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Fibroblast growth factor-1 (FGF-1) promotes adipogenesis by downregulation of Carboxypeptidase A4 (CPA4) - a negative regulator of adipogenesis implicated in the modulation of local and systemic insulin sensitivity

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Abstract

FGF-1 promotes differentiation of human preadipocytes into mature adipocytes via modulation of a BMP and Activin Membrane-Bound Inhibitor (BAMBI)/Peroxisome proliferator-activated receptor (PPARγ)-dependent network. Here, we combined transcriptomic and functional investigations to identify novel downstream effectors aligned with complementary analyses of gene expression in human adipose tissue to explore relationships with insulin sensitivity. RNA-Seq and qRT-PCR analysis revealed significant down-regulation of carboxypeptidase A4 (CPA4) following FGF-1 treatment or induction of differentiation of human preadipocytes in a BAMBI/PPARγ-independent manner. siRNA mediated knockdown of CPA4 resulted in enhanced differentiation of human preadipocytes. Furthermore, expression of CPA4 in subcutaneous adipose tissue correlated negatively with indices of local and systemic (liver and muscle) insulin sensitivity. These results identify CPA4 as a negative regulator of adipogenesis that is down-regulated by FGF-1 and a putative deleterious modulator of local and systemic insulin sensitivity. Further investigations are required to define the molecular mechanism(s) involved and potential therapeutic opportunities.

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Introduction

The fibroblast growth factors (FGFs) are a family of structurally related polypeptides involved in the regulation of a variety of developmental, metabolic, neoplastic and neurological processes as well as associated diseases (Itoh and Ornitz 2011). There are 22 mammalian FGFs which mediate effects via intracrine (intracellular or autocrine), paracrine and endocrine mechanisms. Of these, three FGFs (two paracrine, FGF-1 and FGF-10, and one endocrine, FGF-21) have been demonstrated to play key roles in the development and structural and metabolic remodeling of adipose tissue (Holland et al. 2013, Jonker et al. 2012, Ohta and Itoh 2014).

FGF-1 in particular has been shown to play an important role in adipocyte differentiation and adipose tissue remodeling in mice and humans (Choi et al. 2016, Hutley et al. 2004, Jonker et al. 2012, Lauvrud et al. 2016). Indeed, evidence suggests that FGF-1 acts upstream of the master adipogenic regulator (Hutley et al. 2004), peroxisome proliferator-activated receptor (PPARγ), as well as serving as a downstream effector (Jonker et al. 2012). For example, high fat diet (HFD) induced obesity in mice resulted in a PPARγ-dependent increase in FGF-1 expression in white adipose tissue (Jonker et al. 2012). The physiological importance of the increase in FGF-1 was demonstrated by observations in HFD-fed FGF-1 knockout mice, which exhibited aberrant adipose tissue expansion and an aggressive diabetic phenotype (Jonker et al. 2012). Our own work has demonstrated that FGF-1 promotes differentiation of human preadipocytes via activation of canonical FGF-1 signaling pathways (involving Fibroblast growth factor receptor 1 (FGFR1) / Fibroblast growth factor receptor substrate 2 (FRS2) / Phosphatidylinositol-3-kinase (PI3K) & Extracellular regulated protein kinases (ERK)) in a

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process that involves "priming" of cells through the induction of PPARγ (Hutley et al. 2004, Newell et al. 2006, Widberg et al. 2009).   More recently we identified a key role for BMP and activin membrane-bound inhibitor (BAMBI) as a downstream effector of FGF-1 situated upstream of PPARγ (Luo et al. 2012).   FGF-1 reduced BAMBI expression in preadipocytes and subsequent functional investigations revealed BAMBI knockdown was sufficient to recapitulate the adipogenic effects of FGF-1, including induction of PPARγ, thereby establishing BAMBI as a negative regulator of adipogenesis (Luo et al. 2012).   In addition, we also recently identified a role for carboxypeptidase X-1 (CPX-1), a catalytically inactive member of the carboxypeptidase family, as an FGF-1 effector and positive regulator of adipogenesis situated downstream of FGF-1/BAMBI but independent of PPARγ (Kim et al. 2016).   CPX-1 expression was increased by FGF-1 and functional characterization suggested CPX-1 may promote adipogenesis and adipose tissue remodeling via effects on the extra-cellular matrix (Kim et al. 2016).

In the current study we identify carboxypeptidase A4 (CPA4) as a negative regulator of adipogenesis that is down-regulated by FGF-1 in a BAMBI/PPARγ-independent manner.   In addition, we report that expression of *CPA4* in subcutaneous human adipose tissue (shAT) correlates with both local and systemic insulin sensitivity.   Collectively these findings suggest that characterization of the molecular mechanisms underpinning these observations will provide novel insights into adipose tissue remodeling that may also reveal novel therapeutic strategies to reduce obesity-associated complications.

Materials and Methods

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Reagents

Unless otherwise stated, general reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia), and tissue culture reagents were from Thermo Fisher Scientific Life Sciences (Waverly, VIC, Australia).

Cell culture and siRNA mediated gene knockdown

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes (PAs) or primary human PAs were cultured and differentiated as described (Luo et al. 2012, Newell et al. 2006, Widberg et al. 2009). Cells were cultured and differentiated with or without FGF-1 (1ng/mL) and heparin (90 μg/mL) as indicated. SGBS PAs were transfected at 80% confluence with scrambled (Scr) control siRNA or gene-specific siRNA (50 nM) (Qiagen, Victoria, Australia) using NanoJuice (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. 72 h post-transfection cells were induced to differentiate and RNA was harvested at the indicated time points. Each gene-specific siRNA sequence was selected from a panel of four based on preliminary investigations to determine knockdown efficiency and results were confirmed using distinct gene-specific sequences to confirm the specificity of the observed effects. The siRNA sequences used routinely throughout were: BAMBI-CTGAGGATGCTTCGAAGTGAA; PPARγ-GAGGGCGATCTTGACAGGAAA; CPA4-CCGGCCGATGTATGTACTGAA; Scrambled-AATTCTCCGAACGTGTCACGT.

Library Preparation, Sequencing and RNA-Seq analysis

SGBS PAs were treated as outlined above (±FGF-1 or ±BAMBI siRNA, ±PPARγ siRNA) for 72 h.

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Total RNA was collected using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All other reagents were from Life technologies (Victoria, Australia). rRNA depletion was performed using the RiboMinus™ Eukaryote Kit v2 with 5 mg total RNA. RNA-Seq libraries were prepared using the Ion Total RNA-Seq Kit v2. The Ion Proton system was used for sequencing and templating respectively. Libraries were barcoded using Ion Torrent RNA-Seq barcodes and multiplexed with two libraries run simultaneously on one P1 chip. Reads from each sample were mapped against the human genome (hg19) using TopHat (version 1.1.0). Annotated transcripts were quantified using Cuffdiff (using a generative statistical model of RNA-Seq) and genes used in downstream analysis were supported by at least 10 reads per sample.

Origin of subcutaneous human adipose tissue (shAT) and determination of metabolic parameters

shAT was obtained in a subset of an obese cohort (n=18 ♂ subjects: average age 46±13 year; body mass index (BMI) 36.5±5.5 kg/m2; total body fat 47±17.3 kg) that had been subjected to comprehensive anthropometric and metabolic analysis (Chen et al. 2015). RNA was extracted using Trizol (Invitrogen) in combination with an RNeasy Mini Kit (Qiagen). Gene expression was measured by qRT-PCR. Adipose tissue insulin resistance (ATIR) was determined as the product of fasting insulin and FFA concentrations as originally described (Groop et al. 1989). Hepatic and muscle insulin sensitivity (HIS and MIS) were measured using a two-step hyperinsulinemic-euglycemic clamp with $[6,6-^{2}H_{2}]$ glucose, as described (Chen et al. 2015). Briefly, endogenous glucose production (EGP) suppression during a 2 h

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infusion of low-dose (15 mU/m^2·min) insulin was used to measure HIS, and glucose infusion

rate during the last 30 min of a high-dose (80 mU/m^2·min) insulin infusion clamp was used to

calculate MIS. All studies were conducted at the Clinical Research Facility at the Garvan

Institute of Medical Research (Sydney, Australia) and the study protocol was approved by St

Vincent's Hospital Human Research Ethics Committee (Sydney, Australia).

qRT-PCR

qRT-PCR was performed as described (Widberg et al. 2009). Briefly, cyclophilin was used as

the housekeeping gene and the relative expression of the gene of interest (GOI) was

determined using the calculation = $2^{(Ct\ housekeeping - Ct\ GOI)}$. Primers were as follows (all human):

CPA4 forward (f)-CAATGAAGGGCAAGAACGGAGC/ reverse (r)-GGTCAGGAAAGTCTGCGGCAAT;

PPARγ (f)-GAAACTTCAAGAGTACCAAAGTGCAA / (r)-AGGCTTATTGTAGAGCTGAGTCTTCTC;

AdipoQ (f)-GACCAGGAAACCACGACTCA / (r)-CGATGTCTCCCTTAGGACCA; AdipoR2

(f)-GCCTCTACATCACAGGAGCTGC / (r)-CTGGAGGTTTGAGACACCATG; Glut4

(f)-CTCTCTGGCATCAATGCTGT / (r)-ACCGAGACCAAGGTGAAGAC; Cyclophilin

(f)-CGCGTCTCCTTTGAGCTGTT / (r)-TCTCCAGTGCTCAGAGCACG.

Oil red O staining and quantification

Lipid accumulation was evaluated by Oil Red O staining, essentially as described (Whitehead

et al. 2004), in cells on day 14 of differentiation. Measurement was performed by capturing

images of Oil red O stained cells in situ (10 images/well) and quantifying staining using Image

J software.

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Adiponectin ELISA

Conditioned medium was collected from differentiated SGBS PAs, and adiponectin secretion was measured with a human Total Adiponectin ELISA, according to the manufacturer's instructions (R&D Systems).

Statistical analyses

Statistical analyses were performed in GraphPad Prism 5.0 using Student t test, one-way ANOVA, two-way ANOVA, and linear regressions as appropriate. Data are expressed as mean ± SEM.

Results

CPA4 is down-regulated by FGF-1

To elaborate the FGF-1/BAMBI/PPARγ adipogenic network human SGBS PAs were incubated for 72 h in the absence or presence of FGF-1 or BAMBI siRNA in parallel with either PPARγ siRNA or scrambled (control) siRNA. Total RNA was subjected to expression profiling by RNA-Seq which revealed significant changes in the expression of over 1500 genes, with 598 being downstream of FGF-1 (data not shown). Here we report the characterization of one FGF-1-responsive gene, *CPA4*, which was down-regulated by around 70% following treatment with FGF-1 (Fig 1A & B). This is consistent with our unpublished observations which showed that FGF-1 treatment of primary human PAs down-regulated *CPA4* expression by around 60% (p=0.0598, n=3) (data not shown). In the current study co-treatment with

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PPARγ siRNA was sufficient to reduce *PPARγ* expression by 67% (p<0.05, n=4) (data not shown) but did not affect FGF-1-mediated down-regulation of *CPA4* (Fig 1A & B), suggesting down-regulation of *CPA4* by FGF-1 is independent of PPARγ. Treatment with BAMBI siRNA reduced *BAMBI* expression by 71% (p<0.05, n=4) (data not shown) and this resulted in a significant increase in *CPA4* expression that was also PPARγ-independent (Fig 1A & B).

To validate and extend these observations we performed qRT-PCR analysis of *CPA4* expression from four independent experiments, following treatment with FGF-1 or BAMBI siRNA as above, prior to induction of differentiation (termed 0 h) as well as 24 h post-induction of differentiation.   This analysis confirmed our RNA-Seq findings, with FGF-1 treatment and BAMBI knockdown showing reciprocal effects on *CPA4* expression (Fig 1C). Importantly, induction of differentiation for 24 h reduced *CPA4* expression across all conditions (Fig 1C).

Down-regulation of CPA4 is maintained throughout differentiation

Having established induction of differentiation for 24 h resulted in reduced expression of *CPA4* we explored the temporal profile of *CPA4* expression during differentiation of SGBS PAs and primary human PAs that were differentiated in the absence or presence of FGF-1.   In the absence of FGF-1 treatment induction of differentiation in both cell-types prompted a marked decline in *CPA4* expression, with *CPA4* levels reduced by over 95% after 3 days, which was maintained throughout the differentiation timecourse (Fig 2A & B).   Cells treated with FGF-1 showed similar profiles, with significantly reduced *CPA4* expression at day 0 (Fig 2A & B).   These results prompted us to hypothesize that CPA4 may act as a negative regulator of

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adipogenesis and that down-regulation of *CPA4* may represent an integral part of the adipogenic program as well as the pro-adipogenic effects of FGF-1.

CPA4 is a negative regulator of adipogenesis

To investigate a putative negative role for CPA4 in the process of adipogenesis we characterized the effects of siRNA mediated CPA4 knockdown on the differentiation of SGBS PAs. Cells were transfected with CPA4 or scrambled (control) siRNA for 72 h and then induced to differentiate for 14 days (all in the absence of FGF-1). Knockdown efficiency was maintained throughout the differentiation timecourse, with CPA4 siRNA-treated cells showing a 70% reduction in *CPA4* expression levels in cells harvested at day 14 (Fig 3A). Morphological and quantitative determination of lipid accumulation by Oil red O staining revealed a two-fold increase in lipid accretion in the CPA4 knockdown cells (Fig 3B & C). Analysis of markers of differentiation by qRT-PCR revealed significantly increased expression of early (*PPARγ*), intermediate (*AdipoQ* and *AdipoR2*) and late (*Glut4*) markers of adipogenesis (Fig 3D). Consistent with the increase in adiponectin gene (AdipoQ) expression, we also found that adiponectin secretion was increased more than two-fold in the CPA4 knockdown cells (Fig 3E). Collectively these results demonstrate that CPA4 is a negative regulator of adipogenesis and support a model whereby FGF-1 treatment promotes adipogenesis, at least in part, via down-regulation of CPA4.

CPA4 expression in subcutaneous human adipose tissue (shAT) correlates negatively with local and systemic insulin sensitivity

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The importance of coordinated adipose tissue expansion in the maintenance of whole body metabolic homeostasis is well-established (Acosta et al. 2016, Henninger et al. 2014, Morley et al. 2015) and increasing evidence provides a link between the FGF-1 axis and obesity-related adipose tissue remodeling (Jonker et al. 2012, Ohta and Itoh 2014). Hence, we sought to extend our *in vitro* observations by exploring the relationship between expression of *CPA4* in shAT with indices of adipose tissue and hepatic and muscle insulin sensitivity in a cohort of obese males who had been subject to comprehensive metabolic characterization (Chen et al. 2015). We found that *CPA4* expression correlated positively with adipose tissue insulin resistance (ATIR) (Fig 4A). We also found that *CPA4* expression in shAT correlated negatively with both hepatic and muscle insulin sensitivity (Fig 4B & C). These findings are consistent with a model whereby decreased expression of *CPA4* in adipose tissue promotes 'healthy' adipose tissue remodeling, which, in turn, contributes to the maintenance of both local and systemic insulin sensitivity.

Discussion

Increased understanding of the molecular processes governing adipogenesis and adipose tissue remodeling are expected to facilitate the development of new therapeutic strategies aimed at reducing obesity-associated diseases. We previously described an FGF-1/BAMBI/PPARγ adipogenic network as a potent driver of adipogenesis of human PAs (Kim et al. 2016, Luo et al. 2012). In the current study we have extended our understanding of this adipogenic network. Using a combination of transcriptome profiling and functional studies we have identified CPA4 as a negative regulator of adipogenesis. CPA4 is

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down-regulated by FGF-1 in a manner that is independent of BAMBI or PPARγ and down-regulation of CPA4 is sufficient to enhance adipogenesis. In addition, we also show that *CPA4* expression in human adipose tissue correlates inversely with local and systemic insulin sensitivity. Collectively, these findings establish CPA4 as a putative therapeutic target.

CPA4 belongs to the M14A subfamily of carboxypeptidases, a family of exopeptidases that typically performs a broad range of functions by removing C-terminal amino acids from proteins and peptides. They play key roles in processes including the degradation of proteins within the digestive tract as well as in the biosynthesis of peptide hormones and neuropeptides (Sapio and Fricker 2014). Hence there is considerable interest in defining the distinct roles of the carboxypeptidases, which are recognized as potential drug targets (Arolas et al. 2007). Indeed, elevated CPA4 expression has been linked with increased aggressiveness of cancers including prostate cancer and non-small lung cell cancer (Sun et al. 2016, Witte et al. 2000).

In the current study we provide the first evidence of a role for CPA4 in the regulation of adipogenesis and insulin sensitivity in humans. We initially observed that *CPA4* was down-regulated in response to FGF-1 treatment of SGBS PAs and primary human PAs. This effect was independent of changes in *BAMBI* or *PPARγ* expression. Further studies revealed that *CPA4* expression was down-regulated during the early stage of differentiation and remained low thereafter. These results prompted us to postulate a negative role for CPA4 in adipogenesis, with down-regulation of *CPA4* representing an integral part of the adipogenic program as well as the pro-adipogenic effects of FGF-1. To test this, we

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characterized the effects of CPA4 knockdown on differentiation of SGBS PAs. We found CPA4 knockdown enhanced adipogenesis morphologically, genetically and functionally. Collectively, these findings indicate that down-regulation of *CPA4* is an integral part of the adipogenic program and that forced reduction of *CPA4* is sufficient to promote adipogenesis. Whilst additional studies are required to determine whether overexpression of CPA4 would be sufficient to inhibit adipogenesis it is noteworthy that histone deacetylase inhibitors (HDACi) inhibit adipogenesis (Lagace and Nachtigal 2004) and CPA4 (which was initially named CPA3) was originally identified as a gene that was highly induced by a potent HDACi, Trichostatin A (Huang et al. 1999). Thus, it seems reasonable to speculate that the anti-adipogenic effects of HDACi are mediated, at least partly, via the induction of CPA4.

The molecular role of CPA4 is unclear. Cellular and biochemical characterization have shown it to be secreted from cells in pro-enzyme form (pro-CPA4) and, once activated, CPA4 exhibits optimal activity at neutral pH suggesting it is likely to exhibit regulated activity within the extracellular environment (Tanco et al. 2010). Recombinant human CPA4 has been shown to preferentially cleave hydrophobic C-terminal residues, namely Phe, Leu, Ile, Met, Tyr, and Val, with additional specificity determined by adjacent residues (Tanco et al. 2010). Potential biological substrates include peptides involved in proliferation and differentiation (Tanco et al. 2010). Thus, one possibility is that targeted activity of CPA4 against one or more substrates regulates the process of adipogenesis. This contrasts with our recent findings for the catalytically inactive carboxypeptidase CPX-1, a positive regulator of adipogenesis that is up-regulated by FGF-1 and implicated in extra-cellular matrix

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remodeling (Kim et al. 2016). Further studies are required to establish the molecular details by which CPA4 interferes with adipogenesis.

Reduced insulin sensitivity, or insulin resistance, is a common denominator of obesity-related metabolic and cardiovascular diseases such as type 2 diabetes (Gustafson et al. 2015, Johnson and Olefsky 2013, Sethi and Vidal-Puig 2007). Moreover, an increasing body of evidence indicates that compromised adipose tissue expansion and function is sufficient to reduce both local and systemic insulin sensitivity. For example, adipocyte-specific deletion of mammalian target of rapamycin (mTOR) compromised adipocyte differentiation and adipose tissue expansion and exacerbated obesity-induced insulin resistance and liver steatosis (Shan et al. 2016). Conversely, the benefits of increasing adipogenesis and adipose tissue expansion have also been demonstrated with one example being the improved local and systemic insulin sensitivity in the adipocyte-specific nuclear receptor co-repressor (NCoR) knockout mouse model (Li et al. 2011). Adipocyte-specific deletion of NCoR resulted in de-repression of PPARγ activity leading to increased adipogenesis and enhanced insulin sensitivity (Li et al. 2011). In the current study we found the expression of *CPA4* in shAT from obese men, who exhibited a wide range of insulin sensitivity despite obesity (Chen et al. 2015), correlated inversely with insulin sensitivity of adipose tissue as well as that of liver and muscle. Our findings in humans are consistent with the scenarios outlined above and suggest that down-regulation of *CPA4* may play a fundamental role in 'healthy' adipose tissue remodeling that helps to maintain both local and systemic insulin sensitivity in the context of human obesity (Samocha-Bonet et al. 2014).

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In summary, we have identified CPA4 as a negative regulator of adipogenesis and putative downstream effector of the FGF-1 axis that contributes to adipose tissue remodeling and modulation of insulin sensitivity in human obesity. Further studies are required to elaborate the molecular details and the functional role of CPA4 in these processes. Such studies will provide new insights into the role of carboxypeptidases in adipose tissue (patho)physiology and may ultimately provide novel therapeutic strategies.

Declaration of interest

The authors disclose no potential conflicts of interest.

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Figure Legends

Figure 1 - CPA4 is downregulated by FGF-1 in human PAs. SGBS PAs were treated with Scrambled (Scr), BAMBI and/or PPARγ siRNA in the presence or absence of 1 ng/mL FGF-1 for 72 h. Total RNA was harvested prior to induction of differentiation and subjected to RNA-Seq analysis. **(A)** CPA4 expression profile in UCSC genome browser; **(B)** Quantitative expression of CPA4 in RNA-Seq; **(C)** SGBS PAs were treated as in (A) and total RNA was isolated from cells harvested just prior to (0 h) and 24 h after induction of differentiation and CPA expression was determined by qRT-PCR. All graphs show mean ± SEM; $n = 4$; B – Cuffdiff; C – two way ANOVA; *indicates comparison between control (Scr siR) and treatment (FGF-1 or BAMBI siR) groups where *p<0.05, **p<0.01, ***p<0.001; [#]indicates comparison between 0 h and 24 h within treatment groups where [###]p<0.001.

Figure 2 - CPA4 is downregulated throughout differentiation of human PAs. (A) SGBS PAs and **(B)** primary human PAs were induced to differentiate in the presence or absence of FGF-1, and total RNA was harvested at the indicated time points and subjected to qRT-PCR analysis to detect CPA4 expression. All graphs show mean ± SEM; $n = 3$; A & B – two way ANOVA, *D0 vs D1-D21, [#]-FGF-1 vs +FGF-1; [##]*p<0.01*, ***/[###]*p<0.001*.

Figure 3 - CPA4 knockdown results in increased differentiation of human PAs. SGBS PAs were transfected with scrambled (Scr) or CPA4 siRNA. 72 h after transfection cells were induced to differentiate for 14 days. **(A)** CPA4 mRNA levels determined by qRT-PCR; **(B)** Oil red O staining showing lipid accumulation (100X); **(C)** Quantitation of Oil red O staining; **(D)**

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RNA was harvested and subjected to qRT-PCR to detect PPARγ, AdipoQ, AdipoR2 and Glut4 expression. **(E)** Cell culture media was harvested and secreted (total) adiponectin was measured; All graphs show mean ± SEM; *n* = 3; A, C & E – t-test; D - one way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 4 - CPA4 expression in adipose tissue correlates negatively with insulin sensitivity in adipose tissue, liver and muscle. Graphs show correlations between CPA4 expression in subcutaneous human adipose tissue of obese ♂ subjects (n=18) and **(A)** Adipose tissue insulin resistance (ATIR); **(B)** Hepatic insulin sensitivity (HIS); **(C)** Muscle insulin sensitivity (MIS). Linear regression; *p<0.05.