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A1M, an extravascular tissue cleaning and housekeeping protein

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Key terms: alpha-1-microglobulin (A1M), radical, reductase, heme, antioxidation, organelle-, cell- and tissue protection

ABSTRACT

Alpha-1-microglobulin (A1M) is a small protein found intra- and extracellularly in all tissues of vertebrates. The protein was discovered 40 years ago and its physiological role remained unknown for a long time. A series of recent publications have demonstrated that A1M is a vital part of tissue housekeeping. A strongly electronegative free thiol group forms the structural basis of heme-binding, reductase- and radical-trapping properties. A rapid flow of liver-produced A1M through blood and extravascular compartments ensures clearing of biological fluids from heme and free radicals and repair of oxidative lesions. After binding, both the radicals and A1M are electroneutral and therefore do not present any further oxidative stress to tissues. The biological cleaning cycle is completed by glomerular filtration, renal degradation and urinary excretion of A1M heavily modified by covalently linked radicals and heme-groups. Based on its role as a tissue housekeeping cleaning factor, A1M constitutes a potential therapeutic drug candidate in treatment or prophylaxis of diseases or conditions that are associated with pathological oxidative stress elements.

INTRODUCTION

It has been recognized for many decades that intracellular activities such as macromolecular synthesis, degradation and transport, cell division, cell motility, energy production, etc., require constant maintenance and adjustments. The term "cell housekeeping" was coined to describe these basal activities found in all cells. Extracellular compartments also require maintenance and the term “tissue housekeeping” applies for extracellular processes that are developed to safeguard order, integrity and functionality of the tissues. For example, waste-products, generated from metabolism, dying cells, invading microorganisms, UV-irradiation, etc., not only creates untidiness but also constitute a chemical threat to tissue components and therefore must be removed. Small organic and inorganic radicals, including reactive oxygen species (ROS) make up a large group of these waste-products, and constitute a potential problem during oxidative stress conditions due to the strong reactivity of their unpaired electrons. Failure to maintain intra- and extracellular housekeeping, or overwhelming the housekeeping machinery during stress-conditions, leads to cell damage, organ failure and development of diseases.

Recently, the human plasma and tissue protein A1M (alpha-1-microglobulin, α_1-microglobulin) has emerged as a tissue housekeeping protein with a central role in cleaning of oxidative waste-products, macromolecular repair, and antioxidation protection. A1M was discovered nearly 40 years ago as a peculiar yellow-brown protein purified from human urine [1]. It was soon shown to be conserved in all vertebrates, secreted from liver and most other epithelial cells, and present in blood and all tissues at remarkably constant concentrations [2]. The yellow-brown coloration was demonstrated to consist of very heterogeneous covalent modifications of several amino acid side-groups [3]. The last ten years, a series of reports have revealed A1M:s physiological role as a cell- and tissue protective antioxidant, operating

by clearing extravascular fluids of free radicals and heme-groups, and transporting them to the kidneys for degradation [4](Figure 1). It is the purpose of this review to summarize the structural and functional properties of A1M and briefly discuss its therapeutic potential.

A1M PROTEIN

Structure

Human A1M has a molecular weight of 26 kDa and consists of a peptide chain with 183 amino acids [5, 6] and oligosaccharides in three positions [7]. The protein is well conserved during evolution and A1M homologues have been identified in humans, other mammals, birds, amphibians and fish (reviewed in [4]). A1M is a member of the lipocalin protein family, and the recently published crystal structure of human A1M [8] displays the typical lipocalin structural features (Figure 2). The lipocalins are a group of 40-50 proteins found in all branches of life [9]. They share a common protein fold: a single polypeptide, 150-190 amino acid residues, and forms an antiparallel eight-stranded β-barrel with one closed and one open end. The interior of the barrel often serves as a binding site for small hydrophobic compounds. A1M contains a functionally important side-chain, Cys34, which is found in all species, located on a loop near the lipocalin pocket, is solvent exposed (Figure 2)[8] and involved in the binding/neutralization of target compounds [10].

Expression

The gene for A1M is denoted *AMBP* (*A*lpha-1-*M*icroglobulin-*B*ikunin *P*recursor gene) because it also encodes bikunin, another plasma protein [6]. Bikunin is a Kunitz-type protease inhibitor [11] and a structural component of extracellular matrix [12]. Bikunin is the common subunit of a group of protein/carbohydrate complexes that constitute plasma proteins of the inter-α-inhibitor family [13]. Transcription of the *AMBP* gene produces an mRNA that is

translated into a precursor protein consisting of the A1M and bikunin proteins connected by a linker tripeptide [14]. Before secretion, the A1M and bikunin components are separated by proteolytic cleavage in the Golgi-system [15]. No physical or functional connection has been found between A1M and bikunin after secretion and the reason for co-synthesis is not yet understood.

The *AMBP* gene has been cloned from man [16] and mouse [17], and mapped to a lipocalin gene cluster in the 9q32-33 region in man [16] and to chromosome 4 in mouse [18]. The A1M-exons are separated from the bikunin-exons by a large intron which contains retroposons and other repeated structures, suggesting that it is a recombinatorial hot-spot [17]. This could have provided the basis for a fusion between an ancestral lipocalin gene (A1M) with an ancestral Kunitz inhibitor gene (bikunin). Interestingly, the A1M/bikunin genetic construction is conserved in all species where A1M has been investigated.

Distribution

Liver, blood plasma, and kidney are the main sites of A1M localization [19]. The liver is the major site of synthesis [20] and its expression in liver is regulated by hepatocyte nuclear factors (HNF 1-4) [21-23]. Following synthesis, the protein is secreted to the blood, where it exists in a concentration of approximately 20-50 mg/ml [24]. About 50% of the total plasma A1M is found in a free form, exposing a reduced and functionally important cysteine side chain (Cys34), and the rest is complex-bound mainly with IgA via a reduction-resistant disulfide bond involving Cys34 [25, 26]. Minor complexes with albumin (~ 7%) and prothrombin (~ 1%) have also been identified [25, 26]. Both free A1M and the complexed forms are rapidly equilibrated between the intra- and extravascular compartments and their half-lifes in blood are about 2-3 min [27, 28]. Free A1M passes almost unhindered through

the glomerular membranes to the primary urine, where most of it is reabsorbed by the proximal tubular cells and catabolized [29]. Significant amounts, however, are excreted in the urine and the A1M-concentration in urine is a sensitive, clinically used indicator of tubular renal damage [5].

Besides the liver, A1M mRNA has been detected in most other cell types and the A1M protein has been identified in the perivascular connective tissue of most organs [30, 31], especially abundant in epidermis of skin [32]. It is often co-localized with elastin and collagen [31-33] and binds to collagen *in vitro* [34]. A distribution of A1M at various interfaces between the cells of the body and the ambient environment (blood/tissue, air/tissue, intestinal lumen/villi), as well as at the interface between maternal blood and fetal tissues in placenta [35, 36], is consistent with a protective role of A1M *in vivo* (see below).

Mitochondria

A part of A1M is found intracellularly, localized mainly to the mitochondria, and specifically to Complex I of the respiratory chain [37]. Mitochondrial localization (Figure 4D) and an uptake of exogenously added A1M to mitochondrial Complex I of skin keratinocytes, blood cells and liver was shown by several methods. The functional role of A1M in the mitochondria is not fully characterized, but it was speculated that the protein is involved in assisting the mitochondria to maintain its energy delivery during cell death [37]. A1M may also, at the same time, counteract and eliminate the radicals generated by the mitochondrial respiration to prevent oxidative damage to surrounding healthy tissue [37].

MOLECULAR MECHANISMS

As shown in Figure 1, A1M has a number of mechanistic properties which contributes to its role as a tissue housekeeping protein. These are summarized below.

Reduction

Enzymatic reductase/dehydrogenase properties were recently described for A1M with a wide spectrum of organic and inorganic substrates (Figure 3A) [10, 38]. The protein was capable of reducing the heme proteins cytochrome c and methemoglobin, free iron and the synthetic compounds nitroblue tetrazolium (NBT) [38]. The results thus suggest that A1M is rather unspecific with regard to its substrates. A catalytic reductase effect was seen in the presence of ascorbate, NADH and NADPH [38], but these are probably not the only physiologically relevant electron-donating co-factors, since the reducing activity of A1M has been shown both intra- and extracellularly (see below). The reductase and dehydrogenase activities are dependent on the thiol group of Cys34 and the three lysyl residues of Lys92, 118 and 130. According to the three-dimensional structure of A1M, these four side-chains are located in the vicinity of each other at the open end of the lipocalin barrel (Figure 2). The three Lys residues were proposed to function by creating a positively charged microenvironment around the Cys34, thus lowering the pK_a of the thiol group and favoring its oxidation [38]. In addition, A1M rapidly reduced the synthetic radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and this reaction was also dependent on the free thiol group of Cys34 [10]. Reduction of physiological substrates have been shown both intra- and extracellularly. It was shown that the cell cytosol and thiol groups of cytosolic proteins of the erythroid cell line K562 were reduced by A1M [39]. Furthermore, A1M reduced the number of carbonyl groups on oxidized collagen in a reaction which was entirely dependent on the presence of Cys34,

and partly on Lys92, 118 and 130 [32, 40]. The detailed chemical mechanism of the carbonyl group reduction is not yet known, however.

Heme-binding and degradation

A1M binds heme with $K_d=10^{-6}$ M [41, 42]. The heme-binding is evolutionarily conserved and has been shown to occur to A1M in plasma from man, mouse, rat, guinea pig, cow, chicken and plaice [42]. By titration of the heme-group it was shown that a trimeric A1M/heme complex is formed, where each A1M-molecule binds two heme-groups, *i.e.* $[(heme)_2(A1M)]_3$ [43]. A possible heme-binding site consisting of a Cys-Pro-dipeptide motif similar to those seen in the cytochrome P450 family is located at loop 1 (Cys34-Pro35) [8] (Figure 2). Apart from this non-covalent interaction, a possibly covalent binding was seen which remained after boiling in SDS [41, 42]. A processed form of A1M, called t-A1M (t=truncated), lacking the C-terminal tetrapeptide (Leu-Ile-Pro-Arg) of full-length A1M, was generated by a reaction with lysed red blood cells or with purified hemoglobin (Hb) [41]. The processed t-A1M bound to heme, but was also capable of degrading the heme-group to a heterogeneous chromophore associated with the protein. The t-A1M form is produced *in vivo* and has been found in urine, skin and placenta [41, 44-46]. Together, these reports suggest that A1M contributes to extracellular heme clearance by binding and degradation.

Radical scavenging

The radical scavenging mechanism of A1M was studied using the synthetic radical ABTS [10] and it was shown that A1M reacts with this radical in a reaction that involves Cys34-catalyzed reduction and results in covalent trapping of radical metabolites to several amino acid side-chains on A1M (Figure 3B). A1M was shown to reduce 5-6 molecules of the ABTS-radical in a rapid semi-catalytic reaction, and covalently trapped 3 additional ABTS-radicals

by attaching them onto tyrosyl side-chains. Two of the modified Tyr-residues were identified as Tyr22 and Tyr132 (Figure 2). Besides these two tyrosyl-residues, several lysyl side-chains were shown to be modified by radical trapping. Thus, Lys69 [47], 92, 118 and 130 [3] are covalently modified in human A1M isolated from urine. Furthermore, the tryptophan metabolite 3-OH-kynurenin, which has a propensity to form free radicals [48], was identified as a lysyl modification on urinary A1M from hemodialysis patients [49]. The side-chains of Lys92, 118 and 130 may therefore serve a dual role both in activation of the Cys34 thiol by lowering its pK_a [38] and as electron-donors in the radical-trapping mechanism. Finally, it has also been reported that the Cys34 thiol itself may be covalently modified by unidentified brown-colored oxidation products *in vivo* [50]. The term "radical sink" was used to describe the radical binding property of A1M [10], referring to the fact that both the radicals and the A1M protein itself are electroneutral after the reactions and therefore do not present any further oxidative stress to tissue components. Although this radical trapping mechanism has so far not been proven to occur in vivo, it is likely that it is physiologically relevant *in vivo* (see below for a summary of data obtained *in vivo*) [51, 52]. Furthermore, the presence of a heavily modified and brown-colored A1M-protein in human urine, compared to albumin purified from the same source (Figure 3C), suggests that radical scavenging is a highly A1M-specific reaction and not a non-specific protein modification occurring on plasma and tissue proteins in general.

Up-regulated A1M-expression

The expression of the A1M-gene (*AMBP*) is increased during oxidative stress. Up-regulated expression of A1M, both on mRNA- and protein levels, has been shown in human cell lines, primary keratinocytes, and skin and retinas explants after exposure to stress-conditions with hydrogen peroxide, hydroxyl radicals, Hb and heme [32, 53, 54]. This is supported by human

in vivo data. Thus, Olsson *et al.* [46] showed a correlation between the concentrations in plasma and placenta tissue of A1M and Hb, and between A1M and biomarkers of oxidative stress (protein carbonyl groups and plasma peroxidation capacity), respectively, in preeclampsia (PE), a human disease characterized by oxidative stress (see below). To date, the transcription factors responsible for this upregulation have not been identified. However, it may be speculated that the A1M-gene is regulated by the antioxidant response element (ARE) and thus are regulated via the Keap1/Nrf2 system [55, 56]. This has however so far not been shown.

PROTECTIVE EFFECTS

Employing the molecular mechanisms described above, A1M can protect cells and tissues against internal and external chemical challenge. A unifying model of the physiological role of A1M is that it functions as a "radical sink", continuously cleaning tissues from free radicals and oxidants, including free heme and radicals generated by extracellular Hb, heme and iron, by binding and neutralizing them and delivering the products to the kidneys for degradation and/or excretion. Below, the *in vitro*, *ex vivo* and *in vivo* findings of the A1M protective effects of are summarized.

Cell models: Protection against Hb-, heme- and radical-induced cell- and tissue damage

Several reports describe *in vitro* protective actions of A1M in cell cultures stressed by exposure to Hb-, heme- and free radicals. We found that exogenously added A1M prevented intracellular oxidation and up-regulation of HO-1 induced by heme, H_2O_2 and Fenton reaction-generated hydroxyl radicals in primary keratinocytes [32] and in the erythroid cell line K562 [39]. Further confirmation of this was that silencing of the endogenous A1M expression by addition of siRNA led to an increased cytosol oxidation [39]. In addition, we

showed that exogenous A1M inhibited cell lysis of K562 cells caused by heme and cleared the cells from bound heme. It was shown that 200 µM heme killed 50% of the cells but adding 2 µM A1M lowered the proportion of dead cells to 15% [39]. As discussed above, A1M is expected to act by clearing both intra- and extracellular fluids from radicals and heme-groups. Supporting this, internalization of extracellular A1M by the K562 cells could be shown [39]. Furthermore, exogenous A1M was shown to inhibit the swelling of mitochondria, and to reverse the severely abrogated ATP-production of mitochondria when exposed to heme and H_2O_2 [37] (Figure 4).

Cell models: Protection against irradiation-induced cell damage

A1M added to the culture medium was also shown to inhibit the propagation of cell death induced by irradiation of HepG2 cell monolayers [40, 57]. The cells were irradiated with a low dose of alpha-particles at a small, restricted area. Addition of A1M reduced the amount of dead cells by approximately 50% in irradiated cells (directly hit cells) and by 100% in the cells surrounding the irradiation area, not directly hit by the particles (bystander cells), and completely inhibited the irradiation-induced apoptosis, formation of carbonyl groups and upregulation of the stress-response related genes HO-1, p21 and p53. These results suggests that A1M protects against bystander cell killing by tissue housekeeping, *i.e.* clearing of free radicals and oxidants, released from the irradiated cells.

Organ models: Protection of skin explants and ex vivo perfused placenta.

The protective effects of A1M have been studied in skin explants and in a perfused placenta model [32, 36]. In the skin explants, exogenously added A1M inhibited the heme-induced ultrastructural damage (Figure 5A-C), up-regulation of antioxidation- and cell cycle regulatory genes, and formation of protein carbonyl groups, a marker of oxidative stress [32].

Furthermore, A1M was shown to be localized ubiquitously in the dermal and epidermal layers of skin, and the *AMBP*-gene, which is expressed in keratinocytes, was also up-regulated after exposure to heme and Fenton reaction-generated hydroxyl radicals [32]. Besides skin, A1M has been described to be widely distributed in placenta cells and matrix [35, 46], and its protective effects were investigated in the placenta perfusion model [36]. This model enables studies of cell- and matrix structure and functions including the feto-maternal barrier [58]. While *ex vivo* perfusion with Hb in the fetal circulation led to a significant increase of the perfusion pressure, feto-maternal leakage of Hb, morphological damage, and up-regulation of genes related to immune response, apoptosis, and oxidative stress, simultaneous addition of A1M to the maternal circulation abrogated the Hb leakage (Figure 5D), morphological damage (Figure 6B) and gene up-regulation [36].

The skin and placenta studies also demonstrated a protective effect of A1M *vis-à-vis* extracellular matrix and collagen fibrils. In fact, destruction of collagen fibrils by incubation with Hb, heme or Fenton reaction-generated hydroxyl radicals was almost completely inhibited by A1M (Figure 6). The fibrils could also be repaired by addition of A1M after the destruction already had taken place, most likely the result of hydroxyl radical-clearing [32]. Interestingly, perfusion of placentas with A1M induced a significant up-regulation of extracellular matrix genes, and a remarkable increase was observed in the number of collagen fibrils [36]. Currently, it is not clear how this additional effect of the A1M is related to its reductase-, radical-binding and heme-binding properties.

In vivo models: Protection effects of A1M infusion in rat and sheep models

In two very recent studies [51, 52] we evaluated the protective effect of A1M in two different *in vivo* animal models. In the first study, A1M was shown to prevent kidney damage in rats,

induced by a continuous Hb-infusion [52]. The Hb-infusion caused a rapid and marked increase in glomerular permeability, which was almost totally reversed by A1M.

PE is a serious pregnancy complication associated with oxidative stress, where extracellular Hb has been identified as a plausible causative factor [59, 60]. The therapeutical potential of A1M as a treatment of PE was investigated in a pregnant ewe PE model, a model wherein starvation induces hemolysis and subsequent release of Hb, heme, and free radicals [51]. Late pregnancy ewes were starved for 36 hours and then treated with intravenous A1M or placebo injections. Following the injections, the ewes were re-fed and monitored for an additional 72 hours. Starvation induced PE-like symptoms, *e.g.* increased levels of the hemolysis marker bilirubin, structural damage to the placenta and kidneys and increased glomerular permeability. The observed pathological effects were ameliorated by A1M-infusion.

THERAPEUTIC POTENTIAL OF A1M

Many diseases and conditions are associated with unwanted oxidation of cells and molecules in the tissues and lead to formation of reactive free radicals, which in turn may lead to tissue damage and subsequent development of disease. Potential therapeutical drugs with antioxidant properties have been evaluated during the last decades, but the outcome of these studies have mostly been unsuccessful and there is still a need for developing safe drugs for the treatment or prophylaxis of diseases or conditions that have an oxidative stress element [61]. Based on its role in tissue housekeeping as a heme- and radical clearing agent, A1M is a possible candidate for therapeutic use in clinical situations with a high load of oxidants, radicals, Hb and/or heme. Treatment of preeclampsia, Hb-induced glomerular filtration injury and skin wound healing has been reported as described above. A1M has several advantages over traditional antioxidants/radical scavengers such as vitamins E and C: 1) A1M is a

naturally occurring protein with its own metabolic routes ensuring optimal delivery and clearance; 2) The molar capacity of A1M in binding/buffering radicals is approximately 10:1, compared to 1:1 for vitamins C and E; 3) After binding a maximum load of radicals, and/or reducing oxidants or oxidation products, A1M does not present any oxidative stress to tissue components. Overall, A1M presents as a very interesting therapeutic opportunity for a number of diseases and iatrogen conditions, but it still remains to be investigated in clinical use.

CONCLUDING REMARKS

A1M was discovered almost 40 years ago, purified from human urine [1]. It was soon shown to be conserved in all vertebrates, secreted from liver and most other epithelial cells, and present in blood and all tissues at remarkably constant concentrations [2]. A recent series of reports strongly suggest that A1M has a physiological role as a protective antioxidant, operating by clearing extravascular fluids of free radicals and heme-groups, and transporting them to the kidneys for degradation [4](Figure 1). Based on a role as a tissue housekeeping cleaning factor, A1M is a potential therapeutic drug candidate in treatment or prophylaxis of diseases or conditions that have an oxidative stress element, and future research should be aimed towards both its clinical usefulness and further mechanistic studies.

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FIGURE LEGENDS

Figure 1. Overview of metabolism, biodistribution and protective properties of A1M. From left to right: The main location of synthesis of A1M is the liver, but it is also expressed at a lower rate in most other cells. The expression rate of A1M is upregulated by elevated levels of Hb, heme and free radicals. A1M is synthesized as A1M-bikunin, a precursor protein which is cleaved in the Golgi, and the A1M and bikunin proteins are secreted separately. After secretion, nascent A1M is rapidly extravasated and contributes to tissue housekeeping by radical- and heme-cleaning, tissue repair and mitochondrial protection of wounded cells as described in the text. Finally, heme- and radical-modified A1M is again taken up by the blood-stream via unknown mechanisms, and cleared by glomerular filtration and tubular reabsorption and degradation. The figure was made by Axelform (http://www.axelform.com).

Figure 2. Ribbon representation of three-dimensional crystal structure of human A1M. The molecule is viewed from the side of the lipocalin barrel with the opening to the left. The nine β-strands constituting the lipocalin barrel are marked with yellow color. Side-groups with identified or suggested function have been high-lighted and labelled. Cys34 (red) is an absolute requirement for the reductase and cell-protection activities [38, 40], is involved in radical scavenging [10], and was suggested to participate in heme-binding [8]. Lys69, Lys92, Lys118, and Lys130 (blue) are involved in reductase activity [38] and are covalently modified *in vivo* [3]. Tyr22 and Tyr132 (black) are covalently modified in radical scavenging [10]. The figure is modified from the published crystal structure of human A1M [8].

Figure 3. Reductase and radical trapping mechanisms of A1M.

A. Reductase properties. A1M is represented by a Lipocalin barrel showing the thiolate (S^-) group of Cys34 and Lys92, Lys118 and Lys130, located around the upper rim of the lipocalin pocket. The pK_a of the Cys34 thiolate is lowered by the proximity of the three positively charged lysyl side-chains. Electron-transfer from the thiolate group can reduce various substrates as described in the text. The presence of electron-donating co-factors (NADH, NADPH and ascorbate) regenerates the thiolyl radical making the reduction faster and catalytic. **B. Radical trapping mechanism.** The figure is modified from [10] and based on the reactions with the synthetic radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS). As in the reductase mode shown in panel A, the Cys34 thiolate undergoes a reducing electron-transfer reaction with a substrate, in this case a radical. In the absence of electron-donating co-factors, the resulting thiolyl radical is regenerated by intramolecular reactions with tyrosyl side-chains, producing tyrosyl radicals. The tyrosyl radical then reacts with another free radical substrate, forming a stable Tyr-radical adduct. The tyrosyl and lysyl side-chains of Tyr22, Tyr132, Lys69, Lys92, Lys118 and Lys130 in the A1M-lipocalin pocket have been shown to be targets of covalent radical-adduction. **C. Brown color of human urine A1M**. Fifty (50) mg albumin and A1M were purified from human urine as described [5], dialyzed against water and freeze-dried. Brown-colored heterogeneous covalent modifications of the human urine A1M were identified on Lys69, Lys92, Lys118 and Lys130 [3, 47]. Albumin is shown as a control protein, localized in blood plasma and purified from urine, but unmodified, illustrating the specificity of the radical trapping mechanism of A1M.

Figure 4. A1M protects and binds to mitochondria.

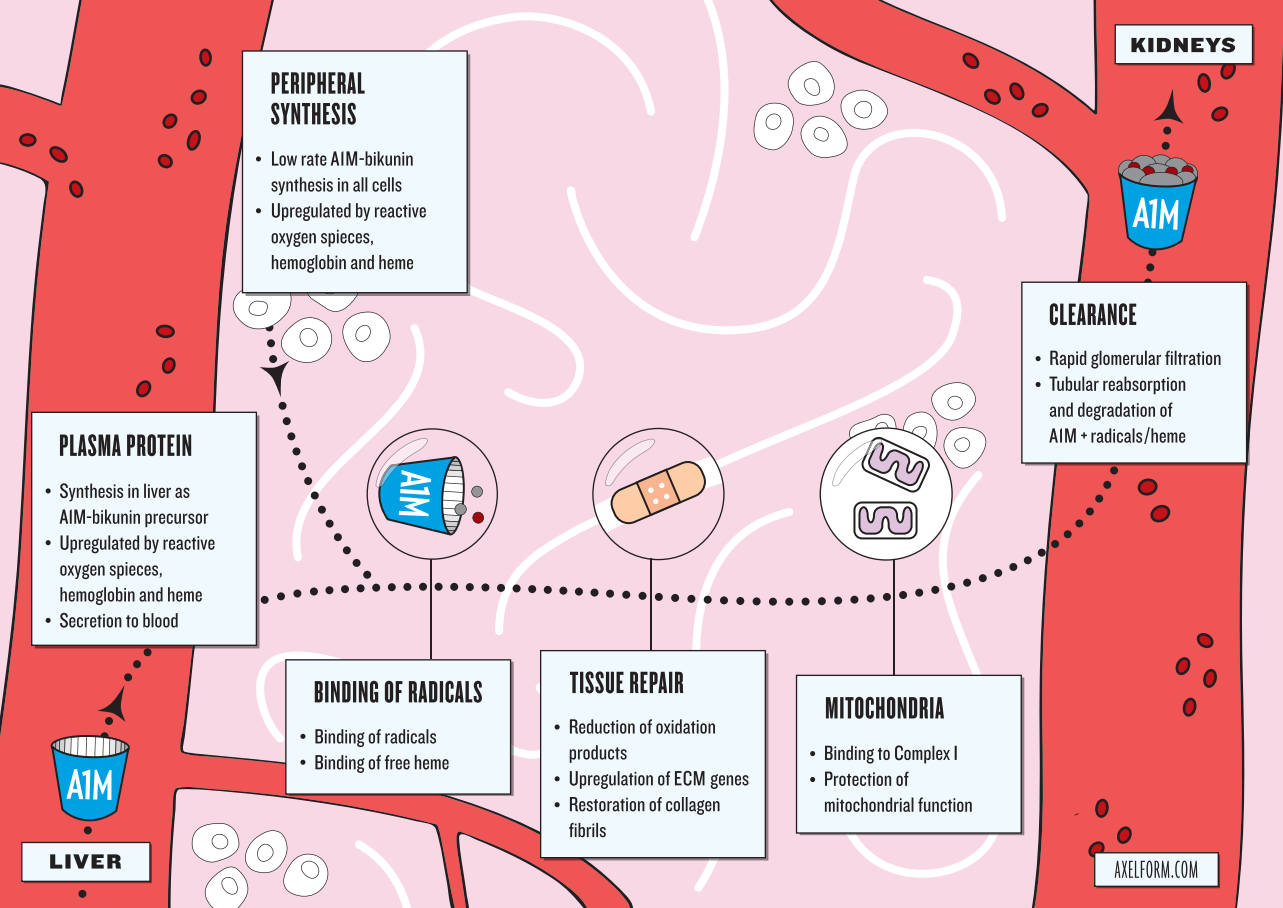
Keratinocytes (**A**-**C**) were incubated for 20 hours at room temperature with buffer only (**A**), with 20 μM heme (**B**) or with 20 μM heme and 10 μM A1M (**C**). The samples were prepared, analyzed and observed as described [37]. Scale bar in **C** indicate 2 μm (is applicable for Figure **A**-**C**). Mitochondrial structures are highlighted with arrows and depicted in details (zoomed pictures). Scale bar in zoomed insert of **C** indicates 0.5 μm (is applicable for zoomed inserts in **A**-**C**). Electron microscopy immunolabeling of human primary keratinocytes with gold-labeled anti-A1M was performed (**D**). A1M is seen to be localized to mitochondria. The samples were prepared and observed as described [37]. Scale bar indicates 100 nm. The effect of A1M on mitochondrial function was investigated by measuring ATP-production of purified mitochondria exposed to heme or H_2O_2, as described in [37]. Mitochondria were incubated with 1-20 μM heme, with or without 10 μM A1M (**E**) or 20-250 μM H_2O_2 with or without 10 μM A1M (**F**) for 30 minutes. $* P < 0.05$. Figure adapted with permission from [37].

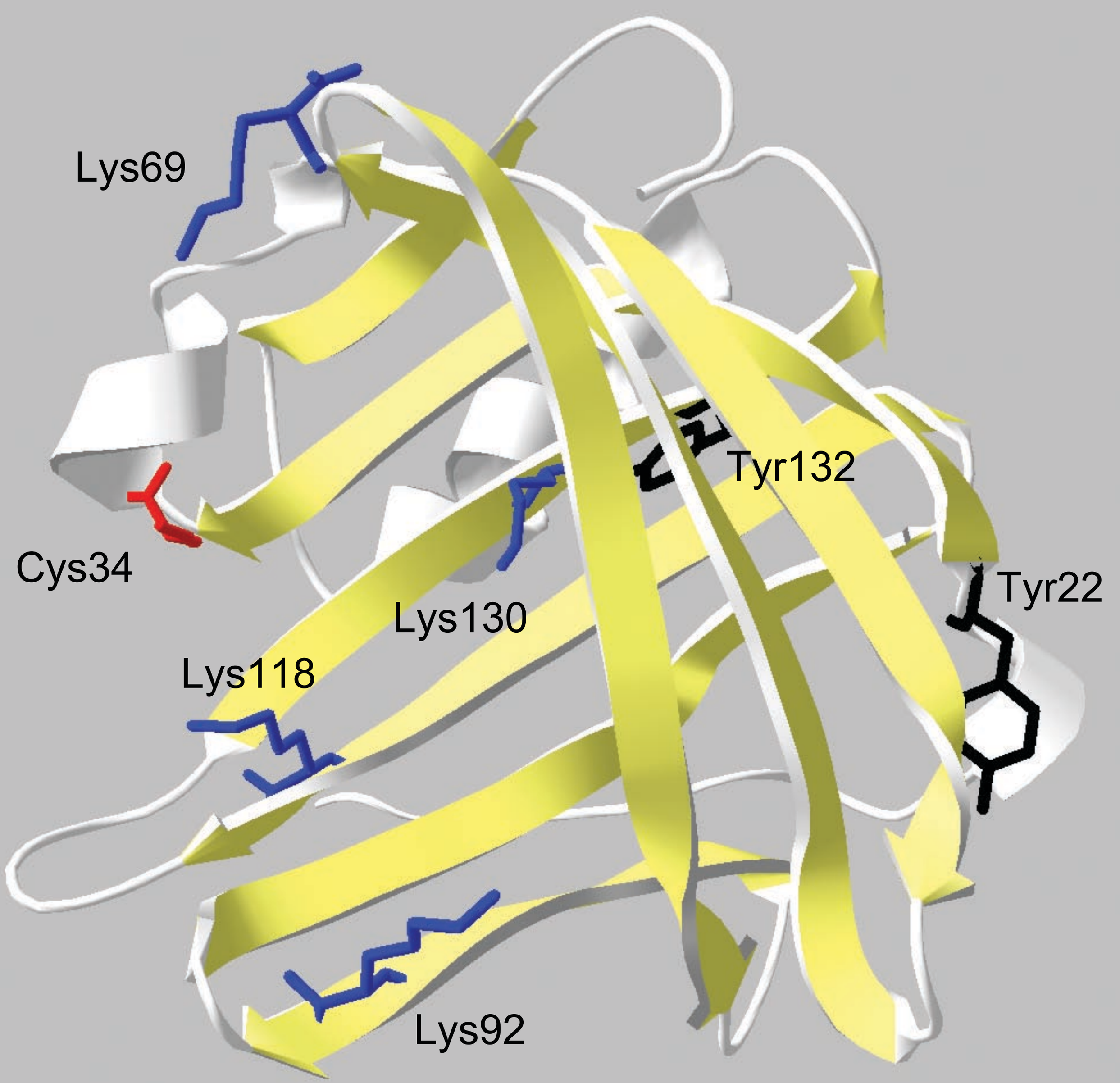
Figure 5. A1M protects cells and tissue.

Skin sections (**A**-**C**) were incubated for 20 hours at room temperature with buffer only (**A**), with 20 μM heme (**B**) or with 20 μM heme and 10 μM A1M (**C**). The samples were prepared, analyzed and observed as described [32]. Abbreviations in figure denote: BM=basement membrane, PM=plasma membrane, ECM=extracellular matrix. Scale bar in **C** indicate 2 μm (is applicable for Figure **A**-**C**). *Ex vivo* placenta perfusion with 3 mg/ml Hb (■), 3 mg/ml Hb + 0.5 mg/ml A1M (▲), 0.5 mg/ml A1M (▼) or perfusion medium only (●). Figure adapted with permission from [32, 36].

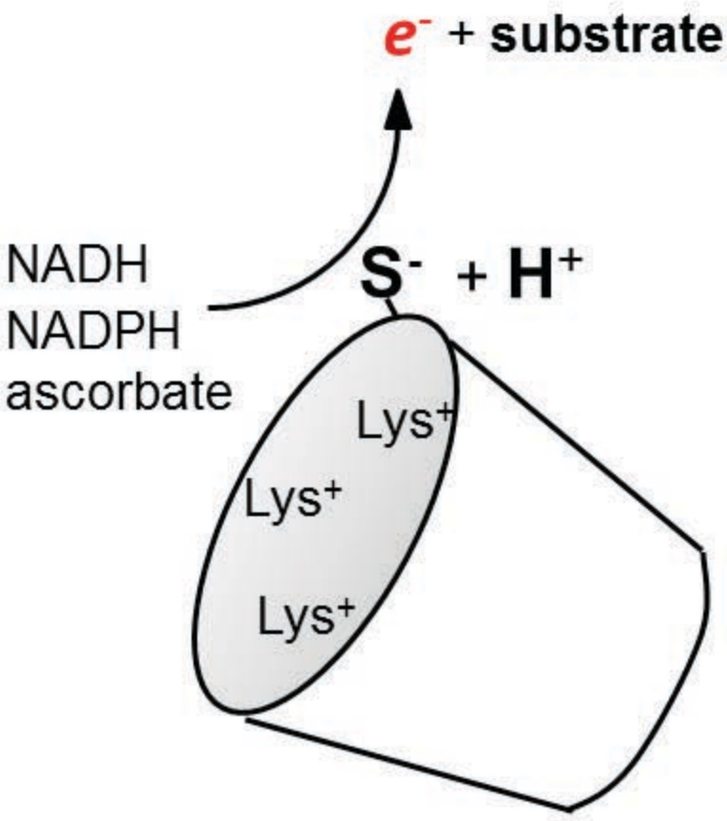
Figure 6. Protection and repair of collagen fibrils.

A. Transmission electron microscopy of purified collagen I [32]. Collagen was incubated with buffer for 24 hours at room temperature to allow fibrillation (**left**). Fibrils were then incubated for a second 24-hour period with 20 μM heme (**middle**). Fibrils incubated with heme were then incubated for a third 24 hour period with 10 μM A1M (**right**). To study binding of A1M to collagen (**right, insert**), collagen was allowed to form fibrils for 24 hours and then incubated with gold-labeled A1M and analyzed as described [32]. **B.** Transmission electron microscopy of placental extracellular matrix after *in vitro* perfusion 2 hours with medium (**left**), 3 mg/ml Hb (**middle**), or 3 mg/ml Hb + 0.5 mg/ml A1M (**right**). The conditions are described in [36]. Figure adapted with permission from [32, 36].

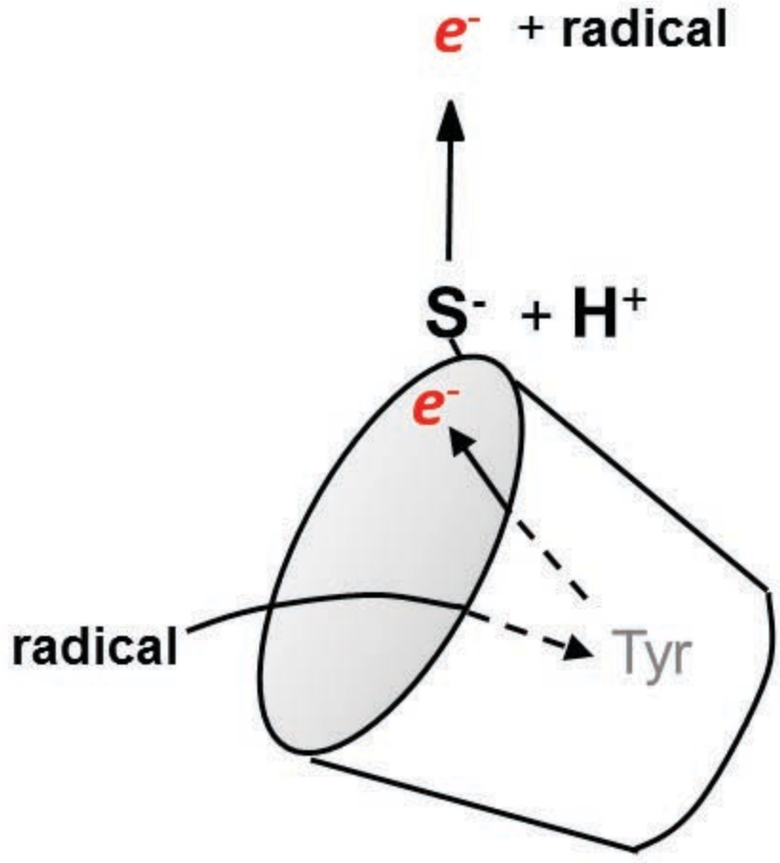

KIDNEYS
PERIPHERAL SYNTHESIS
• Low rate AIM-bikunin synthesis in all cells
• Upregulated by reactive oxygen spieces, hemoglobin and heme
A1M
CLEARANCE
• Rapid glomerular filtration
• Tubular reabsorption and degradation of AIM + radicals/heme
PLASMA PROTEIN
• Synthesis in liver as AIM-bikunin precursor
• Upregulated by reactive oxygen spieces, hemoglobin and heme
• Secretion to blood
A1M
BINDING OF RADICALS
• Binding of radicals
• Binding of free heme
TISSUE REPAIR
• Reduction of oxidation products
• Upregulation of ECM genes
• Restoration of collagen fibrils
MITOCHONDRIA
• Binding to Complex I
• Protection of mitochondrial function
A1M
LIVER
AXELFORM.COM


Lys69
Tyr132
Cys34
Tyr22
Lys130
Lys118
Lys92

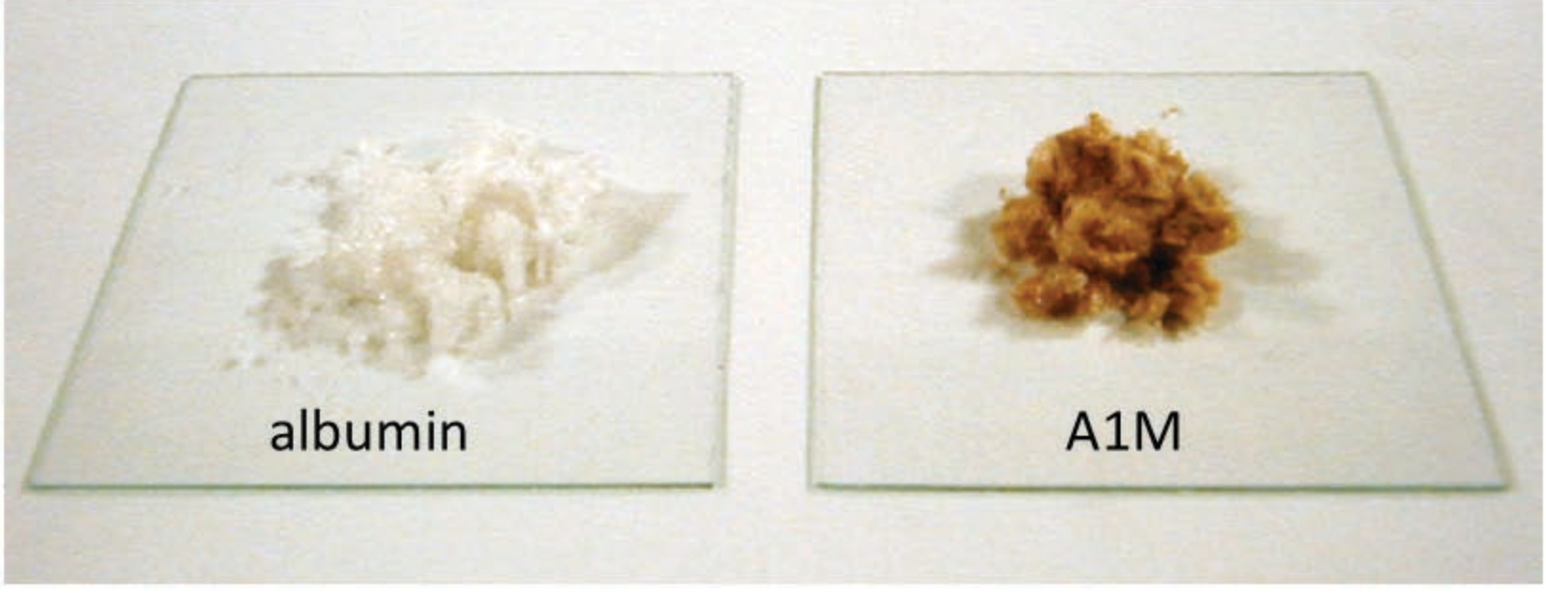
A. Reduction

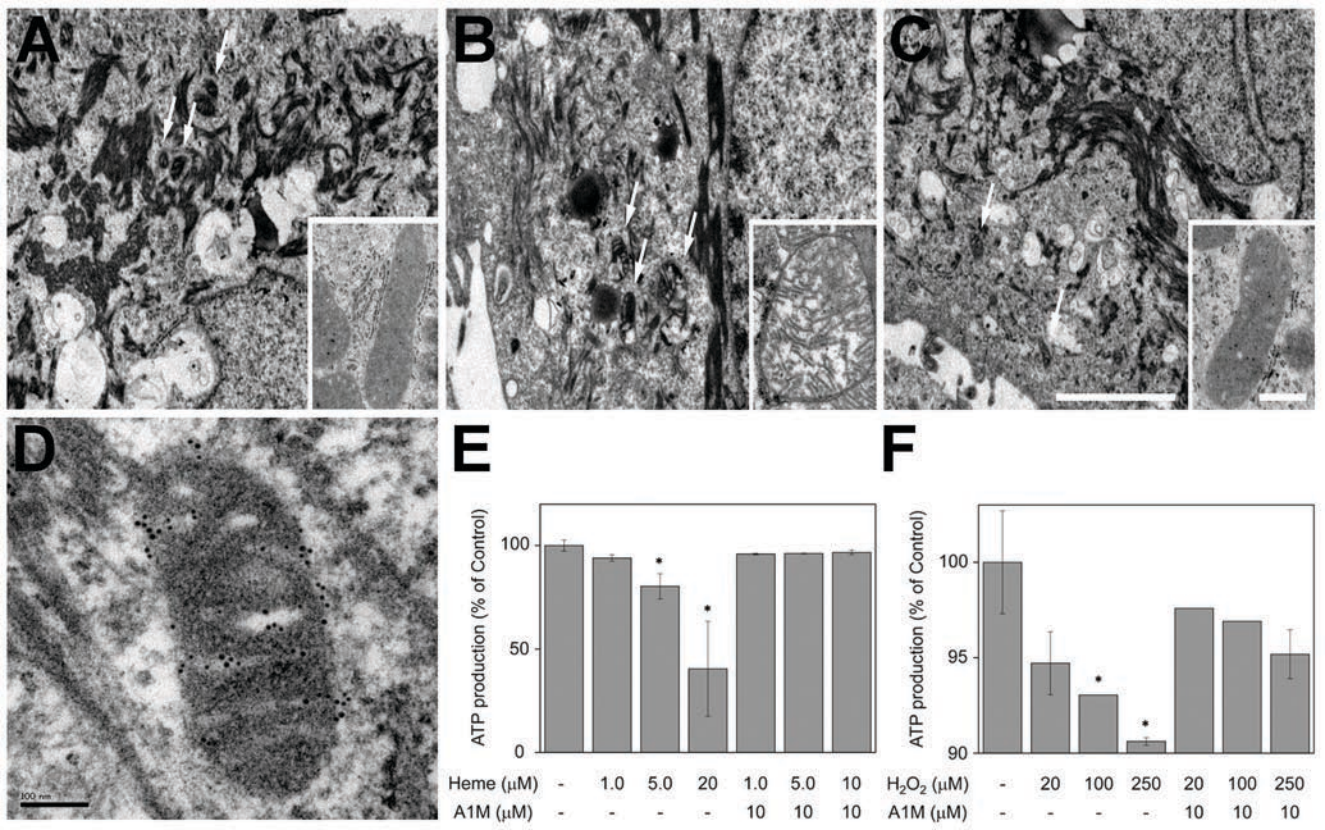


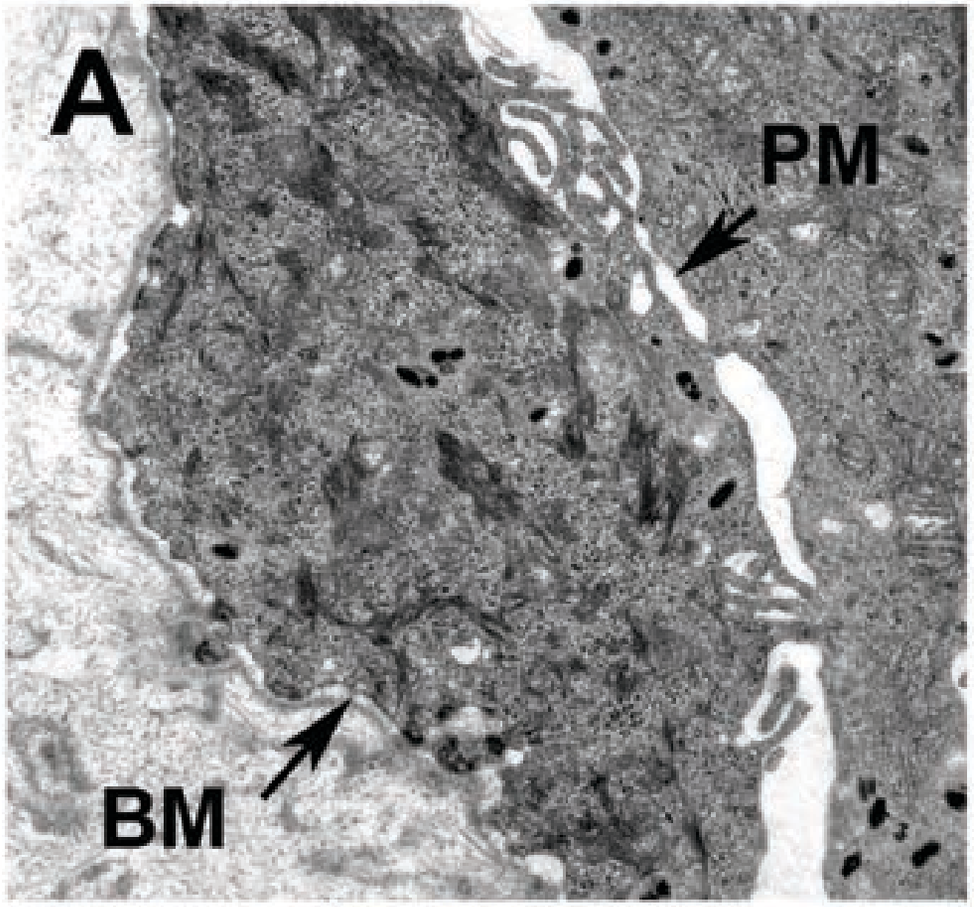
B. Radical trapping

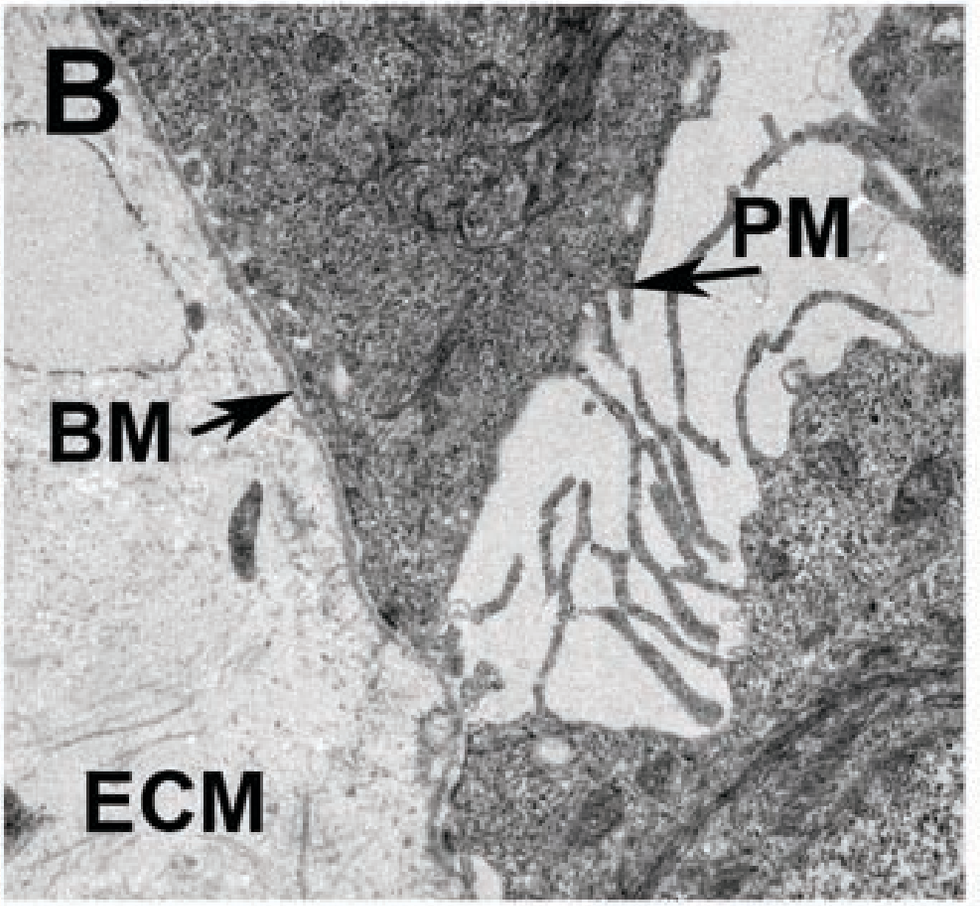


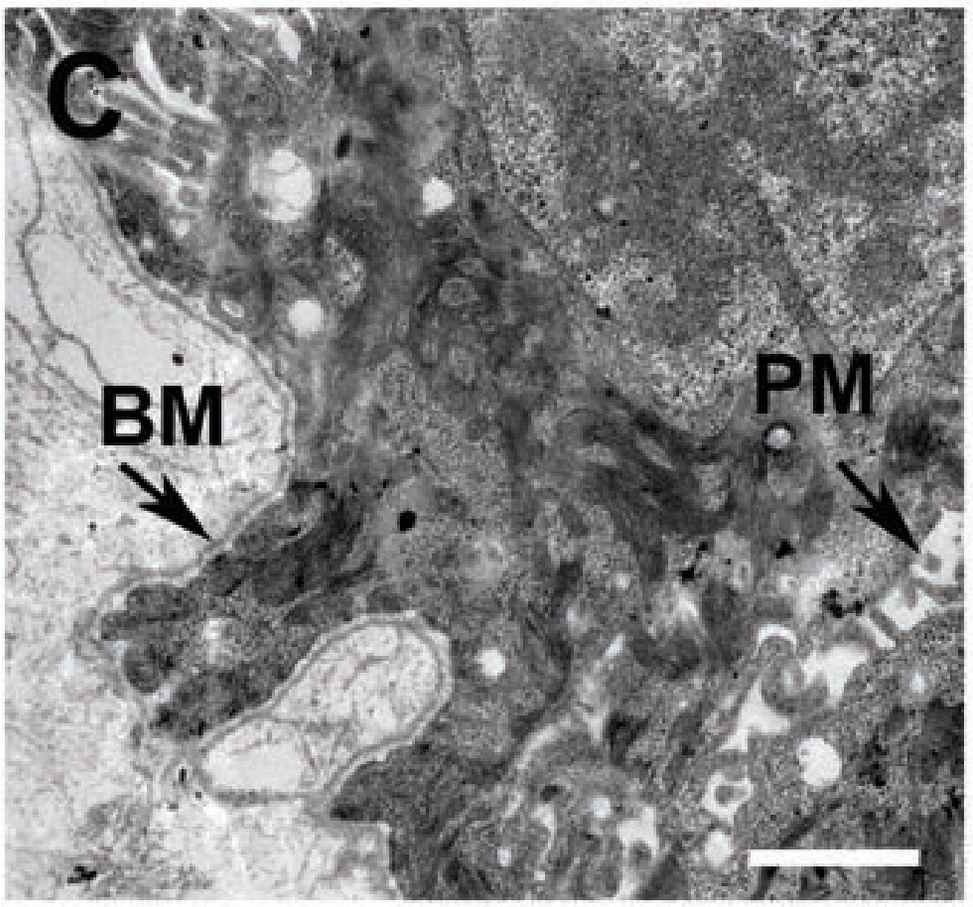
C. *In vivo* modification

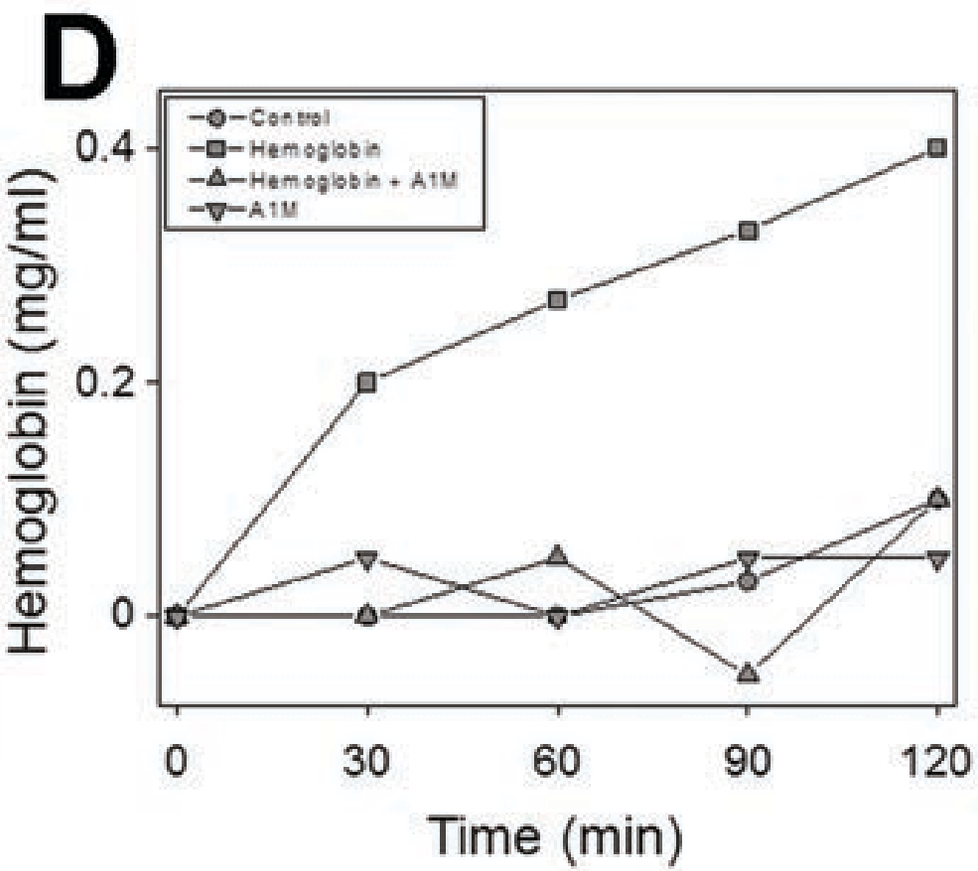



A
B
C
D
100 nm
E
ATP production (% of Control)
100
50
0
Heme (μM) - 1.0 5.0 20 1.0 5.0 10
A1M (μM) - - - - 10 10 10
F
ATP production (% of Control)
100
95
90
H2O2 (μM) - 20 100 250 20 100 250
A1M (μM) - - - - 10 10 10

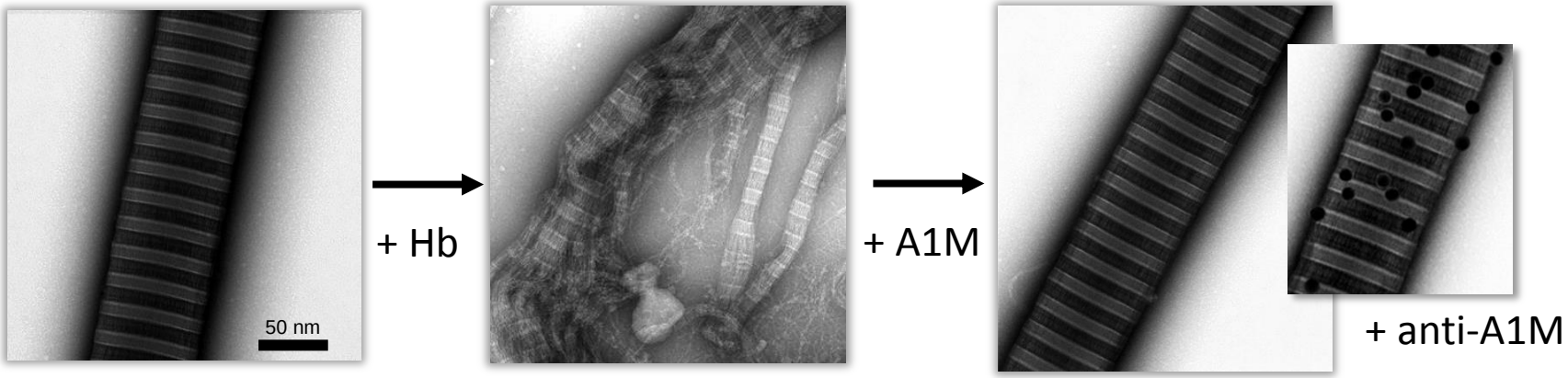

A
PM
BM


B
PM
BM
ECM

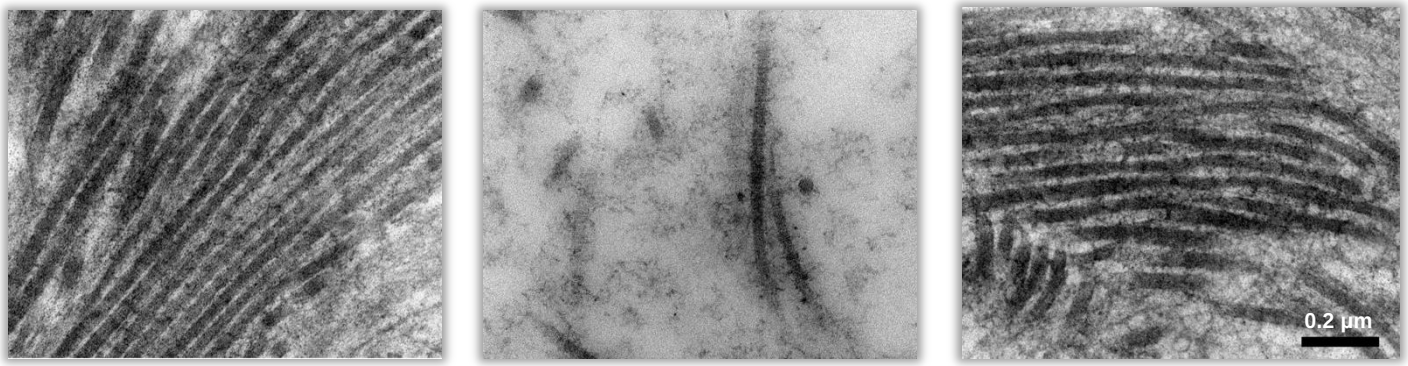

C
BM
PM


D
Control
Hemoglobin
Hemoglobin + A1M
A1M
Hemoglobin (mg/ml)
0.4
0.2
0
0
30
60
90
120
Time (min)

A Purified collagen



B Placenta tissue perfusion



Control Hb Hb + A1M