

Research article

**THE ANTI-APOPTOTIC ACTIVITY OF ALBUMIN FOR
 ENDOTHELIUM IS INHIBITED BY ADVANCED GLYCATION END
 PRODUCTS RESTRICTING INTRAMOLECULAR MOVEMENT**

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Abstract: Human serum albumin (HSA) inhibits endothelial apoptosis in a highly specific manner. CNBr fragmentation greatly increases the effectiveness of this activity, suggesting that this type of protection is mediated by a partially cryptic albumin domain which is transiently exposed by intra-molecular movement. Advanced glycation end-product (AGE) formation in HSA greatly reduces its intra-molecular movement. This study aimed to determine if this inhibits the anti-apoptotic activity of HSA, and if such inactivation could be reversed by CNBr fragmentation. HSA-AGE was prepared by incubating HSA with glucose, and assessed using the fructosamine assay, mass spectrometry, SDS-PAGE and fluorometry. Low levels of AGE in the HSA had little effect upon its anti-apoptotic activity, but when the levels of AGE were high and the intra-molecular movement was reduced, endothelial cell survival was also found to be reduced to levels equivalent to those in cultures without HSA or serum ($p < 0.001$). Survival was restored by the inclusion of native HSA, despite the presence of HSA with high levels of AGE. Also, CNBr fragmentation of otherwise inactive HSA-AGE restored the anti-apoptotic activity for endothelium. Apoptosis was confirmed by DNA gel electrophoresis,

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Abbreviations used: AGE – advanced glycation end product; HSA – human serum albumin; HUVEC – human umbilical vein endothelial cells; PBS – phosphate-buffered saline; SCS – supplemented calf serum

transmission electron microscopy and fluorescence-activated cell sorting analysis, and there was no evidence for direct toxicity in the HSA-AGE preparations. The results are consistent with the proposed role of intra-molecular movement in exposing the anti-apoptotic domain in HSA for endothelium. The levels of AGE formation required to inhibit the anti-apoptotic activity of HSA exceeded those reported for diabetes. Nonetheless, the data from this study seems to be the first example of reduced protein function due to AGE-restricted intra-molecular movement.

Key words: Apoptosis, Cryptic domain, Endothelium, HSA, HSA-AGE

INTRODUCTION

In both isolated tissue culture and in tissue explants, endothelial cells rapidly become apoptotic if deprived of serum [1, 2]. We earlier reported that human serum albumin (HSA) strongly inhibits this process. This protective activity of HSA is dependent upon the native protein configuration and is independent of possible serum contaminants, bound lipids, or radical scavenging activity [2-4]. Although the precise identity of the endothelial receptor responsible for the anti-apoptotic activity of HSA is unknown, it appears to be a G-coupled protein receptor acting via a PI-3 kinase-dependent mechanism [4]. Further reports that HSA displays vasculoprotective activity also support an *in vivo* role for its anti-apoptotic activity [5, 6].

Importantly for this study, CNBr-mediated fragmentation of HSA increases its anti-apoptotic activity for endothelium up to 100 fold [4]. This suggests that the HSA protein domain that binds the anti-apoptotic endothelial receptor is partially cryptic, and is only exposed in a transient conformational subpopulation of molecules (Fig. 1A, B) [4]. This model for the transient exposure of the receptor-binding domain is consistent with the unusually high degree of intra-molecular movement of HSA relative to other proteins [7]. Although we lack further evidence for intra-molecular movement playing a role in the anti-apoptotic activity of HSA, one prediction of this model is that reduced intra-molecular HSA movement would also reduce the degree of protection afforded to the endothelium (Fig. 1C). A further prediction is that such a reduction or inactivation could be reversed by CNBr protein fragmentation (Fig. 1D). In an earlier study, we demonstrated that the intra-molecular movement of HSA is greatly reduced by the formation of advanced glycation end product (AGE) [8]. AGE forms through the reaction of glucose with Lys or Arg residues, to produce highly fluorescent and often cross-linking compounds [8-14]. The aim of this study was to test the predicted effects of AGE formation and CNBr fragmentation in HSA upon its anti-apoptotic activity for endothelium (Fig. 1C, D).

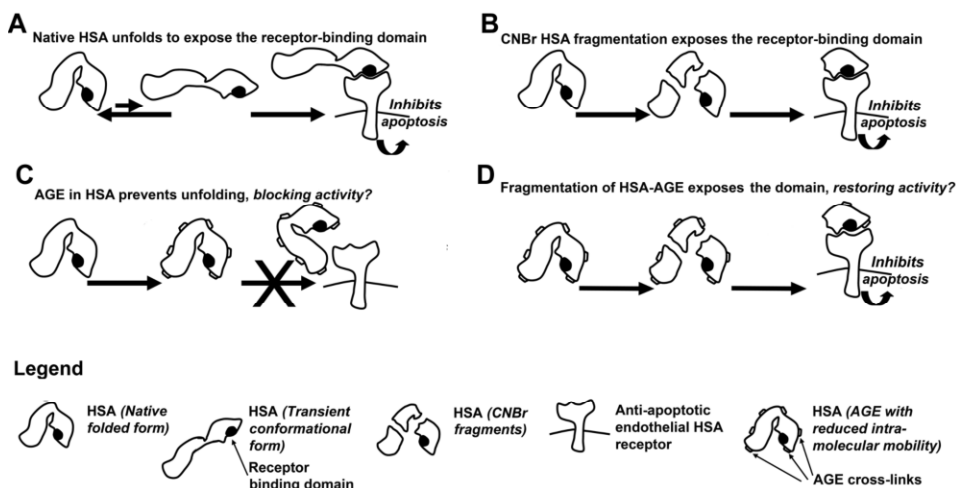


Fig. 1. Diagrams illustrating the current model for the exposure of the HSA protein domain responsible for binding and activating the anti-apoptotic endothelial receptor either through intra-molecular movement (A) or protein fragmentation (B). It also illustrates the tested hypotheses that AGE formation in HSA inhibits its anti-apoptotic activity by reducing intra-molecular movement (C) in a way that is reversible by CNBr fragmentation (D). A – Most of the molecules of native HSA are folded to obscure the protein domain responsible for activating the anti-apoptotic receptor. However, intra-molecular movement generates a small population of transiently unfolded HSA molecules with the receptor binding domain exposed in such a way that the anti-apoptotic receptor can be bound and activated. Once activated, the G-coupled protein receptor inhibits endothelial apoptosis via a PI3 kinase-dependent mechanism [4]. Since only few HSA molecules are in the active transient conformational form at any one time, high but physiologically relevant concentrations of HSA are required to achieve sufficient levels of the active conformational form to protect the endothelium [2-4]. B – Fragmentation of HSA by CNBr exposes the anti-apoptotic receptor-binding domain, greatly increasing its availability to the receptor, thus increasing the anti-apoptotic activity [4]. C – AGE formation in HSA with internal cross-linkages reduces the level of intra-molecular movement [8], and it is hypothesised that this reduces the access of the anti-apoptotic receptor-binding domain to its receptor, with a consequent loss of anti-apoptotic activity. D – It is also hypothesised that the anti-apoptotic activity could be restored to otherwise inactive HSA-AGE via CNBr fragmentation.

MATERIALS AND METHODS

Materials

Tissue culture flasks were purchased from Corning Costar (Edward Keller, VIC, Australia). Supplemented calf serum (SCS), penicillin and streptomycin were obtained from the Commonwealth Serum Laboratories (Melbourne, Australia), and heparin from DBL (Melbourne, Australia). Amphotericin B was purchased from ICN (Seven Hills, NSW, Australia). The endothelial cell growth supplement was prepared from bovine hypothalamus. Proteinase K and RNase

A were from Boehringer Mannheim (Mannheim, Germany), and the HSA was from Calbiochem (San Diego, CA). Phosphate-buffered saline (PBS) tablets were from Oxoid (Bakingstoke, UK). Bradford protein assay reagent was purchased from Biorad (Hercules, CA). All the other reagents used in this study were purchased from Sigma (St. Louis, LO).

HSA-AGE preparation and CNBr fragmentation

The HSA-AGE preparations were made by co-incubating HSA (16 mM) with glucose (50 mM) in PBS containing EDTA (1 mM), PMSF (1 mM), and 0.02% NaN_3 at 40°C for up to 115 days. Since fructosamine is produced early in AGE formation and is readily detected via the colorimetric assay [9-12, 15], progressive glycosylation of HSA was monitored using the fructosamine assay for up to 115 days as detailed elsewhere [8].

Experiments with endothelium were performed with HSA at four levels of AGE formation, designated HSA-AGE 1 to 4. The preparations used were identical to those described in an earlier paper [8]. Matrix-assisted laser desorption ionisation time of flight mass spectrometry yielded a molecular mass of $66,434 \pm 24$ Da for native HSA, while HSA-AGE 1 to 4 had respective molecular masses of $66,911 \pm 33$ Da; $67,048 \pm 21$ Da; $67,354 \pm 31$ Da, and $67,582 \pm 22$ Da [8]. SDS-PAGE demonstrated the maintained integrity of the protein during AGE formation [8]. AGE was detected by analysing fluorescence emission scans from 400 to 500 nm using an excitation wavelength of 370 nm [8]. The emission profiles were characteristic of progressive AGE formation, with maximal fluorescence at about 440 nm, and they respectively measured 5.25, 17.36, 30.05, 48.22 and 62.53 fluorescence units for HSA and HSA-AGE 1 to 4 [8]. A progressive reduction in intra-molecular movement was also demonstrated by fluorometry on the basis of: reduced Trp fluorescence reflecting reduced access of Trp 214 to light; reduced bis-ANS fluorescence reflecting restricted access of bis-ANS to hydrophobic protein domains; and reduced acrylamide quenching of Trp fluorescence reflecting reduced access of acrylamide to Trp 214 [8]. The preparations were concentrated by pressure dialysis and subsequently dialyzed into M199 with antibiotics, and the protein concentrations were determined using the Bradford assay. Samples were then diluted with M199 containing antibiotics to achieve a final concentration of 10% (w/v). CNBr fragments of HSA and HSA-AGE 4 were also prepared for bioactivity determination. Briefly, the protein was dialysed into water to a final concentration of 5 mg/ml. These protein preparations were then added to 90% formic acid to achieve a final formic acid concentration of 70% with CNBr (2 mg/ml). The samples were sealed under Argon gas and then incubated at ambient temperature overnight before extensive dialysis, initially against PBS and finally into M199 with antibiotics. SDS-PAGE was used to confirm protein fragmentation.

Preparation of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase digestion and cultured in M-199 with 20% SCS, 50 µg/ml endothelial cell growth supplement, 30 U/ml heparin, 100 U/ml penicillin, 100 µg /ml streptomycin and 2.5 µg /ml amphotericin B [3, 4, 16], and the experiments were performed with confluent cells from passages 3 to 5.

Culture conditions for the study and quantification of endothelial apoptosis

Confluent HUVEC were washed with M199 in either 75 cm² flasks or triplicate wells in 24-well plates prior to incubation for up to 24 h in M199 in the absence of heparin or growth factor, and with or without HSA (1.3 to 600 µM), HSA-AGE 1 to 4 (600 µM, 4% w/v), or SCS (20% v/v). In some experiments, HUVEC were exposed to both HSA-AGE 4 (600 µM, 4% w/v) and HSA (600 µM, 4% w/v) simultaneously. Experiments with CNBr-HSA and CNBr-HSA-AGE 4 were performed at 150 µM, 1% w/v. Endothelial cells rapidly detach from the culture surface at an early stage of apoptosis, so HUVEC apoptosis can be reliably quantified by simple haemocytometer cell counts of surviving adherent cells [3, 4, 16]. In this study, HUVEC apoptosis was quantified by counting surviving adherent cells in triplicate 24-well or quadruplicate 96-well tissue culture plates [3, 4, 16]. An apoptosis protection index was determined by comparing the adherent cell number with that in parallel cultures with 20% SCS [3]. Student's T-test was used to assess the statistical significance.

Verification of endothelial apoptosis

In necrosis, DNA fragmentation is essentially random, and will produce a smear in DNA gel electrophoresis. This contrasts with internucleosomal fragmentation in apoptotic cells, which produces a 180-base pair ladder effect in agarose gels [3, 4, 16, 17]. Apoptotic DNA fragmentation can also be detected by FACS for DNA, and is seen as a cell population with sub-diploid DNA content [4, 16, 18]. Apoptosis further contrasts with necrosis in that organellar integrity is lost in necrosis but maintained during apoptosis, while apoptotic cells also undergo nuclear and cellular fragmentation. These features are readily detected by transmission electron microscopy (TEM) [19-21]. In addition to these universal TEM features of apoptosis, the apoptotic endothelium seems unique in that apoptotic particles become honeycombed during canalicular fragmentation, in which plasma membranes extend into cells as multiple branching canaliculi [22, 23]. In this study, morphological changes, DNA gel electrophoresis, TEM and FACS were used to verify apoptosis as detailed elsewhere [3, 4, 16, 22, 23].

RESULTS AND DISCUSSION**Loss of HSA protective activity with high levels of AGE formation**

As observed in earlier studies [2-4], HSA protected the endothelium from apoptosis ($p < 0.001$). AGE in HSA had little effect upon HUVEC survival at

low levels of glycosylation, but at higher AGE levels, this protective activity was lost ($p < 0.001$; Fig. 2A). Diluting HSA-AGE 4 in dose response experiments had no effect. In addition, the number of surviving HUVEC exposed to HSA-AGE 4 preparations was never lower than in cells exposed to M199 alone (Fig. 2A), and this was noted over seven separate experiments, an observation at variance with that expected if HSA-AGE were toxic for HUVEC.

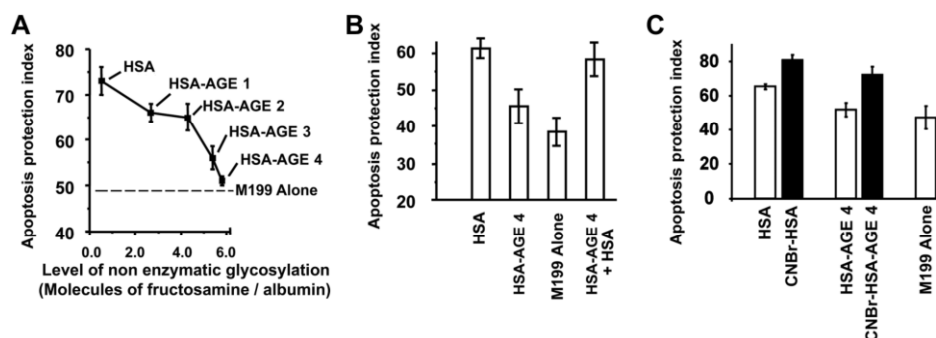


Fig. 2. Graphs showing the effect of AGE in HSA upon the anti-apoptotic activity. A – A graph showing the level of HUVEC apoptosis after 24 h serum-free incubation with preparations of HSA (600 μ M) with increasing levels of AGE relative to M199 alone (dashed line). HSA-AGE 1 and HSA-AGE 2 had almost identical anti-apoptotic activities to HSA, while HSA-AGE 4 was inactive ($p < 0.001$) yielding results not significantly different to a culture of HUVEC with M199 alone. B – A histogram comparing HUVEC apoptosis in culture medium with M199 alone with the effect of HSA, HSA-AGE 4, and HSA-AGE 4 combined with HSA (all at 600 μ M). Native HSA was protective in a serum-free culture with M199 alone, but this activity was absent for HSA-AGE 4. The addition of native HSA to HSA-AGE 4 restored the activity to levels similar to those for HSA alone, indicating that HSA-AGE 4 was not toxic for HUVEC. C – A histogram demonstrating the effect of CNBr fragmentation on the anti-apoptotic activity of HSA and HSA-AGE 4 (protein preparations at 150 μ M). CNBr-HSA was more active than HSA at the concentration tested, while HSA-AGE 4 was inactive. CNBr fragmentation of HSA-AGE 4 restored protective activity to the inactivated protein.

Native HSA prevented the death of HUVEC exposed to HSA-AGE

Further supporting the lack of toxic or pro-apoptotic activity in HSA-AGE was that physiological levels of native HSA (600 μ M, 4% w/v) prevented HUVEC death in the presence of HSA-AGE 4 (600 μ M, 4% w/v; Fig. 2B). If HSA-AGE 4 preparations were directly toxic or apoptotic for HUVEC, native HSA would not have restored HUVEC survival to levels identical to those found with HSA alone.

CNBr fragmentation of HSA-AGE restored its protective activity

CNBr fragmentation of HSA increased its anti-apoptotic activity for endothelium, consistent with the results of earlier studies [4]. Furthermore, CNBr fragmentation of HSA-AGE 4 restored this protective activity to protein previously inactivated by glycosylation (Fig. 2C), supporting the hypothesis

tested (Fig. 1D). This data further supports an absence of toxic activity toward the endothelium in the HSA-AGE preparations studied, as such toxicity would seem unlikely to be lost upon CNBr cleavage. Similar results were obtained in two separate experiments with cells from different donors.

HUVEC exposed to HSA-AGE 4 were apoptotic and not necrotic

The data demonstrating the apoptotic rather than necrotic death of HUVEC in HSA-AGE 4 was consistent with the loss of anti-apoptotic activity in HSA with high levels of AGE. FACS analysis of HUVEC treated with HSA-AGE 4 revealed a significant population of cells with sub-diploid DNA content, characteristic of apoptosis (Fig. 3) [4, 16, 18].

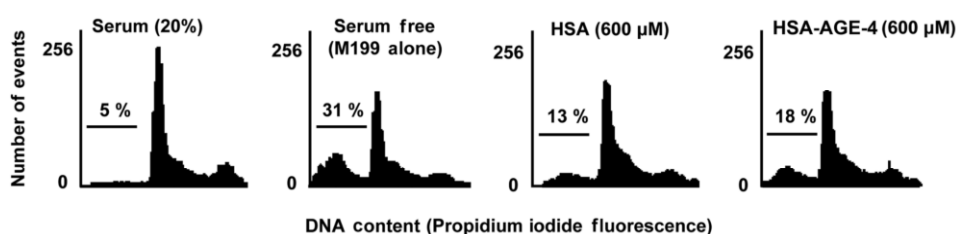


Fig. 3. The results of the FACS analysis for the DNA content of HUVEC cultured with serum (20%), M199 alone, 600 μ M HSA or 600 μ M HSA-AGE-4. G0/G1 and G2/M peaks characteristic of cultured cells were seen, while clear sub-diploid apoptotic peaks were present. The size of the sub-diploid peaks was greatest in the cells deprived of serum and HSA, and this was more pronounced in cells exposed to HSA-AGE 4 than in cells treated with HSA alone. These changes were typical of apoptosis and consistent with apoptotic rather than necrotic death of HUVEC in the presence of HSA-AGE 4.

TEM examination of the detached HUVEC exposed to HSA-AGE 4 revealed ultrastructural features typical of apoptosis in the endothelium, including the formation of small apoptotic bodies, nuclear fragmentation and condensation, maintained mitochondrial integrity, an intact rough endoplasmic reticulum with ribosomal studding, increased cytoplasmic electron density, and canalicular fragmentation (Fig. 4A). The ultrastructure of the adherent cells (Fig. 4B) failed to reveal any sign of apoptosis or cytotoxicity, thus the nuclear material, rough endoplasmic reticulum and mitochondria were intact while the organellar swelling expected in injured cells was absent [19-23]. DNA gel electrophoresis of detached HUVEC in 75-cm² flasks treated with HSA-AGE 4 revealed internucleosomal fragmentation typical of apoptosis (Fig. 4C) [3, 4, 16, 17].

The data is consistent with the proposed model for intra-molecular movement mediating transient exposure of the HSA anti-apoptotic domain for endothelium

In the absence of any data indicating either a pro-apoptotic or toxic effect of HSA-AGE 4 preparations, we interpret our observations as indicating that AGE results in a loss of the normal anti-apoptotic activity of HSA [2-4]. In light of the

significant reduction in the intra-molecular movement of HSA with AGE formation [8], the current data also supports the model proposed for transient exposure of the anti-apoptotic domain of HSA due to intra-molecular movement (Fig. 1A).

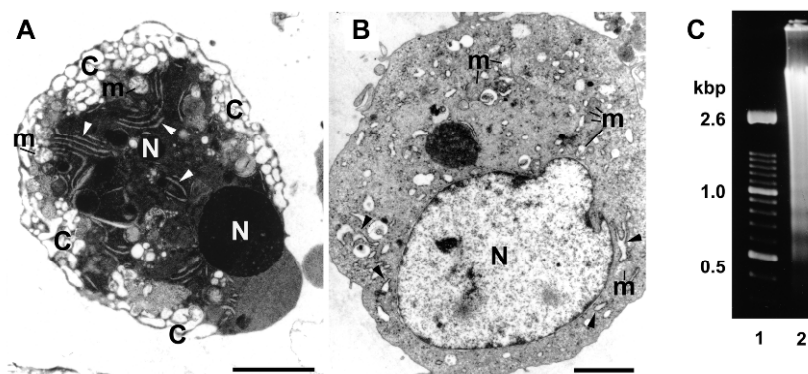


Fig. 4. Transmission electron micrographs of apoptotic detached (A) and non-apoptotic adherent (B) HUVEC exposed to 600 μ M HSA-AGE 4 for 24 h, as well as an ethidium bromide stained agarose gel showing internucleosomal DNA fragmentation in apoptotic HUVEC exposed to 600 μ M HSA-AGE 4 (C). The detached cells (A) had the ultrastructural features of endothelial apoptosis including nuclear (N) and canalicular fragmentation (C), while the mitochondria (m) and rough endoplasmic reticulum (arrow heads) were intact in both detached apoptotic (A) and adherent non-apoptotic cells (B). There was no ultrastructural sign of cellular cytotoxicity in either population. A comparison of DNA extracted from the detached HUVEC exposed to 600 μ M HSA-AGE 4 (Lane 2) with standards (Lane 1) revealed the 180- to 200-base pair internucleosomal fragmentation typical of apoptosis (C). The bars for the electronmicrographs are 5 μ m.

Regarding possible alternative interpretations of our data, it is possible that AGE in HSA increases binding to the anti-apoptotic endothelial receptor in a way which fails to yield receptor signaling. However, this possibility seems excluded by the complete restoration of native HSA's protective activity in the presence of an equivalent quantity of HSA-AGE 4 (Fig. 2B). Also, distortion of the HSA ligand by AGE seems unlikely to increase binding to the specific endothelial receptor. The precise AGE products responsible for the reduced anti-apoptotic activity of HSA have not been determined, but mass spectrometric analysis indicates that anti-apoptotic activity is lost when glycosylation occurs in amino acid residues 1-147 and 572-609, respectively representing the N and C terminal regions [8].

Earlier reports of toxic activity with AGE formation in albumin may reflect differences in the experimental preparations and the toxicity of protein aggregates

At first sight, our results appear at variance to earlier reports of toxic or apoptotic effects on the endothelium of albumin-AGE, and might reflect the use

of bovine endothelium and/or cells by other workers [24-27]. The formation of toxic protein aggregates in albumin-AGE preparations is a more likely explanation. A precedent for this is established by the toxic effect of protein aggregates across a wide range of cells including the endothelium [28-30]. Notably, AGE formation in bovine serum albumin results in amyloid-like protein aggregates [31], and amyloid precipitates are recognized as toxic to a range of cells [28]. Furthermore, we have observed toxic effects of HSA protein aggregates produced by CNBr fragmentation under denaturing conditions, and of HSA aggregates produced by glutaraldehyde treatment (data not shown). While the HSA-AGE preparations in this study did not display detectable toxic activity for the endothelium, similar preparations have been described as having oxidative properties that vary depending on the agents and conditions used for AGE formation [32, 33]. Our current observations indicating the absence of significant oxidative toxicity in HSA-AGE preparations are that: HUVEC survival in the presence of HSA-AGE never fell below the levels seen in serum- or albumin-deprived endothelium; the addition of native HSA overcame HUVEC apoptosis in the presence of HSA-AGE; and the anti-apoptotic activity of otherwise inactive HSA-AGE was restored by CNBr fragmentation.

Loss of anti-apoptotic activity with HSA-AGE formation is unlikely to be significant in diabetes

HSA has a vasculoprotective role *in vivo* [5, 6], while endothelial apoptosis contributes to both delayed diabetic healing [34] and diabetic retinal vasculopathy [35]. However, the levels of HSA glycosylation achieved in diabetes seem significantly lower than those required to inhibit endothelial apoptosis in this study, with approximately two glucose residues per albumin molecule seen *in vivo* [36], and at least 6 fructosamine residues together with additional AGE products seen in HSA-AGE 4 (Fig. 2A). From this, it seems unlikely that the observed effect of AGE formation in HSA, namely in reducing anti-apoptotic activity for endothelium, contributes to vascular disease. However, our study does support the hypotheses tested (Fig. 1C, D), and appears to provide the first reported example of loss of protein activity by AGE-induced restriction in intra-molecular movement.

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