The hepatic acute-phase proteins α_1-antitrypsin and α_2-macroglobulin inhibit binding of transferrin to its receptor

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Transferrin binding to human placental sites was inhibited by the acute-phase proteins α_1-antitrypsin (α_1-AT) and α_2-macroglobulin (α_2-MG), whereas haptoglobin, C-reactive protein and ferritin displayed no such effect. In equilibrium saturation binding assays, the effective acute-phase proteins decreased the apparent affinity of the binding sites for transferrin, but the transferrin binding-site density $B_{max.}$ was not significantly changed. For instance, the addition of 30 μM α_1-AT increased the K_D of transferrin from 8.46 ± 1.51 nM to 21.6 ± 3.04 nM; the $B_{max.}$ values were 1.17 ± 0.18 pmol/mg of protein and 1.04 ± 0.25 pmol/mg of protein respectively. In kinetic studies, α_1-AT decreased the association rate constant k_{+1} of the ^{125}I-transferrin-binding-site complex from $2.18(\pm 0.21) \times 10^7$ $M^{-1} \cdot min^{-1}$ to $3.99(\pm 0.18) \times 10^6$ $M^{-1} \cdot min^{-1}$. In contrast, the dissociation rate constant k_{-1} was not changed (0.0948 ± 0.002 min^{-1}, 0.089 ± 0.0017 min^{-1}). On isoelectric focusing, no alteration in transferrin protein pattern or shift in isoelectric point was detected in the presence of α_1-AT. Inhibition of transferrin binding by the acute-phase proteins α_1-AT and α_2-MG is competitive. Interestingly, inhibition is already present at physiological concentrations. However, full inhibition is only achieved at concentrations above the normal range, which are attained in acute-phase reactions.

INTRODUCTION

The concentrations of certain plasma proteins are distinctly increased in response to infections, malignant diseases and a variety of immunological disorders, in a complex reaction known as the acute-phase response. These acute-phase proteins are mainly produced by hepatocytes on stimulation by cytokines: interleukin 6 (IL-6) stimulates the synthesis of the full spectrum of acute-phase proteins, whereas interleukin 1 (IL-1) and tumour necrosis factor (TNF) have only a partial effect (Perlmutter et al., 1989; Castell et al., 1989; Titus et al., 1991). There are a number of biochemically distinct acute-phase proteins, such as α_1-antitrypsin (α_1-AT), α_2-macroglobulin (α_2-MG), haptoglobin, C-reactive protein and ferritin (van Snick, 1990), and, although their regulations are fairly well understood, their complex effects are still being investigated. One of the most interesting acute-phase proteins is α_1-AT, a 52 kDa glycoprotein produced by hepatocytes and macrophages, being the only acute-phase protein known to be associated with disease in the deficiency state. The main function of α_1-AT is the inhibition of potentially destructive enzymes such as neutrophil elastase (Carell et al., 1982). Moreover, α_1-AT alters phagocyte function by inhibiting monocyte and neutrophil chemokinesis and chemotaxis (Breit et al., 1983). The lack of α_1-AT leads to α_1-AT deficiency which is one of the most frequently inherited disorders associated with chronic liver disease and/or juvenile emphysema (Crystal, 1990). Changes in iron metabolism, which are among the many different physiological responses by which an organism reacts in an acute phase, are still poorly understood.

Iron and its metabolism are essential for all living organisms. Transferrin, the main iron-transport protein in human blood, is a monomeric glycoprotein with a molecular mass of about 80 kDa and a known amino acid sequence (MacGillivray et al., 1982). Its principal function lies in transporting iron from absorption sites (intestine) and storage sites (liver) to sites of utilization (mainly bone marrow) (Putnam, 1975; Aisen and Brown, 1975). Cellular iron uptake is mediated by specific cell-surface transferrin receptors (Jandl and Katz, 1963), which are ubiquitous in the human (Huebers and Finch 1987), but are found in especially large numbers in the placenta (Davies et al., 1981) and reticulocytes (Frazier et al., 1982; Deiss, 1983).

This study was designed to investigate a possible role for acute-phase proteins as mediators of the changes in iron–transferrin metabolism that can cause anaemia during infections and malignant diseases.

MATERIALS AND METHODS

Materials

Human diferric ^{125}I-transferrin (specific radioactivity about 1400 Ci/mmol) was obtained from Amersham International (Amersham, Bucks. U.K.) and human α_1-AT from C.R.T.S., Lille, France. α_1-AT was characterized by isoelectric focusing (IEF) (phenotype MM), the concentration was measured by nephelometry (Behring Diagnostics, Vienna, Austria) and the degree of purification was about 99% as estimated by immunoelectrophoresis. Only minor contamination with albumin was found (< 1%). Human transferrin was a gift from A. Bomford, London. For purification and characterization, see Bomford et al. (1986). α_2-MG, C-reactive protein, haptoglobin, dexamethasone and IL-6 (human recombinant, expressed in *Escherichia coli* activity $> 1 \times 10^7$ i.u./mg) were from Sigma (Munich, Germany). IL-1 was obtained from Collaborative Research (Lexington, MA, U.S.A.), TNF from Boehringer Ingelheim (Ingelheim, Germany), BSA from Serva (Heidelberg, Germany) and purified human ferritin (liver) from Chemicon International (Los Angeles, CA, U.S.A.).

IEF was performed on PhastGel IEF 4–6.5 from Pharmacia LKB, Uppsala, Sweden, employing the PhastSystem of Pharmacia LKB, according to the manufacturer's instructions.

Abbreviations used: α_1-AT, α_1-antitrypsin; α_2-MG, α_2-macroglobulin; $B_{max.}$, maximum density of binding sites; *nh*, pseudo-Hill coefficient; IL, interleukin; TNF, tumour necrosis factor; IEF, isoelectric focusing.

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Membrane preparation and binding experiments

Human placenta (weighing about 600 g) was obtained immediately after delivery and transferred to ice-cold buffer A (50 mM Hepes, pH 7.4, 100 mM KCl, 2 μg/ml trypsin inhibitor, 50 μM phenylmethanesulphonyl fluoride). After removal of connective tissue, the placenta was homogenized with an Ultra-Turax T25 homogenizer, diluted 1:10 (v/v) in buffer A and centrifuged four times at 30000 *g* for 30 min until the supernatant was clear. The rotor ($r_{av.}$ = 10.8 cm) was operated at 4 °C. The pellet was resuspended in buffer A and stored at −70 °C.

Equilibrium binding experiments and kinetic studies were performed as described by Anderson et al. (1986) with minor modifications. Membranes with 0.58–1.8 mg of protein/ml [protein measured as described by Lowry et al. (1951)] were incubated in buffer B (50 mM Hepes, pH 7.4, 100 mM KCl, 50 μM phenylmethanesulphonyl fluoride and 1% BSA) with 32.1–129 pM ^{125}I-transferrin in a final assay volume of 0.25 ml at 37 °C. Non-specific binding was defined by the addition of 1 μM diferric transferrin. For equilibrium saturation binding experiments the specific radioactivity of ^{125}I-transferrin was varied from 1400 Ci/mmol to 1.4 Ci/mmol by addition of unlabelled transferrin. After 2 h or at the times indicated (kinetic studies), the reaction was stopped with 600 μl of ice-cold buffer C [20% (w/v) poly(ethylene glycol) 6000, 10 mM $MgCl_2$ and 10 mM Tris/HCl, pH 7.4]. After centrifugation at 11000 *g* for 15 min and subsequent careful aspiration of the supernatant, the pellet was determined for radioactivity in a LKB 1282 compugamma counter.

IEF

IEF was performed as described (Penhallow et al., 1991) with the following minor differences: 4 μl of each sample was applied on a Pharmacia PhastSystem gel, and the gels were run up to 500 V·h for 25 min in a pH range of 4–6.5.

Data analysis

The binding-inhibition experiments were analysed by non-linear algorithms as described (Graziadei et al., 1989). Equilibrium and kinetic studies were calculated by linear regression. Unless indicated otherwise, the data are given as means ± S.E.M. of *n* determinations.

RESULTS

Inhibition of transferrin binding to its receptor by acute-phase proteins

Figure 1a shows examples of ^{125}I-transferrin-binding-inhibition profiles of the major acute-phase proteins. α_2-MG was the most potent inhibitor, followed by α_1-AT, which was approximately 10 times less active. Remarkably, C-reactive protein, haptoglobin and ferritin showed no inhibitory activity. The inhibition curves for α_2-MG and α_1-AT were monophasic but did not reveal complete inhibition down to the non-specific binding definition. The cytokines IL-1, IL-6 and TNF exhibited no effect, as also did dexamethasone (alone as well as in combination with IL-6) and albumin, one of the most interesting negative acute-phase proteins (van Snick, 1990).

Binding-inhibition studies with unlabelled diferric transferrin and apotransferrin yielded K_i values of 8.16 ± 0.83 nM (n = 9) and 160.3 ± 7.9 nM (n = 3) respectively (Figure 1b). The results of all binding-inhibition experiments are summarized in Table 1.

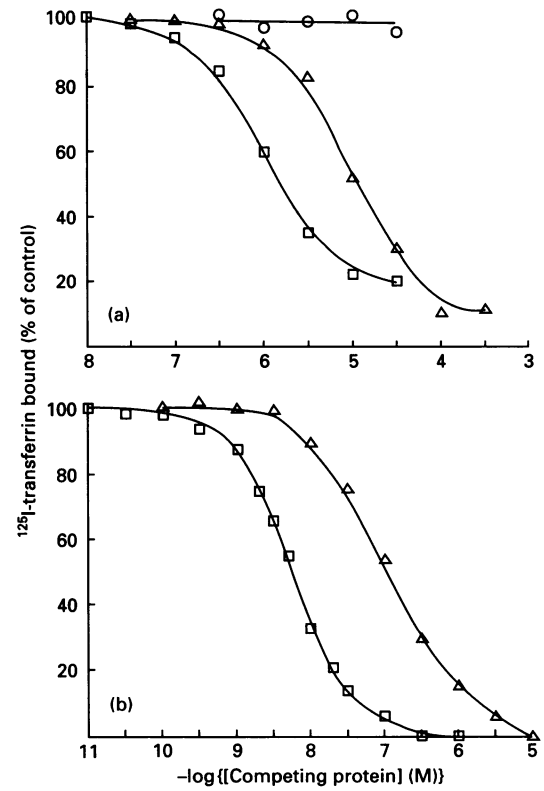

Figure 1 Profile of ^{125}I-transferrin-binding inhibition by (a) acute-phase proteins and (b) transferrin

Human placental membranes (0.58–1.8 mg of protein/ml) were incubated in an assay volume of 0.25 ml with 32.1–128 pM ^{125}I-transferrin in the presence of inhibitor at various concentrations for 120 min at 37 °C. (**a**) α_1-AT (△): K_i = 8.89 ± 0.41 μM, nh = −1.08 ± 0.09; α_2-MG (□): K_i = 0.77 ± 0.016 μM, nh = −1.18 ± 0.12; haptoglobin (○): no effect. (**b**) Diferric transferrin (□): K_i = 8.16 ± 0.83 nM, nh = −0.97 ± 0.05; apotransferrin (△): K_i = 160.3 ± 7.9, nh = −0.83 ± 0.09. The values are taken from different experiments each of which was performed in duplicate.

Table 1 Pharmacological profiles of transferrin-binding sites in human placenta

Human placental membranes (0.58–1.8 mg of protein/ml) were incubated in an assay volume of 0.25 ml with 32.1–129 pM ^{125}I-transferrin in 37 °C for 120 min in the presence of increasing substance concentrations. The values are means ± S.E.M., nh is the pseudo-Hill coefficient and n is the number of experiments, each of which was performed in duplicate. The percentage of maximal inhibition is the percentage of specific control binding at the highest concentration of substance tested. n.e., no effect up to highest concentration (given in parentheses).

Substance	K_i (nM)	nh	Maximal inhibition (%)	n
Diferric transferrin	8.16 ± 0.83	−0.97 ± 0.05	100	9
Apotransferrin	160.3 ± 7.9	−0.83 ± 0.09	100	3
α_1-AT	8890 ± 41	−1.08 ± 0.09	10.3 ± 1.7	4
α_2-MG	770 ± 16	−1.18 ± 0.12	23.6 ± 4.4	3
C-reactive protein	n.e. (1 μM)			2
Haptoglobin	n.e. (50 μM)			2
Ferritin	n.e. (1 μM)			2
IL-1	n.e. (30 μM)			2
IL-6	n.e. (30 μM)			3
TNF	n.e. (1 μM)			3
Dexamethasone	n.e. (100 μM)			3
Albumin	n.e. (100 M)			2

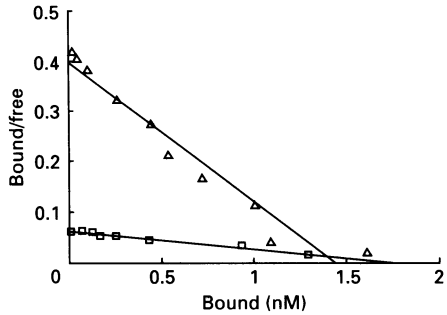

Figure 2 Saturation isotherm of ^{125}I-transferrin binding to human placental membranes in the presence of 30 μM α_1-AT

An assay volume of 0.25 ml containing 0.58–1.8 mg of protein/ml and ^{125}I-transferrin at various specific radioactivities from 1400 Ci/mmol to 1.4 Ci/mmol (addition of unlabelled diferric transferrin) was incubated for 120 min at 37 °C either with transferrin alone (control) or in the presence of 30 μM α_1-AT. The linear regression of the parameters computed from Scatchard transformation yielded the following data. Control: K_D = 7.59 nM, $B_{max.}$ = 1.43 nM, which is equivalent to 1.25 pmol/mg of protein ($r = -0.97$) (△); in the presence of 30 μM α_1-AT: K_D = 24.6 nM, $B_{max.}$ = 1.7 nM, which is equivalent to 1.49 pmol/mg of protein ($r = -0.99$) (□). The values are means of a single experiment performed in duplicate.

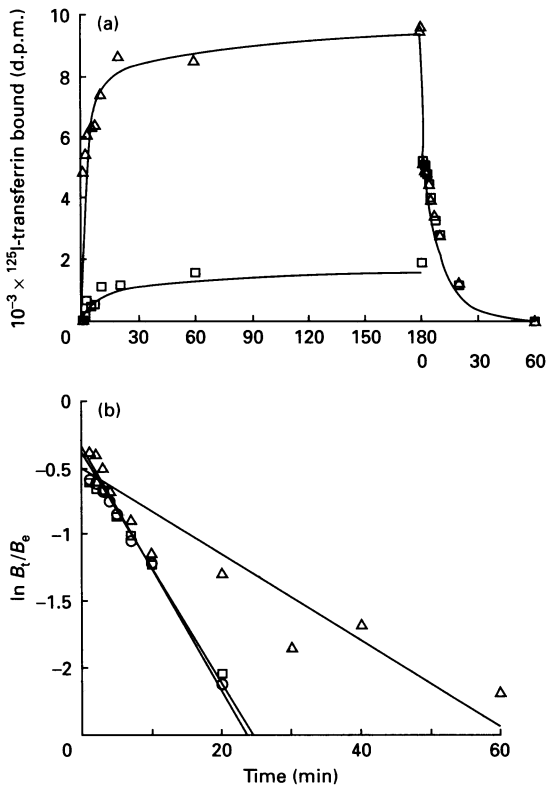

Figure 3 Association and dissociation kinetics of ^{125}I-transferrin binding to human placental membranes in the presence of 30 μM α_1-AT

(**a**) Association and dissociation kinetics. Human placental membranes (1.15 mg of protein/ml) were incubated with 79 pM ^{125}I-transferrin in an assay volume of 0.25 ml at 37 °C for the times indicated, either alone or with the addition of 30 μM α_1-AT. The calculated association rate constant, k_{+1}, was 2.50×10^7 M$^{-1}\cdot$min^{-1} (0–20 min, $r = 0.78$) for the control condition (△) and 3.82×10^6 M$^{-1}\cdot$min^{-1} (0–20 min, $r = 0.89$) in the presence of 30 μM α_1-AT (□). Dissociation was initiated after 180 min incubation by the addition of 1 μM diferric transferrin and 1 μM diferric transferrin with 30 μM α_1-AT. The values are from duplicate determinations. (**b**) Semilogarithmic plot of ln B_t/B_e (bound ligand measured for the indicated time [B_t]/bound ligand [B_e]) versus time. The slope was determined by linear regression and yielded the following dissociation constants: 1 μM transferrin (k_{-1} = 0.0869 min^{-1}, $r = -0.96$) (□), 1 μM transferrin together with 30 μM α_1-AT (k_{-1} = 0.0907 min^{-1}) (△) and 100 μM α_1-AT (k_{-1} = 0.0392 min^{-1}, $r = -0.91$) (○). The values shown are means of duplicate determinations.

Heating of α_1-AT for 30 min at 60 °C decreased its maximal inhibiting potency from 90% to 41%. When the incubation temperature was lowered from 37 °C to 4 °C, no significant change in the K_i value of α_1-AT was detected ($K = 7.11 \pm 0.21$ μM, $n = 2$). Furthermore, in experiments using α_1-AT instead of human placenta membrane particles, no specific binding of ^{125}I-transferrin to α_1-AT could be seen (results not shown).

Equilibrium saturation binding assay

Equilibrium saturation binding experiments with ^{125}I-transferrin on human placenta yielded a K_D value of 8.46 ± 1.51 nM and a $B_{max.}$ of 1.17 ± 0.18 pmol/mg of protein ($n = 6$). The addition of 30 μM α_1-AT to the assay caused the effect shown in Figure 2. The K_D value was significantly increased to 21.60 ± 3.04 nM, whereas the $B_{max.}$ value (1.04 ± 0.25 pmol/mg of protein) remained almost unchanged ($n = 3$). α_2-MG displayed the same property, the addition of 3 μM α_2-MG increasing the K_D value to 18.05 ± 1.64 nM while leaving unchanged the $B_{max.}$ value of 1.14 ± 0.12 pmol/mg of protein ($n = 2$) (not shown).

α_1-AT effects in kinetic studies

^{125}I-transferrin bound to human placental membranes with an association rate constant, k_{+1}, of $2.18(\pm 0.21) \times 10^7$ M$^{-1}\cdot$min^{-1} ($n = 3$). As depicted in Figure 3(a), the addition of 30 μM α_1-AT reduced the bound ^{125}I-transferrin by 17.6 ± 1.2% and decreased the k_{+1} value to $3.99(\pm 0.18) \times 10^6$ M$^{-1}\cdot$min^{-1} ($n = 3$). After the equilibrium had been reached, the addition of 1 μM unlabelled diferric transferrin led to an apparently monophasic decay of radioligand–receptor complex with a dissociation rate constant k_{-1} of 0.0948 ± 0.002 min^{-1} ($n = 3$). In the presence of 1 μM transferrin and 30 μM α_1-AT, the dissociation rate constant was not significantly changed ($k_{-1} = 0.089 \pm 0.017$ min^{-1} [$n = 3$]). α_1-AT alone at a concentration of 100 μM yielded a k_{-1} value of 0.0486 ± 0.0165 min^{-1} ($n = 2$) (see Figure 3b).

The dissociation constants for transferrin, either alone or in the presence of 30 μM α_1-AT, calculated from k_{-1}/k_{+1}, were 4.35 nM and 22.31 nM respectively and were in general agreement with the K_D values obtained by equilibrium saturation analysis and the K_i values derived from binding-inhibition experiments.

Kinetic studies of the addition of 3 μM α_2-MG produced similar effects to those of α_1-AT. The k_{+1} value was decreased [$(k_{+1} = 3.93(\pm 0.32) \times 10^6$ M$^{-1}\cdot$min^{-1} ($n = 2$)], whereas the k_{-1} value was not significantly changed [$k_{-1} = 0.091 \pm 0.002$ min^{-1} ($n = 2$)] (not shown).

These results (in combination with data from equilibrium binding experiments) indicate the α_1-AT and α_2-MG are competitive inhibitors of transferrin binding to its receptor.

IEF

In Figure 4, IEF gels are shown, focusing transferrin alone and in combination with 100 μM α_1-AT. IEF of transferrin is used for detection of the polymorphism of the transferrin molecule (Pascali et al., 1988). Differing degrees of, or major alterations in, sialation are detected by a shift in the isoelectric points. For example, removal of sialic acid produces a more basic isoelectric point (Woodworth and Balin, 1975; Penhallow et al., 1991). In our experiments, no change in the protein pattern or difference in isoelectric point could be detected.

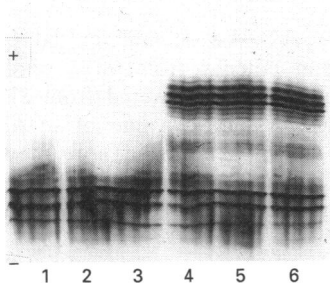

Figure 4 IEF of transferrin and transferrin plus α_1-AT

Diferric transferrin (4 μl of 50 μM), either alone (lanes 1–3) or in combination with 100 μM α_1-AT (lanes 4–6), was applied to the gel and run up to 500 V·h for 25 min. The pH range was from 4 [cathode(+)] to 6.5 [anode (−)]. The addition of α_1-AT produced no difference in the transferrin pattern in IEF.

DISCUSSION

The purpose of the present study was to investigate a possible interference of α_1-AT and other acute-phase proteins with iron metabolism in an attempt to understand the mechanism of acute-phase response-mediated anaemia. For this purpose, we performed radioactive binding experiments with ^{125}I-transferrin using membrane preparations of human placenta, which is generally known to be abundant in surface transferrin receptors. The dissociation constant for this placental transferrin receptor, as obtained in our experiments, whether determined by equilibrium saturation binding or derived from kinetic data, is in general agreement with previous studies (Newman et al., 1982; Trowbridge et al., 1984; Vogel et al., 1987). α_1-AT competitively inhibited ^{125}I-transferrin binding to the placental transferrin receptor in kinetic (Figures 3a and 3b) as well as equilibrium binding experiments (Figures 2a and 2b). α_1-AT did not alter the dissociation rate constant but decreased the association rate constant of ^{125}I-transferrin and, consequently, increased the K_D value. This result was confirmed in equilibrium saturation binding assays, in which α_1-AT again increased the K_D value, but no difference in $B_{max.}$ value was detected. The inhibition caused by α_1-AT was not complete, which is probably due to the limit of solubility of the proteins at the highest concentrations employed in our assays. Indeed, the concentrations of α_1-AT (more than 100 μM), measured by normal laboratory methods (nephelometry), were lower than the weighed-in concentrations used in the experiments. Remarkably, inhibiting by α_1-AT was already seen at low physiological concentrations (normal range 20–50 μM). Maximum inhibiting, however, was attained at concentrations above the normal range, which are achieved in acute-phase situations.

To explain the inhibiting effect of α_1-AT on transferrin, three principal modes of interaction can be considered: (1) alteration of the conformation of the transferrin molecule, and, consequently, a reduction in the affinity of transferrin for its receptor; (2) a change in the molecular mobility of the membrane surface and its receptors and/or change in the fluidity of surrounding membrane lipids; (3) direct competitive inhibition by α_1-AT at the binding site of the transferrin receptor. The first possibility can be rejected by exclusion of specific binding of α_1-AT to the transferrin molecule and vice versa and by showing that, after addition of α_1-AT, neither a change in the protein pattern of transferrin nor a difference in the isoelectric point was seen on IEF (Figure 4). In contradiction to the second assumption, α_1-AT did not lose its inhibitory activity when the incubation temperature was lowered from 37 °C to 4 °C. Therefore it is most likely that α_1-AT competes directly with transferrin for the receptor site. Kinetic and saturation experiments provide further support for this explanation.

α_2-MG, another acute-phase protein, displayed the same inhibitory activity as α_1-AT to transferrin binding. Inhibition was only partial in the physiological range (2–4 μM) and the maximal effect was reached with concentrations higher than the normal values. The inhibition was incomplete, suggesting that, for this protein also, problems of solubility at high concentrations arise, which may be responsible for this phenomenon. Likewise, equilibrium saturation and kinetic studies indicated direct competition of α_2-MG with transferrin for the receptor-binding site. Both α_1-AT and α_2-MG belong to the class of neutrophil proteinase inhibitor proteins. α_2-MG possesses one of the broadest spectrums of inhibiting activities. Because of its large molecular mass (725 kDa), its control of proteinase activity is restricted to the circulation (Travis, 1988), in contrast with α_1-AT which is able to penetrate into tissues.

Two other prominent members of the acute-phase protein family used in our competitive binding experiments, C-reactive protein and haptoglobin, did not inhibit ^{125}I-transferrin binding. In the human, they have distinctly different main functions from those of α_1-AT and α_2-MG. Besides being a diagnostic tool during acute-phase response, C-reactive protein demonstrates the property of complement fixation, enhancement of phagocytosis and chromatin binding (Robey et al., 1984). Haptoglobin, a glycoprotein, binds intravascular free haemoglobin and carries it to the reticuloendothelial system, thus preventing a haemoglobin loss. Since C-reactive protein and haptoglobin exhibited no inhibition of transferrin binding, we suggest that the capacity of α_1-AT and α_2-MG to compete for the transferrin-binding site has to be seen in the context of related molecular structures of the active centres of these two glycoproteins, both of which are neutrophil proteinase inhibitors. It might be of further interest that α_2-MG and α_1-AT have longer half-lives (about 8 and 4 days respectively) than C-reactive protein (only a few hours), haptoglobin (about 2 days) (Farrow and Baar, 1973; Koj, 1974) and the cytokines IL-1, IL-6 and TNF, which are cleared from the circulation within a few minutes (Andus et al., 1991). Anaemia as part of the acute-phase response is a rather prolonged effect and therefore proteins with longer half-lives are probably required.

IL-6, alone or in combination with dexamethasone, has been shown to be the major inducer of the production of acute-phase proteins in human (Castell et al., 1989) and rat (Koj et al., 1984; Geiger et al., 1988; Andus et al., 1988) hepatocytes; it does so by increasing the amount of mRNA levels of these proteins. IL-1 and TNF showed the same effect though to a lesser degree (Dinarello, 1984; Perlmutter et al., 1986). In our experiments, IL-6 and dexamethasone, either alone or in combination, IL-1 and TNF demonstrated no inhibition of transferrin binding. For this reason, we suggest that the involvement of cytokines and dexamethasone in iron–transferrin metabolism is not directly related to cell surface receptors but rather involves regulation of the expression of acute-phase protein genes.

Albumin, a well-known 'negative' acute-phase protein (van Snick, 1990), the amount of which is decreased by cytokines IL-6, IL-1 and TNF during acute-phase response (Koj et al., 1984; Castell et al., 1989), did not have any effect on transferrin binding.

In summary, we have found that α_1-AT and α_2-MG, the hepatic acute-phase proteins, demonstrate an effect on iron–transferrin metabolism that has not been described previously. We have shown that α_1-AT and α_2-MG, both members of the antiproteinase family with half-lives of 4 and 8 days respectively, regulate transferrin binding to its surface receptor

by inhibiting it in a dose-dependent and competitive manner. However, other acute-phase proteins, C-reactive protein and haptoglobin, with different main functions and distinctly shorter half-lives, do not display such an inhibiting activity. We suggest that the inhibiting potential of these two acute-phase proteins, α_1-AT and α_2-MG, could explain the pathophysiology of anaemia during the acute-phase response of infections and malignant diseases.

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