

Glutathione is the main endogenous inhibitor of protein glycation

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Abstract. Glycation is the cause of diabetic complications and contributes to the development of other diseases and aging. Numerous exogenous compounds have been tested for their anti-glycating activity. The aim of this study was to answer the question, which endogenous compounds at physiological concentrations can effectively prevent glycation. A set of endogenous compounds has been tested for the ability to protect albumin from glucose-induced glycation *in vitro* at a concentration of 1 mM and in a physiological concentration range. Only glutathione was found to protect significantly against glycation at physiological concentrations. Glutathione depletion increased the rate of hemoglobin glycation in erythrocytes incubated with high glucose concentrations. These results indicate that the level of glutathione is the main determinant of glycation of intracellular proteins.

Key words: Glycation — Advanced glycation end products — Diabetes — Albumin — Glutathione

Introduction

Glycation is one of the most important non-enzymatic post-translational modifications of proteins. This process is initiated by binding of carbonyl groups of monosaccharides or aldehyde metabolic intermediates to free amino groups of proteins to form Schiff bases. Then the Schiff bases are subject to intramolecular rearrangements resulting in formation of early glycation products (Amadori products). Further transformations of Amadori products yield advanced glycation end products (AGEs) (Tessier 2010; Schalkwijk and Miyata 2012). AGEs formation takes place under normal physiological conditions, but is accelerated in hyperglycemia (type 1 and 2 diabetes mellitus) and diseases involving oxidative stress such as cardiovascular diseases (Cao et al. 2014; Semba et al. 2015), chronic obstructive pulmonary disease (Hoonhorst et al. 2014), cystic fibrosis (Sadowska-Bartosz et al. 2014a) or multiple sclerosis (Sadowska-Bartosz et al. 2013).

Glycation induces structural changes of proteins, which are believed to be responsible for diabetic complications and to contribute to the course of other diseases and aging (Simm et al. 2015; Drenth et al. 2016; Ramasamy et al. 2016). In par-

ticular, the accumulation of AGEs plays an important role in the formation of degenerative changes in the lens of the eye, leading to cataracts or vision loss (Kandarakis et al. 2014).

Therefore, prevention of glycation or acceleration of the removal of glycation products can be expected to ameliorate the course diabetes and other diseases and slow down aging, extending both lifespan and healthspan. Various exogenous substances have been tested for their ability to prevent glycation, including aminoguanidine, pyridoxamine, 2,3-diaminophenazine, pyridoxine, penicillamine, benfotiamine, thiazolidine derivatives, edaravone (Schalkwijk and Miyata 2012), gold and silver nanoparticles (Liu et al. 2014; Ashraf et al. 2016) and nitroxides (Sadowska-Bartosz et al. 2015a). Numerous food constituents, especially flavonoids and other polyphenols were found to be efficient inhibitors of glycation *in vitro* and some of them were reported to be active *in vivo* (Sadowska-Bartosz and Bartosz 2015a). However, attenuation of glycation by exogenous compounds is not easy to achieve. Glycation is a non-enzymatic reaction and can be inhibited on a competitive basis. It is hardly possible to achieve *in vivo* a concentration of an exogenous compound, which would compete for reactive carbonyl groups with amino groups of endogenous compounds, present at concentrations of 10–100 mM. Moreover, exogenous compounds may have side effects precluding their safe use *in vivo*. It may be thus of interest to check, which of endogenous compounds can be the most important in the protection of cellular or extracellular proteins against glycation. The aim of

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this study was to answer this question by comparing protection of a model protein (albumin) *in vitro* against glycation by relevant endogenous compounds present at physiological concentrations.

Materials and Methods

Materials

All basic reagents were from Sigma-Aldrich (Poznań, Poland), unless indicated otherwise. Bovine serum albumin (BSA) was purchased from AppliChem GmbH (Darmstadt, Germany). Spectrophotometric and fluorimetric measurements were made in an Infinite200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times.

Sample preparation

BSA was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, at a final concentration of 90 μ M. Glucose (500 mM) was used to induce glycation. Samples with or without the compounds tested at a concentration of 1 mM or at physiologically relevant concentrations were incubated at closed vials with addition of 1 mM sodium azide as a preservative at 37°C for 6 days (Sadowska-Bartosz et al. 2014b). The use of the high glucose concentration allowed for shortening incubation time necessary to obtain a significant level of glycation. The effects of physiological concentrations of the compounds, chosen on the basis of literature data, on BSA glycation were also tested.

Fluorescence measurements

Fluorescence of sample aliquots (200 μ l) was measured in a microplate reader, at wavelengths of 325/440 nm (AGEs), 330/415 nm (dityrosine), 325/434 nm (*N*-formylkynurenine) and 365/480 nm (kynurenine) (Sadowska-Bartosz et al. 2014b). Formation of amyloid beta-sheet structure was measured using thioflavin T. Shortly, 95 μ l of sample aliquots were added with 5 μ l of 640 μ M thioflavin T in 0.1 M sodium phosphate buffer, pH 7.4, and incubated at room temperature for 1 h. Then the fluorescence intensity was measured at wavelengths of 435/485 nm (LeVine et al. 1999).

AGE assay

AGE content was also evaluated by enzyme-linked immunosorbent assay (ELISA) Kit for AGEs (USCN Life Science Inc., Product No. CEB353Ge), according to the protocol of the manufacturer.

Glycation of hemoglobin in erythrocytes

Peripheral blood from one healthy donor (38-year-old woman) was collected using 3% sodium citrate as an anticoagulant. The study was approved by the Bioethics Committee of the University of Łódź (Poland). The blood was centrifuged (2000 \times g, 10 min, 4°C), plasma and leukocyte buffy coat were aspirated and the erythrocyte pellet was washed three times with 3 volumes of phosphate buffered saline (PBS) *per* 1 volume of a suspension. Half of the erythrocyte suspension was incubated on a shaker with 2,4-chlorodinitrobenzene (CDNB) at 37°C for 1 h, and washed thrice to remove excess CDNB; another half was not treated with CDNB. The hydrophobic compound CDNB enters cells *via* diffusion, is conjugated with glutathione intracellularly and the conjugate formed (2,4-dinitrophenyl-S-glutathione) is pumped out by the multidrug resistance protein 1 (MRP1; ABCC1) (Keppler et al. 1998).

Erythrocytes were suspended to a final hematocrit of 10% in PBS containing 5, 50 or 100 mM glucose and 17 μ M ampicillin at 37°C and incubated on a shaker for 24, 48 and 72 h.

Estimation of glutathione content

The erythrocyte content of reduced glutathione (GSH) was estimated with *o*-phthalaldehyde according to Senft et al. (2000). Hemoglobin was estimated according to Drabkin and Austin (1935).

Estimation of hemoglobin glycation

Glycated hemoglobin was assayed with ELISA Kit for Glycated Hemoglobin A1c (USCN Life Science Inc., Product No. CEA190Hu), according to the protocol of the manufacturer.

Statistical analysis

All the experiments were done at least in triplicate. Data were presented as mean values and standard deviations. Statistical analysis of the data was performed using STATISTICA software package (version 10, StatSoft Inc. 2010, Tulsa, OK, USA, www.statsoft.com). Differences between means were analyzed using Student's *t*-test for independent samples and were considered significant or highly significant at ^a $p < 0.001$, ^b $p < 0.01$ or ^c $p < 0.05$.

Results

We have compared the influence of a set of potential glycation inhibitors on the extent of BSA glycation estimated with simple measurements of the fluorescence of glycoxidation products and the formation of amyloid structures, estimated

with thioflavin. Fifteen amino acids or their derivatives and peptides, four organic acids, two polyamines, three B-group vitamins and three nucleotides were used at 1 mM concentration (as in our previous studies) and at physiologically relevant concentrations (Table 1).

Arginine, glycine, tyrosine, serine, carnosine, urea as well as creatine, spermine and spermidine did not decrease glycation at 1 mM concentration and in the physiological concentration range. All the nucleotides tested, both at physiological concentration for blood plasma and at a concentration of 1 mM (approximate physiological intracellular concentration)

did not alter the glycation process. Cysteine inhibited glycation in a concentration-dependent manner; 1 mM cysteine significantly decreased formation of AGEs ($t = 14.6^a$; $k = 4$ in all cases; $^a p < 0.001$), dityrosine ($t = 40.3^a$), N⁷-formylkynurenine ($t = 17.7^a$), kynurenine ($t = 10.4^a$) and amyloid aggregates ($t = 4.49^b$; $^b p < 0.01$). However, physiological concentrations of cysteine had little effect: 25 μM cysteine decreased AGE formation ($t = 4.62^b$), dityrosine content ($t = 6.35^b$) and N⁷-formylkynurenine content ($t = 14.7^b$), without affecting any other glycoxidation parameters. Lysine at a concentration 1 mM decreased formation of AGEs ($t = 6.42^b$), dityrosine

Table 1. Physiological concentrations of endogenous compounds studied

Compound	Physiological concentration	Reference
<i>Amino Acids and Derivatives, Peptides</i>		
L-arginine	80 ± 20 μmol/l (plasma)	Le Boucher et al. 1997
	125 ± 5.5 μmol/l (plasma)	Hanssen et al. 1998
	60–120 μmol/l (serum)	Suschek et al. 2003
	123.3 ± 44.6 μmol/l (plasma)	Alvares et al. 2012
	80–120 μmol/l (plasma)	Morris 2007
	40–120 μmol/l (plasma)	Kingsbury et al. 1998
	87 ± 3 μmol/l (plasma; Swedish population)	Klassen et al. 2001
	69 ± 3 μmol/l (plasma; Guatemalan population)	
137 ± 8 μmol/l (plasma)	Moss et al. 2004	
Carnosine (β-alanyl-L-histidine)	5 mmol/kg (human skeletal muscle)	Baguet 2010
Creatinine	60–130 μmol/l (serum)	Sadowska-Bartosz et al. 2014a
	115.2 ± 48.8 μmol/l (men; serum)	Wang et al. 2001
	94.6 ± 39.3 μmol/l (women; serum)	
Cysteine	52 ± 11 μmol/l (plasma)	Le Boucher et al. 1997
	25.2 ± 1.5 μmol/l (erythrocyte)	Sekharet al. 2011
Glycine	239 ± 40 μmol/l (plasma)	Evins et al. 2000
	100–330 μmol/l (plasma)	Kingsbury et al. 1998
	248 ± 13 μmol/l (plasma; Swedish population)	Klassen et al. 2001
	210 ± 17 μmol/l (plasma; Guatemalan population)	
	514.7 ± 33.1 μmol/l (erythrocyte)	Sekhar et al. 2011
GSH	1.6 ± 0.5 μmol/l (plasma)	Ashfaq et al. 2006
	2.4 ± 1.0 μmol/l (plasma)	Moriarty et al. 2003
	6.75 ± 0.47 μmol/g Hb (erythrocytes)	Sekhar et al. 2011
	8.46 ± 0.175 μmol/g Hb (erythrocytes)	Giustarini et al. 2013
GSSG	0.11 ± 0.05 μmol/l (plasma)	Ashfaq et al. 2006
	0.2 ± 0.2 μmol/l (plasma)	Moriarty et al. 2003
	0.0132 ± 0.004 μmol/gHb (erythrocytes)	Giustarini et al. 2013
Histidine	82 ± 10 μmol/l (plasma)	Le Boucher et al. 1997
	30–150 μmol/l (plasma)	Kingsbury et al. 1998
	87 ± 6 μmol/l (plasma; Swedish population)	Klassen et al. 2001
	87 ± 3 μmol/l (plasma; Guatemalan population)	
Lysine	188 ± 32 μmol/l (plasma))	Le Boucher et al. 1997
	100–300 μmol/l (plasma)	Kingsbury et al. 1998
	195 ± 9 μmol/l (plasma; Swedish population)	Klassen et al. 2001
	150 ± 8 μmol/l (plasma; Guatemalan population)	
Melatonin	0.042 ± 0.03 nmol/l (plasma)	Caglayan et al. 2001

(continued)

Table 1. Continued

Compound	Physiological concentration	Reference
Methionine	25 ± 4 µmol/l (plasma)	Le Boucher et al. 1997
	25 ± 1 µmol/l (plasma; Swedish population)	Klassen et al. 2001
	24 ± 1 µmol/l (plasma; Guatemalan population)	
Serine	114 ± 19 µmol/l (plasma)	Le Boucher et al. 1997
	90–290 µmol/l (plasma)	Kingsbury et al. 1998
	114 ± 4 µmol/l (plasma; Swedish population)	Klassen et al. 2001
	102 ± 5 µmol/l (plasma; Guatemalan population)	
Tryptophan	44 ± 7 µmol/l (plasma)	Widner et al. 1997
	73 ± 14.9 µmol/l (serum)	Kingsbury et al. 1998
	30–80 µmol/l (plasma)	Klassen et al. 2001
	25 ± 1 µmol/l (plasma; Swedish population)	
	24 ± 1 µmol/l (plasma; Guatemalan population)	
Tyrosine	59 ± 12 µmol/l (plasma)	Le Boucher et al. 1997
	35–102 µmol/l (plasma)	Armstrong and Stave 1973
	39–89 µmol/l (plasma)	Grünert et al. 2013
	30–120 µmol/l (plasma)	Kingsbury et al. 1998
	60 ± 4 µmol/l (plasma; Swedish population)	Klassen et al. 2001
	54 ± 2 µmol/l (plasma; Guatemalan population)	
Urea	5300 ± 1080 µmol/l (men; plasma)	Waters et al. 1967
	4800 ± 1200 µmol/l (women; plasma)	Klassen et al. 2001
	6500 ± 2500 (µmol/l) (plasma; Guatemalan population)	
<i>Organic acids</i>		
Oxaloacetic acid	0.34 ± 0.02 µmol/l (blood)	Laplante et al. 1995
Pantothenic acid	1.1–12 µmol/l (blood)	Wittwer et al. 1989
	4.79–11.05 µmol/l (blood)	Cohenour and Calloway 1972
Pyruvic acid	29 ± 23 mol/l (plasma)	Okada et al. 1998
	33 ± 14 µmol/l (erythrocytes)	Travis et al. 1971
Uric acid	380 µmol/l (plasma)	de Oliveira and Burini 2012
	214–494 µmol/l (plasma)	*
	119–375 µmol/l (women; serum)	Hesse et al. 2002
	155–404 µmol/l (men; serum)	
	0.074 ± 0.016 µmol/g Hb (women; erythrocytes)	Kandár et al. 2014
0.083 ± 0.026 µmol/g Hb (men; erythrocytes)		
<i>Polyamines</i>		
Spermidine	0.32 ± 0.07 µmol/l (serum)	Marton et al. 1973
	72.9 ± 34.9 nmol/l (plasma)	Igarashi et al. 2006
	14.7 (10–24) µmol/l (erythrocytes)	Seghieri et al. 1997
Spermine	0.039 ± 0.029 µmol/l (plasma)	Desser et al. 1981
	30.7 ± 39.5 nmol/l (plasma)	Igarashi et al. 2006
<i>B-group vitamins</i>		
Vitamin B1	0.064 ± 0.12 µmol/l (plasma)	Thornalley et al. 2007
Vitamin B2	0.0027–0.0425 µmol/l (plasma)	Hustad et al. 1999
	0.018 µmol/l (plasma)	Midttun et al. 2007
Vitamin B6	0.0757 µmol/l (plasma)	Peeters et al. 2007
<i>Nucleotides</i>		
AMP	0.067 ± 0.02 µmol/l (human skeletal muscles)	Hellsten et al. 1998
ATP	1.02 ± 0.09 µmol/l (plasma)	Lader et al. 2000
NAD	24.3 µmol/l (whole blood)	Creeke et al. 2007

*This range is considered normal by the American Medical Association

($t = 7.64^a$) and N⁷-formylkynurenine ($t = 3.49^{c}; p < 0.05$); 0.4 mM lysine slightly reduced formation of AGEs ($t = 4.67^b$), dityrosine ($t = 2.91^c$), N⁷-formylkynurenine ($t = 6.8^b$), kynurenine ($t = 2.89^c$) and amyloid β -structures ($t = 3.65^c$). Histidine, methionine and tryptophan at a concentration of 1 mM slightly decreased glycation as evaluated by all parameters measured (AGEs: $t = 19.9^a, 9.92^a$ and 11.6^a , respectively; dityrosine: $t = 32.0^a, 26.0^a$ and 21.0^a , respectively; N⁷-formylkynurenine: $t = 23.6^a, 16.6^a$ and 16.3^a , respectively; kynurenine: $t = 13.4^a$ for histidine and 10.6^a for tryptophan; amyloid formation: $t = 7.18^b, 7.31^b$ and 5.54^b , respectively), but physiological concentration of these amino acids had generally no effect either. Oxidized glutathione (GSSG) at physiological concentrations did not affect glycation; however, 1 mM GSSG caused an increase in AGEs ($t = 5.67^b$), dityrosine ($t = 12.0^a$), N⁷-formylkynurenine ($t = 8.03^b$) and kynurenine ($t = 3.04^c$) levels. Melatonin at a concentration of 1 mM significantly inhibited formation of AGEs ($t = 16.2^a$), dityrosine ($t = 5.12^b$) and N⁷-formylkynurenine, but had no effect in the physiological range of concentrations. Panthothenic acid at a physiological concentration had no effect, with the exception of an increase in amyloid formation ($t = 11.6^a$) but a concentration of 1 mM intensified glycation. Uric acid promoted glycation in concentration-dependent manner.

Vitamin B1 at a concentration of 1 mM significantly increased AGE ($t = 34.1^a$), dityrosine ($t = 20.5^a$), N⁷-formylkynurenine ($t = 22.7^a$) and kynurenine formation ($t = 47.4^a$), and slightly enhanced tryptophan loss ($t = 5.49^b$).

Vitamin B2 at a physiological concentration did not affect most of glycation markers; however, 1 mM riboflavin significantly decreased formation of AGEs ($t = 93.6^a$), dityrosine ($t = 418.4^a$), N⁷-formylkynurenine ($t = 172.7^a$) and kynurenine ($t = 45.5^a$), had no effect on tryptophan loss and increased amyloid formation ($t = 10.3^a$). Vitamin B6 at a concentration of 1 mM inhibited glycation, judging on the basis of all parameters measured but kynurenine and amyloid formation; physiological concentration of vitamin B6 had no effect on glycation.

Pyruvic acid significantly decreased glycation at a supraphysiological concentration of 1 mM (AGE: $t = 12.4^a$; dityrosine: $t = 28.8^a$; N⁷-formylkynurenine: $t = 21.5^a$; kynurenine: $t = 9.72^a$; amyloid formation: $t = 8.52^a$) but was ineffective in the physiological concentration range. GSH significantly decreased the value of all indices of glycooxidation in a concentration-dependent manner, with the exception of kynurenine (AGE: $t = 19.3^a$; dityrosine: $t = 20.7^a$; N⁷-formylkynurenine: $t = 26.5^a$; $t = 9.72^a$; amyloid formation: $t = 14.6^a$) (Table 2).

The level of AGEs estimated by fluorimetric measurements was generally confirmed by estimation of AGEs with an ELISA Kit. Carnosine, GSSG, histidine, melatonin, methionine and vitamin B1 at physiological concentration had no significant impact on AGEs formation. Tryptophan at a concentration of 80 μ M and urea at a concentration of 10 mM slightly inhibited AGEs formation ($t = 3.18^c$ and 2.84^c , respectively) but were ineffective at lower concentrations. Uric acid in a physiological

Table 2. The effect of various additives at physiological concentration and 1 mM on the extent of the glycooxidation of BSA (90 μ M) incubated with 500 mM glucose, estimated with fluorimetric parameters

		AGE	Dityrosine	N ⁷ -Formylkynurenine	Kynurenine	Tryptophan	Amyloid
BSA (control)		3.1 \pm 1.2 ^a	3.6 \pm 1.1 ^a	3.3 \pm 1.2 ^a	8.7 \pm 2.0 ^a	12.3 \pm 10.7 ^a	15.07 \pm 5.78 ^a
BSA+glucose, no additives		100	100	100	100	100	100
<i>Amino Acids and Derivatives, Peptides</i>							
Arginine	1 mM	106.7 \pm 3.2 ^c	109.7 \pm 1.8 ^a	108.9 \pm 5.5 ^c	107.1 \pm 2.4 ^b	105.7 \pm 10.5	71.59 \pm 21.64
	0.05 mM	103.6 \pm 9.7	104.2 \pm 3.0	98.0 \pm 5.2	97.8 \pm 5.3	105.6 \pm 7.6	98.28 \pm 8.03
	0.1 mM	98.0 \pm 4.5	97.3 \pm 6.4	98.7 \pm 4.3	101.4 \pm 5.2	104.2 \pm 9.0	101.28 \pm 4.26
	0.2 mM	96.9 \pm 4.2	95.5 \pm 9.1	97.1 \pm 5.1	97.5 \pm 3.3	109.4 \pm 9.6	107.51 \pm 39.75
Carnosine	1 mM	104.0 \pm 7.6	106.3 \pm 7.8	107.5 \pm 2.9 ^c	121.4 \pm 2.6 ^a	124.8 \pm 10.0 ^c	101.1 \pm 6.27
	2.5 mM	99.9 \pm 3.3	101.6 \pm 4.9	98.3 \pm 2.1	124.0 \pm 9.4 ^c	134.2 \pm 3.0 ^a	111.53 \pm 11.89
	5 mM	96.3 \pm 4.2	104.9 \pm 2.7 ^c	96.4 \pm 5.7	137.5 \pm 4.5 ^a	162.9 \pm 16.4 ^b	96.6 \pm 12.86
	10 mM	105.7 \pm 5.6	96.5 \pm 4.1	98.6 \pm 4.1	148.1 \pm 2.8 ^a	227.83 \pm 11.6 ^a	98.5 \pm 12.68
Creatinine	1 mM	96.1 \pm 1.6 ^c	100.2 \pm 3.3	100.8 \pm 4.4	97.7 \pm 6.5	102.8 \pm 12.8	97.4 \pm 9.68
	0.05 mM	101.6 \pm 5.7	103.5 \pm 3.0	104.3 \pm 2.6 ^c	101.8 \pm 8.8	95.5 \pm 13.6	96.17 \pm 7.32
	0.1 mM	102.2 \pm 10.5	104.4 \pm 6.6	104.4 \pm 7.6	101.0 \pm 10.2	100.1 \pm 11.0	104.93 \pm 6.4
	0.2 mM	100.7 \pm 9.3	106.5 \pm 7.2	108.8 \pm 8.8	96.3 \pm 4.8	107.6 \pm 9.8	106.03 \pm 6.49
Cysteine	1 mM	77.7 \pm 2.7 ^a	69.5 \pm 1.3 ^a	73.0 \pm 2.6 ^a	83.6 \pm 2.7 ^a	71.0 \pm 10.6 ^b	71.32 \pm 11.04 ^c
	0.025 mM	90.9 \pm 3.4 ^b	87.5 \pm 3.4 ^b	87.8 \pm 1.4 ^a	99.0 \pm 7.1	100.0 \pm 18.5	99.34 \pm 4.66
	0.05 mM	86.9 \pm 3.4 ^b	84.8 \pm 1.4 ^a	85.2 \pm 1.5 ^a	98.9 \pm 1.8	108.6 \pm 12.8	104.64 \pm 5.71
	0.1 mM	82.1 \pm 1.3 ^a	78.5 \pm 1.0 ^a	77.6 \pm 2.3 ^a	98.2 \pm 2.6	101.4 \pm 8.9	103 \pm 7.87

(continued)

Table 2. Continued

		AGE	Dityrosine	N ⁷ -Formylkynurenine	Kynurenine	Tryptophan	Amyloid
Glycine	1 mM	115.9 ± 3.0 ^a	111.5 ± 1.6 ^a	112.5 ± 5.9 ^c	113.5 ± 4.9 ^b	106.1 ± 15.0	121.08 ± 6.52 ^b
	0.1 mM	98.8 ± 7.0	100.9 ± 7.7	98.1 ± 3.5	97.2 ± 1.7 ^c	113.2 ± 14.6	103.27 ± 5.98
	0.2 mM	101.1 ± 3.9	96.8 ± 6.6	100.5 ± 3.4	98.2 ± 6.0	119.3 ± 13.1	112.46 ± 15.17
	0.4 mM	101.7 ± 5.6	96.9 ± 2.7	98.0 ± 1.4	97.1 ± 2.8	119.2 ± 18.8	102.87 ± 4.44
GSH	1 mM	64.3 ± 3.2 ^a	66.4 ± 2.8 ^a	67.9 ± 2.1 ^a	102.3 ± 3.5	69.5 ± 12.4 ^c	61.47 ± 4.56 ^a
	5 mM	59.0 ± 1.8 ^a	61.4 ± 2.3 ^a	62.7 ± 5.8 ^a	100.1 ± 2.2	57.9 ± 19.0 ^c	56.12 ± 11.11 ^b
	10 mM	58.4 ± 4.4 ^a	52.7 ± 1.3 ^a	56.8 ± 2.1 ^a	97.8 ± 4.8	51.9 ± 18.4 ^c	51.66 ± 12.13 ^b
	20 mM	55.0 ± 4.5 ^a	47.5 ± 4.4 ^a	53.0 ± 3.7 ^a	100.4 ± 5.3	34.3 ± 19.7 ^b	47.33 ± 7.2
GSSG	1 mM	116.8 ± 5.1 ^b	111.8 ± 1.7 ^a	111.5 ± 2.5 ^b	115.1 ± 8.6 ^c	109.7 ± 13.7	100.27 ± 6.53
	1 μM	98.8 ± 4.8	99.8 ± 2.7	96.4 ± 2.9	105.0 ± 5.1	109.1 ± 11.6	107.87 ± 6.22
	5 μM	102.6 ± 2.8	101.6 ± 8.8	97.4 ± 2.5	87.5 ± 6.8 ^c	110.8 ± 15.3	110.12 ± 11.4
	10 μM	101.8 ± 3.2	98.79 ± 1.1	102.3 ± 2.1	85.9 ± 3.3 ^b	107.8 ± 0.8 ^a	101.37 ± 7.08
Histidine	1 mM	68.5 ± 2.8 ^a	63.5 ± 2.0 ^a	66.7 ± 2.4 ^a	130.6 ± 4.0 ^a	86.2 ± 5.6 ^c	77.04 ± 5.54 ^b
	0.04 mM	98.8 ± 4.9	96.5 ± 3.2	96.3 ± 4.2	100.2 ± 4.0	100.2 ± 6.7	96.37 ± 10.41
	0.08 mM	100.0 ± 6.4	99.1 ± 7.5	97.8 ± 7.0	98.2 ± 3.7	103.2 ± 16.3	96.06 ± 7.74
	0.16 mM	94.2 ± 1.4 ^b	93.7 ± 1.9 ^b	93.8 ± 1.4 ^b	97.6 ± 9.5	103.6 ± 3.7	97.99 ± 7.25
Lysine	1 mM	80.2 ± 5.4 ^b	77.3 ± 5.1 ^b	77.5 ± 11.1 ^c	78.7 ± 5.0 ^b	98.9 ± 13.2	84.05 ± 13.77
	0.1 mM	96.3 ± 7.1	97.6 ± 5.3	96.9 ± 7.0	97.6 ± 4.8	102.8 ± 12.6	101.02 ± 7.3
	0.2 mM	96.8 ± 3.5	98.9 ± 3.1	98.7 ± 2.0	101.4 ± 3.8	100.1 ± 20.3	105.26 ± 6.63
	0.4 mM	93.6 ± 2.4 ^b	92.6 ± 4.4 ^c	92.5 ± 1.9 ^b	94.3 ± 3.4 ^c	107.4 ± 11.5	87.49 ± 6.07 ^c
Melatonin	1 mM	59.8 ± 4.3 ^a	80.0 ± 6.8 ^b	76.5 ± 8.1 ^b	175.5 ± 7.1 ^a	Not measurable	72.11 ± 9.74
	20 pM	98.6 ± 3.6	100.2 ± 8.7	98.2 ± 9.1	118.0 ± 11.5	102.5 ± 8.4	94.73 ± 8.87
	40 pM	99.7 ± 5.1	97.5 ± 2.7	100.6 ± 3.6	122.3 ± 9.9 ^c	102.0 ± 9.5	98.56 ± 13.97 ^b
	80 pM	96.1 ± 4.0	99.0 ± 2.3	100.1 ± 4.5	137.5 ± 5.8 ^a	99.9 ± 9.5	104.51 ± 10.43 ^b
Methionine	1 mM	86.4 ± 2.4 ^a	84.6 ± 1.0 ^a	86.6 ± 1.4 ^a	103.6 ± 3.7	93.9 ± 12.8	66.55 ± 7.92 ^b
	10 μM	98.1 ± 1.9	96.7 ± 1.2 ^b	96.1 ± 1.4 ^b	100.7 ± 1.2	103.6 ± 7.2	97.51 ± 9.51
	25 μM	99.0 ± 3.4	98.0 ± 3.3	97.5 ± 4.1	104.6 ± 3.7	98.4 ± 4.6	100.09 ± 8.32
	50 μM	93.7 ± 1.7 ^b	92.4 ± 1.3 ^a	92.9 ± 0.8 ^a	99.0 ± 2.5	101.6 ± 10.2	96.06 ± 4.49
Serine	1 mM	97.2 ± 4.7	96.3 ± 1.2 ^b	95.7 ± 10.7	100.5 ± 3.9	153.6 ± 11.9 ^b	107.67 ± 4.21 ^c
	0.05 mM	99.9 ± 5.1	98.0 ± 4.0	102.6 ± 1.9	101.5 ± 7.6	123.7 ± 10.8 ^c	110.88 ± 13.4
	0.1 mM	96.1 ± 3.9	101.3 ± 3.9	100.3 ± 4.1	96.3 ± 2.5	127.3 ± 6.2 ^b	106.13 ± 10.18
	0.2 mM	97.0 ± 4.1	100.6 ± 3.9	101.6 ± 5.5	98.0 ± 3.0	130.3 ± 9.7 ^b	103.67 ± 4.78
Tryptophan	1 mM	79.8 ± 3.0 ^a	65.7 ± 2.8 ^a	73.6 ± 2.8 ^a	80.3 ± 3.2 ^a	68.5 ± 4.3 ^a	62.92 ± 11.59 ^b
	20 μM	97.3 ± 2.9	96.3 ± 2.1 ^c	95.2 ± 2.5 ^c	96.1 ± 2.2 ^c	106.0 ± 7.0	99.65 ± 9.67
	40 μM	92.9 ± 3.4 ^c	92.6 ± 1.6 ^b	92.9 ± 1.6 ^b	94.5 ± 6.3	107.1 ± 11.4	100.22 ± 7.85
	80 μM	88.4 ± 2.9 ^b	85.1 ± 2.9 ^a	86.5 ± 3.7 ^b	84.7 ± 4.0 ^b	106.0 ± 6.6	98.08 ± 7.89
Tyrosine	1 mM	712.8 ± 6.7 ^a	843.8 ± 12.3 ^a	727.2 ± 1.4 ^a	1461.1 ± 60.7 ^a	207.4 ± 5.9 ^a	1668.21 ± 40.04 ^a
	0.03 mM	98.5 ± 1.0	98.7 ± 1.3	101.4 ± 5.6	97.2 ± 6.2	97.9 ± 10.8	97.42 ± 3.63
	0.06 mM	116.1 ± 3.6 ^b	114.5 ± 3.2 ^b	114.5 ± 2.0 ^a	123.5 ± 4.0 ^a	102.5 ± 6.3	98.16 ± 3.72
	0.12 mM	123.1 ± 2.2 ^a	119.6 ± 2.6 ^a	120.5 ± 2.5 ^a	134.4 ± 2.0 ^a	97.5 ± 6.5	107.91 ± 9.85
Urea	1 mM	98.0 ± 4.7	92.9 ± 3.4 ^c	97.2 ± 5.4	95.6 ± 3.8	105.4 ± 8.2	98.03 ± 6.01 ^c
	2.5 mM	91.9 ± 5.4	89.7 ± 3.6 ^b	93.4 ± 4.3	91.0 ± 5.1 ^c	107.1 ± 7.3	90.71 ± 12.82 ^b
	5 mM	90.5 ± 3.3 ^b	87.2 ± 2.0 ^a	92.1 ± 3.7 ^c	90.7 ± 4.0 ^c	105.7 ± 11.0	85.97 ± 7.92
	10 mM	86.2 ± 6.0 ^c	82.7 ± 4.4 ^b	82.6 ± 4.7 ^b	85.4 ± 5.3 ^b	103.9 ± 11.2	81.64 ± 9.06
<i>Organic acids</i>							
Oxaloacetic acid	1 mM	102.7 ± 5.9	98.9 ± 4.1	103.0 ± 13.4	78.8 ± 4.9 ^b	101.0 ± 11.8	95.9 ± 13.63
	0.2 μM	97.6 ± 4.3	102.0 ± 4.0	102.0 ± 3.9	99.8 ± 4.5	101.5 ± 17.1	97.58 ± 5.57
	0.35 μM	100.2 ± 7.2	104.2 ± 5.2	107.2 ± 6.2	105.6 ± 6.6	99.5 ± 11.2	98.77 ± 7.76
	0.7 μM	100.7 ± 2.8	100.9 ± 1.9	101.6 ± 1.7	97.7 ± 4.3	103.1 ± 10.0	99.12 ± 11.99

(continued)

Table 2. Continued

		AGE	Dityrosine	N ^ε -Formylkynurenine	Kynurenine	Tryptophan	Amyloid
Pantothenic acid	1 mM	574.6 ± 4.1 ^a	629.7 ± 10.0 ^a	510.2 ± 7.2 ^a	772.1 ± 16.3 ^a	307.8 ± 8.2 ^a	505.71 ± 9.71 ^a
	1 μM	99.1 ± 3.8	97.4 ± 6.2	103.0 ± 4.2	99.4 ± 6.7	109.6 ± 13.4	105.96 ± 8.59
	5 μM	99.9 ± 1.7	101.9 ± 1.9	101.5 ± 2.1	96.7 ± 4.1	116.9 ± 11.5	99.37 ± 12.8
	10 μM	95.2 ± 5.9	101.6 ± 3.5	105.4 ± 5.8	97.0 ± 3.9	125.4 ± 5.9 ^b	152.31 ± 7.78 ^a
Pyruvic	10 μM	99.6 ± 3.7	98.4 ± 2.8	98.5 ± 1.9	99.2 ± 2.0	106.6 ± 7.5	100.27 ± 5.51
	20 μM	99.5 ± 2.5	101.4 ± 2.1	100.6 ± 1.6	99.0 ± 5.2	103.7 ± 8.7	100.81 ± 5.39
	30 μM	97.0 ± 5.6	100.4 ± 1.1	99.0 ± 1.2	98.8 ± 4.0	105.5 ± 7.9	99.01 ± 1.84
	100 μM	93.4 ± 4.3	93.5 ± 1.7 ^b	96.3 ± 2.0 ^c	91.7 ± 5.0 ^c	94.3 ± 9.8	92.18 ± 6.22
Pyruvic acid	5 mM	63.1 ± 5.2 ^a	61.7 ± 2.3 ^a	60.0 ± 3.2 ^a	57.8 ± 7.5 ^a	56.3 ± 5.6 ^a	31.89 ± 13.84 ^b
Uric acid	1 mM	203.0 ± 7.7 ^a	197.9 ± 4.5 ^a	217.9 ± 6.5 ^a	202.8 ± 14.5 ^a	11.09 ± 2.2 ^a	232.81 ± 8.84 ^a
	0.18 mM	122.1 ± 2.6 ^a	122.0 ± 4.2 ^a	133.4 ± 1.9 ^a	128.5 ± 4.7 ^a	34.4 ± 7.1 ^a	124.09 ± 17.42
	0.35 mM	138.4 ± 5.2 ^a	140.8 ± 2.9 ^a	151.2 ± 2.4 ^a	157.5 ± 8.0 ^a	24.0 ± 1.8 ^a	133.43 ± 11.23 ^b
	0.7 mM	170.9 ± 4.7 ^a	185.5 ± 5.5 ^a	171.6 ± 7.1 ^a	198.8 ± 8.4 ^a	18.0 ± 1.3 ^a	166.65 ± 7.51 ^a
<i>Polyamines</i>							
Spermidine	1 mM	123.2 ± 4.4 ^a	1121 ± 5.2 ^c	115.9 ± 6.3 ^c	164.8 ± 10.4 ^a	122.7 ± 20.7	120.77 ± 8.36 ^c
	0.5 μM	97.4 ± 3.8	98.3 ± 3.5	97.2 ± 2.7	97.7 ± 4.7	107.9 ± 12.8	116.7 ± 7.21 ^c
	1 μM	100.4 ± 4.0	96.4 ± 8.2	99.5 ± 7.0	99.7 ± 9.1	111.3 ± 6.3 ^c	106.05 ± 16.38
	2 μM	102.3 ± 2.7	98.7 ± 9.5	99.0 ± 5.3	98.5 ± 8.3	107.9 ± 9.2	108.22 ± 7.78
Spermine	1 mM	111.8 ± 4.6 ^c	110.1 ± 2.8 ^b	116.8 ± 5.3 ^b	131.2 ± 4.2 ^a	190.1 ± 6.1 ^a	114.21 ± 9.08
	20 nM	96.6 ± 4.9	96.3 ± 1.7 ^c	99.6 ± 7.6	97.5 ± 7.9	131.7 ± 12.9 ^c	105.17 ± 6.93
	40 nM	100.8 ± 4.5	100.1 ± 5.1	103.2 ± 0.8 ^b	100.2 ± 3.1	128.9 ± 4.0 ^a	97.92 ± 4.62
	80 nM	96.9 ± 2.6	99.7 ± 8.3	103.1 ± 2.7	98.2 ± 6.3	131.7 ± 10.8 ^b	115.38 ± 10.95
<i>B-group vitamins</i>							
Vit B1	1 mM	247.53 ± 7.5 ^a	196.3 ± 8.1 ^a	240.2 ± 10.7 ^a	1130.1 ± 37.7 ^a	80.98 ± 6.0 ^b	95.98 ± 3.55 ^c
	30 nM	100.8 ± 4.3	100.6 ± 5.2	99.5 ± 4.0	99.3 ± 5.7	107.7 ± 10.9	104.38 ± 5.81
	60 nM	98.7 ± 6.8	99.7 ± 3.3	97.7 ± 6.3	100.5 ± 2.3	105.4 ± 14.2	99.24 ± 12.72
	120 nM	102.5 ± 6.5	101.1 ± 4.2	97.5 ± 4.2	98.3 ± 5.9	108.2 ± 11.9	105.59 ± 2.84
Vit B2	1 mM	11.9 ± 1.6 ^a	16.0 ± 0.4 ^a	13.2 ± 0.9 ^a	12.6 ± 3.3 ^a	104.4 ± 6.4	560.38 ± 77.1 ^a
	10 nM	97.6 ± 5.1	92.1 ± 1.2 ^a	90.7 ± 1.9 ^b	91.6 ± 4.3 ^c	103.2 ± 11.0	100.48 ± 6.81
	20 nM	95.3 ± 4.7	90.8 ± 3.5 ^c	89.2 ± 3.6 ^b	87.7 ± 5.4 ^c	106.0 ± 16.7	98.29 ± 6.5
	40 nM	86.1 ± 3.8 ^b	87.1 ± 2.0 ^a	85.8 ± 2.1 ^a	83.4 ± 8.4 ^c	101.6 ± 8.2	100.09 ± 5.82
Vit B6	1 mM	0 ^a	0 ^a	0 ^a	99.4 ± 7.1	23.3 ± 0.5 ^a	74.58 ± 8.36
	38 nM	99.7 ± 4.5	99.3 ± 3.3	98.0 ± 4.4	100.2 ± 3.9	98.1 ± 6.9	103.57 ± 11.97
	75 nM	100.8 ± 3.0	99.2 ± 5.2	96.5 ± 3.7	97.0 ± 5.0	103.0 ± 9.8	95.53 ± 9
	150 nM	104.3 ± 6.7	100.0 ± 2.3	100.9 ± 5.1	100.1 ± 6.9	112.3 ± 15.8	97.1 ± 9.31
<i>Nucleotides</i>							
AMP	1 mM	107.0 ± 9.3	104.9 ± 6.1	97.2 ± 6.0	105.5 ± 7.6	109.9 ± 12.3	107.84 ± 10.02
	35 nM	105.4 ± 12.6	99.4 ± 3.9	98.2 ± 2.4	96.9 ± 5.4	105.1 ± 13.4	98.72 ± 13.39
	70 nM	107.8 ± 6.7	101.4 ± 2.8	98.2 ± 3.3	100.8 ± 6.7	108.1 ± 14.6	99.91 ± 13.74
	140 nM	104.6 ± 4.4	97.4 ± 3.3	98.3 ± 5.5	97.9 ± 9.9	107.2 ± 11.5	109.95 ± 9.21
ATP	1 mM	103.3 ± 4.0	98.4 ± 3.6	105.1 ± 3.5	81.6 ± 5.9 ^b	103.2 ± 15.8	105.64 ± 7.94
	0.5 μM	102.5 ± 3.5	104.1 ± 4.3	103.5 ± 2.1 ^c	105.1 ± 10.0	95.6 ± 16.2	98.41 ± 7.54
	1 μM	104.0 ± 2.2 ^c	102.8 ± 5.4	103.8 ± 5.9	103.9 ± 3.9	107.3 ± 5.5	103.04 ± 9.41
	2 μM	98.1 ± 4.8	99.8 ± 2.2	102.0 ± 4.9	99.4 ± 7.2	100.5 ± 12.1	108.76 ± 6.12
NAD	1 mM	105.1 ± 9.8	99.6 ± 5.7	104.5 ± 7.3	105.4 ± 6.6	99.7 ± 4.8	104.87 ± 7.33
	10 μM	106.3 ± 1.7	103.3 ± 4.6	105.0 ± 4.5	102.9 ± 4.7	104.0 ± 1.5 ^c	104.65 ± 8.06 ^b
	25 μM	103.8 ± 1.4 ^c	104.5 ± 5.7	104.9 ± 4.6	103.5 ± 5.8	109.7 ± 11.8	102.99 ± 9.53
	50 μM	103.5 ± 4.7	98.9 ± 3.9	101.5 ± 5.4	103.0 ± 6.3	97.9 ± 6.1	106.39 ± 9.87

Data are shown as mean ± SD. Statistical significance of differences: ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$, (paired Student's *t*-test).

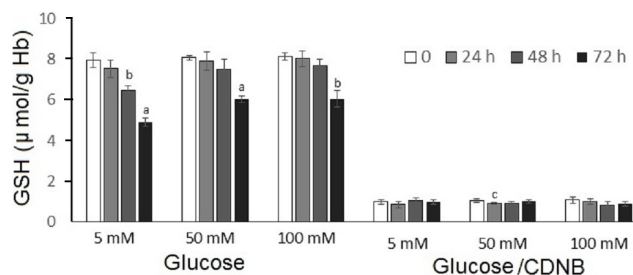


Figure 1. Effect of CDNB (2,4-chlorodinitrobenzene) treatment on the glutathione (GSH) content of erythrocytes. Statistical significance of differences: ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$ (corresponding CDNB-treated vs. non-treated samples).

concentration range significantly increased AGEs generation assayed by ELISA ($t = 32.2^a$ for 0.35 mM uric acid and 49.3^a for 0.7 mM uric acid). Cysteine at a concentration of 0.1 mM slightly reduced generation of AGEs ($t = 4.71^b$) while lower concentrations did not affect the amount of AGEs. Lysine at concentrations of 0.2 and 0.4 mM slightly inhibited glycation ($t = 3.91^b$ and 3.54^c , respectively). Pyruvic acid reduced glycation in a concentration-dependent manner but in a supra-physiological concentration range ($t = 5.97^b$ and 6.25^b for 2.25 and 5 mM pyruvic acid, respectively). GSH protected against glycation in a concentration-dependent manner in the millimolar concentration range, corresponding to its intracellular concentrations ($t = 2.99^c$, 4.65^b and 5.36^b for 5, 10 and 20 mM GSH, respectively; Table 3).

Incubation of erythrocytes in the presence of various glucose concentrations (5, 50 and 100 mM) at 37°C for up to 72 h led to gradual loss of GSH, attenuated by high glucose concentrations. Incubation with CDNB reduced drastically the GSH level (Fig. 1). Incubation of these erythrocytes with glucose did not restore the GSH content. The content of glycated hemoglobin increased with increasing incubation time and increasing glucose concentration. Preincubation of erythrocytes with CDNB promoted hemoglobin glycation as compared with non-treated cells (Fig. 2).

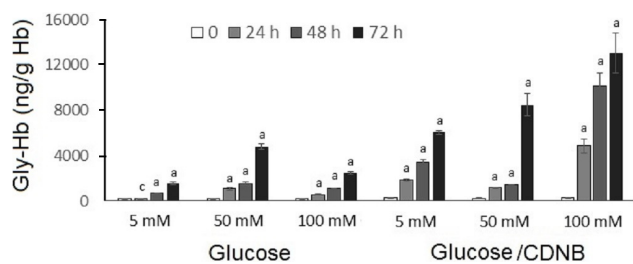


Figure 2. Effect of glutathione depletion and glucose concentration on hemoglobin glycation (Gly-Hb) in erythrocytes incubated *in vitro*; ^a $p < 0.001$, ^c $p < 0.05$.

Table 3. Effect of endogenous compounds on AGE formation of BSA incubated with glucose

System/Additives	AGE (ng/ml)	
BSA (control)	1247 ± 155 ^b	
BSA+glucose, no additives	2318 ± 171	
Carnosine	2.5 mM 5 mM 10 mM	2247 ± 103 2200 ± 127 2266 ± 91
Cysteine	0.025 mM 0.05 mM 0.1 mM	2127 ± 166 2099 ± 79 1841 ± 39 ^b
GSH	5 mM 10 mM 20 mM	1947 ± 129 ^c 1807 ± 83 ^b 1729 ± 83 ^b
GSSG	1 μM 5 μM 10 μM	2178 ± 134 2171 ± 11 2145 ± 152
Histidine	0.04 mM 0.08 mM 0.16 mM	2184 ± 176 2134 ± 195 2152 ± 211
Lysine	0.1 mM 0.2 mM 0.4 mM	2140 ± 97 1926 ± 26 ^c 1849 ± 153 ^c
Melatonin	20 pM 40 pM 80 pM	2308 ± 167 2166 ± 27 2158 ± 63
Methionine	10 μM 25 μM 50 μM	2398 ± 115 2331 ± 120 2135 ± 5
Pyruvic acid	1.125 mM 2.25 mM 5 mM	2011 ± 132 1710 ± 42 ^b 1578 ± 113 ^b
Tryptophan	20 μM 40 μM 80 μM	2248 ± 84 2262 ± 61 1950 ± 105 ^c
Urea	2.5 mM 5 mM 10 mM	2162 ± 108 2048 ± 179 1938 ± 155 ^c
Uric acid	0.18 mM 0.35 mM 0.7 mM	2847 ± 329 9899 ± 370 ^a 31832 ± 1023 ^a
Vit B1	30 nM 60 nM 120 nM	2329 ± 93 2229 ± 88 2161 ± 95

BSA (90 μM) was incubated with 500 mM glucose and physiological concentrations of compounds at 37°C for 6 days. The AGE content was estimated by ELISA. Data are shown as mean ± SD. Statistical significance of differences: ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$, with respect to BSA incubated with glucose without any additives (paired Student's t-test).

Discussion

We examined the protective effect of endogenous compounds on protein glycation *in vitro* using BSA as a model protein. BSA has a 76% similarity in amino acid sequence to human serum albumin (HSA), which is the most abundant human plasma protein (Arasteh et al. 2014). Glycated albumin may be a useful and specific marker of glycemia for pediatric diabetic patients (Lee et al. 2013), diabetic hemodialysis patients (Inaba et al. 2007), diabetic patients with cardiovascular complications (Sato et al. 2013), diabetic patients with advanced chronic kidney disease (Vos et al. 2011) and patients with coronary artery disease (Ma et al. 2015).

Our results confirm that some potential anti-glycating agents, even if active at higher concentrations, are ineffective at physiological concentrations. Administration of L-arginine to rats with experimental diabetes decreased hemoglobin glycation (Méndez and Balderas 2001; Ma et al. 2015); however, in these experiments animals were treated with 10 mM arginine, what suggest that only supraphysiological concentrations may be effective. In our study, arginine was effective at 1 mM concentration and not at lower concentrations. Pyruvate has been reported to prevent cataract development (Hegde and Varma 2005) and protect against fructose-induced formation of high molecular weight aggregates of crystallin (Ramamurthy et al. 2001). However, in our studies its protective activity was evident only at supraphysiological concentrations. GSSG at a high concentration (1 mM) had a pro-glycating effect. Such GSSG concentration is high but may be attained under oxidative stress conditions and may contribute to the pro-glycating effect of oxidative stress. It should be kept in mind that, due to differences in conditions and thus kinetics of glycation, the effect of endogenous compounds on protein glycation *in vivo* may be different than in the artificial *in vitro* system used. Nevertheless, the compounds not effective *in vitro* can hardly be expected to have significant effects *in vivo*. In particular, GSH can be expected to be a better antiglycating agent *in vivo* than *in vitro* since under *in vitro* conditions GSH is partly oxidized to GSSG during 6-day incubation.

From among the compounds studied, only GSH showed an anti-glycating effect *in vitro*, in a cell-free system. In order to check whether prediction from such a system holds within a cell, we studied the effect of glutathione depletion on glycation of hemoglobin in erythrocytes incubated with elevated glucose concentrations for up to 72 h. Incubation with CDNB led to a drastic decrease in erythrocyte GSH level. Hemoglobin glycation proceeded at a higher rate in cells depleted of GSH.

Our results are in accordance with several earlier findings. Ramamurthy and colleagues observed that glutathione

(10 mM) reverses the effect of glucose on myosin function (Zhao et al. 2000). Jain (2008) found a negative correlation between the level of GSH and that of glycated hemoglobin following experimental modulation of GSH level in erythrocytes. Huby and Harding (1988) reported that galactosylation of lens proteins is inhibited by GSH.

However, the anti-glycating action of GSH may be not free of undesired effects. Reaction of GSH with glucose may lead to glycation of the α -NH₂ group of the glutamate residue or -SH group of the cysteine residue glutathione, in GSH. N-1-Deoxyfructos-1-yl glutathione was identified as the major glycation product of GSH glycation by glucose *in vitro*. This compound is a poor substrate for glutathione peroxidase, glutathione reductase and glutathione S-transferase (Linetsky et al. 2005).

Another compound which may contribute to prevention of glycation *in vivo* is ascorbic acid. This compound was not included in the present study as it shows a pro-glycating activity *in vitro* (Sadowska-Bartosz and Bartosz 2015b). In our previous experiments 1 mM ascorbate did not affect hemoglobin glycation in erythrocytes incubated with high glucose (Sadowska-Bartosz and Bartosz 2015b; Sadowska-Bartosz et al. 2015b). However, it cannot be excluded that in cells expressing SVCT transporters, in which intracellular ascorbate concentration may reach millimolar levels, ascorbate may also be a significant anti-glycating agent.

We checked also the effect of uric acid (0.7 mM) on hemoglobin glycation of erythrocytes finding a tendency for an increase, but without statistical significance (not shown).

These results demonstrate that the level of intracellular glutathione may be an important determinant of the rate of glycation of intracellular proteins, though being of no importance for blood plasma proteins due to its low extracellular concentrations. It has been suggested that the action of exogenous antioxidants is based principally on the induction of endogenous antioxidant defense *via* activation of Nrf2 factor rather than direct antioxidant action of exogenous compounds (Forman et al. 2014). A similar situation may exist for glycation of intracellular proteins: keeping high glutathione level may be more important for limiting glycation than exogenous additives, reaching much lower levels *in vivo*. It would be of interest to examine epidemiological data for a possible correlation between the erythrocyte glutathione level and the level of glycated hemoglobin, and between the uric acid level and albumin glycation.

Conflict of interest. The authors declare no conflict of interest.

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