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Simvastatin reduces pentraxin 3 expression and secretion in human endothelial cells

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Acute phase protein pentraxin 3 (PTX3) is increased in patients with atherosclerotic cardiovascular diseases, such as acute coronary syndrome and coronary artery vulnerability, thereby serving as an important biomarker for the prognosis of cardiovascular diseases. This study was designed to evaluate the effects of statins on the expression of PTX3 in human endothelial cells. We observed that PTX3 is a TNFα inducible protein in human endothelial cells. In contrast, treatment with simvastatin reduces basal as well as TNFα induced gene and protein expression of PTX3 in a dose- and time-dependent manner. Our study suggests that reducing PTX3 expression by statins could contribute to its protective effects against endothelial dysfunction and coronary artery plaque destabilization.

1. Introduction

Atherosclerosis is the major cause of cardiovascular and cerebrovascular diseases, which represents a leading cause of death in developed and developing countries (Fang et al. 2018; Libby and Hansson 2015; Rader and Daugherty 2008; Xu et al. 2014). Among all factors causing atherosclerosis, enhanced vascular inflammation and endothelial dysfunction is an initiating key player in all phases of atherosclerosis, from leukocyte adhesion/transmigration, to plaque rupture (Libby et al. 2014). Statins are powerful cardiovascular drugs in treating hypercholesterolemia associated cardiovascular diseases. Previous studies have shown that statins exert protective functions against endothelial dysfunction, the proliferation and migration of smooth muscle cells, foam cell formation, vascular inflammation. Statins also stabilized vulnerable plaques in both mice and human (Zhou and Liao 2009; Ziaeian and Fonarow 2017). The cardiovascular actions of statins are related to both lipid-lowering effects and lipid-independent effects (i.e., pleiotropic effects) (Zhou and Liao 2009).

Pentraxin 3 (PTX3), and two other members of pentraxin family-C reactive protein (CRP) and serum amyloid P, are useful biomarkers for cardiovascular diseases, such as atherosclerosis, angina pectoris, myocardial infarction and heart failure (Casula et al. 2017; Mantovani et al. 2006). Previous studies have shown that the expression and secretion of PTX3 can be stimulated by different inflammatory stimuli, such as TNF-α, IL-1β, and LPS, supporting its critical role in regulating vascular inflammation and innate immunity (Casula et al. 2017; Mantovani et al. 2006). PTX3 is strongly expressed in human atherosclerotic plaques, and colocalized with macrophage and endothelial cells (Napoleone et al. 2002; Rolph et al. 2002; Savchenko et al. 2008). Like high-sensitivity CRP (hs-CRP), PTX3 is significantly upregulated in patients with cardiovascular diseases; therefore, it can be used as a useful early biomarker of cardiovascular diseases (Casula et al. 2017; Mantovani et al. 2006). PTX3 is expressed in major vascular cell types, including endothelial cells, smooth muscle cells, neutrophils, and macrophages (Casula et al. 2017; Mantovani et al. 2006). PTX3 reduces the production of vasodilatory nitric oxide in endothelial cells and directly induce endothelial dysfunction and increase blood pressure in mice via P-selectin and MMP1 pathway (Carrizzo et al. 2015). PTX3 also affects macrophage cholesterol metabolism by increasing lipid uptake and reducing PPAR/LXRa/ABCA1-dependent cholesterol efflux (Liu et al. 2014), two important processes for determining foam cell formation (Xu et al. 2013). Also, the expression of PTX3 is significantly upregulated in patients with acute coronary syndrome, and associates with coronary artery plaque vulnerability, suggesting that PTX3 play an important role in inflammatory disorders including atherosclerosis (Casula et al. 2017; Mantovani et al. 2006).

Statins are HMG-CoA reductase inhibitors which lower total cholesterol and LDL cholesterol by limiting cholesterol synthesis (Zhou and Liao 2009; Ziaeian and Fonarow 2017). Here, we investigated whether PTX3 can be induced by pro-inflammatory cytokine TNFα, and whether simvastatin (Fig. 1) can reduce PTX3 gene and protein expression in human endothelial cells. Our datamining study shows that another lipophilic statin-atorvastatin reduces PTX3 in human endothelial cells. Further studies using simvastatin indicate that simvastatin reduces basal as well as TNFα-induced PTX3 upregulation, suggesting that statins can be exploited as a potential PTX3 inhibitor in treating cardiovascular diseases.

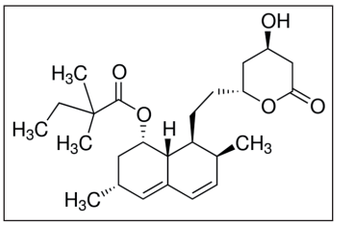

Fig. 1: Chemical structure of simvastatin

2. Investigations and results

2.1. PTX3 is a TNFa inducible protein in human endothelial cells

Previous studies have shown that PTX3 is a pro-inflammatory gene (inducible by multiple inflammatory stimuli) (Rolph et al. 2002). By analyzing the PTX3 gene promoter region, we found two putative NF-kB binding motif in PTX3 promoter (-2000-0 bp) (Fig. 2A). We also found that TNFα stimulated PTX3 expression and secretion in a time-dependent manner, suggesting that PTX3 is a NF-kB target gene (Fig. 2B and C).

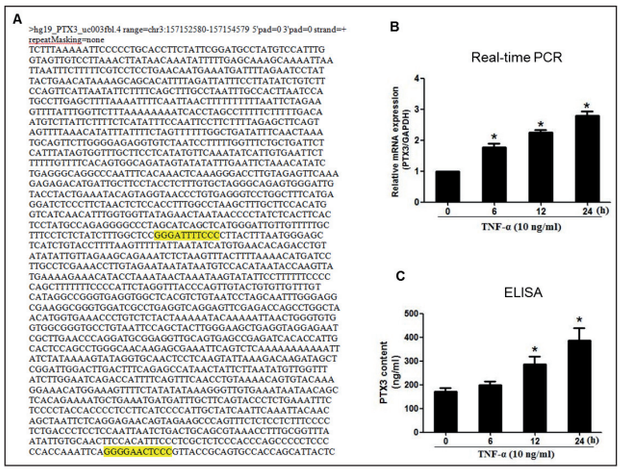

Fig. 2: PTX3 is inducible by TNFα. A, Bioinformatic analysis identified two putative NF-kB binding sites in PTX3 gene promoter. B-C, TNFα induced PTX3 gene (determined by real-time PCR) and protein expression (determined by ELISA) in HUVECs in a dose-dependent manner, n=3, *P<0.05 vs. control (time 0).

2.2. Datamining of atorvastatin on PTX3

Next, we performed a datamining study which investigates the effect of atorvastatin on different endothelial cell transcriptomics (GSE2450) (Boerma et al. 2006). We observed that in both HUVEC and EA.Hy926 endothelial cells, atorvastatin reduces positive control gene CTGF (Eberlein et al. 2001) expression, meanwhile, PTX3 gene expression was also significantly downregulated by atorvastatin (Fig. 3). These data indicate that atorvastatin could affect PTX3 expression in endothelial cells.

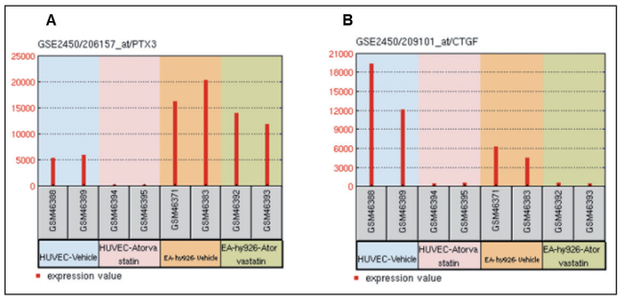

Fig. 3: Datamining of HUVEC and EA.hy926 cells expose to atorvastatin (GSE2450 (Boerma et al. 2006)). PTX3 (A) and CTGF (B) transcript number were selected for presentation. Vehicle was used as control.

2.3. Simvastatin reduces PTX3 mRNA and protein expression in a dose- and time-dependent manner

Next, we evaluated the effect of statin on PTX3 expression under basal conditions. Our real-time PCR and ELISA data have shown that simvastatin reduces PTX3 gene (Fig. 4A and B) and protein expression (Fig. 4C and D) in a dose- and time-dependent manner.

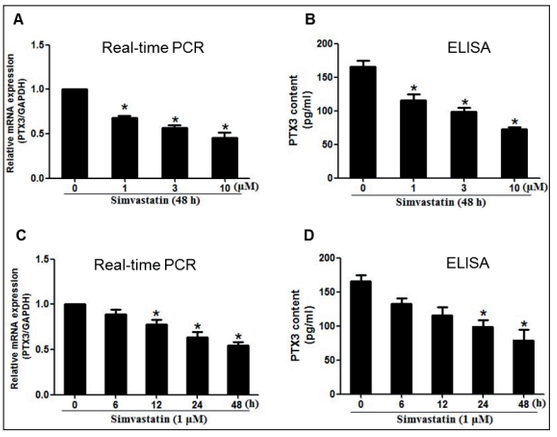

Fig. 4: Simvastatin reduces PTX3 gene and protein expression under basal conditions. HUVECs were treated with vehicle or simvastatin of indicated concentrations or indicated time points before RNA and whole cell lysate were collected for real-time PCR (A-C) and ELISA assay (B-D), respectively, n=3, *P<0.05 vs. control.

2.4. Simvastatin reduces TNFα-induced PTX3 gene and protein expression in a dose- and time-dependent manner

After observing that simvastatin reduces basal PTX3 expression, we next examined whether simvastatin could reduce PTX3 expression in the presence of TNFα, a potent stimuli triggering vascular inflammation. Our data show that simvastatin reduces TNFα induced PTX3 upregulation in HUVECs (Fig. 5).

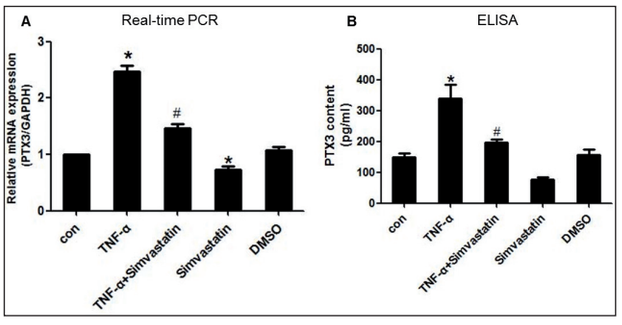

Fig. 5: Simvastatin reduces TNFα-induced PTX3 gene and protein expression. HUVECs were treated with vehicle or simvastatin (1 μM) before stimulation with TNFα for 6 h. RNA and whole cell lysate were collected for real-time PCR (A-C) and ELISA assay (B-D), respectively, n=3, *P<0.05 vs. control.

3. Discussion

Atherosclerotic cardiovascular disease (ACVD), such as atherosclerosis, myocardial infarction etc.) represents the leading cause of death in developed and developing countries (Libby and Hansson 2015; Tabas et al. 2015). A major cause of ACVD is persistent vascular inflammation with limited inflammation resolution (Tabas et al. 2015). Previous studies have shown that PTX3 is an important biomarker and participant of vascular inflammation (Casula et al. 2017; Mantovani et al. 2006). For example, Rolph et al. (2002) have shown that PTX3 is highly expressed in human atherosclerotic plaques, mainly in macrophages and endothelial cells. PTX3 also promotes the expression of tissue factor, thereby participating the coagulation events (Napoleone et al. 2002). In 2011, Yokota et al. have shown that simvastatin reduces the expression of PTX3 and MCP1, two potent mediators of vascular inflammation in rheumatoid fibroblast-like synoviocytes. The downregulating effect of simvastatin on PTX3 expression was fully reversed by mevalonate and geranylgeranylpyrophosphate, rather than farnesyl-pyrophosphate. However, the effect of simvas-

tatin on PTX3 expression and secretion in human endothelial cells remains unknown to date. In light of the potent pro-inflammatory effects of PTX3, we can visualize that downregulating PTX3 could contribute to statin-mediated suppression of vascular inflammation. In the present study, we observed that TNFα promotes PTX3 expression and secretion, supporting PTX3 is a TNFα-inducible protein. Most importantly, we observed that simvastatin reduces basal as well as TNFα-induced PTX3 expression in human endothelial cells. These data, together with recent description of other statins, such as atorvastatin on PTX3 expression (Baetta et al. 2015), collectively suggest that reducing PTX3 expression is a common pharmacological activity of statins.

Pentraxin 3 (PTX3, also known as TNF-inducible gene 14 protein) is a member of the pentraxin superfamily which also include including high-sensitivity C-reactive protein (hs-CRP), serum amyloid P (SAP), and pentraxin 3 (PTX3) (Casula et al. 2017; Mantovani et al. 2006). PTX3 expression can be induced by TNFα and interleukin 1 β (IL-1β) (Casula et al. 2017; Mantovani et al. 2006). PTX3 is produced by several cell types, including dendritic cells (DCs), fibroblasts, smooth muscle cells, endothelial cells (Casula et al. 2017; Mantovani et al. 2006). PTX3 serves as an acute phase response protein, as the blood levels of PTX3, low under normal conditions, but increase rapidly during inflammatory disorders, such as endotoxic shock, sepsis and atherosclerosis (Casula et al. 2017; Mantovani et al. 2006). Increased PTX3 level in the circulating blood is significantly associated with endothelial dysfunction (evidenced by elevated level of asymmetric dimethylarginine) in subjects with non-alcoholic fatty liver disease (Gurel et al. 2016). PTX3 represents a better prognostic marker of PAD than hs-CRP and SAP in evaluating disease severity (Igari et al. 2016). Therefore, PTX3 is a pleiotrophic protein that exerts multiple functions in vascular biology. More therapeutic strategies will be developed in future to identify PTX3 inhibitors in treating cardiovascular diseases.

We recognize that endothelial cells from various sources (vein vs artery) may respond differently. Further functional studies need to be validated in human aortic endothelial cells. Further studies can be performed to evaluate whether hydrophilic statins, such as rosuvastatin can also reduce PTX3 expression in endothelial cells or other vascular cell types, such as smooth muscle cells and macrophages. It remains to be investigated the specific signaling pathways leading to the suppression of PTX3 by statins, and whether overexpression of PTX3 could reverse statin-mediated suppression of vascular inflammation. Due to the fact that there are some conflicting reports showing that PTX3 may also play a protective role under some circumstances, it remains unknown whether reducing PTX3 will cause some unwanted side effects. Last but not least, it will be interesting to investigate whether the PTX3 reducing effects of statins are related to the pleiotropic effects of statins in experimental models and patients with cardiovascular diseases.

In conclusion, we show here that simvastatin reduces PTX3 gene and protein expression in human endothelial cells, which could possibly contribute to statin-mediated anti-atherosclerotic effects. Our study also suggests that PTX3 represent a promising therapeutic target for treating atherosclerosis associated vascular inflammation.

4. Experimental

4.1. Reagents

HUVECs were purchased from Cell Resource Center at the Shanghai Institutes for Biological Sciences (SIBS, Shanghai, China). TNFa was purchased from PeproTech (USA). Simvastatin was purchased from Aladdin(Shanghai, China).

4.2. Cell culture

HUVECs were obtained from Shanghai Institutes for Biological Sciences (SIBS, Shanghai, China). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Scientific). Cells were incubated at 37 °C in 5% CO_2 (Thermo). TNF-α-induced HUVEC inflammation model was established by stimulation with 10 ng/mL TNF-α for different time points. Then, the effect of simvastatin on TNF α induced PTX3 expression and secretion was determined. To test the effect of simvastatin on PTX3 basal expression and secretion, HUVECs were incubated with indicated concentrations of simvastatin for 24 h before RNA and supernatant was collected for real-time PCR and ELISA, respectively.

4.3. Real-time PCR

After treatment with simvastatin with or without TNFα, total RNA was extracted using Trizol reagent. RNA concentration was measured by a Spectrophotometer (Thermo Scientific) by calculating from OD_{260}. After that, total RNA was reverse transcribed to cDNA using a Reverse Transcription Kit from Thermo Scientific following the manufacturer's instructions. Quantitative real-time PCR was then performed with a Bio-Rad iQ5 real-time PCR thermal cycler, using iQ SYBR Green Supermix (Bio-Rad) for relative mRNA quantification. The sequences of all the primers used were: PTX3 forward: CATTGTTCCTGAGGGAGGAATC; PTX3 reverse: AGTCTCCCAGAGAAGGCTAAT; GAPDH-forward: GATTCCACCCATGGCAAATTC; GAPDH-reverse: CTGGAAGATGGTGATGGGATT. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative mRNA expression of target genes. GAPDH was used as the loading control (Wu et al. 2010).

4.4. ELISA

After treatment with TNFα with or without simvastatin for indicated concentrations, PTX3 secretion in the culture supernatant was measured by enzyme-linked immunosorbent assay in accordance with the manufacturer's instructions (Hangzhou Multi-Sciences Biotech, Zhejiang,China).

4.5. Data mining

Data were mined from NCBI GEO database (GSE2450) (Boerma et al. 2006) as previously described (Xu 2017). Top 250 genes regulated by atorvastatin were included in analysis. The expression of PTX3 and CTGF were selected in HUVECs and EA.Hy926 endothelial cell lines exposed to atorvastatin.

4.6. Statistical analysis

All data were expressed as means±SEM. Statistical analysis was performed using GraphPad Prism software. Student's t test and one-way analysis of variance (ANOVA) with post hoc Bonferroni tests were used for comparisons between two groups and multiple comparisons, respectively. In all cases, differences were considered statistically significant with $P < 0.05$.

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Conflicts of interest: None declared.

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