Fructose-3-Phosphate Production and Polyol Pathway Metabolism in Diabetic Rat Hearts

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Previous studies have suggested that polyol-pathway and nonenzymatic glycation may be involved in the development of cardiac myopathy, a well-known manifestation of diabetes. Although the exact etiology of this complication is not fully understood, it is likely to be multifactorial. In this study, we investigated the metabolic consequences of diabetes and the effect of aldose reductase inhibitor (ARI) treatment on cardiac tissues of Sprague-Dawley rats. Perchloric acid (PCA) extracts of hearts from the animals were examined using ^{31}P-nuclear magnetic resonance (NMR), gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC). In ^{31}P-NMR spectra of diabetic animals, a peak resonating at the chemical shift of 5.8 ppm with a coupling constant of 10 Hz was identified as fructose-3-phosphate (F3P). Undetectable in controls (< ~20 nmol/g), this metabolite was present at a concentration of 81.3 ± 16.3 nmol/g wet weight (n = 4) in diabetic rat hearts. GC/MS analysis of these extracts from diabetics also identified a decomposition product of F3P, 3-deoxyglucosone (3DG), at a concentration of 9.4 ± 3.5 nmol/g (n = 3), compared with 0.98 ± 0.43 nmol/g (n = 3) in controls. No evidence was found for the expected detoxification products of 3-DG, 3-deoxyfructose and 2-keto 3-deoxygluconate. Concomitant with the elevation of F3P and 3DG, fructose and sorbitol levels were also elevated in diabetic animals. Surprisingly, ARI treatment was found to have no effect on the levels of these metabolites. These data suggest that either the heart may be unique in its production of fructose or it may not readily transport the ARI sorbinil. Production of the potent glycating agents F3P and 3DG in diabetics suggests that these compounds may be contributing factors in the glycation of cardiac proteins in the diabetic rat heart.

CARDIOMYOPATHY is a well-established manifestation of diabetes.[1-5] The etiology of this complication is likely to be a consequence of a combination of factors such as dysfunction of the autonomic nerves,[6] fibrosis,[7-9] collagen deposition,[9-11] and a multitude of other biochemical changes in the cardiac muscle.[12-19] The cumulative effect of these processes may ultimately distort the normal mechanical performance of the heart. Some of the biochemical alterations documented in diabetic hearts are manifested as a change in various factors such as sacrolemmal Ca^{2+} and Na^{+}-K^{+} pump activity,[15-17] activation of protein kinase C,[19] decrease of glucose transporters,[14] various metabolic abnormalities,[20] and increased nonenzymatic glycation.[21-23] Our interest is in the nonenzymatic glycation hypothesis,[24-26] which states that some of the complications in diabetics are a consequence of the reaction of the carbonyl group of sugars with the amines of proteins, which ultimately rearrange to form advanced glycation end products. This biochemical phenomenon has been well documented in a number of tissues affected by diabetes, including the lens,[27,28] kidney,[25,30] erythrocytes,[31] and heart.[21,23]

Studies in this laboratory have documented that one contributor to the nonenzymatic glycation process is fructose-3-phosphate (F3P), acting either by direct reaction with protein or via its decomposition product 3-deoxyglucosone (3DG).[32,33] F3P is produced by enzymatic phosphorylation of fructose,[34,35] which in turn is produced primarily via the polyol pathway.[35] An increased flux through this pathway,[36] which converts glucose to sorbitol and fructose, has been implicated in the development of diabetic complications in the lens, retina, and peripheral nerves.[36-40] While the exact mechanism by which the polyol pathway contributes to complications is still not clear, its involvement may be a consequence of a number of factors such as increased osmotic stress,[36] depletion of *myo*-inositol,[41,42] oxidative stress,[43] pseudohypoxia,[44] and production of glycating agents.[33] Regardless of the details of the mechanism, the best evidence supporting the involvement of this pathway in diabetic complications comes from studies using aldose reductase inhibitors (ARIs)[36-40] suggesting that these compounds are efficacious in the prevention of cataracts, retinopathy, and loss of motor nerve conduction velocity.[36-40]

Studies of cardiac tissue from diabetics have also suggested possible involvement of the polyol pathway in the development of cardiomyopathy.[4,12,13,45] Given this possibility and potential role of this pathway in development of complications in other tissues, we investigated the metabolic consequences of diabetes and ARI treatment on the levels of polyol pathway sugars in diabetic rat hearts. We examined cardiac tissue from diabetic rats by ^{31}P-NMR, HPLC and GC/MS and detected elevated concentrations of F3P and 3DG. Administration of an ARI to these diabetic animals had no effect on the increased levels of F3P and 3DG or fructose and sorbitol.

MATERIALS AND METHODS

Materials

F3P and 3DG were synthesized as described previously.[32,46] Streptozotocin was obtained from Sigma (St Louis, MO), and sorbinil was kindly donated by Pfizer (Groton, CT).

Animals

Male Sprague-Dawley rats (200 g) were divided into three groups with three to six animals per group: controls, diabetics, and diabetics fed

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Table 1. Characteristics of the Three Animal Groups at Death

Animal (n)	Body Weight (g)	Glucose (mg/dL)	Heart Weight (g)	Mean Water Consumption (g)
Normoglycemics (6)	453 ± 23	62 ± 5	1.6 ± 0.2	39 ± 6
Diabetics (6)	227 ± 92	327 ± 47	1.15 ± 0.19	237 ± 38
Diabetics + ARI (5)	216 ± 45	332 ± 64	1.03 ± 0.16	233 ± 35

sorbinil, an ARI, in drinking water (estimated dosage, 100 mg/kg/d). Diabetes mellitus was induced by intraperitoneal injection of streptozotocin (Sigma) at a dosage of 85 mg/kg in 0.01 mol/L sodium citrate buffer, pH 4.5. After injection of streptozotocin, diabetes was confirmed by measurement of glucose levels in venous blood for 24 hours using the One Touch blood glucose analyzer (Lifescan, Milpitas, CA). The consumption of water by both diabetic groups was similar (Table 1). Rats were killed after 55 days of diabetes with an overdose of pentobarbital. Hearts were dissected and immediately clamp-frozen. Lenses from rats with a duration of diabetes of 30 days were saved. These tissues were stored at −40°C until processed. Characteristics of the animals in the three groups before death are shown in Table 1.

Perchloric Acid Extracts

Frozen hearts from individual animals and pooled lenses from several animals were ground in a mortar chilled with liquid nitrogen. Eight volumes of 5% perchloroacetic acid (PCA) (wt/vol) were added to the ground powder. A 25-μL aliquot of 100-mmol/L methyl phosphonic acid was added as an internal standard for quantitation of phosphorylated metabolite by ^{31}P-nuclear magnetic resonance (NMR). The resultant slurry was centrifuged at 8,000 × *g* for 10 minutes. The supernatant was saved, and the pH was adjusted to between 5 and 6 by addition of potassium hydroxide. Perchlorate salt was precipitated after centrifugation at 5,000 × *g* for 10 minutes. The supernatant was lyophilized, and the dried powder was reconstituted in 1.5 mL D_2O and 150 μL 0.25-mol/L CDTA (1,2-diaminocyclohexanetetraacetic acid; Aldrich, Milwaukee, WI). CDTA was added as a chelating agent to achieve good spectral resolution in NMR spectra. The final pH of the extract was adjusted to 8.0.

Quantitation of Cardiac and Lenticular Sugars

A 0.5-mL aliquot of PCA extracts was passed through a column containing an anion-exchange resin (AG1-X8, Cl^- form; BioRad, Hercules, CA) and a cation-exchange resin (BioRad AG53-X8, H^+ form) to remove charged species. An aliquot of 2-deoxyglucose (Sigma;

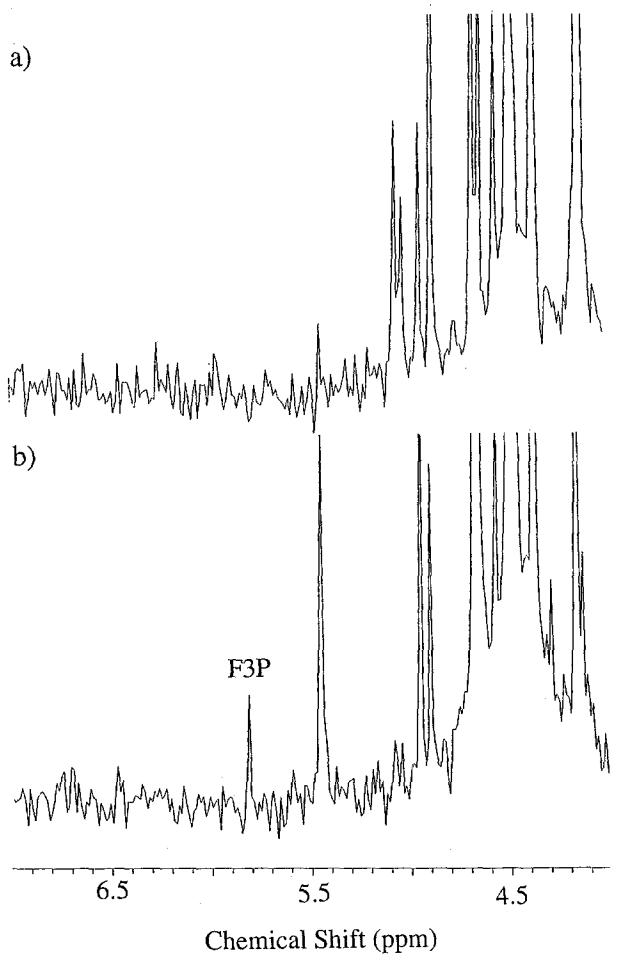

Fig 1. Typical ^{31}P-NMR spectrum of (a) normal and (b) diabetic rat hearts.

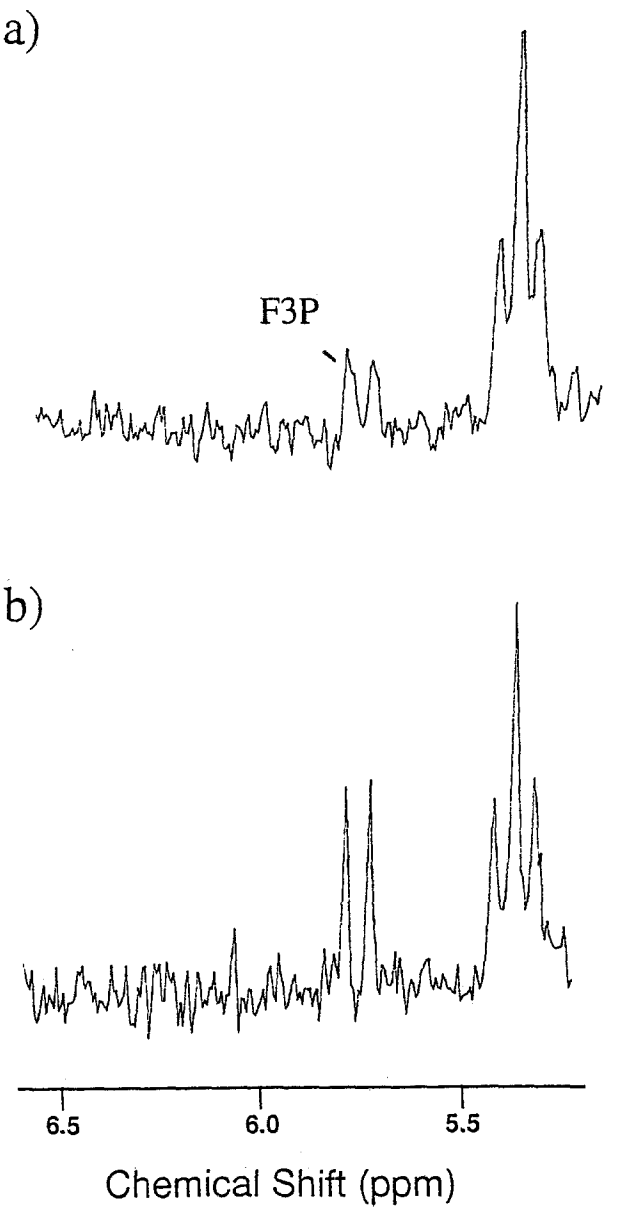

Fig 2. Proton-coupled ^{31}P-NMR spectrum of (a) diabetic rat heart extract and (b) extract spiked with authentic F3P.

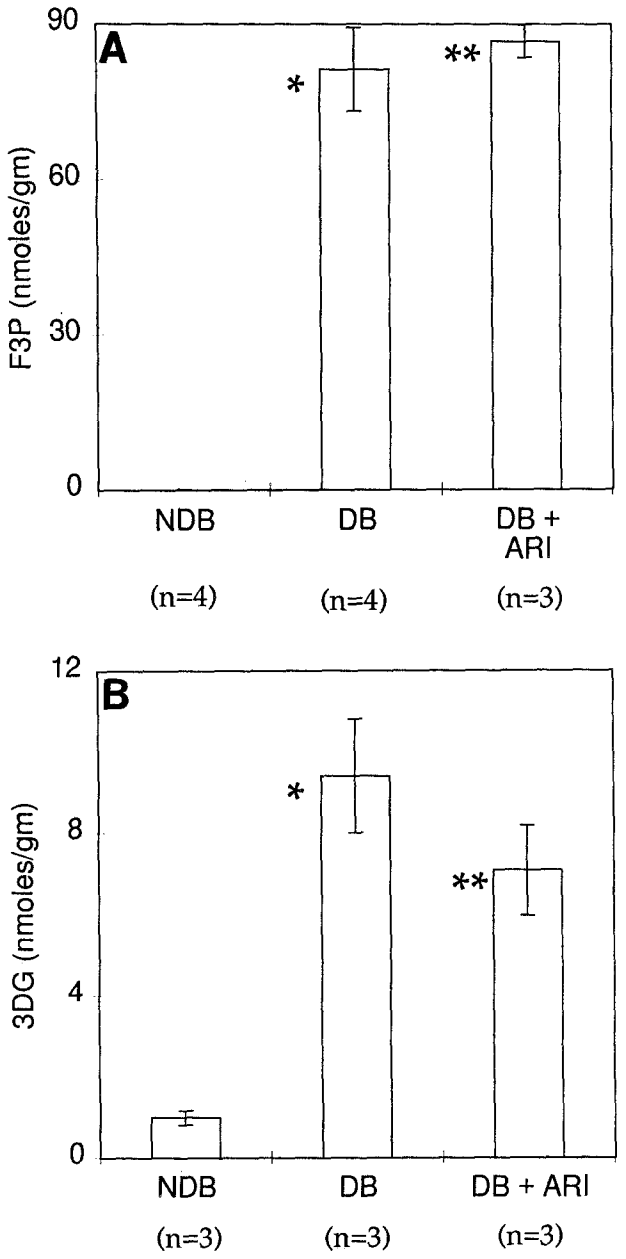

Fig 3. Levels of (A) F3P and (B) 3DG in rat hearts (NDB, nondiabetic; DB, diabetic; DB + ARI, diabetic fed a diet supplemented with ARI). *NDB *v* DB, $P < 1.7 \times 10^{-5}$; **DB *v* DB + ARI, $P < .36$.

8.2 μg, 25 μL 2.0-mmol/L solution) was added as an internal standard. Sugar concentrations were determined using a Dionex (Sunnyvale, CA) high-performance liquid chromatography (HPLC) system equipped with a pulsed amperometric detector (DX-500). Glucose and fructose levels were measured on a Dionex Carbopac PA-1 anion-exchange column using a gradient of 16 to 32 mmol/L sodium hydroxide. The sorbitol level was measured on a Dionex Carbopac MA-1 anion-exchange column using isocratic elution with 600 mmol/L NaOH. Peak areas were normalized to 2-deoxyglucose, and concentrations were determined from the standard curves prepared on the same day.

Quantitation of Cardiac 3DG

3DG was quantified in rat hearts by modification of a procedure previously reported by Yamada et al.[47] A 20-μL aliquot of 10-μmol/L U-^{13}C-3DG was added as an internal standard to 0.5 mL of the heart PCA extract, followed by 1 mL of a 1-mmol/L 2,3-diaminonaphthalene solution (Sigma). This mixture was incubated overnight at room temperature and then extracted with 4 mL ethylacetate, and the organic layer was completely dried by centrifugal evaporation. A 120-μL aliquot of TMS reagent (Tri-Sil reagent; Pierce Chemical, Rockford, IL) was added to make trimethylsilyl derivatives of the 3DG-DAN adduct. The sample vials were capped, vigorously stirred for 30 minutes at room temperature, and then centrifuged on a benchtop centrifuge to settle the salts formed during derivatization. The derivatized sample was transferred to a vial containing a limited-volume insert and sealed under argon. These samples were analyzed using gas chromatography/mass spectrometry (GC/MS).

GC/MS Analyses

A HP 5890 gas chromatograph (GC); Hewlett-Packard, Palo Alto, CA) equipped with a HP 5971 mass selective detector and HP 7673 automatic sampler was used for analysis. GC separation of a 2-μL sample was performed on a fused silica capillary column (DB-5, 25 m × 0.25 mm ID) using a temperature program as follows: injector port at 250°C, and initial column temperature at 150°C for 1 minute and then increased to 290°C at 16°C/min and maintained for 15 minutes. The transfer line was maintained at 250°C. Quantitation of 3DG was performed by selected ion monitoring of *m/z* 295 and 306 for plasma 3DG and *m/z* 299 and 309 for the U-^{13} C-3DG internal standard.

^{31}P-NMR Data Acquisition and Processing

^{31}P-NMR spectra were acquired on a Bruker AM-400 spectrometer (Billerica, MA) at 162 MHz with 60° pulses and a 1.5-second repetition

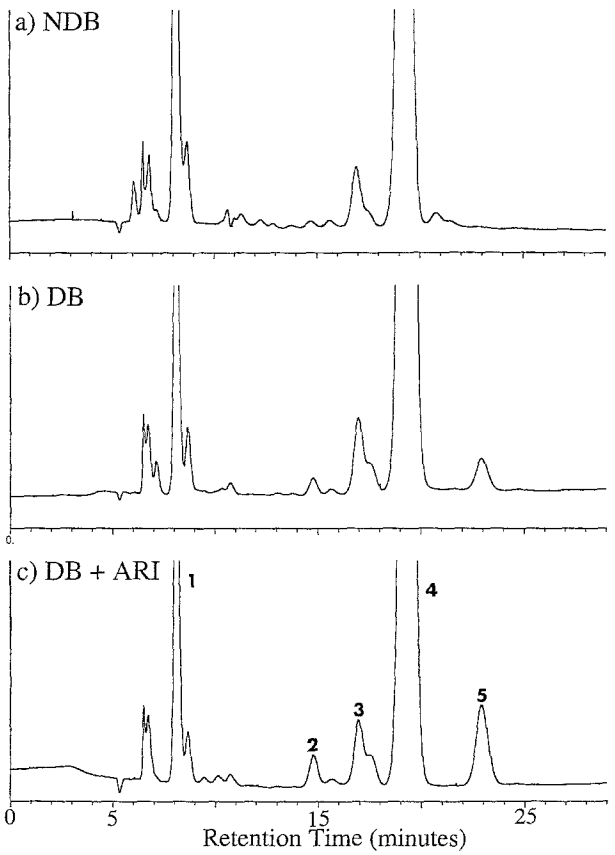

Fig 4. Typical HPLC chromatograms of various sugars in rat hearts from (a) nondiabetics, (b) diabetics, and (c) diabetics fed a diet supplemented with ARI (1, inositol; 2, sorbitol; 3, internal standard; 4, glucose; 5, fructose).

time. Typically, spectra were acquired in blocks of 20,000 scans. Chemical shifts were referenced to the naturally occurring glycerophosphorylcholine peak (0.49 ppm). Identification of F3P was based on chemical-shift analysis and on the ^{1}H-^{31}P spin-spin coupling pattern. F3P was quantified using an external concentration standard. The effects of partial saturation of the signal on the concentration of F3P were found to be within 10% of the measured values. F3P concentration was normalized to the total wet weight of the heart and expressed as nanomoles per gram wet weight.

RESULTS

Figure 1 shows ^{31}P-NMR spectra from control and diabetic rat hearts. A resonance at 5.8 ppm was detectable in the ^{31}P-NMR spectrum from diabetic rats (Fig 1b). Since this resonance is not observed in controls (Fig 1a), its presence in diabetic tissue is likely to be associated with hyperglycemia. The chemical shift of this unknown peak is consistent with F3P, previously observed in various diabetic tissues. However, to rigorously identify this resonance, a proton-coupled ^{31}P-NMR spectrum was spiked with authentic F3P (Figs 2a and b). The spiked extract was analyzed by ^{31}P-NMR at pH 5.4 and 7.5. The peaks of the metabolite at 5.8 ppm and authentic F3P coresonated at these two pH levels, thereby proving conclusively that the observed metabolite is F3P.

F3P was detectable in diabetic hearts as early as 14 days after induction of diabetes. While it was undetectable in normoglycemics, its concentration in diabetics after 55 days of hyperglycemia was 81.3 ± 16.3 nmol/g wet weight (n = 4) (Figs 1 and 3). Concomitant with elevated F3P, diabetic rat hearts also show higher levels of 3DG (Fig 3). The concentration of this carbohydrate was elevated approximately 10-fold in diabetic rats compared with age-matched controls (9.4 ± 3.5 nmol/g *v* 0.98 ± 0.43 nmol/g, n = 3). Diabetic rats fed ARI had concentrations of F3P and 3DG similar to those of diabetics (Fig 3: F3P, 86.7 ± 6.3 nmol/g, n = 3; 3DG, 7.0 ± 2.6 nmol/g, n = 3). GC/MS and HPLC examination of these heart extracts for the reductive and oxidative detoxification products of 3DG, 3-deoxyfructose and 2-keto-3-deoxygluconate, respectively,[48,49] found no trace of these compounds.

Since previous studies on the lens have determined that F3P production is directly related to the concentration of its precursor fructose,[33-35] measurements were performed to determine cardiac levels of this compound along with the other polyol pathway–associated sugars sorbitol and glucose. A typical HPLC chromatogram of these sugars in controls, diabetics, and diabetics fed ARI is shown in Fig 4. In diabetics, the concentration of glucose was elevated over twofold relative to controls, but the concentrations of sorbitol and fructose were increased over 25-fold and 150-fold, respectively, and were unaffected by ARI treatment (Figs 4 and 5).

To evaluate whether the lack of inhibitory activity of the ARI in the heart may be due to insufficient dosage of the animals with ARI, polyol pathway sugar levels were measured in the lens of these animals. Sorbitol and fructose levels were normal-

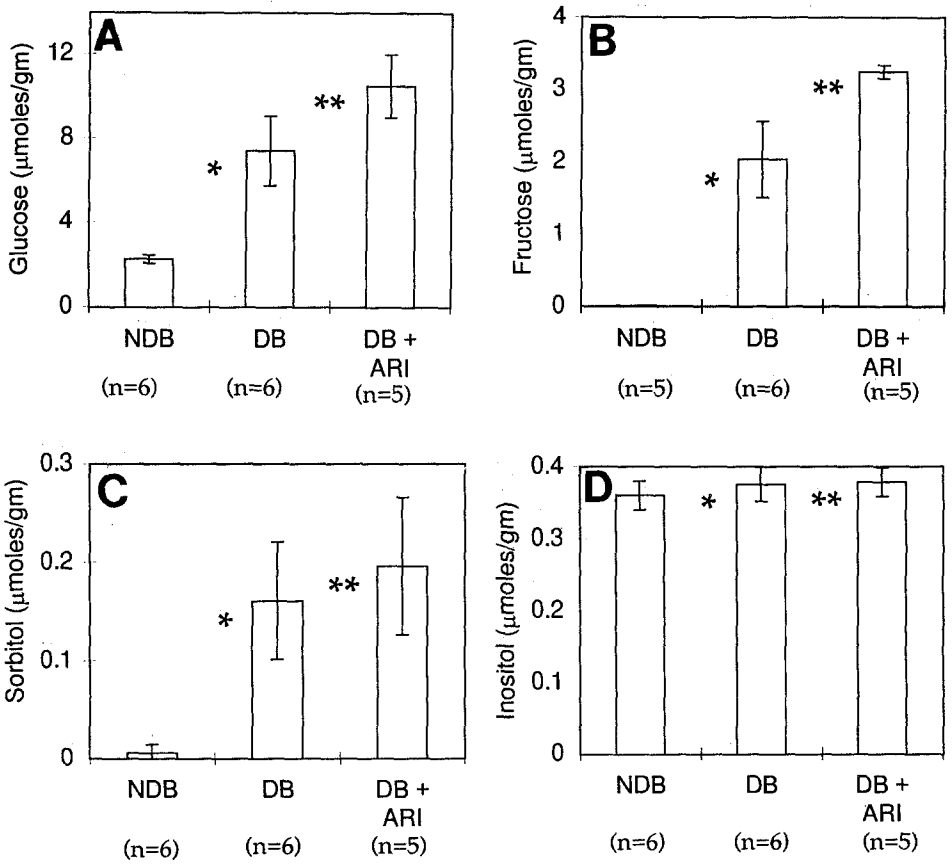

Fig 5. Cardiac levels of the polyol pathway sugars (A) glucose, (B) fructose, (C) sorbitol, and (D) inositol in rat hearts (NDB, nondiabetic; DB, diabetic; DB + ARI, diabetic fed diet supplemented with ARI). Glucose: *NDB *v* DB, $P < .002$; **DB *v* DB + ARI, $P < .09$. Fructose: *NDB *v* DB, $P < .002$; **DB *v* DB + ARI, $P < .09$. Sorbitol: *NDB *v* DB, $P < .01$; **DB *v* DB + ARI, $P < .35$. Inositol. *NDB *v* DB, $P < .39$; **DB *v* DB + ARI, $P < .36$.

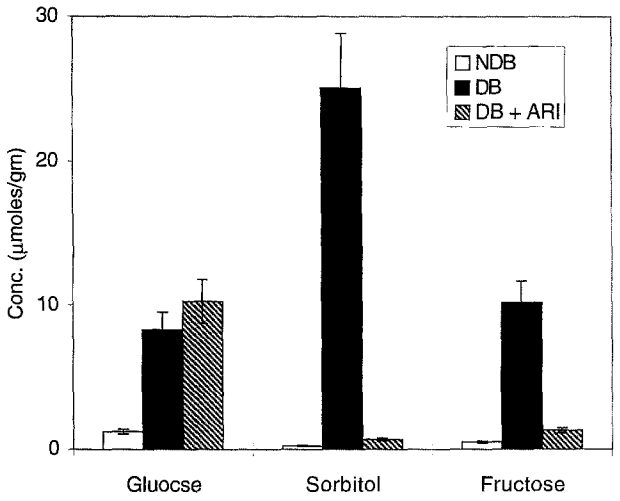

Fig 6. Lenticular levels of the polyol pathway sugars glucose, sorbitol, and fructose. Duration of diabetes, 30 days. Metabolite concentrations were estimated from a pool of 6 lenses to minimize biological variability.

ized in animals treated with ARI, suggesting adequate systemic dosage (Fig 6).

DISCUSSION

An increased risk of cardiac complications independent of any involvement of atherosclerosis has been previously documented for diabetic patients.[2,7] A considerable amount of study has been performed to understand the underlying factors that may contribute to the pathogenesis of this complication. Among the numerous changes noted with the onset of diabetes,[6-23] there is evidence for increased nonenzymatic glycation of cardiac proteins in diabetics.[21-23] Such glycated proteins in diabetic hearts have primarily been documented as borohydride reducible groups with an affinity for the phenylboronate column,[21,23] periodic acid-Schiff base–positive deposits,[7-9] and as advanced glycation end products (AGEs) in the collagen of the interstitium of hearts from diabetics.[10,22] Overall, as these findings are consistent with the accumulation of increased levels of nonenzymatically glycated cardiac proteins in diabetics.

While there are a number of possible contributors to glycation in the diabetic rat heart, this study shows significant production of the reactive glycating agents F3P and 3DG in this tissue, which may contribute to this process.

Given the well-documented reactivity of 3DG toward proteins, this dicarbonyl is generally detoxified in several cell systems by reductive[48] and oxidative[49] mechanisms to 3-deoxyfructose and 2-keto-3-deoxygluconate, respectively. However, these reactions are not evident in the heart. Their absence in the diabetic rat heart suggests that increased 3DG biosynthesis in diabetic hearts is likely to be more deleterious than in other tissues with active detoxification pathways.

Measurements of cardiac levels of sorbitol and fructose (Figs 4 and 5) in the diabetic and ARI-fed diabetic animals suggest an apparent lack of efficacy of sorbinil in this tissue. Similar results have been obtained in other independent studies where the ARIs ponalrestat and statil structurally distinct from sorbinil (Cameron NE, personal communication),[12] did not normalize cardiac fructose levels in diabetic rats. There are several possibilities that may explain this observation. The possibility of inadequate systemic dosage of these animals can be ruled out, based on our measurements of water consumption by ARI-treated animals and the normalization of fructose and sorbitol levels in the lens from these animals. However, it is possible that the ARI may not be transported as effectively into the heart as it is in the lens, or that the half-life of sorbinil in the heart may be much shorter than in the lens, thereby reducing its ability to inhibit AR. Another alternative explanation for these results could be that the rat heart may possess another active route for production of fructose. A precedent for such a possibility is the production of fructose in the porcine lens, which lacks sorbitol dehydrogenase[50] but has millimolar levels of fructose (Lal S, unpublished observations). One possible mechanism for such an alternate source of fructose could be dephosphorylation of phosphorylated fructose (such as fructose-1-phosphate or fructose-6-phosphate) by a nonspecific phosphatase.[51]

In conclusion, this study demonstrates production of the potent glycating agents F3P and 3DG in diabetic rat hearts. Over time, these reactive sugars are likely to have a deleterious effect on heart function. Our experiments also demonstrate that ARI treatment does not prevent accumulation of fructose and sorbitol in the heart, suggesting that the heart may be unique in its pathway of fructose production, or that, unlike other tissues, it may not readily take up and/or retain ARIs.

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