Review Article

Pathological role of dipeptidyl peptidase (DPP) -4 in liver and ameliorative effects of dpp-4 inhibitors

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ABSTRACT

Dipeptidyl peptidase 4 (DPP4) is the target of the gliptins, a recent class of oral ant diabetics. DPP4 (also called cluster of differentiation 26) was previously characterized in immune cells but also has important metabolic functions which are not yet fully understood. Thus, we investigated the function of DPP4 in human white preadipocytes and adipocytes. We found that both cell types express DPP4 in high amounts; DPP4 release markedly increased during differentiation. In preadipocytes, lentiviral DPP4 knockdown caused significant changes in gene expression as determined by whole-genome DNA-array analysis. Metabolic genes were increased, forexample, PDK4 18-fold and PPARγC1α (=PGC1α) 6-fold, and proliferation elated genes were decreased (e.g. FGF7 5-fold). These effects, contributing to differentiation, were not inhibited by the PPARγ antagonist T0070907. Vice versa, the PPARγ agonist pioglitazone induced a different set of genes (mainly FABP4). DPP4 knockdown also affected growth factor signaling and, accordingly, retarded preadipocyte proliferation. In particular, basal and insulin-induced ERK activation (but not Akt activation) was markedly diminished (by around 60%). This indicates that DPP4 knockdown contributes to adipocyte maturation by mimicking growth factor withdrawal, an early step in fat cell differentiation. In mature adipocytes, DPP4 becomes liberated so that adipose tissue may constitute a relevant source of circulating DPP4.

Keywords: Dipeptidyl peptidase 4, adipocytes, pre-adipocytes, hepatocyte, adipokine

INTRODUCTION

Dipeptidyl peptidase 4 (DPP4), also known as adenosine deaminase binding protein or cluster of differentiation 26 (CD26), is a serine exopeptidase able to inactivate peptides composed of proline, hydroxyproline, or alanine as the penultimate residue[1-3]. It has a strong capacity to act in various peptides and is also widely expressed in many specialized cell types, such as endothelial cells, macrophages, and adipocytes. On its physiological aspects, DPP4 inactivates the glucagon-like peptide-1 (GLP-1), an incretin secreted by the gastrointestinal tract[4-6]. Based on the ant diabetic actions of this incretin, several DPP4 inhibitors (named as gliptins) were launched in the market and are being in use for the treatment of type 2 diabetes.[7,8] It is noteworthy that DPP4 also inactivate some cytokines, chemokines, and neuropeptides involved in inflammation, immunity, and vascular function.

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Pathological and physiological role of Dpp-4 in liver and adipose tissues

A recent article published by Ghorpade *et al*.[3] puts light on the clinically evident interaction between liver and visceral adipose tissue (VAT) in obesity-induced metabolic diseases. This crosstalk may involve many unknown circulating factors. Inflammation of VAT in obesity is an already well-recognized pathological process, but the source of this inflammation is still a matter of investigation, and maybe some hepatic-derived circulating factor (a hepatokine) would be involved on it. Ghorpade *et al*. evidenced that obesity in mice stimulates hepatocytes to synthesize and secret DPP4, which

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acts with plasma factor Xa to promote inflammation of adipose tissue macrophages and insulin resistance[9-12]. They demonstrated that soluble DPP4 activates the caveolin-1 pathway in adipose tissue macrophages. In combination with the protease-activated receptor 2 pathway activation by factor Xa, both pathways synergistically stimulate the extracellular signal-regulated protein kinases 1 and 2 and the nuclear factor kappa B, which are distal inflammatory signaling molecules. This finding expands the traditional view of DPP4 as an endothelial product or an adipokine and invites us to look either at the hepatocyte as a source of DPP4, acting as a hepatokine[13].

Curiously, Ghorpade *et al*. experimentally showed that silencing expression of DPP4 on hepatocytes suppressed inflammation of VAT and insulin resistance, but this effect did not occur with sitagliptin, an orally administered DPP4 inhibitor. We have observed that constitutive DPP4 activity was associated with markers of endothelial activation and micro vascular function in humans, and that inhibition of this enzyme was associated with attenuation of endothelial dysfunction and atherogenesis. However, gliptins did not reduce major cardiovascular outcomes in cardiovascular safety trials. All these findings lead us to speculate that genetic silencing and pharmacological inhibition of DPP4 may have different actions on the atherosclerotic process[14].

Although Ghorpade *et al*. suggested that silencing DPP4 expression may have metabolic benefits that are not achievable through "currently available oral DPP4. Inhibitors," (it is essential to consider that there are differences in the way in which gliptins interact with the DPP4. Sitagliptin, alogliptin, and linagliptin form non-covalent interactions with residues in the catalytic site of DPP4 while vildagliptin and saxagliptin form a reversible covalent enzyme-inhibitor complex in which there are slow rates of inhibitor binding and dissociation, resulting in a slow enzyme balance between the active and inactive forms). This balance may further impact on the possible DPP4 inhibitors' ability to mitigate inflammation and insulin resistance promoted by the hepatocyte-secreted DPP4[15].

The Ghorpade *et al*. assured that the anti-inflammatory effect of lowering plasma DPP4 activity by sitagliptin is counteracted by its effect of increasing plasma insulin. The enhancement of insulin secretion following DPP4 inhibition occurs in a glucose-dependent fashion due to the greater availability of GLP-1. This effect is substantially different from the compensatory hyperinsulinemia induced by insulin resistance, a state that distinctly affects the insulin signal transduction pathways[16]. Insulin resistance compromises the phosphatidylinositol 3-kinase pathway, related to insulin-stimulated glucose uptake, but not the mitogen-activated protein kinase pathway, which plays a role in inflammatory responses and may partially explain the link between hyperinsulinemia and inflammation. Perhaps one or more DPP4 substrates (or even an agent per se) are implicated in this apparent deleterious effect.

If the role of DPP4 in the crosstalk between hepatocytes and adipose tissue observed in mice is confirmed in humans, gene-silencing based therapies specifically focused on hepatocyte DPP4 expression could represent a complementary (or even more prominent) therapeutic option to type 2 diabetes than the currently available gliptins[17].

Role of Dpp-4 in adipocytes and pre-adipocytes

DPP4 is the target of the gliptins, a recent class of oral ant diabetics. DPP4 (also called CD26) was previously characterized in immune cells but also has important metabolic functions which are not yet fully understood. Thus, an investigation was made on the function of DPP4 in human white preadipocytes and adipocytes where it was found that both cell types express DPP4 in high amounts; DPP4 release markedly increased during differentiation. In preadipocytes, lentiviral DPP4 knockdown caused significant changes in gene expression as determined by whole-genome DNA-array analysis. Metabolic genes were increased, for example, PDK4 18-fold and PPARγC1α (=PGC1α) 6-fold, and proliferation related genes were decreased (e.g., FGF7 5-fold). These effects, contributing to differentiation, were not inhibited by the PPARγ antagonist T0070907. Vice versa, the PPARγ agonist pioglitazone induced a different set of genes (mainly FABP4)[18]. DPP4 knockdown also affected growth factor signaling and, accordingly, retarded preadipocyte proliferation. In particular, basal and insulin-induced ERK activation (but not Akt activation) was markedly diminished (by around 60%). This indicates that DPP4 knockdown contributes to adipocyte maturation by mimicking growth factor withdrawal, an early step in fat cell differentiation. In mature adipocytes, DPP4 becomes liberated so that adipose tissue may constitute a relevant source of circulating DPP4.

Expression and release of DPP4 in human white (pre) adipocytes

In order to examine the role of DPP4 in the human adipose tissue, the measurement of expression of this enzyme in primary human white adipocytes at mRNA and protein level during differentiation was done. Mature adipocytes were obtained by *in vitro* differentiation of primary cultured preadipocytes, following the protocol described in the Methods section. In accordance with this an observation was made where detection of DPP4 protein in preadipocytes by immunofluorescence with z-stack analysis revealed DPP4 (green signal) localization primarily in the outer cell regions, i.e., in a typical appearance of membrane proteins. Taken together, DPP4 is expressed in preadipocytes and adipocytes, is located primarily in the cell membrane from which it becomes increasingly released during maturation[19].

Gene expression profile after DPP4 knockdown

As described, DPP4 was highly expressed in preadipocytes but was hardly released from these cells. This implies a different function of DPP4 in preadipocytes as compared to mature adipocytes. No clear hints were available what the function of DPP4 in preadipocytes could be so a study was done study to screen for the changes in gene expression using a whole genome oligo microarray (Agilent)[20]. A heat map of four replicate experiments visualizing genes that were altered at least 5-fold. Salient genes altered at least two-fold and forming functional clusters. The most pronounced changes were found in genes involved in lipid metabolism (with an up-regulation in most cases) and in some proliferation related genes (down-regulated in most cases) as summarized. For example, a 5–24-fold up-regulation of FABP4, PDK4 and PPARγ C1α was observed. The transcription factor C/EBPε, a relative of C/EBPα and C/EBPβ, was induced 20-

fold. C/EBPα and C/EBPβ are known to be involved in adipocyte differentiation. C/EBΠε was initially detected in lymphoid and myeloid cells based on its structural similarity to C/EBΠα and C/EBΠβ, and is assumed to regulate their differentiation. The function of C/EBPε in preadipocytes is unknown but, due to its structural similarity, C/EBPε could mimic the effects of C/EBPα and/or C/EBPβ. Other transcription factors were also increased, such as members of the KLF family (e.g., KLF15, −5, −2). Among the genes induced or suppressed at least two-fold, four functional clusters became obvious, namely lipid metabolism, proliferation, structural genes including cell-cell contact and cell migration. Representative metabolic and proliferative genes were selected for further investigation by quantitative polymerase chain reaction (PCR). The gene PPARγ C1α, also known as PGC1α, is a transcription regulator and appears to have a crucial role in cellular energy metabolism, in particular in mitochondrial biogenesis. Therefore, it was confirmed that its up-regulation is also on the protein level by Western blotting [Figure 2e]. DPP4 is a multifunctional protein which actions beyond peptidase activity. Thus, we tested whether the observed effects by the knockdown could be due to the peptidase activity. The latter can be inhibited by sitagliptin (10 μM). No effect of sitagliptin on the expression of the genes responsive to DPP4 knockdown was observed (not shown). This indicates that a non-peptidase function of DPP4 is responsible for the observed regulation of gene expression[21].

Effect of DPP4 knockdown in later stages of differentiation

DPP4 knockdown elicited changes in gene expression in preadipocytes. Thus, the author investigated the effect of DPP4 knockdown also in later stages of adipocyte maturation. Preadipocytes stably transduced with DPP4 shRNA which were differentiated and the expression of the genes of interest was studied by reverse transcription-PCR at Days 0, 6, and 12 of differentiation [Figure 4]. For comparison, the differentiation protocol was also performed with cells expressing non-target control shRNA. Compared to Day 0 of differentiation, the effect of DPP4 knockdown diminished during differentiation despite DPP4 mRNA remained suppressed. At Day 0 of differentiation (i.e., before switching the cells to differentiation medium), the expression rates of the genes shown were higher (or lower in case of FGF7) in DPP4 knockdown cells compared to sh-control cells. The differentiation process caused an increase of the metabolic genes (FABP4, PDK4, PPARγ C1α, PLIN1, and APOE). This effect was more pronounced in the sh-control cells so that after differentiation (Day 12) the expression levels of these metabolic genes were virtually identical in the DPP4 knockdown and in the sh-control cells [Figure 4]. The growth factor FGF7 was decreased in preadipocytes in response DPP4 knockdown, but also for this gene the expression levels became similar in DPP4 knockdown and sh-control cells after differentiation[22].

Effect of DPP4 knockdown on intracellular signaling

For closer investigation of the mechanisms by which DPP4 knockdown exerts the described effects on gene expression, we studied the activation of protein kinase signaling pathways. Growth factor withdrawal is the first step of adipocyte differentiation *in vitro*, and it was reported that *in vivo* an autocrine. EGF-related growth factor, Pref-1, prevents differentiation through activation of ERK. In cultured preadipocytes, we observed a basal activity of the ERK pathway, measured as phosphorylated ERK by Western blotting. Preadipocytes express insulin receptors; insulin receptor expression was not influenced by DPP4 knockdown. ERK phosphorylation was markedly enhanced by stimulation with insulin (100 nM for 10 min). After knockdown of DPP4, insulin-induced ERK phosphorylation was significantly weaker. In contrast, the activation of the pAkt pathway by insulin was not diminished after DPP4 knockdown. Thus, DPP4 knockdown selectively attenuated the growth factor-like signaling of insulin. In line with the described effects on growth factor signaling, DPP4 knockdown prevented further proliferation of the preadipocytes as measured by cell counting over time. Taken together, DPP4 knockdown in preadipocytes diminished the ability of insulin and probably other growth factors to activate the ERK signaling pathway. This mimics growth factor withdrawal, leads to growth arrest and could thereby contribute to initiate the first step of differentiation[23].

DPP-4 as adipokine

DPP4, a novel adipokine, has a higher release from VAT that is particularly pronounced in obese and insulin-resistant patients. DPP4 may be a marker for visceral obesity, insulin resistance, and the metabolic syndrome. A recent study demonstrated that adipocytes release DPP4 in a differentiation dependent manner. Circulating DPP4 concentrations are increased in obese subjects and correlate with fasting plasma insulin, leptin, and adipocyte size in subcutaneous adipose tissue (SAT); however, the tissue source of circulating DPP4 is not known. The study aimed to assess DPP4 expression and release in paired biopsies of SAT and VAT of lean and obese patients and of patients with or without impaired glucose tolerance, as well as DPP4 release from adipose tissue *in vivo*. Because circulating DPP4 is increased in obese patients with the metabolic syndrome, we hypothesized that DPP4 expression and release in VAT are more prominent than in SAT and that VAT DPP4 could be a marker for insulin sensitivity. DPP4 expression was positively correlated with BMI in both SAT and VAT, with VAT consistently displaying higher expression than SAT. The *ex vivo* release of DPP4 from adipose tissue explants was higher in VAT than in SAT in both lean and obese patients, with obese patients displaying higher DPP4 release than lean controls. Net release of DPP4 from adipose tissue was also demonstrated *in vivo* with greater release in obese subjects than in lean subjects and in women than in men. Insulin-sensitive obese patients had significantly lower circulating DPP4 than did obesity-matched insulin-resistant patients. In this experiment, DPP4 positively correlated with the amount of VAT, adipocyte size, and adipose tissue inflammation[24].

CONCLUSION

DPP4 expression, especially in VAT, is negatively the various functions of DPP4 have been widely discussed, among others in the fields of immunology, (neuro-) endocrinology and glucose homeostasis. However, the role of DPP4 in human adipose tissue is still unclear. Our results now revealed a strong expression of this gene in human white preadipocytes and adipocytes and revealed a possible contribution of DPP4 to the adipocyte differentiation process. Furthermore, mature

adipocytes were identified as a potential source of circulating DPP4. Adipocyte maturation is a complex process and involves several different mediators and signaling pathways. Among these are the two master regulators PPARγ and the C/EBP family. Our knockdown experiments revealed changes in the expression of functional gene clusters indicative for adipocyte differentiation. Investigation of signaling pathways identified a potential mechanism by which DPP4 knockdown could contribute to differentiation. It became obvious that basal and insulin-induced ERK phosphorylation was attenuated.

In contrast, the activation of the Akt pathway by insulin was not affected, arguing for a selective action of DPP4 on growth factor signaling through ERK. It should be noted that insulin probably has a dual role in respect to adipocyte differentiation. On the one hand, insulin promotes differentiation and is a component of the differentiation medium. For this effect, the activation of the pAkt signaling pathway appears to be relevant. On the other hand, by activation of ERK insulin behaves like a growth factor and may thereby counteract the onset of differentiation. The role of ERK in adipocyte differentiation is not fully clear,but in the case of the EGF-related growth factor Pref-1, which acts on preadipocytes in an autocrine way, it was clearly shown that ERK activation by Pref-1 prevents differentiation. The effects of DPP4 knock-down were not influenced by inhibition of PPARγ, an important player in adipocyte maturation but acting at a later stage of this process. Accordingly, the set of genes induced by DPP4 knockdown differed from the set induced by PPARγ. The action of DPP4 at an early step in the differentiation process also explains why DPP4 knockdown, in contrast to PPARγ activation, did not promote triglyceride accumulation because the latter most likely is a late event in adipocyte maturation. Beside of metabolic genes, genes encoding extracellular matrix proteins and proteins being involved in cell-cell interaction and migration were altered by DPP4 knockdown. A link between the extracellular matrix could be seen in different studies. The role of DPP-4 as adipokine was seen. DPP4 as a new adipokine that may be a missing link between increased adipose tissue mass in obesity and obesity-associated metabolic diseases.[5] Although much attention has focused on the role of DPP4 in the degradation of GLP-1, our earlier data suggest that DPP4 also exerts direct effects, as it is able to induce insulin resistance in adipocytes and skeletal muscle cells in concentrations that can be found in the circulation of overweight and obese subjects. DPP4 thus may also have local for a better understanding of the regulation of DPP4 in humans with different degrees of obesity and insulin sensitivity, in this study, we measured DPP4 mRNA expression in adipose tissue and correlated it with clinical parameters and adipose tissue measures. DPP4 expression is systematically lower in SAT irrespective of the body fat level, suggesting that there is a depot-specific control of DPP4 expression. The fact that circulating DPP4 and DPP4 expression in adipose tissue both correlate with adipocyte size and adipose tissue inflammation also suggests that proinflammatory adipokines released from enlarged adipocytes could regulate DPP4 release. The findings with DPP4 expression in adipose tissue in relation to BMI have been divergent, with a first report on this subject demonstrating higher DPP4 expression in adipose tissue from obese patients than in that from lean controls[18] and data from a second study describing higher DPP4 expression in lean subjects than in obese ones.[19]Together with our previous publication describing DPP4 as a novel adipokine,[5] we now show in different groups of patients that both DPP4 mRNA expression and DPP4 protein levels are increased in both SAT and VAT from obese subjects patients with a continuous spectrum of BMIs, as well as carefully characterized insulin-sensitive and insulin- resistant morbidly obese patients, we can furthermore demonstrate that associated with insulin sensitivity in both lean and obese subjects. To extend our understanding of how DPP4 is not only expressed in adipose tissue but also released from the tissue, we also studied DPP4 release from adipose tissue explants ex vivo. SAT biopsies from obese patients were characterized by higher DPP4 release than seen in those from lean controls. This set of data corroborates our earlier study showing that enlarged subcutaneous adipocytes from obese patients release higher amounts of DPP4 than do adipocytes from lean controls. In addition, we have now shown that adipose tissue explants from VAT release more DPP4 than do SAT explants, pointing to a possible higher relative contribution by VAT to circulating DPP4 levels[25].

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