Nicorandil decreases oxidative stress in slow- and fast-twitch muscle fibers of diabetic rats by improving the glutathione system functioning

Sarai Sánchez-Duarte¹, Sergio Márquez-Gamíño², Rocío Montoya-Pérez¹, Erick Andrés Vilcaña-Gómez², Karla Susana Vera-Delgado², Cipriana Caudillo-Cisneros², Fernando Sotelo-Barroso², Ma Teresa Melchor-Moreno², Elizabeth Sánchez-Duarte²*¹

¹Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México, and ²Departamento de Ciencias Aplicadas al Trabajo, Universidad de Guanajuato Campus León, León, Guanajuato, México

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*Correspondence
Elizabeth Sánchez-Duarte
Tel: +52-477-267-49-00 (ext. 4833)
E-mail address: elizabeth.sanchez@ugto.mx

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ABSTRACT
Aims/Introduction: Myopathy is a common complication of any diabetes type, consisting in failure to preserve mass and muscular function. Oxidative stress has been considered one of the main causes for this condition. This study aimed to search if Nicorandil, a KATP channel opener, could protect slow- and fast-twitch diabetic rat muscles from oxidative stress, and to unveil its possible mechanisms.

Materials and Methods: Diabetes was induced in male Wistar rats by applying intraperitoneally streptozotocin (STZ) at 100 mg/kg doses. Nicorandil (3 mg/kg/day) was administered along 4 weeks. An insulin tolerance test and assessment of fasting blood glucose (FBG), TBARS, reduced (GSH), and disulfide (GSSG) glutathione levels, GSH/GSSG ratio, and mRNA expression of glutathione metabolism-related genes were performed at end of treatment in soleus and gastrocnemius muscles.

Results: Nicorandil significantly reduced FBG levels and enhanced insulin tolerance in diabetic rats. In gastrocnemius and soleus muscles, Nicorandil attenuated the oxidative stress by decreasing lipid peroxidation (TBARS), increasing total glutathione and modulating GPX1-mRNA expression in both muscle types. Nicorandil also increased GSH and GSH/GSSG ratio and downregulated the GCLC- and GSR-mRNA in gastrocnemius, without significant effect on those enzymes’ mRNA expression in diabetic soleus muscle.

Conclusions: In diabetic rats, Nicorandil attenuates oxidative stress in slow- and fast-twitch skeletal muscles by improving the glutathione system functioning. The underlying mechanisms for the modulation of glutathione redox state and the transcriptional expression of glutathione metabolism-related genes seem to be fiber type-dependent.

INTRODUCTION
Diabetes mellitus (DM) is characterized by hyperglycemia, which begins with impairment of insulin signalling, production deficiency and/or resistance to its effect, leading to metabolic disorders. Once DM is established, numerous organs, tissues, as well as systems, and their physiologic mechanisms could become deteriorated. Diabetic damage to skeletal muscle tissues is known as diabetic myopathy¹². Being the skeletal muscle tissue the main store and consumer of glucose in the body, the physiopathologic mechanisms underlying insulin resistance at its plasmatic membrane and cellular interior are of capital meaning³⁴⁵.

Oxidative stress has an essential participation at the genesis of diabetic complications, as is the case for diabetic myopathy¹⁶. Oxidative stress occurs due to excessive production of reactive oxygen species (ROS). When the antioxidant protection system is insufficient or fails removing ROS, cellular damage happen¹⁷. Bioavailability of antioxidant defenses is crucial to balance ROS production in skeletal muscle³⁹. Disturbances in
The redox state have been associated to the onset of insulin resistance\(^5\), and changes in expression of redox-sensitive genes\(^6\), causing impaired growth\(^4\), likewise structural and functional changes in muscle cells\(^1,10,11\).

It is well known that skeletal muscles can be formed by different type of fibers, having specific functions to stimulation. The metabolic characteristics determining twitch mode. Slow-twitch type I fibers are fatigue resistant due to their high oxidative capacity; conversely, fast-twitch type IIa, IIb, and IIx myofibers tend to fatigue rapidly\(^12\). By the other hand, insulin sensitivity correlates with muscle fiber type; specifically, insulin-stimulated glucose uptake is higher in slow-twitch muscle fibers compared to fast-twitch myofibers\(^13\). Diabetes might affect skeletal muscles differentially, those integrated mainly of fast-twitch fibers exhibit more severe loss of function and atrophy than the ones composed predominantly of slow-twitch fibers\(^14\). Fiber type distribution could play an important role in susceptibility to ROS-induced damage due to diabetes\(^15\).

Nicorandil has been recognized to have clinical significance\(^16\). Researchers have reported effects of nicorandil on cardiovascular diseases, protecting against ischemia-reperfusion injury in heart\(^17–20\), and in skeletal muscle\(^21–23\). Nicorandil has an action linked with its functions as K\(_{ATP}\) channel opener\(^17,24,25\), nitric oxide donor activity\(^18,19\), and cell antioxidant capacity enhancer\(^26–28\). We have recently reported that Nicorandil improves muscle function by reversing fatigue and modulating glutathione redox state in slow chicken skeletal muscle fibers\(^23\). Likewise, in STZ-induced diabetic rats, Nicorandil protects, via a radical scavenging effect, islet \(\beta\)-cells against damage induced by STZ, both \textit{in vivo} and \textit{in vitro}\(^29\). Moreover, Nicorandil has been used to treat diabetic complications such as nephropathy\(^30\), cardiomyopathy\(^31\), and cardiovascular diseases\(^28,32\). Nowadays, whether this protective effect occurs on myopathy diabetic, has yet to be defined.

Accordingly, it was hypothesized that Nicorandil might exert a protective effect in skeletal muscle by reducing oxidative damage induced by chronic hyperglycemia and improving insulin tolerance. Thus, this study is aimed to investigate the effect of Nicorandil on oxidative stress markers (lipid peroxidation, the redox status of glutathione) and mRNA expression of antioxidant status-related genes in slow- and fast-twitch muscles of STZ-induced diabetic rats.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats weighing 95–100 g were selected for this study. All animals were housed in a specific pathogen-free environment, with a 12 h light-dark cycle, 22 ± 2°C. Animals were provided a standard rat chow and water \textit{ad libitum}. Use of animals for experiments was done according to Mexican Regulations for Use and Care of Laboratory Animals (NOM-062-ZOO-1999) and approved by the Institutional Committee on Bioethics in Research of the Universidad de Guanajuato (Code-CIBIUG-P17-2019).

**Experimental protocol**

The animals were randomly distributed into four groups \((n = 6\) each): normoglycemic rats (Control), normoglycemic rats treated with nicorandil (Nicorandil), non-treated diabetic rats (Diabetes) and diabetic rats treated with nicorandil (Diabetes + Nicorandil).

Diabetes was induced by a single intraperitoneal injection of STZ freshly dissolved in citrate buffer (0.5 M, pH 4.5; Sigma-Aldrich, St. Louis, MO, USA), at doses of 100 mg/kg body weight. Blood glucose concentrations were evaluated in samples obtained from the tails, by using a glucometer (Accu-Chek Performa; Roche, Indianapolis, IN, USA). Only rats with FBG levels \(\geq 300\) mg/dL were considered as diabetics. Normoglycemic corroborated rats in the control group received a citrate buffer injection instead of STZ. Treatment for four weeks with Nicorandil (Sigma) started 4 weeks after STZ, by intraperitoneal injection of 3 mg/kg/day. Animals in the Control group were managed similarly as those in the Nicorandil-treated groups. Variations in body weight and FBG were evaluated every week. All groups were evaluated during the same period. Once treatment was complete, the animals of all groups were fasted for 8 h and euthanized using cervical dislocation. Skeletal muscle samples from soleus and gastrocnemius were immediately collected and stored at \(-80\)°C until further processing.

**Insulin tolerance test**

At the end of the experimental protocol, an insulin tolerance test (ITT) was performed for all groups. Rats fasted for 12 h, the glucose concentration was determined at \(t = 0\), before insulin administration (i.p. 0.75 U insulin/kg body weight), then 30, 60, 90 and 120 min after. The blood glucose response was calculated as the area under the curve (AUC-ITT) according to the mathematical TAI model\(^33\).

**Protein assessment**

Protein concentrations in muscle tissue samples were measured by Biuret method\(^34\), using bovine serum albumin as standard.

**Determination of lipid peroxidation levels**

An assay of thiobarbituric acid reactive substances (TBARS) was used as a marker of lipid peroxidation in soleus and gastrocnemius muscles according to the method described by our group\(^23\).

**Glutathione measurement**

Total glutathione (GSH + GSSG), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured by the method of Rahman \textit{et al.}\(^35\).

**RNA isolation and RT-qPCR for mRNA expression analyses**

Total RNA was isolated from muscle samples, previously immersed, and homogenized in TRizol (TRI Reagent; Sigma Aldrich), according to the method described by Chomczynski\(^36\), with minor modifications. Concentration and purity of the extracted RNA were analysed spectrophotometrically at optical densities of 260/280 ratio using the BioPhotometer (Eppendorf,
Hamburg, Germany). Only samples with ratios above 1.7 were used for expression analyses. For each sample, 2 μg of RNA were reverse transcribed into cDNA using a cDNA synthesis kit, according to manufacturer’s instructions (Qiagen, Hilden, Germany). Real-time quantitative polymerase chain reaction (qPCR) was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, CA, USA), using the QuantFast SYBR Green PCR Kit (Qiagen). Primer sequences were designed using information from the Gene Bank of the National Center for Biotechnology Information public database. The sequences of primer sets used were: Glutathione peroxidase 1 (GPX1), forward 5'-CCGGTCGGACATCTTTGAGG-3' and reverse 5'-GATCGCAACTGGGTGAGAA-3'; glutathione reductase (GSR), forward 5'-TTACTGAATGGCGGCGATGT-3' and reverse 5'-CGGCGTTTCCTCATGTTGTC-3'; glutamate-cysteine ligase catalytic subunit (GCLC), forward 5'-TCCA-GATGTCGATTGCCTGC-3' and reverse 5'-CGGGTCGGACATACTTGAGG-3'; β-actin, forward 5'-GTTCACACCATTCTGACTGCT-3' and reverse 5'-CGGACTCATCGTACTCCTGC-3'. The amplification efficiency for each sample, and the relative mRNA expression levels were calculated using experimental standard curves according to the ΔΔCT method, with the endogenous housekeeping gene β-actin as an internal control.

Statistical analysis

The experimental data were expressed as mean ± standard error of the mean (SEM) and analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison test. Statistical significance was set at *P < 0.05 by using GraphPad Prism™ Software version 6 (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of Nicorandil on fasting blood glucose and body weight of diabetic rats

As shown in Figure 1, FBG levels were several times higher in the Diabetes group (>500 mg/dL) compared to the Control group (87.10 ± 6.59 mg/dL; *P < 0.001) (Figure 1a). Nicorandil treatment resulted in significant reduction of FBG levels in diabetic rats (Diabetes + Nicorandil group), dropping by as much as 16% compared to Diabetes group (*P < 0.05), but did not reach normal values (*P < 0.01). No significant difference in FBG was found between Control and Nicorandil groups. Regarding to body weight (Figure 1b), both, the Diabetes and Diabetes + Nicorandil groups had lower body weight than the Control and Nicorandil groups (*P < 0.001). No statistical differences were found in this regard between diabetic rats treated or not with Nicorandil (Diabetes vs Diabetes + Nicorandil; *P > 0.05).

Nicorandil treatment improved insulin tolerance in diabetic rats

Figure 2 shows the results of the ITT performed in 12 h fasted rats, at the end of the 4 weeks Nicorandil’s intervention. For the test, rats were injected with insulin immediately after measuring basal fasting blood glucose levels (t = 0). Insulin reduced blood glucose levels, transiently in the Diabetes group, between 30 and 90 min. The glycemia reduction was more evident and lasting in the Diabetes + Nicorandil group (*P < 0.01; Figure 2a). In Figure 2b, the ITT-AUC (Area Under the Curve for the ITT responses) was plotted for the four groups. Diabetes + Nicorandil group showed to have a remarkably lower ITT-AUC than the Diabetes group (*P < 0.01), suggesting that Nicorandil improves insulin sensitivity.

Figure 1 | Effects of Nicorandil on (a) fasting blood glucose (FBG) and (b) body weight. Data are presented as mean ± SEM (n = 6 per group). *P < 0.05 vs Control group. #P < 0.05 vs Nicorandil group. &P < 0.05 vs Diabetes group.
Nicorandil reduces lipid peroxidation in fast and slow diabetic skeletal muscles

To evaluate oxidative stress in skeletal muscles from STZ-induced diabetic rats, we determined the TBARS levels as an indicator of lipid peroxidation. As shown in Figure 3, a significant increase of TBARS concentration was observed in the gastrocnemius (3a) and soleus (3b) muscles from diabetic rats in comparison to Control group ($P < 0.01$). Nicorandil treatment prevented the increase of lipid peroxidation in both muscle types from the Diabetes + Nicorandil group, when compared to the Diabetes group ($P < 0.01$). Oppositely, Nicorandil did not alter TBARS levels in soleus nor in gastrocnemius muscles in the Control group.

**Effect of Nicorandil on the redox status of glutathione in fast- and slow-twitch skeletal muscle in diabetic rats**

To test whether lower lipid peroxidation levels caused by Nicorandil in diabetic rat muscles were parallel to oxidative status changes, the glutathione defense system was analyzed in the gastrocnemius (Figure 4) and soleus muscle (Figure 5). The redox
status of glutathione changed considerably in diabetic rat muscles compared to control rat muscles ($P > 0.05$). When compared to Control group, the GSH/GSSG ratio in gastrocnemius and soleus muscles of diabetic rats was significantly lower, 71.3% and 94.9%, respectively. Accordingly, reduced concentrations of total glutathione (TGSH) were observed in gastrocnemius and soleus muscles from diabetic rats (32.9% and 82.9%, respectively), compared to those from the Control group, $P < 0.05$. On the other hand, Nicorandil significantly increased the TGSH levels in gastrocnemius muscle (28.70%; $P < 0.05$) and soleus muscle (111.02%; $P < 0.05$) compared to Diabetes group. Consistently, Nicorandil also increased TGSH in gastrocnemius from normoglycemic rats (Nicorandil Group; 45%; $P < 0.01$) compared to Control rats. Further, the GSSG lower with a significantly higher GSH/GSSG ratio (50.38%; $P < 0.05$) was shown in gastrocnemius muscle in the Diabetes + Nicorandil group compared to Diabetes group (Figure 4b). This effect was also observed in soleus muscle, though without statistically significance (Figure 5b).

**Nicorandil modulates the expression of antioxidant status-related genes**

Finally, we examined if glutathione alterations in redox status were related to changes in expression of glutathione metabolism-related genes (GPx1, GCLC and GSR) both in gastrocnemius and soleus muscles (Figure 6). In diabetic animals, expression of GPX1 mRNA was significantly elevated in both gastrocnemius (Figure 6a) and soleus (Figure 6b) muscles (141% and 131%, respectively) compared to Control group. Surprisingly, diabetic rats treated with Nicorandil significantly down-regulated expression levels of GPX1 to the same level as the Control group in both muscle types ($P < 0.05$). However, marked differences between gastrocnemius and soleus were found in mRNA levels of GCLC and GSR; upregulated only in gastrocnemius muscle, exhibiting 257% and 293%, respectively ($P < 0.01$). Meanwhile, treatment with Nicorandil significantly down-regulated expression levels to nearly the same level as the control group (Figure 6a). By contrast, no significant difference was found in mRNA expression of GCLC and GSR in soleus muscle compared to all groups (Figure 6b).

**DISCUSSION**

Hyperglycemia induced oxidative stress, and the increased production of ROS in DM may negatively affect the integrity of skeletal muscle7. The use of pharmacological agents as Nicorandil, a nicotinamide derivative, conjugated with a nitrate moiety, a well-known KATP channel activator38 has shown to be effective in reducing oxidative stress markers in different muscle types. The present study shows that Nicorandil modulates the expression of antioxidant status-related genes in diabetic rat muscles.
tissues. However, the influence of Nicorandil on skeletal muscle under diabetic conditions is not yet fully understood. Therefore, in this study, we investigated the antioxidant effects of Nicorandil in fast and slow skeletal muscles. In our findings, treatment with Nicorandil demonstrated beneficial effects parallel to changes in glycemic levels, insulin tolerance, and oxidative stress levels. Redox balance after Nicorandil preserves the stability of glutathione in favour of its reduced form (GSH) in diabetic skeletal muscle.

As expected, in the present study glycemia (Figure 1a), body weight (Figure 1b) and insulin sensitivity (Figure 2) were significantly affected in STZ-induced diabetic rats compared to control group, as it has been previously shown. The above mentioned alterations were, at least partially, mitigated by Nicorandil. When comparing Diabetes and Diabetes-Nicorandil groups, a decrease in FBG was observed (Figure 1b). As well, insulin tolerance in diabetic rats was improved (Figure 2). Evidence supports benefits of supplemental antioxidants in improving insulin functioning in diabetes. In this sense, protective effects of Nicorandil in diabetes have received attention due to its antioxidative effects, as it protects islet beta-cells against damage induced by STZ both in vitro and in vivo, via a radical scavenging effect. Moreover, considering that the hyperglycemic state in DM results in insulin resistance by insulin-dependent tissues (e.g., skeletal muscle), our findings may be partly related to the fact that Nicorandil prevents palmitate-induced insulin resistance in skeletal muscle cells, as demonstrated by Dymkowska et al.

Elshazly found that Nicorandil reduces insulin resistance in rats with non-alcoholic fatty liver disease (NAFLD) through lipid peroxidation related mechanisms. Similarly, several studies have demonstrated association of diabetes with lipid peroxidation by-products, and function and signalling impairment of insulin. In addition, hyperglycemia increases ROS generation, and eventually to an oxidative stress state, which triggers lipid peroxidation in muscular cells, as observed in soleus and gastrocnemius muscles in our study. Lipid peroxidation considered a marker of oxidative damage has been involved in insulin resistance. The oxidation of membrane lipids substantially alters the physical and biological properties of membranes, fluidity, transport of metabolites and ions, and other functions of membrane proteins. Interestingly, Nicorandil treatment decreased levels of TBARS in both studied muscles (Figure 3), suggesting reduction of ROS generation. Nicorandil as a reductor of the lipid peroxidation and/or oxidative damage, has been previously reported in cardiac tissue for

Figure 5 | Effect of Nicorandil on glutathione redox status in soleus muscles. (a) Reduced glutathione (GSH); (b) glutathione disulphide (GSSG); (c) the redox ratio (GSH/GSSG), and (d) total glutathione (GSH + GSSG). Data are shown as the mean ± SEM. *P < 0.05 vs control group; †P < 0.05 vs Nicorandil group; ‡P < 0.05 vs Diabetes group; n = 6 per group.
protecting and attenuating ischemic damage\textsuperscript{32} and protecting hepatocytes against NAFLD\textsuperscript{40}.

In this study, glutathione (GSH), one of the main antioxidant systems in muscle cells\textsuperscript{49} was evaluated as well. GSH, an abundant cellular thiol, is a significant determinant for maintaining and regulating thiol-redox status. As an antioxidant, this molecule is involved in detoxification of products deriving from ROS-promoted oxidation of lipids, free radicals, and probably other cellular components\textsuperscript{50}. Consistent with our results, in metabolic diseases like diabetes, the redox balance, particularly the GSH synthesis and status, are strongly affected\textsuperscript{31,52}. In diabetic rats, GSSG levels markedly increased, with a significant decline in the GSH/GSSG ratio, and a concomitant decrease in levels of TGSH both in gastrocnemius and soleus muscles, indicative of oxidative state (Figures 4 and 5). The reduction in the GSH/GSSG ratio is an important biomarker of oxidative stress and believed to account for proteins dysfunction required for proper muscle contraction, by altering the redox status and protein activities in muscle cells\textsuperscript{8}. However, we observed the elevation of TGSH levels in both slow- and fast-twitch types of muscle, implying that Nicorandil effectively plays a role in strengthening antioxidant defense, as it has been confirmed in other tissues\textsuperscript{28,39,40}. Recently, Nicorandil has proven to be a regulator of glutathione’s redox status in slow skeletal muscle and to improve muscle function by increasing post-fatigue tension\textsuperscript{23}. Fatigue is common in diabetes as well, accompanied by a marked decrease in the antioxidant capacity\textsuperscript{53}. In our results, Nicorandil promoted both, enhancement of GSH level and,
consequently the elevation of GSH/GSSG ratio in gastrocnemius muscle compared to the Diabetes group (Figure 4c). A similar tendency was observed in soleus muscle though this was not statistically significant (Figure 5c). Since these two muscles differ in their fiber type composition, it is interesting to point out that Nicorandil does not seem to impact their redox state in the same way. Soleus muscle consists of type I slow fibers, compared to gastrocnemius, formed predominantly by fast fibers (mixed type IIa, IIb)12,54. In this context, reports indicate that GSH levels in muscle fibers are correlated to the metabolic profile50. Thus, our results confirm that the redox status of GSH is differentially regulated in a muscle-specific manner. The mechanism underlying the effect of Nicorandil on the levels of GSH may be regulated by different redox balance enzymes. This compound seems to attenuate ROS formation by inducing the expression of thioredoxin (TRX) protein in the vascular tissue of STZ-induced diabetic rats39. TRX is an endogenous redox regulator found in skeletal muscle as an ancillary antioxidant enzyme acting on thiols reducing capacity during oxidative stress1,15. In this regard, the antioxidant protein TRX and GSH are key regulators of redox-sensitive pathways and contribute to protecting skeletal muscle cells from oxidant-mediated dysfunction55. On the other hand, skeletal muscle expresses an extensive battery of crucial antioxidant genes that could contribute to lower levels of GSSG in diabetes, including GPX156, GCLC57 and GSR58. It is known that GSH-coupled antioxidant enzymes such as GPX and GSR participate in the maintenance of redox homeostasis. GSSG is predominantly produced by GPX, an abundant and ubiquitous expressed selenoprotein that uses GSH as a substrate to catalyze the reduction of H2O2 and lipid peroxides, while GSR catalyzes the reaction of GSSG back to its reduced form59. This work was supported in part by grants from Coordinación de la Investigación Científica, Universidad de Guanajuato (ESD-CIIC-237/2019) and PRODEP (grant number: UGTO-PTC-667). Also supported by grants from Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo (RMP).

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DISCLOSURE

The authors declare no conflict of interest.

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