Acceleration of glucose-mediated crosslinking of collagen by free lysine

D. Fujimoto and K. Horiuchi

Department of Chemistry, Hamamatsu University School of Medicine, Hamamatsu 431-31 (Japan), 24 June 1985

Summary. Crosslinking occurred in collagen when it was incubated with glucose. Free lysine accelerated the crosslinking markedly.
Key words. Collagen; crosslink; Maillard reaction.

It is known that reducing sugars such as glucose react nonenzymatically with amino groups in proteins and the condensation products then undergo multiple chemical reactions to form various products, including brown pigments and protein crosslinks[1]. The reactions have received attention from food chemists, since stored and heat-treated foods undergo these reactions and their bioavailability is thus decreased. More recently, it has been shown that the glucose-mediated crosslinking also occurs in vivo. The crosslinking occurs appreciably in the long-lived proteins such as collagen and lens crystallins during aging[2,3]. The reaction appears to be accelerated in diabetes[4]. However, the mechanism of the glucose-mediated crosslinking of proteins has not been elucidated well. This paper reports the acceleration of the glucose-mediated crosslinking of collagen in vitro by free lysine.

Materials and methods. Tibias and femurs were obtained from 6-week-old chicks. The diaphysis was cleaned of marrow, cut into small pieces, washed with a large volume of 0.5 M NaCl at 4 °C, and subjected to demineralization. The demineralized bone was homogenized by a Physcotron homogenizer.

Bone collagen (100 mg wet wt.) was suspended in 0.1 M sodium phosphate buffer, pH 7.2 (1.5 ml) in the presence or absence of D-glucose and L-amino acids at 37 °C for 4 weeks. One drop of toluene was added to inhibit bacterial growth. After incubation bone collagen was collected, washed with water and then digested with cyanogen bromide in 70% formic acid at 20 °C for 3 h[2]. After digestion, each sample was diluted 10-fold in water and centrifuged at 10,000 × g for 20 min. The supernatant was evaporated under reduced pressure and the dried sample was hydrolyzed with 6 M HCl at 110 °C for 24 h. The precipitate was also hydrolyzed in the same manner. Hydroxyproline in the hydrolysate was determined[5] and the amount of collagen was estimated.

Results and discussion. The level of crosslinking in the collagen was assessed by measuring the amount of solubilized peptides after cyanogen bromide digestion[2]. The results are summarized in the table. The chick bone collagen incubated without glucose was solubilized completely by cyanogen bromide treatment. The collagen incubated with glucose alone was also solubilized completely, although SDS-polyacrylamide gel electrophoresis of the solubilized peptides indicated that crosslinking occurred to some extent (data not shown). The collagen incubated with glucose in the presence of free lysine was markedly resistant to solubilization by cyanogen bromide, indicating that the collagen was highly crosslinked and remained insoluble even after cyanogen bromide had cleaved several bonds. The degree of the insolubility depended on the concentration of lysine. Other amino acids (alanine and arginine) had a similar, but lesser effect. The result indicates that free amino acids, particularly lysine, accelerate the glucose-mediated crosslinking in collagen.

The mechanism of the glucose-mediated crosslinking in proteins has not been elucidated well. Pongor et al. proposed an imidazole compound incorporating two glucose molecules and two lysine residues as a possible crosslinking group[6]. Oritani et al. suggested that the crosslinking may be caused by dicarbonyl compounds formed from glucose by the reaction with amino groups[7]. The present finding may favor the latter mechanism. The reaction between glucose and free amino acids also produces dicarbonyl compounds, resulting in the acceleration of crosslinking in the protein.

The glucose-mediated crosslinking may be involved in the aging process of human body[2,3] and in diabetes[4]. It has been thought so far that only the concentration of glucose in the body fluids affects the rate of the crosslinking. The present study suggests that the concentration of free amino acids, particularly that of lysine, may affect the rate of the crosslinking, too.

Cyanogen bromide digestibility of collagen after in vitro incubation

Incubation conditions			Amount solubilized by cyanogen bromide digestion (%)
Glucose (mM)	Amino acid	(mM)	
0	–	0	100
200	–	0	100
200	Lysine	20	79
200	Lysine	60	26
200	Lysine	200	16
20	Lysine	200	100
200	Alanine	200	71
200	Arginine	200	49

Results are the mean of duplicates.

1 Mauron, J., Prog. Fd Nutr. Sci. *5* (1981) 5.
2 Fujimoto, D., Biomed. Res. *5* (1984) 279.
3 Monnier, V.M., and Cerami, A., Science *211* (1981) 491.
4 Monnier, V.M., Kohn, R.R., and Cerami, A., Proc. natn. Acad. Sci. *81* (1984) 583.
5 Woessner, J.F., Archs Biochem. Biophys. *93* (1961) 440.
6 Pongor, S., Ulrich, P.C., Bencsath, F.A., and Cerami, A., Proc. natn. Acad. Sci. *81* (1984) 2684.
7 Okitani, A., Cho, R.K., and Kato, H., Agric. biol. Chem. *48* (1984) 1801.

0014-4754/86/040405-03$1.50 + 0.20/0

Phospholipid composition of cardiac ($Na^+ + K^+$)-ATPases from various species

T. Matsuda and H. Iwata*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565 (Japan), 2 July 1985

Summary. There is a difference in phospholipid composition of cardiac ($Na^+ + K^+$)-ATPase preparations between species which are sensitive to ouabain and those which are not. Sphingomyelin is higher and phosphatidylcholine is lower in the enzymes from sensitive species than in those from insensitive ones. Lysophosphatidylcholine is detectable only in the latter preparations.
Key words. ($Na^+ + K^+$)-ATPase; phospholipid; ouabain.

Phospholipid composition of cardiac ($Na^+ + K^+$)-ATPase preparations from various animals

Species	Relative concentrations of phospholipids (%)						
	SPH	PC	PI	PS	PE	CL	LPC
Bovine	22.2 ± 3.8***	32.0 ± 2.9**	7.0 ± 2.0	4.2 ± 0.5	30.6 ± 1.9	4.0 ± 1.0	ND
Dog	19.2 ± 2.3***	37.8 ± 2.1*	4.9 ± 1.0	5.1 ± 1.2	29.1 ± 1.9	3.9 ± 0.4	ND
Rabbit	17.2 ± 1.0***	40.1 ± 1.2*	3.6 ± 0.5	6.8 ± 0.5	29.7 ± 1.0	2.6 ± 0.4	ND
Guinea pig	13.0 ± 0.7***	40.9 ± 0.3***	5.4 ± 0.6	4.6 ± 0.3	33.3 ± 0.9	2.8 ± 0.1	ND
Rat	4.7 ± 0.4	44.3 ± 0.4	3.1 ± 0.4	2.8 ± 1.1	39.2 ± 1.4	3.7 ± 0.3	2.0 ± 0.3
Mouse	6.6 ± 0.9	47.9 ± 1.5	6.6 ± 0.8	6.9 ± 1.7	28.0 ± 1.6	1.6 ± 0.5	2.4 ± 0.3

Values are means ± SE of 4–6 determinations. The specific activities (μmoles Pi/mg protein/min) of the enzymes were 1.43 ± 0.14 (bovine), 1.76 ± 0.20 (dog.), 1.20 ± 0.04 (rabbit), 1.39 ± 0.18 (guinea pig), 1.04 ± 0.11 (rat) and 1.08 ± 0.22 (mouse) (n = 4–9). Total phospholipid contents (μmoles Pi/mg protein) of these preparations were 1.69 ± 0.15 (bovine), 2.07 ± 0.14 (dog), 1.47 ± 0.35 (rabbit), 1.71 ± 0.06 (guinea pig), 1.33 ± 0.04 (rat) and 0.84 ± 0.07 (mouse) (n = 4–9). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, compared to rat and mouse. ND: not detectable. SPH: sphingomyelin, PC: phosphatidylcholine, PI: phosphatidylinositol, PS: phosphatidylserine, PE: phosphatidylethanolamine, CL: cardiolipin, LPC: lysophosphatidylcholine.

It is known that sensitivity of cardiac ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) to ouabain inhibition varies considerably among animal species and the difference parallels the sensitivity of the heart to the positive inotropic effect of the drug[1-3]. The study of the species difference will provide information of the nature of the interaction of the enzyme with cardiac glycosides. Since the species difference is observed in the highly purified enzyme[1,4], it might be determined by an inherent difference in protein structure. However, the enzymes from different species have similar catalytic subunits[5,6]. On the other hand, phospholipids are present in the purified enzyme preparations and are involved in the conformational change of the enzyme[7]. It is therefore considered that lipids around the enzyme may play a role in ouabain sensitivity of the enzyme. Our recent analysis of Arrhenius plots of cardiac ($Na^+ + K^+$)-ATPase activity has shown that the break point for the enzymes of ouabain sensitive species is 29 °C while that of ouabain insensitive species is 24–25 °C[6]. The finding suggests that annular lipid may contribute to the ouabain sensitivity. We report here the phospholipid composition of cardiac ($Na^+ + K^+$)-ATPase preparations from various animal species.

Materials and methods. Cardiac ($Na^+ + K^+$)-ATPases were partially purified by gentle extraction with SDS from various animals and the activities were determined as described previously[6,8]. Phospholipids of the enzyme preparations were isolated by extraction with chloroform/methanol (1:2, v/v) in the presence of 0.05% butylated hydroxytoluene, and separated by two-dimensional TLC using 0.2 mm Silica Gel aluminium sheet (E. Merck AG) by the method of Esko and Raetz[9]. The discrete phospholipid spots were determined by exposure of the developed sheet to iodine vapor and identified by comparison with standards (Sigma). The sports were scraped off the sheet and the phosphorus content was assayed by the method of Bartlett[10]. Total phospholipid was determined as described previously[11].

Results and discussion. The enzymes used here showed varying degrees of sensitivity to ouabain inhibition as reported previously[6]: the IC_{50} values were 0.3 μM (bovine and dog), 1 μM (rabbit and guinea pig) and 100 μM (rat and mouse) under our assay conditions. The amount of total phospholipids was similar in these enzyme preparations. The table summarizes the distribution of phospholipid classes of cardiac ($Na^+ + K^+$)-ATPase preparations from different animals. The ouabain sensitive group including bovine, dog, rabbit and guinea pig enzymes showed a higher proportion of sphingomyelin and a relatively lower proportion of phosphatidylcholine compared to the insensitive rat and mouse enzymes. Furthermore, lysophosphatidylcholine was detectable only in the insensitive group. To study a possible role of the lipids in the sensitivity to ouabain inhibition, we added sphingomyelin to the rat enzyme or lysophosphatidylcholine to the dog enzyme, but they did not affect the ouabain sensitivity (data not shown). These reconstitution experiments show that phospholipid may not be a crucial determinant of the species difference.

There are two classes of lipids in the membranes. The lipid closely associated with the enzyme protein is referred to as annular lipid and the surrounding lipid is referred to as bulk lipid. Since the annular lipid is only a small part of the membrane lipid, the chemical analysis of crude enzyme preparations yields information about the bulk lipid. The present study indicates that there is a species difference in the phospholipid composition of bulk lipid in cardiac ($Na^+ + K^+$)-ATPase preparations. The species difference reported here seems to be consistent with the temperature-dependence of the enzyme activity, as reported previously[6], but it is not known whether the annular lipids have the same composition as the bulk lipids. Abeywardena et al.[12] have recently reported a good correlation between physical properties of membranes and the sensitivity of ($Na^+ + K^+$)-ATPase to ouabain inhibition. This finding, together with their previous study using a reconstitution system[13], suggested that membrane lipids modulate the sensitivity of the enzyme to ouabain inhibition. The idea seems to be in line with our data, though their finding is different from ours with respect to the phospholipid composition of the enzyme preparation. They found no species variation in the distribution of major phospholipid classes. The inconsistent result might be due to different methods for enzyme preparation and phospholipid separation; they used enzyme preparations which had lower activities than ours, and they did not separate minor phospholipid classes such as sphingomyelin, phosphatidylinositol, phosphatidylserine and lysophosphatidylcholine. In connection with the role of lipids in sensitivity, Akera et al.[14,15] have previously suggested that the low sensitivity of rat heart to cardiac glycosides may result from the lack of a lipid barrier regulating the release of the glycosides from their binding sites. The idea of a lipid barrier may be supported by the present finding that the phospholipid composition of rat cardiac ($Na^+ + K^+$)-ATPase is different from those of the ouabain sensitive enzymes. But the exact mechanism remains unclear.

Acknowledgment. We are indebted to Miss Hiroko Kojima for her excellent technical assistance.

1 Schwartz, A., Lindenmayer, G.E., and Allen, J.C., Pharmac. Rev. *27* (1975) 3.
2 Akera, T., and Brody, T.M., Pharmac. Rev. *29* (1978) 187.
3 Schwartz, A., Whitmer, K., Grupp, G., Adams, R.J., and Lee, S., Ann. N.Y. Acad. Sci. *402* (1982) 253.
4 Periyasamy, S.M., Lane, L.K., and Askari, A., Biochem. biophys. Res. Commun. *86* (1979) 742.
5 Periyasamy, S.M., Huang, W., and Askari, A., Comp. Biochem. Physiol. *76*B (1983) 449.
6 Matsuda, T., and Iwata, H., Biochem. Pharmac. *34* (1985) 2343.
7 Dahl, J.L., and Hokin, L.E., A. Rev. Biochem. *43* (1974) 327.
8 Matsuda, T., Iwata, H., and Cooper, J.R., J. biol. Chem. *259* (1984) 3858.

9 Esko, J.D., and Raetz, C.R.H., J. biol. Chem. *255* (1980) 4474.
10 Bartlett, G.R., J. biol. Chem. *234* (1959) 466.
11 Baba, A., Matsuda, T., and Iwata, H., Biochim. biophys. Acta *482* (1977) 71.
12 Abeywardena, M.Y., McMurichie, E.J., Russell, G.R., and Charnock, J.S., Biochem. Pharmac. *33* (1984) 3649.
13 Abeywardena, M.Y., and Charnock, J.S., Biochim. biophys. Acta *729* (1983) 75.
14 Akera, T., Wiest, S.A., and Brody, T.M., Eur. J. Pharmac. *57* (1979) 343.
15 Akera, T., Yamamoto, S., Chubb, J., McNish, R., and Brody, T.M., Naunyn-Schmiedebergs Arch. Pharmak. *308* (1979) 81.

0014-4754/86/040405-01$1.50 + 0.20/0

Effect of taurine administration on liver lipids in guinea pig

A. Cantafora, A. Mantovani, R. Masella, L. Mechelli and D. Alvaro*

*Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome (Italy), and * II Cattedra di Gastroenterologia, Università La Sapienza, Viale dell'Università 37, I-00185 Roma (Italy), 10 June 1985*

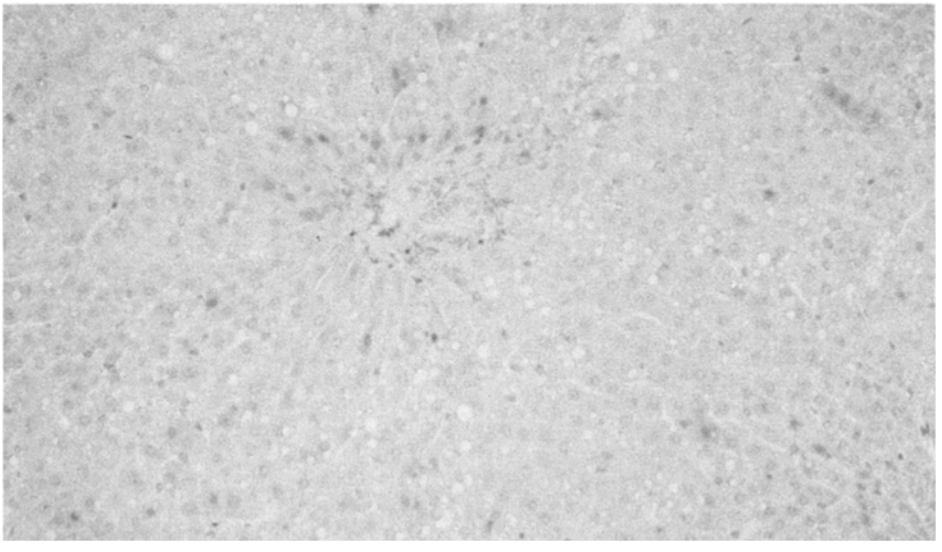
Summary. The oral administration of 0.4% taurine in drinking water for 14 consecutive days showed the following hepatic effects in male guinea pig. The percentage of tauro-conjugated biliary bile acids was increased from 17.2–54.2%; the ratio liver weight/body weight was increased, and fatty change was induced. Liver triglyceride concentration was accordingly increased; diglyceride and phosphatidylcholine concentrations were reduced by the treatment, while phosphatidylethanolamine level was not affected. These changes suggest an adverse effect of taurine administration on phosphatidylcholine hepatic synthesis.
Key words. Taurine; fatty liver; bile acids; lipid metabolism.

Taurine is a sulfur containing amino acid, whose only known physiological role in the liver is the conjugation of bile acids[1]. Its administration to man or to animals with a low biosynthetic capacity for taurine in the liver, e.g., guinea pig or rabbit, can reverse the conjugation pattern of biliary bile acids from glyco-conjugation to tauro-conjugation[2,3]. Beneficial effects have been attributed to taurine administration in the clinical course of acute and chronic hepatitis[4,5], in drug-induced liver diseases[6] and in cirrhosis[7]. More recently, taurine has been administered, at relatively high doses, to children less than two years old to prevent retinal disfunction occurring in the course of long-term parenteral nutrition without taurine[8]. Experimentally taurine feeding alleviated the effects of hepato-toxic agents like naphthylisocyanate[9], carbon tetrachloride[10], and sulfolithocholate[11]. In this last study, however, a 5-day treatment with 0.5% taurine in drinking water induced a mild lipid accumulation and dilatation of endoplasmic reticulum membranes in guinea pig liver as shown by electron microscopy. The growing interest in the clinical use of taurine in adults and children led us to extend this finding by administering a similar dose of taurine for a longer period to guinea pigs.

Materials and methods. Taurine (2-aminoethansulfonic acid) was obtained from E. Merck, Darmstadt, FRG. All chemicals used were of analytical grade. Male Hartley guinea pigs, about 380 g each (purchased from Charles River, Calco, Italy), were housed in metabolic cages. Food and water were available ad libitum. Taurine was administered with drinking water (0.4% solution) which was freely available for 14 consecutive days. On the fifteenth day the animals were sacrificed by decapitation. Liver and bile were collected from each animal at the end of the trial.

Liver lipid analysis. About 2 g of liver were homogenized and their lipids were quantitatively extracted with chloroform-methanol following Folch et al.[8]. The lipid extract was fractionated by thin-layer chromatography and analyzed by gas chromatography following a method derived by Christie et al.[12]. Briefly the extracts were fractionated by thin-layer chromatography on silica gel precoated plates with the following solvent systems; n-hexane-diethyl ether-acetic acid (70:30:1, by vol.) for neutral lipids, and chloroform-methanol-acetic acid-water (65:25:15:4 by vol.) for phospholipids. Bands corresponding to triglycerides (TG), diglycerides (DG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were revealed by iodine vapor, scraped off the plate and analyzed by gas-liquid chromatography as previously described[13].

Biliary bile acid analysis. Bile obtained by gallbladder puncture



Fatty infiltration in the liver of guinea pig treated for 14 days with taurine. H and E stain, × 100.