

## Regulation of Angiopoietin-Like Proteins (ANGPTLs) 3 and 8 by Insulin

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**Objective:** Circulating ANGPTL8 has recently been used as a marker of insulin action. We studied expression and insulin regulation of ANGPTL8 and ANGPTL3 in vivo and in vitro.

**Design and Methods:** Expression of *ANGPTL8* and *ANGPTL3* was studied in 34 paired samples of human liver and adipose tissue. Effects of insulin on 1) plasma concentrations and adipose tissue expression of ANGPTL8 and ANGPTL3 (in vivo 6-h euglycemic hyperinsulinemia; n = 18), and 2) *ANGPTL8* and *ANGPTL3* gene and protein expression in immortalized human hepatocytes (IHH) and adipocytes were measured. Effect of ANGPTL3 on secretion of ANGPTL8 in cells stably overexpressing *ANGPTL3*, -8, or both was determined.

**Results:** *ANGPTL3* was only expressed in the liver, whereas *ANGPTL8* was expressed in both tissues. In vivo hyperinsulinemia significantly decreased both plasma ANGPTL8 and ANGPTL3 at 3 and 6 hours. Insulin increased *ANGPTL8* expression in human adipose tissue 14- and 18-fold at 3 and 6 hours and *ANGPTL8* was the most insulin-responsive transcript on microarray. Insulin also increased ANPGL8 in cultured adipocytes and IHH but the protein mainly remained intracellular. In vitro in IHH, insulin decreased *ANGPTL3* gene expression and secretion of ANGPTL3 into growth medium. Overexpression of ANGPTL8 in CHO cells did not result in its release into culture medium while abundant secretion occurred in cells co-expressing ANGPTL3 and -8.

**Conclusions:** Insulin decreases plasma ANGPTL3 by decreasing *ANGPTL3* expression in the liver. Insulin markedly increases ANGPTL8 in adipose tissue and the liver but not in plasma. These data show that measurement of plasma ANGPTL3 but not -8 reflects insulin action in target tissues. (*J Clin Endocrinol Metab* 100: E1299–E1307, 2015)

Insulin decreases circulating triglycerides (TGs) by inhibiting adipose tissue lipolysis, very low-density lipoprotein production from the liver, and by stimulating intra-

vascular lipolysis of TG-rich lipoproteins in peripheral tissues (1). Angiopoietin-like proteins (ANGPTLs), particularly ANGPTL3, -8 and -4, play a major role in TG

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Abbreviations: CHO, Chinese hamster ovary; IHH, immortalized human hepatocyte; SGBS, Simpson-Golabi-Behmel syndrome; TG, triglyceride.

trafficking and mediate at least some of the effects of insulin (2). Of these, insulin regulation of ANGPTL4 has been best studied. In humans, in vivo hyperinsulinemia decreases circulating ANGPTL4 (3, 4). In vitro, insulin down-regulates the Angptl4 mRNA in 3T3-L1 adipocytes (5) and mRNA and protein concentrations in human hepatocytes (4), likely via the transcription factor FoxO1 (6). Mice lacking Angptl4 (7) and humans carrying sequence variants of *ANGPTL4*, which interfere with either production or secretion of the protein or its ability to inhibit lipoprotein lipase, have reduced levels of TGs (8).

*ANGPTL8*, also known as betatrophin, TD26, hepatocellular carcinoma-associated gene, c19orf80, refeeding-induced fat and liver (9, 10), and lipasin (11), is a recently identified member of the *ANGPTL* gene family, which is expressed in adipose tissue and the liver and found in human plasma (12). *ANGPTL8* is involved in TG storage in adipose tissue given that Angptl8 knockout mice gain less fat than wild-type mice (13), and siRNA-mediated knockdown of this protein decreases the TG content of 3T3-L1 adipocytes (9). Insulin markedly increases *ANGPTL8* expression in 3T3-L1 adipocytes but the protein is not secreted into the culture medium (9). In mouse liver, the Angptl8 transcript increases markedly in response to refeeding (9, 12) but data regarding insulin regulation of plasma *ANGPTL8* protein concentrations are lacking, and it is unknown whether plasma *ANGPTL8* concentration reflects insulin action in human adipose tissue. This knowledge would seem to be of interest given that several recent studies have suggested plasma *ANGPTL8* as a marker of insulin resistance (14–18).

*ANGPTL3* is mainly expressed in the liver in humans (8). Treatment with recombinant *ANGPTL3* increases circulating TGs (19), and *ANGPTL3* inhibits activities of both lipoprotein lipase and endothelial lipase (20, 21) in vitro. In humans, loss-of-function mutations of *ANGPTL3* are associated with decreases in TG, low-, and high-density lipoprotein (22). Insulin decreases *ANGPTL3* mRNA and protein in human hepatoma HepG2 cells (23). However, there are no data on in vivo effects of insulin on circulating *ANGPTL3* in humans.

Secretion of *ANGPTL3* and -8 may be mechanistically interdependent given that coexpression of the two proteins in the liver of mice decreases circulating *ANGPTL3* compared with expression of *ANGPTL3* alone (12). In HepG2 cells coexpression of *ANGPTL3* and -8 increases *ANGPTL8* protein secretion to culture media compared with expression of *ANGPTL8* alone, suggesting that *ANGPTL8* secretion might depend on *ANGPTL3* (12).

In the present study we 1) compared expression of *ANGPTL3*, -8, and -4 in liver and adipose tissue samples from the same subjects; 2) determined effects of in vivo

euglycemic hyperinsulinemia on circulating concentrations of *ANGPTL3*, -8, and -4, and on expression of the respective genes in human adipose tissue biopsies; 3) examined how insulin regulates *ANGPTL3* and -8 gene and protein expression in cells and culture media of IHH and cultured adipocytes; and 4) determined in Chinese hamster ovary (CHO) cells whether *ANGPTL8* secretion depends on *ANGPTL3*.

## Study Design and Methods

### Expression of *ANGPTL3*, -8, and -4 in human liver and adipose tissue

#### Subjects

We recruited 34 subjects undergoing laparoscopic gastric bypass surgery. The inclusion criteria were 1) age 18–65 years; 2) no known acute or chronic disease except for obesity based on history, physical examination, and standard laboratory tests (blood counts, serum creatinine, electrolyte concentrations, and electrocardiogram); and 3) alcohol consumption less than 20 g per day. During the week preceding surgery, blood samples were taken after an overnight fast for measuring plasma glucose, serum insulin, liver enzyme, serum TG, and total and high-density lipoprotein cholesterol concentrations as described (24). At the time of surgery, wedge biopsies of the liver and sc adipose tissue were obtained. One half of the liver biopsy specimen was sent to the pathologist for histopathological assessment, and the rest was immediately frozen in liquid N<sub>2</sub>. Triglyceride content (percentage of hepatocytes with macrovesicular and microvesicular steatosis), stage, grade, and presence of necroinflammation in the liver biopsies were determined under light microscope by a liver pathologist in a blinded fashion (25). Characteristics of the human subjects studied are summarized in Supplemental Table 1.

### Quantification of *ANGPTL3*, -8, and -4 mRNA concentrations

*ANGPTL3*, -8, and -4 gene expressions were determined in the liver and adipose tissue biopsies by qPCR. Total RNA was isolated with the RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and oligo(dT) priming. Power SYBR Green PCR Master Mix and Light Cycler 480 II (Roche Diagnostics) were used for qPCR analysis. Data were normalized to the mRNA of acidic ribosomal phosphoprotein 36B4. Primer sequences are shown in Supplemental Table 2.

### Effects of insulin on plasma ANGPTLs and gene expression in adipose tissue in vivo in humans

#### Subjects

A total of 18 nondiabetic Caucasian women were recruited based on the following inclusion criteria: 1) age 18–60 years; 2) no known acute or chronic disease other than obesity based on history, physical examination and standard laboratory tests (blood counts, serum creatinine, electrolyte concentrations, and electrocardiogram); and 3) body mass index less than 40 kg/m<sup>2</sup>.

Exclusion criteria included pregnancy or treatment with drugs that may alter glucose tolerance.

### **Whole-body insulin sensitivity and effect of insulin on plasma ANGPTL3, -8, and -4 concentrations and ANGPTL4 and -8 gene expression in adipose tissue in vivo**

Whole-body insulin sensitivity was measured using the euglycemic hyperinsulinemic clamp technique, as previously described (26). After an overnight fast, two 18-gauge catheters (Venflon; Viggo-Spectramed) were inserted, one in the antecubital vein for insulin and 20% glucose infusion (Insulin Actrapid; Novo Nordisk), and another retrogradely in a heated hand vein to sample arterialized venous blood. The rate and duration of the continuous insulin infusion were 1 mU/kg per minute for 6 hours. Normoglycemia was maintained by adjusting the rate of 20% glucose infusion based on plasma glucose measurements, from the arterialized venous blood collected every 5 minutes. Whole-body insulin sensitivity was determined from the glucose infusion rate required to maintain normoglycemia between 30 and 360 minutes. Blood samples were collected at 0, 3, and 6 hours for measuring plasma ANGPTL3, -8, and -4 concentrations. Needle aspiration biopsies of sc adipose tissue were taken before and after 3 and 6 hours of hyperinsulinemia for measurement of *ANGPTL8* and *-4* expression. The qPCR was performed as described above, using a geometric mean of the housekeeping genes *36B4* and  *$\beta$ -actin* as reference. *ANGPTL3* was not expressed in adipose tissue.

To compare the insulin responsiveness of *ANGPTL8* to other genes in the human adipose tissue transcriptome, we reanalyzed previously reported microarray data deposited in the GEO database (GSE26637) (27). The microarrays were performed on RNA isolated from adipose tissue biopsies obtained at 0 and 3 hours of in vivo hyperinsulinemia (27). The fold change in gene expression was calculated and 100 genes showing the highest increase were selected for statistical analysis. Significances were evaluated using the Student *t* test and by applying Benjamini-Hochberg multiple-test correction.

The nature and potential risks of the above two studies were explained to all subjects prior to obtaining their written informed consent. The study was carried out in accordance with the declaration of Helsinki. The protocols were approved by the Medicinal Ethics Committee of the Helsinki and Uusimaa Hospital District.

### **Plasma concentrations of ANGPTLs**

Plasma ANGPTL3 and ANGPTL4 concentrations were measured by in-house ELISAs, which have been previously described and validated to be specific, not cross reacting with each others' antigens (28). Plasma ANGPTL8 concentrations were determined using an ELISA (Aviscera Bioscience, Catalog No. SK00528-02) according to the manufacturer's protocol. Each assay included an ANGPTL8 control sample supplied with the ELISA kit. The interassay and intra-assay coefficients of variation were 13% and 7%. The ANGPTL8 ELISA assay did not cross react with ANGPTL3 or -4. Western blot analysis of plasma ANGPTL8 in plasma samples taken before and after 3 and 6 hours of hyperinsulinemia was carried out at the Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, by using a previously characterized antibody (12).

## **Insulin regulation of ANGPTL3 and -8 in vitro**

### **Cell culture**

Immortalized human hepatocytes (IHH) (29) were grown on 12- or 6-well CellBIND Surface cell culture plates (Corning) in William's E medium (GIBCO/Life Technologies) containing 10% fetal bovine serum, 100 U/mL penicillin, 2 mmol/L L-glutamine, 100  $\mu$ g/mL streptomycin, 50nM dexamethasone, and 100nM insulin (Sigma-Aldrich).

Simpson-Golabi-Behmel syndrome (SGBS) adipocytes respond to insulin (glucose uptake) and  $\beta$ -adrenergic stimuli (lipolysis) and thus resemble human primary adipocytes (30). SGBS preadipocytes were grown and differentiated on 6- and 12-well plates as described (30). Briefly, cells were grown in DMEM/F12 containing 10% fetal bovine serum for 3 days until they reached confluency. Differentiation was induced using serum- and albumin-free DMEM/F12 medium with 0.5 mmol/L methyl iso-buthylxantine, 0.1  $\mu$ mol/L cortisol, and 2  $\mu$ mol/L rosiglitazone. Dexamethasone (25 nmol/L), 0.01 mg/mL transferrin, 0.2 nmol/L triiodothyronine, and 20 nmol/L human insulin (Sigma-Aldrich) for 4 days. Thereafter, the cells were cultured for 10 days in DMEM/F12 supplemented with 0.1  $\mu$ mol/L cortisol, 0.01 mg/mL transferrin, 0.2 nmol/L triiodothyronine, and 20 nmol/L human insulin for complete differentiation.

### **Effect of insulin on ANGPTL3 and -8 gene and protein expression in IHH and SGBS cells**

Differentiated SGBS cells and IHH were serum starved in DMEM containing 5mM glucose for 24 hours and then treated with 100nM insulin (Sigma-Aldrich) in the same medium for 24 hours. RNA or proteins were isolated after insulin treatment for the measurement of *ANGPTL3* and -8 mRNAs by qPCR and protein levels by the ELISAs described above. For determination of *ANGPTL3* and -8 mRNA concentrations in cells, total RNA was isolated from insulin-treated and untreated IHH and SGBS cells using the PureLink RNA Mini Kit (Invitrogen). Reverse transcription and qPCR for *ANGPTL3* and -8 were conducted as described above. The data were normalized to the geometric mean of two housekeeping mRNAs, the acidic ribosomal phosphoprotein *36B4*, and succinate dehydrogenase complex, subunit A (*SDHA*).

For measurement of *ANGPTL3* and -8 protein concentrations, growth media and cell lysates of IHH and SGBS adipocytes (lysed in 50mM Tris-HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150mM sodium chloride, and 1mM EDTA) were collected after 24 hours. The media and cell lysates were used for ELISA assays. As a control, adiponectin concentrations were measured using Western blotting using an antibody against adiponectin (Sigma-Aldrich; Catalog. No. A6354).

### **Expression of ANGPTL3 and -8 in CHO cells**

The cDNAs for *ANGPTL3* and *ANGPTL8* preceded by the signal sequence of human albumin were synthesized (Genescript) and subcloned into a proprietary mammalian expression vector containing a CAG promoter and an IRES-pac puromycin selection marker placed after the *ANGPTL* open reading frame. CHO cells were transfected with the *ANGPTL3* or -8 construct alone or both with Fugene 6 (Promega) and selected with 10–400  $\mu$ g/mL puromycin (HyClone/ThermoFisher Scientific). After selection, the cells were adapted to suspension culture in OptiCHO medium (Life Technologies) supplemented with 2 mmol/L

L-alanyl-L-glutamine. Cultures were expanded at 37°C, after which the protein was produced for 9 days at 31°C. Finally, media and cells were collected by centrifugation and Western blots were performed to analyze ANGPTL8 (Antibody from GenScript) and ANGPTL3 using an in-house rabbit polyclonal antibody.

## Results

### Expression of ANGPTLs in human liver and adipose tissue

ANGPTL3 expression (qPCR) was detectable only in the liver, whereas ANGPTL8 and -4 mRNAs were present in both tissues (Figure 1, A–C). In the 34 subjects with paired samples, ANGPTL8 mRNA expression in the liver was approximately 3.3-fold higher ( $P < .001$ ) than in sc adipose tissue (Figure 1B), whereas ANGPTL4 was less abundantly expressed in the liver than in adipose tissue (Figure 1C).

### Effect of in vivo hyperinsulinemia on ANGPTL3, -8 and -4 concentrations in human plasma

The insulin infusion increased serum insulin concentrations from a fasting concentration of  $42 \pm 7$  to  $500 \pm 19$  pmol/l (30–360 min). Plasma glucose averaged  $5.3 \pm 0.1$  mmol/l in the fasting state and  $5.0 \pm 0.02$  mmol/l during euglycemic hyperinsulinemia.

In vivo euglycemic hyperinsulinemia decreased both plasma ANGPTL3 and -8 significantly, when measured with the ELISA assays (Figure 2, A and C). Plasma ANGPTL3 decreased by 26% from a fasting concentration of  $363 \pm 37$  to  $268 \pm 23$  ng/mL units at 3 hours ( $P < .001$ ) and by 38% to  $225 \pm 24$  ng/mL at 6 hours of hyperinsulinemia ( $P < .001$ ). When measured by the ELISA, the fasting concentration of plasma ANGPTL8 was approximately 19-fold lower than that of ANGPTL3 (Figure 2B). Plasma ANGPTL8 decreased by 17% from a fasting concentration of  $20 \pm 4$  to  $16 \pm 4$  ng/mL units at 3 hours ( $P < .05$ ) and by  $18 \pm 16 \pm 3$  ng/mL at 6 hours of hyperinsulinemia ( $P < .05$ ; Figure 2B). When analyzed by Western blotting using a previously characterized antibody (12), no significant change was detected in the amount of ANGPTL8 between the 0-, 3-, and 6-hour time points (Figure 2D). The correlation coefficient between the ANGPTL8 values measured by Western blotting (ANGPTL8/fibronectin) and the ELISA assay was 0.21;  $P = .16$ . In vivo hyperinsulinemia decreased plasma ANGPTL4 by 14% from a fasting concentration of  $5.9 \pm 0.9$  to  $5.1 \pm 0.7$  ng/mL by 3 hours ( $P < .01$ ) and by 24% to  $4.5 \pm 0.5$  ng/mL ( $P < .01$ ) by 6 hours (Figure 2C).

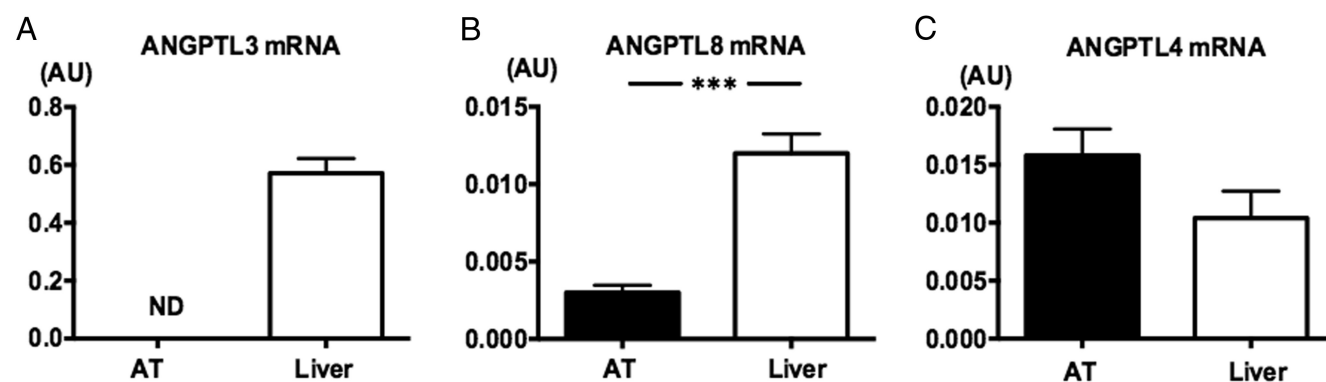
Insulin regulation of ANGPTL3 in hepatocytes in vitro

The insulin-induced decrease of plasma ANGPTL3 could have resulted from down-regulation of gene expression in the liver. When studied in IHH cells in vitro, insulin decreased ANGPTL3 mRNA expression 3.2-fold (Figure 3A). After 24-hour incubation of IHH, most ANGPTL3 was found secreted into the serum-free growth medium. A minor portion was recovered in the cellular fraction (Figure 3D). Secretion of ANGPTL3 into IHH growth medium decreased significantly by 18% and the cellular protein by 25% upon insulin treatment (Figure 3D). Thus, the insulin-induced 26–38% decreases in human plasma could have resulted from insulin inhibition of ANGPTL3 production in the liver.

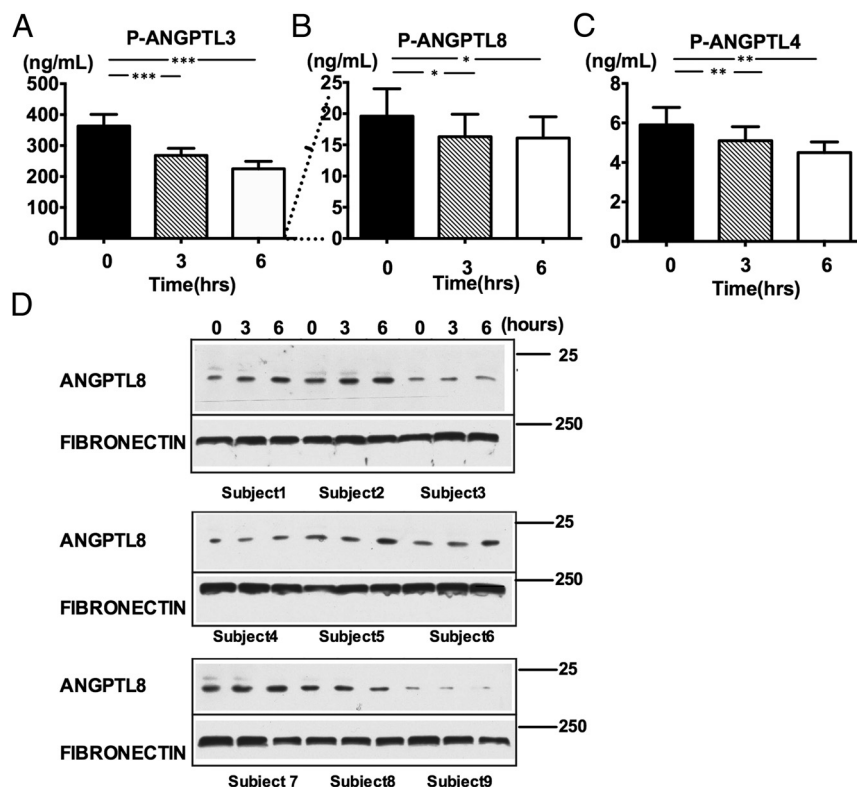
### Insulin regulation of ANGPTL3 in hepatocytes in vitro

Insulin regulation of ANGPTL8 in adipose tissue and hepatocytes in vitro

Reanalysis of microarray data (27) showed that ANGPTL8 was induced 32-fold by in vivo hyperinsulinemia and was the single most insulin-responsive transcript in human adipose tissue (Table 1). The insulin-induced decrease (ELISA) or unchanged concentration (Western blotting) of plasma ANGPTL8 could have been a con-



**Figure 1.** Expression of ANGPTL mRNAs in liver and sc adipose tissue. Relative ANGPTL3 (A), ANGPTL8 (B), and ANGPTL4 (C) mRNA concentrations in the liver and sc adipose tissue (AT) from 34 subjects. The data represent mean  $\pm$  SEM;  $n = 34$ ; \*\*\*,  $P < .001$ , liver vs sc adipose tissue.



**Figure 2.** Effect of insulin on human plasma ANGPTL3, -8 and -4 concentrations in vivo. Plasma ANGPTL3 (A), ANGPTL8 (B), and ANGPTL4 (C) concentrations at 0, 3, and 6 h of insulin infusion in human subjects. The data represent mean  $\pm$  SEM; n = 18; \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ , difference vs 0 hours. Western blots showing plasma ANGPTL8 in nine subjects after 0, 3, and 6 h of insulin infusion (D). The plasma protein fibronectin was used as a loading control.

sequence of insulin action on gene expression in adipose tissue or the liver. To examine this possibility, sc adipose tissue biopsies were collected at 0, 3, and 6 hours of insulin infusion, and *ANGPTL8* mRNA expression was quantified by qPCR. In vivo hyperinsulinemia strikingly induced adipose tissue *ANGPTL8* gene expression by 14- and 18-fold at 3 and 6 hours (Figure 4A). *ANGPTL4* gene expression decreased by 23 and 51% by 3 and 6 hours of hyperinsulinemia compared with the 0-hour time point (Figure 4B).

Given that the effect of in vivo hyperinsulinemia on *ANGPTL8* gene expression in adipose tissue might be indirect and mediated by changes in circulating metabolites such as free fatty acids, we examined effects of insulin on *ANGPTL8* gene expression in vitro in SGBS adipocytes. Insulin increased *ANGPTL8* mRNA concentration 10-fold (Figure 3C) and the cellular ANGPTL8 protein by 32% (Figure 3F). ANGPTL8 accumulated inside the cells, as no ANGPTL was detectable in the medium (Figure 3F). These data imply that, although insulin markedly increases ANGPTL8 locally in adipocytes, this action of insulin does not contribute to circulating ANGPTL8 concentrations.

To determine whether the observed small decrease in circulating ANGPTL8 concentrations (ELISA assay) was

due to insulin action in the liver, we studied the effects of insulin on *ANGPTL8* gene expression in IHH. Insulin increased *ANGPTL8* gene expression 2.2-fold in response to 24-hour treatment (Figure 3B), and the intracellular ANGPTL8 protein by 19% (Figure 3E), but only a negligible amount of ANGPTL8 protein was found in culture medium. These data show that insulin increases ANGPTL8 locally not only in adipose tissue but also in IHH and that this effect does not seem to contribute to extracellular/circulating concentrations of the protein.

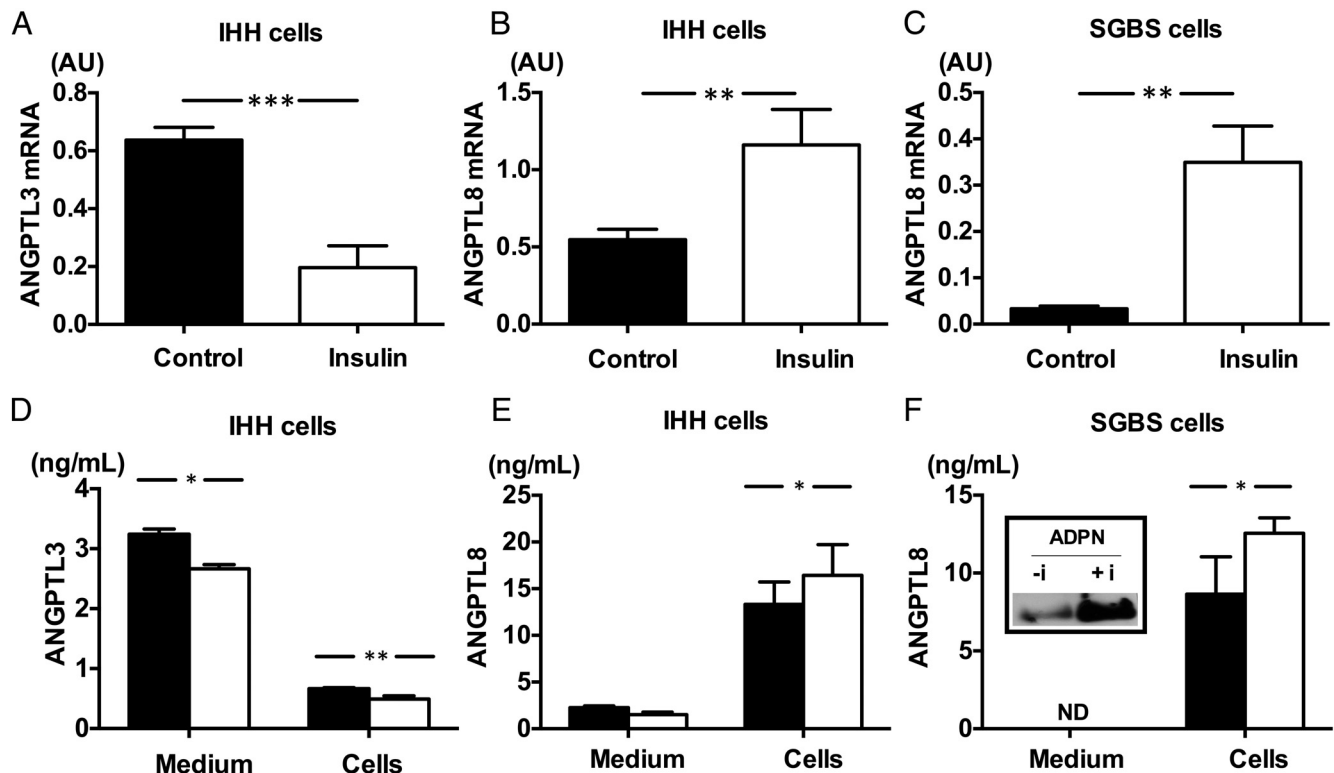
### ANGPTL3 promotes secretion of ANGPTL8 by stably transfected CHO cells

To determine whether ANGPTL3 modulates secretion of ANGPTL8, we created stably transfected CHO cells overexpressing *ANGPTL3*, -8, or both. Cells expressing ANGPTL8 alone were not able to secrete the protein, which accumulated in the cellular fraction as determined by Western blotting (Figure 5). Cells expressing

ANGPTL3 alone secreted this protein abundantly into medium. Abundant secretion of ANGPTL8 into the culture medium was found in cells coexpressing *ANGPTL3* and -8 (Figure 5), demonstrating that ANGPTL3 promotes secretion of ANGPTL8 in CHO cells. A similar result was obtained using the ELISA assays (data not shown). These data are consistent with those reported in HepG2 cells (12), and raise the possibility that the decrease in ANGPTL8 (ELISA assay) was a consequence of the decrease in hepatic ANGPTL3 expression by insulin.

## Discussion

We examined whether and how insulin regulates circulating ANGPTL3 and -8 concentrations and whether changes in their circulating concentrations reflect insulin action in adipose tissue and the liver. Measurement of plasma ANGPTL4 was included to verify that its plasma levels respond to insulin as has been reported. To this end, we measured circulating ANGPTL3 and -8 concentrations and their expression in human adipose tissue biopsies before and after 3 and 6 hours of in vivo euglycemic hyper-



**Figure 3.** Effect of insulin on *ANGPTL3* and *-8* mRNA and protein expression in cultured IHH and SGBS adipocytes. Relative mRNA concentration of *ANGPTL3* and *-8* in control and insulin-treated cultured IHH (A and B). mRNA expression of *ANGPTL8* in control and insulin-treated SGBS adipocytes (C). *ANGPTL3* and *ANGPTL8* protein concentrations in culture medium (Medium) and cell lysates (Cells) of control and insulin-treated IHH (D and E). *ANGPTL8* protein concentrations in culture media and cell lysates of control and insulin-treated SGBS adipocytes (F). The data represent mean  $\pm$  SEM ( $n = 6$ ); \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ . The inset in panel F shows a Western blot of the SGBS adipocyte supernatants from unstimulated (–i) and insulin stimulated (+i) cells, probed for adiponectin.

insulinemia. To explain the observed changes in vivo, effects of insulin on *ANGPTL3* and *-8* gene and protein expression were examined in vitro in IHH and SGBS cells as well as in stably transfected CHO cells overexpressing *ANGPTL3*, *-8*, or both.

The present data are novel in showing that insulin decreases plasma *ANGPTL3* concentrations in vivo in humans. This decrease is likely to reflect insulin action in the liver. Analysis of paired adipose tissue and liver samples in

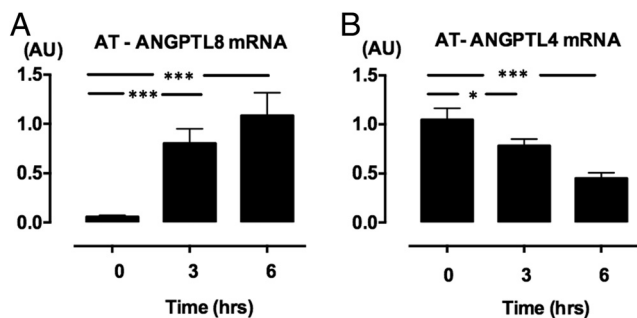
the present study confirmed that *ANGPTL3* is not expressed in human adipose tissue but is found in the liver (8). In IHH cells insulin decreased *ANGPTL3* gene and protein expression. These data are consistent with a previous report showing that insulin down-regulates *ANGPTL3* mRNA and protein expression in human hepatoma HepG2 cells (23). *ANGPTL3* mRNA and protein expression were also reported to be increased in the liver of STZ mice and this increase was attenuated by insulin (31).

**Table 1.** Effect of Insulin on Gene Expression (Microarray Analysis) in Human Adipose Tissue Biopsies

mRNA	Description	Fasting <sup>a</sup> Expression	Insulin-Stimulated Expression	Nominal P Value	Fold Induction by Insulin
ANGPTL8	Angiopoietin-like 8	84	2719	9.3E-04	32.2
NFIL3	Nuclear factor, IL-3 regulated	185	1811	9.0E-03	9.8
PNPLA3	Patatin-like phospholipase domain containing 3	93	757	4.9E-05	8.1
INSIG1	Insulin induced gene 1	510	3494	2.8E-02	6.8
TMEM70	Transmembrane protein 70	239	1439	1.8E-02	6.0
ACLY	ATP citrate lyase	832	4966	1.8E-02	6.0
KIAA0040	KIAA0040	72	407	1.2E-02	5.6
APOC1	Apolipoprotein C-I	338	1889	2.7E-02	5.6
FAM49A	Family with sequence similarity 49, member A	95	426	7.2E-03	4.5
THRSP	Thyroid hormone responsive	5685	25 295	2.9E-04	4.4

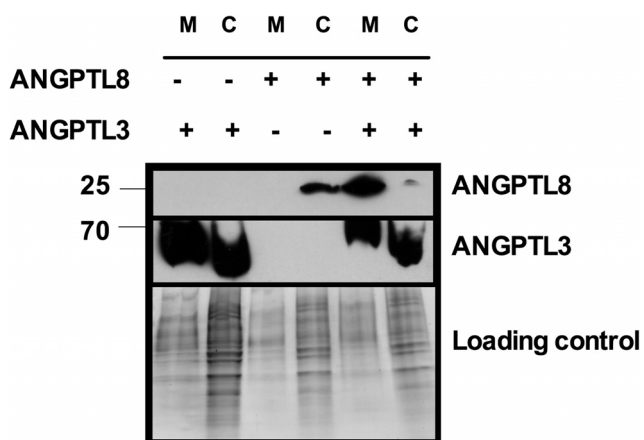
The biopsies were taken before (fasting) and after 3 h (insulin-stimulated) of in vivo euglycemic hyperinsulinemia in 5 normal subjects. Relative expression of the 10 most insulin-induced genes are listed.

<sup>a</sup> Arbitrary normalized intensity value from gene expression array.



**Figure 4.** Insulin induced expression of *ANGPTL* mRNAs in sc adipose tissue. *ANGPTL8* (A) and *ANGPTL4* (B) mRNA expression in human sc adipose tissue before and after 3 and 6 hours' in vivo hyperinsulinemia. \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ , difference compared with the 0-h time point. The data represent mean  $\pm$  SEM from 18 subjects.

Plasma ANGPTL8 concentrations decreased slightly but significantly during euglycemic hyperinsulinemia when measured by ELISA, although no consistent change was detected by Western blotting. Although the antibody used in the Western blot has been validated previously (12), the semiquantitative nature of this technique does not allow detection of small changes. Lack of an increase in circulating ANGPTL8 in vivo is consistent with the in vitro data measuring ANGPTL8 in cells and culture medium in response to insulin. Neither in the study of Ren et al (9) in 3T3-L1 adipocytes nor in the present study in SGBS cells was insulin able to induce secretion of ANGPTL8 into culture media, implying that adipocytes do not contribute to circulating ANGPTL8. In IHH, insulin increased *ANGPTL8* mRNA and protein concentrations but again the protein accumulated intracellularly with



**Figure 5.** Secretion of ANGPTL8 from CHO cells in the presence of ANGPTL3. Culture media (M) and cellular fractions (C) of CHO cells stably transfected with *ANGPTL3*, -8, or both, were analyzed by Western blotting using ANGPTL8 and -3 antibodies. Mobilities of molecular mass markers are shown on the left. For ANGPTL8, the culture media and cell lysates were loaded in equal proportion. Due to robust secretion of the overexpressed ANGPTL3, culture media loading for the anti-ANGPTL3 blot is one tenth of that used for the other medium and cell specimens. A coomassie-stained gel showing sample loading for the ANGPTL8 blot is depicted at the bottom.

only a tiny amount being detected in culture media. The possibility that plasma ANGPTL8 concentration upon hyperinsulinemia is affected by the decreased expression of ANGPTL3 is discussed below.

Lack of an increase in plasma ANGPTL8 was in striking contrast with effects of insulin on *ANGPTL8* in both adipose tissue in vivo and in SGBS cells. Indeed, reanalysis of microarray data from five normal subjects (27) showed that *ANGPTL8* (previously identified as LOC55908 in Additional File 4 of this reference) was the most insulin-responsive transcript (32-fold increase) of all genes examined in human sc adipose tissue. When measured with qPCR in a larger group of subjects, *ANGPTL8* expression was induced 14- and 18-fold at 3 and 6 hours, which is in accord with the studies in 3T3-L1 adipocytes (9). The increase in the *ANGPTL8* transcript was also observed in human SGBS adipocytes in the present study and was accompanied by an increase in cell-associated ANGPTL8 protein. We analyzed the 500-bp proximal promoter region of both *ANGPTL8* and -3, and found both positive (CGCCTC) (32) and negative (T(A/G)TTT) (33) insulin-responsive elements in *ANGPTL8*. However, only negative insulin-responsive DNA elements were observed in *ANGPTL3*, consistent with the observed negative effect of insulin on the expression of this gene/protein.

ANGPTL8 remained intracellular also when overexpressed in CHO cells, but coexpression of *ANGPTL3* and -8 induced secretion of the recombinant ANGPTL8. These data are similar to those in mice in which overexpression of both *ANGPTL3* and -8 but neither cDNA alone induces hypertriglyceridemia (12). The molecular mechanism underlying the interaction between ANGPTL3 and -8 is beyond the scope of the present study. Hypothetically, ANGPTL8 may adopt a transport competent conformation in the endoplasmic reticulum only when it interacts with another, fibrinogen-like domain containing ANGPTL, and may in the absence of the latter be retained by the endoplasmic reticulum quality control apparatus (34, 35). One might thus hypothesize that the decrease in ANGPTL3 in hepatocytes by insulin could have contributed to the decrease in circulating ANGPTL8 concentrations.

Plasma ANGPTL8 concentrations have recently been measured in several studies and suggested to be biomarkers for both type 2 (14–17, 36) and type 1 (37) diabetes, obesity (36), hypercholesterolemia (18), as well as insulin resistance (17). The results have been variable and suggested eg, in obesity either increased (36), unchanged (18) or decreased (15) concentrations. The present data do not exclude the possibility that plasma ANGPTL8 measurements could be useful markers of metabolic abnormalities but suggest that circulating ANGPTL8 concentrations do

not mirror insulin action in either the liver or adipose tissue.

We conclude that insulin acutely decreases circulating ANGPTL3 in humans. This seems to be a consequence of insulin inhibition of *ANGPTL3* gene expression in the liver. Insulin markedly up-regulates *ANGPTL8* both in human adipose tissue in vivo and in SGBS cells and IHH in vitro. However, plasma concentrations of ANGPTL8 do not increase in response to insulin, implying that insulin action on circulating ANGPTL8 does not reflect its action in adipose tissue or the liver.

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