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Cardiovascular Research 53 (2002) 80–88

Cardiovascular Research

www.elsevier.com/locate/cardiores

Up-regulation of K^+ channels in diabetic rat ventricular myocytes by insulin and glutathione

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Received 20 March 2001; accepted 6 August 2001

Abstract

Objective: The cardiac pathogenesis of diabetes mellitus involves oxidative stress that elicits profound changes in myocardial glutathione, an endogenous regulator of cell function. This study examined the role of glutathione in regulating K^+ channel activity in isolated ventricular myocytes from diabetic rats and its relationship to insulin signaling. **Methods and results:** Colorimetric analysis of extracts of ventricular tissue from Sprague–Dawley rats showed that the basal level of reduced glutathione (GSH) was significantly less in rats with experimental diabetes compared with sham controls, consistent with oxidative stress conditions. This change in GSH status paralleled a significant decrease in the activity of γ-glutamylcysteine synthetase, a major pathway involved in GSH homeostasis. Voltage-clamp studies confirmed that, compared with control myocytes, K^+ channels carrying the transient outward current (I_{to}) are down-regulated in the diabetic state and that this electrophysiological change is reversed by in vitro treatment with insulin for 2–3 h. Incubation of diabetic rat myocytes with GSH also normalized I_{to} density compared with untreated myocytes, but with a longer time course than insulin. To determine if up-regulation of I_{to} by insulin was mediated by alterations in myocyte GSH, insulin-responsiveness of diabetic rat myocytes was tested in the presence of 1,3-bis-chloroethyl-nitrosourea, an inhibitor of glutathione reductase, or buthionine sulfoximine, a blocker of γ-glutamylcysteine synthetase. Neither blocker alone altered I_{to} density in diabetic rat myocytes when compared with untreated cells, but each blocked the effect of insulin to up-regulate I_{to}. **Conclusions:** These data suggest that oxidative stress-induced alteration in GSH redox state plays an important role in regulating I_{to} channel function and that GSH homeostasis in ventricular myocytes is functionally coupled to insulin signaling.

Keywords: Diabetes; Free radicals; K^+ channel; Myocytes; Repolarization

1. Introduction

Early cardiovascular complications of diabetes mellitus include significant defects in heart muscle function that are independent of vascular pathology. This condition of diabetic cardiomyopathy is characterized by impaired contractility and abnormal electrical properties [1,2]. Among the more consistent myopathic alterations in cell function that have been reported in experimental models of diabetes is down-regulation of K^+ channel activity underlying the Ca^{2+}-independent transient outward current, I_{to} [3–8] and the quasi steady-state outward current, I_{ss} [6–8]. These changes in ion channel phenotype are proposed to contribute to delayed and abnormal repolarization in the intact heart which may be arrhythmogenic [9]. Moreover, long-term down-regulation of repolarizing K^+ channels may elevate intracellular [Ca^{2+}] and accelerate the progression toward heart failure [10].

The molecular basis for the electrophysiological changes observed in the diabetic heart has been studied in relation to changes in myocardial insulin signaling and glucose utilization, which are markedly impaired in the diabetic state [1,2]. In particular, treatment of isolated ventricular myocytes from diabetic rats with insulin [3–8] or activators of pyruvate dehydrogenase [3,4] increases I_{to}

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Time for primary review 22 days.

0008-6363/02/$ – see front matter
PII: S0008-6363(01)00446-1

density to control levels after a delay of several hours. These data provide important evidence linking myocyte glucose utilization and the activity of cardiac K^+ channels controlling ventricular repolarization. The specific mechanisms involved in this interaction and their alterations during diabetes are not fully understood, but recent evidence suggests that insulin regulation of I_{to} may involve changes in transcription and post-transcriptional processing of channel proteins [8].

A major complication of diabetes that profoundly impacts cardiac function is oxidative stress, which is proposed to result from increased production of reactive oxygen species and deficits in antioxidant defense mechanisms [11–15]. It is clear that excess reactive oxygen species damages cells by reacting with unsaturated lipids, proteins, and nucleic acids [16–18], and it is through such reactions that abnormalities in ventricular function are proposed to develop during uncontrolled diabetes mellitus [11,12]. An important outcome of oxidative stress in the myocardium is a shift in cell redox state, reflected by a decrease in the level of reduced glutathione (GSH) and accumulation of its oxidized form (GSSG) [16,19–21]. The ubiquitous tripeptide GSH plays a major role in regulating many cell proteins susceptible to oxidation, and thus maintenance of a normally high intracellular GSH concentration protects these proteins by maintaining them in a reduced state [19,21]. However, when GSSG accumulates in cells in response to oxidative stress, it reacts with protein thiols (–SH) of cysteine residues, causing formation of mixed or intramolecular disulfides [18,19,21]. This type of modification of redox-sensitive proteins can have important functional effects on overall activity of cells [19,21].

Recent experimental evidence suggests that components of glucose metabolism are involved in GSH homeostasis [17,22–24]. Thus, the purpose of the present study was to examine the relationship of insulin action with GSH metabolism in the control of I_{to} channels in diabetic rat ventricular myocytes. We report that in vitro treatment of isolated myocytes from diabetic rats with GSH up-regulates I_{to} density, similar to the response of cells to insulin. Moreover, the effect of insulin to normalize I_{to} in diabetic rat myocytes is inhibited by blockers of two major enzymes in GSH metabolism. Our data therefore suggest that diabetes-induced alterations in GSH redox state may play an important role in regulating I_{to} channel function and that increasing glucose utilization in diabetic rat myocytes may up-regulate I_{to} density by increasing intracellular GSH.

2. Methods

2.1. Isolation of cardiac myocytes

The institutional review committee at the University of Nebraska Medical Center approved the animal research protocol used in this study and the investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague–Dawley rats weighing 180–200 g were made diabetic by a single intraperitoneal injection of streptozotocin at a dose of 65 mg/kg [3]. Normal rats of similar age and weight used as controls were injected with vehicle only (1 mM citrate buffer, pH 4.5). Diabetic rats in our study exhibited an approximately fourfold greater blood glucose concentration compared with control rats: 20.4 ± 0.9 ($n=23$) versus 4.9 ± 0.2 mM ($n=38$); $P<0.05$. As in our previous studies, body and heart weight in the diabetic group were significantly less than control, but the mean heart weight-to-body weight ratio was not different between groups of rats [3].

Two to four weeks after streptozotocin or vehicle injection, rats were given an overdose of pentobarbital sodium (100 mg/kg, i.p.) and single ventricular myocytes were dissociated from excised, perfused hearts by a collagenase digestion procedure described previously [3]. Dissociated myocytes from both ventricles were suspended in Dulbecco's modified Eagle's medium plus Ham's F-12 (18 mM [glucose]) and stored in an incubator at 37°C until used, usually within 6 h of isolation. In some experiments, myocytes were cultured for 24 h before study. Aliquots of myocytes were transferred to a cell chamber mounted on the stage of an inverted microscope and superfused at 1–2 ml/min with a standard external solution containing (in mM): 138 NaCl, 4.0 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 18 glucose, 5 HEPES, pH 7.4. This solution also contained 0.5 mM $CdCl_2$ to block Ca^{2+} channels.

2.2. Recording techniques

Ionic currents were recorded using the whole-cell configuration of the patch-clamp technique. Briefly, borosilicate glass capillaries were pulled (Sutter Instruments, Model P-87) to an internal tip diameter of 1–2 μm and filled with a pipette solution containing (in mM): 135 KCl, 3 $MgCl_2$, 10 HEPES, 3 Na_2-ATP, 10 EGTA, 0.5 Na-GTP, pH 7.2. Pipettes were coupled to a patch-clamp amplifier (Axopatch 1C, Axon Instruments) and after GΩ seal formation, whole-cell recording conditions were established by rupturing the membrane within the pipette followed by series resistance compensation. During equilibration, whole-cell capacitance was calculated as the area under the capacitative transient divided by the amplitude (−5 mV) of an applied test pulse. A computer program (pClamp, Axon Instruments) controlled command potentials and acquired current signals which were filtered at 2 kHz using a four-pole low-pass Bessel filter. Currents were sampled at 4 kHz by a 12-bit resolution analog-to-digital converter (Axon Instruments) and stored on the hard

disk of a computer. All experiments were done at room temperature (22–24°C).

I_{to} was evoked in each cell by 500-ms depolarizing pulses to test potentials between −40 and +60 mV (0.2 Hz). The holding potential in all experiments was −80 mV and a 100-ms prepulse was applied to −60 mV to inactivate the fast Na^+ current. For each test pulse, I_{to} amplitude was measured as the difference between peak outward current and the steady-state current level at the end of the depolarizing clamp pulse. Data were normalized as current densities by dividing measured current amplitude by whole-cell capacitance (pA/pF).

2.3. Measurement of GSH and related enzymes

The major intracellular redox buffer, GSH, was measured using the enzymatic method of Floreani et al. [25]. Briefly, 50–100-mg tissue samples from the left ventricle were homogenized in 6% metaphosphoric acid. The homogenate was centrifuged (3000×*g*, 4°C, 10 min) and the supernatant collected for assay. Total glutathione (GSH+GSSG) was measured in 100-μl samples of the supernatant by recording the formation of 2-nitro-5-thiobenzoic acid at 412 nm (25°C) in a spectrophotometer (Genesys II) in the presence of 0.25 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 0.4 mM NADPH and 2 U glutathione reductase (type III, Sigma). Oxidized glutathione (GSSG) was determined by derivatizing 150-μl samples of supernatant with 3 μl of undiluted 2-vinylpyridine and assaying 100-μl aliquots of the derivatized sample as above for total GSH. Standard curves for GSH and GSSG were constructed and GSH concentration calculated by subtracting the concentration of GSSG from the total glutathione (GSH+GSSG). Measured concentrations of GSH and GSSG were expressed per gram wet tissue weight and as a ratio, GSH/GSSG. We also measured GSH concentration in isolated myocytes using a modification of the enzymatic technique described above. Briefly, aliquots of myocytes (~$1–2\times10^5$ cells/aliquot) were sonicated in 300 μl of 10% trichloroacetic acid, and centrifuged. The supernatant was added to cuvettes containing 100 μl of 0.05 M EDTA, 590 μl Tris-EDTA buffer (1.0 M Tris, 0.02 M EDTA) and 10 μl of 0.01 M DTNB. After 5-min equilibration, absorbance was read at 412 nm (25°C) and compared with known GSH standards. Measured GSH concentrations were expressed per 10^6 cells, the latter measured by a hemocytometer.

The activities of glutathione reductase and γ-glutamylcysteine synthetase were also determined by spectrophotometric methods. Glutathione reductase activity was measured by the method of Carlberg and Mannervik [26]. Briefly, isolated tissue samples (50–100 mg) from the interventricular septum were homogenized in ice-cold Tris buffer (0.1 M, pH 8.0 with 2 mM EDTA), centrifuged at 6000×*g* at 4°C for 30 min, and the supernatant collected. A 200-μl aliquot of the supernatant was added to a 1-ml cuvette containing KH_2PO_4 buffer (0.2 M, pH 7.0) plus 2 mM EDTA, 20 mM GSSG and 2 mM NADPH. The change in absorbance at 340 nm was monitored for 5 min at 30°C. A unit of glutathione reductase activity was defined as the amount of enzyme catalyzing the reduction of 1 μM NADPH per minute. Specific activity was expressed in milliunits (mU) per mg protein, the latter measured by a commercial kit (Pierce).

γ-Glutamylcysteine synthetase activity was determined by the method of Seelig and Meister [27]. Briefly, tissue samples (50–100 mg) from the septum were homogenized in Tris buffer (0.1 M, pH 8.0 with 2 mM EDTA) and centrifuged at 10,000×*g* at 4°C for 30 min. A 50-μl aliquot of supernatant was added to a reaction mixture containing 0.1 M Tris buffer, 150 mM KCl, 5 mM Na_2-ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM Na_2-EDTA, 0.2 mM 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 mU, defined as the activity converting 1 nM of NADH to NAD per minute. Enzyme activity for each sample was normalized per mg protein as for glutathione reductase.

2.4. Statistical analysis

All results are expressed as a mean±S.E.M. Comparisons of two groups were made using a Student's *t*-test, whereas comparisons of more than two groups were carried out by analysis of variance. When a significant difference among groups was indicated by the initial analysis, individual paired comparisons were made using a modified Bonferroni *t*-test. Differences were considered significant at $P<0.05$.

3. Results

3.1. The GSH system in diabetic rat heart

Significant changes in cardiac content of GSH and GSSG have been documented in experimental models of diabetes, suggesting that the heart is under marked oxidative stress [11,12]. To assess the status of the GSH system in our model, we first compared concentrations of GSH and GSSG in left ventricular tissue samples from control and diabetic rat hearts. Fig. 1A illustrates that mean GSH concentration in the diabetic rat heart (filled bars) was ~35% less than control, whereas the GSSG level was significantly increased in the diabetic rat heart. Therefore, as summarized in Fig. 1B, the cell redox state, as reflected by GSH/GSSG ratio, was decreased ~50% in the diabetic rat heart compared with control. Consistent with these findings, basal GSH concentration measured in isolated myocytes from diabetic rat hearts (75.3±3.9 nM/10^6 cells,

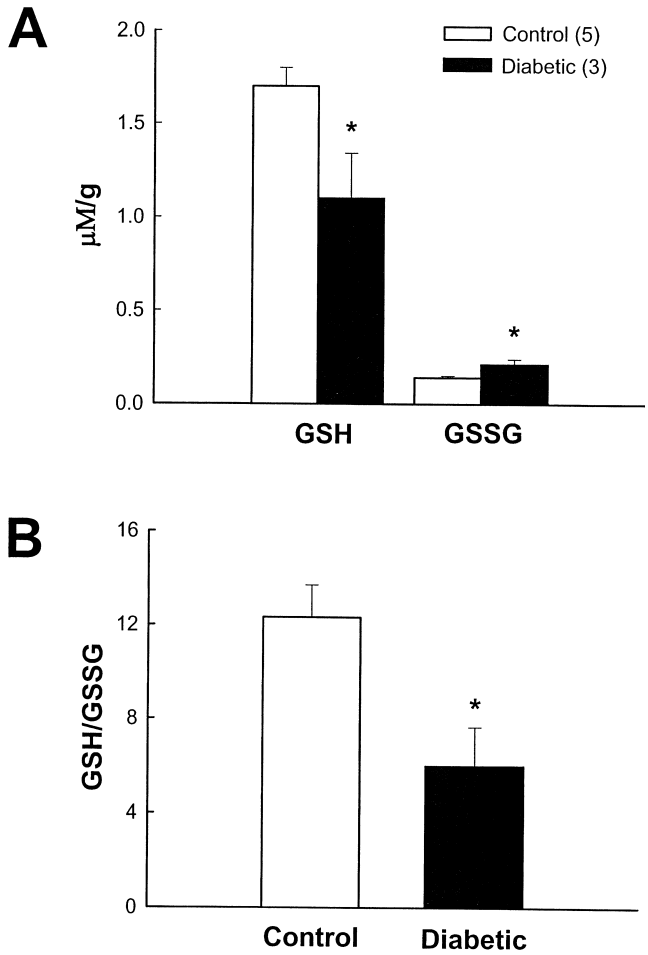

Fig. 1. GSH status in the ventricle. (A) GSH and GSSG levels in left ventricle of control and diabetic rat hearts were measured and expressed in μM per gram wet tissue weight. *$P<0.05$ compared with control. (B) GSH/GSSG ratio is compared for control and diabetic rat ventricular tissue. *$P<0.05$ compared with control. Number of tissue samples analyzed in each group is shown in parentheses.

$n=7$ hearts) was ~21% less than control (95.0±5.0 nM/10^6 cells, $n=8$ hearts; $P<0.05$). These data suggest therefore that the diabetic rat heart is subjected to marked oxidative stress, most likely related to the significant hyperglycemia elicited after streptozotocin injection.

The normal steady-state concentration of GSH in cardiac myocytes, as in most mammalian cells, is in excess of GSSG, through the activities of two major pathways: (i) glutathione reductase, which catalyzes the reduction of GSSG to GSH using NADPH as a source of reducing equivalents [26]; and (ii) γ-glutamylcysteine synthetase, the rate limiting step in de novo GSH synthesis [27]. Therefore, to further explore cellular mechanisms responsible for the decline in GSH in the diabetic rat heart, the activities of these enzymes were measured in tissue samples of septum from each heart. Fig. 2A illustrates that the basal activity of glutathione reductase in the diabetic rat heart (filled bar) was 13% less than control ($P>0.05$).

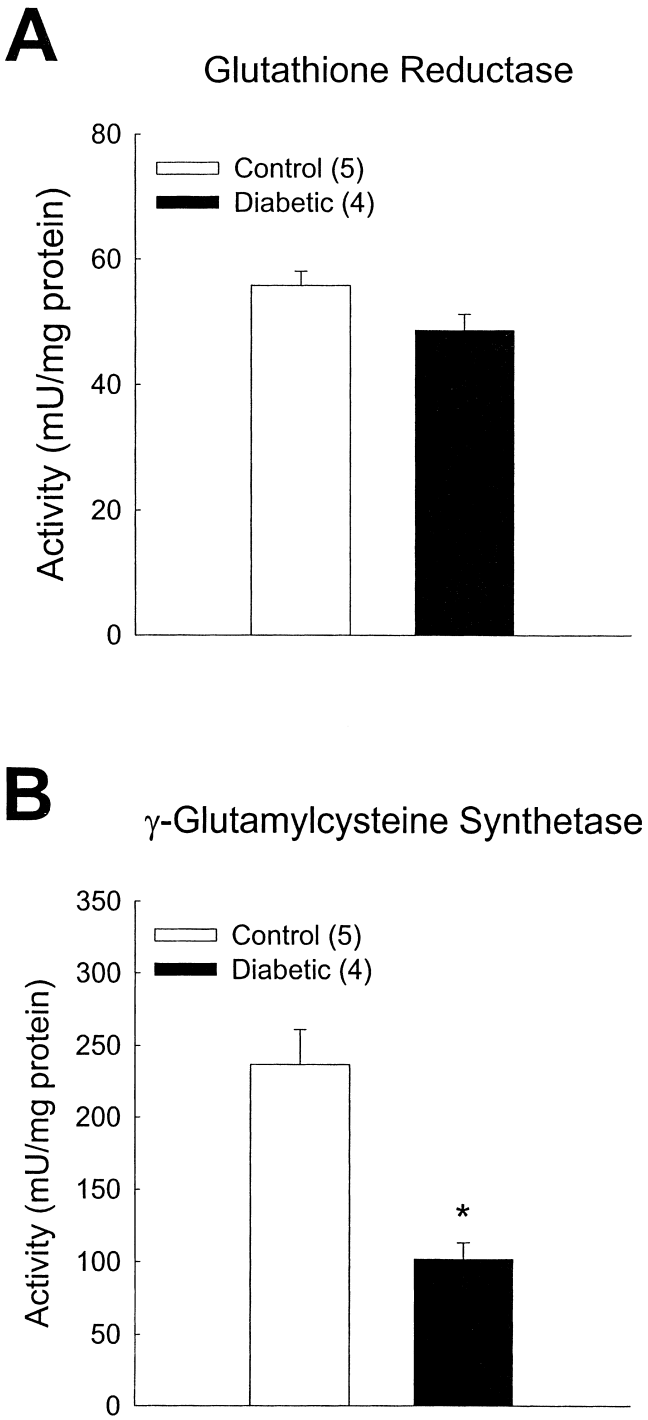

Fig. 2. GSH-related enzymes in heart. (A) Basal activity of glutathione reductase, expressed as mU/mg protein, is compared for tissue samples taken from control (open bar) and diabetic rat hearts. (B) Activity of γ-glutamylcysteine synthetase is compared for control (open bar) and diabetic rat hearts. *$P<0.05$.

A larger difference in activity was found for γ-glutamylcysteine synthetase (Fig. 2B), which was 57% less in the diabetic group (filled bar) compared with control ($P<0.05$). The depressed activities of these pathways in the diabetic state thus provide a mechanistic explanation

for the significant decrease in GSH content of isolated tissues and myocytes from diabetic rat hearts.

3.2. Effects of exogenous GSH on I_{to} in diabetic rat myocytes

Voltage-clamp studies from our laboratory [3–5] and others [6–8] have consistently shown a significant decrease in basal I_{to} density in myocytes from diabetic rat hearts compared with control, with no significant changes in voltage- or time-dependent properties. Down-regulation of I_{to} was confirmed in the present study where maximum I_{to} density (+60 mV) in diabetic rat myocytes was ~30% less than control (data not shown). Moreover, it has been shown that in vitro treatment of diabetic rat myocytes with insulin normalizes I_{to} density after a delay of several hours [3–8]. This insulin-mediated up-regulation of I_{to} was also verified in the present study where treatment of diabetic rat myocytes with 0.1 μM insulin for 2–4 h increased mean maximum I_{to} density (+60 mV) from 18.5±1.4 (untreated myocytes; $n=12$) to 26.3±2.0 ($n=16$) pA/pF ($P<0.05$), a mean value that was not different from control myocytes (26.9±1.4 pA/pF, $n=24$; $P>0.05$). Moreover, in contrast to diabetic rat myocytes, 2–4-h insulin treatment of control myocytes did not alter maximum I_{to} density (untreated, 26.4±2.1 pA/pF, $n=15$; insulin treated, 26.4±4.1 pA/pF, $n=8$; $P>0.05$).

Given that myocardial GSH in diabetic rats was significantly less than control (Fig. 1A), we next tested the hypothesis that GSH controls I_{to} channel density. Fig. 3 summarizes results from experiments where isolated myocytes from diabetic rat hearts were incubated with GSH. Fig. 3A compares raw current traces recorded at test potentials from −40 to +60 mV in an untreated diabetic rat myocyte (upper traces) and another treated with 2 mM GSH for 5 h. In the GSH-treated cell ($C_m=122$ pF), I_{to} amplitude was greater than in the untreated myocyte ($C_m=125$ pF). Fig. 3B plots maximum I_{to} density in untreated (open bars) and GSH-treated diabetic rat myocytes as a function of incubation time. For purposes of comparison, mean data from insulin-treated diabetic rat myocytes are also shown (filled bars). These data illustrate that insulin treatment for 2–4 h (left-hand bars) significantly increased maximum I_{to} density compared with untreated and GSH-treated diabetic rat myocytes. However, as shown by the right-hand bars, GSH treatment for 4–6 h also normalized I_{to} density as did insulin. Fig. 3C compares mean I–V relationships recorded from control myocytes (open circles) with untreated and GSH-treated (4–6 h; filled circles) diabetic rat myocytes, illustrating that GSH-treatment of diabetic rat myocytes for 4–6 h normalized I_{to}. In contrast, GSH-treatment of myocytes from control rats for the same duration did not significantly affect the mean I–V relationship for I_{to}. Specifically, maximum I_{to} density in GSH-treated ($n=8$) and untreated ($n=15$) control myocytes were 25.8±4.4 and 28.3±2.0 pA/pF, respectively ($P>0.05$).

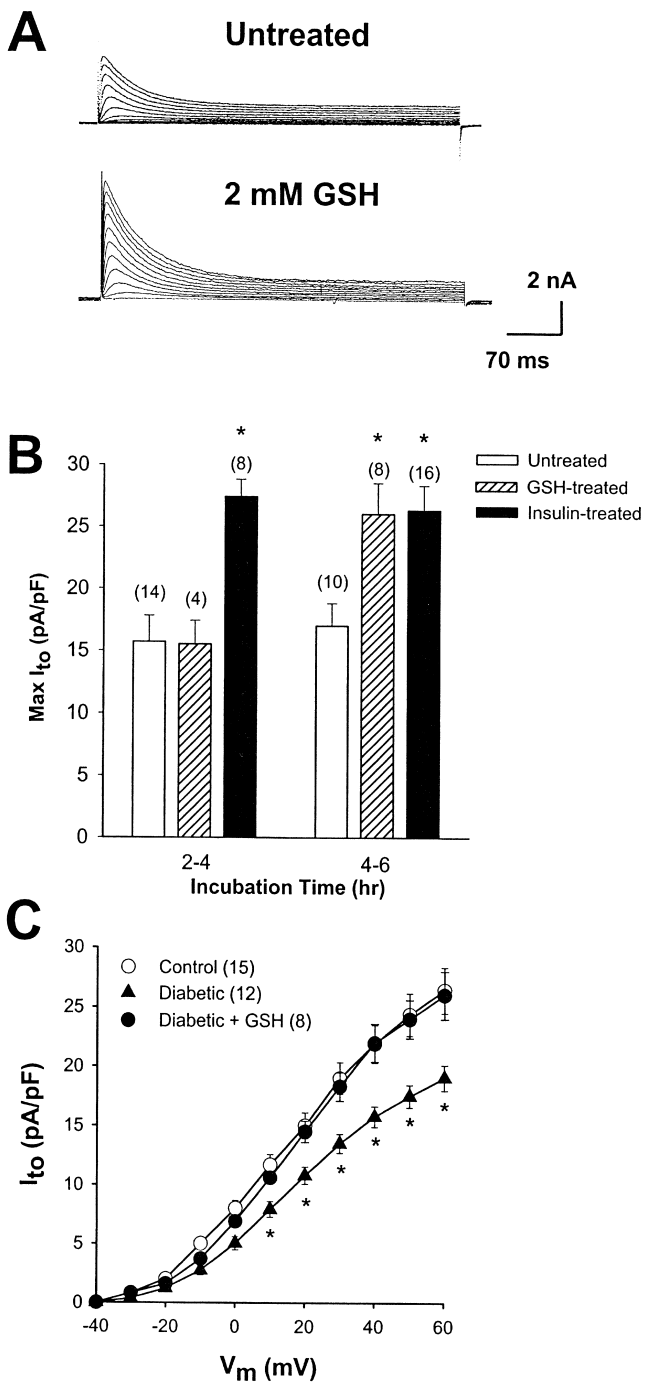

Fig. 3. Up-regulation of I_{to} by GSH. Myocytes from diabetic rats were incubated with 2 mM GSH for up to 6 h. (A) Raw currents measured at test potentials from −40 to +60 mV are compared in an untreated myocyte (upper traces) and another treated with GSH for 5 h. (B) Maximum I_{to} density (+60 mV) is plotted as a function of incubation time in untreated, GSH- and insulin-treated diabetic rat myocytes. Number of myocytes for each group is shown in parentheses. *$P<0.05$ compared with untreated group. (C) Mean I–V relationships are compared in control myocytes (open circles), and in diabetic rat myocytes left untreated or treated with GSH for 4–6 h (filled circles). *$P<0.05$ compared with control.

3.3. Insulin responsiveness and blockers of GSH metabolism

To determine if up-regulation of I_{to} by insulin is mediated by alterations in myocyte GSH, we tested the effects of two different inhibitors of GSH metabolism on the response of diabetic rat myocytes to insulin. In a first group of experiments, myocytes were treated with 0.1 μM insulin in the absence or presence of an inhibitor of glutathione reductase, 1,3-bis-chloroethyl-nitrosourea (BCNU). Fig. 4 shows that the effect of insulin to increase I_{to} density (cross-hatched bars) was blocked when 0.1 mM BCNU was added to the incubation medium (shaded, cross-hatched bar). The presence of BCNU alone (filled bar) did not affect maximum I_{to} density compared with untreated myocytes (open bar), nor did BCNU treatment alter maximum I_{to} density in control myocytes (BCNU-treated, 26.3±3.9 pA/pF, $n=8$; untreated, 28.3±2.0 pA/pF, $n=15$; $P>0.05$). A second, related series of experiments examined the effects of an inhibitor of γ-glutamylcysteine synthetase, buthionine sulfoximine (BSO), on the electrophysiological effects of insulin. Fig. 5A shows that when diabetic rat myocytes were incubated with insulin plus 0.5 mM BSO for 4 h, I_{to} density was significantly increased (shaded, cross-hatched bar) compared with myocytes treated with BSO alone (filled bar). This response was similar to the effect of insulin alone (cross-hatched bar) when compared with untreated myocytes (open bar). However, when myocytes were pre-incubated with BSO for 24 h, subsequent insulin treatment for 2–4 h failed to increase I_{to} (Fig. 5B), while the

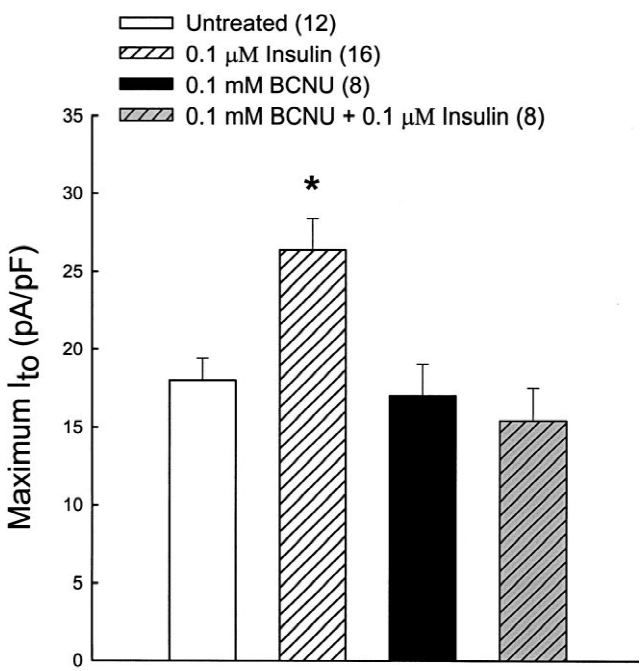

Fig. 4. Inhibitory effect of BCNU (0.1 mM) on insulin-responsiveness of I_{to} in diabetic rat myocytes. *$P<0.05$ compared with untreated group.

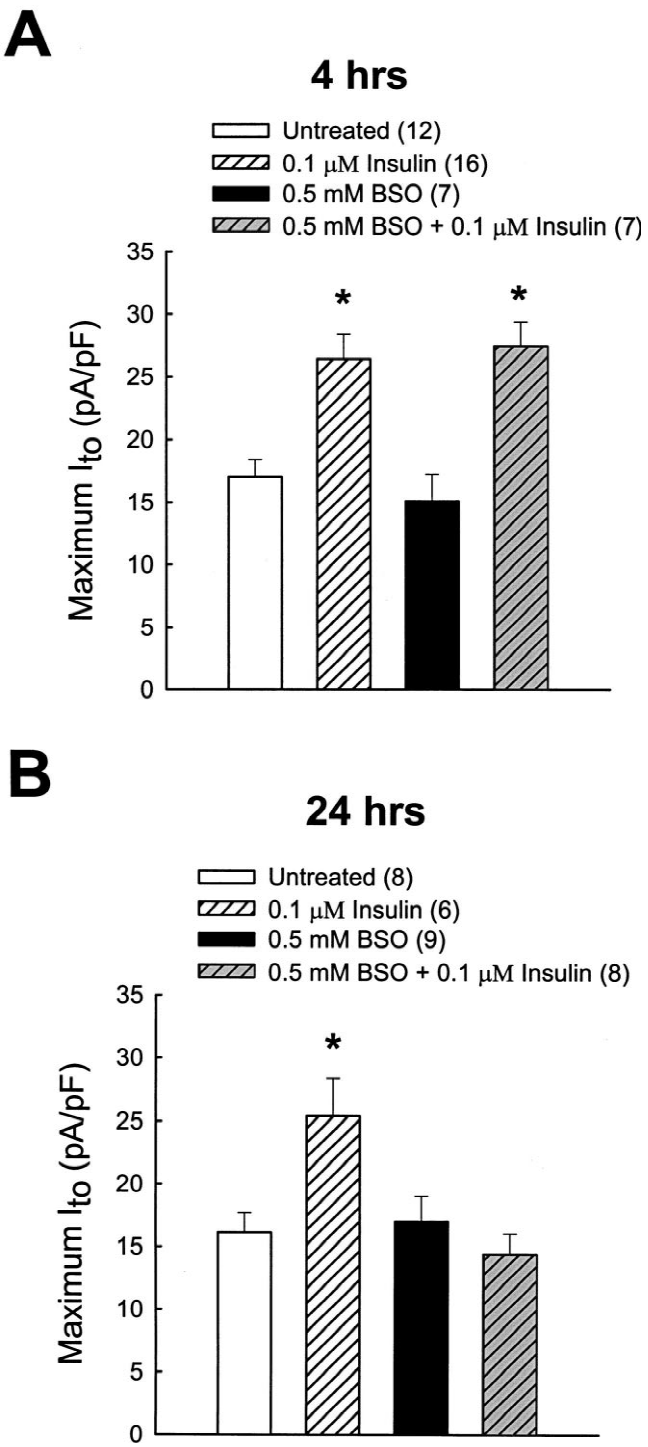

Fig. 5. Effect of BSO on insulin-responsiveness of I_{to}. (A) Diabetic rat myocytes were incubated for 2–4 h with 0.5 mM BSO in the presence or absence of 0.1 μM insulin. Data are also shown for untreated, and insulin-treated diabetic rat myocytes. *$P<0.05$ compared with untreated group. (B) In a separate group of experiments, diabetic rat myocytes were pre-incubated with 0.5 mM BSO for 24 h prior to subsequent 4-h treatment with insulin. As in panel A, data are also shown for untreated, and insulin-treated diabetic rat myocytes studied after 24 h in primary culture. *$P<0.05$ compared with untreated group.

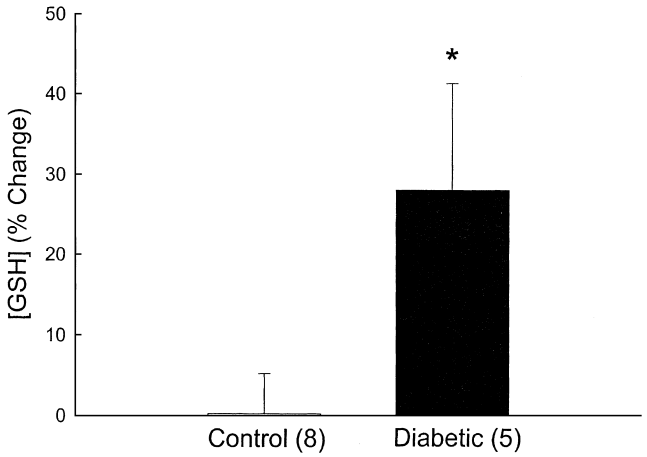

Fig. 6. Insulin-stimulated change in myocyte GSH. Cell suspensions from eight control and five diabetic rat hearts were equally divided into untreated and insulin-treated groups, and GSH measurements made after 3-h incubation. GSH concentration is expressed as a percent change relative to the suspension of untreated myocytes. *$P<0.05$ compared with control group.

insulin-responsiveness of diabetic rat myocytes in the absence of BSO (cross-hatched bar) was maintained after 24 h in culture medium, at least in terms of up-regulating I_{to}. Treatment of diabetic rat myocytes with BSO alone for 24 h did not change basal I_{to} density (filled bar), but a similar treatment decreased I_{to} density in control myocytes (BSO-treated, 15.7±3.9 pA/pF, $n=8$; untreated, 29.4±3.8 pA/pF, $n=8$; $P<0.05$).

To further explore the interaction of insulin signaling with myocyte GSH, a final group of experiments measured the relative change in GSH levels in isolated control and diabetic rat myocytes treated in vitro with insulin for 3 h. In these experiments, myocyte suspensions from each heart were divided equally into untreated and insulin-treated groups, and maintained in primary culture for 3 h. Fig. 6 illustrates that relative to untreated cells, insulin elicited a significantly greater increase in GSH concentration in diabetic rat myocytes (filled bar) compared with controls. Due to the relatively small amount of cell material available in these experiments, we did not measure GSSG.

4. Discussion

4.1. Down-regulation of K^+ channels in diabetic cardiomyopathy

Impaired insulin signaling and glucose utilization are postulated to play major roles in electrical remodeling of the heart in diabetes mellitus. In support of this hypothesis, we [3–5] and others [6–8] have shown that insulin treatment of diabetic rat ventricular myocytes up-regulates I_{to} density to normal levels after a delay of several hours. Moreover, specific abnormalities of glucose utilization, such as depressed pyruvate dehydrogenase activity, have also been linked to diabetic cardiomyopathy [2], and we have shown that agents which directly activate this regulatory enzyme mimic the effects of insulin on I_{to} in diabetic rat myocytes [3]. The mechanisms of insulin-mediated regulation of K^+ channel activity are not fully understood, but recent studies by Shimoni et al. [8] have provided important insights. In particular, they report that insulin-responsiveness of I_{to} in ventricular myocytes from Type I diabetic rats is blocked by inhibitors of protein synthesis and of protein trafficking via the cytoskeleton. Their data are consistent with decreased I_{to} density in non-diabetic models of heart failure [10,28–30] and support the hypothesis that I_{to} channels are down-regulated in chronic disease states by alterations in transcription and surface expression of channel protein [10,30]. In the case of diabetes mellitus, these cellular processes in the heart are proposed to be under the control of the insulin signaling cascade [8].

Evidence from the present study suggests that oxidative stress is involved in the etiology of diabetes-induced down-regulation of I_{to}. Indeed, several experimental studies show that ventricular dysfunction associated with diabetes is linked to increased oxidative damage to myocytes [11–13,15]. While hyperglycemia has been implicated as a main determinant of oxidative stress in diabetes, it remains unclear whether high glucose alone is sufficient to explain decreased I_{to} density in the ventricle. In this regard, our laboratory has recently reported that I_{to} remodeling in ventricular myocytes of euglycemic rats with chronic infarction is reversed in vitro by metabolic activators of glucose utilization, including an insulin mimetic compound [28,29]. These studies suggest the possibility that I_{to} remodeling in vivo is closely linked to chronic insulin deficiency or impaired insulin signaling, which would lead to oxidative stress over time via suppressed endogenous antioxidant defense mechanisms.

The present study also provides new information implicating GSH as playing a key role in regulating I_{to} channel activity, possibly through its control of myocyte redox state. In our diabetic rat model, a significant shift in redox state of the myocardium was indicated by a decreased GSH/GSSG ratio (Fig. 1B), and is consistent with the hypothesis that diabetic conditions profoundly impact GSH-dependent processes through oxidative stress [11,12]. A major consequence of a decreased GSH/GSSG ratio is oxidation of regulatory proteins at cysteine residues, through formation of mixed disulfides with GSSG or intramolecular disulfides [19,21]. For most proteins, this type of oxidative modification can be reversed by re-establishing a normally reduced intracellular environment [19,21], and in our study, we tested whether a redox mechanism plays a role in regulating I_{to} channel activity. Indeed, we found that incubation of diabetic rat myocytes with GSH normalized I_{to} density with a time lag of several hours (Fig. 3), but while these data imply that redox state controls I_{to} density, it is not known what regulatory steps

in overall channel activity are involved. The relatively long time delay for extracellular GSH or insulin to up-regulate I_{to} density is inconsistent with direct redox modulation of the I_{to} channel protein, which would be expected to occur with much faster kinetics. It is more likely, therefore, that myocyte redox state controls regulatory steps involved in transcription of new channel proteins or post-translational trafficking.

4.2. Maintaining cell GSH

Our study also suggests that maintaining myocyte GSH in diabetes mellitus preserves cell function, at least in terms of I_{to} channels. When intracellular GSH levels drop during pathophysiological states, one approach to restore them is to supplement cells with exogenous GSH, as we did in our experiments (Fig. 3). However, mammalian cells do not normally take up intact GSH [31–33]. Instead, it is proposed that extracellular GSH (i) is enzymatically degraded to its constituent amino acids (glutamate, cysteine, glycine), followed by their uptake and re-synthesis to GSH in the cytoplasm [31–36], or (ii) increases cell uptake of cysteine, the rate limiting amino acid in GSH synthesis, by reducing extracellular cystine, the oxidized form of cysteine [34–36]. A second approach to increase cell GSH is to activate the main pathways involved in GSH homeostasis, and data from our study suggest that insulin signaling may play such a role. Under physiological conditions, a relatively high GSH/GSSG ratio is maintained in cardiac cells through the activities of glutathione reductase, which converts GSH from GSSG using NADPH as a source of reducing equivalents, and γ-glutamylcysteine synthetase, the rate limiting step in GSH synthesis [34–36]. In our study, insulin-responsiveness of I_{to} in diabetic rat myocytes was blocked by an inhibitor of each pathway (BCNU, Fig. 4 and BSO, Fig. 5). Blockade of insulin's effect by BSO did require a prolonged preincubation period, which is consistent with other studies using this compound to inhibit γ-glutamylcysteine synthetase [37]. Nevertheless, our experiments (Figs. 4–6) suggest that biochemical steps controlled by insulin signaling are functionally linked to cell GSH, which plays an important role in regulating I_{to} channels. The multiple GSH-related pathways that are potentially affected by the insulin signaling cascade may account for the more rapid effects of in vitro insulin treatment on I_{to} density compared with GSH (Fig. 3), which does not readily cross cell membranes in its intact form [31–33].

The mechanisms of insulin control of myocardial GSH are not completely defined. It may be postulated that insulin increases the supply of amino acid precursors of GSH by increasing the expression or activity of sarcolemmal transporters [35]. Since cysteine availability is rate limiting for GSH synthesis [34–36], the ASCP or x_c^- transporter may be a primary target for insulin action [35]. Second, insulin may increase the expression or activity of glutathione reductase or γ-glutamylcysteine synthetase [35]. In this regard, Mak et al. [15] reported that γ-glutamylcysteine synthetase activity in rat heart 14 weeks after streptozotocin injection is significantly decreased compared with sham controls and that in vivo insulin treatment restores activity to control levels. Finally, insulin-mediated control of glucose utilization may provide essential metabolic co-factors required to maintain cell GSH. Thus, NADPH produced by the pentose pathway may be preferentially utilized by glutathione reductase to recycle GSH from GSSG [17,22,24,38].

In summary, our data identify GSH as a key regulator of I_{to} channels and suggest that diabetes-induced electrical remodeling of the heart involves oxidative stress that profoundly affects cell redox state. The electrophysiological importance of insulin signaling in the heart may thus be related to its apparent control of GSH homeostasis and the protection of vulnerable ion channels and transporters from oxidation. The relevant pathways and molecular signals involved in the redox control of I_{to} or other cardiac ion channels are not well defined and necessitate further study.

Acknowledgements

This study was supported by an American Diabetes Association Research Award to G.J. Rozanski, and by grants from the National Heart, Lung and Blood Institute to G.J. Rozanski (HL66446) and K.P. Patel (HL48023).

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