Short-Term Over-Eating Induces Insulin Resistance in Fat Cells in Lean Human Subjects

Anna Danielsson[1], Siri Fagerholm[1], Anita Öst[1], Niclas Franck[1,3], Preben Kjolhede[2], Fredrik H Nystrom[3], Peter Strålfors[1]

[1] Division of Cell Biology, Department of Clinical and Experimental Medicine, Diabetes Research Centre, University of Linköping, SE58185 Linköping, Sweden.
[2] Department of Clinical and Experimental Medicine, University of Linköping, Linköping, Sweden.
[3] Department of Medicine and Health Sciences, Diabetes Research Centre, University of Linköping, SE58185, Linköping, Sweden.

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Correspondence to Peter Stralfors
Division of Cell Biology
University of Linköping
SE58185 Linköping, Sweden

e-mail: peter.stralfors@liu.se
phone: +46 – 13 224315
fax: +46 – 13 224314

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ABSTRACT

Insulin resistance and type 2 diabetes (T2D) are closely linked to obesity. Numerous prospective studies have reported on weight gain, insulin resistance and insulin signaling in experimental animals, but not in humans. We examined insulin signaling in adipocytes from lean volunteers, before and at the end of a four-week period of weight gain based on a fast-food, high-calorie diet; and we also examined adipocytes from patients with T2D. During diet, subjects gained 10% body weight and 19% total body fat, but stayed lean (BMI=24.3 kg/m^2), and developed a moderate systemic insulin resistance. Similarly to the situation in T2D: (i) the amount of insulin receptors was reduced, and (ii) phosphorylation of IRS1 at tyrosine and (iii) at serine-307 (human sequence, corresponding to murine serine-302) were impaired; while (iv) the amount of IRS1 protein and (v) the phosphorylation of IRS1 at serine-312 (human sequence, corresponding to murine serine-307) were unaffected, by the diet. Contrary to in diabetes and likely due to the on-going weight-gain, phosphorylation of MAP-kinases ERK1/2 became hyperresponsive to insulin. To our knowledge this is the first study of insulin signaling during over-eating in humans and it demonstrates T2D-effects on intracellular insulin signaling already after four weeks of diet and that the effects in humans differ from laboratory animals.

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INTRODUCTION

Type 2 diabetes (T2D) is closely linked to obesity (1) and is the result of reduced sensitivity to insulin in the main target tissues of the hormone and a failure of the insulin producing β-cells of the pancreas to compensate the reduced insulin sensitivity with increased release of insulin. Control of target cells by insulin involves binding to and activation of its cell surface receptor via autophosphorylation of tyrosine residues. These are recognized by a set of signal transducers, particularly the insulin receptor substrate protein-1 (IRS1) that in turn is phosphorylated on tyrosine residues. Thus phosphorylated IRS1 can bind and activate phosphatidylinositol 3´-kinase that activates downstream signaling to metabolic control. Tyrosine phosphorylated IRS1 can also bind Grb2, which initiates a Ras-mediated activation of MAP-kinases ERK1/2 for mitogenic control via phosphorylation of transcription factors. In contrast, the molecular mechanisms behind insulin resistance in humans are poorly understood (2, 3). It has been shown in both human skeletal muscle (4-7) and adipose tissue (8, 9) that insulin-induced phosphorylation of IRS1 on tyrosine residues is impaired in T2D. In adipocytes from patients with T2D we have found that a positive feedback control loop, involving phosphorylation of IRS1 at serine-307 (human sequence, corresponding to serine-302 in the murine sequence), is not fully functional (10, 11). On the other hand, other studies have reported increased phosphorylation of IRS1 at serine-312 (human sequence, corresponding to serine-307 in the murine sequence), which is part of a negative feedback loop, reviewed in (12-16).

There are many studies of obesity in humans, and some have prospectively evaluated effects of weight reduction in obese subjects (17), but very few modern studies have examined lean human subjects during weight gain (18, 19). There are, however, many experimental animal studies of obesity, weight gain, and high-fat feeding that influence metabolic control and insulin sensitivity, reviewed in (12-16). For the first time we now report a study of insulin signaling in adipocytes from healthy human beings subject to high-calorie over-eating.

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MATERIALS AND METHODS

Subjects and study description

Informed consent was obtained from all participating individuals and the procedures have been approved by the Ethics committee at Linköping University and were performed in accordance with the Declaration of Helsinki.

This study originally included 18 young volunteers (20). Participants agreed to increase their body weight 5-15% by eating two fast-food meals a day and restrict their physical activity to 5000 steps a day during four weeks (20). Adipose tissue biopsies were obtained at the start and end of the study period. Due to the leanness of the subjects at the start of the study (Table 1), we could not get enough adipose tissue from several subjects. The small biopsy volumes of tissue also restricted the type and number of analyses. From a subgroup of six subjects (4 females and 2 males) that exhibited increased insulin levels we obtained enough material to analyze selected aspects of insulin signaling.

The subjects were subjected to dual energy X-ray absorptiometry (Hologic 4500, Hologic, Waltham, MA, USA), for analysis of body composition. During the study period the six participants average energy intake about doubled compared with before the intervention (Table 2). During intervention, on average, 37 % of the energy was from fat, 47 % from carbohydrates (43 % of the total carbohydrate intake were mono- and disaccharides (Table 2)) and 15 % from protein. The detailed composition of the diet was based on reports three days before the study (baseline) and another three-day period at the end of the first (period 1) and third (period 2) week of the study. In most cases exact food composition given by the corresponding fast food restaurant could be used as a source of information, but when such information was incomplete we used food composition charts. For food bought at groceries, the manufacturer's information was used. If no such information was available, the foodstuff was substituted with a similar item in the database of Swedish National Food Administration. Total caloric intake and that of macronutrients were also determined for the whole study period, and this was based on all food receipts and on interviews performed after the intervention period.

Subcutaneous adipose tissue was excised by needle aspiration under local anesthesia with mepivacain (Carbocain®, AstraZeneca AB, Södertälje, Sweden). Approximately 10 mL tissue was aspirated in a fan-like area unilaterally in the umbilical region. On the second occasion fat was from the contralateral umbilical region. Blood was drawn in the fasting state for determination of insulin concentrations.

For a comparison with the diabetic state, subjects with or without T2D were recruited consecutively as they were scheduled for elective surgery at the department of Obstetrics and Gynecology at the University Hospital in Linköping. Subcutaneous fat was obtained during abdominal surgery (laparotomy) under general anesthesia. A 1.5 cm broad slice of subcutaneous tissue from the skin to

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the muscle fascia was excised. The only selection criterion for non-diabetic subjects was that they had not been diagnosed with diabetes. Patients with diagnosed T2D were included if they conformed to criteria for the metabolic syndrome: obesity/over weight (BMI>28) and dyslipidemia and/or hypertension. We did not match control subjects for obesity or BMI. Therefore both our non-diabetic subjects and patients with diabetes were not selected for anything but the absence or presence of the disease, respectively.

Materials

Mouse anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA). Rabbit phospho-threonine(308)-protein kinase B and anti-IRS1 polyclonal antibodies were from Upstate Biotech (Charlottesville, VA, USA). Anti-phospho-serine(307)-IRS1, anti-phospho-serine(312)-IRS, and phospho-ERK1/2 polyclonal antibodies from Cell Signaling Techn. (Beverly, MA, USA). Rabbit polyclonal anti-insulin receptor β-subunit and anti-actin antibodies were from Santa Cruz Biotechn. (Santa Cruz, CA, USA).

Isolation and incubation of adipocytes

Adipose tissue, obtained by needle aspiration or by surgical excision, were cut into small pieces and adipocytes isolated after digestion by collagenase (type 1, Worthington, NJ, USA) as described (21). Cells were treated and incubated with the indicated concentration of insulin in supplemented Krebs-Ringer solution as described in (9).

SDS-PAGE and immunoblotting

Cell incubations were terminated by separating cells from medium using centrifugation through dinonylphtalate. To minimize postincubation signaling modifications in the cells and protein modifications, which can occur during immunoprecipitation, the cells were immediately dissolved in SDS and β-mercaptoethanol with protease and protein phosphatase inhibitors, frozen within 10 sec, and thawed in boiling water for further processing (21). Total cell number, cell surface, or cell volume of the adipocytes can be compared. Adipocytes consist of a central lipid droplet occupying >95% of the cell volume and the cytosol is a 200-500 nm thin film beneath the plasma membrane. We have therefore compared total volumes of cells as a good approximation to a comparison based on cytosolic volume or cell surface area. Equal amounts of cells as determined by lipocrit, that is total cell volume, was subjected to SDS-PAGE and immunoblotting (8) and evaluated by chemiluminescence imaging (Las 1000, Image-Gauge, Fuji, Tokyo, Japan). The amount of actin protein was the same in the adipocytes obtained at baseline and at the end of the high-calorie intervention (not shown), and also in adipocytes from non-diabetic controls compared with patients with T2D. Moreover, the amount of insulin receptor and IRS1, and the phosphorylation of IRS1 at tyrosine, protein kinase B, and ERK1/2

were normalized to the amount of actin in each sample; the phosphorylation of IRS1 at serine 307 or serine 312 was normalized to the amount of IRS1 in each sample.

Dose-response data were normalized to percent of maximal effect and the effect of insulin was fitted to experimental data using the sigmoidal dose-response algorithm of GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). Curves were compared statistically using *F*-test with the sigmoidal curve-fitting algorithm in GraphPad Prism 4. When indicated, Student´s t-test was used to compare control and intervention groups. The null hypothesis was rejected if $P<0.05$.

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RESULTS

Whole body effects

Subjects increased their body mass index (BMI) from the low baseline value 22.4 kg/m^2 to a moderate 24.3 kg/m^2, which corresponded to a total body fat increase of 19 %, or 3.4 kg, on average, as determined by dual energy X-ray absorptiometry (Table 1). The fat deposition was paralleled by development of a moderate, but significant, systemic insulin resistance measured both as increased insulin resistance (HOMA) and as decreased insulin sensitivity (QUICKI), and also as increased fasting concentrations of circulating insulin (Table 1).

Insulin signaling in isolated adipocytes

Insulin binding sites have earlier been found to be reduced on adipocytes from obese and insulin resistant subjects (22). We therefore examined the effect of the high-calorie diet on the amount of insulin receptors in the isolated adipocytes. The concentration of insulin receptors was significantly reduced by about 40% at the end of the diet as compared with baseline levels (Table 3), such as could result from reduced FOXO1 activation of insulin receptor transcription (23). A similar reduction of the amount of receptors was found in cells from patients with T2D, compared with non-diabetic subjects (Table 3). It has been reported that the cellular concentration of IRS1 is reduced in adipocytes from patients with T2D (24). There was, however, no significant effect of the high-calorie diet on the concentration of IRS1 protein (Table 3), nor did we detect any difference in the concentration of IRS1 in adipocytes from patients with type 2 diabetes compared with a group of non-diabetic subjects (Table 3).

The concentration of insulin needed for the insulin receptor to phosphorylate IRS1 at tyrosine was low at the start of the high-calorie diet, but the cells required several-fold higher concentrations of insulin at the end of the intervention (Fig. 1A). The maximal effect of insulin on phosphorylation of IRS1 at tyrosine was also reduced (Fig. 1A). A similarly reduced sensitivity to insulin was seen in cells from patients with T2D compared with non-diabetic controls (Fig. 1B). For technical limitations we could not compare the maximal effect of insulin on this number of subjects, but we have previously reported a tendency towards lower maximal effects of insulin in diabetic adipocytes (8).

We have previously reported that in obese patients with T2D a positive feedback control loop, to phosphorylation of IRS1 at serine-307 (human sequence, corresponding to serine-302 in the murine sequence), is attenuated (10, 11). Interestingly, at the end of the high-calorie feeding period insulin-induced phosphorylation at serine 307 was attenuated (Fig. 1C), similarly to our earlier observation in adipocytes from diabetic patients (11). In contrast, neither the basal level of phosphorylation at serine-312 (corresponding to serine-307 in the murine sequence) nor the effect of insulin to induce

phosphorylation at this serine was affected by the high-calorie diet (Fig. 1D,E). This was again similar to the situation in T2D (9).

The high-calorie diet had no significant effect on the downstream phosphorylation of protein kinase B at threonine-308 in response to insulin (Fig. 2A). Likewise, in adipocytes from patients with T2D the sensitivity to insulin for phosphorylation of protein kinase B was similar to in cells from non-diabetic controls (Fig. 2B).

The downstream effect of insulin to phosphorylate MAP-kinases ERK1/2 was enhanced at the end of the high-calorie diet, both the sensitivity to insulin and the maximal response was affected (Fig. 2C).

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DISCUSSION

Several conclusions can tentatively be drawn from this study of high-calorie diet and rapid weight gain in lean human subjects, in comparison with T2D and the metabolic syndrome. First, a significant, albeit moderate, systemic insulin resistance developed already after four weeks on high-calorie diet. Second, this was reflected in altered insulin signal transduction in adipocytes, which in several aspects approached the situation in cells from patients with T2D. Third, insulin downstream mitogenic signaling to phosphorylation and activation of MAP-kinases ERK1/2 was enhanced, in sharp contrast to the situation in T2D. Fourth, these changes in insulin signaling occurred in lean and healthy young people that attained a very low degree of adiposity. Even after diet and weight gain the study subjects were not obese or overweight, hence our findings suggest that, at least on this time-scale, weight gain caused the lowering of insulin sensitivity. This conclusion is a mirror image, at the other end of the spectrum, of clinical observations that obese and insulin resistant people improve their insulin sensitivity by weight reduction and increased physical activity, although remaining obese or overweight (17, 25).

Although it was not possible to match T2D patients to our young and lean over-eating study group, it is very interesting to compare the effects of over-eating on insulin signaling with the situation in adipocytes from patients with T2D. The effects of over-eating on the amount of insulin receptor, and phosphorylation of IRS1 at tyrosine and at serine-307, were similar to the situation in adipocytes from patients with type 2 diabetes (herein and (11)); as was the lack of effect on the amount of IRS1 protein and phosphorylation of IRS1 at serine-312 (herein and (9)). This was, moreover, also found in adipocytes made insulin resistant by incubation with the diabetogenic adipokine RBP4 (9). The normal state of phosphorylation of IRS1 at serine-312 was reflected in the unperturbed levels of IRS1 protein at the end of the diet or in the diabetic state, as phosphorylation of this serine residue in H4IIE rat hepatoma cells has been linked to increased degradation of IRS1 (26). The insulin stimulated phosphorylation of protein kinase B was not affected by the diet, nor in type 2 diabetes (herein) or in response to RBP4 (9). This is similar to what has been found in skeletal muscle of obese and diabetic patients, where muted association of IRS1 (or IRS2) with PI3-kinase does not affect the phosphorylation of protein kinase B in response to insulin (4).

The hyperresponsive effects of the high-calorie over-eating on insulin control of ERK1/2 and mitogenic signaling was the opposite to the situation in T2D or after induction of insulin resistance by RBP4, where in both cases the sensitivity to insulin is reduced (9). This is interesting as cells from the diabetic patients reflect a steady-state situation, whereas here we examined adipocytes obtained at the end of four weeks of high-calorie diet, while the subjects were still on the diet. Hence, at this time point weight gain had not stabilized. The adipose tissues of these subjects should therefore have been in a hypertrophic mode in order to accommodate the increasing storage of triacylglycerol. This is

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likely reflected in the insulin-hyperresponsive ERK1/2, which are part of the major insulin–regulated cell growth-controlling signaling pathway. The MAP-kinase signaling pathway has been shown to be critical for proliferative effects of insulin in H4IIE hepatoma cells (27) and ERK1$^{-/-}$ mice have indeed reduced adiposity and are resistant to high-fat induced obesity, while in embryo fibroblasts from these mice ERK1 is required for adipogenesis (28). In a follow up to this study it will be important to analyze to what extent over-eating affects the number vs. size of the adipocytes, as well as to examine how ERK1/2 respond to insulin in subjects when a new steady-state energy balance and stabilized higher body weight have been attained. It can be noted that studies of animal cells (3T3-L1 adipocytes) have demonstrated that increased activity of ERK1/2 causes a dramatic downregulation of IRS1 protein in the cells (29), this was obviously not the case with human adipocytes.

This feasibility study produced statistically significant results despite a small number of subjects. The study subjects acted as their own controls, before is compared with at the end of the diet. There was dominance for female over male subjects (2:1) that may confound the results, and it will be important to address possible gender differences in isolated adipocytes during over-eating. We have, however, earlier found no difference in insulin signaling when examining adipocytes from male and female patients with T2D (8). The small biopsy volumes of tissue that could be obtained from lean subjects restricted the type and number of analyses that could be done. Results demonstrate, though, that such studies of humans are feasible and, indeed, necessary in order to understand the effects of over-eating on human fat cells. This study was restricted to examination of abdominal subcutaneous fat, as open surgery is required to obtain visceral fat. Visceral fat is often considered to be more associated with disease than subcutaneous fat. We nevertheless did record substantial effects on insulin signaling in the subcutaneous fat. It remains to examine if this is a reflection of similar effects in the visceral fat or if visceral fat responds qualitatively different to over-eating.

Much is known about effects of high-fat feeding and adiposity in experimental animals, especially in transgenic mice over-expressing or being deficient in an array of different proteins, which in many cases have been found to protect or make the animals more susceptible to obesity, insulin resistance and diabetes, reviewed in (12-16). One finding in studies with animals is that metabolic overload from high-calorie over-eating inhibits insulin signaling via increased phosphorylation of IRS1 at serine residues, especially serine-307 (murine sequence, corresponding to serine-312 in the human sequence), reviewed in (12-16). In the fat cells of the subjects herein the phosphorylation of serine-312 was not affected, while phosphorylation of serine-307 (corresponding to serine-302 in the murine sequence) was attenuated, at the end of the high-calorie over-eating period. This compares well with the situation recorded in human T2D. It should also be borne in mind that experimental animals mostly are bred for generations with unlimited food availability and little opportunity for physical

activity and have thus been genetically selected to those conditions that are being studied, which is not true for human beings.

Our study of high-calorie high-fat diet-induced weight gain raises many questions, such as effects on insulin control of glucose uptake, other aspects of insulin signaling, insulin signaling in skeletal muscle, signaling when body weight is at a steady-state after the weight gain, hypertrophia vs hyperplasia of the adipocytes, gender differences, and to what extent visceral and subcutaneous adipocytes respond differently, that should be approached in a larger follow up to this first study. An important further objective would be to quantify and characterize any adipose tissue macrophage infiltration during weight gain. The study of humans impose severe limitations to what can be done, compared with studies of animals. To study weight gain in lean humans, moreover, means that the small biopsy volumes of adipose tissue that can be obtained restrict the type and number of analyses. This study demonstrates that studies of humans are required and that in spite of many limitations important insight and understanding can be obtained.

DISCLOSURE

Authors declare no conflicting interests.

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REFERENCES

1. Colditz GA, Willet WC, Stampfer MJ, et al. (1990) Weight as risk factor for clinical diabetes in women. *Am. J. Epidemiol.* 132: 501-513.
2. Biddinger SB, Kahn CR. (2006) From mice to men: insights into the insulin resistance syndromes. *Ann. Rev. Physiol.* 68: 20.21-20.36.
3. Muoio DM, Newgard CB. (2008) Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nature Rev. Mol. Cell Biol.* 9: 193-205.
4. Kim Y-B, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB. (1999) Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J. Clin. Inv.* 104: 733-741.
5. Goodyear L, Giorgino F, Sherman L, Carey J, Smith R, Dohm G. (1995) Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J. Clin. Inv.* 95: 2195-2204.
6. Cusi K, Maezono K, Osman A, et al. (2000) Insulin resistance differentially affects the PI 3-kinase and MAP kinase-mediated signaling in human muscle. *J. Clin. Inv.* 105: 311-320.
7. Björnholm M, Kawano Y, Lehtihet M, Zierath J. (1997) Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase acitivity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes* 46: 524-527.
8. Danielsson A, Öst A, Lystedt E, et al. (2005) Insulin resistance in human adipocytes downstream of IRS1 after surgical cell isolation, but at the level of phosphorylation of IRS1 in type 2 diabetes. *FEBS J.* 272: 141-151.
9. Öst A, Danielsson A, Liden M, Eriksson U, Nystrom FH, Strålfors P. (2007) Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. *FASEB J.* 21: 3696-3704.
10. Danielsson A, Nystrom FH, Strålfors P. (2006) Phosphorylation of IRS1 at serine 307 and serine 312 in response to insulin in human adipocytes. *Biochem. Biophys. Res. Commun.* 342: 1183-1187.
11. Danielsson A, Öst A, Nystrom FH, Strålfors P. (2005) Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. *J. Biol. Chem.* 280: 34389-34392.
12. Gual P, LeMarchand-Brustel Y, Tanti J-F. (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochemie* 87: 99-109.
13. Um SH, D´Alessio D, Thomas G. (2006) Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab.* 3: 393-402.
14. Hotamisligil GS. (2006) Inflammation and metabolic disorders. *Nature* 444: 860-867.
15. Luca Cd, Olefsky JM. (2008) Inflammation and insulin resistance. *FEBS Lett.* 582: 97-105.
16. Guilherme A, Virbasius JV, Puri V, Czech MP. (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature Rev. Mol. Cell Biol.* 9: 367-377.
17. Petersen KF, Dufour S, Befroy D, Lehrke M, Hendler RE, Shulman GI. (2005) Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* 54: 603-608.
18. Salans LB, Horton ES, Sims EAH. (1971) Experimental obesity in man: cellular character of the adipose tissue. *J. Clin. Inv.* 50: 1005-1011.

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19. Lammert O, Grunnet N, Faber P, et al. (2000) Effects of isoenergetic overfeeding of either carbohydrate or fat in young men. *Br. J. Nutrition* 84: 233-245.
20. Kechagias S, Ernersson Å, Dahlqvist O, Lundberg P, Lindström T, Nystrom FH. (2008) Fast food based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut* 57: 649-654.
21. Strålfors P, Honnor RC. (1989) Insulin-induced dephosphorylation of hormone-sensitive lipase. Correlation with lipolysis and cAMP-dependent protein kinase activity. *Eur. J. Biochem.* 182: 379-385.
22. Olefsky JM. (1976) Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. *J. Clin. Inv.* 57: 1165-1172.
23. Puig O, Tjian R. (2006) Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. *Genes Dev.* 19: 2435-2446.
24. Rondinone CM, Wang L-M, Lonnroth P, Wesslau C, Pierce JH, Smith U. (1997) Insulin receptor substrate (IRS) 1 is reduced and IRS2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin dependent diabetes mellitus. *Proc. Natl. Acad. Sci. USA* 94: 4171-4175.
25. Dixon JB, O´Brien PE, Playfair J, et al. (2008) Adjustable gastric banding and conventional therapy for type 2 diabetes. *J. Am. Med. Assoc.* 23: 316-323.
26. Greene MW, Sakaue H, Wang L, Alessi DR, Roth RA. (2003) Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by serine 312 phosphorylation. *J. Biol. Chem.* 278: 8199-8211.
27. Törnkvist Å, Parpal S, Gustavsson J, Strålfors P. (1994) Inhibition of raf-1 kinase expression abolishes insulin stimulation of DNA synthesis in H4IIE hepatoma cells. *J. Biol. Chem.* 269: 13919-13921.
28. Bost F, Aouadi M, Caron L, et al. (2005) The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. *Diabetes* 54: 402-411.
29. Jager J, Gremeaux T, Cormont M, Marchand-Brustel YL. (2007) Interleukin-1 beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 148: 241-251.
30. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28: 412-419.
31. Katz A, Nambi SS, Mather K, et al. (2000) Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J. Clin. Endocrinol. Metab.* 85: 2402-2410.

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FIGURE LEGENDS

Figure 1. Phosphorylation of IRS1 at tyrosine (A,B) at serine-307 (C) and serine-312 (D,E).

A, B, Adipocytes obtained at baseline (open circles) and at the end (closed circles) of the high-calorie over-eating period (A); or from patients with type 2 diabetes (closed circles), age 35-74 years, average 57; BMI 28-43 kg/m^2, average 35 (n=15) compared with non-diabetic controls (open circles), age 31-82 years, average 55; BMI 23-33 kg/m^2, average 26 (n=16) (B), were incubated with the indicated concentration of insulin for 10 min, when incubations were terminated and equal volumes of whole cells were subjected to SDS-PAGE and immunoblotting for phosphotyrosine. The phosphorylation of IRS1 is expressed as percent of maximal insulin-effect at the start (baseline) of the high-calorie diet, mean±SE (n=6) (A); or as percent of maximal insulin-effect in control cells and in diabetic cells, respectively, mean±SE (B). Samples from the same subject were analyzed on the same gel. The curves were statistically different (P=0.0009 (A); P<0.0001 (B), *F*-test).

C-E, Adipocytes obtained at baseline (open bars) and at the end (closed bars) of the high-calorie over-eating period were incubated with (+) or without (-) 100 nM insulin, as indicated. After 10 min (C, E) or 30 min (D) incubations were terminated and equal volumes of whole cells were subjected to SDS-PAGE and immunoblotting for phosphorylation of IRS1 at serine-307 (C) or serine-312 (D,E) (whereas insulin-stimulated phosphorylation of IRS1 at serine-307 is maximal after 10 min, the phosphorylation at serine-312 requires 30 min (10), we therefore incubated for 10 or 30 min, as indicated). The phosphorylation of IRS1 in response to insulin is expressed as percent of baseline phosphorylation without insulin; mean±SE (C, n=6; D, n=3; E, n=6). Samples from the same subject were analyzed on the same gel. Paired Student´s t-test.

Figure 2. Insulin sensitivity for phosphorylation of protein kinase B (A, B) and Map-kinases ERK1/2 (C).

A,C, Adipocytes obtained at baseline (open circles) and at the end (closed circles) of the high-calorie over-eating period were incubated with the indicated concentration of insulin for 10 min, when incubations were terminated and equal volumes of whole cells were subjected to SDS-PAGE and immunoblotting for phosphorylation of protein kinase B at threonine-308 (A) or phosphorylation of Map-kinases ERK1/2 (C). Phosphorylation is expressed as percent of maximal insulin-effect at the start (baseline) of the high-fat diet; mean±SE (n=6). Samples

from the same subject were analyzed on the same gel. The two curves in (C) were statistically different (P=0.006, *F*-test).

B, Adipocytes from patients with type 2 diabetes (closed circles), age 35-74 years, average 57; BMI 28-43 kg/m^2, average 34 (n=16), and from non-diabetic controls (open circles), age 31-94 years, average 58; BMI 17-33 kg/m^2, average 25 (n=17), were incubated with the indicated concentration of insulin for 10 min, when incubations were terminated and equal volumes of whole cells were subjected to SDS-PAGE and immunoblotting for phosphorylation of protein kinase B at threonine-308. The phosphorylation is expressed as percent of maximal insulin-effect in control cells and diabetic cells, respectively, mean±SE.

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TABLE 1

Characteristics for subjects participating in the high-calorie diet. Values are the average for the participating subjects at the start of the study (baseline) and at the end, or as the ratio of the averages for the values at the end and at baseline. Means±SD (n=6). Paired Student´s t-test. Body composition was determined by dual energy X-ray absorptiometry (Hologic 4500, Hologic, Waltham, MA, USA). BMI, body mass index; HOMA, homeostasis model assessment (30); QUICKI, Quantitative Insulin Sensitivity Check Index (31).

Age years	BMI kg/m^2		Total body fat kg		Insulin pM		HOMA		QUICKI	
	baseline	end	baseline	end	baseline	end	baseline	end	baseline	end
27.3±3.2	22.4±1.8	24.3±2.2	17.6±6.0	21.0±6.7	36.7±15.9	50.3±17.9	1.1±0.5	1.6±0.6	0.385±0.056	0.356±0.038
	P=0.002		P=0.006		P=0.030		P=0.036		P=0.029	

TABLE 2

Characteristics of food intake. Values are the averages for the participating subjects three days before the study (baseline) and during two three-day periods, at the end of the first week (period 1) and at the end of the third week (period 2), of the study. Means±SD (n=6).

Intake	Baseline	Period 1	Period 2
Mono- & disaccharides (g/day)	102±35	270±52	244±45
Total carbohydrates (g/day)	243±49	600±108	566±112
Total energy (MJ/day)	8.54±1.37	23.69±2.77	23.14±4.24

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TABLE 3

Cellular concentration of insulin receptor and IRS1. The amounts of insulin receptor or IRS1 in adipocytes were compared at baseline and after 4 weeks on the high-calorie diet. Samples from the same subjects were analyzed on the same gel by SDS-PAGE and immunoblotting. Results are expressed as percentage of the amount of protein at the start (baseline) of the high-energy diet, mean±SE (n=6). The amounts of insulin receptor or IRS1 were also compared in adipocytes from subjects with T2D (age 33-72 years, average 53 years; BMI 28-48 kg/m^2, average 36 kg/m^2 (n=16)), with cells from non-diabetic controls (age 34-80 years, average 62 years; BMI 20-39 kg/m^2, average 28 kg/m^2 (n=17)). Samples from subjects with and without T2D were analyzed on the same gels, by SDS-PAGE and immunoblotting. Results are expressed as percentage of non-diabetic controls, mean±SE. Paired Student´s t-test.

	Diet (% of baseline)	T2D (% of controls)
Insulin receptor	57±7, P=0.001	55±12, P=0.035
IRS1	103±21	98±30

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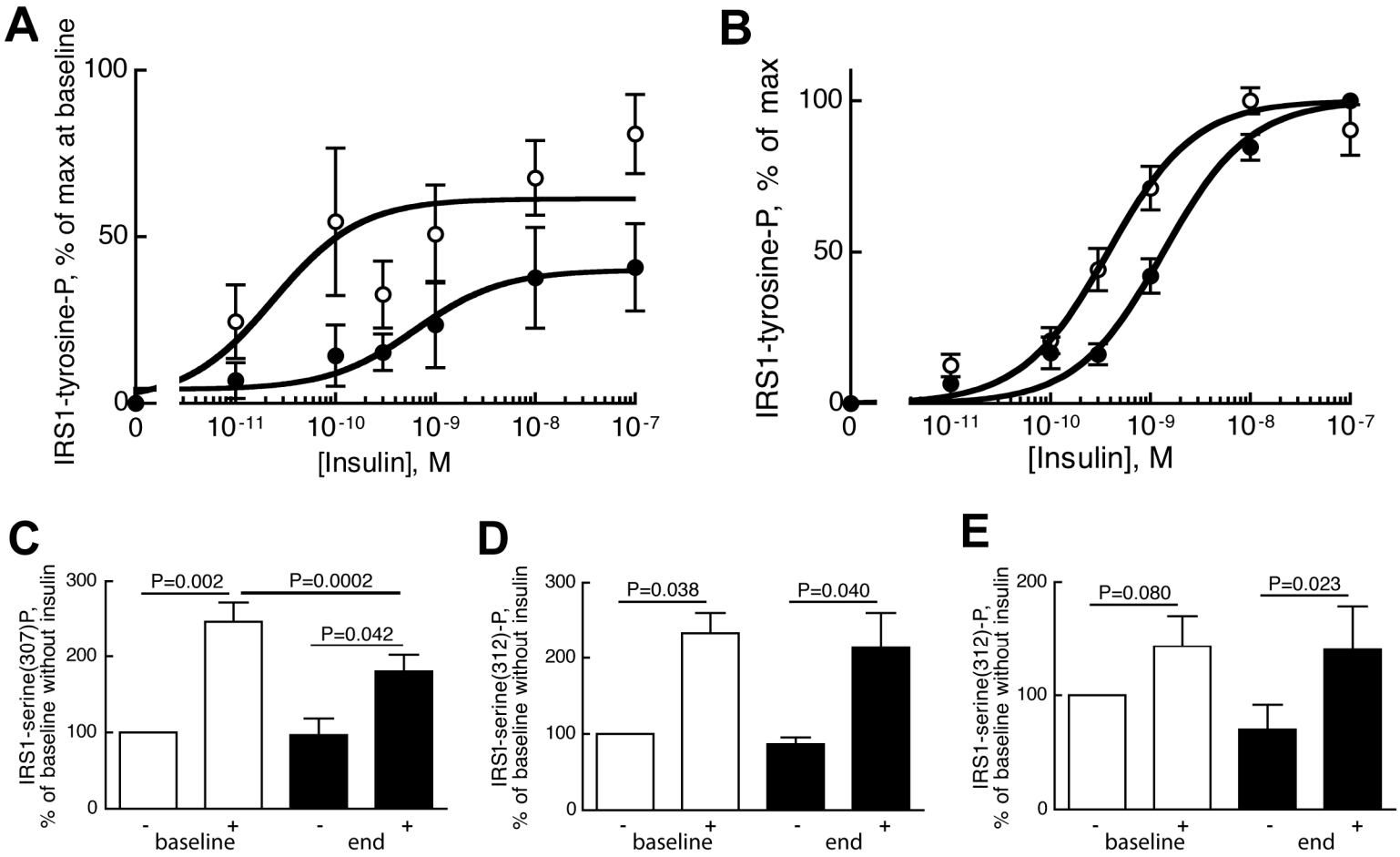
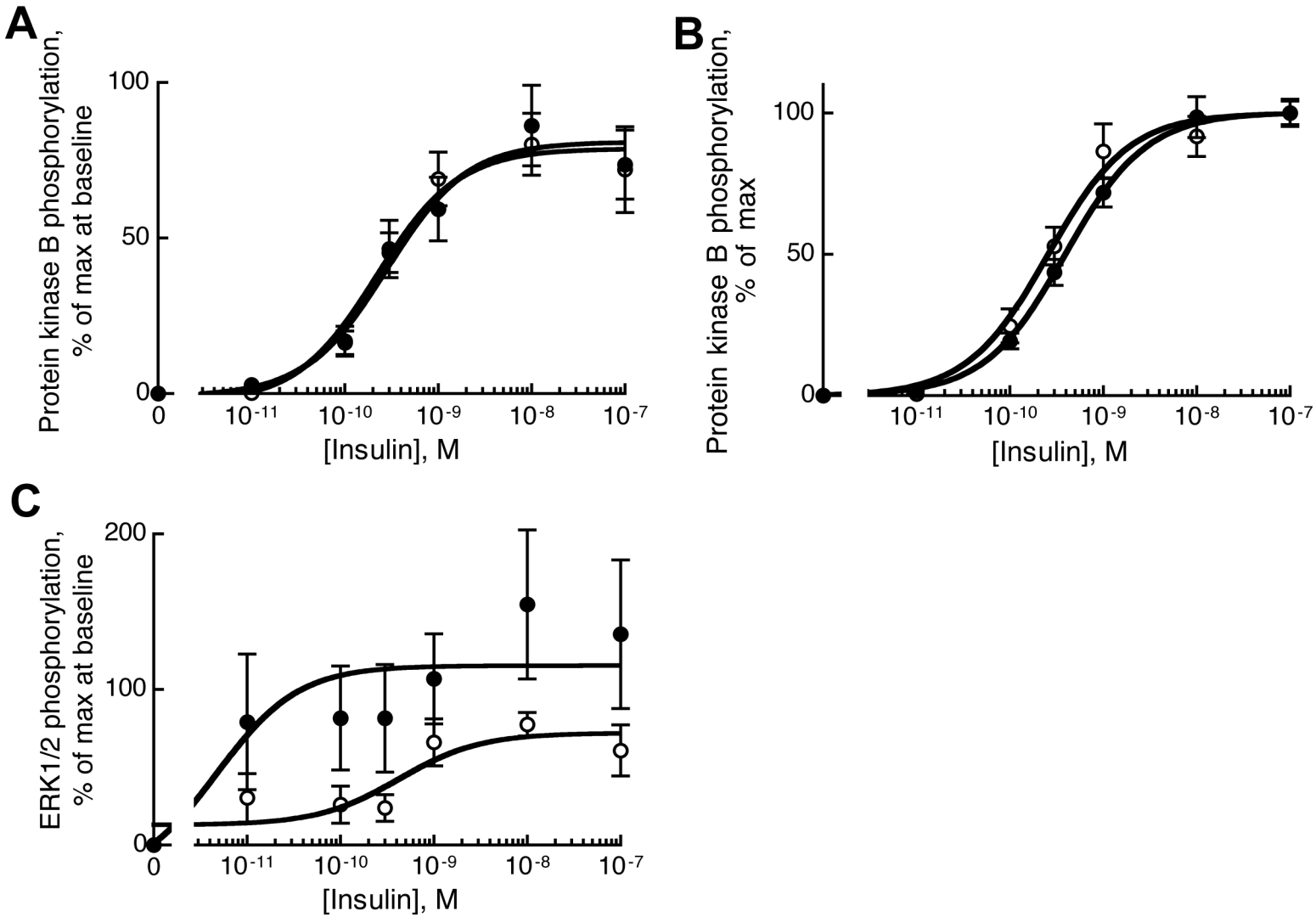
Danielsson et al, Figure 1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