Regeneration of the Heart in Diabetes by Selective Copper Chelation

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Heart disease is the major cause of death in diabetes, a disorder characterized by chronic hyperglycemia and cardiovascular complications. Although altered systemic regulation of transition metals in diabetes has been the subject of previous investigation, it is not known whether changed transition metal metabolism results in heart disease in common forms of diabetes and whether metal chelation can reverse the condition. We found that administration of the Cu-selective transition metal chelator trientine to rats with streptozotocin-induced diabetes caused increased urinary Cu excretion compared with matched controls. A Cu^{II}-trientine complex was demonstrated in the urine of treated rats. In diabetic animals with established heart failure, we show here for the first time that 7 weeks of oral trientine therapy significantly alleviated heart failure without lowering blood glucose, substantially improved cardiomyocyte structure, and reversed elevations in left ventricular collagen and β_1 integrin. Oral trientine treatment also caused elevated Cu excretion in humans with type 2 diabetes, in whom 6 months of treatment caused elevated left ventricular mass to decline significantly toward normal. These data implicate accumulation of elevated loosely bound Cu in the mechanism of cardiac damage in diabetes and support the use of selective Cu chelation in the treatment of this condition.
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CO, cardiac output; ECM, extracellular matrix; EPR, electron paramagnetic resonance; KHB, Krebs-Henseleit bicarbonate buffer; LCM, laser confocal microscopy; LV, left ventricular; LVH, LV hypertrophy; MRI, magnetic resonance imaging; STZ, streptozotocin; TEM, transmission electron microscopy.

Diabetes is accompanied by increased prevalence of left ventricular (LV) hypertrophy (LVH), LV dysfunction, and coronary artery disease (1), but the mechanism by which hyperglycemia or associated metabolic abnormalities lead to or cause heart disease has remained obscure. Four main processes have been implicated in glucose-mediated vascular disease, and it has been suggested that the primary fault that leads to tissue damage is hyperglycemia-driven overproduction of superoxide by the mitochondrial electron-transport chain in endothelial cells (2). Normalizing mitochondrial superoxide production in endothelial cells in vitro was reported to block four pathways of hyperglycemic tissue damage (3). A number of therapeutic studies that used antioxidant or carbonyl-trapping agents have had variable outcomes, indicating that the pathways that lead to in vivo tissue damage may be incompletely understood (4).

The biology of transition metals, such as Zn, Mn, Mo, Cr, V, Fe, and Cu, has previously been evaluated in the context of diabetes. In hemochromatosis, excess myocardial Fe can cause heart disease, and Fe-mediated islet damage results in diabetes (5). Myocardial Fe excess also causes heart disease in hemosiderosis, in association with excessive dietary Fe intake, or ineffective erythropoiesis as in thalassemia or sideroblastic anemia (6,7). Altered cardiac Fe metabolism is implicated in the mechanism of heart disease in additional circumstances (8).

Defective Cu metabolism is said to impair cardiovascular health in at least two known settings, chronic Cu deficiency (9,10) and defective intracellular Cu transport to mitochondrial cytochrome C oxidase caused by missense mutations in the second cytochrome *c* oxidase assembly gene, *SCO2* (11). Thus, Cu deficiency has been implicated as a defect of Cu homeostasis that can lead to cardiac disease (12). By contrast, heart disease is not noted in chronic intracellular Cu overload, e.g., Wilson's disease (13).

Free Fe and Cu ions are the most redox-active in mammalian tissues (14), where they may contribute to tissue damage by generation of reactive oxygen species such as hydroxyl radicals (15). However, the in vivo availability of catalytic Fe and Cu is usually very restricted, which serves as an important antioxidant defense (14).

Whether Fe- or Cu-catalyzed redox reactions may play some role in diabetes complications has been discussed previously (4,16). It has not been established that such mechanisms or, for example, altered transition metal metabolism play a role in the forms of heart disease that complicate the major classes of diabetes, type 1 and type 2. Previously, administration of an Fe chelator and a Cu chelator in a 2-week experiment in diabetic rats was reported to ameliorate decreases in sciatic motor nerve conduction velocity, restore nutritive endoneurial blood flow, decrease systemic arterial pressure, and cause supranormal sciatic nutritive vascular conductance (17).

We used trientine to probe relationships between Fe and Cu and diabetic heart disease, and the effects of Cu removal in diabetic rats and in people with diabetes. Trientine binds Cu^{II} selectively (log $K_f \cong 20$) but also has significant affinity for Zn^{II} (log $K_f \cong 12$), Fe^{II} (log $K_f \cong 8$), and Fe^{III} (18). It is used clinically in the therapy of Wilson's disease, an inherited Cu-transporter defect that causes Cu accumulation and localized organ damage (19,20).

We show here that trientine causes increased urinary Cu output in diabetic rats and humans compared with treated controls. Trientine reversed heart failure in diabetic rats, and diabetic rats and humans demonstrate improved cardiac structure after chronic trientine treatment. Potential mechanisms include regeneration of f-actin and normalization of collagen. Excess loosely bound Cu thus is implicated in the mechanism by which diabetes damages the heart.

RESEARCH DESIGN AND METHODS

Reagents and ethical and regulatory approvals. All reagents were from Sigma unless otherwise stated. All studies were approved by relevant ethics and regulatory committees.

Urinary metal excretion in rats. Male Wistar rats (303 ± 3 g) received an injection of streptozotocin (STZ; 55 mg/kg i.v.) or saline. Diabetes was diagnosed as blood glucose >11 mmol/l and insulin was not replaced. After 7 weeks of diabetes, rats were anesthetized, ureters were catheterized, and urine was collected. Animals were ventilated, and end-tidal CO_2 and body temperature were maintained at 35–40 mmHg and 37°C, respectively, with saline replacement of fluid loss. Trientine (triethylenetetramine dihydrochloride; Fluka) was infused (intravenously, 60 s once hourly) in increasing doses (0.1, 1.0, 10, and 100 mg/kg in 75 μl of physiological saline), whereas controls received saline alone. Urine (15-min aliquots) was centrifuged, and supernatants were diluted (1:25 [vol/vol]) in 0.02 mol/l HNO_3. Metals were determined by graphite furnace-atomic absorption spectrophotometry (Perkin Elmer), and X-band electron paramagnetic resonance (EPR) spectra were obtained (77°K, Varian E3).

Cardiac function in rats. After 6 weeks of diabetes, rats were assigned to one of four groups: "untreated control" ($n = 9$), "untreated diabetes" ($n = 8$), "trientine-treated diabetes" ($n = 8$), or "trientine-treated control" ($n = 11$). Diabetic animals had blood glucoses >20 mmol/l for 6 weeks before receiving trientine (8–11 mg/day) in drinking water or control treatment from weeks 7–13. Cardiac function was measured in isolated perfused working hearts. Animals were anesthetized and heparinized (1,000 IU/kg i.v.), and hearts were excised, then immersed in 4°C Krebs-Henseleit bicarbonate buffer (KHB). Retrograde (Langendorff) perfusion was established (KHB, 37°C, gassed with O_2:CO_2 95:5 [vol/vol]). Working-mode perfusion was then established (preload, 10 cmH_2O; afterload, 55.9 mmHg) with pacing (300 bpm; Digitimer). Intrachamber LV pressure (SP855; AD Instruments), aortic pressure (PX23XL, Stratham Gould), and aortic (Transonic T206) and coronary flows were measured; pressure and flow data were recorded (Powerlab16s, ADI); and ventricular pressure development ($+dP_{LV}/dt$) and relaxation ($-dP_{LV}/dt$) were derived. Atrial filling pressure was decreased (5 cmH_2O) and then increased (seven steps of 2.5 cmH_2O to 20 cmH_2O [final]), and 1-min averages were extracted. Intergroup differences were contrasted (mixed models/repeated measures; SAS v8.1). Filling pressure was then fixed at 10 cmH_2O, and afterload increased from 55.9 mmHg in 9 × 2-min steps. Maximum afterload attained was either 106.7 mmHg (final) or that at which aortic flow became zero. Results were compared by survival analysis (PROC LIFETEST, SAS v8.01).

Extraction of chelatable copper by coronary artery perfusion. Hearts were removed from rats with 7 weeks of diabetes and controls ($n = 10$/group) and perfused until stable (20 min, nonworking mode). Hearts then underwent retrograde perfusion (KHB, 5 mmol/l trientine). Thirty-second fractions (2 min) were analyzed (ICPMS, Elan 6100; Perkin Elmer), and total extracted Cu was calculated.

Cardiac histology. Tissue for histology was obtained after functional analyses, when hearts were arrested in diastole (retrograde infusion 4 ml cold KCl [24 mmol/l]) and dissected, and wet ventricular weights were recorded. For transmission electron microscopy (TEM), ~1-mm^3 blocks of LV subendocardial myocardium (5–6/group) were fixed (2.5% glutaraldehyde, PBS, 2 days), postfixed (1:1 [vol/vol] 1% [wt/vol] OsO_4/PBS, 1 h), embedded (100% 812 epoxy resin), sectioned (70 nm), stained (uranyl acetate [2% {wt/vol}, 20 min] then lead citrate [3 min]), and imaged (Tecnai-12; Phillips). Remaining LV tissue was frozen at 80°C. For β_1 integrin immunochemistry, LV samples were thaw-fixed (4% paraformaldehyde/PBS, 24 h), embedded (6% agar), and vibratomed (120 μm; Campden) for immunohistochemistry. Sections were stained (f-actin, Phalloidin-Alexa Fluor 488; 1:25 in PBS, 3 h; Molecular Probes), washed (PBS), blocked (2% BSA in PBS, 1 h), and immunostained with β_1 integrin Ab [1:50 in 0.1% BSA/PBS (21)]. After washing (0.1% BSA/PBS), sections were stained with secondary CY5 goat anti-rabbit Ab (1:200 0.1% BSA in PBS; overnight, 4°C; Jackson ImmunoResearch). Slides were washed (0.1% BSA/PBS), mounted (Citifluor; Agar Scientific), and stored (dark, 4°C) until imaging (LCM; Leica TCS SP2). For collagen immunohistochemistry, 30-μm LV cryosections (5/group) were dried (2 h), fixed (ice-cold acetone, 10 min), and rehydrated (PBS, 5 min). Sections were blocked (60 min, 10% goat serum in PBS) and immunostained (overnight, 4°C) with either rabbit anti-rat type I collagen Ab or type III collagen Ab (1:100 in 5% goat serum/1% BSA/PBS; Chemicon Australia). After washing (PBS), slides were stained for 2 h with secondary Ab (Alexa Fluor 647 goat anti-rabbit Ab; 1:200; Molecular Probes) and either phalloidin-Alexa Fluor 488 (type I collagen) or phalloidin-rhodamine (1:25; Molecular Probes) diluted in 5% goat serum/1% BSA/PBS. Slides were washed (PBS), mounted (antifade; Dako), and stored (dark, 4°C) until imaging (LCM; Leica TCS SP2). For TEM, four longitudinally orientated images were recorded (×1,600, ×2,650, and ×5,600/LV; $n = 5$–6/group). For β_1 integrin and actin, five optical sections (×630) of longitudinally orientated myocytes/LV ($n = 3$/group) were analyzed. For collagens I and III, three transversely oriented optical sections/LV (×640) were analyzed ($n = 5$/group) and specific areas were measured (ImageJ v1.29x; http://rsb.info.nih.gov/ij), expressed as percentages of totals, and compared (one-way ANOVA, planned comparisons).

Urinary Cu excretion in humans. Men with type 2 diabetes ($n = 20$) and control subjects ($n = 20$) underwent complete 12-day elemental balance studies in a residential metabolic unit where all foods and beverages were provided. Total daily intake (duplicate diets) and excretion (urinary and fecal) of trace metals (Fe, Cu) were determined (ICPMS). Daily baseline measurements were made (6 days), after which oral trientine dihydrochloride (2.4 g once/day; Anstead) or matched placebo was administered (2 × 2 randomized, double-blind protocol) and metal balance was measured (daily for 6 days).

Effect of trientine on LV mass in diabetic humans. The effect of 6 months of trientine or placebo on LV mass in humans with type 2 diabetes was measured (placebo-controlled, parallel-group study). Individuals (30–70 years) who provided written informed consent were eligible for inclusion when they had type 2 diabetes with HbA_{1c} >7%, abnormalities of diastolic filling demonstrated by mitral inflow Doppler with preload reduction (no patient with normal mitral filling proceeded to randomization), LV ejection fraction (echocardiography) ≥50% with evidence of diastolic dysfunction but no regional wall-motion abnormalities, no new medications for >6 months before randomization with no change of β-blocker dose during that period, and normal electrocardiograms (sinus rhythm, normal PR interval, normal T wave and QRS configuration, and isoelectric ST segment). Women were postmenopausal, surgically sterile, or nonlactating and nonpregnant and using adequate contraception. Patients were ineligible when they failed to meet the inclusion criteria or had morbid obesity (BMI ≥45 kg/m^2); type 1 diabetes; significant cardiac valvular disease; autonomic neuropathy; LV wall-motion abnormality (echocardiography); multiple drug allergies; use or misuse of substances of abuse; abnormal tests of electrolyte homeostasis; renal, hepatic, or thyroid function at randomization; or standard contraindications to magnetic resonance imaging (MRI).

Before randomization, patients entered a 4-week single-blind run-in of two placebo capsules twice daily, during which ≥90% compliance was required for progression. Patients who met inclusion criteria were randomized to receive trientine (2× 300 mg twice daily) before meals (total dose 1.2 g/day) or identical placebo capsules. Treatment assignment was performed centrally

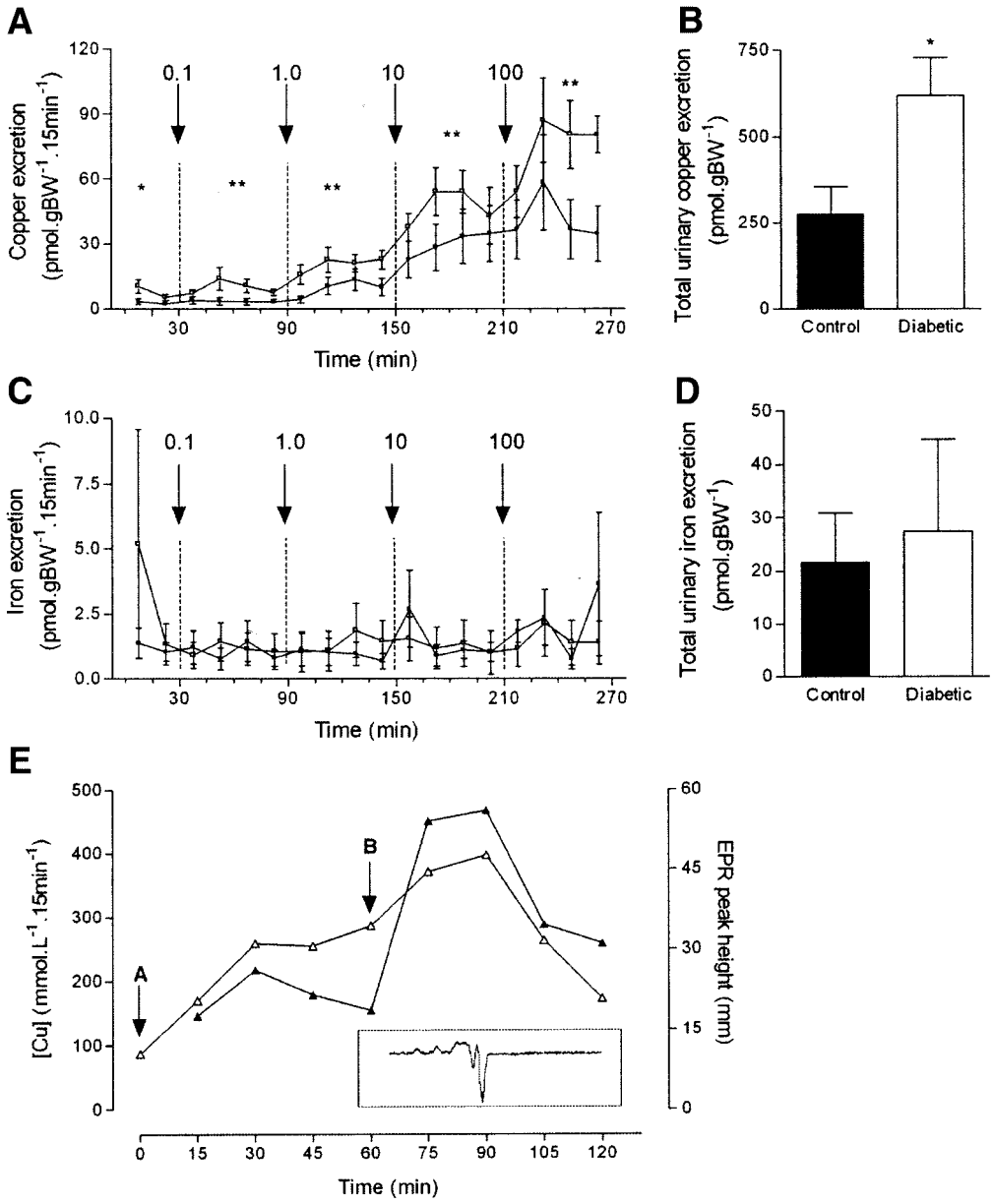

FIG. 1. Trientine elicits increased Cu excretion in rats with diabetes. *A*: Urinary Cu after intravenous trientine (0.1, 1.0, 10, and 100 mg/kg; *arrows*) in control (■; $n = 7$) and diabetic (□; $n = 7$) rats. *B*: Total urinary Cu (mean ± SE; ■, control; □, diabetes). *C*: Urinary Fe excretion after increasing bolus doses of trientine, as in *A*. *D*: Total urinary Fe. *E*: Urinary [Cu] (△) and EPR signal from Cu^{II}-trientine complex (▲) after 10 mg/kg (*A*) and 100 mg/kg (*B*) trientine; *inset*: EPR signal from 75-min urine. *$P < 0.05$, **$P < 0.01$ vs. control. gBW, grams of body weight.

using variable block sizes to ensure balance throughout trial recruitment, and numbered drug packs were prepared and dispensed sequentially to randomized patients. Double-blind treatment was continued for 6 months in each patient. Cu status was monitored by monthly serum Cu and Cu,Zn superoxide dismutase activity (22), and hematological variables relevant to detection of systemic copper deficiency (total blood hemoglobin concentration, mean red cell volume, and mean red cell hemoglobin content) (23).

At baseline and after 6 months of treatment, LV mass was determined using cardiac MRI (24), performed in the supine position with the same 1.5 Tesla scanner (Siemens Vision, Munich, Germany) using a phased-array surface coil. Prospectively gated gradient echo images were acquired in six short axis and three long axis slices with the use of a segmented k-space pulse sequence (repetition time, 8 ms; echo time, 5 ms; flip angle, 10°; field of view, 280–350 mm) with view sharing (11–19 frames/slice). Each slice was obtained during a breath-hold of 15–19 heartbeats. The short-axis slices spanned the left ventricle from apex to base with a slice thickness of 8 mm and interslice gap of 2–6 mm. Long-axis slices were positioned at equal 60° intervals about the LV long axis. LV mass and volume were calculated using guide-point modeling (24), which generates precise and accurate estimations of mass and volume. A three-dimensional mathematical model of the left ventricle was interactively fitted simultaneously to the epicardial and endocardial boundaries of the wall in each slice. Volumes were calculated up to but not beyond the mitral valve plane as defined by that through the hinge points of the mitral valve leaflets. Volume and mass then were calculated from the model by numerical integration (mass = wall volume × 1.05 g/ml). All measurements were performed by a single member of the research team at the end of the 6-month data collection.

Outcome analyses were conducted by intention-to-treat, using a maximum likelihood approach to impute missing-at-random data within a mixed model, and marginal least-squares adjusted means were determined. Changes from baseline were compared between treatment groups in the mixed model with baseline values entered as a covariate. Because there were only two groups in the main effect and no interaction effect, no post hoc procedures were used. In additional analysis, the influence of clinically important differences between the treatment groups at baseline was considered by adjusting for them as covariates in an additional model. All P values were calculated from two-tailed tests, and $P < 0.05$ was considered significant. The effect of treatment on categorical variables was tested using the Mantel-Haenszel test (SAS v8.01, SAS Institute).

RESULTS

Trientine treatment evokes elevated urinary Cu output in diabetic rats. Trientine was administered intravenously to groups of diabetic rats and controls (Fig. 1). Doses were increased stepwise (Fig. 1*A*) until levels similar to those for Wilson's disease were reached (25). Diabetic rats had increased basal urinary Cu excretion

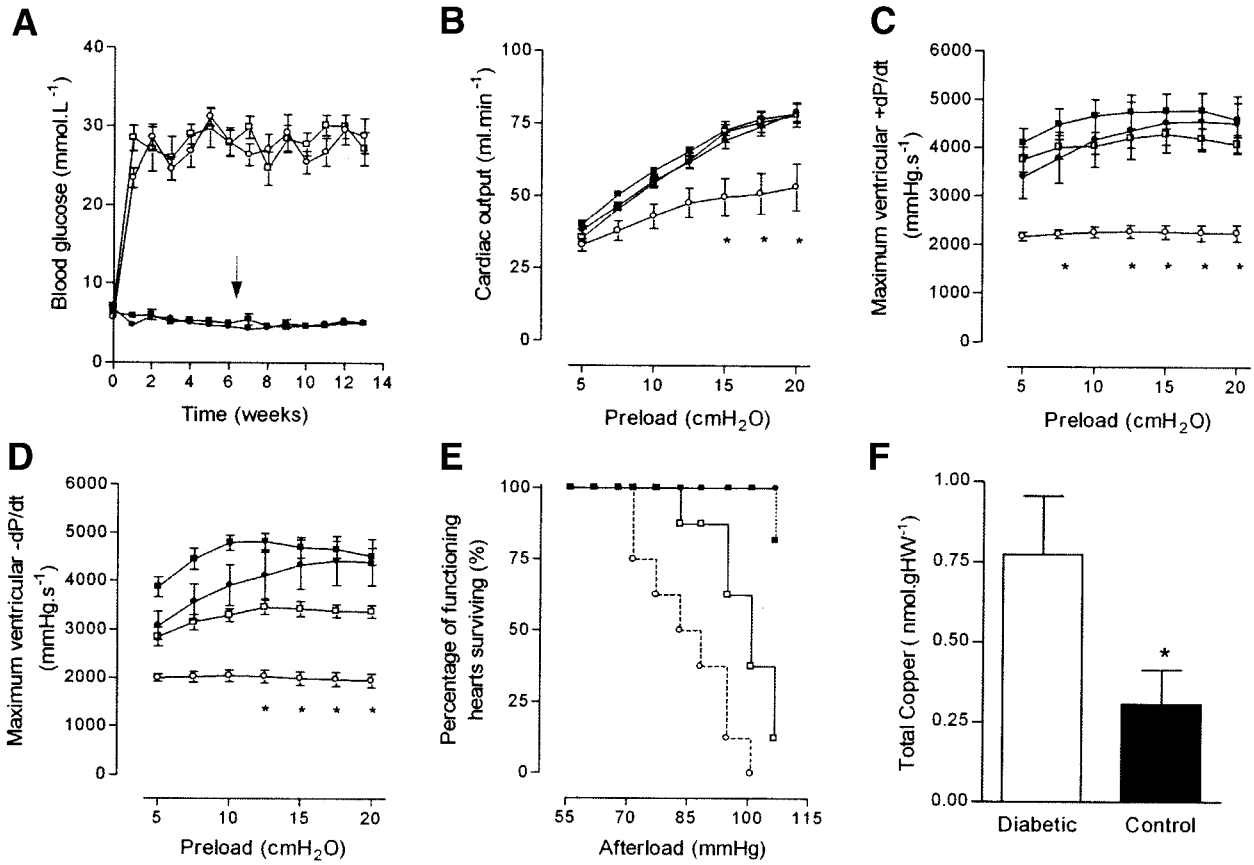

FIG. 2. Trientine improves cardiac function in rats with diabetes and heart failure. Trientine was administered for 7 weeks after 6 weeks of established diabetes. ○, untreated diabetes; ●, untreated control; □, trientine-treated diabetes; ■, trientine-treated control; means ± SE, n = 8–11/group. *A*: Blood glucose; arrow indicates time from which trientine (8–11 mg/day) was administered. *B–E* were derived from isolated perfused working hearts excised after 7 weeks of trientine treatment. *B*: CO with increasing atrial filling pressures (preload). *C*: $+dP_{LV}/dt$ with increasing preload. *D*: $-dP_{LV}/dt$ with increasing preload. *E*: Percentage of hearts pumping at each afterload pressure ($P < 0.05$, Wilcoxon test). *$P < 0.05$, trientine-treated diabetes vs. untreated diabetes. *F*: Total copper in 2-min trientine perfusate (n = 10/group, mean ± SE) normalized to heart weights from diabetic (■) or control (□) animals (*$P < 0.04$). gHW, grams of heart weight.

compared with controls (Fig. 1*A*), whereas basal urinary Fe output was equivalent (Fig. 1*C*). Trientine elicited prompt increases in urinary Cu (Fig. 1*A*) but not Fe (Fig. 1*C*). Trientine caused increased urinary total Cu (Fig. 1*B*) but not Fe (Fig. 1*D*). A Cu^{II}-trientine complex (26) was detected by EPR spectroscopy (Fig. 1*E*), and spectral intensity correlated well with total Cu. Therefore, rats develop increased chelatable systemic Cu^{II} as a consequence of diabetes.

Trientine alleviates heart failure in diabetic rats. Diabetes causes LV dysfunction and heart failure in rats (27,28) and humans (1,29). Here, we used trientine to probe a possible role of increased tissue Cu in diabetic heart disease in rats (Fig. 2). We confirmed that LV dysfunction was well established in STZ-induced diabetic rats after 6 weeks of diabetes (results not shown). Rats that had had untreated diabetes for 6 weeks were treated with trientine for an additional 7 weeks (trientine-treated diabetes). Cardiac function and structure were compared with three additional groups: untreated diabetes, trientine-treated control, and untreated control. Untreated diabetes (30) caused increased cardiac mass/body mass (Table 1) that was partly reversed by trientine, which, however, had no effect on cardiac mass/body mass in treated controls (Table 1).

Compared with untreated controls, diabetic hearts had markedly attenuated cardiac output (CO) that progressively worsened as filling pressure increased (Fig. 2*B*), whereas the CO–filling pressure relationship was normal in trientine-treated diabetes (Fig. 2*B*); thus, 7 weeks of trientine normalized this relationship and both coronary and aortic flows improved (results not shown). Trientine normalized CO in diabetic rats despite persistent hyperglycemia. Thus, increased loosely bound Cu^{II} contributes to heart disease in diabetes.

We derived mean $+dP_{LV}/dt$ at constant afterload and various filling pressures (Fig. 2*C*). $+dP_{LV}/dt$ was decreased in untreated diabetic rats and the disparity from control

TABLE 1
Effects of diabetes and trientine on cardiac mass/body mass ratio in rats

Group	Cardiac mass/body mass ($\times 10^3$)
Untreated control	2.8 ± 0.1
Untreated diabetes	4.7 ± 0.1*
Trientine-treated diabetes	4.1 ± 0.2*†
Trientine-treated control	2.8 ± 0.1

Data are means ± SE. *$P < 0.001$ for diabetes groups vs. respective controls; †$P < 0.05$ for trientine-treated diabetes vs. untreated diabetes.

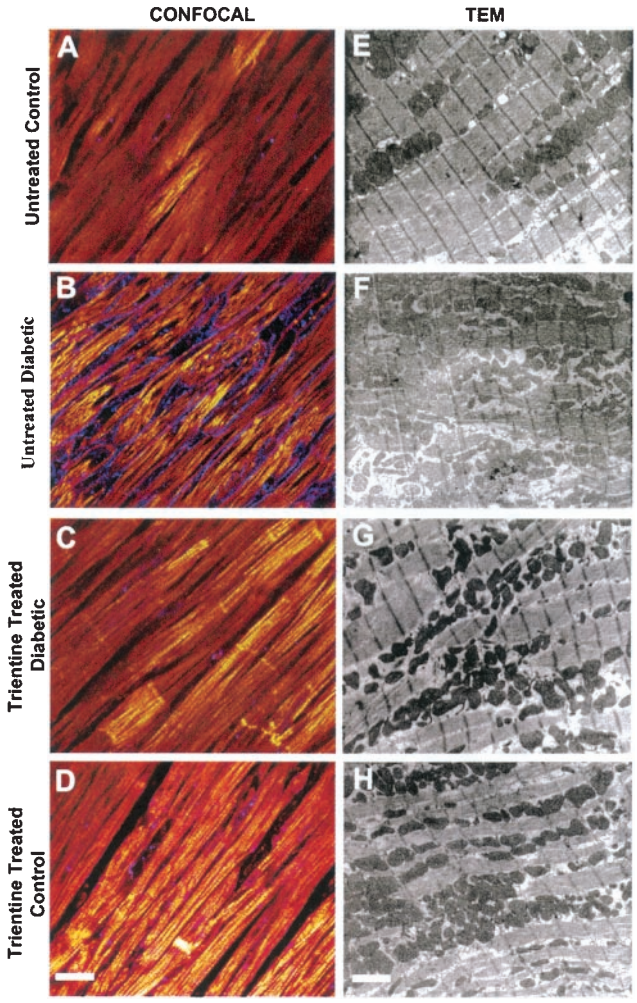

FIG. 3. Trientine improves LV structure in rats with diabetes and heart failure. Images represent five optical sections per heart times three hearts per treatment for LCM and four sections per heart times five to six hearts per treatment for TEM. *A–D*: LCM images of 120-μm sections costained for f-actin (orange) and β_1 integrin (blue) (scale bar = 33 μm). *E–H*: TEM images of corresponding 70-nm sections stained with uranyl acetate/lead citrate (scale bar = 158 nm). *A* and *E*: Untreated control. *B* and *F*: Untreated diabetes. *C* and *G*: Trientine-treated diabetes. *D* and *H*: Trientine-treated control.

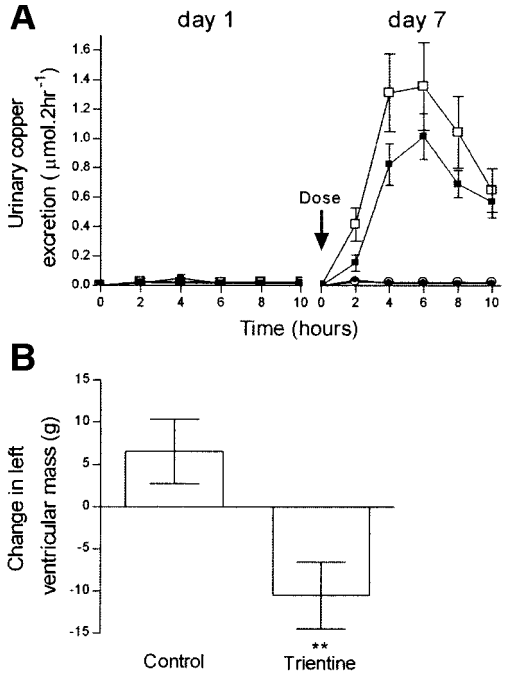

FIG. 4. Effects of trientine on urinary Cu and LVH in humans with type 2 diabetes. *A*: Effects of oral trientine were compared with placebo between men with type 2 diabetes and age-matched control subjects; ○, placebo-treated type 2 diabetes. ●, placebo-treated control; □, trientine-treated type 2 diabetes; ■, trientine-treated control. Urinary Cu excretion (μmol/2 h) on day 1 (baseline) and day 7 after initial 2.4 g of trientine (n = 20) or placebo (n = 20). Basal Cu output was significantly greater in type 2 diabetes than control ($P < 0.001$), and trientine elicited more Cu from type 2 diabetes than from controls ($P < 0.05$). *B*: Trientine or matched placebo was administered to patients with type 2 diabetes (n = 15) or matched type 2 diabetic control subjects (n = 15) in a double-blind, parallel-group study. Groups were well matched for relevant variables (see text). Differences in LV masses (mean ± SE, g) were determined by cardiac MRI. **$P < 0.01$ vs. placebo-treated control subjects.

worsened as filling pressure increased. By contrast, $+dP_{LV}/dt$ in trientine-treated diabetic rats was not significantly different from untreated controls throughout (Fig. 2*C*). Trientine restored $+dP_{LV}/dt$ without lowering blood glucose (Fig. 2*A*). Thus, increased Cu^{II} plays a role in the deterioration of systolic function in diabetes. Peak $-dP_{LV}/dt$ is the maximum rate of fall in LV pressure during the cardiac cycle and a measure of diastolic dysfunction (31). Decreased $-dP_{LV}/dt$ reflects increased stiffness of the LV wall, which may be associated with increased content (32,33) and altered three-dimensional organization (33) of fibrous connective tissue structures such as those formed by collagen. Maximum $-dP_{LV}/dt$ was impaired in diabetic hearts compared with controls (Fig. 2*D*) and restored by trientine, linking elevated Cu^{II} to increased collagen and wall stiffness in diabetes. Each heart was subsequently evaluated in a second protocol, in which preload was maintained at 10 cmH_2O and afterload was increased stepwise either to 106.7 mmHg (final) or until aortic flow ceased (functional failure; Fig. 2*E*). Trientine-treated diabetic hearts were more resistant to the effects of increasing afterload than untreated diabetic hearts ($P < 0.05$). For illustration, when 50% of the untreated diabetic hearts had functionally failed, ~90% of trientine-treated diabetic hearts were still pumping (Fig. 2*E*). Trientine thus restored the ability of diabetic hearts to tolerate both increased afterload and increased preload.

TABLE 2
Effects of diabetes and trientine on type I and type III collagen in left ventricle of rat hearts

Group	Type I collagen (% area)	Type III collagen (% area)
Untreated control	9.0 ± 0.6*	3.9 ± 0.4†
Untreated diabetes	12.1 ± 0.8	6.5 ± 0.9
Trientine-treated diabetes	10.3 ± 0.9*NS	4.5 ± 0.6†
Trientine-treated control	8.6 ± 0.5*	4.0 ± 0.3†

Data are means ± SE; n = 5/group. *$P < 0.01$ and †$P < 0.05$ for untreated diabetes vs. corresponding group means. NS, not significantly different from untreated control.

Trientine elicits elevated coronary venous Cu output in diabetic rats. We perfused 5 mmol/l trientine via the main coronary arteries and measured metal content in the venous outflow (Fig. 2*F*). Trientine elicited acutely increased Cu output from diabetic hearts ($P < 0.04$). Thus, Cu accumulates in diabetic rat hearts. The acute Cu

release suggests that the extra Cu may be in the extracellular matrix (ECM).

Trientine improves cardiac structure in diabetic rats. In preliminary studies, LV tissue from trientine-treated rats was readily discriminated from that of untreated diabetic rats by two blinded histopathologists (results not shown). In the studies reported here, LCM was used to image f-actin and β_1 integrin, the latter being an ECM marker (34). Diabetes caused extensive damage to myocardial structure, with disorganized muscle fibers, decreased f-actin, and markedly increased β_1 integrin (Fig. 3*A* and *B*). By contrast, myocytes from diabetic rats markedly improved after trientine (Fig. 3*C*), with normalized orientation and appearance. LV from trientine-treated controls appeared normal (Fig. 3*D*). Thus, trientine improved cellular histology in diabetes, consistent with the functional recovery. Untreated diabetes increased type I collagen (35,36), but trientine-treated diabetes did not differ from controls (Table 2). Untreated diabetes also increased type III collagen (37), which was normalized by trientine (Table 2). Thus, trientine ameliorated increased collagen in diabetes, consistent with improved CO and $-dP_{LV}/dt$.

Diabetes caused marked ultrastructural damage (28) with focally decreased myocyte volume and loss and disorganization of actin filaments and myocytes, whose mitochondria were swollen (Fig. 3*E* and *F*). Trientine partly restored ultrastructure with improved cellular volume and orientation, restoration of mitochondria, and normalization of ECM (Fig. 3*G*). Myocardium from trientine-treated control rats appeared normal (Fig. 3*H*). Changes in measured cardiac mass (Table 1) therefore reflect the sum of changes in the intracellular and extracellular compartments, and trientine improved cardiac structure, even in severe diabetes.

Trientine elicits elevated urinary Cu output in human diabetes. We measured urinary Cu and Fe excretion in men with type 2 diabetes and matched control subjects. Groups were well matched for age and BMI. In diabetic groups, the median (range) disease duration (years) was 7.5 (range, 1–34) in trientine-treated and 5.9 (range, 1–13) in placebo, mean (SD) fasting plasma glucose (in mmol/l) values were 10.8 (4.3) and 11.5 (3.8), and mean (SD) HbA_{1c} (%) values were 9.1 (1.6) and 9.9 (2.7), respectively (all NS). Urine volumes (2.8 ± 0.2 l/day; control, 2.1 ± 0.2; $P < 0.01$) and basal urinary Cu (0.26 ± 0.04 μmol/day; control, 0.20 ± 0.03; $P < 0.001$) were greater in diabetes (Fig. 4*A*). Trientine- and placebo-evoked 2-h urinary Cu excretions were measured on day 7 after the first drug dose (2.4 g once daily). Trientine elevated Cu output in both groups but more in diabetes ($P < 0.05$; Fig. 4*A*), whereas Fe was unchanged (results not shown); trientine did not increase urine volume in either group (results not shown). Thus, trientine elicited similar urinary Cu responses in diabetic rats and humans, so increased Cu^{II} may occur commonly in diabetes.

Trientine ameliorates LVH in human diabetes. Trientine (600 mg twice daily) or matched placebo was administered orally for 6 months to diabetic adults ($n = 15$/group; Fig. 4*B*). Groups were well matched for age and BMI. Median (range) disease duration (years) was 8 (range, 1–21) in trientine-treated and 10 (range, 1–24) in placebo, mean (SD) HbA_{1c} (%) values were 9.3 (2.0) and 9.3 (1.3), and percentage of women was 56 and 44, respectively (all NS). Groups were well matched for other pharmacotherapies, including β-blockers, calcium antagonists, ACE inhibitors, cholesterol-lowering drugs, antiplatelet agents, and antidiabetic drugs. LV mass was measured by MRI at baseline and after 6 months of treatment; initial masses were increased compared with known values for age-matched control subjects (1). Mean (SD) masses (g) were 207.5 (48.7) in trientine-treated and 202.2 (53.1) in placebo. Mean LV mass decreased by 5% ($P < 0.01$) after 6 months of treatment, whereas that in matched placebo-treated control subjects increased by 3% (Fig. 4*B*), an overall difference of 8% between groups ($P < 0.01$). This effect occurred without change in systolic or diastolic blood pressure and remained significant after LV mass was indexed to body surface area: mean (95% CI) ΔLV mass/body surface area (g/m^2) was −5.56 (−9.64 to −1.48) in trientine treated and 3.49 (−0.63 to 7.61) in placebo ($P < 0.01$). No significant drug-related adverse events occurred. Serum Cu, serum superoxide dismutase activity, and the monitored hematological indexes did not differ significantly between groups (data not shown), indicating that prolonged trientine did not cause Cu deficiency in diabetes.

DISCUSSION

Here, rats with severe diabetes had increased urinary Cu excretion compared with matched controls when treated with trientine, which also elicited increased Cu excretion in diabetic humans, indicating that Cu metabolism is abnormal in diabetes. Most cell Cu is tightly bound (38) and regulated by binding proteins, so intracellular free Cu is essentially undetectable (39). Cu^{II}, which was present in urine from drug-treated diabetic rats, is the most effective divalent ion for binding to organic molecules and the main extracellular Cu ion, whereas Cu^{I} predominates inside cells (14). Trientine binds Cu less strongly than most Cu-binding proteins (14). These observations, taken together with our current findings showing prompt increments in Cu^{II} excretion after trientine administration, indicate that this increased Cu^{II} is unlikely to be released from an intracellular pool. More likely, the Cu^{II} is bound to ECM components, such as collagen; because it is readily extracted by trientine, the increased Cu^{II} must be loosely bound.

Decreased myocyte diameter (40), decreased f-actin (40), and diminished α-actin mRNA (41) have previously been reported in diabetes. However, the exact mechanisms by which diabetes damages the heart remain uncertain, although hyperglycemia, dyslipidemia (42), and hypertension are likely contributors. Free Cu is highly reactive (14). We investigated whether increased Cu^{II} might contribute to diabetic heart disease, where LV hypertrophy and dysfunction contribute to increased rates of heart failure (1,43). Unexpectedly, we discovered that trientine improved cardiac structure and function in diabetic rats, and without lowering blood glucose. Coronary trientine perfusion revealed excess cardiac Cu accumulation, probably in the ECM. Thus, we have implicated increased tissue Cu in a novel mechanism coupling diabetes to LV dysfunction and shown that Cu chelation might confer clinical benefit.

We also showed that 6 months of trientine decreased LV mass in diabetic humans with presymptomatic LVH, a major risk factor for heart failure and death (1). Most trientine-treated diabetic patients also had hypertension, but it did not change systolic or diastolic blood pressure or HbA_{1c}. These findings suggest that trientine could be the first in a new class of therapeutic molecules for diabetic heart failure.

Diabetes increases cardiac collagen content, which contributes to diastolic dysfunction. Here, Cu chelation was effective in the reversal of collagen accumulation in diabetic rat hearts. Damaged f-actin was also improved after this treatment, which could help explain improved CO and $+dP_{LV}/dt$. Thus, trientine may treat both diastolic and systolic dysfunction. The finding of increased β_1 integrin in diabetic hearts and its reversal by trientine support the conclusion that diabetes caused remodeling, in which myocytes move, shed integrin, and then produce more to establish new ECM connections (21) and that this was reversed by the chelator. The molecular mechanisms by which diabetes and trientine affect cardiac collagen and actin remain to be elucidated.

How might diabetes lead to increased extracellular Cu and myocardial damage? One aspect of the mechanism could be increased advanced glycation end products and glycoxidation products (44). Our studies suggest that diabetes might cause two- to threefold increases in ECM Cu. Glycation reportedly increased in vitro Cu binding to collagen by a similar factor (45). ECM Cu trapping could help to explain the different location of tissue damage in diabetes compared with Wilson's disease. Oral administration of the Cu chelator tetrathiomolybdate reportedly decreased neointimal thickening after balloon injury in the rat (46). Impaired glucose tolerance occurs in conditions other than diabetes, notably atherosclerosis, hypertension, obesity, and normal aging, all of which are accompanied by increased tissue advanced glycation end products (47). Increased tissue Cu^{II} could also play a role in the tendency to accelerated cardiovascular disease occurring in these conditions.

In summary, we have shown that rats and humans with diabetes have increased urinary Cu after treatment with trientine, which reversed heart failure and LV damage in diabetic rats and significantly ameliorated LVH in humans. Accumulation of excess tissue Cu^{II}, probably in the ECM, is likely to play a significant role in the mechanism through which diabetes damages the heart. Selective Cu chelation is proposed as a new method for treating LV disease in diabetes and perhaps other conditions associated with lesser degrees of hyperglycemia.

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