EXERCISE AND DIABETES INFLUENCE ANTIOXIDANT ACTIVITY AND GENE EXPRESSION IN FEMALE RATS

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ABSTRACT

The goal of this study was to examine the influence of exercise training and type I diabetes on the activity of aconitase and superoxide dismutase (SOD), on levels of reduced glutathione (GSH), on the expression of genes responsible for glutathione synthesis (Gclc, g-glutamylcysteine synthase; Gss, glutathione synthetase), and on the expression of genes responsible for the formation of two potassium channels (Kcnd2, Ito; KCNQ1, IKS) in cardiac muscle. Forty female Sprague-Dawley rats were divided into four groups: sedentary controls, sedentary diabetics, trained controls, and trained diabetics. Diabetes was induced via streptozotocin injection, and training consisted of swimming up to 2 hours per day for 11 weeks. Aconitase activity and GSH levels were unchanged, but SOD activity was significantly elevated in trained diabetic rats compared with sedentary diabetic rats. Gss expression was significantly downregulated in diabetic rats compared with control rats. Kcnd2 expression was significantly downregulated in trained diabetic rats compared with sedentary diabetic rats, and KCNQ1 expression was consistently downregulated in trained rats compared with sedentary rats and in diabetic rats compared with control rats. These results suggest training alone or combined with diabetes increases antioxidant mechanisms and downregulates the expression of genes related to K⁺ ion channel activity.

Ventricular arrhythmias are a leading contributor to sudden death in chronic disease states that are characterized by cardiac remodeling, including congestive heart failure, myocardial infarction, and diabetes mellitus (Kaprielian et al. 2002, Li et al. 2005, Oudit et al. 2001, Tsuchida and Watajima 1997). Potassium channels are of particular importance because they contribute to the repolarization and duration of the action potential characteristic of cardiac muscle. Recent work has demonstrated that reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radical, play an important role in the electrophysiological changes seen in chronic cardiac disease states. Mitochondrial aconitase, which irreversibly converts citrate to isocitrate in the citric acid cycle, is inactivated by superoxide and hydrogen peroxide (Bulteau et al. 2003), so its activity may be used to evaluate the level of oxidative stress. Superoxide also induces apoptosis via numerous mechanisms, including lipid peroxidation and protein denaturation, and is degraded to H₂O₂ by superoxide dismutase (SOD). Reduced glutathione (GSH), a major free radical scavenger produced by all cells, protects the heart against damage from H₂O₂ (Li et al. 2003), and its levels reflect the degree of oxidative challenge placed upon the tissue. g-glutamylcysteine synthase is responsible for catalyzing the synthesis of g-glutamylcysteine from glutamic acid and cysteine, and glutathione synthetase then catalyzes the synthesis of g-glutamylcysteine from glutamic acid and cysteine, and glutathione synthetase then catalyzes the synthesis of GSH from g-glutamylcysteine and glycine; the second reaction is ATP-dependent (Huang et al. 2000, Rozanski and Xu 2002). The ratio of GSH to its oxidized form, GSSG, is often used to evaluate the ability of the tissue to withstand oxidative stress. Moreover, GSH homeostasis appears to be influenced by glucose metabolism (Rozanski and Xu 2002). In diabetic rat hearts, the production of ROS is increased, and GSH depletion is responsible for the cell death that leads to cardiac disease (Ghosh et al. 2005, Shen et al. 2004). In both the failing heart
(Chen et al. 2005) and the diabetic heart (Ferreira et al. 2003, Fitzl et al. 2001), oxidative stress causes increased production of superoxide in the mitochondria, altering the expression of mitochondrial proteins (Shen et al. 2004). Oxidative stress has been suggested as the cause of elevated expression of redox-sensitive transcription factors in the cardiac tissue of diabetic rats as evidenced by increased activity of nuclear factor-κB and activating protein-1 (Nishio et al. 1998). In addition, overexpression of manganese SOD has been shown to reduce cardiomyopathy in a mouse model of type 1 diabetes mellitus (Shen et al. 2006).

While the benefits of exercise on overall health are well established, exercise is also associated with the production of ROS, potentially causing oxidative damage to cardiac muscle tissue (Ji 1999). In rodents, regular exercise has been shown to increase median lifespan (Holloszy 1993) and to upregulate antioxidant defense mechanisms in rodent skeletal muscle, heart, and liver (Bronikowski et al. 2003, Ji 1999, Ji et al. 1998). In mice, voluntary exercise has been shown to attenuate age-related changes in cardiac gene expression, particularly by minimizing changes in genes associated with inflammation and responses to stress (Bronikowski et al. 2003, Jin et al. 2000). In rats, a distinct difference in cardiac gene expression has been reported between animals subjected to a physiological load (exercise training) and those subjected to a pathological load (myocardial infarction, congestive heart failure) (Jin et al. 2000). Although physiological hypertrophy, a classic adaptation to chronic exercise training, has inconsistent effects on GSH levels in cardiac tissue in rodents (Dhalla and Singal 1994, Kanter et al. 1985, Lennon et al. 2004). However, as mentioned previously, GSH is influenced by glucose metabolism, and exercise training has been shown to influence both glucose metabolism and insulin sensitivity. Trained rats have increased cardiac glucose uptake at rest and during acute exercise compared with sedentary rats (Kainulainen et al. 1989). While the GLUT4 isoform of the glucose transporter is increased in skeletal muscle in response to exercise training (Holloszy 1993), the GLUT1 isoform seems to play a more important role in cardiac muscle (Laybutt et al. 1997).

Potassium channels represent the largest and most diverse group of ion channels (Rasmussen et al. 2004, Roden and George 1997). At least nine different K+ currents have been identified and may be categorized into three groups: the calcium-independent voltage-gated (Kv) transient outward currents (I_{to1}, I_{to2}), the delayed outwardly rectifier currents (I_{ks}, I_{kr}, I_{kor}), and the rapidly activating currents (I_{k1}, I_{kach}, I_{kapt}, I_{kr}) (Nerbonne 2000, Roden and George 1997). These currents vary in density, voltage-dependent properties, and time-dependent properties, and in the heart the distribution of these channels affects the duration of the action potential (Viswanathan et al. 1999). Studies suggest that I_{to} density is decreased in heart failure (Li et al. 2002), as evidenced by decreases in mRNA expression and channel proteins for Kv (Huang et al. 2000, Yao et al. 1999). Increased extracellular levels of GSH upregulate I_{to} in isolated myocytes, possibly by increasing cellular uptake of cysteine, from rats with chronic myocardial infarction (Leopold and Loscalzo 2000, Rozanski and Xu 2002). This upregulation is also dependent upon glucose utilization (Rozanski and Xu 2002, Xu et al. 1996, Xu et al. 2002), and I_{to} current density has been shown to be decreased in the myocytes of animal models of altered glucose metabolism, including streptozotocin (STZ)-induced diabetic rats (Xu et al. 1996), genetically diabetic rats (Tsuchida and Watajima 1997), and insulin-resistant animal models (Shimoni et al. 2000). In rodents, I_{to} is the major determinant of action potential duration (Wang et al. 1994). Kv4.2, also known as Kcnd2, is the most abundant member of the Kv family of Kva subunits and encodes I_{to1} in rats. All members of the Kv4 family are characterized by fast activation and inactivation (Nerbonne 2000). Unlike other transcripts, Kcnd2 is expressed in a steep gradient from the epicardium to the papillary muscles (Dixon and McKinnon 1994). Decreases in Kcnd2 mRNA have been measured in ventricular tissue from rats with STZ-induced diabetes (Nishiyama et al. 2001) and rabbits with heart failure (Rose et al. 2005). KCNQ1, also known as KcLQT1, is one of 5 members of the Kva subunit family for the I_{ks} current (Bubisch et al 1999, Wang et al. 1996, Yang et al. 1997). All members of this family are characterized by very slow activation and very, very slow inactivation (Nerbonne 2000). In the heart KCNQ1 combines with KCNE1 from the b subunit family to form I_{ks} (Barhanin et al. 1996, Sanguinetti et al. 1996). KCNQ1 is widely distributed between individual cardiac cells (Rasmussen et al. 2004), and mutations of this component are responsible for a condition known as long QT syndrome (Wattanasirichaigoon and Beggs 1998, Yang et al. 1997).

The major goal of this study was to examine the influence of exercise training and type 1 diabetes mellitus on the activity of aconitase and SOD and on the ratio of GSH/GSSG in cardiac tissue and to determine if exercise training and type 1 diabetes mellitus alter the expression of the genes responsible.
for the synthesis of GSH, Gclc (g-glutamycysteine synthetase) and/or Gss (glutathione synthetase). A second goal was to determine if exercise training and type 1 diabetes mellitus alter the expression of genes responsible for two different K+ channels, Kcnd2 and KCNQ1. Our results suggest exercise training, alone or combined with diabetes, has little to no phenotypic effect on antioxidant mechanisms but downregulates the expression of KCNQ1. In addition, our results suggest exercise training may attenuate increases in blood glucose over time in insulin-deficient rats.

**MATERIALS AND METHODS**

**Animals**

All methods were consistent with the *Guide for the Use and Care of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985), and were approved by the University of Nebraska at Kearney Institutional Animal Care and Use Committee (IACUC #91003-A). Forty female Sprague-Dawley rats (age 3 weeks) were obtained from Harlan Industries (Indianapolis, IN) and housed in the Bruner Hall of Science Animal Care Facility on the campus of the University of Nebraska at Kearney. Rats were kept under a 12:12 hour light-dark cycle (light from 0700 to 1900) and provided standard rat chow and water ad libitum. At age 4 weeks, type 1 diabetes mellitus (D) was induced in half the rats (n=20) via ip injection of STZ (65 mg/kg in 0.1 M citrate buffer, pH = 4.5). The remaining 20 control (C) rats received vehicle injection. Diabetic status was confirmed by measuring blood glucose levels 24 hours later using a tail-clip method and a glucometer (Roche Diagnostics Corp., Indianapolis, IN). The next week half the rats in each group (10 D rats, 10 C rats) began a daily swim-training regime in 30-35°C water. Exercise-trained (ET) rats began with 15 minutes of swimming on the first exposure, and swim time was progressively increased 15 minutes each week until rats were training 2 hours per day, 5 days per week. Sedentary non-trained (NT) cage mates (n=20) were handled daily and placed in shoulder-deep 30-35°C water weekly to experience similar stresses.

All rats were sacrificed at age 16 weeks using an overdose of sodium pentobarbital (150 mg/kg ip). Prior to sacrifice blood glucose levels were measured in all diabetic animals as described previously. Left ventricle, right ventricle, and septum tissue from all groups of rats were collected, washed, and snap frozen using liquid nitrogen. Samples for gene analysis were stored in 200 ml TRIzol® reagent (Invitrogen Corp., Carlsbad, CA) while samples for enzyme activity and total GSH analysis were simply snap frozen. All samples were stored at -80°C. Skeletal muscle tissue samples from the biceps femoris were also collected, washed, snap frozen in liquid nitrogen, and stored at -80°C.

**Antioxidant enzyme activity**

One tissue sample from each region of the heart from each rat was used to evaluate the activity of aconitase and SOD using kits purchased from Oxis Research™ (Portland, OR; catalog numbers 21041 and 21010, respectively). Samples were read on a Multiskan® MCC plate reader with Ascent® software (Thermo Electron Corporation, Vantaa, Finland). A total protein assay was performed on the same homogenate using a bicinchoninic acid (BCA™) protein assay kit purchased from Pierce (Rockford, IL; catalog number 23225). Activity was expressed in Units/mg protein.

**GSH/GSSG ratio**

One tissue sample from each region of the heart from each rat was used to evaluate the ratio of reduced to oxidized glutathione using a kit purchased from Oxis Research™ (catalog number 21040). Unfortunately, the measurement of GSSG failed; therefore, GSH levels (nmol/mg tissue) only are reported in the results.

**Gene expression**

Cardiac muscle tissue samples were thawed, homogenized in Trizol® reagent, and centrifuged at 4°C for 20 minutes at 12,000 rpm. RNA isolation was performed on the homogenate using a RiboPure™ kit purchased from Ambion, Inc. (Austin, TX). Spectrophotometry and agarose gel electrophoresis analyses were done to verify the quantity and quality of the isolated RNA. Once sufficient RNA (at least 2 mg/ml) was isolated from each region of the heart from 3 rats in each of the 4 groups, the expression of Kcnd2, KCNQ1, Gclc, and Gss were analyzed in triplicate using a 7500 Real Time PCR System (Applied Biosystems Incorp., Foster City, CA). Primers for Kcnd2 (Rn00583375), KCNQ1 (Rn00563101), Gclc (Rn00564188), and Gss (Rn00564188) as well as the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Rn4352338E), were purchased from Applied Biosystems. Each well on the 96 well plate contained 25 ml TaqMan® 2x Universal PCR MasterMix No AmpErase® UNG (Applied Biosystems, kit 4309169), 1.25 ml 40x MultiScribe™ and RNase Inhibitor Mix (Applied Biosystems, kit 4309169), 2 ml sample (concentrated to 2 mg RNA per ml), 2.5 ml primer...
mix, and 19.25 ml DNAse-, RNAse-free distilled water (Invitrogen Corp., Grand Island, NY). Samples were analyzed using the Applied Biosystems universal cycling conditions for TaqMan® gene expression assays of 30 minutes at 48°C followed by 10 minutes at 95°C and then 40 cycles of 15 seconds at 95°C followed by 60 seconds at 60°C. The difference in threshold cycle (CT) was used to calculate the quantitative fold change between groups, and fold changes were then normalized relative to GAPDH transcript levels.

Citrate synthase activity
To evaluate the effectiveness of the training program, citrate synthase activity was measured from tissue samples from the biceps femoris as described by Srere (1969). Briefly, samples were diluted 1:10 in ice-cold 0.1 M Tris buffer (pH 8.0) and homogenized. Following centrifugation for 1 minute at 2,000 rpm and 4 minutes at 4,000 rpm, samples were analyzed in a 96 well plate with each well containing 120 ml 0.1 M Tris buffer, 20 ml 3.0 mM acetyl coenzyme A, 20 ml 1.0 mM dithio-bis-2-nitrobenzoic acid (DTNB), and 20 ml sample. Following a 7 minute incubation at room temperature, the change in absorbance at 414 nm was recorded every 30 seconds for 2 minutes. Next, 20 ml 5.0 mM oxaloacetate was added to each well, and the change in absorbance at 414 nm every 30 seconds for 3 minutes was recorded. The average activity (DOD) per minute before adding oxaloacetate was subtracted from the average activity per minute after adding oxaloacetate. Citrate synthase activity was then calculated by using the extinction coefficient for DTNB (13.6 mmol·cm⁻¹) and by taking into account the dilution of the original tissue (1/100) as follows: (net DOD per minute) / ((13.6mmol) (0.01g)) = activity in mmol/g/min.

Statistical analysis
All data were analyzed using analysis of variance (ANOVA) and Student's t-test (two-tailed). Significance was ascribed for p<0.05. Initial statistical analysis indicated no regional differences (results not shown), so data from all three regions of the heart were combined for further analysis.

RESULTS
Diabetic rats had significantly higher (p<0.001) blood glucose compared with C rats (D 452 ± 96 mg/dl; C 137 ± 9 mg/dl) 24 hours following STZ injection, and blood glucose levels were still elevated in D rats at the time of sacrifice (519 ± 136 mg/dl). Blood glucose levels were not significantly different between NTD and ETD rats 24 hours following STZ injection (NTD 457 ± 93 mg/dl; ETD 441 ± 107 mg/dl) or at sacrifice (NTD 565 ± 87; ETD 460 ± 167 mg/dl), although the increase in blood glucose within NTD over time was significant. ET rats had significantly higher (p<0.01) citrate synthase activity compared with NT rats (ET 23.4 ±11.4 mmol/g/min; NT 11.5 ± 9.2 mmol/g/min). There were no significant differences in body weight between groups (ET 243 ± 24 g; NT 236 ± 24 g; D 232 ±27 g; C 248 ±17 g).

ANOVA indicated significant differences in SOD activity, but not in aconitase activity or GSH levels, between groups (Table 1). SOD activity was significantly elevated in ETD rats compared with NTD rats (Table 1). ANOVA also indicated significant differences in Gss and KCNQ1, but not Gclc or Kcnd2, expression between groups (Figure 1). KCNQ1 was consistently downregulated in D and ET rats compared with C and NT rats, and Kcnd2 expression was significantly downregulated in ETD rats compared with ETC rats (Figure 1).
Antioxidants and gene expression in rats

Figure 1. Fold changes in Gclc, Gss, Kcnd2, and KCNQ1 in cardiac muscle tissue comparing groups of female rats. The difference in threshold cycle (Ct) was used to calculate the quantitative fold change between groups, and fold changes were then normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels. n = 3 for each group. NTC, non-trained control; NTD, non-trained diabetic; ETC, exercise-trained control; ETD, exercise-trained diabetic. * t-test indicates significant difference (p < 0.05). # ANOVA indicates significant differences (p < 0.05).

Table 1. Aconitase activity (Units/mg protein), superoxide dismutase activity (Units/mg protein) and reduced glutathione (nmol/mg tissue) in cardiac muscle tissue of female rats. NTC, non-trained control; NTD, non-trained diabetic; ETC, exercise-trained control; ETD, exercise-trained diabetic. Values are mean ± SD. * t-test indicates significant difference compared with NTD (p < 0.05). # ANOVA indicates significant difference between groups (p < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Aconitase</th>
<th>Superoxide dismutase</th>
<th>Reduced glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC (n=10)</td>
<td>7.28 ± 4.50</td>
<td>0.19 ± 0.11</td>
<td>1.50 ± 0.37</td>
</tr>
<tr>
<td>NTD (n=10)</td>
<td>6.95 ± 3.88</td>
<td>0.22 ± 0.12</td>
<td>1.64 ± 0.39</td>
</tr>
<tr>
<td>ETC (n=10)</td>
<td>8.30 ± 4.22</td>
<td>0.24 ± 0.17</td>
<td>1.44 ± 0.52</td>
</tr>
<tr>
<td>ETD (n=10)</td>
<td>9.08 ± 5.78</td>
<td>0.35 ± 0.22*</td>
<td>1.91 ± 0.59</td>
</tr>
</tbody>
</table>
DISCUSSION

Exercise and diabetes are both known to increase the formation of ROS that can inactivate enzymes and must be degraded by a series of enzymé-controlled reactions in order to minimize the harmful effects of oxidative stress. SOD degrades $O_2^\cdot$ to $H_2O_2$, and GSH is then oxidized to GSSH as $H_2O_2$ is reduced to $H_2O$. Our observation of significant differences in SOD activity, but not in aconitase activity or GSH levels, suggests a significant increase in $O_2^\cdot$ production with ET combined with D, which is consistent with the observation of increased $O_2^\cdot$ production by both myocardial mitochondria and coronary arteries in the diseased heart (Chen et al. 2005). $\gamma$-glutamylcysteine synthase is generally regarded as the rate-limiting enzyme in the formation of GSH (Jung and Thomas 1996). Our results show no differences in Gclc expression, but a general downregulation of Gss was observed in ET and/or D rats compared with NTC rats. Downregulation will not necessarily result in decreased enzyme quantity or activity, of course, because mRNA and resultant protein levels are affected by many factors. Similarly, upregulation of a gene will not necessarily result in increased quantity of that protein because mRNA levels may not predict protein levels or levels of enzyme activity. Without data for GSSG and the ability to calculate the GSH/GSSG ratio, we can only speculate as to whether the differences in expression of Gss are expressed phenotypically as differences in GSH. However, if more ROS are produced and no differences in GSH are observed, it follows that adequate amounts of GSH were produced and subsequently oxidized into GSSG since GSH levels are all within the typical range reported for rat myocardium (Leeuwenburgh et al. 1997).

Our results suggest a downregulation in Kcnd2 expression in ETD rats compared with ETC rats and a general downregulation of KCNQ1 expression in ET rats compared with NT rats. Again, without GSSG data, we can only speculate that these differences may be due to altered GSH. Insulin has been shown to have an electrophysiological effect on myocytes of diabetic rats through its effects on glutathione metabolism (Xu et al. 1996). Recent evidence, however, suggests that the remodeling of $I_0$ in the diabetic rat heart may not be due to changes in the glutaredoxin system but more likely due to changes in the thioredoxin system, particularly due to decreased thioredoxin reductase (Li et al. 2005). Both the thioredoxin and glutaredoxin systems are members of the thioldisulfide oxidoreductase superfamily that act to reduce protein disulfides and protein-mixed disulfides, respectively (Fernandes and Holmgren 2004, Jurado et al. 2003, Paget and Buttnor 2003, Yamawaki et al. 2003). The lack of insulin in diabetic individuals may decrease the availability of NADPH required by the thioredoxin and glutaredoxin systems to reduce the proteins that control $I_0$ density (Li et al. 2003). In addition, while the duration of the cardiac action potential in normal hearts is influenced by the activation of $I_K$ and $I_Ks$, evidence suggests that the increased length of the action potential in diabetic hearts may be due in part to a decrease in $I_0$ (Casis et al. 2000, Xu et al. 1996). This could explain the difference in Kcnd2 expression observed between ETD and ETC rats. Left ventricular hypertrophy is a classic adaptation to ET (Scheuer and Tipton 1997), and hypertrophied hearts also display a prolonged action potential (Lebeche et al. 2006). Alone, KCNQ1 produces rapidly activating $K^+$ currents, and the slowly activating $K^+$ current $I_Ks$ is produced when KCNQ1 combines with minK (Barhanin et al. 1996, Sanguinetti et al. 1996). While the lack of GSSG data make any connection between GSH and KCNQ1 speculative, the observations that mutations in KCNQ1 are associated with increased action potential duration (Wattanasiriachaigoon and Beggs 1998, Yang et al. 1997) and that hypertrophied hearts have longer action potentials may explain the decreased expression of KCNQ1 in cardiac tissue of ET rats relative to NT rats.

Our observation that exercise training attenuated any further increase in blood glucose levels is consistent with the observations of Dall’Aglio et al. (1983), who suggested this attenuation was due to enhanced insulin action. An increase in the sensitivity of glucose transporters during exercise was first observed by Richter et al. (1982), and a subsequent study demonstrated that the enhanced glucose uptake continues in skeletal muscles following cessation of exercise and is caused first by insulin independent mechanisms and then by insulin dependent mechanisms (Garetto et al. 1984). In control settings, most of the GLUT4 isoform transporters are stored intracellularly, and GLUT4 translocation to the cell surface is stimulated by insulin, muscular contractions, and hypoxia (Cartee et al. 1991, Douen et al. 1990, Holloszy and Hansen 1996, Kawanaka et al. 1999). Endurance exercise training appears to cause an adaptive increased in the GLUT4 transporter in skeletal muscles (Friedman et al. 1990, Holloszy 2005, Ploug et al. 1990, Rodnick et al. 1990). These mechanisms may explain the attenuated long-term increase in blood glucose levels in the ETD rats compared with NTD rats.
The influence of gender on our observations must not be overlooked because gender is known to influence the development of disease states. In a transgenic mouse model of hypertrophic cardiomyopathy, for example, female mice with LV hypertrophy continue to display concentric hypertrophy when male mice of the same age display LV dilation (Olsson et al. 2001). In STZ-induced diabetic rats, males exhibit more severe cardiomyopathy (Brown, Anthony et al. 2001, Brown, Walsh et al. 2001) and consistently have attenuated K+ currents compared to female counterparts (Shimoni and Liu 2003a, Shimoni and Liu 2003b). Shimoni and Liu (2003b) observed no change in Ipeak controlled by Kcnq2, in diabetic female rats while Ipeak was significantly reduced in diabetic male rats. Our results indicate significant downregulation of Kcnq2 expression in ETD female rats compared with ETC female rats, and a trend toward downregulation of Kcnq2 expression in ETD female rats compared with NTD female rats (P = 0.08). Therefore, gender-specific differences in the regulation of K+ channels must be taken into consideration.

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LITERATURE CITED


