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Overexpressing arginase-II in pre-adipocytes promotes IL-6 production through p38mapk leading to vascular endothelial inflammatory activation

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Abstract: Both stromal cells including pre-adipocytes and adipose tissue macrophages are involved in adipose inflammation that is mechanistically linked with aging-associated diseases including atherosclerosis and type-II diabetes. Our previous studies demonstrated that arginase-II (Arg-II) promotes macrophage inflammation and endothelial dysfunction contributing to the development of these diseases. Here we investigate the role of Arg-II in pre-adipocyte inflammation and its interaction with endothelial cells**.** Overexpressing Arg-II in 3T3-L1 preadipocytes up-regulates IL-6 with concomitant activation of p38mapk, which is mitigated by the p38mapk inhibitor SB203580. Moreover, 3T3-L1 cells overexpressing Arg-II conditioned medium enhances vascular adhesion molecule-1 (VCAM-1) in endothelial cells, which is inhibited by neutralizing anti-IL-6 antibody. Thus, Arg-II promotes IL-6 production in pre-adipocytes through activation of p38mapk, leading to inflammatory endothelial activation.

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Introduction

Aging represents an important challenging health topic of our society [1]. Aging is highly associated with insulin resistance, type-2 diabetes (T2D), and cardiovascular disease [2]. It has been proposed that chronic low-grade systemic inflammation is the mechanism linking aging to the mentioned diseases [3, 4]. Studies demonstrate that chronic systemic inflammation in aging occurs and associates with endothelial activation [5, 6], which is characterized by enhanced endothelial expression of inflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) that facilitate inflammatory cell adhesion to endothelial cells and further migration of these inflammatory cells into the vascular wall, leading to initiation and progression of vascular disease [7, 8].

Moreover, aging is associated with increased levels of pro-inflammatory cytokines including interleukin-1 (IL-1 , tumor necrosis factor- (TNF-), and IL-6 [9, 10]. Evidences show that adipose tissue inflammation is an important contributor not only to the obesity- but also to age-associated chronic inflammatory status [11-15]. It has been

suggested that adipose tissue is one of the major sources of inflammatory cytokines including IL-6 in obesity and in aging [16, 17]. Adipose tissue contains adipocytes and stromal vascular fraction (SVF), the latter produces major pro-inflammatory mediators when the adipose tissues get inflamed [11, 13]. The dominant cells in SVF are leukocytes and adipose-tissue stromal cells (ATSCs). The former include adipose tissue macrophages (ATMs) and lymphocytes, whereas the latter are mainly fibroblasts and preadipocytes. It has been reported that ATMs produce almost all adipose tissue TNF-α and that preadipocytes are primary source for adipose tissue IL-6 (representing 90% of adipose IL-6 production) [13]. In addition, both of these cytokines can induce endothelial activation, promoting atherosclerotic cardiovascular disease [18], suggesting an age-associated adipose tissue pro-inflammatory status which may link aging to age-associated vascular disease.

Studies in recent years including our own demonstrate that the L-arginine: urea hydrolase arginase is involved in cardiovascular aging and age-associated diseases [19-27]. Two isoforms of arginase exist in the organism, which are encoded by separate genes, i.e., the cytoplasmic type-I arginase (Arg-I) and mitochondrial type-II arginase (Arg-II) [28]. Both isoforms have similar biological functions, one of which is involved in L-arginine metabolism. Arg-I is most abundantly and constitutively expressed in hepatocytes and can be induced in many extrahepatic tissues or cells [29-31]. The primary function of Arg-I is detoxification of ammonia through the urea cycle in the liver [32]. Arg-II is widely expressed in many extrahepatic tissues with the most abundance in the kidney and prostate [31, 33, 34]. Our recent studies report that Arg-II promotes endothelial senescence and inflammation as well as macrophage inflammation, contributing to insulin resistance and atherogenesis [20, 35]. However, a role of Arg-II in adipose tissue inflammation in aging and its interplay with endothelial activation has not been investigated, yet.

p38mapk is a serine/threonine kinase and works as stress sensor and plays a role in activation of transcription factors and *de novo* transcription, stabilization of mRNA and induction of translation [36]. This pathway is also involved in pro-inflammatory cytokine biosynthesis (e.g. TNF-α, IL-1β and IL-6) [37, 38], matrix metalloproteinases (MMPs) and adhesion molecule expression [39] as well as endothelial cell senescence in vascular aging [21].

In the present study, we provide evidence showing that Arg-II activates p38mapk promoting production of IL-6 in preadipocytes, leading to increased endothelial inflammatory adhesion molecule expression.

Materials and Methods

Materials

All chemicals including those used for immunoblotting and anti-tubulin (T5168) antibody were obtained from Sigma (Buchs, Switzerland); RPMI-1640 was purchased from Amimed (Muttenz, Switzerland); p38mapk inhibitor SB203580 (4-[4-Fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]1H-imidazole) were purchased from Calbiochem (Lucerne, Switzerland); Rabbit anti-phospho-p38mapk (Thr180/Tyr182) and mouse anti-p38mapk antibodies were from Cell Signaling Technology (Allschwil, Switzerland); Bio-Rad DC™ protein assay kit was from Bio-Rad Laboratories (Basal, Switzerland); Alexa Fluor680-conjugated anti-mouse IgG (A21057) was from Molecular Probes / Invitrogen (Lucerne, Switzerland); IRDye800-conjugated anti-rabbit IgG (926-32211) were from LI-COR Biosciences (Bad Homburg, Germany); Endothelial cell growth supplement (ECGS) pack was from PromoCell GmbH (Allschwil, Switzerland); Neutralizing rat anti-mouse-IL-6 and rat IgG1 control antibody were from eBioscience (Vienna, Austria).

Cell Culture and Transduction of 3T3-L1 preadipocytes with Recombinant Adenoviral (rAd)

The mouse preadipocyte cell line 3T3-L1 (American Type Culture Collection) was maintained in low glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (FCS). Human umbilical vein endothelial cells (HUVECs) were cultivated as described [35]. For adenoviral transduction, 3T3-L1 cells were transduced with rAds at a multiplicity of infection of 500 as described [40]. To enhance transduction efficiency, rAds were incubated with serum-free medium containing 0.5 μg/ml polylysine (Buchs,Switzerland) for 100 min prior to the addition to 3T3-L1 cells that were pre-washed with phosphate-buffered saline [40]. After incubation at 37℃ for 4 hours with rotation, the fresh medium was added and cells were cultured for an additional 3 days. At day 3 post transduction, cells were serum-starved in 0.2% BSA-DMEM for overnight before the experiments.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Two-step qRT-PCR analysis was performed to evaluate mRNA expression of the endogenous IL-6 in 3T3-L1 cells as described previously [41]. Total RNA was extracted from cells with Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the supplier's protocol. The mRNA expression levels of all genes were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences of mouse origin (m) are as follows:

mIL6F: GACAACCACGGCCTTCCCTA;
mIL6R: GCCTCCGACTTGTGAAGTGGT.
mArg-IIF: CCCCTTTCTC TCGGGGACAGAA;
mArg-IIR: GAAAGGAAAGTGGCTGTCCA.
mGAPDHF :ACCCAGAAGACTGTGGATGG;
mGAPDHR :ACACATTGGGGGTAGGAACA.

Enzyme-Linked Immunosorbent Assay (ELISA)

The protein level of proinflammatory mediators in the conditioned medium of DMEM containing 0.2% BSA was evaluated by using the ELISA MAX Deluxe from BioLegend, according to the manufacturer's instructions (Lucerna Chem AG, Luzern, Switzerland).

Immunoblotting Analysis

Cell lysate preparation, SDS-PAGE, transfer of protein from within the SDS gels to an Immobilon-P membranes (Millipore) were performed as previously described [42]. The resultant membrane was incubated overnight with the corresponding first antibody at 4°C with gentle agitation after blocking with 5% skimmed milk. The protein was then decorated with corresponding anti-mouse (Alexa Fluor 680–conjugated) or anti-rabbit (IRDye 800–conjugated) secondary antibodies. Signals were visualized with Odyssey Infrared ImagingSystem (LI-COR Biosciences). Quantification of the signals was performed in NIH Image 1.62 software.

Interplay between 3T3-L1 cells and endothelial cells

HUVECs were maintained in RPMI-1640 medium supplemented with 5% FCS and ECGS [43]. Cells of passage 1 to 2 (P1 to P2) were treated for 4h with conditioned medium (DMEM containing 0.2% BSA) of 3T3-L1 cells. HUVECs extracts were then prepared and subjected to immunoblotting analysis for expression of VCAM-1 and ICAM-1.

Antibody blocking studies

The conditioned medium of 3T3-L1 cells was pre-treated with control IgG or neutralizing anti-IL-6 antibody for 2h at 37℃ prior to the addition to the HUVECs. HUVECs were then incubated for 4h with this conditioned medium as described above.

Statistics

Data are given as mean ± SEM. In all experiments, n represents the number of experiments or animals. Statistical analysis was performed with unpaired t-test or ANOVA with Bonferroni post test. Differences in mean values were considered significant at $P < 0.05$.

Results

Arg-II promotes IL-6 expression in 3T3-L1 preadipocytes

Our previous study showed that Arg-II promotes endothelial dysfunction and macrophage pro-inflammatory responses, contributing to insulin resistance and atherosclerosis [20, 35]. Here we show that overexpression of Arg-II in 3T3-L1 preadipocytes (**Fig. 1A**) enhanced expression of IL-6 at both mRNA (**Fig. 1B**) and protein levels (**Fig. 1C**). No increase in TNF-α or MCP-1 was, however, detectable (data not shown).

Arg-II promotes IL-6 expression through p38mapk in 3T3-L1 preadipocytes

To evaluate the potential mechanism by which Arg-II promotes proinflammatory responses in preadipocytes, we then examined effects of Arg-II on p38mapk activation. As shown in **Fig. 2A**, overexpression of Arg-II in 3T3-L1 cells induced a time dependent activation of p38mapk as measured by phosphorylation of p38mapk at Thr180/Tyr182 (p-p38mapk), which reached the highest level at 60 to 84 hours post-transduction. Moreover, inhibition of p38mapk with the specific inhibitor SB203580 (10 μmol/L, 84 hours) prevented Arg-II-induced IL-6 protein production in the cells (**Fig. 2B**). These results demonstrate that Arg-II promotes IL-6 production in 3T3-L1 preadipocytes through p38mapk signalling pathway.

Preadipocytes overexpressing Arg-II upregulate endothelial VCAM-1 expression through IL-6

Next we studied the interplay between preadipocytes and endothelial inflammation. For this purpose, conditioned medium from 3T3-L1 preadipocytes overexpressing Arg-II was collected at 84 hours post-transduction with or without treatment with the p38mapk inhibitor SB203580 (10 μmol/L, 84 hours) and applied to human endothelial cells in culture for 4 hours. Conditioned medium from cells overexpressing Arg-II without SB203580 treatment strongly upregulated endothelial VCAM-1 but not ICAM-1 expression, which was not observed when endothelial cells were treated with conditional medium from 3T3-L1 cells overexpressing Arg-II but treated with p38mapk inhibitor SB203580 (**Fig. 3A**). Moreover, incubation of the conditioned medium from preadipocytes overexpressing Arg-II with a neutralizing antibody against IL-6 (10 μg/ml) for 2 hours reduced endothelial VCAM-1 expression (**Fig. 3B**). These results demonstrate that Arg-II promotes IL-6 expression and secretion in pre-adipocytes through activation of p38mapk, leading to enhanced VCAM-1 expression in endothelial cells.

Discussion

Previous studies demonstrated that Arg-II is upregulated in endothelial cells in aging, which promotes endothelial senescence accompanied with eNOS-uncoupling and upregulation of adhesion molecules in aging [20, 21] and Arg-II is also upregulated in macrophages and promotes pro-inflammatory cytokine release including IL-6 and contributes to insulin resistance and glucose intolerance in obesity and atherosclerosis [35]. In the present study, we further show a pro-inflammatory function of Arg-II in preadipocytes, resulting in amplification of endothelial activation. Overexpression of Arg-II in

3T3-L1 pre-adipocytes significantly upregulates expression and release of the pro-inflammatory cytokine IL-6. This is the first report showing the pro-inflammatory role of Arg-II in pre-adipocytes.

This effect of Arg-II in stimulation of IL-6 in the cells is accompanied with activation of p38mapk. Inhibition of p38mapk with the specific inhibitor SB203580 abolishes the IL-6 release, demonstrating that Arg-II enhances IL-6 production in preadipocytes through p38mapk pathway. The crosstalk between Arg-II and p38mapk has also been found in endothelial cells in obesity and aging, which is involved in endothelial dysfunction under these conditions [21, 44]. The underlying mechanisms of how Arg-II activates p38mapk in the cells remain unknown. An enhanced p38mapk activity has been demonstrated in adipocytes from type 2 diabetic patients [45] and in the heart and liver of high-fat diet-fed rats [46] and also in endothelial dysfunction and senescence [21, 44, 47], which points toward the importance of p38mapk in endothelial dysfunction as well as in functional changes of adipocytes.

Another important evidence we provide in this report is the functional consequence of Arg-II-induced preadipocyte inflammation. We show that treatment of endothelial cells with the conditioned medium from preadipocytes expressing Arg-II enhances VCAM-1 levels in endothelial cells. Moreover, the effect of conditioned medium on endothelial VCAM-1 expression is well correlated with the IL-6 levels in the conditioned medium. The conditioned medium with lower IL-6, i.e., that from 3T3-L1 expressing Arg-II in the presence of p38mapk inhibitor did not significantly induce endothelial VCAM-1 expression.

Taken together, the results from *in vitro* cultured cells demonstrate that Arg-II plays an important role in stimulation of inflammatory cytokine IL-6 release from preadipocytes through p38mapk, leading to endothelial inflammatory activation (**Fig. 4**). The study may explore a novel function of Arg-II in adipose tissue inflammation which contributes to age-associated endothelial inflammatory activation, which *in vivo* results should be verified next.

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Author Contributions

Chang Liu and Rong Luo performed experiments, analyzed data, and prepared the figures. Li Cheng and Xiu Li did manuscript editing. Li Cheng and Xiu Li carried out the project design, wrote the manuscript. All authors are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Statments

This work was approved by the Ethical Committee of Chengdu Medical College. All authors participated and reviewed the manuscript and consented to publish this article.

Conflict of interest Statement

The authors declare no conflict of interest.

Figure legends

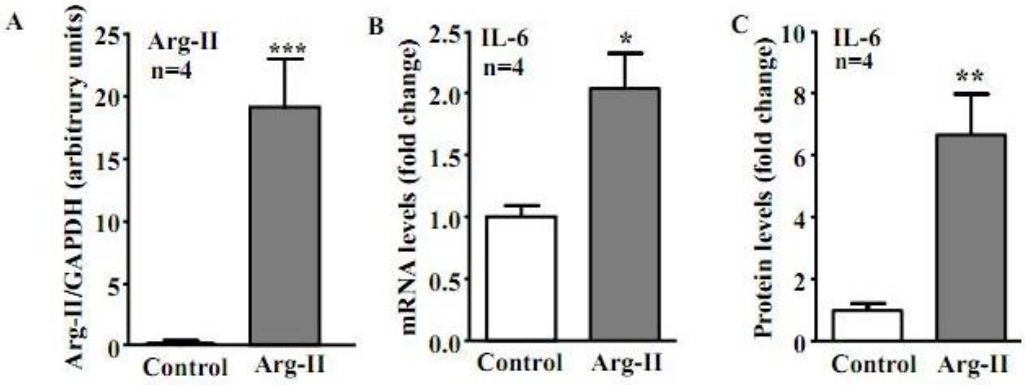

Fig. 1. Arg-II enhances IL-6 expression in preadipocytes. 3T3-L1 preadipocytes were transduced with rAd/CMV-LacZ as Control or rAd/CMV-Arg-II (Arg-II). 72 hours post-transduction (p.t), cells were serum-starved for overnight. RNA was then (84 hours p.t) extracted and conditioned medium was collected for **(A)** qRT-PCR analysis of Arg-II mRNA; **(B)** qRT-PCR analysis of IL-6 mRNA; and **(C)** ELISA for monitoring IL-6 level in conditioned medium. *P<0.05, **p<0.01, and ***P<0.001 vs. Control.

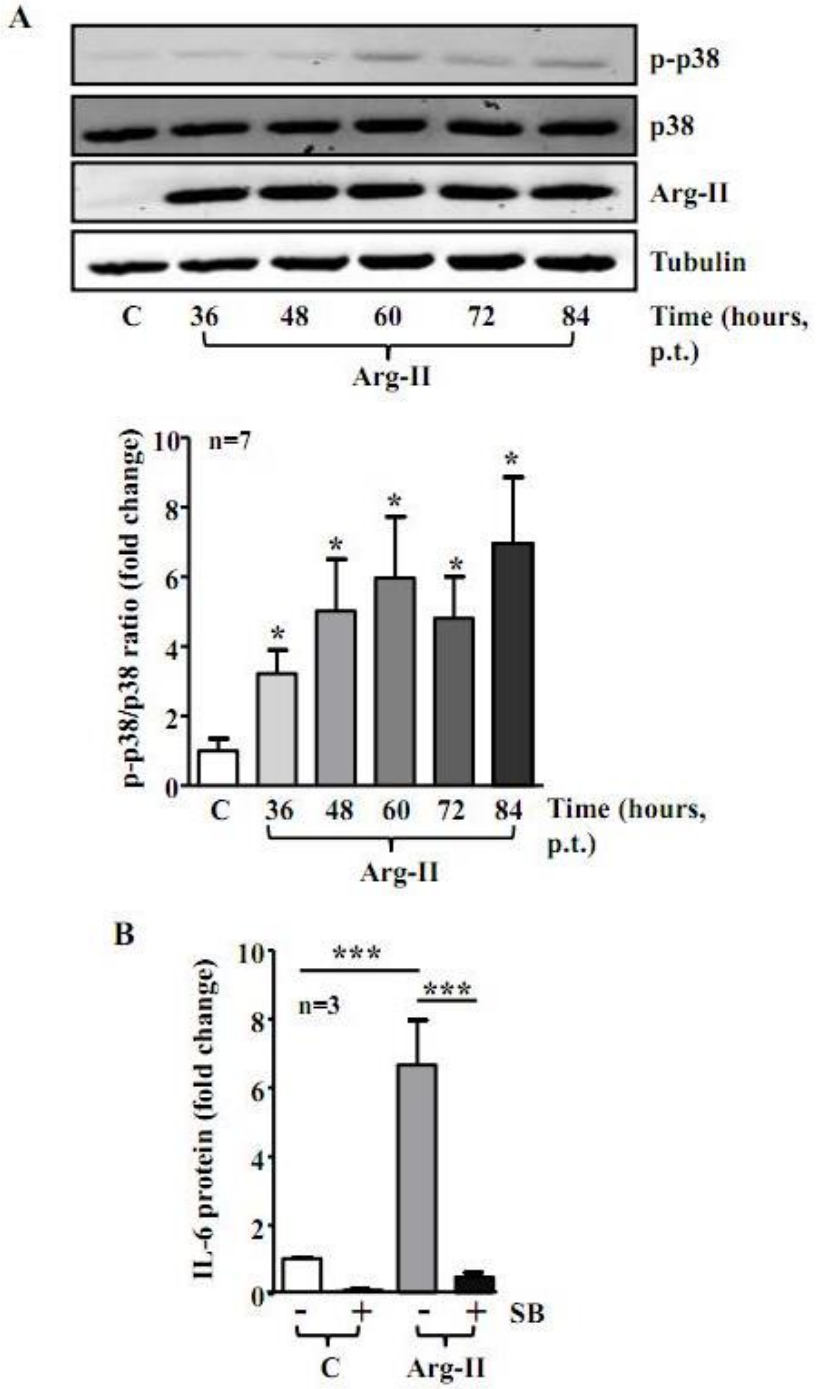

Fig. 2. Arg-II promotes IL-6 expression through p38mapk in preadipocytes. 3T3-L1 cells were transduced as in Fig. 1, cells were serum-starved for overnight prior to protein extraction and/or conditioned medium collection. **(A)** Cell lysates were prepared at different time point (36, 48, 60, 72, 84 hours p.t) post transduction. Shown is immunoblotting analysis of phosphorylated p38 and total p38 levels. Tubulin served as a loading control. Quantification of the signals is shown in the bar graphs below. *P<0.05 vs.C (control)**. (B)** Conditioned medium was collected 48 h post transduction. IL-6 level in conditioned medium was then measured by ELISA. SB, p38mapk inhibitor SB203580 (10 μmol/L, 48h). ***P<0.001 between the indicated groups.

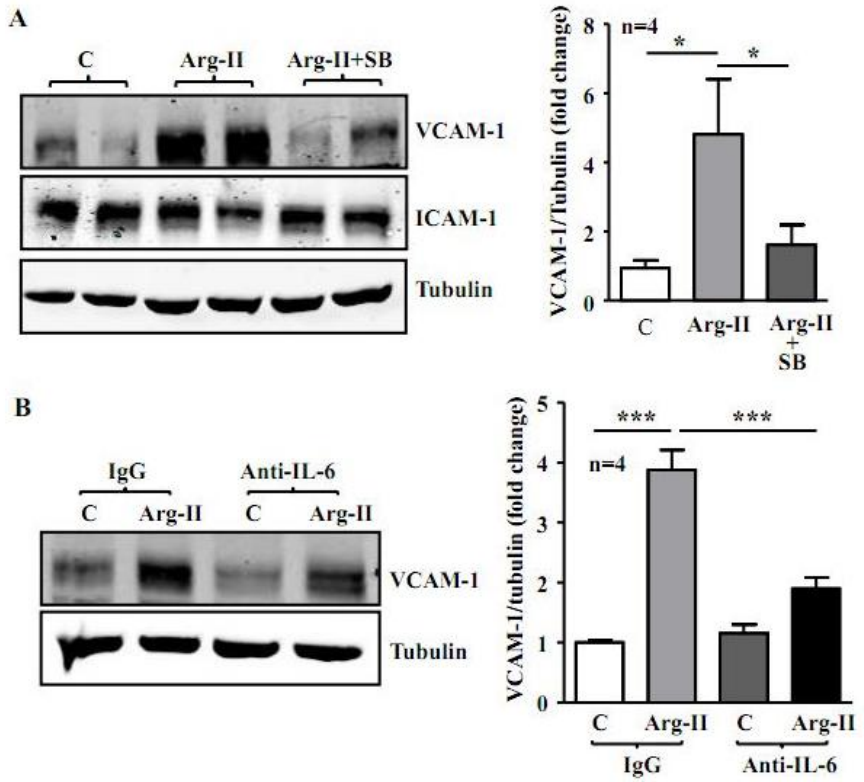

Fig. 3. Preadipocytes overexpressing Arg-II promote endothelial VCAM-1 expression through IL-6. **(A)** Conditional medium of 3T3-L1 cells was prepared as in Fig. 2B and used to treat HUVECs for 4 hours. HUVECs lysates were then prepared and subjected to immunoblotting analysis of VCAM-1 and ICAM-1. Tubulin served as a loading control. **(B)** Expreiments were performed as in (A), except that the conditioned medium were first incubated with control IgG or neutralizing anti-IL-6 antibody for 2 h prior to the addition to HUVECs. Quantification of the signals is shown in the bar graphs in the right panel. *P<0.05, ***<0.001 between the indicated groups.

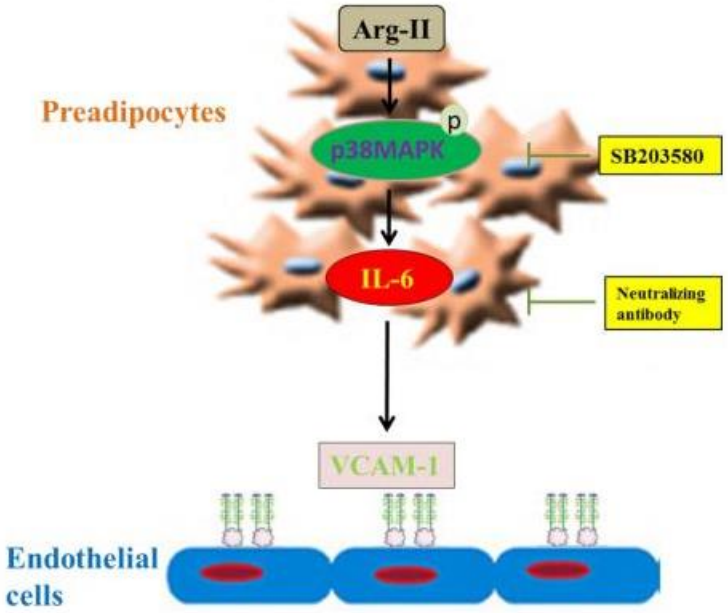

Fig. 4. Summary of the findings. Arg-II promotes IL-6 production in pre-adipocytes through activation of p38mapk, leading to enhanced endothelial adhesion molecule expression, which may contribute to the disorders associated with chronic inflammatory status in aging.

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