Activation of hepatic AMPK by 17β-estradiol suppresses both nuclear receptor Nr2c2/TR4 and its downstream lipogenic targets, reduces gluconeogenic genes and improves insulin signaling

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Abstract
Estrogen replacement reduces the frequency of type 2 diabetes (T2D), an effect which involves the suppression of hepatic glucose production. The objective of this study was to identify the hepatic mechanisms involved in the beneficial effects of 17β-estradiol (E2) on insulin sensitivity in mice exposed to long term (10 months) high fat diet (HFD) feeding. E2 treatment in HFD mice led to significant improvements in glycemic control. In the livers from these animals, Western blotting studies showed that E2 treatment led to significant increases in the activation state of the AMP-activated protein kinase (AMPK), in association with reduction of the nuclear receptor Nr2c2/TR4 both mRNA and protein levels, and suppression of downstream lipogenic gene expression. These effects were in parallel with up-regulation of hormone sensitive lipase (lipo) expression by E2. Increased fasting glucose levels following HFD feeding were associated with increases in the expression levels of the gluconeogenic genes, g6pt1 and pyruvate carboxylase, whilst E2 treatment significantly reduced their expression levels. The insulin signaling pathway was studied in the liver after acute insulin intervention. The phosphorylation states of AKT2 and FOXO1 were both decreased in HFD mice, and E2 treatment reversed these changes. In conclusion, E2 treatment reduced body weight and improved glycemic control in association with activation of hepatic AMPK, reduced expression of its downstream target Nr2c2/TR4, and consequent decreases in lipogenic gene expression. Together with increased triglyceride mobilization, these changes paralleled improved hepatic insulin signaling and reduced gluconeogenic gene expression.

Introduction
A large body of literature suggests that, relative to men, mechanisms exist in pre- and perimenopausal women which are protective against type 2 diabetes (T2D). For example, an age-prevalence study has shown that the total frequency of T2D in women was less than that in men until 59 years, after which it equalized or exceeded the frequency in males [1]. Longitudinal studies have also demonstrated that fasting plasma glucose levels increase with age in females more than in males, particularly from 50 years of age onwards [2]. Menopause itself is an independent risk factor for elevated fasting plasma glucose concentrations and progressive increases in insulin resistance [3, 4]. Conversely, two randomized clinical trials have shown that estrogen replacement reduces the frequency of T2D development [5, 6], reduces fasting levels of glucose and insulin [7-9], and promotes insulin sensitivity and glycemic control in women with T2D [10, 11]. Specifically, E2 replacement suppresses hepatic glucose production [12, 13], even in postmenopausal women with T2D [14]. These observations suggest that the beneficial effects of E2 treatment involve alterations in hepatic insulin resistance.
Hepatic insulin resistance is associated with both lipid accumulation and augmented endogenous glucose production due to increased hepatic gluconeogenesis [15, 16]. Conversely, activation of hepatic adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), a central regulator of cellular energy homeo-
stasis, leads to a number of beneficial effects. For example, AMPK activation results in inhibition of fork-head box protein/transcription factor (FOXO1), a transcription factor regulated by the insulin signaling pathway. In this pathway, binding of insulin to the insulin receptor leads to phosphorylation of insulin receptor substrate (IRS) molecules, and downstream phosphorylation and activation of the intermediate Akt; this suppresses glucose production partly by phosphorylation of FOXO1, leading to its nuclear exclusion and decreased expression of its target gluconeogenic enzymes glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PEPCK) [17, 18]. Accordingly, inhibition of FOXO1 by activated AMPK suppresses gluconeogenic gene expression [19]. In addition, activated AMPK suppresses hepatic lipogenesis via the phosphorylation & inactivation of the product of the lipogenic gene acetyl-CoA carboxylase 1 (ACC1) [19]. Finally, the suppression of lipogenic gene expression due to activation of AMPK by metformin is associated with inhibition of the nuclear receptor Nr2c2/TR4 [20]. Both increased hepatic triglyceride content [21] and raised levels of endogenous glucose production [21, 22] are observed in estrogen receptor (ER) α-knockout (ERKO) mice. Conversely, the promotion of insulin sensitivity in the liver by E2 has been associated with the suppression of lipogenic gene expression, increased leptin receptor expression [23], as well as augmented levels of IRS-1 and activated AKT [24]. These results indicate that E2 signaling is involved in the maintenance of hepatic insulin sensitivity.

To further understand the events by which E2 signaling maintains hepatic insulin sensitivity, a chronic high fat diet–exposed perimenopausal mouse model was employed to correlate the E2-induced suppression in fasting glucose levels with AMPK activation. Activation of AMPK was associated with down-regulated expression of the nuclear receptor Nr2c2/TR4, reduced expression of lipogenic genes, and diminished hepatic triglyceride content. These alterations were associated with reduced gluconeogenic gene expression and changes in phosphorylation status of components of the insulin signaling pathway.

### Table 1. Antibodies and antibody dilutions used for Western blotting studies

| Antibody                                      | Mol.wt (kDa) | Dilution factor | Manufacturer   |
|-----------------------------------------------|--------------|----------------|----------------|
| Phospho-Monophosphate-Activated Protein Kinase (AMPKα) (Thr172) | 62           | 1:1000         | Cell Signaling |
| AMPKα (23A3)                                   | 62           | 1:1000         | Cell Signaling |
| Phospho-Acetyl-CoA Carboxylase pSer79          | 280          | 1:1000         | Thermo         |
| Acetyl-CoA Carboxylase (B.800.8)               | 280          | 1:1000         | Thermo         |
| Phospho-Inulin receptor substrate 2 (IRS 2)(phospho S731) | 170          | 1:1000         | Cell Signaling |
| IRS-2 (L:326) Antibody                        | 170          | 1:1000         | Cell Signaling |
| Phospho-Akt (Ser473) Antibody                 | 62           | 1:1000         | Cell Signaling |
| Akt (pan) (C67E7) Rabbit mAb #4691            | 62           | 1:1000         | Cell Signaling |
| Phospho-Forkhead box protein O1 (Foxo1) (Ser256) Antibody 9461 | 72          | 1:1000         | Cell Signaling |
| FoxO1 (C29H4) Rabbit mAb #2880                | 72           | 1:1000         | Cell Signaling |
| Nuclear receptor subfamily 2, group C, member (2Nr2c2/TR4) (ab109301) | 62          | 1:1000         | Abcam          |
| Beta Actin antibody                           | 42           | 1:2000         | Cell Signaling |
| Anti-rabbit IgG, HRP-linked Antibody          |              |                |                |

### Material and Methods

#### Animals, treatment with E2 and administration of insulin

Seven week-old female C57BL/6 mice (Scanbur BK, Sollentuna, Sweden) and were maintained with free access to food and water in a temperature-controlled room (22–23°C) with a 12 hr-12 hr light-dark cycle. All procedures involving animals were approved by the local ethical committee. After acclimatization for one week, the mice were divided randomly into two groups. Mice belonging to the low fat diet (LFD) group were maintained until 12 months of age on a chow diet (Lactamin, Kimstad, Sweden) containing (in g/100g): 4.5 fat, 69 carbohydrate and 14.5 protein. Mice belonging to the high fat diet (HFD) group were also maintained until 12 months of age (corresponding to 10 months of HFD feeding) on a diet containing (in g/100g): 34.9 fat, 26.3 carbohydrate and 26.2 protein (D12492, Research Diets, New Jersey, USA). With the exception of the hepatic insulin signaling study, at 11 months of age, both groups were divided into treatment and vehicle subgroups. The treatment subgroups (LFD+E2, HFD+E2) were injected subcutaneously (50 µg/kg body weight/day) for 1 month with 17β-estradiol (E2; Sigma-Aldrich, Stockholm, Sweden) prepared in vehicle, whilst the other sub-groups (LFD, HFD) were treated similarly with the same volume of vehicle (90% sesame oil, 10% ethanol). On the final day of treatment, mice were fasted...
overnight and on the following day, were weighed and euthanized by cervical dislocation. Liver tissues were dissected, snap-frozen and stored at -80ºC. The numbers of mice included in each experiment are given in the respective figure legends.

For determination of insulin signaling, at the end of the E2 treatment period, mice in the LFD, HFD and HFD+E2 subgroups were given an intraperitoneal injection of either insulin (2 mU/g; Actrapid, Novo-Nordisk) or a similar volume of saline. All mice were then sacrificed 5 minutes later, and organs were collected as described above. Since no significant changes were observed in E2-treated LFD mice, and so data from this group are not included in insulin signalling study (Fig. 6).

**Table 2. mRNA targets and Assay-on-Demand designations used for RT-PCR studies**

| Gene     | Primer code       | Full name                                      |
|----------|-------------------|-----------------------------------------------|
| SCD-1    | Mm00772290_m1     | Stearoyl-CoA desaturase 1                      |
| FAS      | Mm00662319_m1     | Fatty acid synthase                           |
| ACCt     | Mm0130428_m1      | Acetyl-CoA carboxylase                         |
| CD36     | Mm0135918_m1      | Fatty acid translocase                         |
| CYP7a1   | Mm00484152_m1     | Cholesterol 7a-hydroxylase (cytochrome P450 7A1) |
| LDLR     | Mm00440169_m1     | Low density lipoprotein receptor              |
| G6PC     | Mm00839363_m1     | Glucose 6 phosphatase catalytic               |
| G6PT1    | Mm00484574_m1     | Glucose 6 phosphatase transport 1             |
| G6PC3    | Mm00616234_m1     | Glucose 6 phosphatase catalytic 3             |
| PEPCK    | Mm00440636_m1     | Phosphoenolpyruvate carboxykinase             |
| PC       | Mm01239161_m1     | Pyruvate carboxylase                          |
| LXRα     | Mm00443454_m1     | Liver X receptor α                            |
| Pparg    | Mm00440939_m1     | Proliferator-activated receptor α             |
| Ppargy   | Mm00440945_m1     | Proliferator-activated receptor γ             |
| Stat-3   | Mm00456961_m1     | Signal transducer and activator               |
| SREBP1c  | Mm00550338_m1     | Sterol regulatory element binding protein 1c  |
| TR α     | Mm00617505_m1     | Thyroid receptor isoform α                    |
| TR β     | Mm00437044_m1     | Thyroid receptor isoform β                    |
| LePR     | Mm00440174_m1     | Leptin receptor                               |
| AdipoR1  | Mm01291334_m1     | Adiponectin receptor 1                        |
| AdipoR2  | Mm01184028_m1     | Adiponectin receptor 2                        |
| CPT1b    | Mm00487191_g1     | Carnitine palmitoyltransferase 1b             |
| CPT2     | Mm00487205_m1     | Carnitine palmitoyltransferase 2              |
| Nr2c2/TR4| Mm01182440_m1     | Nuclear receptor subfamily 2, group C, member 2|
| LIPE     | Mm00495359_m1     | lipase, hormone sensitive                     |
| 18S      |                   | Endogenous standard                           |

Plasma insulin levels

Plasma insulin levels were assessed by use of a RIA employing 125I-labeled porcine insulin, guinea pig anti-porcine serum, and rat insulin as a standard (Novo Nordisk).

Western blotting

Snap-frozen liver tissue (approximately 100 mg) was homogenized on ice in 1 ml RIPA buffer and centrifuged at 4°C for 20 minutes at 12000 g. Aliquots of the supernatants were run on 5% or 10% ECL gels (Amersham, GE Healthcare, UK) and transferred to PVDF membranes. After blocking in 5% fat-free dry milk (Bio-Rad Laboratories, Sweden) dissolved in Tris-buffered saline (TBS), membranes were washed with wash buffer (TBS/0.1% (v/v) Tween-20) and 10 mls TBS buffer containing the primary antibody of interest (see Table 1) was added. Membranes were incubated overnight at 4°C, washed 3 times with wash buffer and incubated for 1 h at room temperature in 10 mls TBS containing secondary antibody, according to the manufacturer’s recommendations (see Table 1). The membranes were washed with wash buffer three times, and an ECL detection kit (SuperSignal West Femto, Thermo Scientific Pierce, Nordic Biolabs, Sweden) was used to visualize the bands, followed by documentation with an LAS 3000 system (Fuji Film Co., Tokyo, Japan). ImageJ was used to quantify the band intensities.

**RNA preparation and real-time RT-PCR**

Total RNA was prepared from snap-frozen liver tissue using TRIzol reagent (Life Technologies Europe, Sweden) and further cleaned using RNeasy Minikit and treated with DNase I (Qiagen, Sweden). Purified total RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagents and random hexamer primers (Applied Biosystems, Sweden). The expression of target mRNAs was assessed in triplicate 25 µl reactions 30, 60 and 120 minutes following, an intraperitoneal injection of glucose (2 g/kg body weight).

**Intraperitoneal glucose tolerance test (IPGTT)**

Mice were fasted overnight, after which blood glucose levels were assessed by glucometer (MediSence, Abbott Scandinavia, Solna, Sweden) before, and at 15, 30, 60 and 120 minutes following, an intraperitoneal injection of glucose (2 g/kg body weight).
containing 1x Master Mix and the appropriate Assay-on-Demand (see Table 2) (both from Applied Biosystems, Sweden) with a 7300 Real-Time PCR system. 18S was used for the normalization of expression levels, and the ΔΔCT method was employed to calculate fold changes in target gene expression.

**Triglyceride assay**

Triglycerides were extracted from snap-frozen liver tissues and quantified using a Triglyceride Colorimetric Assay Kit (Cayman Chemical, BioNordika, Sweden), according to the manufacturer’s instructions.

**Statistical analyses**

Prism Graph Pad Software (California, USA) was used to analyze data. Significances were calculated using ANOVA, followed by the Bonferroni post hoc analysis with multiple comparisons. Values of P < 0.05 were considered significant. All values are presented as means ± SEM.

**Results**

**Body weight and glucose tolerance**

HFD feeding for 10 months led to a doubling in body weight, compared to LFD controls (59.71 ± 0.52 g vs. 30.00 ± 0.58 g, respectively; P<0.001) (Fig. 1A). One month of E2 treatment had no effect on body weight in LFD mice (30.33 ± 0.61 g vs. 30.00 ± 0.58 g in controls). However, E2 treatment significantly reduced the body weights of HFD mice, compared with untreated HFD controls (41.01 ± 0.70 g vs. 59.71 ± 0.52 g, respectively; P<0.001) (Fig. 1A). Increased body weight in HFD mice was associated with significantly elevated plasma insulin levels (P<0.001), while E2 treatment in E2-treated HFD mice significantly reduced plasma insulin levels (P<0.001) (Fig. 1B). E2 had no effect on plasma insulin levels in LFD mice.

An IPGTT revealed significantly increased fasting glucose levels in HFD mice (Fig. 1C) and elevated glucose levels at all time points during the test, compared with LFD mice (P<0.05 or P<0.001) (Fig. 1D). E2 had no effect on blood glucose levels in LFD mice, compared with untreated controls (Fig. 1D). However, in HFD mice, E2 treatment resulted in decreased glucose levels during the IPGTT to levels which were significantly lower (P<0.001) than those of untreated HFD mice at all time points (Fig.1D).

**Activation of hepatic AMPK**

AMPK is a key metabolic sensor of cellular energy status which is able to activate pathways associated with increased insulin sensitivity. To understand wheth-

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**Figure 1.** Mice were maintained for 10 months on either an LFD or an HFD, and treated with vehicle or E2 for the final 30 days of each dietary intervention. Immediately after treatment, body weights were measured (A), and plasma levels of insulin were assessed using RIA (B), Fasting blood glucose levels prior to the start of the IPGTT (C), and blood glucose values during an IPGTT were determined as indicated (D). (A-C) ***, P value < 0.001; n = 6-7 (D) †, P value < 0.05, †††, P value < 0.001for HFD vs LFD mice; and ***, P value < 0.001 for HFD+E2 vs HFD mice; n = 6-7.
The improvements in glucose homeostasis in HFD mice following treatment with E2 involved alterations in AMPK, its activation state was studied by assessing the extent of phosphorylation of Thr172 on the α subunit of this enzyme. HFD feeding resulted in a significant (P<0.001) reduction in the ratio of phosphorylated AMPKα to total AMPK, compared with LFD mice (Fig. 2A). E2 administration to HFD mice resulted in a significant (P<0.001) increase in the ratio of phosphorylated AMPKα to total AMPK, compared with untreated HFD mice.

In order to observe whether these alterations in the activation state of AMPK led to functional changes in downstream targets of AMPK, the phosphorylation state of ACC1 was assessed. Employing an antibody against phosphorylated Ser79 of ACC1, a significant (P<0.01) decrease in the level of ACC1 phosphorylation was observed in livers from HFD mice, compared with LFD mice, corresponding to a relative increase in the activation state of ACC1 after HFD feeding (Fig. 2B). Conversely, however, the administration of E2 in HFD mice led to a significant (P<0.01) increase in phosphorylation of ACC1 at Ser79, compared with HFD controls, indicating a relative decrease in the activation of this protein after E2 treatment.

Expression of Nr2c2/TR4

The activation of AMPK by metformin has recently been shown to be associated with the reduced transcriptional activation of lipogenic genes by the nuclear receptor Nr2c2/TR4 [20], and so it was of interest to determine whether the hepatic expression levels of this nuclear receptor changed following E2 treatment. In HFD mice, Nr2c2/TR4 protein levels were significantly (P<0.001) increased (Fig. 3B), whilst administration of E2 in HFD mice led to significant reductions in both the mRNA (P<0.01) and protein (P<0.001) levels of Nr2c2/TR4 (Fig. 3A and 3B, respectively).

Expression of genes regulating hepatic lipid levels and hepatic triglyceride levels

Since mRNA levels of nr2c2/tr4, as well as Nr2c2/TR4...
protein levels, were diminished in the livers of E2-treated HFD mice, it was of interest to examine whether E2 treatment also would lead to reduced lipogenic gene expression. In HFD mice, expression of the lipogenic gene acc1 was significantly (P<0.001) upregulated, with respect to that in LFD mice (Fig. 4A), whilst levels of mRNAs encoding other lipogenic genes (scd1, fas) were unchanged. However, administration of E2 in HFD mice resulted in significant reductions in the expression levels of all three lipogenic genes (scd1 and acc1, P< 0.001; fas, P< 0.05), compared with untreated HFD controls (Fig. 4A). In agreement with the analyses of acc1 mRNA levels, E2 treatment in HFD mice led to significant (P<0.001) reductions in the total levels of ACC1 protein (Fig. 4B). Furthermore, the mRNA levels of hormone sensitive lipase (lipe) were suppressed in HFD mice, compared with LFD controls (lipe; P<0.01), and E2 treatment in HFD mice resulted in increased lipe expression (P<0.05). No changes were observed between any of the groups in the mRNA levels of fatty acid transporter cd36, carnitine palmitoyltransferase (cpt) 1b, cpt2, the cholesterol-metabolizing enzyme cholesterol-7α hydroxylase (cyp7α1) , and of low density lipoprotein receptor (ldlr) (Fig. 4A).

It was of interest to investigate whether the respective decreases and increases in the expression of lipogenic genes and lipe were reflected in alterations in hepatic triglyceride levels. In the livers of HFD mice, triglyceride levels were significantly (P<0.001) increased, compared with those in LFD mice (Fig. 4B). As reported previously [23], E2 treatment in HFD mice led to a significant (P<0.001) decrease in triglyceride levels, in comparison with untreated HFD controls. In LFD mice, E2 had no effect on hepatic triglyceride levels (Fig. 4C).

To identify additional mechanisms underlying these alterations in hepatic triglyceride levels, the expression of a number of transcription factors was examined. In HFD mice, the mRNA levels of pparγ were significantly (P<0.001) reduced, compared with those in LFD mice, whilst E2 treatment did not further affect pparγ expression levels (Fig. 4D). While the levels of ppara mRNA were unchanged after HFD feeding, E2 treatment of HFD mice led to significant (P<0.05) reductions in ppara expression. The expression levels of stat3 were unchanged between the groups (Fig. 4D), whilst the expression of lxrα and srebp1 were not significantly af-
fected by HFD feeding or E2 treatment (Fig. 4D).

Expression of adipokine receptors, gluconeogenic genes and thyroid receptors

Compared with livers from LFD mice, the levels of mRNAs for leptin receptor and adiponectin receptor 2 were significantly (P<0.01 and P<0.05, respectively) reduced in HFD mice (Fig. 5A). E2 treatment in LFD mice resulted in significant (P<0.05 and P<0.05, respectively) reductions in the expression levels of these two genes, relative to those in control LFD mice. Despite this effect of E2, administration of E2 in HFD mice led to a significant (P<0.05) increase in expression levels of leptin receptor, compared with untreated HFD mice (Fig. 5A). No differences were observed in the expression of adiponectin receptor 1 between any of the groups.

Since treatment with E2 in HFD mice significantly suppressed fasting glucose levels (Fig. 1C), the expression of genes involved in hepatic glucose production was examined. While significant (P<0.05) reductions in the expression levels of glucose-6-phosphatase catalytic subunit (g6pc) were observed after HFD feeding, compared with LFD mice, those of glucose-6-phosphate translocase (g6pt1) and of pyruvate carboxylase were significantly (P<0.05 and P<0.05, respectively) increased (Fig. 5B). Importantly, however, treatment with E2 resulted in significantly reduced levels of mRNAs for g6pc, g6pt1 and pyruvate carboxylase (P<0.001, P<0.001 and P<0.01, respectively) in HFD mice, compared with untreated HFD mice. Also, E2 administration in LFD mice led to a significant (P<0.05) reduction in the expression of g6pc, compared with untreated LFD mice (Fig. 5B). No changes were seen in the expression levels of glucose-6-phosphatase catalytic subunit-related protein (g6pc3), or of phosphoenolpyruvate carboxykinase (pepck), between any of the groups.

To identify whether the E2-induced alterations in lipogenic and gluconeogenic gene expression were associated with changes in thyroid hormone signaling, we assessed the expression of thyroid hormone receptor (TR) α and TRβ. The results showed that neither HFD feeding nor E2 treatment had any effect on the expression levels of trα and trβ (Fig 5C).

Hepatic insulin signaling pathway

Since E2 treatment led to improvements in glucose

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**Figure 5.** Expression levels of adipokine receptors (A), gluconeogenic genes (B) and thyroid receptors (C) in the livers of E2-treated or vehicle-treated LFD and HFD mice were assessed by real-time PCR. *, P value < 0.05, ***, P value < 0.01 and ****, P value < 0.001; n = 6-7
tolerance and fasting glucose levels, the abundances and phosphorylation status of components of the insulin signaling pathway were studied in LFD, HFD and E2-treated HFD mice following administration of either saline or insulin for 5 minutes. The fold changes in phosphorylation status of these signaling components in response to insulin administration are shown in Figure 6. IRS2 phosphorylation did not change significantly, although the fold changes in response to insulin were lower in HFD mice compared to either LFD or E2-treated HFD mice (Fig. 6A). However, significant reductions (P<0.001) in the fold-change in insulin-induced phosphorylation were observed in HFD mice, compared with LFD controls, for both AKT2 (Fig. 6B) and FOXO1 (Fig. 6C). Conversely, in E2-treated HFD mice, significant increases in the fold-change in phosphorylation status were observed for AKT2 and FOXO1, compared with HFD mice (P<0.01 and P<0.05, respectively) (Fig. 6B&C). Thus HFD feeding leads to changes in the phosphorylation status of insulin signaling pathway molecules which are consistent with the presence of hepatic insulin resistance, whilst E2 treatment reverses these alterations.

**Discussion**

The HFD mice employed in this study represent a model of peri-menopausal obesity which involves impaired glucose tolerance, raised fasting glucose levels and insulin resistance; these features are accompanied by reduced plasma E2 levels, and the E2 treatment regimen employed was shown previously to normalize plasma E2 concentrations, reduce body weight & restore normal levels of glucose tolerance [23]. The liver plays a central role in the regulation of glucose homeostasis, and the suppression of fasting glucose levels by E2 treatment indicated that the beneficial effects of E2 are associated with improvements in hepatic insulin sensitivity. Accordingly, this study aimed to identify mechanisms which might underlie these beneficial changes.

Since AMPK is a key metabolic sensor of cellular energy status which responds to various stimuli that affect the homeostasis of cellular metabolism [19], it was of interest to determine whether the beneficial effects of E2 treatment involve changes in AMPK activation sta-

![Figure 6. Insulin signaling status in liver: Ratio of phosphorylated/total protein levels of IRS2 (A), Akt2 (B) and FOXO1 (C), in the livers of vehicle-treated LFD and of E2-treated or vehicle-treated HFD mice after acute insulin administration. *, P value < 0.05; **, P value < 0.01 and ***, P value < 0.001; n = 3-4](image-url)
tus. Assessment of the extent of phosphorylation at Thr172 of AMPK demonstrated that the AMPK activation state was reduced in the livers of HFD mice versus LFD mice, whilst AMPK was significantly activated by E2 treatment in HFD mice. AMPK activation has been reported to prevent diet-induced hepatic lipid accumulation by inhibiting mTORC1 signaling and endoplasmic reticulum stress response [25]. Additionally, activation of AMPK leads to the inhibition of the lipogenic protein intermediate ACC1 by phosphorylation on Ser79 and the suppression of lipogenesis [26]. Consistent with this, the proportion of ACC1 which was phosphorylated on this residue was significantly decreased in the livers of HFD mice, compared with LFD mice. Conversely, however, ACC1 phosphorylation was significantly increased in E2-treated HFD mice. These results suggest that one of the mechanisms by which E2 reduces hepatic lipid levels involves the activation of AMPK, a consequence of which is the inactivation of ACC1 and a subsequent reduction in the rate of lipogenesis. This role for E2 in the maintenance of the activation state of AMPK is consistent with the respective reduction and stimulation of the AMPK activation state in the muscle tissue of both ERKO mice and E2-treated ovariectomized mice [22, 27]. These results are also consistent with a report showing that ER interacts directly with the βγ-binding domain of AMPKa [28].

The activation of AMPK by agents such as metformin has recently been shown to lead to the inactivation of the nuclear receptor Nr2c2/TR4, leading to the suppression of downstream lipogenic gene expression [20]. The results of this study show that HFD feeding led to increased protein levels of the nuclear receptor Nr2c2/TR4, while E2 treatment of HFD mice was associated with reductions in Nr2c2/TR4 expression at both the mRNA & protein levels. Since the AMPK-dependent regulation of Nr2c2/TR4 transactivation of lipogenic gene expression may represent an important route by which E2 affects hepatic lipogenesis, the goal of this study was to show that the effects of E2 treatment on AMPK activation and Nr2c2/TR4 expression levels were associated with parallel alterations in hepatic lipid metabolism. Studies of the profile of lipogenic gene expression in this tissue showed that HFD feeding was accompanied by increased acc1 expression, and E2 treatment in HFD mice reduced mRNA levels of the lipogenic genes scd1, fas and acc1, as observed previously [23]. ACC1 protein levels were also significantly reduced. These changes were specific to chronic HFD exposure, since E2 treatment in LFD mice did not affect the expression of any of these genes. Furthermore, these alterations in lipogenic gene expression were associated with parallel changes in hepatic triglyceride levels, since HFD feeding significantly increased in hepatic triglyceride levels, whilst E2 treatment in HFD mice almost normalized triglyceride levels to those seen in LFD mice. Consistent with its lack of effect on lipogenic gene expression, E2 treatment in LFD mice had no effect on hepatic triglyceride levels. Thus HFD feeding led to increased protein Nr2c2/TR4 protein levels, raised lipogenic gene expression and augmented hepatic triglyceride levels. Conversely, reductions in both the mRNA and protein levels of Nr2c2/TR4 following E2 treatment of HFD mice were associated with the suppression of lipogenic gene expression and diminished hepatic triglyceride levels. These data are consistent with the proposal that Nr2c2/TR4 is a potentially important regulator of lipogenic gene expression [20]. Since lipid accumulation in the liver is strongly associated with insulin resistance in this organ [15], our results suggest that the beneficial effects of E2 in this organ involve the AMPK-dependent inhibition of lipogenic gene expression via the modulation of Nr2c2/TR4 levels, and subsequent down-regulation hepatic lipogenesis.

The current report determined the expression of several other transcription factors for which a role in the regulation of hepatic lipogenesis has been described. However, the changes in lipogenic gene expression which were observed in HFD mice and E2-treated HFD mice did not correlate with changes in the expression of either srebp1, a transcription factor which has been identified as being involved in the regulation of lipogenic gene expression, or of lxrα, which regulates SREBP1 expression [29, 30]. Changes have been described in lipogenic gene expression in the absence of alterations in expression of SREBP1 or LXRα [31]. In the case of PPARγ, signaling via this nuclear receptor promotes the expression of genes involved in lipogenesis and glucose metabolism, and suppresses the expression of genes involved in gluconeogenesis and fatty acid oxidation [32]. However, PPARγ is not highly expressed in the liver, and increases in pparγ levels are not universally observed following HFD feeding [33]. It is thus possible that the reduction in pparγ expression in HFD mice that observed in this study represents an adaptation to the chronic dietary conditions in our experiment. E2 treat-
ment did not alter pparα expression in HFD mice, contrary to our previous observations [23]. Conversely, however, pparα is highly expressed in the liver, and promotes the catabolism of lipids, including fatty acid oxidation [34], and it was therefore unexpected that reduced hepatic triglyceride content in E2-treated HFD mice was associated with reduced pparα expression. However, these observations are consistent with previous findings showing that the beneficial metabolic effects of fenofibrate treatment in female mice are seen only following ovariectomy [35], and with results showing that E2 inhibits the induction of genes targeted by fenofibrate activated-PPARα [36]. Furthermore, the lack of any alteration in the expression levels of cpt1b and cpt2 in E2-treated HFD mice also suggests that E2-induced reductions in hepatic triglycerides were not associated increased fatty acid oxidation. Finally, the levels of stat3 expression were unchanged following E2 treatment, despite the role for this protein in leptin receptor signaling [37] and its involvement in the suppression of hepatic steatosis via the down-regulation of SREBP1 expression [38, 39].

Changes in hepatic triglyceride content were also not associated with alterations in the expression of other genes involved in lipid metabolism, including cd36, a gene involved in fatty acid uptake which is associated with the development of fatty liver [40], cyp7a1, which catalyzes the rate-limiting initial step of bile acid synthesis [41], and ldlr, which has been demonstrated previously to be induced by estrogen [42]. However, increased hepatic triglyceride levels in HFD mice correlated with decreased expression levels of lipe, and reduced hepatic triglyceride content in E2-treated HFD mice was associated with increased lipe expression, suggesting that E2 may affect hepatic lipid levels at least in part by regulating triglyceride mobilization.

Our group has previously shown that the promotion of insulin sensitivity by E2 treatment in HFD mice leads to reduced circulating levels of the adipokine adiponectin [23]. AMPK is a major mediator of the beneficial metabolic effects of adiponectin via signaling through adiponectin receptors R1 and R2, of which adiponectin receptor R1 is most highly expressed in the liver [43-45]. However, the alterations that were observed here in AMPK activation were not associated with changes in adiponectin receptor R1 expression. Meanwhile, expression of adiponectin receptor R2 was reduced in HFD mice, compared with LFD mice, and E2 treatment reduced expression of this receptor in LFD mice, with a trend towards reduced levels in E2-treated HFD mice. Together, these observations indicate that the activation of AMPK by E2 is independent of signaling through either of the hepatic adiponectin receptors. It has also been reported previously that HFD mice exhibit increased plasma levels of leptin, which were suppressed by E2 treatment [23]. In the current study, hepatic leptin receptor expression in HFD and E2-treated HFD mice showed a reciprocal pattern of changes, being significantly reduced and increased, respectively. Furthermore, E2 also reduced expression of leptin receptor in LFD mice.

HFD mice exhibited significant increases in fasting blood glucose levels, a parameter which reflects the extent of hepatic glucose production. Therefore gluconeogenic gene expression in this organ was studied. Unexpectedly, the expression levels of g6pc and of pepck in HFD mouse livers were significantly reduced and unchanged, respectively. However, there are contradictory results are reconciled by studies which confirm the lack of correlation between hepatic pepck or g6pc expression levels with fasting glucose levels in either rodent models of type 2 diabetes, or in patients with type 2 diabetes [46]. However, protein levels of pyruvate carboxylase, an enzyme which catalyzes the first committed step of gluconeogenesis, have been shown to correlate closely with plasma glucose concentrations [18]. In this study, a correlation between increased hepatic glucose production in HFD mice and significant increases in pyruvate carboxylase expression were observed. E2 treatment of HFD mice almost normalized fasting glucose levels, and this was associated with significant reductions in the expression levels of g6pc, g6pt1 and pyruvate carboxylase. Our group and others have previously described significant reductions in g6pc expression levels following E2 treatment in HFD mice [23]. In addition, the present data demonstrates that E2 had a suppressive effect on g6pc expression in LFD mice, and in agreement with this, g6pc has been shown to be a direct target gene for signaling through ERα [47]. Furthermore, the expression of g6pc and pepck has been shown to be suppressed by activated AMPK [48], and so E2-induced AMPK activation may provide a mechanistic explanation for the reductions in g6pc expression observed in E2-treated HFD mice. Finally, some of the effects of E2 on hepatic gluconeogenesis may be secondary to its modulation of levels of growth hormone.
and thyroid hormone [50]. In the case of the latter, no changes were detected in the mRNA levels of either 

tra or trβ after E2 treatment in LFD or HFD mice.

The fact the E2 treatment significantly improved glucose tolerance and fasting glucose levels in HFD mice, together with our previous data showing that E2 treatment improves insulin tolerance [23, 51], suggests that E2 improves insulin signaling in organs involved in the regulation of glucose homeostasis. To study this, changes in insulin-dependent phosphorylation of intermediates in the insulin signaling pathway were studied in the liver. Compared with LFD mice, insulin-resistant HFD mice exhibited reduced insulin-dependent phosphorylation of AKT2 & FOXO1. These alterations were accompanied by increased levels of hepatic triglycerides and elevations in the expression of gluconeogenic genes, in association with raised fasting glucose levels. The promotion of insulin sensitivity by E2 treatment in HFD mice was associated with increased insulin-dependent phosphorylation of both AKT2 and FOXO1, in association with reduced hepatic triglyceride content, decreased expression of gluconeogenic genes and lowered fasting glucose levels. The fold changes of IRS2 were not significant. However, increased hepatic IRS2 protein levels in the absence of increased insulin-induced phosphorylation have been described previously in E2-treated ovariectomized rats [52], and the effects of E2 may include the stabilization of IRS-2 by reducing its rate of degradation [53].

In conclusion, improvements in glucose homeostasis induced by E2 treatment in HFD mice are associated with the activation of hepatic AMPK and reductions in expression levels of its downstream targets ACC1 and Nr2c2/TR4, a nuclear receptor involved in the transcriptional regulation of genes involved in lipogenesis. In parallel with these alterations, E2 treatment also led to reductions in lipogenic gene expression and diminished hepatic triglyceride levels. E2-induced reductions in hepatic lipid levels may also be associated with increased triglyceride mobilization. Finally, the positive effects of E2 on glycemic control involve reductions in fasting glucose levels, which occur in parallel with the suppression of gluconeogenic gene expression and improved hepatic insulin signaling.

Declaration of Interest: The authors declare no conflict of interest

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