Recent studies have shown that lipocalin-2, a novel adipokine, is upregulated by insulin via phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways. We investigated the effects of insulin on lipocalin-2 protein production and secretion into conditioned media from human omental adipose tissue explants.

**RESULTS**

Hyperinsulinemic induction in human subjects significantly increased circulating lipocalin-2 levels (*P* < 0.01). Also, in omental adipose tissue 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 phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase inhibitors.

**CONCLUSIONS**

Lipocalin-2 is upregulated by insulin via phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways.

From the 1Endocrinology & Metabolism Group, Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Coventry, U.K., and the 2Department of Endocrinology and Metabolic Diseases, The Medical University of Lodz and Polish Mother's Memorial Research Institute, Lodz, Poland. Corresponding author: Harpal S. Randeva, harpal.randeva@warwick.ac.uk.

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Regulation of lipocalin-2 by insulin

**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}, 10^{-9}, and 10^{-7} mol/l) on lipocalin-2 protein production in human omental adipose tissue explants at 24 h were assessed by Western blotting. Western blot analysis of protein extracts from omental adipose tissue demonstrates that the antibody against lipocalin-2 and the antibody against β-actin recognized bands with apparent molecular weights of 23 and 45 kDa, respectively. Densitometric analysis of lipocalin-2 immune complexes normalized to β-actin revealed that protein levels of lipocalin-2 were significantly increased by insulin (10^{-9} and 10^{-7} mol/l) in human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedmann's ANOVA and post hoc Dunn's test. *P < 0.05; **P < 0.001. C: Dose-dependent effects of insulin (10^{-11}, 10^{-9}, and 10^{-7} mol/l) on lipocalin-2 secretion into conditioned media from human omental adipose tissue explants at 24 h were measured by ELISA. Lipocalin-2 secretion was significantly increased by (10^{-9} and 10^{-7} mol/l) human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedmann'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 adipose tissue explants at 24 h was assessed by Western blotting and compared with insulin (10^{-7} mol/l) without inhibitors. Western blot analysis of protein extracts from omental adipose tissue demonstrates that the antibody against lipocalin-2 and the antibody against β-actin recognized bands with apparent molecular weights of 23 and 45 kDa, respectively. Densitometric analysis of lipocalin-2 immune complexes normalized to β-actin revealed that insulin-induced lipocalin-2 protein production was significantly decreased by LY294002 and U0126 in human omental adipose tissue explants. Data are expressed as percent 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 adipose tissue explants at 24 h was assessed by ELISA and compared with insulin (10^{-7} mol/l) without inhibitors. Lipocalin-2 secretion into conditioned media was significantly decreased by LY294002 and U0126 from human omental adipose tissue explants. Data are expressed as percent 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.
adipose tissue explants was measured by ELISA (R&D Systems, Abingdon, U.K.), according to the manufacturer’s protocol, with an intra-assay coefficient of variation of <5%.

**Primary explant culture**
Adipose tissue explants were cultured with or without the addition of insulin (Sigma-Aldrich), mitogen-activated protein kinase kinase (MEK) inhibitor (U0126; Calbiochem, San Diego, CA), or phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002; Calbiochem) as previously described (6).

**Western blotting**
Protein lysates were prepared and Western blotting was performed as previously described (6). We used monoclonal primary mouse anti-human antibody for lipocalin-2 (Abcam, Cambridge, U.K.) (1:500 dilution) and monoclonal primary rabbit anti-human antibody for β-actin (Cell Signaling Technology, Beverly, MA) (1:1,000 dilution).

**Statistics**
Data were analyzed 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 ± 12.0 pmol/l to 294.6 ± 31.0 pmol/l. Insulin levels remained elevated until the end of the prolonged insulin-glucose infusion (366.0 ± 37.0 pmol/l). Lipocalin-2 levels remained unaltered throughout the control day from 91.2 ± 21.5 ng/ml between 0800 and 1000 h to 82.8 ± 21.9 ng/ml between 0800 and 1000 h the next day (Fig. 1A; P > 0.05).

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

Lipocalin-2 protein production and secretion into conditioned media were significantly increased dose dependently by insulin from human omental adipose tissue explants; these effects were negated by both PI3K inhibitor (LY294002; 50 μmol/l) and MEK inhibitor (U0126; 10 μmol/l) (Fig. 1B–E; *P < 0.05, **P < 0.01, ##P < 0.01). Treatment of adipose tissue 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 mitogen-activated protein kinase signaling 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 insulin resistance 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 h) in healthy subjects because type 2 diabetes and insulin resistance syndromes are 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 by insulin ex vivo and in vivo. Our findings provide novel insights into lipocalin-2 physiology, which may be pertinent to hyperinsulinemic states such as obesity and diabetes.

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