Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake

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Received 24 April 2001; accepted in final form 8 October 2001

Campbell, S. E., and M. A. Febbraio. Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. Am J Physiol Endocrinol Metab 282: E1139–E1146, 2002; 10.1152/ajpendo.00184.2001.—This study examined the roles of the female sex steroids, 17β-estradiol (E2) and progesterone (Prog), on glucose uptake and GLUT4 protein expression. Female Sprague-Dawley rats were either sham operated (C) or ovariectomized and treated with placebo (O), E2 (E), Prog (P), or both hormones at physiological doses (P + E) or the same dose of Prog with a high dose of E2 (P + HiE) via timed-release pellets inserted at the time of surgery, 15 days before metabolic testing. On the morning of day 15, animals received a 300-μCi injection (ip) of 2-deoxy-[14C]glucose and then either exercised on a motorized treadmill for 30 min at 0.35 m/s or remained sedentary in their cages for the same period. Basal glucose uptake was not different between the treatment groups in either the red or white quadriceps. However, glucose uptake was decreased (P < 0.05) in O, P, and P + E rats during exercise in the red quadriceps compared with C rats, whereas E and P + HiE treatment restored glucose uptake. Glycogen content in skeletal muscle followed similar trends, with no differences seen in resting animals. Postexercise red quadriceps glycogen levels were higher (P < 0.05) in the E and P + HiE rats compared with O and P. Treatment of ovariectomized rats with progesterone (P rats) decreased (P < 0.05) GLUT4 content in the red quadriceps by 21% compared with C rats. These data demonstrate that estrogen-deficient animals have a decreased ability for contraction-stimulated glucose uptake and increased glycogen use during aerobic exercise. However, changes in contraction-stimulated glucose uptake could not be explained by altered transporter protein content, since the absence of E2 had no effect on GLUT4 protein content.

ovariectomy; estrogen; progesterone; carbohydrate metabolism

Although the ovarian hormones operate primarily in reproduction, they are also known to influence glucose homeostasis. Altered postprandial glucose tolerance has been correlated with natural fluctuations in the ovarian hormones throughout the menstrual cycle (7, 9), during pregnancy (31), and after menopause (3). In addition, insulin-stimulated glucose uptake is often (6, 35) but not always (34, 40) impaired during the luteal phase of the menstrual cycle. Progesterone is thought to be at least partially responsible for insulin resistance during pregnancy and could possibly contribute to the onset of gestational diabetes mellitus (21, 32). Although the association between insulin resistance and the expression of the insulin-responsive glucose transporter (GLUT4) is well established (1, 18, 20, 33), it is somewhat surprising that no studies have examined the effect of the ovarian hormones on GLUT4 protein expression in insulin-responsive tissue.

Muscle contraction is a far more potent stimulus for glucose uptake than insulin (28). Although it is well known that the mechanisms for insulin- and contraction-mediated glucose uptake are quite different (16), exercise may, nonetheless, provide an excellent model for studying the effects of ovarian hormones on glucose disposal, because it places a major metabolic stress on the contracting skeletal muscle. Surprisingly, few studies have investigated such an effect. Zderic et al. (41) recently demonstrated that glucose disposal is decreased during exercise in the luteal compared with the follicular phase in healthy women. However, because of changes in both the absolute and relative concentrations of the ovarian hormones throughout the menstrual cycle, it is difficult to ascertain the individual effects of estrogen and progesterone from human studies. In rodents, in vitro electrical stimulation of glucose uptake was decreased by ovariectomy compared with intact animals (13), but because the sex steroids were not replaced in this study, it is unknown whether the effect was due to the absence of estrogen or progesterone.

Therefore, the purpose of this study was twofold. First, we sought to examine whether the ovarian hormones alter GLUT4 protein content in insulin-sensitive tissue. Second, we sought to determine whether basal and contraction-stimulated glucose uptake in skeletal muscle was influenced by the ovarian hormones. We hypothesized that GLUT4 protein content would be decreased by ovariectomy, resulting in a concomitant attenuation in glucose uptake in contracting muscle. Furthermore, we hypothesized that estrogen, but not progesterone, treatment would restore GLUT4 and glucose uptake to normal levels.
METHODS

Animals. Female Sprague-Dawley rats 12–15 wk old, weighing 203.1 ± 4.5 (SE) g, were used in these experiments. All animals were housed in a temperature-controlled room (21 ± 2°C) with a 12:12-h light-dark cycle. Water was available ad libitum, and rats were given 20 g standard rat chow/day to control food intake. The amount of rat chow administered daily was selected based on pilot work from our laboratory, which demonstrated that food intake of 10% body weight was sufficient to maintain body weight over the duration of the experiment. This experiment was approved by the Animal Research Ethics Committee of the University of Melbourne.

Experimental design. Rats were bilaterally ovariectomized or sham operated under sodium brieatal anesthesia (60 mg/kg ip). Groups of ovariectomized rats were treated immediately with timed-release hormone pellets (Innovative Research of America) inserted subcutaneously with either 17β-estradiol (E_{2}; 2.5 μg/day; E group), progesterone (Prog; 1.5 mg/day; P group), both hormones at the same doses (P + E), or both hormones with the same dose of Prog but pharmacological levels of E_{2} (25 μg/day; P + HiE). Groups of intact and ovariectomized rats were treated with vehicle-only placebo pellets (C and O, respectively). Animals were treated for 14 days postoperation before metabolic testing, which occurred on day 15. Efficacy of the ovarioctomies and sex steroid treatments were confirmed by plasma E_{2} and Prog RIA kits (Diagnostic Products) at the end of the treatment period. Animals were randomly subdivided into either exercise or resting groups (n = 5 in each group).

Metabolic testing. Animals remained sedentary during the 14-day treatment period. Rats were fasted for 12 h before commencement of metabolic testing. On the morning of day 15, all animals received a 300-μCi injection (ip) of 2-deoxy-[14C]glucose (2-DG). Exercising rats were then run at 0.35 m/s for 30 min on a custom-made motorized treadmill, and resting rats remained inactive in their cages for 30 min. Immediately after removal from the treadmill or cage, animals were suffocated with CO_{2} (80:20 CO_{2}-O_{2}), rendering unconsciousness in <20 s. A midline incision was made, and the diaphragm was cut to ensure death. A cardiac puncture was performed, the blood was spun at 7,000 × g for 2 min, and the plasma was removed and stored at −80°C for analysis of ovarian hormones, insulin, glucose, and lactate. The muscles of the hindlimb were exposed rapidly, the quadriceps were removed and dissected into white and red portions, and all visible connective tissue was removed. The liver and white adipose tissue (WAT) were also removed. All tissues were then snap-frozen in liquid nitrogen and stored at −80°C for later analysis.

GLUT4 protein. Samples of red and white quadriceps muscle were weighed and digested in 1 ml of 1 M NaOH in a shaking water bath at 60°C for 1 h, followed by neutralization with 1 ml of 1 M HCl. The digest was then separated for two different treatments. In the first treatment, 500 μl of digest were deproteinized in equal volumes of Ba(OH)_{2} and ZnSO_{4} and then mixed and spun at 8,500 g for 5 min. The supernatant (1 ml) was recovered and added to 3 ml of water and 10 ml of liquid scintillation cocktail. In the second treatment, 500 μl of digest were deproteinized in 2 ml of 6% perchloric acid, mixed, and spun at 8,500 g for 10 min. The supernatant (2 ml) was recovered and added to 2 ml of water and 10 ml of liquid scintillation cocktail. Radioactivity of both treatments was measured by a β-scintillation counter. The first treatment yielded unphosphorylated 2-DG, and the second treatment measured total 2-DG; hence, the difference gave phosphorylated 2-DG.

Tissue glycogen. Muscle samples to be analyzed for glycogen content were freeze-dried, weighed, powdered, and extracted in 250 μl of 2 M HCl at 100°C for 2 h and then neutralized with 750 μl of 0.667 M NaOH. The extract was then analyzed for glucose, as has been described previously (26). Liver samples to be analyzed for glycogen content were weighed and extracted in 70% perchloric acid and then assayed for glycogen by the amyloglucosidase method (26). Previous work from our laboratory has demonstrated that this latter method is a more sensitive and specific analysis for liver glycogen compared with the former, which is specific for skeletal muscle.

Blood hormone and metabolite assays. Plasma E_{2}, Prog, and insulin were measured by commercially available double-antibody RIA kits (Diagnostic Products and Pharmacia & Upjohn). Plasma glucose and lactate were analyzed using an automated method (Electrolyte Metabolite Laboratory; Radiometer, Copenhagen, Denmark).

Statistics. All statistical comparisons were made using one- or two-way ANOVA, as appropriate, with significance set at the P < 0.05 level. Specific differences were located with a Student-Newman-Keuls’s F-test post hoc comparison. All data statistics were compared using the Statistica software package (Statsoft, Tulsa, OK), and data are reported as means ± SE.
RESULTS

The animal characteristics are presented in Table 1. Plasma E2 and Prog concentrations confirmed the efficacy of the ovariectomies and sex steroid treatments. No significant differences were found among the mean values of either initial or final body weight. Because no differences were found in either the sex steroid levels or weights between resting and exercised animals, these data were pooled.

The plasma glucose, lactate, and insulin data are presented in Fig. 1. There were no differences in plasma glucose or lactate values among the six groups of rats at either rest or after exercise. As expected, however, both glucose and lactate were elevated (P < 0.05) when comparing exercised with rested animals. Plasma insulin values were elevated (P < 0.05) in the P rats, both at rest and during exercise, compared with C, E, P + E, and P + HiE rats. As expected, there was also a decrease (P < 0.05) when exercised and rested animals were compared.

Glucose uptake (Fig. 2) in the red quadriceps was similar among all treatment groups at rest. During exercise, however, there was a decrease (P < 0.05) in the O, P, and P + E rats compared with C rats. Treatment with E2 (E rats) returned glucose uptake to normal, and treatment with a high dose of E2 was able to compensate for the inhibitory effect of Prog on physiological levels of E2. P rats also had decreased GLUT4 content in the red quadriceps (21%, P < 0.05) compared with C rats. There was a similar decrease compared with O rats (18%); however, this did not reach statistical significance (P = 0.054). These effects were not seen in any other treatment groups in these tissues, although there were trends for P + E rats to be decreased compared with C rats (WAT P = 0.07; red quadriceps P = 0.06). No differences were detected in white quadriceps between any treatment groups (Fig. 4).

DISCUSSION

This is the first study to examine the effects of ovariectomy and hormone replacement therapies on GLUT4 protein and contraction-stimulated glucose uptake in vivo. The results demonstrate that the ovarian hormones exert a significant effect on carbohydrate metabolism in contracting skeletal muscle. Estrogen-deficient animals demonstrated impaired contraction-mediated glucose uptake and increased glycogen depletion compared with both control and E2-treated animals. Of note, Prog inhibited the beneficial effect of E2 at physiological concentrations, since P + E rats also demonstrated impaired contraction-mediated glucose uptake and increased glycogen depletion compared with both control and E2-treated animals. The data also demonstrate that Prog treatment decreases GLUT4 protein in insulin-responsive tissue. However, changes in contraction-stimulated glucose uptake could not be explained by altered transporter protein content, since the absence of E2 had no effect on crude membrane GLUT4.

Previous studies using an ovariectomized model have observed that ovariectomy usually results in a relative body weight gain and that this is reversed by treatment with E2 (25, 36). This is thought to be a significant relation between glucose uptake and glycogen content postexercise; r and P values for individual trend lines are presented in Table 2.

There was a mean treatment effect for resting liver glycogen (Fig. 3), with E and P + HiE having higher (P < 0.05) glycogen stores than O and P. This effect was no longer seen postexercise, when hepatic glycogen stores were significantly reduced in all treatment groups.

Ovariectomy (O rats) did not decrease total GLUT4 protein content compared with C rats in any of the tissues. In contrast, treatment of ovariectomized rats with Prog (P rats) decreased GLUT4 content by 28 and 26% in WAT compared with C and O rats, respectively (P < 0.01). P rats also had decreased GLUT4 content in the red quadriceps (21%, P < 0.05) compared with C rats. There was a similar decrease compared with O rats (18%); however, this did not reach statistical significance (P = 0.054). These effects were not seen in any other treatment groups in these tissues, although there were trends for P + E rats to be decreased compared with C rats (WAT P = 0.07; red quadriceps P = 0.06). No differences were detected in white quadriceps between any treatment groups (Fig. 4).

Table 1. Animal characteristics

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<thead>
<tr>
<th></th>
<th>C</th>
<th>O</th>
<th>E</th>
<th>P</th>
<th>P + E</th>
<th>P + HiE</th>
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<tr>
<td>Final body wt, g</td>
<td>217 ± 4</td>
<td>214 ± 4</td>
<td>212 ± 5</td>
<td>214 ± 5</td>
<td>214 ± 5</td>
<td>200 ± 6</td>
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<tr>
<td>Plasma E2, pg/ml</td>
<td>35.8 ± 3.9</td>
<td>11.8 ± 0.8</td>
<td>10.7 ± 1.3</td>
<td>10.7 ± 1.3</td>
<td>10.7 ± 1.3</td>
<td>8.3 ± 0.7</td>
</tr>
<tr>
<td>Plasma Prog, ng/ml</td>
<td>35.8 ± 3.9</td>
<td>11.8 ± 0.8</td>
<td>10.7 ± 1.3</td>
<td>10.7 ± 1.3</td>
<td>10.7 ± 1.3</td>
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Values are means ± SE. E2, 17β-estradiol; C, control; O, ovariectomized placebo; E, ovariectomized and treated with E2; P, ovariectomized and treated with progesterone (Prog); P + E, ovariectomized and treated with Prog and E2; P + HiE, ovariectomized and treated with Prog and pharmacological levels of E2. P < 0.05 compared with C (a), O (b), E (c), P (d), P + E (e), and P + HiE (f).
result of neuroendocrine responses that increase food intake and reduce physical activity (36). Although previous studies have found that either ovariectomy or hormone replacement can adversely affect glucose and/or insulin homeostasis (5, 25), neither study controlled for food intake. Hence, it was not possible to directly attribute the observed metabolic dysfunction to alterations in the ovarian hormone levels. To our knowledge, this is the first study that has controlled food intake for the duration of the treatment to eliminate the effect of weight gain on metabolic dysfunction. Despite the maintenance of body weight, Prog-treated animals still developed insulin resistance, because their plasma insulin concentration was higher in the absence of normal plasma glucose concentrations (Fig. 1), whereas ovariectomized animals also had slightly elevated insulin levels ($P = 0.11$ compared with C rats), indicating that the ovarian hormones have direct effects on insulin sensitivity.

Contraction-stimulated glucose uptake in red skeletal muscle was decreased in the absence of estrogen. This observation is consistent with previous studies that have observed that estrogen increases basal glucose uptake in rat skeletal muscle in vivo (25) and insulin-stimulated mouse skeletal muscle in vitro (29).
Of note, in these previous studies, Prog treatment had no effect. In contrast, in the present study, Prog treatment not only impaired contraction-mediated glucose uptake when solely administered but impaired glucose uptake in rats treated with physiological concentrations of both E2 and Prog, and the intact C rats. This may best be explained by the fact that the intact rats were cycling normally and were therefore exposed to elevated Prog levels (40–50 ng/ml) for only ~12 h during the proestrus phase of their 4- to 5-day cycle. Furthermore, control rats were killed at various times throughout the estrous cycle so that a more representative collection of data points could be obtained; therefore, these pooled results incorporate the changing levels of the ovarian hormones. Conversely, the P + E rats were exposed continuously to high levels of Prog; therefore, the P + E rats would have a greater tendency to exhibit any inhibitory characteristics of Prog.

The mechanism(s) for the actions of the ovarian hormones on glucose uptake is not readily apparent from the present data. By measuring the effects of the ovarian hormones on GLUT4, we sought to determine whether any alterations in glucose transport were attributable to transporter protein content. We observed that Prog treatment suppressed GLUT4 expression in red quadriceps and WAT, whereas a similar trend (WAT P = 0.07; red quadriceps P = 0.06) was observed in rats receiving both hormones at physiological levels (P + E). It is possible that the decrease in GLUT4 protein content in skeletal muscle could be the result of relative inactivity of P and P + E rats compared with E rats, since a decrease in physical activity has previously been demonstrated to negatively affect GLUT4 protein content (24). However, the decrease in GLUT4 content in WAT was similar, and often greater, indicating that the effect of Prog is independent of the relative physical activities of the rats (Fig. 4). Although our observations with respect to GLUT4 are novel and important, we cannot attribute alterations in glucose transport to changes in GLUT4 protein content, since ovariectomy markedly decreased contraction-mediated glucose uptake but had no effect on GLUT4 protein content in red quadriceps muscle. It is possible, therefore, that the ovarian hormones may influence signaling molecules and/or GLUT4 trafficking. In the present study, we were not able to measure GLUT4 protein content in subcellular and/or plasma membrane fractions of the muscle samples because of insufficient muscle sample size to complete all analyses. Our

Table 2. Trendline r and P values

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<tr>
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<th>C</th>
<th>O</th>
<th>E</th>
<th>P</th>
<th>P+E</th>
<th>P+HiE</th>
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<tbody>
<tr>
<td>r</td>
<td>0.802</td>
<td>0.809</td>
<td>0.854</td>
<td>0.674</td>
<td>0.976</td>
<td>0.933</td>
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<tr>
<td>P</td>
<td>0.10</td>
<td>0.09</td>
<td>0.06</td>
<td>0.21</td>
<td>0.005</td>
<td>0.02</td>
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Trend line r and P values for correlation performed on glucose uptake and glycogen content in red quadriceps postexercise.
GLUT4 measures made on total crude membrane fractions can neither confirm nor refute the possibility that the ovarian hormones may affect GLUT4 translocation from the subcellular pools to the plasma membrane. Indeed, there a paucity of data that have examined such an effect, and further research in this area is warranted. Of note, however, Weiner et al. (37) have demonstrated that estrogen increases calcium-dependent nitric oxide synthase activity in skeletal muscle. This may offer a possible mechanism for the effects of the ovarian hormones on contraction-mediated glucose uptake, since nitric oxide has been implicated in augmenting glucose uptake in both rat (8) and human (2) skeletal muscle. Further research that examines the effect of the ovarian hormones on GLUT4 translocation during muscle contraction may shed further light on the current observations.

It is also possible that the ovarian hormones primarily affected lipid metabolism and that the changes we observed in carbohydrate metabolism were secondary to changes in fat metabolism. If this was the case, one would expect lipid metabolism to be increased with ovariectomy, since glucose uptake was reduced in O compared with C animals. To the contrary, we have recently observed that carnitine palmitoyltransferase I and β-3-hydroxyacyl-CoA dehydrogenase activity, enzymes representative of fatty acid flux into the mitochondria and β-oxidation, respectively, are also reduced with ovariectomy (unpublished observation). Therefore, it appears that the effect of the ovarian hormones on carbohydrate metabolism is unlikely to be secondary to altered fat metabolism.

Glycogen levels in the red quadriceps were also different postexercise between the treatment groups, with E and P + HiE rats having higher glycogen content postexercise compared with O and P rats. Interestingly, these two groups also had higher glucose uptake, suggesting that the increase in glucose uptake was enough to result in a glycogen-sparing effect. There was a trend for a positive relation between glucose uptake and glycogen content in all groups (Fig. 5). If these data were pooled across groups, this relation was statistically significant (r = 0.876; P < 0.01), suggesting that the differences in glucose uptake between the animals might explain a significant portion (76%) of the variability in postexercise glycogen concentrations. It is, however, interesting to observe that P rats, the group with the weakest correlation, were also the group that exhibited insulin resistance. This suggests that these animals were perhaps less able to match glucose uptake to cellular carbohydrate metabolism. It is important to note that an increase in glucose uptake does not necessarily result in glycogen sparing in humans (10). This may be because rats can oxidize more glucose compared with humans (15, 38), an obvious interspecies difference.

The observation of differing hepatic glycogen content when comparing the treatment groups in the rested animals is consistent with previous studies that have found increased glycogen storage in E2-treated animals, E and P + HiE groups (4, 23). This is likely because of increased glycogen synthase activity (29). The results do, however, differ in that the P and P + E rats had lower hepatic glycogen, where previously Prog has been found to increase glycogen storage (19, 23).
This is perhaps because we controlled for food intake and therefore limited the opportunity for these animals to supercompensate their glycogen stores. The increase in glycogen content previously seen with Prog treatment was thought to be the result of hyperinsulinemia (19, 23). Insulin levels in this study were not as elevated as in previous studies, because we controlled for food intake, and this could also result in less glycogen storage. Interestingly, there was no longer a treatment effect on hepatic glycogen concentrations postexercise, suggesting an increase hepatic glycogenolysis in E and P + HiE rats compared with the other groups. Because there was a significant increase in glucose uptake in the E and P + HiE rats, with no corresponding change in plasma glucose values, it is likely that the hepatic glucose production was increased in response to increased peripheral demand.

Considerable evidence exists linking Prog to insulin resistance (7, 22, 31), but the precise mechanisms involved in this peripheral insulin resistance are not fully understood. Of note, however, insulin resistance has previously been linked to abnormalities in glucose uptake, often via alterations in GLUT4 (12, 20, 39). Reduced basal and insulin-stimulated glucose transport rates in isolated adipocytes have been associated with a marked depletion of GLUT4 protein in insulin-resistant patients (12). This was not seen in skeletal muscle (11, 27), since only the vastus lateralis and rectus abdominis have been studied. Differential regulation of GLUT4 in individual muscles has demonstrated that the vastus lateralis and rectus abdominis respond minimally to diabetes in rats, whereas soleus and heart muscles have a marked suppression in glucose disposal in the diabetic compared with baseline condition (14). Thus it remains possible that GLUT4 depletion could cause insulin resistance in muscles. Indeed, a recent study has demonstrated that, in the absence of muscle, GLUT4 results in severe insulin resistance (42). Although in the present study we observed a statistically significant suppression in the quadriceps muscle, it was only in the red portion. When red and white muscles were pooled, the decrease in GLUT4 content was no longer significant. Type I fibers are more insulin sensitive, with a greater capacity for stimulated glucose uptake (17, 20, 32) and, hence, are perhaps more susceptible to insulin-resistant pathologies.

Of particular interest in these results is the inhibition of the beneficial effects of E2 by Prog at physiological concentrations, seen in glucose uptake. This inhibition was abolished with pharmacological concentrations of E2, demonstrating that, in high enough concentrations, E2 can override the inhibitory effect of Prog. This could have significant clinical effects when considering long-term treatment of amenorrheic or postmenopausal women with oral contraceptives or hormone replacement therapy. In these situations, it may be prudent to treat with an estrogen-based therapy vs. either Prog or combination therapies. Conversely, if a combination hormone replacement therapy is being considered, our data suggest the utilization of elevated levels of E2 to offset any inhibition by Prog. It is interesting to note that similar results have been found in clinical studies. Women using combination therapy demonstrated poorer glucose homeostasis and insulin sensitivity than those using estrogen alone (7, 22).

In conclusion, the absence of the ovarian hormones in female rats resulted in a decrease in glucose uptake and glycogen content postexercise in red skeletal muscle. Treatment with E2 restored these parameters to control levels, whereas treatment with Prog alone had no effect. These data suggest that estrogen deficiency results in a limited capacity for glucose uptake in times of metabolic stress, forcing the cell to seek an alternative fuel source. If ovariectomized animals are treated with both E2 and Prog in physiological concentrations, Prog inhibits the oxidative effect of E2, and this is only restored with pharmacological concentrations of E2. The data also demonstrate that Prog treatment decreases GLUT4 protein in insulin-responsive tissue but that changes in contraction-stimulated glucose uptake could not be explained by altered transporter protein content, since the absence of E2 had no effect on GLUT4 protein.

We thank Dr. Anne Thorburn, Dr. Kirsten Howlett, Dr. Sofianos Andrikopoulos, and Tamara Konopka for valuable advice and technical expertise.
REFERENCES


