Original Article
α-Naphthoflavone modulates inflammatory response in adipocytes-macrophages interaction through NFκB signaling

Yanmei Sun¹, Minghua Xie², Tingting Huang², Xu Zhang², Sicong Lei², Qun Shi², Suqing Wang², Cuifang Fan¹*, Jie Zhang³,⁴*

¹Department of Obstetrics and Gynecology, Renmin Hospital, Wuhan University, China; ²Department of Nutrition and Food Hygiene, School of Public Health, Wuhan University, China; ³Department of Urology, Renmin Hospital, Wuhan University, China; ⁴Hubei Key Laboratory of Kidney Disease Pathogenesis and Intervention, Huangshi, China. *Equal contributors.

Received August 31, 2014; Accepted October 17, 2014; Epub October 15, 2014; Published November 1, 2014

Abstract: Objective: Our previous study demonstrated that α-naphthoflavone (α-NF) inhibits mouse 3T3-L1 pre-adipocytes differentiation via PPARγ, a key transcription factor in adipogenesis. Due to the critical role of inflammation in adipogenesis, we speculated that the suppression role of α-NF in adipogenesis might involve in modulation of cytokines secretion raised by adipocyte differentiation cocktail. Therefore, the present study aims to investigate the role of α-NF in modulating of inflammatory response during adipocytes differentiation and adipocyte-macrophage interaction. Methods: Conditioned medium from different doses of α-NF treated 10-day differentiated 3T3-L1 adipocytes were collected to culture RAW264.7 macrophages. Conditioned medium from activated macrophages and α-NF pre-treated macrophage were used to investigate the effects of α-NF in adipocytes differentiation. Cultured cells and medium were harvested for RT-PCR, Western blot and ELISA. Results: α-NF dose-dependently decreased TNF-α and IL-6 and increased IL-10 expression induced by IDM (Insulin, dexamethasone, isobutylmethylxanthine) in 3T3-L1 pre-adipocytes. Conditioned medium from α-NF treated 3T3-L1 differentiated cells inhibited inflammatory response in mouse macrophage cell line RAW264.7 in contrast to IDM control medium. NFκB activation elicited by IDM was suppressed by α-NF in a dose-response manner. Consequently, decreased TNF-α and increased IL-10 secretion, downstream targets of NFκB signaling pathway, were observed with α-NF in macrophages. Finally, Conditioned medium from α-NF pre-treated, LPS-activated macrophages ameliorated the suppression of 3T3-L1 adipogenesis by LPS activated macrophages. Conclusion: Our results suggest that α-NF regulates inflammation response in both adipocytes and macrophages and adipocyte-macrophage interaction which contributes to pre-adipocyte differentiation.

Keywords: Adipocyte-macrophage interaction, α-NF, condition medium, inflammation

Introduction

Obesity is a state of low-grade chronic inflammation characterized by abnormal cytokine production, increased synthesis of acute phase reactants and activation of pro-inflammatory signaling pathways [1-3]. The imbalance of pro- and anti-inflammatory status has been linked to an increased risk of developing insulin resistance, type 2 diabetes and cardiovascular diseases [4-6]. Moreover, macrophage infiltration is a vital event in the initiation of pathologic obesity and macrophage secreted factors impair human adipogenesis [7]. Available evidence from obese mice and humans revealed that macrophages, adipocytes and pre-adipocytes produce a variety of adipokines and cytokines including TNF-α and IL-6 [8], contribute to the elevation of circulating inflammatory markers in obesity. Local interaction under different conditions [9, 10] suggesting the cross-talk between adipocytes and macrophages is a potential mechanism that aggravates inflammatory changes in obese adipose tissue.

Flavonoids are a large family of plant secondary metabolites that are typical dietary component although they are not considered as nutritive
α-Naphthoflavone modulates adipocyte-macrophage interaction

elements. They have a wide arrange of biological activities including anti-oxidative, anti-inflammatory, and anti-cancer [11-13]. Alpha-naphthoflavone (α-NF) is a synthetic flavonoid and used as an antagonist for the aromatic hydrocarbon receptor (AhR) [14]. Our previous study demonstrated that α-NF inhibit IDM induced 3T3-L1 adipogenesis [15]. Due to the important role of adipocyte-macrophage interaction in pre-adipocyte differentiation and obese development, we hypothesize that the suppression of α-NF on 3T3-L1 differentiation might involve in the modulation of inflammation in adipocyte-macrophage interaction. Therefore, the present study is to investigate the role of α-NF in the inflammatory response during 3T3-L1 adipogenesis, the interaction between adipocytes and macrophages, and the possible underlying mechanism.

Materials and methods

Cell culture and preparation of conditioned medium

Mouse 3T3-L1 pre-adipocyte was from the Chinese Academy of Science (Shanghai, China) and Mouse macrophage cell line RAW264.7 was a kind gift from Prof. Ouyang Jingping (Wuhan University). Mature adipocytes were differentiated from 3T3-L1 pre-adipocytes as described in our previous study [15]. Adipocyte-conditioned medium was prepared by differentiating 3T3-L1 cells in 6-well plates for 10 days with different does of α-NF treatment and control medium was from IDM differentiated cells alone. Macrophage-conditioned medium was collected from the 24 h LPS activated RAW264.7 macrophage with or without α-NF pre-treatment. Control medium was RPM1-10% FBS kept at 37°C for 24 h in the absence of macrophages. All conditioned media were pooled from at least three individuals and store at -80°C until used.

Measurement of secreted cytokines by ELISA

TNF-α, IL-10 levels in cells culture medium were measured with ELISA kits according to the manufacturer’s instructions (R&D systems).

qRT-PCR

Cells harvested in 1 ml of Trizol reagent (Invitrogen), RAN extraction and qRT-PCR were performed as previously described [15]. Generally, 1 µg of total RNA was used for cDNA synthesis and qPCR was performed in 96-well plates with the SYBR Green kit (ABI) in a Step-one Plus real-time PCR detection system. Gene expression was quantified by the comparative cycle threshold method. Details of primer sets are available upon request.

Western blot

Whole cell lysates were isolated as previously described [15]. A total of 50 µg protein was separated by 12% SDS-PAGE. Western blot was performed using antibody against NFκB (1:2000, CST) and β-actin (1:5000, Sigma).

Figure 1. α-NF suppressed pro-inflammatory cytokines, while increased anti-inflammatory cytokines expression during 3T3-L1 adipogenesis. A: The inflammatory cytokines expression after 10 days differentiation in 3T3-L1 cells. B: The concentration of inflammatory cytokines after 10 days differentiation in 3T3-L1 cells cultured medium. *: Compared with non-treatment (NT) group, P < 0.05; **: Compared with NT group, P < 0.01; #: Compared with IDM group, P < 0.05; ##: Compared with IDM, P < 0.01.
α-Naphthoflavone modulates adipocyte-macrophage interaction

Oil red O staining and quantification

Detail performance and measurement were described elsewhere [15]. The critical points in ORO are: Working solution should be prepared freshly and filtered by a 0.45 μm filter; ORO dye outside the cells should be wash out by running water before quantification.

Statistical analysis

Data were presented as the mean ± SEM and analyzed with one-way ANOVA by PRISM. The post-hoc tests were performed once ANOVA revealed significant. Statistical significance was set at \( P < 0.05 \).

Results

α-NF inhibits pro-inflammatory cytokines expression in 3T3-L1 cells upon differentiation

Our previous study showed that α-NF inhibited adipogenesis in 3T3-L1 pre-adipocytes. Considering the key role that the inflammatory cytokines play in the pre-adipocytes differentiation and obesity related insulin resistance, we measure the effects of α-NF on the inflammatory cytokines in 3T3-L1 cells during adipogenesis. Our results showed that IDM cocktail significantly increased pro-inflammatory cytokines TNF-α and IL-6, and repressed anti-inflammatory cytokine IL-10 in both mRNA expression and secretion (Figure 1A, 1B). In contrast to IDM cocktail, α-NF exhibited reverse effects on these cytokines expression and production (Figure 1A, 1B), although it did require higher dose of α-NF for IL-6 and IL-10 expression to reach the significant level.

Conditioned medium from α-NF treated 3T3-L1 differentiated cells suppresses pro-inflammatory cytokines production in RAW264 macrophages

Adipocytes-macrophages interaction plays a vital role in initiation and progression of obesity and associated chronic diseases. α-NF suppressed pre-adipocytes differentiation at dose-
α-Naphthoflavone modulates adipocyte-macrophage interaction

7771

and it also modulated inflammatory response induced by IDM differentiated cocktail IDM (Figure 1). Therefore, we collected 3T3-L1 adipocytes conditioned medium (αCM) after 10 days differentiated with different dose of α-NF treatment to culture RAW-264.7 macrophages for 24 hours. We found that αCM up-regulated the pro-inflammatory cytokines expression in macrophage, α-NF dose-dependently repressed TNF-α and IL-6 expression compared with IDM control (Figure 2A). ELISA revealed the similar impact of α-NF on TNF-α and IL-10 production in condition medium (Figure 2B).

Conditioned medium from α-NF treated 3T3-L1 differentiated cells inhibits NF-κB activation in RAW264 macrophages

TNF-α, IL-6 and IL-10 are downstream targets of NF-κB. The modulating role of α-NF in TNF-α, IL-6 and IL-10 production prompts us to observe the NF-κB activation. NF-κB is a canonical pro-inflammatory signaling pathway. Once activated by the extracellular signals, NF-κB rapidly translocates from the cytoplasm to the nucleus and activates target gene expression. We found significantly elevated nucleus NF-κB and concurrently repressed cytoplasm NF-κB expression after IDM hormone cocktail treatment (Figure 2C, 2D). α-NF suppressed NF-κB activation induced by IDM in dose-dependent manner (Figure 2C, 2D).

To further investigate the role of α-NF in modulating NF-κB target genes, we used pyrrolidine dithiocarbamate (PDTC), a specific antagonist of NF-κB, to block NF-κB activation. We found that PDTC did suppress IDM induced NF-κB activation and α-NF exerted a synergistic effect (Figure 3A, 3B). For the downstream target genes expression, α-NF and PDTC exhibited synergistic suppression on TNF-α production (Figure 3D), but not on its mRNA expression (Figure 3C). Furthermore, PDTC ablated IL-10 secretion elicited by α-NF (Figure 3D), suggest-
α-Naphthoflavone modulates adipocyte-macrophage interaction

α-NF pre-treated macrophages conditioned medium improves 3T3-L1 cells adipogenesis suppressed by LPS

To study the effects of α-NF in adipocyte-macrophage interaction on pre-adipocyte differentiation, we collected macrophage conditioned medium (mCM) from LPS activated RAW264 macrophages (100 ng/ml LPS, mCM) and α-NF (5 mg/ml) pre-treated activated macrophages (LPS+ α-NF, amCM) to culture 3T3-L1 cells with IDM for 8 days. Results showed that mCM from LPS activated macrophages significantly inhibited 3T3-L1 cells differentiation induced by IDM, while amCM from α-NF pre-treated activated macrophage partly improved this inhibition (Figure 4A, 4B). Real-time RT-PCR revealed that the key transcription factor PPARγ in adipogenesis was suppressed by mCM and partially rescued by α-NF pre-treatment (Figure 4C). We further observed that the pro-inflammatory cytokines (TNF-α, IL-10) expressions were up-regulated by LPS but repressed by α-NF pre-treatment in RAW264.7 macrophages (Figure 4D). ELISA revealed that α-NF production in macrophage conditioned medium was consistent with mRNA expression (Figure 4E).

Discussion

The present study demonstrated α-NF not only suppresses inflammatory response in 3T3-L1 adipocytes upon differentiation, also dramatically change pro-inflammatory and anti-inflammatory balance in macrophages in the presence of mature adipocytes conditioned medium. Further, α-NF suppresses IDM induced inflammation through deactivating NFκB signaling in macrophages. Meanwhile, α-NF ameliorates the inhibition of adipogenesis by LPS activated macrophage conditioned medium.

These findings suggest that α-NF can suppress inflammatory response and regulate adipocyte differentiation and inflammation through a mechanism including NFKB. α-NF, as a structural analog of flavone, is an antagonist to the aromatic hydrocarbon receptor (AhR) [16] and a potent antiplatelet flavonoid [17]. α-NF is also reported to induce vasorelaxation in endothelium [14] and attenuate B[a]P-induced migration and invasion of vascular smooth muscle cells [18]. Adipose tissue inflammation has been regarded as an important central event in the initiation and maintenance of obesity via adipogenesis. α-NF suppressed inflammatory response during adipogenesis and also in macrophages which elicited by mature adipocytes
α-Naphthoflavone modulates adipocyte-macrophage interaction

condition medium. In particular, α-NF decreased pro-inflammatory cytokines TNF-α, IL-10 expressions and increased anti-inflammatory cytokine IL-10 secretion. Pro-inflammatory cytokines, such as TNF-α and IL-6 are major regulators of adipose tissue metabolism. TNF-α can reduce lipid accumulation via inhibition of lipoprotein lipase and stimulation of hormone sensitive lipase. TNF-α also suppresses glucose uptake via GLUT4 and IRS-1. IL-10, as an anti-inflammatory cytokine, is essential for maintaining the integrity and homeostasis of adipose tissue by repressing pro-inflammatory responses and limit unnecessary tissue damage caused by inflammation.

Numerous studies suggest that cross-talk between adipocytes and macrophages promote pro-inflammatory cytokines production [19]. Macrophages in obese individual stimulated by pro-inflammatory cytokines lead to insulin resistance and macrophages block insulin action in adipocytes [20]. Further, macrophages in adipose tissue inhibit human pre-adipocytes differentiation via repression of transcriptional factors involved in adipogenesis [21]. Our finding showed that fully differentiated adipocytes CM stimulated pro-inflammatory responses in macrophage and α-NF suppressed this response in dose-dependent manner. Notably, α-NF elevated anti-inflammatory cytokines IL-10 release which repressed by aCM. The effects of α-NF on inflammatory responses in macrophages were parallel with NFκB deactivation. PDTC, as a selective NFκB inhibitor, abolished α-NF elicited IL-10 expression and secretion suggesting the important role of NFκB signaling in α-NF regulation.

TNF-α, mainly secreted by macrophages, is a strong suppressor of adipogenesis [22]. Conditioned medium from activated macrophages stimulated by LPS dramatically inhibited 3T3-L1 cells differentiation with concurrent suppression on PPARγ, in agreement with previous reports. Conditioned medium from macrophages, which pre-treated with α-NF, not only significantly suppressed TNF-α secretion, also increased PPARγ expression. However, 3T3-L1 differentiation repressed by mCM was only partially restored by α-NF pre-administration. Macrophages produce a huge variety of biomolecules including cytokines, chemokines and growth factors, it cannot be ruled out that other macrophage secreted factors may also be induced during the adipocytes-macrophages interaction. Furthermore, extracellular matrix remodeling plays a vital role in adipogenesis [23, 24] and decreased expression of fibronectin is the characteristics of differential initiation in 3T3-L1 pre-adipocytes. Cytokines secreted by activated macrophage might participate in regulating fibronectin expression.

In summary, our findings demonstrated that α-NF regulates inflammation responses in adipocytes-macrophages interaction which contributes to pre-adipocytes differentiation via NFκB pathway.

Acknowledgements

This work was supported by Hubei Provincial Natural Science foundation (2012FFB04428) and Hubei Provincial Health foundation (X6-B63). Suqing Wang was supported by National Natural Science Foundation of China (3097-2463).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cuifang Fan, Department of Obstetrics and Gynecology, Renmin Hospital, Wuhan University, China. Tel: 13971050592; E-mail: 359568289@qq.com; Dr. Jie Zhang, Department of Urology, Renmin Hospital, Wuhan University, China. Tel: 13707233066; E-mail: zhangjie888@sian.com

References

α-Naphthoflavone modulates adipocyte-macrophage interaction


