NON-ENZYMATIC GLYCOSYLATION OF HUMAN SERUM LIPOPROTEINS

Elevated ϵ-lysine glycosylated low density lipoprotein in diabetic patients

E. SCHLEICHER, T. DEUFEL and O. H. WIELAND

Klinisch-Chemisches Institut und Forschergruppe Diabetes, Akademisches Lehrkrankenhaus München-Schwabing, 8000 München 40, Kölner Platz 1, FRG

Received 14 April 1981

1. Introduction

The 'minor hemoglobins' (HbA_{1a-c}) were the first examples to show that glucose can react non-enzymatically with proteins in the human body to form stable glycosyl–protein adducts. Other proteins have been observed to undergo non-enzymatic glycosylation, such as the eye lens crystallins [1], red cell membrane proteins [2,3], serum albumin [4–6]. The amount of HbA_{1a-c} is increased in diabetic patients depending on the level of hyperglycemia; this holds also for glycosylated albumin [7,8], and erythrocyte membrane proteins [2,3]. Apart from the diagnostic value of HbA_1 and glycosyl–albumin as tools for long-term control of diabetes, protein glycosylation has attracted special interest with regard to the role it may play in the development of the late complications of diabetes. In these studies we have found that glucose is covalently bound to ϵ-amino groups of lysine of human apo-lipoproteins upon incubation, in vitro. Moreover, we could show that the level of glycosylated apoprotein B (apo-B) of the low density lipoproteins (LDL) is increased in the serum from diabetic patients.

2. Materials and methods

Human serum albumin and reagents for the quantitative determination of apo-A and apo-B by immunodiffusion were from Behringwerke (Marburg). Immobilised heparin (lipotype) was purchased from Panchem (Kleinwallstadt). Chemicals used for polyacrylamide gel electrophoresis were from Serva (Heidelberg), all other chemicals were from Merck (Darmstadt). HPLC analysis was done on a Waters apparatus (Milford USA). D-[U-^{14}C]Glucose was from Radiochemical Centre (Braunschweig). To remove any radioactive contaminants which are prone to react rapidly with albumin (unpublished) and could invalidate the in vitro labelling experiments [9], the preparation was pre-purified by dissolving 6.9 μmol D-[U-^{14}C]glucose in 1 ml phosphate-buffered saline (PBS) containing 2 mg human serum albumin. After 36 h incubation at 37°C the albumin was precipitated by acetone, the supernatant evaporated, and the glucose purified by gel-filtration on Sephadex G-25.

2.1. *Purification of lipoproteins*

Human serum lipoproteins for incubation studies were purified by ultracentrifugation essentially according to [10]. Apo-A and B content was estimated by radial immunodiffusion. LDL from normal and diabetic patients were isolated as follows: 0.5 ml serum was applied to a heparin column (Panchem) and the β-lipoproteins were eluted according to the supplier's instructions. After adjusting the density to 1.065 by extensive dialysis the β-lipoproteins were flotated by ultracentrifugation at 15°C for 18 h at 150 000 × g. LDL and VLDL were separated under the same conditions at a density of 1.006. LDL was precipitated and delipidated according to [11].

LDL from patients was also purified by immunoadsorption [12] on a column containing Sepharose-bound anti-apoprotein B immunoglobulin. The immobilized anti-apoprotein B was kindly provided

Dedicated to Professor Helmuth Holzer on the occasion of his 60th birthday

by Professor W. Stoffel (Köln). Serum samples of 1 ml were applied and recycled 5 times for complete absorption. After washing with PBS, the apo-B containing lipoproteins were eluted with 0.2 M glycine–HCl buffer (pH 2.8). VLDL was separated by ultracentrifugation and LDL was flotated at a density of 1.06.

2.2. *Determination of protein (lysine)-bound glucose*

Protein bound glucose was determined as in [13]. The method is based on the formation of furosine (ε-*N*-(2-furoylmethyl)-L-lysine) upon hydrolysis of the proteins in 6 N HCl which is separated on HPLC and quantified by its UV absorbance against a synthetic fructose–lysine standard. As a control, protein samples were treated prior to hydrolysis at pH 7.8 with ~10 mg $NaBH_4$ for 60 min at room temperature to reduce the ketoamine linkages, and dialyzed against PBS. After reduction no furosine peak was detectable.

2.3. *SDS–polyacrylamide gel electrophoresis*

Electrophoresis was done on 10% polyacrylamide gels containing 0.1% SDS as in [14]. Lipoproteins were dissolved in 100 μl 0.2% SDS containing 3 mM Tris–HCl buffer (pH 8.2) and 125 mM β-mercaptoethanol to reduce SH-bonds by heating to 95°C for 5 min followed by 1 h at 37°C. Prior to application of protein the gels were subjected to pre-electrophoresis for ~2 h The proteins were separated at 2.5 mA/gel for 1.5 h and stained overnight with Coomassie brilliant blue.

3. Results

3.1. *In vitro incorporation of glucose into lipoproteins*

Purified LDL and HDL from normal human serum incorporated glucose via ε-aminolysine groups in a time-dependent manner when incubated in vitro in a high glucose medium. This is illustrated in fig.1 which further indicates that most of the ^{14}C-radioactivity taken up is recovered by furosine analysis as lysine-bound glucose. The small remaining part may be attributable to other ketoamine linkages, e.g., of the N-terminal amino acids. As can also be seen in fig.1 small amounts of protein bound glucose were already present in the native lipoproteins, i.e., at zero time of incubation.

The dependence of lipoprotein glycosylation on the glucose concentration of the medium displayed also a linear relationship over a 10-fold range up to 55 mmol/l glucose (fig.2). These studies further indicate that the incorporation of glucose into LDL and HDL is by ~25% lower compared with human serum albumin. Further proof for covalent incorpora-

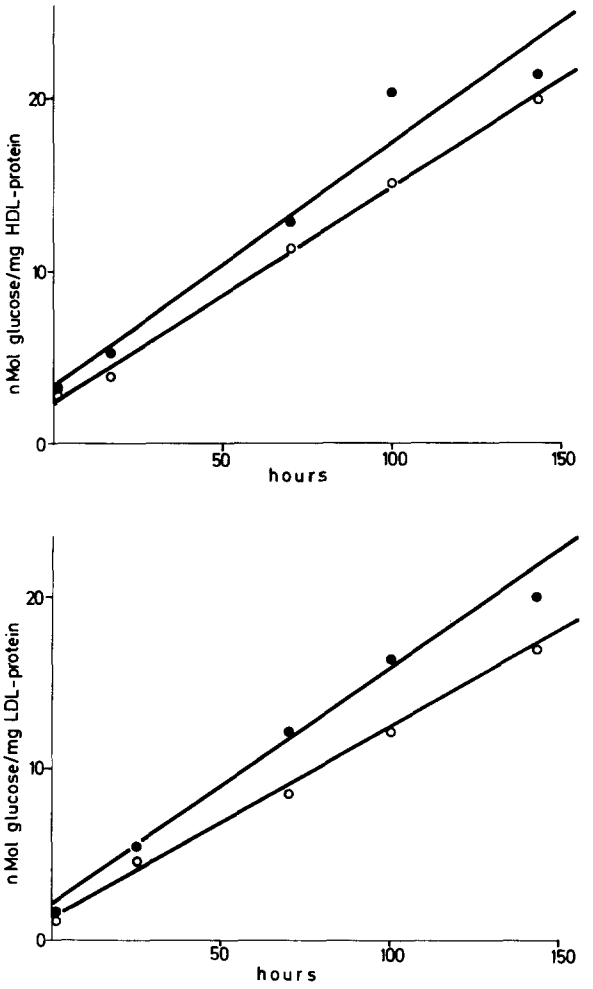

Fig.1. Time dependent incorporation of [U-^{14}C]glucose into HDL– and LDL–protein. HDL and LDL with a protein content of 2.55 mg apo-A and 2.45 mg apo-B, respectively were incubated at 37°C in 5 ml PBS (pH 7.4) containing 27.5 mmol [U-^{14}C]glucose/l with a specific radioactivity of 9.13×10^5 dpm/μmol, and 0.02% NaN_3. At the times indicated 1 ml samples were withdrawn, dialyzed against isotonic NaCl solution, and the protein precipitated and delipidated by acetone as in section 2. The precipitate was taken up in 6 N HCl, hydrolyzed and analyzed for furosine content as in [13]. A sample of the hydrolysate was taken for measurement of radioactivity using a Packard liquid scintillation counter. Data calculated from radioactivity (•) or from furosine (○).

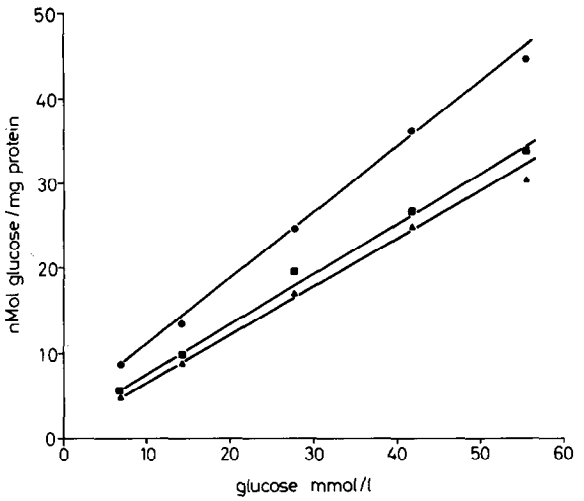

Fig.2. Concentration dependent incorporation of glucose into human serum albumin (•), LDL (■), and HDL (▲). Incubation was performed for 8 days at 37°C in 0.4 ml PBS (pH 7.4) containing 0.02% NaN_3 and increasing concentrations of glucose as given on the abscissa. Protein was 1 mg/ml. For further treatment see fig.1.

tion of [^{14}C]glucose into the protein moiety of the lipoprotein fractions was obtained by studying the distribution of radioactivity of the protein bands after SDS–polyacrylamide gel electrophoresis. As shown in fig.3(a,b) the bulk of radioactivity of LDL and VLDL, respectively, coincided with the fractions corresponding to apo-B. Small amounts of label appeared at the position of apo-E, and also in the apo-C region of VLDL. The VLDL-fraction also contained some albumin as indicated in fig.3(b). Fig.3(c) shows the results of the corresponding experiments with HDL. The radioactive label comigrates with the apoproteins A_1 and A_2, respectively. Both apoproteins seem to be glycosylated at about the same rate.

3.2. *Increase of glycosylated LDL in diabetes*

In order to establish if lipoprotein glycosylation is enhanced also in vivo at increased glucose concentrations we have examined the LDL fractions obtained from serum of diabetic patients. From the data in fig.4 it is clear that the LDL apo-B of diabetics contains a higher proportion of lysine bound glucose when compared with non-diabetic persons. Based on an apo-B M_r = 255 000 [15] normal apo-B is calculated to contain ~0.5 mol glucose/mol protein while in the diabetic with the highest level of glycosylated LDL this ratio was increased to 1.9.

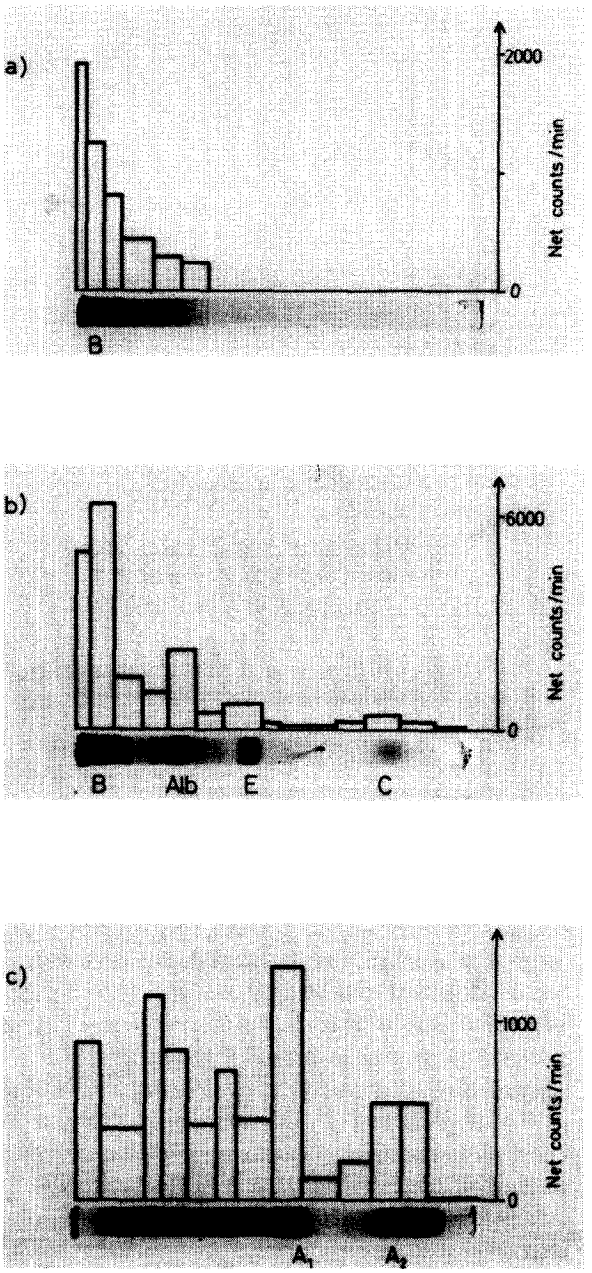

Fig.3. Analysis of [^{14}C]glucose-treated lipoproteins by SDS–polyacrylamide gel electrophoresis. VLDL (0.86 mg protein/ml), LDL (0.38 mg protein/ml), and HDL (1.76 mg/ml) were incubated at 37°C in PBS containing 31.1, 48.4 and 27.5 mmol [U-^{14}C]glucose/l (spec. radioact. 8–10 × 10^6 dpm) for 8 days (VLDL 13 days). After dialysis, acetone precipitation and delipidation the proteins were treated for electrophoresis as in section 2.3. (a) LDL; (b) VLDL; (c) HDL. LDL and HDL were kindly provided by Dr Münscher, Behringwerke (Marburg). A_1, A_2, B, C, E indicate the position of the corresponding apoproteins, Alb = albumin.

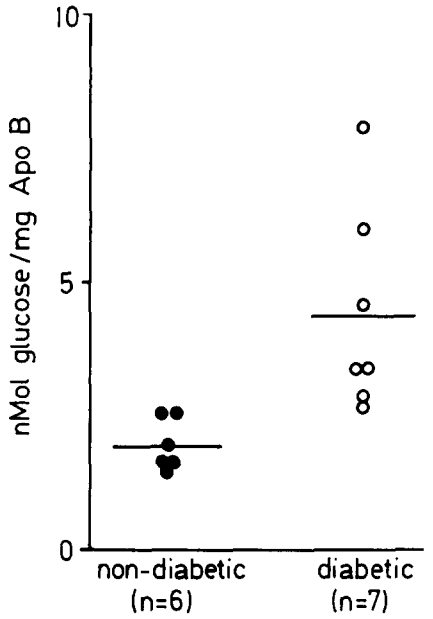

Fig.4. Glycosyl–LDL levels in non-diabetics (•) and diabetic patients (○). For methodological and analytical details see section 2.

4. Discussion

The discovery of a specific LDL-receptor pathway and its defect in familial hypercholesterolemia has provided new insight into the regulation of cholesterol metabolism and its inter-relationships with the development of atherogenesis [16]. The binding of lipoproteins to their receptors is determined by the protein moieties and can be changed by selective chemical modification of lysine or arginine residues of the apoproteins [17]. Of particular interest in these findings is that chemical modification of lysine residues of apo-B by acetylation [18] or malondialdehyde treatment [19] results in an increased cellular LDL-uptake and cholesterol accumulation via receptors (scavenger receptors) different from the specific receptors for native LDL. The observation on LDL-glycosylation described here is the first example to show that covalent modification of lysine groups of apo-B can take place in vivo. If this would also challenge the normal cellular LDL–receptor interactions, the increased level of glycosylated LDL in diabetic patients might help to a better understanding of the high incidence of vascular complications in diabetes. Cell culture studies on the properties of glycosylated lipoproteins are now under way.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg and the Fritz-Thyssen Stiftung, Köln.

References

[1] Stevens, V. J., Rouzer, C. A., Monnier, V. M. and Cerami, A. (1978) Proc. Natl. Acad. Sci. USA 75, 2918–2922.
[2] Bunn, H. F. and Briehl, R. W. (1970) J. Clin. Invest. 49, 1088–1095.
[3] Schleicher, E., Scheller, L. and Wieland, O. H. (1981) Biochem. Biophys. Res. Commun. 99, 1011–1019.
[4] Bailey, A. J., Robins, S. P. and Tanner, M. J. A. (1976) Biochim. Biophys. Acta 434, 51–57.
[5] Day, J. F., Thorpe, R. and Baynes, J. W. (1979) J. Biol. Chem. 254, 595–597.
[6] Dolhofer, R. and Wieland, O. H. (1979) FEBS Lett. 103, 282–286.
[7] McFarland, K. F., Catalano, E. W., Day, J. F., Thorpe, S. R. and Baynes, J. W. (1979) Diabetes 28, 1011–1014.
[8] Dolhofer, R. and Wieland, O. H. (1980) Diabetes 29, 417–422.
[9] Trueb, B., Holenstein, C. G., Fischer, R. W. and Winterhalter, K. H. (1980) J. Biol. Chem. 255, 6717–6720.
[10] Weisgerber, K. H., Bersot, T. P. and Mahley, R. W. (1980) J. Clin. Invest. 66, 901–907.
[11] Gressner, A. M., Köster-Eiserfunke, W., Van de Leuv, E. and Greiling, H. (1980) J. Clin. Chem. Clin. Biochem. 18, 279–285.
[12] Stoffel, W. and Demont, T. (1981) Proc. Natl. Acad. Sci. USA 78, 611–615.
[13] Schleicher, E. and Wieland, O. H. (1981) J. Clin. Chem. Clin. Biochem. 19, 81–87.
[14] Laemmli, U. K. (1970) Nature 227, 680–685.
[15] Zampighi, G., Reynolds, J. A. and Watt, R. M. (1980) J. Cell Biol. 87, 555–561.
[16] Goldstein, J. L. and Brown, M. S. (1977) Annu. Rev. Biochem. 46, 897–930.
[17] Mahley, R. W., Innerarity, T. L. and Weisgraber, K. H. (1980) Ann. NY Acad. Sci. 348, 265–277.
[18] Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) Proc. Natl. Acad. Sci. USA 76, 333–337.
[19] Fogelman, A. M., Shechter, J., Seager, J., Hokom, M., Child, J. S. and Edwards, P. A. (1980) Proc. Natl. Acad. Sci. USA 77, 2214–2218.