Redox Biochemistry and Chemistry 15 (2026) 100071

Contents lists available at ScienceDirect

Redox Biochemistry and Chemistry

journal homepage: www.journals.elsevier.com/redox-biochemistry-and-chemistry

Uratylated albumin activates endothelial cells to induce monocyte adhesion and the release of pro-inflammatory cytokines

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ARTICLE INFO

Keywords:
Uric acid
Oxidation
Albumin uratylation
Post-translational modification
Endothelial dysfunction

ABSTRACT

Albumin, the most abundant serum protein in mammals, is critical for transporting low-water-soluble metabolites, ions, and xenobiotics, as well as maintaining osmotic balance. This protein is highly susceptible to post-translational modifications, which can alter its structure and function. Reactive oxygen species (ROS) play a significant role in inducing such modifications, leading to protein dysfunction and contributing to inflammatory and cardiovascular pathologies. Previous studies have demonstrated a link between uric acid (UA) oxidation products, particularly urate hydroperoxide (HOOU), and albumin modifications through a process termed uratylation. These albumin adducts result from the addition of a 140 Da product mass predominantly on lysine and amine from N-terminal residues. Therefore, the aim of this study was to investigate the structural and functional consequences of uratylation on albumin and its effects on endothelial cells. Uratylation promotes a decrease in enthalpy change of denaturation of albumin and binding of Anilino-Naphthalene-Sulfonic acid (ANS), suggesting slight changes in protein structure. Uratylated albumin reduced human umbilical vein endothelial cell (HUVEC) migration, increased intercellular adhesion molecule-1 (ICAM-1) expression and, consequently, monocyte adhesion (THP-1), indicating endothelial activation. Furthermore, the presence of uratylated albumin stimulated the release of tumor necrosis factor-α (TNF-α). These findings provide novel insights into the mechanistic pathways connecting uric acid oxidation, post-translational modification on albumin and inflammation, highlighting the potential role of uratylated albumin in endothelial dysfunction and atherogenesis.

1. Introduction

Albumin is the most abundant serum protein in mammals responsible for the transport of low water-soluble metabolites, ions, xenobiotics and in maintaining osmotic balance within the circulatory system [1,2]. Due to its high concentration, it is particularly susceptible to post-translational modifications. The most commonly reported modifications include oxidation, nitrosylation, glycation, truncation, and dimerization [3–7]. Reactive oxygen species (ROS) can induce irreversible structural modifications and alter albumin activity [5]. Glycation and oxidation often alter conformation and stability leading to a loss of function in albumin [8–11]. Modified circulating serum albumin is associated with ongoing increase in endothelial damage by the induction of inflammatory mediators and monocytes migration into the intima, enhanced expression of adhesion molecules [6,12] and cardiovascular morbidity [13,14]. Turner et al. identified a 140-Da mass addition on ε-amino from lysine and α-amino of the N-terminal glycine residues in albumin incubated with uric acid in presence of lactoperoxidase/hydrogen peroxide. This new reported post-translational modification, named uratylation, was also found when albumin was incubated with uric acid and activated neutrophils [15].

Uric acid or its unprotonated form, urate, has been widely associated with endothelial dysfunction, vascular damage and cardiovascular disease [16–18]. Urate is the main substrate for plasma peroxidases [19]. We and other groups characterized the mechanism and kinetics of the oxidation of uric acid by the heme-peroxidases myeloperoxidase,

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https://doi.org/10.1016/j.rbc.2026.100071
Received 11 June 2025; Received in revised form 14 January 2026; Accepted 17 January 2026
Available online 19 January 2026

lactoperoxidase and peroxidasin [20–23]. One-electron oxidation of urate by these proteins generates urate free radical that can further combine with superoxide, also abundant in the inflammatory milieu, to form urate hydroperoxide, a more stable intermediate, but with powerful oxidant capacity [24,25]. Therefore, oxidation of urate either by inflammatory or vascular peroxidases is likely a key event in the pathogenesis of cardiovascular disease and uratylation of plasma albumin may be a hallmark in this process.

Likewise, uratylation on albumin could affect protein conformation and function. Therefore, this study compared physicochemical properties including transition temperature (T_m), enthalpy for denaturation, UV absorption and hydrophobic interactions, as well as alterations in secondary structure and aggregation of native and uratylated albumin. The impact of albumin modification on endothelial cells was evaluated by assessing cellular adhesion and migration after incubating cells with uratylated albumin. Activation of endothelial cells by uratylated albumin was measured by the expression of intercellular adhesion molecule (ICAM-1), adhesion of monocytes onto them and release of pro-inflammatory cytokines. The investigation of the effects of uratylation on albumin's structure may help clarify the mechanisms connecting uric acid to inflammatory and cardiovascular diseases.

2. Results & discussion

2.1. Physicochemical properties of native and uratylated albumin using differential scanning calorimetry

Initially, we evaluate the influence of uratylation on the physicochemical properties of albumin. Thus, the transition temperature (T_m) was assessed by differential scanning calorimetry (DSC), a useful technique for investigating protein unfolding [26]. Heat capacity (Cp) was recorded, in a thermogram, as a function of temperature, corrected for the Cp differences between the native and denatured states. For straightforward reversible unfolding processes, the thermodynamic parameters of denaturation, such as enthalpy change, can be directly derived from the thermogram [27]. The T_m values were 57.5°C and 57.8°C for the modified and the native protein, respectively, consistent with the previously reported T_m 58°C for native albumin [28,29]. This demonstrates that uratylation is not enough to affect this parameter (Fig. 1A). However, a much lower enthalpy change (ΔH) of denaturation was detected for uratylated albumin (ΔH = 50.42 ± 3.89 kcal/mol) when compared to the native protein (ΔH = 86.76 ± 1.37 kcal/mol) (Fig. 1B), suggesting disturbances in protein's structural integrity [30] This is aligned with previous studies showing that oxidative modifications or binding of small molecules can lead to subtle alterations in albumin structure [10,11,31]. Of relevance, denatured or modified albumin render the protein more susceptible to degradation [32].

2.2. Effect of uratylation on conformational changes in albumin

Human serum albumin contains three structurally similar α-helix subunits called domain I, II and III. The Trp214 situated at the domain II contributes to the spectroscopy features of the protein [33–35] and modifications on albumin structure often lead to alterations in absorbance and intrinsic fluorescence [34,35]. Absorption spectra of native albumin or albumin previously incubated with urate hydroperoxide (~1:50 protein:oxidant) for 48 h at 37°C were very similar among different incubations (data not shown). Alterations on spectroscopy features of albumin may be subtle and hard to verify when modified albumin is not isolated from the remaining native one. In fact, alterations in albumin absorbance or fluorescence by glycation are usually seen by using a very large proportion of the sugar over protein [36]. The two main limitations of this study are: 1) the restricted amount of purified urate hydroperoxide obtained in each synthesis (around 1 mM) restraining the proportion of oxidant over protein; 2) the inability to separate the modified from the native albumin, which can

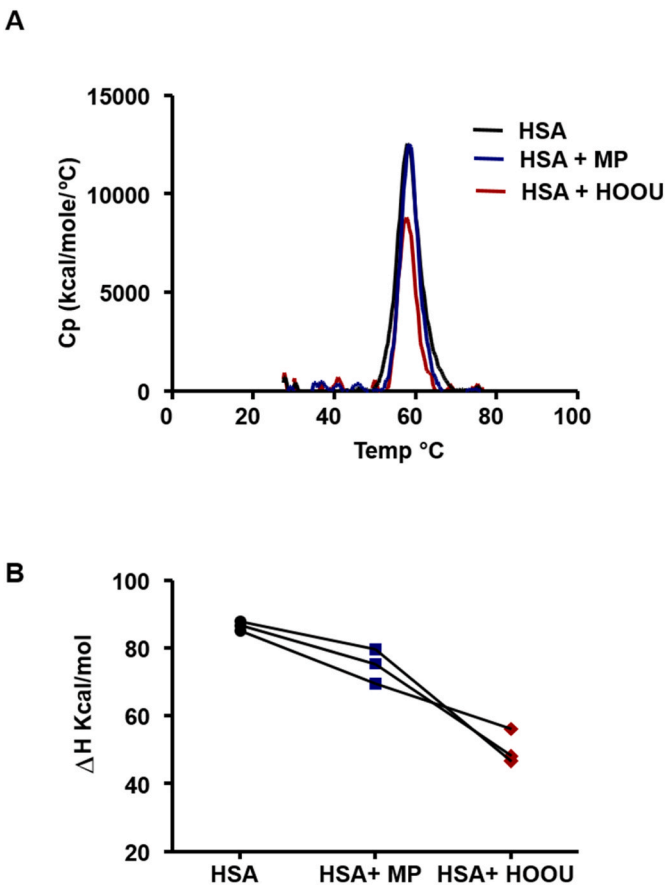

Fig. 1. Differential scanning calorimetry (DSC) of native and uratylated albumin. Human serum fatty acid free albumin (5 mg/mL,72 μM) was incubated with urate hydroperoxide (500 μM, (~1:7 protein:oxidant equivalents) for 30 min at 25°C in 50 mM phosphate buffer, pH 7.4. (A) DSC was determined by VP-DSC (Microcal, USA) operating with scan rate 60°C/h. (B) Enthalpy changes of albumin pre-ttreated with buffer (HSA), with vehicle control (HSA + MP) or with 500 μM urate hydroperoxide (HSA + HOOU, 1:7 protein: oxidant). Data are representative of 3 independent experiments.

underestimate the physicochemical alterations in the uratylated protein. Since glycation on lysine residues of albumin is better detected following several hours to days of reaction [37,38], our next experiments were carried out always for 48 h incubation at 37°C. Additionally, we maintained the highest proportion of oxidant over protein that our synthesis allowed (100 oxidants over 1 protein) and, eventually, a half of this proportion (50 oxidants over 1 protein).

Because total protein absorbance was not sensitive to detect possible conformational changes in uratylated albumin, we used Anilino-Naphthalene-Sulfonic acid (ANS), a fluorophore that binds to domain III with high affinity and detects changes in protein hydrophobicity [39–41]. An increase in ANS fluorescence intensity and a shift toward blue wavelength are usually observed after ANS binding to either exposed hydrophobic region or hydrophobic clusters [39,42]. The intensity of ANS (10 μM) fluorescence was lower when it was incubated with 1 μM uratylated albumin at both ~1:50 and 1:100 protein:oxidant equivalents (Fig. 2). In agreement, a previous study showed a decrease in ANS fluorescence when it was incubated with glycated or heated albumin [34]. In summary, our data reveals that uratylation may induce changes in protein structure, leading to decreased ANS binding [39,43]. In spite of that, no alterations were found in the circular dichroism (CD) spectra (Suppl Fig. S1), indicating that there were no deep alterations in the secondary structure of the protein [26,28]. Interestingly, a previous study using molecular dynamics simulations reported only a 10 % reduction in α-helix content upon glycation [44]. This suggests that

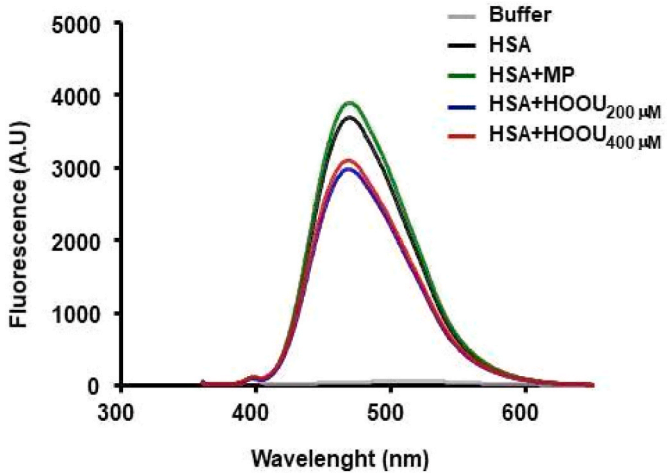

Fig. 2. ANS fluorescence spectra in native and uratylated albumin. Albumin (0.25 mg/mL) was incubated with 200 or 400 μM urate hydroperoxide (~1:50 or 1:100 protein:oxidant equivalents), or with evaporated mobile phase (MP, vehicle control) in 50 mM phosphate buffer pH 7.4, for 48 h at 37°C. Samples containing the final concentration of 1 μM albumin were incubated with 10 μM ANS and the fluorescence spectra were acquired in a Hitachi F7000 fluorimeter (Hitachi, Tokyo, Japan) at 25°C using an excitation wavelength λexc = 380 nm. Traces represent one of three independent experiments (n = 3).

albumin's secondary structure is relatively stable even under chemical modifications.

Since uratylation disturbed the binding of ANS to albumin, we evaluated whether it could promote aggregation of the protein. No differential high molecular weight bands were found between native or modified albumin (1:100 protein:oxidant). However, it did promote the appearance of a new band at ~75 kDa (Fig. 3A), that represents 4 % of the total protein from that sample (Fig. 3B). Although uratylation covalent adducts can promote a mass increment, it would not be as high as ~10 kDa. In fact, this modification may affect electrophoretic mobility, resulting in subpopulations visible on the denaturing gel. The band observed at ~75 kDa is therefore likely related to the modification. However, it may also result from incomplete sample denaturation or heat-induced effects. This band may disappear under different electrophoresis or denaturation conditions. Additionally, we know that albumin glycation leads to structural changes that affect protein compaction [44]. We confirmed that this band was albumin and no other sample contaminant by LC/MS/MS (Suppl Fig. S2). However, the amount of protein was too low to confirm the uratylation adducts in this band.

Size-exclusion chromatography analysis of the oligomerization state of uratylated albumin shows an elution profile comparable to that of the unmodified protein. Therefore, the presence of the ~75 kDa band may result from partial denaturation of the formed oligomers rather than reflecting a different subpopulation presenting a ~10 kDa mass increment in solution (Suppl Fig. S3).

2.3. Effect of uratylated albumin on endothelial cells

Post-translational modifications and oxidation on albumin may impair protein function, ultimately leading to tissue damage [3,13]. For instance, glycated albumin stimulates inflammatory signaling and pro-oxidant effects in murine HL-1 cardiomyocytes, suggesting a putative role in the pathogenesis of heart failure [13]. Here, we investigated whether uratylated albumin could affect Human Umbilical Vein Endothelial Cells (HUVECs) migration through the Wound Healing test [45]. The ability to migrate is a characteristic of endothelial cells and is a complex and important process for the maintenance and integrity of the endothelial tissue, being fundamental in healing processes, angiogenesis, vasculogenesis, and in the restoration of blood vessels [46]. Uratylated albumin, which was modified using equivalent ~1:50 or 1:100 protein:oxidant for 48 h at 37°C, decreased cell migration when compared with the native albumin (HSA). The albumin incubated with MP was not different from HSA (Fig. 4A and B). Uratylated albumin did not affect adhesion of HUVECs cells (Fig. 4C).

Oxidative modifications on serum albumin lead to the activation of neutrophils, inducing the production of oxidants and the release of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [13,47]. Here, we evaluated whether uratylated albumin could activate HUVECs

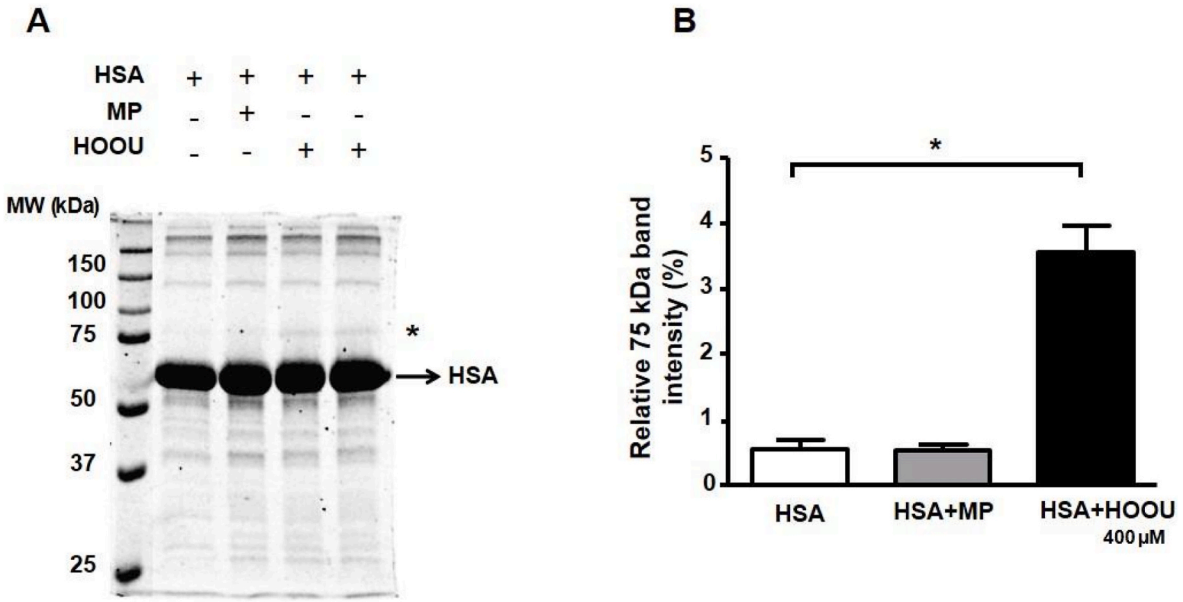

Fig. 3. Evaluation of the formation of uratylated albumin aggregates. Albumin (0.25 mg/mL) was incubated with urate hydroperoxide (400 μM) (1:100 protein: oxidant equivalents) or with evaporated mobile phase (MP, vehicle control) in 50 mM phosphate buffer pH 7.4 for 48 h at 37°C. (A) After incubation, 10 μg of protein were separated in a reducing SDS-PAGE. (B) The relative amount of the 75 kDa band was compared using ImageJ and it was expressed as the percentage of total protein densitometry from the specific lane. Data are the mean ± S.E.M from 3 independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni's post-test, *p < 0.05 compared to the native HSA group.

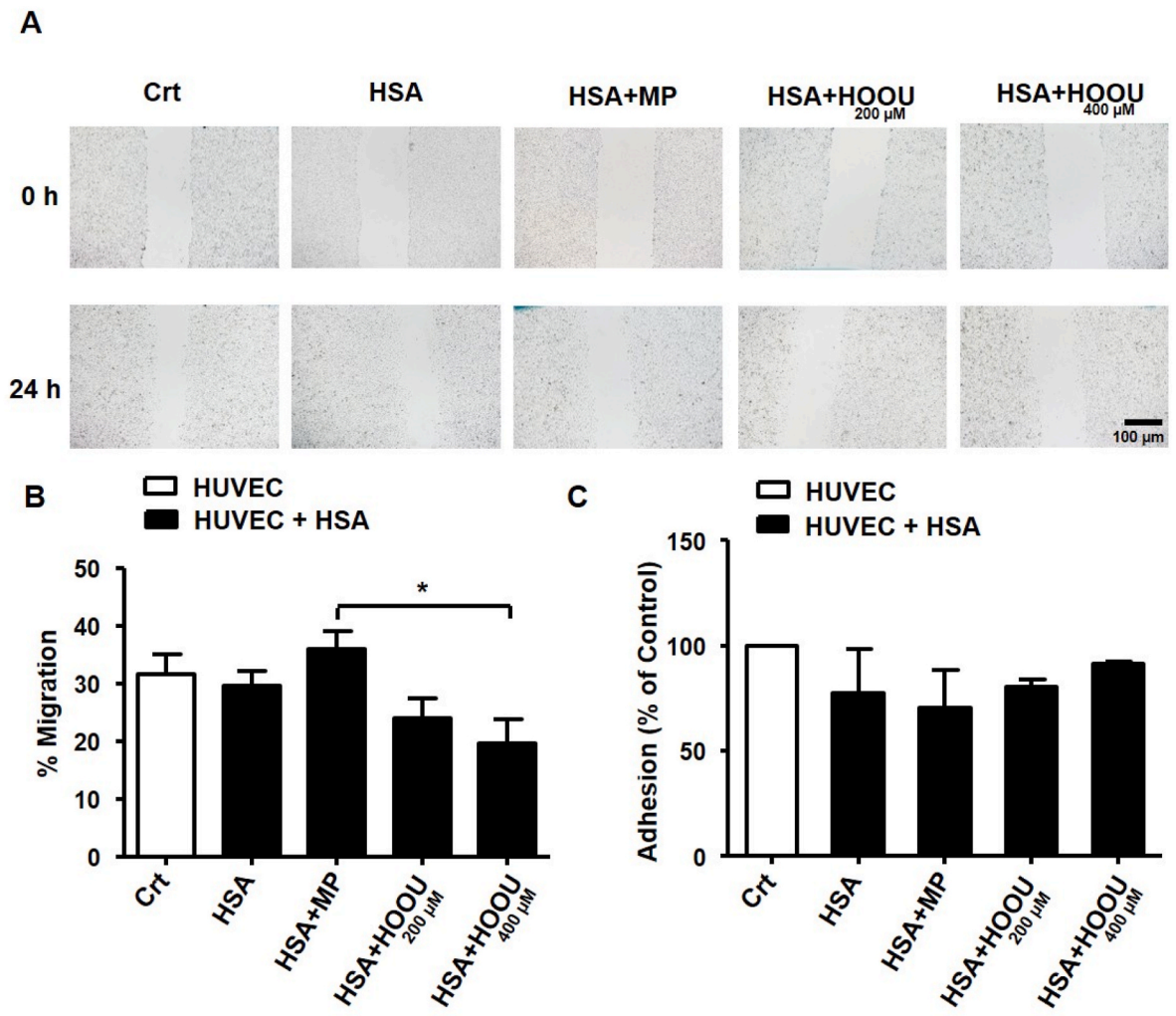

Fig. 4. Effect of uratylated albumin on endothelial cell migration (A, B) and adhesion (C). HUVECs (5×10^5 cells/well) were cultured in 6 well plates with RPMI medium. After two days, the medium was replaced with RPMI containing 0.2 % BSA. Following 24 h, a scratch was introduced into the cell monolayer. Control cells received no treatment (Ctr), while other groups were treated with native albumin (HSA), vehicle-treated albumin (HSA + MP), or uratylated albumin (HSA + HOOU) (~1:50 and 1:100 protein:oxidant equivalents), using 50 µg of protein. All treatments were performed during 24 h in RPMI culture medium. (A) Images of scratch were taken in an inverted light microscope (Nikon) using 4× magnification before (0 h) and after (24 h) the incubation period. Images are representative of 3 independent experiments. (B) The distance of the scratch was assessed using ImageJ software and the percentage of migration was calculated as depicted in the methods section. (C) For the adhesion assay, HUVECs (2.5×10^5 cells/well) were seeded in 96-well plates and incubated for 24 h with RPMI medium alone (Ctr), native albumin (HSA), albumin treated with vehicle (HSA + MP), or uratylated albumin (50 µg modified protein). After incubation, cells were washed with PBS and stained with crystal violet. Each data represents the mean ± S.E.M. of 3 independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni's post-test, *$p < 0.05$ compared to the HSA group.

and stimulate monocytes adhesion to them. Incubation of HUVECs with uratylated albumin (50 µg, for 24 h) led to a significantly higher adhesion of THP-1 monocytes when compared to HUVECs with no treatment (Ctr), treated with native albumin or with albumin pre-incubated with vehicle (MP) (Fig. 5A and B). A similar effect was displayed by the positive controls LPS or TNF-α.

In agreement to a higher increase in monocytes adhesion, HUVECs pre-treated with LPS, TNF-α or uratylated albumin (1:100 protein: oxidant) presented a significant increase in the expression of the intercellular adhesion molecule-1 (ICAM-1) (Fig. 6A and B; Suppl Fig. S4). In accordance, Magzal et al. demonstrated an increase in ICAM-1 and VCAM-1 mRNA expression in HUVECs treated with a modified (cysteinylated) albumin from plasma of hemodialysis patients when compared to HUVECs treated with unmodified albumin from healthy controls [3]. ICAM-1 mediates the interaction between monocytes and endothelial cells, facilitating leukocyte adhesion and migration into the sub-endothelium [48,49]. Upregulation of ICAM-1 by uratylated albumin highlights a putative mechanism for endothelial inflammation, which is closely related to the initiation and progression of atherosclerosis [49].

2.4. Uratylated albumin induces the release of TNF-α

The induction of monocyte adhesion to HUVECs by uratylated albumin was accompanied by an increase in TNF-α release (Fig. 7A). Since TNF-α was assessed in co-cultured HUVECs-THP-1 supernatants, we cannot distinguish the source of the cytokine. Previous studies demonstrated that oxidized albumin induced mRNA transcription of the pro-inflammatory cytokines IL-6, IL-8 and IL-1β in HUVECs [3] Likewise, glycated albumin increased IL-6 and TNF-α mRNA transcription in cardiomyocytes [13]. We also found a significant increase in TNF-α mRNA in HUVECs treated with uratylated albumin (Fig. 7B). However, TNF-α levels from HUVECs supernatants were below the detection limit in all groups (data not shown). This suggests that uratylated albumin is able to activate and prime HUVECs, but the final release of mature TNF-α is dependent on a secondary stimulus, as the contact of THP-1. From the

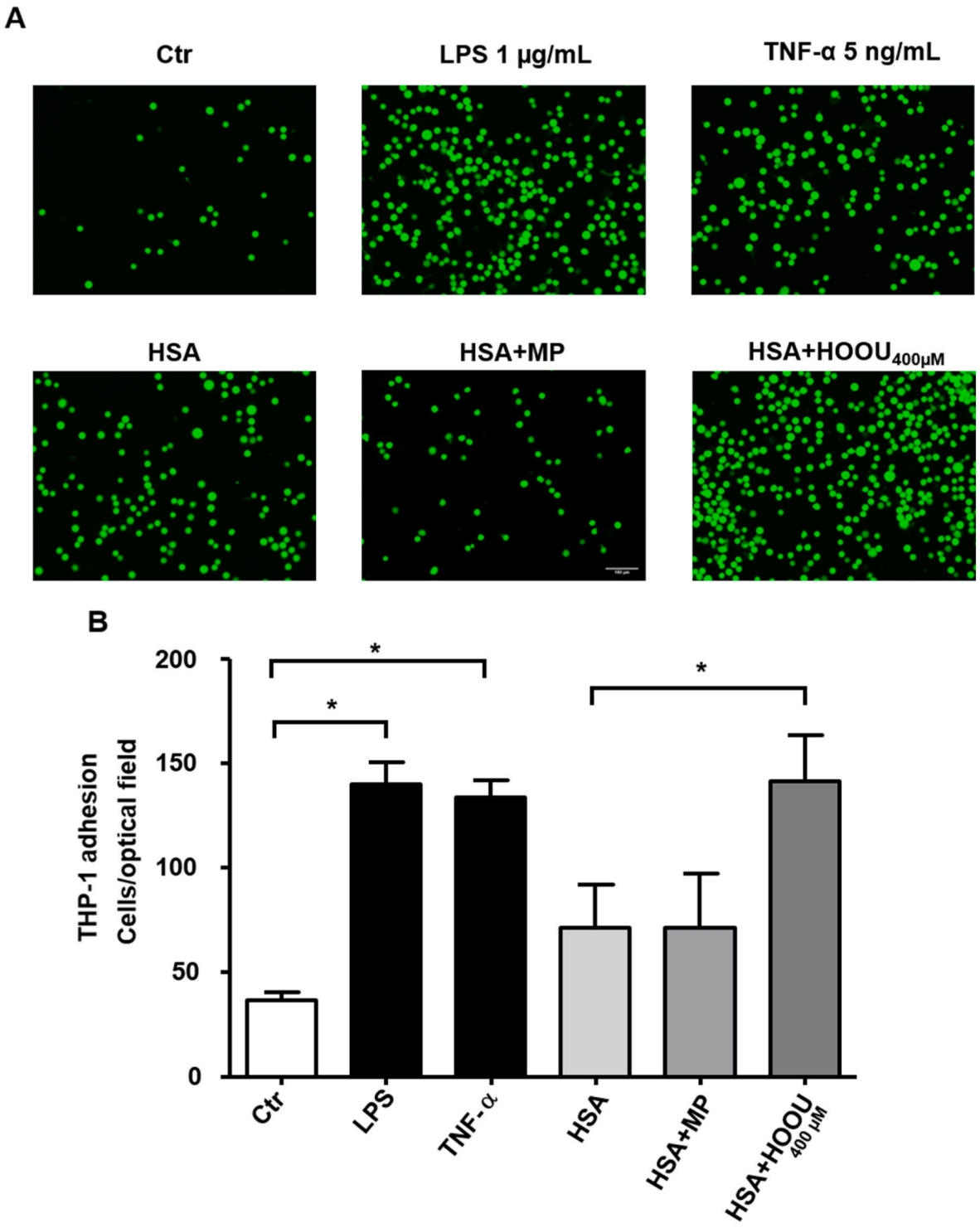

Fig. 5. Uratylated albumin increases monocyte adhesion to endothelial cells. HUVECs (5×10^4) were seeded in 24-well plates for 2 days. Subsequently, cells were washed with PBS and treated or not (Ctr) with TNF- α (5 ng/mL), LPS (1 µg/mL), 50 µg native albumin (HSA), albumin treated with vehicle (HSA + MP) or albumin previously treated with urate hydroperoxide (HSA + HOOU), equivalent 1:100 protein:oxidant for 48 h at 37°C. Cells rested in RPMI culture medium for 24 h. After incubation, the supernatant was removed and THP-1 cells (4×10^5/well), previously stained with calcein-AM (10 nM), were added. After 1 h incubation, non-adhered THP-1 were removed by washing with PBS. Imagens were taken by EVOS FLoid Imaging System (A). THP-1 cells/optical field were counted by Image-J software (B). Bar graphs represent mean ± S.E.M. of 3 independent experiments. Statistical analyses were performed by one-way ANOVA followed by Bonferroni's post-test, *$p < 0.05$ compared to the control or HSA groups.

present study, it was not possible to identify the exact mechanisms by which uratylated albumin is priming HUVECs and inducing monocyte adhesion. However, it is well documented that modification of albumin by oxidation, addition of carboxymethyl or glycation adducts on Lys residues promotes the binding to the Receptor for Advanced Glycation End products (RAGE) [50–52]. In silico studies revealed that glycated Lys64, Lys^{233}, Lys^{262}, Lys^{378} and Lys^{525} promote the strongest complex with RAGE [51] and several Lys residues were target to uratylation in

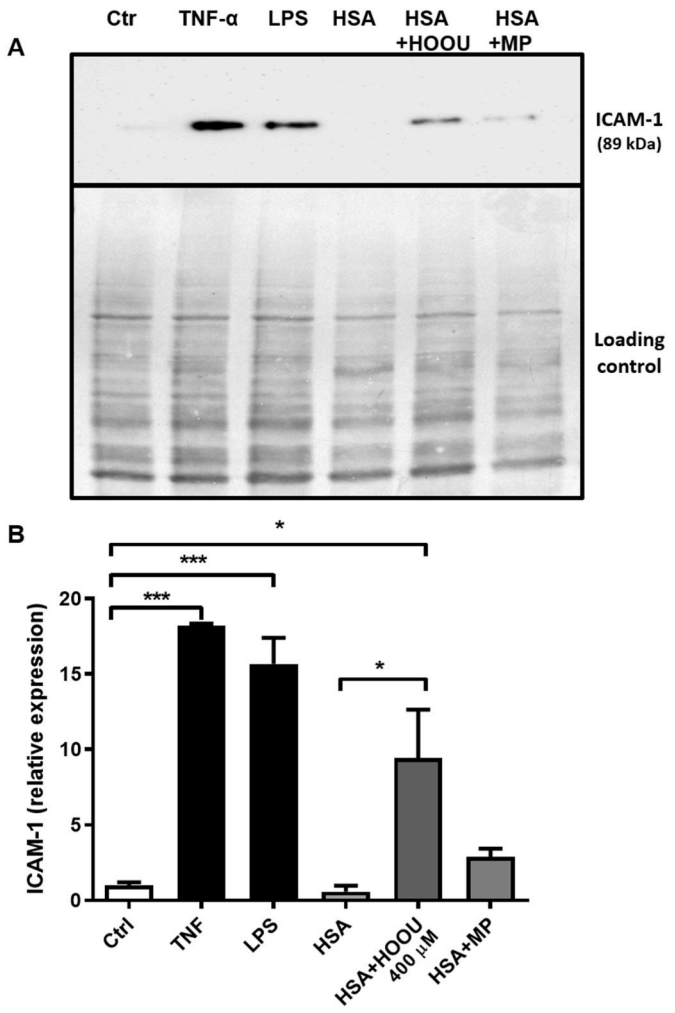

Fig. 6. ICAM expression in HUVECs after treatment with uratylated-albumin. HUVECs 5×10^4 were seeded in 24-well plates for 2 days. Subsequently, cells were washed with PBS and treated or not (Ctr) with native albumin (HSA), albumin previously incubated with vehicle (HSA + MP), uratylated albumin (HSA + HOOU 50 μg modified protein, previously incubated for 48 h with urate hydroperoxide in an 1:100 protein:oxidant), TNF-α (5 ng/mL) or LPS (1 μg/mL) in RPMI culture medium. The supernatant was removed after 24 h, cells were lysed and processed for western blotting. The ICAM band was normalized by total protein using comassie. Bar graphs represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed by ordinary one-way ANOVA and Bonferroni's multiple comparisons test. *p < 0.05 compared to the control or HSA group. ***p < 0.0001 compared to control.

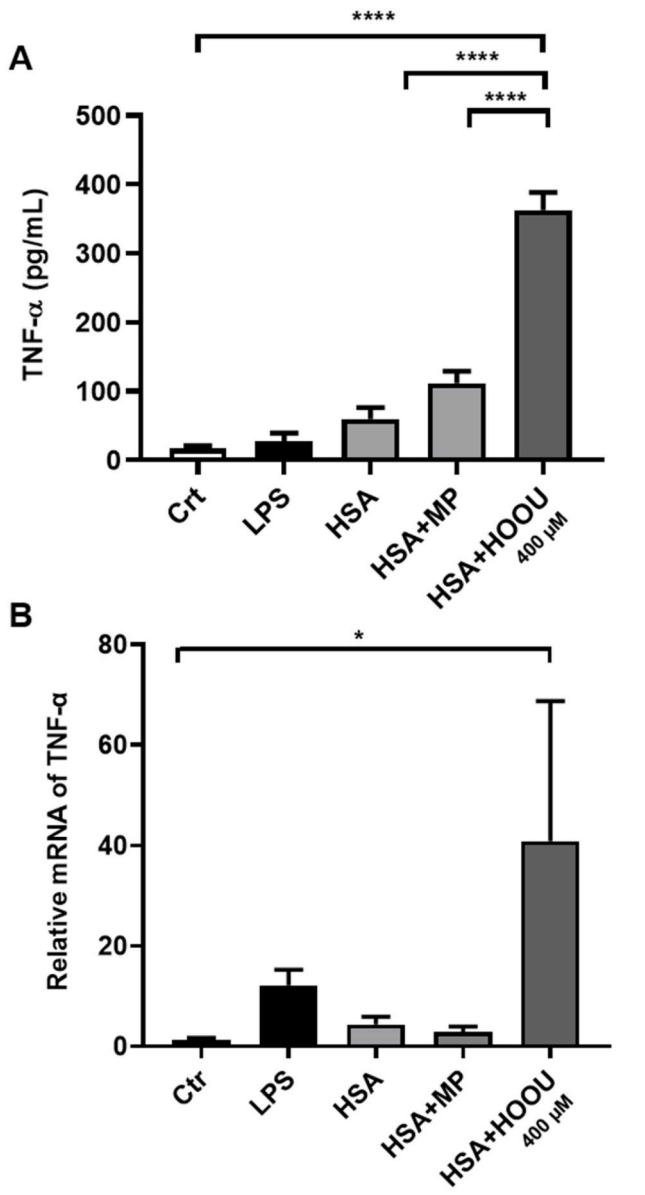

Fig. 7. Uratylated-albumin induced TNF-α. (A) HUVECs 5×10^4 were seeded in 24-well plates for 2 days. Subsequently, cells were washed with PBS and treated or not (Ctr) with native albumin (HSA), albumin previously incubated with vehicle (HSA + MP), uratylated albumin (50 μg modified protein, previously incubated for 48 h with urate hydroperoxide 1:100 protein:oxidant) in RPMI culture medium for 24 h. The supernatant was removed, and THP-1 cells (4×10^5/well) were added and incubated for 1 h. TNF-α released from co-cultured cells was determined by ELISA. (B) Relative mRNA expression of TNF-α in HUVECs. Gene expression was analyzed by quantitative real-time PCR using GAPDH as the endogenous reference gene. Data are presented as relative mRNA levels. Bar graphs represent the mean ± S.E.M. of 3 independent experiments. Statistical analysis was performed by ordinary one-way ANOVA and Newman-Keuls multiple comparisons test. *p < 0.05 and ***p < 0.0001 compared to the control group or HSA or HSA + MP.

albumin, including the Lys^{525} [15]. In spite of uratylation being a different adduct from glycation, it likely promotes similar alterations in albumin structure and both, simulation and binding assays that investigate the interaction of RAGE with uratylated albumin should be carried out. RAGE is a multi-ligand receptor of the immunoglobulin superfamily. Binding to this receptor activates NADPH oxidase, PI3K-Akt and MAPKs pathways, culminating activation of nuclear factor-κB and transcription of cytokines in endothelial cells, monocytes, and vascular smooth muscle cells [3,53,54].

Several studies have demonstrated that post-translational modifications, such as oxidation and glycation, on human serum albumin are associated with various pathological conditions, including cardiovascular diseases, diabetes, and chronic kidney disease [55–57]. Elevated levels of advanced oxidation protein products (AOPP) and modified albumin were found in plasma from patients with idiosyncratic drug-induced liver injury [58]. Furthermore, oxidized albumin in hypoalbuminemic and hemodialysis patients has been shown to induce increased expression of inflammatory cytokines in HUVECs and primary peripheral blood leukocytes [3,59].

Nevertheless, this is the first study to demonstrate that the recently reported modification on albumin, uratylation, induces endothelial cells activation, monocytes adhesion and may trigger inflammation. Notably, the effects of uratylated albumin on endothelial cells suggest a potential involvement in atherogenesis and corroborates previous studies on the

role of uric acid oxidation products in endothelial cell dysfunction and subclinical atherosclerosis [20,60,61].

3. Conclusion

In conclusion, this study reveals that uratylation of albumin leads to structural and conformational changes in the protein. However, isolation of the modified protein is challenging, making it difficult to precisely confirm the structural changes and its effects. Uratylated albumin decreased HUVECs migration, activated endothelial cells, inducing monocyte adhesion, which may be in the genesis of vascular dysfunction. Together, these findings widen our understanding on the mechanistic pathways by which uric acid can contribute to the development of cardiovascular disease and bring a deeper understanding of the biochemical mechanisms involving inflammation and oxidative stress.

4. Materials and methods

Human serum albumin fatty acid free (A3782, Sigma Aldrich), RPMI 1640 medium (with and without phenol red). Fetal bovine serum (FBS) was purchased from Vitrocell (Campinas, Brazil). Solvents were purchased from JTBaker (Thermo Fisher Scientific, Life Technologies, Waltham, MA, USA). All other reagents were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany.

4.1. Formation of uratylated albumin by urate hydroperoxide

Urate was oxidized as previously described by Patricio et al., 2015 [62]. Briefly, urate (20 mM) solution was prepared in 40 mM NaOH. Riboflavin (500 μM) was dissolved in a sodium phosphate buffer (10 mM; pH 6) containing 100 μM diethylenetriaminepentaacetic acid (DPTA). The reaction was carried out in plate with a 340 mm diameter well, with a total volume of 4 mL sodium phosphate buffer (10 mM; pH 6.8), urate (1.5 mM) and riboflavin (100 μM) under UV light. The light source was equipped with six lamps, 15 mW (GE.).

Urate hydroperoxide was separated in a preparative HPLC (Shimadzu, Tokyo, Japan) through a TSK-Gel Amide column (10 μm; 21.5 mm × 30 cm, TOSOH Bioscience, Tokyo, Japan), mobile phase of 30 % ammonium acetate (10 mM, pH 6.8) and 70 % acetonitrile, with constant flux of 6 mL/min in an isocratic mode during 30 min. Excess of acetonitrile from urate hydroperoxide collected peak and from mobile phase (MP) collected samples was evaporated with inert gas as described by Mineiro et al., 2020 [60]. The evaporated mobile phase (MP) was used as the vehicle in this study, and has been reported as non-toxic under comparable conditions [60]. Urate hydroperoxide concentration was measured by its absorbance at 308 nm ($\varepsilon_{308nm} = 6400\ M^{-1}\ cm^{-1}$), as previously described [62].

For the physicochemical assay, the amount of protein required for modification was 5 mg/mL (calorimetry). Unless stated, the other experiments were carried out using a concentration of 0.25 mg/mL albumin with 200 or 400 μM urate hydroperoxide (~1:50 or 1:100 protein to oxidant, respectively) previously incubated in 50 mM phosphate buffer pH 7.4 for 30 min at 25°C or 48 h at 37°C. The reaction was stopped by filtration using ultra Amicon filters (10 kDa). Separation of uratylated albumin proved to be challenging and, therefore, all analysis were carried out using a mixture of native and modified albumin.

4.2. Differential scanning calorimetry

The phase transition temperature (T_m), and enthalpy change (ΔH) of the unmodified and previously modified albumin (5 mg/mL plus 500 μM urate hydroperoxide, ~1:7 albumin to urate hydroperoxide incubated for 30 min at 25°C, in 50 mM phosphate buffer pH 7.4) was determined on a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal, Northampton, MA, USA). The reference cell was filled with 50 mM phosphate buffer pH 7.4. Samples were heated at a constant scan rate of 60°C/h and the temperature was scanned from 25 to 80°C. The data were analyzed using the software Origin®MicroCal, provided by the manufacturer and the graphs were plotted using the Graphpad prism 5.0 program.

4.3. UV–Vis absorbance

Albumin (0.5 mg/mL) was incubated with a single dose of urate hydroperoxide (400 μM, 1:100 protein to oxidant) for 48h at 37°C. Subsequently, samples were washed with phosphate buffer and filtered in 10 kDa filters cut-off (3 times). Samples protein were quantified by Bradford [63] and normalized to 0.1 mg/mL to assess absorbance of modified and unmodified protein using a microplate reader (Biotek Synergy, Biotek, Winooski, VT, USA).

4.4. ANS binding studies

Conformational changes of modified and unmodified albumin were evaluated using Anilino-Naphthalene-Sulfonic acid (ANS). Albumin (0.25 mg/mL, 3.76 μM) reacted with urate hydroperoxide (200 or 400 μM, 1:50 or 1:100 protein to oxidant) for 30 min at 25°C. This albumin (1 μM) solution was incubated with 10 μM ANS in phosphate buffer. The fluorescence spectra of ANS were recorded from 390 to 550 nm with excitation at 370 nm.

4.5. Albumin aggregation assay

Albumin (0.25 mg/mL, 3.76 μM) was incubated with urate hydroperoxide (400 μM, 1:100 protein to oxidant) in 50 mM phosphate buffer pH 7.4 for 48 h at 37°C. Samples were separated in a reducing 12 % SDS-PAGE. Aliquots of the samples (25 μg) were incubated in sample buffer (62 mM Tris-HCl, pH 6.8 containing 10 % glycerol, 2 % SDS, 0.01 % bromophenol blue, 20 mM of β-mercaptoethanol) for 5 min at 95°C and then applied on the gel. Coomassie was used for gel staining.

4.6. Cell culture

A selection of immortalized human umbilical vein endothelial cell line (HUVEC) was kindly provided by Prof. Francisco Laurindo's group from INCOR (São Paulo – Brazil). HUVECs were maintained in RPMI medium containing 10 % fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (30 mg/mL) at 37°C in a 5 % CO_2 atmosphere. For experiments, confluent adherent cells were harvested using a solution containing trypsin (0.1 %) and EDTA (0.5 mM) prepared in phosphate-buffered saline solution (PBS; 10 mM, pH 7.4). After centrifugation and counting, cells were seeded on culture plates in the appropriate concentration. HUVECs were used between passages 2 and 10. THP-1 cells (ATCC TIB-202) were maintained in RPMI medium containing 10 % fetal bovine serum (FBS), 0.05 mM β-mercaptoethanol, streptomycin (100 mg/mL), and penicillin (30 mg/mL) at 37°C in a 5 % CO_2 atmosphere.

4.7. Evaluation of cell migration and adhesion of HUVEC cells

HUVECs (5×10^5 cells/well) were harvested and seeded in 6-well plates. After 2 days, the culture medium was replaced by RPMI containing 0.2 % BSA. After 24 h, a scratch was made in the cell monolayer [64]. Subsequently, cells were washed with PBS and incubated in RPMI medium without phenol red and FBS containing native or modified albumin (50 μg). For modification, albumin (0.25 mg/mL, 3.76 μM) was incubated with urate hydroperoxide (400 μM), equivalent to 1:100 protein:oxidant, during 48 h at 37°C. Cells were incubated with the specific treatments for 24 h at 37°C and 4 % CO_2. Images were acquired at the end of the incubation period. Reference marks were made in the plates and the images were captured in an inverted light microscope. ImageJ software was used to measure the distance of each scratch using

%migration = (initial distance – final distance)/initial distance*100 %. The images of scratch were taken in an inverted light microscope (Nikon) using 4 × magnification before and after the incubation period.

4.8. Evaluation of monocyte (THP-1) adhesion to HUVEC

HUVEC cells (5×10^4 cells/well) were plated in 24-well plates and cultivated during 3 days. Subsequently, cells were washed with PBS and incubated in RPMI medium without phenol red. Albumin (0.25 mg/mL, 3.76 μM) was previously modified with urate hydroperoxide (400 μM, 1:100 protein to oxidant) for 48 h at 37°C, 50 μg of native or modified albumin was added to the HUVECs. HUVECs were incubated with LPS (1 μg/mL) or TNF-α (5 ng/mL) for positive control samples. After 24 h, cells were washed twice with PBS and incubated with THP-1 cells (4×10^5/well) for 1 h. THP-1 cells were previously stained with 10 nM Calcein-AM (Invitrogen) for 30 min. After incubation, cells were washed with PBS until the complete removal of non-adherent cells. Imagens were taken by EVOS FLoid Imaging System (Thermo Fisher Scientific). THP-1 cells/optical field were counted by Image J.

4.9. Evaluation of ICAM-1 expression in HUVEC cells

HUVEC cells (5×10^4 cells/well) were plated in 24-well plates and cultivated for 3 days. Subsequently, cells were washed with PBS and incubated in the presence of native or modified albumin (50 μg), TNF- α (5 ng/mL) or LPS (1 μg/mL) in RPMI culture medium for 24 h without phenol red and FBS. Albumin (0.25 mg/mL, 3.76 μM) was previously modified with urate hydroperoxide (400 μM, 1:100 protein to oxidant) for 48 h at 37°C. Then, cells were washed with PBS, lysed and 20 μg of total protein content was mixed with Laemmli buffer and heated at 95°C for 10 min and loaded on 10 % SDS-PAGE gel. Proteins were transferred to PVDF membranes with a Trans-Blot Turbo Transfer System (Bio-Rad). Blot membranes were blocked for 1 h with 5 % milk in PBS and incubated with primary antibodies (anti-ICAM-1, Ab109361, 1:1000), overnight at 4°C. HRP-conjugated secondary antibodies were incubated at 1:10000 dilution for 1 h at room temperature. Membranes were incubated with Clarity Western ECL Substrate (Bio-Rad Laboratories) and proteins were detected using chemiluminescence mode on the ChemiDoc Imaging System (Bio-Rad Laboratories).

4.10. Measurement of TNF-α in HUVEC supernatant using ELISA assay

HUVECs (5×10^4 cells/well) were plated in 24-well plates and cultivated for 3 days. Subsequently, cells were washed with PBS and incubated in the presence of native or modified albumin (50 μg) or LPS (1 μg/mL) in RPMI culture medium for 24 h without phenol red and FBS. Albumin (0.25 mg/mL, 3.76 μM) was previously modified with urate hydroperoxide (400 μM, 1:100 protein to oxidant) for 48 h at 37°C. Then, cells were washed with PBS twice and incubated with THP-1 cells (4×10^5 cells/well) for 1 h. After incubation, cell supernatants were collected and centrifuged (1400 rpm for 10 min at 4°C) to remove THP-1 cells and cell debris. The supernatants from HUVECs treated with albumin and without co-culture were also collected to TNF-α measurements. The quantification of TNF-α in cell culture supernatant was performed using the Human TNF-α ELISA Kit (ab46088) from Abcam, according to the manufacturer's instructions. Briefly, 100 μL of each sample was added to the ELISA plate with Biotinylated anti TNF-α antibody and incubated for 3 h at room temperature. (18–25°C). After washing, a secondary antibody conjugated Streptavidin-HRP solution was added and incubated for 30 min and then washed. After washing, Chromogen TMB substrate was added and incubated for 15 min. The reaction was stopped, and absorbance was measured at 450 nm using a microplate reader. TNF-α concentrations in the cell culture supernatant were calculated using a standard curve generated with known concentrations.

4.11. qRT-PCR

HUVEC cells (5×10^4 cells/well) were plated in 24-well plates and cultivated for 3 days. Subsequently, cells were washed with PBS and incubated in the presence of native or modified albumin (50 μg) or LPS (1 μg/mL) in RPMI culture medium for 24 h without phenol red and FBS. Albumin (0.25 mg/mL, 3.76 μM) was previously modified with urate hydroperoxide (400 μM, 1:100 protein to oxidant) for 48 h at 37°C. Then, cells were washed with PBS twice and the RNA was extracted using 500 μL the Trizol® Reagent (Life technologies) and 1 μg of RNA was used to cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad) accordingly to the manufacturer's protocols. Quantitative real-time polymerase chain reaction was performed in a 10 μL reaction using iTaq Universal SYBR® Green Supermix (Bio-Rad) according to the manufacturer's protocol. SYBR fluorescence was analyzed by QuantStudio™ 3 Real-Time PCR System (Thermo Fisher). The information of primers used to amplify each gene is as follows: GAPDH (endogenous reference gene) - Fwd 5′- CCAGCAAGAGCACAAGAGGAA-3′, Rvs 5′-ATGGTACATGACAAGGTGCGG-3'. hTNF-α - Fwd 5′-CCGAGGCAGTCAGATCATCTT-3′, hTNF-Alpha- Rvs- 5′-AGCTGCCCCTCAGCTTGA-3'. The fluorescence was collected for each amplification cycle and the data analyzed using the $2^{-\Delta\Delta CT}$ method for the relative quantification. Expression of the target genes was calibrated against the conditions found in control cells.

4.12. Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed by one-way analysis of variance (ANOVA) with repeated measures where appropriate (GraphPad Prism 5.0 or OriginPro 8.5). Statistical significance was assessed through post-test comparisons with Bonferroni, Tukey or Student's t-test. Results with $p < 0.05$ were considered significant.

CRediT authorship contribution statement

Railmara Pereira da Silva: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Beatriz Pereira da Silva:** Methodology. **Antônio Paulo Siqueira Pratti:** Methodology. **Bianca Dempsey:** Methodology. **Caroline Dutra Lacerda:** Methodology. **Gustavo Penteado Battesini Carretero:** Methodology. **Álbert Souza Peixoto:** Methodology. **Litiele Cezar Cruz:** Methodology. **Camilla Adan:** Methodology. **Iolanda Midea Cuccovia:** Resources. **Flavia Carla Meotti:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Funding

Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP): CEPID Redoxoma 2013/07937-8; Young Investigator—2, grant number 2018/14898-2. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 303572/2022-1). R.P.S, B.P.S., A.S*.P. and* B.D received a fellowship from FAPESP.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Professor Dr. Francisco Laurindo (Incor) for providing the HUVEC cell line and Prof. Shaker Chuck Farah for the support on size-exclusion chromatography. We acknowledge BioRender.com for the

support in the creation of the Graphical Abstract Image.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rbc.2026.100071.

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