Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA

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Ghosh, Sanjoy, Dake Qi, Ding An, Thomas Pulnilkunnil, Ashraf Abrahani, Kuo-Hsing Kuo, Richard B. Wambolt, Michael Allard, Sheila M. Innis, and Brian Rodrigues. Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA. Am J Physiol Heart Circ Physiol 287: H2518–H2527, 2004. First published July 29, 2004; doi:10.1152/ajpheart.00480.2004.—Diabetic patients are particularly susceptible to cardiomyopathy independent of vascular disease, and recent evidence implicates cell death as a contributing factor. Given its protective role against apoptosis, we hypothesized that dietary n-6 polyunsaturated fatty acid (PUFA) may well decrease the incidence of this mode of cardiac cell death after diabetes. Male Wistar rats were first fed a diet rich in n-6 PUFA [20% (wt/wt) sunflower oil] for 4 wk followed by streptozotocin (STZ, 55 mg/kg) to induce diabetes. After a brief period of hyperglycemia (4 days), hearts were excised for functional, morphological, and biochemical analysis. In diabetic rats, n-6 PUFA decreased caspase-3 activity, crucial for myocardial apoptosis. However, cardiac necrosis, an alternative mode of cell death, increased. In these hearts, a rise in linoleic acid and depleted cardiac glutathione could explain this “switch” to necrotic cell death. Additionally, mitochondrial abnormalities, impaired substrate utilization, and enhanced triglyceride accumulation could have also contributed to a decline in cardiac function in these animals. Our study provides evidence that, in contrast to other models of diabetic cardiomyopathy that exhibit cardiac dysfunction only after chronic hyperglycemia, n-6 PUFA feeding coupled with only 4 days of diabetes precipitated metabolic and contractile abnormalities in the heart. Thus, although promoted as being beneficial, excess n-6 PUFA, with its predisposition to induce obesity, insulin resistance, and ultimately diabetes, could accelerate myocardial abnormalities in diabetic patients.

Obesity is a common nutritional condition in the Western world, with attendant disorders, such as hypertension, insulin resistance, and type 2 diabetes. Over time, patients who develop diabetes are susceptible to heart disease, a leading cause of death in these patients (38). Factors that largely account for this increased incidence of cardiovascular dysfunction include large and small coronary vessel disease (38). However, a significant number of diabetic patients continue to suffer from left ventricular dysfunction and clinically overt congestive heart failure in the absence of vascular defects (8, 38). Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal contractile protein and enzyme function, (38), and, more recently, apoptosis, a regulated energy-dependent cell suicide mechanism (14, 16). Given that animal models of diabetes [e.g., the Zucker diabetic fatty rat (50) and the streptozotocin (STZ) diabetic rat (24)] also display progressive myocardial abnormalities in the absence of vascular changes but in the presence of accelerated cell death, it is possible that this loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy, and eventual contractile impairment.

Increase in plasma glucose during diabetes has been suggested to predispose cardiomyocytes to death by apoptosis (16). However, in diabetes, cardiac energy production is almost exclusively from fatty acids, which are supplied in excess to the heart and have been implicated in cardiac lipid overload and cell death (13, 37). Thus, in vitro, palmitic acid, a saturated fatty acid, causes intracellular accumulation of ceramide and reactive oxygen species in cardiomyocytes and can precipitate apoptotic cell death in the presence or absence of hyperglycemia (13, 27). Recently, we demonstrated that feeding a 20% (wt/wt) palm oil diet (rich in palmitic acid) to diabetic rats enhances cardiac apoptosis in vivo (17).

The majority of studies that have looked at the relations between lipotoxicity and cardiovascular complications of diabetes have utilized lard or other sources of saturated dietary fat rich in palmitic acid (7, 17, 26). However, in humans, increased awareness of obesity and its cardiovascular complications have led to an indiscriminate substitution of atherogenic saturated cooking fats with “heart-friendly” refined vegetable oils, such as sunflower oil, rich in n-6 polyunsaturated fatty acids (PUFA) (42). In several studies, n-6 PUFA conferred protection against arrhythmias (32) and coronary artery disease (12) and, at least in human primary fibroblasts and Leydig cells, prevented apoptosis (4, 31).

Substantial data exist on the role of saturated fatty acid in the development of cardiac apoptosis and, ultimately, cardiomyopathy in animal models of diabetes. However, in light of the current dietary recommendations, the impact of the more relevant n-6 PUFA on cardiac function and morphology, especially during diabetes, is not clearly defined. We hypothesized that, given the role of saturated fatty acids in accelerating cardiac apoptosis after diabetes, switching to an n-6 PUFA-rich diet may well be protective against cell death. Instead of preventing cardiac cell death, our data for the first time suggest that, during diabetes, along with other morphological and functional abnormalities, n-6 PUFA converts the mode of cellular demise to necrosis, an alternate form of cell death.
MATERIALS AND METHODS

Experimental animals. This investigation conforms to the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication No. 85-23, Revised 1985] and the University of British Columbia. Previous studies have established that feeding 20% (wt/wt) sunflower oil for 4 wk increases the n-6 PUFA content of circulating lipoproteins (35), a major source of fatty acid to the heart. To investigate potential beneficial effects of n-6 PUFA, male Wistar rats (220–240 g) were first fed an n-6 PUFA diet [AIN-76A supplemented with 20% (wt/wt) sunflower oil; Research Diets; Table 1] for 4 wk to increase cardiac PUFA content [PUFA control (PC) group]. Energy contribution from fats in this diet was 40% of total energy intake, similar to human consumption (21). Some PUFA-fed animals were then given a moderate dose of STZ (55 mg/kg iv), a selective β-cell toxin (36). With this dose of STZ, there is only a partial destruction of β-cells with ~50% reduction in plasma insulin levels and stable hyperglycemia [PUFA diabetic (PD) group] within 24 h (39). An acute time period (4 days) was chosen on the basis of previous studies that demonstrated peak incidence of cardiac apoptosis after 3 days of STZ-induced diabetes in normal chow-fed rats (14). PUFA feeding was continued subsequent to STZ administration, and after 4 days of diabetes, rats were anesthetized with pentobarbital sodium (65 mg/kg ip), blood was collected, and hearts were excised for histological, morphological, and biochemical analysis. Additional control [normal control (NC) group] and STZ [normal diabetic (ND) group] animals fed normal laboratory chow [5% corn oil; PMI Feeds; Table 1] were utilized to compare the effects of n-6 PUFA feeding and diabetes.

Separation and characterization of cardiac lipids. Total cardiac lipids were extracted and solubilized in chloroform-methanol-ace- tone-hexane (4:6:1 vol/vol/vol/vol). Separation of cardiolipin, tri-glycerides (TG), and fatty acids was achieved with an HPLC (2690 Alliance HPLC, Waters, Milford, MA) equipped with an autosampler and a column heater. Fatty acids were quantified as their respective methyl esters, with heptadecaenoic acid (17:0) used as the internal standard, with a Varian 3400 gas-liquid chromatograph equipped with a flame ionization detector, a Varian Star data system, and an SP-2330 capillary column (30 m x 0.25 mm ID; Supelco, Bellefonte, PA) (23). Fatty acids, TG, and cardiolipin were expressed as micrograms per milligram of protein.

Table 1. Composition of experimental diets

<table>
<thead>
<tr>
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<th>PUFA Diet</th>
<th>Normal Diet</th>
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<tr>
<td>Protein, g/100 g</td>
<td>24.1</td>
<td>23.4</td>
</tr>
<tr>
<td>Carbohydrate, g/100 g</td>
<td>44.9</td>
<td>49.9</td>
</tr>
<tr>
<td>Fat, g/100 g</td>
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<td>4.5</td>
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<tr>
<td>Fiber</td>
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<td>8.3</td>
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<tr>
<td>kcal/g</td>
<td>4.6</td>
<td>4.0</td>
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<tr>
<td>Fatty acids, g/100 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>3.8</td>
<td>1.6</td>
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<tr>
<td>PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-6</td>
<td>10.6</td>
<td>1.2</td>
</tr>
<tr>
<td>n-3</td>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Polyunsaturated fatty acid (PUFA) diet ingredients are as follows: casein, 80 mesh, ta-methionine, LoDex 10, cornstarch, sucrose, cellulose, BW200, corn oil, sunflower oil, mineral mix (dicalcium phosphate, magnesium oxide, potassium citrate, potassium sulfate, sodium chloride, potassium KSO4·12H2O), cupric carbonate, potassium iodate, ferric citrate, monoglycerides, sodium selenite, zinc carbonate), vitamin mix [vitamin A, vitamin D3, vitamin E, menadione sodium bisulfite, biotin (1%), cyanocobalamin (0.1%), folic acid, nicotinic acid, calcium pantotenate, pyridoxine·HCl, riboflavin, thiamin·HCl], and choline bitartrate. Normal diet ingredients are as follows: ground corn, soybean meal, beet pulp, fish meal, wheat, cane molasses, ground oats, mineral mix (see PUFA diet) and vitamin mix (see PUFA diet).

Cardiac caspase-3 activity. To investigate the contribution of the apoptotic pathway, caspase-3 activity, the prime effector of cardiomyocyte apoptosis, was estimated (Molecular Probes). Caspase-3 is a cysteine-containing protease crucial in the “execution” phase of apoptosis and breaks down the contractile proteins in the heart (20). Briefly, ventricular tissue (200 mg) was placed in cell lysis buffer and homogenized (4°C). Subsequently, the suspension was centrifuged at 12,000 g for 3 min, and the supernatant was separated and reacted with the aminomethylcoumarin (AMC)-derived substrate Z-DEVD-AMC. Caspase-3 activity is proportional to the amount of AMC released and is expressed as micromoles of AMC released per milligram of protein.

Cardiac necrosis. Necrosis leads to a loss of cell membrane integrity with release of lactate dehydrogenase (LDH), a major source of heart necrosis [ND and PD] were kept for 4 days before being killed. Measurements were obtained before death from fed animals. FFA, free fatty acids; TG, triglycerides. *Significantly different from PC, P < 0.05. †Significantly different from NC, P < 0.05. ‡Significantly different from all groups, P < 0.05.

Estimation of GSH. Cardiac GSH content of frozen, ground tissue was measured using a commercially available kit (Cayman Chemicals). GSH was reacted with 5,5′-dithiobis-2-nitrobenzoic acid to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The concentration of GSH was measured using a commercially available kit (Cayman Chemicals). GSH was reacted with 5,5′-dithiobis-2-nitrobenzoic acid to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The difference between total GSH and GSSG was the reduced GSH. Protein assay was performed according to the Bradford method. Values are expressed as nanomoles of GSH per milligram of protein.

ATP assay. ATP from heart tissue was extracted using 2.5% TCA, pH was adjusted to 7.8 with 1 M KHCO3, and ATP (nmol/mg protein) was measured by a luciferase-luciferin assay kit (Sigma).

Electron microscopy. Morphological evaluation of hearts was carried out using transmission electron microscopy. Briefly, left ventricular tissue was fixed in 1.5% glutaraldehyde and paraformaldehyde,
Heart and substrate oxidation. Hearts from halothane (2–3%)-anesthetized rats were perfused in the working mode with modified Krebs-Henseleit buffer (including 1.0 mM [9,10-3H]palmitate prebound to 3% BSA, 5.5 mM [U-14C]glucose, 2.0 mM calcium, and 100 U/l insulin) at a preload of 11.5 mmHg, as described previously (28). An afterload of 80 mmHg was maintained for the first 30 min. During this time, samples of perfusate and haemine hydroxide were taken every 10 min for measurement of glucose and fatty acid oxidation. Subsequently, the afterload was clamped off, causing the peak systolic pressure to increase to ~135 mmHg, and the perfusion was continued for another 20 min. Heart function [rate-pressure product (RPP) – heart rate (mmHg·min⁻¹·10⁻³)] was measured using a physiograph (Direcwin, Raytech).

Serum measurements. Serum samples were stored at −20°C until they were assayed. Diagnostic kits were used to measure glucose (Sigma), insulin and leptin (Linco), and nonesterified fatty acids (Wako).

Statistical analysis. Values are means ± SE. One-way ANOVA followed by Tukey’s test or the Holm-Sidak test was used to determine differences between group mean values. The level of statistical significance was set at \( P < 0.05 \).

RESULTS

General characteristics of experimental animals. Table 2 describes the general characteristics of the experimental groups. There was no difference in food intake (data not shown) between rats fed the n-6 PUFA diet and those fed normal chow for 4 wk. However, the n-6 PUFA diet increased body weight compared with normal chow. Circulating TG were higher in the PC than in the NC group, and serum insulin was increased in the PC group (likely as a consequence of insulin resistance) compared with the NC group, but there was no overt hyperglycemia in the PC group. STZ precipitated loss of circulating insulin and caused overt hyperglycemia in the ND and PD groups. Interestingly, in contrast to the ND group (361 ± 9 and 358 ± 9 g before and after STZ, respectively), cut into small blocks (~1 mm³), and fixed for 8 h at 4°C. After it was washed, the tissue was postfixed with 1% osmium tetroxide and further treated with 1% uranyl acetate and dehydrated using increasing concentrations of ethanol (50–100%). Blocks were embedded in molds, polymerized, and sectioned at ~100 nm. Sections were stained with 1% uranyl acetate and Reynold’s lead citrate. Images of the longitudinal sections were obtained with an electron microscope (model H7600, Hitachi).
diabetes in PUFA-fed animals was associated with a profound loss of body weight (450 ± 11 and 393 ± 11 g before and after STZ, respectively). This loss in body weight could not be attributed to any change in food or fluid intake but could be a result of excessive lipolysis and loss of adipose tissue mass.

Additionally, dietary n-6 PUFA feeding for 4 wk increased serum leptin levels compared with the NC group, whereas diabetes reduced leptin in the ND and PD groups. n-6 PUFA feeding induced a 6- to 7-fold increase in total cardiac free fatty acids and a 40- to 80-fold increase in total cardiac TG in the PC and PD groups and indicated a cardiac lipid overload in these animals.

Free PUFA content in the heart. Although several classes of fatty acids (e.g., saturated and monounsaturated) were measured, only the cardiac PUFA content in the free form is described in Fig. 1. Feeding 20% (wt/wt) sunflower oil successfully magnified n-6 (mainly linoleic and arachidonic acid) and n-3 (mainly eicosapentaenoic and docosahexaenoic acid) PUFA content in the heart. However, the increase in n-6 PUFA was substantially greater than the increase in n-3 PUFA and was reflected in an approximately twofold higher n-6-to-n-3 PUFA ratio in the PC group. Diabetes had no further effect on the n-6 or n-3 PUFA content.

Cardiac cell death. Previous studies have associated cardiac lipid overload to cell death via apoptosis. To determine cardiac apoptosis after n-6 PUFA feeding, caspase-3 activity, crucial for myocardial apoptosis, was estimated and determined to be unchanged in the PC and PD groups compared with the NC group (Fig. 2). In the ND group, the caspase-3-like activity was ~2.5-fold higher than control and indicates acceleration of apoptotic cell death, as previously described (14). Inasmuch as apoptosis can proceed in a caspase-3-independent manner, TdT-mediated dUTP nick end labeling (TUNEL) on myocytes was also performed and found to be increased in ND hearts but was insignificant in PD hearts (data not shown).

Absence of apoptosis may not signify lack of cell death, inasmuch as cell demise may also progress via a necrotic mechanism. Cardiac necrosis was absent in NC, ND, and PC hearts.

Fig. 3. Effect of 4 days of diabetes on cardiac cell death in animals fed a moderately high-PUFA diet for 4 wk. Formalin-fixed heart sections were treated with Masson’s trichrome stain. Black arrows, severe disruption of contractile apparatus (hypercontracted muscle and thickened fibers); white arrows, focal necrosis (gray discoloration) in positive control (isoproterenol-treated) hearts (E, ×400) and PD hearts (D, ×600). Cardiac necrosis was absent in NC (A, ×400), ND (B, ×400) and PC (C, ×400) hearts. Bottom right: lactate dehydrogenase (LDH) activity in serum collected at death. Values are means ± SE of 4 rats in each group. *Significantly different from all other groups, P < 0.05.
pathway. Incidence of necrosis by measurement of serum LDH revealed higher circulating levels of this enzyme marker only in PD hearts (Fig. 3, bottom right). However, inasmuch as serum LDH alone does not substantiate the incidence of cardiac necrosis, cardiac ventricles were stained with Masson’s trichrome (9). In hearts treated with isoproterenol, a β-agonist, severe disruption of the contractile apparatus, together with focal necrosis, was observed (Fig. 3E). No such morphology was evident in NC, ND, and PC hearts (Fig. 3, A–C). After induction of diabetes, PD hearts showed necrotic changes, which, on higher magnification, demonstrated extensive gray patches and focal necrosis with hypercontracted muscle fibers (Fig. 3D), characteristic of contraction band necrosis (3).

Cardiac GSH. Factors such as GSH levels, which dictate the redox environment of the cell and also the mode of cell death (11), were also measured. PUFA feeding was associated with a significant decrease in GSH. Interestingly, diabetes for 4 days in normal chow-fed rat hearts could not decrease cardiac GSH, whereas superimposition of diabetes in PUFA-fed animals led to a further decrease in cardiac GSH levels (Fig. 4A). Although cardiac GSSG was marginally increased in ND hearts compared with NC hearts, this difference did not reach significance. PUFA feeding had no effect on GSSG. However, induction of diabetes in the PD group increased cardiac GSSG almost threefold (Fig. 4B). Evaluation of the GSH-to-GSSG ratio revealed the lowest values in the PD group, indicating substantial oxidative stress in this group (Fig. 4C) (47).

Cardiolipin and mitochondrial morphology. Cardiolipin is a mitochondrial membrane phospholipid that is essential for optimal functioning of oxidative phosphorylation enzyme complexes and ATP generation (49). Diabetes in animals fed normal chow and high-fat diets did not alter cardiolipin levels compared with the respective controls (Fig. 5, top). n-6 PUFA feeding induced a profound loss of cardiac cardiolipin levels (Fig. 5, top), together with a drop in total ATP: 8.9 ± 0.6, 8.1 ± 0.8, and 6.9 ± 0.6 nmol ATP/mg protein in the NC, ND, and PC groups, respectively, vs. 5.1 ± 0.2 nmol ATP/mg protein in the PD group (P < 0.05). Inasmuch as changes in cardiolipin have also been associated with mitochondrial structural alterations, we scrutinized mitochondrial morphology in all the above groups. Figure 5A depicts a mitochondrion with a double membrane and lamellar cristae, which are typical in NC, ND, and PC hearts. A novel observation in this study was abnormal condensed mitochondria, but only in the PD group (Fig. 5B).

Glucose and palmitate oxidation. Inasmuch as condensed mitochondria suggest lack of energy production, glucose and palmitate oxidation (Fig. 6) in the isolated heart were studied in parallel with heart function. At low afterload (80 mmHg), rates of oxidation of exogenous glucose decreased in the ND and PC groups, with a parallel increase in palmitate oxidation in these groups. Interestingly, in the PD group, although glucose oxidation dropped further compared with the ND and PC groups, palmitate oxidation remained unchanged. To meet the energy demand at high afterload (135 mmHg), all except the PD group increased their glucose oxidation. With regard to fatty acid oxidation, only the NC group was able to increase palmitate oxidation, suggesting that, in the ND, PC, and PD groups, fatty acid oxidation was already operating at its maximum at low afterloads.

Electron microscopy and ultrastructural morphology. In the milieu of increased intracardiac fatty acid levels (Table 2) and in the absence of any further increase in fatty acid oxidation, we hypothesized that intracardiac TG will build up in PD hearts. Figure 7 demonstrates ultrastructural morphology of hearts isolated from the different groups. As expected, although lipid droplets were visible in both n-6 PUFA-fed groups, only PD hearts demonstrated abundant lipidlike vacuoles. Additionally, NC, ND, and PC hearts showed highly packed myofibrils separated by rows of mitochondria. Myofibrillar sarcomeres in these three groups exhibited clear Z-lines, whereas PD hearts demonstrated more diffuse Z-lines, indicat-
ing myofibrillar damage. These findings are consistent with necrosis in PD hearts (Fig. 3) and could precipitate an early contractile defect.

Estimation of heart function. Cardiac mechanical function measured as the RPP of the different groups is summarized in Fig. 8. There were no differences in RPP between the groups at normal workload (80 mmHg). Subsequent to an increase in workload to 135 mmHg, RPP increased in all groups. Compared with the NC group, neither fat feeding nor STZ-induced diabetes affected heart function in response to this high workload. Interestingly, superimposition of diabetes for 4 days in the PD group induced the smallest increase in cardiac function.

DISCUSSION

Numerous clinical and epidemiological reports have confirmed that diabetic patients are particularly susceptible to cardiomyopathy, independent of vascular disease, and recent evidence points to cell death as a potential contributing factor (38). Indeed, in a recent study, using TUNEL assay, we detected a fivefold increase in apoptosis in the diabetic heart and further validated this observation by identifying an increase in the activity of caspase-3, the proteolytic enzyme involved in the execution of apoptosis (17). In this study, an increase in cardiac caspase-3 activity was also observed in the ND group. Despite this augmented apoptosis in the diabetic heart, the number of cells affected in this and other studies (14, 16) is relatively minor and may explain the lack of functional changes at this early time point. However, given that apoptotic cells are cleared rapidly by phagocytes (20) and in the absence of any significant regeneration of the adult myocardium, low cell death rates over chronic periods of hyperglycemia may still result in progressive loss of myocytes and contractile failure (20).

Interestingly, compared with the ND group, superimposition of diabetes in 20% (wt/wt) PUFA-fed rats did not change caspase-3 activity or TUNEL-positive myocytes, and the prevalence of apoptosis in this group is questioned. At least in cell lines, n-6 PUFA is also known to block apoptosis (4, 31). Overall, our data suggest that the type, rather than the amount, of fatty acid controls lipotoxic apoptosis (30). Indeed, feeding a 20% (wt/wt) palm oil diet [rich in palmitic acid, which is converted to proapoptotic ceramide (30)] increases apoptosis in control hearts, a process that was further aggravated after induction of diabetes (17).

Although apoptosis is the predominant form of cellular demise, the diabetic heart is also characterized by necrosis, an “accidental” form of cell death (16). Assessment of serum markers or histochemical detection of necrosis did not reveal any necrotic change in NC, ND, and PC hearts. However, the PD group demonstrated a rise in serum LDH and severe
cardiac necrosis, as evidenced by light microscopy and appropriate staining. Furthermore, ultrastructural evidence demonstrated diffuse Z-lines, suggesting myofibrillar damage in PD hearts, indicative of necrotic changes. In an effort to determine the elements that induce necrosis, we measured the cardiac free fatty acid species and determined that sunflower oil feeding increased cardiac n-6 PUFA, predominantly linoleic acid. In clinical studies, accumulation of linoleic acid has been strongly associated with an increased inflammatory response in endothelial cells, in addition to an augmented risk of myocardial infarction and necrosis (46). Additionally, in vitro, linoleic acid precipitates necrosis via proinflammatory pathways in various cell types (44, 45).

Oxidative stress has also been implicated in the etiology of several lipotoxic and diabetic complications (8, 13) and can arise as a result of an imbalance between the production of free radicals and their neutralization by antioxidants such as GSH (8, 47). In ventricular myocytes, under conditions of oxidative stress, GSH is converted to GSH disulfide (GSSG) and the GSH-to-GSSG ratio decreases (i.e., a high GSH-to-GSSG ratio maintains a normal reducing intracellular environment). Interestingly, n-6 PUFA feeding induced a loss of reduced GSH, which decreased further with diabetes, an effect that could be directly linked to an increased conversion of reduced GSH to GSSG under conditions of augmented oxidative stress (47). A major functional impact of a decreased GSH-to-GSSG ratio is oxidation of regulatory proteins at cysteine residues (11, 47). For caspase activation and propagation of the apoptotic pathway, an intact cysteine moiety, which can easily be oxidized under increased oxidative stress and GSH-depleted conditions (19), is necessary. Overall, our data suggest that increased cardiac n-6 PUFA content and depleted cardiac GSH operate in concert to allow a “switch” to necrosis in PD hearts.

Compromised myocardial energy status may also dictate necrotic death. Cardiolipin, a mitochondrial membrane phospholipid essential for optimal functioning of oxidative phosphorylation enzyme complexes and ATP generation (49), was estimated, along with total myocardial ATP levels. In myocardial ischemia, disappearance of total cardiolipin resulted in loss of oxidative phosphorylation activity (29) and could explain the drop in mitochondrial ATP synthesis. Total cardiolipin decreased almost sixfold after n-6 PUFA feeding, with a significant drop of ATP only in PD hearts, which could have contributed to cardiac necrosis (40). Changes in cardiolipin have been associated with mitochondrial morphological alterations. In Barth’s syndrome, a genetic disorder characterized by dilated cardiomyopathy, a lack of cardiolipin in mitochondria has been described, together with abnormal mitochondria with concentric cristae (10). A novel observation in this study was that, analogous to Barth’s syndrome, n-6 PUFA feeding for 4 wk, together with 4 days of hyperglycemia, led to similar changes in some mitochondria. Interestingly, abnormal condensed mitochondria have been recently recognized in skeletal muscle and cardiac atrial neurons from diabetic patients (25). Given that control and diabetic PUFA groups showed similar changes in cardiolipin, it could be assumed that the altered mitochondrial morphology in PD animals is a consequence of an additional effect of hyperglycemia; i.e., when hyperglycemia sets in, there is augmented oxidative stress and extensive damage to the mitochondria already compromised by depleted cardiolipin levels (34).

Structural damage to the mitochondria is associated with derangements in fuel utilization and could lead to cardiac contractile impairment. Compared with glucose, fatty acids are the preferred substrate consumed by the heart, providing ~70% of its energy requirements. With hypoinsulinemia or insulin resistance, which characterizes ND and PC animals, respectively, glucose utilization is compromised, and the heart is forced to use higher levels of fatty acids (37). Interestingly, PD animals, characterized by a combination of insulin resistance initially followed by eventual hypoinsulinemia after STZ, demonstrated a precipitous drop in cardiac glucose utilization with no further increase in fatty acid oxidation. Despite these changes in substrate preference, heart function at low afterloads remained unchanged. At higher afterloads, glucose and fatty acid oxidation increased in the NC group. In ND and PC hearts, the only increase was that of glucose oxidation, inasmuch as, presumably, fatty acid oxidation was already operating at its maximum. The PD group alone failed to increase its glucose oxidation in response to a higher energy

Fig. 6. Glucose and palmitate oxidation at low (A, 80 mmHg) and high (B, 135 mmHg) afterloads. During measurement of heart function, samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of substrate oxidation. Values are means ± SE of 4 rats in each group. NC, normal chow diet before diabetes induction; ND, normal chow after diabetes induction; PC, PUFA-rich diet before diabetes induction; PD, PUFA-rich diet after diabetes induction. *Significantly different from low afterload, P < 0.05. †Significantly different from all other groups. #Significantly different from NC.
demand, an aspect that could have contributed to the drop in cardiac function. Interestingly, when diabetic hearts are perfused with glucose and fatty acids, addition of dichloroacetate, a stimulator of glucose oxidation, to the perfusion buffer improved heart function (33). More recently, shifting substrate utilization from fatty acids to glucose using trimetazidine improved left ventricular function in patients with diabetes and ischemic cardiomyopathy (15), and in transgenic db/db mice, which overexpress GLUT-4 glucose transporters, glucose metabolism and contractile function were normalized (5). Our study provides novel evidence that, in contrast to other models of diabetic cardiomyopathy, which exhibit cardiac dysfunction only after chronic hyperglycemia, n-6 PUFA feeding coupled with only 4 days of diabetes can precipitate metabolic and contractile abnormalities in the heart.

The heart and other nonadipose tissues have an inadequate ability to handle excess lipids, and accumulation of TG has been proposed to impair cardiac function (48, 50). In our study, n-6 PUFA feeding substantially increased cardiac fatty acid in the PC and PD groups, but only PD hearts demonstrated a considerable increase in lipid droplets. Given that fatty acid oxidation is likely operating at a maximum in PC hearts, the excess circulating fatty acids in PD hearts are likely channeled toward TG synthesis and could explain the increase in lipid

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Fig. 7. Ultrastructural cardiac morphology. Representative electron micrograph of hearts from NC, ND, PC, and PD animals are depicted. Scale bar, 1 μm. M, mitochondria; Nu, nucleus; L, lipid-like vacuoles. White arrows, Z-lines.

Fig. 8. Cardiac function at low (80 mmHg) and high (135 mmHg) afterloads. Isolated hearts were perfused in working mode at an afterload of 80 mmHg for the first 30 min. Then afterload was clamped off, causing peak systolic pressure to increase to \( \sim 135 \) mmHg, and perfusion was continued for another 20 min. Values are means ± SE of 4 rats in each group. *Significantly different from all other groups at afterload \( \sim 135 \) mmHg, \( P < 0.05 \).

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Fig. 8. Cardiac function at low (80 mmHg) and high (135 mmHg) afterloads. Isolated hearts were perfused in working mode at an afterload of 80 mmHg for the first 30 min. Then afterload was clamped off, causing peak systolic pressure to increase to \( \sim 135 \) mmHg, and perfusion was continued for another 20 min. Values are means ± SE of 4 rats in each group. *Significantly different from all other groups at afterload \( \sim 135 \) mmHg, \( P < 0.05 \).
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droplets in these hearts. Interestingly, in Zucker fatty rats, when glucose utilization is compromised (e.g., during fasting), increased lipid availability is associated with cardiac lipid accumulation (48). It is unclear why PD hearts were unable to increase fatty acid oxidation, even in the presence of excess fatty acids. In this regard, leptin, a liporegulatory hormone, increases fatty acid entry and TG storage in adipose tissue but activates fatty acid oxidation and decreases TG accumulation in the heart (2). Although leptin was high in PC animals, PD animals demonstrated low insulin and leptin levels, factors that could have contributed to fatty acid being channeled from oxidation to increased cardiac TG assembly.

Although the n-6 PUFA-rich diet was utilized as a beneficial regimen in this study, sunflower oil induced obesity and insulin resistance in a similar manner to other saturated fat diets. However, insulin resistance, hyperinsulinemia, and impaired glucose tolerance, which characterize obese humans, are usually associated with transition to frank diabetes when secretion of insulin is no longer able to overcome insulin resistance and impaired glucose disposal. In rodents fed high-fat diets, insulin resistance does not progress to hyperglycemia in the absence of genetic defects (36). Thus our present model, in which a low dose of STZ was used to precipitate overt hyperglycemia after insulin resistance, is a close approximation of the human condition, where there is a gradual evolution of insulin resistance to hyperglycemia.

In summary, chronic caloric excess of n-6 PUFA when coupled with acute diabetes of only 4 days precipitated mitochondrial abnormalities, a steep drop in GSH, altered substrate utilization, and myocardial TG deposition. Given that these hearts also demonstrated necrosis and extensive myocardial cell loss, a feature that is predominant only in chronic diabetes (1, 14, 16, 24), our data suggest that this mode of cell death in PUFA-fed diabetic hearts is an important factor in accelerating diabetic cardiomyopathy. Although these effects of n-6 PUFA in the diabetic animal would seem contrary to accepted belief as being beneficial, in countries such as Israel, with high dietary n-6 PUFA consumption, there is an excessive incidence of obesity, insulin resistance, hypertension, and type 2 diabetes (6). Thus the dilemma with recommending vegetable oils such as sunflower oil as a source of PUFA is that they contain high levels of n-6 PUFA but negligible amounts of n-3 PUFA, the primary candidate for conferring cardiovascular benefit. Given the concurrent scarcity of fish oil in the Western diet, the main source of n-3 PUFA, the n-6-to-n-3 PUFA ratio has been amplified from a traditional balance of 1:1 to an astounding 15:1 to 20:1 in the modern diet (41); thus advocating diets rich amplified from a traditional balance of 1:1 to an astounding 15:1 to 20:1 in the modern diet (41); thus advocating diets rich

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