Non-Enzymatic Glycosylation of Myosin:
Effects of Diabetes and Ageing

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Abstract. The influence of diabetes mellitus, streptozotocin-induced diabetes and ageing on the non-enzymatic glycosylation of myosin from cardiac and skeletal muscles was investigated. In cardiac muscle, and to a lesser extent also in skeletal muscles of the rat, non-enzymatic glycosylation of myosin increases with the age, as measured in 6-, 12- and 29-month-old animals. Skeletal muscle myosin from diabetic humans and also that from diabetic rat cardiac muscle are more glycosylated when compared with control myosin preparations. Ca²⁺-ATPase activity of myosin is lower in muscles of diabetic individuals as compared with control muscles.

Key words: Non-enzymatic glycosylation (Glycation) — Myosin — Diabetes mellitus — Streptozotocin-diabetes — Ageing — Muscle

Introduction

Non-enzymatic glycosylation (NEG) of plasma and tissue proteins has been demonstrated in healthy subjects and especially in diabetic patients (Mayer and Friedman 1983; Kennedy and Baynes 1984; Reiser 1990). NEG has been studied mainly in long-lived proteins like collagens, lens crystallins and basic nerve myelin. Less attention has been devoted to NEG of intracellular proteins. The latters have been shown to be glycated in vivo and in vitro; the question remains open how the cellular functions of intracellular proteins are altered by glycation.

Myosin is a basic constituent of muscle and is also present in non-muscle cells. The properties of glycated myosin were briefly studied by Yudkin et al. (1989) who found slightly more glycated myosin in the hearts of diabetic subjects, and by Brown et al. (1990) who demonstrated a decrease of actin activated ATPase of myosin after glycation with simple sugars in vitro. The purpose of this study is to report on the effects of diabetes and ageing on myosin glycation and its ATPase activity. In order to evaluate this further, myosin isolated from rat cardiac muscle
was glycate also in vitro in the presence of ribose.

**Materials and Methods**

*Patients and animals*

Myosin was isolated by autopsy from skeletal muscles of healthy subjects (65 yr) which died by an accident, a patient with diabetes mellitus (70 yr), duration of diabetes 8 yr, glycaemia 18 mmol/l, cardiac and skeletal (thigh) muscles of control rats and the same muscles of rats injected with streptozotocin (50 mg/kg b.w., i.v., killed 9 months later, glycaemia 21 mmol/l).

*Myosin preparation*

Myosin was isolated from muscles as described earlier (Syrový and Gutmann 1977). Ca$^{2+}$-ATPase activity was measured at 27°C in a medium containing 0.05 mol/l Tris-HCl, pH

**Table 1. Myosin glycation levels**

<table>
<thead>
<tr>
<th>Source of myosin</th>
<th>n</th>
<th>Glycation expressed as amount of fructosamine (nmol/g myosin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human skeletal muscle, control</td>
<td>3</td>
<td>20.0 ± 1.5</td>
</tr>
<tr>
<td>Human skeletal muscle, diabetic</td>
<td>3</td>
<td>25.5 ± 1.6</td>
</tr>
<tr>
<td>Rat skeletal muscle, control (6-month-old)</td>
<td>3</td>
<td>18.0 ± 2.0</td>
</tr>
<tr>
<td>Rat skeletal muscle, control (12-month-old)</td>
<td>3</td>
<td>19.1 ± 1.7</td>
</tr>
<tr>
<td>Rat skeletal muscle, control (29-month-old)</td>
<td>3</td>
<td>21.6 ± 2.0</td>
</tr>
<tr>
<td>Rat cardiac muscle, control (6-month-old)</td>
<td>3</td>
<td>21.7 ± 1.9</td>
</tr>
<tr>
<td>Rat cardiac muscle, control (12-month-old)</td>
<td>3</td>
<td>23.0 ± 1.5</td>
</tr>
<tr>
<td>Rat cardiac muscle, control (29-month-old)</td>
<td>3</td>
<td>27.7 ± 1.2</td>
</tr>
<tr>
<td>Rat skeletal muscle, diabetic (12-month-old)</td>
<td>3</td>
<td>21.0 ± 0.9</td>
</tr>
<tr>
<td>Rat cardiac muscle, diabetic (12-month-old)</td>
<td>3</td>
<td>36.1 ± 2.1</td>
</tr>
</tbody>
</table>

Means ± S.E.M are shown. *p < 0.05; significant difference between muscle from control and diabetic individuals.
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7.5; 0.025 mol/l KCl, 5 mmol/l ATP, 10 mmol/l CaCl₂ and 0.25 mg protein/ml. Inorganic phosphate liberated in the reactions was measured by the method of Fiske and Subbarow (1925).

Glycation assay procedure

To determine the amount of sugar covalently bound to protein, the fructosamine assay was used (Johnson et al. 1982) with 1-deoxy-1-morpholinofructose as standard.

In vitro glycation

To glycate myosin (5 mg/ml) it was incubated with 0.5 mol/l KCl, 10 mmol/l HEPES, pH 7.2, 0.1 mol/l D-ribose, 3 mmol/l sodium azide, 2 mmol/l 2-mercaptoethanol, and 2 mmol/l phenylmethylsulfonylfluoride at 21°C for the indicated time. Prior to the glycation assay, the samples were dialyzed extensively against 0.5 mol/l KCl, 10 mmol/l HEPES, pH 7.2 for 48 h to remove free sugar.

Results

The extent of myosin NEG was studied in skeletal and cardiac muscles of control 6-month, 12-month and 29-month-old rats, in cardiac and skeletal muscles of diabetic rats, and human skeletal muscles from normal and diabetic subjects (Table 1). The results show that myosin of control muscles of both species studied is slightly glycated. There is no significant difference in the glycation of myosin between skeletal and cardiac muscles of adult control rats (6-months old), but glycation increases with the age of the animals, especially in cardiac myosin.

Figure 1. Ketoamine formation in cardiac myosin in the presence of 100 mmol/l ribose (12-month-old rat, • = control; ○ = diabetic). Each point represents mean of two determinations.
Myosin from muscles of diabetic individuals is more glycated than that of control individuals. This is true for skeletal muscle from diabetic humans and also for cardiac muscle from diabetic rats, the difference in this respect between control skeletal muscle and skeletal muscle from diabetic rats being insignificant.

It seemed of interest to ascertain the extent of myosin glycation in vivo. The results are presented in Fig. 1. Incubation of myosin with 0.1 mol/l ribose resulted in non-enzymatic attachment of the sugar to the cardiac myosin from both the control and the diabetic rats. NEG increased the amount of ketoamine bound to cardiac myosin of control and diabetic rats 2.6 and 1.9 times, respectively.

The potential significance of myosin glycation on its cellular function was assessed by comparing Ca\(^{2+}\)-ATPase activity of myosins from muscles of control and diabetic subjects. Myosin Ca\(^{2+}\)-ATPase activity was decreased in all muscles of diabetic subjects as compared with the controls. The most marked difference in Ca\(^{2+}\)-ATPase activity of cardiac myosin (32%) was observed between control and diabetic rats (Table 2).

Table 2. Myosin Ca\(^{2+}\)-ATPase activity

<table>
<thead>
<tr>
<th>Source of myosin</th>
<th>n</th>
<th>Ca(^{2+})-ATPase activity ((\mu)moles P(_{i})/mg protein.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human skeletal muscle, control</td>
<td>3</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>Human skeletal muscle, diabetic</td>
<td>3</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>Rat skeletal muscle, control</td>
<td>3</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>(12-month-old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat cardiac muscle, control</td>
<td>3</td>
<td>0.81 ± 0.05(a)</td>
</tr>
<tr>
<td>(12-month-old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat skeletal muscle, diabetic</td>
<td>3</td>
<td>0.81 ± 0.09</td>
</tr>
<tr>
<td>(12-month-old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat cardiac muscle, diabetic</td>
<td>3</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>(12-month-old)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means ± S.E.M are shown. \(a p < 0.05\); significant difference between muscles from control and diabetic individuals.

Discussion

NEG of proteins has been demonstrated in numerous studies to occur in vivo, and even in vitro, similar products are formed. Less clear is, however, which proteins are glycated in vivo; how NEG alters cellular function of the individual proteins;
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and how this type of protein modification is influenced under various physiological and pathophysiological conditions

The present study confirms and extends the results showing that cardiac muscle in diabetic subjects contains slightly higher levels of glycated myosin as compared with control subjects (Yudkin et al. 1989). In streptozotocin-induced diabetes in rats, the levels of myosin glycation (measured as ketoamine formation) are higher in cardiac muscle than in skeletal muscles. This may be due to the fact that the entry of glucose into the skeletal muscle cells depends on the presence of insulin, while being insulin independent in the rat cardiac muscle. Other factors, such as differences in glycation rate, or myosin turnover, may be involved. Also, one must be careful in comparing results obtained with muscles from different species, and data concerning diabetes mellitus and streptozotocin-induced diabetes.

It is evident from Table 1 that the levels of myosin glycation in cardiac muscle of control rats increase with the age. Myosin is a protein with a relatively long half-life (29 days, see Obinata et al. 1981), and this half-life is probably not the same for various myosins. The turnover rate of muscle proteins decreases with ageing (Millward et al. 1975). Our results cannot be used to explain why myosin glycation increases with the age. Conditions which favour or hinder glycation of proteins may change with ageing. Moreover, mean glucose levels rise with the increasing age (Harding et al. 1989). Oxidative reactions and oxidative stress may also be important for the higher glycation of proteins in older individuals (Wolff et al. 1991).

Ca\(^{2+}\)-ATPase activities of all myosins from muscles of diabetic subjects were lower when compared with those of the corresponding myosins from control animals. The most pronounced reduction in myosin Ca\(^{2+}\)-ATPase activity was found for cardiac muscle myosin from diabetic rats (by 32%). Our results suggest that for myosin from muscles of diabetic subjects (which is more glycated than that from control subjects), ATPase activity may be reduced as a result of glycation of lysine or other aminoacids (e.g., arginine) located near or in the active moiety of the enzyme protein, or due to another still unknown mechanism. The amino acid composition of the enzyme active site is not known, nevertheless, 6-15 lysine residues/100 amino acid residues have been reported in different parts of the myosin molecule from various muscles (Leger et al. 1975, McNally et al. 1989, Tong and Elzinga 1990).

In diabetic rats, the maximum velocity of ventricular contraction is decreased and this change is accompanied by a reduced Ca\(^{2+}\)-myosin ATPase activity (Fein et al. 1951). The diabetes-associated decrease in myosin ATPase activity is due to a change in myosin isoenzyme composition, V\(_3\) myosin becomes the predominant isoenzyme (Dillman 1980). It is thus possible that in diabetes, both the glycation of myosin and the change in myosin isoenzyme distribution contribute to the decrease of myosin ATPase activity.
ATPase activity of myosin from cardiac and skeletal muscles of control rats of various ages was not studied, since it has already been known that developmental changes of myosin ATPase are due to redistribution of isomyosins with different enzymatic activities (Syrový 1987) and thus it would be difficult to distinguish between the influence of glycation and the effects of ageing itself.

Decreased actin-activated myosin ATPase activity upon nonenzymatic glycosylation has recently been demonstrated in vitro (Brown et al. 1990). In their study, myosin was incubated with the sugar for 48 hours, at 37°C and pH 8.0, i.e. under conditions when the enzyme is not stable. It would be desirable to perform a detailed study on the effect of myosin glycation in vitro on its enzymatic activity, provided appropriate conditions can be defined.

In summary, this study has shown that the glycation of myosin isolated from cardiac and/or skeletal muscles of humans and rats with diabetes mellitus, from subjects with experimentally induced diabetes and during ageing increase. Future studies are to be designed to compare glycation of isomyosins and to localize the glycation site of myosin.

Acknowledgements. We wish to thank Dr. P. Hník for his help in preparing the manuscript, and Mrs. J. Běmová for skillful technical assistance; Prof. O. Benešová kindly provided muscles from old rats, and Dr. L. Dvořáková supplied samples of human muscles.

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Final version accepted February 25, 1992