GLYCATION AS A POST-TRANSLATIONAL MODIFICATION OF MYOSIN STRUCTURE AND FUNCTION; ROLE OF GLUTATHIONE; GLYCATION AND AGING

A Thesis in
Physiology

by
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ABSTRACT

The aims of the studies conducted in this thesis were to investigate the effect of the post-translational modification glycation on myosin function in order to better understand the mechanisms underlying the aging-related slowing of skeletal muscle. Non-enzymatic glycosylation (glycation) has been recognized as an important post-translational modification underlying alterations of structure and function of extracellular proteins. The effect of glycation on intracellular proteins is, on the other hand, less well known in spite of the vital importance of intracellular proteins for cell, tissue, and organ function. The aim of this study was to explore the effects of glycation on the structure and function of skeletal muscle myosin. Myosin was incubated for up to 30 minutes with glucose and subsequently tested for structural and functional modifications by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and a single fiber in vitro motility assay, respectively. MALDI spectra revealed glycation-related structural alterations as evidenced by disappearance of specific Lys-C proteolysis products and appearance of higher mass peaks that are attributed to crosslinking by glucose. This change was paralleled by a significant reduction in in vitro motility speed, suggesting a structure-related decline in myosin mechanics in response to glucose exposure. There is complete reversal of motility speed after reaction with the Schiff base-cleaving agent hydroxylamine hydrochloride.

Glutathione (GSH) is an abundant tripeptide in skeletal muscle and is known for its antioxidant function and ability to protect cells from toxicity of xenobiotics. Earlier studies have suggested that abundant thiol compounds may inhibit the process of glycation. In order to understand the effect of GSH on glycated myosin function, we used a single fibre in vitro motility assay. Myosin function responded to glucose exposure in a dose-dependent manner, i.e., motility speeds were reduced by 10, 34, and 90% of pre-incubation values after 30 minutes exposure to 1, 3 and 6 mM glucose concentration, respectively. The 30-minute 6 mM glucose incubation was followed by a 20-minute 10 mM GSH incubation. After glucose exposure (0.10 ± 0.07 µm/s, n=3), GSH treatment restored (p<0.001) motility (0.98 ± 0.06 µm/s, n=3) close to pre-incubation levels (1.12 ± 0.06 µm/s, n=3).

Antibodies against Advanced Glycation Endproducts (AGEs) were used to quantify the amount of AGEs in SOL and EDL muscle cross sections from male and female Wistar rats of 5 different age-groups (3-6 months, 11-12 months, 16-19 months, 20-25 months and 27-30 months). Confocal microscopy was used to detect the primary anti-AGE-RNAse antibody. Intracellular AGEs showed a significant increase (p<0.001) in both SOL and EDL muscles of males and females across age groups. However, sarcolemmal AGE formation was significant (p<0.05) across age-groups only in the fast-twitch EDL muscle of male rats. Immunoblotting showed myosin along with 2 other proteins of ~57 kD, to be glycated in all age groups of both muscle types in both the sexes. Cross sections were double labelled for Type I and Type IIA fiber types in EDL and SOL muscles respectively. The increase in AGEs was irrespective of fiber type in both SOL and EDL muscles.
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to amma and anna
INTRODUCTION

Myosin

Myosin is a major structural component of skeletal muscle, and it is considered to be the molecular motor that converts free energy derived from its hydrolysis of ATP into mechanical work. The myosin molecule comprises a heavy chain (MyHC) of ~220 kD, 2 light chains (MyLC) of ~20 kD. The heavy chain is divided into three structurally and functionally different regions i.e., 1) the globular head, consisting of 3 domains (~25 kD, the N-terminus; ~50 kD and ~20 kD) which contains the nucleotide binding site and the actin binding site; 2) the neck, an α-helical extension of the head on to which an essential, and a regulatory light chains are bound; 3) the tail, an α-helical extension of the neck to the C-terminus, which intertwines with the tail of another myosin molecule in an α-helical coiled coil fashion contributing to the formation of the backbone of the thick filament. The light chains have been classified into essential (MyLC1 and MyLC3) and regulatory (MyLC2) light chains based on the conditions required for their differential dissociation from the heavy chain. The globular head, neck, along with the 2 light chains constitute the myosin subfragment - (S1).

The cyclic interaction of myosin with the thin filament, which comprises mainly actin and actin associated proteins (tropomyosin, troponin I, T and C), results in the generation of force. The binding of ATP to myosin dissociates the interaction between the proteins (Lynn and Taylor, 1971). This is followed by hydrolysis of ATP that facilitates the rebinding of actin and myosin. This constitutes the cross bridge cycle.

The thick and the thin filaments are organized in regular arrays of contractile units called sarcomeres. The ability of skeletal muscle to generate force and motion can be attributed to the mechanical interaction between these two filaments. Studies using single muscle fibers have shown that the expression of myosin isoforms is the major, although not the only determinant for the wide range of shortening velocities under which muscle cells operate. That is, there is a close relationship between the maximum velocity of unloaded shortening ($V_0$), the actin-activated ATPase activity of myosin, and the myosin isoform expression of single muscle fibers in different species (Barany, 1967; Greaser, et al., 1988; Sweeney, et al., 1988; Larsson, et al., 1993; Bottinelli, et al., 1994; Reggiani, et al., 1997). Ten distinct MyHC isoforms that have been described include the embryonic, fetal, β/slow (type I), α-cardiac, slow tonic, type IIa, IIx and IIb, two superfast isoforms, one in the extraocular and one in the jaw-closing muscle of cat and dog (Schiaffino and Reggiani, 1996). Rat hindlimb muscles express four of the isoforms viz., type I, IIa, IIx, and IIb. Typically, MyHC expressed in a fiber is correlated with its contractile properties, such that the $V_0$ values are of the order I<IIa<IIx<IIb (Bottinelli, et al., 1991). During the aging process, a fast- to slow MyHC isoform transition has been reported in both fast- and slow-twitch rodent muscles (Larsson, 1986; Larsson and Edstrom, 1986; Larsson et al., 1993; Ansved and Larsson, 1989; Larsson and Ansved, 1995).
Glycation

Glycation or non-enzymatic glycosylation of proteins occurs by a chemical reaction of reducing sugars with primary amino groups in proteins to form a Schiff's base linkage (Watanabe H., 1992). That is, the aldehyde groups of free, unbound sugars react with free amino groups of proteins, which further undergo various rearrangements and free radical mediated oxidation to generate advanced glycation end products (Avigad, et al., 1996; Lal, et al., 1996). The rearrangement of the Schiff's bases gives rise to Amadori products. The Amadori product subsequently degrades into α-keto aldehyde compounds. These secondary compounds can react with proteins to form crosslinks as well as chromo/fluorophoric adducts called Maillard products or advanced glycation end products (AGEs) (Wolff, et al., 1991). This reaction is a common post-translational modification of proteins. There is considerable evidence linking hyperglycaemia, the most obvious metabolic abnormality in diabetes, with accelerated formation of irreversible non-enzymatic AGEs (Boekel, 1991; Brownlee, 1995). Glycation of tau protein has also been reported to be a mechanism for the induction of oxidative stress in Alzheimer's disease (Yan, et al., 1994).

The earliest protein study that led to the detection of glycation in pathology was hemoglobin (Hb). Trivelli, et al., (1971), reported that the early stage of the glycation reaction occurs in vivo, through a study of minor Hbs in diabetes. Many of the in vitro studies of glycation have been done on bovine serum albumin (BSA) and collagen, perhaps reflecting the availability, reactivity and natural abundance of the proteins. Reaction of glucose with BSA has been reported to be proportional to both the glucose concentration and the incubation time in vitro (Shaw and Crabbe, 1994) and to alter the response of the protein when different detection methods are used (Wu, et al., 1996). Various methods have been devised to detect and characterize the product of carbohydrate and protein at different stages leading up to the formation of AGEs, such as chromatography, electrophoresis, fluorescence and immunochemical techniques (Makita, et al., 1991; Shaw and Crabbe, 1994; Wu, et al., 1996).

Due to the immense importance of myosin in muscle contraction, any modification of the motor protein will have significant consequence on motor performance. Hence, glycation of myosin may impact the contractile properties of skeletal muscle. Myosin, a stable protein with high lysine content (~11%) and a long turnover rate is an ideal candidate for the slow process of glycation (Alliegro, 1998). Myosin ATPase activity has been reported to decrease upon incubation of myosin with various sugars, like glucose, fructose, galactose, ribose and glyceraldehyde (Syrovy and Hodny, 1993). Decreased myosin ATPase activity has been reported in response to incubation of myosin with glucose 6-phosphate (Avigad, et al., 1996) and myosin S1 head with glucose (Brown and Knull, 1992). The two lysine-rich regions of myosin, and potential targets for glycation, are functionally the two most important parts of the myosin molecule viz., the actin-binding site and the ATPase pocket.

Glutathione

The ubiquitous tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine), is the most prevalent intracellular thiol, and is known to function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells. Glutathione is synthesized intracellularly by the consecutive
actions of γ-glutamylcysteine synthetase and GSH synthetase (Meister and Anderson, 1983). GSH is primarily synthesized in the liver and released into the circulation for transport to peripheral tissues (Powers and Lennon, 1999)). The reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG) by GSH peroxidase. GSH and glutathione disulfide (GSSG) constitute the most important redox buffer in animal cells both in the cytosol and in the organelles (Csala, et al., 2001). The intracellular levels of GSH in mammalian cells is in the millimolar range (0.5-10 mM), whereas micromolar concentrations are typically found in blood plasma (Meister and Anderson, 1983).

GSH is a non-enzymic antioxidant that has several antioxidant functions. GSH serves as a substrate for glutathione peroxidase (GPX), and also scavenges singlet oxygen and hydroxyl radicals (Jain, 1998)). The GPX system is seen as essential in skeletal muscle where oxidative stress and lipid peroxidation increase dramatically with increased work, exercise, infection, or disease (Powers and Lennon, 1999).

Oxidative stress caused primarily by reactive oxygen species (ROS) can be viewed as the imbalance between the antioxidants and the ROS tending towards production of more ROS. The ground state oxygen molecule is unreactive with most organic substances and ROS are defined as any species which are more reactive than the ground state oxygen molecule. The univalent reduction of oxygen results in a series of cytotoxic oxygen species such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH.) (Ji, 1990). Protein modifications elicited by 1) a direct oxidative attack on amino acid side chains or 2) the modification of side chains with lipid peroxidation products or 3) modification of side chains with reducing sugars, can all lead to the formation of protein carbonyl derivatives (Stadtman and Berlett, 1998), which can be used as a convenient marker of oxidative protein damage under conditions of oxidative stress in exercise (Westerblad and Allen, 1992; Radak, et al., 2000; Smolka, et al., 2000), aging (Stadtman, 2001; Moskovitz, et al., 2002), and various pathologies (Kang, et al., 2002).

In skeletal muscle, unlike many tissues, antioxidant enzyme activities, including GPX, appear to increase with age. 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 increased glycation of extra cellular protein, Hb in response to a GSH deficiency (Jain, 1998). Further, advanced glycation end products have been known to alter the GSH redox state in human neuroblastoma cells, generally toward more oxidizing conditions (Deuther-Conrad et al., 2001).

**Aging and AGEs**

Advanced Glycation Endproducts are the result of chemical reaction of reducing sugars with primary amino groups, i.e., aldehyde groups of free unbound sugars react preferentially with free amino groups of proteins to reversibly form Schiff base adducts. These structures may undergo further Amadori rearrangements and free radical mediated oxidation to finally generate irreversible advanced glycation end products (Avigad, et al., 1996; Lal, et al., 1996).

Because of the wide range of pathological implications of AGEs, several compounds that inhibit their formation have been evaluated. The most studied and potent inhibitor is aminoguanidine (amG), a nucleophilic hydrazine compound which is more reactive than the ε-
amino group of lysine in proteins. Consequently, it preferentially binds to the carbonyl of amadori product-derived fragmentation products and forms unreactive triazines (Brownlee, 1995). Another compound, β-mercaptoethanol, has been found to restore ATPase activity of glycated myofibrils proportional to the increasing concentration of the compound (Syrovy and Hodny, 1993). An agent cleaving glucose-derived crosslink in vitro and in vivo, N-phenacylthiazolium bromide (PTB) has been shown to reduce glycosylation markers in diabetic erythrocytes (Vasan, et al., 1996). The investigators also claim to offer PTB as a potential therapeutic approach for the removal of established AGE crosslinks. Glycation inhibitors are particularly significant for the study of aging, since glycation of proteins in response to oxidative stress has been regarded as an important biochemical mechanism underlying the pathophysiology of aging (Brownlee, 1995).

Post-translational modifications such as non-enzymatic glycation have been shown to affect protein function (Watanabe H, 1992; Shaw and Crabbe, 1994; Wu, et al., 1996). Aging of skeletal muscle is characterized by profound changes in the ultrastructural and functional properties of the muscle fiber. Aging-related changes in the contractile properties at the motor unit (Edstrom and Larsson, 1987; Larsson and Ansved, 1995), cellular (Larsson, 1986; Edstrom and Larsson, 1987; Larsson, et al., 1997) and the molecular levels (Hook and Larsson, 2000) are well documented. Due to the immense importance of myosin in muscle contraction, any modification of the motor protein has great consequence on the activity of the individual. Hence, glycation of myosin may impact the contractile properties of skeletal muscle. Actin is another myofibrillar protein known to be affected by glycation in vitro (Kuleva and Kovalenko, 1997).

The maximum velocity of unloaded shortening (V₀) is a very important design parameter of skeletal muscle since muscles develop their maximum power at approximately one-third of V₀ (Rome, et al., 1990). V₀ of a muscle is proportional to the myosin ATPase activity (Barany, 1967). Studies have reported a ~50% decline in V₀ in rat and human type I fibers with aging (Larsson, et al., 1997; Degens, et al., 1998; Yu, et al., 1998). However, the decline in motility speed with aging at the molecular level is less dramatic than at the cellular level (Hook, et al., 1999). In human fibers expressing IIa myosin from old subjects, Larsson et al., (1997) showed an ~ 30% decline in V₀ as compared to the young which was not paralleled at the molecular level i.e., absence of an aging related slowing of motility speed in the in vitro motility assay in fibers expressing type IIa myosin (Hook, et al., 1999). This difference reflects an aging-related change at the cellular level in structural or thin filament associated proteins.

Proteins undergo a number of post-translational modifications during aging which may affect enzymatic activity; stability and digestibility (see Mooradian and Wong, 1991). To date, the large majority of projects studying the effects of glycation on protein structure and function have focused on extra-cellular proteins, such as collagens, lens crystallin, hemoglobin, myelin and albumin perhaps reflecting the availability, reactivity, and natural abundance of the proteins. However, there have been no studies documenting modifications of non-myofibrillar proteins with respect to aging in the muscle fibers. Consequently, there is a significant need to understand the contribution of myofibrillar and non-myofibrillar protein modifications to the slowing of skeletal muscle during the aging process.
SPECIFIC AIMS

The specific aims of the studies conducted in this thesis are as follows:

1. To investigate the effect of the post-translational modification, glycation on myosin structure and function

2. To study the role of the antioxidant glutathione in relation to the process of myosin glycation

3. To detect Advanced Glycation Endproducts in the fast- and slow-twitch skeletal muscles of rats with respect to age
MATERIALS AND METHODS

1. Animal care and muscle dissection

The first two studies were carried out using young (2-4 months), and young (2-4 months) Wistar and Sprague Dawley rats and in addition to the young, the third study used adult (11 months) and old (20, 27-30 months), male and female Wistar rats. For the third study, rats were obtained from the breeder (Mollegaard Breeding & Research Centre, Denmark) at the age of two months. The animals were sacrificed and tissues were harvested at five different ages, i.e., 3-6 months, 11-12 months, 16-19 months, 20-24 months and 27-30 months.

All animals were housed in the barrier facility at the Karolinska hospital at room temperature on a 12:12 h light and dark cycle and fed ad libitum. The animals were anaesthetized with fentanyl-fluanisone (0.2-0.3 ml/kg im) followed by pentobarbital sodium (30 mg/kg ip). The skin over the lower part of the limbs was removed, and the soleus and the extensor digitorum longus (EDL) muscles were gently dissected free from the surrounding tissue and clamped at approximately the in situ length. The muscle was subsequently weighed, frozen in isopentane chilled with liquid nitrogen, and stored at -80°C pending use. Following the removal of muscles, the animals were euthanized by excising the heart.

The use of animal material in this study was approved by the ethical committees at the Karolinska Hospital, Stockholm, Sweden, and the Pennsylvania State University, University Park, USA.

2. Muscle fiber preparation

The muscle fibers used in the studies were membrane permeabilized by chemical skinning. Chemical skinning: Small bundles of approximately 25-50 fibers were dissected free from the muscle and were tied to glass micro-capillary tubes. The bundles were then placed in skinning solution (50% (v/v) glycerol, 4 mM MgATP, 1 mM free Mg2+, 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate and sufficient KCl to adjust ionic strength to 180 mM ) at 3°C for 24 hours and treated with a cryoprotectant (sucrose) for long-term storage at -80°C (Larsson, et al., 1993; Frontera and Larsson, 1997; Larsson, et al., 1999). Prior to use in the in vitro motility assay, a sucrose-treated bundle was transferred to a 2.0 M sucrose solution for 30 minutes and subsequently incubated in solutions of decreasing sucrose concentrations (1.5 - 0.5 M). The bundle was then stored in skinning solution at -20°C and single fibers were dissected and were used within two weeks.

3. Actin purification

Actin was purified essentially as described by (Pardee and Spudich, 1982). Briefly, rabbit skeletal muscles from the back and hind legs were dissected, minced and extracted with Guba Straub solution (0.3 M KCl, 0.15 M KH2PO4, pH 6.5). The residue was pelleted by
centrifugation at 5000 g for 10 minutes, then re-suspended and extracted two times in buffer and four times in acetone. The obtained acetone powder was allowed to dry and then stored at -20°C before being further processed.

Acetone powder was extracted (2 mM Tris-HCl pH 7.6, 0.5 mM ATP, 0.1 mM CaCl₂, and 0.25 mM DTT) and filtered. The filtrate was centrifuged at 100,000 g for 3 hours and actin was polymerized from the supernatant by addition of KCl and MgCl₂ to a final concentration of 10 mM and 2 mM respectively. The filamentous actin (F-actin) was pelleted at 100,000 g for 3 hours, and subsequently re-suspended and homogenized in G-buffer (5 mM Tris-HCl pH 7.6, 0.5 mM ATP, 0.2 mM CaCl₂, 0.5 mM DTT) followed by overnight dialysis against G-buffer. After two additional days of repeated homogenization and dialyses, the G-actin solution was centrifuged for 2 hours to remove the remaining debris. The purity of actin was determined by SDS-PAGE, and the actin concentration was calculated using an extinction coefficient of 0.63 and absorbance reading at 290 nm. For long-term storage, the actin solution was added drop-wise to liquid nitrogen, and the acquired granules were kept at -80°C pending use. One day before the experiment, actin was polymerized and fluorescent-labeled with rhodamine-phalloidin (Rh-Ph; Molecular Probes Inc., OR).

4. Single fiber in vitro motility assay

A fiber segment from the Soleus muscle, was placed on a glass slide between two strips of grease; and a cover slip, pre-coated with 0.1% nitrocellulose in amyl acetate, was placed on top, thus creating a flow-cell of about 2.5 µl volume. Myosin was extracted from the fiber segment through the addition of high-salt buffer (0.5 M KCl, 25 mM Hepes pH 7.6, 4 mM MgCl₂, 4 mM EGTA, 2 mM ATP, 1% β-mercaptoethanol). After a 30-minute incubation on ice, a low-salt buffer (25 mM KCl, 25 mM Hepes pH 7.6, 4 mM MgCl₂, 1 mM EGTA, 1% β-mercaptoethanol) was applied, followed by BSA (1 mg/ml) in low-salt buffer. To block non-functional myosin molecules, unlabelled F-actin filaments in low-salt buffer (15 µM) were sonicated for 1 minute and infused into the flow-cell. To remove F-actin from the functional myosin heads, low-salt buffer containing 2 mM ATP was applied, followed by low-salt buffer. Subsequently, Rh-Ph labelled actin filaments in low-salt buffer (20 nM), and low-salt buffer were added to the flow cell. Filament movement was initiated by adding motility buffer (2 mM ATP, 0.1 mg/ml glucose oxidase, 23 µg/ml catalase, 2.5 mg/ml glucose in low-salt buffer).

The flow-cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70, Olympus America Inc. NY) and the fluorescent-labelled actin filaments were visualized through an x60 objective (NA 0.7) with illumination from a 100 W mercury lamp. The temperature of the flow-cell was thermostatically controlled at 25 °C (Bionomic Controller, BC-100, 20/20 Technology, USA) by a thermometer probe (HH21, Omega Engineering Inc., USA) placed 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 Inc., USA) and recorded on VCR tape (Hook, et al., 1999; Hook and Larsson, 2000; Hook, et al., 2001).
5. 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 bi-directionally. Using an image analysis package (OPTIMAS 6.0, Optimas Corp., USA), a filament was tracked from the centre of mass, and the speed was calculated from 20 frames at an acquisition rate of 1 frame/sec. The average speed and standard deviation of the 10 filaments were calculated. Since the standard deviation in this group of filaments was small (between 10 and15% of the mean), the average speed was taken as representative of the muscle fiber (Hook, et al., 1999; Hook and Larsson, 2000; Hook, et al., 2001).

6. Incubations

Two segments of a fiber were used to obtain the pre- and post-incubation values, i.e., myosin isolated from the first fiber segment was assayed to obtain pre-incubation data and myosin from the second segment was incubated with selected solutions to obtain post-incubation data. The extracted myosin from the second halves of slow- and fast-twitch fibers was exposed to 6 mM glucose for 15, 20 and 30 minutes and 10, 20 and 30 minutes respectively. Control incubations with sucrose (a non-reducing sugar used as an osmotic control) and low-salt buffer were carried out for 30 minutes on both slow and fast myosin. Hydroxylamine hydrochloride, an agent that cleaves Schiff base linkage between reducing sugars and proteins, was used to test the reversal of early glycation of myosin. That is, after 30 minutes of glucose exposure and motility speed measurements, the myosin was incubated for an additional 20 minutes in a buffer containing hydroxylamine hydrochloride, and motility speeds before and after incubation with hydroxylamine hydrochloride were compared. Slow myosin was incubated with 10 mM GSH for 20 minutes following a 30-minute exposure to 6 mM glucose, to test the efficacy of the antioxidant in reversing the effect of glucose on motility. Control incubations of low salt buffer and 10 mM GSH for 50 minutes were performed.

7. Mass spectrometry

Myosin was extracted from soleus muscle of Wistar rats according to the method of Svensson et al, ((Svensson, et al., 1997)1997). That is, muscle samples were homogenized in Guba-Straub solution and extracted on ice for 20 minutes. The samples were then centrifuged and the supernatant was diluted in twelve volumes of low-salt buffer. After 30 minutes of incubation on ice, the residue that was pelleted by centrifugation was re-suspended in 12 volumes of low-salt ATP buffer. After a final round of 30-minute incubation on ice and centrifugation, the pellet, which contained myosin was dissolved in buffer, stored on ice and used within two days. Protein concentration was estimated using the modified method of Lowry et al. (1951). The extracted myosin (3-4 µg in 5 µL) was incubated with 6 mM D-(+)-glucose (Sigma >99.5% pure, HPLC grade) for 30 minutes and Schiff base linkages between glucose and protein were stabilized by adding 50 mM sodium cyanoborohydride as reducing agent.
Myosin samples were treated by addition of 10 µL of dithiothreitol solution (55 mM in 100 mM ammonium bicarbonate, pH 8.2) for 4 h at 56°C to reduce disulfide bonds. After cooling to room temperature, 0.5 µL of sequencing grade endoproteinase Lys-C from *Lysobacter enzymogenes* (100 ng/µL in 50 mM Hepes, pH 8.0; Roche Molecular Biochemicals, Indianapolis, IN) was added and the solution was incubated at 37°C for 24 hours. Blank digestions were performed without myosin to verify that assigned peaks in the MALDI mass spectra did not arise from autoproteolysis of Lys-C. MALDI measurements were performed on a Voyager-DE STR time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, MA) operating in positive linear mode. Aliquots (0.5 µL) of digests were applied to a thin film of α-cyano-4-hydroxycinnamic acid (CHCA) matrix on the MALDI sample target and were allowed to dry under ambient conditions. On-plate desalting was achieved by adding 5 µL of 0.01% trifluoroacetic acid, which was removed after 10 seconds. Ions formed by a pulsed UV laser beam from a nitrogen laser (337 nm) were accelerated at 20 KeV. Mass spectra were obtained as averages of 256 laser shots, and three independent MALDI mass measurements were made from each sample to evaluate reproducibility.

Average isotopic masses of Lys-C digestion products were calculated from the SwissProt sequence using PAWS software v. 8.1.1 for MacOS (ProteoMetrics, Inc.).

8. **Immuno-blot**

Myosin heavy chain (MyHC) isoforms were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from ~5 cross-sections (8 µm) of muscle samples. Equal volumes from each sample of ~5 cross sections (8 µm thickness) were loaded in each lane. The acrylamide concentrations in the stacking and running gels were 3.5% and 12% (w/v), respectively, and the gel matrix included 10% glycerol. A constant current (16 mA per gel) was used and the gels were run for 5 h at 10°C (see Larsson et al. 1995). The separating gels (160 x 180 x 0.75 mm) were silver-stained (Giulian, et al., 1983). In separate unstained gels for immunoblotting analyses, proteins were transferred electrophoretically to nitrocellulose membrane as described by Towbin et al. 1979). Pre-stained markers were used as positive controls to ensure successful transfer. Immobilization of the proteins on the nitrocellulose membrane was confirmed using the Blot-Fast stain kit (Bio-Rad), The blots were first incubated with the anti-AGE RNAse primary antibody, visualized by the Alkaline-Streptavidin Phosphatase reaction. The color produced due to this reaction was used to detect the presence of glycation in a qualitative test. The primary antibody was visualized with a secondary antibody (goat anti-mouse IgG) followed by reaction with alkaline phosphatase – streptavidin and final color development with NBT/BCIP.

9. **Immunofluorescence and confocal microscopy**

**Immunofluorescence technique:** Soleus and EDL muscle cross-sections (8 µm) were mounted on cover glasses and allowed to air-dry for 30 minutes. Sections from the different age-groups within a gender were placed on the same glass and treated under identical conditions. All steps were performed at room temperature unless otherwise specified. Sections were fixed in acetone at -20°C followed by a pre-incubation in 3% BSA in Phosphate Buffered Saline (PBS) to
prevent non-specific binding. A chamber was created encircling all the sections on each slide using a hydrophobic marker to ensure uniform exposure of antibodies to all the sections within the chamber. The cross-sections were processed for immunocytohistochemistry with the following antibodies: anti-AGE RNAse reactive with AGEs, A4.951 reactive with the β/slow (type I) myosin heavy chain (MyHC) isoform (Schiaffino, et al., 1989), and A4.74 reactive with the IIa MyHC isoform (Schiaffino, et al., 1989). Double labeling was performed by incubating the sections with the primary IgG antibodies. All sections were stained with the anti-AGE RNAse polyclonal antibody (1:400) and the slow-twitch soleus with the anti-fast (A4.74) (1:10) and the fast-twitch EDL with the anti-slow (A4.951) (1:3) MyHC monoclonal antibodies for 30 minutes each. This was followed by 30-minute incubations with the secondary antibodies labeled with a fluorophore (Alexa fluor 568 (1:50), anti-rabbit IgG and Alexa fluor 488 (1:200), anti-mouse IgG) to detect the primary antibodies. Slides were washed 2 times with PBS between incubations. After a final wash with PBS, sections were mounted using mowiol as mounting medium with DABCO as an anti-fade agent.

Confocal microscopy: Sections were scanned at a 60x lens magnification and 1 zoom factor using a confocal laser scanning microscope (Olympus, FV200 attached to an Olympus IX70 microscope). A Krypton laser was used to visualize Alexa fluor 568 and an Argon laser for Alexa fluor 488. An XY scan was obtained in a plane close to the top surface of the section, away from the cover-slip as determined by an initial XZ scan. Fluorescent intensities of the scans were measured using the Fluoview software (v. 2.0.32, Olympus America). The fluorescent intensities were measured for the whole muscle fiber, i.e., the sarcolemma and in the interior of the muscle fiber, and the interior of the muscle fiber, i.e., excluding the sarcolemmal region, separately. The fluorescent intensities typically differed significantly between the sarcolemma and the cytoplasm of the muscle fiber. Therefore, the staining intensities of these regions are treated separately. The sarcolemmal intensity was calculated as the difference between the whole fiber cross-section and the cytoplasm intensities. The intracellular fluorescent intensities were normalized to the cross sectional area of the fibers. Multiple regions of the muscle section were scanned to yield the average intensity of the section. Intensities are presented in arbitrary units.

10. SDS-PAGE

Myosin heavy chain (MyHC) isoforms were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from a separate part of the single fibre segments analysed in the in vitro motility assay. The total acrylamide and bis concentrations were 4% (w/v) in the stacking gel and 12% in the running gel, while the gel matrix included 30% glycerol. Sample loads were equivalent to 0.1 mm of the fibre segment. The separating gels (160 x 180 x 0.75 mm) were silver-stained (see (Giulian, et al., 1983; Larsson, et al., 1993; Larsson, et al., 1994). The cross-sections used for immuno-blot were run on 12% gels. The acrylamide concentrations in the stacking and running gels were 3.5% and 12% (w/v), respectively, and the gel matrix included 10% glycerol. A constant current (16 mA per gel) was used and the gels were run for 5 h at 10°C (see (Larsson, et al., 1995). The separating gels (160 x 180 x 0.75 mm) were silver-stained (Giulian, et al., 1983).
11. Statistics

Means and standard deviations of means (SD) of data collected were calculated from individual values by standard procedures. A two-tailed t-test was used for comparisons of two groups. A one-way ANOVA was used for comparisons among multiple groups. Two-way ANOVA was used to test for gender and aging differences. Differences were considered significant at $p<0.05$. 
**RESULTS**

Papers I, II and III refer to Appendices A, B and C respectively.

**Effects of glucose exposure on myosin structure (Paper I)**

MALDI mass spectra of Lys-C digests of myosin showed numerous peaks attributable to digestion products. The myosin heavy chain, with a mass of 223 kDa, is expected to give about 202 proteolysis products of < 6 kDa after reduction of disulfides assuming 100% cleavage efficiency. Myosin contains 201 lysine residues and offers numerous potential sites for glycation.

MALDI spectra of digests of control and glucose-incubated myosin show great similarity. Several of the peaks observed in spectra of control myosin digests were either not present or were less abundant in spectra of digests of glucose-treated myosin. One prominent peak at m/z 4196 disappeared upon glucose treatment. Examination of the amino acid sequence suggests this proteolysis product corresponded to amino acid residues 147-184, which is the consensus ATP binding domain (calculated m/z = 4198). A peak at m/z 2004 was reduced in abundance relative to neighboring peaks in the MALDI spectra of digests of control myosin, and is assigned to amino acids 1263-1279, i.e., the coiled-coil region of the protein. Concomitant appearance of a peak at m/z 2169 is attributed to the reduced Schiff base adduct (theoretical mass increase = 164 Da).

No peaks were observed corresponding to products of proteolytic cleavage corresponding to a Schiff base product glycated at either Lys-146 or Lys-184 (theoretical m/z = 4491 and 4906) uncleaved by Lys-C at the site of modification.

**The speed of actin filaments propelled by different myosin isoforms (Paper I)**

Single 2-4 mm muscle fiber segments were dissected from the fast-twitch extensor digitorum longus (EDL) and the slow-twitch soleus muscles from 2-4 month male Wistar and Sprague-Dawley rats. Muscle fibers were divided into two groups based on the myosin heavy-chain (MyHC) isoform expression, a fast-twitch group expressing type IIB MyHC isoform, or a combination of IIx and IIB MyHC isoforms, and a slow-twitch group expressing the β/slow (type I) MyHC isoform.

Myosin heavy-chain isoform expression had a strong impact on actin filament speed and no overlap in speed was observed between the slow and fast isoforms. The average motility speeds for the type I, IIxb, and IIB MyHC isoforms were 1.12 ± 0.43 µm/s (n=11), 4.20; 5.57 µm/s (n=2), and 5.51 ± 1.29 µm/s (n=7), respectively. A significant (p<0.001) strain related difference in actin motility speed propelled by type I myosin was observed between Wistar (1.42 ± 0.33 µm/s) and Sprague Dawley (0.81 ± 0.17 µm/s) rats.
Changes in actin motility pattern in response to glucose incubation of myosin (Papers I and II)

The criterion for acceptance of motility recordings from the single muscle fiber in vitro motility assay has been determined to be a bi-directional movement by at least 90% of the total number of moving actin filaments. All pre-incubation and control post-incubation recordings fulfilled this criterion. In glucose post-incubation preparations, on the other hand, an increased deviation from the bi-directional linear movement of actin filaments was observed with increasing duration of glucose exposure.

Effects of glucose, sucrose and low-salt buffer incubations on actin motility speed (Paper I)

Incubations for 30 minutes with low-salt buffer, with or without β-mercaptoethanol, had no significant impact on motility speed. These values were therefore pooled and are presented as control post-incubation data. In the slow myosin, motility speed after 30 minutes of incubation with low-salt buffer or sucrose (0.79 ± 0.19 µm/s, n=3; 1.25 µm/s, n=1) did not differ significantly from pre-incubation motility speed (0.82 ± 0.33 µm/s, n=3; 1.12 µm/s, n=1). Similar to the slow myosin, in vitro motility speed on fast myosin after 30 minutes of exposure to the low-salt buffer (6.42 ± 0.32 µm/s, n=3) showed no difference when compared to the pre-incubation values (5.78 ± 0.25 µm/s, n=3). In the slow myosin in vitro motility preparations, actin filament speed decreased to 87 ± 16, 82 ± 14, and 48 ± 22% (p<0.05) of pre-incubation values after 15, 20 and 30 minutes of glucose exposure respectively. A similar result was observed in the fast myosin preparations, in which motility speed after 10, 20 and 30 minutes exposure to glucose decreased to 89 ± 3, 65 ± 26, and 21 ± 33% (p<0.05) compared with pre-incubation values. Incubation of type I myosin with glucose in low-salt buffer containing β-mercaptoethanol did not affect post-incubation motility speed (1.31 µm/s, n=1) when compared with the pre-incubation value (0.90 µm/s, n=1).

Dose-response relationship (Paper II)

In order 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 minutes. A graded and significant reduction in motility speed from pre-incubation values was observed in all 3 concentrations, thus confirming a dose-response relationship. After the 1 mM glucose incubation, motility speed decreased (p<0.05) 10 % from pre- (1.02 ± 0.07 µm/s, n=3) to post-incubation values (0.93 ± 0.05 µm/s, n=3). After the 3 mM glucose incubation, motility speed decreased (p<0.05) 34% from pre- (1.22 ± 0.26 µm/s, n=3) to post-incubation values of (0.81 ± 0.15 µm/s, n=3). Finally after the 6 mM glucose incubation, motility speed decreased (p<0.001) by more than 90 % from pre- (1.12 ± 0.04 µm/s, n=3) to post-incubation values (0.10 ± 0.07 µm/s, n=3).
Effects of hydroxylamine hydrochloride and GSH on glucose incubated myosin (Papers I and II)

Exposure of myosin to 10 mM hydroxylamine hydrochloride for 20 minutes restored the slowing of motility speed observed after 30 minutes of incubation with glucose, i.e., the pre-incubation in vitro motility speed of filaments (0.98 ± 0.25 µm/s, n=16) decreased after exposure to glucose (0.21 ± 0.23 µm/s, n=17), and was restored to pre-incubation levels (1.04 ± 0.26 µm/s, n=17) after exposure to hydroxylamine hydrochloride. GSH incubation resulted in similar responses in motility speeds as hydroxylamine incubation. In all preparations, incubations for 30 minutes with 6 mM glucose reduced motility speed (p<0.001) by more than 90%. Subsequent incubation with 10 mM GSH for 20 minutes restored motility speed (0.98 ± 0.06 µm/s, n=3) to almost pre-incubation levels (1.12 ± 0.06 µm/s, n=3). Post-glucose low salt buffer incubations did not, on the other hand, increase motility speed (0.03 ± 0.05 µm/s, n=3) to pre-incubation values (1.13 ± 0.04 µm/s, n=3). Further, a 16 to 20% decrease (p<0.001) in motility speed was observed in response to a 50-minute incubation with GSH in low salt buffer or with low salt buffer alone. In addition, incubation with hydroxylamine hydrochloride and GSH reduced the relative proportion of the randomly to bi-directionally moving actin filaments.

In the GSH experiments extraction of similar amounts of myosin was ensured in each preparation by controlling the length of the fiber to 2 mm. This control of the amount of myosin extracted enabled us to obtain a consistent post-glucose incubation response.

Anthropometric analysis (Paper III)

The body weight (BW) and muscle weight (MW) of the animals used in the study showed aging related differences. The body weight increased significantly (p<0.001) in males but not in females during aging. In both males and females, there was an aging-related change in soleus and EDL muscle weights. Significant gender-related differences were observed in BW and MW (p<0.001), the males were heavier and had larger soleus and EDL muscles than the females.

Immuno-blot analysis (Paper III)

Proteins from muscle cross sections were separated electrophoretically in a 12% gel. Equal volumes from each sample of ~5 cross sections (8 µm thickness) were loaded in each lane. Three bands reacted positively to the antibody from samples of the different age groups. Myosin as well as two other bands of protein with sizes of ~57 and 57.5 kDa, showed glycation in all the age groups of both soleus and EDL muscle samples.

Measurement of muscle protein glycation (Paper III)

An aging-related increase in the amount of cytoplasmic Advanced Glycation Endproducts was observed in both soleus and EDL. In the slow-twitch soleus, an aging-related increase (p<0.001) in the amount of glycated cytoplasmic proteins were observed in both male and female
rats. In the oldest age-group (27-30 months), the amount of glycated cytoplasmic proteins was slightly higher (p<0.05) in the females (4867 ± 400) than in the males (4222 ± 158). In the fast-twitch EDL, an aging-related increase (p<0.01) in glycated cytoplasmic proteins was observed in the males, but not in the females and the fluorescent signal was significantly lower (p<0.01) females (3190 ± 486) than in the males (4406 ± 632).

There were no significant differences in the sarcolemmal intensities between males and females in soleus or EDL. Sarcolemmal intensity was not significantly different in soleus among the different age groups whereas in the fast twitch EDL fibers a significant (p<0.05) difference was found in males. Double labeling of sections to identify type IIa MyHC in soleus and type I MyHC fibers in EDL muscle sections was used to test for muscle fiber type specific differences in the formation of AGE in the different age-groups and genders. Type IIa MyHC fibers in soleus were labeled with A4.74 primary antibody and Alexa fluor 488 secondary antibody. Type I MyHC fibers in EDL were labeled with A4.74 primary antibody and Alexa fluor 488 secondary antibody. There were no significant differences in fluorescence intensities of Alexa fluor 568 between type IIa (labeled with A4.74) and unlabelled type I fibers in soleus and between type I (labeled with A4.951) and unlabeled fibers in EDL.
DISCUSSION

Effect of glucose on myosin structure

Glycation of proteins resulting in crosslinking has been used to justify the biochemical point of view of many pathologies including complications due to diabetes (Brownlee, 1995; Syrovy and Hodny, 1992) and alzheimer’s disease (Yan et al., 1994). To date, the large number of projects that have studied the effects of glycation on protein structure and function have focused on extra-cellular proteins, such as collagens, lens crystallin, hemoglobin, myelin and albumin. Several techniques such as HPLC, fluorescence, ELISA, and mass spectrometry have been employed to study the AGE modifications and crosslinks of these proteins (Oimomi et al., 1987; Nicholls and Mandel, 1989; Myint et al., 1995; Shamsi and Nagaraj, 1999; Turk et al., 1999; Fujimori, 1989; Saito et al., 1997).

In this study, structural modifications of myosin were tested using MALDI mass spectrometry and myosin structure was shown to be altered in response to glucose exposure. The dominant structural modification of myosin was the selective disappearance of specific proteolytic fragments upon incubation of myosin with glucose. Reactive protein side chains, particularly those involving lysine residues, undergo a reversible reaction with glucose to form Schiff base adducts. Schiff base adducts may undergo spontaneous reactions during sample handling including Amadori rearrangements or cleavage of the Schiff base by nucleophiles such as thiols. In order to explore the sites on the protein, that are modified by reaction with glucose, the glycated protein is treated with sodium cyanoborohydride which forms a reduced adduct that is not susceptible to displacement. After reduction of disulfide bridges, the myosin undergoes proteolysis by endoproteinase Lys-C, which hydrolyzes the protein on the C-terminal side of lysine residues. In the case of myosin, proteolysis is expected to form more than 200 peptide fragments. However, lysine side chains that have been modified by glycation are not cleaved by the endoproteinase. As a result, the enzyme will not cleave at sites, where it would have hydrolyzed myosin that had not undergone glycation. This should result in the near-complete disappearance of the corresponding proteolysis product, with the appearance of at least one new digestion product from glucose-treated protein whose mass is higher than the disappeared proteolysis product.

MALDI spectra of digests of control and glucose-incubated myosin show great similarity, although several of the peaks observed in spectra of control myosin digests were either not present or were less abundant in spectra of digests of glucose-treated myosin. One prominent peak at m/z 4196 disappeared upon glucose treatment, whose amino acid sequence suggests a correspondence to the amino acid residues 147-184 in the consensus ATP binding domain (calculated m/z = 4198). The reduced abundance of the proteolysis product corresponding to amino acids 1263-1279 and the appearance of a glycated Schiff base product points to glycation at Arg-1268 or Arg-1275. Furthermore, the appearance of several peaks > 4 kDa for the glucose-treated myosin but not in controls suggests two possibilities: (1) glycation blocked proteolysis at sites where lysine modification occurred, leading to higher mass products of glycation, and/or (2) glycation led to formation of crosslinks between proteolysis products, which would also result in formation of higher mass products. These high mass products were not observed in significant abundance in digests of glucose-treated myosin as would be expected from early stage glycation products. These data point to the fact that the observed structural modifications of myosin by glucose were in the early stages of this process.
Effect of glucose on myosin function

An aging-related decline in function of skeletal muscle at the cellular (Li and Larsson, 1996; Larsson et al., 1997; Degens et al., 1998) and molecular levels (Hook et al., 1999) has been repeatedly documented. In an attempt to understand the underlying mechanism of this aging-related slowing of muscle fibers, the possibility of a post-translational modification of the motor protein myosin viz., glycation, as a modulator of myosin function was considered. A decrease in synthesis and turnover rate of muscle proteins with aging (Balagopal et al., 1997) and the long half-life of myosin (29-30 days) (Kay, 1978) renders them more susceptible to post-translational modifications.

The major findings from this study are 1) the dramatic effect of both fast and slow myosin function in response to glucose exposure; and 2) the reversal of the glycation induced changes in myosin function after incubation with the glucose-cleaving agent hydroxylamine hydrochloride. A significant reduction in actin filament speed was observed in both slow and fast myosin after the incubation with glucose, demonstrating an isoform-independent decline in the mechanical performance of the motor protein. Siemankowski et al., (1985) have shown in kinetic studies on actomyosin preparations, that the rate-limiting factor in the cross-bridge cycle is the release-rate of ADP from myosin, and not the ATPase activity. Furthermore, Sweeney and co-workers (1998) showed that either removal or reversal of the positive charge from three adjacent lysine residues within the loop 1 sequence resulted in a slowing of the motility speed. Based on these results, it is reasonable to conclude that the decrease in actin sliding speed in the present study is the result of glycation-induced structural alterations in, or close to, the ATPase hydrolytic cleft. Additional evidence in support of the specificity of glucose effect on myosin function is the lack of change in motility speed in response to 30 minutes of incubation with sucrose. Sucrose was used as an osmotic control in equimolar quantities of glucose. Sucrose is a non-reducing sugar and therefore does not possess the ability to react with lysine in the same way as glucose.

Hydroxylamine hydrochloride is a nucleophilic reagent that is more reactive than lysine side chains in binding aldehyde and ketone groups. As a result, various derivatives of hydroxylamine hydrochloride are useful for displacing carbonyl adducts from lysine residues in proteins (El-Saleh et al., 1984; El-Saleh et al., 1986). The restoration of motility by the use of hydroxylamine hydrochloride implies a reversal of the Schiff base formation. Since Schiff base formation occurs in the early reaction of glycation, we surmise that the modification of myosin by glucose in our experiments is in the initial phase of the glycation process.

Although it has been demonstrated that the speed of actin filament movement is largely independent of the density of myosin immobilized to the surface, provided that myosin density exceeds a lower limit (Uyeda et al., 1990; Uyeda et al., 1991), a more recent study has shown that on a myosin-poor surface the filaments moved unevenly and with a wider distribution in motility speed (Hook, 1999). The observation of decreasing linearity in actin filament motion with increasing incubation time may be explained by a glycation-related decrease in the number of functionally working myosin molecules. This transformation in F-actin motion pattern was not observed in control incubations.

Biochemical experiments on glucose- and glucose-6-phosphate-exposed myosin in solution that demonstrated a decrease in actin-activated ATPase activity (Avigad et al., 1996; Brown and Knill, 1992), and cell physiological experiments using the skinned fiber preparation showed reduced ATPase activity in parallel with a decrease in the specific tension after
incubation with physiological levels of glucose-6-phosphate (Patterson, et al., 2000). It is therefore suggested that the targeting of reducing sugars to the lysine-rich ATPase catalytic site and the actin-binding region of the myosin molecule is the most likely mechanism for causing the reduction in \textit{in vitro} motility speed.

A dose-related response in myosin function to glucose concentration is also observed in our study. There is a striking similarity in the response of myosin function to 15 minutes of 6 mM glucose exposure (Ramamurthy et al., 2001) and to 30 minutes of 1 mM glucose exposure. Considering the fact that modifications in multiple sites on the myosin molecule can contribute to the decline in function (Siemankowski et al., 1985; Sweeney et al., 1998, ), it can be surmised that greater concentration of glucose for a shorter duration or a greater duration of exposure to a lower concentration of glucose affects increasing number of sites on the myosin molecule.

**Role of glutathione in myosin glycation**

It is well known that GSH is an important antioxidant. In addition to the antioxidant functions, GSH is also a reactive nucleophile that plays an important role in the breakdown of exogenous and endogenous electrophilic toxins. GSH has several antioxidant functions. First, it readily interacts with a variety of radicals, including hydroxyl and carbon radicals, by donating a proton (Yu, 1994). A second important antioxidant function of GSH is to act as a co-substrate of GPX in the elimination of both H$_2$O$_2$ 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 (Ji, 1992). The reducing power to the Glutathione Reductase system in skeletal muscle is mainly provided by NADP-specific isocitrate dehydrogenase (Lawler and Demaree, 2001).

In skeletal muscle, unlike many tissues, antioxidant enzyme activities, including GPX, appear to increase with age (Ji, 1990; Ji, et al., 1998). 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 focussing on the role of GSH in relation to glycation of intracellular proteins. The only study published so far has demonstrated an increased glycation of extra cellular proteins in response to a GSH deficiency (Jain, 1998).

The restoration of motility by GSH after incubation with a reducing sugar implies a reversal of Schiff base formation. GSH is the most abundant intracellular non-protein thiol. Strong nucleophiles such as hydroxylamine can displace carbonyl groups from protein side chains. GSH is capable of reacting with reactive carbonyl groups, forming hemithioacetals, as is the case with $\alpha$-oxoaldehydes (Thornalley, 1998; Vander Jagt, 2001). 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.

Post-translational modification by glycation and free radical damage of proteins are seen to interact in a synergistic manner i.e., glycation is accelerated by free radicals (Wolff et al., 1991; Kristal and Yu, 1992) and oxidative stress has been reported to be increased in response to binding of AGEs to the AGE receptors (Wolff et al, 1991; Lander et al., 1997; Yan et al., 1994a, 1994b). GSH levels are known to increase during the aging process (Leeuwenburgh, 1994), perhaps in response to an increase in oxidative stress (see (Polidori, 2000). However, in spite of
the increase in GSH content during the aging process, glycation of myofibrillar proteins is found to increase with age. Deuther-Conrad et al., (2001) report that AGEs cause a dose-dependent and long-term increase in GSSG in vitro. This could, in part, explain the decrease in efficacy of GSH in controlling the formation of AGEs in aged tissue. 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.

**Advanced Glycation Endproducts and aging**

The major findings from the study are as follows 1) there is a progressive increase in AGEs in the intracellular regions irrespective of muscle type and in the sarcolemmal regions in fast-twitch and not slow twitch fibers, 2) myosin along with 2 other proteins of ~57kD, is glycated irrespective of age, 3) myosin is glycated irrespective of fiber type. In the course of glycation of many proteins, sugars react preferentially with ε-amino groups of lysines and myosin especially contains high amount of lysine residues, often arranged in clusters or neighbouring histidines, an arrangement which may facilitate amino group reactivity (Bai, et al., 1989; Hunt and Wolff, 1991). Glycation is known to alter myofibrillar protein function during aging (Syrovy and Hodny, 1992; Brownlee 1995).

In the present study, we have used an AGE specific antibody, raised against an epitope which is formed after incubation of glucose with RNAse (Verbeke, et al., 1997). Tissue AGEs which form in vivo appear to contain a common immunological epitope which cross reacts with AGEs prepared in vitro, supporting the concept that immunologically similar AGE structures are formed upon incubation of sugars with different proteins (Horiuchi, et al., 1991). Makita et al., (1991) have shown AGE modified BSA by glucose, glucose 6-phosphate, and fructose react to the anti-AGE-RNAse antibody in a similar fashion.

The abundance of content and decrease in synthesis and turnover rate (Balagopal, et al., 1997) of myosin and other myofibrillar proteins renders them susceptible to post-translational modifications such as glycation. Using the anti AGE-RNAse antibody, a progressive and significant increase in AGEs across the different age groups was observed irrespective of fiber type and sex. In the different age groups, the fluorescent intensities of AGEs appear to be distributed evenly in the cross section of the muscle fibers. This implies an even spread of glycated proteins within the muscle fiber. Functional changes in the muscle fiber with aging have been observed predominantly in fibers expressing type I MyHC isoform (Larsson, et al., 1997; Degens, et al., 1998; Yu, et al., 1998; Thompson and Brown, 1999). However, the presence of AGEs does not appear to differ between fiber types. Type I and IIa fibers appear to be equally modified as reflected by the observed fluorescent intensities.

Western blot data showed that myosin is persistently glycated in all the age groups. Although thin filament proteins (Kuleva and Kovalenko, 1997) and sarcoplasmic reticulum (SR) associated proteins (Williams, et al., 1998) are equally susceptible to post-translational modifications, we did not observe positive reactions of the AGE antibody with actin. Another interesting observation in this study is the progressive increase in glycation at the sarcolemmal region. Glycation of sarcolemmal proteins is not a well explored field of study, since most studies involving glycation of skeletal muscle proteins have focused on myofibrillar proteins (Syrovy and Hodny, 1993; Kuleva and Kovalenko, 1997; Ramamurthy, 2001). However, two other bands of ~57 kD showed glycation in all age groups, which could potentially belong to the
class of sarcolemmal or SR associated proteins. The identity of these proteins remains to be established.

The sarcolemma of the muscle fibers in a cross section is inseparably attached to the endomysium. This layer of connective tissue is primarily composed of collagen fibers, an important structural component of the muscle tissue. Glycation of collagen has been well studied with respect to hyperglycemia (Fujimori, 1989; Howard, et al., 1996; Turk, et al., 1999) and aging (Tsuru, et al., 2002). Collagen is a long-lived protein and highly susceptible to modification by glycation. Crosslinking of collagen fibers by glycation causes reduced elasticity or increased stiffness (Kuzuya, et al., 2001; Sell, et al., 2001; Verzijl, et al., 2002) and glycation inhibitors have been shown to restore collagen function (Sell, et al., 2001).

The changes in \( V_0 \) observed at the cellular level (Degens et al., 1998; Larsson et al., 1997; Yu et al., 1998) in skeletal muscle fibers unparalleled at the molecular level (Hook et al., 1999), could potentially involve modifications of thin filament proteins and/or the endomysium. Muscle membrane foldings have been observed in a subpopulation of freeze dried fibers expressing the fast myosin isoform from old men (Larsson, et al., 1997). Although the selective expression of this morphological change in the specific population cannot be explained, the potential of collagen crosslinking due to glycation causing these foldings, eventually contributing to a lower \( V_0 \) is high.

In conclusion, glycation of muscle proteins plays an important role in affecting muscle function in the aging process.
REFERENCES


APPENDIX A

Paper I: Changes in myosin structure and function in response to Glycation

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Abstract

Non-enzymatic glycosylation (glycation) has been recognized as an important post-translational modification underlying alterations of structure and function of extracellular proteins. The effect of glycation on intracellular proteins is, on the other hand, less well known in spite of the vital importance of intracellular proteins for cell, tissue, and organ function. The aim of this study was to explore the effects of glycation on the structure and function of skeletal muscle myosin. Myosin was incubated for up to 30 minutes with glucose and subsequently tested for structural and functional modifications by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and a single fiber in vitro motility assay, respectively. MALDI spectra revealed glycation-related structural alterations as evidenced by disappearance of specific Lys-C proteolysis products and appearance of higher mass peaks that are attributed to crosslinking by glucose. This change was paralleled by a significant reduction in in vitro motility speed, suggesting a structure-related decline in myosin mechanics in response to glucose exposure. Further evidence that early glycation products form in the regulatory regions of the myosin molecule is derived from the fact that there is complete reversal of motility speed after reaction with the Schiff base-cleaving agent hydroxylamine hydrochloride. Thus glycation of skeletal muscle myosin has a significant effect on both the structural and functional properties of the protein; a finding that is of importance in understanding the mechanisms underlying the impairment in muscle function associated with aging and diabetes.

Introduction

Skeletal muscle myosin generally accounts for 15-25% of the total body protein, and the coding sequence for the adult myosin isoforms is among the most heavily amplified in mammalian species (1). Myosin is considered to be the molecular motor that converts free energy derived from the hydrolysis of ATP into the mechanical work that drives muscle contraction. Studies using single muscle fibers have shown that the expression of myosin isoforms is the major, although not the only determinant for the wide range of shortening velocities under which muscle cells operate. There is a close relationship between the maximum velocity of unloaded shortening, the actin-activated ATPase activity of myosin, and the myosin isoform expression of single muscle fibers in different species (2-7).

Skeletal muscle myosin has been reported to have a half-life as long as 29-30 days (8). Also, an aging-related decrease in the myosin turnover rate has been observed in human muscle (9). The slow turnover rate makes myosin a potential target for post-translational modifications. For example, changes due to non-enzymatic glycosylation (glycation) have been suggested to play a major role in the functional and morphological changes associated with diabetes and aging (10). Glycation of proteins occurs by a chemical reaction of reducing sugars with primary amino groups, i.e., aldehyde groups of free unbound sugars react preferentially with free amino groups of proteins to reversibly form Schiff base adducts. These structures may undergo further Amadori rearrangements and free radical mediated oxidation to finally generate irreversible advanced glycation end products (11, 12).

To date, a large number of projects that have studied the effects of glycation on protein structure and function have focused on extra-cellular proteins, such as collagens, lens crystallin, hemoglobin, myelin and albumin. Significantly less scientific attention has been focused on the
effects of glycation on intracellular protein structure and function in spite of the fact that intracellular glucose metabolites such as glucose-6-phosphate and glyceraldehyde-3-phosphate have been reported to be more potent glycation agents than glucose (11, 13). Intracellular proteins, such as myosin, may accordingly be susceptible to glycation. The two most lysine-rich regions of myosin, and potential targets for glycation, are the actin-binding site and the ATPase pocket. These are two functionally very important parts of the myosin molecule. However, the effects of glycation on myosin structure and function remain to be established, and there is accordingly a compelling need for a detailed understanding of the effects of glycation-induced post-translational modifications of myosin on regulation of muscle contraction.

It is hypothesized that: (1) glycation primarily affects myosin function via an effect on the nucleotide-binding site, and (2) glycation of myosin has a significant impact on myosin structure and function independent of myosin isoform. To test these hypotheses we have employed: (1) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to analyze glycation-induced structural modifications of myosin and (2) an in vitro motility assay that allows detailed studies of actomyosin function after myosin and myosin-associated proteins have been extracted from a short single muscle fiber segment (14, 15, 16). These methods are advantageous because minute quantities of myosin can be used to study post-translational modifications in specific myosin isoforms. Preliminary results from this study have been presented in short form elsewhere (17, 18).

Materials and Methods

Animal care and muscle dissection

The study was carried out on young (2-4 months) male Wistar (n=3) and Sprague Dawley (n=1) rats. The animals were kept in conventional facilities at 20-22°C, with constant humidity and a 12-hour light/12-hour dark cycle, and were fed standard laboratory chow and tap water ad libitum. The rodents were anaesthetized by intramuscular injections of fentanyl-fluanisone (0.2-0.3 ml/kg) followed by pentobarbitone (30 mg/kg) administered intraperitoneally. The skin over the right and left hind limb was removed, and the soleus and extensor digitorum longus (EDL) muscles were dissected from surrounding tissue. The use of animal material in this study was approved by the ethical committees at both the Karolinska Hospital, Stockholm, Sweden and the Pennsylvania State University, USA.

Mass spectrometry

Myosin was extracted from soleus muscle according to the method of Svensson et al, (19). That is, muscle samples were homogenized in Guba-Straub solution and extracted on ice for 20 minutes. The samples were then centrifuged and the supernatant was diluted in twelve volumes of low-salt buffer. After 30 minutes of incubation on ice, the residue that was pelleted by centrifugation was re-suspended in 12 volumes of low-salt ATP buffer. After a final round of a 30-minute incubation on ice and centrifugation, the pellet, which contained myosin was dissolved in buffer, stored on ice and used within two days. Protein concentration was estimated
using the modified method of Lowry et al. (20). The extracted myosin (3-4 µg in 5 µL) was incubated with 6 mM D-(+)-glucose (Sigma >99.5% pure, HPLC grade) for 30 minutes and Schiff base linkages between glucose and protein were stabilized by adding 50 mM sodium cyanoborohydride as reducing agent. Samples were transported to the Penn State Intercollegiate Mass Spectrometry Center for matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analyses. Myosin samples were treated by addition of 10 µL of dithiothreitol solution (55 mM in 100 mM ammonium bicarbonate, pH 8.2) for 4 h at 56 °C to reduce disulfide bonds. After cooling to room temperature, 0.5 µL of sequencing grade endoproteinase Lys-C from *Lysobacter enzymogenes* (100 ng/µL in 50 mM Hapes, pH 8.0; Roche Molecular Biochemicals, Indianapolis, IN) was added and the solution was incubated at 37 °C for 24 hours (Fig. 1). Blank digestions were performed without myosin to verify that assigned peaks in the MALDI mass spectra did not arise from autoproteolysis of Lys-C.

 MALDI measurements were performed on a Voyager-DE STR time-of-flight mass spectrometer (Perseeptive Biosystems, Framingham, MA) operating in positive linear mode. Aliquots (0.5 µL) of digests were applied to a thin film of α-cyano-4-hydroxycinnamic acid (CHCA) matrix on the MALDI sample target and were allowed to dry under ambient conditions. On-plate desalting was achieved by adding 5 µL of 0.01% trifluoroacetic acid, which was removed after 10 seconds. Ions formed by a pulsed UV laser beam from a nitrogen laser (337 nm) were accelerated at 20 KeV. Mass spectra were obtained as averages of 256 laser shots, and three independent MALDI mass measurements were made from each sample to evaluate reproducibility (21).

 The reported amino acid sequence for the β/slow myosin isoform from Wistar rat was obtained from the SwissProt sequence database (accession number P02564). The SwissProt entry identifies the ATP-binding site at amino acid residues 178-185, and actin-binding regions corresponding to residues 655-677 and 757-771. Average isotopic masses of Lys-C digestion products were calculated from the SwissProt sequence using PAWS software v. 8.1.1 for MacOS (ProteoMetrics, Inc.).

### Muscle fiber preparation

Small bundles of approximately 25-50 fibers were dissected free from the muscle and were tied to a glass microcapillary tube. The bundles were then placed in skinning solution at 3°C for 24 hours and treated with a cryoprotectant (sucrose) for long-term storage at -80°C (5, 22, 23). Prior to use in the *in vitro* motility assay, a sucrose-treated bundle was transferred to a 2.0 M sucrose solution for 30 minutes and subsequently incubated in solutions of decreasing sucrose concentrations (1.5 - 0.5 M). The bundle was then stored in skinning solution at -20°C and used within two weeks.

### In vitro motility assay

Actin was purified from rabbit skeletal muscle as previously described (24), and was fluorescent-labeled with rhodamine-phalloidin (Rh-Ph; Molecular Probes Inc., OR). A muscle fiber segment, 2-4 mm in length, was placed on a glass slide between two strips of grease; and a coverslip, pre-coated with 0.1% nitrocellulose in amylacetate was placed on top, creating a flow-
cell of about 5 µl volume. Myosin was extracted from the fiber segment through addition of high-salt buffer (0.5 M KCl, 25 mM Hapes pH 7.6, 4 mM MgCl₂, 4 mM EGTA, 2 mM ATP, 1% β-mercaptoethanol). After a 30-minute incubation on ice, a low-salt buffer (25 mM KCl, 25 mM Hapes pH 7.6, 4M MgCl₂, 1 mM EGTA, 1% β-mercaptoethanol) was applied, followed by BSA (1 mg/ml) in low-salt buffer. To block non-functional myosin molecules, unlabelled F-actin filaments in low-salt buffer (15 µM) were sonicated for 1 minute and infused into the flow-cell. To remove F-actin from the functional myosin heads, low-salt buffer containing 2 mM ATP was applied, followed by low-salt buffer. Subsequently, Rh-Ph labeled actin filaments in low-salt buffer (20 nM) and low-salt buffer were added, and to initiate filament movement, motility buffer (2 mM ATP, 0.1 mg/ml glucose oxidase, 23 µg/ml catalase, 2.5 mg/ml glucose in low-salt buffer) was infused.

The flow-cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70, Olympus America Inc. NY) and the fluorescent-labeled actin filaments were visualized through an x60 objective (NA 0.7) by illumination from a 200-watt mercury lamp. The temperature of the flow-cell was thermostatically controlled (Bionic Controller, BC-100, 20/20 Technology, USA) by a thermometer probe (HH21, Omega Engineering Inc., USA) placed 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 Inc., USA) and recorded on VCR tape (14).

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 exception of the preparations incubated with glucose, in which a larger fraction of the filaments moved randomly, recordings and analyses were only performed from preparations in which >90% of the filaments moved bi-directionally. Using an image analysis package (OPTIMAS 6.0, Optimas Corp., USA), a filament was tracked from the center of mass and the speed was calculated from 20 frames at an acquisition rate of 5 or 1 frame(s)/sec, depending on the fiber MyHC composition. The average speed and standard deviation of the 10 filaments were calculated. Since the standard deviation in this group of filaments was small (between 10-15% of the mean), the average speed was taken as representative of the muscle fiber (14-16).

Incubations and motility speed analyses

Pre-incubation data were obtained from motility speed measurements on myosin extracted from a single fiber as described above. To acquire post-incubation values, the extracted myosin was incubated with low-salt buffer (with or without β-mercaptoethanol), glucose (with or without β-mercaptoethanol), sucrose, or hydroxylamine hydrochloride (post-glucose). Motility speed was analyzed before and after the respective incubations. The following two methods of obtaining post-incubation data were tested and compared:

1. Myosin from one fiber segment was used to obtain both pre- and post-incubation values. Following pre-incubation measurements, the labeled actin was removed from the flow-cell by increasing the ionic-strength of the buffer. After proper reconditioning and incubation of
the exposed myosin, labeled actin filaments were re-introduced into the experimental chamber and post-incubation motility speed measurements were carried out.

2. Two segments of a fiber were used to obtain the pre- and post-incubation values, i.e., myosin isolated from the first fiber segment was assayed to obtain pre-incubation data and myosin from the second segment was incubated with selected solutions to obtain post-incubation data.

Pilot experiments using the first method described, showed that there were no differences in the motility speeds between pre- and the post- incubation values with low-salt buffer. However, on occasion, removal of the labeled actin from the pre-incubation preparation proved difficult and hence interfered with post-incubation measurements. Therefore, the second method - using two segments of the same fiber - was chosen to study the effects of glucose exposure on actomyosin performance, since this method eliminated the ambiguity due to the interference created by the presence of actin filaments remaining from the pre-incubation preparation. Furthermore, the accuracy of using separate preparations from the same muscle fiber gains support from a previous study in our lab, which has shown that no significant difference exists between in vitro motility speeds of two halves of a single fiber assayed under identical conditions (15).

The extracted myosin from the second halves of slow- and fast-twitch fibers was exposed to 6 mM glucose for 15, 20 and 30 minutes and 10, 20 and 30 minutes respectively. After selective blockage of inactive myosin with fragmented F-actin, fluorescent-labeled actin was added and motility recorded. A relatively high concentration of glucose representative of extra-cellular levels of 6 mM, was used to compensate for the duration of time that myosin could be kept functional in the in vitro preparation. Control incubations with sucrose (a non-reducing sugar used as an osmotic control) and low-salt buffer were carried out for 30 minutes on both slow and fast myosin. Hydroxylamine hydrochloride, an agent that cleaves Schiff base linkage between reducing sugars and proteins, was used to test the reversal of early glycation of myosin. That is, after 30 minutes of glucose exposure and motility speed measurements, the myosin was incubated for an additional 20 minutes in a buffer containing hydroxylamine hydrochloride, and motility speeds before and after incubation with hydroxylamine hydrochloride were compared. Changes in post-incubation motility speed are expressed either as percent changes from pre-incubation values or as individual motility speed values.

**Statistics**

Means and standard deviations of means (SD) of data collected from both Wistar and Sprague Dawley rats were calculated from individual values by standard procedures. A two-tailed t-test was used for comparisons of two groups. Differences were considered significant at p<0.05.
Results

Effects of glucose exposure on myosin structure

MALDI mass spectra of Lys-C digests of myosin showed numerous peaks attributable to digestion products. The myosin heavy chain, with a mass of 223 kDa, is expected to give about 202 proteolysis products of < 6 kDa after reduction of disulfides assuming 100% cleavage efficiency (25). Myosin contains 201 lysine residues and offers numerous potential sites for glycation.

MALDI spectra of digests of control and glucose-incubated myosin show great similarity (Fig. 2). Several of the peaks observed in spectra of control myosin digests were either not present or were less abundant in spectra of digests of glucose-treated myosin. One prominent peak at m/z 4196 disappeared upon glucose treatment (Fig. 2). Examination of the amino acid sequence suggests this proteolysis product corresponded to amino acid residues 147-184, which is the consensus ATP binding domain (calculated m/z = 4198). A peak at m/z 2004 was reduced in abundance relative to neighboring peaks in the MALDI spectra of digests of control myosin, and is assigned to amino acids 1263-1279, i.e., the coiled-coil region of the protein.

Concomitant appearance of a peak at m/z 2169 is attributed to the reduced Schiff base adduct (theoretical mass increase = 164 Da).

No peaks were observed corresponding to products of proteolytic cleavage corresponding to a Schiff base product glycated at either Lys-146 or Lys-184 (theoretical m/z = 4491 and 4906) uncleaved by Lys-C at the site of modification. Most proteolysis products from control myosin had molecular masses in the 1-4 kDa range, and nearly all of these showed similar relative abundances in digests of glucose-treated myosin. Glucose treatment was also followed by formation of more high molecular mass (> 4 kDa) proteolysis products, which might be expected if Lys-C failed to cleave at the site of a glycated lysine residue or if glycation led to intramolecular cross-linking. Masses of these high mass products did not match any calculated values for Schiff base glycation products. Furthermore, these peaks were not observed in MALDI spectra of digests of myosin treated with hydroxylamine hydrochloride after incubation with glucose.

The speed of actin filaments propelled by different myosin isoforms

Single 2-4 mm muscle fiber segments were dissected from the fast-twitch extensor digitorum longus (EDL) and the slow-twitch soleus muscles from 2-4 month male Wistar and Sprague-Dawley rats. Muscle fibers were divided into two groups based on the myosin heavy-chain (MyHC) isoform expression, a fast-twitch group expressing type IIB MyHC isoform, or a combination of IIX and IIB MyHC isoforms, and a slow-twitch group expressing the β/slow (type I) MyHC isoform.

Myosin heavy-chain isoform expression had a strong impact on actin filament speed and no overlap in speed was observed between the slow and fast isoforms (Fig. 3). The average motility speeds for the type I, IIXB, and IIB MyHC isoforms were 1.12 ± 0.43 μm/s (n=11), 4.20; 5.57 μm/s (n=2), and 5.51 ± 1.29 μm/s (n=7), respectively. A significant (p<0.001) strain related difference in actin motility speed propelled by type I myosin was observed between Wistar (1.42 ± 0.33 μm/s) and Sprague Dawley (0.81 ± 0.17 μm/s) rats. The reason for this
strain-difference is not known. This observation deserves further scientific attention, but this is beyond the scope of the present study.

Changes in actin motility pattern in response to glucose incubation of myosin

The criterion for acceptance of motility recordings from the single muscle fiber in vitro motility assay has been determined to be a bi-directional movement by at least 90% of the total number of moving actin filaments. All pre-incubation and control post-incubation recordings fulfilled this criterion. In glucose post-incubation preparations, on the other hand, an increased deviation from the bi-directional linear movement of actin filaments was observed with increasing duration of glucose exposure (Fig. 4). Owing to the random-like nature of their motility, the speed of these filaments was not included in the analysis. However, a fraction of the bi-directionally moving filaments expressed a uniform but wavy motion, and a comparative analysis of the motility speed of these did not reveal a significant difference from the speed of linear bi-directionally-moving filaments. Hence, these filaments were included in the analyses of glucose post-incubation recordings.

Effects of glucose, sucrose and low-salt buffer incubations on actin motility speed

Incubations for 30 minutes with low-salt buffer, with or without β-mercaptoethanol, had no significant impact on motility speed. These values were therefore pooled and are presented as control post-incubation data. In the slow myosin, motility speed after 30 minutes of incubation with low-salt buffer or sucrose (0.79 ± 0.19 μm/s, n=3; 1.25 μm/s, n=1) did not differ significantly from pre-incubation motility speed (0.82 ± 0.33 μm/s, n=3; 1.12 μm/s, n=1; Fig. 5A). Similar to the slow myosin, in vitro motility speed on fast myosin after 30 minutes of exposure to the low-salt buffer (6.42 ± 0.32 μm/s, n=3) showed no difference when compared to the pre-incubation values (5.78 ± 0.25 μm/s, n=3; Fig. 5B). In the slow myosin in vitro motility preparations, actin filament speed decreased to 87 ± 16, 82 ± 14, and 48 ± 22% (p<0.05) of pre-incubation values after 15, 20 and 30 minutes of glucose exposure respectively. A similar result was observed in the fast myosin preparations, in which motility speed after 10, 20 and 30 minutes exposure to glucose decreased to 89 ± 3, 65 ± 26, and 21 ± 33% (p<0.05) compared with pre-incubation values. Incubation of type I myosin with glucose in low-salt buffer containing β-mercaptoethanol did not affect post-incubation motility speed (1.31 μm/s, n=1) when compared with the pre-incubation value (0.90 μm/s, n=1; Fig. 5A).

Effects of hydroxylamine hydrochloride on glucose incubated myosin

Exposure of myosin to 10 mM hydroxylamine hydrochloride for 20 minutes restored the slowing of motility speed observed after 30 minutes of incubation with glucose, i.e., the pre-incubation in vitro motility speed of filaments (0.98 ± 0.25 μm/s, n=16) decreased after exposure to glucose (0.21 ± 0.23 μm/s, n=17), and was restored to pre-incubation levels (1.04 ± 0.26 μm/s, n=17) after exposure to hydroxylamine hydrochloride (Fig. 6). In addition, incubation with hydroxylamine hydrochloride reduced the relative proportion of the randomly to bi-directionally...
moving actin filaments (Fig 7). Post-glucose incubation for 20 minutes with low-salt buffer did not change motility from the 30-minute glucose-incubation levels.
Discussion

The major findings from this study are as follows: (1) structural modifications of myosin are documented by the selective disappearance of specific proteolytic fragments upon incubation of myosin with glucose, reflecting a relationship in myosin structure and function in response to exposure to a reducing sugar; (2) the dramatic effect of both fast and slow myosin function in response to glucose exposure; and (3) the reversal of the glycation induced changes in myosin function after incubation with the glucose-cleaving agent hydroxylamine hydrochloride.

Reactive protein side chains, particularly those involving lysine residues, undergo a reversible reaction with glucose to form Schiff base adducts. Schiff base adducts may undergo spontaneous reactions during sample handling including Amadori rearrangements or cleavage of the Schiff base by nucleophiles such as thiols. In order to explore the sites on the protein, that are modified by reaction with glucose, the glycated protein is treated with sodium cyanoborohydride which forms a reduced adduct that is not susceptible to displacement. After reduction of disulfide bridges, the myosin undergoes proteolysis by endoproteinase Lys-C, which hydrolyzes the protein on the C-terminal side of lysine residues. In the case of myosin, proteolysis is expected to form more than 200 peptide fragments. However, lysine side chains that have been modified by glycation are not cleaved by the endoproteinase. As a result, the enzyme will not cleave at sites where it would have hydrolyzed myosin that had not undergone glycation. Analysis of the products of proteolytic digestion is accomplished using MALDI mass spectrometry, which indicates the molecular masses of the digestion products. Extensive glycation of a specific lysine side chain should result in the near-complete disappearance of the corresponding proteolysis product, with the appearance of at least one new digestion product from glucose-treated protein.

MALDI analyses show that glucose treatment resulted in 100% loss of the proteolysis product corresponding to amino acid residues 147-184 in the consensus ATP-binding region of the protein. The reduced abundance of the proteolysis product corresponding to amino acids 1263-1279 and the appearance of a glycated Schiff base product points to glycation at Arg-1268 or Arg-1275. Furthermore, the appearance of several peaks > 4 kDa for the glucose-treated myosin but not in controls treated with hydroxylamine suggests two possibilities: (1) glycation blocked proteolysis at sites where lysine modification occurred, leading to higher mass products of glycation, and/or (2) glycation led to formation of crosslinks between proteolysis products, which would also result in formation of higher mass products. These high mass products were not observed in significant abundance in digests of glucose-treated myosin that were subsequently treated with hydroxylamine, as would be expected from early stage glycation products. Further studies are underway to isolate and characterize these high mass products and identify other myosin sites that are reactive to glycation.

A significant reduction in actin filament speed was observed for both slow and fast myosin after the incubation with glucose, demonstrating an isoform-independent decline in the mechanical performance of the motor protein. The gradual decrease in motility speed correlated with the duration of glucose exposure, although a marked drop in speed was observed after approximately 20 minutes. A glycation-induced alteration in the myosin structure is proposed, since a complete reversal of actin sliding speed was observed after incubation with hydroxylamine hydrochloride. Hydroxylamine hydrochloride is a nucleophilic reagent that is more reactive than lysine side chains in binding aldehyde and ketone groups. As a result, various derivatives of hydroxylamine hydrochloride are useful for displacing carbonyl adducts from
lysine residues in proteins (26, 27). The restoration of motility by the use of hydroxylamine hydrochloride implies a reversal of the Schiff base formation. Since Schiff base formation occurs in the early reaction of glycation, we surmise that the modification of myosin by glucose in our experiments is in the initial glycation phase. Additional evidence provides strong support for the argument that the observed changes in myosin function are caused by an early formation of glycation products between the reducing sugar glucose and lysine residues in the myosin heavy chain domain. This is evidenced by the lack of change in motility speed in response to 30 minutes of incubation with the non-reducing sugar sucrose and the inhibition of the effect of the reducing sugar glucose by β-mercaptoethanol.

Oxidative damage by free radicals has been implicated as an important factor in the impairment of protein function (28). However, it is unlikely that oxidative damage of myosin contributes to the observed slowing in in vitro motility speed after 30 minutes of glucose incubation, since motility was retained after 50 minutes of incubation (30 minutes with glucose and subsequently 20 minutes with hydroxylamine hydrochloride), without the use of the reducing agent β-mercaptoethanol. Further, it is unlikely that the effect of hydroxylamine hydrochloride incubation was non-specific, since a similar 20 minute-incubation with low-salt buffer, did not restore motility to pre-incubation levels.

An interesting observation was the decreasing linearity in actin filament motion with increasing incubation time, i.e., filaments displayed a gradual change from a linear to a more random movement with longer exposure to glucose. This transformation in F-actin motion pattern was not observed in control incubations, and may be explained by a glycation-related decrease in the number of functionally working myosin molecules. In a previous study, we have shown that actin filament directionality is dependent on the density of interacting myosin. In the center of the flow-cell where the concentration of myosin was high, the filaments moved bi-directionally, while in the periphery of the myosin streak where the myosin-density was lower, actin filaments demonstrated a more random motion (15). This observation is supported by a study in which a microlithographic material was used to pack myosin with high density and on which actin filaments glided in a smooth and linear movement. Conversely, on a myosin-poor surface the filaments moved unevenly and with a wider distribution in motility speed (29). However, it has been demonstrated that the speed of actin filament movement is largely independent of the density of myosin immobilized to the surface, provided that myosin density exceeds a lower limit (30, 31). It is further suggested that the targeting of reducing sugars to the lysine-rich ATPase catalytic site and the actin-binding region of the myosin molecule is the most likely mechanism for causing the reduction in in vitro motility speed. This is supported by biochemical experiments on glucose-exposed myosin in solution that demonstrated a decrease in actin-activated ATPase activity (11, 32), and by cell physiological experiments using the skinned fiber preparation. These experiments showed reduced ATPase activity in parallel with a decrease in the specific tension after incubation with physiological levels of glucose-6-phosphate (33). Kinetic studies on actomyosin preparations, however, have shown that the rate-limiting factor in the cross-bridge cycle is the release-rate of ADP from myosin, and not the ATPase activity (34). The correlation between ATPase activity, ADP release and actomyosin mechanics has been further investigated using various chimeras of two loop structures, one located close to the ATPase catalytic site (loop 1) and another in the actin-binding domain (loop 2). Structural changes in loop 1 demonstrated a significant impact on the rate of ADP release and actin filament speed (35), while alteration in the loop 2 sequence had a strong influence on the actin-activated ATPase activity, but no effect on the in vitro motility speed (36). Furthermore,
Sweeney and co-workers (1998) showed that either removal or reversal of the positive charge from three adjacent lysine residues within the loop 1 sequence resulted in a slowing of the motility speed. Based on these results, it is reasonable to conclude that the decrease in actin gliding speed in the present study is the result of glycation-induced structural alterations in, or close to, the ATPase hydrolytic cleft. These are probably parallel to a similar change in the actin binding site, although structural modifications in this region are of less functional importance.

Non-enzymatic glycosylation has been known to be an important post-translational modification underlying aging-related alterations of protein structure, function and digestibility (37). To our knowledge this study is the first to show that glycation of skeletal muscle myosin has a significant effect on both protein structure and function. Given the reported increase in glycated myosin from old rodent muscle and in muscle of diabetic subjects (38-40), the results of this study may prove useful both in the understanding of the mechanisms underlying the aging-related decrease in motility speed propelled by type I myosin observed at the cellular and molecular levels in rodents and humans (14, 15, 41-45), and in the pathophysiological changes of muscle function associated with diabetes (38).
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References


Figure legends

Fig 1. Schematic depiction of the chemistry of stabilization of Schiff base between glucose and myosin and subsequent digestion of the protein by endoproteinase Lys-C for measurement by MALDI MS.

Fig 2. MALDI mass spectra of Lys-C digests of control myosin (A) and glucose-treated myosin (B). Protein was reduced with dithiothreitol before 24-h digestion. The increased abundance of peaks in the m/z 4000-8000 region is of particular note. Insets show narrow region of MALDI mass spectra of Lys-C digests of control myosin (A) and glucose-treated myosin (B). The peak at m/z 4196 in the control digest is assigned to amino acid residues 147-184 which is the consensus ATP binding domain. Treatment with glucose, as described in the text, leads to complete disappearance of this proteolysis product.

Fig 3. The distribution of average actin motility speeds on myosin extracted from type I single muscle fibers of Sprague-Dawley (hatched bars rising from right) and Wistar (hatched bars rising from left) rats and from type IIxb and IIb fibers of Sprague-Dawley rats (filled bars).

Fig 4. Depiction of actin filament motion pattern on type I myosin showing decreasing directionality with increasing duration of 6 mM glucose incubation, and the reversal in directionality after incubation with 10 mM hydroxylamine hydrochloride. Vertical bar represents 20 µm and horizontal bar 25 µm.

Fig 5. Percentage decreases in the speed of actin filaments sliding over slow (A) and fast (B) myosin incubated with low-salt buffer (○), 6 mM glucose (●), 6 mM glucose with β-mercaptoethanol (▲), and 6 mM sucrose (■). Values are expressed as means ± SD.

Fig 6. In vitro motility speeds on slow myosin in pre-incubated (unfilled bar), glucose incubated (hatched bar) and subsequently hydroxylamine hydrochloride incubated (filled bar) preparations. Values are expressed as means ± SD.

Fig 7. Proportion of linear bi-directional (hatched bars) and random (open bars) moving filaments after 0, 15 and 30 minutes of glucose exposure and subsequent 20 minutes of hydroxylamine hydrochloride (20 minutes) exposure.
Measure masses of digestion products using MALDI MS
FIGURE 2
Figure 3

Motility speed, µm/s

Number of filaments

0 1 2 3 4 5 6 7 8 9

0 10 20 30 40 50

*  

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<table>
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<tr>
<th>Incubation time, minutes</th>
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FIGURE 5

Incubation time, minutes

Change in motility speed, %

A

B
**FIGURE 6**

![Graph showing motility speed (µm/s) over incubation time (minutes). The graph compares different incubation times with error bars to indicate variability.](image-url)
FIGURE 7

Incubation time, minutes

Relative number, %
APPENDIX B

PAPER II: Glutathione reverses early effects of glycation of myosin function

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Submitted
Summary

Non-enzymatic glycosylation (glycation) has been recognized as an important post-translational modification underlying alterations of structure and function of extracellular proteins during both ageing and diabetes. Intracellular proteins may also be affected by this modification, and glycation has been suggested to contribute to the ageing-related impairment in skeletal muscle function. Glycation is the chemical reaction of reducing sugars with primary amino groups, i.e., aldehyde groups of free unbound sugars preferentially react with free amino groups of proteins to form reversible Schiff base adducts. Rearrangement of these adducts results in the formation of irreversible Advanced Glycation End-products. Glutathione (GSH) is an abundant tripeptide in skeletal muscle and is known for its antioxidant function and ability to protect cells from toxicity of xenobiotics. Earlier studies have suggested that abundant thiol compounds may inhibit the process of glycation. In order to understand the effect of GSH on glycated myosin function, we used a single fiber in vitro motility assay, i.e., where myosin is extracted from a single muscle fiber segment to propel fluorescent-labelled 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 pre-incubation values after 30 minutes exposure to 1, 3 and 6 mM glucose concentration, respectively. The 30-minute 6 mM glucose incubation was followed by a 20-minute 10 mM GSH incubation. After glucose exposure (0.10 ± 0.07 µm/s, n=3), GSH treatment restored (p<0.001) motility (0.98 ± 0.06 µm/s, n=3) close to pre-incubation levels (1.12 ± 0.06 µm/s, n=3). It is concluded that glucose modifies myosin function in a dose-dependent manner and that GSH reverses the effect of glucose on myosin function.
Introduction

Non-enzymatic glycosylation (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 (2, 18).

It is well known that glycation of proteins is an important mechanism underlying ageing- 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 ageing-related decrease in myosin turnover rate (3), and the lysine-rich regions of the actin-binding site, along with the catalytic domain, makes myosin a potential target for modification by glycation. For instance, increased amounts of glycated myosin have been reported in skeletal muscles of old rodents (32).

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 employed where myosin was extracted from single muscle fiber segments. The effect on actin motility speed of 30-minute incubations with 6 mM glucose (with or without β-mercaptoethanol) and with a low-salt buffer or a non-reducing 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 approximately 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 minutes of glucose exposure, MALDI mass spectra of Lys-C digest 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 non-protein 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 non-enzymic antioxidant that has several antioxidant functions. GSH serves as a substrate for glutathione peroxidase (GPX), and also scavenges singlet oxygen and hydroxyl radicals (16). The GPX system is seen as essential in skeletal muscle where oxidative stress and lipid peroxidation increase dramatically with increased work, exercise, infection, or disease (29). GSH and glutathione disulfide (GSSG) constitute the most important redox buffer in animal cells both in the cytosol and in the organelles (5). Further, the GSH redox status in human neuroblastoma cells is altered by Advanced Glycation End products (AGEs) due to a decrease in the concentration of the reduced form of GSH (7).

GSH and glucose 6-phosphate dehydrogenase deficiency is reported to increase protein glycation in hemoglobin (13). GSH has also been shown to inhibit glycation of eye lens proteins by dehydroascorbate through a mechanism attributed to reduction of dehydroascorbate to ascorbate (26). A subsequent study showed that GSH inhibited protein glycation by glucosamine
(1). However, the influence of GSH on the effect of glycation on skeletal muscle myosin function has not been studied to date. We hypothesize that GSH plays an important role in reversing the effect of early glycation of myosin \textit{viz.}, formation of Schiff base between myosin and glucose.

In order to test this hypothesis, we used the single fiber \textit{in vitro} motility assay to explore the effects of GSH treatment of myosin that was extracted from single Soleus muscle fibers of rats, after a 30-minute 6 mM glucose incubation.
Materials and Methods

Animal care and muscle dissection

The study was carried out using young (2-4 months) male Wistar (n=3) rats. The animals were kept in conventional facilities at 20-22°C, with constant humidity and a 12-hour light/12-hour dark cycle. All were fed standard laboratory chow and tap water ad libitum. The rodents were anaesthetized by intramuscular injections of fentanyl-fluanisone (0.2-0.3 ml/kg) followed by pentobarbitone (30 mg/kg) administered intraperitoneally. The skin over the right and left hind limbs was removed, and the Soleus muscle was dissected from surrounding tissue. Following the removal of muscles, the animals were euthanized by excising the heart. The use of animal material in this study was approved by the ethical committees at the Karolinska Hospital, Stockholm, Sweden, and the Pennsylvania State University, USA.

Muscle fiber preparation

Small bundles of approximately 25-50 fibers were dissected free from the muscle and were tied to glass micro-capillary tubes. The bundles were then placed in skinning solution at 3°C for 24 hours and treated with a cryoprotectant (sucrose) for long-term storage at -80°C (8, 19, 21). Prior to use in the in vitro motility assay, a sucrose-treated bundle was transferred to a 2.0 M sucrose solution for 30 minutes and subsequently incubated in solutions of decreasing sucrose concentrations (1.5 - 0.5 M). The bundle was then stored in skinning solution at -20°C and was used within two weeks.

In vitro motility assay

Actin was purified from rabbit skeletal muscle as previously described (27), and was fluorescent-labelled with rhodamine-phalloidin (Rh-Ph; Molecular Probes Inc., OR). A fiber segment from the Soleus muscle, was placed on a glass slide between two strips of grease; and a cover slip, pre-coated with 0.1% nitrocellulose in amyl acetate, was placed on top, thus creating a flow-cell of about 2.5 µl volume. Myosin was extracted from the fiber segment through the addition of high-salt buffer (0.5 M KCl, 25 mM Hepes pH 7.6, 4 mM MgCl₂, 4 mM EGTA, 2 mM ATP, 1% β-mercaptoethanol). After a 30-minute incubation on ice, a low-salt buffer (25 mM KCl, 25 mM Hepes pH 7.6, 4 mM MgCl₂, 1 mM EGTA, 1% β-mercaptoethanol) was applied, followed by BSA (1 mg/ml) in low-salt buffer. To block non-functional myosin molecules, unlabelled F-actin filaments in low-salt buffer (15 µM) were sonicated for 1 minute and infused into the flow-cell. To remove F-actin from the functional myosin heads, low-salt buffer containing 2 mM ATP was applied, followed by low-salt buffer. Subsequently, Rh-Ph labelled actin filaments in low-salt buffer (20 nM), and low-salt buffer were added to the flow cell. Filament movement was initiated by adding motility buffer (2 mM ATP, 0.1 mg/ml glucose oxidase, 23 µg/ml catalase, 2.5 mg/ml glucose in low-salt buffer).

The flow-cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70, Olympus America Inc. NY) and the fluorescent-labelled actin filaments were visualized through an x60 objective (NA 0.7) with illumination from a 100 W mercury lamp. The temperature of the flow-cell was thermostatically controlled at 25 °C (Bionomic Controller, BC-100, 20/20 Technology, USA) by a thermometer probe (HH21, Omega Engineering Inc.,
USA) placed 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 Inc., USA) and recorded on VCR tape (10-12).

Based on observations from 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. 2001; 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. Using an image analysis package (OPTIMAS 6.0, Optimas Corp., USA), a filament was tracked from the centre of mass, and the speed was calculated from 20 frames at an acquisition rate of 1 frame/sec. The average speed and standard deviation of the 10 filaments were calculated. Since the standard deviation in this group of filaments was small (between 10-15% of the mean), the average speed was taken as representative of the muscle fiber (10-12).

**Incubations and motility speed analyses**

Pre-incubation data were obtained from motility speed measurements on myosin extracted from a single fiber as described above. To acquire post-incubation values, the extracted myosin was incubated with: 1) 6 mM glucose (Sigma >99.5%, HPLC grade, without β-mercaptoethanol) for 30 minutes and 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 minutes, 2) low-salt buffer (with β-mercaptoethanol) for 50 minutes, or 3) 10 mM GSH (without β-mercaptoethanol) for 50 minutes. Further, to obtain post-recordings for a dose-response relationship, 30-minute incubations of myosin with 1, 3, and 6 mM glucose (without β-mercaptoethanol) were performed. Motility speed was analysed before and after the respective incubations.

Post-incubation data were obtained from the following method: Two segments (2 mm each) of a fiber were used to obtain the pre- and post-incubation values, i.e., myosin isolated from the first fiber segment was assayed to obtain pre-incubation data and myosin from the second segment was incubated with selected solutions to obtain post-incubation data. In previous experiments we have shown that there is no significant difference in motility speed when myosin is extracted from two adjacent segments of the same fiber (11). In addition, we have shown the same effects on motility speed in response to glucose exposure when (1) the same segment of the fiber was used for both pre- and post-incubation experiments, as when (2) two separate segments of the same fiber was used for pre- and post-incubation experiments, respectively (30). However, on occasion, removal of the labelled actin from the pre-incubation preparation proved difficult and hence interfered with post-incubation measurements when the first method, (using the same segment for both pre- and post-incubation 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 pre-incubation preparation (30).

**Separation and identification of myofibrillar protein isoforms**

Myosin heavy chain (MyHC) isoforms were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from a separate part of the single fiber segments analysed in the *in vitro* motility assay. The total acrylamide and bis concentrations were 4% (w/v) in the stacking gel and 7% in the running gel, while the gel matrix included 30% glycerol. Sample loads were equivalent to 0.1 mm of the fiber segment. The separating gels (160 x 180 x 0.75 mm) were silver-stained (see (9, 20, 21)).

**Statistics**

Means and standard deviations of means (SD) of data collected were calculated from individual values by standard procedures. A one-way ANOVA and two-tailed paired t-test were used for comparison. Differences were considered significant at p<0.05.
**Results**

**Myosin heavy chain (MyHC) isoform expression**

All the Soleus fibers included in this study expressed 100% of the β/slow (type I) MyHC isoform based on protein separations using very sensitive silver stained 7% SDS-PAGE.

**Dose-response relationship**

In order 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 minutes. A graded and significant reduction in motility speed from pre-incubation values was observed in all 3 concentrations, thus confirming a dose-response relationship (Fig. 1). After the 1 mM glucose incubation, motility speed decreased (p<0.05) 10% from pre- (1.02 ± 0.07 µm/s, n=3) to post-incubation values (0.93 ± 0.05 µm/s, n=3). After the 3 mM glucose incubation, motility speed decreased (p<0.05) 34% from pre- (1.22 ± 0.26 µm/s, n=3) to post-incubation values of (0.81 ± 0.15 µm/s, n=3). Finally after the 6 mM glucose incubation, motility decreased (p<0.001) by more than 90% from pre- (1.12 ± 0.04 µm/s, n=3) to post-incubation values (0.10 ± 0.07 µm/s, n=3).

**Effects of glucose and GSH incubations on actin motility speed**

In all preparations, incubations for 30 minutes with 6 mM glucose reduced motility speed (p<0.001) by more than 90%. Subsequent incubation with 10 mM GSH for 20 minutes restored motility speed (0.98 ± 0.06 µm/s, n=3) to almost pre-incubation levels (1.12 ± 0.06 µm/s, n=3, Fig. 2). Post-glucose low salt buffer incubations did not, on the other hand, increase motility speed (0.03 ± 0.05 µm/s, n=3) to pre-incubation values (1.13 ± 0.04 µm/s, n=3). Further, a 16 to 20% decrease (p< 0.001) in motility speed was observed in response to a 50-minute 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 an unspecific degradation of the motor protein.

There was a greater and more consistent reduction in motility speed in response to glucose incubation in the present study than in our previous report (30). This was due to the more consistent amount of myosin extracted from each fiber (Ramamurthy et al., 2001, 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 post-glucose 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

Bi-directional 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 post-glucose incubation recordings. After the 30-minute-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 a bi-directional motility pattern. This is in accordance with previous observations after 30 minutes of incubation with 6 mM glucose (30). Depiction of deviations from linearity of post-glucose-incubation and restoration of linearity of subsequent GSH incubation is demonstrated in figure 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 minutes 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 bi-directional movement of actin filaments were affected by the glucose incubation. After 30 minutes of incubation of myosin with glucose, motility speed dropped significantly by ~80% in both fast (type IIxb and IIb) and slow (type I) myosin isoforms, and bi-directionality 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 minutes of 6 mM glucose exposure. However, incubation with 10 mM GSH for 20 minutes restored motility to almost pre-incubation values, whereas post-glucose 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 the 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 co-substrate 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 to 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 ageing process. However, there are to our knowledge no studies focussing on the role of GSH in relation to glycation of intracellular proteins. The only study published so far has demonstrated an increased glycation of extra cellular proteins in response to a GSH deficiency (13). Further, advanced glycation end products have been known to alter the GSH redox state in human neuroblastoma cells, generally toward more oxidising conditions (7).
It is well known that GSH is an important antioxidant. In addition to the 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 hemithioacetals, 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 ageing rodent skeletal muscle (31, 32). During aging, GSH and total glutathione content is known to increase in skeletal muscles of rats (23). There is also an observed concomitant increase in the oxidative stress in skeletal muscles during aging (see (28)) as measured by the malondialdehyde levels in healthy active elderly women. Based on these observations, it can be surmised that the increase in GSH content could primarily be targeted towards combating the increased oxidative stress, and it could potentially also play a role in the reversal of early glycation products in skeletal muscle. However, in spite of the increase in GSH content during the aging process, glycation of myofibrillar proteins are found to increase with age. Deuther-Conrad et al., (2001) report that AGES cause a dose-dependent and long-term increase in GSSG in vitro. This could, in part, explain the decrease in efficacy of GSH in controlling the formation of AGES in aged tissue.

Non-enzymatic glycation has been implicated in the pathophysiology of diabetes and ageing (32, 35, 37). The present results suggest that GSH, in addition to its antioxidant function, could play an important role in preventing 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.
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Figure legends

**Figure 1.**  **Dose response curve for 30 minutes incubation.** Motility speed in response to 30-minute incubations with 1, 3, or 6 mM glucose. The graph is based on pre- and post-incubation measurements from three different fiber preparations at the 1, 3 and 6mM glucose incubations. * and *** denote p<0.05 and p<0.001, respectively.

**Figure 2.**  **GSH restores post-glucose motility speed.** Effects of (1) a 6mM glucose 30-minute glucose incubation followed by a 20-minute 10 mM GSH incubation (open bars) or low salt buffer (grey bars), (2) a 50 minute low salt buffer incubation (hatched bars), and (3) a 50 minute 10 mM GSH (black bars) incubation.

**Figure 3.**  **Qualitative changes in actin motility pattern.** Depiction of actin filament motion pattern on type I myosin showing decreasing directionality with increasing duration of 6 mM glucose incubation, and the reversal in directionality after incubation with 10 mM GSH for 20 minutes.
References


FIGURE 2

The figure shows a bar graph representing the change in motility speed (%) over different incubation times (min). The y-axis represents the change in motility speed (%) ranging from 0 to 120, and the x-axis represents the incubation time in minutes ranging from 0 to 80. The bars indicate the mean change in motility speed with error bars representing the standard deviation.
APPENDIX C

PAPER III: Detection of aging-related increase in Advanced Glycation End products in fast- and slow- twitch skeletal muscles in the rat

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MANUSCRIPT IN PREPARATION
Introduction

Post-translational modifications such as non-enzymatic glycation have been shown to affect protein function (Shaw and Crabbe, 1994; Watanabe et al., 1992; Wu et al., 1996). Glycation is a chemical reaction of reducing sugars with primary amino groups, i.e., aldehyde groups of free unbound sugars react preferentially with free amino groups of proteins to reversibly form Schiff base adducts. These structures may undergo further Amadori rearrangements and free radical mediated oxidation to finally generate irreversible advanced glycation end products (AGEs) (Avigad et al., 1996; Lal et al., 1996).

Aging of skeletal muscle is characterized by profound changes in the ultrastructural and functional properties of the muscle fiber. Aging-related changes in the contractile properties at the motor unit (Edstrom and Larsson, 1987; Larsson and Ansved, 1995), cellular (Larsson and Edstrom, 1986; Edstrom and Larsson 1987; Larsson et al., 1997) and the molecular level (Hook and Larsson, 2000) are well documented. Myosin is a major structural component of skeletal muscle, and it is considered to be the molecular motor that converts free energy derived from its hydrolysis of ATP into mechanical work. Due to the immense importance of myosin in muscle contraction, any modification of the motor protein has great consequence on the activity of the individual. Hence, glycation of myosin may impact the contractile properties of skeletal muscle. Actin is another myofibrillar protein known to be affected by glycation in vitro (Kuleva and Kovalenko 1997).

The maximum velocity of unloaded shortening ($V_0$) is an important parameter of skeletal muscle since muscles develop their maximum power at approximately one-third of $V_0$ (Rome et al., 1990). $V_0$ of a muscle is proportional to the myosin ATPase activity (Barany, 1967). Studies have reported a ~50% decline in $V_0$ in rat and human type I fibers (Degens et al., 1998; Larsson et al., 1997; Yu et al., 1998). However, the decline in motility speed at the molecular level is less dramatic than at the cellular level (Hook et al., 1999). In human fibers expressing IIA myosin from old subjects, Larsson et al., (1997) showed an ~ 30% decline in $V_0$ as compared to the young which was not paralleled at the molecular level i.e., absence of an aging related slowing of motility speed in the in vitro motility assay in fibers expressing type Ila myosin (Hook et al., 1999). This difference is reflects an aging related change at the cellular level in the structural or thin filament associated proteins.

Proteins undergo a number of post-translational modifications during ageing which may affect enzymatic activity; stability and digestibility (see Mooradian and Wong, 1991). To date, a large number of projects that have studied the effects of glycation on protein structure and function have focused on extra-cellular proteins, such as collagens, lens crystallin, hemoglobin, myelin and albumin perhaps reflecting the availability, reactivity, and natural abundance of the proteins. However, there have been no studies documenting modifications of non-myofibrillar proteins with respect to aging in the muscle fibers. Consequently, there is a need to understand the contribution of myofibrillar and non-myofibrillar protein modifications to the slowing of skeletal muscle during the aging process. We hypothesized that 1) AGEs increase in the skeletal muscle fiber irrespective of fiber type with aging, 2) there is an aging-related accumulation of sarcolemmal proteins with aging primarily in the fast fibers.
Materials and Methods

Animals and tissue preparation: The experiments were carried out on male and female Wistar rats. The rats were obtained from the breeder (Mollegaard Breeding & Research Centre, Denmark) at the age of two months. All animals were housed in the barrier facility at the Karolinska hospital at room temperature on a 12:12 h light and dark cycle and fed ad libitum. The animals were sacrificed and tissues were harvested at five different ages, i.e., 3-6 months, 11-12 months, 16-19 months, 20-24 months and 27-30 months. The animals were anaesthetized with fentanyl-fluanisone (0.2-0.3 ml/kg im) followed by pentobarbital sodium (30 mg/kg ip). The skin over the lower part of the limbs was removed, and the soleus and the extensor digitorum longus (EDL) muscles were gently dissected free from the surrounding tissue and clamped at approximately the in situ length. The muscle was subsequently weighed, frozen in isopentane chilled with liquid nitrogen, and stored at -80°C pending use. Following the removal of muscles, the animals were sacrificed by excising the heart. The use of animal material in this study was approved by the ethical committees at the Karolinska Hospital, Stockholm, Sweden, and the Pennsylvania State University, USA.

Immunofluorescence technique: Soleus and EDL muscle cross-sections (8 µm) were mounted on cover glasses and allowed to air-dry for 30 minutes. Sections from the different age-groups within a gender were placed on the same glass and treated under identical conditions. All steps were performed at room temperature unless otherwise specified. Sections were fixed in acetone at -20°C followed by a pre-incubation in 3% BSA in Phosphate Buffered Saline (PBS) to prevent non-specific binding. A chamber was created encircling all the sections on each slide using a hydrophobic marker to ensure uniform exposure of antibodies to all the sections within the chamber. The cross-sections were processed for immunocytohistochemistry with the following antibodies: anti-AGE RNAse reactive with AGEs (Verbeke et al., 1997), A4.951 reactive with the β/slow (type I) myosin heavy chain (MyHC) isoform (Schiaffino et al., 1989), and A4.74 reactive with the Ila MyHC isoform (Schiaffino et al. 1989). Double labeling was performed by incubating the sections with the primary IgG antibodies. All sections were stained with the anti-AGE RNAse polyclonal antibody (1:400) and the slow-twitch soleus with the anti-fast (A4.74) (1:10) and the fast-twitch EDL with the anti-slow (A4.951) (1:3) MyHC monoclonal antibodies for 30 minutes each. This was followed by 30- minute incubations with the secondary antibodies labeled with a fluorophore (Alexa fluor 568 (1:50), anti-rabbit IgG and Alexa fluor 488 (1:200), anti-mouse IgG) to detect the primary antibodies. Slides were washed 2 times with PBS between incubations. After a final wash with PBS, sections were mounted using mowiol as mounting medium with DABCO as an anti-fade agent.

Confocal microscopy: Sections were scanned at a 60x lens magnification and 1 zoom factor using a confocal laser scanning microscope (Olympus, FV200 attached to an Olympus IX70 microscope). A Krypton laser was used to visualize Alexa fluor 568 and an Argon laser for Alexa fluor 488. An XY scan was obtained in a plane close to the top surface of the section, away from the cover-slip as determined by an initial XZ scan. Fluorescent intensities of the scans were measured using the Fluoview software (v. 2.0.32, Olympus America). The fluorescent intensities were measured for the whole muscle fiber, i.e., the sarcolemma and in the interior of the muscle fiber, and the interior of the muscle fiber, i.e., excluding the sarcomemal region,
separately. The fluorescent intensities typically differed significantly between the sarcolemma and the cytoplasm of the muscle fiber. Therefore, the staining intensities of these regions are treated separately. The sarcolemmal intensity was calculated as the difference between the whole fiber cross-section and the cytoplasm intensities. The intracellular fluorescent intensities were normalized to the cross sectional area of the fibers. One to two regions of the muscle section were scanned to yield the average intensity of the section. Samples that showed pathology were not included in the analysis. Female sol and EDL samples of age group 16-19 months and EDL of 11-12 months were not included. Intensities are presented in arbitrary units.

**SDS-PAGE and immuno-blot:** Myosin heavy chain (MyHC) isoforms were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from five cross-sections (8µm) of each muscle cross-section. The acrylamide concentrations in the stacking and running gels were 3.5% and 12% (w/v), respectively, and the gel matrix included 10% glycerol. A constant current (16 mA per gel) was used and the gels were run for 5 h at 10°C (see Larsson et al. 1995). The separating gels (160 x 180 x 0.75 mm) were silver-stained (Giulian et al. 1983).

Equal volumes from each sample of ~5 cross sections (8 µm thickness) were loaded in each lane. Pre-stained markers were used as positive controls to ensure successful transfer. In separate unstained gels for immunoblotting analyses, proteins were transferred electrophoretically to a nitrocellulose membrane as described by Towbin et al. (1979). Immobilization of the proteins on the nitrocellulose membrane was confirmed using the Blot-Fast stain kit (Bio-Rad). The blots were first incubated with the anti-AGE RNAse primary antibody. The primary antibody was visualised with a secondary antibody (goat anti-mouse IgG) followed by reaction with alkaline phosphatase – streptavidin and final color development with NBT/BCIP. The color produced due to this reaction was used to detect the presence of glycation in a qualitative test.

**Statistics:** Means and standard deviations of means (SD) were calculated from individual values by standard procedures. A one-way ANOVA was used for comparisons between groups. Two-way ANOVA was used to test for gender and aging differences. Differences were considered significant at p<0.05.
RESULTS

Animals: Significant gender-related differences were observed in body and muscle weights, i.e., males were heavier and had larger muscle masses than the females irrespective of age (p<0.001; Table 1). An aging-related increase (p<0.001) in body weight was observed in the males but not in the females, but the increase in body weight was not paralleled by a significant change in the weight of the fast-twitch EDL or the slow-twitch soleus muscles (Table 1).

Electrophoresis and immuno-blot analysis: The electrophoretic separation of proteins from the muscle cross sections was performed in a 12% SDS PAGE gel (Fig. 1a). Fig 1b shows the bands that reacted positively to the antibody from samples of the different age groups. Myosin as well as two other bands of protein with sizes of ~57 and 57.5 kDa, showed glycation in all the age groups of both soleus and EDL muscle samples.

Measurement of muscle protein glycation: An aging-related increase in the amount of cytoplasmic Advanced Glycation Endproducts was observed in both soleus (fig 2) and EDL (fig 3). In the slow-twitch soleus, an aging-related increase (p<0.001) in the amount of glycated cytoplasmic proteins were observed in both male and female rats (fig 4). In the oldest age-group (27-30 months), the amount of glycated cytoplasmic proteins was slightly higher (p<0.05) in the females (4867 ± 400) than in the males (4222 ± 158). In the fast-twitch EDL, an aging-related increase (p<0.01) in glycated cytoplasmic proteins was observed in the males, but not in the females (fig 5) and the fluorescent signal was significantly lower (p<0.01) females (3190 ± 486) than in the males (4406 ± 632).

There were no significant differences in the sarcolemmal intensities between males and females in soleus or EDL. Sarcolemmal intensity was not significantly different in soleus among the different age groups (fig 6) whereas in the fast twitch EDL fibers a significant (p<0.05) difference was found in males (fig 7). Double labeling of sections to identify type IIA in soleus and type I in EDL muscle sections was used to test for muscle fiber type specific differences in the formation of AGE in the different age-groups and genders. Fig (8 a) shows type IIA fibers in soleus labeled with A4.74 primary antibody and Alexa fluor 488 secondary antibody. Fig (9 a) shows type I fibers in EDL labeled with A4.74 primary antibody and Alexa fluor 488 secondary antibody. There were no significant differences in fluorescence intensities of Alexa fluor 568 between type IIA (labeled with A4.74) and unlabelled type I fibers in soleus (fig 8 b) and between type I (labeled with A4.951) and unlabelled fibers in EDL (fig 9 b).
DISCUSSION

The major findings from the study are as follows 1) there is a progressive increase in AGEs in the intracellular as well as the sarcolemmal regions of the muscle fiber 2) myosin along with 2 other proteins of ~57kD, is glycated irrespective of age 3) myosin is glycated irrespective of fiber type. In the course of glycation of many proteins, sugars react preferentially with ε-amino groups of lysines and myosin especially contains high amount of lysine residues, often arranged in clusters or neighbouring histidine, an arrangement which may facilitate amino group reactivity (Hunt and Wolff, 1991; Bai et al., 1989). Glycation is known to alter myofibrillar protein function during aging (Syrovy and Hodny, 1992; Brownlee 1995).

In the present study, we have used an AGE specific antibody, raised against an epitope which is formed after incubation of glucose with RNAse (Verbeke et al., 1997). Tissue AGEs which form in vivo appear to contain a common immunological epitope which cross reacts with AGEs prepared in vitro, supporting the concept that immunologically similar AGE structures are formed upon incubation of sugars with different proteins (Horiiuchi et al., 1991). Makita et al. (1991) have shown AGE modified BSA by glucose, glucose 6-phosphate, and fructose react to the anti-AGE-RNAse antibody in a similar fashion.

The abundance of content and decrease in synthesis and turnover rate (Balagopal et al., 1997) of muscle proteins renders them susceptible to post-translational modifications such as glycation. Using the anti AGE-RNAse antibody, a progressive and significant increase in AGEs across the different age groups was observed irrespective of fiber type and sex. In the different age groups, the fluorescent intensities of AGEs appear to be distributed evenly in the cross section of the muscle fibers. This implies an even spread of glycated proteins within the muscle fiber. Functional changes in the muscle fiber with aging have been observed predominantly in fibers expressing type I MyHC isoform (Degens et al., 1998; Larsson et al., 1997; Yu et al., 1998). However, the presence of AGEs does not appear to differ between fiber types. Type I and Ila fibers appear to be equally modified as reflected by the fluorescent intensities observed.

Western blot data showed that myosin is persistently glycated in all the age groups. Although thin filament proteins (Kuleva and Kovalenko, 1997) and sarcoplasmic reticulum (SR) associated proteins (Williams et al., 1998) are equally susceptible to post-translational modifications, we did not observe positive reactions of the AGE antibody with actin. Another interesting observation in this study is the progressive increase in glycation at the sarcolemmal region. Glycation of sarcolemmal proteins is not a well explored field of study, since most studies involving glycation of skeletal muscle proteins have focused on myofibrillar proteins (Syrovy and Hodny, 1993; Kuleva and Kovalenko, 1997; Ramamurthy et al., 2001). However, two other bands of ~57 kD showed glycation in all age groups, which could potentially belong to the class of sarcolemmal or SR associated proteins. The identity of these proteins is to be established.

The sarcolemma of the muscle fibers in a cross section is inseparably attached to the endomysium. This layer of connective tissue is primarily composed of collagen fibers, an important structural component of the muscle tissue. Glycation of collagen has been well studied with respect to hyperglycemia (Fujimori, 1989; Howard et al., 1996; Turk et al., 1999) and aging (Tsuru et al. 2002). Collagen is a long-lived protein and highly susceptible to modification by glycation. Crosslinking of collagen fibers by glycation causes reduced elasticity or increased
stiffness (Kuzuya et al., 2001; Verzijl et al., 2002; Sell et al., 2002). Glycation inhibitors have been shown to restore collagen function (Sell et al., 2002).

The changes in $V_0$ observed at the cellular level (Degens et al., 1998; Larsson et al., 1997; Yu et al., 1998) in skeletal muscle fibers unparalleled at the molecular level (Hook et al., 1999), could potentially involve modifications of thin filament proteins and/or the endomysium. Muscle membrane foldings have been observed in a subpopulation of freeze dried fibers expressing the fast myosin isoform from old men (Larsson et al., 1997). Although the selective expression of this morphological change in the specific population cannot be explained, the potential of collagen crosslinking due to glycation causing these foldings, eventually contributing to a lower $V_0$ is high.

We believe that glycation of myosin along with other muscle proteins contribute to the aging related decline in muscle function. The increase in the amount of glycated proteins, including myosin in muscle fibers reflects an aging related change. In conclusion, glycation of muscle proteins plays an important role in affecting muscle function in the aging process.
Table 1: Body and muscle weights of male and female rats of different age groups. Values are means ± SD.

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td></td>
<td>BW (gms)</td>
<td>Sol (gms)</td>
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<tr>
<td>3-6</td>
<td>375 ± 005</td>
<td>0.18 ± 0.02</td>
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<tr>
<td>11-12</td>
<td>625 ± 115</td>
<td>0.18 ± 0.00</td>
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<tr>
<td>16-19</td>
<td>592 ± 070</td>
<td>0.20 ± 0.05</td>
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<tr>
<td>20-25</td>
<td>604 ± 091</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>27-30</td>
<td>608 ± 056</td>
<td>0.19 ± 0.03</td>
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<tr>
<td>p-value</td>
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a) Electrophoretic separation of proteins from ~5 cross sections (8 µm) of EDL muscle of male rats on 12% gels. *lanes 1 and 4*, 3-6 months; *lanes 2 and 5*, 20-25 months; *lanes 3 and 6*, 27-30 months, lane 7, MW marker. b) Photograph of immunoblot of AGE modified myofibrillar proteins of EDL muscle cross-sections. The membrane was treated with anti-AGE-RNase primary antibody to identify AGE modified proteins. *lane 1*, MW marker; *lane 2*, 3-6 months; *lane 3*, 20-25 months.
Confocal images of soleus muscle cross-sections of a) 3-6 month- b) 11-12 month- c) 16-19 month- d) 20-25 month- e) 27-30 month old male rats showing an increase in AGEs in the fibers. Alexa fluor 568 was used to obtain the fluorescent signal.
Confocal images of EDL muscle cross-sections of a) 3-6 month- b) 11-12 month- c) 16-19 month- d) 20-25 month- e) 27-30 month old male rats showing an increase in AGEs in the fibers. Alexa fluor 568 was used to obtain the fluorescent signal.
Cytoplasmic fluorescent intensities in soleus muscle of a) male and b) female rats across age groups. There was a significant difference \( p<0.001 \) among age groups in both males and females and between genders \( p<0.05 \) in the oldest age-group. Values are mean ± SD.
Cytoplasmic fluorescent intensities in EDL muscle of a) male and b) female rats across age groups. There was a significant difference (p<0.001) among age groups in both males and females, and between males and females (p<0.01) in the oldest age-group. Values are mean ± SD.
Sarcolemmal fluorescent intensities in soleus muscle of a) male and b) female rats across age groups. Sarcolemmal fluorescent intensities were obtained by the difference in the intensities of cytoplasm and the whole cell. There was no significant difference among age groups in either sex. Values are mean ± SD.
Sarcolemmal fluorescent intensities in EDL muscle of a) male and b) female rats across age groups. Sarcolemmal fluorescent intensities were obtained by the difference in the intensities of cytoplasm and the whole cell. Males showed a significant difference (0.05) but not the females. Values are mean ± SD.
Double labeling of soleus muscle cross-section of 20-25 month old male rat. a) the type IIa MyHC isoform was labeled with the A4.74 primary antibody and Alexa fluor 488 secondary antibody b) labeled with anti AGE-RNAse primary antibody and Alexa fluor 568 secondary antibody. There was no significant difference in the intensity of Alexa fluor 568 between fiber types.
Double labeling of EDL muscle cross-section of 20-25 month old male rat. a) the type I MyHC isoform was labeled with the A4.951 primary antibody and Alexa fluor 488 secondary antibody b) labeled with anti AGE-RNAse primary antibody and Alexa fluor 568 secondary antibody. There was no significant difference in the intensity of Alexa fluor 568 between fiber types.
Vita

Bhagavathi Ramamurthy is a native of Bangalore, the garden city of India. The first born of Uma and Ramamurthy, she did all her schooling in Bangalore. With an interest in life science, she discontinued professional training in engineering to study Biology. With MS and MPhil degrees in Zoology, she arrived in the US to pursue a doctorate in Physiology. In her spare time, she is interested in sports, painting, reading, and social service.