slides, fixed for 10 min in 0.5% parafomaldehyde at room temperature, and rinsed with PBS (18). VARCs were fixed in 3% paraformaldehyde followed by permeabilization with 0.2% Triton X-100 (15). Both tissue sections and cultured cells were incubated overnight at 4°C with the following monoclonal antibodies (mAbs) or affinity purified polyclonal antibodies (pAbs): guinea pig pAbs against dystrophin (anti-DYS11, kindly provided by J. C. Perriard, Institute of Cell Biology, ETH Zurich, Switzerland); mouse mAbs against sarcomeric myosin heavy chains, which binds to all sarcomeric isoforms (1:5000, 3 h, room temperature; Alexis Immunoresearch Laboratories Inc., West Grove, PA) or Cy3-conjugated rabbit anti-guinea pig antibody (Dako, Glostrup, Denmark) combined with rhodamine-phalloidin or fluorescein isothiocyanate-phalloidin (Molecular Probes, Inc., Eugene, OR). When used, the anti-DYS11 was tested for specificity by histochemistry and in immunoblots (results not shown).

**Cardiac cell apoptosis**

The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) technique as previously described (20, 21). A marker of nuclei, 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes), was used to counterstain DNA.

**Characterization of myofibrillar structures**

Immunocytochemistry

Heart left ventricles were removed rapidly in 0°C, frozen in liquid nitrogen, and stored at −70°C. Cryosections (5–5 μm) were prepared, collected on gelatin precoated slides, fixed for 10 min in 0.5% paraformaldehyde at room temperature, and rinsed with PBS (18). Cells were fixed in 3% paraformaldehyde followed by permeabilization with 0.2% Triton X-100 (15). Both tissue sections and cultured cells were incubated overnight at 4°C with the following monoclonal antibodies (mAbs) or affinity purified polyclonal antibodies (pAbs): guinea pig pAbs against dystrophin (anti-DYS11, kindly provided by J. C. Perriard, Institute of Cell Biology, ETH Zurich, Switzerland); mouse mAbs against myomesin [clone B4, kindly provided by J. C. Perriard, Institute of Cell Biology, ETH Zurich, Switzerland]; mouse mAb against sarcomeric myosin heavy chains, which binds to all sarcomeric isoforms (1:5000, 3 h, room temperature; Alexis Immunoresearch Laboratories Inc., West Grove, PA) or Cy3-conjugated rabbit anti-guinea pig antibody (Dako, Glostrup, Denmark) combined with rhodamine-phalloidin or fluorescein isothiocyanate-phalloidin (Molecular Probes, Inc., Eugene, OR). Before use, the anti-DYS11 was tested for specificity by histochemistry and in immunoblots (results not shown).

**Table 1.** Body weight, heart weight, glucose, and free fatty acids in the blood of control and diabetic rats after 2 wk of treatment with STZ (five animals per group; mean values ± SD)

<table>
<thead>
<tr>
<th></th>
<th>BWbeg (g)</th>
<th>BWend (g)</th>
<th>HWend (mg)</th>
<th>HW/BWend (mg/g)</th>
<th>Nonfasted blood glucose (mmol/liter)</th>
<th>Nonfasted free fatty acids in the blood (μmol/liter)</th>
</tr>
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<tbody>
<tr>
<td>Control rats</td>
<td>172 ± 4</td>
<td>246 ± 6</td>
<td>868 ± 74</td>
<td>3.53 ± 0.16</td>
<td>12.24 ± 0.8</td>
<td>266 ± 114</td>
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<td>STZ rats</td>
<td>173 ± 4</td>
<td>246 ± 6</td>
<td>868 ± 74</td>
<td>3.53 ± 0.16</td>
<td>12.24 ± 0.8</td>
<td>266 ± 114</td>
</tr>
<tr>
<td>Significance</td>
<td>ns</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>ns</td>
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BWbeg, Body weight before treatment; BWend and HWend, body and heart weight after treatment; HW/BWend, ratio of heart over body weight after treatment; ns, not significant.
Derived from measurements performed on myocytes extracted in 12.5 molar potassium hydroxide from formalin-fixed heart tissue (14). At least 30 myocytes were measured from each animal (five STZ-animals and five control animals). Maximum myocyte length (± sd) was on average $132 ± 21$ (controls) and $138 ± 22 \mu m$ (STZ hearts); maximum cell width was $27 ± 17$ and $27 ± 15 \mu m$, respectively. Thus, myocyte length was $4.2\%$ larger in STZ hearts without reaching significance, whereas cell width was identical in STZ hearts and controls. In accordance, the mean profile area independently derived from measuring cell circumference was $5.8\%$ larger in STZ hearts than controls ($3654 ± 502 \text{ vs. } 3454 ± 512 \mu m^2$) but did not reach significance. The heart weight is primarily determined by the myocyte mass. To estimate the ratio of heart mass to body weight from the myocyte mass, the maximum myocyte volume was calculated, assuming that they possess an idealized cylindrical shape. The mean myocyte volume (± sd) was $76.0 ± 4.1 \mu m^3$ for STZ hearts and $74.1 ± 4.7 \mu m^3$ for controls. The myocyte volume from STZ hearts yielded a $57\%$ higher value of the ratio to body weight than the myocyte volume from the control hearts ($P < 0.01$). This value of $57\%$ is close to that of $43\%$ we found for heart weight over body weight. Thus, the larger mean myocyte volume independently supports the relative cardiac hypertrophy derived from heart weight.

In addition, we found the kidney mass to be increased as well. The combined kidney weight of the STZ rats was on average $989 ± 29$, compared with $938 ± 13 \text{ mg}$ ($P < 0.01$) in controls, yielding a $45\%$ ($P < 0.001$) higher kidney to body mass ratio for the diabetic animals. Similar differences in kidney weights have been reported for STZ rats after 2 wk (23).

Finally, the nonfasted blood glucose and free fatty acid levels were on average twice as high in STZ rats, compared with controls (Table 1). The difference in blood glucose was highly significant, whereas the interindividual scatter of free fatty acids was too large to reach significance.

**Reduced myocyte contact in diabetic rats**

The myocardial structure was then analyzed in cardiac muscle sections. In the confocal microscope pictures of Figs. 1, A and B, and 2, A and B, the myocyte boundaries are outlined by immunofluorescent staining for dystrophin in green. In control hearts the myocytes are densely packed together, leaving little intercellular space (Fig. 1A). The dystrophin at the inner side of the surface membrane (sarcolemma) appears in yellow, resulting from overlapping with the red staining of intracellular actin by phalloidin-rhodamine. In contrast, the myocytes appear much more loosely packed in STZ hearts, leaving more intercellular space in between (Fig. 2B). Noticeably, most of the dystrophin staining associated with individually separated myocytes appears in green (almost no overlap with intracellular actin). Lack of cellular coherence was associated with increased intercellular space as reflected by the empty black space. Some myofibrillar cross-striation can be discerned in both controls and STZ-hearts.

Along with dystrophin (green), the sections in Fig. 2, A and B, are immunostained for connexin43 in red, which is mostly localized in the region of the intercalated discs marking the end-to-end connections of myocytes arranged in series. At lower magnification, the $Z$-line regions of control hearts are more regularly arranged in register (Fig. 2A) than the STZ heart in which they are individually scattered all over (Fig. 2B). At higher magnification, a reduction in immunofluorescence of connexin43 as well as of dystrophin is apparent in the STZ hearts (Fig. 2B). In the bottom row (Fig. 2C), connexin43 staining in red is combined with green staining for the sarcomeric marker myomesin. The left panel displays stronger and broader connexin43 staining in a control section than the diabetic heart sections in the middle and right panels. Furthermore, variable sarcomeric disarray was evident in the diabetic heart sections.

Collagen and extracellular matrix material was not visibly increased in trichrome-stained STZ heart sections (data not shown), as has been reported in the literature (12, 22).

**Apoptosis in the in vivo diabetes model**

As an indication for apoptosis, the relation of TUNEL-positive to total nuclei stained with DAPI was counted in sections from five control and five STZ hearts (Fig. 1C). Seven fields containing some 10,000 nuclei each were evaluated per section. TUNEL-positive nuclei averaged $0.0021 ± 0.0010\%$ in controls and increased by $3.3\text{-fold to } 0.0069 ± 0.0015\%$ ($P < 0.001$) in STZ hearts. In a number of separate sections, TUNEL-positive nuclei appeared only in cross-striated myocytes counterstained against myomesin. We cannot exclude, however, that some TUNEL-positive nuclei may stem from cells other than myocytes. Similar numbers of TUNEL-positive nuclei and TUNEL-positive nuclei with concomitant DAPI labeling were reported by Melvin et al. (19).

**FIG. 1. Reduced myocyte contact and enhanced apoptosis in diabetic rat hearts.** Double staining of dystrophin (A and B) in green and actin in red (yellow indicates overlapping of the two staining) and nuclei labeling (C) with the TUNEL assay and counterstaining with DAPI in cardiac muscle cross-sections of control (A) and diabetic (B and C) rats. Note the dense cell packing and almost complete overlap of actin staining with dystrophin (yellow) in control (A) as opposed to STZ hearts (B). The circles highlight apoptotic nuclei (C). Representative images of five animals per group.
positive cardiac nuclei have also been reported in the literature for control and STZ-treated animals (22).

High glucose inhibits myofibrillar formation in cultured adult rat cardiomyocytes

VARCs in primary culture undergo drastic remodeling when maintained in serum-containing milieu (17, 24). After settling of the rod-shaped myocytes on the substratum, most of the contractile structures are disassembled within the first 2 d. Over the following 5–6 d, the myocytes grow radially, and new myofibrils are formed until spontaneous beating activity sets in. The cultured myocytes increase in volume 2-to 3-fold, compared with their initial size; in other words, they hypertrophy in vitro. After 10 d in control culture at 5.5 mmol/liter glucose, new myofibrils fill out almost the entire cell body (Fig. 3A). However, exposure of the myocytes to 33.3 mmol/liter glucose for 8 d drastically reduces myofibrillar formation (Fig. 3B). The residual contractile structures, marked by immunostaining for the sarcomeric M-line protein myomesin, are concentrated in the central cell area. The myocytes are still filled out by an actin cytoskeleton lacking any cross-striation.

Glucose transporters and glucose uptake in cultured adult rat cardiomyocytes

It was important to clarify whether the myofibrillar impairment is due to high glucose in the myocytes, in other words, whether the high glucose in the medium is able to enter the cells, or whether high glucose down-regulated the glucose transporters so that the structural damage of the cells may rather be due to glucose starvation as observed in mouse blastocytes (25). Densitometric analysis of the insulin-dependent main glucose transporter GLUT4 in immunoblots revealed no change of protein level between myocytes cultured for 10 d in either 5.5 or 33.3 mmol/liter glucose medium (Fig. 4A). In addition, there was no apparent difference in confocal microscope pictures of GLUT4 translocation from cytosolic vesicles to the cell membrane in the low- and high-glucose myocytes on 30-min challenge with insulin (Fig. 4B). Subsequently we measured uptake of radioactively labeled glucose and 2-deoxyglucose (the latter of which is transformed only to deoxyglucose-6-phosphate and not further metabolized). Deoxyglucose was equally taken up from 10 through 40 min by both low- and high-glucose-conditioned myocytes (Fig. 4B). Finally, the possible effect of preincubation at different glucose concentrations on subsequent acute glucose uptake was tested. Therefore [14C]glucose was offered for 60 min in medium containing 5.5 mmol/liter glucose and 33.3 mmol/liter. The acute response was similar,
independently of the glucose concentrations in the culture medium before the acute experiment (Fig. 4C). Thus, the intracellular glucose level of VARCs in culture is reflected by the ambient glucose concentration in the medium.

**Myosin heavy chain and actin expression in cultured adult rat cardiomyocytes**

The detailed study on the level of the major contractile proteins reveals a graded reduction of the sarcomeric myosin heavy chains (myosin-HC) and cardiac α-actin to about half, depending on both the duration and time point when the high glucose was added to the culture medium (Fig. 5). In contrast, the nonsarcomeric smooth muscle α-actin did not change at all. The most drastic reduction of myofibrillar proteins occurred when high glucose was present over the entire culturing period of 10 d, including the most intensive remodeling phase during the early days. Shorter periods of high glucose during a later phase had a milder effect, but it may still be detrimental in the long run by disrupting the rebuilding of myofibrillar structures during the normal turnover. Despite these changes, the myocytes retained their autonomous beating activity (data not shown).

**Protection of myofibrillar destruction by antioxidant treatment**

Increased oxidative stress has been proposed to be implicated in the complications of diabetes (12). Production of reactive oxygen species and cell death can be drastically reduced by NAC, a thiol-containing radical scavenger and glutathione precursor, in STZ rat hearts and isolated VARCs (12). We therefore tested whether down-regulation of myosin-HC protein, as assessed from immunoblots, and destruction of myofibrils by high glucose in VARCs can be prevented by the addition of a combination of NAC and vitamin E throughout the whole culture period (Fig. 6). Myofibrillar appearance was assessed according to the criteria defined in Materials and Methods. The antioxidant regimen completely prevented both myosin-HC down-regulation and myofibrillar damage. In fact, the antioxidants led to an increase of the values for myosin-HC accumulation and myofibrillar intactness above 100%, which was set for the untreated control VARCs. These results indicate that oxidative stress can fully account for the impairment of sarcomeric myosin accumulation and myobrillar viability and that the antioxidative regimen even improves assembly of sarcomeric myosin and myofibrils in untreated VARCs in long-term culture.

**Discussion**

The prevalence of diabetes has dramatically increased in Western societies and with it the frequency of diabetes-
related heart failure. In the present study, we observed that STZ-induced diabetes in rats leads to a specific cardiomyopathy characterized in vivo by cardiomyocyte apoptosis and loss of intercellular contact. In the in vitro model of isolated VARC in culture, high glucose inhibited the formation of myofibrils that can be prevented by antioxidants.

In an animal model of obesity, apoptosis induced by increased concentrations of lipids (lipoapoptosis) appears to contribute to cardiac dysfunction (26–29). Here we show that cardiomyocyte apoptosis is also ongoing in an animal model of diabetes. It is likely that the underlying mechanisms of this apoptotic process are similar in both animal models and could be due to the deleterious effects of dyslipidemia. Indeed, we have previously shown in isolated cardiomyocytes that the saturated palmitic acid induces apoptosis via de novo ceramide formation and activation of the mitochondrial apoptotic pathway (11). The increase in nonfasted free fatty acid blood levels after 2 wk of STZ treatment (Table 1), although not significant, seems to be in line with this hypothesis. In contrast, high glucose has no effect on the intrinsic apoptosis rate of VARCs in culture (11). Because cardiomyocyte demisision by apoptosis is considered to contribute to the development of heart failure in humans (8, 30), it is conceivable that the observed increase in apoptosis during STZ-induced diabetes may contribute to the development of cardiomyopathy.

For the in vivo study, we used the STZ-induced diabetic rats, characterized by insulin deficiency. It is now widely accepted that pancreatic islet β-cell death occurs in both type 1 and 2 diabetes, leading to absolute or relative insulin deficiency (for review, see Ref. 31). Therefore, diabetes induced by STZ can be considered as a model of both diabetes types. Nevertheless, the model has its limits for each type. On the one hand, a single injection of STZ usually does not lead to an autoimmune reaction as observed in type 1 diabetes, and on the other hand, the animals lack a significant resistance to insulin action as seen in type 2 diabetes. Nevertheless, metabolic changes typically observed in patients with diabetes, characterized by hyperglycemia and increased circulating free fatty acids, were induced. Thus, eventually the heart will be exposed to the major compounds of a diabetic milieu.

Adult cardiomyocytes in long-term culture undergo drastic morphological transitions (32). After attachment, the contractile and cytoskeletal structures are almost completely degraded. This is followed by reexpression of some fetal genes and regeneration of the myofibrillar apparatus. Similar changes in protein expression can occur in vivo during the remodeling process of cardiac tissue. Therefore, the deleterious effects of glucose on myofibrillar formation and structure may be partly due to acceleration of dedifferentiation.

The low number of TUNEL-positive nuclei, both in controls and STZ hearts, corresponds to recently published values for the same in vivo diabetic rat model (22). These authors reported the highest number of TUNEL-positive myocyte nuclei (0.025%) to occur 3 d after STZ administration, which then diminishes to 0.016% at 10 d, and 0.0072% at 28 d, whereas the baseline level was constant at 0.002% in untreated animals. No signs of necrosis were detected at all. Because the process of apoptosis seems to be completed within a few hours, leaving no inflammatory traces, its manifestation as TUNEL-positive nuclei may underestimate the true amount of cell death (33). In addition, TUNEL-positive nuclei in cardiomyocytes were also reported to indicate active DNA processing in repair mechanisms that need not necessarily result in apoptotic completion (discussed in Ref. 34). Yet in the long term, cardiomyocyte demise may well contribute to diabetic heart failure.

Our results indicate that the disturbed formation of myofibrils in cultured VARCs is mediated by oxidation and not by intracellular glucose deprivation. Application of antioxidants was sufficient for effective prevention of the glucose-induced myofibrillar degeneration and was even able to improve myofibrillar formation in untreated control myocytes (Fig. 6). This latter finding may not be surprising because VARCs in culture are exposed to a much higher oxygen tension, responsible for some reactive oxygen species production, than in the heart tissue in vivo (around five times lower than in VARCs) (35, 36). We have earlier shown that aminoxydine fails to prevent the deleterious effect of high glucose on the myofibrils (11). This suggests that formation of nitric oxide and/or advanced glycation end products does not play a major role in the destructive process.

When considering developing a therapeutic stratagem from these observations, one has to bear in mind that the animal model develops diabetes very fast and covers only a short period of the disease. Diabetes in patients develops slowly and represents a chronic disease complicated by additional factors promoting diabetic cardiomyopathy including angiopathy, endothelial dysfunction, and autonomous dysregulation. Therefore, the potential benefit of the proposed therapeutic stratagems remains to be shown.

In the long run, cardiomyopathy is invariably associated...
with pathological remodeling of the heart. This maladaptive process is associated with changes in structural tissue organization and alterations of intracellular Ca\(^{2+}\) handling and affects metabolism, eventually leading to heart failure characterized by contractile dysfunction and risk for hazardous arrhythmias. This may be partly due to alterations of the gap junctions, which provide for electrical coupling of adjacent cardiomyocytes (for review, see Ref. 37). Expression of connexin43, the main ventricular connexin isoform, is decreased in diabetic rat hearts concomitant with a loss of contact among the cardiomyocytes. These observations could explain the disorganization of the heart tissue in diabetic cardiomyopathy.

In conclusion, our results show that a diabetic milieu leads to structural alteration of the heart at multiple levels including apoptosis, cell-cell contacts, and contractile structures. An understanding of the pathway of these glotoxic and lipotoxic effects is required for opening the door to new therapeutic stratagems. Based on current thinking, unsaturated fatty acids and antioxidants appear as promising approaches.

Acknowledgments

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