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**LIVER X RECEPTOR (LXR) ACTIVATION NEGATIVELY REGULATES VISFATIN
EXPRESSION IN MACROPHAGES**

**Thérèse Hèrvée Mayi^{1,2,3,4}, Elena Rigamonti^{1,2,3,4}, François Pattou^{1,5,6}, Bart Staels^{1,2,3,4}
and Giulia Chinetti-Gbaguidi^{1,2,3,4}**

¹ Univ Lille Nord de France, F-59000, Lille, France

² Inserm, U1011, F-59000, Lille, France

³ UDSL, F-59000, Lille, France

⁴ Institut Pasteur de Lille, F-59019, Lille, France

⁵ Department of Endocrine Surgery, University Hospital, Lille, France

⁶ U859 Biotherapies for diabetes, INSERM, Lille, France

Address correspondence to:

Bart Staels

Inserm UR 1011, Institut Pasteur de Lille

1, rue du Professeur Calmette, BP 245, Lille 59019, France

Tel: +33-3-20-87-73-88; Fax: +33-3-20-87-73-60

E-mail : bart.staels@pasteur-lille.fr

ABSTRACT

Adipose tissue macrophages (ATM) are the major source of visfatin, a visceral fat adipokine upregulated during obesity. Also known to play a role in B cell differentiation (pre-B cell colony-enhancing factor (PBEF)) and NAD biosynthesis (nicotinamide phosphoribosyl transferase (NAMPT)), visfatin has been suggested to play a role in inflammation.

Liver X Receptor (LXR) and Peroxisome Proliferator-Activated Receptor (PPAR) γ are nuclear receptors expressed in macrophages controlling the inflammatory response. Recently, we reported visfatin as a PPAR γ target gene in human macrophages. In this study, we examined whether LXR regulates macrophage visfatin expression. Synthetic LXR ligands decreased visfatin gene expression in a LXR-dependent manner in human and murine macrophages. The decrease of visfatin mRNA was paralleled by a decrease of protein secretion. Consequently, a modest and transient decrease of NAD⁺ concentration was observed. Interestingly, LXR activation decreased the PPAR γ -induced visfatin gene and protein secretion in human macrophages.

Our results identify visfatin as a gene oppositely regulated by the LXR and PPAR γ pathways in human macrophages.

KEYWORDS - nuclear receptors, adipocytokines, visfatin, inflammation, macrophages

INTRODUCTION

Visfatin/PBEF/NAMPT is a cytokine secreted by adipocytes and preferentially by macrophages in adipose tissue, which circulates in both human and murine plasma [1]. Visfatin was originally described as a cytokine-like molecule that promotes pre-B cell colony formation *in vitro* [2]. Later on, visfatin was identified as nicotinamide phosphoribosyl transferase (NAMPT), a rate-limiting enzyme in the synthesis of nicotinamide adenine dinucleotide (NAD⁺) from nicotinamide, thus acting as an extra- and intracellular NAD biosynthetic enzyme that converts nicotinamide (NAM, a form of vitamin B3) to nicotinamide mononucleotide (NMN), a NAD precursor, in mammals [3]. Visfatin expression is induced by inflammatory stimuli such as interleukin-1beta (IL-1 β), tumor necrosis factor alpha (TNF α) and interleukin-6 (IL-6) in monocytes and macrophages [4-6]. Conversely, visfatin has been shown to regulate the production of IL-1 β , IL-6 and TNF α in peripheral blood mononuclear cells, suggesting a potential role in the pathogenesis of inflammation-related disorders.

Many macrophage functions are regulated by transcription factors such as the nuclear receptors, including the Liver X Receptors (LXR α and LXR β) and the Peroxisome Proliferator-Activated Receptor (PPAR) γ [7]. LXR and PPAR γ are ligand-activated nuclear receptors inhibiting the inflammatory response and participating in the control of transcription of genes involved in lipid and glucose metabolism. After activation by their ligands, LXR and PPAR γ heterodimerize with Retinoic X Receptor (RXR) and bind to specific DNA sequences called LXR response elements (LXRE) or PPAR response elements (PPRE), respectively [8]. LXR are activated by natural (oxysterols) or synthetic ligands such as T0901317 and GW3965, while PPAR γ is activated by natural 15-deoxy-D12;14-prostaglandin J2 (15d-PGJ2), and synthetic ligands, glitazones and the GW1929 compound [8]. Furthermore, when activated by their agonists, LXR and PPAR γ inhibit the cytokine-induced expression of inflammatory genes

by negatively interfering with the NF- κ B, STAT and AP-1 signaling pathways in a DNA-binding independent manner (for review [8]).

Recently, we have reported that visfatin is a PPAR γ target gene in human macrophages [9]. Here we investigated whether visfatin expression is also regulated by LXR activation. We demonstrate that LXR activation decreases macrophage visfatin gene expression in a LXR-dependent manner. This leads to a transient decrease in NAD⁺ concentration. Interestingly, LXR activation also decreases visfatin gene expression and protein secretion induced by PPAR γ agonists.

MATERIALS AND METHODS

Cell culture

Monocytes from healthy normolipidemic or obese humans were isolated by Ficoll gradient centrifugation and primary macrophages obtained after 10 days of differentiation [10]. Macrophages were incubated in the presence of LXR ligands T0901317 (1 μ M), GW3965 (1 μ M) or DMSO for the indicated times (3, 6, 12 or 24h) or with T0901317 (0.25, 0.5, 1 μ M) for 24h. In some experiments, macrophages were co-treated with T0901317 (1 μ M) and recombinant human TNF α or human IL-1 β (5 ng/ml, Promokines) for 6h. In parallel experiments, macrophages were pre-treated with T0901317 (1 μ M) for 6h and then stimulated with IL-1 β (5 ng/ml) for 2h. Where indicated, macrophages were incubated with the PPAR γ ligand GW1929 (600 nM) for 24h and then treated with T0901317 (1 μ M) for a further 6h.

Murine bone marrow-derived macrophages (BMDM) were prepared from C57BL/6J mice as described [11] and treated with T0901317 (2 μ M) for 24h.

Human ATM were isolated as previously reported [9] from visceral adipose tissue biopsies of obese patients undergoing bariatric surgery (ABOS project approved by the Ethical committee and the Federation of clinical research of CHRU of Lille, France). ATM were cultured for 24h in Endothelial Cell Basal Medium (Promocell) supplemented with 0.1% BSA [12] before treatment with T0901317 (1 μ M) or GW3965 (1 μ M).

RNA extraction and analysis

Total cellular RNA was extracted from macrophages and ATM using *Trizol* (Invitrogen, France) or RNeasy micro kit (Qiagen), respectively, and reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). cDNAs were quantified by quantitative polymerase chain reaction (Q-PCR) on a MX 4000 apparatus (Agilent Biotechnologies) using Brilliant II SYBR $\text{\textcircled{R}}$ Green QPCR Master Mix kit

supplemented with specific primers for human visfatin (5'-GCCAGCAGGGAATTTTGTTA-3' forward and 5'-TGATGTGCTGCTTCCAGTTC-3'reverse), mouse visfatin (5'-TCCGGCCCCGAGATGAAT-3' forward and 5'-GTGGGTATTGTTTATAGTGAGTAACCTTGT-3' reverse) and human/mouse cyclophilin (5'-GCATACGGGTCCTGGCATCTTGTC C-3'forward and 5'-ATGGTGATCTTCTTGCTGGTCTTGC-3' reverse). Visfatin mRNA levels were normalized to cyclophilin mRNA.

Short-Interfering RNA

Human macrophages were transfected with specific siRNA for human LXR α and LXR β (SMARTpool siRNA) and non-silencing control siRNA using the transfection reagent DharmaFECT Reagent 4 (Dharmacon). 16h after transfection, cells were incubated with T0901317 (1 μ M) or vehicle (DMSO) and harvested 24h later.

Measure of visfatin protein secretion

Human macrophages and ATM were treated with LXR ligands T0901317 (1 μ M), GW3965 (1 μ M) or DMSO for 24h. Supernatants were collected and extracellular visfatin concentrations measured using a commercially available ELISA kit (Phoenix Pharmaceuticals, Germany).

Measure of NAD cellular content

Total nicotinamide adenine dinucleotide (NADt = NAD+NADH) levels were determined in cell lysates using a specific NADH/NAD quantification kit (Biovision research products). Briefly, human macrophages were treated with T0901317 (1 μ M) or DMSO for 6 or 24h and the NAD/NADH ratio was calculated as (NADt-NADH)/NADH. NAD levels were normalized to protein content. Results are expressed as percentage, the control non-stimulated

cells being expressed as 100%. All assays were done in triplicate in at least three independent experiments.

Statistical analysis

Statistical differences between groups were analysed by Student's t tests and were considered significant when $p \leq 0.05$.

RESULTS

LXR activation decreases visfatin gene expression in a LXR-dependent manner in macrophages

To determine whether LXR might affect the expression of visfatin in macrophages, Q-PCR analysis was performed on RNA isolated from primary human macrophages treated during 3, 6, 12 and 24h with LXR ligands. Treatment with either T0901317 (1 μ M) or GW3965 (1 μ M) resulted in a significant repression of visfatin gene expression, reaching a plateau of about 50% inhibition after 3 or 6h treatment with GW3965 or T0901317, respectively (figure 1A). Treatment with increasing concentrations of T0901317 (0.25, 0.5 and 1 μ M) did not further inhibit the expression of visfatin, which was almost maximal at the concentration of 0.25 μ M (figure 1B). To investigate whether regulation of visfatin expression by LXR can also be extended to mouse macrophages, Q-PCR analysis was performed on RNA isolated from BMDM treated with T0901317 (2 μ M) (figure 1C). Visfatin mRNA expression was decreased by T0901317 also in murine BMDM. Similar results were obtained upon LXR activation in the murine macrophage cell line RAW264.7 (data not shown).

Since it has been reported that visfatin is a visceral fat adipokine mainly produced by ATM, the expression of visfatin mRNA in macrophages and visceral ATM was compared. Q-PCR analysis revealed that visfatin was 6-fold higher expressed in ATM than in monocyte-derived macrophages (MDM) from the same obese donors (figure 1D). To determine whether LXR activation affects the expression of visfatin in ATM, Q-PCR analysis was performed on ATM treated with T0901317 (1 μ M) or GW3965 (1 μ M) for 24h. Visfatin mRNA was decreased by LXR agonists to a similar extent as in MDM (figure 1E).

To address whether the inhibitory effect of LXR ligands on visfatin gene expression is mediated by LXR, a siRNA approach was used. The effect of T0901317 (1 μ M) was analyzed in the presence or in the absence of siRNA targeting LXR α/β . Visfatin induction upon LXR

activation was completely lost in LXR α/β siRNA-transfected macrophages compared to scrambled siRNA-transfected cells (figure 1F), indicating that the reduction of visfatin mRNA expression by T0901713 occurs via LXR activation.

LXR activation decreases visfatin gene expression induced by inflammatory cytokines

Since the expression of visfatin has been shown to be induced by inflammatory stimuli in macrophages [13], we decided to investigate whether LXR activation could block the induction of visfatin by inflammatory cytokines. Macrophages were activated or not with TNF α or IL1- β during 6h. As expected, visfatin expression was stimulated by these cytokines. Moreover, the stimulatory effect of TNF α on visfatin gene expression was less prominent than IL1- β , consistent with previous data derived from rheumatoid arthritis synovial fibroblasts [14]. Co-treatment of activated-macrophages with T0901317 for 6h repressed the cytokine-induced visfatin expression (figure 2A).

To determine whether LXR activation can also prevent cytokine-induced visfatin expression, macrophages were pre-treated for 6h with T0901317 (1 μ M) and subsequently activated with IL1- β for 2h. Our results show that pre-treatment with LXR ligand reduced the IL-1 β -induced visfatin mRNA (figure 2B).

LXR activation decreases visfatin protein secretion and intracellular NAD⁺ concentration in human macrophages

To determine whether LXR agonists also regulate visfatin protein levels, the secretion of visfatin was analyzed in MDM and ATM treated or not with LXR ligands. Treatment with either T0901317 (1 μ M) or GW3965 (1 μ M) for 24h significantly decreased visfatin release in MDM and ATM (figure 3A and 3B).

To investigate whether the inhibition of visfatin by LXR ligands could affect the intracellular NAD^+ concentration, macrophages were treated or not with T0901317 (1 μM) for 6 or 24h and intracellular NAD^+ level determined by enzymatic assay. LXR activation reduced the basal level of intracellular NAD^+ concentration (figure 3C).

LXR activation decreases PPAR γ -induced visfatin gene expression and protein secretion in human macrophages.

We previously reported that PPAR γ agonists induce the expression and secretion of visfatin in a PPAR γ -dependent manner in human macrophages [9]. To investigate whether LXR activation could repress the induction of visfatin by PPAR γ activation, macrophages were treated with the PPAR γ agonist GW1929 (600 nM) for 24h and further activated for 6h in the presence or absence of T0901317 (1 μM). LXR activation clearly reduced visfatin gene expression and protein secretion induced by the GW929 compound (figure 4A and 4B). These results suggest that a potential negative LXR/PPAR γ cross-talk exists on the regulation of visfatin expression in macrophages.

DISCUSSION

In adipose tissue (AT), visfatin is an inflammatory response cytokine mainly produced by macrophages [1]. In line, visfatin has been suggested to act as an inflammatory mediator, expressed in monocytes and lipid loaded- macrophages within unstable atherosclerotic lesions where it potentially plays a role in plaque destabilization [4, 15]. We previously reported that PPAR γ positively regulates visfatin expression in human macrophages [9]. The objective of this study was to determine whether the nuclear receptor LXR controls the visfatin expression in macrophages.

Our results show that LXR activation with both LXR ligands GW3965 and T0901317 down-regulates the expression of visfatin in macrophages, reaching a maximal effect very rapidly (6h). Interestingly, we also found that LXR activation decreases the expression of visfatin mRNA in murine macrophages (BMDM).

Since visfatin is a visceral adipose fat adipokine mainly produced by ATM [1], visfatin gene expression was compared in monocyte-derived macrophages (MDM) and visceral ATM isolated from the same obese donors. Visfatin mRNA level was found to be 6-fold higher in ATM than in MDM and was also down-regulated by LXR activation. Specifically knock-down of LXR α/β with siRNA demonstrated that the LXR agonists repress visfatin gene expression in a LXR-dependent manner. Previous studies have shown that visfatin expression is induced by inflammatory stimuli such as IL1- β and TNF α in macrophages [13]. Interestingly, pre-treatment or co-treatment of inflammatory macrophages with LXR ligand T0901317 blocked the visfatin gene induction by these cytokines, probably via mechanisms resembling its anti-inflammatory properties. Thus, the regulation of visfatin, itself an “inflammatory cytokine-like” adipokine could contribute to the inflammatory response to inflammatory cytokines such as IL1- β and TNF α via AP-1 and NF- κ B activation [16] and to the anti-inflammatory action of LXR agonists.

LXR agonists reduced visfatin protein secretion and mRNA levels in MDM and ATM to a similar extent. LXR activation reduced intracellular NAD⁺ concentration to a comparable extent as previously observed in NIH-3T3 murine fibroblasts transduced with visfatin specific shRNA, leading to 20-40% of NAD reduction, whereas cells over-expressing visfatin increased total intracellular NAD⁺ levels by 15-25%[17]. We suggest that the slightly decrease of intracellular NAD⁺ concentration by LXR agonists is the consequence of visfatin mRNA reduction. Based on the large body of evidence suggesting a major role for NAD in controlling cell survival, altered NAD metabolism can directly affect macrophage lifespan [17]. NAD⁺ is a cofactor of many deacetylases of the Sirtuin family, especially SIRT1. For instance low to moderate overexpression of SIRT1 in transgenic mouse hearts attenuated age-dependent increases in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers. In contrast, a high level of Sirt1 increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy [18]. SIRT6, another member of the sirtuin family, has been identified as a NAD-dependent enzyme able to increase TNF α production in macrophages by acting at a post-transcriptional level [19]. Taken together these observations suggest that NAD⁺ can exert pro- and/or anti-inflammatory properties depending on the activated sirtuins.

Interestingly, LXR activation blocks visfatin gene expression and secretion induced by PPAR γ . To our knowledge, this is the first gene regulated in an opposite manner by LXR and PPAR γ in macrophages. Further studies are required to determine the molecular mechanisms behind this negative cross-talk between LXR and PPAR γ .

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FIGURE LEGENDS

Figure 1: LXR activation decreases visfatin gene expression in macrophages in a LXR dependent manner.

Human monocyte-derived macrophages (MDM) were incubated or not (control) with (A) LXR ligands T0901317 (1 μ M), GW3965 (1 μ M) for the indicated times, or (B) with T0901317 (0.25, 0.5, 1 μ M) for 24h. (C), Mouse BMDM were treated or not with T0901317 (2 μ M) for 24h. (D), MDM and ATM were isolated from the same obese patients. (E), ATM were treated with T0901317 (1 μ M) or GW3965 (1 μ M) for 24h. (F) Macrophages were transfected with non-silencing control or LXR α/β siRNA and then treated with or without T0901317 (1 μ M) for 24h. Visfatin mRNA was analyzed by Q-PCR and normalized to cyclophilin mRNA. Results are representative of those obtained from 3 independent macrophage preparations and are expressed relative to the control cells set as 1. Each bar is the mean value \pm SD of triplicate determinations. Statistically significant differences are indicated (t-test; * p <0.05; ** p <0.01; *** p <0.001).

Figure 2. LXR activation decreases cytokine-induced visfatin gene expression in human macrophages.

(A) MDM were stimulated for 6h or not with human TNF α or IL-1 β (5 ng/ml) in the presence or in the absence of T0901317 (1 μ M). (B) Macrophages were pre-treated for 6h or not with T0901317 (1 μ M) and then activated with IL-1 β (5 ng/ml) for 2h. Visfatin mRNA was analyzed by Q-PCR and normalized to cyclophilin mRNA. Results are representative of those obtained from 3 independent macrophage preparations and expressed relative to the levels in untreated cells set as 1. Each bar is the mean value \pm SD of triplicate determinations. Statistically significant differences are indicated (control vs cytokines * p <0.05; ** p <0.01; cytokines vs LXR agonists § p <0.05; §§ p <0.01).

Figure 3: LXR activation decreases visfatin secretion and affects intracellular NAD concentration in human macrophages.

MDM (A) and ATM (B) were treated or not with T0901317 (1 μ M) or GW3965 (1 μ M) for 24h and visfatin protein secretion quantified in supernatant by ELISA. (C) Macrophages were treated or not with T0901317 (1 μ M) during 6 or 24h and NAD concentrations measured by an enzymatic cycling reaction assay and normalized to protein levels and expressed as percentage, the control non-stimulated cells being expressed as 100%. Results are representative of 3 independent macrophage preparations. Each bar is the mean value \pm SD of triplicate determinations. Statistically significant differences are indicated (* p <0.05; ** p <0.01; *** p <0.001).

Figure 4: LXR activation blocks PPAR γ -induced visfatin gene expression and secretion in human macrophages.

Macrophages were treated with the PPAR γ ligand GW1929 (600 nM) during 24h followed by the addition of T0901317 (1 μ M) for 6h, or with each compounds alone and visfatin mRNA (A) and protein secretion (B) measured. Each bar is the mean value \pm SD of triplicate determinations. Statistically significant differences are indicated (control vs PPAR γ agonists * p <0.05; ** p <0.01; PPAR γ agonists vs PPAR γ /LXR agonists §§ p <0.05; §§§ p <0.01).

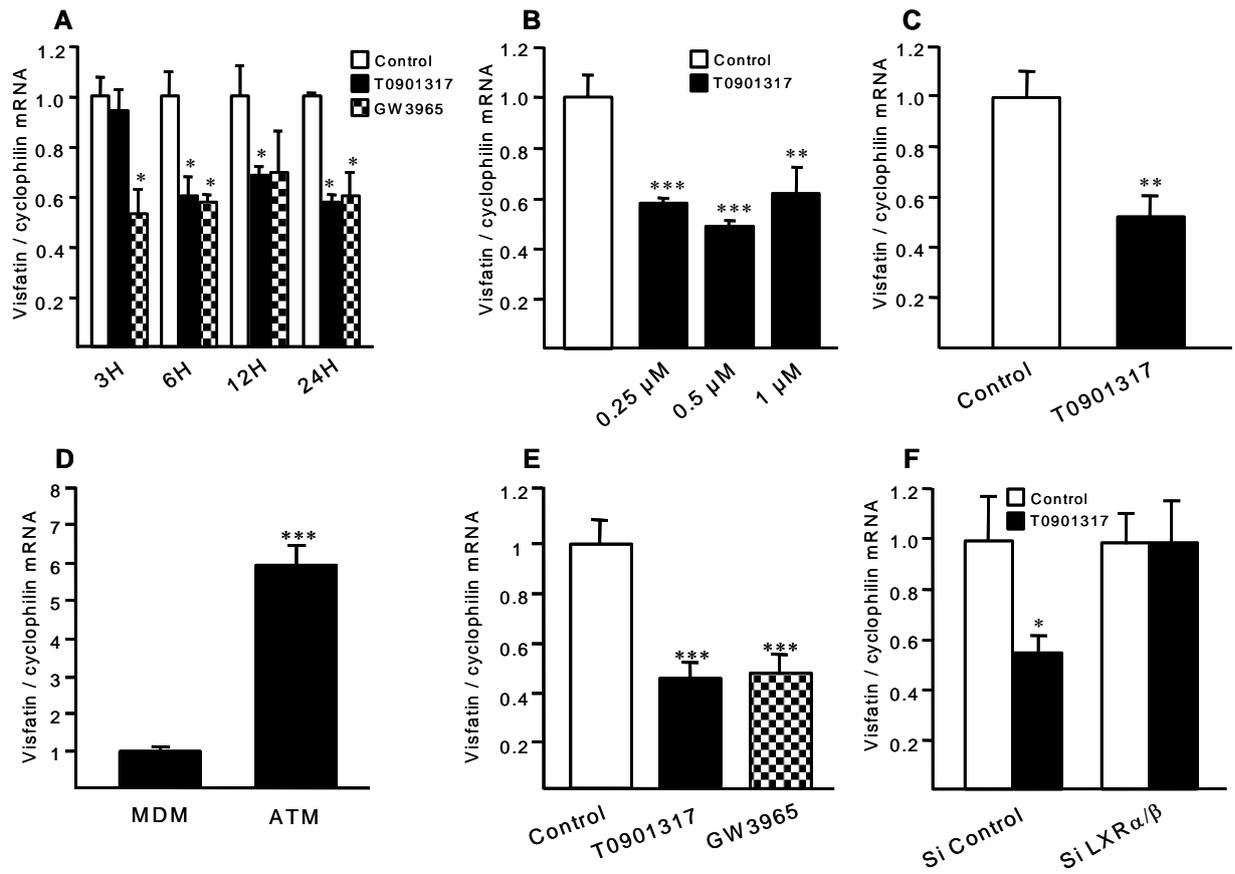


Figure 1

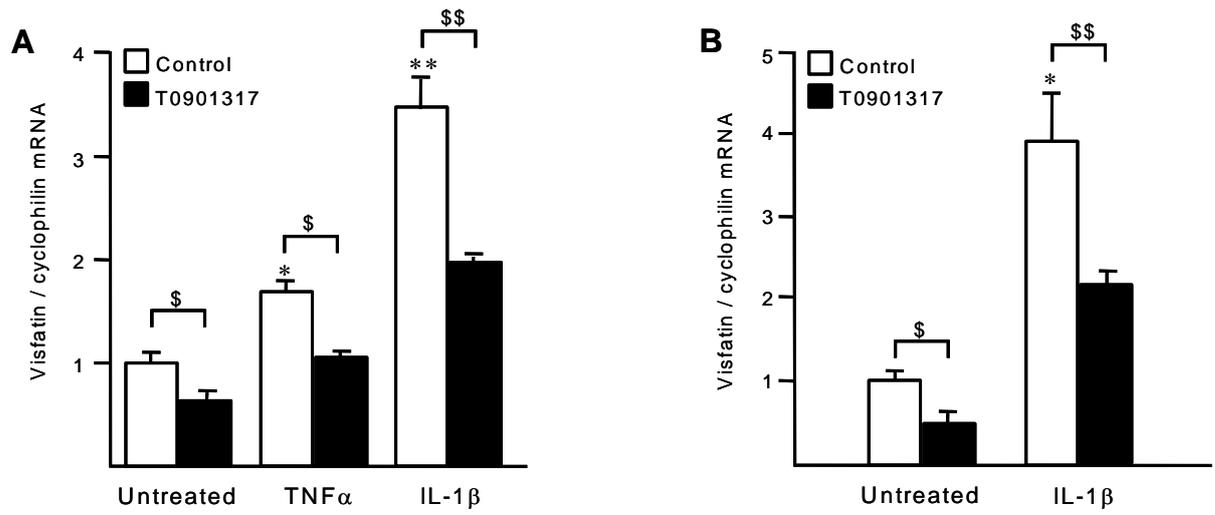


Figure 2

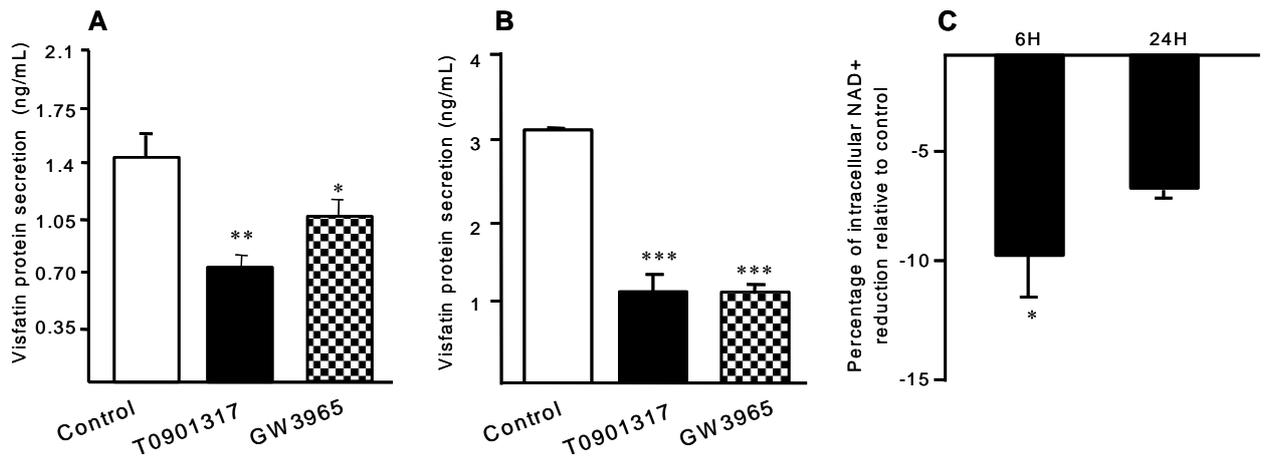


Figure 3

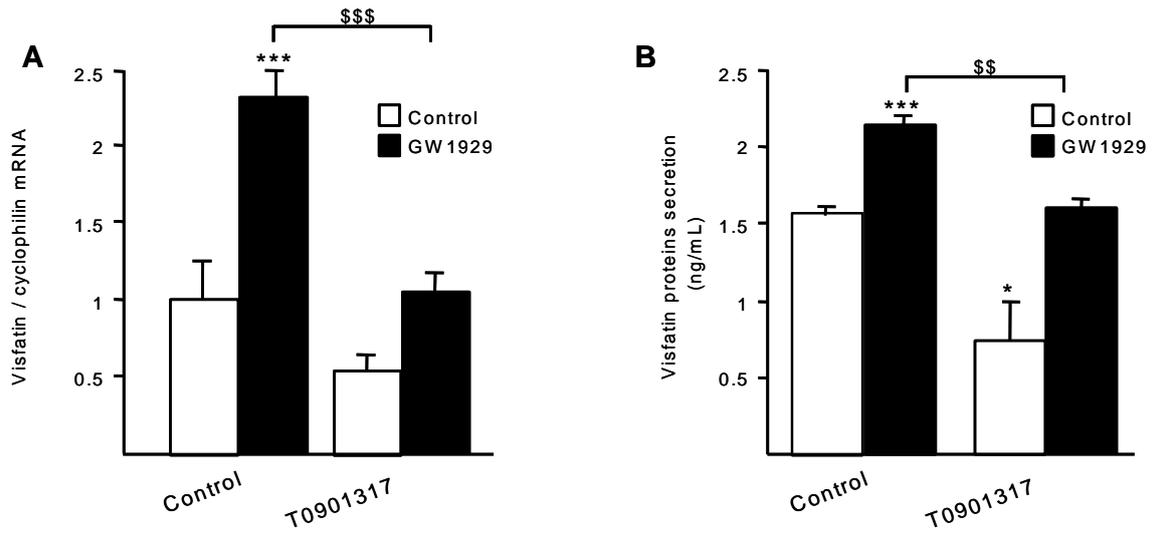


Figure 4