Macrophages are novel sites of expression and regulation of Retinol Binding Protein-4 (RBP4)

Short Title: Retinol binding protein-4 in macrophages

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SUMMARY

Obesity is linked to a low-level chronic inflammatory state that may contribute to the development of its metabolic associated complications. Retinol-binding protein 4 (RBP4) is an adipokine associated with parameters of obesity including insulin resistance indices, body mass index, waist circumference, lipid profile, and recently, with circulating inflammatory factors. Due to the infiltration of adipose tissue in obesity by macrophages derived from circulating monocytes and, on the other hand, the existence of a close genetic relationship between adipocytes and macrophages, we decided to examine if RBP4 is expressed in monocytes and/or in primary human macrophages. While we did not detect expression of RBP4 in undifferentiated monocytes, RBP4 expression became evident during the differentiation process of monocytes into macrophages and was highest in differentiated macrophages. Once we demonstrated the expression of RBP4 in macrophages we checked if RBP4 expression could be regulated by inflammatory stimuli such as tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), or the endotoxine Lipopolisacharide (LPS). We observed that while RBP4 expression was strongly inhibited by TNF-α and endotoxin LPS it was not affected by IL-6. Our results highlight the complexity behind the regulation of this adipokine and demonstrate that RBP4 expression in macrophages could be modulated by inflammatory stimuli.

Keywords: RBP4, primary human macrophages, inflammatory markers, obesity

Obesity induces an inflammatory state that is implicated in many of its metabolic complications, including insulin resistance, type 2 diabetes and atherosclerosis. The monocyte/macrophage system, which plays a prominent role on the onset of the atherosclerotic lesions, has recently been linked to obesity in humans, where circulating monocytes infiltrate adipose tissue (AT) and differentiate into macrophages (Weisberg *et al.* 2003). Monocytes could also be associated with global insulin resistance, in a mice model in which the deletion in monocytes/macrophages cells of Ikβ Kinase (IKK-β), a central coordinator of inflammatory responses through activation of NF-κβ, hampers the development of systemic insulin resistance (Arkan *et al*. 2005).

Retinol binding protein-4 (RBP4) is an adipokine associated with obesity and a potential candidate to link glucose uptake in adipocytes to systemic insulin resistance (Yang *et al.* 2005). Moreover, elevated circulating RBP4 levels have also been recently associated with circulating inflammatory factors as C reactive protein (CRP) and IL-6 in obese children (Balagopal *et al.* 2007). Since adipocytes and macrophages seem genetically close, both cell types express similar sets of genes including numerous cytokines (Lee and Evans 2002, Makowski *et al.* 2001, Endemann *et al.* 1993), we decided to explore if RBP4 is also expressed in human monocytes/macrophages, and if so, whether it could be regulated by inflammatory stimuli.

Monocytes were isolated from peripheral blood mononuclears cells of healthy men (n=6, age: 39.4±4.8 years, BMI: 30.8±3.8 kg/m^2) with the RosetteSep Human Monocyte Enrichment Cocktail Kit (StemCell Technologies, Vancouver, Canada.) according to the manufacturer's directions. Monocytes at a density of 3x10^6 cells/well, in 3ml of RPMI-1640 media, were differentiated into macrophages with 10ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF) (Sigma, St. Louis, Missouri, U.S.A.) for five days. Expression of CD68, a macrophage-specific gene used as a control of differentiation (Holmess and Simmons 1993), was strongly increased by day 5 compared to day 1 (2.09x10^4±1.9x10^4 vs. 6.84x10^{-1}±1.82x10^{-1}; p=0.034). At day 5 of differentiation (Fig. 1A) the cell culture medium was replaced and macrophages were incubated with TNF-α 50ng/ml, IL-6 50ng/ml, or endotoxin Lipopolysaccharide (LPS) 1µg/ml, from *Escherichia coli* 026:B26 (Sigma) for 48-hours. As a positive control for adipokine production in monocytes/macrophages, we quantified mRNA expression and protein secretion of resistin (Patel *et al.* 2003). Total-RNA was isolated from

cells with RNeasy® Mini Kit, (QIAGEN Science, Maryland, U.S.A). Reverse transcription was performed with one μg of total RNA with Reverse Transcription System Kit (Promega Corp., Madison, U.S.A.). Real-Time quantitative PCR for RBP4, Resistin, and CD68 was performed using Lightcycler technology with LightCycler® FastStart DNA master SYBR Green I Kit (Roche Diagnostics, Basel, Switzerland). To avoid detection of non-specific PCR products, the purity of each amplified product was confirmed by agarose gel analysis and by melting curve analysis. RBP4 protein was measured in the cell culture medium using Human RBP4 ELISA Kit (AdipoGen, Seoul, Korea) with a limit detection of 1ng/ml and a cross reactivity with human RBP4 of 100%, and without cross reactivity with human resistin. Resistin was measured using Human Resistin ELISA Kit (BioVendor Laboratory Medicine, Inc., Czech Republic) with a limit detection of 0.2 ng/ml. The statistical analysis was performed with the SPSS/PC+ (v.15 for Windows) and nonparametric tests for related samples. The level of significance was $p \leq 0.05$. The Hospital's ethics committee approved the study and informed consent was obtained from each subject.

The expression of RBP4, at mRNA and protein levels, which was not detectable at the start of the differentiation process (monocytes), appeared at day 4 and was highest at day 5 (macrophages) (Fig. 1B). As we expected, the expression at mRNA and protein levels of resistin was evident in monocytes and was highest in macrophages (Fig. 1C). When macrophages (day 5), were incubated with inflammatory stimuli, RBP4 expression and protein levels were significantly down-regulated by TNF-α and LPS, but remained unchanged in the incubation with IL-6 (Fig 2A). Also as we expected, resistin was up-regulated by all the stimuli, with LPS having the maximum effect (Fig. 2B) (Lehrke *et al.* 2007).

In this communication we report for first time the expression of RBP4 in human macrophages and its regulation by some inflammatory stimuli. This finding of RBP4 adipokine expression in human macrophages adds to the growing number of similarities between macrophages and adipocytes regarding the expression of cytokines and adipokines. What could be the clinical significance of the expression of RBP4 in macrophages? In general, due to the various functions that macrophages perform in different tissues and in the formation of the atherosclerotic wall during the onset of the atherosclerotic lesions (Linton and Fazio 2003), alterations in the expression and release of adipokines

such as RBP4 by macrophages could be of biological importance. Thus, it has been suggested that expression of resistin in monocytes-derived macrophages may contribute to acceleration of atherogenesis promoting lipid accumulation in human macrophages (Weibin *et al.* 2006). Since macrophages are a minor fraction of the cells in a tissue, the effects of these alterations will probably be mediated through local signalling. In this sense, paracrine signalling by macrophages has been pointed out as the most likely mechanism behind the hampering in the development of systemic insulin resistance in mice (Arkan *et al.* 2005).

In particular, taking in consideration that in obesity macrophages could account for 40-50% of the total cells in AT (Weisberg *et al.* 2003) and a significant positive relationship between mRNA RBP4 expression in AT and macrophages infiltration has been found by Yao-Borengasser et al. (Yao-Borengasser *et al.* 2007), local alterations in RBP4 release by macrophages in AT could have a significant biological effect in this tissue.

In addition, we found that the RBP4 expression in macrophages was regulated by inflammatory stimuli. The expression of resistin in macrophages was up-regulated by all pro-inflammatory agents, in agreement with the increasing implication of this adipokine in inflammation and inflammation-related diseases (Shanshan and Yingying 2006). RBP4 expression was strongly down-regulated by incubation of macrophages with TNF-α and LPS, whereas incubation with pro-inflammatory cytokine IL-6 had no effect (Fig. 2A). What could be the mechanism behind this differential regulation? Although TNF-α and LPS bind to different receptors in the cell surface, TNFR1/R2 and toll-like receptors (TLR), respectively, they trigger overlapping signal transduction pathways that activate similar transcription factors including activator protein-1 (AP-1) and nuclear factor kB (NF-*k*B) (Tesz *et al.* 2007). However, IL-6 activates different intracellular signalling pathways such as the Janus kinase (JAK) that activates transcription factors of the signal transducer and activator of transcription (STAT) pathways (Tesz *et al.* 2007). In agreement with our observation, TNF-α and LPS (but not IL-6) have been reported to be implicated in decreasing peroxisome proliferator-activated receptor gamma (PPARγ) levels in adipocytes (Mrácek *et al.* 2004, Zhang *et al.*1996). Interestingly, PPARγ is a master regulator of adipogenesis and is induced during the differentiation of monocytes into macrophages at the same late stage in which we see induction of RBP4 expression (Vosper *et al.*

2001). On the other hand, TNF-α down-regulates RBP4 and PPARγ in adipocytes, where treatment with PPARγ ligand troglitazone increases both RBP4 and PPARγ (Sell and Eckel 2007). The above data plus our observation of down-regulation of RBP4 by inflammatory stimuli and the high similarity in gene expression between adipocytes and macrophages could indicate also a positive link between RBP4 and PPARγ in macrophages.

Interestingly, similar to our results in macrophages, TNF-α strongly down-regulates production of RBP4 in primary human adipocytes (Sell and Eckel 2007). On the other hand, in comparison with lean controls, lower levels of mRNA RBP4 expression in AT were found in obese women (Janke *et al*. 2006). These findings suggest that RBP4 expression, in adipocytes and macrophages, may be down-regulated in states associated with elevated levels of pro-inflammatory mediators such as in obesity. These above observations appear to be contrary to the potential role that has been suggested for RBP4 as adipokine up-regulated in obesity. However the link between this adipokine and human obesity is not fully understood. Klöting N et al. found that elevated systemic RBP4 levels correlated positively with adipose mRNA RBP4 and inversely with insulin sensitivity in obese subjects (Klöting *et al*. 2007). Moreover, subjects treated with agents that enhance insulin sensitivity have decreased systemic RBP4 levels (Hammarstedt *et al.* 2008, Lin *et al.* 2008). However RBP4 systemic levels do not always correlate with RBP4 expression in adipose tissue (Yao-Borengasser *et at.* 2007, Janke *et al.* 2006). Furthermore, although it is generally accepted that TNF-α induces insulin resistance, it down-regulates the RBP4 expression in isolated adipocytes (Sell and Eckel 2007) and in macrophages (Figure 2A). In addition, agents that enhance insulin sensitivity, increased RBP4 expression in AT and in isolated adipocytes (Yao-Borengasser *et at.* 2007, Sell and Eckel 2007). It has been suggested that other tissues may contribute to systemic RBP4 levels in humans (Yao-Borengasser *et al.* 2007), and probably other factors are implicated in alter the RBP4 production.

In summary, we report for first time the appearance of RBP4 during differentiation of human monocytes into primary macrophages. This observation may have clinical significance due to the importance of tissue macrophages derived from circulating monocytes in inflammatory related pathologies such as, atherosclerosis and obesity. Moreover, regulation of this adipokine by TNF-α and

LPS provide evidence that RBP4 may be involved in inflammatory responses and our results highlights the complexity behind the regulation of this adipokine.

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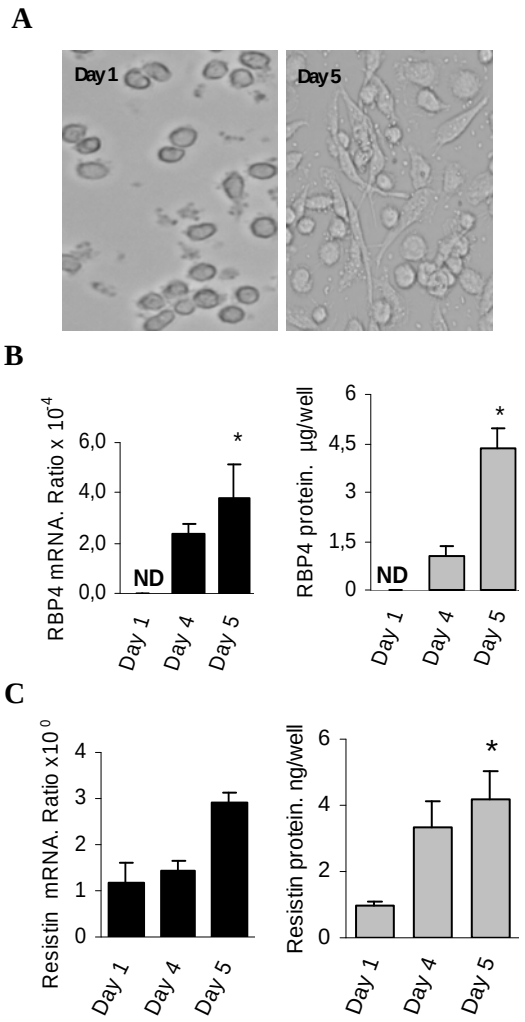
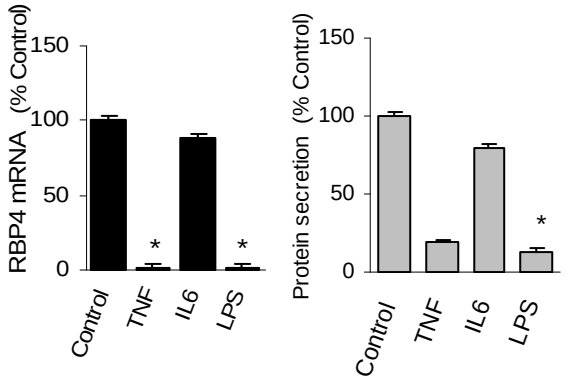
Figure 1



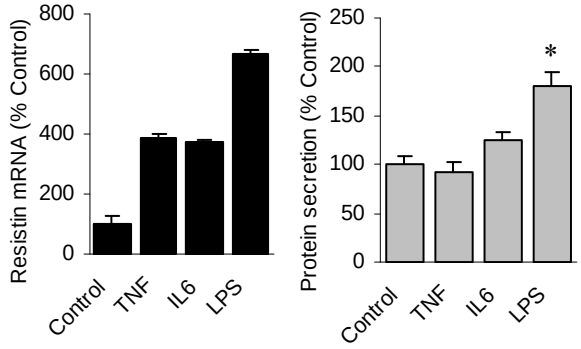
RBP4 and Resistin expression during differentiation of human circulating monocytes into macrophages. **(A)** Cell morphology at day 1 after plating and at day 5 of differentiation (X 400). **(B)** RBP4 and **(C)** Resistin mRNA expression and protein secretion during differentiation. Ratio expresses mRNA gene/mRNA β-actin gene. Values are means ± SEM. ND: not detected. *Indicates statistically significant difference ($p \leq 0.05$) by Friedman *U* test.

Figure 2

A



B



(A) RBP4 and **(B)** Resistin production in macrophages treated with TNF-α, IL-6, and LPS. Results are expressed as the % of the values of untreated macrophages (control). *Indicates statistically significant difference compared with control ($p \leq 0.05$) by Wilcoxon signed rank sum test.