Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 588-593

ADVANCED GLYCATION OF RAT LIVER HISTONE OCTAMERS: AN IN VITRO STUDY

A. Gugliucci[1]

Department of Anatomy, University of Montreal, Montreal, Quebec, Canada

Received July 15, 1994

The question whether histones could accumulate AGE products and whether this reaction could take place even on the native histone octamer, the basic nucleosome unit, was addressed in this study. In vitro AGE formation on rat liver native histones octamers by incubation with different sugars was assessed. We provide evidence for the in vitro formation of both pentosidine and total AGE fluorescence on histone octamers in a time and sugar concentration dependent fashion. Ketoses (D-fructose and D-ribose) were more potent than aldoses. D-glucose-6-phosphate was three times as effective as D-glucose in generating AGE fluorescence. Advanced glycation of histone octamers led to the formation of cross links. For all sugars included in this study a similar pattern was observed: H2A and H3 bands disappear from electrophoretic runs.

Glycation of proteins, a reaction which is accelerated in diabetes mellitus, may alter protein structure and function through covalent attachment of sugar residues to ε-amine groups of lysine (1-2). In vitro glycation of DNA leads to mutations and transcription alterations in prokaryotes and has been suggested to play a role in increased terathogenesis as seen in diabetic offspring (2-5). Histones, which are present in the same amounts as DNA in nuclei (6), could theoretically be a target for glycation, at least at the same rate than DNA (7). Their high content in lysine and long half life would greatly favour the reaction but on the other hand their compact arrangement in nucleosomes would restrict this interaction.

Only recently, early glycation of isolated histones was first studied in vitro and an apparent increase in early glycation of histones in liver tissue from diabetic patients was documented (8-11). With time, early glycation adducts go on to generate several fragmentation products including 3-deoxyglucosone, a highly reactive aldehyde. This compound reacts in turn with free amino groups to form advanced glycation end products (AGE).

AGE products may be involved in the molecular pathogeny of diabetic complications (2). One of the recently characterised AGE products is pentosidine. In

[1] Correspondence and reprint requests should be addressed to Alejandro Gugliucci,MD, PhD, Department of Anatomy, University of Montreal, CP 6128 succ centre ville, H3C 3J7, Montreal, Quebec, Canada. Fax: (514) 343 2459, E-mail: gugliuca@alize.ere.umontreal.ca.

0006-291X/94 $5.00

this work we set out to study to what extent histones could accumulate AGE products and whether this reaction could take place even on the native histone octamer, the basic nucleosome unit. For that purpose we studied in vitro AGE formation on rat liver native histones octamers by incubation with different sugars. We compared the rates of AGE formation on histone octamers by following total AGE fluorescence formation, pentosidine accumulation and electrophoretical changes in a kinetic study.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St Louis MO, USA)

Histone purification

Total histones were purified from livers of 200 g male Sprague-Dawley rats as described previously (12). Briefly, nuclei were first isolated from liver homogenates by ultracentrifugation on a gradient of sucrose according to the method of Bobel and Potter (13). Histones were separated from DNA by ultracentrifugation and the natural histone octamer complex was isolated as previously described (12). Washed nuclei were extracted for 1 h with 0.75 mol/l NaCl, 10 mmol/l Tris/HCl, pH 7.4, containing 1 mmol/l phenylmethylsulfonyl fluoride. This removes loosely bound H1 and H5. The residual nucleohistone was sedimented by ultracentrifugation at 150 000 x g for 16 h in a Beckman XL 70 ultracentrifuge (Bioanalytical System Group, Mississauga, Ont, Canada). The pellet was homogenised in the same buffer containing 2 mol/l NaCl and after adjusting DNA concentration to 4 mg /ml, the native histone octamer was extracted for 1 h. The DNA was pelleted by ultracentrifugation and the supernatant, containing the core histone octamers was conserved.

Glycation of core histones

Histone octamers were adjusted to 1 mg/ml and incubated under sterile conditions (after passage through a 0.2 μm Millipore membrane, Millipore, Mississauga, Ont, Canada) in 2 mol/l NaCl, 10 mmol/l Tris/HCl, pH 7.4, containing 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l NaN_3 and either 0.1 or 0.5 mol/l of the following sugars: D-glucose, D-glucose-6-phosphate, D-fucose, D-fructose and D-ribose. As controls, histones were incubated in buffer alone or in buffer containing the same concentrations of D-sorbitol. At time 0 and at the indicated times aliquots of each incubation were taken, dialysed overnight against 2 mol/l NaCl, 10 mmol/l Tris/HCl, pH 7.4 and frozen at -80° C. Incubations were pursued up to 30 days.

Analysis of AGE products in histone octamers

AGE products were measured by their characteristic fluorescence properties, using a Turner 430 spectrophluorometer (AMSCO Inst. Carpinteria CA, USA). Histones were adjusted to 100 μg/ml and fluorescence was measured at excitation maximum of 370 nm and emission maximum of 440 nm for total AGE products and at excitation maximum of 335 nm and emission maximum of 385 nm for pentosidine (14). In both cases fluorescence was expressed in arbitrary units taking 1 as the fluorescence of each sample at time 0.

Electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions (PAGE-SDS) was run on 12% gels as described (15) using a Protean II xi Slab cell from BioRad (Bio Rad Laboratories, Mississauga, Ont, Canada). Gels were stained with either Coomasie Brilliant Blue R 250 or silver stained as previously described (15).

Proteins were measured by the method of Bradford (16).

RESULTS

Incubation of histone octamers in the presence of sugars led to the accumulation of *AGE specific fluorescence* in a time-dependent fashion (Fig. 1a).

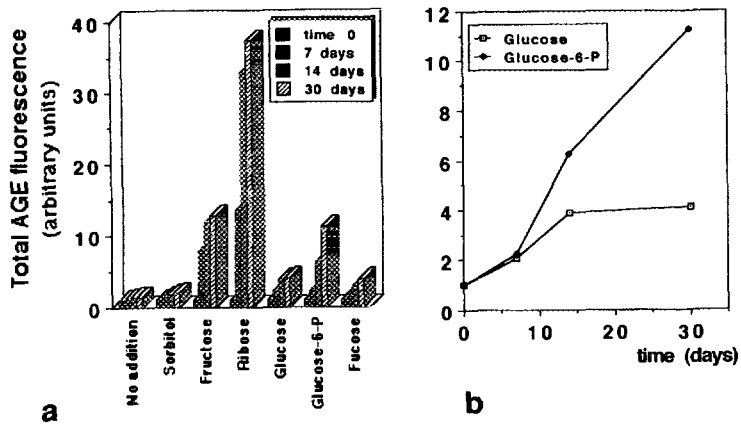

FIGURE 1. **Advanced glycation of histone octamers. Effect of time and sugar species on total AGE fluorescence.** Rat liver histone octamers were isolated as described in Methods and incubated in the presence of 0.5 mol/l of the sugars shown. Total AGE fluorescence was measured at excitation maximum of 370 nm and emission maximum of 440 nm. Values are the mean of two experiments measured in triplicate with a CV lower than 5%. a) Overall comparison of the effect of different sugars. b) Comparison of glucose and glucose-6-phosphate.

Ketoses (fructose and ribose) were more effective as glycating agents. Glucose and fucose, which have roughly the same percentage of open chains, gave essentially the same profiles. Glucose-6-phosphate was much more effective than glucose (Fig 1b). A byphasic temporal course is apparent for glucose, fucose and glucose-6-phosphate.

When *pentosidine fluorescence* was analyzed, a similar, though less pronounced increase in fluorescence was demonstrated (Figs. 2a and 2b).

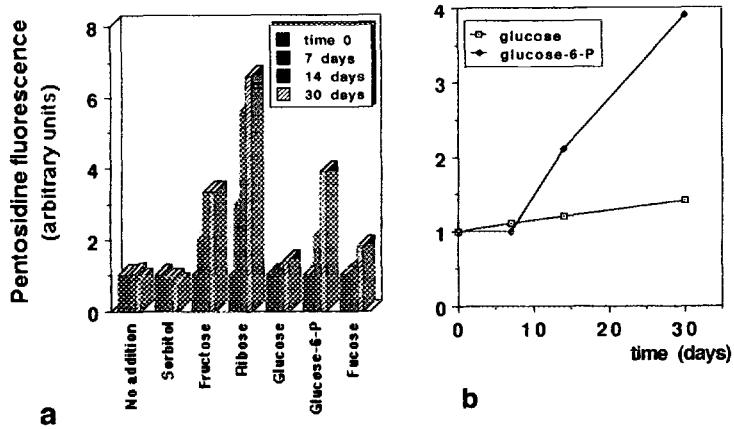

FIGURE 2. **Advanced glycation of histone octamers. Effect of time and sugar species on pentosidine formation.** Rat liver histone octamers were isolated as described in Methods and incubated in the presence of 0.5 mol/l of the sugars shown. Pentosidine fluorescence was measured at excitation maximum of 335 nm and emission maximum of 385 nm. Values are the mean of two experiments measured in triplicate with a CV lower than 8%. a) Overall comparison of the effect of different sugars. b) Comparison of glucose and glucose-6-phosphate.

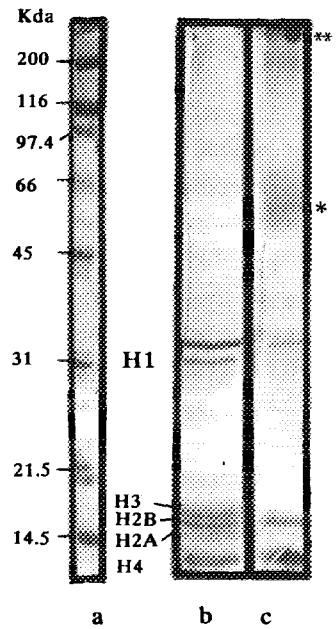

FIGURE 3. **Cross-linking of histones due to advanced glycation.** Control and glycated histones were applied to a 12% polyacrylamide gel with a stacking gel of 4%. Each lane contained 10 μg protein. Gel was silver stained as described in Methods. a) Molecular weight standards. b) Rat liver histones incubated for two weeks with sorbitol. c) Rat liver histones incubated for two weeks with glucose as described in Methods. Advanced glycation of histones led to the disappearance of H2A and H3 bands as well as to the the appearance of polymers of about 60 Kda (*) and highly polymerized forms of over 200 Kda (**).

In both cases a similar pattern of AGE accumulation was shown when lower concentrations (0.1 mol/l rather than 0.5 mol/l) of sugars were employed . Maximal increment amounted to aproximately one third that obtained with higher sugar concentrations (data not shown).

Advanced glycation of histone octamers led to *cross-linking of individual histone molecules* as shown in Figure 3. In Figure 3b rat liver histones were incubated for two weeks in the presence of sorbitol, as a control. Incubation with glucose for the same period is seen in Figure 3c. Regardless of the nature of the reducing sugar employed a similar pattern was observed: mainly the progressive dissapearence of H2A and H3 bands and the appearence of high molecular weight polymers.

DISCUSSION

Studies on glycation of isolated histones are quite recent, with the determination of early glycation (ketoamine formation in the presence of glucose) on individual histones (8-11). Histones are extremely abundant, rich in lysine and usually have the same life span as the cell (6), all of which make them a potential target for glycation. This reaction could occur during their rapid synthesis in the S

phase of the cell cycle, or during transcription. We wondered to what extent glycation could occur on the native histone nucleosome octamer. This prompted us to assess AGE formation on the native histone octamer in a comparative kinetic study. Besides glucose, which is not particularly high at the intracellular level,we included glucose-6-phosphate and fructose whose levels have been shown to be elevated in some target cells in diabetic complications. Glucose-6-phosphate, moreover, has been shown to alter DNA mutation rate in prokaryotic strains which accumulate this intermediate (5), and glyceraldehyde-3-phosphate can alter DNA in vitro being 200-fold more potent as a glycating agent than equimolar amounts of glucose (17). Ribose was also included since it has been shown to be an extremely potent glycating agent, given its high percentage of open chain form (18).

We provide evidence for the in vitro formation of both pentosidine and total AGE fluorescence on histone octamers in a time and sugar concentration dependent fashion. The kinetic patterns are essentially the same, total fluorescence amounting, however, near five-fold pentosidine fluorescence. As predicted on the basis of their respective reactivities (18), ketoses (fructose and ribose) were more potent than aldoses. When histones were glycated with hexoses a byphasic profile of AGE formation was demonstrated which is in accordance with previous reports on early glycation of histones by glucose (10). This was not evident for ketoses, suggesting different patterns of reaction.

Glucose-6-phosphate was three times as effective as glucose in generating AGE fluorescence as has been reported for other proteins (1), highlighting its putative role if this process occurs in vivo.

Advanced glycation of histone octamers led to the formation of cross links. This reaction became already apparent with only one week of incubation and increased with time. For all sugars included in this study, a similar pattern was observed: H2A and H3 bands disappear from electrophoretic runs under denaturing and dissociating conditions while high molecular weight polymeric forms appear. This would suggest the existence of particularly vulnerable sites on these molecules, even when they are packed in the nucleosome octamer structure. As expected, increased advanced glycation paralleled a significant decreased affinity for Coomassie stain (which binds to basic residues) but not to silver stain. If glycation of histones occurs in vivo, as has been previously reported (8), given their long half life, they could be the site of advanced glycation. This reaction can take place even after normalization of the initial high sugar insult (1-2). As lysine residues are constantly available for histone acetylation (6) they might as well be free and become glycated in the appropriate conditions. It is interesting to note here that glycation of nuclear components, as demonstrated by immunohistochemistry, has been previously reported (19). Although it is true that the sugar concentrations used in this work are not encountered in vivo, it is also certain that the times of reaction in vivo can be much longer than those employed in the present study. Hence, similar rections, though to a lesser degree, could conceivably take place given the long half-life of histones. Indeed a 7-fold increase in intracellular AGE has recently been demonstrated in culture cells incubated during only one week with 30 mmol/l glucose (20).

Our work thus provides evidence that histone octamers can easily accumulate AGE products in vitro, leading to cross-linking of individual histone components. The impact of such results and their potential significance for the in vivo situation remains however to be established.

Acknowledgments: I am greatly indebted to Dr. M. Bendayan for providing laboratory facilities as well as for fruitful discussions and encouragement throughout the elaboration of this study. This work was supported by the Medical Research Council of Canada and the Association du diabète du Québec.

REFERENCES

1. Brownlee M (1992) In: International Textbook of Diabetes Mellitus pp 669-682. John Wiley & Sons Ltd USA.
2. Vlassara, H., Bucala, R. and Striker, L. (1994) Lab. Invest., 70, 138-151.
3. Bucala, R., Model,P., Russel, M. and Cerami, A. (1985) Proc. Natl. Acad. Sci. USA, 82, 8439-8442.
4. Lee, A.T. and Cerami, A. (1987) Proc. Natl. Acad. Sci. USA, 84, 8311-8314.
5. Lee, A.T. and Cerami, A. (1991) Mutation Res. 249, 125-133.
6. McGhee, J.D. and Felsenfeld G. (1980) Ann. Rev. Biochem. 49, 1115-1156.
7. Bojanovic, J.J., Jevtovic A.D., Pantic, Dugandzic, S.M. and Javonovic D.S. (1970) Gerontologia, 16, 304-312.
8. Jobst, K., Lakatos, A., and Horvath, A. (1991) Clin. Chim. Acta, 200, 231-232.
9. Jobst, K., Lakatos, A., and Horvath, A (1990) Acta histochem., 888, 183-185.
10. Lakatos, A., and Jobst, K. (1991-1992) Acta Biochim. biophys. Hung., 26, 33-38.
11. Liebich, H.M., Gesele, E., Wirth., C., Wöll, J., Jobst, K. and Lakatos, A. (1993) Biol. Mass Spectrometry, 22, 121-123.
12. Ruiz Carrillo, A. and Jorcano, J.L. (1979) Biochemistry, 18, 760-768.
13. Blobel, H. and Potter J. (1966) Science, 154, 1662-1665.
14. Njoroge,F.G. and Monnier, V.M. (1989) Prog Clin Biol Res 304, 85-107.
15. Edelstein, S. and Bollag D.M. (1991) in Protein Methods , pp. 95-139 Wiley-Liss, Inc, NY.
16. Bradford, M.M. (1976) Anal. Biochem, 72, 248-254.
17. Mullokandov, E.A., Franklin, W.A. and Brownlee, M. (1994) Diabetologia, 37, 1 45-149.
18. Bunn, H.F. and Higgins, P.J. (1981) Science, 213, 222-224.
19. Kelly, S.B., Olerud, J.E., Witztum, J.L., Curtiss, L.K., Gown, A.M. and Odland, G.F. (1989) J. Invest. Dermatol, 93, 327-332.
20. Brownlee, M. Glycation and diabetic complications. (1994) Diabetes 43, 836-841.