Progesterone increases skeletal muscle mitochondrial H$_2$O$_2$ emission in nonmenopausal women

Daniel A. Kane,1,2 Chien-Te Lin,1,2 Ethan J. Anderson,1,3 Hyo-Bum Kwak,1,2 Julie H. Cox,1,2 Patricia M. Brophy,1,2 Robert C. Hickner,1,2,4 P. Darrell Neufer,1,2,4 and Ronald N. Cortright1,2,4

1The East Carolina Diabetes and Obesity Institute, 2Department of Exercise and Sport Science, 3Department of Pharmacology and Toxicology, 4Department of Physiology, East Carolina University, Greenville, North Carolina

Submitted 1 July 2010; accepted in final form 20 December 2010

were incubated in P4 (60 nM), E2 (1.4 nM), or both. P4 alone sied from five nonmenopausal women in the early follicular phase drial function, saponin-permeabilized vastus lateralis myofibers biop-
In the present study, it was hypothesized that a link between skeletal muscle mE_{44202}, insulin sensitivity, and/or the menstrual cycle hormones E2 and P4 would exist in women. To this end, serum E2 and P4 were measured in a group of eight insulin-resistant and 24 insulin-sensitive subjects on the same day they were biopsied for skeletal muscle mitochondrial function analyses. Additionally, nonmenopausal female subjects were biopsied in the menstrual cycle early follicular phase for ex vivo incubation experiments using elevated midluteal phase-relevant (i.e., ovulatory) E2 (11, 41), or both. Our findings reveal that serum levels of P4 influence the mitochondrial O2 flux (JO₂) and mE_{44202} linked to insulin resistance. This effect may be an acute, posttranslational phenomenon as defined by Stern et al. (46). Group A subjects were therefore described as insulin sensitive (IS, HOMA-IR < 3.60) or insulin resistant (IR, HOMA-IR > 3.60; Table 1).

Skeletal muscle biopsies were obtained from the lateral aspect of the vastus lateralis by the percutaneous needle biopsy technique, with constant suction under local subcutaneous anesthesia (1% Lidocaine). A portion of each biopsy sample was flash-frozen in liquid N₂ for subsequent protein analysis. The remaining portion of the biopsy (~50 mg wet wt) was transferred to ice-cold physiological relaxing buffer (buffer X) for transport, on ice, to the laboratory (~5 min) for dissection, permeabilization, and mitochondrial function assays for both Groups A and B.

Preparation of permeabilized human myofibers. This technique is partially adapted from previous methods (26, 48) and has been described previously (3–5, 25). The technique was used for both Groups A and B. After dissection, connective tissue was removed, and fiber bundles were separated with fine forceps under a binocular dissecting microscope in ice-cold 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 5 DTT, 20 tauanine, 5.7 ATP, 15 Pcr, 6.56 MglCl₂-6H₂O (pH 7.4, 295 mOsm). After separation, myofiber bundles were placed in 4°C buffer X containing 30 μg/ml saponin for 30 min and then washed individually in ice-cold buffer Z, containing (in mM) 110 K-MES, 35 KCl, 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5 DTT, 20 tauanine, 5.7 ATP, 15 Pcr, 6.56 MglCl₂-6H₂O (pH 7.4, 295 mOsm) until analysis (~1 h). To determine the acute effects of E2 and P4 on mitochondrial function, washes for the permeabilized myofibers obtained from subjects in Group B contained hormone treatments: two of the Z washes contained 60 nM P4, two contained 1.4 nM E2, two contained 60 + 1.4 nM P4 + E2, and two contained vehicle (DMSO, 0.2%). Fibers from both Groups A and B used in the H₂O₂ emission experiments were briefly washed in cold buffer Z containing 10 mM Na-pyrophosphate prior to analysis to prevent Ca²⁺-independent contraction.

The concentrations of P4 and E2 used in the ex vivo incubation experiments were chosen in consultation with the serum clinical reference values specified in Tietz Textbook of Clinical Chemistry and Molecular Diagnostics (41). These luteal-phase reference values for nonmenopausal women are as follows (in nM): 6.4–79.5 P4 and 5.3–70.2 E2. These luteal-phase reference values were partially adapted from previous methods (26, 48) and have been described previously (3–5, 25). The technique was used for both Groups A and B. After dissection, connective tissue was removed, and fiber bundles were separated with fine forceps under a binocular dissecting microscope in ice-cold buffer X, containing (in mM) 60 K-MES, 35 KCl, 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5 DTT, 20 tauanine, 5.7 ATP, 15 Pcr, 6.56 MglCl₂-6H₂O (pH 7.4, 295 mOsm). After separation, myofiber bundles were placed in 4°C buffer X containing 30 μg/ml saponin for 30 min and then washed individually in ice-cold buffer Z, containing (in mM) 110 K-MES, 35 KCl, 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5 DTT, 20 tauanine, 5.7 ATP, 15 Pcr, 6.56 MglCl₂-6H₂O (pH 7.4, 295 mOsm) until analysis (~1 h). To determine the acute effects of E2 and P4 on mitochondrial function, washes for the permeabilized myofibers obtained from subjects in Group B contained hormone treatments: two of the Z washes contained 60 nM P4, two contained 1.4 nM E2, two contained 60 + 1.4 nM P4 + E2, and two contained vehicle (DMSO, 0.2%). Fibers from both Groups A and B used in the H₂O₂ emission experiments were briefly washed in cold buffer Z containing 10 mM Na-pyrophosphate prior to analysis to prevent Ca²⁺-independent contraction.

Mitochondrial respiration and H₂O₂ emission measurements in permeabilized human myofibers. O₂ consumption rate was measured by polarographic high-resolution respirometry (Oroboros O₂ K Oxygraph, Innsbruck, Austria) at 30°C in air-saturated (~220–150 μM O₂) buffer Z + 20 mM creatine hydrate and 50 μM N-benzyl-p-toluene sulfonamide (BTS, an inhibitor of myosin II ATPase) under the following protocol: 25 μM palmityl carnitine + 1 mM malate.
followed by sequential additions of 2 mM ADP, 10 μM cytochrome c, 2 mM glutamate, 3 mM succinate, 10 μg/ml oligomycin (inhibitor of mitochondrial ATP synthase), and finally 2 μM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a protonophoric uncoupler). With regard to the acute E2 and P4 incubation experiments, neither oligomycin nor FCCP were added due to time constraints associated with multiple testing.

H2O2 emission was measured at 30°C in buffer Z during state 4 respiration (10 μg/ml oligomycin) by continuously monitoring oxidation of Amplex red (excitation/emission λ = 563/587 nm) using a Fluorolog-3 (Horiba Jobin Yvon, Edison, NJ) spectrophotometer under the following protocol: 25 μM palmitoylcarnitine + 1 mM malate followed by sequential additions of 2 mM glutamate, 3 mM succinate, and 10 mM glyceraldehyde. At the conclusion of each experiment, permeabilized fiber bundles were washed in distilled H2O to remove salts and then freeze-dried in a lyophilizer (LabConco). Mitochondrial respiration rates (Jo2) are expressed as picomoles per minute per milligram dry weight and H2O2 emission rates (mE1H2O2) as picomoles per minute per milligram dry weight.

As with the buffer Z washes, respective treatments of P4 and/or E2 conditions were also created by adding the hormones (dissolved in DMSO) to the respective experimental chamber/cuvette (final DMSO concentration < 2.0%). Neither O2 consumption nor Amplex red fluorescence (standard curve) was differentially affected by any of the treatment conditions in the absence of biological sample.

Optimization of the saponin-permeabilized myofiber preparation for human female subjects. Pilot data collected with permeabilized fibers from female subjects by use of standard protocols (27) exhibited abnormally low rates of Jo2. This was accompanied by a more than 40% increase in complex I-linked Jo2 (i.e., glutamate + malate substrates) after addition of 10 μM cytochrome c (Supplemental Fig. 1), indicating disruption of the outer mitochondrial membranes, likely due to excessive permeabilization. We therefore tested lower concentrations of saponin and found that 30 μg/ml saponin (vs. 50 μg/ml) resulted in optimal mitochondrial function in permeabilized fibers from these human subjects (Supplemental Fig. 1; supplementary material is found with the online version of this paper at the Journal’s website). Moreover, it was determined that the percent coefficient of variation (%CV) for repeated measurements of respirometric O2 flux (Jo2) during state 3 respiration in four myofibers permeabilized with 30 μg/ml saponin was less than one-half the %CV of the Jo2 measured under the same conditions but permeabilized with the standard 50 μg/ml saponin.

Statistics. Data are presented as means ± SE. Statistical analyses were performed with GraphPad Prism (GraphPad Software), using two-way ANOVA (as appropriate) with Bonferroni’s post hoc method for analysis of significance among groups. Pearson bivariate correlations and variable adjustments for %BF were performed using ANCOVA with SPSS 17 software. Dietary record data were processed using Nutritionist Pro software (Axxya Systems). The α-level of statistical significance was set a priori at P < 0.05.

RESULTS

Subject data are presented in Tables 1 and 2. Dietary record assessments indicated no difference between groups with regard to body mass (kg) proportional daily energy (kcal/kg), fiber (g/kg), or macronutrient intake (i.e., g carbohydrate, fat, or protein; P = 0.85, data not shown).

Mitochondrial H2O2 emission and respiratory O2 flux in permeabilized myofibers from insulin-sensitive and insulin-resistant subjects. We hypothesized that serum levels of E2 or P4 would associate with measured mE1H2O2 and/or HOMA-IR in the Group A subjects. Interestingly, only serum P4 concentration (nM, log transformed) correlated with P-C/MG-S-supported mE1H2O2 (r = 0.53; P < 0.01; Fig. 1). This supports the possibility for P4 as the sex steroid responsible for increasing mE1H2O2 and not E2.

The rates of P-C/MG-S-supported mE1H2O2 in permeabilized myofibers from the IR women were more than 50% greater than that of the IS women (P < 0.05; Fig. 2A). This supports a link between mE1H2O2 and insulin resistance. Initially, we did not observe an effect of obesity (%BF) on rates of mE1H2O2 (data not shown). However, it has been suggested that cytokines originating from adipose tissue may influence insulin sensitivity (reviewed in Ref. 35). Interestingly, statistically controlling for the potential secondary effect of adiposity (%BF; ANCOVA) on skeletal muscle mE1H2O2 and HOMA-IR revealed a difference in the mE1H2O2 between IS and IR women (P < 0.01; Fig. 2A, inset). This supports the hypothesis that skeletal muscle mE1H2O2 influences insulin resistance and further suggests that adipose modulates insulin sensitivity.

To examine whether the link between mE1H2O2 and insulin resistance might be mirrored in mitochondrial respiration and/or coupling, we measured Jo2 in permeabilized fibers from IS and IR women and subsequently calculated ratios of respiratory control (Table 3). While no differences in Jo2 were detected with insulin resistance, after adjusting for %BF, a difference in the uncoupling control ratio (UCR, ratio of uncoupled Jo2 to oligomycin-inhibited Jo2) was detected (P < 0.05; Table 3). This suggests that skeletal muscle mitochondrial coupling may be greater in IR than in IS women and that this relationship may be confounded by adiposity. Furthermore, when expressed relative to Jo2, the rate of mE1H2O2 was still significantly greater in the IR than the IS women (P < 0.05; Fig. 2B), suggesting that the increase mE1H2O2 with IR was independent of differences in Jo2.

Acute ex vivo effects of P4 and E2 on mitochondrial function in permeabilized myofibers. To test the acute, ex vivo effects of E2 and P4 on mitochondrial function, skeletal muscle fibers were incubated in either 1.4 nM E2, 60 nM P4, or both for 1–2 h after permeabilization but before and during the experimental measurements. As Fig. 3 illustrates, a trend, manifested in significant main effects for steroid hormones on Jo2 was present in the respirometric experiments. Compared...
suggests that P4 exerts an inhibitory affect on complex I (+G) and possibly also complex II (+S). Interestingly, when combined with E2, P4 (i.e., E2 + P4) did not significantly inhibit JO2 (Fig. 3). When combined with the results of E2 treatment alone (i.e., no effect on JO2), the respirometric data with E2 + P4 suggest that E2 may prevent the inhibitory effects of P4 on JO2.

When rates of mEH2O2 were measured in the Group B muscle fibers treated with P4 and/or E2 acutely postpermeabilization, significant differences were observed after the addition of succinate and also glycerolphosphate (Fig. 4). Compared with control (DMSO), E2 + P4 treatment resulted in significantly greater rates of mEH2O2 during state 4 respiration supported by either P-C/MG + succinate (+S; P < 0.05) and P-C/MG + glycerolphosphate (+Gp, P < 0.01; Fig. 4). Moreover, P4 alone significantly increased mEH2O2 compared with DMSO during P-C/MG + Gp (P < 0.01; Fig. 4). Inter-

Table 3. JO2 and control in insulin-sensitive and insulin-resistant women

<table>
<thead>
<tr>
<th>Substrate Conditions</th>
<th>Insulin Sensitive (n = 23)</th>
<th>Insulin Resistant (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-C/M</td>
<td>8.8 ± 1.2</td>
<td>8.4 ± 2.2</td>
</tr>
<tr>
<td>P-C/M3</td>
<td>49.7 ± 2.7</td>
<td>42.2 ± 4.9</td>
</tr>
<tr>
<td>P-C/MG</td>
<td>145.5 ± 7.8</td>
<td>147.8 ± 14.1</td>
</tr>
<tr>
<td>P-C/MGS</td>
<td>207.8 ± 9.7</td>
<td>198.0 ± 17.7</td>
</tr>
<tr>
<td>P-C/MGSO</td>
<td>49.7 ± 2.8</td>
<td>40.3 ± 5.0</td>
</tr>
<tr>
<td>P-C/MGSO</td>
<td>253.6 ± 12.0</td>
<td>245.9 ± 21.8</td>
</tr>
<tr>
<td>RCR</td>
<td>7.2 ± 0.8</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td>ACR</td>
<td>1.2 ± 0.0</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>UCR</td>
<td>5.2 ± 0.2</td>
<td>6.4 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. JO2, respiratory O2 flux (pmol·s⁻¹·mg dry wt⁻¹); Substrates: P-C/M, 25 μM palmitoylcarnitine + 1 mM malate; P-C/M3, P-C/M + 2 mM ADP; P-C/MG, State 3 + 2 mM glutamate; P-C/MGS, P-C/MG, G, State 3 + 3 mM succinate; P-C/MGSO, P-C/MGS + 10 μM oligomycin; P-C/MGSOU, P-C/MGS + 2 μM FCCP. RCR, respiratory control ratio = [JO2 (P-C/M)]/JO2 (P-C/M3)⁻¹; ACR, adenylate control ratio = [JO2 (P-C/MGSO)]/JO2 (P-C/M)⁻¹; UCR, uncoupling control ratio = [JO2 (P-C/MGSOU) - JO2 (P-C/MGSO)]/JO2 (P-C/MGSO)⁻¹. *P < 0.05 vs. Insulin Sensitive.
estingly, however, E2 alone did not increase mEH2O2 (Fig. 4). Because additions of succinate and glycerophosphate are known to elicit reverse electron flow-mediated superoxide production at complex I (34), these data suggest that P4 increases the potential for complex I-linked mitochondrial H2O2 production. Furthermore, the protection conferred by E2 with regard to the inhibitory effects of P4 on J02 (Fig. 3) was not paralleled in the mEH2O2 measurements (Fig. 4). Taken together, these data support a model whereby E2 prevents P4-inhibited complex I-linked, and possibly complex II-linked J02, and conversely a model whereby E2 does not attenuate a P4-mediated increase in complex I-linked mEH2O2 within the context of elevated menstrual cycle levels of E2 and P4 (Fig. 5).

DISCUSSION

In this study, it was hypothesized that in women the ovarian steroid hormones E2 and P4 influence insulin sensitivity via alterations in the production of mitochondrial H2O2 in skeletal muscle. The novel findings presented here demonstrate that menstrual cycle-relevant concentrations of E2 and P4 (11, 41) can directly affect mitochondrial function in skeletal muscle. Furthermore, a clear relationship emerged between P4 measured in serum and mEH2O2 measured in biopsy samples. Finally, these results provide further evidence for a link between skeletal muscle mEH2O2 and insulin resistance in humans.

As early as 1963, Chance et al. (13) reported that high concentrations of P4 (i.e., mM) exhibited an inhibitory, “rotenone-like” effect on complex I-linked respiration and pyridine nucleotide reduction in mitochondria isolated from pigeon heart. A review (45) of this, and subsequent publications regarding posttranslational effects of experimental steroid hormone concentrations on the function of isolated mitochondria questioned the physiological relevance of these findings based on the supraphysiological concentrations of steroid hormones employed. To clarify discrepancies in the earlier literature, the current study utilized permeabilized myofibers from women in the early follicular phase of the menstrual cycle incubated with high late-follicular phase-relevant concentrations of E2 (1.4 nM) and midluteal phase-relevant P4 (60 nM) (11, 41). In agreement with the results of reports demonstrating an inhibitory effect of P4 on mitochondrial respiration in animals (13, 16, 20, 21), we observed a significantly lower J02 in fibers incubated with P4 alone but not when combined with E2. In light of the results of our recent study linking mEH2O2 to skeletal muscle insulin resistance (3), the observed increase in mEH2O2 after acute ex vivo treatment with P4 and E2 + P4 (but not E2) provides a potential link between the ovarian sex steroids and the reduced insulin sensitivity reported during the luteal phase of the menstrual cycle (see summary Fig. 5) and perhaps also pregnancy, when P4 levels are at their greatest naturally (28).

The results of the current study support the notion of an inhibitory effect of P4 alone on skeletal muscle mitochondrial respiration but not in combination with E2. In support of the present findings, although in slight contrast, a study of the effects of 150 μM P4 and 36 μM E2 on mitochondria isolated from mouse liver reported that state 3 J02 supported by succinate (complex II substrate) was reduced with P4 alone, although the reduction occurred in combination with E2 (21). When the mitochondria were supplied exclusively with glutamate + malate (complex I substrate), both state 3 and state 4 J02 were significantly lowered by treatment with either P4 alone or in combination with E2 compared with controls (21). These effects of P4 or E2 + P4 on mitochondrial J02 were not observed during TMPD + ascorbate respiration, which supplies electrons exclusively to complex IV (21), suggesting complex I, or possibly complex II, as one of the sites of action by P4. In another study, when mitochondria isolated from male rat livers were incubated briefly (1 min) with 30 μM E2, both state 3 and FCCP-uncoupled J02 supported by the complex I substrates glutamate + malate were significantly reduced from controls (33). However, E2 treatment had no effect on the mitochondrial membrane potential (∆Ψ) (33). Furthermore, no effect of E2 on mitochondrial H2O2 production was observed with or without rotenone present (33). This is in contrast to findings from another group demonstrating increased mitochondrial ROS in cultured cells treated for 15 min with greater than 360 nM E2 (17). Even more recently, it was shown that adding P4 to preparations of isolated rat liver mitochondria during experimental measurements decreased the ∆Ψ, the calcium retention capacity, and the capacity for complex I-linked state 3 J02 (16). However, as with most of the investigations into the nongenomic effects of female sex steroids on mitochondrial function, the P4 concentrations used were un-
physiological relative to levels found in circulation; in this case, anywhere from 80 to 150 μM, or over 1,000 times greater than the luteal-phase serum P4 concentration in women (41). Some insight into the rapid, nongenomic nature of late pregnancy-relevant P4 concentrations (1–50 μM) on respiration and glucose metabolism was recently provided (20). In their study, Gras et al. (20) demonstrated inhibition of respiration in isolated muscle strips from male rats that was not matched by the expected compensatory increase in insulin-stimulated glucose transport into the tissue. Importantly, this was a rapid phenomenon, which persisted even when inhibitors of transcription, protein synthesis, and the nuclear progesterone receptor were included in the preparation, strongly supporting the nongenomic nature of P4 on skeletal muscle metabolism (20). Moreover, it was determined that the progesterone receptor membrane component 1, the putative cell-surface mediator of progesterone’s nongenomic effects, is clearly present in rat skeletal muscle (20). Whether the membrane progesterone receptor may mediate or is necessary for the observed increase in mEH2O2 in the current study will require further research. It should be noted, however, that the acute incubation experiments in the current study were conducted on permeabilized myofibers, such that sarcolemmal steroid receptors should not necessarily be required for the observed effects of E2 or P4 on mitochondrial function.

In the current study, we chose to address the inconsistencies raised by supraphysiological concentrations of the female sex steroids on isolated mitochondria by introducing concentrations of E2 and P4 relevant to the female menstrual cycle (11, 41) to test for an inhibitory effect of P4 alone on mitochondrial Jo2 in permeabilized myofibers. P4 is one of the female reproductive hormones most associated with pregnancy, as even its namesake implies (2). While extending the findings of the current study to the increase in P4 during the luteal phase of the menstrual cycle and/or pregnancy (30) might predict a decrease in the basal metabolic rate accompanying either the luteal phase or pregnancy, the results of the current study also demonstrate that E2 can prevent the inhibitory effects of P4 on mitochondrial Jo2. Because E2 also increases during the luteal phase [relative to the early follicular phase (11, 30, 41)] and also during pregnancy (30), this may explain why basal metabolic rate does not decrease in the face of increasing P4 during the luteal phase (8, 43, 51) or pregnancy (18), even when maternal and fetal mass are adjusted for (22). The novel finding of the potential ability for E2 to counteract the inhibitory effects of P4 on respiration may also explain why in the current study we found no relationship between serum P4, E2, or the P4/E2 ratio and Jo2 in the Group A subjects (i.e., the IS vs. IR subjects; data not shown).

Many reports describe a reduction in insulin sensitivity with elevated ovarian sex steroids in both humans and animal models (reviewed in Ref. 28), although it increasingly appears that estrogens are involved in the maintenance of insulin sensitivity, at least in mice (6, 39, 40). Indeed, the reported benefits of hormone replacement therapy on insulin sensitivity in women suggests a role for estrogen in maintaining or minimally modulating insulin sensitivity within a narrow physiological range of estrogen levels (28). In the current study, concentrations of serum P4 and E2 were measured in subjects for whom menstrual cycle did not dictate the day on which skeletal muscle biopsies were performed for the subsequent mitochondrial function assays. A significant correlassation between succinate-supported mEH2O2 and serum P4 was observed (Fig. 1). Because both succinate and glycerophosphate are known to stimulate reverse electron flow-mediated superoxide production at complex I (34), these results suggest a relationship between P4 and complex I-mediated H2O2 in skeletal muscle mitochondria. Considering our recent report demonstrating that the antidiabetic drug metformin selectively attenuates succinate-supported mEH2O2 in rodent skeletal muscle (25), it would seem likely that P4 may exert its effects on insulin sensitivity through a similar mechanism in skeletal muscle, namely, complex I-linked mEH2O2. Metformin has been suggested to reduce reverse electron flow and superoxide production at complex I while allowing forward electron flow and supported respiration (25). Unfortunately, instrumental and tissue availability constraints in the current study prevented parallel experiments aimed at pinpointing the site of ROS generation at complex I (e.g., substrates supplying forward vs. reverse electron flow at complex I with and without rotenone). Nevertheless, rotenone was added after the P-C/MGS condition in the mEH2O2 experiments involving the acute E2 and P4 incubations. The addition of rotenone elicited a relatively small increase (~25%) in mEH2O2 that was similar across treatments (data not shown), which would seem to suggest no qualitative difference in the nature of mEH2O2 influenced by E2 and/or P4. However, these data are difficult to interpret, as both forward and reverse electron flow substrates were present with rotenone. Future studies may benefit from additional protocols in which singular substrates supporting either forward or reverse electron flow through complex I are tested with and without rotenone.

Purely positivist application of the present findings to all scenarios is unlikely, however, even with regard to women’s health. For example, reduced insulin sensitivity and predisposition toward metabolic syndrome are well documented in postmenopausal women, a condition in which ovarian sex steroid levels are markedly reduced (12). Mitigating factors during menopause, such as age, increased abdominal adiposity, and physical activity levels highlight the complex relationship between systemic hormonal glycemic control and the role of ovarian sex steroids at different stages throughout life (12). Indeed, the results of the current study also suggest that %BF may affect the relationship between skeletal muscle mEH2O2 and insulin resistance in nonmenopausal women (Fig. 2), supporting the reported influence of adipose on insulin sensitivity (35). Moreover, changes in the serum concentrations of E2 and P4 in women may be dictated by factors such as smoking (52), dietary fiber (42), and fat (19), but typical changes in E2 and P4 are primarily associated with the menstrual cycle in nonpregnant women (30). It is also important to note that peak levels of E2 and P4 do not normally occur during the same phases of the female menstrual cycle, E2 peaking at or about ovulation midcycle and P4 peaking during midluteal phase. Thus, the high levels of both E2 and P4 employed simultaneously in the acute incubation experiments of the present study represent a potential limitation to extrapolating the results to the menstrual cycle in anything more than a general sense. Further research may clarify this issue with larger, longitudinal studies monitoring women throughout the menstrual cycle. Nevertheless, we have demonstrated an association between high P4 and the mEH2O2 linked to skeletal muscle insulin resistance. Indeed, insulin sensitivity is shown to decrease during both the luteal phase and during pregnancy (28), two conditions in which P4 and E2 levels are elevated.

E533
relative to the follicular phase of the menstrual cycle. If, in fact, high physiological levels of E2 and P4 link conditions such as pregnancy and the luteal phase of the menstrual cycle to skeletal muscle insulin resistance, the next relevant question is which are responsible: E2, P4, or both? In a study involving stable isotope dilution and indirect calorimetry, d’Eon et al. (14) were able to measure glucose uptake and estimate skeletal muscle glucose oxidation during exercise while manipulating the blood levels of E2 and P4 in healthy women. They discovered opposing actions of E2 and P4, the former reducing estimated muscle glycogen utilization and the rate of glucose disappearance from the blood. In contrast, increasing blood levels of P4, in addition to E2, increased the estimated muscle glycogen utilization, but not the rate of glucose disappearance from the blood. These results are supportive of the present findings in that the presence of ovarian sex steroids in combination may alter the singular effect of each alone on cellular metabolism. Indeed, our results demonstrate that, with regard to skeletal muscle mitochondrial J02, P4 alone significantly reduced respiration supported by the multisubstrate combination P-C/MGS (Fig. 3). However, even when P4 was present, E2 preserved J02 (Fig. 3). This is again in agreement with previous reports indicating that E2 preserves mitochondrial function in neuronal cells challenged with proapoptotic factors (32), inhibitors of succinate dehydrogenase (30), high calcium (36), and oxidative stress (32, 50). Although further research will be necessary to reveal the exact mechanism by which E2 exerts its effects on mitochondrial maintenance of function, studies indicate that E2 exerts a direct antioxidant effect on isolated mitochondria (9), and there is additional evidence from other laboratories involving isolated mitochondria that E2 can directly enhance the activity of the manganese-containing superoxide dismutase (37).

The results of the current study, however, do not support the notion of E2 as a direct antioxidant when in combination with progesterone (Fig. 4), although E2 alone did not increase the rate of mE1H2O2 (Fig. 4). Therefore, the present findings suggest that P4 may be related to the insulin resistance observed during conditions of elevated sex steroids. If mE1H2O2 is linked to insulin resistance in skeletal muscle (3), the finding that acute exposure of permeabilized myofibers to P4 or P4 + E2 increased mE1H2O2 (Fig. 4) permits speculation about potential mechanisms whereby P4 influences skeletal muscle insulin sensitivity via mE1H2O2. Because the women in the present study were fasted, their serum P4 levels may actually reflect how their skeletal muscle will respond to metabolic challenges known to reduce insulin sensitivity, such as consuming a large meal (24), prolonged fasting [e.g., 1–2 days (15)], sleep restriction (44), or marathon running (49). During the protocols used in the current investigation, rates of mE1H2O2 were in fact stimulated progressively through the addition of various substrates used in mitochondrial oxidative phosphorylation, and not in the absence or progressive lowering of substrates. If the measurements of mE1H2O2 are viewed not as a resting level enzymatic activity assay but rather as how the mitochondria in skeletal muscle will respond to an influx of substrate, the implications may be more physiologically relevant. Perhaps the effects of P4 on skeletal muscle are pleiotropic, conditionally specific, and evident experimentally only under extreme conditions, such as a high rate of mitochondrial substrate flux. From an evolutionary perspective, P4 might serve as a regulator of substrate provision associated with pregnancy. Indeed, when a woman becomes pregnant, the rise in P4 that accompanies the luteal phase continues and increases during gestation, as often does insulin resistance (23). Teleologically, the rise in P4 during pregnancy and an increase in skeletal muscle mE1H2O2, and, perhaps in turn, mated insulin sensitivity, may have more to do with satisfying the energetic needs of the developing fetus than any pathological condition in carbohydrate metabolism. The rise in P4 may therefore set the stage for a means to divert substrate away from the mother’s skeletal muscle following a meal. While changes in glycemic control during situations of elevated ovarian sex steroids are most certainly the combined result of multiple hormones acting on multiple tissues, the results of the present study support the notion that the direct effects of P4 on skeletal muscle could play an important role (20, 28). Establishing the exact degree to which progesterone mitigates insulin sensitivity in skeletal muscle, and perhaps additional tissues, will require further research.

To conclude, the results of the current study support a model in which high luteal phase-relevant levels of P4 increase mE1H2O2 and decrease J02 in skeletal muscle and in which late-follicular-phase levels of E2 remove the inhibitory effects of P4 on J02 but not mE1H2O2 (Fig. 5). Furthermore, the results of this study clearly suggest a link between mE1H2O2 and insulin resistance in women. Whether the model can explain a causative role for ovarian sex steroids in the etiology of insulin resistance and type 2 diabetes will require further research. Last, these results highlight the need to take into account female menstrual cycle status and the influence of ovarian sex steroids when studying mitochondrial function, even with ex vivo experimental designs.

ACKNOWLEDGMENTS
We thank the subjects who participated in this study. D. A. Kane also thanks C. L. Tweedie for tissue processing and C. G. R. Perry for helpful comments.

Present address for D. A. Kane: Dept. of Human Kinetics, St. Francis Xavier University, Antigonish, NS, Canada B2G 2W5 (e-mail: dkane@stfx.ca).

GRANTS
This study was supported by National Institutes of Health Grants R01 DK-075880 (R. N. Cortright) and DK-074825 and DK-073488 (P. D. Neufer).

DISCLOSURES
No conflicts of interest are reported by the authors.

REFERENCES


