Insulin regulation of glutathione and contractile phenotype in diabetic rat ventricular myocytes

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Diabetes is associated with a unique cardiomyopathy that is independent of vascular disease and that contributes to a greater propensity for heart failure and arrhythmias in diabetic individuals than the general population (32, 40). The onset of clinical diabetic cardiomyopathy is marked by a slowing in relaxation kinetics and diastolic dysfunction; however, as the syndrome progresses, systolic dysfunction emerges, exacerbating the overall decline in ventricular performance (14). The depressed contractile features of the diabetic heart are proposed to be due to perturbations in Ca2+ handling, actin-myosin interactions, or a combination of both (32, 40). Although changes in expression of Ca2+ handling and myofilament proteins have been shown in experimental models of diabetic cardiomyopathy, it is also well recognized that posttranslational modification of these proteins can significantly affect contractile performance in the absence of or in addition to changes in expression. One type of protein modification that may play a central role is the reversible oxidation of amino acid side chains, particularly those of cysteine and methionine residues (9, 10, 36, 38). These residues are susceptible to modification by a variety of reactive oxygen species and their by-products.

Clinical and experimental studies suggest that ventricular dysfunction associated with diabetes mellitus is linked to a multitude of metabolic disturbances that result in increased production of reactive oxygen species and pathophysiological changes in myocyte function (8, 12). In mammalian cells, the reduced form of the tripeptide glutathione (GSH) is an important buffer against reactive oxygen species and hydroperoxides. For example, GSH is a radical scavenger that directly neutralizes carbon-centered radicals, superoxide anion, and hydroxyl radicals (10, 48). GSH is also an essential cofactor for inactivation of hydroperoxides by glutathione peroxidase and for the conjugation of cytotoxic by-products of lipid peroxidation by glutathione S-transferase (10). Recent studies have shown that disease states for which oxidative stress is a contributing factor, such as diabetes, elicit dynamic and profound alterations in cardiac GSH, which concurrently impact cell redox state (11, 46, 50). Most notably, GSH depletion and increased content of oxidized cellular proteins have been shown to play important roles in the pathogenesis of chronic diabetes (8, 11, 50). Many of the pathogenic changes elicited in early stages of diabetes can be reversed or prevented by manipulating endogenous pathways affecting intracellular GSH concentration ([GSH]), indicating that GSH is essential to cell viability and normal cell function (50).

It has recently been proposed that pathways of glucose metabolism are involved in the control of myocardial GSH (1, 31, 35, 42, 50). In the case of diabetes, the well-documented decrease in myocardial insulin signaling and glucose utilization (19, 34) are likely factors contributing to alterations in GSH

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status. Accordingly, insulin replacement therapy in Type 1 diabetic models maintains normal cardiac GSH levels (31, 43, 51). Recent data from our laboratory also provide functional evidence for a link between glucose metabolism and cell GSH. For example, upregulation of voltage-gated K+ channels in diabetic rat ventricular myocytes by insulin is blocked by inhibitors of GSH metabolism and is mimicked by exogenous GSH (50). These and related studies (35) support the notion that an important effect of insulin signaling pathways on cardiac function is the regulation of [GSH] and the control of cell redox state.

The present study examined mechanisms of GSH regulation in the setting of diabetic cardiomyopathy. Our data suggest that GSH depletion in the diabetic heart involves loss of function of major GSH enzymes and decreased substrate availability for glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting step of the pentose pathway. This shift in GSH status is associated with depressed myocyte mechanical function and Ca2+ transients. Furthermore, in vitro insulin treatment of diabetic rat myocytes normalizes [GSH], mechanical properties, and Ca2+ transients via pathways involving G6PD and GSH synthesis. Contraction and Ca2+ handling are also normalized by exogenous GSH or N-acetylcysteine (NAC). These results support the hypothesis that an essential function of glucose metabolism in the heart is the supply of NADPH via the pentose pathway to maintain adequate GSH levels for the redox control of intracellular Ca2+ handling and contraction.

MATERIALS AND METHODS

Induction of diabetes and isolation of myocytes. Animal experimentation was approved by the Animal Care and Use Committee of the University of Nebraska Medical Center, and this investigation conformed with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996). Male Sprague-Dawley rats weighing 220–225 g were made diabetic by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg/kg. Blood glucose levels were measured 3 days after STZ injection to confirm hyperglycemia. Normal rats of similar age and weight injected with vehicle only (1 mmol/l citrate buffer, pH 4.5) were used as controls. Diabetic rats at the end of our study period exhibited a nearly fourfold increase in blood glucose concentration compared with control rats (diabetic: 21.9 ± 0.7 mmol/l, n = 30; control: 5.5 ± 0.3 mmol/l, n = 26; P < 0.05), which was correlated with a significant decrease in immunoreactive serum insulin levels (diabetic: 0.3 ± 0.1 ng/ml; control: 2.0 ± 0.2 ng/ml; P < 0.05) as measured by a commercial kit (Alpo Diagnostics, Uppsala, Sweden). As in our previous study (49), body weight for the diabetic group (269.9 ± 3.0 g) was significantly less than for control animals (293.4 ± 2.9 g; P < 0.05). With this duration of diabetic conditions, heart weight-to-body weight ratio remains essentially unchanged from control (49).

Four to five weeks after STZ or vehicle injection, rats were given an overdose of pentobarbital sodium (150 mg/kg ip), and single ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the dissociated myocytes from all ventricular regions were suspended in 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50). The atria were discarded after the digestion procedure, and the ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion protocol described previously (27, 35, 49, 50).

Measurement of intracellular GSH. Intracellular [GSH] was measured by fluorescence microscopy using the probe monochlorobimane (mBCl; Molecular Probes, Eugene, OR) as described previously (27). Briefly, glass coverslips with attached myocytes were transferred to a culture dish filled with a loading solution containing (in mmol/l) 138 NaCl, 4.0 KCl, 1.2 MgCl2, 1.8 CaCl2, 18 glucose, 5 HEPES, and 0.4 mBCl. This solution also contained 2 mmol/l probenecid to inhibit transporter-mediated export of the GSH-bimane adduct. Cells were loaded with mBCl for 50–60 min at room temperature and then transferred to a recording chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Melville, NY). After washout of extracellular probe, myocytes were illuminated with light at 360 nm by means of a DeltaScan monochromator system (Photon Technology International, Lawrenceville, NJ). A 15 × 15-μm optical sampling window was positioned over the center of each myocyte, and emission fluorescence (460 nm) was collected with a photometer assembly. For all experiments, only Ca2+-tolerant, rod-shaped myocytes with clear cross striations were chosen for GSH measurement because these empirical criteria are correlated with the functional integrity of isolated myocytes (35, 49, 50). [GSH] was calculated from calibration curves generated with graded concentrations of GSH (7.8–1,000 μmol/l) plus rat liver glutathione-S-transferase (0.2 U/ml) to generate the fluorescent GSH-bimane adduct (27). Fluorescence data were collected from each myocyte for 30–60 s to verify steady-state conditions. If fluorescence varied by more than 5% during this recording period, data from that myocyte were excluded from analysis. Measured fluorescence was converted to [GSH] (in amol/μm2) using the calibration curve, the known dimensions of the sampling window, and an estimate of cell thickness derived from a previous study (27).

GSH was also measured in myocyte lysates using a commercial kit (Oxis Research, Portland, OR), which is based on an enzymatic recycling assay described by Tietze (45) and used in our previous studies (50). Briefly, suspensions of isolated myocytes were sonicated in 5% metaphosphoric acid to precipitate proteins, centrifuged at 4°C for 20 min (3000 g), and the supernatant was collected for assay. Total glutathione [GSH + oxidized glutathione (GSSG)] was measured in 100-μl samples of supernatant by recording the formation of 2-nitro-5-thiobenzoic acid at 412 nm (25°C) in the presence of 5,5′-dithio-bis(2-nitrobenzoic acid), NADPH, glutathione reductase, and EDTA. GSSG was determined by derivatizing 150-μl samples of supernatant with 1-methyl-2-vinylpyridium trifluoromethane sulfonate and assaying 100-μl aliquots of the derivatized sample as above for total GSH. Standard curves for GSH and GSSG were constructed, and the [GSH] was calculated by subtracting the GSSG concentration from total glutathione (GSH + GSSG). Measured concentrations of GSH and GSSG were expressed in micromoles per liter per milligram protein and as a ratio (GSH/GSSG).

Mechanical properties and intracellular Ca2+ transients. Mechanical properties of isolated ventricular myocytes were assessed with a video-based edge-detection system (IonOptix, Milton, MA). Briefly, cells were placed in a perfusion chamber mounted on the stage of an inverted microscope (X-40; Zeiss, Thornwood, NJ) and superfused with room temperature (25°C) standard external solution containing (in mmol/l) 138 NaCl, 4.0 KCl, 1.2 MgCl2, 1.8 CaCl2, 18 glucose, and 5 HEPES, pH 7.4. Cells were field stimulated (10 V, 10 ms) at 0.5 Hz through a pair of platinum wires placed on opposite sides of the chamber. The extent of myocyte shortening and rates of shortening and relengthening were measured by IonWizard software (version 5.0; IonOptix).

To measure Ca2+ transients, myocytes attached to laminin-coated glass coverslips were loaded with Fura 3 (5 μmol/l) for 30 min at 37°C using the same external solution as for contraction studies. At the end of the incubation period, cells were washed with external solution to remove extracellular Fura 3 and placed in a chamber on the stage of a laser confocal microscope (confocal LSM 410; Zeiss) equipped with an argon-krypton laser (25-mW argon laser, 0.5% intensity, ×100 lens). Cells superfused with room temperature external solution were then field stimulated at 0.5 Hz (10 V, 10 ms), and...
changes in fluorescence intensities were determined. Fluor 3 was excited by light at 488 nm, and fluorescence was measured at wavelengths of >515 nm. The rate of increase of fluorescence was determined with linear regression analysis, and fluorescence decay was measured as the time to 50% decrease from the peak value.

**Enzymes regulating GSH.** In a separate group of rats, the activities of glutathione reductase, γ-glutamylcysteine synthetase, and G6PD were measured in ventricular tissue samples by spectrophotometric methods. Glutathione reductase activity was measured by the technique of Carlberg and Mannervik (4). Briefly, 50- to 100-mg tissue samples were homogenized in ice-cold Tris buffer (0.1 mol/l, pH 8.0, with 2 mmol/l EDTA) and centrifuged at 4°C (6,000 g) for 30 min. A 200-μl aliquot of supernatant was added to a cuvette containing KH2PO4 buffer (0.2 mol/l, pH 7.0) plus 2 mmol/l EDTA, 20 mmol/l GSSG, and 2 mmol/l NADPH. The change in absorbance at 340 nm was monitored for 5 min at 30°C by a spectrophotometer (Thermo- Spectronic, Waltham, MA). A milliunit (mU) of glutathione reductase activity was defined as the amount of enzyme catalyzing the reduction of 1 nmol NADPH per minute.

γ-Glutamylcysteine synthetase activity was determined by the method of Seelig and Meister (37). Ventricular tissue samples were prepared as above, and a 50-μl aliquot of supernatant was added to a reaction mixture containing 0.1 mol/l Tris buffer and (in mmol/l) 150 KCl, 5 Na2ATP, 2 phosphoenolpyruvate, 10 l-glutamate, 10 l-cysteine, 20 MgCl2, 2 Na2EDTA, 0.2 NADH, 17 μg pyruvate kinase and 17 μg lactate dehydrogenase. The change in absorbance at 340 nm was monitored for 5 min at 37°C, and γ-glutamylcysteine synthetase activity was expressed in milliunits, defined as the activity converting 1 nmol of NADH to NAD per minute.

G6PD activity was measured with a commercial kit (OxisResearch, Foster City, CA). Briefly, ventricular tissue samples were homogenized in supplied diluent and centrifuged at 4°C (6,000 g) for 30 min. A 50-μl aliquot of supernatant was added to a reaction mixture containing NADP, glucose-6-phosphate, and 6-phosphogluconic acid. The change in absorbance at 340 nm in the presence of both substrates was measured at 37°C for 5 min. A second aliquot of supernatant was added to a reaction mixture containing NADP plus 6-phosphogluconic acid alone, and absorbance was again measured at 340 nm. G6PD activity was calculated by subtracting the rate of change in absorbance with 6-phosphogluconic acid alone from that measured with the combined substrates to eliminate the contribution of 6-phosphogluconate dehydrogenase to total NADPH production, thus yielding a more accurate estimation of rate-limiting G6PD activity. The net change in absorbance was expressed in milliunits, defined as the enzyme activity producing 1 nmol of NADPH per minute. Activities for γ-glutamylcysteine synthetase, glutathione reductase, and G6PD were normalized per milligram protein, measured by a commercial kit (Pierce Biotechnology, Rockford, IL).

Before intracellular GSH, Ca2+ transients, or mechanical properties were measured, isolated myocytes were maintained in culture conditions for up to 6 h. However, Fig. 2A shows that GSH/GSSG was significantly decreased in the diabetic group by 33% (1.6 ± 0.08, n = 5 hearts) compared with control (2.4 ± 0.1 amol/μm3 (n = 108 myocytes from 9 rats) and 2.3 ± 0.1 amol/μm3 (n = 122 myocytes from 8 rats), respectively. We also compared the change in [GSH] observed with our fluorescence microscopy technique with measurements of GSH/GSSG in suspensions of isolated myocytes using a standard spectrophotometric assay (45, 50). These latter analyses showed that GSH/GSSG was significantly decreased in the diabetic group by 33% (1.6 ± 0.08, n = 5 hearts) compared with control (2.4 ± 0.1, n = 6 hearts; P < 0.05), verifying conditions of oxidative stress.

Because cellular GSH content is determined by the balance of GSH utilization and production, we measured the activities of two major enzymes involved in controlling GSH levels. In Fig. 1, B and C, basal activities of γ-glutamylcysteine synthetase and glutathione reductase in ventricular tissue samples from control and diabetic rats are compared. As shown, these enzymes were mainly inhibited in the diabetic group compared with control, although the activities were not uniformly decreased throughout the heart. The largest changes were observed in samples from left ventricle, where γ-glutamylcysteine synthetase and glutathione reductase were decreased by 52 and 33%, respectively (P < 0.05). By comparison, tissue samples from the right ventricle showed the least amount of change. Although mean glutathione reductase activity in the right ventricle of diabetic rats was decreased from control by 31%, this change did not reach statistical significance (P = 0.09). Our group (27) previously found no significant regional variations in [GSH] in control or GSH-depleted myocytes. Thus, in the fluorescence microscopy experiments outlined below, we used a mixed population of ventricular myocytes and did not analyze regional differences in the control of [GSH].

As shown above in Fig. 1A, the decreased [GSH] in diabetic rat myocytes compared with control was stable under basal culture conditions for up to 6 h. However, Fig. 2A shows that [GSH] was upregulated by insulin or the pyruvate dehydrogenase activator DCA. Specifically, addition of 100 nmol/l insulin or 1.5 mmol/l DCA to the culture medium significantly increased [GSH] in diabetic rat myocytes but with different time courses. After 1–2 h of incubation with insulin, [GSH] was not different from untreated myocytes from the same group (2.4 ± 0.1 amol/μm3, n = 62), whereas the same duration of DCA treatment significantly increased [GSH] to near control levels (see Fig. 1A). By 3–4 h, both insulin and

**RESULTS**

Upregulation of GSH by insulin and DCA. Figure 1A compares mean data from fluorescence microscopy measurements of [GSH] in ventricular myocytes from control and diabetic rats. In general, mean [GSH] in diabetic rat myocytes was 30–40% less than in controls (P < 0.05) and was unchanged for up to 6 h of culture after cell isolation without any further experimental treatment. Moreover, diabetes-induced GSH depletion was similar throughout the 4- to 5-wk period of diabetic conditions examined in this study: mean [GSH] in myocytes from diabetic rats studied at 4 and 5 wk was 2.4 ± 0.1 amol/μm3 (n = 108 myocytes from 9 rats) and 2.3 ± 0.1 amol/μm3 (n = 122 myocytes from 8 rats), respectively. We also compared the change in [GSH] observed with our fluorescence microscopy technique with measurements of GSH/GSSG in suspensions of isolated myocytes using a standard spectrophotometric assay (45, 50). These latter analyses showed that GSH/GSSG was significantly decreased in the diabetic group by 33% (1.6 ± 0.08, n = 5 hearts) compared with control (2.4 ± 0.1, n = 6 hearts; P < 0.05), verifying conditions of oxidative stress.

Statistical analysis. All results are expressed as means ± SE. Comparisons of two groups were made with Student’s t-test, whereas more than two groups were compared by ANOVA. When a significant difference among groups was indicated by the initial analysis, individual paired comparisons were made by Dunnett’s t-test. Differences were considered significant at P < 0.05.
DCA had significantly increased [GSH] to control levels, whereas at the 5- to 6-h time point [GSH] remained elevated in insulin-treated cells but was decreased in the DCA-treated group ($P < 0.05$). Figure 2B shows that [GSH] in control myocytes was not significantly affected by insulin treatment up to 5–6 h; however, as in diabetic rat myocytes (Fig. 2A), DCA treatment for 5–6 h decreased [GSH] in control cells. Because of the depletion of GSH elicited by prolonged exposure to DCA, we conducted subsequent experiments using insulin as a metabolic agonist to further define the mechanisms of GSH regulation.

**Pathways regulating GSH.** The cellular effectors mediating upregulation of [GSH] by insulin in diabetic rat myocytes were examined in an additional series of experiments with blocking...
agents that target the GSH system at different sites. We first tested the effects of two structurally distinct inhibitors of G6PD, DHEA (30 μmol/l) and 6-AN (1 mmol/l). G6PD is the rate-limiting enzyme of the pentose pathway that generates NADPH required by glutathione reductase to convert GSSG to GSH (4, 9, 36–38). As shown in Fig. 3A, DHEA and 6-AN each blocked the effect of insulin to normalize [GSH] in the diabetic rat myocytes involved pathways in addition to G6PD. This suggested that insulin-sensitive regulation of [GSH] in diabetic rat myocytes involved pathways in addition to G6PD. Thus additional experiments were done in diabetic rat myocytes pretreated with buthionine sulfoximine (50 μmol/l) for 24 h and exposed to insulin in the presence of DHEA or 6-AN. Figure 3B shows that pretreatment with buthionine sulfoximine decreased basal [GSH] by 22% from untreated diabetic rat myocytes (untreated: 1.6 ± 0.88, n = 6 hearts; insulin-treated: 2.2 ± 0.2, n = 5 hearts; P < 0.05; DHEA-treated: 1.4 ± 0.1, n = 6 hearts; DHEA plus insulin: 1.6 ± 0.1, n = 6 hearts). However, fluorescence microscopy studies showed that each inhibitor alone significantly decreased [GSH] compared with that shown for untreated diabetic myocytes. This suggested that insulin-sensitive regulation of [GSH] in diabetic rat myocytes involved pathways in addition to G6PD. Thus additional experiments were done in diabetic rat myocytes pretreated with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (50 μmol/l) for 24 h and exposed to insulin in the presence of DHEA or 6-AN. Figure 3B shows that pretreatment with buthionine sulfoximine decreased basal [GSH] by 22% from untreated diabetic rat myocytes (P < 0.05) but that insulin still significantly increased [GSH], a response that was completely blocked by the addition of DHEA or 6-AN. In contrast to their effects on diabetic rat myocytes, neither DHEA nor 6-AN alone for 3–4 h significantly altered [GSH] in control myocytes (DHEA-treated: 3.6 ± 0.2 amol/μm³, n = 24; 6-AN-treated: 3.5 ± 0.2 amol/μm³, n = 21; untreated: 3.9 ± 0.2 amol/μm³, n = 21), whereas buthionine sulfoximine similarly decreased [GSH] by 24% (untreated: 3.8 ± 0.2 amol/μm³, n = 10; buthionine sulfoximine-treated: 2.9 ± 0.1 amol/μm³, n = 10; P < 0.05).

Because data from the DHEA and 6-AN experiments in diabetic rat myocytes suggest that G6PD plays a key role in GSH homeostasis, we assayed tissue samples from septum to determine whether this enzyme contributed to GSH depletion under diabetic conditions. However, as shown in Fig. 3C, G6PD activity in our diabetic model was not different from control. Finally, we assessed the effects of insulin on the other major GSH-related enzymes. Figure 3D shows that insulin treatment of diabetic rat myocytes significantly increased γ-glutamylcysteine synthetase activity but had no effect on glutathione reductase. In control myocytes, both enzymes were unaffected by insulin (data not shown) as was [GSH] (Fig. 2B). Likewise, insulin treatment of control cells had no significant effect on GSH/GSSG (insulin treated: 2.4 ± 0.1, n = 5 hearts; untreated: 2.4 ± 0.1, n = 6 hearts).

**Insulin regulation of mechanical properties and Ca²⁺ transients.** The functional impact of diabetes-induced GSH dysregulation was analyzed in relation to changes in mechanical properties and Ca²⁺ transients as measured in field-stimulated myocytes at room temperature. Our model of diabetes, as in other studies (6), was marked by significant decreases in

![Fig. 3. Effect of glucose-6-phosphate dehydrogenase (G6PD) and γ-GCS inhibitors on GSH response to insulin.](http://ajpheart.physiology.org/cgi/content/10.1152/ajpheart.00059.2006)
myocyte mechanical activity and prolonged kinetics of Ca\(^{2+}\) transients when compared with controls. Figure 4A shows superimposed length changes in a control and diabetic rat myocyte paced at 0.5 Hz. In addition to a marked decrease in the extent of shortening, the diabetic rat myocyte was characterized by profound decreases in the rate of shortening and relengthening compared with control. These changes in mechanical properties correlated closely with alterations in evoked Ca\(^{2+}\) transients (Fig. 4B). The line scans (Fig. 4B, top) illustrate that the evoked Ca\(^{2+}\) release from control and diabetic rat myocytes was uniform throughout the length of these cells. However, the traces (Fig. 4B, bottom) show that the rate of Ca\(^{2+}\) rise, the peak Ca\(^{2+}\) release, and the Ca\(^{2+}\) decay kinetics were suppressed in the diabetic rat myocyte compared with the control cell. Mean data from cell length and Ca\(^{2+}\) transient studies are summarized in Table 1, which also shows the effect of insulin treatment. In our contraction studies, insulin treatment of diabetic rat myocytes for 3–4 h normalized the rates of cell shortening (−dL/dt) and relengthening (+dL/dt) and percent shortening, whereas these parameters were unaffected by insulin in control myocytes. Similar to regulation of GSH (Fig. 3B), the effect of insulin on mechanical parameters in diabetic rat myocytes was blocked by pretreating cells with DHEA plus buthionine sulfoximine. Insulin also normalized kinetic parameters and peak Ca\(^{2+}\) release in Ca\(^{2+}\) transient studies.

Because insulin normalized [GSH], Ca\(^{2+}\) transients, and mechanical properties in diabetic rat myocytes, we next tested the functional role of GSH by incubating myocytes for 3–4 h with exogenous GSH or NAC, a GSH precursor. Figure 5A shows concentration-response data for GSH and NAC on mechanical properties (−dL/dt, +dL/dt, %shortening) of diabetic rat myocytes. The changes in mechanical properties elicited by GSH and NAC were similar over the concentration range of 0.1–10 mmol/l, but significance only occurred at 10 mmol/l, which was the test concentration used in all subsequent experiments. Figure 5B shows that, in contrast to diabetic rat myocytes, neither 10 mmol/l GSH nor NAC significantly affected mechanical parameters in control myocytes. Although these exogenous reductants significantly improved mechanical function in diabetic rat myocytes, they did not normalize all measured parameters. Specifically, in diabetic rat myocytes treated with GSH, −dL/dt (139.8 ± 9.4 μm/s) and percent shortening (10.8 ± 0.3%) were significantly less than in comparably treated control myocytes (201.5 ± 27.6 μm/s and 12.3% ± 0.5%, respectively; P < 0.05). +dL/dt in GSH-treated diabetic cells was also less than in controls (143.0 ± 10.2 vs. 169.8 ± 17.3 μm/s); but this difference was not significant (P = 0.16). Similar differences in mechanical properties were also observed in NAC-treated diabetic and control myocytes (data not shown).

Parallel confocal experiments were conducted to assess the effects of GSH and NAC on Ca\(^{2+}\) transients. Figure 6A shows that GSH and NAC treatment of diabetic rat myocytes increased the rate of Ca\(^{2+}\) rise and peak Ca\(^{2+}\), whereas it decreased the Ca\(^{2+}\) decay time. Similar to insulin experiments, neither GSH nor NAC significantly affected the kinetics of Ca\(^{2+}\) transients in control myocytes (Fig. 6B). However, in contrast to the effects on mechanical properties (Fig. 5), GSH and NAC both normalized parameters of the Ca\(^{2+}\) transients. Specifically, in diabetic rat myocytes treated with GSH, the rates of Ca\(^{2+}\) rise [103.1 ± 8.4 fluorescence intensity in arbitrary units (fau)/s], peak Ca\(^{2+}\) (2.06 ± 0.19 fau), and Ca\(^{2+}\) decay time (368.3 ± 20.0 ms) were not significantly different from GSH-treated control myocytes (119.1 ± 8.5 fau/s, 1.95 ± 0.10 fau, and 347.7 ± 29.6 ms, respectively). Similar findings were observed in diabetic and control myocytes treated with NAC (data not shown).

**DISCUSSION**

Oxidative stress and GSH in diabetic heart. Intracellular GSH is an essential tripeptide that directly or indirectly regulates a number of biological processes, such as antioxidant defenses, DNA synthesis, ion transport, enzyme activity, transcription, and signal transduction (9, 37, 38). Much of the control of these processes by GSH is mediated by its regulation of the redox state of protein thiol groups of cysteine residues,
Table 1. Effect of in vitro insulin treatment on myocyte mechanical properties and Ca\(^{2+}\) transients

<table>
<thead>
<tr>
<th></th>
<th>Control length, (\mu m)</th>
<th>(-dL/dt, \mu m/s)</th>
<th>(+dL/dt, \mu m/s)</th>
<th>%Shortening</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Untreated</td>
<td>126.5 ± 6.8</td>
<td>113.6 ± 7.3</td>
<td>189.7 ± 10.6</td>
<td>98.5 ± 7.6*</td>
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<tr>
<td>Insulin</td>
<td>121.5 ± 8.5</td>
<td>108.9 ± 6.5</td>
<td>192.0 ± 10.2</td>
<td>169.8 ± 15.6</td>
</tr>
<tr>
<td>DHEA + BSO + Ins</td>
<td>129.1 ± 8.1</td>
<td>103.2 ± 6.7</td>
<td>190.2 ± 14.2</td>
<td>87.3 ± 8.8*</td>
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<tr>
<td>Rate of Ca(^{2+}) rise, fau/s</td>
<td></td>
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<tr>
<td>Control</td>
<td>2.9 ± 0.16</td>
<td>1.52 ± 0.10*</td>
<td>38.1 ± 2.3</td>
<td>50.1 ± 2.3*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.99 ± 0.13</td>
<td>2.05 ± 0.15</td>
<td>35.7 ± 2.7</td>
<td>40.2 ± 4.5</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.08 ± 0.16</td>
<td>1.52 ± 0.10*</td>
<td>38.1 ± 2.3</td>
<td>50.1 ± 2.3*</td>
</tr>
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</table>

Values are means ± SE. Mean data for mechanical studies based on 20–28 control myocytes and 26–35 diabetic rat myocytes. Mean data for Ca\(^{2+}\) transients based on 37 untreated and 32 insulin-treated myocytes in the control group and 32 untreated and 27 insulin-treated myocytes from the diabetic group. Myocytes were pretreated with 100 nmol/l insulin for 3–4 h. \(-dL/dt\), maximum rate of myocyte shortening; \(+dL/dt\), maximum rate of relengthening; DHEA, dehydroepiandrosterone; BSO, buthionine sulfoximine; fau, fluorescence intensity in arbitrary units measured as \(F_{\text{max}} - F_{\text{b}}\) (where \(F\) is fluorescence). *\(P < 0.05\) compared with untreated control.

...which determine the structure and activity of many proteins required for normal cell activity (36). Under oxidative stress conditions, free thiols can undergo a number of different modifications to yield unique chemical intermediates that can lead to a gain or loss of protein function. GSH has been shown to control the biological activity of proteins by preventing modification of thiols through detoxification of reactive oxygen species or by directly participating in the reduction of thiol oxidation of thiols through detoxification of reactive oxygen species. In mammalian species or by directly participating in the reduction of thiol modification of thiols through detoxification of reactive oxygen species. GSH is the principal thiol in the cell, and its activities are essential for cell homeostasis. 

Glucose metabolism and GSH. Although glutathione reductase and \(\gamma\)-glutamylcysteine synthetase are generally considered major enzymes involved in GSH homeostasis, upstream accessory pathways such as G6PD and pyruvate dehydrogenase have also been shown to be key factors affecting [GSH]. G6PD is the rate-limiting enzyme of the cytosolic pentose pathway that generates NADPH utilized by glutathione reductase (9, 36–38). Recent data show that targeted deficiency of G6PD in mice leads to GSH depletion, accumulation of GSSG, and impaired Ca\(^{2+}\) handling in ventricular myocytes (24). In experimental models of diabetes, variable changes in the activity of this enzyme have been reported (23, 51), and in our study we found no change in basal G6PD activity in diabetic hearts compared with control (Fig. 3C). Nevertheless, this enzyme played a key role in insulin-mediated normalization of [GSH], as evidenced by inhibitory effects of DHEA and 6-AN (Fig. 3A). Residual \(\gamma\)-glutamylcysteine synthetase activity also contributed to GSH upregulation by insulin in diabetic rat myocytes, such that, when this enzyme was inhibited along with G6PD, insulin had no effect (Fig. 3B). Together, these findings indicate that an essential function of glucose metabolism in the heart, independent of ATP production, is the regulation of GSH levels for redox control of cell function.

In contrast to variable changes in G6PD, there is general agreement that the activity of pyruvate dehydrogenase is decreased in diabetic rat heart due to an increased state of phosphorylation mediated by pyruvate dehydrogenase kinase (19). DCA inhibits pyruvate dehydrogenase kinase, thereby stimulating overall pyruvate dehydrogenase activity and promoting glucose and pyruvate oxidation (1, 42). Moreover, DCA increases the supply of substrate for active pyruvate dehydrogenase by a concomitant increase in glucose and pyruvate uptake (30). In the present study, we found that DCA rapidly increased [GSH] to control levels in diabetic rat myocytes but that this effect was transient (Fig. 2A). Although we did not examine the mechanism for the decline in [GSH] with prolonged DCA exposure, which was also observed in control myocytes, previous studies suggest that this response may be due to glutathione-\(S\)-transferase-catalyzed conjugation of intracellular DCA with GSH (21), which would be expected to cause GSH depletion.

Despite evidence of its influence on GSH, the link between pyruvate dehydrogenase and GSH homeostasis is not fully understood. Experiments in isolated rat hearts suggest that increased pyruvate dehydrogenase activity leads to citrate ac-
cumulation that increases NADPH production by cytosolic isocitrate dehydrogenase or inhibits phosphofructokinase, thereby diverting glycolytic flux to NADPH-generating G6PD (42). Compared with DCA, insulin in our experiments produced a delayed but more sustained increase in [GSH] over a 6-h incubation period (Fig. 2A). The reason for the lag in response to insulin may simply be due to a dose effect; i.e., a higher insulin concentration would elicit a more rapid response. However, Lloyd et al. (30) reported that insulin and DCA exert quantitatively different effects on substrate oxidation in isolated rat heart. In particular, they showed that DCA exerts a more robust stimulation of pyruvate oxidation than insulin. This may be due to different mechanisms controlling the phosphorylation state of pyruvate dehydrogenase; i.e., DCA inhibits pyruvate dehydrogenase kinase (1, 42), whereas insulin increases pyruvate dehydrogenase phosphatase activity (29, 30). Alternatively, the different time course of GSH upregulation may be related to the more diverse metabolic effects of insulin on the myocardium compared with DCA, such as increased expression or activity of hexokinase (33) or γ-glutamylcysteine synthetase (20) (Fig. 3D). There is no present compelling evidence that glutathione reductase is directly regulated by insulin (44), despite this enzyme being essential for GSH homeostasis. Indeed, our study found no significant change in glutathione reductase activity in the presence of insulin (Fig. 3D).

Cellular functions of GSH. GSH is a key molecule that regulates a multitude of physiological processes (9, 37, 38) through thiol-disulfide exchange reactions that directly involve GSH or that are catalyzed by the oxidoreductase glutaredoxin,
which requires GSH as cofactor (15). Recent data suggest that GSH and glutaredoxin are important regulators of pathophysiological signals activated by oxidative stress, such as apoptosis (7, 22). We also found evidence of regulation of myocyte Ca\(^{2+}\) handling and mechanical properties by GSH in the present experiments (Figs. 5 and 6). Our findings are consistent with studies of G6PD deficiency in nondiabetic myocytes (24), which show similar degrees of GSH depletion and mechanical dysfunction as in our diabetic model. However, it is not known whether regulation of myocyte Ca\(^{2+}\) handling is mediated by the antioxidant or reducing properties of GSH. In the former case, GSH may protect Ca\(^{2+}\) handling proteins from oxidation by directly scavenging reactive oxygen species or by acting as cofactor for inactivation of hydroperoxides by glutathione peroxidase (10, 48). Alternatively, GSH may directly reduce oxidized protein thiols or participate as a cofactor for glutaredoxin-catalyzed thiol reduction (15). The same possible mechanisms also apply to NAC, which increases [GSH] in depleted myocytes (28) and which showed effects similar to GSH in the present study (Figs. 5 and 6). Additional investigation is necessary to better define the antioxidant and reducing effects of exogenous reductants.

It is clear that diabetes elicits significant contractile dysfunction in cardiomyocytes that is accompanied by Ca\(^{2+}\) dysregulation. Experimental studies of long-term diabetes (e.g., >8 wk; Ref. 6) generally show decreased expression of ryanodine receptors (RyR2), sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), and the Na\(^+\)/Ca\(^{2+}\) exchanger, which are postulated to contribute to an abnormal contractile phenotype (2, 6). Although some studies have shown a decrease in L-type Ca\(^{2+}\) current (3), most diabetic models report little change (6, 39). At earlier stages of the disease, as represented by the present study, RyR2 expression and SERCA2a expression are not significantly altered from control (2, 52), although Na\(^+\)/Ca\(^{2+}\) exchanger expression may still be decreased (17). However, the relatively rapid (3–4 h) normalization of Ca\(^{2+}\) transients

![Figure 6. Effect of exogenous GSH and NAC on Ca\(^{2+}\) transients.](image-url)
and mechanical properties by insulin (Table 1), GSH, and NAC (Figs. 5 and 6) in our study is more consistent with posttranslational changes in Ca\(^{2+}\) handling proteins than with changes in expression, and the effects of exogenous GSH and NAC further imply a redox mechanism. Indeed, experimental evidence shows that several Ca\(^{2+}\) handling proteins in myocytes are acutely modulated by redox-specific mechanisms. For example, exogenous thiol-reactive compounds inhibit L-type Ca\(^{2+}\) channels (5, 13) and enhance Ca\(^{2+}\)-induced Ca\(^{2+}\) release from RyR2 (18) in a manner that is reversed by reducing agents such as dithiothreitol. These studies suggest that a pathophysiological shift in myocyte redox state, such as occurs in diabetes, promotes oxidative damage of specific Ca\(^{2+}\) handling proteins that contribute to contractile dysfunction. However, the precise modulation of protein damage by GSH or other endogenous reductants in the setting of diabetes is not well understood. Our study thus provides important functional insights into the repair capabilities of endogenous redox networks such as those utilizing GSH.

In summary, our data suggest that diabetes-induced loss of function of GSH regulatory enzymes contributes to depletion of GSH in ventricular myocytes. We postulate that insulin signaling regulates myocardial GSH through a coordinated activation of pathways involved in GSH synthesis and NADPH production. The status of GSH in turn impacts cardiomyocyte Ca\(^{2+}\) handling proteins and mechanical properties. Thus, it may be postulated that diabetes-mediated GSH depletion contributes to functional alterations in redox-sensitive Ca\(^{2+}\) handling proteins and that signaling pathways related to insulin are protective by virtue of their control of GSH availability for antioxidant and reducing functions.

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