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IJPBS |Volume 3| Issue 2 |APR-JUN |2013|627-632

Research Article
Biological Sciences

STUDY OF THE ANTI-INFLAMMATORY EFFECTS OF RECOMBINANT HUMAN GALECTIN-3 IN THE CONTEXT OF METABOLIC PATHOLOGIES AND CHRONIC INFLAMMATION IN OBESITY

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ABSTRACT

Obesity and its associated metabolic pathologies are the most common metabolic diseases. A key emerging feature of obesity is the relationship between obesity and chronic inflammation, with increased cytokine production and acute-phase inflammatory signaling in the adipose tissue. For this reason, adipose tissue is no longer considered an inert depot of fat cells capable of storing energy, but also an active endocrine organ capable of secreting a diverse array of pro-inflammatory cytokines, all of which are influential in insulin resistance. In this work, we have examined the role of recombinant galectin-3 during LPS-induced activation of macrophages and adipocytes, in the context of chronic inflammation observed in adipose tissue and infiltration of macrophages. The recombinant galectin-3 was used for studies on the anti-inflammatory effects in macrophages and adipocytes. The data presented in this paper clearly reveal that galectin-3 treatment does not modify the basal expression of cytokines, but attenuates the LPS-primed cytokine production in both cell systems. Therefore, galectin-3 plays a critical role in a variety of inflammatory disorders.

KEY WORDS

Human galectin-3, LPS, inflammation, obesity, macrophages, adipocytes

INTRODUCTION

Galectins are animal proteins characterized by conserved carbohydrate-recognition domains which bind β-galactoside glycans (Liu FT *et al.,* 2012). Production of several members of the galectin family, including galectin-1, galectin-3, galectin-9 and galectin-12, is altered in obesity and diabetes in both animals and humans (Kurose Y *et al.,* 2013). In adipose tissue, Gal-3 is expressed by both adipocytes and infiltrating macrophages (Rhodes DH *et al.,* 2013). A functional role for members of the galectin family in modulating obesity and its associated inflammation *(Wellen KE et al., 2003)* and metabolic dysregulation has been demonstrated using knockout mouse models. Studies indicate that galectin-3 knockout mice develop more severe pathology in a variety of models of metabolic disease, including atherosclerosis and kidney damage, suggesting an important protective effect of galectin-3 in response to nutrient overload and dysregulated metabolism (Iacobini C *et al.,* 2009). A clinical study in human volunteers further demonstrated that LPS increases TNF-α and IL-6 levels in adipose tissue causing insulin resistance (Starr ME *et al.,* 2009). Galectin-3 has been widely studied for its involvement in inflammatory responses (Norling LV *et al.,* 2009). Adipose tissue not only secretes proinflammatory molecules, but it also responds to proinflammtory signals by expressing a diverse array of cytokines, chemokines, complement factors and growth factors (Schäffler A *et al.,* 2006)LPS represents a major pathogen-associated molecular pattern (PAMP) on the outer membrane of

Gram-negative bacteria, consisting of hydrophobic lipid A, the 0-polysaccharide chain, and a core oligosaccharide. The signaling cascade involves activation of Mitogen Activated Protein Kinase (MAPK), Extracellular signal-regulated kinases (ERK1/2), p38, and c-Jun amino-terminal kinases (JNK), which further turn on the expression of many inflammatory genes including NADPH oxidase and inducible NOS (iNOS; - Takeda K *et al.,* 2003) producing bactericidal ROS, superoxide, hydrogen peroxide and nitric oxide, respectively. It has been reported that TLR4 is expressed not only on macrophages but also on adipocytes (Lin Y *et al.,* 2000). As it can be surmised by the above discussion, given the extreme diversity of activities exerted by galectins, the potential impact of modulating this family of mediators in obesity and its comorbidities needs to be carefully assessed using specific pharmacological tools.

MATERIALS & METHODS

3T3-L1 adipocyte culture

3T3-L1 preadipocytes were seeded in 24-well plates and cultured according to standard conditions. Briefly, cells were grown in 5% CO_2 in medium containing 10% fetal calf serum in the presence of 1% penicillin-streptomycin mixture. Two days after reaching confluence (day 0), cells were induced to differentiate with a medium containing 10% fetal bovine serum (GIBCO), 1.7 µM insulin, 1 µM dexamethasone, and 0.5 mM IBMX for 48 h. Thereafter, fresh medium containing only insulin was added every 2 days for another 6 days. On day 8, cells were fully differentiated and medium was changed to insulin-free medium containing 10% fetal bovine serum. The medium was then replaced again with fresh DMEM supplemented with 10% (v/v) FBS every other day for the following 10–12 days.

Treatments

On the 10^{th} day after differentiation, the cells were pretreated for 24 h with 0.5% FBS containing medium. Then, the cells were treated with recombinant Gal-3 (20 µg/ml) for 24 h in the presence of LPS (1 µg/ml) and harvested 24 h later. In the control plates, the medium was changed but no treatment was performed. The culture medium and adipocytes were collected in tubes and stored at -80°C, for further analysis.

RAW 264.7 cells

RAW 264.7 cells (American Tissue Culture Center TIB 71) were propagated in T-75 culture flasks (Costar, Cambridge, MA) in Dulbecco's minimal essential medium supplemented with 25 mM glucose (DMEM, high glucose), 10% FCS and gentamicin. Before use, cells were detached at 37°C with trypsin-EDTA (GIBCO, Grand Island, NY) and plated in 96-well microtiter plates at a density of 200,000 cells/200-µl well or in 35-mm petri dishes at a density of 10^7cells/ml in 1 ml. Medium was changed every 24 h, and cells were used after 48–72 h of incubation.

Coculture of adipocytes and macrophages

Mature adipocytes and RAW264.7 were co-cultured in a transwell system (Corning Inc., Acton, MA, USA) with a 0.4-mm porous membrane to separate the upper and lower chambers. Then, $1x10^5$ differentiated 3T3-L1 cells were cultured in the lower chamber, whereas $5x10^4$ RAW cells were cultured in the upper chamber (Yamashita A *et al.,* 2007). The cells were treated with 1 µg/ml of *Escherichia coli* LPS (Sigma, St. Louis, MO, USA) and/or 20 µg/ml recombinant galectin-3 to compare the pro- and anti-inflammatory cytokine expression and alteration of signaling pathways.

Determination of IL-6, TNF-α and IL-8 levels in the adipocyte culture medium and in the macrophage conditioned medium by ELISA

Cell supernatants were collected following indicated treatments. For detection of IL-6 levels in differentiated 3T3-L1 cells, cell supernatant was collected and concentrated using YM3 centricon tubes from Millipore. For all other ELISAs, cell supernatant removed directly from cells (NOT concentrated via YM3 centricon tubes) was used to determine IL-6 levels *via* ELISA. The latter was not concentrated since inducible levels of IL-6 are much higher than basal levels. ELISA was performed according to the manufacturer's protocols. All samples were run in triplicate, and the mean value was used for analysis. The protein concentration of

3T3-L1 adipocytes was determined by the Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as a standard. The results are expressed in pg or ng/ml.

RESULTS

Release of IL-6, TNF-α and IL-8 into adipocyte conditioned medium in response to LPS and Gal-3

Adipocytes are known to produce cytokines that limit insulin signaling. Most of the cytokines are produced at low basal levels in unstimulated adipocytes. LPS acting through TLR4 stimulates the enhanced synthesis and release of the adipocytokines. We therefore evaluated the basal expression of adipocytokines and tested the effects of Gal-3 in LPS-induced adipocytokine production. Figure 17 shows that the IL-6 levels in the adipocyte culture medium after 24 h were almost 8-fold higher upon LPS challenge than in the control group ($P < 0.05$). Interestingly, the IL-6 concentrations were lower in the LPS+Gal-3 group compared with LPS ($P< 0.05$).

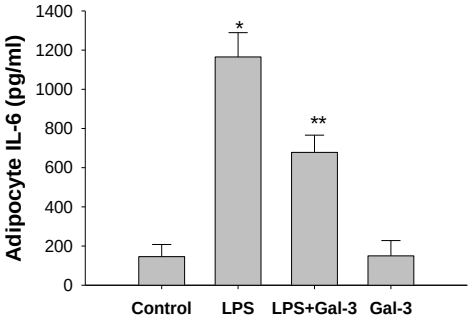


Figure IL-6 release in the culture medium of 3T3-L1 adipocytes treated with LPS (1µg/ml), LPS (1µg/ml) + Gal-3 (20µg/ml) and Gal-3 (20µg/ml). n = 6 for all groups. Values are means ± SE. *p < 0.05 in relation to control, **p < 0.05 in relation to LPS.

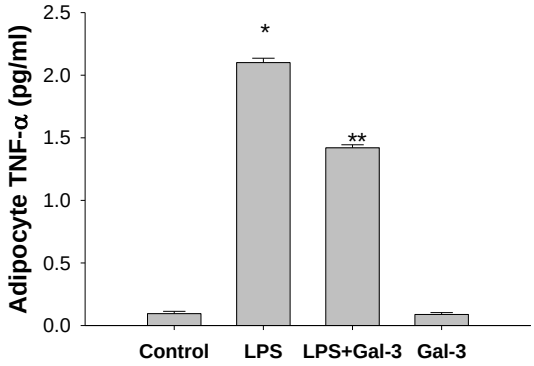


Figure TNF-α release in the culture medium of 3T3-L1 adipocytes treated with LPS (1µg/ml), LPS (1µg/ml) + Gal-3 (20µg/ml) and Gal-3(20µg/ml). n = 6 for all groups. Values are means ± SE. *p < 0.05 in relation to control, **p < 0.05 in relation to LPS.

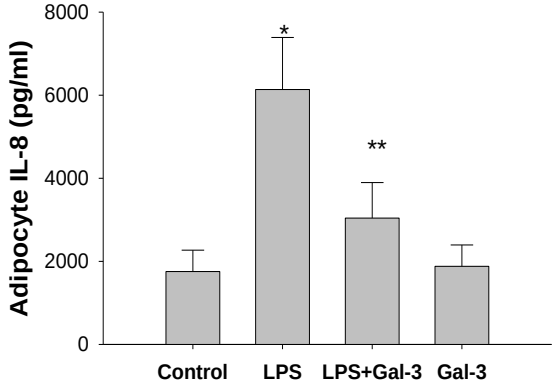
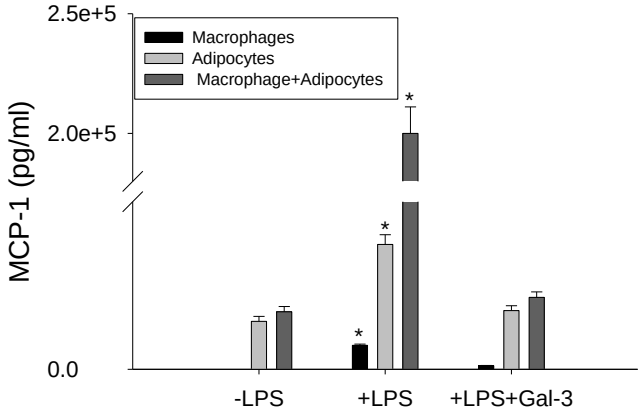


Figure IL-8 release in the culture medium of 3T3-L1 adipocytes treated with LPS (1µg/ml), LPS (1µg/ml) + Gal-3 (20µg/ml) and Gal-3(20µg/ml). n = 6 for all groups. Values are means ± SE. *p < 0.05 in relation to control, **p < 0.05 in relation to LPS.

Cytokine secretion in 3T3-L1 adipocyte-RAW264.7 macrophage co-culture are enhanced upon LPS stimulation, but alleviated by galectin-3 treatment

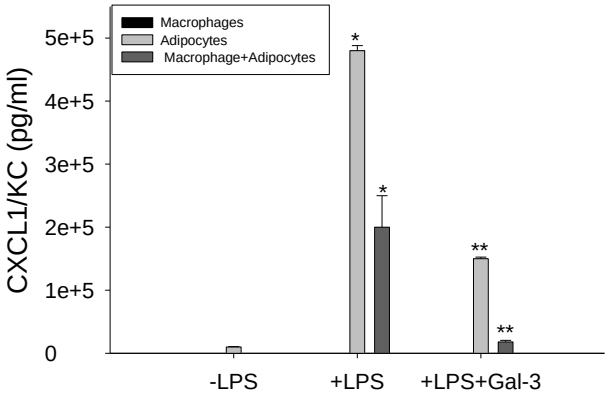
To define the role of galectin-3 as a pathogen pattern recognition receptor and the immunological significance related to the interplay between galectin-3 and LPS in a mixed culture system consisting of adipocytes and macrophages, we tested the effects of 1 µg/ml *E.coli* LPS on the production of cytokines viz. a viz. MCP-1 (**Figure**) and CXCL1/KC (**Figure**). As expected, protein expressions of all these molecules were markedly upregulated in adipocyte–macrophage co-cultures stimulated with endotoxin as compared with co-cultures without endotoxin stimulation or with each cell type alone (P≤0.05), except for CXCL1/KC, where adipocyte-macrophage co-culture showed lower concentration compared to adipocytes alone.

Effect of Gal-3 on LPS-stimulated MCP-1 production in cocultured macrophages and adipocytes compared to isolated cultures



MCP-1 expression analysis in 3T3-L1 adipocytes co-cultured with RAW264.7 macrophages with or without LPS stimulation and also tested the effect of recombinant galectin-3 in alleviating the inflammatory cytokine production in response to LPS.

Effect of Gal-3 on LPS-stimulated CXCL1/KC production in cocultured macrophages and adipocytes compared to isolated cultures



CXCL1/KC production from macrophage culture, adipocyte culture and adipocyte–macrophage co-cultures/mixed cultures stimulated with LPS and exposed to Gal-3.

Notably, the major source of all these molecules appeared to be 3T3-L1 adipocytes, as adipocytes alone produced detectable amounts of these molecules when stimulated with endotoxin, whereas

RAW macrophages produced trace amounts of these proteins regardless of the presence or absence of endotoxin. Baseline productions of all these cytokines were very low in each culture. When the cells were stimulated with LPS, all of these cytokines were mainly produced by adipocytes. However, production was markedly upregulated when co-cultured cells were stimulated with LPS. Similar to our earlier observations with isolated cultures of adipocytes and macrophages, Gal-3 treatment led to a drop in the cytokine levels in the co-culture system upon LPS stimulation.

CONCLUSION

Obesity is associated with a state of chronic and low-grade inflammation. Various studies have provided clear evidence that obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that there is cross talk between macrophages and adipocytes. It is therefore important to elucidate the changes in proinflammatory signals during cross talk and to evaluate endogenous natural compounds that can lower the proinflammatory signals and understand the signaling mechanisms associated with the changes.

In this direction, using isolated cultures of adipocytes, macrophages and cocultures, we present the first comprehensive description of changes in LPS-induced inflammatory cytokine production and consequent anti-inflammatory effects of Gal-3 in 3T3-L1 adipocytes, macrophages and cocultures stimulated with LPS. The adipocytes treated with LPS showed enormous response in production of inflammatory cytokines that included IL-6, TNF-α, IL-8 and IL-10, which was prevented to a significant extent by 20 µM Gal-3. Taken together, Gal-3 reduces LPS-induced IL-6 and MCP-1 release in adipocytes. The relevance of Erk-1/-2 signaling and JNK-signaling in adipose tissue physiology has already been demonstrated in the regulation of insulin sensitivity and lipolysis (Zu L *et al.,* 2009). Stimulation of 3T3-L1 adipocytes by LPS induces adipocytic insulin resistance *via* involvement of JNK (Davis JE *et al.,* 2009). Moreover, LPS induces lipolysis in adipocytes *via* TLR4 and Erk-1/-2 signaling (Zu L, He J *et al.,* 2009). Regarding TLR4, it was demonstrated that stimulation of adipocytes by LPS attenuates insulin signaling by decreasing phosphorylation of AKT (Song MJ *et al.,* 2006).

It is well known that pro-inflammatory cytokines induced by LPS, such as TNF-α, IL-6 and IL-1β play a key role in the process of inflammatory diseases. The pharmacological inhibition of these inflammatory mediators is an important target in the treatment of metabolic pathologies associated chronic inflammation in obesity. In this study, our data revealed that Gal-3 inhibited TNF-α, IL-6 and IL-1β production in a dose-dependent manner in LPS-stimulated RAW 264.7 cells.

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