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Action of Simvastatin on the Attenuation of Inflammation in 3T3-L1 Adipocytes is related to PPARs Modulation

Mariana Kuniyoshi[1], Renata Nakamichi[1], Silmara de Melo Carmona[1], Maria Aparecida da Glória[1], Maria Aparecida Dalboni[2], Beata Marie Redublo Quinto[1*] and Marcelo Costa Batista[1,3]

[1]Department of Medicine/Discipline of Nephrology, Federal University of Sao Paulo, Sao Paulo, Brazil

[2]Postgraduate Medicine Department, Ninth of July University, Sao Paulo, Brazil

[3]Department of Medicine of Israeli Hospital Albert Einstein, Sao Paulo, Brazil

[*]**Corresponding author:** Quinto BMR, Department of Medicine/Discipline of Nephrology, Federal University of Sao Paulo, Sao Paulo, Brazil, E-mail: marie.quinto@unifesp.br

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Abstract

Visceral obesity is the main foundation for the development of Metabolic Syndrome (MS), representing the pathophysiological basis related to the epidemic burden of cardiovascular disease. The increase in the secretion of biologically active molecules such as leptin, a pro-inflammatory adipokine, results in a significant impact on the progression of the MS. Based on its pleiotropic and anti-atherogenic actions, the use of statins targeting inflammation has been advocated as a strategy for reducing atherosclerotic vascular disease. This study aimed to investigate the *in vitro* effect of simvastatin on leptin secretion and expression in mature 3T3-L1 adipocytes after stimulation with TNF-α. Besides, we analyzed the potential effects of simvastatin on the modulation of receptors activated by peroxisome proliferators-alpha (PPAR-α) and gamma (PPAR-γ) in such conditions.

Our results consolidate the concept of MS as an inflammatory disease caused by increased production of mediators of pro-inflammatory proteins, specifical leptin in adipocitary cell models. Treatment with simvastatin reversed the impact of the inflammatory process induced by TNF-α administration in adipocytes. We have also demonstrated that there was a reduced expression of PPAR-α and PPAR-γ receptors when submitted to the inflammatory stimulus. The treatment of those cells with simvastatin reversed the attenuation in the expression of these receptors in cells exposed to TNF-α.

We reiterate, at the cellular level, the integral inflammatory state of obesity-related MS and the association between increased syntheses of leptin in this condition. We suggested the beneficial effect of simvastatin in the attenuation of the inflammatory response associated with increased TNF-α and a possible relationship of PPARs in the modulation of this response.

Keywords: Obesity; Inflammation; Statins; Adipokines; PPAR receptors

Introduction

Cardiovascular diseases are the leading cause of death in the world, resulting in more than 17 million deaths annually, according to the World Health Organization (WHO) [1]. Chronic inflammation characterized by overproduction of adipokines in the hallmark of obesity and has been described as a risk factor associated with Cardiovascular Disease (CVD) occurrence [2,3].

Leptin, a protein secreted by adipocytes, acts on the central nervous system promoting lower food intake and modulates different actions on the inflammatory pathway, mainly through the NFκB system (factor Nuclear kappa B) [4,5]. Increased leptin levels, as observed in obesity, is associated with increased Plasminogen Activator Inhibitor-1 (PAI-1), a specific inhibitor of tissue Plasminogen Activator (tPA), resulting in an increased risk of thrombosis. These abnormalities are possible links between increased adipose mass, pro-inflammatory, and prothrombotic state, thus acting in the promotion of CVD [6-8].

The peroxisome proliferator-activated receptor is a transcription factor belonging to the nuclear receptor family that binds to specific agonists, also known as ligands or activators of PPARs, which have been involved in a wide variety of functions, including homeostasis of lipids and inflammatory responses and are activated in response to polyunsaturated fatty acids, arachidonic acid, and its eicosanoid metabolites (prostaglandins, thromboxines, leukotrienes), considered key elements in the inflammatory process and immune response [9].

Statins inhibit the enzyme 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A reductase (HMGCoA), which catalyzes the conversion of HMG-CoA to mevalonic acid, a precursor of cholesterol [10]. Because of their great ability to reduce

plasma LDLc, these drugs are essential to a drug in the primary and secondary prevention of cardiovascular events. Currently, there is interest in the search for effective therapeutic methods to attenuate the inflammatory process present in atherosclerosis to revert the cardiovascular burden of obesity. The share of drugs used primarily in the treatment of dyslipidemia in pleiotropic actions, as observed with inhibition of 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A reductase (HMGCoA), has been focused on more recent studies.

This study aimed to investigate the simvastatin effect on secretion and expression of leptin in mature 3T3-L1 adipocytes under an inflammatory state. We also speculate if the potential benefits, observed from statins are related to modulation of PPARs-α and γ in such conditions.

Materials and Methods

Cell culture

Murine fibroblasts of the immortalized 3T3-L1 cell line in the 7th passage were obtained from the American Type Culture Collection and were grown in DMEM-HG (Dulbecco's modified Eagle's medium-high glucose, Gibco, NY, USA) containing 10% (v/v) Fetal Bovine Serum (FBS, Gibco, NY, USA). Confluent cells were induced to differentiate into adipocytes, according to a previously established protocol [11].

Treatment

The differentiated adipocytes were pre-treated with Simvastatin (10 µM) (Sigma Aldrich, St. Louis, MO, USA) or the vehicle control (non-supplemented DMEM HG medium) for 24 h at 37°C in an incubator containing 5% CO_2 and ideal humidity conditions. After the simvastatin treatment, the cells were exposed to TNF-α (10 ng/mL), sealed and placed at 37°C for 24 h. Serial dilutions of simvastatin had been previously tested to obtain a working concentration of 10 µM **(Figure 1)**.

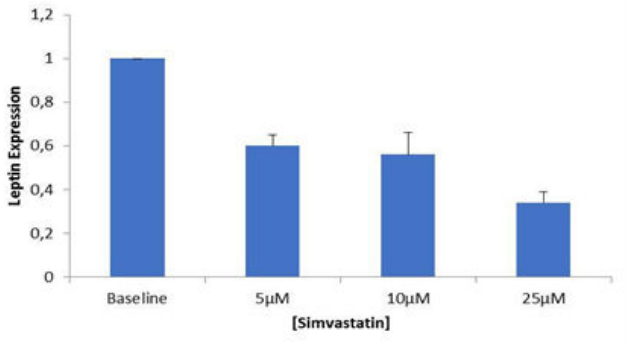

Figure 1: Effect of different concentrations of simvastatin on leptin expression in 3T3-L1 adipocytes.

Measurement of leptin by ELISA

The cells were lysed as previously described by Wang et al. [12]. The secretion of leptin in 3T3-L1 adipocytes was determined by measuring concentrations in the cell culture medium using a commercial ELISA kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol.

RNA extraction

Total mRNA from mature adipocytes was extracted with the QuantiTeck kit (Qiagen, Hilden, NRW, German) according to the manufacture's protocol. After, cDNA was prepared using 1 µg of total RNA by the appropriate kit (RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Scientific, Pittsburgh, PA, EUA).

RT-PCR analysis of leptin

An aliquot of cDNA was mixed with the specific primers for the genes of interest, along with deoxynucleotides, Taq polymerase, and the fluorescent stain SybrGreen (Quantitect kit, Qiagen, Hilden, NRW, German).

The primers to the leptin gene were 3' - CATCTGCTGGCCTTC TCCAA - 5' and 5' - ATCCAGGCTCTCTGGCTTCTG- 3' and HPRT 5' - CTCATGGACTGATTATGGACAG - 3' and 5' - GCAGGTCAGCAAAG AACTTATA-3' . Primers for the genes of interest were designed using the Primer Express® program (Applied Biosystems, Gibco , NY, USA) and were based on the gene sequences obtained from GenBank. Realtime quantitative PCR was performed in the QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fischer Scientific). Reactions utilizing the primer for the housekeeping gene HPRT (hypoxanthine phosphoribosyltransferase) were systematically performed, and all values obtained for the genes of interest were normalized with HPRT expression.

Immunofluorescence

Immunofluorescence was performed by washing the cells once with ice-cold PBS, followed by fixation with 4% paraformaldehyde at 4°C for 30 min. The cells were washed three times with ice-cold PBS. After, cells were washed with glycine 0.1 M for 2 min and blocked with 1% Bovine Serum Albumin (BSA). The cells were incubated with saponin 0.01% for 15 min and then incubated with primary antibodies as indicate overnight at 4°C. The following primary antibodies were used: Ob-R, mouse monoclonal antibody 1:50 dilution (Santa Cruz), PPAR-α, mouse monoclonal antibody 1:50 dilution (Millipore) and PPAR-γ rabbit monoclonal antibody 1:50 dilution (Millipore). Primary antibodies were detected with TRITC-conjugated anti-mouse antibody and FITC-conjugated anti-rabbit antibody for 2 hat room temperature. The coverslips were mounted with buffered glycerol and examined with 40X or 100X oil immersion objectives on inversion microscope (OLYMPUSBX60), using the software NIS Elements F.

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software. The results were expressed as mean ± standard deviation. The statistical significance of the differences between variables studied was determined by Analysis of

Variance (ANOVA) complemented by Bonferroni's test. The level of statistical significance was 0.05 for all tests.

Result

Expression of leptin

It was observed that the inflammatory stimulus increased the expression of leptin. Pretreatment of the cells with simvastatin and subsequent incubation with TNF-α resulted in a statistically significant decrease in leptin expression **(Figure 2 and Table 1)**.

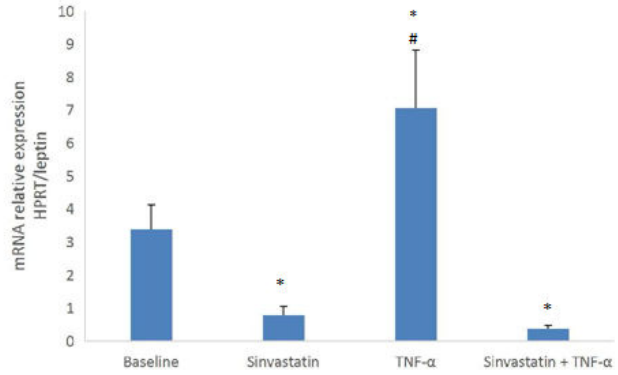

Figure 2: Expression of leptin in 3T3-L1 adipocytes pretreated with simvastatin and stimulated with TNF-α. * versus Baseline; # versus Simvastatin, p<0.05 (n=5).

Secretion of leptin

The leptin secretion of in 3T3-L1 adipocytes exposed to TNF-α increased significantly, after a period of 6 h incubation compared to the baseline group **(Figure 3 and Table 1)**.

Table 1: Leptin expression and secretion in adipocytes pretreated 3T3-L1 with simvastatin and stimulated with TNF-α.

Groups	Relative mRNA HPRT/leptin	Secretion (pg/mL)
Baseline	3,4 ± 0,73	8,92 ± 0,26*
Simvastatin	0,79 ± 0,25•	6,36 ± 0,75*
TNF-α	7,05 ± 1,7•†	13,40 ± 4,3
Simvastatin + TNF-α	0,37 ± 0,09•	6,86 ± 0,67*
*p<0.05 vs TNF-α; •p<0.05 vs Baseline; †p<0.05 vs Simvastatin		

In contrast, there was no difference in the leptin secretion in the group pretreated with simvastatin and stimulated with TNF-α, compared with the baseline group, but there was a reduction statistically significant compared with the group of cells only stimulated by TNF-α **(Figure 3 and Table 1)**.

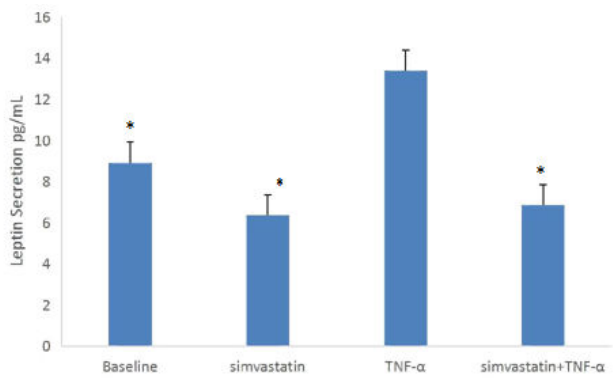

Figure 3: Secretion of leptin in 3T3-L1 adipocytes pretreated with simvastatin and stimulated with TNF-alpha. *versus TNF-α, p<0.05.

Analyzing the correlation of the variables, we observed that the expression of leptin is associated with the levels of secretion **(Figure 4)**.

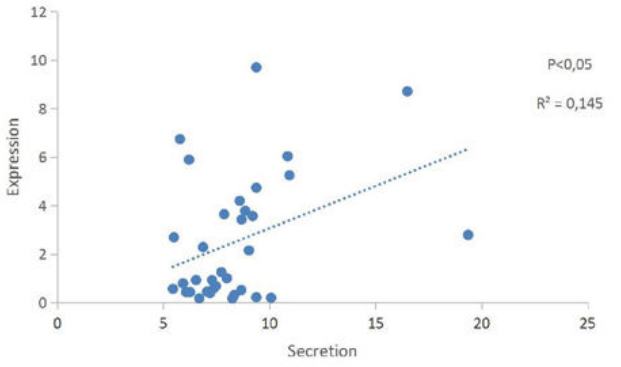

Figure 4: Correlation between mRNA expression and secretion leptin.

Leptin receptor expression by immunofluorescence

The intensity fluorescence of the leptin receptor (Ob-R) under inflammatory stimulus with TNF-α increased compared with the baseline group and with the group pretreated with simvastatin, depicted by an increase of 1.39 times in fluorescence intensity. In the group treated with simvastatin and pre-treated with simvastatin and submitted to a stimulus with TNF-α we observed a decrease of 2.08 and 1.92 times, respectively, in the OB-R expression compared with baseline group **(Figure 5)**.

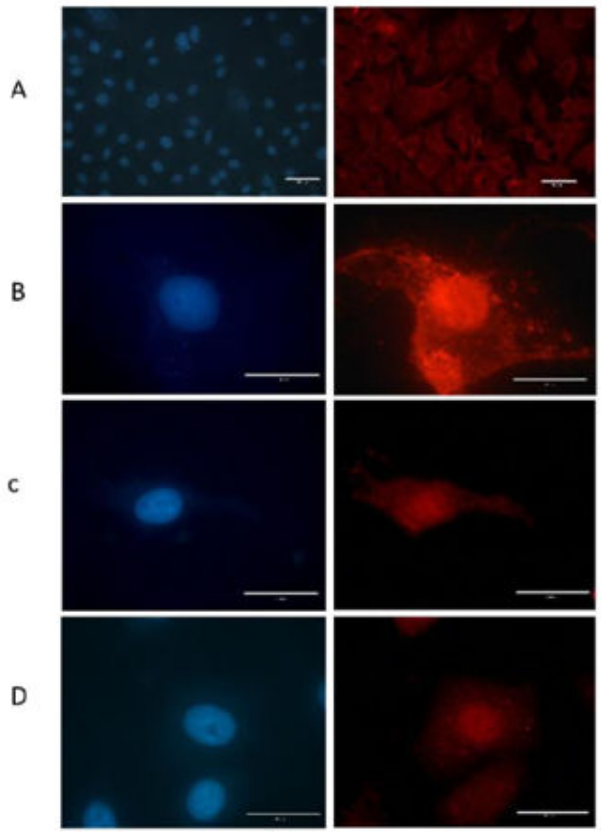

Figure 5: Analysis of Ob-R expression in the different groups, by immunofluorescence: (A) Ob-R baseline (B) Ob-R incubated with TNF-α for 6 h, (C) Ob-R incubated with simvastatin 24 h (D) OB-R incubated with simvastatin and TNF-α.

Expression of PPAR-γ and PPAR-α receptors by immunofluorescence

The analysis of fluorescence showed an increase in the expression of PPAR-γ and PPAR-α receptors in the simvastatin group and the group pre-treated and stimulated with TNF-α when compared with the baseline group. The image obtained indicates a decrease in the intensity of fluorescence on both receptors in the group submitted to the inflammatory stimulus.

On the simvastatin group, the increase in fluorescence intensity of PPAR-α and PPAR-γ was 1.72 and 1.19 times respectively, compared with the image analysis of the baseline group. The group pre-treated with simvastatin and incubated with TNF-α presents an increase in fluorescence intensity of 1.94 times and 2.15 times of PPAR-α and PPAR-γ, respectively, compared with the baseline group **(Figure 6)**.

In summary, we observed an increase in leptin expression and secretion in 3T3-L1 cells when exposed to an inflammatory stimulus. In contrast, the inflammatory stimulus promoted by TNF-α resulted in attenuation on PPAR-α and PPAR-γ receptor expression. The pretreatment of these cells using the HMG-CoA and subsequently exposed to inflammatory stimulus resulted in reduced expression and secretion of leptin. In this condition, compared with the baseline group, the expression of PPARs increased, suggesting the protective role of simvastatin in the inflammatory process.

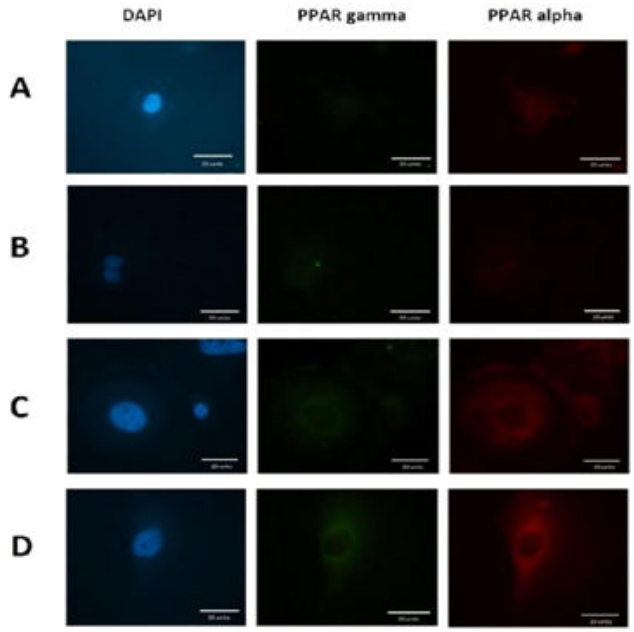

Figure 6: Analysis of expression of PPAR-α and PPAR-γ receptors in the different groups by immunofluorescence: (A) Baseline (B) incubated with TNF-α for 6 h (C) incubated with simvastatin for 24 h, (D) pre-treated with simvastatin for 24 h and incubated with TNF-α for 6 h.

Discussion

Visceral obesity is responsible for an inflammatory response, primarily mediated by TNF-α which affects many biological processes including differentiation, apoptosis, and energy metabolism modulating the inflammation [9]. The adipose tissue has been indicated as an important effector in the perpetuation of the inflammatory state and the prothrombotic effect associated with inflammation [13-16].

We observed that TNF-α is active in the 3T3-L1 adipocytes demonstrated by a dose-dependent response on leptin secretion in these cells. As well as, this effect was also demonstrated by Simons et al. and Kirchgessner et al. [17,18].

Recently, studies using obesity animal models suggested that leptin is not only a gain weight marker but also a player on the development of a systemic inflammatory state and insulin resistance under such conditions. Studies reported an increase in plasma levels of leptin associated with dyslipidemia, hepatic steatosis, and insulin resistance [19,20]. Moreover, it has been also reported that high levels of leptin in obese individuals induce expression of CRP (C Reactive Protein) in vascular endothelium, perpetuating the development of chronic conditions and inflammatory diseases [21-23]. Leon-Cabrera et al. demonstrated that plasma leptin levels influence the

inflammatory state and contribute to abnormalities in energy expenditure, suggesting that leptin may be used as a marker for recognition events related to metabolic disorders associated with obesity [24]. Liu et al. observed that SOCS3 (cytokine signaling suppressor related to activation of leptin) stimulated inflammatory condition and apoptosis of adipocytes, enhancing the participation of suppressing the phosphorylation of JAK2/STAT (signal transducer and transcription activator), resulting in attenuation of leptin signaling [25,26]. This observation indicates that the leptin inhibitory mechanism on the JAK2/STAT pathway contributes to adipocyte apoptosis.

In our study, an increase in leptin secretion was demonstrated after 6 h of incubation with TNF-α. Although clinical studies and animal models have shown the association between hyperleptinaemia and high levels of TNF-α, studies *in vitro* with cells do not reproduce this result homogeneously [27,28]. In a preliminary analysis, our group conducted a study using 3T3-L1 fibroblasts differentiated into adipocytes and stimulated with TNF-α for 48 h [6]. This stimulation period was disregarded for further analysis since, after 24 h of incubation with TNF-α, the expression of leptin reached undetectable levels. Besides, the viability of these cells decreased significantly in this period of exposure to cytokine. Zhang et al. showed an increase in leptin secretion when adipocytes were incubated with TNF-α for 6 h [28]. However, the levels of adipokines gradually declined, achieving undetectable values in 48 h. This finding was further supported by Kirchgessner et al. that observed a 5.5-fold higher secretion in comparison with the baseline leptin in 3T3-L1 adipocytes in the first 6 h of exposure, followed by a rapid decline to undetectable levels after 24 h of inflammatory stimuli [29].

We used simvastatin, HMG-CoA reductase inhibitor, on the pre-treatment of adipocytes and we assessed the expression and secretion levels of leptin after the inflammatory stimulus. It was observed that the increased leptin secretion and expression under inflammatory conditions was reduced with the use of simvastatin. In agreement with our findings, some studies have evidenced the ability of statins in suppressing the inflammatory response by reducing the secretion of leptin [30,31]. Using 3T3-L1 cells, Maeda et al. found that the administration of simvastatin reduced leptin secretion [30]. Similarly, Shih et al. observed a decreased leptin secretion treating human coronary artery smooth muscle cells with atorvastatin previously incubated with angiotensin II as inflammatory stimuli [31]. The effect of HMG-CoA reductase inhibitors was also studied on Ob-R receptors, indicating that its expression was attenuated compared with cells only stimulated with TNF-α. We have not found studies that investigated the use of statins and reduced expression of Ob-R receptor.

Considering the important role of PPARs in the control of biological processes related to lipid metabolism and inflammation, we also studied the action of simvastatin on the peroxisome proliferator-activated receptors and we found that pre-treatment with simvastatin reversed the reduction in expression of PPARs induced by stimulation with TNF-α. The reversal of the inflammation state through the use of statins may be related to its action in other ways, such as suggested by Takemoto et al., regulating the synthesis of eNOS, decreasing ADMA synthesis (endothelial injury marker) although statins and PPAR agonists share properties in the regulation of inflammatory genes [32-34]. The PPARs and leptin are important elements involved in the regulation of energy metabolism as shown by Törüner et al. by demonstrating that PPAR-γ agonists decreased leptin levels in rats submitted to high-calorie diet [35]. According to the authors, the use of thiazolidinediones (PPAR-γ agonists) in cultured adipose cells reduced the expression of leptin [36,37]. Additionally, it has been suggested that PPARγ activation decreases the leptin receptor's expression and inhibits their transduction pathways [38,39].

Few studies report the action of statins on expression or secretion of leptin in 3T3-L1 adipocytes and its possible correlation with the PPARs. Our results emphasize the ability of simvastatin in reducing leptin secretion and expression, suggesting that the anti-inflammatory effect of statins may be associated with the PPARs activation.

Conclusion

In conclusion, we demonstrated that the inflammatory stimulus determines the increase in the leptin expression and secretion level, and is associated with the expression of its receptor on the 3T3-L1 cell line. The pre-treatment with simvastatin in 3T3-L1 adipocytes attenuates the secretion and expression of leptin under basal conditions and inflammatory stimulus. This observation highlights the potential beneficial effect of statins on adipose tissue as an important therapeutic target in metabolic syndrome. We also demonstrated that inflammatory stimulus by TNF-α action resulted in attenuation of PPAR-α and γ expression in 3T3-L1 cells. The pre-treatment of the adipocytes with simvastatin determined an increase in the expression of these receptors. We suggest the involvement of activation of the PPAR receptors in the modulation of leptin with a potential beneficial effect of the use of HMG-CoA reductase inhibitors in this mechanism.

Acknowledgment

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