Ex vivo and in vivo Regulation of Lipocalin-2, a Novel Adipokine, by Insulin

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Submitted 5 July and accepted 30 September 2008.

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**Objective** Lipocalin-2, a novel adipokine, has been shown to be elevated in obese, insulin resistant and diabetic subjects. We therefore sought to study the *ex vivo* and *in vivo* effects of insulin on Lipocalin-2 levels in humans.

**Research Design and Methods** We investigated the *in vivo* effects of insulin (hyperinsulinemia) on circulating Lipocalin-2 levels by ELISA via a prolonged insulin-glucose infusion. The *ex vivo* effect of insulin on adipose tissue (AT) Lipocalin-2 protein production and secretion into conditioned media were assessed by western blotting and ELISA, respectively.

**Results** Hyperinsulinemic induction in human subjects significantly increased circulating Lipocalin-2 levels ($P < 0.01$). Also, in omental AT explants, insulin caused a significant dose-dependent increase in Lipocalin-2 protein production and secretion into conditioned media ($P < 0.05, P < 0.01$, respectively); these effects were negated by both PI3K and MEK inhibitors.

**Conclusions** Lipocalin-2 is up-regulated by insulin *via* PI3K and MAPK signalling pathways.

**Abbreviations:** AT-Adipose Tissue; ELISA-Enzyme-Linked ImmunoSorbent Assay; IR-Insulin Resistance; RIA-Radio Immunoassay
Obesity and the metabolic syndrome are associated with serious cardio-metabolic sequelae including insulin resistance (IR), hyperinsulinemia, diabetes, dyslipidemia and cardiovascular disease (1).

The metabolic syndrome is associated with visceral obesity. Adipose tissue (AT) produces cytokines termed ‘adipokines’ that are implicated in the pathogenesis of the metabolic syndrome (2).

Recently, Yan et al. established Lipocalin-2 as a novel adipokine, highly expressed by AT in murine models of obesity (3). Also, they demonstrated that Lipocalin-2 levels are increased by dexamethasone and tumour necrosis factor-alpha; and is reduced by rosiglitazone in murine adipocytes (3).

More recently, Wang et al. reported elevated levels of Lipocalin-2 in obesity and diabetes (4).

We therefore studied the effects of acute and chronic hyperinsulinemia on circulating Lipocalin-2 levels via a prolonged insulin-glucose infusion in humans. We also assessed the effects of insulin on Lipocalin-2 protein production and secretion into conditioned media from human visceral AT explants.

RESEARCH DESIGN AND METHODS

Subjects—We measured circulating Lipocalin-2 in six healthy subjects [age: (mean ± SD): 26·5 ± 8 years, BMI: 23·2 ± 2·5 kg/m²]. In order to account for the possible diurnal variation in Lipocalin-2 levels, we obtained a daily control curve by measuring fasting Lipocalin-2 levels at 30 minute intervals from 0800 to 1000 hours. Subsequently, Lipocalin-2 levels were measured at 2-hourly intervals until 2400 h and then at 0400 h on day 2. On the following day the same subjects were subjected to a prolonged insulin-glucose infusion for 26 hours beginning at 0800 hours. Insulin (Human Actrapid) was administered intravenously as a priming dose of 0·04 U/kg followed by continuous infusion of 0·5 mU/kg/minutes. By choosing this rate of insulin infusion we expected to achieve hyperinsulinemia with an approximate four to six fold elevation of basal insulinemia (5).

Fasting blood samples were drawn at 30 minute intervals between 0800 and 1000 hours on day 1 and day 2 of the prolonged insulin-glucose infusion (the first and the last two hours of the infusion). Intermediate blood samples were taken at 2-hourly intervals until 2400 h and then at 0400 h on day 2. Glucose levels were maintained between 4·0 and 6·0 mmol/l.

For AT explant studies; after an overnight fast, AT was obtained (0800-1000 hrs) from six surgical patients [age: (mean ± SD): 27·5 ± 7 years, BMI: 23·8 ± 2·8 kg/m²]. Samples were placed into sterile containers containing Medium 199 (Sigma-Aldrich, Gillingham, UK) for primary AT culture.

Exclusion criteria for all subjects included known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes mellitus, hypertension (blood pressure, >140/90 mmHg), renal impairment (serum creatinine, >120 µmol/L). None of the subjects were on any medications for at least 6 months prior to the study, including glucocorticoids, anti-diabetic and anti-obesity drugs, lipid lowering agents or anti-hypertensive medication. The Local Research Ethics Committee approved the study and all patients involved gave their informed consent, in accordance with the guidelines in The Declaration of Helsinki 2000.

Assays—Serum insulin levels were measured by RIA (Pharmacia, Milton Keynes, UK). Lipocalin-2 in serum and conditioned media from human omental AT explants were...
measured by ELISA (R&D Systems, Abingdon, UK), according to manufacturer’s protocol, with an intra-assay coefficient of variation of < 5%.

**Primary Explant Culture**—AT organ explants were cultured with or without the addition of insulin (Sigma-Aldrich, Gillingham, UK), MEK inhibitor (U0126) [Calbiochem, San Diego, USA] and/or PI3K inhibitor (LY294002) [Calbiochem, San Diego, USA] as previously described (6).

**Western Blotting**—Protein lysates were prepared and western blotting performed as previously described (6). We used monoclonal primary mouse-anti-human antibody for Lipocalin-2 (Abcam, Cambridge, UK) [1:500 dilution] and monoclonal primary rabbit-anti-human antibody for beta-actin (Cell Signalling Technology Inc., Beverly, USA) [1:1000 dilution].

**Statistics**—Data were analysed by Student’s t-test, Mann-Whitney U test and/or Friedman’s ANOVA according to the number of groups compared. $P < 0.05$ was considered significant.

**RESULTS**

Insulin infusion resulted in elevation of fasting insulinemia from $78.1 \pm 12.0 \text{pmol/L}$ to $294.6 \pm 31.0 \text{pmol/L}$. Insulin levels remained elevated until the end of the prolonged insulin-glucose infusion ($366.0 \pm 37.0 \text{pmol/L}$). Lipocalin-2 levels remained unaltered throughout the control day from $91.2 \pm 21.5 \text{ng/ml}$ between 0800 and 1000 hours to $82.8 \pm 21.9 \text{ng/ml}$ between 0800 and 1000 hours the next day (Figure 1A; $P > 0.05$).

There was a profound effect of insulin on Lipocalin-2 levels over 26 hours of insulin infusion: from $103.2 \pm 17.9 \text{ng/ml}$ between 0800 and 1000 hours to $159.7 \pm 34.5 \text{ng/ml}$ between 0800 and 1000 hours the following day (Figure 1A; $P < 0.01$). The increase in Lipocalin-2 levels was relatively acute approaching maximal values at 4 hours (Figure 1A: $180.8 \pm 45.7 \text{ng/ml}$, $P < 0.01$) and persisting throughout the entire period of hyperinsulinemia.

Lipocalin-2 protein production and secretion into conditioned media was significantly increased dose dependently by insulin from human omental AT explants; these effects were negated by both PI3K inhibitor (LY294002; 50µM) and MEK inhibitor (U0126; 10µM) [Figure 1B-E: *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$]. Treatment of AT explants with either LY294002 or U0126 alone did not affect Lipocalin-2 levels (data not shown).

**CONCLUSIONS**

Our findings highlight the involvement of both PI3K and MAPK signalling pathways in insulin induced Lipocalin-2 production and may explain the increased Lipocalin-2 levels in hyperinsulinemic subjects (4). Our observations may have clinical/therapeutic applications, given that Lipocalin-2 promotes IR in adipocytes and hepatocytes (3).

It should be emphasized that our study utilized relatively small numbers of subjects because of the challenge imposed by the prolonged insulin-glucose infusion study. Additionally, we studied a relatively short-term effect of hyperinsulinemia (24 hours) in healthy subjects; type 2 diabetes and insulin resistance syndromes being more chronic states of hyperinsulinemia. Nevertheless, our observations are highly consistent and significant and raise interesting questions on the mechanisms regulating Lipocalin-2 production.

In conclusion, we show for the first time the potent and robust regulation of Lipocalin-2 *ex vivo* and *in vivo*, by insulin. Our findings provide novel insights into Lipocalin-2 physiology, which may be pertinent to hyperinsulinemic states such as obesity and diabetes.
ACKNOWLEDGEMENTS

The General Charities of the City of Coventry funded this study. HSR would like to acknowledge S. Waheguru, University of Warwick for his continual support.

Disclosure statement: All authors have nothing to disclose and there is no duality of interest.
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Figure Legends

Figure 1. (A) Mean concentrations of Lipocalin-2 in ng/ml in all subjects, before and after insulin infusion. Data are means ± SD. Group comparison by Student’s t-test. **P < 0.01. (B) Dose dependent effects of insulin (10^{-11} M, 10^{-9} M, 10^{-7} M) on Lipocalin-2 protein production in human omental AT explants at 24 hours was assessed by western blotting. Western blot analysis of protein extracts from omental AT demonstrate that the antibody against Lipocalin-2 and the antibody against β-actin recognised bands with apparent molecular weights of 23kDa and 45kDa, respectively (Figure 1B-inserts). Densitometric analysis of Lipocalin-2 immune complexes having normalized to β-actin respectively revealed that protein levels of Lipocalin-2 were significantly increased by insulin (10^{-9} M, 10^{-7} M) in human omental AT explants. Data are expressed as % difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman’s ANOVA and post hoc Dunn’s test. *P < 0.05, ***P < 0.001. (C) Dose dependent effects of insulin (10^{-11} M, 10^{-9} M, 10^{-7} M) on Lipocalin-2 secretion into conditioned media from human omental AT explants at 24 hours were measured by ELISA. Lipocalin-2 secretion was significantly increased by (10^{-9} M, 10^{-7} M) from human omental AT explants. Data are expressed as % difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison by Friedman’s ANOVA and post hoc Dunn’s test. *P < 0.05, ***P < 0.001. (D) Effect of PI3K (LY294002) and MEK (U0126) inhibitors on insulin induced Lipocalin-2 protein production in human omental AT explants at 24 hours was assessed by western blotting; compared to insulin (10^{-7} M) without inhibitors. Western blot analysis of protein extracts from omental AT demonstrate that the antibody against Lipocalin-2 and the antibody against β-actin recognised bands with apparent molecular weights of 23kDa and 45kDa, respectively (Figure 1D-inserts). Densitometric analysis of Lipocalin-2 immune complexes having normalized to β-actin respectively revealed that insulin induced Lipocalin-2 protein production was significantly decreased by LY294002 and U0126, respectively, in human omental AT explants. Data are expressed as % difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Differences between groups were assessed using the Mann-Whitney U test. **P < 0.01, ###P < 0.01. (E) Effect of PI3K (LY294002) and MEK (U0126) inhibitors on insulin induced Lipocalin-2 levels in conditioned media from human omental AT explants at 24 hours was assessed by ELISA; compared to insulin (10^{-7} M) without inhibitors. Lipocalin-2 secretion into conditioned media was significantly decreased by LY294002 and U0126, respectively, from human omental AT explants. Data are expressed as % difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Differences between groups were assessed using the Mann-Whitney U test. **P < 0.01, ###P < 0.01.