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Glycation of the Muscle-Specific Enolase by Reactive Carbonyls: Effect of Temperature and the Protection Role of Carnosine, Pirydoxamine and Phosphatidylserine

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Abstract Reactive carbonyls such as 4-hydroxy-2-nonenal (4-HNE), trans-2-nonenal (T2 N), acrolein (ACR) can react readily with nucleophilic protein sites forming of advanced glycation end-products (AGE). In this study, the human and pig muscle-specific enolase was used as a protein model for in vitro modification by 4-HNE, T2 N and ACR. While the human enolase interaction with reactive α-oxoaldehyde methylglyoxal (MOG) was demonstrated previously, the effect of 4-HNE, T2 N and ACR has not been identified yet. Altering in catalytic function were observed after the enzyme incubation with these active compounds for 1–24 h at 25, 37 and 45 °C. The inhibition degree of enolase activity occurred in following order: 4-HNE > ACR > MOG > T2 N and inactivation of pig muscle-specific enolase was more effective relatively to human enzyme. The efficiency of AGE formation depends on time and incubation temperature with glycating agent. More amounts of insoluble AGE were formed at 45 °C. We found that pirydoxamine and natural dipeptide carnosine counteracted AGE formation and protected enolase against the total loss of catalytic activity. Moreover, we demonstrated for the first time that phosphatidylserine may significantly protect enolase against decrease of catalytic activity in spite of AGE production.

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Abbreviations

ACR	Acrolein
4-HNE	4-hydroxy-2-nonenal
MOG	Methylglyoxal
T2 N	Trans-2-nonenal
PBS	Phosphate buffered saline pH 7.4

1 Introduction

Intracellular oxidative stress is a source of reactive low-molecular-weight carbonyl compounds such as: α-oxoaldehydes, 2-alkenals and their derivatives [13, 17, 45, 50]. These compounds are formed in cells in many ways e.g., by glucose autooxidation, from triose phosphate metabolites of glycolytic pathway and from amino acids conversion or lipids peroxidation. The glycation process known as the Maillard reaction, is also a source of these compounds [23, 45, 47, 48]. The carbonyl stress in living cells is closely correlated with the rise of level of reactive α-oxo-aldehydes, including 3-deoxyglukoson, glyoxal, methylglyoxal (MOG) and increase of α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal (4-HNE), trans-2-nonenal (T2 N), acrolein (ACR) (Fig. 1). Reactive carbonyl species (RCS), as strong electrophiles, are able to modify nucleophilic centers of cellular macromolecules. RCS can react with the ε-amino group of lysine, the aminoguanidine group of arginine, the imidazole moiety of histidine or sulfhydryl group of cysteine [27, 37, 57]. Proteins undergo such modifications during Maillard reaction e.g., glycation process. In the early stages of Maillard reaction, RCS

create Schiff base, ketoamine and tiohemiacetals, respectively. Irreversible and multiple changes lead to advanced glycation end-products (AGE) formation [2, 49]. Stable chemical AGE structures are formed in most proteins of long half-life time, such as structural proteins. In the consequence of collagen or laminin modification, the large and insoluble AGE deposits have been cumulated in extracellular matrix [28, 32]. AGE are abundantly present in the circulatory system because of albumin glycation or other serum proteins with high turnover numbers [29, 55, 56]. Moreover, the AGE products are also produced inside cells [8, 42, 44]. Glycating agents which belong to the α-oxoaldehydes are 20 000 times more reactive than glucose in proteins modifications [48]. Under physiological conditions, the intracellular level of reactive carbonyls is kept in low range by efficiency of the catabolic systems. The methylglyoxal level in human plasma oscillates from 100 to 600 nM [18]. The cytosolic MOG concentration in normal mammalian cells is less than 10 μM [7] and the level of 4-hydroxynonenal (4-HNE) does not exceed of 1 μM [13]. However, in the oxidative stress conditions, the accumulation of 4-HNE in membranes reaches 5–10 mM value [51]. The MOG and 4-HNE as small compounds diffuse easily from formation sites and they can pass among subcellular compartments, thus further propagating glycation damage and consequently they have potential of negative influence on many cell processes [10, 51].

Enolase was chosen as a model protein to investigate glycation process because of its abundance and multifunctional properties in human, mammals and lower organisms [33, 41]. This protein is commonly known as 2-phospho-D-glycerate hydrolyase (EC 4.2.1.11) in glycolysis/glyconeogenesis pathway but takes part in many nonenzymatic processes. The crucial role of the basic amino acids residues e.g. histidine, lysine and arginine in enolase catalytic function was described in details in many previous reports [12, 33, 38]. Arginine also plays an important role in the association of enolase monomers to

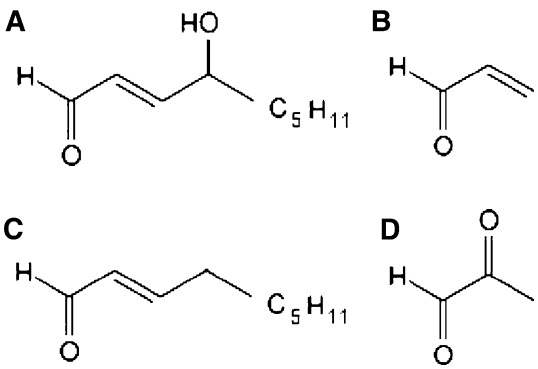

Fig. 1 Chemical structures of reactive carbonyls: 4-hydroxy-2-nonenal (**a**), acrolein (**b**), trans-2-nonenal (**c**), methylglyoxal (**d**)

the dimeric structure of the native enzyme [33]. The enolase of vertebrates occurs as hetero- or homodimer formed from α, β or γ type of subunits. Enolase α is ubiquitous isoenzyme in adipose tissue, liver, kidney, spleen, pancreas and lung; γ isoenzyme is located in neuroendocrine tissues and neurons, whereas enolase β is muscle-specific. The ββ dimer expresses over 90% of total enolase activity in skeletal muscles in human and mammals [30, 33]. Previous investigations proved that this protein is more susceptible for lysine and arginine glycation than other enzymes of glycolysis pathway. It is suggested that enolase plays role of reactive carbonyls scavenger what probably protects other intracellular proteins against glycation process [11]. In our recent study on in vitro glycation of human β-enolase by methylglyoxal, the kinetic data indicate that MOG decreased the enolase specific activity, although the enzyme maintained partially the ability to conversion of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) in spite of AGE formation [35].

The aim of this report is the characterization of muscle-specific enolase glycation products formed upon protein incubation with 4-hydroxy-2-nonenal, trans-2-nonenal and acrolein as more reactive aldehydes than methylglyoxal. The inhibitory effect of these glycating agents on human and pig muscle-specific enolase is determined and analysis of AGE obtained at various conditions is performed. Also the protecting role of some intracellular compounds e.g., carnosine, pirydoxamine and phosphatidylserine on muscle-specific enolase during glycation process is determined.

2 Materials and Methods

2.1 Materials

All chemical reagents were of the highest purity and were purchased from Sigma–Aldrich and Merck. Methylglyoxal (40% aqueous solution) was distilled before use. Human striated muscles for enolase isolation were from histologically normal tissue obtained as the postoperative material from the Department of Vascular, General, and Transplantation Surgery of Wroclaw Medical University, and approval of the study was granted by the Bioethics Commission of Wroclaw Medical University.

2.2 Methods

2.2.1 Purification of Enolase From Pig and Human Skeletal Muscle, Enolase Activity Assay and Protein Determination

Enolase was prepared from the frozen skeletal muscle. The purification of enzyme was performed from crude extract

by heat treatment, precipitation of proteins in 60–80% saturated ammonium sulfate and using three steps of exchange-ionic liquid chromatography [4]. Enolase concentration was determined spectrophotometrically at 280 nm using the absorption coefficient $A^{0.1\%} = 0.89$. This value was determined for rabbit muscle enolase (1 mg mL^{-1}) [4].

Enolase catalytic activity was assayed spectrophotometrically at the room temperature. The enolase substrate e.g., 2-phosphoglycerate acid (2-PGA) was added at 1 mM concentration to 50 mM imidazole–HCl buffer, pH 6.8, containing 3 mM $MgSO_4$ and 0.4 M KCl. The conversion of the 2-PGA to phosphoenolpyruvate (PEP) was followed at 240 nm for 1 min. One unit of enolase activity is defined as the amount of protein which catalyses the synthesis of 1 μM PEP within 1 min under these conditions. The molar absorption coefficient was taken as 1,520 cm^2 mol^{-1} to calculate from ΔA_{240}/min amount of micrograms of product formed from 2-PGA. The specific activity was expressed as units per milligram of enzyme [4]. Pure enolase was stored in stabilizing buffer (7.5 mM imidazole–HCl buffer, pH 6.8, containing 2.5 mM $MgSO_4$, 50 mM NaCl, and 50% glycerol) and stored at 4 °C.

2.2.2 *Prediction of Glycation Sites of Human and Pig Muscle-Specific Enolases*

Total amount of nucleophilic side residues in the monomer of human and pig muscle-specific enolase was obtained from ExPASy Proteomics Server (P13929 and Q3ZC09 UniProtKB/Swiss-Prot accession number for human and pig enolase, respectively). The prediction of lysine residues as glycation sites at enolase monomer was carried out by NetGlycate 1.0 server which predicts access of ε-amino groups of lysines for glycation in mammalian proteins (ExPASy Proteomics Server). Structural 3D graphics of enolase dimer with solvent accessible of basic residues was evaluated using molecule visualization MarvinSpace 5.3.6 tool (ChemAxon Kft., Hungary). The sequence of human alpha-enolase (PDB entry 3B97) and their three-dimensional model, reported by Kang [19] was used for our modeling.

2.2.3 *Glycation of Enolase From Human and Pig Skeletal Muscle*

All experiments were performed in phosphate buffered saline (PBS) pH 7.4, acc. to Pietkiewicz et al. [35]. The control samples for each glycation experiment contained native enolase only in PBS. Chemical modification was carried out in the dark, using 15 μM of enolase and an excess of reactive aldehyde. The high molar level of glycation factor was used for maximal saturation of accessible reactive amino acid residues in the dimeric enolase molecule. The effect of temperature on the amount of AGE products was assayed on the pig muscle-specific enolase glycated by 4-HNE, ACR and MOG at 25, 37 and 45 °C. The modification of enolase by acrolein was performed in 280 and 840 molar excess of ACR to protein, to compare the effect of glycation factor concentration on the yield of AGE production. The incubation time was varied and had been depended on total loss of catalytic activity of enolase. Modification was stopped by addition of 4 mg mL^{-1} lysine solution to bind the excess of modifier. Aliquots of reaction mixture containing 5–20 μg of enolase were collected at various time intervals to measure of enzyme specific activity and for SDS/PAGE analysis. Carnosine and piridoxamine were added as inhibitors of glycation in equimolar ratios to glycation factor in the reaction mixture. The protective role of phosphatidylserine (PS) was examined at concentration 100 times higher than that for enolase. The experiment was performed at 37 °C with 300 molar excess of MOG to protein after previous 30 min of preincubation with PS. In order to determine the ability of free amino group present in PS molecule to react with MOG as a reactive carbonyl compound, additional experiments were performed. Samples of 6.75 μM enolase were modified with 1 mM MOG at 37 °C after previous of enzyme preincubation with PS at concentration varied from 0.25 to 4 mM. After 5 h of incubation, the enzyme catalytic activity was spectrophotometrically assayed as well as level of uncoupled MOG was determined at 284 nm acc. to Lo et al. [26].

2.2.4 *SDS/PAGE Analysis of Glycation Products*

The enolase-derived AGE were tested with the SDS–PAGE electrophoresis according to Laemmli [22]. Samples of the reaction mixtures were incubated for 5 min at 95 °C in 0.068 M Tris–HCl buffer pH 6.8 containing 2% (w/v) SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.025% bromophenol blue. Electrophoresis was performed in 0.0025 M Tris–HCl, 0.192 M glycine and 0.1% SDS electrode buffer pH 8.3, in the 4% stacking and 12% resolving gel (MiniPROTEAN® System, Bio-Rad). The products were visualized by staining with Coomassie Brilliant Blue R-250. Gel images were analyzed using Aida Image Analyzer software (Raytest) in order to perform of densitogram assays.

3 Results and Discussion

Nonenzymatic modification of proteins, nucleic acids and lipids by reducing sugars and short chain aldehydes changes properties of modified molecules in irreversible mode.

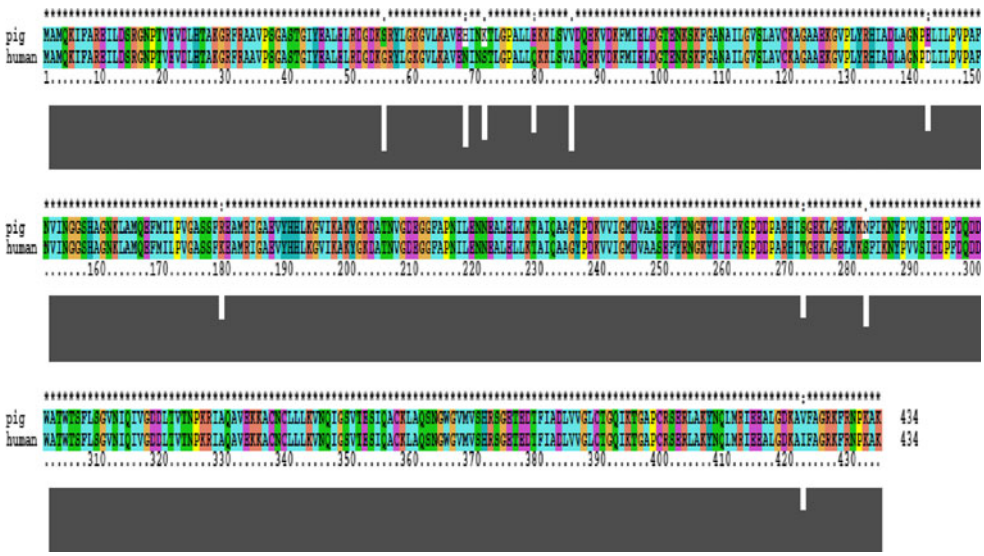

Fig. 2 The amino acid sequence alignment of pig and human β-enolases performed by ClustalX 2.0.12 application

Glycation occurs during organism aging and also it is a main reason of some pathological changes concerned with hyperglycemia [2, 36, 53]. Enolase as the most common cell protein may undergo glycation more frequently than other glycolytic enzymes [11]. This enzyme belongs to high evolutionarily conservative proteins [33, 34]. Over 83% of amino acid sequence identity and 91% of sequence homology observed between human isoenzymes was reported [33, 34]. We have established the high similarity in sequences of human and pig β-enolases (Fig. 2). Probably, the solvent accessibility of amino acids residues susceptible for glycation is also very similar in both enolases.

3.1 Prediction of Nucleophilic Residues of Muscle Enolase Available to Glycation by Reactive Carbonyls

The amount of total nucleophilic sites in the monomer of human and pig enolases are summarized in Table 1. The crystal structure of human and pig muscle-specific enolases are not revealed yet. However, on the basis of high identity of human β- and α-enolases, we can adopt 3D model of alpha-isoenzyme to visualize the solvent exposed nucleophilic centers: 14 of lysine and 16 of arginine residues, from total 38 and 17, respectively, existed in α-monomer (Fig. 3). According to web proteomic tool, probably 14 from 38 of lysine residues presented in pig (about 37% of total content) and 13 from 37 (about 35% of total) in human β-enolase monomer can be the dominant potential glycation sites. There are K5, K64, K71, K80, K92, K126, K162, K202, K228, K256, K335, K358, K394, K420 for pig muscle-specific and K5, K64, K80, K92, K126, K162, K202, K228, K256, K335, K358, K394, K420 for human muscle-specific enzyme. Over 88% of total arginine residues amount in human β-enolase—R8, R14, R31, R49, R131, R182, R252, R268, R326, R399, R402, R411, R425 and R428 were established as available to solvent and these same with additional of R178 were found for pig enzyme. After association of monomers to dimer, part of nucleophilic centers will be hidden in the interface area. Moreover, all of histidine and cysteine residues were accessed for glycating agents (data not included in Fig. 3). Considering the obtained structural data and glycation prediction analysis, the distinct molar excesses of reactive aldehydes were applied to modify the available amino-acid residues in the native dimeric molecules of human and pig β-enolases. Mostly, the glycation experiments were done with a 300:1 molar ratio of RCS to protein. Similar conditions were previously reported for in vitro modifications of bovine serum albumin, human Cu,Zn-superoxide dismutase, 3-phosphoglyceric aldehyde dehydrogenase and lactate dehydrogenase [20, 24, 31, 56].

Table 1 The total amount of basic amino acids and cysteine residues in the monomer of human and pig β-enolases in comparison with of their contents in human α-enolase

Enolase monomer	Amino acid content			
	K	R	H	C
Pig β-enolase	38	18	8	6
Human β-enolase	37	17	7	6
Human α-enolase	38	17	6	6

3.2 The Human Muscle-Specific Enolase is Less Susceptible to Inactivation by Reactive Aldehydes than Pig Enzyme

The inhibition of β-enolase catalytic activity by reactive aldehydes during glycation process was established in

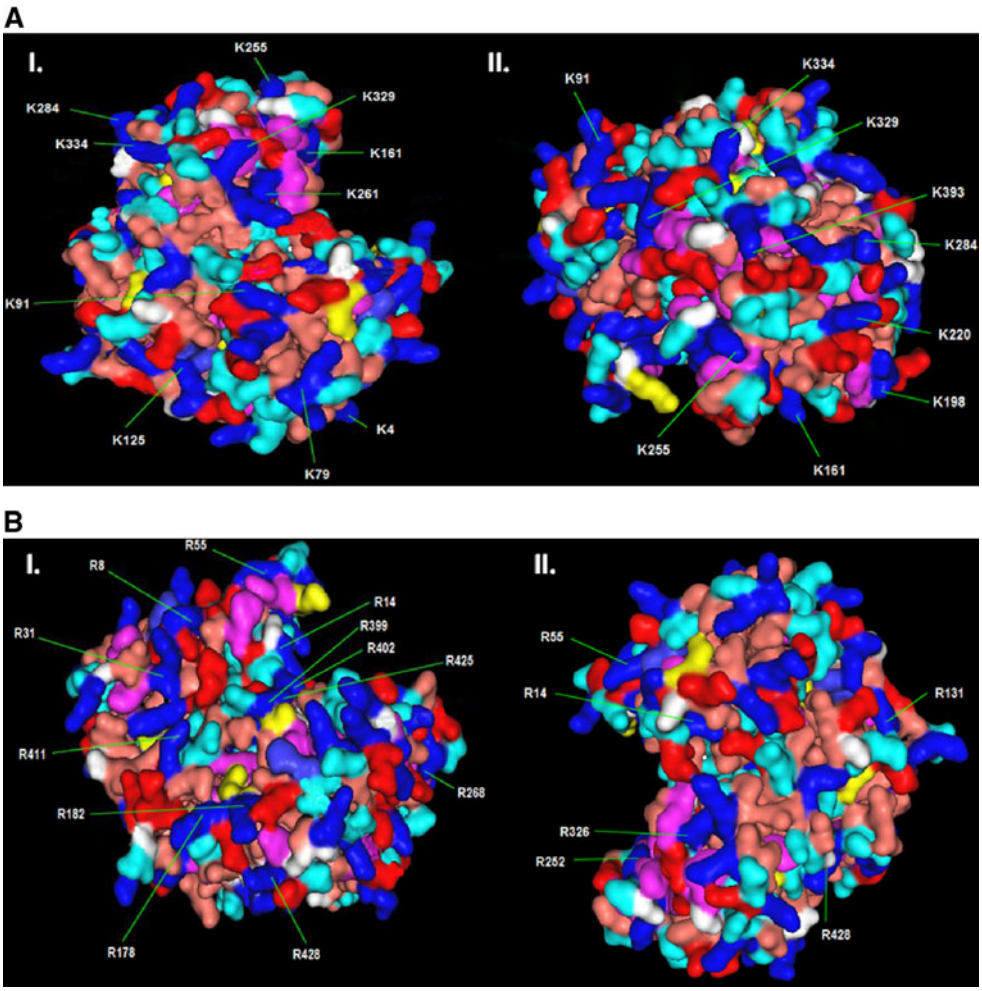

Fig. 3 The presentation of human α-enolase dimer with lysine (**a**) and arginine (**b**) residues susceptible to glycation. A view of the molecule in front (I) and rotated 225° for lysine and 135° for arginine visualization (II)

following order: 4-HNE > ACR > MOG > T2 N (Fig. 4). These differences resulted from various electrophilicity of reactive carbonyls [27]. Since 4-hydroxy-2-alkenals are more reactive with nucleophilic compounds compared with reactivity of α,β-unsaturated aldehydes lacking the hydroxyl group [9, 50], the 4-HNE was stronger enolase inhibitor than T2 N and ACR. The 300 molar excess of 4-HNE caused the decrease of about 95% of human β-enolase catalytic activity after incubation in 37 °C at 8 h (Fig. 4a). Similar effect was observed for glycated pig β-enolase, however, the pig enzyme was more sensitive to inhibition by reactive aldehydes (Fig. 4b). We can classify MOG and T2 N to gentle inhibitors of muscle-specific enolase. The SDS/PAGE analysis of human muscle-specific enolase derivatives after modification with 4-HNE, T2 N, ACR and MOG revealed various AGE components of the reaction mixtures (Fig. 4c). The most heterogenic AGE adducts, with Mw 110, 200, 250 and 280 kDa and spread indistinct band with proteins Mw over 280 kDa, as well as broad band of adducts with lower molecular weight with Mw 48–52 kDa were observed in the sample after incubation with 4-HNE for 8 h at 37 °C (Fig. 4c, lane 3). Glycation with MOG, T2 N and ACR produced the AGE with Mw < 250 kDa (Fig. 4c, lanes 4–6). Part of AGE in the samples 3–6 (Fig. 4a, b) did not enter into the resolving 12% gel as insoluble fraction. Significant amount of native enolase in the presence of T2 N not undergo glycation (Fig. 4c, lane 5), which corresponded with weak inhibitory effect of T2 N in kinetic experiments observed.

Differences in composition of AGE mixtures observed in Fig. 4c were most likely due to different mechanisms of RCS-protein interaction. Protein modification by MOG follows by Schiff base formation with lysine and arginine residues and further rearrangement of intermediates in the next steps of Maillard process [48, 49]. On the contrary, the α,β-unsaturated carbonyls are capable to interact as a bifunctional electrophiles with protein amino groups either by their carbonyl group or unsaturated bond, to form Schiff base or Michael adducts, respectively [5]. Michael adducts are created as thioether derivatives with sulfhydryl group of cysteines in the first stage of 4-HNE modification. On the other hand the 4-HNE also binds nitrogen of histidines imidazol moiety and ε-amino group of lysines [27, 40, 50]. Usually, these reaction products are converted into cyclic

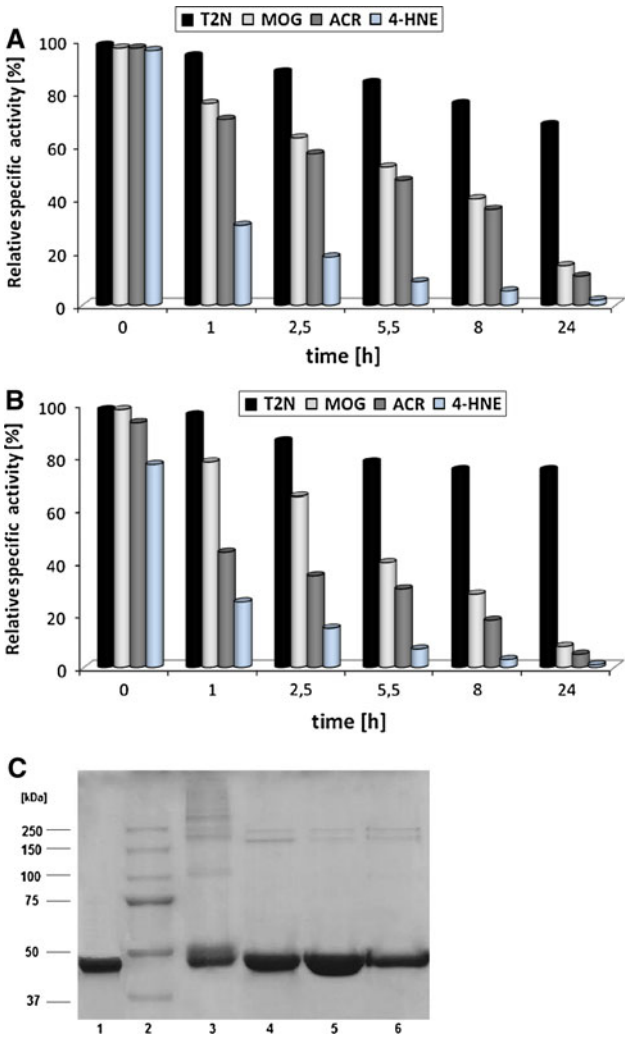

Fig. 4 Influence of reactive aldehydes on the catalytic activity of human (**a**) and pig (**b**) muscle-specific enolase after incubation at temperature 37 °C (with 300:1 molar ratio of glycating agent to protein); the experiment was done once and fitted using sigmaplot software. SDS/PAGE pattern (**c**) of AGE obtained after the human enolase glycation at 8 h–native enolase (1), protein Mw standards (2), modification with 4-HNE (3), MOG (4), T2 N (5) and ACR(6)

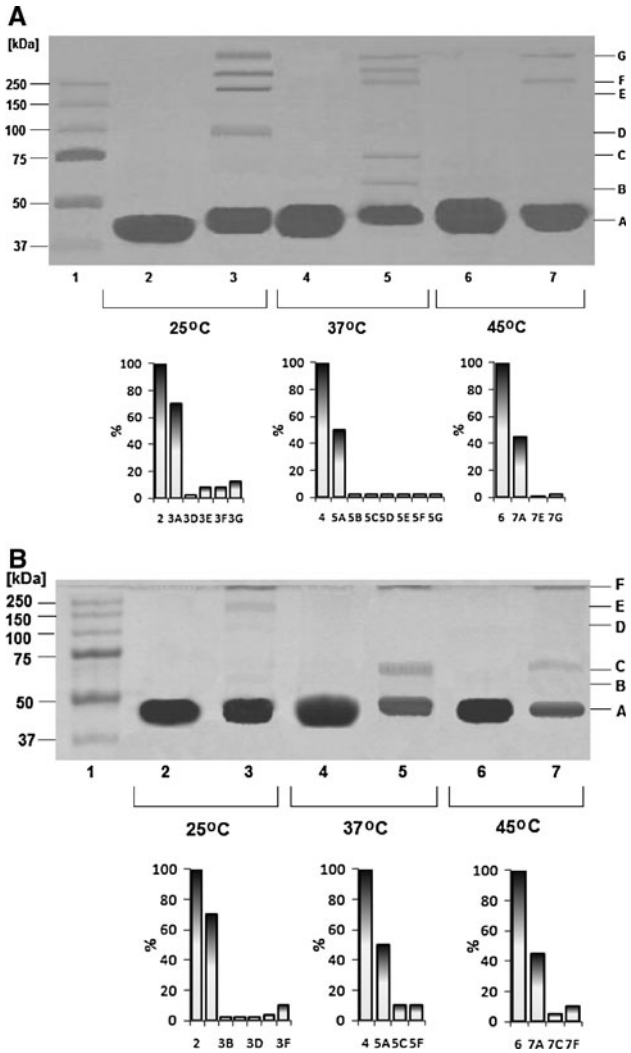

Fig. 5 The SDS/PAGE analysis of AGE obtained after pig β-enolase modification with MOG (**a**) and 4-HNE (**b**) after 24 h modification with 300:1 molar excess of glycation factor to protein. Band A: lanes 2,4,6–control samples with native enolase; lanes 3,5,7–samples with modified enolase; bands B-G: AGE adducts. Panel below the SDS/PAGE presents the densitometric analysis of protein bands

mono- and bishemiacetal derivatives. Additionally, the oxidation steps and intermolecular cyclizations lead to intra- and intermolecular cross-links creation due to the presence of Michael adducts with free ε-amino lysine groups [6, 40, 50]. Recent studies show that acrolein reactivity with nucleophilic protein regions is much higher than that of glyoxal or methylglyoxal. Acrolein generates Michael products in similar to 4-HNE mechanism or may form the Schiff base with N-terminal amino group or ε-amino group of lysines [9, 51, 52]. Acrolein reacts with thiol groups at much higher rate than with other nucleophilic residues, so the main fraction of glycation products of proteins is made up by Michael adducts [5]. Second carbonyl of ACR molecule is untouched during addition, so the resulting compounds can take a part in further reactions leading to the formation of intra- and intermolecular cross-links [21, 43].

Due to the fact, that modification of human and pig muscle enolases by T2 N caused insignificant inhibition of catalytic activity and less amount of AGE formation compared with 4-HNE effect, therefore only more active modifiers e.g., 4-HNE, MOG and ACR were used in the next experiments. The increase of incubation temperature from 25 to 45 °C accelerated the loss of enzyme catalytic activity (data not shown) and facilitated the formation of AGE-enolase derivatives (Fig. 5). The AGE composition after glycation of pig β-enolase with methylglyoxal for 24 h (Fig. 5a) was different from those observed after

incubation with 4-hydroxynonenal (Fig. 5b). Some high-molecular-weight AGE as insoluble fraction was produced, which did not enter to the 12% resolving gel. Therefore, pig muscle-specific enolase glycation with MOG at 45 °C permitted to detect only two bands of adducts in soluble fraction, with Mw over 250 kDa (Fig. 5a, bands F,G on lane 7). More amount of high-molecular-weight AGE aggregates remained in stacking gel during modification with 4-HNE (Fig. 5b, band F) and only enolase-derived adduct with Mw 70 kDa was observed in soluble fraction when incubation temperature increased to 37 or 45 °C (Fig. 5b, band C of lanes 5 and 7, respectively). Control samples with native enolase incubated at the same time and temperatures were shown in lanes 2, 4 and 6 in Fig. 5a, b.

Modification of pig β-enolase by acrolein followed also on temperature- and concentration-depended manner (Fig. 6). It is reasonable that inactivation of enzyme catalytic activity was more effective at higher excess of glycating agent and with elevation of temperature incubation (Fig. 6a, c). Furthermore, the higher excess of acrolein significantly changed the electrophoretic mobility of native enolase (band A in Fig. 6d, lanes 3, 5, 7) and the more reach pattern of high-molecular-weight AGE was performed at molar ratio 840:1 of ACR to protein in comparison with 280:1 relation (Fig. 6b, d).

It is noteworthy that the almost total loss of enzyme catalytic activity had not being observed before 23 h of enolase modification with 280 molar excess of ACR at 45 °C (Fig. 6a). The rise of ACR level up to 840 molar excess resulted decrease of enolase activity. Enolase of reaction mixture achieved about 3% of control sample activity in just after 1.5 h of modification at 45 °C (Fig. 6c). The nonmodified enolase amount in the reaction samples was significantly diminished with increase of temperature (Fig. 6b, d, band A, lanes 3,5,7).

According to densitometric analysis of AGE formation at 45 °C (Fig. 6b graph for 45 °C), the nonglycated fraction of enolase received after incubation with 280:1 molar excess of ACR, remained only 60% amount of control sample (incubated without ACR). These results corresponded with SDS–PAGE profile observed on band A in Fig. 6b, lane 7 vs. 6. When the level of ACR increased to molar ratio 840:1, amount of nonglycated enolase diminished to less than 50% of native enolase in the control sample (Fig. 6d graph for 45 °C). It was demonstrated also in electrophoretic analysis (band A in Fig. 6d, lane 7 vs. 6).

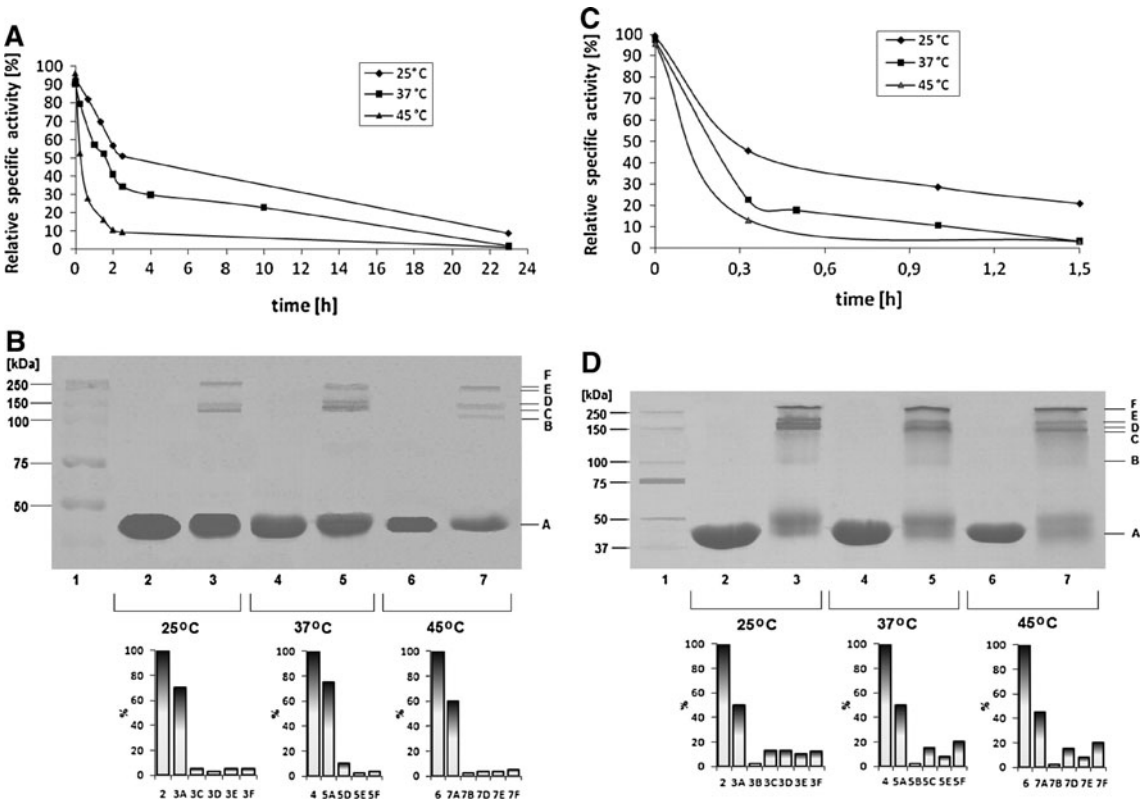

Fig. 6 Modification of pig β-enolase by acrolein: the enolase activity inhibition at (**a**) 280:1 and (**c**) 840:1 molar excess of ACR to protein for 23 h and 1.5 h incubation time, respectively; electrophoretical detection of AGE observed in samples (**a**) and (**c**) are shown in panel b and d, respectively. Band A: lanes 2,4,6–control samples with native enolase; lanes 3,5,7–samples with modified enolase at 25, 37 and 45 °C, respectively; bands B–F: AGE adducts. Graphs below of the each SDS/PAGE pattern presents the densitometric analysis of protein bands obtained in various temperature incubation

Some of these products did not enter to the 12% resolving gel because of their low solubility, similarly to AGE formed by 4-HNE. The diffused of protein band A in Fig. 6d (lane 3, 5 and 7), indicated the creation of lower molecular-weight AGE derivatives with Mw range 48-55 kDa. Similar effect of 4-HNE during glycation of human β-enolase was demonstrated in Fig. 4c, lane 3.

The level of AGE formed in the cells can be limited by glycation inhibitors [39]. A natural dipeptide carnosine (β-alanyl-L-histidine) belongs to this family. The important role of carnosine in peroxide dismutase protection against glycation by 4-HNE was reported previously [25]. This dipeptide is present in cells of skeletal muscle, brain and other human tissues [16]. Particularly the high level of carnosine, in the range of milimolar concentrations was detected in striated muscle [16]. Therefore it can be an effective scavenger of reactive carbonyls and may prevent proteins from the in vivo glycation in muscle tissue. Recent studies show also that pirydoxamine—a natural intermediate of B_6 vitamin metabolism, inhibits effectively protein glycation. Pirydoxamine may binds Amadori products (early glycation products) or interact with reactive carbonyl compounds which appear during carbohydrates oxidation and lipids peroxidation in the cells [1, 54]. It is noteworthy that deficiency of pirydoxamine can intensify the glycation process.

Our kinetic experiments demonstrated that human β-enolase maintains its catalytic activity in almost 70–90% in the presence of both preventing factors during glycation with MOG at 37 °C (Fig. 7a, lanes 2 and 3 vs lane 4). The partial decrease of AGE level in the reaction mixture during human β-enolase glycation by MOG in the presence of carnosine and pirydoxamine was observed (Fig. 7b, lanes 2 and 3 vs lane 4). The enolase-derived adducts with Mw 100 kDa and broad band with Mw over 150 kDa were less intensive for samples preincubated with carnosine or pirydoxamine, compared to those observed for positive controls without both scavengers of MOG. In the absence of protecting agents, insoluble fraction of AGE on the border of stacking and resolving gel of SDS/PAGE analysis contained significant more products.

Glycation of muscle-specific enolase proceeds in interesting way in the presence of phosphatidylserine (PS). The PS has protected human β-enolase against loss of catalytic activity during glycation by MOG (Fig. 8a, lane 2 vs. 1). Although enolase-derived high-molecular-weight AGE were also formed in these conditions, their amount was smaller than that observed in absence of PS, what has been shown in SDS/PAGE analysis (Fig. 8b, lane 4 vs. 5). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are dominant lipid components of inner leaflets of cell plasma membranes. Although PE can be covalently modified by reactive alkenals [3], the PS molecules are not

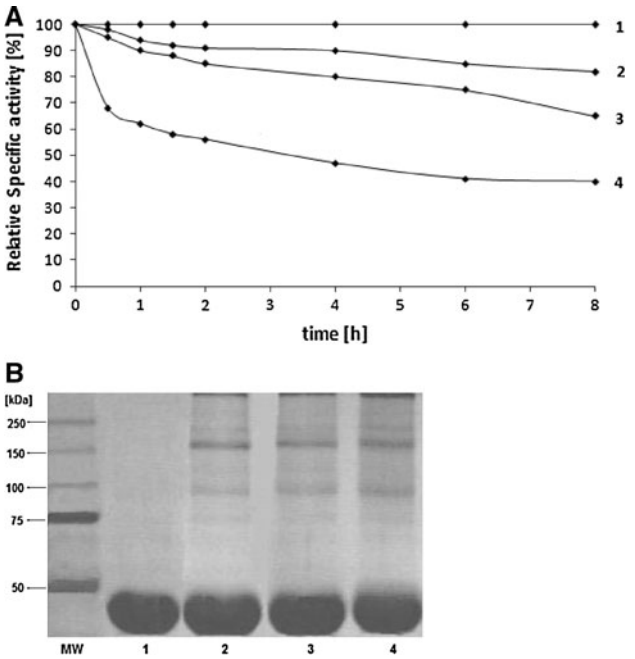

Fig. 7 Glycation of human β-enolase by MOG (1:300 mol/mol) in the presence of pirydoxamine and carnosine: **a** protection against enolase inactivation by MOG–lane 1 nonmodified enolase (native), modification of enzyme by MOG in the presence of carnosine (lane2) and pirydoxamine (lane 3); lane 4–glycation with MOG without scavengers; **b** SDS/PAGE analysis of AGE after 24 h modification: lane 1- nonmodified enolase; glycation in the presence of pirydoxamine (lane 2) and carnosine (lane 3); modification with MOG in the absence of protecting agents (lane 4). The molar amounts of protecting agents were equivalent with MOG

susceptible to modification, possibly due to a low accessibility of the primary amino group [14]. In additional experiment, the ability of free PS to reduce of MOG excess in reaction mixtures has been checked in more gentle conditions of glycation, i.e. with molar ratio 1:150 of enzyme to glycating factor to get distinctive PS influence on enolase activity protection. The modification of 6.75 µM enolase with 1 mM MOG at 37 °C for 5 h resulted in decrease of enzyme activity to 72.5% of control sample (nonmodified enolase) without PS (Fig. 9a). After 15 min preincubation of enolase with 1 mM PS and glycation with 1 mM MOG during 5 h, enzyme maintained 86% of relative catalytic activity. The concentration rise of PS to 4 mM allowed to maintain almost 100% of enolase activity (Fig. 9a). Moreover, as shown in Fig. 9b, the enolase modification with 1 mM MOG after previous the enolase preincubation with PS in concentration ranges from 0.25 to 4 mM caused the increase of uncoupled MOG level in each reaction mixture. It is noteworthy that the presence of some level of uncoupled MOG in sample containing only enzyme and glycating factor in that conditions indicated that the enolase modification with MOG without PS resulted also in the total saturation of all

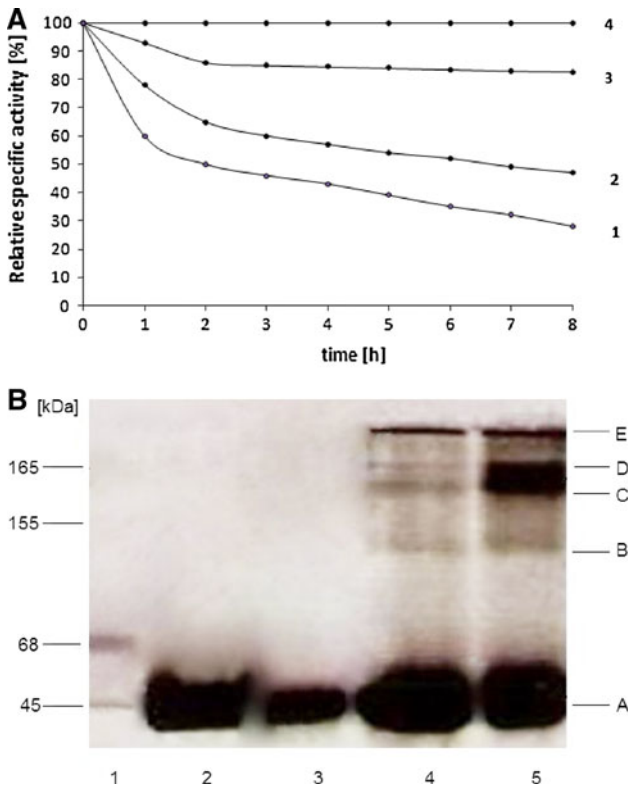

Fig. 8 Effect of phosphatidylserine on modification of human muscle-specific enolase by methylglyoxal (1:300 mol/mol) at 37 °C: **a** kinetic relationship: 1—enolase modified by MOG; 2—enolase exposed to glycation by MOG after 30 min of preincubation with phosphatidylserine; 3—enolase incubated with phosphatidylserine; 4—nonmodified enolase (native); **b** the SDS–PAGE pattern of glycated products: lane 1—protein Mw standards; 2—nonmodified enolase (native); lane 3—native enolase incubated with phosphatidylserine (control sample), lane 4—enolase exposed to glycation with MOG after 30 min preincubation with phosphatidylserine, lane 5—enolase modified with MOG without protecting agent

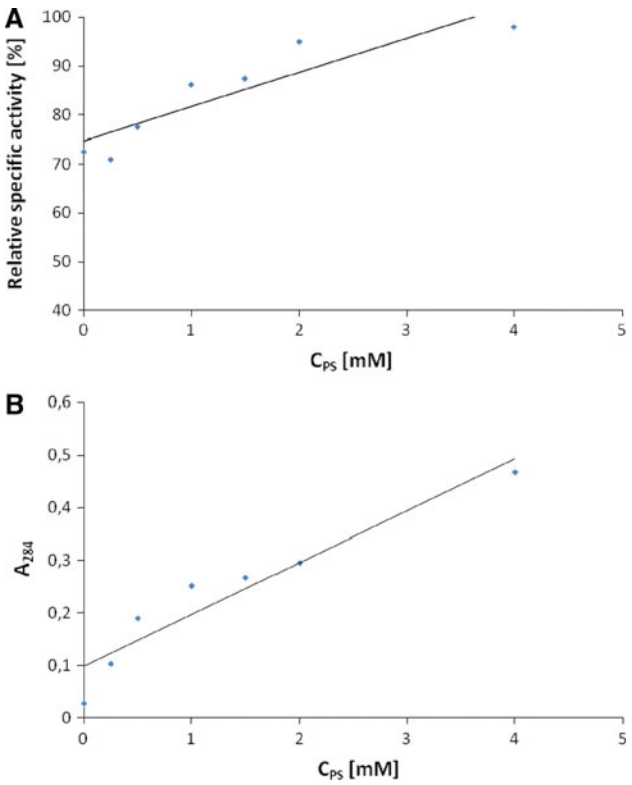

Fig. 9 Effect of phosphatidylserine concentration on modification of 6.75 μM human muscle-specific enolase with 1 mM MOG at 37 °C for 5 h: **a** the influence of PS concentration at the range from 0.25 mM to 4 mM on specific activity of enolase incubated with MOG related to control sample contained a native enzyme; **b** the dependence of uncoupled MOG level on PS concentration in reaction mixture after 5 h enolase modification with MOG; control samples contained only enolase and appreciate concentration of PS

accessible basic residues in dimer of protein. It is in accordance with our prediction by using NetGlycate 1.0 Server (Expasy) described in § 3.1. We suppose that PS does not compete with protein basic residues in glycation process but it is able to disturb the access of glycating factor molecules to these protein sites. Furthermore, the coupling of MOG with amino group of PS does not occur.

Most likely PS has the important influence on the biological properties of proteins by protecting them against modification with glycating agents. The influence of membrane phospholipids on properties of glycolysis pathway enzymes have been described in many reports [15, 46]. However, the interactions between free phospholipids and reactive carbonyls have been not established, yet. The results of our experiments represents novel and essential role of phosphatidylserine in the living cells but the explanation of this phenomenon requires further studies.

4 Conclusions

Modification of basic amino acid side groups by reactive carbonyls permit to enolase catalytic function disorders and caused AGE formation. The high molecular AGE products derived from enolase may lead to in vivo accumulation of insoluble protein deposits in muscle cells and other tissue in aging and hyperglycemic processes. Trans-2-nonenal and methylglyoxal are gently modifiers of human and pig muscle-specific enolase compared to 4-hydroxy-2-nonenal and acrolein. The different composition of AGE resulted from various mechanisms of protein modification by either ACR and 4-HNE or by MOG. Physiological dipeptide carnosine appeared at high molecular level in muscle tissue can play important role for enolase prevention from glycation. Deficient of pirydoxamine may facilitate the muscle enolase modification by reactive dicarbonyls. The ability of phosphatidylserine to muscle enolase protection against inactivation during glycation process suggests the new role of this phospholipid in the muscle cells.

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