Glutathione reverses early effects of glycation on myosin function

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Submitted 29 October 2002; accepted in final form 13 April 2003

Ramamurthy, B., A. Daniel Jones, and L. Larsson. Glutathione reverses early effects of glycation on myosin function. Am J Physiol Cell Physiol 285: C419–C424, 2003.—Nonenzymatic glycosylation (glycation) has been recognized as an important posttranslational modification underlying alterations of structure and function of extracellular proteins during aging and diabetes. Intracellular proteins may also be affected by this modification, and glycation has been suggested to contribute to aging-related impairment in skeletal muscle function. Glycation is the chemical reaction of reducing sugars with primary amino groups resulting in the formation of irreversible advanced glycation end products. Glutathione is an abundant tripeptide in skeletal muscle. To understand the effect of glutathione on glycated myosin function, we used a single-fiber in vitro motility assay in which myosin is extracted from a single muscle fiber segment to propel fluorescent-labeled actin filaments. Myosin function responded to glucose exposure in a dose-dependent manner, i.e., motility speeds were reduced by 10, 34, and 90% of preincubation values after 30-min exposure to 1, 3, and 6 mM glucose, respectively. The 30-min 6 mM glucose incubation postincubation values after 30-min exposure to 1, 3, and 6 mM glucose (with or without β-mercaptoethanol) and with a low-salt buffer or a nonreducing sugar (6 mM sucrose) was measured for both fast and slow myosin. Thirty minutes of exposure to the reducing sugar without β-mercaptoethanol had a dramatic effect on both the actin motility speed and the pattern of filament movement. Motility speed decreased by ~80%, and filaments demonstrated a random motility pattern rather than the linear motion observed in the control experiments. On the other hand, the additional incubations had no impact on either motility speed or motility pattern (30). After 30 min of glucose exposure, matrix-assisted laser desorption ionization (MALDI) mass spectra of Lys-C endoproteinase digests of treated myosin revealed structural modifications in the catalytic domain of the motor protein. Thus glycation of sarcomeric myosin had a significant effect on both the structural and functional properties of the motor protein (30).

The tripeptide GSH is the most abundant nonprotein thiol located in the cytosol and mitochondria of mammalian cells (24). GSH is primarily synthesized in the liver and released into the circulation for transport to peripheral tissues (29). It is a nonenzymic antioxidant that has several antioxidant functions. GSH serves as a substrate for glutathione peroxidase (GPX) and also in vitro motility; skeletal muscle fibers; speed of contraction

NONENZYMATIC GLYCOXYLATION (glycation) of proteins occurs by means of a chemical reaction of reducing sugars with primary amino groups, i.e., aldehyde groups of free unbound sugars react preferentially with free lysine residues to form reversible Schiff base adducts. These structures may undergo further Amadori rearrangements and free radical-mediated oxidation to finally generate irreversible advanced glycation end products (AGEs) (2, 18).

It is well known that glycation of proteins is an important mechanism underlying aging- and diabetes-related alterations in protein structure, function, and digestibility (4, 25). It is also known that proteins with a slow turnover rate are preferential targets for post-translational modifications. Myosin, the molecular motor protein in skeletal muscle that converts chemical energy into mechanical work, has been reported to have a half-life as long as 29–30 days (17). The aging-related decrease in myosin turnover rate (3) and the lysine-rich regions of the actin-binding site, along with the catalytic domain, make myosin a potential target for modification by glycation. For instance, increased amounts of glycated myosin have been reported in skeletal muscles of aged rodents (31).

In a recent series of experiments, it was demonstrated that glycation of myosin impairs myosin function by altering the structural properties of the motor protein (30). A single-fiber in vitro motility assay was used in which myosin was extracted from single muscle fiber segments. The effect on actin motility speed of 30-min incubations with 6 mM glucose (with or without β-mercaptoethanol) and with a low-salt buffer or a nonreducing sugar (6 mM sucrose) was measured for both fast and slow myosin. Thirty minutes of exposure to the reducing sugar without β-mercaptoethanol had a dramatic effect on both the actin motility speed and the pattern of filament movement. Motility speed decreased by ~80%, and filaments demonstrated a random motility pattern rather than the linear motion observed in the control experiments. On the other hand, the additional incubations had no impact on either motility speed or motility pattern (30).

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collaboration. The study was carried out with young (2–4 mo) male Wistar rats (n = 3). The animals were kept in conventional facilities at 20–22°C, with constant humidity and a 12:12-h light-dark cycle. All were fed standard laboratory chow and tap water ad libitum. The skin was transferred to a 2.0 M sucrose solution for 30 min and then incubated in solutions of decreasing sucrose concentrations (1.5–0.5 M). The bundle was then stored in a low-salt buffer (2 mM ATP) for long-term storage at 4°C.

The flow cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70, Olympus America), and the fluorescent-labeled actin filaments were visualized through a ×60 objective (numerical aperture 0.7) with illumination from a 100-W mercury lamp. The temperature of the flow cell was thermostatically controlled at 25°C (Bio-nomic Controller, BC-100, 20/20 Technology) by a thermoelectric system (HH211, Ometron, USA) in contact with the surface of the glass slide next to the flow cell. Actin movement was filmed with an image-intensified SIT camera (SIT 66, DAGE-MIT) and recorded on VCR tape (10–12).

As observed in previous experiments, the amount of myosin extracted from a single fiber segment has an impact on the effects of the reducing sugar glucose on motility speed (Ramamurthy et al., unpublished observations). In an attempt to achieve similarity in the amounts of extracted myosin in the 2.5-μl experimental chamber, the length of the fiber segment was kept constant (2 mm) in all experiments. Motility data analysis. From each single-fiber preparation, 10 actin filaments moving with constant speed in an oriented motion were selected for speed analysis. With the exception of preparations incubated with glucose, in which a larger fraction of the filaments moved randomly, recordings and analyses were performed only on preparations in which >90% of the filaments moved bidirectionally. With an image analysis package (OPTIMAS 6.0, Optimas), a filament was tracked from the center of mass and the speed was calculated from 20 frames at an acquisition rate of 1 frame/s. The average speed and standard deviation of the 10 filaments were calculated. Because the standard deviation in this group of filaments was small (between 10 and 15% of the mean), the average speed was taken as representative of the muscle fiber (10–12).

Incubations and motility speed analyses. Preincubation data were obtained from motility speed measurements on myosin extracted from a single fiber as described in Motility data analysis. To acquire postincubation values, the extracted myosin was incubated with 1) 6 mM glucose (Sigma; >99.5%, HPLC grade, without β-mercaptoethanol) for 30 min followed by either 10 mM GSH (Sigma; >98.0%) in low-salt buffer (without β-mercaptoethanol) or low-salt buffer [with 1% (130 mM) β-mercaptoethanol] for 20 min; 2) low-salt buffer (with β-mercaptoethanol) for 50 min; or 3) 10 mM GSH (without β-mercaptoethanol) for 50 min. Furthermore, to obtain postincubation recordings for a dose-response relationship, 30-min incubations of myosin with 1, 3, and 6 mM glucose (without β-mercaptoethanol) were performed. Motility speed was analyzed before and after the respective incubations.

Postincubation data were obtained by the following method. Two segments (2 mm each) of a fiber were used to obtain the pre- and postincubation values, i.e., myosin isolated from the first fiber segment was assayed to obtain...
RESULTS

MyHC isoform expression. All of the soleus fibers included in this study expressed 100% of the b/slow (type I) MyHC isoform based on protein separations with very sensitive silver-stained 7% SDS-PAGE; n denotes the number of fibers used in the experiments.

preincubation data and myosin from the second segment was incubated with selected solutions to obtain postincubation data. In previous experiments we showed (11) that there is no significant difference in motility speed when myosin is extracted from two adjacent segments of the same fiber. In addition, we showed (30) the same effects on motility speed in response to glucose exposure when the same segment of the fiber was used for both pre- and postincubation experiments as when two separate segments of the same fiber were used for pre- and postincubation experiments, respectively. However, on occasion, removal of the labeled actin from the preincubation preparation proved difficult and hence interfered with postincubation measurements when the first method (using the same segment for both pre- and postincubation measurements) was employed. Therefore, the second method using two segments of the same fiber was chosen. This method eliminated the ambiguity due to the interference created by the presence of actin filaments remaining from the preincubation preparation (30).

Separation and identification of myofibrillar protein isoforms. Myosin heavy chain (MyHC) isoforms were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from a separate part of the single fiber segments analyzed in the in vitro motility assay. The total acrylamide and bis-acrylamide concentrations were 4% (wt/vol) in the stacking gel and 7% in the running gel, whereas the gel matrix included 30% glycerol. Sample loads were equivalent to 0.1 mm of the fiber segment. The separating gels (160 × 180 × 0.75 mm) were silver stained (see Refs. 9, 20, 21).

Statistics. Means ± SD of data collected were calculated from individual values by standard procedures. A one-way ANOVA and a two-tailed paired t-test were used for comparison. Differences were considered significant at P < 0.05.

Dose-response relationship. To test the effect of increasing glucose concentrations on motility speed, myosin extracted from a 2-mm fiber segment was incubated in 1, 3, and 6 mM glucose for 30 min. A graded and significant reduction in motility speed from preincubation values was observed for all three concentrations, thus confirming a dose-response relationship (Fig. 1). After the 1 mM glucose incubation, motility speed decreased (P < 0.05) 10% from preincubation (1.02 ± 0.07 μm/s, n = 3) to postincubation (0.93 ± 0.05 μm/s, n = 3) values. After the 3 mM glucose incubation, motility speed decreased (P < 0.05) 34% from preincubation (1.22 ± 0.26 μm/s, n = 3) to postincubation (0.81 ± 0.15 μm/s, n = 3) values. Finally, after the 6 mM glucose incubation, motility decreased (P < 0.001) by >90% from preincubation (1.12 ± 0.04 μm/s, n = 3) to postincubation (0.10 ± 0.07 μm/s, n = 3) values.

Effects of glucose and GSH incubations on actin motility speed. In all preparations, incubations for 30 min with 6 mM glucose reduced motility speed (P < 0.001) by >90%. Subsequent incubation with 10 mM GSH for 20 min restored motility speed (0.98 ± 0.06 μm/s, n = 3) to almost preincubation levels (1.12 ± 0.06 μm/s, n = 3; Fig. 2). Postglucose low-salt buffer incubations did not, on the other hand, increase motility speed (0.03 ± 0.05 μm/s, n = 3) to preincubation values (1.13 ± 0.04 μm/s, n = 3). Furthermore, a 16–20% decrease (P < 0.001) in motility speed was observed in response to a 50-min incubation with GSH in low-salt buffer or with low-salt buffer alone (Fig. 2). However, these changes were significantly smaller than the effects of glucose exposure, and it is suggested that they represent a nonspecific degradation of the motor protein.

There was a greater reduction in motility speed, which was more consistent, in response to glucose...
incubation in the present study than in our previous report (30). This was because of the more consistent amount of myosin extracted from each fiber (Ramamurthy et al., unpublished observations). In the present study, extraction of similar amounts of myosin was ensured in each experiment by controlling the length of the fiber to 2 mm in each preparation. This control of the amount of myosin extracted enabled us to obtain a consistent postglucose incubation response as well as to test the dose-response relationship of myosin with 1, 3, and 6 mM glucose.

Changes in actin motility pattern in response to glucose and subsequent GSH incubations. Bidirectional movement by at least 90% of the total number of moving actin filaments has been determined to be one of the criteria for acceptance in this single-fiber in vitro motility assay. All recordings fulfilled this criterion except for the postglucose incubation recordings. After the 30-min 6 mM glucose exposure, the large majority of the actin filaments showed no motility, but the few filaments that were moving typically showed a random rather than bidirectional motility pattern. This is in accordance with previous observations after 30 min of incubation with 6 mM glucose (30). Deviations from linearity of postglucose incubation and restoration of linearity of subsequent GSH incubation are depicted in Fig. 3.

DISCUSSION

The results from this study confirm and extend our previous observations on the effect of glycation on myosin function. The dominant findings of this study are as follows: 1) glycation of myosin has a dramatic effect on myosin velocity and directionality of motion; 2) there is a significant dose-response relationship between myosin function and glucose concentration; and 3) GSH reverses the early modifications of myosin by a reducing sugar.

Glycation of myosin has an impact on both structure and function of the motor protein. As shown in a recent study (30). Structural analyses using MALDI mass spectrometry demonstrated the disappearance of a Lys-C digestion product corresponding to the ATPase site of myosin after 30-min incubation with a reducing sugar, with concomitant appearance of higher-molecular-mass peptides. This change was paralleled by a significant impairment in myosin function, i.e., motility speed and bidirectional movement of actin filaments were affected by the glucose incubation. After 30 min of incubation of myosin with glucose, motility speed dropped significantly by ≈80% in both fast (type IIx and IIb) and slow (type I) myosin isoforms and bidirectionality of the actin filaments was lost. These dramatic changes in myosin function were confirmed in the present study, i.e., motility speed was reduced by >90% after 30 min of 6 mM glucose exposure. However, incubation with 10 mM GSH for 20 min restored motility to almost preincubation values, whereas postglucose buffer incubation had no effect on motility. This would therefore confirm that we are dealing with a GSH-specific effect.

GSH is primarily synthesized in the liver and released into the circulation for transport to peripheral tissues (6). The membrane-bound enzyme γ-glutamyltransferase cleaves GSH into its amino acids for transport into the cell. These amino acids are utilized for intracellular synthesis of GSH (24). GSH and GSSG play a very important role as redox buffers in the cytosol as well as in the organelles. Tissue levels of GSH vary widely between different tissues, with the highest concentrations in the eye lens (≈10 mM) and the lowest concentrations in fast-twitch skeletal muscles (≈0.5 mM). However, there are muscle type-specific differences in GSH and total glutathione content (GSH and GSSG) related to the oxidative capacity of the muscle. GSH contents in the slow-twitch oxidative rat soleus (≈3 mM) is higher than that found in erythrocytes as well as in lung and brain tissue (15).

GSH has several antioxidant functions. First, it readily interacts with a variety of radicals, including hydroxyl and carbon radicals, by donating a proton (36). A second important antioxidant function of GSH is to act as a cosubstrate of GPX in the elimination of both H2O2 and other organic peroxides. In this reaction, GSH donates a pair of protons and two GSH molecules are oxidized to form GSSG. GSSG is converted back to GSH through the catalytic action of glutathione reductase, with NADPH providing the reducing power (15). The reducing power for the glutathione reductase system in skeletal muscle is mainly provided by NADP-specific isocitrate dehydrogenase (22).

In skeletal muscle, unlike many tissues, antioxidant enzyme activities including GPX appear to increase with age (14, 16). This can be attributed to an increase in the insults of oxidative stress and other oxidative stress-mediated modifications in the cells, such as glycation, during the aging process. However, there are to our knowledge no studies focusing on the role of GSH in relation to glycation of intracellular proteins. The only study published so far has demonstrated an in-
creased glycation of extracellular proteins in response to a GSH deficiency (13). Furthermore, AGEs have been known to alter the GSH redox state in human neuroblastoma cells, generally toward more oxidizing conditions (7).

It is well known that GSH is an important antioxidant. In addition to its antioxidant functions, GSH is also a reactive nucleophile that plays an important role in the breakdown of exogenous and endogenous electrophilic toxins. The present results demonstrate that GSH reverses the formation of early glycation products. The restoration of motility by GSH after incubation with a reducing sugar implies a reversal of Schiff base formation. Strong nucleophiles such as hydroxylamine can displace carbonyl groups from protein side chains. GSH is capable of reacting with reactive carbonyl groups, forming hemithioacetalts, as is the case with α-oxoaldehydes (33, 34). We propose that GSH degrades early glycation products as a strong nucleophile that displaces glucose, and potentially other carbonyl compounds, from the Schiff base adducts at the lysine residues of myosin.

In skeletal muscle, increased amounts of glycated myosin and a glycation-induced decline in myosin ATPase activity have been reported in aging rodent skeletal muscle (31, 32). During aging, GSH and total glutathione content is known to increase in skeletal muscles of rats (23). A concomitant increase in the oxidative stress in skeletal muscles during aging (see Ref. 28) as measured by malondialdehyde levels is also observed in healthy, active elderly women. In light of these observations, it can be surmised that the increase in GSH content could primarily be targeted toward combating the increased oxidative stress, and it could potentially also play a role in the reversal of early glycation products in skeletal muscle. However, despite the increase in GSH content during the aging process, glycation of myofibrillar proteins is found to increase with age. Deuther-Conrad et al. (7) report that AGEs cause a dose-dependent and long-term increase in GSSG in vitro. This could, in part, explain the decrease in GSH content during the aging process, as a potential mechanism for the increase in GSSG content during the aging process, in healthy, active elderly women. In light of these observations, it can be surmised that the increase in GSH content could primarily be targeted toward combating the increased oxidative stress, and it could potentially also play a role in the reversal of early glycation products in skeletal muscle. However, despite the increase in GSH content during the aging process, glycation of myofibrillar proteins is found to increase with age. Deuther-Conrad et al. (7) report that AGEs cause a dose-dependent and long-term increase in GSSG in vitro. This could, in part, explain the decrease in GSH content during the aging process, as a potential mechanism for the increase in GSSG content during the aging process.

Nonenzymatic glycation has been implicated in the pathophysiology of diabetes and aging (31, 35, 37). The present results suggest that GSH, in addition to its antioxidant function, could play an important role in preventing the progress of glycation of intracellular proteins. This is, to our knowledge, the first report documenting the direct effects of GSH in reversing early glycation effects on myosin.

We are grateful to Jeffrey T. Heckman for excellent technical assistance.

DISCLOSURES

This study was supported by grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (AR-45627, AR-47318), the Muscular Dystrophy Association (US), the European Commission, and the Swedish Medical Research Council (08651).

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AJP-Cell Physiol • VOL 285 • AUGUST 2003 • www.ajpcell.org


