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The lipocalin α_1-microglobulin protects erythroid K562 cells against oxidative damage induced by heme and reactive oxygen species

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Abstract

α_1-Microglobulin is a 26 kDa plasma and tissue glycoprotein that belongs to the lipocalin protein superfamily. Recent reports show that it is a reductase and radical scavenger and that it binds heme and has heme-degrading properties. This study has investigated the protective effects of α_1-microglobulin against oxidation by heme and reactive oxygen species in the human erythroid cell line, K562. The results show that α_1-microglobulin prevents intracellular oxidation and up-regulation of heme oxygenase-1 induced by heme, hydrogen peroxide and Fenton reaction-generated hydroxyl radicals in the culture medium. It also reduces the cytosol of non-oxidized cells. Endogeneous expression of α_1-microglobulin was up-regulated by these oxidants and silencing of the α_1-microglobulin expression increased the cytosol oxidation. α_1-microglobulin also inhibited cell death caused by heme and cleared cells from bound heme. Binding of heme to α_1-microglobulin increased the radical reductase activity of the protein as compared to the apo-protein. Finally, α_1-microglobulin was localized mainly at the cell surface both when administered exogeneously and in non-treated cells. The results suggest that α_1-microglobulin is involved in the defence against oxidative cellular injury caused by haemoglobin and heme and that the protein may employ both heme-scavenging and one-electron reduction of radicals to achieve this.

Keywords: *Antioxidation, α_1-microglobulin, red cell, ROS, heme.*

Abbreviations: *ABTS, 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid)diammonium salt; α_1m, α_1-microglobulin; AGP, α_1-acid glycoprotein; DTT, dithiothreitol; G3DPH, glyceraldehyde-3-phosphate dehydrogenase; Hb, haemoglobin; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NEM, N-ethylmaleimide; PI, propidium iodide; ROS, reactive oxygen species; t-α_1m, truncated α_1-microglobulin; 5-IAF, 5-iodoacetamido-fluorescein.*

Introduction

Heme is an essential molecule in aerobic living systems, participating in many oxygen-dependent biological reactions [1]. Heme consists of protoporphyrin IX and a ferrous (Fe^{2+}) iron atom which has high affinity for free oxygen (O_2). It binds to various apo-proteins forming heme-proteins, the function of which are determined by the identity of the peptide core [2]. The most abundant human heme-protein is haemoglobin, the major oxygen-carrying system in the blood. Haemoglobin consists of four globin subunits ($\alpha_2\beta_2$), each carrying a heme group in its active centre [3]. Normally, haemoglobin is found strictly within red blood cells in blood.

Severe hemolysis, blood clotting and other circumstances associated with accumulation of free haemoglobin leads to release of heme. This has pro-inflammatory, pro-oxidant and toxic effects on cells and tissue components [4,5]. Free heme is a source of reactive oxygen species (ROS) which are highly reactive and, unless counteracted by

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anti-oxidant defence mechanisms, can damage lipids, proteins and DNA through oxidative modification, cross-linking and fragmentation (reviewed in [6]). Being a hydrophobic molecule, heme can interact with cell and organelle membranes where it has toxic and cytolytic effects through both oxidative and non-oxidative mechanisms. Thus, heme-toxicity is implicated in a number of pathologies associated with elevated levels of haemoglobin and heme-proteins.

The most well-described haemoglobin-detoxification system is haptoglobin, which binds to extracellular haemoglobin in blood plasma [7,8]. The resulting haptoglobulin–haemoglobin complex is cleared from blood by binding to the macrophage receptor CD163 [9]. This receptor also has a weak affinity for free haemoglobin [10], but there is no evidence that is binds to free heme. Several heme-detoxification systems exist in humans (reviewed in [7] and [6]). Free heme in blood is sequestered by albumin and the high-affinity plasma protein haemopexin [11]. The haemopexin–heme complex is cleared from the circulation by the hepatocyte receptor CD91 [12]. In the cells, heme oxygenase-1 (HO-1) catabolizes cellular heme to free iron, biliverdin and CO and is up-regulated by most cells in response to heme and ROS exposure [13,14].

Our group has focused on α_1-microglobulin (α_1m), a protein that recently has been implicated as an extracellular heme- and haemoglobin antagonist. α_1m, also known as protein HC [15], is a 26 kDa plasma and tissue protein belonging to the lipocalin superfamily, which consists of 40–45 members, distributed throughout the animal, plant and bacterial kingdoms [16,17]. The lipocalins share a characteristic eight-stranded β-barrel fold [18], forming a hydrophobic pocket that acts as a ligand-binding site. α_1m is synthesized mainly in the liver, secreted into the blood stream [19] and transported to the extravascular compartments [20]. In a number of recent reports, α_1m has been described to be involved in the defence against pathological oxidation caused by haemoglobin, heme and ROS. It was shown that α_1m can bind heme and that a processed form of α_1m (t-α_1m), generated by the exposure to erythrocyte membranes or purified haemoglobin, has heme-degrading properties [21]. α_1m co-localizes with heme and t-α_1m is continuously formed in chronic leg ulcers, an inflammatory condition where free heme and iron released after hemolysis are considered to be pathogenic factors [22]. α_1m has also been shown to have reductase properties, reducing cytochrome c, methemoglobin and free iron [23] and reactions between α_1m and the stable synthetic ABTS-radical resulted in reduction of the radical and covalent trapping to side-chains of α_1m [24]. Finally, it was reported that haemoglobin and ROS induced an increased expression of α_1m in liver and blood cell-lines [25].

The human erythroid cell line, K562, which can be induced to produce haemoglobin [26,27], also expresses and secretes small amounts of α_1m. The expression and secretion of α_1m was increased when haemoglobin, heme or ROS was present in the cell medium [25], suggesting that α_1m may be part of an anti-oxidative auto-protection mechanism in these cells. Here, we have investigated the protective effects of α_1m in K562 cells against heme- and ROS-induced cytosol oxidation, HO-1 expression and cell death.

Materials and methods

Materials

Cell culture media and supplements were obtained from GIBCO (Paisley, Scotland, UK). Ascorbate and α_1-acid glycoprotein (AGP) were from Sigma-Aldrich Sweden AB and human serum albumin (HSA) was from Roche Diagnostics GmbH (Mannheim, Germany). Hemin (Ferriprotoporphyrin IX chloride) was purchased from Porphyrin Products, Inc. (Logan, UT) and a 10 mM stock solution prepared by dissolving in dimethyl sulphoxide (Sigma-Aldrich). 'Heme' and 'hemin' are sometimes used to designate free protoporphyrin IX with a bound Fe^{2+} or Fe^{3+} atom, respectively; in this article, 'heme' is used regardless of the iron oxidation state. Hydrogen peroxide was from Acros Organics (Geel, Belgium). Ammonium iron (III) sulphate dodecahydrate ($NH_4Fe(SO_4)_2 \times 12H_2O$) was from Merck (Darmstadt, Germany). A mixture of Fe^{3+}, ascorbate and hydrogen peroxide was used to generate hydroxyl radicals by the Fenton-reaction. Different concentrations of this mixture were tested (not shown) and 10 μM Fe^{3+}, 100 μM ascorbate and 20 μM hydrogen peroxide was shown to induce significant cytosol oxidation without affecting the cell viability. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) and 5-iodoacetamidofluorescein (5-IAF) were obtained from Molecular Probes (Leiden, Netherlands). Propidium iodide (PI), N-ethylmaleimide (NEM), dithiothreitol (DTT) and 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) di-ammonium salt (ABTS) were from Sigma-Aldrich. Recombinant human α_1m was expressed in *E.coli*, purified and refolded as described by Kwasek et al. [28]. Mouse monoclonal antibodies against human α_1m (BN11.10) were prepared as described [29].

Cell culture

The human erythroid cell line K562 was cultured in RPMI 1640 with GlutaMAX I medium containing 10% foetal bovine serum (FBS) (Life Technologies AB), 100 μg/ml antibiotics (pencillin and streptomycin) and 0.25 μg/ml antimycotics (amphotericin B

and Fungizone®). 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.

Determination of intracellular oxidation

The redox-sensitive probe H_2DCFDA was added to 0.5–1.0 × 10^6 K562 cells/ml in serum-free medium to a final concentration of 3 μM. After 30 min, the cells were washed twice in phosphate buffered saline (PBS, 10 mM Na-phosphate pH 7.4, 125 mm NaCl) and suspended in fresh medium. Heme, hydrogen peroxide, ascorbate, α_1m or AGP were added as indicated in the figure legends and the cells were incubated for various times. After incubation the fluorescence intensity of the suspension was quantified using flow cytometry (BD FACSAria™, BD Biosciences, Palo Alto, CA). The analysis was performed on 10 000 cells using a Coherent® Sapphire™ Solid State Laser (excitation: 488 nm, emission: band pass filter 530/30 nm).

Fluorescent labelling of oxidized thiol proteins

K562 cells were washed and suspended in PBS to 0.5–1.0 × 10^6 cells/ml and incubated with heme, $NH_4Fe(SO_4)_2$, hydrogen peroxide, ascorbate or α_1m as indicated in the figure legends. Reversibly oxidized thiol proteins were then monitored as described [30]. Briefly, protein thiols in their reduced state were blocked by resuspending in a buffer containing 100 mM NEM (N-ethylmaleimide) and incubating at room temperature for 15 min. After lysing the cells, excess NEM was removed by desalting through a Micro Bio-Spin® 6 Chromatography Column (Bio-Rad Laboratories, Hercules, CA). The oxidized thiols were then reduced with 1 mM DTT (dithiothreitol) and the resulting free protein thiols were labelled by adding 200 μM 5-IAF. Excess 5-IAF was removed by desalting through a Micro Bio-Spin® 6 Chromatography Column and samples containing 60 μg protein were run on a 10% SDS-PAGE. The gel-electrophoresis was carried out as described by Laemmli [31] in the dark, with a constant voltage of 200 V. After completion of electrophoresis, gels were scanned using a Molecular Imager® FX (Bio-Rad, excitation: 488 nm, emission: 530 nm). Oxidized thiol proteins were quantified by measuring the pixel density of relevant bands, using Adobe Photoshop CS3.

Silencing of the α_1m gene

Three siRNA-targeting α_1m nucleotides were purchased from Sigma-Aldrich and evaluated for their ability to inhibit α_1m expression in human K562 cells. The best results, evaluated by real time PCR analysis of the α_1m/glyceraldehyde-3-phosphate dehydrogenase (G3DPH) mRNA ratio (see below), were obtained with the α_1m siRNA pair (NM_001633/1): 5′-CCUAUGUGGUCCACACCAA -3′ and 5′-UUGGUGUGGACCACAUAGG -3′. This siRNA species was subsequently used for all experiments. The transfection of siRNA was conducted according to the protocol from OZ Biosciences (Marseille, France). Briefly, siRNA was diluted in culture medium, containing Lullaby®-siRNA transfection reagent (OZ Biosciences), to a final concentration of 5 nM. This solution was incubated for 20 min in room temperature and added to a pellet of 2 × 10^6 cells drop-by-drop. The cells were then cultivated under standard conditions. After 24 h, the cells were washed, loaded with H_2DCFDA, oxidized with heme and analysed with flow cytometry as described above. Alternatively, cells were resuspended in serum free medium with or without heme, according to the figure legends, and analysed with real time PCR.

RNA isolation and real-time PCR

Total RNA was isolated from K562 cells using the acid guanidinium phenol chloroform method supplied by QIAGEN Sciences (Maryland, USA). 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 min 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 K562 cells exposed to heme, hydrogen peroxide or a mixture of $(NH_4)Fe(SO_4)_2$, hydrogen peroxide and ascorbate. Human G3DPH was used to normalize the expression of α_1m and HO-1 which are depicted in the figure as ΔΔCt. Primers were designed accordingly: α_1m forward primer 5′-CACTCGTTGGCGGAAAGG-3′, reverse primer 5′-ACTCATCATAGTTGGTGTGGAC-3′; HO-1 forward primer 5′-CAACAAAGTGCAAGATTCTG-3′, reverse primer 5′-AAAGCC-CTACAGCAACTG-3′; G3DPH forward primer 5′-TGGTATCGTGGAAGGACTC-3′, reverse primer 5′-AGTAGAGGCAGGGATGATG-3′. The expression was analysed using iQ SYBR Green Supermix (Bio-Rad). Amplification was performed at 55°C for 40 cycles in iCycler Thermal Cycler (Bio-Rad) and data analysed using iCycler iQ Optical System Software.

Fluorescence microscopy

K562 cells were washed and suspended in PBS to 0.5–1.0 × 10^6 cells/ml and incubated with human α_1m as indicated in the figure legends. The cells were washed, placed on ice and resuspended in blocking solution (5.4 mM KCl; 1.2 mM KH_2PO_4; 0.8 mM $MgSO_4$;

5.6 mM D-glucose; 127 mM NaCl; 10 mM Hepes; 1.8 mM $CaCl_2$; pH 7.3; 1% BSA; 5% goat serum) for 15 min. First, cell surface staining was carried out by incubating for 15 min on ice with mouse monoclonal antibodies against α_1m (5 μg/ml). This was followed by washing and incubating for 15 min on ice with goat anti-mouse IgG F(ab′)$_2$ fragments (Alexa Fluor® 594; Invitrogen, Eugene, OR). After washing, total staining of the cells (cell surface + cytosol) was performed by suspending in ice-cold Na-medium, fixation with 1% BD CellFIX (BD Biosciences, Belgium) on ice for 15 min and at room temperature for 45 min, followed by permeabilization in 0.02% Triton-X and blocking in 1% BSA, 5% goat serum, 0.2% Tween-20. The cells were then stained at 4°C overnight with mouse monoclonal antibodies against α_1m at 5 μg/ml. Subsequently, goat anti-mouse IgG F(ab′)$_2$ fragments (Alexa Fluor® 488; Invitrogen, Eugene, OR) were applied for 1 h at room temperature. Cells were mounted using ProLong Gold AntiFade Reagent with DAPI (Invitrogen). Visual inspection and recording of images was performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, using a Plan Apochromat 100 × objective.

Cell viability assay

K562 cells were washed and suspended in PBS to 0.5–1.0 × 10^6 cells/ml and incubated with heme, human α_1m and/or AGP as indicated in the figure legends. After incubation the nucleus-staining dye PI was added to a final concentration of 10 μM and the fluorescence intensity of the suspension was quantified using flow cytometry (BD FACSAria™, BD Biosciences, Palo Alto, CA). The analysis was performed on 10 000 cells using a Coherent® Sapphire™ Solid State Laser (PE-chanel, filter-setting 556 LP and 576/26 BP).

Absorbance scanning of solubilized cells

K562 cells were washed, suspended in PBS to 0.5–1.0 × 10^6 cells/ml and incubated with heme or proteins in various steps as indicated in the figure legends. After incubation, the medium was saved and the cells were washed and solubilized with buffer containing 50 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1% NP-40; 1 μg/μl pepstatin; 5 μg/μl antipain; 10 μg/μl leupeptin. Both solubilized cells and medium were then analysed visually and spectrophotometrically by reading the absorbance spectra (300–700 nm).

Gel chromatography

K562 cells were washed and incubated (0.5–1.0 × 10^6 cells/ml) with buffer or 50 μM heme, then washed and incubated for 2 h with buffer or 10 μM α_1m as indicated in the figure legends. After incubation, cells and culture medium were separated by centrifugation and culture medium was analysed by gel-chromatography. This was performed on a 25 ml-Superose 12 HR 10/30 column (GE Healthcare) using a Fast Performance Liquid Chromatography (FPLC) apparatus (Bio-Rad) equipped with a 0.5 ml sample injection loop, monitoring the eluate at 280 and 405 nm and collecting 0.5 ml fractions. The column was equilibrated and eluated with 20 mM Tris-HCl, pH 8.5; 0.1 M NaCl; 0.02% NaN_3.

ABTS assay

The reductase activity of α_1m was analysed by reaction with the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)-radical (ABTS-radical) as described previously [24]. Briefly, ABTS-radical was prepared by oxidation of 7 mMmM ABTS with 2.45 mM potassium disulphite in water for at least 5 h in the dark and using the resulting ABTS-radical solution within 24 h. Heme (50 μM) was incubated with 0.5–1.0 × 10^6 K562 cells, the cells were washed and then incubated 2 h with 10 μM α_1m as indicated in the figure legends. Supernatant aliquots (> 5 μl) were then added to a 35 μM solution of ABTS-radical in 25 mM Na-phosphate, pH 8, giving a final concentration of 3 μM α_1m. The absorbance of the ABTS-radical was read every 10 s at 735 nm, for a total of 3 min. The rate of reduction of the ABTS-radical was estimated by linear regression of the first five points, i.e. 40 s of the reaction, including time-point zero.

Statistical analysis

Results from triplicate experiments are presented as mean ± SD. Statistical analysis was performed in the computer program Origin (Microcal Software, Inc., Version 6), comparing groups with Student's *t*-test.

Results

Anti-oxidation effects of α_1m in K562 cells

K562 cells were cultured with different concentrations of heme (5–20 μM) for 2 h and the generation of ROS was evaluated by measuring the amount of oxidized cytosolic H_2DCFDA (Figure 1A). A slight but significant increase of the relative fluorescence intensity was seen with 5 μM heme and a clear increase was seen with 10 and 20 μM. The time-dependence of the cytosol oxidation was studied using 10 μM heme (Figure 1B). The addition of heme induced a rapid increase in the relative fluorescence intensity, which was sustained throughout the incubation period. From the dose and time experiments 10 μM heme for 2 h was chosen for further oxidation experiments. The effects of α_1m were examined by adding 2, 5 or 10 μM α_1m to the K562 cells, prior to the addition of heme (Figure 1C). Ten micromolar heme was then added and the cells were

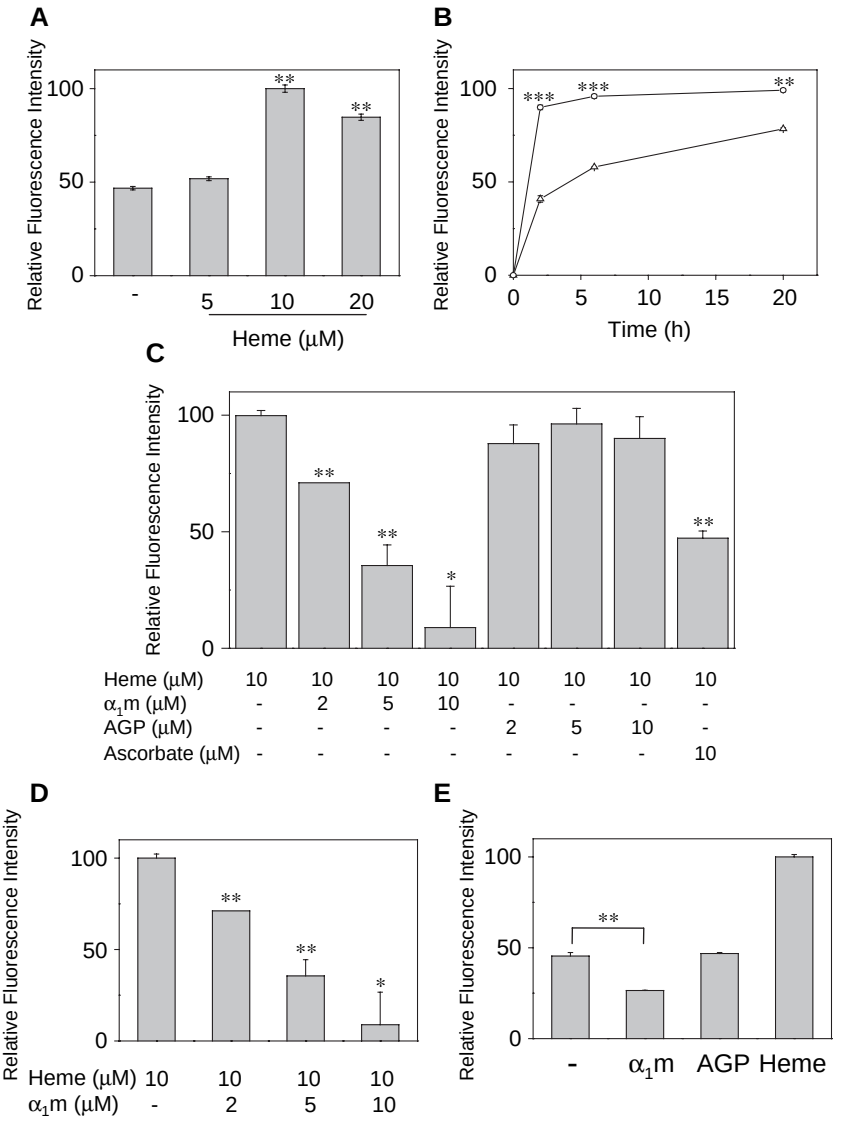

Figure 1. Heme-induced intracellular oxidation. K562 cells were labelled with 3 μM of the oxidation-sensitive probe H_2DCFDA for 30 min, washed and resuspended in fresh medium. (A) The cells were cultured with heme (5–20 μM) for 2 h and analysed with flow cytometry. (B) Cells were incubated in buffer only (Δ) or with 10 μM heme (○). The cell suspension was collected after 0, 2, 6 or 20 h and analysed with flow cytometry. (C) α_1m (2, 5 or 10 μM), AGP (2, 5 or 10 μM) or ascorbate (10 μM) were added to the cells prior to the addition of 10 μM heme. The cells were incubated for 2 h and analysed with flow cytometry. (D) Cells were incubated with 10 μM heme for 30 min, washed twice with PBS and then incubated with α_1m (2, 5 or 10 μM) for 2 h and analysed with flow cytometry. (E) The cells were cultured for a period of 2 h with either culture medium only, heme (10 μM), α_1m (10 μM) or AGP (10 μM). The cell suspension was collected and analysed with flow cytometry. The relative fluorescence intensity of 10 000 cells (excitation 488 nm, emission 530 nm) was plotted as mean of triplicates ± SD, 100% defined as the mean fluorescence intensity (MFI), induced by 10 μM heme. Statistical analysis was performed in the computer program Origin (Microcal Software, Inc., Version 6), comparing groups with Student's *t*-test. $^{\star}p < 0.05$; $^{\star\star}p < 0.01$; $^{\star\star\star}p < 0.001$.

incubated for 2 h. A dose-dependent reduction in relative fluorescence by up to ~90% was seen when α_1m was added. In control experiments, no inhibition of the fluorescence was seen with the lipocalin AGP at the same concentrations as α_1m and 10 μM ascorbate reduced the fluorescence by ~50% (Figure 1C).

The results demonstrate that α_1m inhibits cytosol oxidation by heme. We also investigated whether α_1m could reduce the cytosol in cells that had been pre-incubated with heme (Figure 1D). The cells were incubated with 10 μM heme for 30 min and all the unbound heme was washed away. α_1m (2, 5 or 10 μM) was added and the cells were incubated for 2 h. The results revealed that α_1m was able to significantly reduce cytosol oxidation in a dose-dependent manner. To test whether α_1m could reduce the cytosol of 'resting', non-stimulated cells, these were incubated with α_1m (10 μM) for a period of 2 h (Figure 1E). Addition of α_1m to the cells resulted in a clear reduction in the fluorescence intensity as compared to cells without α_1m. Heme, on the other hand, increased the fluorescence as expected. The reduction

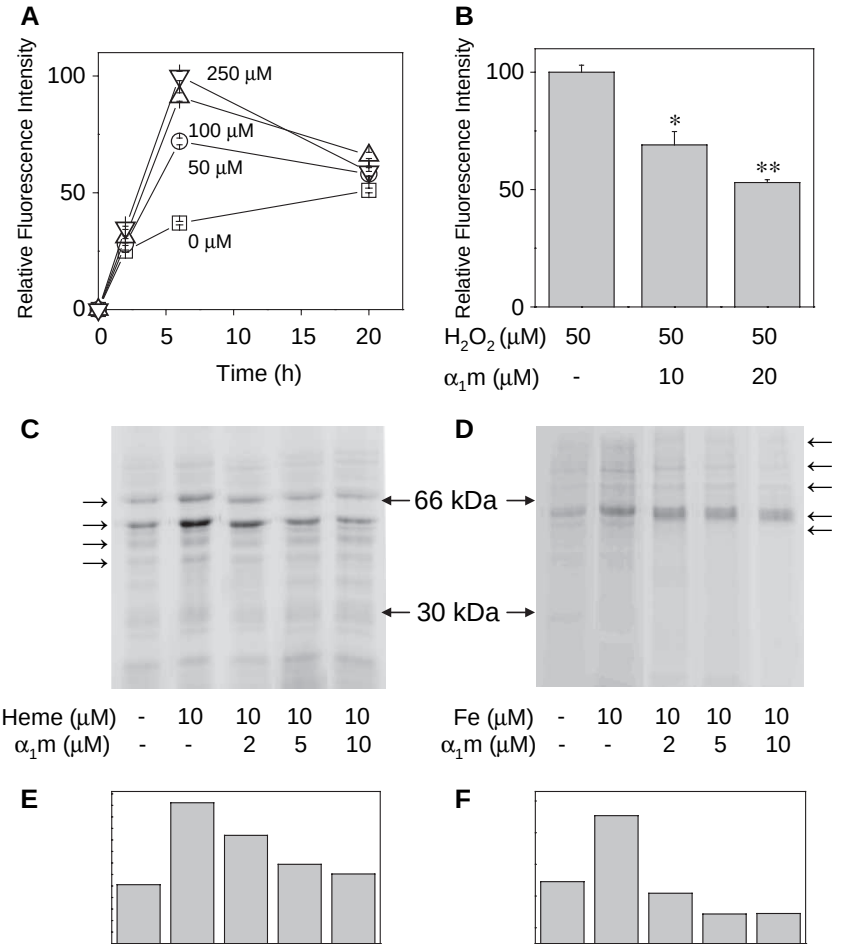

Figure 2. Inhibition of H_2O_2- and Fenton reaction-induced intracellular oxidation. (A) and (B) inhibition of H_2O_2 and Fenton reaction induced intracellular oxidation. K562 cells were labelled with 3 µM of the oxidation-sensitive probe H_2DCFDA for 30 min, washed and resuspended in fresh medium. The cells were cultured with H_2O_2 (50–250 µM) for a period of 0–20 h and the cell suspension analysed with flow cytometry (A). Ten or 20 µM α_1m were added to the cells prior to the addition of 50 µM H_2O_2. The cells were cultured for 6 h and the cell suspension analysed with flow cytometry (B). The relative fluorescence intensity of 10 000 cells (excitation 488 nm, emission 530 nm) was plotted as mean of triplicates ± SD, 100% is defined as the mean fluorescence intensity (MFI), induced by 250 µM (A) or 50 µM (B) H_2O_2. Statistical analysis in (A) and (B) was performed in the computer program Origin (Microcal Software, Inc., Version 6), comparing groups with Student's *t*-test. $^{\star}p < 0.05$; $^{\star\star}p < 0.01$. (C)–(F) Intracellular protein thiol-oxidation was measured by SDS-PAGE according to Baty et al. [30], described in the Materials and methods section. α_1m, 2–10 µM, was added to cells prior to the addition of 10 µM heme and visualized by fluorimetry (C) and quantified by pixel intensity analysis (E). α_1m, 2–10 µM, was added prior to the addition of a mixture containing 10 µM $(NH_4)Fe(SO_4)_2$ + 100 µM ascorbate + 20 µM H_2O_2 (shown as Fe in the figure) and visualized by fluorimetry (D) and quantified by pixel intensity analysis (F). One representative experiment is shown in (C) and (E), and (D) and (F), respectively.

of unspecific background oxidation seen with α_1m was not observed when adding the control protein, AGP.

We investigated whether the anti-oxidative effects of α_1m were restricted to cells oxidized with heme or could also be directed against other oxidants. H_2O_2 (50–250 µM) was used to induce oxidation for a period of 0–20 h (Figure 2A). H_2O_2 induced elevated levels of H_2DCFDA up to a peak at 6 h after which the levels decreased. Simultaneous incubation of cells with α_1m (10 or 20 µM) and 50 µM H_2O_2 for 6 h showed a dose-dependent reduction in the fluorescence induced by H_2O_2, demonstrating the inhibitory effects of α_1m (Figure 2B).

To further examine the anti-oxidative effects of α_1m, intra-cellular protein thiol oxidation was measured. Two, five and ten micromolar α_1m was added prior to adding 10 µM heme to K562 cells and these were then incubated for 6 h. Intracellular proteins labelled with the disulphide-specific fluorescent probe 5-IAF were separated by SDS-PAGE. The oxidized thiols were visualized by fluorimetry (Figures 2C and D) and the strongest bands quantified by measuring pixel density (Figures 2E and F). Heme induced an increased protein thiol oxidation (Figure 2C and E) and the thiol label intensity of the four strongest bands (migrating as 50, 55, 60 and 66 kDa; marked by arrows) was inhibited by α_1m in a dose-dependent manner down to the level of resting cells. The weakly stained bands showed less upregulation by heme and less inhibition by α_1m (not shown), perhaps because of non-specific binding of the probe. Furthermore, α_1m also inhibited non-heme induced protein thiols: a

mixture of Fe^{3+}, ascorbate and hydrogen peroxide (10 μM, 100 μM and 20 μM, respectively) was used to generate hydroxyl radicals by the Fenton-reaction. The mixture resulted in an increased level of oxidized protein thiols (Figure 2D and F) and the thiol label intensity of the five strongest bands (migrating as 50, 60, 66, 80 and 120 kDa; marked by arrows) decreased by the addition of α_1m.

The heme oxygenase 1 (HO-1)-gene is up-regulated in K562 cells as a result of heme exposure [6]. We analysed the effect of α_1m on oxidant-induced HO-1 expression (Figure 3A). As expected, HO-1 was up-regulated by 10 μM heme, 50 μM H_2O_2 or a mixture of 10 μM Fe^{3+}, 20 μM H_2O_2 and 100 μM ascorbate and the up-regulation was reversed by 10 μM α_1m. The expression of the house-keeping gene G3DPH was not affected by any of the additions.

Rescue from cell death

The effects of heme and α_1m on cell viability was investigated by adding 20–500 μM heme to K562 cells for 4 h. The viability of the cells was determined using staining with PI and FACS-analysis. A clear dose-dependence of the cell death was observed (Figure 3B) up to almost 100% at 500 μM heme. No effect was seen using corresponding amounts of the heme solvent, DMSO (not shown). The ability of α_1m to

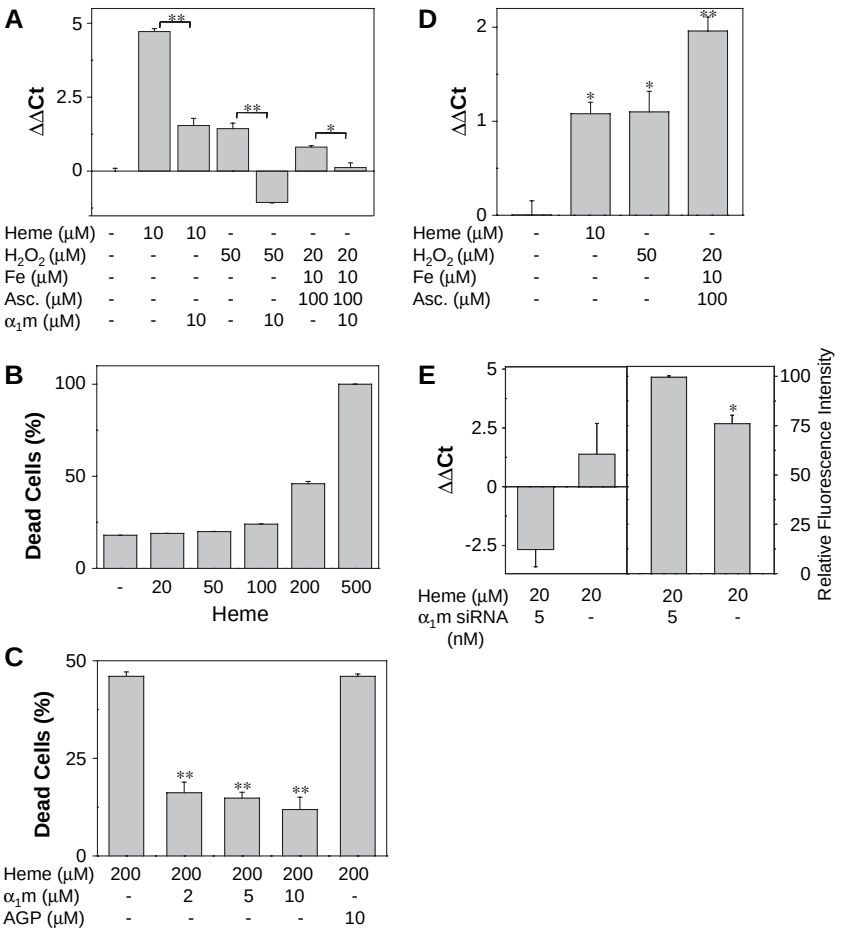

Figure 3. (A) Inhibition of oxidant-induced HO-1-expression. Real-time PCR was used to examine the expression of the HO-1 mRNA in K562 cells exposed to heme, hydrogen peroxide or a mixture of $(NH_4)Fe(SO_4)_2$, hydrogen peroxide and ascorbate (Fenton reaction). The HO-1 expression was also analysed with the addition of α_1m to all the conditions. (B) and (C) Inhibition of heme-induced cell death. K562 cells were cultured with heme, with or without α_1m or AGP for 4 h. The cell suspension was collected, mixed with 10 μM PI (final concentration) and analysed with flow cytometry. The percentage PI-positive cells (= dead cells) of 10 000 cells (PE-channel, filtersetting 556 LP and 576/26 BP) was plotted as mean of triplicates ± SD. (D) Up-regulation of α_1m. Real-time PCR was used to examine the expression of the α_1m mRNA in K562 cells exposed to heme, hydrogen peroxide or a mixture of $(NH_4)Fe(SO_4)_2$, hydrogen peroxide and ascorbate (Fenton reaction). (E) Silencing of α_1m-expression. K562 cells were transfected with 5 nm α_1m-specific siRNA, cultured for 24 h, washed, loaded with H_2DCFDA as described in the Materials and methods section (right panel) and exposed to 20 μM heme for 2 h. Cells were then analysed by real time-PCR (left panel) or flow cytometry (right panel). RNA-extraction, cDNA-preparation and PCR-amplification were performed as described in the Materials and methods section. The relative fluorescence intensity of 10 000 cells (excitation 488 nm, emission 530 nm) was plotted as mean of triplicates ± SD, 100% is defined as the mean fluorescence intensity (MFI), induced by α_1m in cells challenged by 20 μM heme. All expression levels were normalized against G3DPH and are depicted in the figure as ΔΔCt. Statistical analysis was performed in the computer program Origin (Microcal Software, Inc., Version 6), comparing groups with Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

rescue cells from death was examined by adding α_1m (2, 5 or 10 μM) or AGP (10 μM) to the cells, prior to the addition of 200 μM heme and incubating for 4 h. Approximately 70% of the dead cells could be rescued with the addition of α_1m at the highest concentrations (Figure 3C). No significant effect was observed with AGP.

Analysis of endogeneous α_1m in K562 cells

It was shown previously that small amounts of α_1m are secreted from K562 cells and that the secretion was increased after incubation with haemoglobin or ROS [25]. Figure 3D shows real-time PCR-analysis of α_1m-mRNA in K562 cells incubated with medium, 10 μM heme, 50 μM H_2O_2 or a mixture of 10 μM Fe^{3+}, 20 μM H_2O_2 and 100 μM ascorbate. All these oxidants induced an increase in the α_1m mRNA levels. To investigate the anti-oxidative effects of the endogeneous α_1m, we silenced the α_1m-gene by adding α_1m-specific siRNA and challenged the cells with 20 μM heme. The α_1m mRNA was partially silenced (Figure 3E, left panel), resulting in a significant increase of cytosol oxidants as measured by the H_2DCFDA probe (Figure 3E, right panel).

Since endogeneously produced α_1m is secreted from the cells, both this and exogeneously added α_1m [25] is found in the cell-medium, outside the cells. Using fluorescence microscopy, we investigated the cellular localization of α_1m (Figure 4). Most of the endogenous protein was detected as a patchy staining on the surface of the cells, as shown by incubation with anti-α_1m before (Figure 4A, centre) and after (Figure 4A, right) permeabilization and fixation. Weak staining of intra-cellular compartments was also observed after permeabilization (Figure 4A, right). Approximately 60% of the cells were positive for α_1m staining. However, a much stronger staining was obtained after cells had been incubated with exogeneous α_1m and also in this case most of the staining was located at the cell surface ($\sim$90% of the cells) in a patchy pattern, as shown by incubation with anti-α_1m before (Figure 4B, centre) and after (Figure 4B, right) permeabilization and fixation.

Heme scavenging and radical reduction by α_1m

The mechanisms of the cytoprotective effect of α_1m were studied by analysing the interactions between the protein and cell-bound heme. Cells were incubated with 10 μM heme for 30 min, excess heme washed off and α_1m or the control protein AGP added to a concentration of 2 or 10 μM and incubated for 2 h. The supernatants were saved, cells washed and solubilized and both media and solubilized cells analysed by spectrophotometry (Figure 5A, except AGP) and visually (Figure 5B). Heme incorporated to the cells was seen as a strong brown-colouring of the cells; the typical absorbance spectrum with a non-

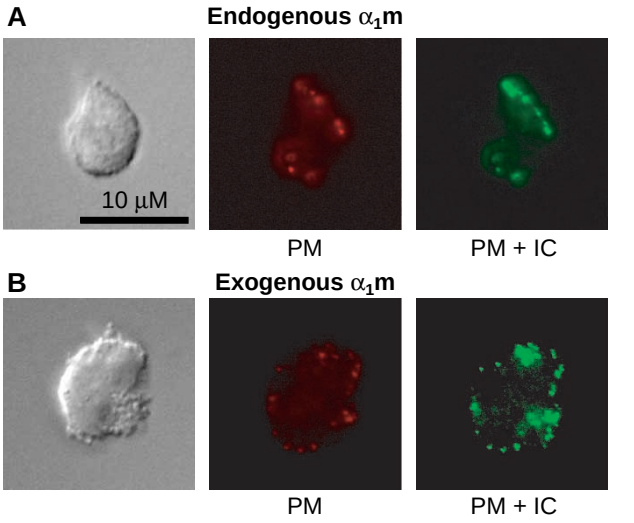

Figure 4. Localization of α_1m in cells. Cells incubated with either medium only (A) or 10 μM α_1m (B) for 2 h were washed, placed on ice and subjected to plasma membrane (PM) staining (centre) with mouse monoclonal antibodies against α_1m, followed by goat anti-mouse IgG F(ab′)$_2$ fragments (Alexa Fluor® 594; red). The cells were then put on ice, fixed and permeabilized and subjected to total staining (plasma membrane+intracellular, PM+IC) with mouse monoclonal antibodies against α_1m followed by goat anti-mouse IgG F(ab′)$_2$-fragments (Alexa Fluor® 488; green). Cells were mounted and visual inspection and recording was performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera using a Plan Apochromat 100× objective. The picture is representative for three separate experiments. Sizebar is 10 μM.

distinct peak was detected around 400 nm. When adding α_1m, the heme was almost completely removed from the cells and instead found in the medium. The control protein AGP at the same molar concentrations had much less effect on the cell-bound heme (Figure 5B).

We next analysed whether the heme removed from the cells was bound to α_1m. Cells were incubated with 10 μM heme for 30 min, washed extensively and then incubated with 10 μM α_1m in serum-free medium for 2 h. The supernatant was removed and separated by gel-chromatography on a Superose 12 column (Figure 6A). Most of the heme (abs 405 nm) was co-eluted with the protein (abs 280 nm), suggesting that it is bound to α_1m. Furthermore, increased amounts of dimeric and higher-order aggregates of α_1m were seen after heme-binding, as compared to α_1m incubated with cells but without heme.

It was shown recently that α_1m can reduce the ABTS-radical, a stable organic radical, in a semi-catalytic manner, leading to formation of free, reduced ABTS and covalent attachment to side-groups on the protein [24]. We investigated this property of the protein after incubation with cells and/or heme. The disappearance of the ABTS-radical (abs 735 nm) was seen with α_1m alone or α_1m incubated with heme or heme plus cells (Figure 6B). The reduction rate was slightly, but significantly increased when α_1m was

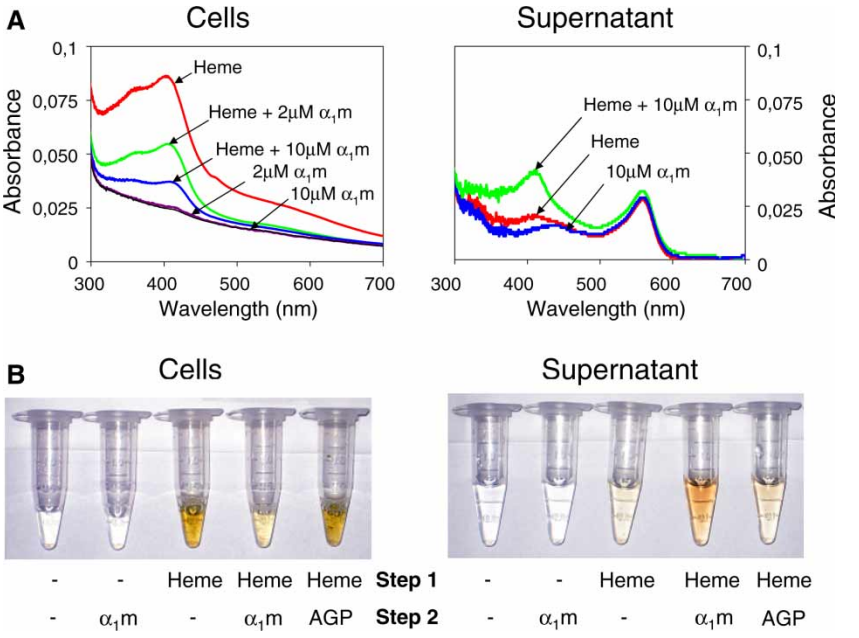

Figure 5. Heme accumulation in cells. K562 cells were cultured with either buffer or 10 μM heme for 30 min, washed and resuspended in fresh culture medium. (A) The cells were then incubated with α_1m (2 or 10 μM) for 2 h, after which time the culture medium was saved. The cells were washed and solubilized by suspending in a buffer containing 1% NP-40. The culture medium and cell suspension were then analysed spectrophotometrically by reading the absorbance spectra (300–700 nm). (B) The various cultures were also analysed visually. Cells were incubated with either buffer or 10 μM heme for 30 min (Step 1), washed and then incubated with buffer, 10 μM α_1m or 10 μM AGP for 2 h (Step 2), washed and solubilized as described above.

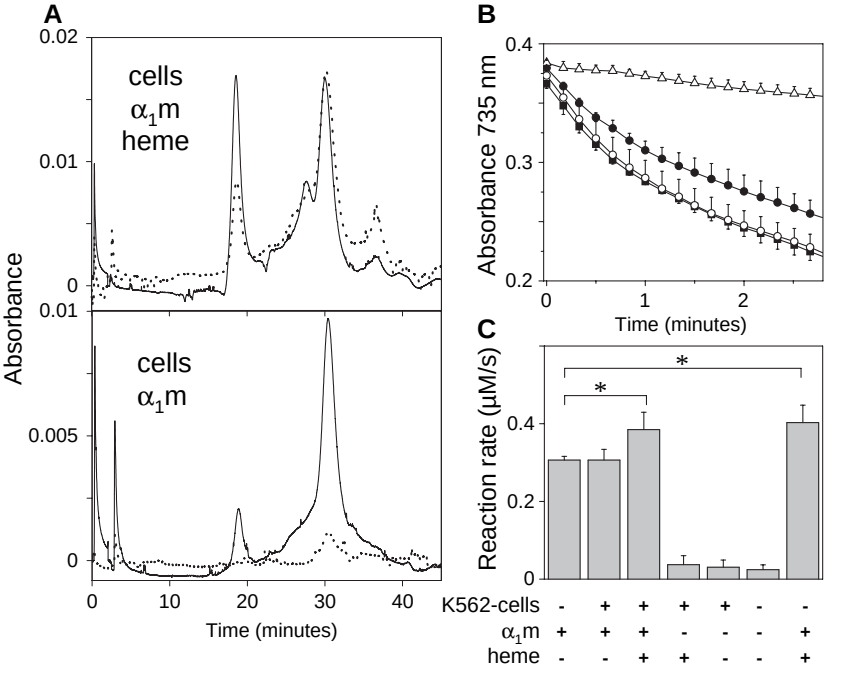

Figure 6. Biochemical and redox properties of α_1m-heme complex. K562-cells ($0.5–1 \times 10^6$) were incubated with buffer or 50 μM heme for 30 min, washed and then incubated with 10 μM α_1m for 2 h. After centrifugation, the supernatants were analysed. (A) Gel-filtration was performed on a 25 ml-Superose 12 HR 10/30 column using a Fast Performance Liquid Chromatography (FPLC) apparatus equipped with a 0.5 ml sample injection loop, monitoring the eluate at 280 (solid line) and 405 nm (dot line) and collecting 0.5 ml fractions. The column was equilibrated and eluated with 20 mM Tris-HCl, pH 8.5; 0.1 m NaCl; 0.02% NaN_3. (B) ABTS-radical reduction activity was measured by mixing cell supernatants (Δ: cells+heme; ●: cells+α_1m; ○: cells+heme+α_1m), giving a final concentration of 3 μM α_1m, with 35 μM ABTS-radical in 25 mM Na-phosphate, pH 8, reading the absorbance at 735 nm at regular intervals. Control reaction: 3 μM α_1m+10 μM heme without cells (■). (C) The reaction rates were calculated as the absolute values of the slopes of a line drawn by regression analysis of the points during the first 40 s, including time-point zero. Identical numbers of cells were used for the comparison of the ABTS-reduction rates. All values represent the mean and SD from three separate experiments. Statistical analysis was performed in the computer program Origin (Microcal Software, Inc., Version 6), comparing groups with Student's *t*-test. * $p < 0.05$.

incubated with heme-loaded cells or with 10 μM heme ($p < 0.05$) (Figure 6C). Background activity, i.e. a very slow disappearance of the ABTS-radical, was seen with the controls.

Discussion

Accumulating experimental evidence suggests that extracellular, extravascular haemoglobin and the downstream release of heme have oxidative toxic effects that contribute to cell injury [32–34]. It has been shown previously that α_1m has heme-binding and heme-degrading properties [21] as well as reductase properties [23,24]. We therefore hypothesized that α_1m may counteract heme-induced and possibly ROS-induced cellular oxidation. This was indeed shown in this work, i.e. α_1m could reduce the heme-, hydrogen peroxide- and Fenton reaction-induced intracellular oxidation. No inhibition was seen by the control protein AGP, suggesting that the effect seen with α_1m is not a result of non-specific protein buffering. Possible mechanisms for the prevention of cytosol oxidation may be scavenging of heme, ROS and other downstream oxidants, but also that α_1m may be part of redox buffering mechanisms, i.e. reducing oxidized intracellular components.

The results show a dose-dependent increase of cell death with increasing concentrations of extracellularly added heme. Heme is a lipid-soluble molecule that interacts rapidly with the plasma membrane and other hydrophobic cell constituents [35]. Most of the cellular heme (i.e. after washing of the cells incubated with heme) was found in the cytosolic compartment (not shown), most likely bound to soluble macromolecules. The mechanism for the lysis is not known, but it has for instance been suggested that heme interferes with ion transport over the plasma membranes, leading to lysis of the erythrocytes [36]. The heme-induced lysis was inhibited by α_1m at much lower concentrations. Sixty per cent of the cells killed by heme were rescued from lysis by an α_1m:heme ratio of 1:100. This suggests that catalytic mechanisms besides scavenging of the added heme are involved in the inhibition by α_1m.

K562 cells express α_1m [25]. The fluorescence microscopy experiments showed that endogeneous α_1m is mainly localized to the cell surface. Exogeneously added α_1m was also found mainly at the plasma membranes of the cells. Physiologically, an extracellular or cell-surface localization of an antioxidant may be strategically advantageous, primarily by counteracting external sources of oxidation. Thus, heme and ROS, arising from hemolysis or tissue necrosis, are directly exposed to reduction or scavenging by secreted or plasma membrane-bound α_1m. Reduction of extracellular components are likely to shift the redox equilibrium over the plasma membrane, leading also to reduction of the cytosol. This may be the mechanism behind the finding that addition of α_1m to the culture medium reduced the cytosol of cells under normal culture conditions, e.g. with no oxidants added.

K562 cells were chosen as a model for the studies of cytoprotection mechanisms of α_1m because they were reported to be capable of turning on haemoglobin synthesis when heme is added to the culture medium [27]. This would allow studies of the effects of α_1m on oxidative stress from endogeneous as well as external sources. However, the K562 cells, in our hands, could not be induced to synthesize haemoglobin and the anti-oxidation effects were investigated by adding heme exogeneously. In spite of this, the results also suggest that α_1m may be part of auto-protection mechanisms against internal oxidative loads. Silencing of the α_1m-gene in the cells resulted in an increase of cytosol oxidants as measured by the H_2DCFDA probe. Furthermore, the expression of the α_1m-gene was up-regulated by addition of oxidants (heme and ROS). These results suggest that α_1m contributes to the cellular redox buffer system. To study the possible physiological relevance of such an endogeneous function of the lipocalin, we are currently investigating the expression and localization of α_1m in primary erythroid stem cell clones, where heme- and haemoglobin presents an intracellular oxidative load.

The heme degradation enzyme HO-1 was up-regulated by the same oxidation conditions that also resulted in an increased expression of α_1m (heme, hydrogen peroxide and Fenton reaction-induced hydroxyl radicals). The up-regulation of HO-1 was reversed by α_1m, which can be expected since α_1m inhibited the cytosol oxidation caused by these oxidants. HO-1, a well-known heme detoxification enzyme, catabolizes heme to free iron, biliverdin and CO and is up-regulated in many cells in response to heme and ROS exposure [13,14,37]. Thus, α_1m and HO-1 may have similar physiological functions: participation in cytoprotection by anti-oxidation and elimination of heme. However, the two proteins differ in many respects, for example heme is bound by α_1m ('scavenging') [21,22,38], but it is not likely that it is converted to iron, biliverdin and CO. Instead, heme reacts with α_1m forming heterogeneous brown covalent modifications of the protein (see below). Besides elimination of the pro-oxidant heme, α_1m has antioxidant effects of its own, as shown in this paper, whereas the anti-oxidative effects of HO-1 are explained by the downstream production of bilirubin [37,39]. Furthermore, the sites of synthesis are different: the major part of α_1m is secreted by the liver and distributed to the extracellular fluids in all organs [20], whereas the current view is that HO-1 is synthesized locally and mainly remains intracellular.

What is the role of α_1m in relation to the plasma haemoglobin- and heme-scavengers, haptoglobin and

hemopexin? The two latter proteins are found in high concentrations in blood, bind to haemoglobin and heme and the complexes are cleared from the circulation via binding to macrophage- and hepatocyte scavenger receptors CD163 and CD91, respectively [9,12]. We propose that α_1m has a role as an anti-oxidant and heme-scavenger in extravascular fluids rather than in blood (besides a possible cellular role, as discussed above). It is secreted to blood from the liver, but its concentration in blood is comparatively low, 1–2 μM [26], due to a very rapid equilibrium ($T_{1/2} = 2$–3 min) between intra- and extravascular compartments [20,40]. It is rapidly cleared from the circulation and thus from the extravascular compartment by tubular cells in the kidneys [20]. Although not yet shown conclusively, the uptake of α_1m in the tubular cells is most likely mediated by the multi-ligand receptor megalin, a member of the LDL-receptor family [41]. It is not known, however, whether there is a selective uptake of α_1m modified by heme or other radicals, compared to unmodified α_1m.

Several experiments indicate that the binding of heme to α_1m is covalent. It was previously shown that denaturation does not dissociate the heme-group from the protein [21]. The binding may involve a similar covalent trapping mechanism, as recently described for ABTS-radical [24]. It was shown that α_1m reduces the ABTS-radical in a pseudo-catalytic way in a series of reactions that are dependent upon the thiol group at C34. The reduction reactions are followed by trapping of ABTS-radicals by covalent attachment to several side-groups on α_1m. Proteolytic digestion and LC-MS analysis of the α_1m-heme complex will hopefully show whether this hypothesis is true and reveal the structure of tentative protoporhyrin-components and their localization on the α_1m protein core.

One potential physiological function of α_1m could be to act as a 'radical sink' using its reductase and scavenging activities. The protein is yellow-brown *in vivo* and the colour was shown to be caused by heterogeneous modifications of several amino acid side-chains [42–44]. The yellow-brown modifications could be degradation products of organic radicals reduced and trapped by α_1m in the human body. One such radical may be kynurenin, a tryptophan metabolite found to be covalently associated with amniotic α_1m in haemodialysis patients [44]. Other possible targets are tyrosine radicals and oxidation products of tyrosine such as dopa, which promote oxidation in biological systems [45,46] and were shown to react with α_1m [24]. As discussed above, it seems plausible that α_1m operates mostly extravascularly, rather than in plasma. It may also be speculated that radical reduction and scavenging is particularly important in fluids enriched in waste-products, such as urine, cerebrospinal fluid and amniotic fluid. α_1m, purified from urine or amniotic fluid, is more intensely coloured than plasma α_1m, supporting this notion. The objects of future studies should be to identify as many as possible of the radical targets of α_1m in normal and pathological conditions.

In conclusion, oxidative reactions of heme and ROS cause modifications of cytosolic molecules, up-regulation of HO-1 as well as cell lysis and may be involved in different pathological conditions. The results in this investigation together with a series of recent publications [21,22,24] suggest that α_1m is involved in the defence against cellular oxidative injury caused by extracellular haemoglobin and heme.

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