Modulation of estrogen signaling in hepatic and vascular tissue

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Administration of 17-β-estradiol to an insulin resistant mouse model acutely improves hepatic insulin sensitivity

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Abstract

Prolonged 17-β-estradiol (E_2) administration affects insulin sensitivity. However, it is unknown whether E_2 influences insulin sensitivity directly or indirectly e.g. via modulation of plasma free fatty acid levels and/or intra-hepatic lipid levels. Therefore, acute effects of E_2 administration were studied by performing a hyperinsulinemic-euglycemic clamp in an insulin resistant mouse model (APOE*3-Leiden mice, which had been fed a high fat diet for 13 weeks). Six hours after E_2 administration, estrogen receptor mediated transcription was induced predominantly in liver, but plasma triglyceride, insulin, free fatty acid and intra-hepatic lipid levels were unaffected. During the hyperinsulinemic clamp, the hepatic glucose production was significantly inhibited in the E_2 treated mice as compared to control mice (4.5 ± 11 *versus* 34 ± 29 $\mu mol\cdot min^{-1}\cdot kg^{-1}$; P=0.013), whereas the peripheral glucose disposal rate was significantly lower in the E_2 treated mice (29.8 ± 8.9 *versus* 50.9 ± 26.4 $\mu mol\cdot min^{-1}\cdot kg^{-1}$; P=0.017). The E_2-induced increased sensitivity of liver was accompanied by a significant decrease in the expression of hepatic genes involved in gluconeogenesis. Thus, administration of E_2 to an insulin resistant mouse model acutely improves hepatic insulin sensitivity at the expense of peripheral insulin sensitivity, through mechanisms independent of plasma lipid levels and hepatic lipid accumulation.

Introduction

17-β-estradiol (E_2) is a sex hormone that plays a major role in the establishment and maintenance of the reproductive tract and mammary glands [1-4]. In addition, E_2 is implicated in the regulation of a host of physiological processes including lipid and glucose metabolism [5-8]. E_2-deficiency, such as occurs after menopause in humans, is associated with many features of the metabolic syndrome including central obesity, insulin resistance and dyslipidemia (for review see [9]). Conversely, hormone replacement therapy has been associated with a reduction in the incidence of diabetes, which is a major complication associated with the metabolic syndrome [10,11]. However, it's obvious that menopause occurs as a function of aging, and aging itself is also associated with an increased incidence of the metabolic syndrome. Therefore, controversy remains regarding the role of E_2 in the metabolic syndrome.

More direct evidence for the effects of estrogens on glucose and lipid homeostasis has been obtained in mouse models. Ovariectomized mice become obese and insulin resistant [5]. Similarly, estrogen receptor α ($ER\alpha^{-/-}$) and aromatase knockout mice (ArKO, an estrogen-

deficient model) develop insulin resistance and impaired glucose tolerance [12-16]. However, also in these estrogen (receptor) deficient mouse models the glucose and lipid phenotypes require weeks to months to develop, implying involvement of many additional metabolic pathways. Thus, it is evident that estrogens have a multitude of effects on different processes in different organs, which apparently interact at multiple levels to achieve metabolic regulation. Consequently, it remains unclear to which extent the effects of E_2 on insulin sensitivity are the consequence of direct effects of E_2 and/or of indirect effects, e.g. related to long term effects of E_2 on triglyceride tissue distribution and ensuing tissue function.

To examine the short-term effects of E_2 on insulin sensitivity, we have studied the acute effects of E_2 in an insulin resistant mouse model. Male APOE*3-Leiden mice were used, which on a high-fat diet, develop many features of the metabolic syndrome, including obesity, hyperlipidemia, and insulin resistance [17]. Our results indicate that E_2 administration improves hepatic insulin sensitivity within several hours of administration at the expense of peripheral insulin sensitivity, independent of plasma and intra-hepatic lipid levels.

Results

Body weight and plasma parameters

To induce features of the metabolic syndrome, two groups of male APOE*3-Leiden mice were put on a high fat diet for a period of 13 weeks. This resulted in obese mice with a body weight of 35-37 grams. In addition, the mice exhibited hypercholesterolemia (4.5-4.7 mmol/l) and

Table 1. Plasma parameters in overnight-fasted APOE*3-Leiden mice fed a high fat diet that received E_2 or vehicle for 6 hrs

	Basal		Hyperinsulinemic	
	Vehicle	**6 hrs E_2**	**Vehicle**	**6 hrs E_2**
Bodyweight (gr)	35 ± 5	37 ± 3		
TG (mmol/l)	1.2 ± 0.6	1 ± 0.6		
Chol (mmol/l)	4.5 ± 1.6	4.7 ± 2.3		
Glucose (mmol/l)	7.9 ± 2.4	8.3 ± 1.6	8.9 ± 3.4	9.7 ± 2.8
Insulin (ng/ml)	1.5 ± 1.1	1.7 ± 1.1	4.8 ± 2[*]	5.6 ± 3.6[*]
FFAs (mmol/l)	1.4 ± 0.4	1.2 ± 0.5	0.9 ± 0.4[*]	0.7 ± 0.2[*]
GIR (μmol · min^{-1} · kg^{-1})			26.3 ± 14.2	25.7 ± 13.9

Plasma levels were measured during basal and clamp conditions. Body weight was measured just before the clamp. Values represent the mean ± SD of 8 mice per group. * p< 0.005 compared to basal conditions

moderate hyperinsulinemia (1.5-1.7 ng/ml) (table 1). Acute administration of E_2 did not affect body weight, plasma triglycerides (TG), cholesterol, insulin and free fatty acids (FFA) levels (table 2).

ER activity in vivo

The biodistribution and peak activation time of E_2 after bolus injection depends on the type and site of administration and dissolvent used. To determine the tissues that are activated and time course of activation by E_2 in our hands, E_2-activity was monitored *in vivo* using a luciferase (luc) reporter system and a highly sensitive CCD camera. Non-invasive optical imaging was performed at different time points after E_2 injection in male transgenic reporter mice, in which the luc gene was driven by an estrogen response element (ERE) containing promoter (ERE-Luc mice), (Fig. 1). At time point t = 6 hours, *in vivo* luc expression peaked and was almost exclusively limited to liver. Therefore, the t = 6 hours after treatment point was taken as the moment to assess the acute effects of E_2 administration.
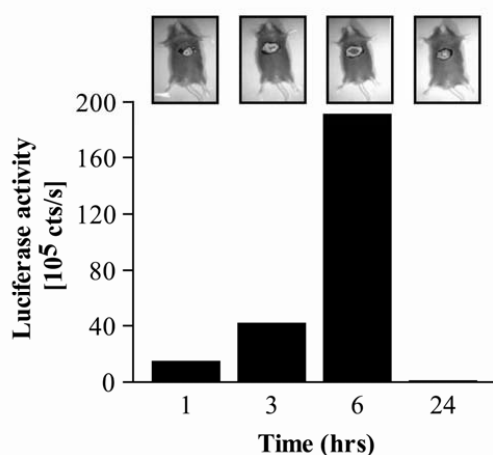

Figure 1. E_2 induced ER mediated transcription in ERE-Luc transgenic male mice.
Optical imaging of bioluminescence emitted from E_2 (50 μg/kg, sc) treated ERE-Luc transgenic male mice in time

Insulin sensitivity after E_2-treatment

To determine the acute effect of E_2 administration on insulin sensitivity, a hyperinsulinemic-euglycemic clamp study was performed six hours after E_2 administration. During hyperinsulinemic conditions, no significant differences were observed in plasma glucose levels (table 2). FFA levels were suppressed (P = 0.002) to a similar extent in both vehicle and E_2 treated mice (table 2). The clamp results showed a significantly lower insulin-mediated whole-body glucose uptake in the E_2 treated mice as compared to control mice (29.8±8.9 *versus* 50.9±26.4 μmol · min^{-1}· kg^{-1}; P=0.017; Fig 2A). Moreover, no significant insulin-mediated suppression of hepatic glucose production (HGP) was observed in control mice, indicative for a state of hepatic insulin resistance. In contrast, in the E_2 treated mice, HGP was significantly suppressed under

hyperinsulinemic conditions, from 39.3 ± 11.5 to 4.5 ± 11.2 μmol $\cdot$ min^{-1} $\cdot$ kg^{-1} (P=0.0002; Fig 2B). Thus, acute E_2 treatment attenuates peripheral insulin sensitivity with regard to glucose disposal, but improves hepatic insulin sensitivity with regard to suppression of HGP.
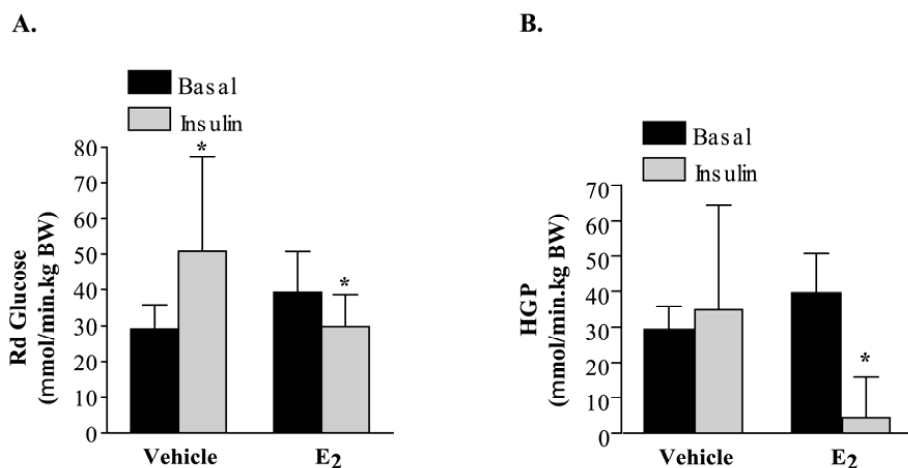
A. **B.**

Figure 2. Peripheral and Hepatic insulin sensitivity E_2 treated APOE*3-Leiden mice
Hyperinsulinemic-euglycemic clamp of APOE*3-Leiden male mice six hours after vehicle or E_2 (100 µg/kg, sc) treatment. (A) Basal and insulin-mediated stimulation of whole-body glucose uptake (B) Basal and insulin-stimulated rates of HGP. Data are means$\pm$SD, n=8. *$P<0.05$, using nonparametric Mann-Whitney tests.

Hepatic lipid content after E_2 treatment

Male APOE*3-Leiden mice on a high-fat diet develop steatosis, which may be causally related to hepatic insulin resistance. Since hepatic insulin sensitivity improved acutely after E_2
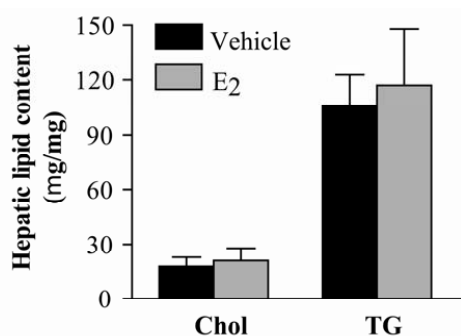

Figure 3. Effect acute E_2 administration on hepatic lipid content.
Hepatic TG and cholesterol content was analyzed under hyperinsulinemic conditions in E_2 versus control treated APOE*3-Leiden male mice. Values represent the mean$\pm$SD of 8 mice. *, statistically significant difference of $P<0.05$ compared with vehicle treated mice.

treatment, hepatic TG and cholesterol content were analyzed. As depicted in Fig 3, the E_2 treated mice did not exhibit a change in hepatic lipid content compared to the vehicle treated group (cholesterol content: 17.8 ± 5.1 *versus* 21 ± 6.8 µg/mg; TG content: 106 ± 16.8 *versus*

117±30.9 µg/mg, respectively) indicating that E_2 improves hepatic insulin sensitivity independently of a change in hepatic lipid content.

Hepatic mRNA expression levels

To examine the improved sensitivity of the liver to insulin-mediated inhibition of HGP in more detail, mRNA levels of relevant genes were analyzed by taqman analysis. Short-term induction of hepatic ER activity led to a 2.3- fold reduction of hepatic peroxisomal proliferators-activated receptor-γ coactivator-1α (PGC-1α) RNA levels (P = 0.0002) (Fig 4A). The expression of PhosphoEnolPyruvateCarboxyKinase (PEPCK) and Glucose-6-phosphatase (G6P) were unchanged (Fig 4A). In addition the expression of Glycogen Phosphorylase (GP) was 1.7-fold decreased (P=0.0016) (Fig 4A).
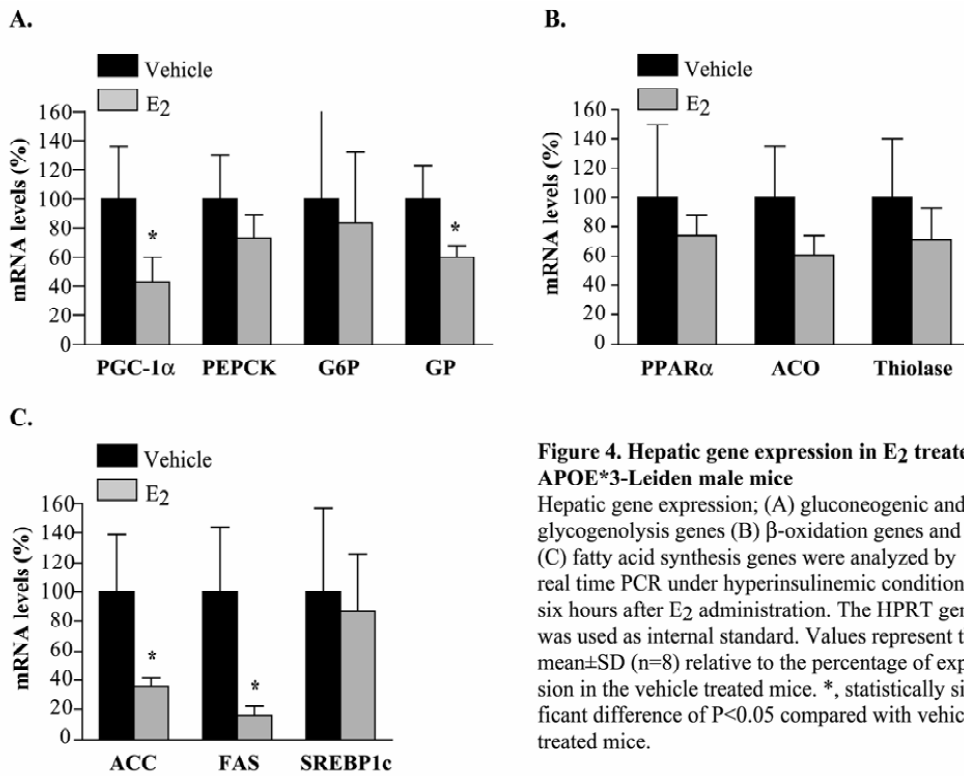

Figure 4. Hepatic gene expression in E_2 treated APOE*3-Leiden male mice
Hepatic gene expression; (A) gluconeogenic and glycogenolysis genes (B) β-oxidation genes and (C) fatty acid synthesis genes were analyzed by real time PCR under hyperinsulinemic conditions, six hours after E_2 administration. The HPRT gene was used as internal standard. Values represent the mean±SD (n=8) relative to the percentage of expression in the vehicle treated mice. *, statistically significant difference of P<0.05 compared with vehicle treated mice.

Since the hepatic glucose pathway is linked to the hepatic lipogenic pathway and since it has been shown that long-term modulation of E_2 signaling affects expression of enzymes involved in fatty acid β-oxidation and fatty acid synthesis, genes involved in these pathways were also analyzed. The expression of PPARα, Acyl-CoA oxidase (ACO, catalyzing the initial step of peroxisomal β-oxidation) and thiolase (catalyzing the final step of β-oxidation) were not significantly affected by acute E_2 administration (Fig 4B). On the other hand, acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), both key enzymes involved in *de novo* synthesis of fatty acids were significantly repressed (6.3- and 2.8-fold, respectively), while the sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor able to activate lipogenic genes like FAS and ACCα was unchanged (Fig 4C).

Discussion

The present study demonstrates for the first time that E_2 acutely improves hepatic insulin resistance with regard to HGP, whereas it attenuates peripheral insulin sensitivity with regard to glucose disposal in an insulin resistant mouse model. The improvement in hepatic insulin resistance was associated with decreased hepatic expression of the transcription factor PGC-1α and the glycogenolysis enzyme GP. Simultaneously, plasma FFA levels and hepatic lipid content were not affected. Thus E_2 has acute effects on hepatic insulin sensitivity, independent of hepatic lipid accumulation.

Our results show that there are specific differences with respect to insulin sensitivity between acute and long-term administration of E_2. In rat and mice models, long-term E_2 modulation results in an improvement of both hepatic and peripheral insulin sensitivity with respect to glucose output and disposal [16,18,19]. In contrast, short-term E_2 administration improves hepatic insulin sensitivity but attenuates peripheral insulin sensitivity. Since long-term E_2 administration does affect TG distribution and body weight, it is possible that the long-term effects of E_2 on peripheral insulin sensitivity are a long term consequence of these physiological changes. Specifically, the preventive effect of estrogen on steatosis would positively affect hepatic insulin sensitivity, since steatosis and hepatic insulin resistance are highly correlated. However, since E_2 will affect many organs and also affects neuro-endocrine signaling, it is likely that the net effect of long-term E_2 administration is the result of multiple complex interactions.

The APOE*3-Leiden mice, fed a high fat diet for 13 weeks, are highly resistant to insulin-mediated suppression of HGP, as demonstrated by no reduction in HGP by insulin

(Fig 2B). In comparison, chow-fed age-matched APOE*3-Leiden mice show 40-50% suppression of HGP by insulin. The single dose of E_2 acutely increased the insulin-mediated suppression of HGP in the fat-fed APOE*3-Leiden mice at least to the level of chow-fed APOE*3-Leiden mice. Thus a single dose of E_2 has a highly potentiating effect on insulin sensitivity of HGP.

Peripheral glucose disposal is approximately 150% increased by insulin in chow-fed age-matched APOE*3-Leiden mice. In high fat-fed APOE*3-Leiden mice it is only increased by 50%. Thus peripheral glucose disposal is also insulin resistant. This situation is further deteriorated by administration of E_2. At the moment, we have no explanation for this phenomenon. Since acute E_2 treatment did not reveal ER activity in muscle and adipose tissue in living ERE-Luc reporter mice, is seems likely that non-transcriptional E_2-mediated processes play a role. However, it is also possible that the bioluminescence method is not sensitive enough to detect limited, but potentially physiologically relevant ER activation in skeletal muscle and adipose tissue.

Under physiological insulin sensitive conditions, insulin reduces HGP through inhibition of hepatic gluconeogenesis and glycogenolysis. In contrast, under insulin resistant conditions, insulin is unable to suppress HGP and this has been associated with a failure in the down regulation of hepatic genes involved in glucose output [20]. In the present study, acute administration of E_2 in insulin resistant mice did not change expression levels of G6P and PEPCK (Fig 4A), which both play important roles in gluconeogenesis. However, the transcriptional coactivator protein PGC-1α, identified as an important inducer of gluconeogenesis [21], was significantly reduced upon acute administration of E_2 (Fig 4A). This apparent discrepancy indicates that under these conditions, down regulation of PGC-1α is not a dominant effect in the down regulation of G6P and PEPCK gene expression. More in line with the observed effect of E_2, expression of GP, an enzyme involved in glycogenolysis was significantly decreased. Whether this effect is directly related to down regulation of PGC-1α remains to be determined. Our data demonstrate that acute E_2 treatment regulates hepatic glucogenic genes, which could at least partly explain the observed E_2 mediated improvement of insulin-mediated inhibition of HGP.

Acute E2 administration did not affect intrahepatic TG levels in high fat-fed APOE*3-Leiden mice. In contrast, long term modulation of E_2 signaling is known to affect intrahepatic lipid levels. For example, constitutive E_2 deficiency, such as occurs in male aromatase knockout mice results in severe hepatic steatosis [13]. This phenotype has been associated

with decreased expression of genes involved in fatty acid β-oxidation [22,23] such as ACO and Thiolase, but also with increased expression of genes involved in de novo synthesis of fatty acids, including FAS and ACCα [13].

We found that acute E_2 treatment in insulin resistant APOE*3-Leiden did not change mRNA levels of genes involved in hepatic β-oxidation (Fig 4B). However, the fatty acid synthesis genes, FAS and ACCα were clearly suppressed (Fig 4C). Nevertheless, E_2 mediated suppression of FAS and ACCα did not result in decreased intrahepatic TG levels. It is possible that the six hour time window in the current study may not be sufficient to detect differences in intrahepatic TG flux. Alternatively, the effects of reduced FAS and ACCα gene expression are overruled by other compensatory transcriptional and/or post-transcriptional mechanisms.

In conclusion, administration of E_2 results in an acute improvement of hepatic insulin sensitivity with respect to HGP in obese, hyperlipidemic and insulin resistant mice fed a high-fat diet. This effect is independent of body weight, plasma lipid levels and/or intrahepatic TG content. Concomitantly, the administration of E_2 acutely impairs peripheral insulin sensitivity, through mechanisms not understood. These data demonstrate that acute and long-term administration of E_2 differentially affects tissue-specific insulin sensitivity.

Methods

Animals

All animal work was approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands and the experimental protocols complied with the national guidelines for use of experimental animals. 32 Wks old APOE*3-Leiden male mice (n=16) generated in the animal facility of TNO-Prevention and Health, were housed under standard conditions in conventional cages with free access to water and food. At the age of 19 wks, they were fed a high fat diet containing 45.4% fat (Hope Farms, Woerden, The Netherlands) for 13 wks.

Bioluminescent reporter imaging.

The experiment was carried out in male transgenic reporter mice, in which the luciferase gene was driven by an estrogen response element containing promoter (ERE-Luc mice) [24]. At time point 1, 3, 6 and 24 hours after s.c injection of 100 μg/kg 17 β-estradiol (dissolved in sesam oil, Sigma) in the neck, bioluminescent signals (BLS) were measured by Xenogen

IVIS imaging system (IVIS 100). The living mice were intraperitoneally injected with the luciferase substrate, luciferin, at a dose of 150 mg/kg body weight approximately 5 minutes before imaging. The mice were anaesthetized with isoflurane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were quantified by using the Living Image software (Xenogen Corp, Almeda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). BLS from the region of interest (ROI) was expressed using the pseudo colour scale (Red most intense and Blue least intense luminescence) and the data were presented as the cumulative photon counts collected within each ROI.

Plasmaparameters

Blood samples were taken via tail bleeding in paraoxon-coated capillaries, to prevent lipolysis [25]. Plasma was collected by centrifugation at 4°C. Plasma levels of total Chol, TG and FFAs were determined enzymatically using commercially available kits and standards (Sigma Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany). Plasma insulin was measured by ELISA (Mercodia Ultrasensitive mouse insulin ELISA, Mercodia, Sweden). Levels of plasma glucose were determined were determined using a commercially available kit (Sigma; Boehringer Mannheim, Mannheim, Germany). During the clamp experiment, whole-blood glucose was measured by a Freestyle hand glucose analyzer (Disetronic, Vianen, The Netherlands). All plasma parameters were determined according to the manufacturers' instructions.

Hyperinsulimemic euglycemic clamp

Male APOE*3-Leiden mice fed a high fat diet, fasted overnight (food withdrawn at 05.00 hour p.m.) were given 17β-estradiol (s.c, 100μg/kg) (Sigma, E8875) (n=8) or vehicle (sesame oil, Sigma) (n=9) at 06.00 hour a.m. Six hours after treatment insulin-mediated suppression of endogenous (hepatic) glucose production was studied by performing a hyperinsulinemic-euglycemic clamp analysis using ^{3}H-D-Glucose as tracer. The clamp analysis and calculations were performed as described previously [26]. After the last blood sample, mice were sacrificed, livers were taken out and immediately frozen using liquid nitrogen and stored at -80°C until further analysis.

Calculations

Total plasma ^{3}H-glucose radioactivity was determined in10-µl plasma and in supernatants after trichloric acid (20%) precipitation and water evaporation to eliminate $[^3$H]-H$_2$O. The rates of glucose oxidation were determined as previously described [27]. Under steady state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance (Ra; *i.e.* endogenous glucose production plus exogenous D-glucose infusion). Ra glucose was calculated as the ratio of the rate of infusion of [3-^{3}H] glucose (dpm) and the steady-state plasma [^{3}H] glucose specific activity (dpm/µmol glucose). The hepatic glucose production was calculated as the difference between the rate of glucose disappearance and the infusion rate of exogenous D-glucose.

Hepatic lipid analysis

Liver samples were homogenized in PBS. Protein content was measured by BCA protein assay kit (Pierce) at 562 nm. Hepatic lipids were extracted according to Bligh and Dyer [28]. After dissolving the lipids in 2% Triton X-100, the contents of Chol and TG in liver tissues were determined as described above.

Table 2. Primer sequences of genes used for mRNA quantification

Gene	Forward primer	Reverse primer
HPRT	5'-TTGCTCGAGATGTCATGAAGGA	5'-AGCAGGTCAGCAAAGAACTTATAG
FAS	5'-GGCATCATTGGGCACTCC	5'-GCTGCAAGCACAGCCTCT
ACC1	5'-GCCATTGGTATTGGGGCT	5'-CCCGACCAAGGACTTTGTT
SREBP1c	5'-GGAGCCATGGATTGCACA	5'-CCTGTCTCACCCCCCAGCA
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
PEPCK	5'-CCATGAGATCTGAGGCCACA	5'-GTATTTGCCGAAGTTGTAGCCG
G6P	5'-CAGGTCGTGGCTGGAGTCTT	5'-GACAATACTTCCGGAGGCTGG
GP	5'-GCGGTGACCGGTGTAGCAA	5'-CTTGTCTGGTTCTAGCTCGCTG
PGC1	5'-TTTTTGGTGAAATTGAGGAATGC	5'-CGGTAGGTGATGAAACCATAGCT
PPARα	5'-CCTCAGGGTACCACTACGGAGT	5'-GCCGAATAGTTCGCCGAAA
ACO	5'-GCCACGGAACTCATCTTCGA-3'	5'-CCAGGCCACCACTTATGGA-3'
Thiolase	5'-GGCAGGTTGTCACGCTACTCA-3'	5'-ATGGATACCACGCCGTAAGC-3'

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse

transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [29]. PCR primer sets (table 2) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. Since HPRT did not respond to the estrogen treatment, it was used as the standard housekeeping gene. The differences in relative gene expression numbers was calculated by C_t ($C_{t(HPRT)} - C_{t(target\ gene)}$). The date was verified by use of another independent housekeeping gene, cyclophilline.

Statistical analysis

Results are presented as mean ± SD values for the number of animals indicated. Differences between the experimental groups were determined by Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Acknowledgements

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