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TNF-α modulates statin effects on secretion and expression of MCP-1, PAI-1 and adiponectin in 3T3-L1 differentiated adipocytes

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ABSTRACT

Purpose: Systemic inflammatory conditions, as seen in obesity and in the metabolic syndrome, are associated with high plasmatic levels of proatherogenic and prothromboticadipokines and low levels of adiponectin. Inhibitors of HMG-CoA reductase have beneficial effects in reducing cardiovascular events attributed predominantly to its lipid-lowering effects and recent studies suggest that these effects might be due to its anti-inflammatory properties. Based on the pleiotropic properties of simvastatin we studied the effects of this drug on the secretion and expression of adiponectin, PAI-1 and MCP-1 in mature adipocytes under baseline conditions and after an inflammatory stimulation.

Materials and methods: The differentiated adipocytes were incubated with 10 μM simvastatin or vehicle and TNF-α 10 ng/mL or vehicle were added to treatment media. After 24 h of incubation, the media was harvested and the proteins of interest were analyzed by Multiplex method. Gene expression was analyzed by real time-PCR.

Results: The addition of TNF-α increased the expression and secretion of MCP-1 and PAI-1. However, stimulation did not interfere with the secretion of adiponectin, despite having significantly reduced its expression. Our data also demonstrated that simvastatin reduced the expression and secretion of MCP-1, under baseline (770.4 ± 199.9 vs 312.7 ± 113.7 and 1.00 ± 0.14 vs 0.63 ± 0.13, $p < 0.05$, respectively) and inflammatory conditions (14945 ± 228.7 vs 7837.6 ± 847.4 and 24.16 ± 5.49 vs 14.97 ± 2.67, $p < 0.05$, $p < 0.05$, respectively). Simvastatin also attenuated the increase in expression and secretion of PAI-1 induced by TNF-α (16898.6 ± 1663.3 vs 12922.1 ± 843.9 and 5.19 ± 3.12 vs 0.59 ± 0.16, respectively $p < 0.05$), but under baseline conditions had no effect on the expression or secretion of PAI-1. The statin increased the expression of adiponectin under baseline conditions and inflammatory stimulation (1.03 ± 0.08 vs 4.0 ± 0.96 and 0.77 ± 0.19 vs 2.16 ± 0.23, respectively, $p < 0.05$) and also increased the secretion of this adipokine but only with the inflammatory stimulus (5347.7 ± 1789.3 vs 7327.3 ± 753.6, $p < 0.05$).

Conclusions: Our findings suggested that simvastatin counteracted the stimulatory effect of TNF-α on secretion and expression of MCP-1, PAI-1 and adiponectin, implying a potential anti-atherogenic effect during the inflammatory process; these pleitropic effects were more pronounced with HMG-CoA reductase inhibitor.

1. Introduction

Atherosclerosis is one of the principal causes of mortality in the world. Inflammation, the physiopathologic basis of atherosclerosis, is not included among the classic risk factors but does appear to be an additional important therapeutic target in the reduction of cardiovascular risk [1,2].

The increase in the secretion of biologically active molecules such as MCP-1 (monocyte chemoattractant protein-1) [3–5] and PAI-1 (plasminogen activator inhibitor-1) [6–8] has an ominous impact on the progression of atherogenesis. In contrast, adiponectin, the most abundant gene product of adipocytes, has a protective effect against the development of atherosclerotic disease, and its levels are reduced in metabolic syndrome [9,10].

The implication of drugs used primarily for treatment of dyslipidemia in pleiotropic and anti-atherogenic actions [11,12], additionally to modifying effects on the lipid profile, has been the focus of more recent studies and can lead to a therapeutic

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approach to inflammation as a strategy for reducing cardiovascular risk [13,14]. Ridker et al. demonstrated that the use of rosuvastatin, a HMG-CoA reductase inhibitor, leads to a significant decrease in coronary risk in non-dyslipidemic individuals with elevated C reactive protein, a marker of systemic inflammation [15].

The objective of this study was to investigate the "in vitro" effect of simvastatin on the secretion and expression of adiponectin, PAI-1 and MCP-1 in mature adipocytes derived from the 3T3-L1 cell line, under baseline conditions and after an inflammatory stimulation, mimicking the inflammatory milieu of metabolic syndrome.

2. Methods

2.1. Cell culture

Murine fibroblasts of the immortalized cell line 3T3-L1, in the 11th passage were stored in liquid nitrogen, at -120 °C until thawed, plated and grown in DMEM-HG (*Dulbecco's Modified Eagle Medium high glucose,* Gibco) culture medium supplemented with 10% fetal bovine serum (10% FBS, Gibco). On reaching confluence, the cells were harvested after trypsinization and distributed into 6-well plates. The fibroblasts, then in their 12th passage, were grown to 90% confluence, at which time differentiation into adipocytes step then initiated, according to a previously established protocol [16]. In order to induce 3T3-L1 cells to differentiate into adipocytes, on the 1st day (D_0), the culture medium was substituted with DMEM HG medium containing 10% FBS, 160 nM insulin, 250 μM dexamethasone and 0.4 mM 3-isobutyl 1-methylxanthine (IBMX, Sigma) (differentiation medium). On D_4, after 96 h of incubation, differentiation medium was substituted with DMEM HG medium containing 160 nM insulin and 10% of FBS (*feeding* medium). After 72 h (D_7), the latter medium was refreshed, and at D_{10}, the cells differentiated into adipocytes were incubated for 24 h in culture medium containing simvastatin (treatment medium) or culture medium without drug (control medium). TNF-α (Sigma), used to mimic the inflammatory state at the final concentration of 10 ng/mL [16] was added with the treatment medium or the control medium in 2 of the 6-well cell culture plates. Previously, adipocytes were incubated with TNF-α (1, 10, 25 and 50 ng/mL) and we verified that at 25 and 50 ng/mL of TNF- α, the concentration of adiponectin in medium decreased, but generated a decrease of 35% in cell viability. During the whole process, the cells were kept in at 37 °C incubator, at 5% CO_2 and at ideal humidity conditions.

2.2. Treatment

On the 10th day after the start of differentiation, the culture medium in the wells was replaced with medium containing simvastatin (Sigma), vehicle (non-supplemented DMEM HG medium), simvastatin + TNF-α and TNF-α only. Each of the 6-well plates was considered a distinct treatment group ($n = 6$). The medium without drug and without stimulus was considered the baseline control; the medium without drug and with added TNF-α (10 ng/mL) was called the control with stimulus.

Previously, simvastatin was diluted and activated as followed: in a 1.5-mL tube, 5 mg simvastatin (Sigma) were dissolved in 190 μL DMSO and 810 μL Milli-Q water. NaOH, 0.1 M, was added to the tube, which was then heated at 70 °C for 2 h [17]. This solution was serially diluted with DMEM medium, in order to obtain a medium with 10 μM simvastatin.

After serial dilutions to reach the final work concentration of 10 μM simvastatin the final percentage of DMSO was less than 0.002%.

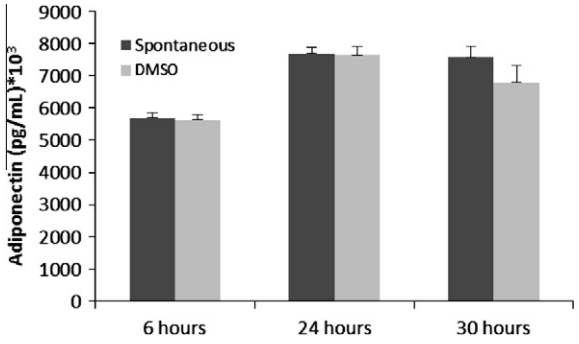

Fig. 1. Effect of DMSO on adiponectin secretion.

A control assay was performed to verify the influence of DMSO at 0.002% on adiponectin production after 6, 24 and 30 h of incubation (Fig. 1).

2.3. Dose effects of simvastatin on MCP-1 and PAI-1 release by adipocytes

To choose optimal simvastatin concentration on adipokines release by adipocytes, cells ($N = 4$) were incubated with increasing doses of simvastatin (0.1, 10, 100 μM) for 24 h. Concentrations of MCP-1 and PAI-1 were determined in the 24 h culture supernatants of adipocytes. In this preliminary protocol, our results suggest that simvastatin had no dose-dependent effect (Fig. 2) and, therefore, 10 μM concentration was chosen to carry out the study. Under TNF-α stimulating condition simvastatin either had no dose-dependent effect (data not shown).

Based on a pilot time-response experiment (12, 24, 30 h) were adipocytes raised maximum production of MCP-1 and PAI-1 after 24 h of incubation with TNF-α; all assays were carried out during this period (data not shown).

2.4. Characterization of differentiation of fibroblasts into adipocytes by oil red staining

On D_4, the fibroblasts began to acquire the phenotype of adipocytes, with accumulation of fat droplets in the cytoplasm. On D_{10}, about 70% of the fibroblasts had differentiated into adipocytes. The formation of lipid droplets, a morphologic appearance characteristic of adipocytes, was confirmed by oil red (Sigma) staining. The process of the differentiation of 3T3-L1 fibroblasts into adipo-

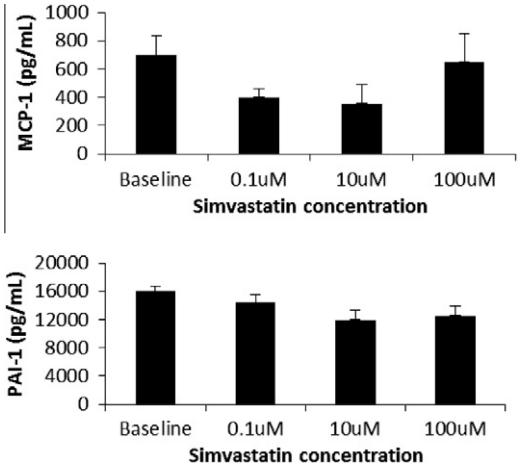

Fig. 2. Dose-response effect of simvastatin on MCP-1 and PAI-1 adipocyte's release.

cytes was evaluated every 24 h starting at D_0, by observing the morphologic appearance of the cells with a light microscope. Adipocytes were identified by oil red staining of lipid droplets which are characteristically found in the cytoplasm of adipocytes [18]. Initially, 60 mg of oil red stain were dissolved in 20 mL of isopropanol. Next, 300 μL of this solution were diluted in water in 6:4 proportions. Culture medium was aspirated and the *wells* were washed twice with phosphate-buffered saline (PBS). Next, 4% paraformaldehyde (diluted in PBS), was added in a volume sufficient to cover the cells. After 30 min at room temperature, the medium was aspirated and the wells were washed 3 times with PBS. The cells were then incubated in oil red solution (300 μL) for 2 h, at 37 °C. Afterward, the medium was aspirated, and the plates were washed thrice with distilled water and placed into the incubator, at 37 °C to dry. After this step, the cells were examined with optic microscope and photographs were taken.

2.5. Determination of cell viability

We could observe by trypan blue (Sigma) exclusion that from the 4th to the 10th day, at the beginning of differentiation, cell viability was about 95% (data not shown). The cells were trypsinized and suspended in 10 mL of PBS. A 25-μL aliquot of this cell suspension was mixed with 75 μL trypan blue stain. From this mixture, 10 μL were placed in a Neubauer chamber to count viable nucleus cells stained in red.

2.6. Analysis of the secretion of MCP-1, PAI-1 and adiponectin by adipocytes

The secretion of the adipokines was determined by the quantification of the concentrations of MCP-1, PAI-1 and adiponectin in the cell culture medium, using the Luminex xMAP method, with the multiplex Mouse Adipocyte Panel kit (Linco, Millipore), and according to the manufacturers' instructions. MCP-1 and PAI-1 were assayed simultaneously. The quantification of adiponectin was done separately, after the samples had been diluted 500 times in DMEM medium.

2.7. Gene expression and secretion of proteins of interest

The primers for the proteins of interest were designed using the Primer Express® program (Applied Biosystems, USA), based on gene sequences obtained at GenBank. Each pair was analyzed utilizing nucleotide-nucleotide Blast (available at http://www.ncbi.nlm.nih.gov/ BLAST/). Total RNA was extracted from the cultured adipocytes with the QuantiTect kit (Qiagen), in accordance with the manufacturer's instructions. Next, cDNA was prepared using 1 μg total RNA and the appropriate kit (RT-PCR kit, GLT). 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). Real-time quantitative PCR was performed in a thermocycler (ABI 7700). The specificity of the product was confirmed by melting point curve analysis, as well as by conventional agarose gel electrophoresis. The products were quantified and compared to controls based on the analysis of the number cycles necessary to obtain a given fluorescence value in the log-linear phase of the reaction. Reactions utilizing the primer for the housekeeping gene HPRT were systematically performed, and all values obtained for the genes of interest were normalized with HPRT expression.

2.8. Statistical analysis

The statistical analyses were performed using the software SPSS 16.0. The results for each individual group were expressed as a mean ± standard deviation. The t-test for independent samples was used for comparison between baseline control and control after TNF-α. Statistical significance of differences between the variables studied was determined by analysis of variance (ANOVA), complemented by Bonferroni's test. In all tests, the level of statistical significance considered was 0.05.

3. Results

3.1. Effect of TNF-α on the expression and secretion of the adipokines

The cells incubated with TNF-α increased the expression and secretion of MCP-1 by adipocytes approximately 19 and 24 times, respectively. The inflammatory stimulus also induced an increase in the expression and secretion of PAI-1. Unlike what was observed in the experiments with other adipokines, there was no difference between the concentrations in culture medium of 3T3-L1 cells for adiponectin under baseline conditions or TNF-α stimulus. However, TNF-α significantly decreased the expression of adiponectin. There was a correlation between the expression and secretion of MCP-1 ($r = 0.93$; $p < 0.001$) and of PAI-1 ($r = 0.64$; $p < 0.001$), but not between expression and secretion of adiponectin.

3.2. Effect of simvastatin on MCP-1

In the adipocytes, simvastatin induced a statistically significant decrease in the secretion as well as expression of MCP-1 under baseline conditions (770.4 ± 199.9 vs 312.7 ± 113.7 and 1.00 ± 0.14 vs 0.63 ± 0.13, $p < 0.05$, respectively) and after stimulation with TNF-α (14,945 ± 228,7 vs 7837.6 ± 847.4 and 24.16 ± 5.49 vs 14.97 ± 2.67, $p < 0.05$, respectively) (Table 1) (Fig. 3).

3.3. Effect of simvastatin on PAI-1

Under baseline conditions, the statin didnot exert an effect on the expression or secretion of PAI-1. Under inflammatory stimulus,

Table 1
Effect of TNF-α stimulus on the secretion and expression of MCP-1, PAI-1 and Adiponectin.

	Spontaneous	Simvastatin control	TNF-α Stimulus	Simvastatin + TNF-α
Secretion (pg/mL)				
MCP-1	770.4 ± 199.9	312.7 ± 113.7*	14945.0 ± 228.7	7837.6 ± 847.4**
PAI-1	14371 ± 1011	17538.9 ± 1538	16898.6 ± 1663.3	12,922 ± 843.9**
Adiponectin (x10^3)	5643 ± 121.2	6801 ± 533.2	5347.7 ± 1789.3	7327.3 ± 753.6**
Relative mRNA expression				
MCP-1	1.0 ± 0.14	0.63 ± 0.13*	24.16 ± 5.49	14.97 ± 2.67**
PAI-1	0.93 ± 0.12	1.06 ± 0.09	5.19 ± 3.12	0.59 ± 0.16**
Adiponectin	1.03 ± 0.08	4.0 ± 0.96*	0.77 ± 0.19	2.16 ± 0.23**

* Spontaneous vs Simvastatin control, $p < 0.05$.
** TNF- α stimulus vs Simvastatin + TNF-α, $p < 0.05$.

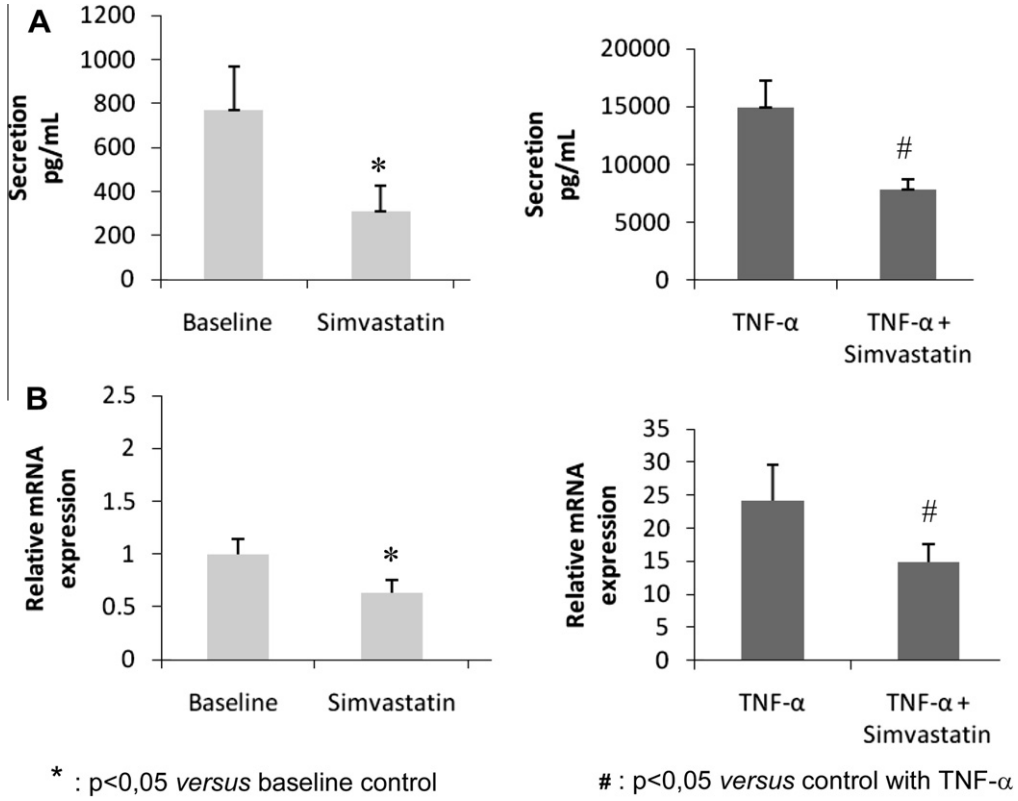

Fig. 3. Effects of lipid profile-modifying drugs on secretion (A) and expression (B) of MCP-1 in 3T3-L1 adipocytes ($n = 6$) under normal condition on left (gray) and under inflammatory stimulation on right (black).

simvastatin reduced both the expression and secretion of PAI-1 (16898.6 ± 1663.3 vs 12922.1 ± 843.9 and 5.19 ± 3.12 vs 0.59 ± 0.16, respectively $p < 0.05$) (Table 1) (Fig. 4).

3.4. Effect of simvastatin on adiponectin

The statin increased the expression of this adipokine under both baseline conditions and inflammatory stimulus (1.03 ± 0.08 vs 4.0 ± 0.96 and 0.77 ± 0.19 vs 2.16 ± 0.23, respectively, $p < 0.05$) and increased the secretion of adiponectin only after the inflammatory stimulus (5347.7 ± 1789.3 vs 7327.3 ± 753.6, $p < 0.05$) (Table 1) (Fig. 5).

4. Discussion

In the present study, we observed that TNF-α, is active in differentiated 3T3-L1 adipocytes, simulating the inflammatory *millieu* of obesity and metabolic syndrome by increasing the expression and secretion of MCP-1 and PAI-1. These findings corroborate the hypothesis that this tissue is of utmost importance in the perpetuation of the inflammatory status and pro-thrombotic effect associated with inflammation. However, TNF-α did not interfere with the secretion of adiponectin, despite having significantly reduced its expression. The discrepancy between the effects of TNF-α on adiponectin gene expression and secretion may be due to differences in the half-life of adiponectin protein in the culture medium, relative to the half-life of adiponectin mRNA. Additionally, the measurement of adiponectin secretion was relied upon accumulation of this protein in the culture medium over 24 h and doesnot account for the rate of degradation following release. This result concurs with the finding of Wang et al., who demonstrated that incubation with TNF-α for 24 h did not alter the secretion of this adipokine by 3T3-L1 mature adipocytes, even reducing its gene expression [19].

Our data also demonstrated that simvastatin markedly reduced the expression and secretion of MCP-1, under baseline and inflammatory conditions. In fact, Han and coworkers observed that the use of simvastatin diminished the expression of MCP-1 in the monocytes of normocholesterolemic patients [3]. Inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibits the synthesis of mevalonic acid [11], and consequently the production of intermediate isoprenoids [4,11], which are activators of intracellular signaling proteins, responsible for the initiation of inflammatory cascades. It is well documented that this effect can be reversed by the administration of mevalonate [20]. Additionally, the action of the statin has been related with the inhibition of NF-κB transcription factor pathway, which is essential in the gene expression of numerous atherogenic mediators, including MCP-1 [12].

Regarding to PAI-1, the present work showed that simvastatin only attenuated the increase in the expression and secretion of PAI-1 induced by TNF-α, an observation contrary to a study by Laumen et al., who reported that rosuvastatin was able of decrease the secretion of PAI-1 by 50% under baseline conditions, in 3T3-L1 adipocytes [17]. Such divergence could be attributed to pharmacologic differences between rosuvastatin and simvastatin. Although few reported studies related to the action of the HMG-CoA inhibitor on the secretion of PAI-1 by 3T3-L1 adipocytes we suggested that the statin had a beneficial effect on adipose tissue, since the drug attenuated the pro-thrombotic state induced by TNF-α. In fact, our group had already demonstrated that treatment of peripheral blood mononuclear cells isolated from critically ill patients with simvastatin resulted in an attenuation of spontaneous pro-inflammatory cytokine production concurring with potential benefit of statins in this scenario [21].

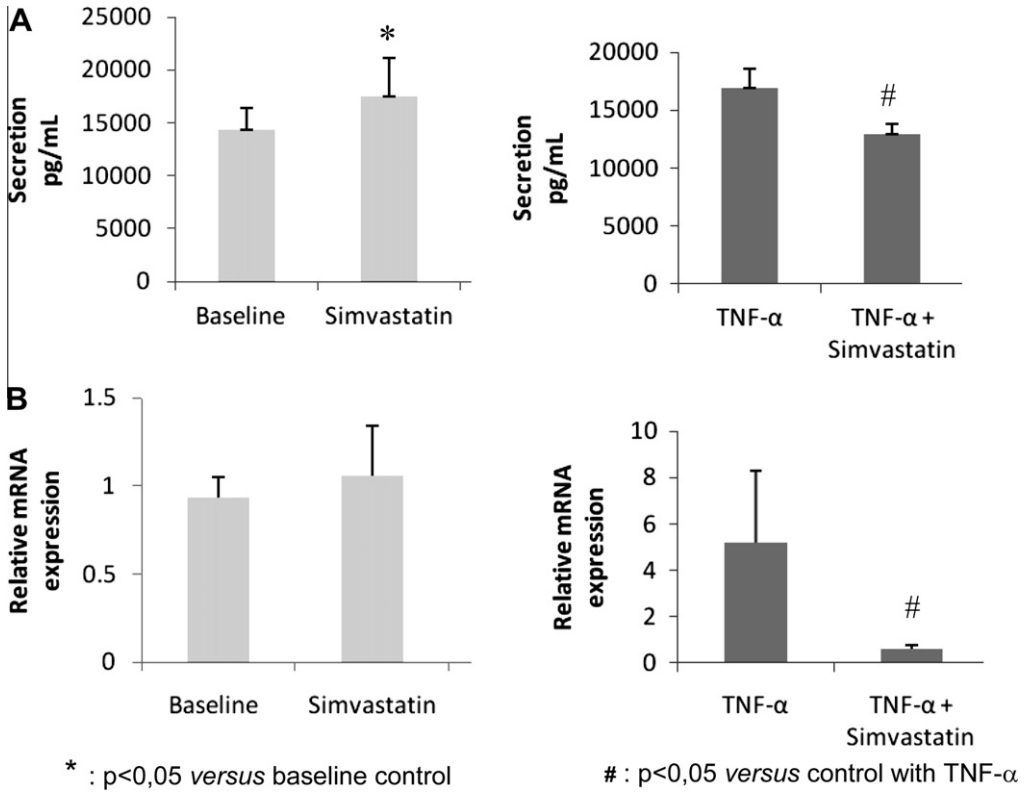

Fig. 4. Effects of lipid profile-modifying drugs on secretion (A) and expression (B) of PAI-1 in 3T3-L1 adipocytes ($n = 6$) under normal condition on left (gray) and under inflammatory stimulation on right (black).

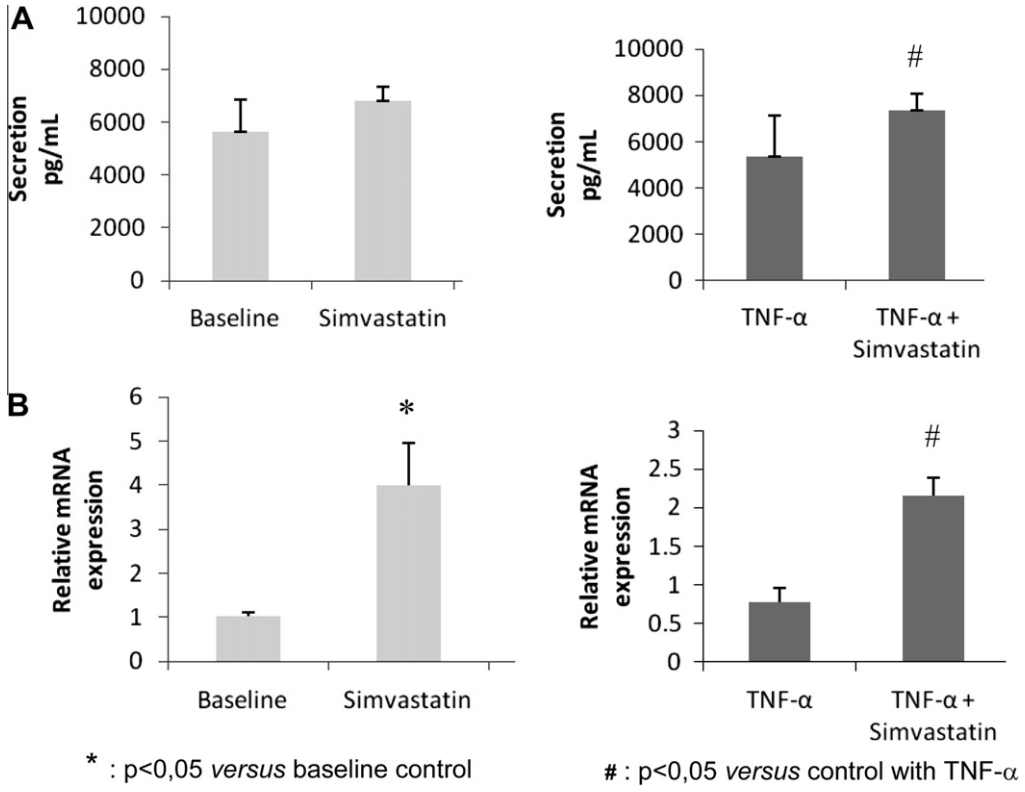

Fig. 5. Effects of lipid profile-modifying drugs on secretion (A) and expression (B) of adiponectin in 3T3-L1 adipocytes ($n = 6$) under normal condition on left (gray) and under inflammatory stimulation on right (black).

In addition, simvastatin increased the expression of adiponectin under both baseline and inflammatory stimulation, but only increased the secretion of this adipokine with the inflammatory stimulus. It is known that adiponectin is very abundant in the plasma, even under baseline conditions, but mechanisms of stimuli that act on its secretion have been poorly elucidated. Our data corroborates the study conducted by Sakamoto and coworkers, who demonstrated that the use of pravastatin (other HMG-CoA reductase inhibitors) was capable of significantly increase the adiponectinemia in dyslipidemic patients [22].

It has been described that TNF-α induce the nuclear translocation of NF-κB and upregulate the expression and secretion of MCP-1, PAI-1 and PCR [23]. The simvastatin effect in diminishing the mRNA expression and secretion of MCP-1, observed in our results could be attributed to the increased production of adiponectin once it is known that this adipokine inhibits the NF-κB pathway [24]. Others transcriptions factor have been described to be altered by statins. KFL4 a member of the Kruppel-like family mediates the anti-inflamatory effects of statins on endothelial cells. In this cells statins upregulate extracellular signal-regulated kinase (Erk5) by the MEK5/mitogen activated protein kinase pathway [25]. Birsoy et al. reported the characterization of KLF4 as an essential early regulator of adipogenesis. In their work it was demonstrated that KLF 4 is expressed in 3T3-L1 and knockdown of KLF4 inhibits adipogenesis, downregulates CCAAT/enhancer-binding protein (C/EBPbeta) levels, wich then stimulate the expression of PPAR γ [26].Based on the fact that antiatherogenic effects of PPAR agonists and statins have common characteristics, Yano et al. suggested that a common mechanism may exist via crosstalk of their pathway [27,28]. This study demonstrated that statins suppressed LPS-induced expression of TNF-α and MCP-1 and activation of NF-KB and activator protein-1 (AP-1) through PPARα- and PPARγ-dependent pathways. Therefore, statins might suppress inflammatory responses via activation of both PPARα and PPARγ, thereby including antiatherogenic actions [27].

In conclusion our findings demonstrated simvastatin counteracted the stimulatory effect of TNF-α on secretion and expression of MCP-1, PAI-1 and adiponectin, suggesting a potential anti-atherogenic effect during the inflammatory process involved in metabolic syndrome as well as in the progression of atherosclerosis. The translation of such observations to clinical setting requires further studies.

5. Competing interests

The authors declare that they have no competing interests.

6. Authors' contributions

BMRQ and SMVL contributed to the conception and design, collection, assembly of data and manuscript writing. BMRQ, ABR, MTZ, MAD and MCB contributed to data analysis and interpretation, and manuscript writing. LO contributed to the collection and assembly of data. RN carried out part of experiments and contributed to the manuscript writing. BMRQ and MCB contributed to the study conception, provision of study materials, data analysis and manuscript writing. All authors read and approved the final manuscript.

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