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Up-regulation of α_1-microglobulin by hemoglobin and ROS in hepatoma and blood cell lines

Magnus G. Olsson[a], Maria Allhorn[a], Tor Olofsson[b] and Bo Åkerström[a*]

Departments of [a]Clinical Science and [b]Laboratory Medicine, Lund University, Lund, Sweden

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[*]To whom correspondence should be addressed:

Department of Clinical Science, Section for Clinical and Experimental Infection Medicine,

Lund University, BMC, B14

S-221 84 Lund, Sweden

Tel.: + 46 46 222 8578

Fax: + 46 46 157756

Email: Bo.Akerstrom@med.lu.se

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ABSTRACT

α_1-Microglobulin is a 26 kDa glycoprotein synthesized in the liver, secreted to the blood and rapidly distributed to the extravascular compartment of all tissues. Recent results show that α_1-microglobulin has heme-binding and heme-degrading properties and it has been suggested that the protein is involved in the defence against oxidation by heme and reactive oxygen species. In the present study the influence of hemoglobin and reactive oxygen species (ROS) on the cellular expression of α_1-microglobulin was investigated. Oxy- and methemoglobin, free heme and Fenton reaction-induced hydroxyl radicals induced a dose-dependent up-regulation of α_1-microglobulin on both mRNA- and protein levels in hepatoma cells and an increased secretion of α_1-microglobulin. The up-regulation was reversed by addition of catalase and ascorbate, and by reacting hemoglobin with cyanide which prevents redox reactions. Furthermore, the blood cell lines U937 and K562 expressed α_1-microglobulin at low levels, and this expression increased up to eleven-fold by the addition of hemoglobin. These results suggest that α_1-microglobulin-expression is induced by ROS, arising from redox reactions of hemoglobin or from other sources and are consistent with the hypothesis that α_1-microglobulin participates in the defence against oxidation by hemoglobin, heme and reactive oxygen species.

Keywords: α_1-microglobulin, hemoglobin, ROS, up-regulated expression, hepatoma cells, blood cells.

INTRODUCTION

Hemoglobin is the major oxygen carrier of blood, but has a number of toxic, potentially dangerous side effects. Hemoglobin is a tetramer consisting of four globin subunits ($\alpha_2\beta_2$), each carrying an iron-containing heme group in its active center [1]. Oxyhemoglobin, i.e. ferrous (Fe^{2+}) hemoglobin binding oxygen (O_2), is known to undergo spontaneous intramolecular oxidation-reduction reactions, in which the iron is oxidized to the ferric (Fe^{3+}) form (methemoglobin) and the oxygen is reduced to superoxide. Further downstream reactions lead to formation of ferryl (Fe^{4+}) hemoglobin, free heme and various reactive oxygen species (ROS) [2]. ROS include hydrogen peroxide and the free radicals superoxide and hydroxyl radicals. All these compounds are toxic since they can induce oxidative damage on for example matrix molecules, cell membranes and other tissue components [3].

The overwhelming part of hemoglobin is found strictly compartmentalized within erythrocytes. The auto-oxidation of oxyhemoglobin and downstream free-radical formation is largely prevented by the intracellular inhibitors superoxide dismutase, catalase, and glutathione peroxidase [4, 5]. In spite of this, slow auto-oxidation occurs intracellularly. Oxidized hemoglobin forms which are unstable and easily denatured are found deposited together with free heme and iron on the cytosolic face of the erythrocyte membrane [6]. Hemoglobin is also found extracellularly in plasma at normal concentrations up to around 5 mg/L, mainly as a result of hemolysis [1]. Plasma contains haptoglobin [7], a high-affinity binder of oxyhemoglobin and inhibitor of auto-oxidation; iron- and heme-binding proteins such as transferrin, albumin, and hemopexin; and antioxidants such as vitamin E and ascorbic acid. However it is generally agreed that these systems are not sufficient to protect against hemoglobin-mediated oxidative cell and tissue damage during increased extravascular hemolysis and general hemolytic pathologic disorders.

α_1-Microglobulin (α_1m) is a 26 kDa glycoprotein, first isolated from human urine [8]. It is an evolutionarily well-conserved plasma and tissue protein, which has been identified in mammals, birds, amphibians and fish [9, 10]. α_1m, also known as protein HC [11], is known to be mainly synthesised in the liver, although a minor expression of the protein has been seen in the pancreas and kidney [12, 13]. α_1m is co-expressed with bikunin, a plasma protein with proteinase inhibitor activity and a component of the extracellular matrix [14]. The α_1m-bikunin gene (*AMBP*) is transcribed and translated into an α_1m-bikunin precursor protein [15]. This is cleaved in the Golgi, and the two proteins are secreted separately into the blood stream [16], α_1m in a free form and bikunin covalently bound to one or two so called heavy chains. Since no functional connection between the two proteins has been observed, the reason for the co-expression is unknown. After secretion, α_1m is rapidly distributed to different tissues where it is found in the extravascular compartments [17]. Human α_1m exists in a free form and as high molecular weight complexes bound to IgA [18], albumin and prothrombin [19]. α_1m is a member of the lipocalin superfamily, a group of proteins from animals, plants and bacteria, with diverse functions but a well-conserved three-dimensional structure. Lipocalins are folded into an eight-stranded β-barrel [20] creating a hydrophobic pocket which can carry small ligands. Purified α_1m is brown-coloured by modification of three lysyl residues located close to the opening of the lipocalin pocket. Several recent reports suggests that α_1m is involved in the defence against hemoglobin and free heme. Thus, α_1m binds heme [21] and exposure to erythrocyte membranes or purified hemoglobin leads to the formation of a truncated form of α_1m (t-α_1m), which has heme-degrading properties. In chronic leg ulcers, an inflammatory condition in which hemoglobin, heme and iron are pathogenic factors, α_1m is co-localized with heme, binds heme and t-α_1m is formed [22].

In the present work we have investigated the influence of hemoglobin on the expression of α_1m in hepatoma and blood cell lines. The results suggest that an increased expression of α_1m is induced by free hemoglobin, both in the liver and in peripheral cells, and that the up-regulation is dependent upon ROS-formation.

EXPERIMENTAL PROCEDURES

Reagents and proteins

Cell culture media and supplements were obtained from GIBCO (Paisley, Scotland, U.K.). Human plasma α_1m was prepared as described [19]. Human hemoglobin was from Sigma Chemical Co. (St. Louis, MO, USA) and shown spectrophotometrically [23] to contain 70-80% methemoglobin. This preparation is henceforth called "methemoglobin". All concentrations of met- and oxyhemoglobin are given per tetramer. Ascorbate was from Sigma. Bovine liver catalase was from Roche Diagnostics GmbH (Mannheim, Germany). Hemin (Ferriprotoporphyrin IX chloride) was purchased from Porphyrin Products, Inc (Logan, Utah, USA) and a 2 mM stock-solution prepared by dissolving in dimethyl sulphoxide (Sigma). Hydrogen peroxide was from Acros Organics (Geel, Belgium). Ammonium iron (III) sulfate dodecahydrate was from Merck (Damstadt, Germany). ECL ™ Western blot detection reagents and developing equipment were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). Polyclonal antibodies against human α_1m and bikunin were prepared in rabbits as described by Berggård and Bearn et al [24] and mouse monoclonal antibodies against human α_1m (BN11.10) as described [25]. Rabbit anti-human bikunin was made by AgriSera (Vännäs, Sweden) using recombinant bikunin prepared as described by Falkenberg et al. [26]. Rabbit anti-mouse immunoglobulin and rabbit anti-human α_1-acid glycoprotein were purchased from Dako (Dakopatts, Denmark). Goat anti-rabbit immunoglobulin and goat anti-human α_1m were prepared as described [27]. Polyclonal swine anti-rabbit immunoglobulin/HRP was from Dako. Protein LG was purified according to Kihlberg et al. [28] and coupled to Sepharose as described [29]. Proteins were labelled with ^{125}I (Bio-Nuclear AB, Stockholm, Sweden) using the chloramin-T method [30]. The labelled proteins were separated from free iodide by gel filtration on Sephadex G-25 column (PD-10, Amersham Biosciences AB, Uppsala, Sweden). The specific

activities were approximately 0.3 MBq per μg protein for plasma α_1m and 0.5 MBq per μg protein for goat anti-rabbit immunoglobulin and rabbit anti-mouse immunoglobulin.

Preparation of oxyhemoglobin and cyanhemoglobin

Red blod cells from 50 ml human blood were isolated by centrifugation (1200 xg, 10 minutes) and washed 4 times with 10 volumes of phosphate buffered saline (PBS, 10 mM phosphate, pH 7.4; 120 mM NaCl and 3 mM KCl). The blood cells were then lysed by resuspension in hypotonic buffer (20 volumes H_2O : 1 volume PBS) on ice. The membranes were separated from the cytosol by centrifugation (14000 xg, 20 minutes) and the supernatant was dialysed 3 times against 15mM Tris-HCl, pH 8.0 in 4°C. Two-hundred ml of DEAE-Sephandex A-50 (Amersham Biosciences AB, Uppsala, Sweden) was packed in a column and the dialysed supernatant was applied to the gel and separated by a gradient consisting of 15 mM Tris-HCl, pH 8.0 and 15 mM Tris-HCl, pH 8.0 + 0.2 M NaCl. Fractions were collected and the absorbance was measured at 280 nm, 577 nm and 630 nm to identify and determine the concentration of oxyhemoglobin.

Cyan-hemoglobin was prepared by mixing hemoglobin (1 mM) with 10 mM KCN and incubating for 10 minutes in 20°C. The solution was desalted on a Sephadex G-25 column, eluting with 20 mM Tris-HCl, pH 8.0.

Cell culture

The human erythroid cell line K562, the human histiocytoma cell line U937 and the human hepatoma cell line HepG2 were cultured in RPMI 1640 with GlutaMAX™I medium (Life Technologies AB, Täby, Sweden) containing 10 % fetal bovine serum (FBS)(Life Technologies AB, Täby, Sweden) and 100 μg/ml antibiotics (penicillin and streptomycin). When grown under serum-free conditions, all other constituents were kept the same. The cells

were incubated at 37°C in an atmosphere of 5 % CO_2, and the medium was changed regularly. The adherent HepG2 cells were trypsinated as described [31].

Analysis of α_1m in cell media and cells

HepG2 cells were grown to 60-90 % confluency and the medium changed to serum-free medium. Methemoglobin, oxyhemoglobin, cyan-hemoglobin, catalase, heme, $(NH_4)Fe(SO_4)_2$, hydrogen peroxide or ascorbate were added as indicated in the Figure legends and the cells were incubated for various times. Culture medium was collected and concentrated by ultra-filtration in Microcon Cells (Cut-off: 10kDa; Millipore, Bedford, MA, USA) or precipitation with acetone. When using the latter method, 0.8 ml acetone was added to 0.2 ml sample, incubated (2h, -20°C), centrifuged (20000 xg, 4°C, 30 min.) and dissolved in PBS (125 mM NaCl, 10 mM phosphate, pH 7.4). α_1m was then analysed by radioimmunoassay (RIA), lactate dehydrogenase (LDH), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described below. The cells were harvested and homogenized with buffer containing 50 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1 % NP40; 1 μg/μl pepstatin, 5 μg/μl antipain; 10 μg/μl leupeptin, or with QIAzol™ Lysis reagent (QIAGEN Sciences, Maryland, USA) supplemented with 0.8 % polyacryl carrier (Molecular Research Center Inc., Cincinnati, OH, USA). The homogenized cells were then analysed with the Bradford protein assay or by real-time PCR as described below.

K562 or U937 cells were grown in suspension to 1.0 – 2.0 x 10^6 cells per ml and the medium changed to serum-free medium. Hemoglobin and hydrogen peroxide were added as indicated in the Figure legends and the cells incubated for 24 hours. The culture medium was collected, centrifuged (600 xg, 4°C, 10 min.), concentrated by an affinity gel chromatography column and α_1m analysed by RIA, SDS-PAGE and Western blot as described below.

Pulse-chase experiment

HepG2 cells, cultured to approximately 80 % confluency were washed and cultured in methionine-free medium (Dulbecco′s Modified Eagle Medium without L-methionine, Cat. no. 21013-024, GIBCO™, Paisley, Scotland, U.K.), substituted with 2 mM cysteine and 1 μM methionine plus, in some cases, 15 μM methemoglobin. After 1 hour [^{35}S] methionine (L-[^{35}S] methionine SJ1015, 37TBq/mmol, Amersham Biosciences, Buckinghamshire, England) was added (1.85 MBq/ml) and "chased" by the addition of unlabelled methionine (L-methionine, Prod. No. 37131, BDH Chemicals Ltd, Poole, England) to 2 mM after 15 minutes. The chase was terminated after 15, 30, 60 and 120 minutes by collecting the medium, rinsing the cells with PBS and solubilizing with 1 ml 50 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1% NP40; 1 μg/μl pepstatin, 5 μg/μl antipain; 10 μg/μl leupeptin. The solubilized material was scraped off the dishes and centrifuged for 5 minutes at 2290 xg. Culture medium and solubilized cells were analysed by immunoprecipitation with antibodies against α_1m or bikunin as described below.

RIA and total protein analysis

RIA was performed as described [32]. Briefly, goat antiserum against human α_1m (0.2 ml, dil. 1:6000) was mixed with ^{125}I-labelled α_1m (0.1 ml, appr. 0.05 pg/ml) and unknown samples or standard α_1m-concentrations (0.2 ml). The dilutions were done in 0.1M sodium phosphate, pH 7.5 + 0.1 % BSA (RIA buffer). After incubating overnight at room temperature, antibody-bound α_1m was precipitated by adding 0.3 ml bovine serum and 1.6 ml 15 % polyethylene glycol in RIA-buffer, centrifuging at 1500xG for 40 min, and analysing the ^{125}I-activity of the pellets in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences). Total protein in cell-homogenates was determined by Bradford protein assay. This assay was performed by adding one ml of Bradford reagent (containing 4.2 μM Coomassie

Brilliant Blue G (Sigma-Aldrich, St. Louis, MO, USA), 5 %(v/v) EtOH, 6 % (v/v) H_3PO_4 dissolved in H_2O) to 100 µl of each sample, and incubating at room temperature for 5 min before determination of absorbance at 595 nm using a UV spectrophotometer (Beckman DU640; Beckman Instruments, Palo Alto, CA, USA). Albumin was used as a standard and plotting the absorbance at 595 nm versus protein concentration generated a standard curve.

LDH-analysis

LDH-concentrations in the culture media were used to estimate the degree of cytolysis during the experiments. LDH was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega (Madison, WI, USA). The analysis was performed according to the instructions from the manufacturer.

Immunoprecipitation

Immunoprecipitation of pulse-chase media was accomplished as follows. Non-specific material was first removed by incubating the metabolically labelled media or solubilized cells with 20 µl protein LG-Sepharose for 2 hours in 4°C and centrifugation for 2 minutes at 8000 xg. The supernatants were then incubated with 10 µl of the appropriate antisera overnight in 4°C. 20 µl protein LG-Sepharose were then added, the samples centrifuged (8000 xg, 2 minutes) and the pellets washed repeatedly with rinsing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02 % NaN_3, 0.6 M KCl, 5 mM EDTA, 2 % TritonX-100) and once with PBS. The precipitates were then boiled with sample buffer, centrifuged and the supernatants separated by SDS-PAGE. Finally, the gels analysed on a Fuji FLA 3000 phosphoimaging system (Fujifilm Sweden AB, Stockholm, Sweden).

SDS-PAGE and Western blot

SDS-PAGE (T = 12 %, C = 3.3 %) was performed as described by Laemmli [33]. The gels were run under reducing conditions using a high molecular weight standard (Rainbow markers, Amersham Biosciences, Buckinghamshire, England). The separated proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA, USA) as described [34]. The membranes were then incubated with the appropriate antibodies and Western blot was performed using ^{125}I-labelled secondary antibodies as previously described by Wester et al [35], and developing the images on the membranes using Fuji FLA 3000 phosphoimaging system (Fujifilm Sweden AB, Stockholm, Sweden). Alternatively, the ECL™ system (Boehinger, Mannheim GmbH, Mannheim, Germany) was employed using horseradish peroxidase-labelled secondary antibodies. The membranes were developed using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA) in a ChemiDoc XRS detection unit (BioRad Labs, Hercules, CA, U.S.A.).

Affinity chromatography

Mouse monoclonal antibodies against human α_1m (BN11.10) were immobilized to Affigel Hz (Bio-Rad Labs, Hercules, CA, USA) at 20 mg/ml, following instructions from the manufacturer. One ml gel was packed in a 10 ml plastic column, rinsed with PBS, pre-eluted with 9 ml 0.1 M glycine-HCl, pH 2.3 and equilibrated with PBS. Centrifuged (as described above) culture medium was applied to the column, which was subsequently washed with 50 ml PBS, and α_1m eluted with 6 ml 0.1 M glycine-HCl, pH 2.3. Eluted fractions were neutralized with one-tenth volume 1 M Tris-HCl, pH 8.0 and analysed by RIA and Western blot. The concentrations of α_1m in culture medium with additions of hemoglobin were

corrected by subtracting the RIA-values of the same non-conditioned medium (i.e. not exposed to cells) after affinity chromatography and elution.

RNA isolation and Real-Time PCR

Total RNA was isolated from HepG2 cells using the acid guanidinium phenol chloroform method supplied by QIAZOL Sciences. The OD ratio (optical density at 260 nm/280 nm) of RNA was always greater than 1.8. Reverse transcription was performed on 3 μg total RNA at 42°C for 60 minutes in the presence of 0.5 μg oligo$(dT)_{18}$ primer, 200 U reverse transcriptase and 20 U RiboLock™ Ribonuclease inhibitor in reaction buffer (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas GMBH, St. Leon-Rot, Germany). Real-time PCR was used to examine the expression of the α_1m and heme oxygenase-1 (HO-1) mRNA in hepatoma cells exposed to oxyhemoglobin, hydrogen peroxide or a mixture of $(NH_4)Fe(SO_4)_2$, hydrogen peroxide and ascorbate. Human glyceraldehyde-3-phosphate dehydrogenase (G3DPH) was used to standardize the expression of α_1m and HO-1. Primers were designed accordingly: α_1m forward primer 5′-CACTCGTTGGCGGAAAGG-3′, reverse primer 5′-ACTCATCATAGTTGGTGTGGAC-3′; HO-1 forward primer 5′-CAACAAA-GTGCAAGATTCTG-3′, reverse primer 5′-AAAGCCCTACAGCAACTG-3′; G3DPH forward primer 5′-TGGTATCGTGGAAGGACTC-3′, reverse primer 5′-AGTAGAGGCA-GGGATGATG-3′. The expression was analyzed using iQ SYBR Green Supermix (Bio-Rad). Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad) and data analyzed using iCycler iQ Optical System Software.

Statistical analysis

Statistical analysis was performed using Origin™ (Microcal Software, Inc, Version 5). Comparison of two groups was made using Student's *t* test. The results from one representative experiment were presented as the mean of quadruples ± sd or duplicates ± sd.

RESULTS

Up-regulation of α_1-microglobulin expression in HepG2 cells

HepG2 cells were incubated with 1, 5, 15, 30 or 50 μM methemoglobin or oxyhemoglobin for 6 hours (Figure 1A). The culture medium was collected and the secretion of α_1m investigated by RIA. Resting HepG2 cells expressed α_1m as expected. The addition of oxyhemoglobin to the cells resulted in a dose-dependent increase of the α_1m expression. The addition of methemoglobin also showed a dose-dependent increase, except for the highest concentrations, 30 and 50 μM, which showed a decrease in the expression of α_1m. To determine whether the latter was due to cytotoxicity of methemoglobin, cytolysis was estimated by LDH-determination of the culture media. A clear increase of the LDH-release was seen with 30 and 50 μM methemoglobin compared to background levels (Figure 1A). Thus, cytotoxic effects of 30 and 50 μM methemoglobin may explain the lower α_1m-secretion in these experiments, and 5 or 15 μM methemoglobin and 30 μM oxyhemoglobin were used for further experiments. The time-dependence of the expression was studied using 5 μM methemoglobin and 30 μM oxyhemoglobin (Figure 1B).The concentration of α_1m accumulated in the medium with time as expected, and a significantly increased α_1m-concentration was seen by oxyhemoglobin after 1 hour and by methemoglobin after 2 hours. When separated by SDS-PAGE, α_1m migrates as a band of approximately 33 kDa, even though it has a molecular weight of 26 kDa [19]. Western blot analysis of the HepG2 culture media showed increased amounts of the 33 kDa α_1m band when HepG2 cells were exposed to hemoglobin (Figure 1C), supporting the previous findings. The intensity of the band also increased with time (not shown) and similar results were obtained with polyclonal and monoclonal antibodies against α_1m (not shown). Bikunin, expressed from the same gene as α_1m, was seen in the media as a broad band around 45 kDa and high molecular weight bands

around 200 kDa, representing glycosylated, free bikunin and heavy chain-linked bikunin, respectively [36]. The intensity of the bikunin bands clearly increased with hemoglobin added to the cells. As expected, α_1-acid glycoprotein, used as a control protein, was expressed at similar levels at the various conditions.

Oxidant-dependent up-regulation of α_1-microglobulin expression

To investigate if the up-regulation is dependent upon redox-reactions of the heme-chelated iron of hemoglobin, we studied the effects of cyan-hemoglobin. The strong bindning of CN^- effectively prevents redox-reactions of the iron atom. HepG2 cells were exposed to 5 μM methemoglobin, 30 μM oxyhemoglobin and 30 μM cyan-hemoglobin for 6 hours and the α_1m-concentration of the culture media determined by RIA (Figure 2A). As seen before, both met- and oxyhemoglobin induced an approximately two-fold higher expression of α_1m. In contrast, the expression remained at the same level as resting cells in the presence of cyan-hemoglobin. Furthermore, catalase (0.1 μM) significantly inhibited the up-regulation induced by 5 μM methemoglobin (Figure 2B), suggesting that hydrogen peroxide is involved in the signalling pathway. Ascorbate, a reducing agent and radical scavanger also inhibited the methemoglobin-induced up-regulation. The addition of free heme to the cell culture media, increased the concentrations of α_1m in the media in a dose-dependent (Figure 2C) and time dependent (not shown) manner. This supports the view that the globin-chains of hemoglobin are not involved in the regulatory mechanism. Finally, a mixture of Fe^{3+}, ascorbate and hydrogen peroxide (10 μM, 100 μM and 20 μM, respectively) induced an almost two-fold increase in the α_1m-concentration (Figure 2D). This mixture generates hydroxyl radicals in the Fenton-reaction [37]. The effects of the three components of the Fenton-reaction were tested individually in control experiments (Figure 2D). Hydrogen peroxide increased the expression of α_1m, but not as much as the Fenton-reaction. The ferric ion, which is a strong

oxidant by itself, had no effect even at five-fold higher concentration. Ascorbate had a small inhibitory effect on the α_1m-expression. Taken together, the experiments shown in Figure 2 suggest that the ROS-species hydrogen peroxide and hydroxyl radicals, but not the non-ROS oxidant Fe^{3+}, can induce an up-regulation of α_1m-expression in the HepG2 cells.

Up-regulation of α_1-microglobulin-bikunin precursor

The effects of hemoglobin (methemoglobin) on the processing of the α_1m-bikunin precursor were studied in a pulse-chase experiment. Cultured HepG2 cells, labelled with [^{35}S]methionine, were exposed to 15 μM methemoglobin and cultured for 120 minutes. α_1m and bikunin in solubilized cells and culture medium were analysed by immunoprecipitation followed by SDS-PAGE and phosphoimaging. As expected the results from the solubilized cells showed an immediate appearance of the 45 kDa precursor protein with both α_1m (Figure 3A) and bikunin (3B) antibodies. The yield of the precursor increased somewhat when exposing cells to hemoglobin. A minor precursor protein band at approximately 40 kDa, followed the same pattern as the 45 kDa band, but was only seen when using α_1m antibodies. Methemoglobin induced an increased yield of a high molecular weight band when using bikunin antibodies. In the medium, methemoglobin induced a strong increase in the concentration of free 33 kDa α_1m and a high molecular weight α_1m-form (>200 kDa), not seen in control cells, after 60 minutes. (Figure 3C). It can be speculated that the latter is uncleaved α_1m-bikunin, linked to heavy chains of the inter-α-inhibitor species [36]. Weak α_1m-bands of approximately 40 kDa and 66 kDa were also observed. Bikunin followed the same pattern as α_1m in the medium, i.e. exposure to methemoglobin resulted in increased concentrations of a 45 kDa band and high molecular weight bands around 200 kDa after 60 minutes (Figure 3D).

To confirm the results on the mRNA level, the expression of α_1m mRNA was investigated with real-time PCR after exposing hepatocytes to various oxidants. An upregulated expression of the α_1m mRNA was seen after adding oxyhemoglobin, hydrogen peroxide or the mixture of Fe^{3+}, hydrogen peroxide or ascorbate as compared to the resting cells (Figure 4). As expected, HO-1 expression was also induced when exposing the cells to oxyhemoglobin, hydrogen peroxide or the mixture of Fe^{3+}, hydrogen peroxide or ascorbate. The G3DPH-gene expression level was unchanged in all cells. Agarose gel electrophoresis of the RT-PCR products revealed the expected sizes (not shown).

Up-regulation of α_1-microglobulin expression in blood cell lines

The effect of methemoglobin and hydrogen peroxide on α_1m protein expression was investigated in U937 cells, a histiocytic cell-line. Cells were exposed to 15 μM methemoglobin or 50 μM hydrogen peroxide for 24 hours and the culture medium was analysed by RIA and Western blot after anti-α_1m affinity chromatography purification. The RIA showed that resting U937 cells indeed expressed a low amount of α_1m, and that 15 μM methemoglobin and 50 μM hydrogen peroxide increased the level of expression about 4.5-fold (Figure 5A). Western blotting confirmed the patterns seen with RIA, i.e. a small amount of the 33 kDa α_1m band was seen in the medium of resting cells, whereas medium from cells exposed to methemoglobin showed a clear increase in the expression (Figure 5B). Another band at approximately 66 kDa, probably the α_1m-dimer, followed the same pattern as the 33 kDa band. A weaker band just below 30 kDa was seen in the medium of exposed and resting cells with similar intensity.

We next investigated whether methemoglobin could induce expression of α_1m in K562 cells, an erythroid cell line. Culture medium from K562 cells, exposed to 5 or 15μM

methemoglobin, or 50 μM hydrogen peroxide for 24 hours, was applied to anti-α_1m affinity chromatography and the eluate was analysed by radioimmunoassay. The results showed that a very small amount of α_1m was synthesised in resting cells but increased up to eleven-fold by addition of hemoglobin (Figure 5A). The increase in α_1m protein expression in response to hemoglobin was dose dependent. The eluates were also studied by Western blotting (Figure 5B). A 33 kDa α_1m band was seen when the cells were cultured in the presence of hemoglobin but not in the resting cells. A 50 kDa band was seen in all lanes with similar intensity. This may correspond to non-specifically stained mouse immunoglobulin heavy chain eluted from the column.

Comparison of α_1-microglobulin expression between cell lines

To obtain an indication of the physiological relevance of the blood cell expression, the expression levels of α_1m in the hepatoma cells and blood cells were compared. The expression levels in resting blood cells were very low when comparing to hepatoma cells (Table 1). Adding hemoglobin, the relative increase of the expression was higher in blood cells than in HepG2 cells, even though the absolute levels were still higher in HepG2 cells than in both U937 and K562 cells. When comparing U937 and K562 cells, the U937 cells expressed more α_1m than K562 cells.

DISCUSSION

We have investigated the effects of hemoglobin and ROS on the expression of the lipocalin α_1m. It can be concluded that methemoglobin, oxyhemoglobin, hydrogen peroxide and hydroxyl radicals induce an up-regulation of α_1m in hepatoma cells, a white blood cell line and a red blood cell line. In the hepatoma cells, an increased production of α_1m-mRNA, α_1m-bikunin precursor protein and secreted α_1m and bikunin can be seen.

The results in this study indicate that ROS, which can be formed as a result of redox reactions of hemoglobin, cause the up-regulation of α_1m. Thus, the up-regulation induced by methemoglobin was inhibited by catalase, suggesting involvement of hydrogen peroxide. The up-regulation was also inhibited by cyanide, a strong chelator that "freezes" the heme-bound iron in the ferric (Fe^{3+}) form and thus inhibits redox reactions of the hemoglobin that are necessary for the formation of ROS. Furthermore, hydrogen peroxide alone, as well as a mixture of Fe^{3+}, ascorbate and hydrogen peroxide, which generate hydroxyl radicals in the Fenton reaction [37], also up-regulated the α_1m expression. Finally, free heme generated a significant up-regulation of α_1m, supporting the view that downstream ROS rather than the globin-chains of hemoglobin induce the up-regulation of α_1m.

Both oxy-and methemoglobin induced an increased α_1m-expression. It has not been the purpose of this work to delineate the more or less complex pathways, but both hemoglobin-forms are highly oxidative molecules which may undergo redox reactions leading to ROS- and free radical-production. Thus auto-oxidation of oxyhemoglobin, peroxidase activity of methemoglobin, formation of ferryl(Fe^{4+})-hemoglobin or degradation of the hemoglobins to free heme and iron, have been described [reviewed in 38-40]. The up-regulation by methemoglobin was inhibited by catalase, suggesting that hydrogen peroxide generated by the cells [41, 42] and thus the peroxidase activity of methemoglobin may be important for the signal induction. The peroxidase activity may lead to oxidation of various biological

substrates, including amino acid side-chains and membrane lipids and other cellular components, and subsequent propagation of the oxidative signal to the nucleus.

It has been shown in other studies that ROS can influence the regulation of gene expression. The heme-degrading protein HO-1, for example, is up-regulated by stimuli that increase the amount of ROS [43, 44]. Furthermore, it has been shown that scavengers of ROS, such as N-acetyl cysteine, inhibit or reduce the magnitude of HO-1 induction by oxidative stress [45]. Although the exact mechanism of redox signalling, in particular the intracellular targets of ROS, are less clear, changes in the cellular redox state may affect the regulation of many genes via different redox sensor systems [46]. It has for instance been reported that HO-1 gene expression is induced by the Ras pathway via c-Jun N-terminal kinases (JNK) [47]. Even though the pathway for ROS-mediated up-regulation of α_1m is not investigated here, it could be speculated that it is regulated by the hepatocyte nuclear factor-4 (HNF-4), a transcription factor responsible for α_1m expression under normal, resting conditions [48]. HNF-4 was shown to be involved in ROS-induced up-regulation of iNOS [49]. Therefore, it would be of interest to investigate if the ROS-induced increased α_1m-expression is regulated by a similar HNF-4-dependent pathway.

There have been a few reports during the years showing that α_1m, besides its major site of synthesis, is also produced in peripheral organs such as pancreas and kidney [12, 13]. In this study we report the synthesis of α_1m in the blood cell lines U937 and K562, supporting the possibility of a peripheral synthesis of α_1m in blood cells. Earlier studies have reported contradictory results regarding the α_1m expression in lymphocytes and other white blood cells. The reports have shown the presence and synthesis of α_1m and, in one case, purification of the protein from lymphocyte culture medium [50, 51], while others have reported negative results [52]. The data obtained in this study offer a possible explanation to this, i.e. the most positive results may be explained by the presence of oxidants during cell cultures. Several

prior immunohistochemical reports [53, 54] have shown a particular occurrence of α_1m in a blood cell type referred to as histiocytes. These results are supported by our results from the U937 cells, which have been characterized as a histiocytic cell line, since the data in this study indicate that there is a low basal expression of α_1m during "normal" conditions.

The results from the pulse-chase experiment indicate that α_1m is processed normally in the HepG2 cells and that the expected 33 kDa α_1m-form is secreted. Furthermore, this is also the size of α_1m secreted from blood cells. These results indicate that the oxidative conditions have no major effects on the post-translational processing of the α_1m-bikunin precursor. This is in contrast to a recently published work, where α_1m-expression was up-regulated by oxalate in kidneys [55], and the major secretion product was an α_1m band of approximately 60-65 kDa, proposed to be uncleaved α_1m-bikunin. This discrepancy is difficult to explain, but neither the cells nor the culture conditions are related, since oxalate is not considered to be a pro-oxidant but is involved in kidney stone formation.

A series of recent publications suggests that α_1m is involved in the defence against heme and hemoglobin [21, 22]. The results in this investigation further support this view since an up-regulated expression of α_1m by downstream ROS may be regarded as a feed-back response contributing to extracellular protection against oxidative damage.

LIST OF ABBREVIATIONS

α_1m, α_1-microglobulin; ROS, reactive oxygen species; AMBP, α_1-microglobulin/bikunin precursor; t-α_1m, truncated α_1-microglobulin; G3DPH, glyceraldehyde-3-phospate dehydrogenase; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; metHb, methemoglobin; oxyHb, oxyhemoglobin; CN-Hb, cyanhemoglobin; Hb, hemoglobin; JNK, c-Jun N-terminal kinases; HNF-4, hepatocyte nuclear factor 4.

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LEGENDS TO FIGURES

Figure 1. Protein expression of α_1m, measured in HepG2 cell culture medium. The cells were cultured to approximately 80 % confluency, washed twice with PBS and resuspended in serum-free medium. **A:** Cells were cultured with 1, 5, 15, 30 or 50 μM methemoglobin (metHb); 1, 5, 15, 30 or 50 μM oxyhemoglobin (oxyHb) or medium only for a period of 6 hours. The concentrations of met- and oxyhemoglobin are given per tetramer. Culture medium was collected, concentrated and the concentration of α_1m (gray) determined by RIA. The cells were homogenised and the total protein concentrations determined with Bradford protein assay. The amount of LDH (stripes) present in the culture medium was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay. The results from one representative experiment are plotted as the mean of quadruples ± sd. **B:** Cells were cultured with 5 μM methemoglobin (○); 30 μM oxyhemoglobin (Δ) or medium only (□) for a period of 1-6 hours. After the incubation period, the medium was collected, concentrated and the concentration of α_1m determined by RIA. The cells were homogenised and the total protein concentration measured with Bradford protein assay. The results from one representative experiment are plotted as the mean of quadruples ± sd. **C:** Cells were cultured with 5, 15 μM oxyhemoglobin or culture medium only for 6 hours. Culture medium was collected and concentrated. A quantity of 15 μg total protein per lane was separated by SDS-PAGE (12%) and transferred to PVDF membranes. The membranes were incubated with rabbit anti-α_1m serum, rabbit anti-bikunin or rabbit anti-α_1-acid glycoprotein followed by goat anti-rabbit immunoglobulin labelled with ^{125}I and developed using a Fuji FLA 3000 phosphoimaging system. Control cells are shown in lane 1, cells exposed to 5 μM oxyhemoglobin are shown in lane 2 and cells exposed to 15 μM oxyhemoglobin are shown in lane 3.

Figure 2. Oxidation-dependent up-regulation of α_1m expression in HepG2. The cells were cultured to approximately 80 % confluency, washed twice with PBS and resuspended in serum-free medium. **A:** Cells were cultured with 5 μM methemoglobin, 30 μM oxyhemoglobin, 30 μM cyan-hemoglobin (CN-Hb) or culture medium only for 6 hours. The culture medium was then collected, concentrated and the concentration of α_1m was determined by RIA. The cells were homogenised and the total protein concentration measured with Bradford protein assay. The results from one representative experiment are plotted as the mean of quadruples ± sd. **B:** The cells were cultured with culture medium only, 5 μM methemoglobin, 5 μM methemoglobin + 0.1 μM catalase or 5 μM methemoglobin + 100 μM ascorbate for a period of 6 hours. The results from one representative experiment are plotted as the mean of quadruples ± sd. **C:** Cells were cultured with 15 or 50 μM heme, or culture medium for 1 hour. The medium was collected, concentrated and the concentration of α_1m determined by RIA. The cells were homogenised and the total protein concentration measured with Bradford protein assay. The results from one representative experiment are plotted as the mean of quadruples ± sd. **D:** Cells were cultured with culture medium, 10 μM $(NH_4)Fe(SO_4)_2$ + 100 μM ascorbate + 20 μM H_2O_2, 50 μM $(NH_4)Fe(SO_4)_2$, 500 μM ascorbate or 20 μM H_2O_2 for 6 hours. The medium was collected, concentrated and the concentration measured by RIA. The cells were homogenised and the total protein concentration measured with Bradford protein assay. The results from one representative experiment are plotted as the mean of quadruples ± sd. **, $P< 0.01$; *, $P< 0.05$.

Figure 3. Pulse-chase of α_1m-bikunin in HepG2 cells. Cells were cultured to 80 % confluency and washed with PBS. The cells were then cultured for 1 hour in serum-free/low methionine medium only or with 15 μM methemoglobin. [^{35}S]methionine was added and the cells cultured for another 15 min. Unlabelled methionine was added to 2 mM and the cells cultured for 15, 30, 60 or 120 minutes. α_1m and bikunin were then immunoprecipitated from solubilized cells and culture medium and analysed by SDS-PAGE (12%) and phosphoimaging.

Figure 4. mRNA expression of α_1m in HepG2 cells. Cells cultured to 80 % confluency were washed in PBS, resuspended in serum-free medium and exposed to culture medium only, 15 μM oxyhemoglobin, 100 μM hydrogen peroxide or 10 μM $(NH_4)Fe(SO_4)_2$ + 100 μM ascorbate + 20 μM H_2O_2 (=Fenton) for 2 hours. Total RNA was extracted from homogenized cells, cDNA was prepared using reverse transcription and expression of α_1m (black), HO-1 (grey) and G3DPH (stripes) was analysed using real-time PCR. Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler and data analyzed using iCycler iQ Optical system Software.

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Figure 5. Expression of α_1m in U937 and K562 cell culture media. U937 or K562 cells (1-2 x 10^6 cells/ml) were resuspended in serum-free medium. **A:** The cells were cultured with culture medium; 5 or 15 μM methemoglobin, or 50 μM H_2O_2 for 24 hours. The medium was collected and concentrated by anti-α_1m affinity chromatography. The α_1m concentration of eluted pooled fractions was determined by RIA. The bars show mean ± sd of duplicate samples from a representative experiment. ***, $P< 0.001$; **, $P< 0.01$. **B:** Cells were cultured with 0 (lanes 1), 5 (lane 2) or 15 μM (lanes 3) methemoglobin for 24 hours. The culture

medium was collected and applied to anti-α_1m affinity chromatography and the eluted fractions were separated by SDS-PAGE. The proteins were then transferred to a PVDF membrane and incubated with a mixture of monoclonal antibodies against α_1m, followed by rabbit anti mouse immunoglobulin labelled with horseradish peroxidase. The results were visualised by using the ECL system.

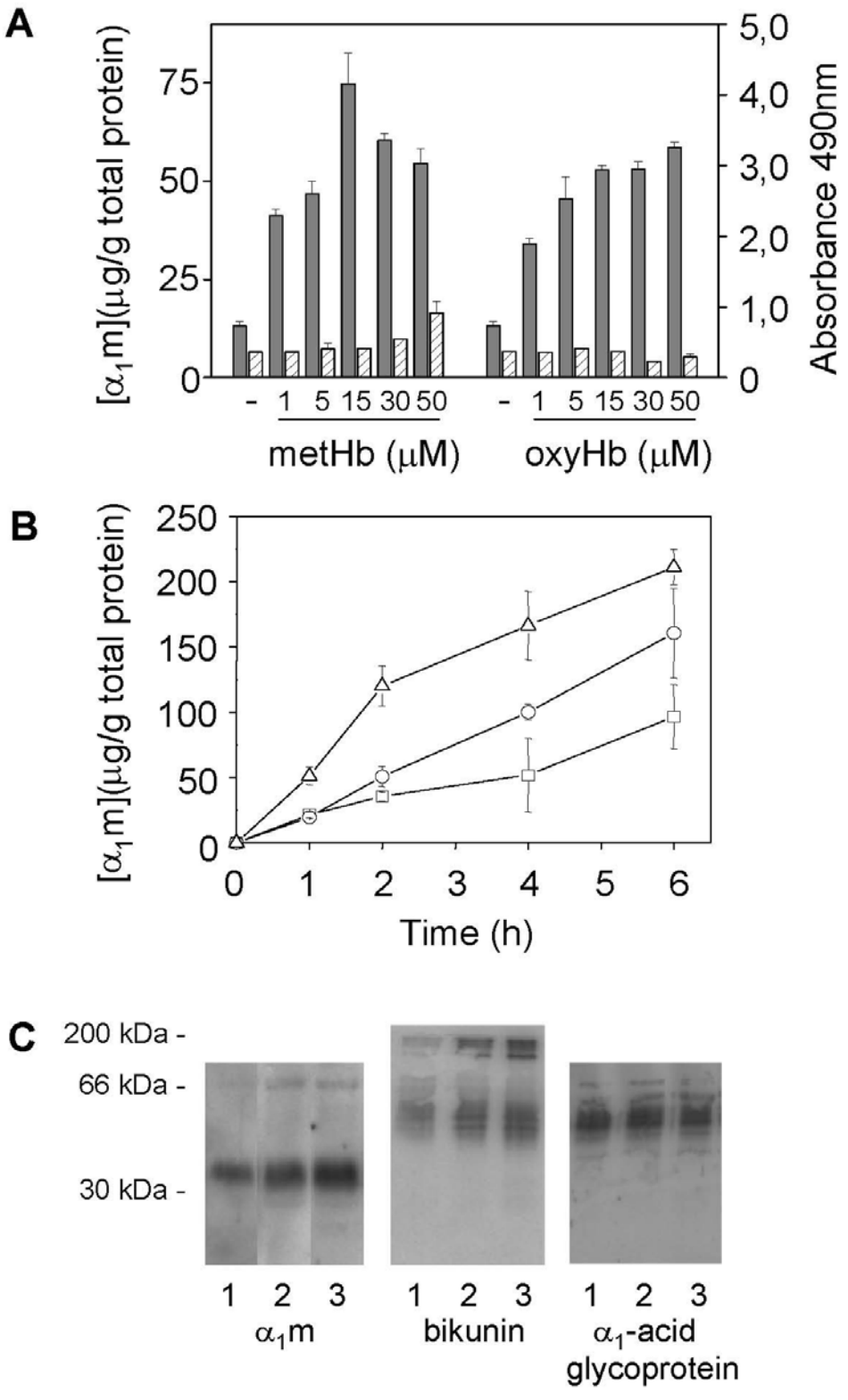

A
[α1m](µg/g total protein)
75
50
25
0
5,0
4,0
3,0
2,0
1,0
0
Absorbance 490nm
- 1 5 15 30 50
metHb (µM)
- 1 5 15 30 50
oxyHb (µM)
B
[α1m](µg/g total protein)
250
200
150
100
50
0
0 1 2 3 4 5 6
Time (h)
C
200 kDa -
66 kDa -
30 kDa -
1 2 3
α1m
1 2 3
bikunin
1 2 3
α1-acid
glycoprotein

Figure 1

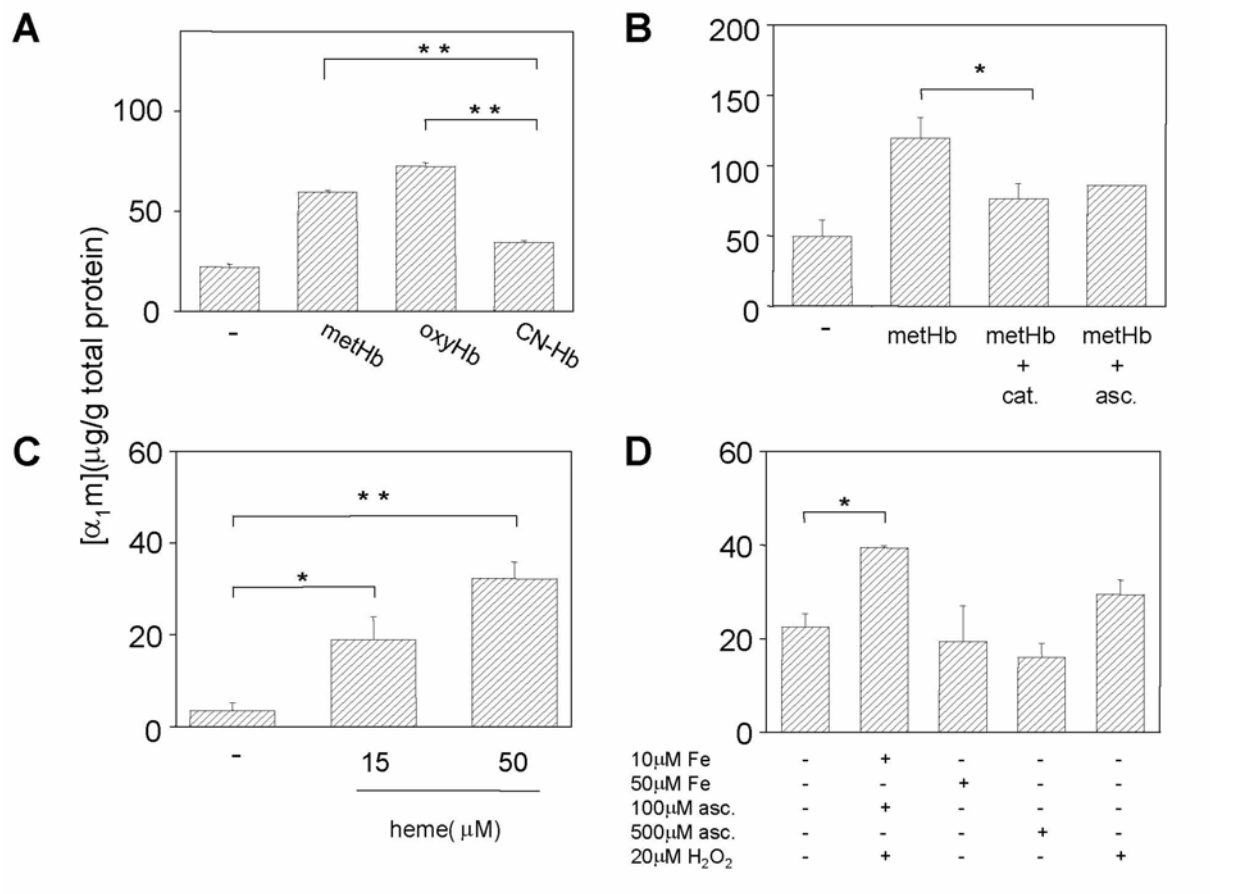

A
B
C
D
[α1m](μg/g total protein)
100
50
0
200
150
60
40
20
- metHb oxyHb CN-Hb
metHb
metHb + cat.
metHb + asc.
15
50
heme(μM)
10μM Fe
50μM Fe
100μM asc.
500μM asc.
20μM H2O2

Figure 2

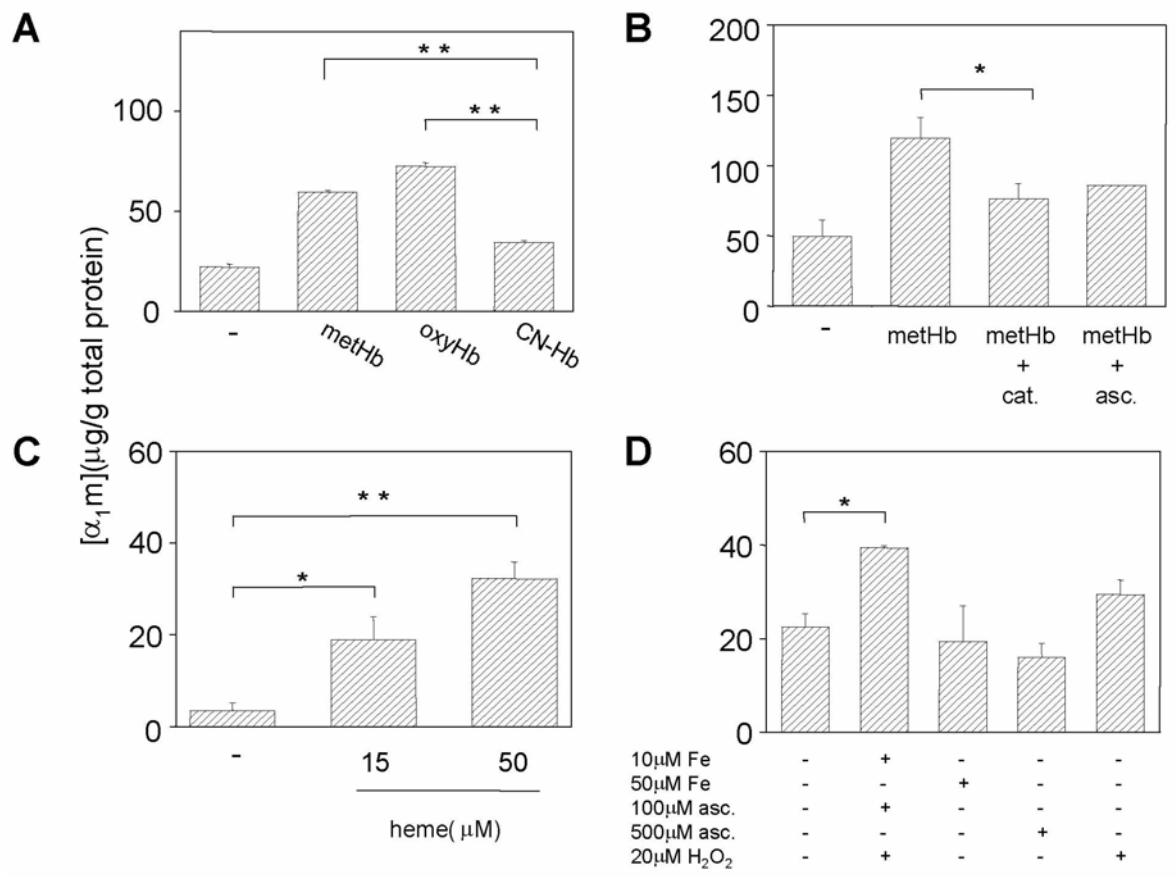
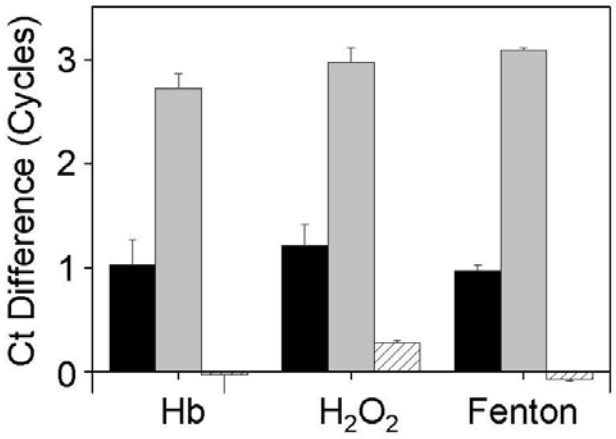

A
B
C
D
[α1m](μg/g total protein)
100
50
0
200
150
60
40
20
- metHb oxyHb CN-Hb
metHb
metHb + cat.
metHb + asc.
15
50
heme(μM)
10μM Fe
50μM Fe
100μM asc.
500μM asc.
20μM H2O2

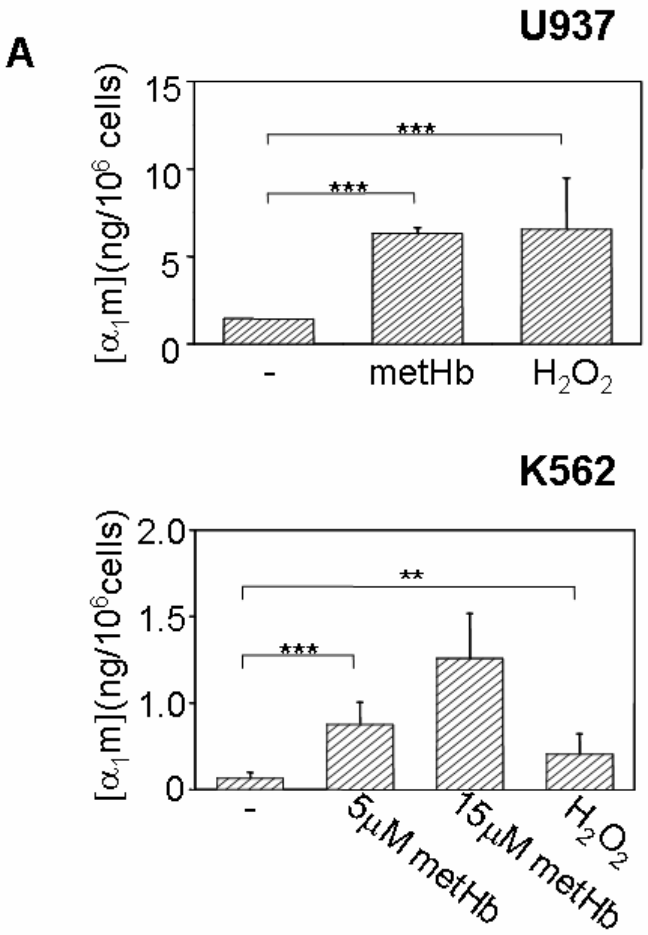
Figure 3



Ct-values ± SEM as measured by Real Time PCR.

	α_1m	HO-1	G3DPH
Control	18.65 ± 0.36	24.04 ± 0.11	17.48 ± 0.04
15µM Hb	17.62 ± 0.24	21.29 ± 0.12	17.51 ± 0.24
100µM H_2O_2	17.44 ± 0.21	21.06 ± 0.14	17.21 ± 0.02
10µM Fenton	17.68 ± 0.05	20.95 ± 0.02	17.56 ± 0.01

Figure 4


A
U937
[α1m](ng/10^6 cells)
15
10
5
0
***
***
-
metHb
H2O2
K562
[α1m](ng/10^6cells)
2.0
1.5
1.0
0
**
***
-
5μM metHb
15μM metHb
H2O2

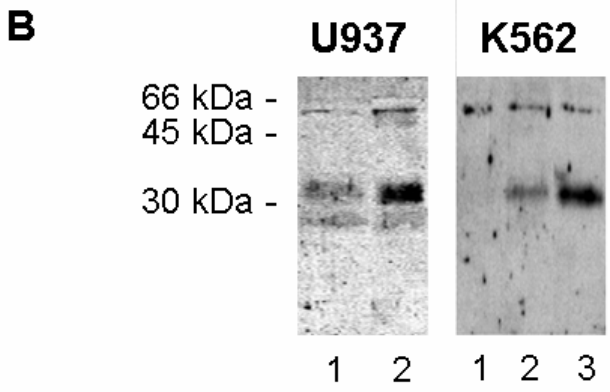

B
U937
K562
66 kDa -
45 kDa -
30 kDa -
1 2
1 2 3

Figure 5