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Human apolipoprotein A-I Gly26Arg stimulation of inflammatory responses via NF-kB activation: Potential roles in amyloidosis?

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ABSTRACT

The cascade of molecular events leading to Human apolipoprotein A–I (apoA–I) amyloidosis is not completely understood, not even the pathways that determine clinical manifestations associated to systemic protein deposition in organs such as liver, kidney and heart. About twenty natural variants of apoA–I were described as inducing amyloidosis, but the mechanisms driving their aggregation and deposition are still unclear. We previously identified that the mutant Gly26Arg but not Lys107-0 induced the release of cytokines and reactive oxygen species from cultured RAW 264.7 murine macrophages, suggesting that part of the pathogenic pathway could elicit of an inflammatory signal. In this work we gained deep insight into this mechanism and determined that Gly26Arg induced a specific pro-inflammatory cascade involving activation of NF-κB and its translocation into the nucleus. These findings suggest that some but not all apoA–I natural variants might promote a pro-oxidant microenvironment which could in turn result in oxidative processing of the variants into a misfolded conformation.

1. Introduction

High density lipoproteins (HDL) are transporters implicated in the movement of cellular cholesterol through the plasma compartment toward the liver. The major protein component of HDL in human is the apolipoprotein A–I (apoA–I). An extensive research supports that low amounts of circulating lipid-poor apoA–I, either recently synthesized or as a catabolic product of the HDL, are essential to ensure the efficiency of the protein in the reverse cholesterol transport pathway [1]. On the other hand, it is well-recognized that the flexible structure of the lipid-free form implies a risk of suffering minor conformational shifts, generating intermediate states prone to aggregate *in vivo* or to lose the physiological functions. This behavior is typical of proteins that could undergo amyloid-like misfolding. Though not deeply characterized, many factors have been suggested as participants in these events: either mutations of the protein sequence or modifications in the microenvironment that could introduce transient or permanent arrangements in the spatial protein organization.

Non-hereditary amyloidosis due to apoA–I with the native sequence was detected in senile plaques [2]. In a previous paper, we suggested that a cellular microenvironment associated with chronic inflammation, as a pro-oxidant milieu or in mildly acidic pH, promotes misfolding, aggregation, and increased binding of apoA–I to extracellular matrix elements; this fact may thus favor protein deposition as amyloid-like complexes [3,4]. In addition, the hereditary form of apoA–I amyloidosis is described as a late-onset condition resulting in systemic deposition of amyloid in tissues [5].

In spite of the growing body of evidence focused on α-synuclein or the Aβ peptide misfolding, little is known about structural features participating in pathological pathways elicited by apoA–I.

Abbreviations: ApoA–I, apolipoprotein A–I; BSA, bovine serum albumin; COX, cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; HDL, high density lipoproteins; IκB, inhibitor protein of NF-κB; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCP-1, monocytic chemotactic protein-1; MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; p65, functionally active subunit of NF-κB; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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Patients with gene mutations affecting residues 1–75 suffer mainly hepatic or kidney dysfunction [6], whereas mutations in amino acids 173–178 cause amyloidosis of the heart, larynx, and skin [5,7]. Though not prevalent, apoA–I deposits in testis, uterus, and peripheral nerves were reported [5]. The first form of apoA–I amyloidosis described was due to the mutant Gly26Arg which induces peripheral neuropathy [8], renal failure [9,10], and hepatic dysfunction [11]. Protein deposits were mainly interstitial rather than glomerular, and proteinuria was a minor aspect of the disease. In previous studies, and in order to gain insight in this pathology, we analyzed two apoA–I natural variants: Lys107-0 (a unique deletion mutant inducing amyloidosis associated with severe atherosclerosis [12] and Gly26Arg. We demonstrated that Lys107-0 was significantly less stable and more prone to aggregate than the protein with the native sequence (wild-type, Wt), and that the acquisition of a positive charge (in Gly26Arg) induces loss of stability and a more relaxed structure. However its aggregation tendency seems to be similar to that of the Wt. In this same study we determined that Gly26Arg induced ROS, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β production from RAW 264.7 murine macrophages cells. Interestingly, Wt apoA–I and Lys107-0 did not induce this pro inflammatory situation [13].

Like other inflammatory diseases, human peripheral neuropathies are thought to be caused by attacks by the immune system on targets within peripheral neurons. Macrophages within nerve lesions may express and elaborate a multitude of regulatory molecules [14] enhancing a local inflammatory landscape. With this thought in mind, we hypothesize that part of the pathological pathways of the Gly26Arg variant could be due to subtle changes in protein conformation that initiate and/or perpetuate a local inflammatory condition associated with organ dysfunction [13].

Continuing with that study, the objective of this manuscript is to gain deeper knowledge of the pathways that could be implicated in the pro-inflammatory response of this apoA–I mutant.

2. Materials and methods

2.1. Chemicals

His-purifying resin was purchased from Novagen (Darmstadt, Germany). The rest of chemicals was of the highest analytical grade available obtained from Sigma-Aldrich (St Louis, MO): guanidine hydrochloride (GndHCl), lipopolysaccharide (LPS), polymyxin B sulfate, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

2.2. Cloning, expression and purification of Wt apoA–I

The Wt form and the substitution mutant Gly26Arg were obtained from protocols carried out according to Ramella et al. [3]. Briefly, the cDNA for human apoA–I and the variant Gly26Arg were inserted into a pET-30 plasmid, and transformed into BL21 (DE3) expression host cells (Novagen). The expression and purification of proteins were carried out following the methodology described previously [3].

2.3. Protein structure analysis

The probable conformational shift that could occur to apoA–I variants during the cellular incubation times was analyzed. ApoA–I variants were incubated by 72 h at 37 °C, at a final concentration of 1 mg/mL, in 20 mM Tris pH 7.4 with agitation. Following, proteins were spun (30 min at 15.000 rpm). Supernants (Spnt) and pellets were separated and taken to the initial volume. Intrinsic fluorescence of the Trp residues was measured on an Olis upgraded SLM4800 Spectrofluorometer (ISS Inc, Champaign, IL). Excitation wavelength was set to 295 nm and emission registered between 310 and 420 nm. Spectra were compared with respect to the proteins without incubation.

2.4. Cell culture and MTT cell viability assay

RAW 264.7 murine macrophages (ECACC, Salisbury, UK) were cultured at 37 °C using a humidified incubator (5% CO_2) in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. During the experiments, cells had less than 20 passages. Cell viability assay was performed according to Ramella et al. [13]. Briefly, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, cells incubated at 37 °C during 20 min. The precipitate (formazan) was solubilized in dimethyl sulfoxide (DMSO) after removing the medium and absorbance measured at 490 nm on a microplate reader.

Cultured RAW 264.7 macrophages (6×10^4 cells/well, 24 h, 37 °C) were treated according to the previous protocol [13]. ApoA–I variants (0.1 and 1 μg/mL) or LPS (0.1 and 1 μg/mL) were added and cellular redox activity quantified 24 h later by measuring the conversion of the tetrazolium salt MTT into its formazan product, which was solubilized in dimethyl sulfoxide (DMSO) and absorbance measured at 490 nm on a microplate reader [13].

2.5. Determination of nitric oxide (NO) and prostaglandin (PG) E_2 production

RAW 264.7 macrophages were plated and treated as described previously [13] and then treated as commented above (presence of apoA–I variants, 0.1 or 1.0 μg/mL, 37 °C, 24 h) and LPS was used as a positive control. As the antibiotic Polymyxin B binds specifically and sequesters the LPS, it was added to the proteins in order to avoid any minor contamination from the LPS that could remain from the bacterial purification stocks. The supernatants were collected and the presence of nitrites determined in them with the aid of Griess reagent (Sigma-Aldrich, St Louis, MO). Nitrite production was assessed as the index of nitric oxide generation [15]. PGE_2 production was determined with the aid of a specific enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI) used according to the manufacturer's instructions.

2.6. Preparation of cytosolic and nuclear fractions from RAW 264.7 cells

RAW 264.7 cells were scraped and washed (×2) with phosphate-buffered saline (PBS). Protein extraction was performed with the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit according to the manufacturer's instructions (Fermentas Life Science, Opelstrasse, Germany). Proteins were determined by the Bradford method using bovine serum albumin (BSA) as standard.

2.7. Analysis for cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)

Assays were performed by Western Blot according to the methods previously described by Giner et al. [16]. Briefly, 30 mg of proteins were loaded onto 10% sodium dodecyl sulfate polyacrylamide electrophoresis gel and transferred onto polyvinylidene difluoride membranes at 125 mA for 90 min. Membranes were then blocked in PBS-Tween 20 containing 3% w/v defatted milk. Membranes were incubated with anti- iNOS or anti−COX-2 polyclonal antibodies (1/1000, Cayman, MI, USA) to detect these proteins. The blots were washed and incubated with peroxidase−COnjugate anti-rabbit and anti-mouse immunoglobulin G (1/20000 dilution;

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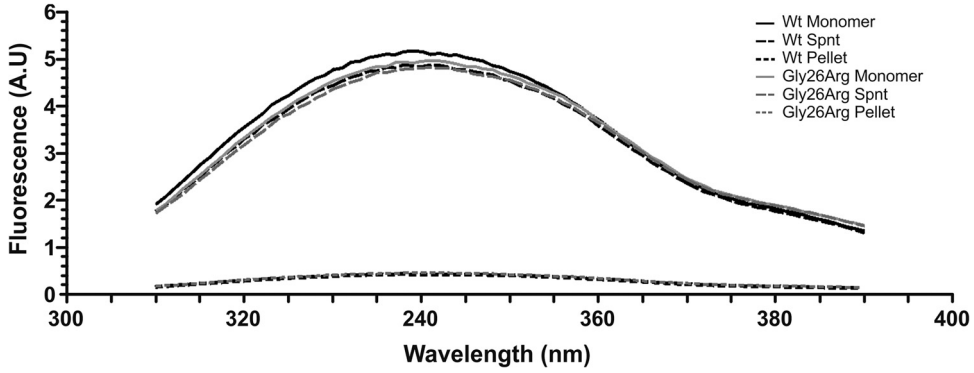

Fig. 1. Characterization of apoA–I variants' conformation. Wt and Gly26Arg were incubated at 1.0 mg/mL for 72 h at 37 °C; following proteins were spun for 30 min at 15,000 rpm. Trp fluorescence emission spectra were registered on the Spnt (long dashed lines) and the pellets (short dashed lines) at the same dilution aliquots. Continuous lines are the spectra measured of the freshly prepared proteins. Dark and grey lines correspond to Wt and Gly26Arg respectively. Excitation was set at 295 nm and emission was recorded between 310 and 390 nm.

Cayman, MI, USA) and immunoreactive bands visualized by chemiluminescence (GE Healthcare, Fairfield, CT) [16].

2.8. Western Blot analysis for p65 nuclear factor (NF)-κB and its inhibitor IκBα

In the case of nuclear factors, membranes were incubated with the corresponding polyclonal antibodies: anti-p65 (SC-7151) or anti-IκBα (SC-1643) from Santa Cruz Biotechnologies (Santa Cruz, CA) at dilution of 1/500. β-Actin (1/12000, Sigma-Aldrich, St Louis, MO) and PARP (1/400, Santa Cruz Biotechnologies) were used as total protein loading control and nuclear protein loading control, respectively. The blots were treated according to the report by Andújar et al. as cited above [16].

2.9. Immunocytochemistry for NF-κB p65 localization

The effect of apoA–I on the nuclear translocation of p65 was examined by immunocytochemistry according to the previous report cited by Giner et al. [16]. Briefly, cells (1×10^5 cells/mL) were untreated or treated with LPS (1 μg/mL) and apoA–I variants (1 μg/mL) and then fixed in a solution of methanol:acetic acid (95:5) for 20 min at −20 °C. After washing in PBS, cells were permeabilized with Triton X-100 (0.3%) at 4 °C overnight and then blocked with 5% BSA for 15 min. Slides were incubated (rabbit polyclonal anti-p65, 1:500 dilution, 2 h in a humectation chamber at 37 °C) and then slides washed with PBS and incubated at 37 °C for 40 min with a secondary goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Samples were analyzed under a fluorescent microscope and images captured with a camera (Nikon Microscope Eclipse E800, Badhoevedorp, The Netherlands).

2.10. Transient transfection and NF-κB-dependent reporter gene expression assay

To test the effect of apoA–I on the transcriptional activity of NF-κB, RAW 264.7 cells (5×10^5 cells per well) were placed in 24-well plates and transiently transfected with pNFκB-Luciferase expression plasmid (0.8 mg) and the control plasmid TK-Renilla (0.2 mg), both kindly donated by Dr. Lidija Klampfer. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used to perform the transfections, following the manufacturer's instructions. 24 h after transfection, cells were treated with the apoA–I variants (1 μg/mL) for 8 h. The luciferase assay was performed with the Dual-Luciferase Assay System, following the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was normalized to TK-Renilla activity as a control for transfection efficiency.

2.11. Data analysis

Unless otherwise stated, data are shown as means ± SD, being representative of at least three measurements performed under exact conditions. Statistical analyses used ANOVA followed by Dunnett's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, USA).

3. Results

3.1. Protein conformation

Due to the nature of pro-amyloidogenic proteins, which could be sensitive to aggregate under mild conditions, it is clue that a fresh homogeneous and monomeric conformation is used in each experimental design. Thus, proteins were resuspended in guanidine hydrochloride and exhaustively dialyzed before being used en each experiment. However, it is worth testing the hypothesis that, during the incubation times, protein could be misfolded, giving rise to aggregates which should be responsible at least in part for the events assayed. In order to check whether such misfolded conformations could take part in this set up, we incubated Wt and Gly26Arg at high concentrations (1 mg/mL) and analyzed Trp fluorescence (representing in average the 4 native residues in position 8, 50, 72, and 108) [13,4]. Fig. 1 shows (as we have previously described) similar spectra of Wt and Gly26Arg in the samples freshly prepared [13]. Interestingly, these spectra are almost indistinguishable from the soluble fractions obtained after incubation (long-dashed lines in the Figure). Moreover, no Trp-associated intensity is detected in the pellet, indicating that during this incubation period both proteins preserved their structure and no aggregated species are formed. Transmission electron microscopy confirmed that no aggregates were observed, either for Wt or Gly26Arg under these conditions (not shown).

3.2. Cytotoxicity

Our research included an assessment of the cytotoxicity of the LPS and apoA–I variants on RAW 264.7 cells using the MTT assay. Treatment with LPS and variants of apoA–I for 24 h did not show relevant cytotoxicity against RAW 264.7 macrophages (viability higher than 85%). Percentages of cell viability are shown in Fig. 2.

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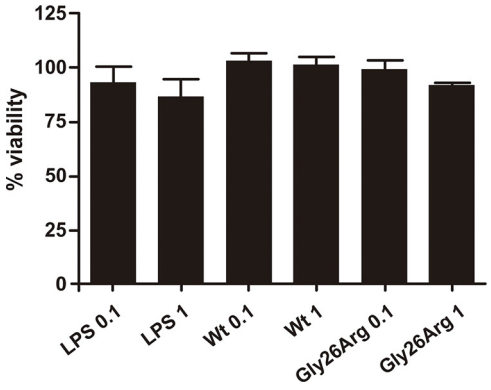


Fig. 2. Effect of apoA–I Gly26Arg on cell viability on RAW 264.7 macrophages. Viability was determined by MTT assay following incubation of cells for 24 h with either apoA–I Wt or Gly26Arg. Numbers in the legend of each bar represent protein concentration used (either 0.1 or 1 μg/mL). Values represent percentage of live cells compared to the blank group treated with vehicle only. Data represent the mean ± SD of at least three different experiments.

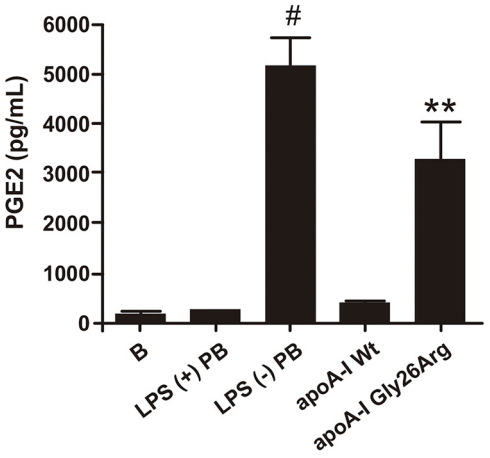


Fig. 4. Effect of apoA–I Gly26Arg on PGE2 production in RAW 264.7 murine macrophages. The apoA–I natural variant was tested at 1 μg/mL in the presence of 50 μg/mL polymyxin B, and LPS (positive control) was tested at 1 μg/mL in the absence (LPS (-) PB) or the presence (LPS (+) PB) of 50 μg/mL polymyxin B. Values represent production of PGE2 in pg/mL and are expressed as mean ± SD, n = 3. **P < 0.01 compared to Wt apoA–I, #P < 0.01 compared to blank.

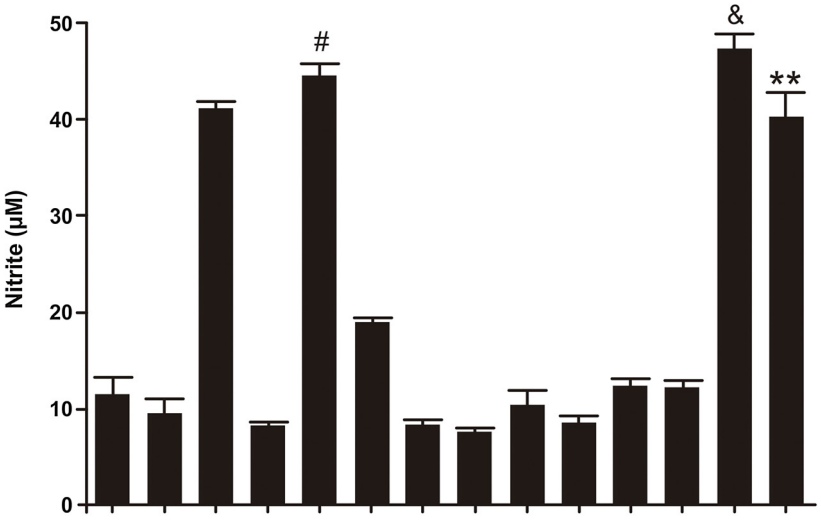
3.3. *Effect of apoA–I variants on the NO and PGE_2 production in RAW 264.7 cells*

To exclude the possibility that endotoxin contamination in the preparation of apoA–I variants might contribute to increase nitrite production, cells were pretreated with polymyxin B (50 μg/mL), an antibiotic that binds to and deactivates LPS. Nitrite formation in the medium, which reflects NO generation, was measured by Griess' reaction. As shown in Fig. 3, polymyxin B significantly inhibited nitrite production in LPS-induced macrophages, but contamination of the samples was minimal since apoA–I variants tested in the presence and absence of polymyxin B showed no difference in NO production.

Production of NO and PGE_2 was determined in the culture supernatant. As shown in Figs. 3 and 4, NO and PGE_2 production after 24 h treatment with 1 μg/mL of apoA–I Wt did not induce significant cell activation, whereas incubation with Gly26Arg resulted in a marked increase of both pro-inflammatory mediators. ApoA–I Gly26Arg at 0.1 μg/mL did not activate RAW 264.7 macrophages.

3.4. *Effect of apoA–I variants on the expression of iNOS and COX-2 proteins in RAW 264.7 cells*

Levels of iNOS and COX-2 were analyzed in cytosolic extracts from RAW 264.7 cells by Western blot techniques. As shown in Fig. 5, treatment with 1 μg/mL of apoA–I Gly26Arg induced expression of iNOS and COX-2, as compared with Wt which did not elicit expression of these enzymes. ApoA–I variants did not stimulate RAW 264.7 cells at 0.1 μg/mL (data not shown).



LPS (μg/mL)	-	-	0.1	0.1	1.0	1.0	-	-	-	-	-	-	-	-
PB (μg/mL)	-	50	-	50	-	50	-	50	-	50	-	50	-	50
apoA-I Wt (μg/mL)	-	-	-	-	-	-	0.1	0.1	1.0	1.0	-	-	-	-
apoA-I Gly26Arg (μg/mL)	-	-	-	-	-	-	-	-	-	-	0.1	0.1	1.0	1.0

Fig. 3. Effect of apoA–I Gly26Arg on NO production by RAW 264.7 macrophages. Release of nitrites was determined in the culture supernatant with Griess reagent. Compounds were assayed at 0.1 and 1 μg/mL in absence or presence of 50 μg/mL polymyxin B (PB). Values are expressed as nitrite production (μM). Data represent the mean ± SD of at least three different experiments. & P < 0.01 compared to apoA–I Wt in the absence of PB ** P < 0.01 compared to apoA–I Wt in the presence of PB, #P < 0.01 compared to blank (cells under the same conditions but in the presence of polymyxin B).

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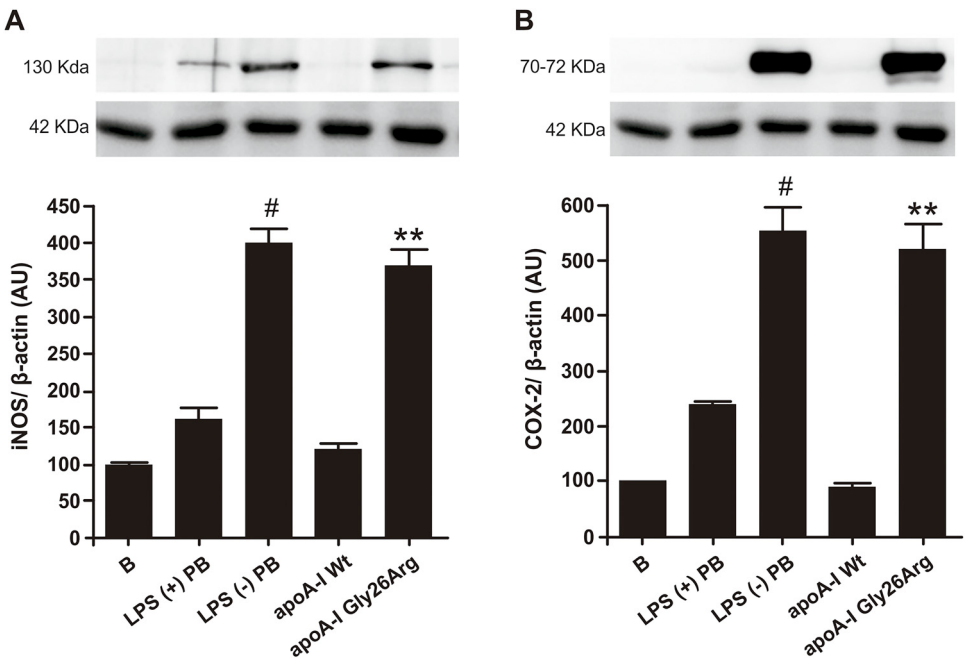

Fig. 5. Effect of apoA–I Gly26Arg on iNOS (panel A) and COX-2 (panel B) expression in RAW 264.7 macrophages. ApoA–I Gly26Arg and Wt were tested at 1 μg/mL in the presence of 50 μg/mL polymyxin B (LPS(+)PB) and LPS (positive control) was tested at 1 μg/mL in the absence or presence of 50 μg/mL polymyxin B (LPS(-)PB). Total protein extracts were subjected to Western blot analysis with iNOS- and COX-2- specific antibodies. iNOS and COX for each sample were determined by densitometry and normalized to β-actin (the 42 kDa band in the upper panel) expressed as arbitrary units (AU). Values are the mean ± SD, n = 5. **P < 0.01 compared to apoA–I Wt, #P < 0.01 compared to blank.

3.5. *Effect of apoA–I Gly26Arg on IκBα degradation and p65 translocation in RAW 264.7 cells*

By Western blot techniques, we determined that treatment of cells with apoA–I Gly26Arg resulted in a significant decrease in cytosolic levels of IκBα (Fig. 6) and translocation of p65, as suggested by increased levels of this protein in the nucleus (Fig. 7). Under our experimental conditions these effects were not observed when cells were incubated with Wt.

3.6. *Effect of apoA–I variant on p65 translocation into the nucleus in RAW 264.7 cells*

The effect of apoA–I-induced nuclear translocation of p65 was determined by Western blot techniques. The apoA–I Gly26Arg (added to cells for 60 min at 1 μg/mL) induced nuclear translocation of p65 in RAW 264.7 cells, as suggested by a significant increase in the levels of p65 in the nucleus, compared with apoA–I Wt (Fig. 7). Immunofluorescence assays further corroborated this finding. In non-stimulated cells (Blank) and in cells in the presence of apoA–I Wt, p65 was localized mainly in the cytoplasm, whereas in cells treated with LPS or apoA–I Gly26Arg, p65 underwent nuclear translocation from the cytoplasm to the nucleus (Fig. 8).

3.7. *Effect of apoA–I variant on LPS-mediated NF-κB transcriptional activity in RAW 264.7 cells*

RAW 264.7 macrophages transiently transfected with NF-κB-Luc plasmids were used to perform a NF-κB reporter gene assay in order to examine the effect of apoA–I variant on LPS-induced NF-κB activation. As shown in Fig. 9, subsequent treatment of the transfected cells with apoA–I Gly26Arg for 8 h increased the luciferase activity over the basal level; instead this effect was not observed when pre-treating the cells with the apoA–I Wt.

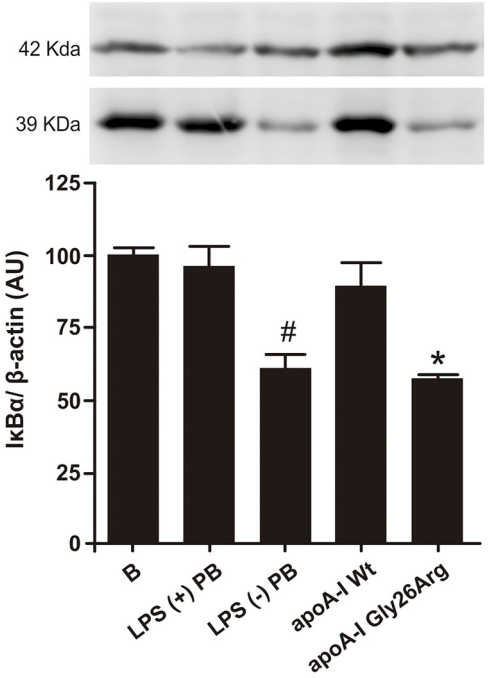

Fig. 6. Effect of the apoA–I Gly26Arg natural variant on IκBα expression in RAW 264.7 macrophages. Gly26Arg and *wt* were tested at 1 μg/mL for 30 min in presence of 50 μg/mL polymyxin B and LPS was tested at 1 μg/mL in the absence or presence of 50 μg/mL polymyxin B (positive and negative control, respectively). Total protein extracts were analyzed by Western blot of IκBα specific antibody. IκBα content for each sample was determined by densitometry and normalized to β-actin as arbitrary units (AU). Results are expressed as mean ± SD, n = 3. **P < 0.01 compared to apoA–I *wt*, #P < 0.01 compared to blank. Figures are representative of three similar experiments with similar results.

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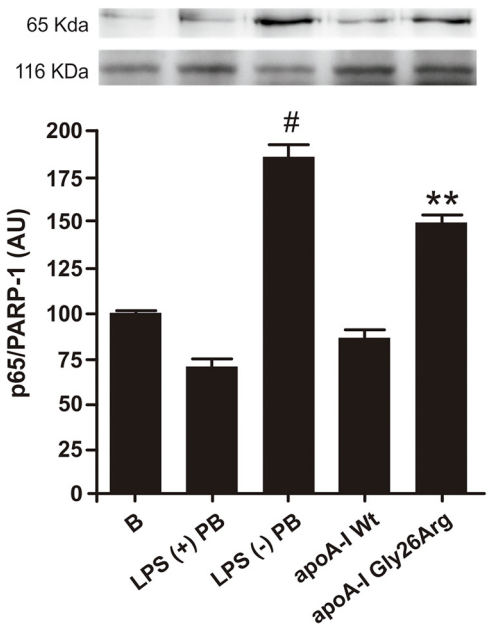

Fig. 7. Effects of two apoA–I natural variants and LPS on p65 translocation to the nucleus in RAW 264.7 macrophages. ApoA–I Gly26Arg were tested at 1 μg/mL by 60 min in the presence of 50 μg/mL polymyxin B and LPS was tested at 1 μg/mL in the absence or presence of 50 μg/mL polymyxin B (positive and negative control, respectively). Total protein extracts were subjected to Western blot analysis of p65 specific antibody. The p65 content for each sample was determined by densitometry and normalized to PARP-1 (the 116 kDa band in the upper panel) as arbitrary units (AU). Results are expressed as mean ± SD, n = 3. **P < 0.01 compared to apoA–I *wt*, #P < 0.01 compared to blank. Figures are representative of three similar experiments with similar results.

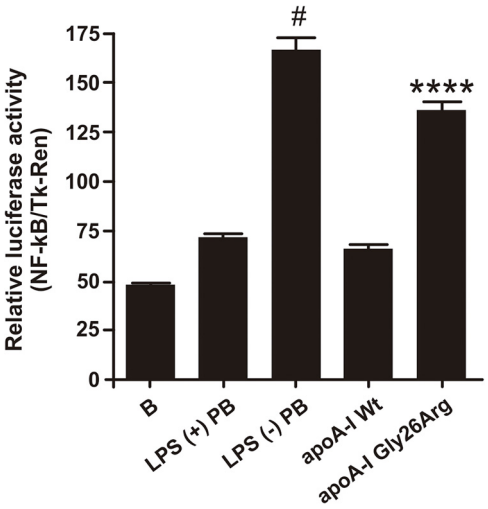

Fig. 9. Effect of apoA–I variant on LPS-mediated NF-kB transcriptional activity in RAW 264.7 cells. ApoA–I Gly26Arg elicits NF-kB activity in RAW 264.7 macrophages, as shown by the luciferase assay. The activity of Luciferase was normalized to TK-*Renilla* activity. Results are expressed as mean ± SD, n = 4. ****P < 0.0001 compared to apoA–I Wt, #P < 0.0001 compared to blank.

4. Discussion

Crosstalk among cells and proteins suggests the following questions: Could protein misfolding be a consequence of environment landscape? Or do misfolded conformations elicit cell activation into a pro-inflammatory scenario? Finally, could both events interact in order to reactivate local organ dysfunction?

The dual role played by apoA–I in the pro-versus anti-inflammatory cascades is intriguing and challenging. Extensive research has well documented from *in vivo* and *in vitro* models the function of apoA–I as inhibiting monocyte recruitment [17], increasing endothelial Nitric Oxide synthase activity [18], and neutralizing the endotoxin lipopolysaccharide [19]. Moreover apoA–I is able to decrease the production of both TNF-α and IL-1β, by blocking activation of monocytes by stimulated T cells [20]. But, on the

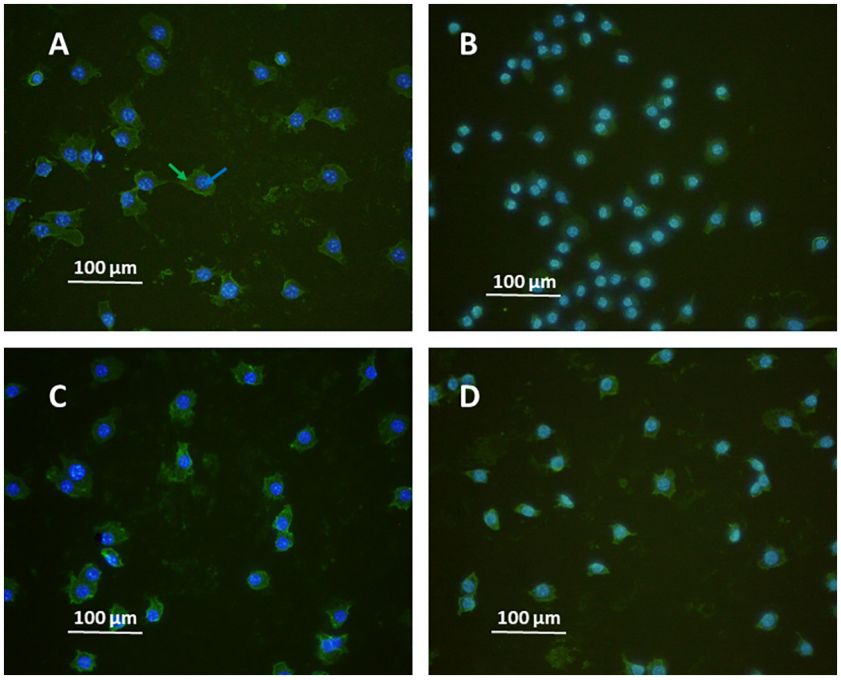

Fig. 8. Cellular localization of p65 by immunocytochemistry analysis. ApoA–I Gly26Arg induces p65 translocation to the nucleus in RAW 264.7 macrophages. p65 was indicated by the presence of fluorescent green (Alexa Fluor® 488) shown with a green arrow and DNA by fluorescent blue (DAPI), indicated by a blue arrow. A) Control (non stimulated cells). B), C), and D) Cells treated with LPS, Wt and Gly26Arg, respectively. In non-stimulated cells (blank) and apoA–I Wt-treated RAW 264.7 cells, p65 was localized mainly in the cytoplasm, whereas after treatment with apoA–I Gly26Arg and LPS (positive control), p65 underwent nuclear translocation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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other side, it was extensively demonstrated that HDL lipoproteins including the proteins and lipids components could suffer both the loss of function and in addition the becoming of pro-inflammatory under chronic pro-inflammatory landscapes [21]. Myeloperoxidase (MPO)-mediated oxidation results in the impairment of two central physiological functions of apoA–I, like ATP binding cassette transporter A1-dependent cholesterol release from cells [22] and lechitin:cholesterol acyltransferase activation [23].

In addition, MPO-oxidized apoA–I is present as amyloid structures in atherosclerotic plaques, indicating that alterations in the protein sequence are events required to shift the equilibrium among function and pathogenicity [24]. In this trend, studies with natural mutants of apoA–I identified in patients clearly show that the minor conformational shift induced by the single mutations could result in protein involvement in many pathways that are common in the cytotoxicity elicited by other amyloid proteins [3,25], including the capability to elicit cellular responses affecting its micro- environmental behavior [26].

One interesting observation from our studies is that monomeric Gly26Arg conformation is able to elicit pro-inflammatory cascades. An interesting challenge in the amyloidosis field is to determine which (if any especial) is the protein conformation that is highly toxic to cells; although unlikely, it could be possible that during incubation time some yield of insoluble species could be formed and interact with cells; in order to discard this possibility, we incubated proteins at a concentration (1 mg/mL) which is about 1000 times higher than that used in the experiments performed here. As Fig. 1 shows, no shift from the native structure could be detected under these conditions and in agreement with our previous results. Significantly longer incubation periods, higher concentrations [4], or acidic pH [3] are required to obtain a small yield of protofibrillar complexes. Moreover, these aggregates are reversible (not shown), and thus they are not likely to occur and/or to be tested in this system.

In addition, it is worth considering that higher amount of apoA–I circulates as lipid-bound complexes. Although it could be hypothesized that this conformation could be involved in the genesis of toxic aggregates, it was strongly stated that lipid bound protein is more stable [27], and the binding to lipids may involve a protective mechanism against misfolding, such as the sequestration of the amyloidogenic segments *via* the native protein-lipid and protein-protein interactions [28].

The close association between expression of inflammatory mediators and chronic pathologies was described previously. COX-2, expressed by macrophages, may generate prostaglandins during acute inflammatory demyelination of the peripheral nerve [29]. Demyelination and axonal injury was also associated with LPS-activated microglia releasing inflammatory cytokines such as IL-1β, IL-6 and TNFα, and increased expression of inducible nitric oxide synthase (iNOS) and production of ROS [30]. Moreover, amyloid neurodegenerative disorders have also been associated with cytokine-mediated neuritic plaques [31,32]. Among other pieces of evidence, it was shown that the inflammatory response elicited by the challenge of microglia-like cells with the $A\beta_{1\text{-}42}$ peptide included upregulation of IL-1β and monocytic chemotactic protein-1 (MCP-1), and a decrease in cell viability [33]. Aggregated α-synuclein was also suggested to induce transformation of microglia cells into a macrophage-like phenotype involving proliferation, increased expression of cell surface receptors, and production of ROS and pro-inflammatory mediators such as TNF-α [18,22].

In a previous work we have shown that apoA–I Gly26Arg mutant (but not Lys107-0) elicited the *in vitro* production of IL-1β, TNFα, and ROS from the murine macrophage RAW 264.7 cell line [13], and now, we got deeper insight in the pathways involved in the macrophages activation. We showed that Gly26Arg induced the production of NO and PGE2, as a consequence of the increase in the iNOS expression and COX-2 enzymes, respectively. Again Lys107-0 did not appear to be active as inducing these pathways (not shown).

As mentioned above, the activation of the transcription of NF-κB is involved in the expression of cytokines, iNOS, and COX-2 among other pro-inflammatory proteins. NF-κB is a ubiquitous transcription factor that plays a crucial role in regulation of genes implicated in cell growth, differentiation, and death [34]. As it was extensively indicated, this signaling pathway is involved in most of the inflammatory cellular responses [35]. Our results show that in the presence of Gly26Arg the RAW264.7 macrophages reduce the cytosolic content of IκB and induce the translocation of p65 NF-κB to the nucleus, indicating that this regulation mechanism is involved in the pro-inflammatory pathway of this mutant.

Macrophages within inflammatory nerve lesions produce regulatory molecules that contribute to immune deregulation leading to peripheral neuropathies [14]. RAW 264.7 macrophages used in our studies were activated with LPS to simulate an inflammatory scenario. As other studies point out, it seems clear that activation of these macrophages after LPS challenge occurs through the phosphorylation and subsequent degradation of IκBα which, in turn, leads to translocation of p65 NF-κB to the nucleus, initiating the transcription of multiple pro-inflammatory mediators such as TNFα, IL-1β, IL-2, IL-6, IL-8, and enzymes such as iNOS and COX-2 [36–38]. As shown by our results, apoA–I Gly26Arg mutant at the dose tested perfectly mimics the pro-inflammatory effect of LPS on macrophages.

5. Conclusions

Altogether, results herein presented strongly support the role of mutant-specific activation of the NF-κB pro-inflammatory cascade in organ-damage events involving chronic co-morbidity associated with apoA–I amyloidosis. We have previously stated that, even though apoA–I is not inherently amyloidogenic, it could be involved in the development of non-hereditary amyloidosis as it is oxidative or proteolytically processed within a pro-inflammatory microenvironment [3]. On the other hand, we herein confirm and expand our knowledge, what indicates that the microenvironment could instead be a *victim* of the pathogenicity of variant proteins that elicit cellular events triggering cell damage. Thus, it is attractive to hypothesize that misfolded proteins (*i.e.*, natural mutant variants) could elicit a pro-inflammatory landscape that effectively yields oxidized pro-amyloidogenic protein conformations which thus act as a positive feedback-like mechanism amplifying local pathogenicity. The finding that the mutant Lys107-0 does not reproduce this effect [13] and behaves instead similar to the Wt in the pro-inflammatory response supports in addition that different pathways could be involved in the amyloidosis due to apoA–I variants. Further work will be required to solve this hypothesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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