

Glycosidated ferritin induces the release of microparticles positive for Toll-like receptors derived from peripheral blood CD14+ cells

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Abstract: Both increased serum ferritin levels and Toll-like receptor (TLR) activation show independent association with the inflammatory processes. During inflammation, cell activation and apoptosis are accompanied by the release of membrane-derived microparticles (MPs), which are considered to be mediators of intercellular communication as they induce specific responses in target cells. The aim of this study was to determine whether glycosidated and glycosidated ferritin induce *in vitro* release TLR microparticles from CD14+ peripheral blood mononuclear cells. Peripheral blood mononuclear cells were stimulated with glycosidated, glycosidated and native ferritin. The release of microparticles from CD14+ cells, the presence of TLR2+ and TLR4+ on the microparticles surface and the presence of interleukins-6 and -8 (IL-6 and IL-8) inside the microparticles after stimulation were determined by flow cytometry. The role of nuclear factor κ B (NF- κ B) was evaluated by pretreatment of the cells with the Bay 11-7085 inhibitor. Glycosidated and glycosidated ferritin induced the release of microparticles from CD14+ cells, the majority of which expressed TLR2+ and TLR4+ on their surface and contained IL-6 and IL-8. These effects were dependent on NF- κ B activation. Our findings show that glycosidated and glycosidated ferritin might be involved in the release of microparticles and stimulation of inflammatory responses.

Key words: microparticles; Toll-like receptor (TLR); ferritin; glycation; inflammation

INTRODUCTION

Increased levels of serum ferritin and inflammatory markers such as tumor necrosis factor alpha (TNF α) and C-reactive protein (CRP) are frequently related to systemic inflammatory diseases [1-4]. Chronic subclinical inflammation is also mediated by p38 mitogen-activated protein kinase (p38 MAPK), reactive oxygen species (ROS) [5], protein kinase C (PKC) and nuclear factor- κ B (NF- κ B) among other factors [6]. In patients with diabetes mellitus this leads to an increased risk of atherosclerosis, a risk factor for vascular complications in diabetes [7]. Previous work has shown that ferritin genes are susceptible to induction in the course of plaque formation [8], however the potential mechanisms by which ferritin could be

involved in these inflammatory events are currently unknown.

Toll-like receptors (TLR) constitute a family of proteins essential to the innate immune system. TLR2 and TLR4 bind to components of Gram-positive and Gram-negative bacteria, respectively, but they are also activated by endogenous ligands, including hyaluronic acid fragments, necrotic cells, serum amyloid A, advanced glycation end-products (AGEs) and extracellular matrix components. Therefore, TLR2 and TLR4 may play important roles in inflammatory disorders, including atherosclerosis and diabetes mellitus, as shown by the increased expression of TLR2 and TLR4 in monocytic cells under hyperglycemic conditions and in macrophages from human atherosclerotic

plaques and murine models of atherosclerosis [6]. In particular, oxidized low-density lipoprotein (oxLDL) and AGE-products of LDL (AGE-LDL) trigger TLR4-dependent signaling pathways and induce production of the proinflammatory cytokine IL-6 in human and mouse macrophages [6]. Recently, it was shown that glycated ferritin induces the *in vitro* activation and expression of TLR2 and TLR4 in CD14⁺ blood peripheral macrophages [9].

During inflammatory processes, cell activation and apoptosis are accompanied by the release of membrane-derived microparticles (MPs) [10]. MPs are submicron vesicles shed from a variety of cells that contain surface molecules and cytoplasmic constituents of their parent cells and are considered as biomarkers for several cardiovascular and inflammatory disorders. MPs are also mediators of intercellular communication as they induce specific responses in target cells [11]. Furthermore, plasma cell-derived MPs and urine cell-derived MPs appear to be positively associated with adverse clinical events [12, 13].

The relationship between TLRs, glycooxidated ferritin and elevated ferritin serum levels has not been completely elucidated. Therefore, in this study we examined the *in vitro* effects of glycated and glycooxidated ferritin on the composition of the surface and internal content of MPs released from circulating venous CD14⁺ monocytes from healthy adult volunteers.

MATERIALS AND METHODS

Experiments were performed using a pool of fasting venous blood samples drawn from five healthy adult volunteers. The donors of blood samples signed an informed consent form and were clinically assessed to ensure that they did not have any inflammatory disorder and had not undergone any steroid and/or antiinflammatory therapy. Blood samples were drawn into heparinized tubes for cell stimulation and flow cytometry. All procedures were conducted in accordance with international ethical standards. The study was approved by the Bioethics and Research Committee of the Department of Medicine and Health Sciences, University of Sonora.

Materials

PerCP/Cy5.5 anti-human CD14 (clone HCD14), FITC anti-human CD282 (TLR2) (clone TL2.1), PE anti-human TLR4 (clone HTA125), FITC anti-human IL-6 (clone MQ2-13A5) and PE anti-human IL-8 (clone E8N1) were obtained from BioLegend (San Diego, CA). Fixation buffer (Cat. 420801), permeabilization wash buffer 10x (Cat. 421002) and cell staining buffer (cat. 420201) were also obtained from BioLegend (San Diego, CA). Calibration 1.0 and 0.1 μ M particle size latex fluorescent beads (Cat. L2778 and L9904, respectively), ferritin type I, from horse spleen (Cat. F4503), zymosan A from *Saccharomyces cerevisiae* (Cat. Z4250), Bay 11-7085 [14] (Cat. B5681) and D-(+)-glucose (Cat. 5767) were obtained from Sigma-Aldrich® (Saint Louis, MO). All other reagents used were of the highest grade available.

Native ferritin modification by glycation and glycooxidation

A total of 1 mg native ferritin was dissolved in 1 mL of 0.1 mmol/L phosphate buffer solution (PBS), pH 7.4, containing 1 mmol/L EDTA, 0.1 mg/mL chloramphenicol and 3 mmol/L NaN₃. The protein sample was then incubated with 0.4 M glucose at 37°C for 1 week under a nitrogen atmosphere. Glycated ferritin was then reduced with NaBH₄ for 1 h at 4°C and dialyzed against 0.1 M PBS containing 0.1 mmol/L EDTA for 24 h [15]. For oxidation, glycated ferritin (1 mg/mL) was dialyzed against 5 mmol/L CuSO₄ in PBS for 24 h at 37°C in the dark. Next, the reaction was stopped by incubation with PBS containing 200 μ mol/L EDTA and 40 μ mol/L 3,5-Di-*tert*-4-butylhydroxytoluene for 24 h. The protein glycation process was monitored by SDS/PAGE (data not shown) [9, 15]. Finally, the glycated, glycooxidated and non-modified ferritin concentrations were measured using the Bradford technique [16].

Cell stimulation

A total of 1x10⁶ mononuclear cells from the heparin tubes were adjusted to 100 μ L using 100 mM (pH 7.4) and PBS in flow cytometry tubes. To test TLR2 and TLR4 activation, non-stimulated and cells stimulated with 50 ng/mL glycated ferritin (Gly-ferritin), 50 ng/mL glycooxidated ferritin (GlyOx-ferritin) and 50 ng/

mL native ferritin were incubated at room temperature for 2 h. Stimulation assays under the same conditions using 50 ng/mL zymosan A were performed as positive controls for TLR2 and TLR4 expression [14]. To test the role of NF- κ B, cells were also pretreated with 15 μ mol/L Bay 11-7085 for 30 min [17]. Subsequently, the supernatants were collected and processed for flow cytometry. All assays were performed in triplicate.

Fluorescence-activated cell sorting (FACS) analysis of microparticles

MPs contained in supernatants from the stimulated and non-stimulated cell suspensions were incubated with 20 μ L of anti-CD14-PerCP/Cy5.5, anti-TLR2-FITC and anti-TLR4-PE [18, 19] for 30 min at room temperature in the dark. FACS analysis was performed using 1 and 0.1 μ M particle size latex fluorescent beads as a control for microparticle size. A total of 100000 MP events per assay were evaluated using a FACScalibur flow cytometer (Becton Dickinson, San Diego, CA) and Summit Software Informer[®]. To determine the content of IL-6 and IL-8 in the CD14+ MPs, MP surface staining was performed for anti-CD14-PerCP/Cy5.5. The MPs were fixed in 0.5 mL/tube fixation buffer (BioLegend Cat. No. 420801) in the dark for 20 min at room tempera-

ture. To permeabilize the MPs, permeabilization wash buffer (Cat. No. 421002; 1X in DI water) was added to the samples. Finally, the fixed and permeabilized MPs were incubated with the anti-IL-6-FITC and anti-IL-8-PE [18, 19] for 30 min at room temperature in the dark.

Statistical analysis

All statistical analyses were performed using the SPSS 20 software (Armonk, NY). Data are presented as the mean \pm standard deviation (SD), unless otherwise indicated. The Student's *t*-test was used to compare mean expression of basal control and the average expression produced by the stimulation of cells. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Glycated and glycooxidated ferritin increases the release of MPs from CD14+ cells

The specific MPs were identified by their forward scatter and side scatter using 0.1 μ m and 1.0 μ m latex fluorescent beads to determine the region of MPs that they represent (Fig. 1A). The percentage of MPs

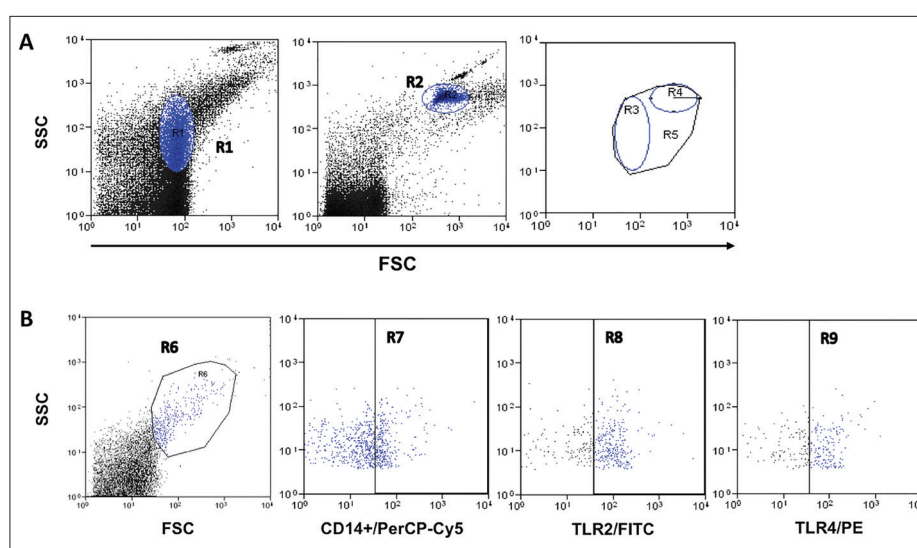


Fig. 1. Representative FACS dot plot of microparticles. **A** – Calibrator beads (blue dots) from 0.1 μ m and 1.0 μ m ('R1 and R2', respectively) are visualized in a forward scatter/side scatter logarithmic representation; 'R5' was selected as the MPs region. **B** – MPs are defined as events (size 0.1-1.0 μ m) gated in the 'R6' window of the cell supernatants (left panel). Representative FACS dot plot of CD14+ MPs are in the 'R7' window. 'R8' and 'R9' correspond to the CD14+/TLR2+ and CD14+/TLR4+ MPs, respectively.

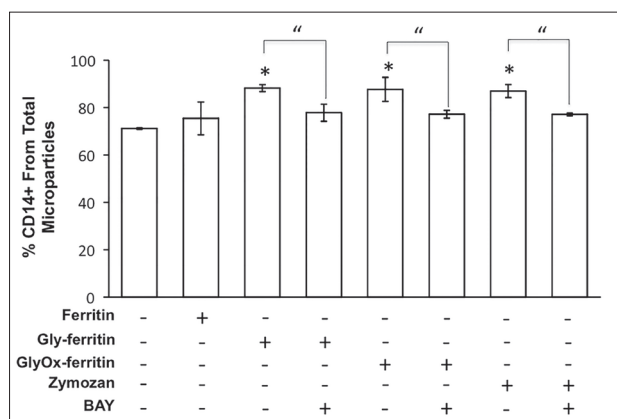


Fig. 2. CD14+ microparticles. The blood mononuclear cells were stimulated with 50 ng/mL of ferritin, Gly-ferritin, GlyOx-ferritin or zymosan A, and were pre-treated for 30 min with 15 μ M BAY to inhibit the NF- κ B pathway. The graph represents the mean \pm SD of three independent experiments and is expressed as the percentage of CD14+ MPs from the total MP value. (*) denotes the significant difference versus the basal control; the inverted commas (") denote significant difference between the stimulus and BAY pretreatment, with $P \leq 0.05$ by the Student's *t*-test.

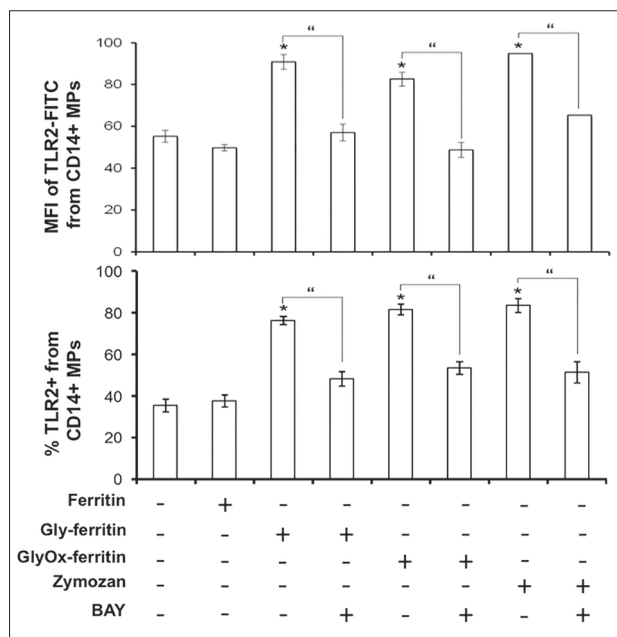


Fig. 3. TLR2 expression in CD14+ microparticles. The blood mononuclear cells were stimulated with 50 ng/mL of ferritin, Gly-ferritin, GlyOx-ferritin or zymosan A, and pre-treated for 30 min with 15 μ M BAY to inhibit the NF- κ B pathway. The graphs represent the mean \pm SD of three independent experiments and are expressed as the medium fluorescence intensity of TLR2+ in CD14+ MPs (upper panel), and as the percentage of TLR2+ MPs from CD14+ total MPs (lower panel). (*) denotes comparisons versus basal; inverted commas (") are comparisons between the stimulus and BAY pretreatments, with $P \leq 0.05$ by the Student's *t*-test.

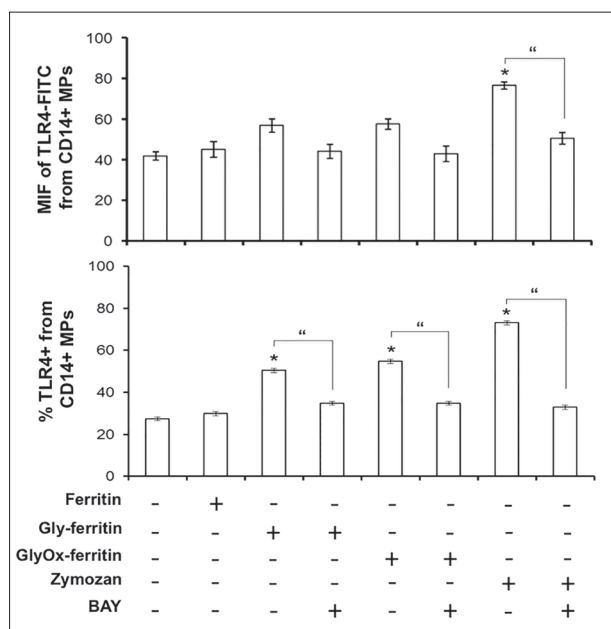


Fig. 4. TLR4 expression in CD14+ microparticles. The blood mononuclear cells were stimulated with 50 ng/mL of ferritin, Gly-ferritin, GlyOx-ferritin and/or zymosan A and pre-treated for 30 min with 15 μ M BAY NF- κ B inhibitor. The graph represents the mean \pm SD of three independent experiments and is expressed as the medium fluorescence intensity of TLR4+ in CD14+ MPs (upper panel), and as the percentage of TLR4+ MPs from the CD14+ total MPs (lower panel). (*) Denotes comparisons versus basal, and inverted commas (") are comparisons between the stimulus and BAY pretreatments, with $P \leq 0.05$ by the Student's *t*-test.

derived from CD14+ was significantly increased from the basal control when cells were stimulated with 50 ng/mL Gly-ferritin or 50 ng/mL GlyOx-ferritin. Also, the positive control for activation zymosan A produced a significant increase in the MPs from CD14+. The observed effect of non-modified ferritin was comparable to the baseline (Fig. 2).

Glycated and glycoxidated ferritin increases the expression of TLR2 and TLR4 in CD14+ microparticles

Stimulation of peripheral blood cells with Gly-ferritin and GlyOx-ferritin induced a significant increase in the percent expression of TLR2 on the surface of CD14+ microparticles (Fig. 3, lower panel). Intensity of the TLR2 expression was also estimated by the mean intensity fluorescence (MFI), which significantly increased (Fig. 3, upper panel). Stimulation of CD14+ with Gly-ferritin and GlyOx-ferritin produced

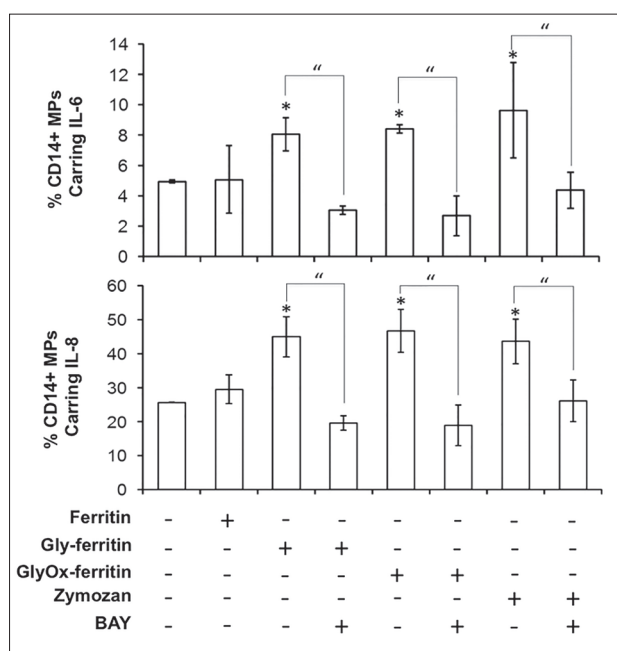


Fig. 5. Microparticles are CD14+ carrying IL-6 and IL-8. Blood mononuclear cells were stimulated with 50 ng/mL ferritin, Gly-ferritin, GlyOx-ferritin and/or zymosan A and were pre-treated for 30 min with 15 μ M BAY NF- κ B inhibitor. The graph represents the mean \pm S.D. of three independent experiments and is expressed as the percentage of MPs from CD14+ carrying IL-6 (upper panel) and IL-8 (lower panel). The asterisk (*) denotes comparisons versus basal; inverted commas (") are comparisons between the stimulus and BAY pretreatments, with $P \leq 0.05$ by the Student's *t*-test.

a discrete but significant rise in TLR4 expression on the surface of total CD14+ microparticles (Fig. 4, lower panel). However, no differences in MIF were observed upon stimulation with Gly-ferritin and GlyOx-Ferritin, and only the positive control zymosan A significantly increased the TLR4-FITC MIF on the CD14+ microparticles (Fig. 4, upper panel).

Role of NF- κ B in TLR2 and TLR4 expression in CD14+ microparticles

To explore the role of nuclear factor- κ B (NF- κ B) in the production of MPs, the CD14+ cells were first incubated with the NF- κ B inhibitor, Bay 11-7085 [14]. The BAY inhibitor abolished the release of MPs from CD14+ cells and the percentage of CD14+ cells was comparable to the basal control (Fig. 2). Pretreatment with BAY significantly reduced the percent expression and MIF of TLR2 in CD14+ MPs previously induced by Gly-ferritin and GlyOx-ferritin (Fig. 3). We also

observed that pretreatment with the BAY inhibitor affected the percent expression of TLR4 in CD14+ MPs stimulated with Gly-ferritin, GlyOx-ferritin and the zymosan A control (Fig. 4, lower panel). Furthermore, the MIF of TLR4 in CD14+ MPs was significantly induced by zymosan A (Fig. 4, upper panel).

Glycated and glycooxidated ferritin increases IL-6 and IL-8 expression in CD14+ MPs

To evaluate the activation of TLR signaling pathways, we measured the content of IL-6 and IL-8 in CD14+ MPs. The cells were pretreated with native ferritin, Gly-ferritin, GlyOx-ferritin or the positive control zymosan A. To determine the role of NF- κ B in the production of cytokines, the cells were pretreated with the NF- κ B inhibitor BAY after stimulation as described above [14]. Stimulation of peripheral blood cells with Gly-ferritin and GlyOx-ferritin induced a significant increase in the percentage of IL-6 and IL-8 in CD14+ MPs (Fig. 5, upper and lower panel, respectively). Also, the positive control zymosan A significantly increased the percentage of IL-6 and IL-8 in CD14+ MPs (Fig. 5, upper and lower panel, respectively). Pretreatment with BAY significantly reduced the expression of IL-6 and IL-8 in CD14+ MPs, previously induced by Gly-ferritin and GlyOx-ferritin (Fig. 5, upper and lower panel, respectively). We observed the same effect of pretreatment with BAY on the proportional expression of cytokines in CD14+ MPs stimulated with zymosan A (Fig. 5, $p < 0.05$).

DISCUSSION

Studies have shown that ferritin can act as an acute phase reactant; also, ferritin has been linked to certain metabolic diseases and their components [2, 3, 20]. The latter assumption has been derived only from statistical correlations, with the mechanisms through which ferritin participates in such disorders remaining unclear [3, 21]. On the other hand, it has been shown that ferritin can undergo oxidative damage by carbonyl compounds, such as methylglyoxal or ROS, which have been related to the increase in the iron content of cells that can be deleterious to cells [21, 22].

Advanced glycation end-products (AGEs) are a diverse group of highly oxidant compounds with pathogenic significance in diabetes as well as in several other chronic diseases. AGEs are created through a nonenzymatic reaction between reducing sugars and free amino groups of proteins, lipids or nucleic acids [7]. AGEs of low density lipoprotein are capable of activating an inflammatory-type response in different target cells, activating the MyD88-dependent signaling NF- κ B pathway when recognized by TLRs, primarily TLR2 and TLR4 [6, 23]. Recent evidence suggests that *in vitro* glycated ferritin is capable of activating TLR2 and TLR4 expression and inducing the release of the proinflammatory cytokines IL-6 and IL-8 in human peripheral blood macrophages [9].

AGEs may also stimulate the release of MPs from vascular endothelial cells [24]. Depending on their content, the MPs could represent markers for treatment and prognosis of chronic and inflammatory diseases [5]. This could be because the MPs are released from activated cells by outward blebbing and vesiculation of the plasma membrane following breakdown of the cytoskeleton of the parental cells. MPs carry cytoplasmic and/or nuclear components of the parental cell that can alter the activity of recipient cells through the transfer of their cargo [5, 25, 26]. For these reasons it is important to determine the expression of specific molecules on the surface and within the MPs.

In this study, we looked at the effects of glycated and glycoxidated ferritin on the percentage of MPs derived from monocytes and the expression of TLR2 and TLR4 on their surface. The internal IL-6 and IL-8 contents of MPs released from circulating CD14+ monocytes from healthy volunteers were also studied under the described experimental conditions. To this end, the protein was modified *in vitro* using the method described by Rivandi et al. [15]. We stimulated human peripheral blood monocytes (PBMCs) with glycated and glycoxidated ferritin for 2 h and an increase in the number of monocyte-derived MPs was observed, whereas stimulations with the native protein did not alter the number of MPs released from PBMCs. This suggests that monocytes respond to modified ferritin by secreting MPs. This response could be similar to that induced by ox-LDL in endothelial cells as shown in a previous report [24]. However, based on the design of the present study it has to be stated

that the influence of glycated ferritin on CD14+ cells might be not only direct but also indirect. Namely, as peripheral blood mononuclear cells, and not purified CD14+ cells, were used in the experiments there was the possibility that some of the observed effects were mediated through CD14- cells that were responsive to the influence of glycated ferritin.

We found that glycated and glycoxidated ferritin increased the number of MPs with TLR2 and TLR4 on their surface. The expression of TLR2 and TLR4 was decreased in the presence of the BAY inhibitor, suggesting that ferritin, modified by glycation and glycoxidation, activates the NF- κ B signaling pathway of monocytic cells. These data are consistent with previous reports, indicating that the release of MPs from macrophages is stimulated by activation of the NF- κ B signaling pathway with TLR ligands [27, 28].

MPs are considered an information vector for other target cells because they can transfer pro- and antiinflammatory cytokines and also transfer chemokine and cytokine receptors [29-31]. We ascertained the presence of IL-6 and IL-8 in monocyte-derived MPs and found that glycated and glycoxidated ferritin increases the number of MPs released by monocytes containing IL-6 and IL-8. Furthermore, this stimulation increases the concentration of IL-6 and IL-8 within these MPs in a manner that is dependent on the NF- κ B signaling pathway.

Our results showed that the MPs released by CD14+ monocytes after stimulation with glycated and glycoxidated ferritin express TLR2 and TLR4 on their surface and carry out inflammatory messages based on their IL-6 and IL-8 contents. In diverse metabolic diseases, increased levels of cytokines in MPs may be important because of their potential effects on target cells [25, 26, 30, 31].

CONCLUSIONS

In this study, the *in vitro* release of MPs from circulating CD14+ cells from healthy volunteers was induced by glycated and glycoxidated ferritin. The majority of MPs expressed TLR2 and TLR4 on their surface and contained IL-6 and IL-8. These effects were dependent on NF- κ B activation. These findings reveal the potential role for modified ferritin to stimulate

an inflammatory response that could contribute to the inflammation observed in diseases where ferritin levels and inflammation markers are increased, such as diabetes mellitus [1, 3, 4, 20]. A limitation of our *in vitro* system is the fact that we observed phenomena outside of the organism. However, with this approach, we now have better insight into the potential role of ferritin in inflammatory diseases. Further studies are necessary to confirm this hypothesis.

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Authors' contribution: López-Soto LF and Galván-Moroyoqui JM contributed equally to this work. López-Soto LF, Galván-Moroyoqui JM and Soto-Guzmán A provided the research direction and protocols and performed the experimental work, performed the statistical analysis and provided the research report. Martínez-Soto JM and Rosales-Ruiz AP performed the experimental work Almada-Balderrama M and Camacho Villa AY provided the protocols and performed the experimental work. Álvarez-Hernández G and Bolado Martínez E validated and analyzed the data. Candia-Plata MC designed the study, oversaw the study and is the guarantor of the manuscript.

Conflict of interest disclosure: The authors have no conflicts of interest, neither financial nor academic.

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