Physiol. Res. 49: 403-409, 2000

Changes of Acute-Phase Proteins in Streptozotocin-Induced Diabetic Rats

L. SASO, P. TOMMASINO, G. ITALIANO[1], E. GRIPPA, M.G. LEONE, M.T. GATTO, B. SILVESTRINI

Department of Pharmacology of Natural Substances and General Physiology, University of Rome, Rome and [1]Fidia Research Laboratories, Abano Terme, Italy

Received July 19, 1999
Accepted January 20, 2000

Summary

Quantitative and qualitative changes of serum proteins, apart from glycation, have not been sufficiently studied in streptozotocin-induced diabetic rats (D), the most common experimental model for diabetes. Thus, we decided to analyze the serum of diabetic rats by concanavalin A-blotting in comparison with rats with acute inflammation induced by fermented yeast (Y), in which characteristic alterations of serum proteins have been described. Two months after the streptozotocin treatment, the blood glucose levels were highly elevated (456±24 vs. 124±10 mg/dl, $p<0.001$, $n=12$), the body weight was significantly lower than normal (279±10 vs. 392±6 g, $p<0.001$, $n=12$), and serum proteins appeared to be highly glycated ($p<0.001$) when analyzed by the fructosamine assay, without any significant change in the total serum protein concentration. Analysis by concanavalin A-blotting, revealed a significant decrease of α_1-inhibitor-3 (α_1-I_3, $p<0.05$) and an increase of the β chain of haptoglobin (β-Hp, $p<0.05$) in both D and Y rats ($n=3$) compared with control animals. However, acute inflammation caused a marked rise of two prominent acute phase proteins, α_2-macroglobulin and hemopexin, which did not change appreciably in diabetic rats. Further work will be necessary to evaluate the physiopathological significance of these phenomena which could result from changes of both concentration and glycosylation of the aforementioned proteins.

Key words

Streptozotocin • Acute phase protein(s) • α_1-inhibitor-3 • Haptoglobin • Glycosylation

Introduction

It is known that, in clinical or experimental diabetes, serum proteins are subject to quantitative and qualitative changes:

1. A lower serum albumin concentrations, mainly due to augmented renal excretion, and increased serum levels of some acute phase proteins (APP) such as fibrinogen, α_1-acid glycoprotein, α_1-antitrypsin, haptoglobin (Hp), ceruloplasmin and C-reactive protein have been reported (McMillan 1989).

2. Qualitative changes, such as glycation, i.e. the non-enzymatic reaction between glucose and proteins in different tissues, detectable by the classical fructosamine assay (Johnson *et al.* 1983), have been extensively studied. It is established that glycation of specific proteins can be partly responsible for diabetic complications, such as cataract (crystallins), atherosclerosis (collagen and

Fax+420 2 24920590
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

lipoproteins), neuropathies (myelin and plasma proteins) and osteoporosis (osteocalcin) (Kennedy and Lyons 1997).

3. Enzymatic glycosylation, consisting of the conjugation of complex carbohydrate moieties to polypeptide chains, is known to play an important role in both physiological and pathological conditions (Dennis *et al.* 1999). In previous studies, we demonstrated that in inflammation, beside the characteristic quantitative changes of APP, specific changes of glycosylation occur (Silvestrini *et al.* 1989, Saso *et al.* 1993, 1999). Similar phenomena have been described in clinical diabetes (Nishio *et al.* 1995, Rellier *et al.* 1999) but only occasionally in streptozotocin-induced experimental diabetes (Samaniego *et al.* 1981, Chapman *et al.* 1991).

Thus, the aim of this study was to evaluate changes in the concentration and glycosylation of selected serum proteins of rats with experimental diabetes induced by streptozotocin, in comparison with the known alterations of the serum protein pattern of rats with generalized inflammation induced by fermented yeast (Silvestrini *et al.* 1967, Saso *et al.* 1999).

Materials and Methods

Reagents

Sodium phosphate, sodium chloride, 2-mercaptoethanol, concanavalin A (Con A, cat. N° C-7275), horseradish peroxidase (cat. N° P-8125), 4-chloro-1-naphthol, bovine serum albumin (BSA, Cohn's fraction V), Nonidet P-40 [octylphenoxypoly (ethoxyethanol)], sodium hydroxide, polyoxyethylene-sorbitan monolaurate (Tween-20, cat. N° P-7949), streptozotocin and nitro blue tetrazolium (NBT, cat. N° N-6876) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, N,N,N',N'-tetramethylenediamine (Temed), N,N'-diallyl-tartardiamide (DATD), N,N' methylenebis(acrylamide) (Bis), glycine, 2-mercaptoethanol, sodium dodecyl sulfate (SDS) and ammonium persulfate were obtained from Bio-Rad (Richmond, CA, USA). Prestained high molecular weight protein standards were from GIBCO/BRL/Life Technologies (Merelbeke, Belgium). Tris and phosphoric acid (85 %) were from Aldrich Chemical Co. (Milwaukee, WI, USA). Coomassie blue G was from United States Biochemical Corp. (Cleveland, OH, USA). Nitrocellulose paper (0.45 μm pore size) was from Schleicher and Schuell, Inc. (Keene, NH, USA).

Induction of diabetes by streptozotocin

We used 24 male Sprague-Dawley rats (Charles River, Calco, Italy) with an initial body weight of about 200 g. Laboratory chow (GRF18, Italiana Mangimi, Milan, Italy) and water were available *ad libitum*. Housing was controlled for temperature, humidity, and 12 h light-dark cycle. Diabetes was induced in 12 rats by injecting intraperitoneally a single dose of 65 mg/ml/kg of streptozotocin prepared in a citrate-phosphate buffer (28 mM citric acid and 44 mM dibasic sodium phosphate), pH 4.5. Twelve control rats (N) received an injection of a single dose of 1 ml/kg of the buffer. Animals were sacrificed 2 months after the induction of diabetes. Experiments were performed according to the F.E.L.A.S.A. guidelines concerning animal care and use.

Induction of acute inflammation

Inflammation was induced in Wistar rats of about 200 g body weight by injection of fermented brewer's yeast as previously described (Silvestrini *et al.* 1967). Briefly, yeast was suspended in sterile double distilled water at a concentration of 100 mg/ml, incubated for 15 h at 37 °C, kept for 24 h at 4 °C prior to its use and injected subcutaneously in the dose of 10 ml/kg body weight. After 48 h, blood was withdrawn by cardiac puncture under light anesthesia and allowed to clot at room temperature for about 2 h. Serum was obtained by centrifugation at 2000x *g* for 10 min. Samples were stored at –20 °C until used.

Blood glucose

Blood glucose levels were measured using Glucopat and Glucoscot reagent strips (Menarini, Italy).

Serum protein content

Serum protein content was determined by the dye-binding assay of Bradford (1976) as modified by Macart and Gerbaut (1982). Briefly, 0.1 μl of serum were added in triplicate to 100 μl of the Bradford reagent (0.01 % Coomassie blue G, 5 % ethanol, 10 % phosphoric acid and 0.003 % SDS in double distilled water) in 96-well assay plates (Falcon 3911, Becton Dickinson, Oxnard, CA, USA). The calibration curve was prepared by adding increasing amounts (1-10 μg) of BSA to the same volume of the Bradford reagent. Hence, the absorbance of the wells was measured at 595 nm using an automatic microplate reader (model 3550 from Bio-Rad, Hercules, CA, USA), connected to a personal computer

equipped with the software Microplate Manager® (Bio-Rad) for data analysis.

Analysis by the fructosamine assay

The glycation of serum proteins was measured according to Johnson *et al.* (1983). Briefly, aliquots of 50 µl of serum were added to 450 µl of 100 mM carbonate buffer pH 10.8 at 22 °C, containing 0.5 mM NBT, and the samples were incubated for 1 h at 37 °C. Hence, aliquots of 100 µl were pipetted in quadruplicate onto a 96-well plate and the absorbance of the wells was measured at 595 nm as described above.

Analysis by concanavalin A-blotting

Aliquots of 0.3 µl of serum were fractionated by electrophoresis on 7.5 % *T* polyacrylamide gels (PAGE) in the presence of SDS (Laemmli 1970) and the proteins were electrophoretically transferred to nitrocellulose paper and stained with concanavalin A (Silvestrini *et al.* 1989). Densitometric analysis was performed using the scanner Hewlett Packard IIC, interfaced with a personal computer equipped with the image analysis software Winbasic 4® from Advanced American Biotechnology (Fullerton, CA, USA).

Statistical analysis

Data were analyzed by Student's t-test (normal distribution) or Mann-Whitney test (non-normal distribution) using the software Sigma-Stat 2.0 for Windows '95 (SPSS, Chicago, IL, USA). Differences with $p<0.05$ were considered as statistically significant.

Table 1. Changes of different physiological parameters following the induction of diabetes

	Controls	Diabetes
Number of animals	12	12
Body weight (g)	362±6	279±10**
Blood glucose (mg/dl)	124±10	456±24**
Glycation of serum proteins (A595 nm)	0.44±0.02	0.59±0.06**
Concentration of serum proteins (mg/dl)	5450±589	5360±832

*Significantly different from controls: ** $p<0.01$ by Student's t-test*

Results

A decrease of about 30 % of the body weight was observed in diabetic rats (D) compared with control animals (N), two months after the injection of streptozotocin (Table 1). Blood glucose levels were high (>400 mg/dl) in diabetic rats compared with normal levels of about 120 mg/dl in controls (Table 1).

Protein glycation, evaluated by the fructosamine assay, was highly increased in diabetic rats compared to controls (Table 1). The protein concentration of the samples, which is known to affect the results of the fructosamine assay (Thomas and Muller 1990) was not sgnificantly different in the two groups (Table 1).

When serum samples from diabetic rats and controls were analyzed by concanavalin A-blotting, many different bands were noted (Fig. 1A). The intensity of several bands differed in the two groups (N, lanes 1-5; vs. D, lanes 6-10), indicating changes in the concentration and/or affinity for concanavalin A (Fig. 1A). The densitometric analysis of the blot revealed a 25 % decrease for α_1-inhibitor-3 (α_1-I_3) and a 25 % increase for the β chain of haptoglobin (β-Hp) (Fig. 1B).

The serum protein pattern of diabetic rats was different from that of rats with yeast-induced acute inflammation (Fig. 1 vs. Fig. 2) which, together with the changes of α_1-I_3 and β-Hp, exhibited a marked increase of the acute phase proteins α_2-macroglobulin (α_2-M) and hemopexin (HPX) (Fig. 2B).

Discussion

We observed specific protein changes by lectin-blotting using concanavalin A (Fig. 1) in streptozotocin-induced diabetic rats (D), together with extensive non-enzymatic glycosylation due to marked hyperglycemia lasting about 2 months (Table 1), The serum protein pattern of diabetic rats was different both in normal rats (N) and rats with acute inflammation induced by injection of fermented yeast (Y). Both D and Y rats had a moderate decrease of α_1-inhibitor-3 (α_1-I_3) and an increase of the β-chain of haptoglobin (Hp) (Figs 1 and 2). However, two prominent acute phase proteins (APP), such as α_2-macroglobulin (α_2-M) and hemopexin (HPX) (Engler and Mege 1986), were elevated in Y but not in D rats, compared to normal Wistar (CTRL) and Sprague-Dawley (N) rats, respectively.

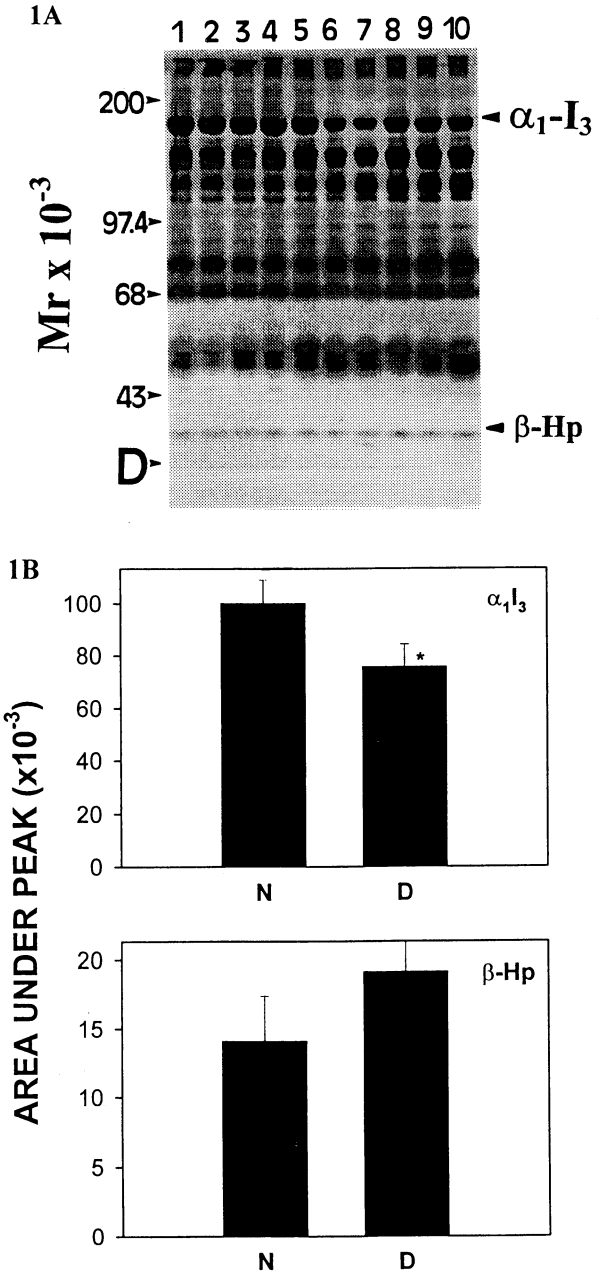

Fig. 1. *Analysis of serum of diabetic rats by concanavalin A-blotting.* ***(A)*** *Aliquots of 0.3 μl of serum from control Sprague-Dawley rats (N, 1-5) and streptozotocin-induced diabetic rats (D, lanes 6-10) were fractionated by SDS-PAGE on 7.5% T polyacrylamide gels and the proteins were electrophoretically transferred onto nitrocellulose paper and stained with concanavalin A. α_1-I_3, α_1-inhibitor-3; α_2-M, α_2-macroglobulin; HPX, hemopexin, β-Hp, β chain of haptoglobin. The molecular weight (Mr) standards were myosin (200 Kd), phosphorylase b (97.4 Kd), bovine serum albumin (68 Kd), and ovalbumin (43 Kd). D, dye front.* ***(B)*** *Densitometric analysis of the blot shown in Fig. 1A. D (n=12), streptozotocin-induced diabetic rats; N (n=12), control Sprague Dawley rats. α_1-I_3, α_1-inhibitor-3; β-Hp, β chain of haptoglobin. Data are means ± S.D. * $p<0.05$ according to Student's t test.*

α_1-I_3 belongs to the family of α-macroglobulins together with α_2-macroglobulin (α_2-M), α_1-macroglobulin, ovostatin and pregnancy zone protein (Sottrup-Jensen 1989). They are large glycoproteins present in the circulation both of vertebrates and invertebrates, acting as sophisticated proteinase inhibitors (Sottrup-Jensen 1989). α_2-M is also known to function as a carrier for interleukins and growth factors (Matsuda *et al.* 1989, Lysiak *et al.* 1995). Despite their common function, the regulation of these proteins is very different even in relatively close species such as the rat, in which is a prominent APP (Koj *et al.* 1988), and man, in which it is not (Housley 1968). Interestingly, diabetes is one of the few pathological conditions characterized by quantitative changes of α_2-M (James *et al.* 1980, Pozzilli *et al.* 1980, Ceriello *et al.* 1989, Skowron-Szlosarczyk *et al.* 1992). α_1-I_3, which is present like α_1-M in rodents but not in humans, is a negative APP, i.e. its concentration diminishes upon inflammation. Furthermore, the serum levels of α_1-I_3 were reduced in both experimental diabetes and acute inflammation (Figs 1 and 2), while α_2-M and HPX behaved differently (Fig. 2). This is also in agreement with a previously reported reduction of α_1I_3 in skeletal muscles of diabetic rats (Stauber *et al.* 1981, 1983, Komori *et al.* 1992). Although the physiopathological significance of these changes is still unknown, it is possible to speculate that variations of α_1-I_3 levels compensate other alterations of serum proteins, as described in hypoalbuminemic rats (Goto *et al.* 1988, Stevenson *et al.* 1998).

The increase of β-Hp in diabetic rats (Fig. 1) is consistent with the augmented concentration of this protein in diabetes, as described by other authors (Jonsson and Wales 1976, McMillan 1989, Rema *et al.* 1996), a phenomenon that could influence serum viscosity (McMillan 1989) and possibly be involved in the pathogenesis of diabetic retinopathy (Rema *et al.* 1996).

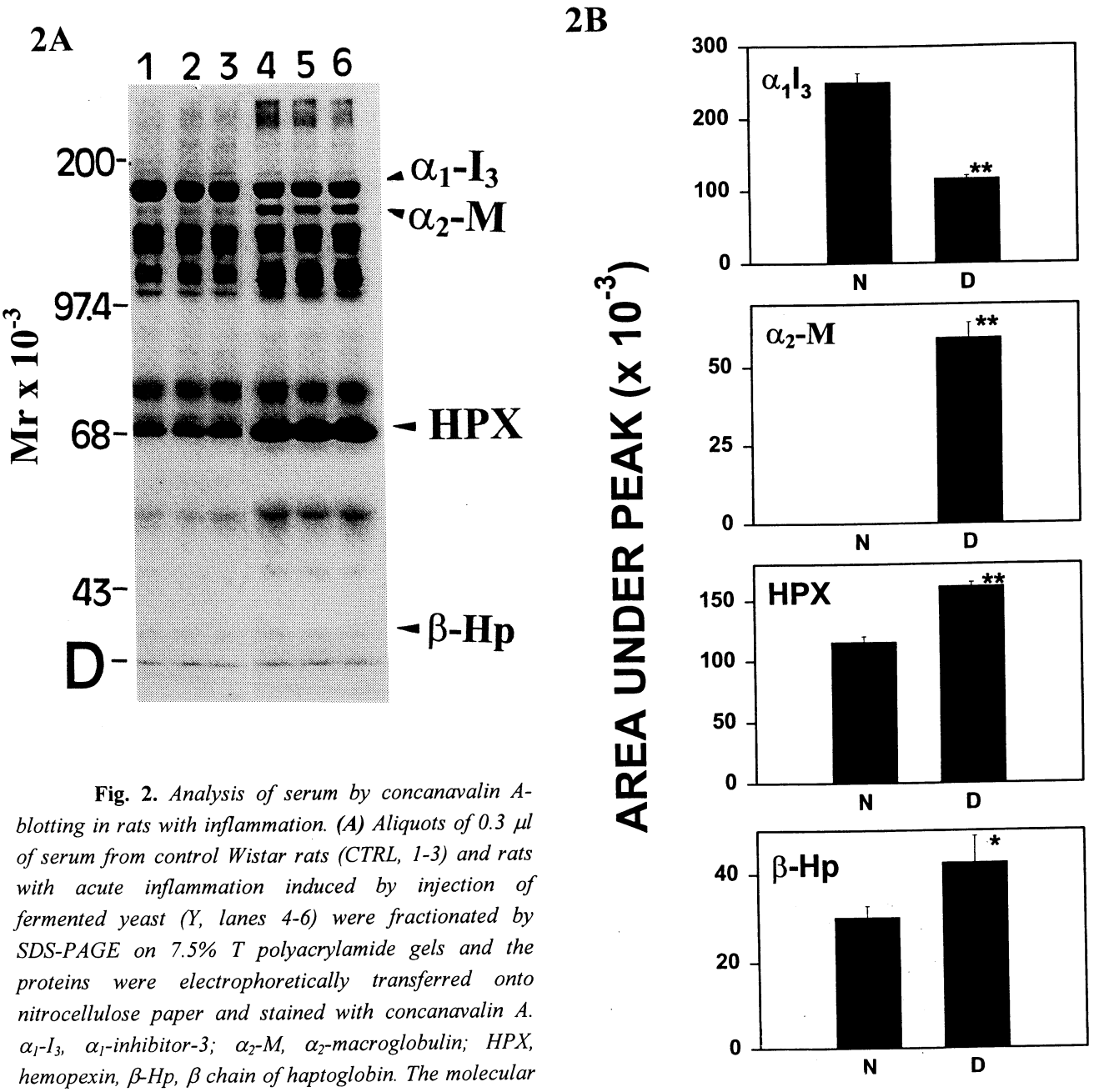

Fig. 2. *Analysis of serum by concanavalin A-blotting in rats with inflammation.* ***(A)*** *Aliquots of 0.3 µl of serum from control Wistar rats (CTRL, 1-3) and rats with acute inflammation induced by injection of fermented yeast (Y, lanes 4-6) were fractionated by SDS-PAGE on 7.5% T polyacrylamide gels and the proteins were electrophoretically transferred onto nitrocellulose paper and stained with concanavalin A. α_1-I_3, α_1-inhibitor-3; α_2-M, α_2-macroglobulin; HPX, hemopexin, β-Hp, β chain of haptoglobin. The molecular weight (Mr) standards were myosin (200 Kd), phosphorylase b (97.4 Kd), bovine serum albumin (68 Kd), and ovalbumin (43 Kd). D, dye front.* ***(B)*** *Densitometric analysis of the blot shown in Fig. 2A. CTRL (n=3), control Wistar rats. Y (n=3), inflamed rats; α_1-I_3, α_1-inhibitor-3; α_2-M, α_2-macroglobulin; HPX, hemopexin, β-Hp, β chain of haptoglobin. Data are mean ± S.D. ** $p<0.001$ and * $p<0.05$, according to Student's t test.*

Finally, it is necessary to stress that some of the protein changes described here could be due, besides quantitative alterations, to variations in the affinity of the carbohydrate moieties of proteins for concanavalin A, due to changes of enzymatic glycosylation. This would not be surprising for haptoglobin which is known to be abnormally glycosylated in different diseases (Saso *et al.* 1993, Turner 1995), including experimental diabetes (Chapman *et al.* 1991). However, with a few exceptions (Chapman *et al.* 1991, Nishio *et al.* 1995), the changes of enzymatic glycosylation have not been examined in diabetes, despite their possible involvement in pathological complications of the disease, as has been shown for glycation.

In conclusion, we reported characteristic qualitative and quantitative changes of APP in diabetic rats compared to control rats and rats with acute inflammation, but further work will be necessary to elucidate their pathophysiological significance.

Acknowledgements

This work was partially supported by a grant of the Noopolis Foundation.

Abbreviations

α_1-I_3, α_1-inhibitor-3; α_2-M, α_2-macroglobulin; APP, acute phase protein(s); β-Hp, β chain of haptoglobin; Con A, concanavalin A; CTRL, control Wistar rats; D, streptozotocin-induced diabetic rats; N, control Sprague-Dawley rats; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Y, rats with yeast-induced inflammation.

References

BRADFORD MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976.

CERIELLO A, GIUGLIANO D, QUATRARO A, STANTE A, DELLO RUSSO P, TORELLA, R: Increased α_2-macroglobulin in diabetes: a hyperglycemia related phenomenon associated with reduced antithrombin III activity. *Acta Diabetol Lat* **26**: 147-154, 1989.

CHAPMAN AE, COPELAND P, DAVIDSON S, CALHOUN JC: Diabetic BB/Wor rat haptoglobin exhibits a probable structural abnormality in Asn-linked oligosaccharides. *Biochim Biophys Acta* **1077**: 265-272, 1991.

DENNIS JW, GRANOVSKY M, WARREN CE: Protein glycosylation in development and disease. *Bioessays* **21**: 412-421, 1999.

ENGLER R, MEGE F: Biochemical characteristics of acute phase proteins in the rat. In: *Marker Proteins in Inflammation*, Vol 3, Walter de Gruyter Co, Berlin, New York, 1986, pp 231-241.

GOTO K, SAITO A, NAGASE S, SINOHARA H: Acute phase response of plasma proteins in analbuminemic rats. *J Biochem (Tokyo)* **104**: 952-955, 1988.

HOUSLEY J: α_2-Macroglobulin levels in disease in man. *J Clin Pathol* **21**: 27-31, 1968.

JAMES K, MERRIMAN J, GRAY RS, DUNCAN LJ, HERD R: Serum α_2-macroglobulin levels in diabetes. *J Clin Pathol* **33**: 163-166, 1980.

JOHNSON RN, METCALF PA, BAKER JR: Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin Chim Acta* **127**: 87-95, 1983.

JONSSON A, WALES JK: Blood glycoprotein levels in diabetes mellitus. *Diabetologia* **12**: 245-250, 1976.

KENNEDY AL, LYONS TJ: Glycation, oxidation, and lipoxidation in the development of diabetic complications. *Metabolism* **46**:14-21, 1997.

KOJ A, MAGIELSKA-ZERO D, KURDOWSKA A, BERETA J: Proteinase inhibitors as acute phase reactants: regulation of synthesis and turnover. *Adv Exp Med Biol* **240**: 171-181, 1988.

KOMORI K, ROBINSON KA, BLOCK NE, ROBERTS RC, BUSE, MG: Phosphorylation of the rodent negative acute-phase protein α_1-inhibitor-3 by the insulin receptor tyrosine kinase. *Endocrinology* **131**: 1288-1296, 1992.

LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685, 1970.

LYSIAK JJ, HUSSAINI IM, WEBB DJ, GLASS WF, ALLIETTA M, GONIAS SL: α_2-macroglobulin functions as a cytokine carrier to induce nitric oxide synthesis and cause nitric oxide-dependent cytotoxicity in the RAW 264.7 macrophage cell line. *J Biol Chem* **270**: 21919-21927, 1995.

MACART M, GERBAUT L: An improvement of the Coomassie Blue dye binding method allowing an equal sensitivity to various proteins: application to cerebrospinal fluid. *Clin Chim Acta* **122**: 93-101, 1982.

MATSUDA T, HIRANO T, NAGASAWA S, KISHIMOTO T: Identification of α_2-macroglobulin as a carrier protein for IL-6. *J Immunol* **142**: 148-152, 1989.

MCMILLAN DE: Increased levels of acute-phase serum proteins in diabetes. *Metabolism* **38**: 1042-1046, 1989.

NISHIO Y, WARREN CE, BUCZEK-THOMAS JA, RULFS J, KOYA D, AIELLO LP, FEENER EP, MILLER TB Jr, DENNIS JW, KING GL: Identification and characterization of a gene regulating enzymatic glycosylation which is induced by diabetes and hyperglycemia specifically in rat cardiac tissue. *J Clin Invest* **96**: 1759-1767, 1995.

POZZILLI P, Di MARIO U, JAVICOLI M, CHINCA M, ORSINI M: α_2-macroglobulin: its variability in diabetes. *Horm Metab Res* **12**: 409-410, 1980.

RELLIER N, RUGGIERO-LOPEZ D, LECOMTE M, LAGARDE M, WIERNSPERGER N: In vitro and in vivo alterations of enzymatic glycosylation in diabetes. *Life Sci* **64**:1571-1583, 1999.

REMA M, MOHAN V, SNEHALATHA C: Acute phase serum proteins in diabetic retinopathy. *Indian J Ophthalmol* **44**: 83-85, 1996.

SAMANIEGO FC, BERRY F, DICE JF: Selective depletion of small basic non-glycosylated proteins in diabetes. *Biochem J* **198**:149-157, 1981.

SASO L, SILVESTRINI B, GUGLIELMOTTI A, LAHITA R, CHENG CY: Abnormal glycosylation of α_2-macroglobulin, a non-acute-phase protein in patients with autoimmune diseases. *Inflammation* **17**:465-479, 1993.

SASO L, LEONE MG, MO MY, GRIPPA E, CHENG CY, SILVESTRINI B: Differential changes in α_2-macroglobulin and hemopexin in brain and liver in response to acute inflammation. *Biochemistry (Moscow)* **64**:839-844, 1999.

SILVESTRINI B, CATANESE B, CIOLI V, BURBERI S, SCORZA BARCELLONA P: Brewers yeast-induced inflammation in rats: investigation on some humoral and functional changes. *Boll Chim Farm* **106**: 385-397, 1967.

SILVESTRINI B, GUGLIELMOTTI A, SASO L, CHENG CY: Changes in concanavalin A-reactive proteins in inflammatory disorders. *Clin Chem* **35**:2207-2211, 1989.

SKOWRON-SZLOSARCZYK S, PODWYSOCKI B, WYSOCKA E, PACZYNSKI A: Blood serum angiotensin-converting enzyme, α_2-macroglobulin and triglycerides in patients with diabetes. *Mater Med Pol* **4**: 268-270, 1992.

SOTTRUP-JENSEN L: α-macroglobulins: structure, shape, and mechanism of proteinase complex formation. *J Biol Chem* **264**: 11539-11542, 1989.

STAUBER WT, GAUTHIER F, ONG SH: Identification and possible regulation of muscle cell lysosomal protease activity by exogenous protease inhibitors. *Acta Biol Med Ger* **40**: 1317-1322, 1981.

STAUBER WT, ONG SH, FRITZ VK, ESNARD F, GAUTHIER F: Protease inhibitor localization in control and streptozotocin-diabetic skeletal muscles. *Histochem J* **15**: 1079-1086, 1983.

STEVENSON FT, GREENE S, KAYSEN GA: Serum α_2-macroglobulin and α_1-inhibitor-3 concentrations are increased in hypoalbuminemia by post-transcriptional mechanisms. *Kidney Int* **53**: 67-75, 1998.

THOMAS L, MULLER TH: Impact of changes in plasma protein concentration on fructosamine values. *Wiener Klin Wochenschr* Suppl 180: 79-82, 1990.

TURNER GA: Haptoglobin: a potential reporter molecule for glycosylation changes in disease. *Adv Exp Med Biol* **376**: 231-238, 1995.

WEBB DJ, HUSSAINI IM, WEAVER AM, ATKINS TL, CHU CT, PIZZO SV, OWENS GK, GONIAS SL: Activated α_2-macroglobulin promotes mitogenesis in rat vascular smooth muscle cells by a mechanism that is independent of growth-factor-carrier activity. *Eur J Biochem* **234**: 714-722, 1995.

Reprint requests

B. Silvestrini, Department of Pharmacology of Natural Substances and General Physiology, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy. Fax: +39-06-49912480, E-mail: silvestrini@uniroma1.it