



ISSN: 2277- 7695

TPI 2015; 3(12): 44-48

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Received: 09-01-2015

Accepted: 12-02-2015

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## Glycated ferritin induces activation and expression of tlr2 and tlr4 in human peripheral blood macrophages

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### Abstract

Recently it has been proposed that serum ferritin might play a role in the pathogenesis of metabolic diseases, however so far unknown mechanism by which ferritin involved in these disorders. It has been reported that ferritin can be modified by glycation and peroxidation, through a process that occurs naturally but is increased in subjects with diabetes, called non-enzymatic glycation. This work was carried out modifying ferritin using an in vitro model of glycation process and used these modified protein by glycation as a stimulus in peripheral blood mononuclear cells (PBMN). We observed that ferritin glycated activates the expression of TLR2 and TLR4 in cells CD14+ derived from PBMN cells and further these PBMN cells increase the secretion of pro-inflammatory cytokines IL-6 and IL-8. Our results suggest that the activation of the TLR signaling pathway, is stimulated with glycated ferritin, increasing the production of pro-inflammatory cytokines in the supernatants of the PBMC, these data strongly suggesting that this modified protein acts as a potent inflammatory stimulus activating the CD14+ cells, it acting in a similar way as is done by advanced glycation endproducts, by activating TLRs and induction of cytokine secretion, which suggests that it could play a key role in the onset of chronic inflammation observed in patients with diabetes and which could contribute to the development of vascular complications in these patients.

**Keywords:** TLRs, glycation, ferritin, inflammation, macrophages.

### 1. Introduction

Diabetes mellitus type 2 (DM2) is a heterogeneous group of complex metabolic condition characterized by increased blood glucose that affects the action and/or insulin secretion, which cause dysfunction of multiple organs or tissues [1]. Complications of type 2 diabetes are often associated with macro and microvascular complications, mostly due to the accelerated atherogenesis, oxidative stress and the inflammatory state of these patients [1]. In DM2, the glycation of proteins and lipids produced by continuous hyperglycemic state, contributing to the formation of advanced glycation end products (AGEs), which are especially accumulate at sites of atherosclerotic lesions binding to RAGE receptors (receptor for AGEs) in endothelial cells, which interaction activates intracellular signaling pathways that mediate a pro-inflammatory effect on them through the activation of toll-like receptors [2, 3]. A modification by glycation of various proteins in DM2 patients is considered as inductors in the development of atherosclerosis and has been considered one of the main therapeutic targets [4-6]. Recently it has been reported in patients with DM2 elevated serum ferritin, which has suggested that alterations in the metabolism of iron could be part of the metabolic syndrome of DM2, which is associated with insulin resistance, hyperinsulinemia, hyperglycemia, dyslipidemia, hypertension and central obesity [7]. It has also been reported that hyper-ferritin correlates with the onset of vascular complications in patients with type 2 diabetes, such as diabetic retinopathy, diabetic nephropathy and vascular dysfunction [8-10], however, these reports only show a qualitative correlation between serum ferritin with complications of DM2 and classical markers of disease, such as hyperinsulinemia and hyperglycemia, whereas the biochemical mechanisms by which serum ferritin may contribute to T2DM have not been explored. Ferritin is the protein responsible for intracellular iron storage and detoxification [11-15]. Ferritin molecule is a heteropolymer of 24 subunits of two different types: L and H, with a molecular weight of 20 kDa each, formed by four helical chains. Variations in the content of subunits that make up the molecule determine the existence of different isoforms, which are divided

into 2 groups: isoferritins acidic (H-rich chains) located in the heart, red blood cells, and monocytes, and basic isoferritins predominant in the liver (rich in L chains), spleen, placenta and granulocytes [16]. Elevated serum ferritin concentration and the state of hyperglycemia could increase the rate of glycation and glycooxidation of ferritin due to the high probability of glucose-ferritin interaction in peripheral circulation. This modified ferritin could cause effects similar to those observed in glycated proteins induce other state hyperglycemia of T2DM, as such, this study was carried out in vitro glycation of ferritin, in order to use it as an AGE and determine whether it could trigger an inflammatory response type on CD14+ peripheral blood mononuclear cells (PBMC), through the measuring the expression of TLR2 and TLR4 and the production of IL-6 and IL-8 cytokines.

## 2. Materials and Methods

### 2.1 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 Bio Legend. Fixation Buffer (Cat. 420801), Ferritin Type I: from horse spleen (Cat. F4503), LPS (Lipopolysaccharides from *Salmonella enterica* serotype enteritidis; Cat. L7770), Zymosan A, from *Saccharomyces cerevisiae* (Cat. Z4250), D-(+)-Glucose (Cat. 5767) were obtaining from SIGMA-ALDRICH®. All the others reagents used were of the highest grade available.

### 2.2 Native ferritin modification by glycation.

1 mg of native ferritin was dissolved in 1 mL of 0.1 M of phosphate buffer solution (PBS) containing 1 mmol/L EDTA, 0.1 mg/mL chloramphenicol and 3 mmol/L NaN<sub>3</sub>. Then was incubated with 0.4 M glucose at 37 °C by 1 week under nitrogen atmosphere, after was reduced with NaBH<sub>4</sub> by 1 h at 4 °C and dialyzed against 0.1 mol/L PBS containing 0.1 mmol/L EDTA for 24 h to remove free glucose Ravandi A *et al.* [20]. Finally, glycated ferritin concentrations were measured with micro Bradford technique and the protein glycation process was evaluated by SDS/PAGE An SH *et al.* [17].

### 2.3 Analysis of ferritin modification

Samples were treated with 5 µl of 4X concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 10 min at 100 °C prior to electrophoresis. Each sample was subjected to SDS-PAGE as described by Laemmli [27], using an 18% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250 [17].

### 2.4 Cytometric análisis

Stimulated and non-stimulated cells were incubated with 20 µL of the mAb anti-CD14-PerCP/Cy5.5, anti-TLR2-FITC and anti-TLR4-PE for 30 min at room temperature in dark. Fluorescence-activated cell sorting (FACS) analysis was conducted, evaluating 20,000 events per assay by using a FACScalibur flow cytometer from Becton Dickinson and Summit Software Informer®.

### 2.5 Statistical analysis

All statistical analyses were performed using the SPSS 20 software (Armonk, NY). Data are presented as the mean ± standard deviation (SD), unless otherwise indicated. Student's

*t*-test was used to compare mean expression of basal control and the average expression produced by the stimulation of cells. For all analyses,  $P < 0.05$  regarded as significant.

## 3. Results

### 3.1 Electrophoretic mobility of glycated ferritin

Ferritin modification by glycation was analyzed by SDS-PAGE. Ferritin mobility in its native form was observed, which is separated in the heavy chain and light chain as expected (figure 1, line1) [17]. Ferritin aggregates was observed after glycation (figure 1, lane 2, arrowhead) and shows that the L-chain diminishes (figure 1, lane 2) while H-chain is not modified. These results suggested that glycation was done in L-chain of ferritin and conduces to aggregates formation of ferritin glycated.

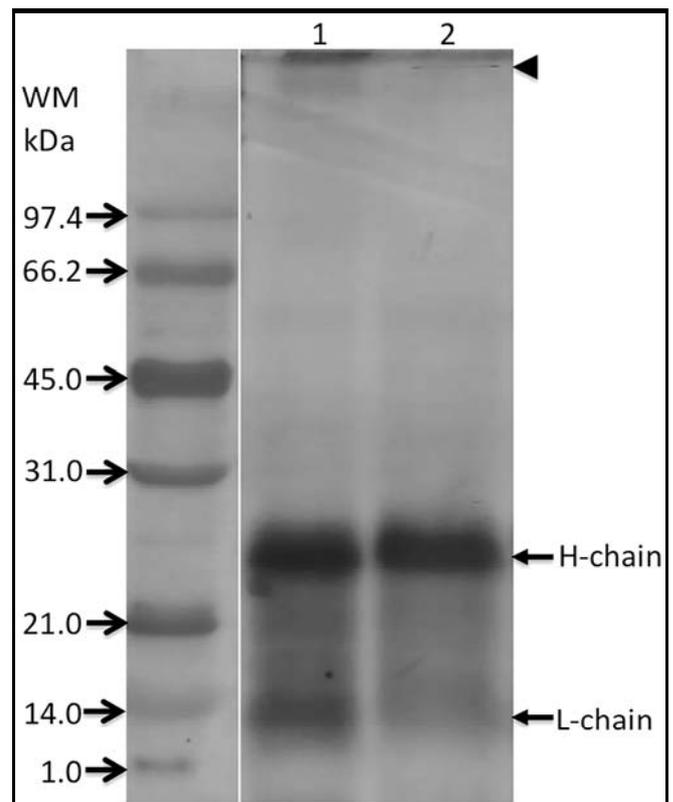
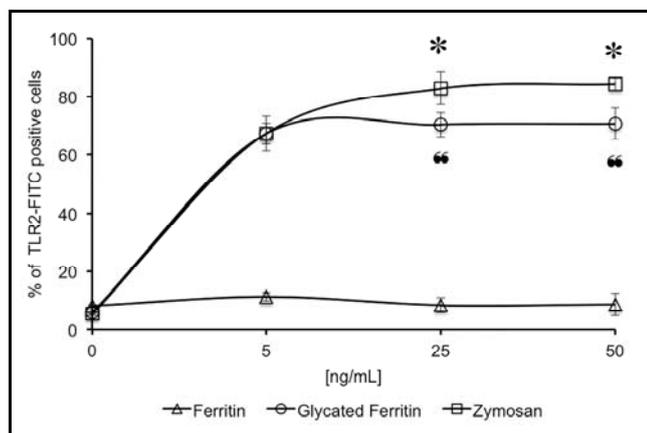


Fig. 1: Electrophoretic mobility of glycated ferritin.

### 3.2 Percentage of TLR2 expression in CD14+ cells exposed to glycated ferritin

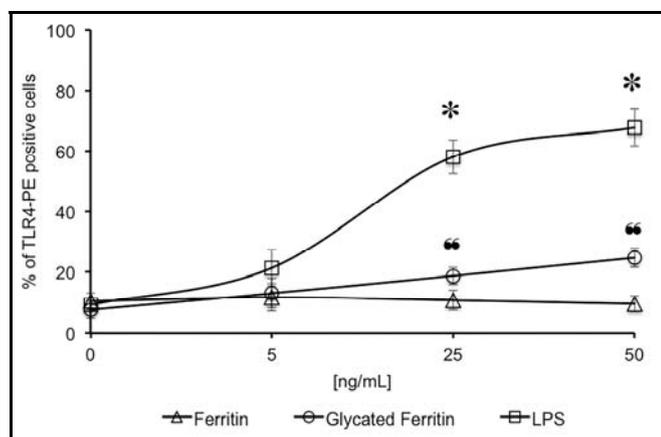
The effect of glycated ferritin on CD14+ cells was determined by flow cytometry. PBMC cells (Peripheral Blood Mononuclear) were incubated with glycated ferritin (5-50 ng/mL), for 2 h. As shown in Fig. 2 the stimuli of CD14+ cells with glycated ferritin resulted in a dose-dependent increase in the percentage of expression of TLR2 on cell surface (figure 2; symbol O,  $P \leq 0.05$ ), from the concentration of 25 and 50 ng/mL of glycated ferritin. To examine the effects of native ferritin on the expression of TLR2, the PMN cells were treated with ferritin without modification (5-50 ng/mL), for 2 h. We observed that ferritin not modify the expression of TLR2 in CD14+ cells (figure 2; symbol Δ,  $P > 0.05$ ). Zymosan was utilized as positive control for TLR2 stimulation [18]. As expected Zymosan induces a significantly increase of TLR2 on surface of CD14+ cells (figure 2; symbol □,  $* P \leq 0.05$ ) [18]. These results suggest that the modification of ferritin by glycation induces the activation of CD14+ cells.



**Fig. 2:** Percentage of TLR2 expression in CD14+ cells exposed to glycated ferritin

### 3.3 Percentage of TLR4 expression in CD14+ cells exposed to glycated ferritin

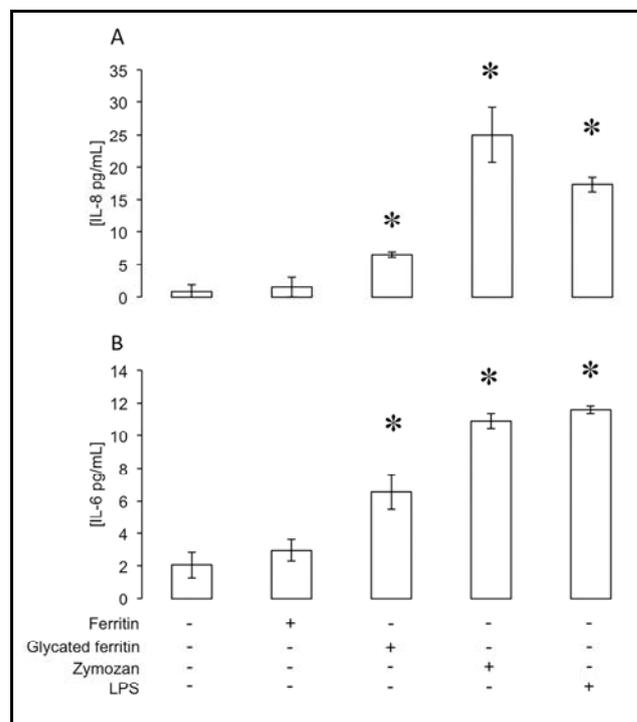
We also evaluate the expression of TLR4 in PBMC cells with the same experimental conditions utilized in figure 2. We observed that modification of ferritin by glycation increase significantly the percentage of TLR4 in CD14+ cells (figure 3; symbol O,  $P \leq 0.05$ ), after stimulation with 25 and 50 ng/mL of glycated ferritin. The negative control (native ferritin) had a similar effect to the previous treatment observed in figure 2. LPS (lipopolysaccharide) was used as positive control for stimulation of TLR4 and we observed that induces a significantly increase of TLR4 on surface of CD14+ cells (figure 3; symbol □,  $* P \leq 0.05$ ). Similarly at the previous result with TLR2 these results suggest that glycated ferritin induces the activation of CD14+ cells.



**Fig. 3:** Percentage of TLR4 expression in CD14+ cells exposed to glycated ferritin.

### 3.4 Effect of glycated ferritin on IL-8 and IL-6 production,

The effect of glycated ferritin on IL-8 and IL-6 secretion was measured by ELISA assay. Glycated ferritin significantly induced IL-6 and IL-8 secretion, after 2h of treatment of PBMC cells (figure 4A and 4B respectively,  $* P \leq 0.05$ ) with glycated ferritin at 50 ng/mL of concentration. IL-8 and IL-6 secretion after treatment with native ferritin was not affected at 50 ng/mL (figure 4a and 4B respectively). Positive controls significantly induced IL-6 and IL-8 production, after 2h of treatment of PBMC cells (figure 4A and 4B respectively,  $* P \leq 0.05$ ) with zymosan or LPS at 50 ng/mL of concentration. These results together with those shown in the expression of TLRs (figure 2 and 3) suggest that glycated ferritin could act as an inflammatory stimulus for PBMC cells.



**Fig. 4:** Effect of glycated ferritin on IL-8 and IL-6 production.

## 4. Discussion

It has been reported that ferritin is susceptible to peroxidation and glycation with  $H_2O_2$  and this methylglyoxal respectively, and this modifications have been linked to a process that happens naturally in healthy subjects and observed is exacerbated in diabetic patients, this process is the non-enzymatic glycation [17, 19]. In our study we used the glycation of ferritin by the method reported by Ravandi and colleagues [20]. We first investigated whether the structure of ferritin was affected by glycation. In the SDS-PAGE analysis a diminish intensity of the band that correspond to light chain of ferritin was observed (figure 1, line 2), also protein aggregation was observed (figure 1, line 2, arrowhead) whilst in its native form ferritin showed normal electrophoretic mobility as expected [21]. These results showed that ferritin glycation occur in similar way to previously reports, in which the formation of aggregates and a decrease in the intensity of the band corresponding to the light chain was observed in SDS-PAGE [17, 19]. Several studies have reported that glycated or oxidized LDL (Light Density Lipoprotein) and other modified endogenous proteins could stimulate the activation and signaling through toll-like receptors [22-25]. Considering this background, this study was conducted in vitro glycation of ferritin, in order to use it as an AGE and determine whether it could trigger an inflammatory response type on peripheral blood mononuclear cells (PBMC), through the measuring the expression of TLR2 and the TLR4 production of cytokines IL-6 and IL-8 [18, 22]. Glycated ferritin shows that acts as a damage-associated molecular pattern (DAMP) stimulating expression of TLR2 and TLR4 in the surface of the CD14+ cells [23-25]. These results suggest that glycation of this protein could activate the signaling pathway of these receptors to induce expression, similar to as reported so modified LDL is with this process [18, 26]. To establish whether glycated ferritin could be activated in PBMC signaling pathways dependent on NF- $\kappa$ B [22, 26], the concentration of pro-inflammatory cytokines was quantified in the supernatants of PBMC stimulated with glycated ferritin. Our results suggest that the activation of the

TLR signaling pathway, which ends with the NF- $\kappa$ B translocation to the nucleus<sup>[18]</sup>, is stimulated with glycated ferritin, increasing the production of pro-inflammatory cytokines in the supernatants of the PBMC, strongly suggesting that this modified protein acts as a potent inflammatory stimulus activating the CD14<sup>+</sup> cells, causing an inflammatory response, which could have an important role in the onset of atherosclerosis as has been reported is carried out with lipoproteins modified by glycation, which use a toll-like receptors to initiate the inflammatory process<sup>[22, 23]</sup>.

## 5. Acknowledgments

This project was supported in part by a CONACYT, México fellowship (#149110 to JM). We are indebted with Department of Medicina y Ciencias de la Salud, UNISON in part by supporting this project. We thank to CONACYT, for their support through a scholarship to Martínez-Soto JM (exp. 2072096145). We are grateful to M. in C. García Villa D, Soto C, for their support in sampling and handling of the cytometer respectively.

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