17β-Estradiol upregulates the expression of peroxisome proliferator-activated receptor α and lipid oxidative genes in skeletal muscle

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Abstract

This study examined the actions of 17β-estradiol (E2) and progesterone on the regulation of the peroxisome proliferator-activated receptors (PPARα and PPARγ) family of nuclear transcription factors and the mRNA abundance of key enzymes involved in fat oxidation, in skeletal muscle. Specifically, carnitine palmitoyltransferase I (CPT I), β-3-hydroxyacyl CoA dehydrogenase (β-HAD), and pyruvate dehydrogenase kinase 4 (PDK4) were examined. Sprague–Dawley rats were ovariectomized and treated with placebo (Ovx), E2, progesterone, or both hormones in combination (E+P). Additionally, sham-operated rats were treated with placebo (Sham) to serve as controls. Hormone (or vehicle only) delivery was via time release pellets inserted at the time of surgery, 15 days prior to analysis. E2 treatment increased PPARα mRNA expression and protein content (P<0.05), compared with Ovx treatment. E2 also resulted in upregulated mRNA of CPT I and PDK4 (P<0.05). PPARγ mRNA expression was also increased (P<0.05) by E2 treatment, although protein content remained unaltered. These data demonstrate the novel regulation of E2 on PPARα and genes encoding key proteins that are pivotal in regulating skeletal muscle lipid oxidative flux.

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Introduction

The ovarian hormones exert significant control on cellular lipid homeostasis in metabolically active tissues such as the liver and skeletal muscle (for review see Campbell & Febbraio 2001a). In skeletal muscle, ovariectomy suppresses the maximal activity of both carnitine palmitoyltransferase I (CPT I) and β-3-hydroxyacyl CoA dehydrogenase (β-HAD), key enzymes in lipid oxidation. The activity of these enzymes is up-regulated following treatment with 17β-estradiol (E2) in red gastrocnemius (type I muscle fiber), but not white gastrocnemius or vastus medialis (predominantly type II muscle fibers) (Campbell & Febbraio 2001b, Beckett et al. 2002). The mechanism by which E2 regulates the activity of lipid oxidative enzymes in type I oxidative skeletal muscle remains unclear. However, in aromatase-deficient (ArKO) mice, exogenous E2 is necessary to maintain the gene expression and enzyme activity of the hepatic β-oxidation pathway (Nemoto et al. 2000, Tada et al. 2001), suggesting a capacity for E2 to regulate the expression of genes involved in lipid oxidation.

The actions of E2 and progesterone on the expression of genes encoding proteins of particular importance in influencing the flux of fatty acids and glucose through the mitochondrial pathway in type I muscle fibers has yet to be analyzed. Therefore, this study aimed to examine the mRNA
abundances of CPT I, β-HAD, and pyruvate dehydrogenase kinase 4 (PDK4) following ovariectomy with subcutaneous infusion of E$_2$, progesterone and combined E$_2$ and progesterone (E+P) in red gastrocnemius muscle. PDK4 is of interest as the protein product of this gene phosphorylates and inactivates the pyruvate dehydrogenase complex, a key regulatory site for glucose oxidative flux (Sugden et al. 2000).

Skeletal muscle contains both functional estrogen (Katzenellenbogen et al. 1993) and progesterone (Shughrue et al. 1988) receptors. These receptors, as has been described in non-classical sex steroid target organs including vascular and adrenal tissue, influence the expression of a variety of genes (Gargett et al. 2002, Serova et al. 2002). However, one characteristic feature of the genes involved in fatty acid oxidation is their sensitivity and regulation by the peroxisomal proliferator-activated receptor (PPAR) family of transcription factors (Gulick et al. 1994, Mascaro et al. 1998, Wu et al. 1999). Both the PPARα and PPARγ isoforms, which are present in skeletal muscle (Lemberger et al. 1996, Loviscach et al. 2000), contribute to regulating the expression of many genes necessary for lipid uptake and oxidation in skeletal muscle (Camirand et al. 1998, Minnich et al. 2001, Muoio et al. 2002b). Cross-talk between the nuclear receptors is not uncommon, and it might be speculated that the ovarian hormones may interact with the PPARs in their regulation of cellular lipid metabolism. Indeed, in estrogen sensitive tissue, E$_2$ has been demonstrated to induce the formation of a prostaglandin D$_2$ metabolite capable of acting as a ligand for PPARγ (Ma et al. 1998), suggesting that the effects of E$_2$ on lipid metabolism could be realized through this signaling pathway. Therefore, a second aim of the current study was to examine the actions of E$_2$ and progesterone on skeletal muscle gene expression and protein abundance of PPARα and PPARγ.

Materials and methods

Experimental design

Female Sprague–Dawley rats, 12–15 weeks old and weighing 203·1 ± 4·5 g (mean ± S.E.) were used in these experiments. All animals were housed in a temperature-controlled room (21 ± 2 °C) with a light/darkness cycle of 12 h. Water was available ad libitum and rats were given 20 g standard rat chow per day, to control food intake. All experimental interventions were formally approved by the Animal Research Ethics Committee, University of Melbourne.

Rats were bilaterally ovariectomized or sham-operated under sodium brieatal anesthesia (60 mg/kg, i.p.). Groups of ovariectomized rats were treated immediately with time release hormone pellets (Innovative Research of America, Sarasota, FL, USA) inserted subcutaneously, administering either 17β-estradiol (E$_2$) (2·5 µg/day), progesterone (1·5 mg/day), or both hormones in combination (E+P). Groups of ovariectomized (Ovx) and sham-operated (Sham) rats were treated with vehicle-only placebo pellets. The Sham group rats were tested on varying days of their estrous cycle to get an inclusive group of ‘control’ animals. Rats were treated for fourteen full days post-operation, and on the morning of day 15 they were suffocated with CO$_2$ (80:20, CO$_2$:O$_2$). A midline incision was made and the diaphragm was cut to ensure death. A cardiac puncture was performed, the blood spun at 7000 g for 2 min, and the plasma removed and stored at −80 °C for analysis of the sex steroids. Efficacy of surgical ovariectomy and steroid treatments were determined with the analysis of plasma E$_2$ and progesterone using RIA kits (Diagnostic Products Corporation, Los Angeles, CA, USA) at the end of the treatment period. Furthermore, plasma insulin was also measured using a commercially available RIA kit (Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden) and free fatty acids (FFA) were determined by spectrophotometric assay analysis for free fatty acids (FFA-C test kit, Wako Chemicals, Neuss, Germany). The muscles of the hindlimb were rapidly exposed, the gastrocnemius removed and the red portion dissected out. Tissues were snap frozen in liquid N$_2$ and stored at −80 °C for further analysis.

Real-time PCR

Tissue RNA was extracted using a modified acid guanidinium thiocyanate–phenol–chloroform extraction method for total RNA (Chomczynski & Sacchi 1987). Samples (0·5 µg/µl RNA) were reverse transcribed to cDNA using the commercially available Promega Reverse Transcription Kit (Promega, USA). The primers were designed using
the Primer Express software package version 1.0 (Applied Biosystems, Foster City, CA, USA) using gene sequences obtained from GenBank (see Table 1 for GenBank accession numbers and sequences). BLAST search for each primer confirmed homologous binding to the desired mRNA. Real-time PCR was used to quantify mRNA expression using SYBR Green technology. Briefly, 12 ng cDNA were amplified with forward and reverse primers and SYBR Green Universal PCR Master Mix (Applied Biosystems) for 40 cycles of PCR using the GeneAmp 5700 sequence detection system (Applied Biosystems). Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double stranded DNA. Tissue mRNA levels were quantified using critical threshold emission increases above a threshold level (10 times the S.D. of the background). Non-specific changes in gene expression were corrected for running the constitutively expressed β-actin mRNA (Whitley et al. 2000), and results were corrected with reference to the amount of β-actin mRNA. End products were checked for contamination and ‘mispriming’ by verifying the denaturing peaks and ensuring the presence of only one product.

### Immunoblotting

Samples were thawed in ice-cold lysis buffer (40 µl/mg muscle) containing 1% SDS, 1% IGEPAL (Sigma, USA), and Complete Protease Inhibitor Cocktail (Sigma), then homogenized with a Polytron PT 1200 (Kinematica, Littau-Lucerne, Switzerland). Homogenates were spun at 10 000 r.p.m. (4 °C, 15 min) and the supernatants removed and analyzed for total protein (BCA protein assay kit, Pierce, Rockford, IL, USA). Denatured total proteins, 120 µg from each sample, were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for 2 h at 50 mA/membrane (Nova Blot, Pharmacia Biotech, USA). Membranes were blocked for 1 h with 5% skim milk in Tris-buffered saline (50 mM Tris–HCl, 750 mM NaCl, 0·25% Tween), and were incubated overnight at 4 °C with either a polyclonal anti-PPARα or anti-PPARγ antibody (1 in 200 dilutions) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed (4 × 5 min), followed by a 60-min incubation with anti-rabbit IgG conjugated to horse-radish peroxidase (1 in 8000 dilution), then washed again. Immunoreactive bands were detected using enhanced chemiluminescence (Super-Signal, Pierce). An internal control of previously extracted rat muscle was used in each gel to normalize for variation in signal observed across the membranes. The resulting autoradiographs were analyzed by laser scanning densitometry (GS710 Calibrated Imaging Densitometer, BioRad, USA) and quantified with QuantityOne (BioRad), version 4.1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank locus</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>X00351</td>
<td>Forward: 5′-GAC AGG ATG CAG AAG GAG ATT ACT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5′-TGA TCC ACA TCT GCT GGA AGG T-3′</td>
</tr>
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<td>β-HAD</td>
<td>AF095449</td>
<td>Forward: 5′-TCG TGA CCA GGC AAT TCG T-3′</td>
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<td></td>
<td></td>
<td>Reverse: 5′-CCG ATG ACC GTC ACA TGC T-3′</td>
</tr>
<tr>
<td>PDK4</td>
<td>AF034577</td>
<td>Forward: 5′-GGG ATC TCG CCT GGC ACT TT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5′-CAC ACA TTC ACG AGG CAG CA-3′</td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5′-CTG GAC AAG AGG CGA ACA CA-3′</td>
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Table 2 Characteristics of the animals in the various experimental groups. Results are means ± S.E.M. (n=8 per group)

<table>
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<tr>
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<th>Sham</th>
<th>Ovx</th>
<th>E₂</th>
<th>Progesterone</th>
<th>E₂+Progesterone</th>
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<tbody>
<tr>
<td>Final body weight (g)</td>
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<td>219±4</td>
<td>216±4</td>
<td>216±5</td>
<td>218±4</td>
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<tr>
<td>Plasma FFA (mmol/l)</td>
<td>0·69±0·06</td>
<td>0·70±0·04</td>
<td>0·76±0·06</td>
<td>0·64±0·05</td>
<td>0·73±0·04</td>
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<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>34·9±4·2</td>
<td>14·4±1·0*</td>
<td>45·0±2·4†</td>
<td>15·5±0·7*</td>
<td>49·9±1·9†</td>
</tr>
<tr>
<td>Plasma progesterone (ng/ml)</td>
<td>25·0±6·3</td>
<td>7·5±0·6*</td>
<td>5·6±0·9*</td>
<td>44·3±1·1†</td>
<td>44·5±1·9†</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td>44·9±4·1</td>
<td>51·0±3·5</td>
<td>46·6±3·5</td>
<td>52·1±2·3</td>
<td>44·6±2·9</td>
</tr>
</tbody>
</table>

*P<0·05 compared with Sham; †P<0·05 compared with Ovx.

Statistics

All data are reported as means ± S.E.M. Statistical comparisons were performed using ANOVA, with a Neuman–Keuls F test for post hoc comparison. Significance was accepted at P<0·05.

Results

Animal characteristics

The animal characteristics are presented in Table 2 (n=8 per group). Plasma E₂ and progesterone concentrations confirmed the efficacy of the surgeries and sex steroid treatments. No effort was made to control for the ovarian status of the sham operated (Sham) animals, thus considerable variation is demonstrated in both the plasma E₂ and progesterone values from this group. No significant differences were found among the mean values of either initial or final body weight. Plasma insulin levels were also similar between all treatment groups (Table 2).

PPARα gene expression and protein abundance

The gene expression and protein abundance of PPARα in the various treatment groups are presented in Fig. 1. The absence of the ovarian hormones in the ovariectomized (Ovx) rats lowered (P<0·05) the mRNA expression of PPARα compared with sham-operated (Sham) rats. Administration of E₂ to ovariectomized animals markedly increased PPARα mRNA abundance in comparison with both the Ovx and Sham rats (P<0·05). Similarly, administration of progesterone, or a combination of both hormones, resulted in elevated (P<0·05) PPARα mRNA levels compared with ovariectomy, although not to the same extent. The protein abundance of PPARα

![Figure 1](image-url)
followed a very similar pattern to its mRNA expression, although the only significant increase in protein was observed in the E2-treated rats compared with Ovx rats ($P<0.05$). There was, however, a trend for lower PPAR$\alpha$ protein content in Ovx rats compared with Sham rats ($P=0.073$).

**PPAR$\gamma$ gene expression and protein abundance**

The gene expression and protein abundance of PPAR$\gamma$ in the various treatment groups are presented in Fig. 2. E$_2$ treatment resulted in greater ($P<0.05$) PPAR$\gamma$ gene expression, compared with both Ovx and Sham rats. However, PPAR$\gamma$ mRNA levels were similar between Ovx and Sham rats, and in progesterone and E+P rats. Interestingly, this increase in gene expression did not translate into an increase in protein abundance, indicating that post-transcriptional regulation may attenuate any effect of E$_2$ on PPAR$\gamma$. Protein abundance of PPAR$\gamma$ was similar across all treatment groups.

**CPT I, $\beta$-HAD and PDK4 gene expression**

The gene expressions of CPT I, $\beta$-HAD, and PDK4 are presented in Fig. 3. The pattern of mRNA expression of all three enzymes was similar to that of both PPAR$\alpha$ mRNA expression and protein abundance, providing further evidence of their PPAR$\alpha$ responsiveness. Specifically, treatment of ovariectomized rats with E$_2$ resulted in a ~sevenfold increase ($P<0.05$) in CPT I mRNA expression compared with both the Ovx and Sham groups. Although progesterone and E+P rats also appear to increase mRNA expression of CPT I these values were not statistically significant ($P=0.29$ and $P=0.39$ respectively). There were also no significant differences in expression of $\beta$-HAD mRNA. Conversely, PDK4 gene expression was decreased ($P<0.05$) in Ovx rats compared with all treatment groups, while E$_2$ rats had increased ($P<0.05$) mRNA abundance compared with all treatment groups, but in particular, a 23-fold increase compared with Ovx rats.

**Discussion**

The results from this study demonstrate that E$_2$ is a potent regulator of PPAR$\alpha$ mRNA and protein, in addition to enhancing the expression of genes critical in the regulation of lipid oxidation in skeletal muscle. The marked actions of E$_2$ are exemplified by the dramatic increases in CPT I and PDK4 mRNA. The importance of the ovarian hormones in maintaining PPAR$\alpha$ levels is further emphasized by the decrease in mRNA expression, and a trend for lower protein content, in Ovx rats compared with Sham rats. Conversely, the results of progesterone treatment alone did not differ from Sham rats; however, when given in combination
with E\textsubscript{2} it did suppress the up-regulation of mRNA expression and protein content of PPAR\textalpha{} observed in E\textsubscript{2}-treated rats. This is similar to previously observed effects of progesterone in skeletal muscle lipid metabolism (Campbell & Febbraio 2001b), and indicates that these differences may result from transcriptional regulation. Furthermore, these results highlight the interaction between E\textsubscript{2} and progesterone \textit{in vivo}, suggesting that cross-talk between the ovarian hormones likely moderates the effect of E\textsubscript{2} on metabolism. Together, these data demonstrate significant regulation of skeletal muscle lipid oxidation genes and PPAR\textalpha{} by the ovarian hormones.

Little is known about the regulation of the expression of the genes encoding for PPAR\textalpha{} and PPAR\textgamma{}. Expression of PPAR\textalpha{} is strongly induced at the transcriptional level by glucocorticoids (Lemberger \textit{et al.} 1994), although this is attenuated by the presence of insulin (Steineger \textit{et al.} 1994). However, the pathway involved in this regulation is still unknown. Importantly, changes in this study occurred in the absence of changes in insulin levels, and thus are more likely to be due to a direct effect of the ovarian hormones. Alternatively, the expression of PPAR\textgamma{} is thought to be trans-activated by the CCAATT enhancer binding protein alpha (C/EBP\textalpha{}) (Hamm \textit{et al.} 2001). E\textsubscript{2} has previously been linked with C/EBP\textalpha{} in the control of some of its target genes (Calkhoven \textit{et al.} 1997). An interesting finding of the current study was the specific control by E\textsubscript{2} on protein levels of the PPARs in skeletal muscle. Despite similar enhancement in the gene expression of both PPAR\textalpha{} and PPAR\textgamma{}, E\textsubscript{2} resulted in a selective up-regulation of the protein abundance of PPAR\textalpha{} alone. Recent data suggests that PPAR\textalpha{} ligand binding, which initiates dimerization with the nuclear binding partner retinoid X receptor (RXR), minimizes protein degradation by stabilizing the PPAR\textalpha{} protein (Hirotani \textit{et al.} 2001). It is tempting to speculate that in skeletal muscle E\textsubscript{2} may generate a PPAR\textalpha{} specific ligand, in contrast to the previous evidence for a PPAR\textgamma{} ligand in the mallard duck uropygial gland (Ma \textit{et al.} 1998). Further studies to examine the nature of E\textsubscript{2} activated PPAR ligands in skeletal muscle are required, particularly given the clinically important ability of PPAR ligands to decrease insulin resistance (Ye \textit{et al.} 2001).

Continuous administration of E\textsubscript{2} up-regulated the expression of all analyzed genes, with the
exception of β-HAD. Indeed, E2 administration increased the gene expression of PDK4 (25-fold increase) and CPT I (7-fold increase) compared with Ovx rats. These two enzymes represent important regulatory sites in the coordinated control of lipid oxidative pathways in skeletal muscle. PDK4 functions by phosphorylating and inactivating pyruvate dehydrogenase (PDH), which catalyzes the oxidation of pyruvate to acetyl-CoA (Sugden & Holness 1994). Previous studies have demonstrated regulation of PDK4 gene expression in response to exercise (Muioio et al. 2002a), starvation (Sugden et al. 2000, Holness et al. 2002b), and insulin treatment (Wu et al. 1999), in parallel to modifications in skeletal muscle lipid oxidation elicited by these treatments. Concomitantly, CPT I catalyzes the initial and rate-limiting step in the transport of fatty acid into mitochondria, thus activation of this gene increases fatty acid oxidation by promoting their entry into the mitochondria. Up-regulation of both of these enzymes will allow for a greater lipid oxidative capacity and provide a molecular mechanism for the impact of E2 on lipid oxidation observed previously in rodent studies examining lipid oxidation either at rest or during exercise with hormonal treatment (Campbell & Febbraio 2001b). However, these actions of E2 cannot be generalized to other tissues and may vary markedly depending upon the administered E2 dosage. Normalized expression of lipid metabolic genes has previously been demonstrated in the liver of estrogen-deficient aromatase knockout mice supplemented with exogenous E2 (Nemoto et al. 2000, Toda et al. 2001). In contrast, higher doses of E2 delivered via subcutaneous pellet at markedly higher doses than those used in the present study (5 mg/day vs 2·5 µg/day) have been shown to suppress liver CPT I mRNA (Gower et al. 2002). It is unclear whether these results demonstrate tissue-specific control or are due to a greater infused dose.

While these data demonstrate a significant response to E2 alone, the data suggest complex interplay in the gene expression response of E2 in the presence of progesterone. In the sham-treated animals, despite comparable E2 levels (see Table 2), the presence of approximately 4-5-fold higher progesterone levels appears to markedly alter the mRNA response. Sham-treated animals had lower levels of all genes, although only PDK4 and PPARα were significantly suppressed. However, the actions of progesterone appear not to be entirely inhibitory as progesterone administration alone tended to result in greater mRNA levels than in both the sham and ovariectomized groups. Thus, these data demonstrate that the relationship between the ovarian hormones and lipid oxidative genes is complex, suggesting either a complex interplay between the ovarian hormones themselves or the involvement of additional factors regulating the expression of the analyzed genes.

Despite the significant impact of the ovarian hormones in determining gene abundance, caution is necessary when extrapolating from alterations in mRNA expression to protein content. Many factors, including mRNA stability, translation efficiency, post-translational modifications and abundance of relevant ligands, exert significant control on the final abundance and activity of the gene product (Orphanides & Reinberg 2002). However, the changes in CPT I mRNA are broadly consistent with the previously observed changes in maximal enzyme activities following identical treatment protocols (Campbell & Febbraio 2001b). While previously our data demonstrated significant suppression of CPT I in Ovx rats compared with the sham-operated controls (Sham), the gene responses despite being lowered by Ovx failed to reach statistical significance. In the present study, no significant alterations in β-HAD gene abundances were demonstrated, despite our previous demonstration of suppressed maximal enzyme activity following Ovx treatment when compared with either Sham or E2 treatment. Little is known of the transcriptional control of β-HAD, with the absence of significant alteration in gene expression suggesting regulation of this gene is exerted predominantly via post-translational mechanisms.

There are several possible mechanisms by which E2 treatment may mediate the observed alterations in the expression of PPARα, PDK4 and CPT I. In addition to acting directly via the receptors located within skeletal muscles (Katzenellenbogen et al. 1993), there is the possibility of indirect regulation through alterations in nutrients and/or hormones. PDK4 has been shown to be regulated by alterations in fatty acid availability, induced by either high fat feeding, starvation or fat infusion (Holness et al. 2002b), in addition to body mass reduction, insulin availability and insulin resistance (Majer et al. 1998, Rosa et al. 2003). However, in
the present study no alterations in either plasma fatty acids or insulin were demonstrated. Given the substantial role that PPARα plays in the regulation of the analyzed genes and lipid metabolism (Mascaro et al. 1998, Wu et al. 1999, Minnich et al. 2001, Ye et al. 2001, Muoio et al. 2002b), it is tempting to attribute the effects seen in this study as due, in part, to the elevated levels of PPARα following E2 treatment. However, recent data demonstrate that this relationship is complex. First, the actions of E2 on lipid metabolism may act independently of PPARα (Djouadi et al. 1998). In PPARα knockout mice, inhibition of cellular fatty acid oxidation resulted in 100% mortality in male, but only 25% mortality in female mice. The pre-treatment of males with E2 reversed this effect, demonstrating that E2 signaling pathways regulate lipid metabolism independently of PPARα (Djouadi et al. 1998). Secondly, the expression of PDK4 is normally activated by fasting, high-fat feeding and exercise in PPARα knockout mice (Holness et al. 2002a, Muoio et al. 2002a). These data are further supported by the demonstration that administration of the PPARα agonist, WY14,643, fails to enhance PDK4 protein expression in soleus muscle (predominantly type I muscle fibers) (Holness et al. 2002a). Additional factors implicated in the control of PDK4, including insulin and plasma free fatty acids, were unaltered in both the present study and previous analysis in similarly treated rats (Campbell & Febbraio 2001b). Clearly, further analysis is required to determine whether PPARα contributes in any part to the E2-mediated response and the nature of the factors involved in the regulation of lipid-oxidative genes following E2 administration.

To our knowledge no previous studies have identified E2 as a transcriptional activator of members of the PPAR family in skeletal muscle. These studies demonstrated marked regulation of the PPARα isoform in skeletal muscle by E2. Furthermore, E2 administration markedly regulated CPT1 and PDK4, genes encoding key proteins in regulating lipid oxidative flux. It is unclear from the present findings whether there is a functional relationship between the ovarian hormones, PPAR gene expression, and the subsequent regulation of lipid metabolism. However, in this context, it would be interesting to further investigate the interaction between the ovarian hormones and PPAR activated fatty acid gene expression.

Acknowledgement

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References


