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Regulation of factors involved in modulation of angiogenesis in cells associated with cardiovascular disease.

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Introduction

1.1. Angiogenesis

Angiogenesis is the physiological process involving vasculogenesis, intussuseption, bridging and/or enlargement of capillaries from pre-existing ones, whereas arteriogenesis means the remodeling of arteriolar anastomoses to functional arteries. Lymphangiogenesis is the generation of new lymphatic vessels from pre-existing lymphatic vessel [1]. The process of angiogenesis is involved in embryogenesis, growth and development, as well as in inflammation and wound healing. Additionally, it is also a fundamental step in tumor growth and their transition of from benign to malignant tumours. Small blood vessels consist exclusively of endothelial cells, lager vessels by contrast are surrounded by smooth muscle cells. Numerous factors such as vascular endothelial growth factor (VEGF), pigmeth epthelium derived factor (PEDF), placental growth factor (PIGF), angiopoetin (Ang) -1 and interleukin- 8 (IL-8) influence vascular growth.

1.1.1. Angiogenesis during adipose tissue development

Angiogenesis, increased number and/or size of blood vessels, is essential to supply growing adipose tissue with oxygen and nutrients. Adipogenesis is highly associated with angiogenesis in the early stages of adipose tissue development. In vitro studies showed that adipose tissue explants and adipocytes differentiation induce blood vessel formation [2,3]. Additionally in several studies it was demonstrated that angiogenesis inhibitors in mice have a negative impact on adipose tissue growth [4]. Numerous factors are involved in adipogenesis regulation in adipose tissue including placental growth factor (PlGF), basic fibroblast growth factor (FGF-2), angiopoetin -2 (Ang-2), leptin, thrombospondin 1 (TSP-1), osteonectin, adiponectin, resistin, tissue factor (TF), tumour necrosis factor alpha (TNF-α), insulin-like growth factor (IGF), hepatocyte growth factor (HGF) etc. Nevertheless VEGF mRNA level is not markedly modulated by obesity in mice, it is expressed at a high level during adipocytes differentiation and in expanding adipose tissue [5] [6]. Generally it is assumed that the VEGF/ vascular endothelial growth factor receptor (VEGFR) system accounts for

most of the angiogenic activity in adipose tissue, making it an attractive target to reduce obesity [5].

1.2. Obesity

Obesity is a growing worldwide metabolic disorder, because overweight persons are at higher risk of developing a range of diseases such as cardiovascular disorders (e.g. artherosclerosis), cancer, insulin resistance, hypertension and diabetes type 2 meaning reduced life expectancy and which have a huge economic and societal consequences. But the exact mechanisms linking obesity to various diseases are poorly understood. Obesity is a medical condition in which adipose tissue mass, size and number of mature adipocytes is increased and is defined by the Body mass index (BMI) which is a value that is calculated as the body weight (kg) divided by the square of the height (m^2) and is used as a measurement of body fat in clinical an epidemiological studies. The BMI values are divided into four main categories: underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5- 24.9 kg/m^2), overweight (BMI 25.0- 29.9 kg/m^2), obese (Class I) (BMI 30- 34.9 kg/m^2), obese (Class II) (BMI 35- 39.9 kg/m^2), obese (Class III) (BMI > 40 kg/m^2) (Table 1). The BMI criteria for Asia and Oceania are slightly different: overweight (BMI > 23 kg/m^2) and obesity (BMI >25 kg/m^2)

Category	BMI (kg/m^2)
Underweight	less then 18
Health weight	18.5 - 24.9
Over weight	25.0 - 29.9
Obese (Class I)	30.0 - 34.9
Obese (Class II)	35.0 - 39.9
Obese (Class III)	≥ 40 kg/m^2

Table 1 Classifications for weight status based on Body Mass Index (BMI).

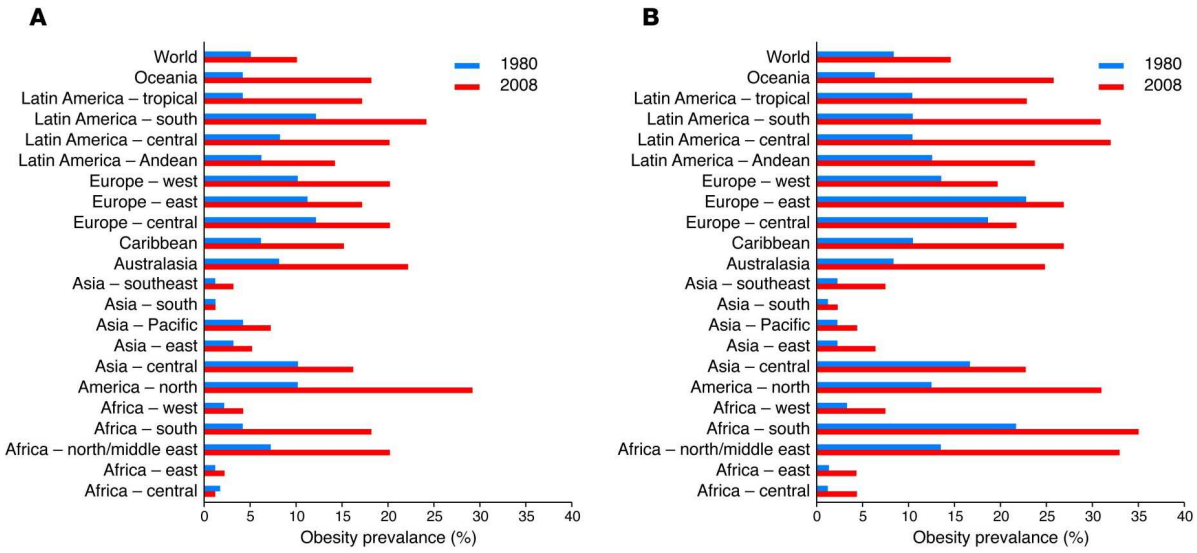


Figure 1 Worldwide change in prevalence of obesity in men (A) and women (B) between 1980 and 2008 [7].

1.2.1. The growing epidemic of obesity

In 2008, according to a study of the World Health Organisation (WHO) 1.5 billion adults over 20 years were overweight, 200 million of men and 300 million of women were obese and in 2010 about 43 million of children under 5 years were overweight [8] (Figure 1). Obesity is no longer just a problem of the Western industrialised world; the largest increases in obesity are seen in low and middle income countries [7,9]. A sharp increase of the total calorie intake was observed in the last decades (Figure 2). Commonly obesity is caused by a combination of excessive and energy-dense food intake, lack of physical activity and genetic susceptibility, in rare cases obesity is primarily caused by genes, endocrine disorders, medication or physical illness but the biology of obesity is very complex and not yet totally understood. Thus there is urgent need for understanding the pathophysiology of excess adiposity.

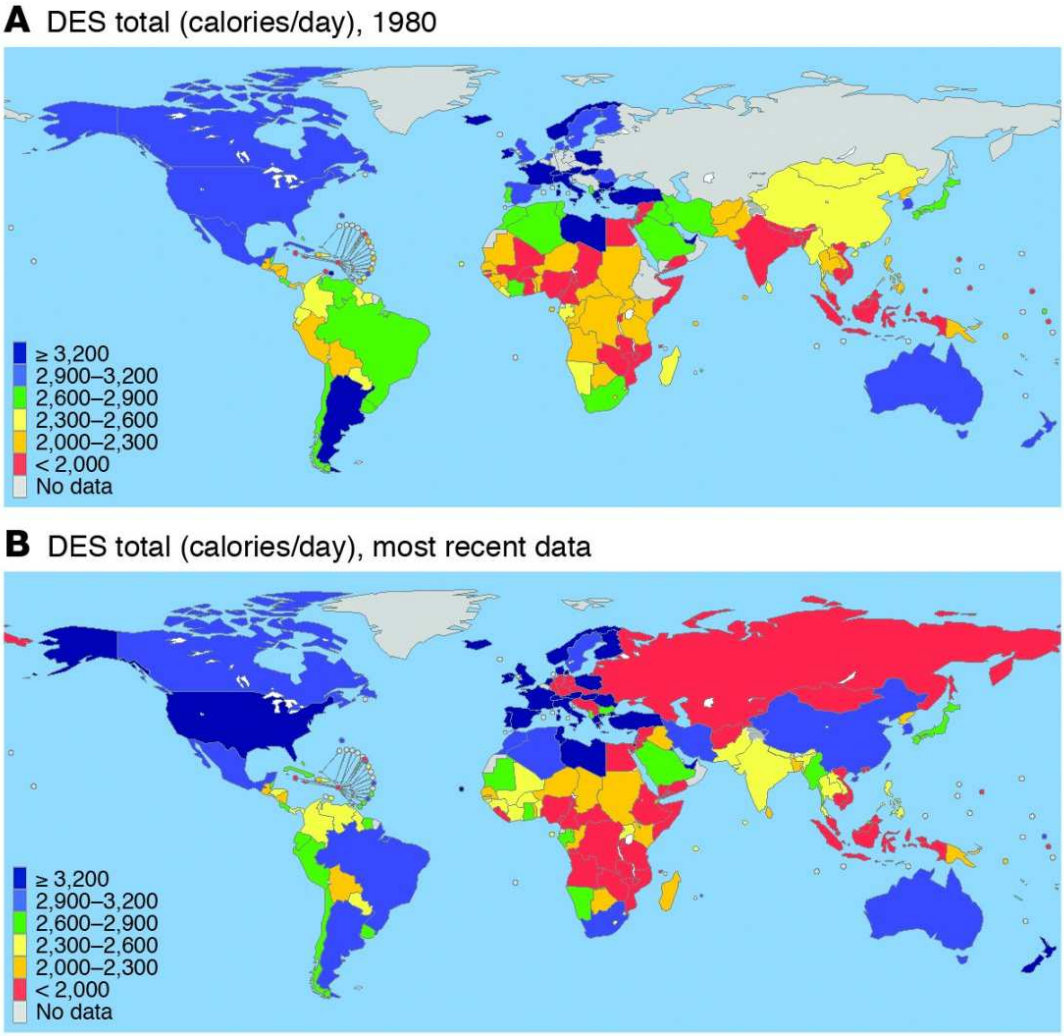


Figure 2 Worldwide estimates of average caloric intake in 1980 (A) and 2008 (B) [8].

1.2.2. Obesity treatment

Dieting, physical exercise and other lifestyle modifications are mainstays in obesity prevention and treatment. However, epidemiological studies have shown that it is very difficult to loose weight and the recidivism rate for regain weight after weight loss is very high [10]. Anti-obesity medication is largely ineffective and associated with strong adverse reactions; even obesity surgery cannot prevent weight regain livelong [11]. Surgical therapy for obesity (bariatric surgery) can be an effective therapeutic modality for subjects with morbid obesity to enable substantial, clinical significant weight loss. Evidence shows that bariatric surgery paired with healthy food choices and regular exercise results in improvement or resolution of morbidities associated with sever obesity [12]. The International Federation for Surgery of Obesity (I.F.S.O.)

has developed guidelines for appropriate selection of candidates for bariatric surgery (Table 2). The criteria for bariatric surgery include patients with a BMI greater than 40 kg/m² above the age/sex-defined weight. To justify bariatric surgery in subjects with a BMI between 35 and 40 kg/m² obesity associated comorbidities must be present and conservative weight management must have failed [13]. Surgical therapy for obesity can be classified as malabsorptive, restrictive and as a combination of the two. Currently the standard bariatric surgeries are Roux-en-Y gastric bypass, laparoscopic gastric band, biliopancreatic bypass and the sleeve gastrectomy [13].

1.2.3. The Roux-en-Y gastric bypass

In Europe the laparoscopic Roux-en-Y gastric bypass is the most commonly performed bariatric surgery. The laparoscopic Roux-en-Y gastric bypass is a combination of restrictive and malabsorptive components. A small stomach pouch is created transecting the upper part of the stomach, next the pouch is attached to a Y-shaped section of the small intestine bypassing the lower stomach, the duodenum and the first portion of the jejunum. As a result the Roux-en-Y gastric bypass restricts food intake and reduces the absorption of nutrients.

Indications for bariatric surgery (according to IFSO)
➢ BMI > 40 kg/m² for more than three years or a BMI > 35 kg/m² in combination with high-risk comorbidities, such as sleep apnea, diabetes mellitus, or degenerative joint disease. Bariatric surgery should be considered as a treatment of last resort after dieting, exercise, psychotherapy, and drug treatments have failed
➢ endocrine dysfunction, patients addicted to alcohol and drugs are not suited for bariatric surgery
➢ serious metabolic disease such as metabolic syndrome or sleep apnea place additional urgency on weight reduction and are an indication for bariatric surgery
➢ endogenous depression generally is a contraindication for bariatric surgery but not reactive depression
➢ operations for weight reduction before the age of 18 should not be performed

Table 2 Criteria for bariatric surgery establish by the International Federation for the Surgery of Obesity (IFSO)

1.2.4. Adipose tissue and inflammation

Half a century ago initial assessments pointed to the involvement of inflammation in obesity, including a report that described increased fibrinogen plasma level in obese patients [14]. The inflammatory concept attracted great interest of the scientific community when the enhanced expression of tumour necrosis factor alpha (TNF-α) in adipose tissue of obese rodents was demonstrated in the 1990s [15]. Since then several groups demonstrated the secretion and production of various cytokines, chemokines, hormones, and other inflammatory factors referred to as adipokines, identifying adipose tissue as one of the greatest endocrine organs in the body [16]. Beside TNF-α macrophage chemoattractant protein-1(MCP-1), interleukin-6 (IL-6), leptin, and resistin are highly expressed adipokines by obese adipose tissue and are suspected to be fundamental for the pathophysiology of obesity [17,18]. It is now well established that adipose tissue, beside its role in energy storage, also functions as an important endocrine organ that secretes a variety of cytokines, hormones and other proteins such as, interleukin-6 (IL-6), IL-8, leptin, resistin and plasminogen activator inhibitor-1 (PAI-1)[19] referred to as adipokines [17,18]. Obesity is causally linked to a low-grade inflammation state [11,14] which is involved in the development of obesity-linked disorders, in particular metabolic dysfunctions. Excess adiposity and adipocytes dysfunction engender an unregulated expression and secretion of adipokines which has been linked to the development of various disease processes through altered immune response (Figure 3). According to this there is considerable interest to develop a better understanding of the immunoregulatory function of adipose tissue. Adipokines have been identified to either contribute to the resolution of inflammation or promote inflammatory response and metabolic dysfunction. Therefore it is assumed that an imbalance of pro- and anti-inflammatory adipokines secreted by adipocytes entail metabolic dysfunctions. Lately much attention has been paid to developing a better understanding of the immunoregulatory function of adipocytes and adipose tissue. New adipokines have been identified to either cause inflammatory responses and metabolic dysfunction or contribute to the removal of inflammation and have positive effects on obesity linked disorders [20]. These insights suggest that an imbalance of pro- and anti-inflammatory adipokines contributes to the development of metabolic dysfunction.

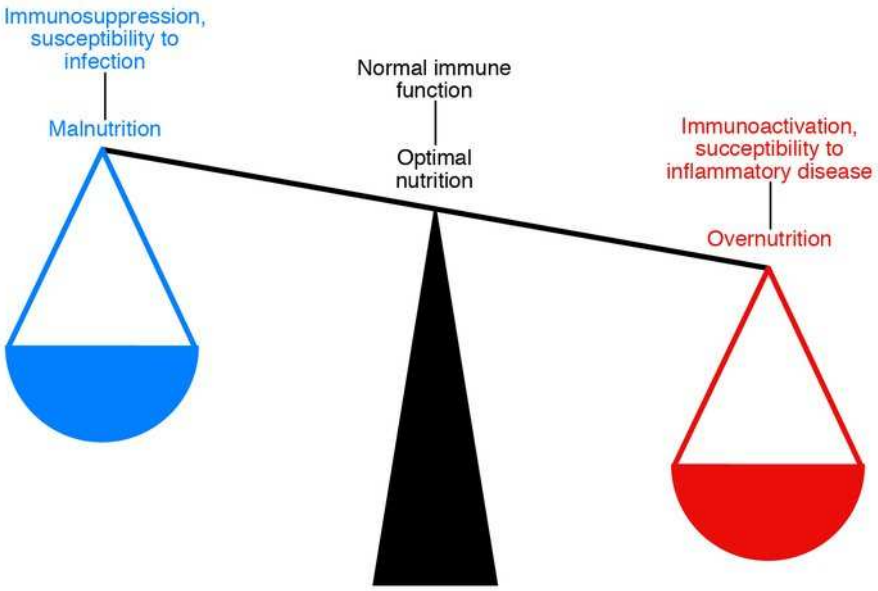


Figure 3 Linkage between metabolism and immunity [21].

1.2.5. Categories of adipose tissue

Adipose tissue can be categorized in three different categories: lean with normal metabolic function, obese with light metabolic dysfunction and obese with complete metabolic dysfunction. As adipose tissue growths, adipocytes undergo hypertrophy increasing their triglyceride storage. With mild obesity, adipose tissue maintains relatively normal metabolic function, low levels of immune cell activation and sufficient vascular function. However, expansion of adipose tissue promotes the transition to a metabolically dysfunctional phenotype. M2 type macrophages are mainly found in lean adipose tissue, whereas in states of obesity adipose tissue M1 macrophages and T cells are accumulated. Anti-inflammatory adipokines are mainly produced by lean adipose tissue; a great number of proinflammatory factors are secreted by adipose tissue in obese patients [22].

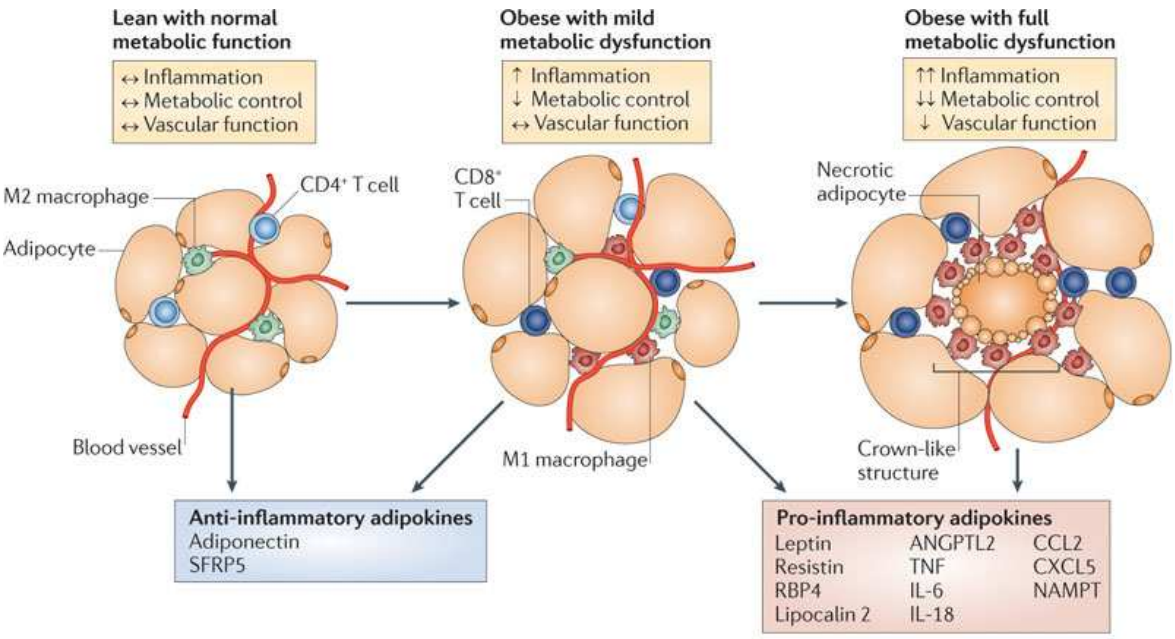


Figure 4 Phenotypic modulation of adipose tissue [22].

1.2.6. Clinical observations

Especially increased visceral fatty tissue (Figure 4) in obese patients leads to a chronic low grade inflammatory state [14,15] which has a crucial role in pathogenesis of obesity-induced disorders. Excess adiposity - in particular visceral adiposity- and adipocytes dysfunction result in deregulated expression of adipokines and has been linked to development of various diseases processes through varied immune response. Clinical and epidemiological studies have described a considerably connection between the development of low grad inflammation and metabolic diseases, especially in the field of obesity and type 2 diabetes. Obese individuals demonstrate an increased level of the pro-inflammatory marker C- reactive protein (CRP) in the blood [23]. An increased CRP and IL-6 level is a sign for the development of type 2 diabetes in various populations [16,17]. In addition weight loss leads to a reduction of the pro-inflammatory adipokines [24].

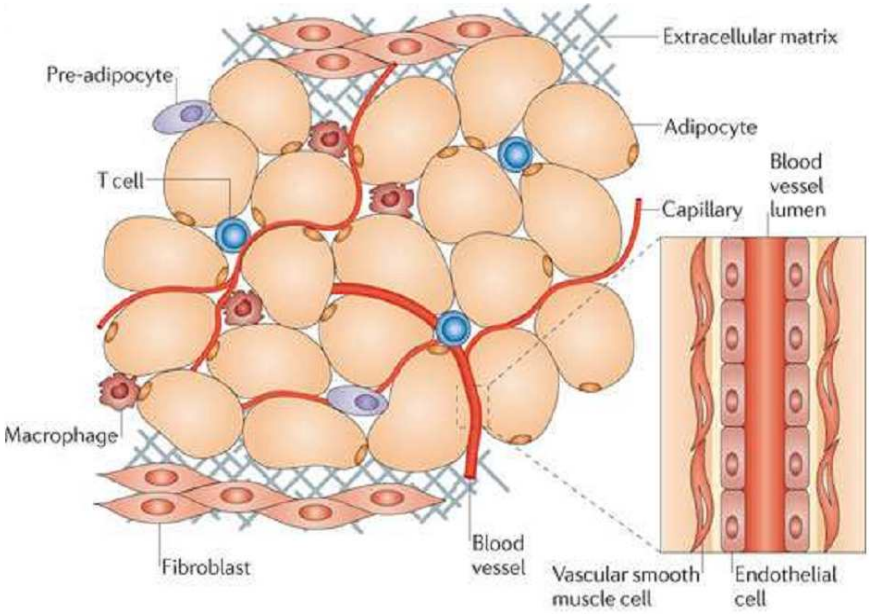


Figure 5 Components of adipose tissue [22].

1.2.7. The adipokine concept

Adipose tissue is a very complex and highly active metabolic and endocrine organ. Besides adipocytes, adipose tissue contains preadipocytes, fibroblasts, nerve tissue, immune cells, connective tissue and vascular cells (Figure 5). as described above adipose tissue was originally considered as an energy storage organ but it was recently detected that it also expresses and secretes factors with important endocrine functions the so-called adipokines [17,18]. Following the onset of obesity, the cellular composition of adipose tissue can be modified including changes in localization, number an phenotype of cells resulting in a difference of the adipokine secretion status. The expression pattern of adipokines is depended on the site of the adipose tissue depot. Visceral and subcutaneous adipose tissue represent the most important fat depot and produce a different adipokine composition [25,26] (Figure 6). A recent study showed that subcutaneous adipose tissue has significant higher mRNA expression of leptin, TNF-α and NFκB. In contrast visceral adipose tissue has higher expression of complement factor C3. No differences were observed for the expression of IL-8 and IL-1β [25].

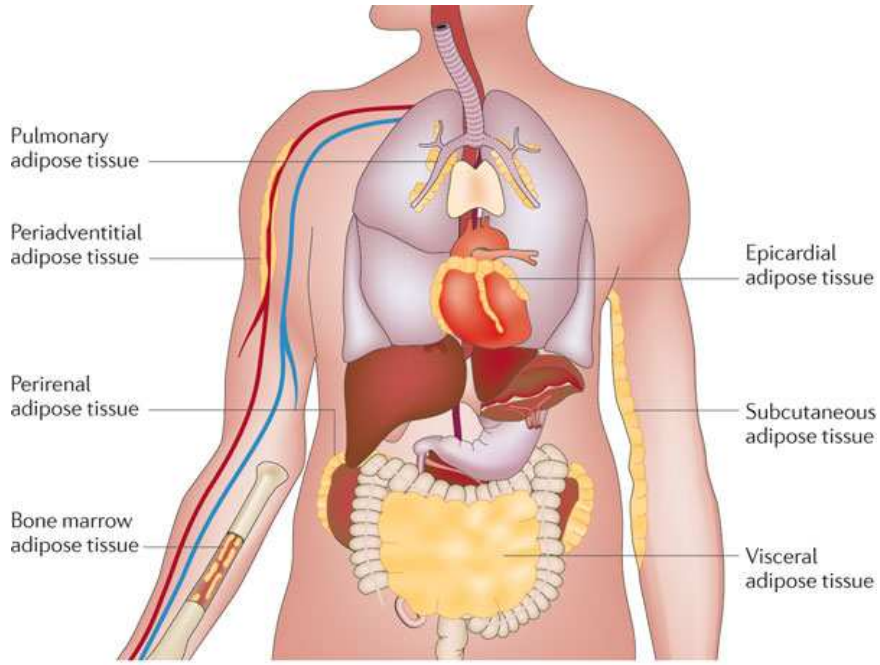


Figure 6 Adipose tissue depots in human body [22].

1.3. Biology of the pigment epithelium-derived factor (PEDF)

The pigment epithelium-derived factor (PEDF), member of the serpins (serin protease inhibitors), is a non-inhibitory, multifunctional protein with neurotrophic, antiangiogenic, antitumorigenic, anti-inflammatory, antioxidant and antithrombic properties [27,28]. The glycoprotein PEDF was initially detected by Joycs Tombran-Tink and Lincoln Johnsonn in conditioned medium from fetal human retinal pigment epithelium (RPE) cells as a factor with effective neuronal differentiation property [29].

1.3.1. Human PEDF gene and protein

The human PEDF is encoded by the 15.6kb *SERPINF1* gene [30], which is localized on chromosome 17p13.1. The *SERPINF1* gene is composed of 8 exons and 7 introns [31]. A 200bp promoter is localized upstream of the PEDF gene which is the suggested binding site for the transcription factors HNF4, CHOP and USF [32]. PEDF is a single chain protein, consisting of 418 amino acid residues, it reveals a high proportion of structural homology comparing the amino acid sequences with other serin protease inhibitors [30]. However, it does not display any protease inhibitor

features [33]. PEDF is not exclusively expressed in the eye but in various tissues such as the spinal cord, brain, skeletal muscles [34], adipose tissue [35], bone [36], heart [37] and liver [38] and also in human plasma high concentrations of PEDF were found. Although PEDF is expressed in many tissues, it has no influence on viability as PEDF null mice are viable and fertile [39]. Investigating which cells express PEDF retinal pigment epithelial cells [29], hepatocytes [40,41] , Müller cells [42], smooth muscle cells macrophages [42,43] , various tumor cells [43], cardic myocytes and fibroblasts [44] and adipose tissue [45] have so far been described as cellular source of PEDF. Famulla et al. identified adipocytes as the main source of PEDF, moreover they demonstrated that increasing PEDF protein expression and secretion positively correlates with the differentiation stage of adipocytes [45]. Furthermore two other studies found a connection between human obesity and PEDF plasma concentration, suggesting adipocytes as the main source of PEDF [46,47].

The human *SERPINF1* gene encodes for a 418 amino acids peptide with a molecular weight of 50 kDa, secreted as a monomeric glycoprotein [48]. The N-terminus of the PEDF protein contains a leader sequence (residues 1-19) inducing protein secretion out of the cell [49]. Two PEDF epitopes with distinct functions were identified: the 34 amino acid peptide (residues 24-57) acts on endothelial cells, induces apoptosis and thereby has antiangiogenic properties; a 44 amino acid peptide (residues 58-101) is suggested to be responsible for the neurotrophic properties of PEDF [49]. The 34 amino acid epitope binds the non-integrin 37/67 kDa laminin receptor procuring the PEDF induced inhibition of angiogenesis [50]. Additionally the 80 kDa phospholipase-linked membrane protein independent phospholipase A2 (PLA2)/ adipose triglyceride lipase (ATGL) was identified as a PEDF receptor. The enzymatic activity of the receptor is stimulated by binding PEDF whereby survival and differentiation of neurons is promoted through inducing lipid metabolism on neuronal surfaces [51].

1.3.2. Crystal structure of PEDF

Simonovic *et al.* solved the crystal structure of human glycosylated PEDF. The three-dimensional structure includes 3 beta sheets and 10 alpha helices. (Figure 7) Furthermore the structure reveals a noteworthy asymmetric charge distribution with a high density of acid residues concentrated on the one site of the molecule and basic residues on the opposite side resulting in a polar 3D structure. The heparin- and proteoglycan-binding sites are supposed to be located on the basic side of the protein [52].

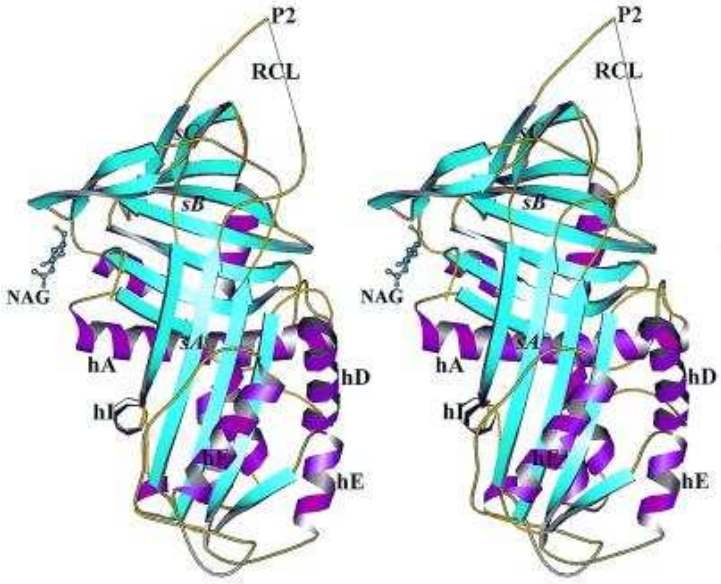


Figure 7 Stereoscopic model of glycosylated PEDF [52]; α-Helices are shown in magenta (hA, hD-F, hl), strands of β-sheets are shown in cyan (sA-C), NAG are represented as ball-and-sticks. The reactive center loop is labeled as RCL, and N-acetylglucosamine is labeled as NAG.

1.3.3. PEDF as an antiangiogenic factor

As mentioned above PEDF is a multifunctional pleiotropic protein with antiangiogenic, antitumorigenic, anti-inflammatory, antioxidant and antithrombic properties [27,28] and has a widespread expression throughout various human tissues such as the adipose tissue, liver, bone, skeletal muscle and plasma. PEDF is a protein with strong antiangiogenic features; it has a stronger antiangiogenic impact than other antiangiogenic factors such as thrombospondin, angiostatin or endostatin [43]. PEDF oppresses ischemia-induced angiogenesis and VEGF- and basic (b) FGF-

induced migration and proliferation of endothelial cells [53]. Additionally PEDF inhibits angiogenesis interacting with vascular endothelial growth factor VEGF signalling [53-55]. Various findings suggest that the antiangiogenic activity of PEDF is interceded via two different mechanisms; via the onset of apoptosis in endothelial cells and via disturbence of the pro- and antiangiogenic factors balance.

PEDF induces apoptosis exclusively in activated endothelial cells of newly formed vessels but not in pre-existing vessels by activating the FAS-FAS ligand death pathway. Activated endothelial cells under the influence of pro-angiogenic factors solely express the FAS receptor and are sensitized toward Fas-mediated apoptosis. Endothelial cells stimulated with PEDF display increased FASL expression leading to caspase-dependent induction of apoptosis [56]. In cultured endothelial cells PEDF induces apoptosis by activating p38 MAPK-dependent cleavage of caspase 3, 8 and 9 [57] and by sequential induction of PPAR and p53 [58]. Furthermore PEDF counteracts the effect of VEGF enhancing the cleavage of VEGF receptor 1 by the gamma secretase, resulting in inhibition of VEGF-induced angiogenesis [59]. There is convincing evidence that the expression of PEDF is regulated by hypoxia. Under anoxic conditions mRNA and protein expression was reduced up to 50 % in human cardial fibroblasts and myocytes [44]. As a consequence of low oxygen concentration the hypoxia-inducible transcription factor (HIF) is stabilized and transported into the nucleus [60]. Under hypoxic conditions PEDF production decreased in retinoblastoma and retinal pigment epithelium cells, but solely at the protein level and not at the mRNA level [43,61]. Notari et al suggested that PEDF is a substrate for matrix metalloproteinase (MMPs), MMP-2 and -9, which are hypoxia-activated and by proteolytically degrading of PEDF influence its posttranslational regulation [61]. Many indications suggest that the expression of the proangiogenic factor VEGF is upregulated under hypoxic conditions [60]. As well as in human cardiac fibroblasts and myocytes as in the retina and retinoblastoma cells anoxic conditions not only result in increased expression of the proangiogenic factor VEGF but also in decrease of antiangiogenic PEDF expression [43,44].

1.3.4. PEDF as an antioxidant, antithrombotic and anti-inflammatory factor

Recently it became evident that PEDF protects endothelial cell against inflammatory activation and injury, which is pivotal for the development of atherosclerosis, by inhibiting TNF alpha induced IL-6 expression via suppression of reactive oxygen species (ROS) generation by NADPH oxygenase [62,63]. Recently Park et al. identified PEDF as an endogenous inhibitor of the canonical Wnt pathway [64] [Figure 8]. The canonical Wnt signalling pathway is a highly conserved pathway. The intracellular signalling cascade is initiated by the binding of a Wnt ligand to the coreceptor complex consisting of the Frizzled (Fz) receptor and the low-density lipoprotein receptor related protein 5 (LRP5) or LRP6, causing the activation of the Dishevelled (Dsh) protein. The activated Dsh inhibits a second complex of proteins that includes axin, glycogen synthase kinase 3 (GSK-3), and the protein adenomatous polyposis coli (APC). The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signalling molecule. After this the GSK-3 is no longer able to phosphorilate β-catenin, releasing it for the axin/GSK3/APC complex and leading to β-catenin accumulation and migration to the nucleus. The nuclear β-catenin interacts with the T-cell factor (TCF) and thereby the transcription factors of multiple target genes including genes relevant for angiogenesis, inflammation and fibrosis are activated. PEDF inhibits the Wnt/ β-catenin pathway by binding to the LRP6, a Wnt coreceptor, with high affinity and in this way blocks the Wnt signalling induced by Wnt ligand [64]. Additionally it was observed that PEDF plays a causal role in evolution of insulin resistance [45].

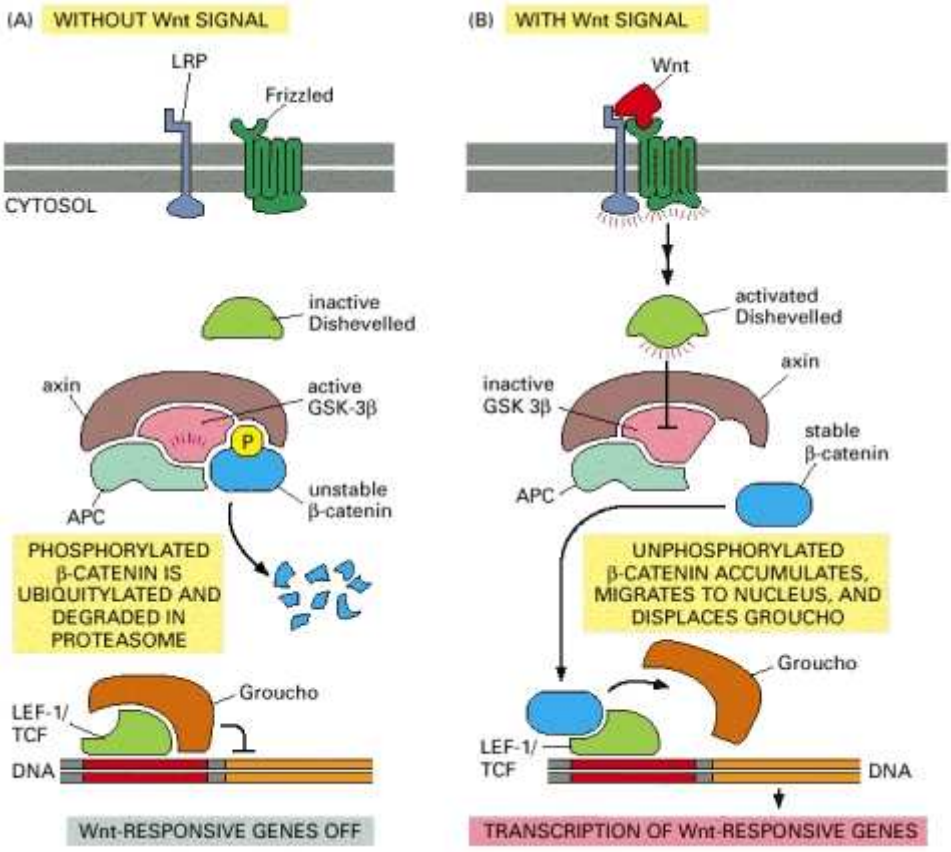


Figure 8 A model of the Wnt activation of the β-catenin signalling pathway. (Molecular Biology of the Cell, 4th edition, Bruce Alberts).

1.4. Vascular endothelial growth factor (VEGF)

The vascular endothelial growth factor (VEGF) family represents important signaling molecules, involved in the modulation of various pathophysiological processes in the vasculature. They regulate vasculogenesis during embryonic development and angiogenesis and vascular maintenance in adults. VEGFs are involved in cardiovascular biology, tissue regeneration, ovarian angiogenesis, endochondrial ossification, survival of haematopoetic stem cells and erythropoietin regulation and are important regulators of tumor angiogenesis, psoriasis and retinal neovascularization [65-67].

1.4.1. Biology of the VEGF family

The VEGF gene family consists of numerous members including VEGF-A, -B, -C, -D, -E, -F and PlGF (placental growth factor) (Figure 9). In 1989 VEGF-A (also called VEGF) was the first VEGF family member to be discovered, subsequently four other members of the human VEGF family (VEGF -B, -C, -D and PlGF) have been identified [68,69]. Additionally viral VEGF homologues called VEGF-E and snake venom VEGFs (VEGF-F) have been detected [70,71]. VEGF is a homodimeric glycoprotein consisting of two identical 23kDa subunits [67]. All members of the VEGF family regulate downstream signaling by binding to 3 different tyrosine kinase receptors (VEGF receptor, VEGFR 1-3) on cell surface, causing them to dimerize and become activated through transphosphorylation [72]. Neuropilin-1 (NRP1) is a membrane-bound coreceptor for VEGF tyrosine kinase receptors [73].

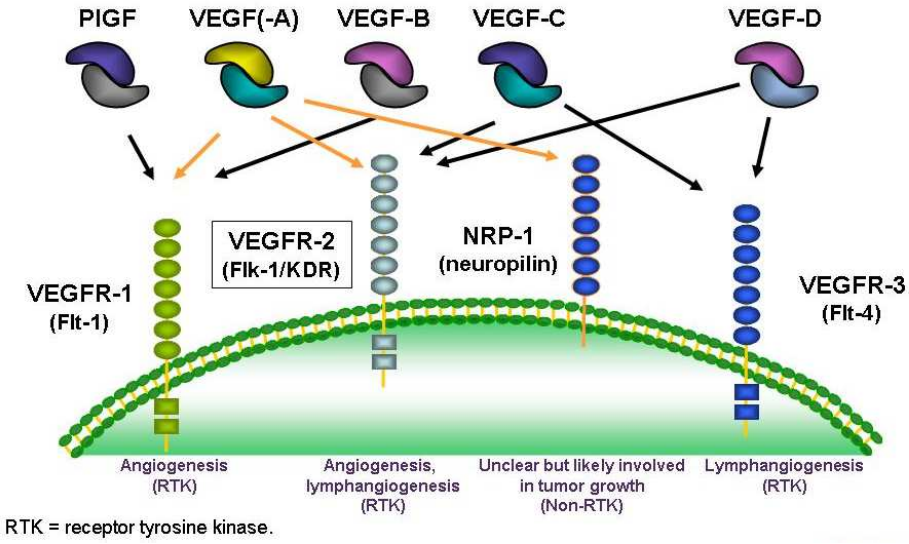


Figure 9 The VEGF family and its receptors [67].

1.4.2. Expression and regulation of VEGF

VEGF in adults is expressed in every vascularised tissue particularly in large blood vessels in skeletal muscle, fenestrated and sinusoidal blood vessels and in secretory and endocrine organs. Therefore low physiological VEGF level is assumed to be

essential for the maintenance of vascular homeostasis [74]. Much higher levels of VEGF are crucial for vasculogenesis and angiogenic mechanisms [72]. VEGF is a central stimulator of angiogenesis, its binding to VEGF receptors promotes endothelial cell migration and proliferation, two key features required for angiogenesis [75].

Expression, availability and activity of VEGF is strictly regulated through a large number of stimuli and by diverse mechanisms such as hypoxia, transcription factors, oncogenes, inflammatory cytokines, mechanical forces and cell stretch [66]. Hypoxia stimulates the secretion of VEGF via up-regulation of hypoxia inducible factor-1 α (HIF-1α) [76,77]. Numerous growth factors and cytokines such as TNF-α, IL-6, IL-1, platelet-derived growth factor-BB (PDGF-BB), PlGF, HGF, TGF-β, FGF, IGF-1 up-regulate VEGF-A expression [76,78]. Under normal conditions VEGF synthesis is regulated by the Hippel-Landau tumor suppressor protein, which under normal conditions ubiquitinates the most important transcriptional activators HIF-1α and thereby causes its degeneration [79,80].

1.5. Interleukin-8

The chemokine IL-8 is a member of the CXC chemokine subfamily and is encoded by the IL-8 gene, located on chromosome 4q13–q21 in humans, composed of four exons, three introns, and a promoter region [81]. The chemokine is secreted by several cell types such as macrophages, epithelial cells and endothelial cells; endothelial cells store IL-8 in the Weible-Plade bodies [82]. IL-8 is expressed in numerous normal and tumor cells and its main function is to initiate and amplify acute inflammatory reactions [83]. There is increasing evidence that IL-8 plays an important role in the pathogenesis of cancer by modulating angiogenesis, tumor growth, and metastasis [84-88]. One of the pivotal roles of IL-8 is the induction of chemotaxis in neutrophiles and lymphocytes but many different cell types (macrophages, mast cells, keratinocytes and endothelial cells) also respond to the chemokine [89,90]. IL-8 binds to the high- affinity receptors CXCR1 and CXCR2 belonging to CXC chemokine receptors, a large family of G protein-linked receptors, capable of inducing angiogenic behavior in endothelial cells [91,92]. A great number of molecular pathways are involved in the IL-8-induced angiogenic behavioral responses in ECs

(e.g., survival, migration, generation of a branching morphogenesis and proliferation of EC) and are relatively unexplored. Mechanisms involved in IL-8 mediated angiogenesis include activation of MMPs and differential expression of antiapoptotic genes [93], Rac-dependent cell retraction and gap formation [94], RhoA-dependent gap formation, CXCR2-mediated VEGFR transactivation [95] and EGFR transactivation [94]. Recently it has been shown that PEDF downregulates IL-8 production in hormone-refractory prostate cancer cells (HRPC) through inactivation of NF-κB and up-regulation of PPARγ (Figure 10) [96].

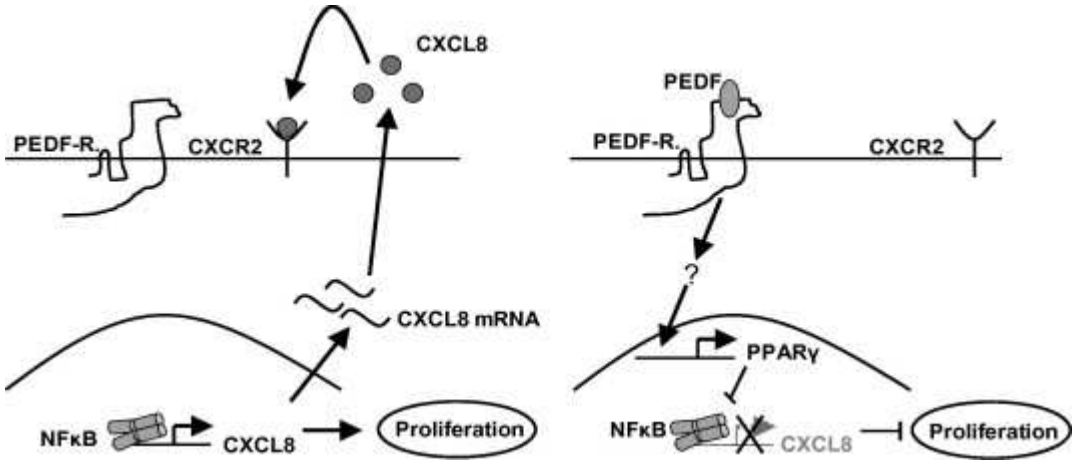


Figure 10 Model established by Hirsch et al for PEDF signaling in human hormone-refractory prostate cancer cells (HRPC). In HRPC, NFκB transcription factor up-regulates IL8 expression (Left panel). IL8 binds the CXCR2 receptor and induces proliferation of HRPC. PEDF interacts with PEDFR and up-regulates PPARγ (Right panel). PPARγ inhibits transcriptional activity of NFκB resulting in repressed IL8 expression and reduced cell proliferation [96].

1.6. Aims of the project

The aim of the study was to investigate the regulation and autokrine function of PEDF in human visceral and subcutaneous preadipocytes/adipocytes and to determine the parakrine effects on cells that are involved in angiogenesis such as HCASMC and HUVEC. Recently it has been demonstrated that PEDF is massively expressed in human adipose tissue and increases during adipogenesis [45]. The aim of this project was to comprehend the significance of PEDF for angiogenesis and adipositas progression in more detail. Further studies could lead to a new and effective therapy which blocks the progression of adiposity. The aims of the present project consist of several parts as follows:

Part I: The aim of this project was to investigate a possible role of PEDF in obesity. In a first step we studied the regulation of the expression of PEDF in human visceral and subcutaneous preadipocytes and adipocytes isolated out of adipose tissue.
Part II: As obesity is associated with a generalized inflammatory state we tested whether inflammatory mediators affect the expression of PEDF in preadipocytes and adipocytes.
Part III: Considering that adipose tissue in vivo is always exposed to an inflammatory hypoxic milieu and as anoxia is a critical modulator in various organs and tissues modulating angiogenesis we studied a possible influence of anoxia on the expression of PEDF in preadipocytes, adipocytes and adipose tissue.
Part IV: In a further step, we investigated whether the stimulation of human coronary artery smooth muscle cells (HCASMC) with PEDF (400ng/ml) affects the expression and protein level of VEGF.
Part V: It has been shown that PEDF downregulates IL-8 production in hormone-refractory prostate cancer cells (HRPC) through PEDF receptor/phospholipase A2 induced inactivation of NF-κB and up-regulation of PPARγ The third aim of the project was to study a possible effect of PEDF on IL-8 mRNA expression and protein production in HUVEC.

Aims of the study

- to study the regulation of PEDF expression in human visceral and subcutaneous preadipocytes, adipocytes
- to test whether inflammatory mediators affect the expression of PEDF in preadipocytes, adipocytes
- to investigate the possible influence of anoxia on the expression of PEDF in adipocytes
- to examine the effect of PEDF on VEGF expression in HCASMC
- to elucidate the effect of PEDF on IL-8 expression level in HUVEC

1. Materials and Methods

1.1. Cell culture

2.1.1. Standard growth conditions

All cell lines were grown in 75 cm² and 225cm² tissue culture flasks (Iwaki) or in 24 well cell-culture plates in a humidified incubator (Thermo Scientific Heraeus Cytoperm 2) under the conditions of 37℃ and 5% CO_2. 24-well-plates were obtained from Greiner Bio One.

2.1.2. Splitting and cell counting

For cell splitting, medium was aspirated and cells were shortly washed with PBS without Ca and Mg. Cells were then incubated for 2 to 6 minutes with trypsin at 37℃ and resuspended in the appropriate medium. Cells were spun down for 5 minutes at 1500 rpm. The resulting cell pellet was resuspended in the appropriate medium. Cell concentrations were determined with a Neubauer counting chamber.

2.1.3. Isolation and cultivation of human preadipocytes

Visceral and subcutaneous adipose tissue was obtained from patients undergoing a laparoscopic Roux-en-Y gastric bypass surgery. All subjects were of Caucasian origin and did not suffer from acute acute infection, cancer or any other consuming disease. All human material was obtained and processed according to the recommendations of the hospital's Ethics Committee and Security Board, which included obtaining informed consent.

Primary cultures of human preadipocytes were prepared from adipose tissue of subcutaneous and visceral origin obtained as described above. Tissue was dissected from skin, visible blood vessels, and fibrous material, minced into small pieces (1 to 2 mm), and digested in Hanks' balanced salt solution containing 0.1% collagenase type IV (both from Sigma) for 60 minutes at 37℃ in a shaking incubator (WTB Binder BFED-53). After they were filtered through a 70-µm nylon mesh (BD Falcon), cells were centrifuged for 10 minutes at 1000 rpm, washed once with DMEM/F-12 containing 20% fetal calf serum (Biochrom), passed through a 40-µm nylon mesh (BD

Falcon), and centrifuged for 5 minutes at 1500 rpm. The isolated sedimented cell fraction was incubated with erythrocyte lysis buffer containing 154 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA (all from Merck) for 10 minutes at room temperature. After centrifugation for 5 minutes at 1500 rpm the resulting cell pellet was resuspended in DMEM/F-12 containing 20% fetal calf serum. Cell concentration was determined with a Neubauer counting chamber and cells were seeded into 24-well plates. All cells were grown in a humidified incubator (Thermo Scientific Heraeus Cytoperm2) under the conditions of 37℃ and 5% CO2. After cell adhesion for 24 hours, preadipocytes exhibited the characteristic fibroblast like shape (Figure 11). Medium was replaced every other day.

2.1.4. Adipocyte differentiation

After adhesion of preadipocytes for 24 hours, adipose differentiation was induced with serum-free DMEM/F-12 containing 33 µmol/L biotin, 17 µmol/L pantothenate, 1 nmol/L triiodothyronine, 100 nmol/L dexamethasone, 500 nmol/L insulin, 1 µmol/L pioglitazone, and 0.25 µmol/L isobutyl-methylxanthine, for the first 3 days, (all from Sigma). Half of the medium was replaced every other day; differentiation was verified by staining with Sudan III, in which differentiated adipocytes were defined as cells whose cytoplasm was filled completely with lipid droplets (Figure 12). Only cultures with >90% adipocytes were used for further adipocyte experiments. All cell culture media and buffers contained 100 U/mL penicillin, 100 U/mL streptomycin, 0.25 µg/mL fungizone, and 2 mmol/L L-glutamine (all from Cambrex).

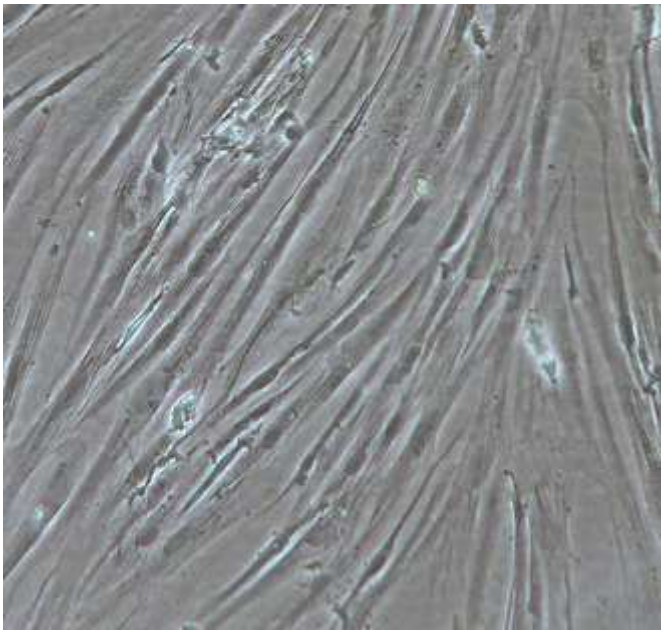


Figure 11 Phase contrast micrographs of human subcutaneous preadipocytes.

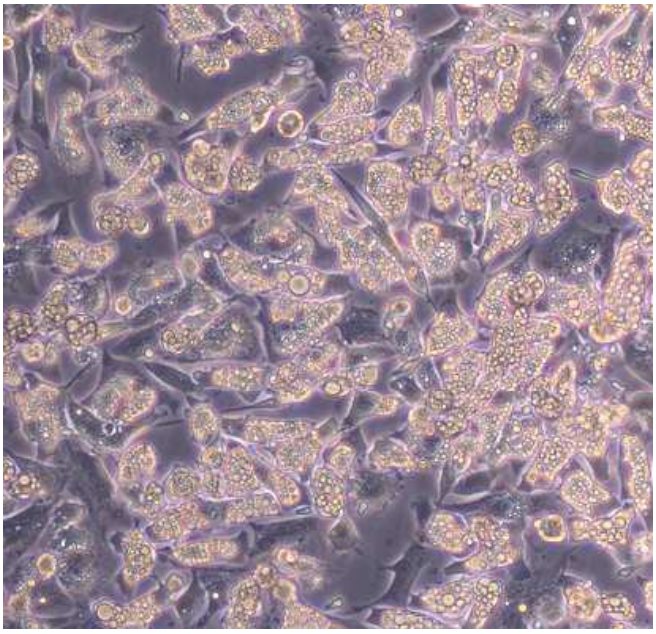


Figure 12 Phase contrast micrographs of human subcutaneous adipocytes.

2.1.5. Principles of adipocyte differentiation

Committed preadipocytes undergo growth arrest and consequently terminal differentiation into adipocytes, which is associated with an increase in expression of adipocytes genes, lipid- metabolizing protein and adipocyte fatty acid binding protein.

During the growth phase, preadipocytes are morphologically similar to fibroblasts, when they are treated with a combination of isobutyl-methylxanthine (IBMX), dexamethasone and insulin, cells undergo a drastic cell shape change, start to adopt a rounded phenotype. Within 4-8 days preadipocytes begin to accumulate lipids in form of intracellular lipid droplets and subsequently acquire the morphological and biochemical characteristics of mature white adipocytes. Treatment of cells with dexamethasone activates CCAAT/enhancer-binding protein β (C/EBPβ) transcription factor, which plays a key role in the complex transcriptional cascade of adipocyte differentiation. IBMX inhibits soluble cyclic nucleotide phosphodiesterases, resulting in increased intracellular cyclic adenosine monophosphate (cAMP) levels. At the nuclear level, treatment with IBMX results in activation of the transcription factor C/EBP δ which induces transcription of C/EBP α and peroxisome proliferator-activated receptor-γ (PPAR-γ). C/EBPα, β and δ are members of a transcription factors family that interact with the CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine) box motif, which occurs in several gene promoters [97]. PPAR-γ is a ligand-activated transcription factor which shares structural homology with the nuclear hormone receptor superfamily. PPARs induced transcriptional regulation requires heterodimerization with the retinoid X receptor (RXR). When activated by a ligand, the dimer increases transcription by binding to specific DNA sequence elements called peroxisome proliferator response element (PPRE) in the promoter region of target genes [98]. Within 3 days of exposure to inducers, the cells undergo two rounds of mitosis, and then mitotic clonal expansion is termed, which is required for differentiation. Insulin or insulin-like growth factor-1 promotes adipocyte differentiation by activating PI3-kinase and Akt activity. Modulation of the activity of the forkhead transcription factor Foxo1 appears to be necessary for insulin to promote adipocyte differentiation. C/EBP α and PPAR direct the final phase of adipogenesis by activating expression of adipocyte-specific genes, such as fatty acid synthetase, fatty acid binding protein, leptin and adiponectin. Biotin is a coenzyme in the metabolism of fatty acids and leucine, and it plays a role in gluconeogenesis [99]. Pantothenic acid is an essential nutrient required to synthesize coenzyme-A (CoA), as well as to synthesize and metabolize proteins, carbohydrates, and fats [100]. Triiodothyronine increases the production of the Na^+/K^+-ATPase and, in general, increases the turnover of different endogenous macromolecules by increasing their synthesis and degradation [101]. Pioglitazone stimulates the nuclear receptor

peroxisome proliferator-activated receptor gamma (PPAR-γ) and to a lesser extent PPAR-α. It modulates transcription of insulin-sensitive genes involved in lipid metabolism control in adipose tissue, muscle and liver [102].

2.1.6. Staining of human adipocytes with Sudan III

Saturated (approximately 1%) stock solution of oil red O in 99% isopropanol was prepared. 60 ml of stock solution was diluted with 40 ml 1% dextrin. Solution was left to stand over night and then filtered.

Adipocytes were washed 3 times with PBS. Then cells were fixed with 7.5% formaldehyde for 30 min, after that cells were again washed 3 times with PBS and 3 times with 50% ethanol. Thereafter cells were stained for 40 min with Sudan III solution and washed three times with 50% ethanol and washed two times with tap water. (Figure 13)

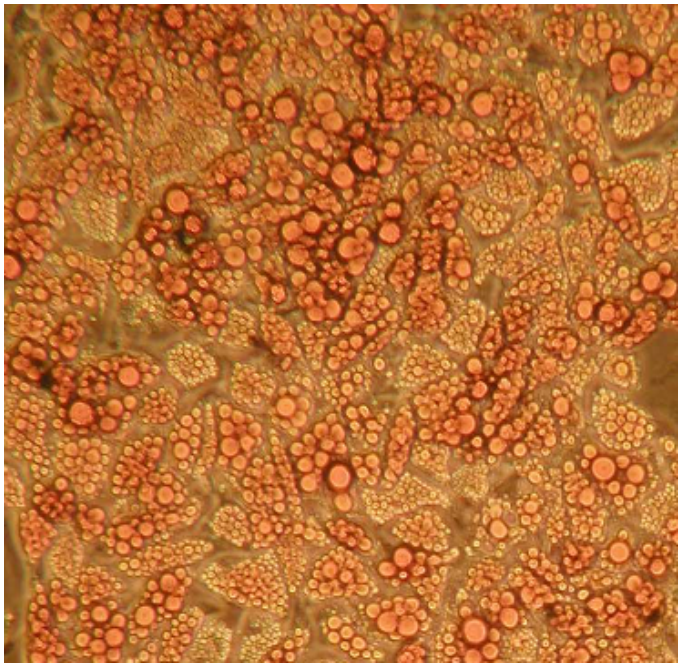


Figure 13 Phase contrast micrographs of human adipocytes stained with Sudan III.

2.1.7. Stimulation of human preadipocytes and adipocytes

Preadipocytes and adipocytes were incubated in serum-free DMEM/F-12 for 24 hours before treatment with the cytokines. Thereafter, the medium was replaced with fresh DMEM/F-12 and recombinant human (rh) OSM (R&D Systems), rhIL-6 (Invitrogen), rhIL-8 (Invirtogen), TNF-α (R&D Systems), SFRP5 (R&D Systems), rhIL-10 (Invitorgen), rhIL-4 (R&D Systems), rhIL-18 (Invitorgen), rhIL-33 (Biovison), rh-Leptin (Prospec) was added at the concentrations indicated. After incubation for 4, 24 and 48h culture supernatants were collected and used immediately or stored at –20℃. Cells were lysed for mRNA isolation.

Cytokine	Concentration
OSM	100 ng/ml
IL-6	100 ng/ml
IL-8	100 ng/ml
TNF-α	2000 U/ml
SFRP5	100 ng/ml
IL-18	100 ng/ml
IL-33	100 ng/ml
Leptin	100 ng/ml

Table 3 Used cytokines and concentrations for stimulation of primary HVPAC, HVAC, HSPAC and HVAC.

2.2. Isolation and cultivation of human coronary artery smooth muscle cells (HCASMC)

Human coronary artery smooth muscle cells (HCASMC) (Figure 14) were derived from tunica intima and tunica media of normal coronary arteries, obtained from patients undergoing heart transplantation. HCASMC were isolated by explant techniqueas described below. Human coronary artery was cut in small pieces and placed on a petri dish coated with 1% gelatine. Tissue pieces were covered with

minimal essential medium (M199; Sigma) containing 20% fetal calf serum (FCS), 20 ng/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, 100U/ ml streptomycin, 0,25µg/ml fungizone and 2 mM L-gluatin (all Cambrex) and incubated at 37℃ in a humidified atmosphere of 5% CO^2: 95% a ir for one to four weeks. After cell attachment the pieces were removed. Cells were cultured in cell culture flasks coated with 1% gelatine in M199 containing 20% FCS 100 U/ml penicillin, 100U/ ml streptomycin, 0,25µg/ml fungizone and 2 mM L-glutamin at 37℃ in humidified atmosphere of 5% CO^2:95% air. Cells were used at low passages. For anoxia experiments cells were cultivated in Anaerocult® IS special incubation bags (Merck).

2.2.1. Stimulation of human coronary artery smooth muscle cells (HCASMC)

24 hours (h) before treatment with the respective cytokine HCASMC were incubated in M199 containing 0.1% bovine serum albumin (BSA; Sigma). Thereafter medium was replaced with fresh M199 containing 0.1% BSA and recombinant human PEDF obtained from Millipore (Bedford) at the indicated concentrations for time periods between 4h and 72h. For anoxia experiments cells were cultivated in Anaerocult® IS special incubation bags (Merck).

Figure 14 Phase contrast micrographs of human coronary artery smooth muscle cells (HCASMC).

2.3. Isolation and cultivation of human umbilical vein endothelial cells (HUVEC)

HUVECs (Figure 15) were isolated from normal human umbilical cords. An adaptor was inserted into the lumen of umbilical cord vessel vein and fixed with sterilized cable-binder. A 20ml-syringe filled with HBSS (Sigma) was connected to the adaptor and the vessel was flushed with 20ml HBSS. Another syringe filled with collagenase solution was connected to the adaptor. The vessel was slowly filled with collagenase solution (collagenase type IV (Sigma), 2mg/ml in HBSS modified) until it leaked out at the other end of the vessel, then the other end of the vessel was closed with a haemostat and filling of the vessel was continued. The umbilical cord vessel was incubated 50 minutes at room temperature; the haemostat was opened after placing the vessel over a centrifuge tube to collect the perfusate. The vessel was flushed with 20ml HBSS, which was and added it to the perfusate. This was then centrifuged for 5 minutes at 1500 rpm. The supernatant was carefully discarded and the pellet was resuspended in M199 (Sigma) containing antibiotics, 20% FCS, 5U/ml heparin, 5µg/ml endothelial cell growth supplement ECGS. Resuspended cells were transferred into an appropriate tissue culture flask, coated with 1% Gelatine and incubated at 37℃, 5% $CO_{2.}$ Medium was changed after 24 hours.

2.3.1. Stimulation of human umbilical vein endothelial cells (HUVEC)

For stimulation M199 containing 20% FCS was replaced with fresh M199 containing 1.25% FCS and recombinant human PEDF obtained from Millipore (Bedford) at the indicated concentrations for time periods between 4h and 72h. For anoxia experiments cells were cultivated in Anaerocult® IS special incubation bags (Merck).

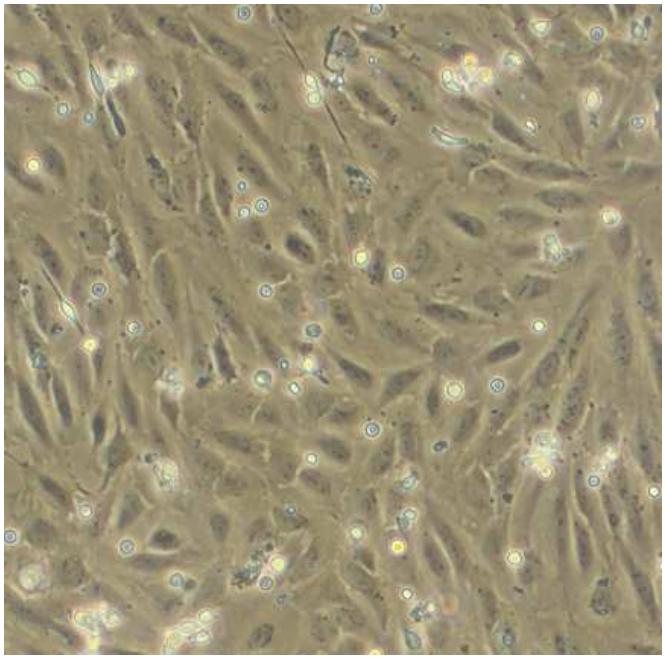


Figure 15 Phase contrast micrographs of human umbilical vein endothelial cells (HUVEC).

2.4. RNA analysis

2.4.1. mRNA purification and determination of RNA concentration

Cells were stimulated as described above, supernatant was removed, and mRNA was isolated with the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. Briefly cells were lysed with a mixture of lysis/binding buffer and PBS. The entire sample was pipetted into the High Pure filter tube and centrifuged. The filter tube was removed from the collection tube, the flow-through was discarded and the filter tube was again combined with the collection tube. The samples were incubated for 15 min at RT with a mixture of DNAse incubation buffer and DNAse I working solution, thereafter samples were washed once with wash buffer I and twice with wash buffer II always followed by centrifugation after each washing step. After the last washing step the filter tube was removed from the Collection tube, combined to a nuclease free, sterile microcentrifungation tube and elution buffer was added. The tube was centrifuged for 1 min at 8000 rpm, afterwards the filter was removed and the RNA containing tube was stored at -80℃. For determination of RNA concentration, 1µl of each RNA sample was examined on the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific).

2.4.2. Synthesis of complementary DNA by reverse transcription

mRNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). After determination of RNA concentration, the appropriate volume for 1µg of RNA was calculated. The RNA was prepared in Eppendorf cups and filled up with nuclease-free water to 10 µl. 2.0µl Random Hexamer Primer and 1.0µl Anchored-oligo(dT)18 Primer were added per sample and incubated 10 min at 65℃ for annealing. Masterm ix was prepared according to Table 4 and was then added to the RNA samples and the program (10 minutes on 25℃, 1 hour on 50℃, 5 minutes on 85℃) was starte d.

1x master mix	volume [µl]
Reaction Buffer	4.0
RNase Inhibitor	0.5
Deoxynucleotide Mix	2.0
Reverse Transcriptase	0.5

Table 4 Mastermix for cDNA synthesis

2.4.3. Quantitative Real-Time PCR

The general principle of a hydrolysis probe assay (Figure 16) is basically the same as for standard PCR: cDNA is denaturated, annealed with primers and a desired fragment within the gene of interest is amplified. In addition, real-time PCR exactly quantifies the amount of PCR product in real time, simultaneously to the amplification. This is possible by addition of a short nucleotide probe labelled with a fluorescent reporter as well as a quencher. The 3´-end of the probe is phosphorylated, so that it can not be extended during PCR. When the probe is intact the quencher molecule is close enough to the reporter to suppress the reporter fluorescent signal. As the primers bind and are extended 5´- nuclease activity of the Taq polymerase cleaves the hydrolysis probe and reporter and quencher are

released separately. In the cleaved probe the dye is able to emit fluorescence, correlating to the amount of PCR product. Consequently, the total amount of PCR product can be measured at every time point during the process. Upon comparison with a housekeeping gene e.g. human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the exact amount of the gene of interest expressed in the cell population can be determined.

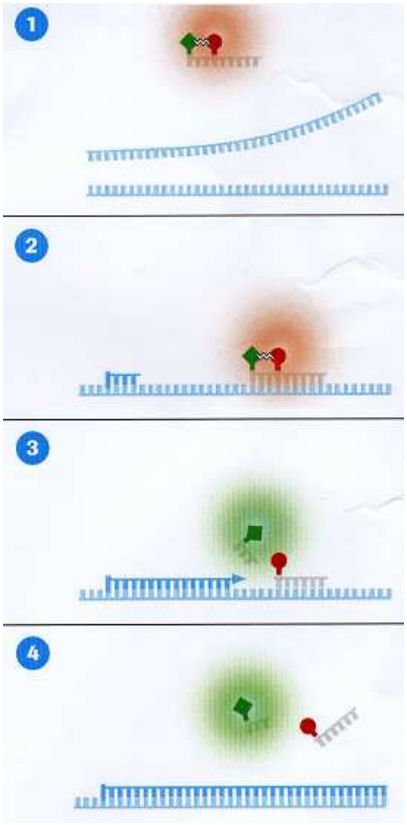


Figure 16 Mechanism of hydrolysis probe assay (Roche)

Specific mRNA levels for PEDF VEGF IL-8 and GAPDH were determined by real time PCR. Samples were analyzed in triplicates on a 96-well reaction plate (Roche, Basel, Switzerland) applying the real-time LightCycler Probe Master, LightCycler TaqMan Master UniversalProbeLibrary kit (Table 5). Real-time polymerase chain reaction (PCR) was performed with the LightCycler 480 Real-Time PCR. Primers, shown in Table 6, were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com). Thermal program shown in Table 7 was used. The amplification conditions consisted of an initial incubation at 95℃ for 10 minutes, followed by 70 cycles of 95℃ for 10 seconds, the respective annealing temperature of 60℃ for 20 seconds and 72 ℃ for 20 seconds and a final

cooling to 40℃. Data was analyzed using LightCycle r 480 Software Version 1.5 Service Pack 3 (Roche).

master mix	volume [µl]
forward primer	0,04
reverse primer	0,04
Universal ProbeLibrary probe	0,2
LightCycler 480 Probes Master	10
dH2O	9

Table 5 Real-time PCR master mix

Target	fwd-Primer	rev-Primer	Annealing temperature
GAPDH	5'-agccacatcgctcagacac –'3	5'-gcccaatacgaccaaatcc-'3	60℃
VEGF	5'-ctacctccaccatgccaagt-'3	5'-ccacttcgtgatgattctgc-'3	60℃
IL8	5'-agacagcagagcacacaagc-'3	5'-atggttccttccggtggt-'3	60℃
PEDF	5'-gtgtggagctgcaccgtat-3'	5'-tccaatgcagaggatagca-3'	60℃

Table 6 Primer sequences for quantitative Real-time PCR

Cycles	temperature [℃]	time
1	95	10min
70	95	10sec
	60	20sec
	72	20sec
1	40	10sec

Table 7 Thermal cycling program for qRT-PCR

2.5. Antigen assays

2.5.1. Enzyme-linked immunosorbent assays

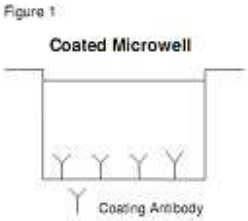
IL8 and VEGF-A antigen in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies against human VEGF-A (Quantikine Immunoassay; eBioscience.) and human IL8 (Quantikine ELISA kit; R&D Systems), respectively. All measurements were performed in triplicates.

2.5.2. VEGF ELISA

VEGF antigen was determined by a specific ELISA (Figure 17) with a standard range from 15.6 to 1000 pg/ml according to manufacturer's instructions. In short the pre-coated microwell strips were washed twice with wash buffer. Samples and standards were properly diluted with sample diluent, added to the wells and incubated at room temperature for 2 hours, on a microplate shaker set at 100 rpm. Test wells were empted and the plate was washed, the VEGF conjugate was added to the wells, incubated for 2 hour. Following a washing step substrate solution for colour development was applied for 20 min and the reaction was stopped by adding 50 µL of stop solution to each well. The ELISA was read using a TECAN Sunrise microplate reader (Tecan) at 450nm and analysed with deltaSoft (Tecan). VEGF-A concentrations read from the standard curve were multiplied by the dilution factor.

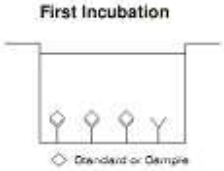
An anti-human VEGF-A coating antibody is adsorbed onto microwells.

Figure 1



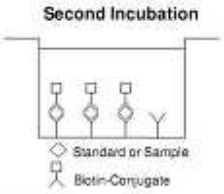
Human VEGF-A present in the sample or standard binds to antibodies adsorbed to the microwells.

Figure 2



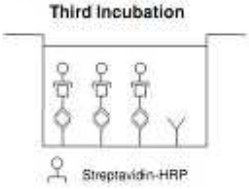
Following incubation unbound biological components are removed during a wash step A biotin-conjugated anti-human VEGF-A antibody is added and binds to human VEGF-A captured by the first antibody.

Figure 3



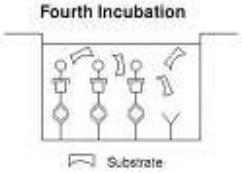
Following incubation unbound biotin-conjugated anti-human VEGF-A antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGF-A antibody.

Figure 4



Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 5



A coloured product is formed in proportion to the amount of human VEGF-A present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human VEGF-A standard dilutions and human VEGF-A sample concentration determined.

Figure 6

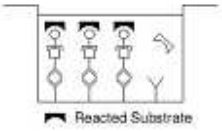


Figure 17 Principles of the VEGF-A ELISA (eBioscience)

2.5.3. IL-8 ELISA

IL-8 antigen was detected in cell conditioned media using the human IL8 Quantikine ELISA kit (R&D Systems). This ELISA has a standard range from 31.2 to 2000 pg/ml according to manufacturer's instructions. Samples and standards were properly diluted with assay diluent, added to the wells and incubated at room temperature for 2 hours. Test wells were emptied and the plate was washed 4 times with wash buffer, the IL-8 conjugate was added to the wells, incubated for 1 hour. Following a washing step 200 µl of substrate solution for colour development was applied for 30 min and the reaction was stopped by adding 50 µL of stop solution to each well. The ELISA was read using a TECAN Sunrise microplate reader (Tecan) at 450nm and analysed with deltaSoft (Tecan). IL-8 concentrations read from the standard curve were multiplied by the dilution factor.

2.6. Statistical analysis

Data was compared statistically by t-test and ANOVA. Values of $P<0.05$ were considered significant.

3. Results

3.1. Part I: Basal expression of PEDF in human visceral and subcutaneous adipocytes.

Human visceral adipocytes derived from human visceral fat expressed 24% more mRNA for PEDF than human visceral preadipocytes (Figure 18). Whereas human subcutaneous adipocytes derived from human subcutaneous fat expressed just 5% more mRNA for PEDF than human subcutaneous preadipocytes. The examined visceral and subcutaneous adipose tissue derived from 14 overweight donors (BMI > 35kg/m^2). The differences between PEDF expression in HVAC and HVPAC was statistically significant, using t-test for paired samples ($p<0.03$), one-way ANOVA revealed a significant difference (* $p<0.01$). Differences between PEDF expression in HSAC and HSPAC was statistically significant, using one-way ANOVA (* $p<0.05$).

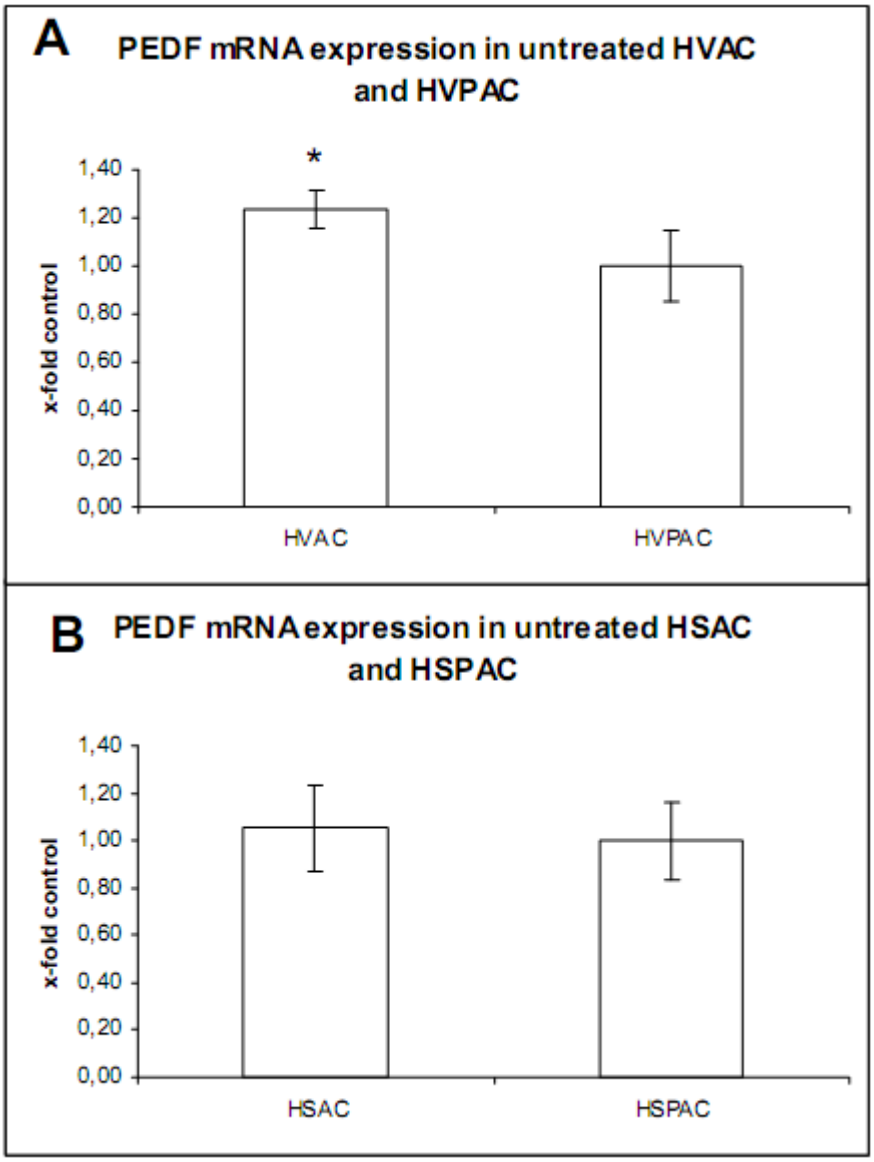


Figure 18 Basal PEDF mRNA expression level in HSADI, HVADI (A), HSPAC and HVPAC (B). RNA was prepared from confluent monolayers of untreated HSADI, HVADI, HSPAC and HVPAC. PEDF mRNA expression of was analyzed by quantitative real-time PCR using glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene as described in METHODS AND MATERIALS. Results are calculated as x-fold of HVPAC, HSPAC respectively which was set as 1 fold. Values represent means +/- SD of 14 determinations with cells obtained from 14 different donors and gave similar results. ($p<0.05$)*

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3.2. Part II: Effect of inflammatory cytokines on PEDF expression.

HSADI, HVADI, HSPAC and HVPAC were treated with different cytokines at the indicated concentrations (Table 3) for 4, 24, and 48 h. RNA was prepared from confluent monolayers of untreated and treated HSADI, HVADI, HSPAC and HVPAC and RT-PCR using specific primer for PEDF and GAPDH as performed as described in METHODS AND MATERIALS.

Treatment of the HSAC, HVAC, HSPAC and HVPAC with the indicated inflammatory cytokines (Table 3) for 4, 24 and 48h did not result in a significant change of the PEDF mRNA-expression. (Data not shown)

3.3. Part III: Influence of stimulation with PEDF on VEGF production in visceral adipocytes.

Effect of PEDF on VEGF expression in visceral adipocytes under normoxic conditions.

Under normoxic conditions PEDF treatment increased VEGF expression significantly in human visceral adipocytes. If cells were treated with PEDF at a concentration of 400 ng/ml for 24, 48 or 72 hours, VEGF expression was increased up to 6 fold (Figure 19).

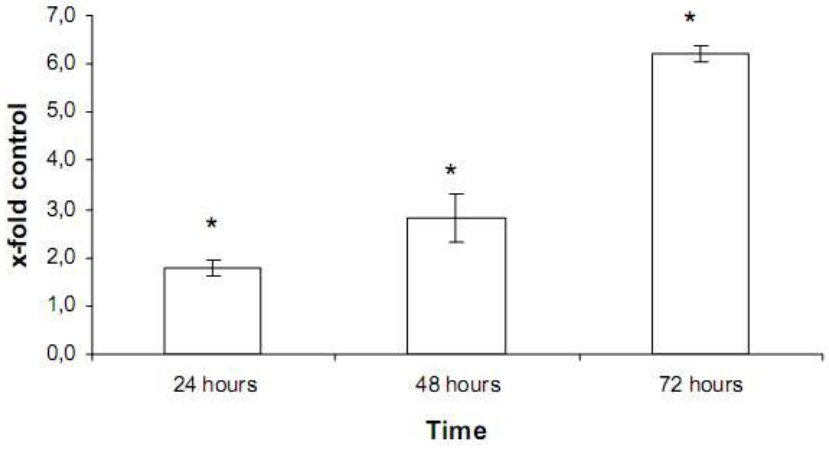

Figure 19 VEGF mRNA expression in human primary adipocytes cultivated under normoxic conditions. Confluent monolayers of HVADI were incubated for 24, 48, or

72 h in absences or in presence of PEDF at a concentration of 400 ng/ml. Whole cells were lysed, mRNA was prepared and analysed by RealTime-PCR with primers specific for VEGF and GAPDH as described in METHODS AND MATERIALS. VEGF mRNA level was normalized according to the respective GAPDH. mRNA levels are given as fold of control which was set as 1-fold. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD. (p<0.5)*

Effect of PEDF on VEGF protein production in visceral adipocytes under normoxic conditions.

Under normoxic conditions PEDF increased VEGF protein production significantly in human visceral adipocytes. If cells were treated with PEDF at a concentration of 400 ng/ml for 24, 48 or 72 hours, VEGF production was increased up to a third (Figure 20).

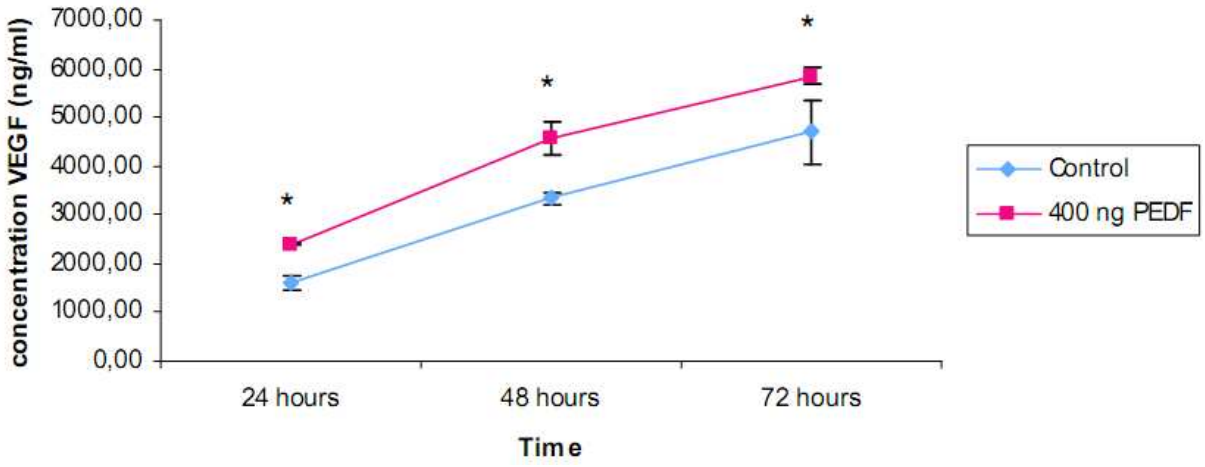

Figure 20 Secretion of VEGF in human primary adipocytes cultivated under normoxic conditions. Confluent monolayers of HVADI were incubated for 24, 48, or 72 h in absences or in presence of PEDF at a concentration of 400 ng/ml. Conditioned media were collected and VEGF antigen was determined by ELISA as described in MATERIALS AND METHODS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD (p<0.005).*

Effect of PEDF on VEGF expression in visceral adipocytes under anoxic conditions.

Under anoxic conditions PEDF treatment did not influence VEGF expression significantly in human visceral adipocytes after 24 and 72h incubations but after 48h PEDF treatment did influence VEGF expression significantly (Figure 21).

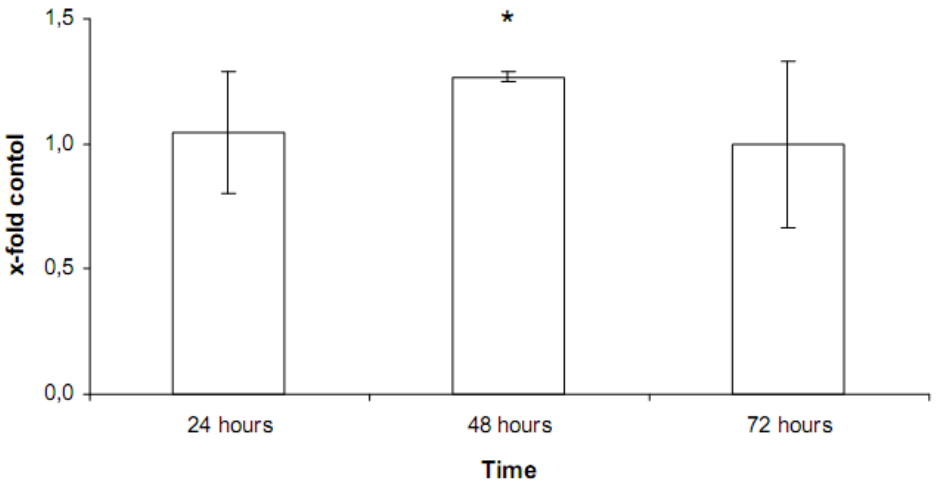

Figure 21 Expression of VEGF in human primary adipocytes treated with 400 ng/ml PEDF for 24, 48, 72 hours cultivated under anoxic conditions. Confluent monolayers of HVADI were incubated for 24, 48, or 72 h in absences or in presence of PEDF at a concentration of 400 ng/ml. Whole cells were lysed, mRNA was prepared and analysed by RealTime-PCR with primers specific for VEGF and GAPDH as described in METHODS AND MATERIALS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. VEGF mRNA levels was normalized according to the respective GAPDH. mRNA levels are given as fold of control which was set as 1-fold. Values represent the mean value +/- SD (p<0.05).*

Effect of PEDF on VEGF protein production in visceral adipocytes under anoxic conditions.

Under hypoxic conditions VEGF protein production in control cells was the same as in PEDF treated cells. Hypoxia increased VEGF production in control cells and in cells treated with PEDF (400 ng/ml) in comparison with PEDF treated cells under normoxic conditions and normoxic control cells (Figure 22).

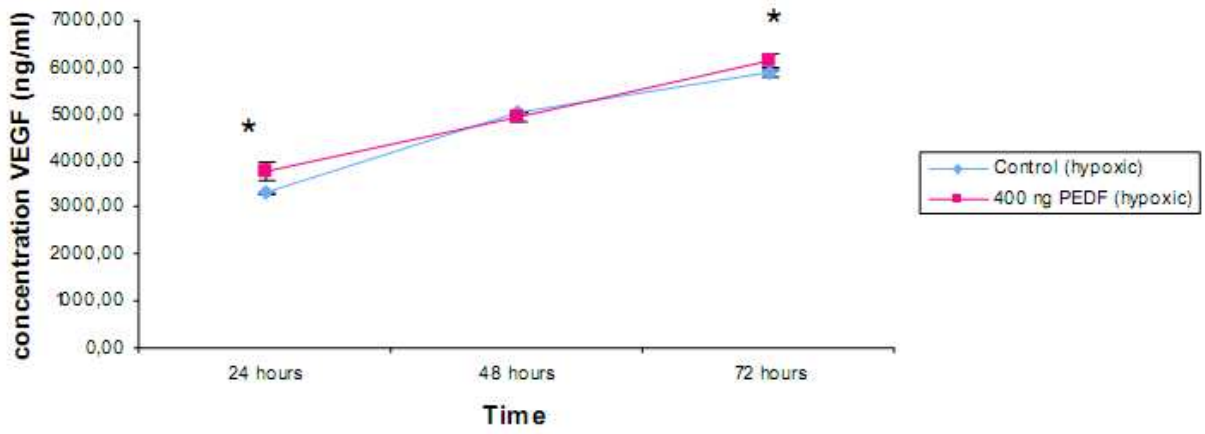

Figure 22 Secretion of VEGF in human primary adipocytes treated with 400 ng/ml PEDF for 24, 48, 72 hours cultivated under normoxic and hypoxic conditions. Confluent monolayers of HVADI were incubated for 24, 48, or 72 h in absences or in presence of PEDF at a concentration of 400 ng/ml. Conditioned media were collected and VEGF antigen was determined by ELISA as described in MATERIALS AND METHODS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD. (p<0.05)*

3.4. Part IV: Effect of PEDF on expression and protein level of VEGF in HCASMC.

Effect of PEDF on mRNA expression level of VEGF in HCASMC under normoxic conditions

When HCASMC under normoxic conditions were treated with PEDF at a concentration of 4, 40, 400, 800 ng/ml for time period of 4, 24, 48 and 72h a dose and time dependent effect on VEGF mRNA production was observed (Figure 23).

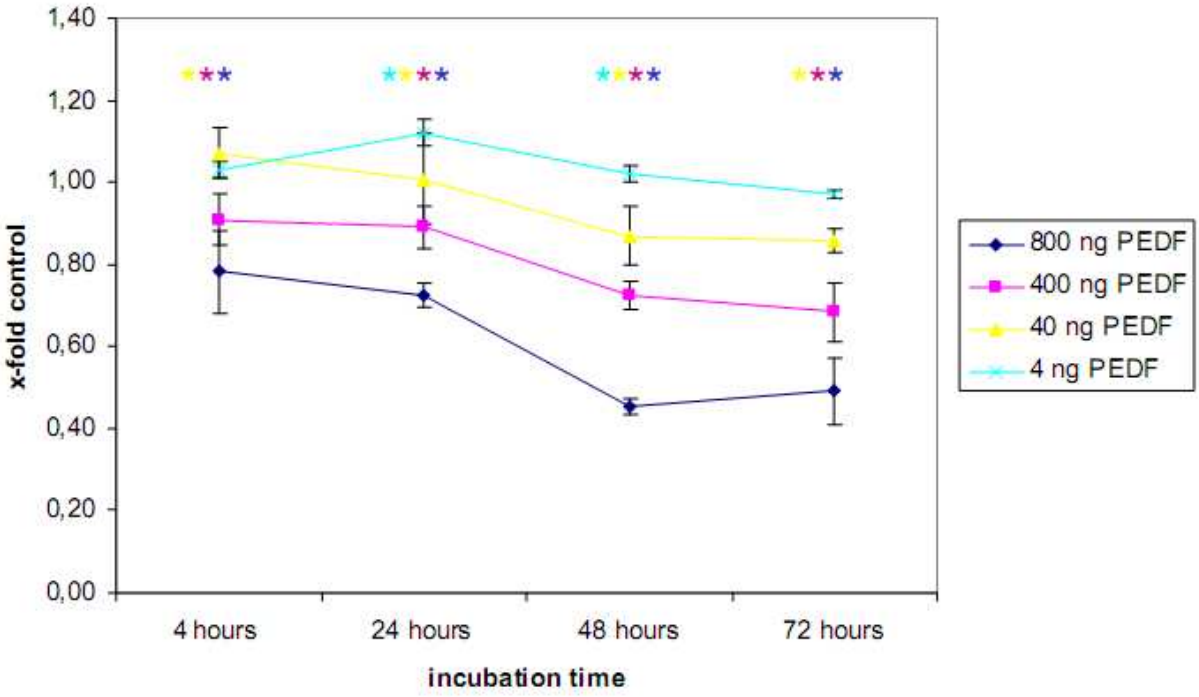

Figure 23 Decrease of VEGF mRNA expression by human coronary artery smooth muscle cells treated with PEDF is dose and time dependant. Confluent monolayers of HCASMC were incubated for 12, 24, 48, or 72 h in absences or in presence of PEDF at a concentration of (A) 4, (B) 40, (C) 400 or (D) 800 ng/ml. Whole cells were lysed, mRNA was prepared and analysed by RealTime-PCR with primers specific for VEGF and GAPDH as described in METHODS AND MATERIALS. Experiments were performed 5-times with cells obtained from 5 different donors and gave similar results. A representative experiment is shown. VEGF mRNA level was normalized according to the respective GAPDH. mRNA levels and are given as fold of control witch was set as 1-fold. Values represent the mean value +/- SD (p<0.01).*

Effect of PEDF on VEGF protein level in HCASMC under normoxic conditions is dose and time dependant.

VEGF ELISA revealed that stimulation of HCASMC with PEDF decreases VEGF protein production. When HCASMC were incubated with PEDF 4 ng/ml under normoxic conditions the protein level of VEGF was only slightly diminished, but treatment with 40, 400 or 800 ng/ml revealed a significant dose and time dependant decrease of VEGF production. If HCASMC were incubated with 40, 400, 800 ng PEDF for 48 hours VEGF level was inhibited by 14% (4 ng/ml), 26% (40 ng/ml), 27% (400 ng/ml), and 41% (800 ng/ml) (Figure 24).

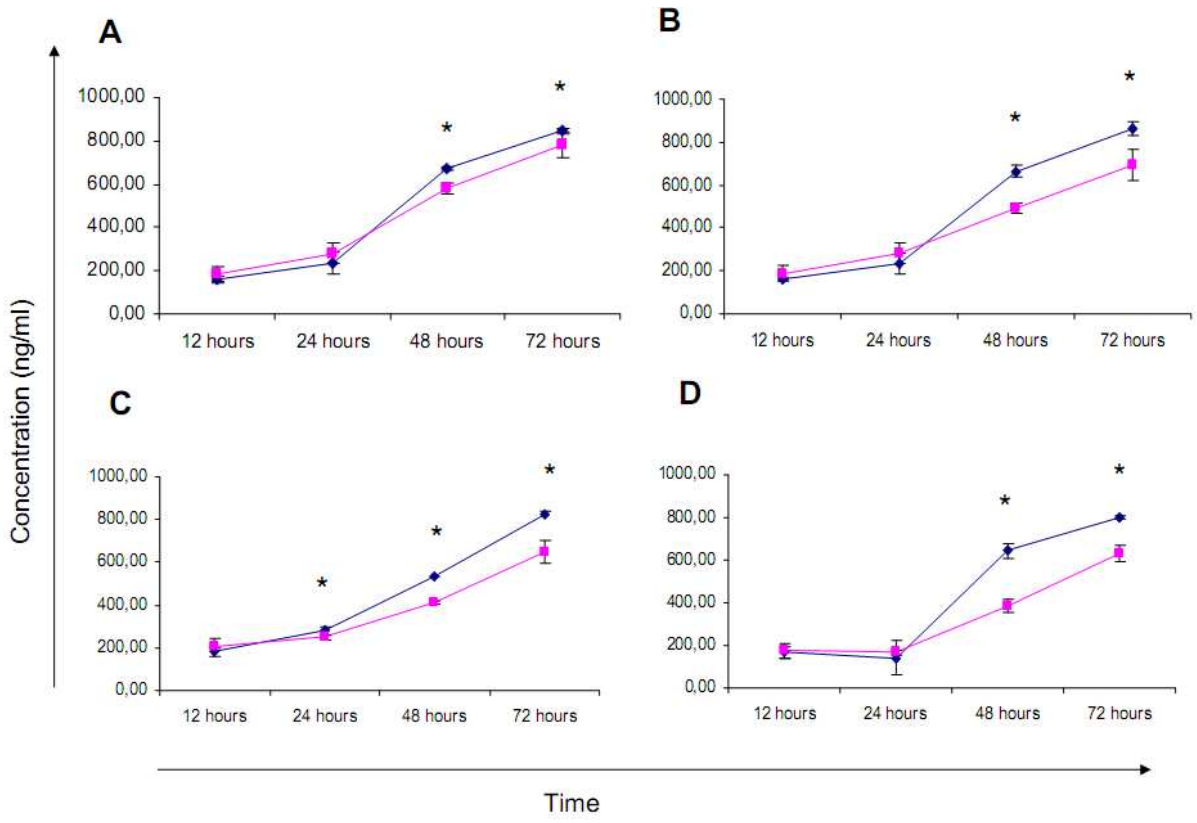

Figure 24 Effect of PEDF on VEGF protein production level in human coronary artery smooth muscle cells is dose-and time-dependent. Control is shown in blue, PEDF treated cells in pink. (F=7.60, p<0.05). Confluent monolayers of HCASMC were incubated for 12, 24, 48, or 72 h in absences or in presence of PEDF at a concentration of (A) 4, (B) 40, (C) 400 or (D) 800 ng/ml. Conditioned media were collected and VEGF antigen was determined by ELISA as described in MATERIALS

AND METHODS. Experiments were performed 5-times with cells obtained from 5 different donors and gave similar results. A representative experiment is shown. VEGF concentrations are given as fold of control witch was set as 1-fold. Values represent the mean value +/- SD (p<0.05).*

Effect of PEDF on VEGF protein level in HCASMC under hypoxic conditions.

When HCASMC under hypoxic conditions were treated with PEDF at a concentration of 400 ng/ml, VEGF protein production after 48 h was equal to the level in not treated cells. However, VEGF protein in both, hypoxic PEDF treated and not treated cells was significantly higher than in normoxic PEDF treated cells (Figure 25).

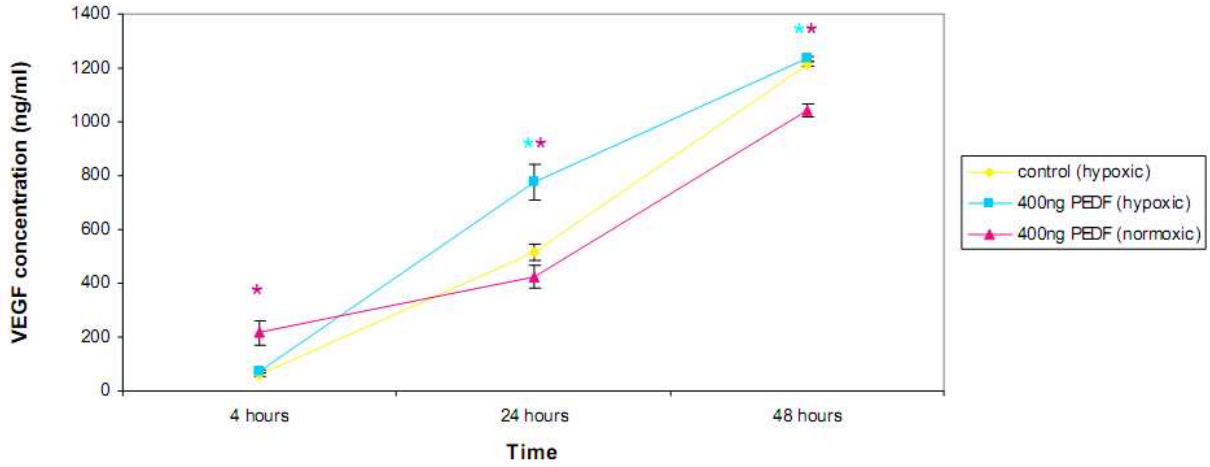

Figure 25 Effect of 400 ng PEDF and hypoxia for time periods of 4, 24 and 48h on VEGF protein production level in human coronary artery smooth muscle cells. Combination of PEDF and hypoxia induced VEGF upregulation. Confluent monolayers of HCASMC were incubated for 12, 24 or 48 h in absences or in presence of PEDF at a concentration of 400 or ng/ml, under normoxic (pink) and hypoxic (yellow, blue) conditions. Conditioned media were collected and VEGF antigen was determined as described in MATERIALS AND METHODS. . Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD. (p<0.001)*

Effect of PEDF on mRNA expression level of IL-8 in HCASMC

When HCASMC were treated with PEDF at a concentration of 400 ng/ml IL-8 mRNA production was diminished up to 78% (Figure 26)

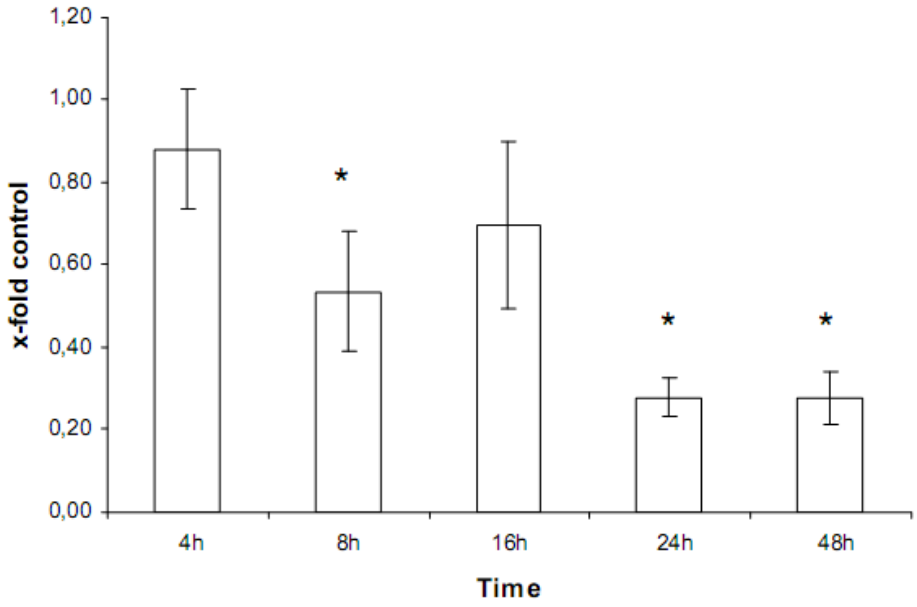

Figure 26 Effect of PEDF stimulation on IL-8 expression in HCASMC. Confluent monolayers of HCASMC were incubated for 4, 8, 16, 24 or 48 h in absence or in presence of PEDF at a concentration of 400 ng/ml. Whole cells were lysed, mRNA was prepared and analysed by RealTime-PCR with primers specific for IL-8 and GAPDH as described in METHODS AND MATERIALS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. IL-8 mRNA level was normalized according to the respective GAPDH. mRNA levels are given as fold of control witch was set as 1-fold. Values represent the mean value +/- SD. (p<0.05)*

Effect of PEDF on IL-8 protein production level in HCASMC

When HCASMC were treated with PEDF at a concentration of 400 ng/ml IL-8 protein production was diminished up to 37% (Figure 27)

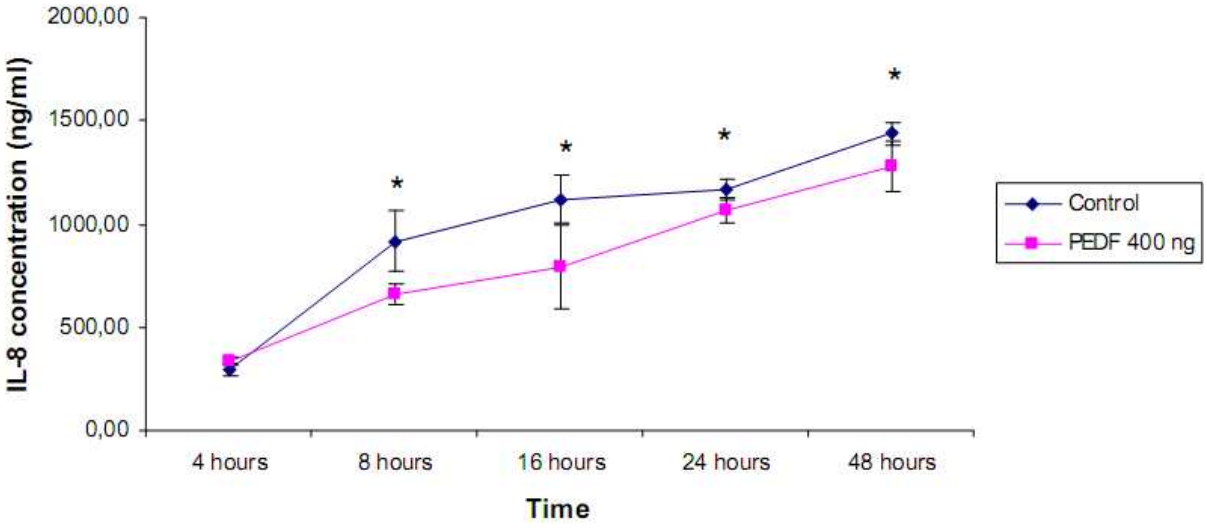

Figure 27 Effect of PEDF stimulation on IL-8 secretion after 4, 8, 16, 24 and 48 hours in HCASMC. Confluent monolayers of HCASMC were incubated for 4, 8, 16, 24 or 48 h in absences or in presence of PEDF at a concentration of 400 ng/ml. Conditioned media were collected and IL-8 antigen was determined by ELISA as described in MATERIALS AND METHODS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD. (p<0.05)*

3.5. Part V: Effect of PEDF on IL-8 expression and protein production in HUVEC.

Effect of PEDF on the IL-8 m RNA level in HUVEC

A significant decrease in IL8 mRNA was observed in HUVEC treated with 400ng/ml human recombinant PEDF (Figure 28).

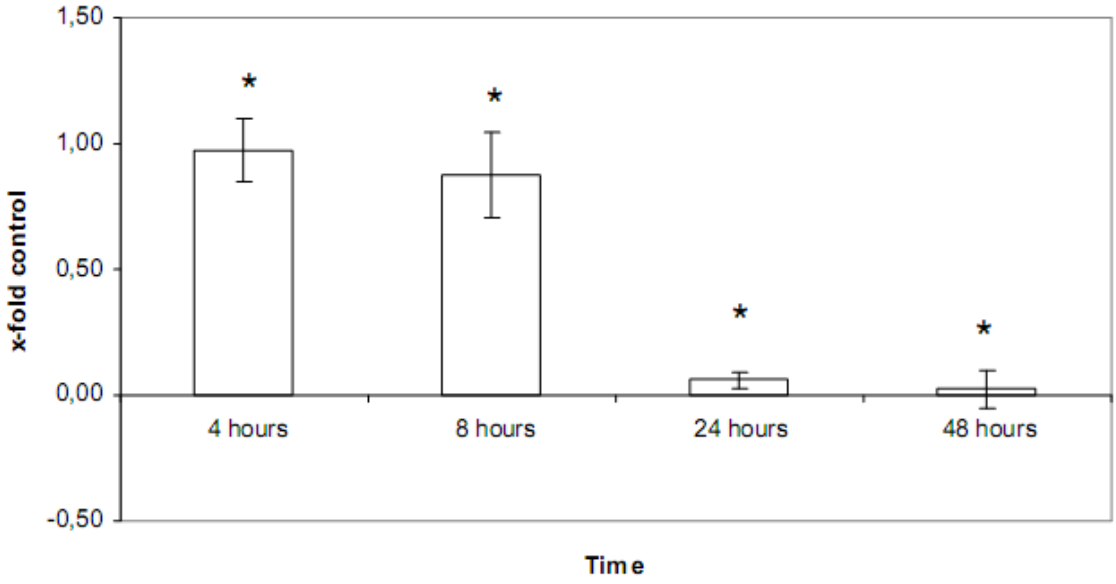

Figure 28 IL-8 mRNA expression decreases after stimulation with 400ng PEDF for 4, 8, 24 and 48h in HUVEC. Confluent monolayers of HUVEC were incubated for 4, 8, 24 or 48 h in absences or in presence of PEDF at a concentration of 400 or ng/ml, under normoxic conditions. Whole cells were lysed, mRNA was prepared and analysed by RealTime-PCR with primers specific for IL-8 and glyeraldehyd-3-phosphate (GAPDH) as described in METHODS AND MATERIALS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. IL-8 mRNA level was normalized according to the respective GAPDH. mRNA level and are given as fold of control witch was set as 1-fold. Values represent the mean value +/- SD. (p<0.05)*

Effect of PEDF on the IL-8 protein production level in HUVEC

A significant decrease in IL8 protein production was observed in HUVEC treated with 400ng/ml human recombinant PEDF (Figure 29).

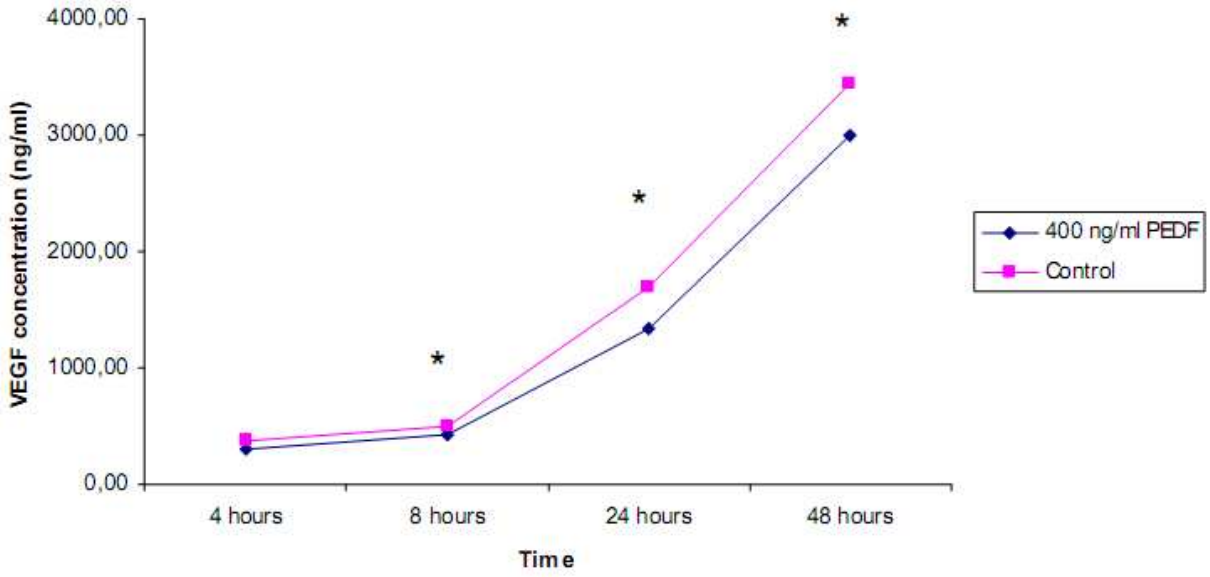

Figure 29 IL-8 mRNA expression decreases after stimulation with 400ng PEDF for 4, 8, 24 and 48h in HUVEC. Confluent monolayers of HUVEC were incubated for 4, 8, 24 or 48 h in absences or in presence of PEDF at a concentration of 400 or ng/ml, under normoxic conditions. Conditioned media were collected and IL-8 antigen was determined by ELISA as described in MATERIALS AND METHODS. Experiments were performed 3-times with cells obtained from 3 different donors and gave similar results. A representative experiment is shown. Values represent the mean value +/- SD. (p< 0.05)*

4. Discussion

In context of obesity adipose tissue secretes a variety of inflammatory adipokines such as IL-6, PAI-1 and TNF- α. A novel adipokine is PEDF, which has been shown to be expressed and secreted by adipocytes, differentiated out of human mesenchymal stem cells [103] and 3T3-L1 mouse adipocytes [104,105]. Recent studies revealed PEDF as one of the most abundant adipokines in supernatant of 3T3-L1 adipocytes and a significant increase in expression and secretion of PEDF during differentiation of human adipocytes was shown. Since *Crowe et al*. [104] demonstrated that PEDF expression in mice correlates with obesity the role of adipose tissue in PEDF secretion became evident. Furthermore PEDF protein levels are positively regulated with oxygen tension in anoxic cardiac myocytes as well as in hypoxic retinal glia cells [37,41]. *Famulla et al*. could show that in adipocytes hypoxia negatively regulates PEDF expression and release [45]. PEDF is involved in many physiological and pathological processes of the human organism. However, little is known about the regulation of PEDF expression and secretion and its impact on angiogenesis. These aspects, which were the focus of the present work, will be discussed in the following.

Basal PEDF expression in human visceral and subcutaneous preadipocytes and adipocytes.

The expression pattern of PEDF during differentiation from human preadipocytes to adipocytes is not yet understood in detail. A number of studies point to a decrease in PEDF mRNA expression during differentiation in 3T3-L1 cells [84,105], whereas other studies showed a differentiation dependant increase of PEDF mRNA in human adipocytes [103,106]. *Famulla et al* showed that in primary human adipocytes isolated out of subcutaneous adipose tissue from lean or moderately overweight women PEDF secretion and expression increased significantly during differentiation process [45]. *Crowe et al* demonstrated in mice that PEDF secretion in adipose tissue correlates with obesity and diabetes [104]. In contrast to other systems we didn’t examine whole-tissue PEDF expression which does not account for the heterogeneous cellular composition of adipose tissue. The number of dendritic cells

increases in adipose tissue with overall body fat mass, which seems to be a source of PEDF [107] therefore further processing of adipose tissue is essential for accurate results. In our system of in vitro differentiated adipocytes we used subcutaneous and visceral preadipocytes isolated out of adipose tissue from patients undergoing gastric surgery who had a BMI over 35 kg/m². In the present study PEDF expression in human visceral adipocytes, preadipocytes has been characterized in detail. Visceral adipocytes expressed 24% more PEDF mRNA than visceral preadipocytes whereas subcutaneous adipocytes expressed just 5% more PEDF mRNA than subcutaneous preadipocytes. Additionally, two other studies could show that PEDF plasma levels in human correlate with obesity [104]. We showed in our study that the PEDF mRNA level in subcutaneous preadipocytes and adipocytes differs slightly and that visceral adipocytes express a higher PEDF mRNA level than visceral preadipocytes. *Famulla et al* were able to show that subcutaneous adipocytes derived from in lean people express significantly more PEDF than subcutaneous preadipocytes, leading to the suggestion that in overweight patients subcutaneous preadipocytes are an important PEDF source and possible are responsible for the higher PEDF level in obesity [45].

Effect of inflammatory cytokines on PEDF expression

Treatment of the HSAC, HVAC, HSPAC and HVPAC with the inflammatory cytokines did not affect PEDF expression in human adipose tissue. Although visceral and subcutaneous adipocytes express gp130, the receptor for all gp130 ligands, none of the gp130 cytokines used in our study resulted in a significant change in PEDF expression. Further studies should be conducted to verify these results also on the protein level.

Influence of PEDF on VEGF expression in visceral adipocytes

Modulation of angiogenesis in adipose tissue may constitute a strategy to affect obesity [2]. In the present study we demonstrated that PEDF treatment under normoxic conditions increases VEGF expression and protein production in human visceral adipocytes. This was in contrast to treatment with PEDF under hypoxia

where production the of angiogenic stimulator VEGF was not influenced. We observed that VEGF protein secretion in visceral adipocytes treated under hypoxic conditions was 20% higher than under normoxic conditions. This was in contrast to our results *Wree et al.* recently published that in white adipocytes VEGF expression does not change upon hypoxic stimulation [108]. PEDF expression and production by adipocytes are elevated in rodent obesity models, also in human overweight patients adipose tissue releases PEDF, leading to a higher PEDF level in the bloodstream. Adipocytes used for this investigation were isolated from adipose tissue derived from overweight patients and therefore probably were exposed to a higher PEDF level. In further investigations VEGF expression and production in adipocytes treated with PEDF derived from lean patients should be used as comparison.

Effect of PEDF stimulation on VEGF mRNA and protein level in HCASMC

There are many inhibitors and stimulators of angiogenesis and among them, VEGF appears as a primary angiogenic stimulator [109]. Several studies showed that PEDF can induce differentiation and inhibition of angiogenesis in several tumors and disrupts the balance between pro- and anti-angiogenic triggers. *Guan et al* initially demonstrated that PEDF is involved in tumor angiogenesis, by downregulation of VEGF and bFGF and enhanced the expression of angiogenic inhibitor thrombospondin 1 (TSP-1) [110]. It was demonstrated in mice that substances inhibiting the angiogenic effect of VEGF also inhibited the growth of adipose tissue.[47] Therefore it seems warranted to acquire more information on the expression and functional role of pro- and antiangiogenic factors. In this study, we explore whether PEDF as angiogenic factor could be a possible target in the regulation of adipose tissue growth. The general aim of this project was to comprehend the significance of PEDF for adiposity and angiogenesis. The antiangiogenic action of PEDF was identified by Dawson and colleagues [43]. In the eye it was demonstrated that PEDF is a major inhibitor of development of vascular network counteracting the proangiogenic effect of VEGF [111-114]. Additionally antioxidant, anti-inflammatory and anticancer activities of PEDF have been shown [48,63,115]. Signalling pathways mediating the broad activities of PEDF have not yet been identified. Previous studies demonstrated that VEGF is highly expressed in HCASMC. The present study under normoxic conditions identified PEDF as an in

vitro inhibitor of proangiogenic factors VEGF in HCASMC, as well on mRNA level as on protein production level. We observed a reduction of VEGF production in a dose- and time dependant manner. PEDF mediated signalling in HCASMC may lead to inhibition of angiogenesis. Interestingly under hypoxic conditions VEGF protein level in HCASMC was not influenced by treatment with PEDF. It remains to be elucidated whether the unchanged VEGF secretion under hypoxic conditions occurs by a ROS-dependent mechanism.

Effect of PEDF on IL-8 mRNA expression and secretion in HCASMC and HUVEC.

Recently a study from *HO et al* showed that PEDF induces apoptosis in primary macrophages and endothelial cells by increasing PPARγ expression and transcriptional activity [58]. *Hirsch et al* were able to show that PEDF treatment inhibits production of IL-8 mRNA and protein in human hormone-refractory cancer cells [96]. They demonstrated that PEDF limits IL-8 production through inactivation of NFκB and activation of PPARγ. PPAR γ is a nuclear fatty acid receptors with ligand-dependent transcriptional activity, regulating various aspects of energy homeostasis [58]. In the present study we examined the in vitro effect of PEDF on IL-8 production in HCASMC and HUVEC and were able to demonstrate a significant decrease in mRNA expression and secretion decrease in both cell types. Decreased IL-8 production is probably involved in the antiangiogenic impact of PEDF. Further investigations are needed to test if in HACSMC and HUVEC PPARγ upregulation and suppressed NFκB-mediated transcription are the involved pathways in reduced production of IL-8 as it was shown in human refractory prostate cancer cells [58,96].

Conclusion and outlook

The present study confirms the importance of PEDF for various mechanisms of angiogenesis. Our study demonstrated that under normoxic conditions PEDF increased VEGF mRNA and protein production significantly in human visceral adipocytes whereas in HCASMC treated with PEDF a dose and time dependent decrease of VEGF mRNA and protein production was observed. These results suggest a cell-specific effect of PEDF that needs further investigations. Also, PEDF reduces the expression of IL-8, another antiangiogenic factor. IL-8 mRNA and protein production was diminished after PEDF treatment as well in HCASMC as in HUVEC. Our results indicate that PEDF may play a role in obesity progression. Furthermore in adipose tissue PEDF secretion may lead to upregulation of proangiogenic factor VEGF. Further research is required to estimate whether blockade of PEDF signalling may be a promising tool for obesity therapy.

5. Abstract

5.1. Summary

Introduction The proportion of overweight and obese people has increased significantly over the last few decades, nowadays one of three adults is overweight and one of ten is obese [8]. Overweight and obese patients are at higher risk of developing a number of diseases such as cardiovascular diseases such as arthrosclerosis, diabetes mellitus, high blood pressure and cancer and have an increased risk of premature mortality [116]. Today overweight and obesity is the fifth most frequent cause of death worldwide [8]. Adipose tissue, besides its role in energy storage, is now also seen as an important endocrine organ that produces and secretes a variety of cytokines, hormones and other proteins [117]. Elevated plasma levels of adipose tissue derived factors, called adipokines, are found in obese patients and are associated with the development and progression of cardiovascular diseases [118,119]. Recently it has been shown that the protein pigment epithelium derived factor (PEDF) a 50kDa member of the serpin family is expressed by adipocytes. PEDF is a multifunctional protein with antiangiogenic, antithrombotic, neuroprotective, antitumorigenic and antiinflammatory properties [103]. It has been demonstrated that PEDF is massively expressed in human adipose tissue, expression level increases significantly during adipogenesis and its plasma level is elevated in overweight patients [45].

Aim It is the aim of this project to characterize the possible role of PEDF in obesity and to investigate the parakrine effects of PEDF on cell types involved in modulation of angiogenesis such as human coronary artery smooth muscle cells (HCASMC) and human umbilical vein endothelial cells (HUVEC).

Methods (I) The first step was to study the regulation of PEDF expression in human visceral and subcutaneous preadipocytes and adipocytes. (II) As obesity is associated with a generalized inflammatory state we tested whether inflammatory mediators affect the expression of PEDF in preadipocytes and adipocytes. (III) As anoxia is a critical modulator in various organs and tissues, modulating angiogenesis we also studied a possible influence of PEDF in combination with anoxia on the expression patterns of adipocytes. (IV) Further on we stimulated human coronary artery smooth muscle cells (HCASMC) with PEDF to investigate the inhibition of VEGF on RNA and protein level. (V)In addition it was aim of our project to study a

possible correlation between PEDF level and Interleukin 8 (IL-8) in HUVEC and HCASMC.

Results PEDF mRNA expression was significantly increased during adipogenesis of primary visceral adipocytes. Stimulation of visceral and subcutaneous preadipocytes and adipocytes with inflammatory cytokines showed no effect on the PEDF mRNA level. Furthermore, PEDF significantly increased VEGF expression and secretion in human visceral adipocytes. In visceral adipocytes treated with PEDF under normoxic conditions, protein expression was significantly increased in parallel with PEDF secretion. In contrast to VEGF increase in adipocytes, VEGF secretion was significantly reduced by PEDF in HUVEC and HCASMC. Additionally in HCASMC and HUVEC PEDF reduced secretion and expression of IL-8.

Conclusion PEDF is one of the most abundant secreted cytokines by adipose tissue and regulates angiogenic processes by influencing VEGF production in HUVEC, HCASMC and HVADI as well as IL-8 secretion in HUVEC and HCASMC. Because of these various actions, PEDF seems to be an important adipokine significantly involved in the modulation of angiogenesis, obesity progression and obesity-related disorders.

5.2. Zusammenfassung

Einleitung Der Anteil übergewichtige und adipöser Menschen ist in den vergangenen Jahrzehnten stark angestiegen, mittlerweile ist bereits jeder dritte Erwachsene übergewichtig und jeder zehnte sogar adipös [8]. Übergewicht und Adipositas begünstigen die Entwicklung einer ganzen Reihe von Krankheiten wie Herz-Kreislauf-Erkrankungen z.B. Artherosklerose, Diabetes mellitus, Bluthochdruck, Krebs und erhöhen das Risiko eines vorzeitigen Todes [116]. Übergewicht und Adipositas stellen mittlerweile die fünfthäufigsten Todesursache weltweit dar [8]. Fettgewebe ist nicht nur ein Speicherorgan für Energie, sondern wirkt auch als aktives endokrines Organ, das eine Vielzahl von Zytokinen, Hormonen und anderen Proteinen sezerniert [117]. Die vom Fettgewebe ausgeschütteten Faktoren werden Adipokine genannt, kommen im Plasma adipöser Patienten in erhöhter Konzentration vor und werden mit der Entstehung und Progression von Herz-Kreislauf-Erkrankungen in Verbindung gebracht [118,119]. Eines der von Adipozyten exprimierten Proteine ist der pigment epithelium derived factor (PEDF), ein 50kDa gorßes Mitglied der Serpin Familie. PEDF ist ein pleiotropes Protein mit antiangiogenen, antithrombotischen, neuroprotektiven, antitumorigenen und antiinflammatorischen Eigenschaften [103]. Erst kürzlich wurde gezeigt, dass PEDF im humanen Fettgewebe massiv exprimiert wird, die Expression des PEDF während der Adipogenese signifikant ansteigt und dass PEDF im Plasma übergewichtiger Menschen in erhöhter Konzentration vorkommt [45].

Ziel Ziel dieses Projekt ist es eine mögliche Rolle von PEDF bei Adipositas zu charakterisieren und die parakrine Wirkungen von PEDF auf, an der Angiogenese beteiligte Zelltypen, wie human coronary artery smooth muscle cells (HCASMC) und auf human umbilical vein endothelial cells (HUVEC), zu untersuchen.

Methoden (I) Zu diesem Zweck wurde die Expression von PEDF in, aus viszeralem und subkutanem Fettgewebe isolierten Präadipozyten und Adipozyten untersucht. (II) Zusätzlich wurde untersucht, ob die Expression des PEDF in Präadipozyten und Adipozyten durch inflammatorische Zytokine beeinflusst wird. (III) In vivo herrscht im Fettgewebe ein entzündliches, mit Sauerstoff minderversorgtes Milieu deshalb wurde untersucht ob PEDF unter hypoxische Bedingungen das Expressionsmuster von Adipozyten beeinflusst. (IV) Weiters wurden HCASMC mit PEDF stimuliert um die Hemmung der Genexpression von Vascular Endothelial Growth Factor (VEGF) auf

RNA- und Proteinebene zu untersuchen. (V) Ebenso sollte untersucht werden ob die Expression von IL-8 in HUVEC durch Stimulation mit PEDF beeinflusst wird.

Ergebnisse Die PEDF Expression ist während der Adipogenese von primären visceralen Adipozyten signifikant angestiegen. Die Stimulation von humanen visceralen und subkutanen Preadipozyten und Adipozyten mit inflammatorischen Zytokinen führte zu keiner signifikanten Veränderung der Expression von PEDF auf mRNA Niveau. Expression und Sekretion des VEGF in humanen visceralen Adipozyten sind nach Inkubation mit PEDF unter normoxischen Bedingungen signifikant angestiegen. Im Gegensatz zum dem Anstieg der VEGF Expression in Adipozyten wurde die VEGF Expression und Sekretion in HUVEC und HCASMC durch Behandlung mit PEDF signifikant reduziert. Zusätzlich konnte gezeigt werden, dass in HCASMC und HUVEC als Folge von PEDF Stimulation die Expression und Sekretion von IL-8 abfällt.

Schlussfolgerung PEDF ist eines von Fettgewebe in hohen Konzentrationen ausgeschüttetes Zytokin, das an der Regulation der Angiogenese beteiligt ist. In HUVEC, HCASMC and HVADI beeinflusst PEDF die Produktion von VEGF und außerdem die Sekretion von IL-8 in HUVEC und HCASMC. Durch seine zahlreichen Wirkungsweisen könnte der PEDF in der Modulation der Angiogenese von Bedeutung sein, ebenso könnte der PEDF eine wichtige Rolle haben bei der Progression von Adipositas und von Erkrankungen die im Zusammenhang mit Übergewicht stehen.

6. Appendix

6.1. References

1. Plate K. From angiogenesis to lymphangiogenesis. *Nat Med*. 2001;7:151-2.
2. Castellot JJ, Jr., Karnovsky MJ, Spiegelman BM. Differentiation-dependent stimulation of neovascularization and endothelial cell chemotaxis by 3T3 adipocytes. *Proc Natl Acad Sci U S A*. 1982;79:5597-601.
3. Montesano R, Mouron P, Orci L. Vascular outgrowths from tissue explants embedded in fibrin or collagen gels: a simple in vitro model of angiogenesis. *Cell Biol Int Rep*. 1985;9:869-75.
4. Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R, Folkman MJ. Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci U S A*. 2002;99:10730-5.
5. Voros G, Maquoi E, Demeulemeester D, Clerx N, Collen D, Lijnen HR. Modulation of angiogenesis during adipose tissue development in murine models of obesity. *Endocrinology*. 2005;146:4545-54.
6. Claffey KP, Wilkison WO, Spiegelman BM. Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem*. 1992;267:16317-22.
7. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, Singh GM, Gutierrez HR, Lu Y, Bahalim AN, Farzadfar F, Riley LM, Ezzati M. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet*. 2011;377:557-67.
8. "WHO: Obesity and overweight" World Health Organization *http://www.who.int/mediacentre/factsheets/fs11/en/index.html*. 2011.
9. de Onis M, Blossner M, Borghi E. Global prevalence and trends of overweight and obesity among preschool children. *Am J Clin Nutr*. 2010;92:1257-64.
10. Leibel RL. Molecular physiology of weight regulation in mice and humans. *Int J Obes (Lond)*. 2008;32 Suppl 7:S98-108.
11. Sjostrom L, Narbro K, Sjostrom CD, Karason K, Larsson B, Wedel H, Lystig T, Sullivan M, Bouchard C, Carlsson B, Bengtsson C, Dahlgren S, Gummesson A, Jacobson P, Karlsson J, Lindroos AK, Lonroth H, Naslund I, Olbers T, Stenlof K, Torgerson J, Agren G, Carlsson LM. Effects of bariatric surgery on mortality in Swedish obese subjects. *N Engl J Med*. 2007;357:741-52.
12. McMahon MM, Sarr MG, Clark MM, Gall MM, Knoetgen J, 3rd, Service FJ, Laskowski ER, Hurley DL. Clinical management after bariatric surgery: value of a multidisciplinary approach. *Mayo Clin Proc*. 2006;81:S34-45.
13. Mechanick JI, Kushner RF, Sugerman HJ, Gonzalez-Campoy JM, Collazo-Clavell ML, Guven S, Spitz AF, Apovian CM, Livingston EH, Brolin R, Sarwer DB, Anderson WA, Dixon J. American Association of Clinical Endocrinologists, The Obesity Society, and American Society for Metabolic & Bariatric Surgery Medical guidelines for clinical practice for the perioperative nutritional, metabolic, and nonsurgical support of the bariatric surgery patient. *Endocr Pract*. 2008;14 Suppl 1:1-83.
14. Ogston D, McAndrew GM. Fibrinolysis in Obesity. *Lancet*. 1964;2:1205-7.
15. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*. 1993;259:87-91.
16. Rocha VZ, Libby P. The multiple facets of the fat tissue. *Thyroid*. 2008;18:175-83.

17. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K. Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol*. 2003;14:561-6.
18. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res*. 2005;96:939-49.
19. Ahima RS. Adipose tissue as an endocrine organ. *Obesity (Silver Spring)*. 2006;14 Suppl 5:242S-249S.
20. Ouchi T, Sakai K, Fukuzawa H, Liu XJ, Higuchi I, Tamenori Y, Nagaya K, Iwayama H, Yao M, Zhang D, Ding D, Kuleff AI, Stoychev SD, Demekhin PV, Saito N, Ueda K. Three-Electron Interatomic Coulombic Decay from the Inner-Valence Double-Vacancy States in NeAr. *Phys Rev Lett*. 2011;107:053401.
21. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest*. 2005;115:1111-9.
22. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*. 2011;11:85-97.
23. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. *JAMA*. 1999;282:2131-5.
24. Esposito K, Di Palo C, Marfella R, Giugliano D. The effect of weight loss on endothelial functions in obesity: response to Sciacqua et al. *Diabetes Care*. 2003;26:2968-9.
25. Samaras K, Botelho NK, Chisholm DJ, Lord RV. Subcutaneous and visceral adipose tissue FTO gene expression and adiposity, insulin action, glucose metabolism, and inflammatory adipokines in type 2 diabetes mellitus and in health. *Obes Surg*. 2010;20:108-13.
26. Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab*. 1998;83:847-50.
27. Yamagishi S, Matsui T, Nakamura K. Atheroprotective properties of pigment epithelium-derived factor (PEDF) in cardiometabolic disorders. *Curr Pharm Des*. 2009;15:1027-33.
28. Tombran-Tink J, Barnstable CJ. PEDF: a multifaceted neurotrophic factor. *Nat Rev Neurosci*. 2003;4:628-36.
29. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res*. 1991;53:411-4.
30. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc Natl Acad Sci U S A*. 1993;90:1526-30.
31. Tombran-Tink J, Pawar H, Swaroop A, Rodriguez I, Chader GJ. Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. *Genomics*. 1994;19:266-72.
32. Xu X, Zhang SS, Barnstable CJ, Tombran-Tink J. Molecular phylogeny of the antiangiogenic and neurotrophic serpin, pigment epithelium derived factor in vertebrates. *BMC Genomics*. 2006;7:248.
33. Becerra SP, Sagasti A, Spinella P, Notario V. Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop. *J Biol Chem*. 1995;270:25992-9.
34. Bilak MM, Corse AM, Bilak SR, Lehar M, Tombran-Tink J, Kuncl RW. Pigment epithelium-derived factor (PEDF) protects motor neurons from chronic glutamate-mediated neurodegeneration. *J Neuropathol Exp Neurol*. 1999;58:719-28.
35. Drevon CA. Fatty acids and expression of adipokines. *Biochim Biophys Acta*. 2005;1740:287-92.

36. Quan GM, Ojaimi J, Li Y, Kartsogiannis V, Zhou H, Choong PF. Localization of pigment epithelium-derived factor in growing mouse bone. *Calcif Tissue Int*. 2005;76:146-53.
37. Rychli K, Kaun C, Hohensinner PJ, Dorfner AJ, Pfaffenberger S, Niessner A, Bauer M, Dietl W, Podesser BK, Maurer G, Huber K, Wojta J. The anti-angiogenic factor PEDF is present in the human heart and is regulated by anoxia in cardiac myocytes and fibroblasts. *J Cell Mol Med*. 2010;14:198-205.
38. Sawant S, Aparicio S, Tink AR, Lara N, Barnstable CJ, Tombran-Tink J. Regulation of factors controlling angiogenesis in liver development: a role for PEDF in the formation and maintenance of normal vasculature. *Biochem Biophys Res Commun*. 2004;325:408-13.
39. Doll JA, Stellmach VM, Bouck NP, Bergh AR, Lee C, Abramson LP, Cornwell ML, Pins MR, Borensztajn J, Crawford SE. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nat Med*. 2003;9:774-80.
40. Chung C, Shugrue C, Nagar A, Doll JA, Cornwell M, Gattu A, Kolodecik T, Pandol SJ, Gorelick F. Ethanol exposure depletes hepatic pigment epithelium-derived factor, a novel lipid regulator. *Gastroenterology*. 2009;136:331-340 e2.
41. Lange J, Yafai Y, Reichenbach A, Wiedemann P, Eichler W. Regulation of pigment epithelium-derived factor production and release by retinal glial (Muller) cells under hypoxia. *Invest Ophthalmol Vis Sci*. 2008;49:5161-7.
42. Baba H, Yonemitsu Y, Nakano T, Onimaru M, Miyazaki M, Ikeda Y, Sumiyoshi S, Ueda Y, Hasegawa M, Yoshino I, Maehara Y, Sueishi K. Cytoplasmic expression and extracellular deposition of an antiangiogenic factor, pigment epithelium-derived factor, in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol*. 2005;25:1938-44.
43. Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science*. 1999;285:245-8.
44. Rychli K, Huber K, Wojta J. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. *Expert Opin Ther Targets*. 2009;13:1295-302.
45. Famulla S, Lamers D, Hartwig S, Passlack W, Horrighs A, Cramer A, Lehr S, Sell H, Eckel J. Pigment epithelium-derived factor is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. *Int J Obes (Lond)*. 2010.
46. Jenkins A, Zhang SX, Gosmanova A, Aston C, Dashti A, Baker MZ, Lyons T, Ma JX. Increased serum pigment epithelium derived factor levels in Type 2 diabetes patients. *Diabetes Res Clin Pract*. 2008;82:e5-7.
47. Yamagishi S, Adachi H, Abe A, Yashiro T, Enomoto M, Furuki K, Hino A, Jinnouchi Y, Takenaka K, Matsui T, Nakamura K, Imaizumi T. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. *J Clin Endocrinol Metab*. 2006;91:2447-50.
48. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem*. 2009;106:769-75.
49. Filleur S, Volz K, Nelius T, Mirochnik Y, Huang H, Zaichuk TA, Aymerich MS, Becerra SP, Yap R, Veliceasa D, Shroff EH, Volpert OV. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer Res*. 2005;65:5144-52.
50. Bernard A, Gao-Li J, Franco CA, Bouceba T, Huet A, Li Z. Laminin receptor involvement in the anti-angiogenic activity of pigment epithelium-derived factor. *J Biol Chem*. 2009;284:10480-90.

51. Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R, Meyer C, Notario PM, Saravanamuthu S, Nueda ML, Sanchez-Sanchez F, Escribano J, Laborda J, Becerra SP. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. *J Biol Chem*. 2006;281:38022-37.
52. Simonovic M, Gettins PG, Volz K. Crystal structure of human PEDF, a potent anti-angiogenic and neurite growth-promoting factor. *Proc Natl Acad Sci U S A*. 2001;98:11131-5.
53. Duh EJ, Yang HS, Suzuma I, Miyagi M, Youngman E, Mori K, Katai M, Yan L, Suzuma K, West K, Davarya S, Tong P, Gehlbach P, Pearlman J, Crabb JW, Aiello LP, Campochiaro PA, Zack DJ. Pigment epithelium-derived factor suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth. *Invest Ophthalmol Vis Sci*. 2002;43:821-9.
54. Qiu J, Terasaki PI, Miller J, Mizutani K, Cai J, Carosella ED. Soluble HLA-G expression and renal graft acceptance. *Am J Transplant*. 2006;6:2152-6.
55. Crawford SE, Stellmach V, Ranalli M, Huang X, Huang L, Volpert O, De Vries GH, Abramson LP, Bouck N. Pigment epithelium-derived factor (PEDF) in neuroblastoma: a multifunctional mediator of Schwann cell antitumor activity. *J Cell Sci*. 2001;114:4421-8.
56. Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med*. 2002;8:349-57.
57. Chen L, Zhang SS, Barnstable CJ, Tombran-Tink J. PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. *Biochem Biophys Res Commun*. 2006;348:1288-95.
58. Ho TC, Chen SL, Yang YC, Liao CL, Cheng HC, Tsao YP. PEDF induces p53-mediated apoptosis through PPAR gamma signaling in human umbilical vein endothelial cells. *Cardiovasc Res*. 2007;76:213-23.
59. Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem*. 2006;281:3604-13.
60. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med*. 2003;9:677-84.
61. Notari L, Miller A, Martinez A, Amaral J, Ju M, Robinson G, Smith LE, Becerra SP. Pigment epithelium-derived factor is a substrate for matrix metalloproteinase type 2 and type 9: implications for downregulation in hypoxia. *Invest Ophthalmol Vis Sci*. 2005;46:2736-47.
62. Yamagishi S, Inagaki Y, Nakamura K, Abe R, Shimizu T, Yoshimura A, Imaizumi T. Pigment epithelium-derived factor inhibits TNF-alpha-induced interleukin-6 expression in endothelial cells by suppressing NADPH oxidase-mediated reactive oxygen species generation. *J Mol Cell Cardiol*. 2004;37:497-506.
63. Yamagishi S, Nakamura K, Ueda S, Kato S, Imaizumi T. Pigment epithelium-derived factor (PEDF) blocks angiotensin II signaling in endothelial cells via suppression of NADPH oxidase: a novel anti-oxidative mechanism of PEDF. *Cell Tissue Res*. 2005;320:437-45.
64. Park K, Lee K, Zhang B, Zhou T, He X, Gao G, Murray AR, Ma JX. Identification of a novel inhibitor of the canonical wnt pathway. *Mol Cell Biol*. 2011;31:3038-51.
65. Doyle B, Caplice N. Plaque neovascularization and antiangiogenic therapy for atherosclerosis. *J Am Coll Cardiol*. 2007;49:2073-80.
66. Ho QT, Kuo CJ. Vascular endothelial growth factor: biology and therapeutic applications. *Int J Biochem Cell Biol*. 2007;39:1349-57.

67. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 2004;25:581-611.
68. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-9.
69. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A*. 1991;88:9267-71.
70. Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. *J Biol Chem*. 1998;273:31273-82.
71. Yamazaki Y, Takani K, Atoda H, Morita T. Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2). *J Biol Chem*. 2003;278:51985-8.
72. Yla-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. *J Am Coll Cardiol*. 2007;49:1015-26.
73. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669-76.
74. Maharaj AS, Saint-Geniez M, Maldonado AE, D'Amore PA. Vascular endothelial growth factor localization in the adult. *Am J Pathol*. 2006;168:639-48.
75. Brasen JH, Kivela A, Roser K, Rissanen TT, Niemi M, Luft FC, Donath K, Yla-Herttuala S. Angiogenesis, vascular endothelial growth factor and platelet-derived growth factor-BB expression, iron deposition, and oxidation-specific epitopes in stented human coronary arteries. *Arterioscler Thromb Vasc Biol*. 2001;21:1720-6.
76. Rissanen TT, Vajanto I, Hiltunen MO, Rutanen J, Kettunen MI, Niemi M, Leppanen P, Turunen MP, Markkanen JE, Arve K, Alhava E, Kauppinen RA, Yla-Herttuala S. Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in ischemic skeletal muscle and its regeneration. *Am J Pathol*. 2002;160:1393-403.
77. Tuomisto TT, Rissanen TT, Vajanto I, Korkeela A, Rutanen J, Yla-Herttuala S. HIF-VEGF-VEGFR-2, TNF-alpha and IGF pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array. *Atherosclerosis*. 2004;174:111-20.
78. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005;438:932-6.
79. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J*. 2000;19:4298-309.
80. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 1992;359:843-5.
81. Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol*. 1989;143:1366-71.
82. Wolff B, Burns AR, Middleton J, Rot A. Endothelial cell "memory" of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. *J Exp Med*. 1998;188:1757-62.
83. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol*. 1994;56:559-64.
84. Wang Y, Yang J, Gao Y, Du Y, Bao L, Niu W, Yao Z. Regulatory effect of e2, IL-6 and IL-8 on the growth of epithelial ovarian cancer cells. *Cell Mol Immunol*. 2005;2:365-72.

85. Mizukami Y, Jo WS, Duerr EM, Gala M, Li J, Zhang X, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, Chung DC. Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. *Nat Med*. 2005;11:992-7.
86. Boldrini L, Gisfredi S, Ursino S, Lucchi M, Mussi A, Basolo F, Pingitore R, Fontanini G. Interleukin-8 in non-small cell lung carcinoma: relation with angiogenic pattern and p53 alterations. *Lung Cancer*. 2005;50:309-17.
87. Zhang W, Stoehlmacher J, Park DJ, Yang D, Borchard E, Gil J, Tsao-Wei DD, Yun J, Gordon M, Press OA, Rhodes K, Groshen S, Lenz HJ. Gene polymorphisms of epidermal growth factor receptor and its downstream effector, interleukin-8, predict oxaliplatin efficacy in patients with advanced colorectal cancer. *Clin Colorectal Cancer*. 2005;5:124-31.
88. Itoh Y, Joh T, Tanida S, Sasaki M, Kataoka H, Itoh K, Oshima T, Ogasawara N, Togawa S, Wada T, Kubota H, Mori Y, Ohara H, Nomura T, Higashiyama S, Itoh M. IL-8 promotes cell proliferation and migration through metalloproteinase-cleavage proHB-EGF in human colon carcinoma cells. *Cytokine*. 2005;29:275-82.
89. Kohidai L, Csaba G. Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNF-alpha) in the unicellular Tetrahymena pyriformis. *Cytokine*. 1998;10:481-6.
90. Kobayashi Y. Neutrophil infiltration and chemokines. *Crit Rev Immunol*. 2006;26:307-16.
91. Schraufstatter IU, Chung J, Burger M. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *Am J Physiol Lung Cell Mol Physiol*. 2001;280:L1094-103.
92. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, Otterson MF, Ota DM, Lugering N, Domschke W, Binion DG. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *J Biol Chem*. 2003;278:8508-15.
93. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol*. 2003;170:3369-76.
94. Schraufstatter IU, Trieu K, Zhao M, Rose DM, Terkeltaub RA, Burger M. IL-8-mediated cell migration in endothelial cells depends on cathepsin B activity and transactivation of the epidermal growth factor receptor. *J Immunol*. 2003;171:6714-22.
95. Petreaca ML, Yao M, Liu Y, Defea K, Martins-Green M. Transactivation of vascular endothelial growth factor receptor-2 by interleukin-8 (IL-8/CXCL8) is required for IL-8/CXCL8-induced endothelial permeability. *Mol Biol Cell*. 2007;18:5014-23.
96. Hirsch J, Johnson CL, Nelius T, Kennedy R, Riese W, Filleur S. PEDF inhibits IL8 production in prostate cancer cells through PEDF receptor/phospholipase A2 and regulation of NFkappaB and PPARgamma. *Cytokine*. 2011;55:202-10.
97. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J*. 2002;365:561-75.
98. Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S, Palmer CN, Plutzky J, Reddy JK, Spiegelman BM, Staels B, Wahli W. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev*. 2006;58:726-41.
99. Lin S, Cronan JE. Closing in on complete pathways of biotin biosynthesis. *Mol Biosyst*. 2011;7:1811-21.
100. Ragaller V, Lebzien P, Sudekum KH, Huther L, Flachowsky G. Pantothenic acid in ruminant nutrition: a review. *J Anim Physiol Anim Nutr (Berl)*. 2011;95:6-16.

101. Chatonnet F, Picou F, Fauquier T, Flamant F. Thyroid hormone action in cerebellum and cerebral cortex development. *J Thyroid Res*. 2011;2011:145762.
102. Smith U. Pioglitazone: mechanism of action. *Int J Clin Pract Suppl*. 2001:13-8.
103. Chiellini C, Cochet O, Negroni L, Samson M, Poggi M, Ailhaud G, Alessi MC, Dani C, Amri EZ. Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation. *BMC Mol Biol*. 2008;9:26.
104. Crowe S, Wu LE, Economou C, Turpin SM, Matzaris M, Hoehn KL, Hevener AL, James DE, Duh EJ, Watt MJ. Pigment epithelium-derived factor contributes to insulin resistance in obesity. *Cell Metab*. 2009;10:40-7.
105. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics*. 2002;1:213-22.
106. Zvonic S, Lefevre M, Kilroy G, Floyd ZE, DeLany JP, Kheterpal I, Gravois A, Dow R, White A, Wu X, Gimble JM. Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics*. 2007;6:18-28.
107. Le Naour F, Hohenkirk L, Grolleau A, Misek DE, Lescure P, Geiger JD, Hanash S, Beretta L. Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *J Biol Chem*. 2001;276:17920-31.
108. Wree A, Mayer A, Westphal S, Beilfuss A, Canbay A, Schick RR, Gerken G, Vaupel P. Adipokine Expression in Brown and White Adipocytes in Response to Hypoxia. *J Endocrinol Invest*. 2011.
109. Pierce EA, Avery RL, Foley ED, Aiello LP, Smith LE. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc Natl Acad Sci U S A*. 1995;92:905-9.
110. Guan M, Yam HF, Su B, Chan KP, Pang CP, Liu WW, Zhang WZ, Lu Y. Loss of pigment epithelium derived factor expression in glioma progression. *J Clin Pathol*. 2003;56:277-82.
111. Aiello LP. Angiogenic pathways in diabetic retinopathy. *N Engl J Med*. 2005;353:839-41.
112. Boehm BO, Lang G, Volpert O, Jehle PM, Kurkhaus A, Rosinger S, Lang GK, Bouck N. Low content of the natural ocular anti-angiogenic agent pigment epithelium-derived factor (PEDF) in aqueous humor predicts progression of diabetic retinopathy. *Diabetologia*. 2003;46:394-400.
113. Gao G, Li Y, Zhang D, Gee S, Crosson C, Ma J. Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization. *FEBS Lett*. 2001;489:270-6.
114. King GL, Suzuma K. Pigment-epithelium-derived factor--a key coordinator of retinal neuronal and vascular functions. *N Engl J Med*. 2000;342:349-51.
115. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. *FASEB J*. 2006;20:323-5.
116. Knight JA. Diseases and disorders associated with excess body weight. *Ann Clin Lab Sci*. 2011;41:107-21.
117. Ahima RS, Flier JS. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab*. 2000;11:327-32.
118. Rexrode KM, Pradhan A, Manson JE, Buring JE, Ridker PM. Relationship of total and abdominal adiposity with CRP and IL-6 in women. *Ann Epidemiol*. 2003;13:674-82.
119. Alessi MC, Bastelica D, Morange P, Berthet B, Leduc I, Verdier M, Geel O, Juhan-Vague I. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and

BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes*. 2000;49:1374-80.

6.2. Index of Figures and Tables

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6.3. Abbreviations

Ang-1	angiopoetin -1
Ang-2	angiopoetin -2
APC	adenomatous polyposis coli
ATGL	adipose triglyceride lipase
BMI	body mass index
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCAAT	cytidine-cytidine-adenosine-adenosine-thymidine
C/EBPβ	CCAAT/enhancer-binding protein β
CHOP	C/EBP homologous protein
CoA	coenzyme-A
CRP	c- reactive protein
DMEM	dulbecco's modified eagle medium
Dsh	dishevelled protein
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
FGF-2	basic fibroblast growth factor
Fz	frizzled
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase 3
HCASMC	human coronary artery smooth muscle cells
HGF	hepatocyte growth factor
HIF	hypoxia-inducible transcription factor
HNF4	hepatocyte nuclear factor 4
HRPC	hormone-refractory prostate cancer cells
HSADI	human subcutaneous preadipocytes
HSPAC	human subcutaneous preadipocytes
HVADI	human visceral adipocytes
HVPAC	human visceral preadipocytes
HUVEC	human umbilical vein endothelial cells
kDA	kilodalton
IBMX	3-isobutyl-1-methylxanthin

IGF	insulin-like growth factor
IL-1	interleukin 1
IL6	interleukin 6
IL8	interleukin 8
LPP5	low density lipoprotein related protein
LRP6	low density lipoprotein related protein 6
MCP-1	macrophage chemoattractant protein 1
MMPs	matrix metalloproteinase
mRNA	messenger RNA
NADPH	nicotinamid-adenin-dinukleotid-phosphat
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OSM	oncostatin m
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEDF	pigment epithelium derived factor
PlGF	placental growth factor
PLA2	phospholipase A2
PPARγ	peroxisome proliferator-activated receptors
PPRE	peroxisome proliferator response element
ROS	reactive oxygen species
RXR	retinoid X receptor
SFRP5	secreted frizzled related protein 5
TCF	t-cell factor
TF	tissue factor
TNF-α	tumour necrosis factor alpha
TSP-1	thrombospondin 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
USF	ubiquitous basic helix–loop–helix-leucine zipper transcription factor

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8. Curriculum Vitae

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