# Circulating follistatin is liver-derived and regulated by the glucagon-to-insulin ratio

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## Abstract

Context: Follistatin is a plasma protein recently reported to increase under conditions with negative energy balance such as exercise and fasting in humans. Currently, the perception is that circulating follistatin is a result of para/autocrine actions from various tissues. The large and acute increase in circulating follistatin in response to exercise suggests that it may function as an endocrine signal.

Objective: Here, we assessed origin and regulation of circulating follistatin in humans.

Design /interventions: First, we assessed arterial-to-venous difference of follistatin over the splanchnic bed at rest and during exercise in healthy humans. To evaluate the regulation of plasma follistatin we manipulated glucagon-to-insulin ratio in humans at rest, as well as in cultured hepatocytes. Finally, the impact of follistatin on human islets of Langerhans was assessed.

Results. We demonstrate that in humans the liver is a major contributor to circulating follistatin both at rest and during exercise. Glucagon increases and insulin inhibits follistatin secretion both in vivo and in vitro mediated via the secondary messenger cAMP in the hepatocyte. Short-term follistatin treatment reduced glucagon secretion from islets of Langerhans, whereas long-term follistatin treatment prevented apoptosis and induced proliferation of rat β-cells.

Conclusions. In conclusion, in humans, the liver secretes follistatin at rest and during exercise and the glucagon-to-insulin ratio is a key determinant of circulating follistatin levels. Circulating follistatin may be a marker of the glucagon-to-insulin tone on the liver.

## 1. Introduction

Follistatin is a secreted protein that inactivates members of the TGF-β family including activins, growth and differentiations factors (GDF), and bone morphogenetic proteins (BMP) (1-5). As follistatin was discovered in high amounts in follicular fluids (5) and binds activin in the pituitary, thereby inhibiting follicle stimulating hormone secretion (6), it has mostly been linked to reproductive physiology. Other functions of circulating follistatin have, however, emerged as it is also associated with the regulation of muscle growth (7) and inflammation (8). In addition, follistatin has been linked to metabolic diseases as it is elevated in plasma in patients with type 2 diabetes (9), non-alcoholic fatty liver disease (NAFLD) (10) and non-alcoholic steatohepatitis (NASH) (10). Follistatin is expressed in a broad range of cells, but no specific tissue or organ is identified as the source of circulating follistatin. Consequently, plasma follistatin is often regarded as spill-over from autocrine or paracrine actions (6).

The auto- and paracrine view of circulating follistatin is challenged by the observation that plasma follistatin acutely increases about 7-fold after an exercise bout (11) which suggest an endocrine function. In mice, exercise-induced follistatin is most likely liver-derived (11), which further supports that follistatin functions as a signal between the liver and peripheral tissues.

The regulatory mechanism for exercise-induced follistatin secretion has not been elucidated, but expression of follistatin is induced by glucagon in cultured hepatocytes (12). Glucagon acts on the liver via glucagon receptors abundantly expressed on the hepatocytes (13;14) and during exercise plasma glucagon increases to stimulate hepatic glucose production (15). Although the main function of glucagon is believed to be glucoregulation, recent data demonstrate that glucagon acts as a secretagogue for hepatic FGF-21 secretion (16-18). The ability of glucagon to promote secretion of

72 hepatic signalling molecules reveals perspectives regarding endocrine signalling between the liver
73 and other organs.

74 Recent data suggest that follistatin may influence beta cell survival as β-cell specific delivery of
75 follistatin results in increased proliferation (19). In addition, when β-cells are exposed to follistatin
76 for 72h the gene expression changes to a pattern associated with more mature β-cells phenotype
77 (20). Thus, follistatin may interact with the endocrine pancreas.

78 In the present study the following hypotheses are tested: 1) in man, follistatin is secreted from the
79 liver both at rest and during exercise; 2) follistatin secretion depends on the glucagon-to-insulin
80 ratio; 3) *in vitro*, follistatin interacts with cells of the endocrine pancreas to modulate hormone
81 secretion and/or cell β-cell survival.

## 2. Material and Methods

### 2.1 Liver vein catheterisation trial

This study has been described (18). In brief, ten healthy males (age 22.9 ±0.8, BMI 22.6±0.5, $VO_2$ max 51.0±1.2 ml/kg/min.) performed 2h of bicycle exercise at 60% $VO_2$max in semi-supine position with catheters placed in an antecubital vein, a hepatic vein and brachial artery allowing for arterial-venous measurements over the liver. After the exercise bout, the subjects rested in supine position for 4h. The subjects arrived after an over-night fast and remained fasted throughout the trial, but had free access to water. Splanchnic blood flow was assessed by the indocyanine green method (21). Splanchnic follistatin production was calculated as ([follistatin (ng/l)]$_{artery}$ − [follistatin (ng/l)]$_{hepatic\ vein}$) x splanchnic plasma flow (l/min).

### 2.2 Glucagon infusion trial

This study has been described (18). Ten healthy males went through four experimental protocols (test days 1-4). For test day 3 (somatostatin infusion), four additional subjects were recruited as four of the original participants were not available. Characteristics of subjects in trial 1, 2 and 4: age 22.9±0.4, BMI 22.3±0.5, subjects in trial 3: age 23.0±0.5, BMI 22.3±0.5. Test day 1: 1h glucagon infusion (GlucaGen, Novo Nordisk) at 6 ng/kg/min. Test day 2: 2h somatostatin infusion (Octreotide, Hospira Nordic) at 100 ng/kg/min (started 10 min prior to the glucagon infusion, in total 130 min) and 1h glucagon infusion at 6 ng/kg/min. Test day 3: 2h (130 min) of somatostatin infusion at 100 ng/kg/min (same as test day 2). Test day 4: saline infusion at the same rate as the glucagon infusion rate. In all four trials, the duration of the experiments was 8h and the subjects remained fasted throughout the trial but had free access to water.

### 2.3 Intralipid infusion trial

This study has been described (22). Fourteen subjects were recruited to the trials, however blood samples were only available for seven subject for this report. In brief, the subjects went through two protocols in fasted conditions; 1) saline infusion with heparin, 2) intralipid infusion in combination with heparin. Only time points prior to the bolus of endotoxin (at 6h) were included in this report. In both trials, the subjects remained fasted throughout the trial, but had free access to water.

### 2.4 Islet and beta cell experiments

Human islets were kindly provided by the Cell Isolation and Transplant Centre of the University of Geneva, through JDRF award 31-2008-413 (ECIT Islet for Basic Research Program). Human intact islets were cultured on 804G-matrix coated dishes.

Cells and culture conditions: Islets of Langerhans were isolated by collagenase digestion of pancreas from adult male Wistar rats followed by Ficoll (Histopaque- 1077, SigmaeAldrich, St. Louis, MO) purification (23). Rat β-cells were sorted as previously described (24). Acute insulin and glucagon secretion: human islets were washed, pre-incubated and incubated for insulin or glucagon secretion assays as described in details in reference (25). Data are mean of six for insulin secretion experiments, and data are mean of four for glucagon secretion experiments.
Detection of apoptosis and proliferation: Cell death was measured by TUNEL assay and proliferation was assessed by BrdU incorporation (26). Data are mean of six experiments.

### 2.5 Hepatocyte and HepG2 cells experiments

Human hepatocytes were isolated from surgical liver biopsies of patients undergoing segmental hepatectomies (n=2). Human liver tissue (between 15-20g) was removed during liver resection for benign or malignant diseases. Human hepatocyte isolation was performed using a two-step collagenase perfusion method (27). Briefly, resected liver was perfused with $Ca^{2+}$-free buffer washing solution (10 ml/min, during 20 min) followed by collagenase solution (type I, 1 mg/ml at 5

ml/min, during 15 min; Sigma, St Louis, MO, USA). Hepatocytes were released by mincing and shaking the liver in a Petri dish containing cold RPMI (Invitrogen, Basel, Switzerland) and 10% fetal bovine serum (FBS; Invitrogen). The cell suspension was filtered through a sterile 100 µm nylon mesh into a beaker placed on ice and then transported to the Surgical Research Unit, University of Geneva Medical School, where human hepatocytes were purified by sedimentation on 30% Percoll isodensity solution (Pharmacia Biotech AB, Uppsala, Sweden) (28). Viability was assessed by trypan blue exclusion. Cells were cultured in DMEM/F12 medium (Invitrogen) containing 2% Fetal bovine serum (Invitrogen), $1\times10^{-6}$ mol/l dexamethasone (Sigma-Aldrich GmbH, Basel, Switzerland), $1\times10^{-8}$ mol/l 3,3' triiodo-L-thyronine, $1\times10^{-8}$ mol/l human insulin (Huminsulin, Lilly France S.A.S, Strasbourg, France), 5 µg/ml apotransferrin (Sigma-Aldrich GmbH), $15\times10^{-3}$ mol/l Hepes.

HepG2 cells (DMSZ, Braunschweig, Germany) were grown in RPMI 1640 containing 11 mM glucose and 10 % FBS, 1 % glutamine, 1% penicillin/streptomycin. Cells were starved 3h in RPMI 1640 before insulin (100 nM) or forskolin (20 µM) was added. In a second set of experiments, cells were starved in RPMI 1640 without glucose. The Quantitect Primer assay Hs_FST_1_SG was used for real-time quantitative PCR analysis. Data are mean of six experiments.

### 2.6 Ethical committee approval

The human *in vivo* studies were approved by the Scientific Ethics Committee of the capital region of Denmark (Hormone infusion trial and exercise trial with liver vein catheterisation: H-1-2012-129, Intralipid infusion trial: (22)) in accordance with the Helsinki Declaration. All subjects provided written informed consent to participate.

### 2.7 Plasma measurements

Blood samples were obtained in tubes containing EDTA for analysis of hormones and in tubes containing aprotinin for analysis of glucagon. All blood samples were immediately spun at 4 $^{o}$C at 3000 g for 15 min and the plasma fractions were stored at – 80 $^{o}$C until analysis. Follistatin (Figure 1, 2 and 3F) was measured by ELISA (R&D systems, Minneapolis, USA) with an intra-assay coefficient of variation (CV) of <5% and inter-assay CV <5%. According to R&D systems this assay measures total follistatin concentration (follistatin 288, follistatin 300 and follistatin 315). All samples were run in duplicate in accordance with the protocol from the manufacturer.

### 2.8 Statistical analysis

Data are presented as means ± (SEM). One-way ANOVAs with Dunnett's post hoc tests were applied for the analysis of hormones over time. £ denotes significant by one-way ANOVA. Significance by one-way ANOVA and Dunnett's post hoc test are denoted as follows: *** $p<0.0001$, ** $p<0.01$, * $p<0.05$. † denotes significant by Student's t-test ($p<0.05$).

## 3 Results

### 3.1 Follistatin is secreted from the splanchnic circulation at rest and during exercise.

Here, we evaluated the splanchnic release of follistatin in humans by measuring arterial-to-hepatic vein (a-hv) differences at rest and during exercise in ten healthy human subjects. At rest, both the a-hv difference of -52.4 ± 15.7 ng/l (p=0.009) and the net hepatic follistatin production (a-hv x plasma flow) of 55.7 ± 20.9 ng/min (p=0.03) (Figure 1A and B, resp.) indicates a constant hepatic follistatin secretion.

Follistatin increases both in the hepatic vein and artery in response to exercise (Figure 1C). Plasma follistatin remains at baseline level during the exercise bout (0-120 min) and then increases 5-fold (p<0.0001) the first 2h into the recovery. During the last 2h of recovery, follistatin reaches a plateau of ~ 7600 ng/l (p<0.0001). The a-hv difference is negative during the entire trial (significant by Student's test, p<0.05, except t=60 min, p=0.09) ( 1D), demonstrating a constant hepatic secretion of follistatin from the splanchnic bed. During exercise, the a-hv difference of follistatin increases markedly and peaks at 636 ng/l at 180 min (p=0.02, post hoc test at 180 min p=0.05). During the 3h recovery, the a-hv of follistatin remains stable although with a small decrease at time point 360 min. When accounting for the hepatic plasma flow, the splanchnic production of follistatin is constant at ~650 ng/min (mean) during the recovery ( 1E).

Because glucagon stimulation induces follistatin expression in hepatocytes (12), we analysed the correlation between the exercise-induced plasma glucagon and insulin responses and plasma follistatin. Peak hepatic follistatin production correlates with nadir insulin ($r^2$=0.45, p=0.03) and peak glucagon-to-insulin ratio ($r^2$=0.58, p=0.01) ( 1F and H), whereas there is no significant correlation with the glucagon peak ($r^2$=0.21, p=0.18) ( 1G).

### 3.2 Glucagon-to-insulin ratio regulates circulating follistatin

To investigate whether glucagon and insulin acutely regulate circulating follistatin, the glucagon-to-insulin ratio was manipulated in physiologically relevant doses and durations in four different trials (see reference (18)  4 for details); 1: high glucagon/high insulin, 2: high glucagon/low insulin, 3: low glucagon/low insulin, and finally 4: control trial with saline infusion.

In the high glucagon/high insulin setting, both glucagon and insulin increases to ~100 pmol/l during the 1h glucagon infusion. Initially there is no regulation of plasma follistatin, whereas there is a slow ~ 1.7-fold increase ($p=0.01$) in the last part of the trial (2A). However, this kinetic profile does not resemble exercise-induced follistatin secretion. During high glucagon/low insulin (as observed during exercise), glucagon increases to ~100 pmol/l whereas insulin decreases to below baseline level. Follistatin increases rapidly with a kinetic profile similar to the exercise response; plasma follistatin increases from ~1200 ng/l at 60 min to its peak at ~4100 ng/l at 180 min (~4-fold increase, $p<0.0001$) (  2B). In contrast to the exercise response, plasma follistatin then decreases from 180 min which indicates a different termination of the follistatin secretion than observed with exercise. During low glucagon/low insulin, both glucagon and insulin are suppressed to below baseline level. Only a minor increase (1.4 –fold) in plasma follistatin is observed ($p=0.003$) (Figure 2C). During saline infusion, there is no regulation of plasma follistatin which remains at ~1600 ng/l ($p=1.0$) (Figure 2D).

As both glucagon infusion (29) and exercise (30) stimulates lipolysis in the adipose tissue with a subsequent increase in plasma free fatty acids (29), we evaluated the effect of free fatty acids on follistatin secretion in healthy males. During intralipid infusion with a prime bolus of heparin both triglycerides and plasma FFA increase substantially to 3000 µmol/l and 2500 µmol/l (22), while

circulating follistatin decreases by ~50% (p=0.01) (Figure 2E). During the control trial (saline infusion), there is no change in circulating follistatin (p=0.7) (Figure 2E).

### 3.3 Glucagon-stimulated follistatin expression and secretion in hepatocytes

To further investigate the regulation of follistatin in liver cells, we stimulated HepG2 (human hepatocarcinoma) cells with forskolin (an adenylate cyclase activator) and insulin. Forskolin increases follistatin mRNA expression at 4h, whereas this expression is reduced by insulin at 2, 4 and 8h ($p<0.05$) (Figure 3A-C). Starvation of HepG2 cells by glucose withdrawal for 4 and 20h increases hepatic follistatin mRNA expression ($p<0.05$), which is inhibited by insulin ($p<0.05$) (Figure 3D-E). Furthermore, we stimulated human primary hepatocytes from two independent donors with glucagon. In the presence of 10 nM glucagon, follistatin was detected in the media 2 and 4 h after stimulation, whereas the control cells did not produce detectable amounts of follistatin at these time points (Figure 3F). After 8h, control cells produced detectable amount of follistatin demonstrating that the liver secretes follistatin under normal conditions (Figure 3F). At 8, 12 and 24h there is no difference between glucagon-stimulated and controls cells. Collectively these data demonstrate that hepatocytes secrete follistatin, and that cAMP and insulin have opposing effects on its expression.

### 3.4 *In vitro* effects of follistatin on cells of the endocrine pancreas

Next, we evaluated the acute effects of follistatin exposure on pancreatic islets within a relevant time course (hours) that mimic the human exercise response. The effect of follistatin on pancreatic insulin and glucagon secretion was tested using whole human islets from healthy donors in static culture. The cells were treated with follistatin (50 ng/ml) for 1, 2, 6, and 24h in the presence of high and low glucose concentrations. Follistatin treatment does not alter insulin secretion at high or low glucose (Figure 4A). In contrast, follistatin treatment acutely suppresses basal glucagon secretion at

high glucose, whereas it has no effect on glucagon secretion at low glucose (Figure 4B). In addition, we investigated the impact of long-term follistatin exposure on β-cell survival and apoptosis. Sorted rat β-cells were treated with follistatin for 24 h, and cell death and proliferation was assessed by TUNEL and BrdU assays, respectively. At both doses of follistatin treatment increased the proliferative rate of rat β-cells equally (Figure 4C). In addition, at both doses follistatin decreased spontaneous cell death in rat β-cells with the most pronounced effect at 10 ng/ml follistatin (Figure 4D).

## 4 Discussion

The present study demonstrates that: 1) in humans, follistatin is secreted from the splanchnic circulation. As the *in vitro* data demonstrate follistatin secretion by hepatocytes, it seems that the liver is a major contributor to circulating follistatin levels; 2) both *in vivo* and *in vitro* data establish changes in the glucagon-to-insulin ratio as the key regulatory mechanism for hepatic follistatin production; 3) follistatin acutely regulates glucagon secretion from human islets of Langerhans within a time frame equivalent to the regulation observed in humans, while prolonged follistatin treatment decreases rat β-cell death and increases proliferation. Collectively, we demonstrate that follistatin is a liver-derived signal responsive to changes in the glucagon-to-insulin ratio that may interact with the endocrine pancreas.

### 4.1 Origin of circulating follistatin in man

Follistatin exists in two major isoforms where the 288 isoform is considered a tissue-bound isoform while 315 isoform is considered to be circulating is plasma (31-33). Although the expression of follistatin in the liver is described (34), the finding that circulating follistatin is liver-derived in humans is novel. We previously investigated the potential contribution of skeletal muscle to exercise-induced circulating follistatin in humans; however we did not detect any arterial-to-venous difference across an exercising leg, and no regulation of the follistatin gene was detected in the skeletal muscle upon exercise (11). Thus, skeletal muscle does not seem to secrete follistatin to any significant extent. In cultured white adipose tissue, follistatin is secreted into the media suggesting a potential contribution to systemic levels (35). However, while follistatin gene expression is markedly increased in the liver in response to exercise in mice (11), no increase was detected in tissues such as adipose tissue, muscle, heart, kidney and spleen (11). Although follistatin may be secreted from the adipose tissue, it is not likely contributing to the exercise-induced increase in

systemic follistatin. Based on existing data, the marked increase in circulating follistatin in response to exercise is most likely due to hepatic secretion, which places follistatin as one of the few identified hepatokines (36).

### 4.2 Regulation of hepatic follistatin secretion

The induction of plasma follistatin observed with high glucagon/low insulin resembles the exercise-induced follistatin increase. The hepatic secretion of follistatin seems to be controlled by the glucagon-to-insulin ratio: glucagon infusion (with high glucagon and high insulin) only increases plasma follistatin moderately, whereas glucagon infusion with a concomitant suppression of insulin increases plasma follistatin markedly. Interestingly, this phenomenon is also observed for FGF-21 (18), demonstrating a similar regulatory mechanism for these two hepatokines *in vivo*. In response to exercise FGF-21 peaks 30 min after the end of the exercise and returns to baseline levels 2h after the exercise bout (18). Exercise-induced follistatin increases 1h after exercise and remains elevated up to 6h after exercise (11). Thus, although FGF-21 and follistatin share regulatory mechanism they have distinct kinetic profiles. Although speculative, this raises the possibility that FGF-21 influences hepatic follistatin secretion via intra-hepatic autocrine/paracrine signalling.

In cultured human hepatocytes, glucagon stimulation rapidly promotes secretion of follistatin into the media. In addition follistatin is present in the media of (unstimulated) controls cells after 8h, indicating a constant secretion of follistatin from hepatocytes. Glucagon and insulin have opposite effects on intracellular cAMP levels, and our data point toward intracellular cAMP as a key regulatory mechanism for hepatic follistatin secretion. Starvation of cells increases intracellular cAMP (37), and we found that glucose withdrawal from hepatocytes *per se* increased follistatin gene expression. Likewise, forskolin, an adenylate cyclase activator, stimulated follistatin expression in HepG2 cells, which was also demonstrated in rat granulosa cells (38). In agreement

with this, the presence of a cAMP response element and AP-1 and AP-2 binding sites were demonstrated in the promoter region of the follistatin gene (38).

During exercise follistatin increases ~5 fold whereas experimentally-induced follistatin secretion through modulation of the glucagon-to-insulin ratio only increases ~4-fold, leaving room for other modulatory stimuli. Importantly, the kinetics of follistatin secretion are similar in the two conditions. Adrenalin acts on the liver and signals via cAMP, and could stimulate follistatin secretion. However, conflicting results are reported; phenylephrine treatment of primary rat hepatocytes increased follistatin expression (12), whereas bolus administration of adrenalin in mice did not (11). Based on the present data, we speculate that, in addition to changes in glucagon-to-insulin ratio, adrenalin might also promote follistatin secretion.

In humans, circulating follistatin increases with prolonged fasting (39). Prolonged fasting and exercise are characterised by an increase in the glucagon-to-insulin ratio and elevated FFA (40). Thus, elevated FFA could confound our interpretation of the regulation of hepatic follistatin secretion. However, experimental elevation of FFA in healthy young subjects decreases circulating follistatin, i.e. FFA *per se* does not stimulate follistatin secretion. Hence, the induction of follistatin observed with fasting (39) is likely explained by the increase glucagon and decrease insulin levels.

### 4.3 Effects of follistatin

Recently, Zhao et al. provided novel data on follistatin's interaction with the β-cells using an adeno associated virus delivery of follistatin to the β-cells (19). Using this approach, it was demonstrated that β-cell specific follistatin overexpression in db/db mice promotes β-cell proliferation and maintenance of pancreatic islet mass (19). In addition, it rescued hyperglycemia, relieved diabetic symptoms and prolonged life span, indicating secondary whole-body effects of the treatment. Another study reported that long-term (72h) follistatin treatment increases expression of genes

associated with a mature β-cell phenotype (20). In line with those reports we find that long-term (24h) follistatin treatment induced β-cell survival by increasing proliferation and reducing cell death in isolated rat β-cells. On the other hand, deletion of the follistatin 315 isoform in mice does not change β-cell mass or proliferation (41). In the present study short-term follistatin stimulation is able to impair glucagon secretion *in vitro*, indicating that in states of energy abundance (high glucose) follistatin may decrease glucagon secretion from the islets of Langerhans. Such a condition could occur when food is available shortly (hours) after an exercise challenge where circulating follistatin is increased 5-7 fold. Consequently, follistatin could represent a feedback signal to the α-cell to decrease glucagon secretion and, in turn, reduce hepatic glucose production. In contrast, long-term (48h) exposure of human islets to follistatin does not change glucagon mRNA levels (42). These data suggest that follistatin's effect on glucagon secretion is time dependent. Several studies have suggested the existence of a signal from the liver to the endocrine pancreas (43-45). Liver-specific insulin receptor knockout (LIRKO) mice display increased β-cell mass explained by increased proliferation (45), whereas no α-cell proliferation is observed. By use of the parabiosis model, *El Ouaamari et al.* found that the β-cell proliferation is promoted by a circulating factor (most likely a protein) derived from hepatocytes (45). Our data suggest that the liver may communicate to the endocrine pancreas via follistatin secretion potentially offering a mechanism for hepatokine-induced β-cell proliferation.

Follistatin is a known inhibitor of myostatin actions (1), and skeletal muscle overexpression of follistatin results in a hypermuscular phenotype (7). Importantly, treatment of mice (46) and ducks (47) with repeated injections of recombinant follistatin induces a hypertrophic response in skeletal muscle tissue. In addition, follistatin treatment induces skeletal muscle repair after injury and immobilization (46). As follistatin is induced during conditions of energy deprivation (e.g. exercise and fasting) *in vivo*, it could be speculated that follistatin acts a as liver-to-muscle signal to spare the

muscle from breakdown e.g. via myostatin inhibition during such conditions. The possible effects and regulation of exercise-induced follistatin is summarized in figure 5.

### 4.4 Follistatin in disease

In addition to being acutely regulated circulating follistatin is increased in the patients with type 2 diabetes and correlate with markers of insulin resistance such as fasting glucose, HbA1c and 2h glucose during an OGTT (9). As circulating follistatin is increased during states of energy deprivation such as prolonged fasting (39) and exercise, conditions associated with increased gluconeogenesis, it could be speculated that circulating follistatin acts in negative feedback loop to regulate hepatic gluconeogenesis. Surprisingly, deletion of the (circulating) follistatin 315 isoform in mice results in augmented gene expression of GLUT2, PEPCK and G6P (48), consequently suggesting that circulating follistatin suppresses gluconeogenesis. Alternatively, follistatin may act to increase β-cell mass as compensatory mechanism to combat increasing blood glucose. Circulating follistatin is also elevated in patients with NAFLD and NASH (10). Interestingly, follistatin 315-deletion in mice results in hepatic steatosis associated with increased hepatic gene expression of LDL receptor, LPL, ACC1 and FAS suggestive of increased lipid uptake and de novo lipogenesis in these mice (48). In addition, mice with transgenic overexpression of follistatin (in skeletal muscle) show resistance to diet-induced hepatic steatosis (49). These data indicate that circulating follistatin reduce hepatic lipid uptake and synthesis, and consequently suggest that circulating follistatin could counteract development of hepatic steatosis in NAFLD and NASH.

## 5. Conclusions

In conclusion, the liver is a major contributor to the circulating levels of follistatin in humans and its secretion is under control by the glucagon-to-insulin ratio. These data suggest that circulating follistatin may be a marker of the glucagon-to-insulin tone on the liver.

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## Figure legends

### Figure 1

**Splanchnic plasma follistatin regulation during exercise in humans (n=10).** At rest, the arterial-venous difference (A) and net production of follistatin (B) is different from zero. Exercise increases plasma follistatin (C), and the arterial-venous difference of the splanchnic circulation (D) and net hepatic production is increased (E). Peak hepatic follistatin production correlate with nadir insulin (F) and peak glucagon/insulin ratio (H), but not with peak glucagon (G) £ significant by one-way ANOVA, * significant by one-way ANOVA and Dunnett's post hoc test (*** $p<0.0001$, ** $p<0.01$, * $p<0.05$). † denotes significant by Student's t-test. Data are mean ± SEM. Figure (A) and (B) present individual data. Hatched bar (1C) indicates duration of the exercise bout.

### Figure 2

**Regulation of plasma follistatin by glucagon and insulin in humans (n=10).** Infusion of glucagon increases follistatin slightly (A), whereas a combined somatostatin-glucagon infusion increases plasma follistatin markedly (B). Infusion of somatostatin (C) only increases plasma follistatin marginally. Infusion of saline does not change plasma follistatin (D). Infusion of intralipid decreases plasma follistatin (E), whereas there no change during the saline infusion (E). £ significant by one-way ANOVA, * significant by one-way ANOVA and Dunnett's post hoc test (*** $p<0.0001$, ** $p<0.01$, * $p<0.05$). Data are mean ± SEM.

**Figure 3**

**Regulation of follistatin in hepatocytes.** In HepG2 cells, follistatin mRNA expression is induced by forskolin, whereas it is inhibited by insulin at 2h (A), 4h (B) and 8h (C). In HepG2 cells, starvation induces follistatin mRNA expression which is inhibited by insulin at 4h (D) and 20h (E). In primary human hepatocytes, glucagon stimulates follistatin secretion (F, black bars) compared with control conditions (F, white bars). † denotes significant by Student's t-test. Data are mean ± SEM.

**Figure 4**

**Follistatin treatment modulates insulin and glucagon secretion.** Human islets were cultured for 0 to 24 h on 804G matrix-coated dishes in the presence of 50 mM follistatin. A: Insulin secretion: human islets were incubated for 60 min at 2.8 mM glucose (open bars) followed by 60 min at 16.7 mM glucose (closed bars) (n = 6). B: Glucagon secretion: human islets were incubated for 60 min at 16.7 mM glucose (closed bars) followed by 60 min at 2.8 mM glucose (open bars) (n = 4). In sorted rat β-cells, 24h of follistatin treatment at 10 ng/ml or 50 ng/ml increases proliferation (C) and decreases cell death (D) (n=4).* marks $p < 0.05$ compared to control, as tested by ANOVA followed by Bonferroni post hoc test. † denotes significant by Student's t-test. Data are presented as mean ± SEM.

Figure 5

Schematic presentation of regulation and potential actions of follistatin. Liver-derived follistatin is regulated by the glucagon-to-insulin ratio during exercise, a mechanism dependent on cAMP in the

409 hepatocyte. Circulating follistatin may interact with the endocrine pancreas to increase β-cell
410 survival and decrease glucagon secretion during energy abundance. Circulating follistatin may also
411 interact with skeletal muscle to induce hypertrophy, or to spare the muscle from breakdown under
412 energy deprived conditions.

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Figure 1

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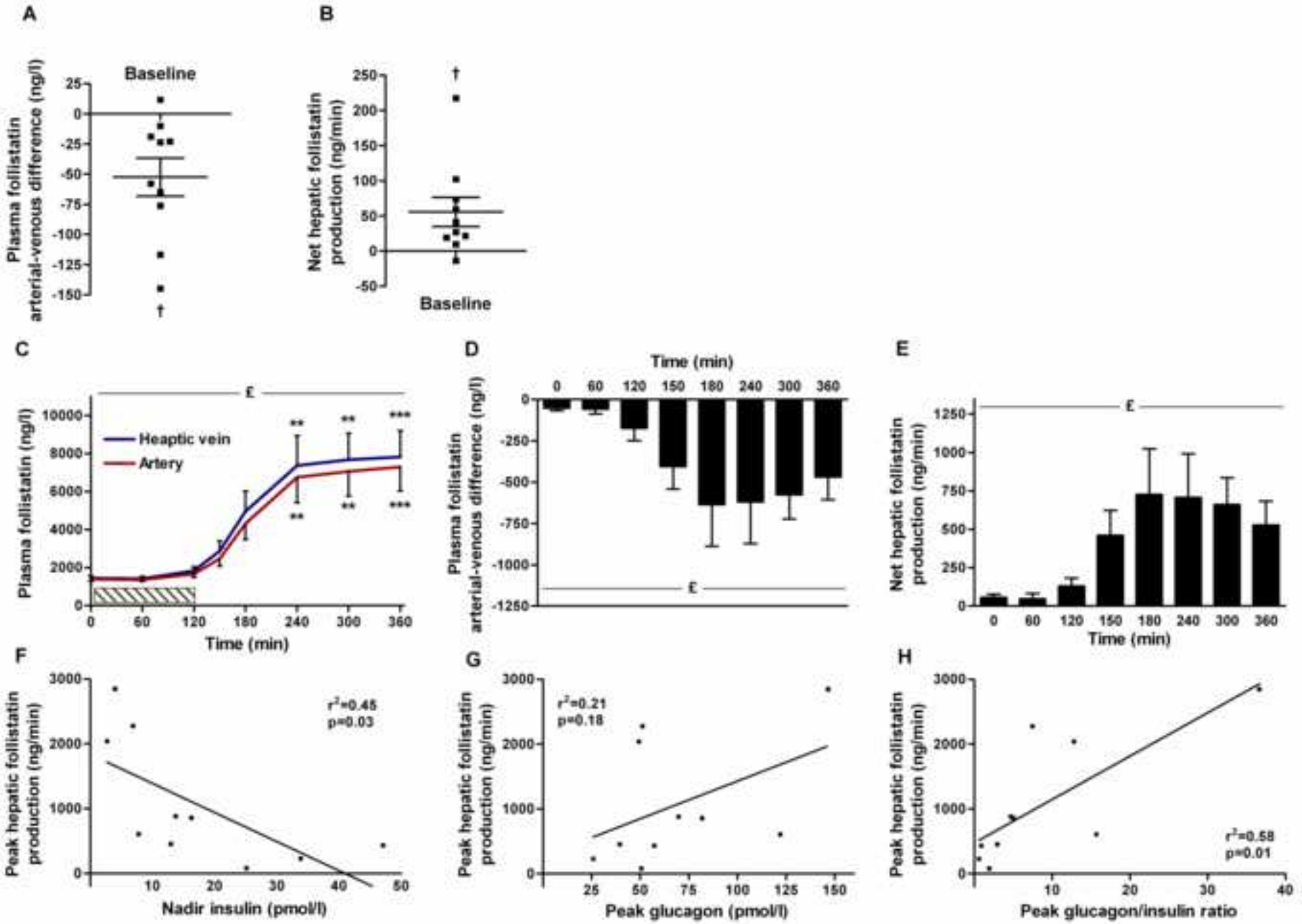
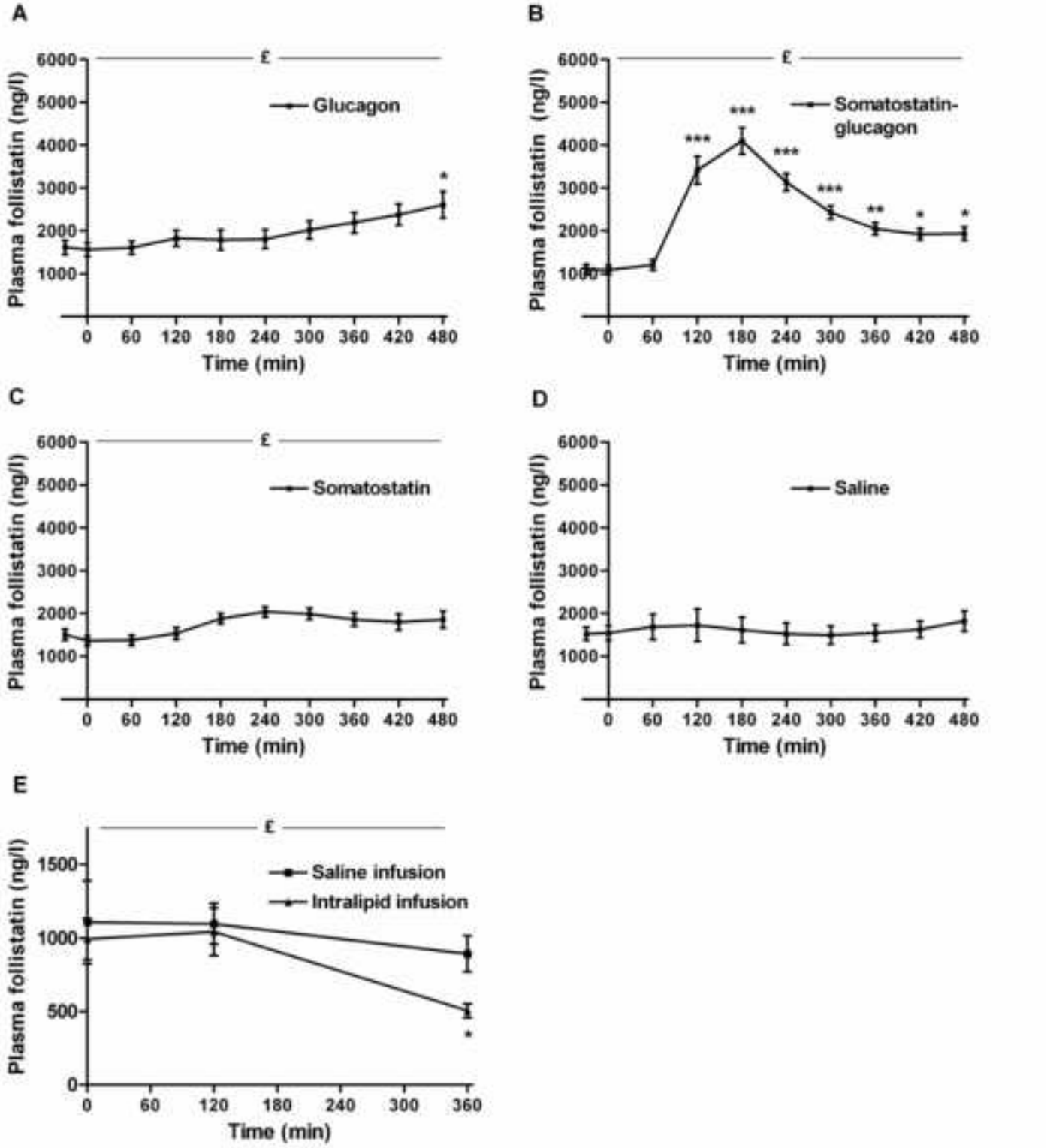


Figure 2





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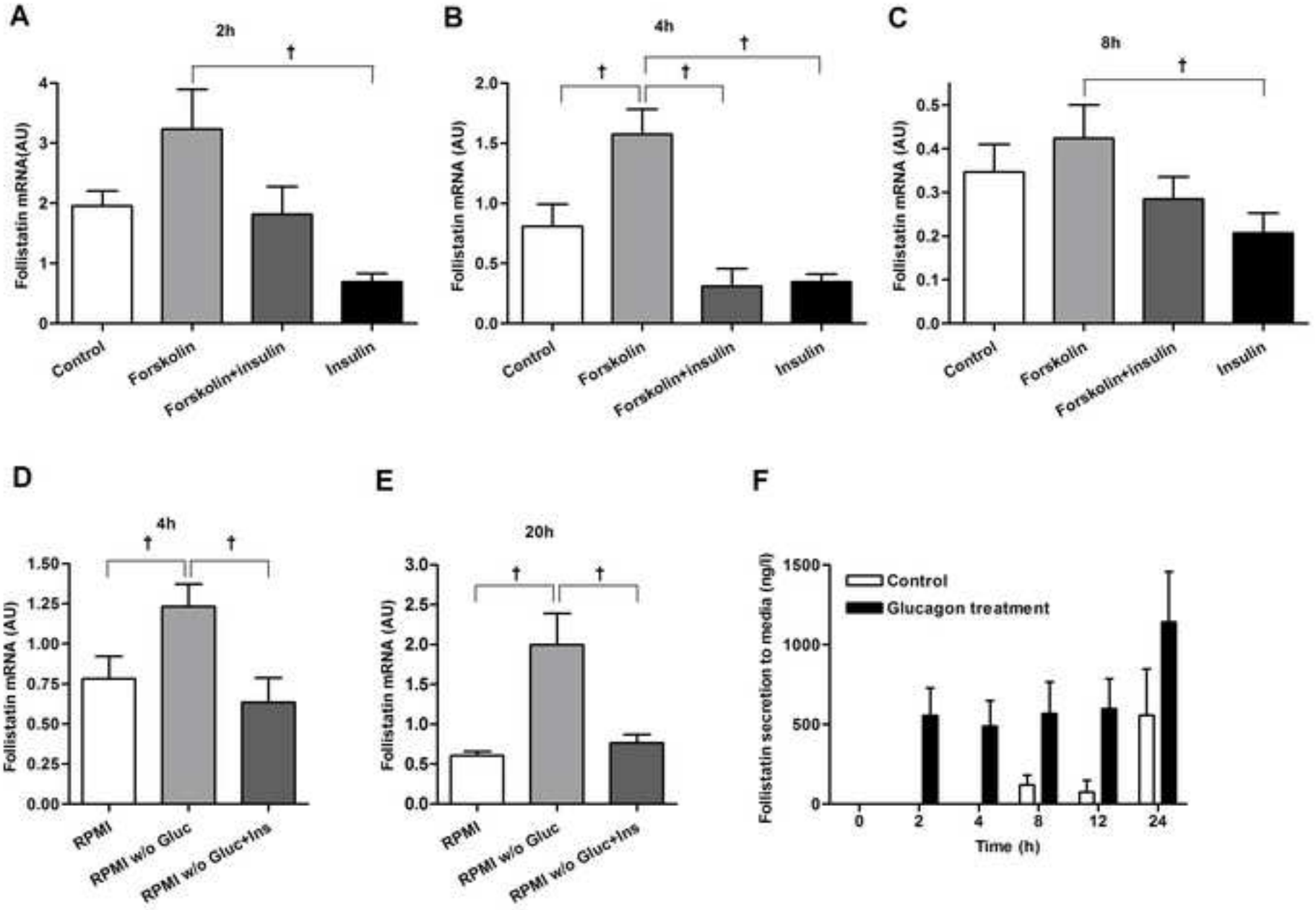

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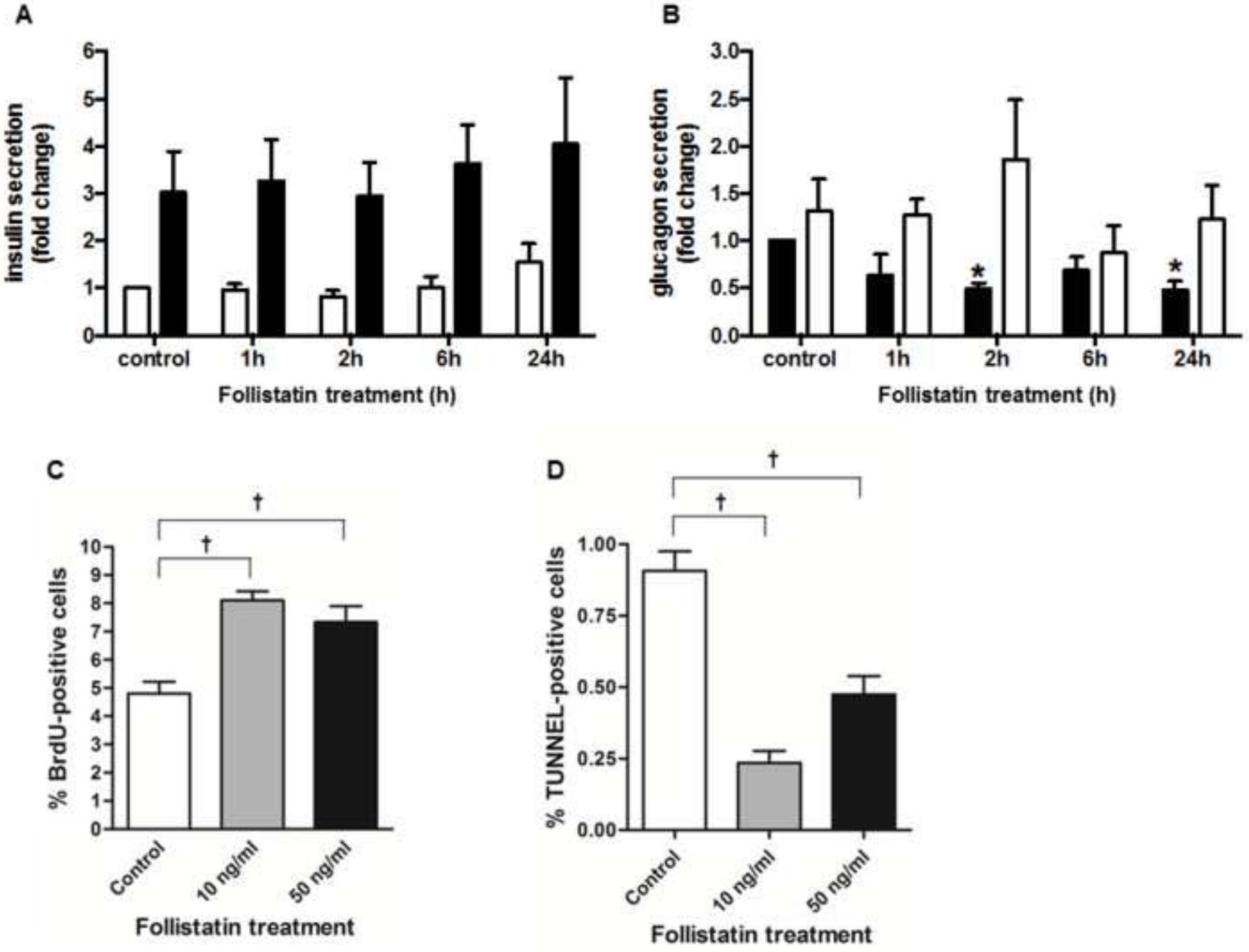

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