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Generation, validation and humanisation of a novel insulin resistant cell model

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ABSTRACT

Insulin resistance is a characteristic of type 2 diabetes and is a major independent risk factor for progression to the disease. In particular, insulin resistance associates with increased body fat and almost certainly contributes to the dramatic increase in risk of type 2 diabetes associated with obesity. Therefore, in order to design truly effective insulin sensitising agents, targeted at the mechanism of disease development, we aimed to generate an obesity-related insulin resistant cell model. Rat hepatoma cells were grown in the presence of serum isolated from obese rodents or obese human volunteers, and the insulin sensitivity of the cells monitored over time by measuring a well-characterised insulin regulated gene promoter. Higher insulin concentrations were required to fully repress the gene in the cells grown in obese rodent serum compared with those grown in serum from lean rodents (almost a 10-fold shift in insulin sensitivity). This was reversed by restoration of normal growth medium, while the insulin resistance was prevented by pioglitazone or metformin. Meanwhile, growth of cells in serum collected from obese human volunteers with diabetes also reduced the insulin sensitivity of the rat cells. No clinical marker predicted the degree of insulin resistance that was generated by the human serum. We have developed a novel insulin resistant cell model for the study of the molecular development of obesity-linked insulin resistance, screen for compounds to overcome obesity-related insulin resistance and potentially search for novel serum biomarkers of insulin resistance.

1. Introduction

Insulin resistance (IR) is one of the two major characteristics of type 2 diabetes, with IR itself being a major independent risk factor for progression to the disease [1–3]. In addition IR is highly likely to be the link between obesity and type 2 diabetes [4–6]. However the early stages of the molecular development of IR remain poorly characterised. Several protein kinases that contribute to, or alter, insulin signalling are regulated by lipids and adipokines, and this has led to the hypothesis that obesity promotes insulin resistance by altering intracellular insulin signalling. For example, ceramide inhibits insulin action [7,8], and fatty acids and TNF-α induce kinases that antagonise IRS signalling (e.g. JNK [9,10] and PKC [7], for excellent review see [11]). In support of this, IRS1, PKC and GSK3 signalling (key for specific actions of insulin) have all been found to be abnormal in a variety of tissues isolated from obese volunteers with diabetes [11–15], while inhibition of GSK3 has been shown to ameliorate IR in cells, animal models and human muscle [16,17]. Finally, there are now several mouse models of IR (see [18,19] for review), many generated by interfering with different aspects of insulin signalling, suggesting that the same clinical phenotype of IR in the population may result from a variety of signalling problems. Clearly, if a spectrum of molecular problems is present in the human insulin resistant population then this has implications for the efficacy of a single therapy combating a specific molecular target.

Reversing IR in the human population at risk of developing type 2 diabetes would slow down the development of the hyperglycemia that defines type 2 diabetes and also the associated health problems such as micro- and macro-vascular disease, retinopathy, neuropathy and kidney disease. Two of the major anti-diabetes therapies (glitazones and biguanides) have beneficial effects on type 2 diabetes, in part due to their insulin sensitising properties.

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However these drugs are weak insulin sensitisers, lose potency with use, and are not effective in all type 2 diabetes. Therefore the search has begun for more effective insulin sensitisers. As such, there is a need for an *in vitro* or cell based approach to aid in the early development of these agents. Since compounds that improve sensitivity in healthy/normal cells would not necessarily be effective in an insulin resistant tissue it would be beneficial to have a cell model that mirrored the clinical problem. The generation of insulin resistant cells by knockout of a single molecule (e.g. cells lacking IRS1) would not necessarily be a model of all, or even the majority of, human IR, as the identity of the major molecular reason for insulin resistance in humans remains unclear.

Therefore, we aimed to develop an unbiased cell model of obesity induced insulin resistance. The rat hepatoma cell (H4IIE) was grown in serum collected from obese rodents or human volunteers (obese with a diagnosis of type 2 diabetes), and insulin sensitivity of the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter monitored. This gene product is rate controlling for hepatic glucose production [20] is abnormally regulated in animal models of obesity and IR [21,22], and overexpression of PEPCK generates hyperglycemia in mice [23]. The novel model generated will hopefully enhance our understanding of the development of obesity induced IR and could provide a preclinical tool in the search for insulin sensitising interventions.

2. Methods and materials

2.1. Materials

Actrapid (human insulin) was purchased from Novo Nordisk A/S (Bagsværd, Denmark). 2x Universal PCR Master Mix, No AmpErase UNG from Applied Biosystems, Complete™ protease inhibitor cocktail tablets from Roche, and 8-(4-chloro-phenylthio)-cAMP from Calbiochem, (Darmstadt, Germany). All other chemicals were of the highest grade obtainable.

2.2. Animals

Diets were purchased from research diets, New Brunswick, NJ (HF diet D12331 and control diet D12328, http://www.research-diets.com/pdf/Data-20Sheets/D12331.pdf) and 15 male Sprague Dawley rats (10 weeks of age) were placed on a high fat (HF) diet for 12 weeks. 15 littermates were given normal chow (Con) for the same period. Serum was prepared from both the diet induced obese (DIO) and normal chow fed control animals, and this was aliquoted and stored at −20 °C until required. Male FaFa (obese Zucker) and Fa/+ (Lean Zucker) rats were purchased from Harlan Laboratories, UK, at 6–8 weeks of age and sacrificed within 2 weeks. Serum was prepared immediately and all serum from each group combined, then aliquoted and snap frozen.

2.3. Cell culture

H4IIE cells, a rat hepatoma cell, and HL1C (a H4IIE line with a stable insertion of a PEPCK promoter, chloramphenicol acetyl transferase gene construct [24]), were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 1000 mg/l glucose, 1% (v/v) penicillin/streptomycin, and 5% (v/v) FBS, in a 37 °C 5% CO_2 incubator.

2.4. Exposure of cells to experimental serum

Rat liver cells (H4IIE or HL1C) were exposed to one of four different sera: (1) 2.5% foetal calf serum (FCS) plus 2.5% rat serum obtained either from rats fed normal chow (Control Serum) or from rats following a high fat diet (High Fat Serum); (2) 2.5% FCS plus 2.5% serum from lean *Fa/+* rats (Control Serum) or *FaFa* obese rats (Obese Serum); (3) 5% rat serum obtained either from lean *Fa/+* rats (Control Serum) or *FaFa* obese rats (Obese Serum); (4) 5% human serum from either lean volunteers (Control Human Serum) or obese volunteers with type 2 diabetes (CASE Human Serum).

In brief, cells were cultured and passaged as normal in the experimental sera. Cells were serum starved for 16 h (rat sera) or 3 h (human sera) prior to measurement of insulin sensitivity. Hormones were added as described in figure legends, and protein (for CAT Assay, HL1C) or RNA (for Taqman, H4IIE) harvested after 3 h.

2.5. CAT assay

Cells were isolated by trypsinisation, washed in PBS and pelleted by centrifugation at 1400 rpm for 5 min. Cell lysate was prepared by sonication in 0.25 M TRIS–HCl, pH 7.5, total protein assessed by Bradford assay, and general cellular protein removed by heating at 65 °C for 10 min and centrifugation. Supernatants were tested for CAT activity as described previously [24], and data expressed as Units of CAT activity per mg of cellular protein.

2.6. RNA isolation and real-time quantitative reverse transcriptase PCR

Following hormone treatments total cellular RNA was extracted from H4IIE cells using TRIreagent™ (Sigma, Poole, Dorset, UK) according to the manufacturer's instructions. cDNA was synthesized from total cellular RNA using Superscript™ III reverse transcriptase kit (Invitrogen, Paisley, UK). Real-time PCR analysis was carried out using ABIPrism7700 sequence detector (Applied Biosystems, Warrington, UK). Primer sequences to rat PEPCK (forward – ACA GGC AAG GTC ATC ATG CA; reverse – TGC CGA AGT TGT AGC CAA AGA; probe – FAM-ACC CCT TCG CTA TGC GGC CC-TAMRA), rat actin (forward – TCT GTG TGG ATT GGT GGC TCT A; reverse – CTG CTT GCT GAT CCA CAT CTG; probe – FAM-CCT GGC CTC ACT GTC CAC CTT CCA-TAMRA) and rat cyclophilin (forward – TTA CTA GGT CTG GCA GGA AGA TTA AAG; reverse – CTG CAT CTC TTG TCT CCA ATG TG; probe – FAM- AGA GGA CCA AGG CGT TAT CGA A-TAMRA) were purchased from Sigma–Aldrich, Poole, Dorset, UK. PCR reactions were carried out using the following cycling conditions – 50 °C – 2 min 1×, 95 °C – 10 min 1× followed by 40 cycles of 95 °C – 15 s, 60 °C – 1 min.

2.7. Human volunteers/recruitment

Twenty control (BMI < 25, no diabetes) subjects were recruited from the general Tayside population and 20 obese (BMI > 30) subjects with type 2 diabetes (cases) were recruited from the diabetes clinic (Table 1). All subjects were male, non-smokers and taking no other medication known to influence insulin sensitivity. They were naïve to anti-hyperglycaemics in order to reduce potential confounding effects of long term intervention. Unfortunately, it was impossible to recruit cases who were not prescribed a statin, and cessation of this treatment for the duration of the current study was not justified. Hence, the lower total and LDL cholesterol compared to the controls (Table 1) were clearly due to the effects of HMG CoA reductase inhibitors taken by all cases. Other basic clinical parameters of these groups are shown in Table 1. Volunteers were fasted overnight prior to a visit to the Clinical Research Centre where 200 ml of fasted whole blood was collected. Serum was prepared and stored at −20 °C in 10 ml aliquots. BMI, blood pressure and waist measurements were taken during the visit.

2.8. Clinical and biochemical measurements

Insulin, CRP, adiponectin, leptin and TNF-α concentrations were determined using the sandwich ELISA principle. Insulin kits

Table 1
Baseline characteristics (mean (SD)) of the human volunteers.

	Controls ($n = 20$)	Cases ($n = 20$)	p
Age (Years)	50.85 (5.96)	56.7 (3.70)	0.001
Weight (Kg)	77.51 (9.12)	106.67 (16.55)	<0.0001
Height (cm)	181.1 (7.45)	174.7 (6.81)	0.007
Waist (cm)	88.25 (6.47)	117.95 (12.85)	<0.0001
BMI (kg/m^2)	23.45 (1.32)	34.95 (5.00)	<0.0001
Hip (cm)	99.7 (3.88)	115.95 (8.06)	<0.0001
Waist:hip ratio	0.88 (0.06)	1.02 (0.05)	<0.0001
Systolic BP (mm Hg)	126.1 (16.06)	139.6 (17.97)	0.017
Diastolic BP (mm Hg)	78 (9.07)	83.25 (8.01)	0.059
Pulse (bpm)	61.4 (10.22)	74.75 (13.22)	0.001
Total cholesterol	5.50 (0.93)	4.42 (0.74)	<0.0001
LDL (mmol/l)	3.39 (0.84)	2.37 (0.54)	<0.0001
HDL (mmol/l)	1.55 (0.38)	1.19 (0.35)	0.006
Trigs (mmol/l)	1.27 (1.09)	2.14 (1.41)	0.039
HOMA-IR[a]	0.75 (0.45–1.26)	2.15 (1.50–3.08)	<0.0001
Insulin[a]	5.69 (3.35–9.68)	15.11 (10.26–22.24)	<0.0001
CRP[a]	532.57 (189.49–1496.84)	2345.40 (1117.04–4924.55)	<0.0001
Adiponectin[a]	7033.40 (4301.97–11499.10)	4205.06 (2591.94–6822.13)	0.002
Leptin[a]	1198.39 (188.27–7627.97)	12,976.36 (7142.59–23574.93)	<0.0001
TNF-α[a]	1.13 (0.74–1.74)	1.08 (0.79–1.47)	0.687

[a] Non-normally distributed data presented as geometric means (range of SD).

were supplied by Mercodia, Uppsala, Sweden (Cat #10-1113-01). CRP and TNF-α were measured using specific kits (Cat # DCRP00 and HSTA00D, respectively), leptin and adiponectin were measured using R&D Systems DuoSet (all from R&D Systems, Abingdon, UK) ELISA kits modified in house. Briefly, HRP-conjugated antibodies were prepared by activating the antibody (supplied in the un-biotinylated form by R&D systems) with SATA (N-succinimidyl-S-acetylthioacetate) at a molar ratio of 30:1. The HRP (Biozyme Labs, Cardiff, UK) was activated with sSMCC (sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate) also at a molar ratio of 30:1. The conjugates were prepared by combining antibody and enzyme at ratio of 3 moles HRP: 1 mole antibody. IIR reagent (SLI, Sussex, UK) was added at a final concentration of 0.05 mg/ml to the reagent diluents and a fluorogenic substrate, QuantiBlu, from Pierce, Il, USA, was used. The leptin assay used the suggested sample dilution of 1:20 and the supplied calibrator. The adiponectin assay used a sample diluted 1:150 and recombinant adiponectin (R&D systems) was used to calibrate the assay. In method comparison studies both assays correlated well to the R&D Quantikine versions of the assays ($r > 0.9$ by Passing-Bablock). All assays were subjected to a vigorous in-house validation process. Assay precision was established over five runs using serum quality controls of high, medium and low concentrations. Intra- and inter-precision for each assay were determined as follows: insulin 1.8–4.2% and 3.9–5.0%CV; CRP 2.4–3.0% and 3.4–10.0%CV; Leptin had an inter-assay imprecision <8% and intra-assay imprecision of <5% on all controls. Adiponectin showed inter-assay imprecision <8% and intra-assay imprecision of ≤13% on all controls. The TNF-α assay showed an inter-assay imprecision <8% and intra-assay imprecision of ≤13% on the low, medium and high controls. Clinical samples were randomised over two 96-well plates for each assay.

Other biochemical measurements were performed by the Clinical Biochemistry Department, Ninewells Hospital.

2.9. Statistical analysis

Non-normally distributed data was log transformed, and presented as the geometric mean. All other data are presented as the mean ± SD. To determine if effects on PEPCK repression were independent of baseline differences in the two treatment groups a linear regression model was used. In the first model, age, BMI, and case/control status were used as covariates. In the second model, all other covariates were added to the first model in turn. Regression modelling for waist:hip ratio, hip, waist, weight and height were not included as they are strongly correlated with BMI. Analysis of cholesterol, LDL and HDL were excluded as all cases were taking HMG CoA reductase inhibitors. The statistical analysis was carried out using Stata SE version 10.

3. Results

3.1. Diet induced obesity

HL1C cells were grown in DMEM containing either 2.5% FCS + 2.5% DIO-derived serum, or 2.5% FCS + 2.5% Con serum. The cell morphology and growth rate during culture and passage (every 2 or 3 days) was similar in both cases (data not shown). HL1C cells contain a CAT reporter with expression under the control of the PEPCK gene promoter [24,25]. In these cells CAT expression is normally low but can be induced 5–10-fold by exposure to glucocorticoid and cAMP for 3 h. However the induction can be completely blocked by including insulin in the culture medium (Fig. 1 and [24]). Repression by insulin is dose-dependent with an EC50 of approximately 0.1 nM, and >80% inhibition at 1 nM insulin (Fig. 1A and [24,26–28]). Growth in DIO-derived rat serum for 9 days did not produce any change in the insulin sensitivity of the HL1C cells (Fig. 1A), however exposure to DIO-derived serum for 24 days resulted in a right shift of the insulin dose response curve (Fig. 1B). The EC50 for the insulin regulation of PEPCK-CAT increased to around 1 nM, although complete inhibition of the gene promoter was still achieved by 10 nM insulin (Fig. 1B). The experiment was repeated with fresh DIO-derived serum and the insulin sensitivity of the HL1C cells was found to be reduced following 2 weeks of growth in the test serum (Fig. 1C), but did not reduce further between 2 and 3 weeks of exposure (Fig. 1C). This suggested that there is a maximal effect of the serum on insulin sensitivity, and this occurs around 2 weeks of growth in DIO-derived serum.

3.2. Genetic predisposition to obesity

We next obtained serum from 8 to 10 week old FaFa rats (obese), and age-matched Fa/+ (lean) rats. HL1C cells were grown in either 2.5% FaFa or 2.5% Fa/+ serum (supplemented with 2.5% FCS in each case), for 3 weeks prior to analysis of PEPCK gene

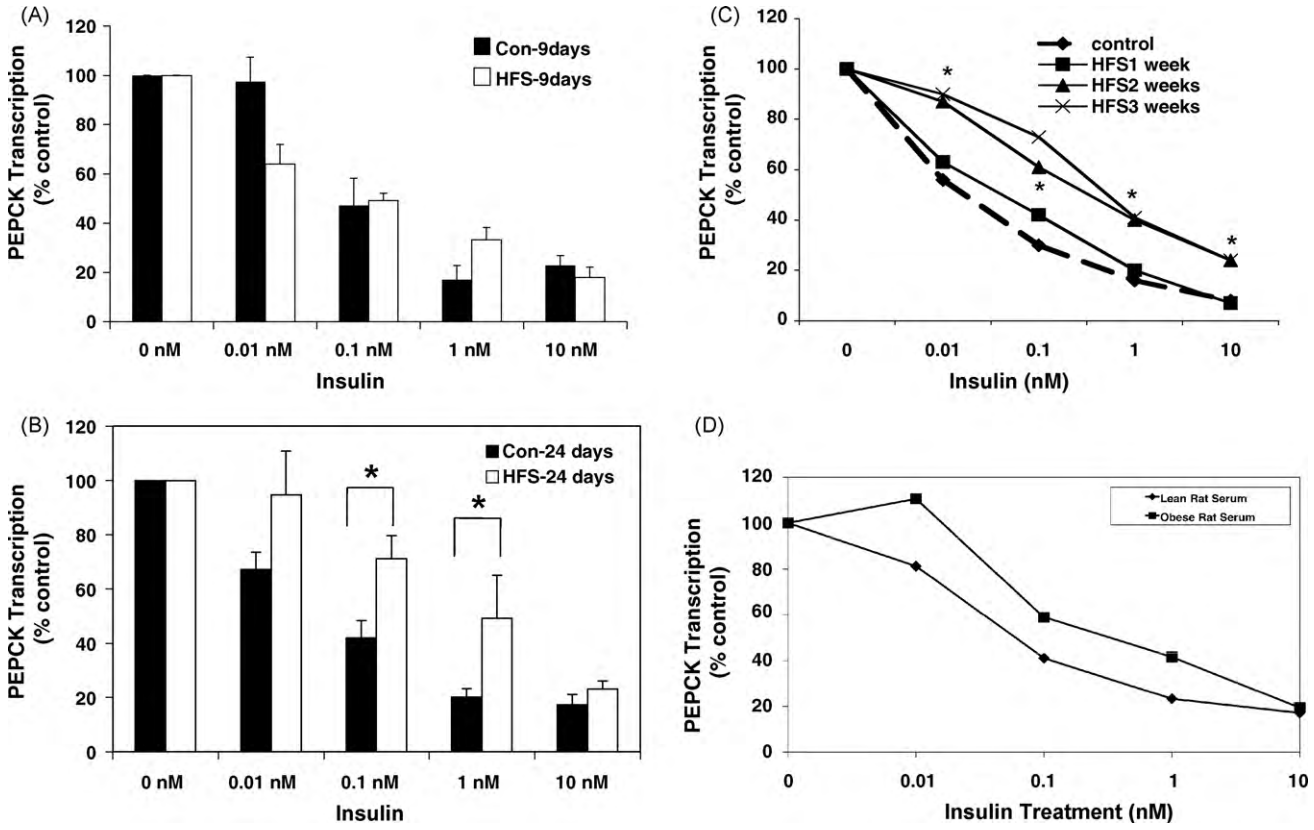

Fig. 1. Comparison of the insulin sensitivity of the PEPCK gene promoter in cells grown in serum from obese or lean rodents. HL1C cells were grown in serum isolated from obese SD rats that had been on a high fat diet for 3 months (HFS), or lean chow fed littermates (Con). After 9 days (A) or 24 days (B), cells were serum starved for 16 h prior to exposure to 8CPT-cAMP (0.1 mM), dexamethasone (500 nM) and insulin as indicated for 3 h. Protein was harvested and CAT reporter assayed as a measure of PEPCK gene promoter activity as detailed under Methods. $^*p < 0.05$, comparison as shown. (C) A direct comparison of insulin regulation of the PEPCK gene promoter in cells exposed to HFS for 1, 2 or 3 weeks. $^*p < 0.05$, comparison of 3 weeks and control. (D) HL1C cells were grown for 3 weeks in serum isolated from obese Fa/Fa or lean Fa/+ rats prior to analysis of insulin regulation of the PEPCK gene promoter as above. Data is presented relative to the CAT activity in the appropriate Dex/cAMP induced control.

promoter regulation (Fig. 1D). Once more insulin sensitivity of the HL1C cells was reduced in cells grown in serum from this leptin receptor deficient IR rat model, with the EC50 for repression of the PEPCK gene promoter shifting from around 0.1–1 nM insulin (Fig. 1D).

3.3. Endogenous gene regulation

The PEPCK-CAT reporter gene is more sensitive to insulin than the endogenous PEPCK gene promoter, with the EC50 for PEPCK mRNA repression by insulin closer to 1 nM (Fig. 2). However, growth of the H4IIE cell line in serum from obese Fa/Fa rats also reduced the insulin sensitivity of the endogenous PEPCK gene promoter, relative to cells grown in Fa/+ lean serum, with a significant difference between the response to 1 or 10 nM insulin (Fig. 2). The serum from Fa/Fa rats also made the PEPCK mRNA levels more sensitive to glucocorticoid and cAMP induction, with enhanced induction of PEPCK mRNA in the presence of these agents relative to that seen in cells exposed to serum from lean littermates (Fig. 2 c.f. 3rd and 4th bar, 8-fold induction in Fa/+ control cells and 12-fold induction in Fa/Fa treated cells, $p < 0.05$, and Fig. 3 c.f. around 15-fold induction of PEPCK gene in cells exposed to FaFa, serum with the around 8-fold induction seen in control cells from Fig. 2).

3.4. Reversibility

In order to establish whether normal insulin sensitivity could be restored to the 'resistant' cells, we generated insulin resistance by culturing cells for 3 weeks in 5% Fa/Fa serum then examined the effect of removing the FaFa serum (Fig. 3A). The insulin sensitivity of cells grown in FaFa serum for three weeks was directly compared with insulin resistant cells that had been placed in 5% FCS for an additional week beyond the 3 week

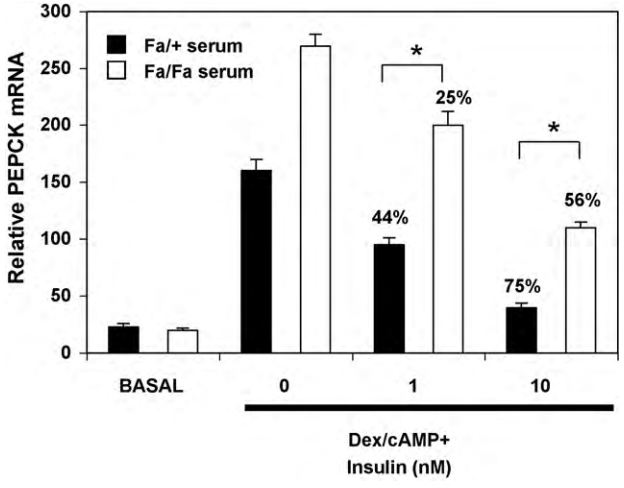

Fig. 2. Comparison of the effect of growing cells in serum from obese or lean rodents on insulin regulation of PEPCK mRNA. HL1C cells were grown for 3 weeks in serum (2.5%) isolated from obese Fa/Fa or lean Fa/+ rats prior to serum starvation and exposure to glucocorticoid (Dex), cAMP and insulin as in Fig. 1. After exposure to hormones total RNA was isolated and PEPCK mRNA levels were quantified by RT-PCR. Data is presented as relative amount of PEPCK mRNA (average ± SEM) after normalisation to cyclophilin mRNA in each sample. Percent inhibition by insulin is indicated in each case. $^*p < 0.05$.

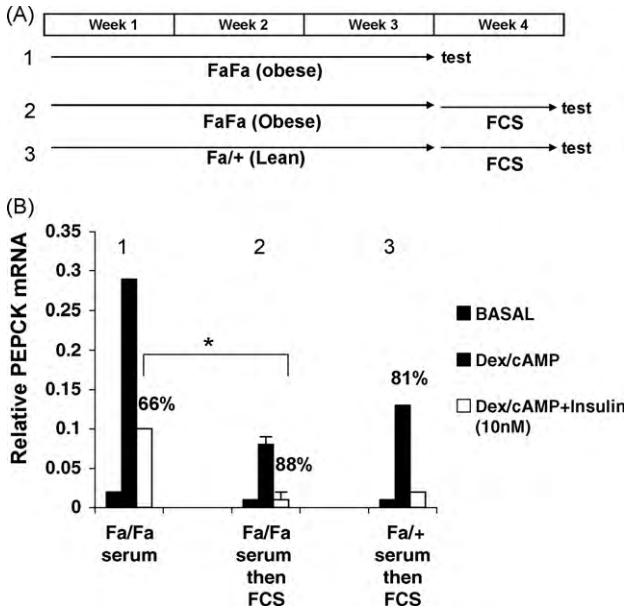

Fig. 3. Measurement of insulin sensitivity of cells after growth of insulin resistant cells in standard serum. (A) H4IIE cells were grown for 3 weeks in 5% Fa/Fa serum. RNA was harvested from half of the cells (Group 1) following exposure to hormones as in Fig. 1, the remainder were placed in 5% FCS for 1 week prior to hormone exposure and RNA isolation (Group 2), while RNA was also isolated from H4IIE cells grown for 3 weeks in Fa/+ lean serum followed by 1 week in FCS prior to hormone exposure (Group 3). (B) Relative PEPCK mRNA (average ± SEM) for the samples isolated above are shown following normalisation for actin mRNA in each sample. Percent inhibition by insulin is indicated for each group. $^{*}p < 0.05$.

resistance generating period (Fig. 3, first two bar charts). The super-induction of the Dex/cAMP response in cells grown in FaFa serum for 3 weeks was completely reversed by replacing the FaFa serum with FCS for 1 week (Fig. 3, 15-fold induction vs 8-fold induction). In addition the response to insulin of the cells was fully restored after replacement of the FaFa serum with FCS for 1 week (Fig. 3 c.f. 66% inhibition in FaFa serum becoming 88% inhibition, $p < 0.05$). Indeed, the hormone responses of cells grown in 5% FaFa serum for 3 weeks, followed by 1 week in FCS were almost identical to that of cells grown in 5% Fa/+ lean serum then 5% FCS for an equivalent time (Fig. 3 FaFa then FCS vs Fa/+ then FCS). In summary, one week after removal of the Fa/Fa serum normal insulin sensitivity had been restored to the cells, showing that the resistance is reversible.

3.5. Effect of insulin sensitisers

Cells were incubated with Fa/Fa or Fa/+ serum ±0.1 mM metformin, or ±1 μM pioglitazone. Drugs were replaced at every cell passage (along with fresh serum), and after 3 weeks the insulin sensitivity of the cells established (Fig. 4). The presence of metformin reduced PEPCK gene transcription, irrespective of the growth conditions or hormone challenge. Indeed 3 weeks growth in the presence of metformin reduced basal PEPCK transcription, partly reduced the induction by glucocorticoids and cAMP, while restoring full repression by insulin (Fig. 4, compare open bars with hashed bars). The same response was seen whether the cells had been grown in Fa/+ or FaFa serum, hence this drug appeared to mimic the action of insulin and directly repress the gene irrespective of the presence of insulin resistance.

In contrast, growth in pioglitazone prevented the reduction in insulin sensitivity of the cells exposed to Fa/Fa serum without any effect on cells grown in Fa/+ serum (Fig. 4, compare open bars and filled bars). Basal, induced and insulin-repressed levels of PEPCK mRNA in the cells grown in Fa/+ serum (LEAN) were almost identical ±pioglitazone. Meanwhile, this drug restored insulin sensitivity of the Fa/Fa serum treated cells to that of the Fa/+ serum treated cells (Fig. 4, c.f. 1 nM treated cells in lean serum, insulin repression is 78% without and 73% with pio, while in cells in Obese Serum, insulin repression is 65% without and 82% with pio, $p < 0.05$), indicating that pioglitazone improved insulin sensitivity rather than simply repressing PEPCK transcription.

This suggests that the cell model can identify agents likely to improve insulin sensitivity, including those that have no effect on insulin sensitivity in healthy cells, but also distinguish between insulin sensitising agents and drugs that simply repress the PEPCK gene in an insulin mimetic type manner.

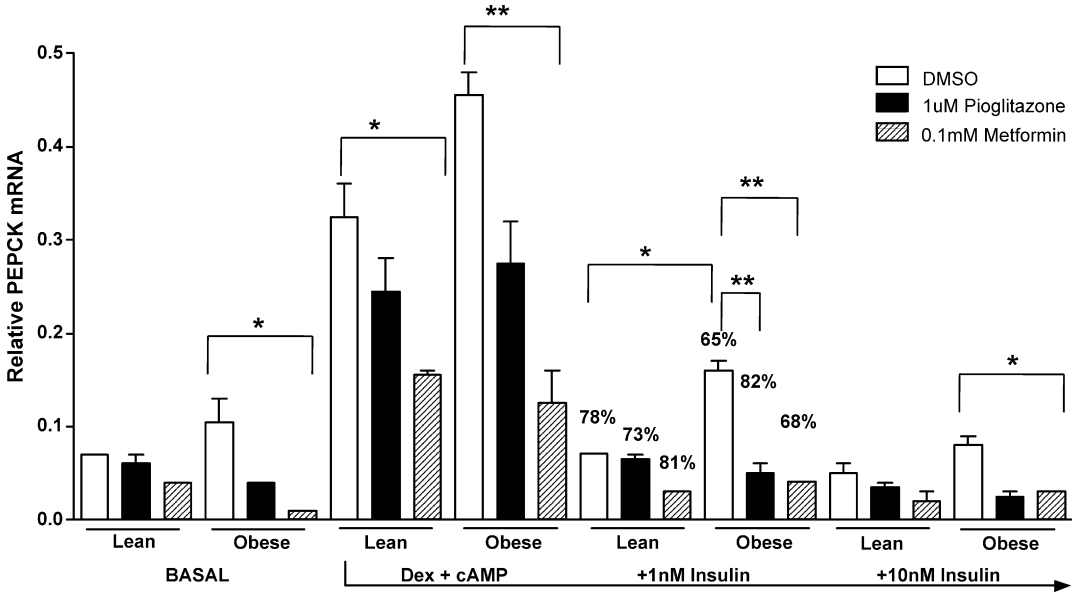

Fig. 4. Effect of the presence of the anti-diabetes drugs, pioglitazone and metformin. H4IIE cells were grown for 3 weeks in 5% Fa/Fa serum, in the presence or absence of pioglitazone or metformin as indicated. Cells were serum starved for 16 h prior to 3 h exposure to dexamethasone (500 nM), 8CPT-cAMP (0.1 mM) ±insulin as indicated, and RNA was isolated. PEPCK mRNA levels (average ± SEM) are presented for each cell treatment after correction for actin mRNA. Percent inhibition by 1 nM insulin in each condition is presented. $^{*}p < 0.05$, $^{**}p < 0.01$ for comparisons as indicated.

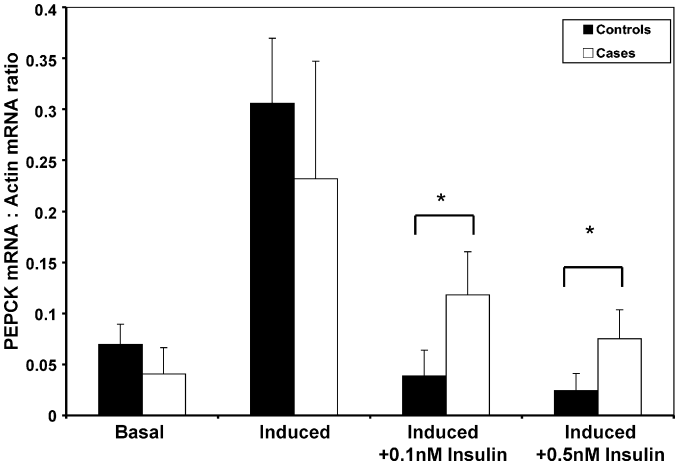

Fig. 5. Comparison of the effect of growing cells in serum from obese or lean humans on insulin regulation of PEPCK mRNA. H4IIE cells were grown for 3 weeks in 5% serum collected from 40 human volunteers, 20 who were obese and diabetic (cases), 20 who were lean and non-diabetic (controls). After 3 weeks in the serum the 40 different pools of cells were serum starved for 3 h prior to 3 h exposure to dexamethasone (500 nM), 8CPT-cAMP (0.1 mM) ±insulin as indicated, and RNA was isolated. PEPCK mRNA levels (average ± SEM) are presented (n = 20, assayed at least in duplicate for each condition) after correction for actin mRNA.

3.6. Analysis of human serum donated by obese diabetic volunteers

Serum was donated by 20 obese (BMI > 30) volunteers with type 2 diabetes (being treated by diet alone), and 20 lean healthy volunteers. H4IIE cells were cultured for 3 weeks in each of the forty serum samples (5%, v/v), prior to analysis of insulin repression of PEPCK mRNA. The mean response to 0.1 nM or 0.5 nM insulin of H4IIE cells cultured in serum from patients was lower than that of cells cultured in serum from lean controls (0.1 nM $p < 0.0001$, 0.5 nM $p < 0.0001$, Fig. 5). However there was no difference in the magnitude of gene induction by glucocorticoids/cAMP, or in the response to higher concentrations of insulin, between controls and cases. In analysis of covariance, the effect on PEPCK repression by 0.1 nM insulin remained significant ($p = 0.003$) even after adjusting for baseline differences in BMI and age between the two groups (Table 2). Inclusion of other covariates (e.g. leptin, adiponectin, TNF-α) into this model did not remove the strong association with diabetes status on insulin suppression of PEPCK, consistent with the fact that no single biomarker measured in this study explained the insulin resistance generated by serum of obese patients with diabetes (Table 2). Interestingly there was a broad range of PEPCK repression by insulin across the 20 individual samples from the obese patients with diabetes, but once again there was no clinical or biochemical parameter associated with this variable response (data not shown).

Table 2
Linear regression models. The dependent variable is percent repression of PEPCK by 0.1 nM insulin. Model 0 is the comparison by case/control status. Model 1 adds age and BMI as covariates. Model 2 adds each additional biochemical parameter to model 1. The p-values represent the coefficient p-values in the model.

	Coefficient p-value case–control	Age	BMI	Other
Model 0	<0.001	–	–	–
Model 1	0.003	0.41	0.25	–
Model 2				
Glucose	0.029	0.45	0.30	0.06
HbA1c	0.012	0.42	0.29	0.56
Insulin	0.003	0.38	0.20	0.52
HOMA-IR	0.003	0.38	0.21	0.57
Triglycerides	0.004	0.44	0.28	0.92
Leptin	0.005	0.41	0.30	0.92
Adiponectin	0.003	0.46	0.22	0.65
TNF-α	0.005	0.40	0.17	0.29
CRP	0.002	0.52	0.18	0.39
Systolic BP	0.003	0.49	0.29	0.77
Diastolic BP	0.004	0.47	0.28	0.63
Pulse	0.004	0.42	0.26	0.94

4. Discussion

The underlying reasons for the variation in insulin sensitivity in the healthy population are not entirely clear, but are likely to be due to a combination of polygenetic determinants and lifestyle choices (diet and exercise). It is widely reported that high serum triglyceride and free fatty acids (obesity) promote accumulation of fat in muscle and liver resulting in defective cellular metabolism and signalling [5,6,8,9,11,14,29–31]. In addition, molecules abnormally expressed in obesity, such as leptin, IL6, TNF-α, resistin, adiponectin, and an amazing array of others (for review see [5,11,32,33]) are reported to regulate aspects of insulin action by promoting reduced signalling capacity (e.g. by downregulating IRS1). Finally, hyperglyceamia and hyperinsulinaemia are also linked to defective cellular responses to insulin, although this has implications as to whether insulin resistance is an early or late event on the pathway from obesity to type 2 diabetes.

With this complexity in mind we hypothesised that serum from obese individuals contained multiple 'resistance causing' factors, and it was possible that focussing on a single agent would not be truly representative of the human disease. Therefore we took the unbiased simple approach of culturing insulin sensitive hepatoma cells in serum from obese animals or obese humans. Growing these cells in serum from either DIO rats, genetically derived obese rats (FaFa), or obese humans with diabetes, resulted in reduced insulin sensitivity of the signalling pathway that regulates the PEPCK gene promoter. This gene is downstream of IRS1, PI 3-kinase, PDK1 and PKB signalling [21,24,27,34,35], as are several other key metabolic genes that are repressed by insulin in liver [28,36,37]. Therefore something in the serum from obese animals and volunteers antagonises the function of this very important insulin signalling pathway. The resistance is not a complete loss of insulin action (increasing insulin will fully repress the gene), while the change in insulin sensitivity takes 2–3 weeks to develop and is quite variable between serum from different individuals. All of this is consistent with what we know about the common human disease, where a shift in insulin sensitivity rather than complete loss of response is observed, and the disease develops slowly and at a variable rate between individuals.

Interestingly the PEPCK gene promoter lends itself particularly well to this type of system. The response to hormones is robust and reproducible, while the range of response to insulin covers two orders of magnitude, with a large signal to noise ratio. We have investigated the G6Pase gene promoter in the same experiments and found the responses to be much more variable and less amenable to quantitation of insulin sensitivity (data not shown). Similarly, the analysis of insulin regulation of physiological processes such as intracellular signalling or glucose transport in muscle and adipocytes is too variable and too steep a response curve to allow their use in this type of cell based assay. Finally, it should be remembered that even in genetic models of obesity there is a variation in the degree of obesity and insulin resistance from animal to animal, and this is exaggerated in the diet induced rodent model. We always used pooled rodent serum in our studies but it can be seen from the data that batch-to-batch variation in the degree of effect on insulin sensitivity was obtained (cf Figs. 2 and 4), and this emphasises the need to use age-matched lean littermate controls for these studies. This presumably reflects distinct concentrations of the resistance causing agent(s), and probably explains much of the variation across the obese human cases.

We believe that this novel model will prove useful in many ways. Firstly, it will be possible to quantify in detail the insulin signalling network within resistant cells, establishing whether a single molecular defect is responsible for the phenotype or it is due to a more global reduction of the pathway capacity. Secondly, it is possible to use the model to distinguish compounds that restore insulin sensitivity to resistant cells from those that simply mimic insulin action on PEPCK gene transcription. It also provides the opportunity to establish that compounds are effective in insulin resistant cells and not just normal cells, prior to moving to animal testing. Thirdly, although challenging, the model provides a tool to confirm the requirement for a given serum component in the response. For example, although we could find no statistical association between insulin, glucose, leptin, TNF-α or triglycerides levels in human sera to subsequent insulin response in the cells cultured in the sera, it would be possible to directly compare whole serum and serum depleted of these components to establish whether any one or more of these components was necessary for resistance generation. At the same time we can use the model to examine the effects of chronic exposure to these levels of the candidate factors, individually and in combination, and hence whether any of them are sufficient, necessary or combine to provide the effect observed with the complete sera. Finally, we have shown that this cell model system can be used to differentiate serum of obese patients with diabetes from serum of healthy controls. The model could therefore be used to assess response to established and novel insulin sensitising therapies, and to potentially distinguish between distinct 'insulin resistance causing factors' in human serum. The current analysis could be extended to a proteomic, transcriptomic and metabolomic screen of these sera, in the hope of identifying markers of early stage type 2 diabetes, although this may require larger data sets to increase the power of detection of individual components. Of course this would be even more effective if similar cellular assays on myocytes, β-cells and adipocytes were available as part of a screen. The ideal scenario would be to have individual serum markers for tissue specific insulin resistance as well as the most appropriate intervention.

In summary, we have developed a cellular model of insulin resistance that we hope will aid the understanding of the molecular development of obesity induced hepatic insulin resistance. We believe that it also provides a unique preclinical tool.

Conflicts of interest

M.K.H., G.Z.F., J.D.B., D.C., A.M.T., C.E.G. were all employees of Wyeth Pharmaceuticals during the preparation of this manuscript.

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