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Nonenzymatic Glycation of Histones In Vitro and In Vivo

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Abstract Purified histones in solution, purified nuclei, or whole endothelial cells in cell culture were used to study the reactivity of histones with various sugars. The sugar incubation of purified histones produced nonenzymatic glycation and formation of histone cross-links showing disappearance of individual histone molecules and appearance of dimers and polymers in SDS–PAGE. In solution, core histones react considerably faster with sugars as compared to H1 histones. In sugar-incubated nuclei where histones are nucleosomally organized, H1 histones, which are located at the periphery of the nucleosome, and H2A-H2B dimers, which are associated with the central $H3_2$-$H4_2$ tetramer, are more reactive as compared to H3 and H4 histones, which are most protected from the glycation reaction. Our in vivo experiments using endothelial cells show that high concentrations of ribose are able to generate protein cross-links paralleled by apoptotic cell death. High concentrations of glucose or fructose do not increase histone glycation or cell death, even after 60 days of incubation of endothelial cells. In long-time glucose- or fructose-treated cells, under nondenaturing and nonreducing SDS–PAGE conditions part of the H3 histones shifted away from their normal location. Because it is known that the mitochondrial production of reactive oxygen species (ROS) increases after hyperglycaemia, we hypothesize that ROS could be responsible for the formation of a disulphide bridge between the side chain of the cysteine residues of H3 molecules. J. Cell. Biochem. 85: 24–34, 2002.

Key words: histones; nonenzymatic glycation; advanced glycation end products; protein cross-link; reducing sugars

Hyperglycemia seems to induce advanced protein glycation, a process involving the nonenzymatic modification of tissue proteins. The formation of these so-called advanced glycation end products (AGEs) may play a pivotal role in the pathogenesis of age-related disorders affecting connective tissue, lens, blood vessels, and nerves [Araki et al., 1992]. Recent reports provide evidence that the formation of AGEs may also be involved in the development of Alzheimer's disease [Smith et al., 1994; Vitek et al., 1994]. AGEs accumulate on proteins in diabetic patients with unphysiologically high blood sugar concentrations and also in healthy controls with normal blood sugar concentrations as a function of age. Accumulation of AGEs depends on sugar concentration, duration of sugar "incubation," and rate of protein turnover.

AGEs on extracellular molecules are exclusively glucose-derived. Because of the lesser intracellular concentration of glucose as compared to extracellular levels it was assumed that the formation of intracellular AGEs must be of long duration. Recently, however, it was found that glucose has the slowest rate of glycosylation product formation of any naturally occurring sugar. Inside cells, however, there are a number of other glycating sugars such as fructose, glucose-6-phosphate, and glyceraldehyde-3-phosphate. It is now known that the rate of AGE formation by these intracellular sugars is considerably faster than the rate of AGE formation by glucose [Bunn and Higgins, 1981; DeBellis and Horowitz, 1987; Monnier et al., 1991]. In vitro experiments have shown that after 5 days the level of fructose-derived AGEs is ten times greater than that of glucose-derived AGEs [McPherson et al., 1988]. More striking is the observation that in endothelial cells cultured for only 7 days in media with sixfold higher (30 mM) glucose concentrations than normal (5 mM) intracellular AGE content increases 14-fold as compared to endothelial cells grown in normal medium [Giardino et al., 1994].

A number of recent observations show that histones are highly susceptible to nonenzymatic

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glycation. Gugliucci [1994] provides evidence for the in vitro formation of both pentosidine and total AGE fluorescence on histones. The same group demonstrated that histones from the liver of diabetic rats show AGE levels threefold higher than those of their age-matched controls. They also showed that AGEs increase with the duration of diabetes and tend to increase with age as well [Gugliucci and Bendayan, 1995]. In the study by Cervantes-Laurean et al. [1996], ADP-ribose was shown to be a potent in vitro agent of histone glycation and glycoxidation. This group demonstrated that the incubation of ADP-ribose with histones at pH 7.5 results in the formation of ketoamine glycation conjugates. Quite recently, Jobst and Lakatos [1996] presented data which suggest that nonenzymatic glycation occurs and AGEs form in liver cell histones of diabetic individuals. The results of Wondrak et al. [2000] demonstrate that the carbonyl content of nuclear proteins increases following alkylating stress with preferential histone H1 carbonylation in vivo. Also HMG proteins seem to be a target for nonenzymatic glycation rather than enzymatic glycosylation [Medina and Haltiwanger, 1998].

The aim of our study was to compare the reactivity of the various histone variants with regard to the nonenzymatic glycation reaction in vitro and in vivo. We were able to demonstrate that in vitro core histones are much more reactive than H1 histones. When histones were nucleosomally organized, however, histone glycation was clearly diminished. Under these conditions H1 histones, which are located at the periphery of the nucleosome, are clearly more reactive as compared to core histones. Our in vivo experiments with endothelial cells show that high concentrations of ribose were able to generate histone protein cross-links paralleled by apoptotic cell death. Incubation with high concentrations of glucose and fructose for up to 2 months does not cause visible protein cross-links but induces reversible oxidation of histone H3.

MATERIALS AND METHODS

Isolation of Histone H1, Core Histone, and Total Histone From Mouse Liver Tissues

Frozen mouse livers (10 g) were thawed and homogenized in 60 ml of ice-cold 10 mM Tris/HCl (pH 7,4), 3 mM $MgCl_2$, 0.32 M sucrose, 0.1 mM mercaptoethanol, and 1 mM PMSF (solution A) using a Potter homogenizer. After centrifugation (1,000*g*, 10 min, 4°C), further homogenization of the tissue was achieved using a Dounce homogenizer. The cells were pelleted by centrifugation at 1,000*g* for 10 min at 4°C and the remaining pellet was homogenized in 60 ml of 10 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 2.2 M sucrose, 0.1 mM mercaptoethanol, and 1 mM PMSF (solution B) using a Dounce homogenizer. Thirty milliliters of the cell lysate was layered on to a 5 ml cushion of solution B and nuclei were pelleted by centrifugation at 113,000*g* for 60 min at 4°C. The pelleted nuclei were resuspended in solution A and centrifuged at 1,000*g* for 10 min at 4°C. The nuclei were used for incubation with different sugars or for preparation of histones. For preparation of total histones, the nuclear pellet was directly extracted with 0.2 M H_2SO_4. For the preparation of histone H1, the nuclear pellet was first homogenized and extracted with four volumes of 5% (w/v) $HClO_4$ for 60 min on ice with occassional vortex-mixing. Homogenization was achieved using a Dounce homogenizer. $HClO_4$-insoluble material was subsequently removed by centrifugation at 17,000*g* for 20 min at 4°C. The soluble H1 histones were precipitated by adding ice-cold TCA to a final concentration of 20% (w/v) for 1 h at 4°C. The precipitated histones were recovered by centrifugation at 17,000*g* for 20 min at 4°C. The pellet was washed with acidified acetone (1% concentrated HCl) and acetone, then dissolved in water containing 50 μM mercaptoethanol and lyophilized [Talasz et al., 1998]. For core histone preparation, the $HClO_4$-insoluble material was extracted with four volumes of 0.2 M H_2SO_4 for 1 h on ice and further processed as indicated above.

Glycation of Histones

H1 histones, core histones or total histones at a final concentration of 1 mg/ml were incubated with various sugars using 50 mM sodium phosphate buffer (pH 7.4), 0.015% NaN_3 and glucose (200 mM), fructose (200 mM), or ribose (200 mM). Reaction aliquots of 50 μl were collected at different time points after start of incubation, SDS–PAGE sample buffer was added, and the aliquot was stored immediately at −20°C. In the case of fluorescence measurements, the aliquots were dialyzed against water.

Incubation of Intact Nuclei With Various Sugars

Intact nuclei prepared from mouse livers were resuspended at a final protein concentration of 6 μg/μl in 50 mM sodium phosphate buffer (pH 7.4), 0.015% NaN_3 and Complete (Roche Diagnostics, Austria) as a protease inhibitor cocktail and incubated in the presence of 200 mM glucose, 200 mM fructose or 200 mM ribose at 37°C. The nuclei suspension was slowly rotated during the whole period of incubation, and the integrity of nuclei was monitored by phase-microscopy at the end of the incubation time. Incubation was stopped at various time points, aliquots were collected, SDS–PAGE sample buffer was added and aliquots were stored at −80°C. The nuclear proteins were separated on SDS–PAGE as described by Laemmli [1970]. Protein concentrations were determined using the bicinchoninic acid procedure [Smith et al., 1985] with the BCA protein assay kit (Pierce, Rockford).

SDS–PAGE and Detection of Histone Glycation

The histones were separated on SDS–PAGE (5% stacking gel, 15% separating gel) using a Mini Protean II slab gel unit (Bio-Rad Laboratories, USA), thereafter gels were stained with Coomassie blue, destained by diffusion and dryed. In the case of immunodetection of glycated histones, the SDS–PAGE-separated proteins were transferred on to nitrocellulose membrane Hybond ECL (Amersham Pharmacia Biotech, England) using a vertical Western blot equipment (Bio-Rad Laboratories, USA). Towbin blot buffer was used (25 mM Tris, 193 mM Glycin, 20% (v/v) methanol, and 0.1% SDS) with 2 h of blotting at 150 mA. After immobilization on nitrocellulose, the proteins were reversible stained with 0.2% (w/v) Ponceau S solution in 3% (w/v) acetic acid for 5 min to consider equal protein transfer. Sugars in glycoconjugates were detected using the DIG glycan detection kit (Roche Diagnostics, Austria) according to the instructions of the manufacturer with some variations. Briefly after blotting, the nitrocellulose filters were oxidized with 10 mM sodium metaperiodate in acetate buffer (pH 5.5) for 20 min, washed three times with PBS (50 mM potassium phosphate pH 6.5, 150 mM NaCl) and incubated with 1 μl DIG-*O*-3-succinyl-ε-aminocaproic acid hydrazide dissolved in 5 ml sodium acetate buffer pH 5.5 for 1 h. After three washes with TBS (50 mM Tris/HCl pH 7.5, 150 mM NaCl) the filters were incubated in blocking solution for 2 h at room temperature and thereafter washed three times in TBS. Digoxigenin labeled glycoconjugates are subsequently detected using anti-DIG antibody conjugated with horseradish peroxidase (1:10,000) (Roche Diagnostics, Austria) visualized using ECL detection reagents supplied by Amersham or anti-DIG antibody conjugated with alkaline phosphatase (1:1,000) (Roche Diagnostics, Austria) visualized using 4-Nitroblue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate.

Cell Culture and Incubation of ECV304 Cells With Various Sugars

Human endothelial cells ECV304 [Takahashi et al., 1990] were used for cell culture experiments. The cells were grown in monolayer culture and cultivated in Medium 199 (Life Technologies, Austria) supplemented with 10% (v/v) heat-inactivated FCS, penicillin (60 μg/ml), and streptomycin (100 μg/ml) in the presence of 5% CO_2. Cells were incubated with 200 mM glucose or 200 mM fructose for short-term (10 days) and long-term (60 days) incubations. In the case of ribose, we used 100 mM for short-term (7–10 days) and 25 mM for long-term (60 days) incubations. Because of the hypertonic conditions applied (200 mM glucose or fructose and at least 280 mM from sodium ions and cations), an appropriate correction of osmolarity was made using 200 mM mannitol-treated cells as control. Cell viability was routinely controlled by Trypan blue exclusion assay. Detection of the typical apoptotic features nuclear fragmentation and condensation of the chromatin at the nuclear membrane were used for measurement of apoptotic cell death. Briefly, detached cells were harvested and resuspended in phosphate buffered normal saline (pH 7.4). One hundred microliters of cell suspension were fixed with 100 μl of 3% paraformaldehyde for 5 min at 4°C, washed with destilled water, dropped (10 μl) onto a slide, and dried at room temperature. For inspection of chromatin, cells were stained with 8 μg/ml of Hoechst 33258.

For denaturing, reduced SDS–PAGE prepared nuclei of ECV 304 cells were lyzed in sample buffer containing 5% mercaptoethanol, 2% SDS and treated at 95°C for 5 min.

Nondenaturing, nonreduced SDS–PAGE was achieved using sample buffer without mercaptoethanol and omitting treatment at 95°C.

Analysis of AGE Products in Histones

AGE products were measured by their characteristic fluorescence properties using Ascent multiwell fluorescence reader (Labsystems Oy, Finland). For total AGE products, fluorescence was measured at an excitation maximum of 370 nm and an emission maximum of 440 nm and at an excitation maximum of 335 nm and an emission maximum of 380 nm for pentosidine [Sell and Monnier, 1989; Gugliucci and Bendayan, 1995].

Measurement of Lactate

Lactate was measured using the L-lactic acid kit (UV method) from Roche Diagnostics according to the instructions of the manufacturer. The volumes were adapted for reaction and measurement in 96-well plate (total volume was 260 μl/well). Reading of absorbance was at 340 nm with the iEMS photometer reader (Labsystems Oy, Finland).

RESULTS

Incubation of Histones With Various Sugars Causes the Disappearance of Individual Histone Molecules and the Formation of Dimers and Polymers

We first investigated whether incubation of histones in solutions with high sugar concentrations could induce the formation of advanced glycation end products. Figure 1 shows SDS–PAGE analysis of core histones (Fig. 1A,B) and H1 histones (Fig. 1C,D) stained with Coomassie blue (Fig. 1A,C) and immunostained for glycoconjugate detection (Fig. 1B,D) following treatment with 200 mM glucose for up to 35 days. After 6 days of treatment, core histone dimers are clearly visible in SDS–PAGE and Western blot (Fig. 1A,B, 6 days). The H3 and H4 histones are not as reactive with glucose as compared to H2A and H2B because the appearance of cross-linked histone molecules after 6–26 days of glucose incubation is accompanied by the progressive disappearance of H2A and H2B core histones. When H1 histones were analyzed, a similar but less pronounced reactivity with glucose was detected (Fig. 1C,D).

Ribose compared to glucose is clearly more reactive as a glycating agent, because 24 h after commencing incubation nearly the complete core histones are glycated and cross-linked to polymers (Fig. 2A,B). The reactivity of H1 histones with ribose is delayed as compared to core histones. The whole bulk of H1 histones,

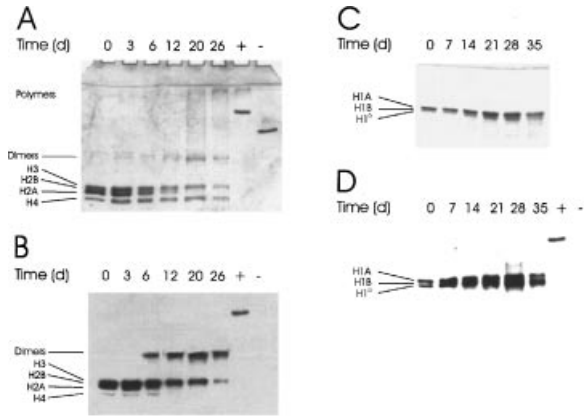

Fig. 1. Incubation of core and H1 histones with 200 mM glucose. **A**: Coomassie blue-stained SDS–PAGE of core histones incubated with glucose for 0, 3, 6, 12, 20, and 26 days. Formed dimers and polymers are clearly visible after 20 days of incubation. **B**: Same samples as in (A) after Western blotting and immunostaining for glycoconjugate detection. Dimers were visible after 6 days of incubation, whereas polymers were not detectable. **C**: Coomassie blue-stained SDS–PAGE of H1 histones incubated with glucose for 0, 7, 14, 21, 28, and 35 days. **D**: Same samples as in (C) after Western blotting and immunostaining for glycoconjugate detection. + transferrin as positive control, – creatinase as negative control.

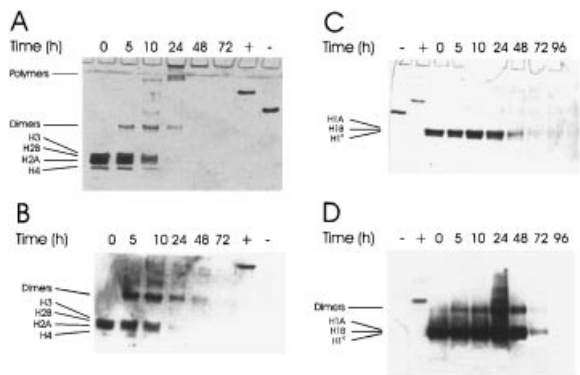

Fig. 2. Incubation of core and H1 histones with 200 mM ribose. **A**: Coomassie blue-stained SDS–PAGE of core histones incubated with glucose for 0, 5, 10, 24, 48, and 72 h. Formed dimers and polymers are clearly visible after 5 and 10 h of incubation. **B**: Same samples as in (A) after Western blotting and immunostaining for glycoconjugate detection. Dimers and polymers were visible after 5 h of incubation. **C**: Coomassie blue-stained SDS–PAGE of H1 histones incubated with 200 mM ribose for 0, 5, 10, 24, 48, 72, and 96 h. **D**: Same samples as in (C) after Western blotting and immunostaining for glycoconjugate detection. + transferrin as positive control, – creatinase as negative control.

however, is glycated and cross-linked after 72–96 h (Fig. 2C,D). Interestingly, we did observe the presence of a significant amount of periodate-reactive material in the Western blot on both the core and the H1 histones without sugar incubation (Figs. 1 and 2, timepoint 0). The faster reaction of core histones with sugars as compared to H1 histones was demonstrated in a mixture of total histones (core and H1 histones) incubated with 200 mM ribose. Whereas 72 h after incubation nearly all the cores had disappeared, H1 histones were detectable even after 120 h (Fig. 3).

The method used for detecting glycoconjugates is based on the metaperiodate reaction with cis-diols generating aldehyde groups which react with biotin hydrazide. It must be emphasized, however, that this method is a standard procedure for glycan detection that provides no specificity for nonenzymatically glycated species. Therefore, both enzymatically and nonenzymatically glycation are detectable with this method.

Increase in Total AGE- and Pentosidine-Induced Fluorescence After Histone Incubation With Sugars

To clarify whether histone incubation with sugars can cause accumulation of advanced glycation end products (AGE) we studied in vitro AGE formation on mouse liver histones by incubating them with various sugars. We compared the rates of AGE formation between core and H1 histones by measuring total AGE and pentosidine fluorescence. The two fluorescence measurements showed that ribose was more effective in glycating histones than was glucose (Fig. 4A,B). It is also obvious that core histones are more reactive than H1 histones,

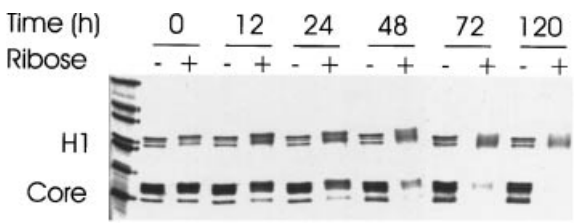

Fig. 3. Incubation of total histones (core and H1 histones) with 200 mM ribose. Coomassie blue-stained SDS–PAGE of total histones which were incubated with (+) or without (–) 200 mM ribose for 0, 12, 24, 48, 72, and 120 h. Core histones disappeared totally after 120 h of ribose incubation, whereas H1 histones were still visible at that time. Molecular marker proteins were run on the first lane.

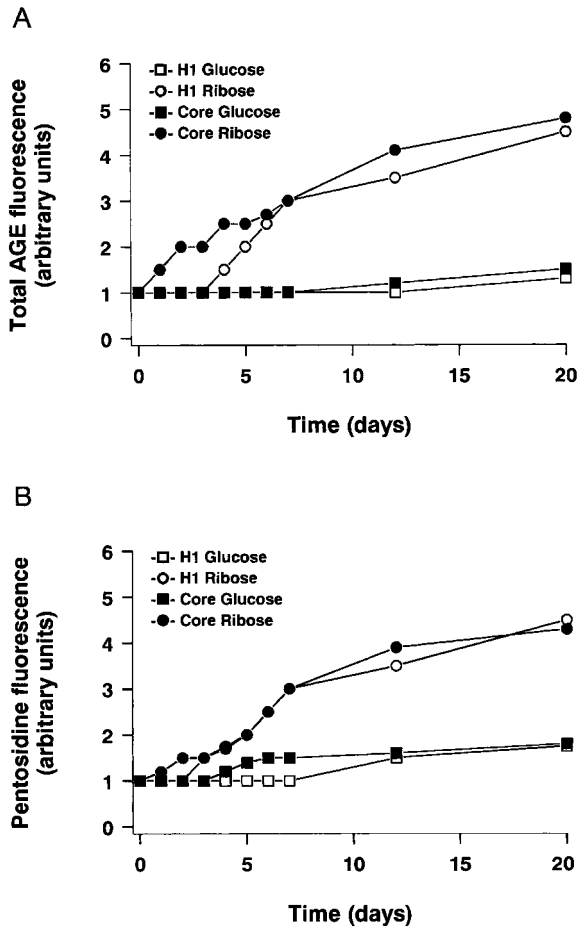

Fig. 4. Advanced glycation end products (AGE)- and pentosidine-induced fluorescence after histone incubation with glucose or ribose. **A**: The rates of AGE formation were compared for core and H1 histones by measuring fluorescence at an excitation maximum of 370 nm and an emission maximum of 440 nm. **B**: The rates of pentosidine formation were compared for core and H1 histones by measuring fluorescence at an excitation maximum of 335 nm and an emission maximum of 380 nm. Mean values were calculated from three independent experiments.

because both the AGE- and pentosidine-induced fluorescence increased earlier in the case of core histones. The results of fluorescence measurements seem to be consistent with our SDS–PAGE analysis. Therefore, we can conclude that the disappearance of histones and the formation of dimers and polymers caused by cross-linking of individual histone molecules must indeed be the result of advanced glycation.

Histones Organized in Chromatin Structure are Partly Protected From Glycation

Next, we wanted to know whether histone proteins which are organized in vivo in a higher-

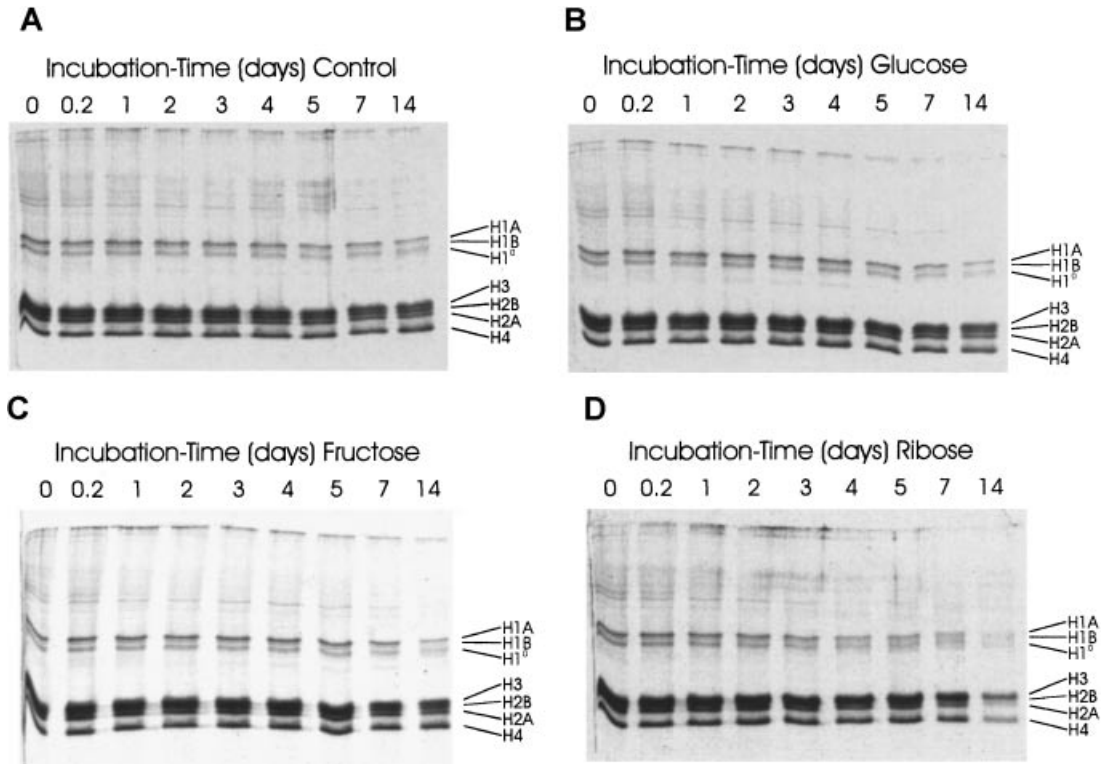

Fig. 5. Incubation of nuclei from mouse liver cells with various sugars. Intact nuclei prepared from mouse livers were resuspended at a final protein concentration of 6 μg/μl in 50 mM sodium phosphate buffer (pH 7.4) containing 0.015% NaN_3 and a protease inhibitor cocktail. Nuclei were incubated at 37°C without addition of sugar (Control) (**A**) or in the presence of 200 mM glucose (**B**), 200 mM fructose (**C**), 200 mM ribose (**D**) for the indicated time. The nuclear proteins were separated on SDS–PAGE. Coomassie blue-stained gels are shown.

order nucleosomal structure bound to DNA would also react with different sugars as compared to histones in solution. We therefore prepared nuclei of mouse liver and incubated them with glucose, fructose or ribose (Fig. 5A–D). The intranuclear sugar concentration corresponds most probably to the used extranuclear sugar concentration because the nuclear membrane is fully permeable for molecules up to 5 kDa. The results in Figure 5 indicate that the glycation of histone octamere and H1 linker histones organized in chromatin is delayed as compared to glycation of histones in solution. This delay is best visible in ribose-incubated nuclei (Fig. 5D). Ribose treatment of histones organized in nuclear chromatin results in nearly total disappearance of individual H2A, H2B, and H1 histone molecules after 2 weeks of incubation, whereas H3 and H4 histones are only diminished but clearly visible at that time (Fig. 5D, 14 days). Interestingly, in sugar incubation of nuclei H1 histones were more susceptible to glycation as compared to core histones, which is in contrast to the incubation of sugars with histones in solution. The nucleosomal organization of chromatin could explain the higher susceptibility of H1 to glycation, because H1 histones are located more outside the nucleosome whereas core histones are more central and therefore seem to be better protected from glycation.

Effects on Histones of Endothelial Cells Treated With High Concentrations of Sugars

Endothelial cells (ECV304) were used to investigate histone glycation and cross-link formation following incubation with various sugars at high concentrations. Glucose (200 mM), fructose (200 mM), and ribose (100 mM) were chosen as glycating agents. Because the rate of nonenzymatic glycation is a function of sugar concentration, time of exposure and rate of protein turnover, we used the highest possible sugar concentrations which do not lead to cell death even after 60 days of incubation. The half-life of histones within nonproliferating cells

ranges between 4 and 5 months, potentially allowing the accumulation of nonenzymatic histone damage with aging. It seems to be reasonable, therefore, to incubate cells for a period long enough to accumulate the probably occurring nonenzymatic histone modifications.

Following treatment, nuclei were prepared, lyzed in SDS sample buffer, and analyzed directly on SDS–PAGE, followed by Coomassie blue staining or Western blot with glycation immunostaining. Whereas cells treated for 10 days with high concentrations (200 mM) of glucose or fructose grew normally and showed high cell viability (96%), cells treated with 50, 100, or 200 mM ribose died in a typical apoptotic manner within 5–10 days (data not shown). In the SDS gel, the treatment with 100 mM ribose resulted in less protein of H1 and core histones, the complete disappearance of other unknown proteins (Fig. 6A) and in cross-linked protein polymers which could not enter the separating or stacking gel. The glycation immunostain revealed the disappearance of histones but also of unknown glycated proteins. Glucose- or fructose-treated cells showed an increase in bulk $H1^0$ histone as compared to control cells (Fig. 6A), but no significant change in protein glycation or the disappearance of proteins. We therefore prolonged the period of sugar incubation to up to 60 days. For ribose we had to diminish the concentration to 25 mM, because higher concentrations resulted in rapid apoptotic cell death. The result of a 60-day incubation is shown in Figure 6B. Neither in Coomassie blue-stained SDS gel nor in glycation immunostain were significant differences detected in the bulk protein concentrations or the glycation content as compared to control cells. Neither specific disappearance of proteins nor formation of cross-links was observed (Fig. 6B). Our results demonstrate that incubation of endothelial cells for 8 weeks with high glucose or fructose or moderate ribose concentrations does not result in direct protein-sugar reaction, as was the case for histones in solution or in nuclei. Since we use unspecific detection of glycoconjugates in Western blots we conclude that differences occur neither in enzymatically nor nonenzymatically glycation.

However, proteins can be influenced indirectly by exposure to high sugar concentrations. It is known from experiments with cultured bovine aortic endothelial cells that hyperglycaemia increased the mitochondrial production

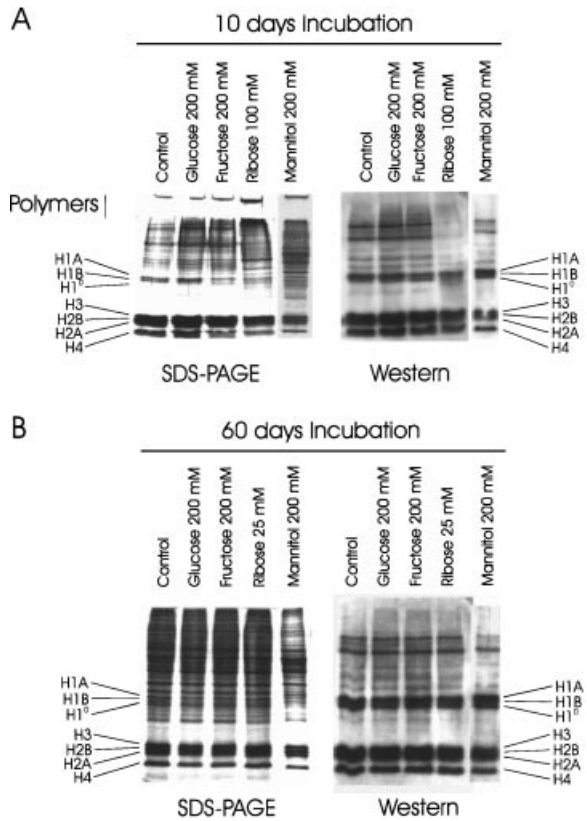

Fig. 6. Treatment of endothelial cells with various sugars. Endothelial cells (ECV 304) grown in monolayer culture were exposed to various sugars at high concentrations for a short (**A**) or long (**B**) incubation period. A: Ten days of incubation with 200 mM glucose, 200 mM fructose or 100 mM ribose. B: Sixty days of incubation with 200 mM glucose, 200 mM fructose or 25 mM ribose. Coomassie blue-stained SDS–PAGE and Western blots with immunostaining for glycoconjugate detection are shown. Mannitol (200 mM) was used as hyperosmolar control.

of reactive oxygen species [Giardino et al., 1996] via the transport of cytoplasmic pyruvate into mitochondria. To demonstrate increased glycolysis in cells treated with high sugar concentrations extracellular lactate measurement was used. In glucose- and fructose-treated cells the lactate concentrations reached significantly higher levels as compared to ribose-treated or control cells (Fig. 7A), indicating a higher level of glycolysis which is known to result in increased reactive oxygen species concentration. Denaturing, reducing SDS–PAGE treating the samples with 2% SDS, 5% mercaptoethanol at 95°C for 5 min were not able to show reversible oxidation of proteins, because this treatment reduced all oxidized forms of cell

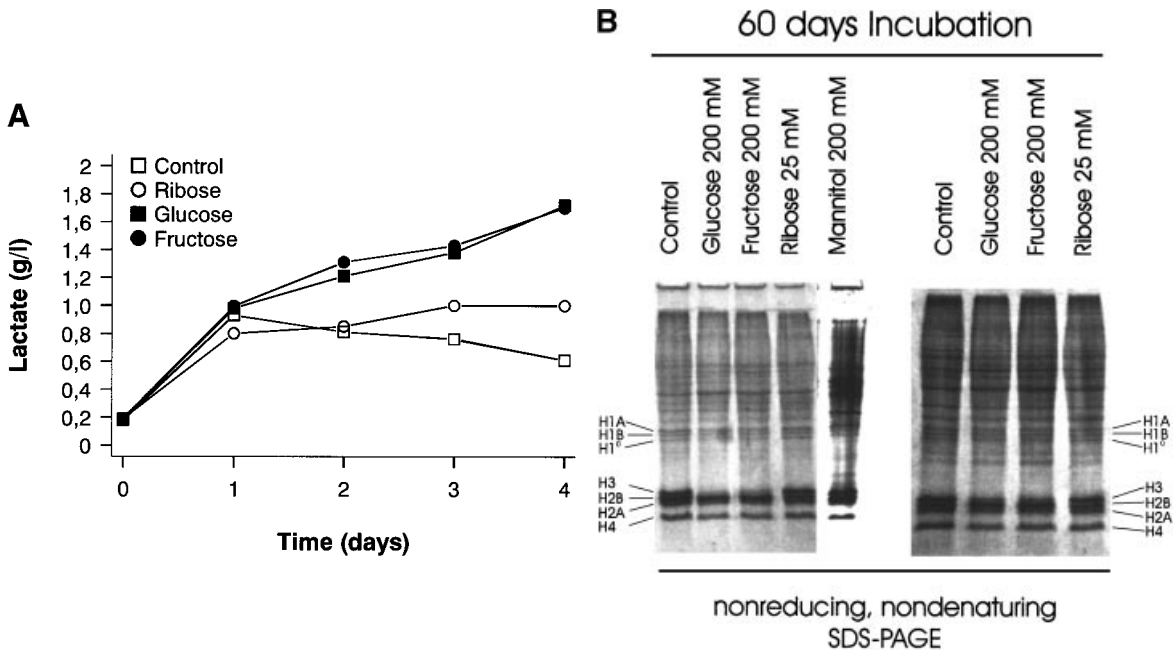

Fig. 7. Increased glycolysis and oxidized histone H3 in endothelial cells treated with high glucose or fructose. **A**: Extracellular lactate concentration as a sign of increased glycolysis. Measurement of lactate produced from endothelial cells treated with high glucose, fructose or ribose concentrations. Mean values were calculated from four independent experiments. **B**: Nuclear proteins from endothelial cells treated for 60 days with high concentrations of sugars were separated on SDS–PAGE using nonreducing and nondenaturing conditions (see Materials and Methods). Mannitol (200 mM) was used as hyperosmolar control. Coomassie blue-stained SDS–PAGE from two independent experiments are shown.

proteins. To obtain more information about the content of oxidized proteins we used nondenaturing, nonreduced SDS–PAGE [Manabe, 2000]. In glucose- or fructose-treated cells we indeed found partial or total loss of H3 histones and a significant reduction in H2A histones (Fig. 7B), indicating a reversible oxidation which induced a change in the running behavior on SDS gel. Because of the hypertonic conditions applied (200 mM glucose or 200 mM fructose plus at least 280 mM from sodium ions and cations) we used an equally hypertonic control with mannitol to make our results comparable. Also under 200 mM mannitol, the cells were viable at least for 60 days and showed differences neither in the normal SDS/PAGE nor in the nonreducing, nondenaturing SDS–PAGE as compared to control cells without mannitol (Figs. 6A,B and 7B).

Glycation Pattern of Tissues From Newborn or Adult Mice

Because the half-life of histones within nonproliferating mouse cells ranges between 4 and 5 months [Commerford et al., 1982], the accumulation of nonenzymatic histone glycation with aging is conceivable. We therefore examined the possibility that histone glycation increases with tissue age, comparing liver and brain of newborn and adult mice. Differences in the expression pattern between newborn (1–2 days) and adult (6–8 months) mice in liver and brain can be demonstrated for the histone $H1^0$, which was nearly not expressed in tissues from newborn mice (Fig. 8, lanes 1–4). Using glycoconjugate detection we demonstrated that no increase in the accumulation of glycated histones or in the formation of cross-links occurred in healthy adult mice (Fig. 8, lanes 5–8). This may reflect a very low glycation reactivity in mice with normal glucose metabolism or an efficient degradation of oxidized histones.

DISCUSSION

This report analyzes the reactivity of H1 and core histones with various sugars during the sugar incubation of purified histones in solution, purified nuclei or whole cells in cell culture. The sugar incubation of purified histones leads to nonenzymatic glycation, the formation of advanced glycation end products with protein

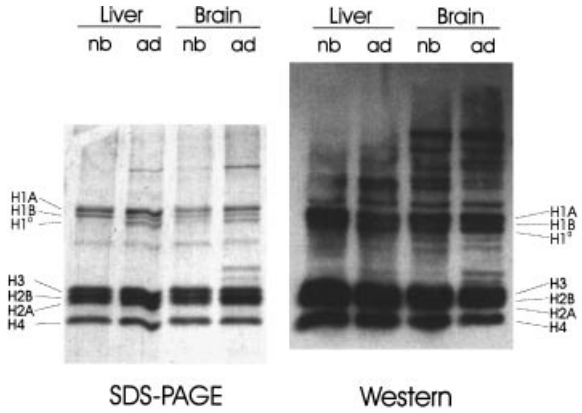

Fig. 8. Histone protein pattern from newborn and adult mouse brain and liver. Coomassie blue-stained SDS–PAGE and Western blots with immunostaining for glycoconjugate detection are shown. Nb, newborn mice (1 day), ad, adult mice (6–8 months).

cross-links and the disappearance of individual histone molecules in SDS gels. In contrast to H1 histones, core histones, especially H2A, followed by H2B, H3, and H4 show high reactivity. Pentosidine emerges as an end product of the Maillard reaction common to glucose, fructose, and ribose. For the formation of pentosidine cross-links both arginine and lysine residues are necessary [Grandhee and Monnier, 1991]. Therefore, the decreased reactivity of purified H1 histones which are very lysine-rich (29%) can be explained by an only minor arginine content of about 1% as compared to core histones with an arginine content of 11–14%. Furthermore, it is well known that proteins contain more or less reactive amino groups [Watkins et al., 1985]. Therefore, in core histones as compared to H1 histones more lysines and arginines with enhanced reactivity can be postulated.

In contrast to pure histones in solution, histones organized in chromatin structure, as in whole nuclei or cells, are clearly protected from glycation reaction, as can be seen in a delayed disappearance of H1 and core histones. When histones are organized in chromatin the H1 histones, which are located at the periphery of the nucleosome [Pruss et al., 1995; Crane-Robinson, 1997], are clearly more reactive as compared to core histones. The core histone octamer which is the central part of the nucleosome has a central kernel of $H3_2$-$H4_2$ tetramer associated with two H2A-H2B dimers [Pruss et al., 1995; Ramakrishnan, 1997]. In nuclei, the central $H3_2$-$H4_2$ tetramers are the most protected histones with regard to the glycation reaction. We can therefore conclude from our studies that for glycation in nuclei the accessibility of the histones in the chromatin structure seems to be more important than the chemical reactivity of the various histone variants. Interestingly, the formation of polymers and cross-links in sugar-treated nuclei is not as clearly visible in SDS gel as compared to sugar-treated histones. An explanation for this phenomenon could be the activity of the 20S proteasome-soluble proteinase complex, which is not only found in the cytosol but also in the nucleus of mammalian cells [Coux et al., 1996].

Our in vivo experiments with endothelial cells show that only high ribose concentrations (100 mM) are able to generate protein cross-links paralleled by the disappearance of various nonhistones and histones, in particular H1 histones (see Fig. 6A), resulting in apoptotic cell death after 7–10 days. For this reason, it is unlikely that cross-links of histone proteins alone are responsible for cell death. High fructose (200 mM) or glucose (200 mM) treatment of endothelial cells does not cause cell death showing no signs of increased glycation in SDS gels even after 60 days of treatment. A few possible mechanisms explained our observations: (1) The high extracellular sugar concentrations do not induce comparable high intracellular or intranuclear sugar concentrations which are necessary for the glycation reaction because of increased glycolysis. (2) The histones organized in chromatin structure are protected from the glycation reaction and (3), increased activity of the intranuclear proteasome complex may be responsible for efficient degradation of glycated or cross-linked histones.

The measured increase in lactate concentration during the highly concentrated sugar treatment reflects an elevated glycolysis as compared to control cells. It is well known that hyperglycaemia increases glycolysis, which generates high cytoplasmic pyruvate and NADH concentrations. Cytoplasmic NADH can donate reducing equivalents to the mitochondrial electron transport chain through two shuttle systems [Nishikawa et al., 2000], and it can reduce pyruvate to lactate, which leaves the cell to provide a substrate for hepatic gluconeogenesis. Experiments with cultured

bovine aortic endothelial cells have shown that hyperglycaemia increases the mitochondrial production of reactive oxygen species (ROS) by transporting cytoplasmic pyruvate into mitochondria [Giardino et al., 1996]. The sulfur-containing amino acids, cysteine and methionine, are especially sensitive to almost all forms of ROS-mediated oxidation although the side chains of other amino acids like arginine, lysine, proline are also known to be oxidatively modified [Berlett and Stadtman, 1997]. Under even mild oxidation conditions cysteine residues are converted to disulfides. Most biological systems, however, contain disulfide reductases that can convert the oxidized forms of cysteine residues back to their unmodified forms. Our findings that under nondenaturing and nonreducing conditions a considerable part of the H3 histones shifted away from their normal location in the SDS gel only in long-time glucose- and fructose-treated cells could be explained with a ROS-induced formation of a disulphide bridge between the side chain of the cysteine 110 of the two H3 molecules of a nucleosome [Camerini-Otero and Felsenfeld, 1977; Luger et al., 1997]. This reversible oxidation can be interpreted as the first sign of cell damage preceding irreversible oxidation processes and AGE formation.

It has been reported that histones organized in nucleosomes protect DNA from iron-mediated damage [Enright et al., 1992]. In an analogous manner, histones could be one of the first targets of nonenzymatic glycation or oxidation in the nuclei and could serve to protect the chemical integrity of DNA. However, because AGEs do accumulate on long-lived proteins as a function of time the protection mechanism could be overcome leading to irreversible DNA damage with age. Enzymatic post-translational modifications of histones are involved in the regulation of multiple chromatin functions, thus it is plausible that accumulation of irreversible oxidatively modified histones during aging or diabetes may alter chromatin structure and function in turn leading to changes in gene expression. Such a mechanism has been proposed to play a role in some of the pathologies associated with diabetes [Brownlee, 1995].

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