Cellular accumulation of heat shock protein (Hsp) 72i in fetuses of trained rats

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Abstract Forty-five Sprague-Dawley rats (60–80 days old) were randomly placed into one of three groups: sedentary pregnant control (PC); prepregnancy trained animals that exercised throughout pregnancy (PR); and nonpregnant trained animals (NPR). Each exercising animal ran at approximately 60–70% aerobic capacity ($\dot{V}O_{2max}$) for 1 hour/day up to and including day 18 of gestation (term = 21 days). On day 20 of gestation, fetuses were excised from each pregnant animal and scrutinized for gross abnormalities. In 3 randomly chosen fetuses from each litter, brain, heart, kidney, hind limb, and placental tissues were removed to assess the accumulation of the inducible isoform of the 70-kilodalton heat shock protein (Hsp 72i). No significant differences were detected between fetal hearts, hind limbs, or placental tissues of PC or PR groups. No Hsp 72i signal could be detected in fetal kidney or brain tissues from either pregnant group. Results indicate that maternal core temperature did not reach the threshold that would induce either gross fetal abnormalities or a fetal heat shock protein response. However, fetal and placental growth was reduced by the exercise protocol.

INTRODUCTION

Fetal temperature regulation is dependent on maternal temperature, fetal metabolism, and uterine blood flow (Lindqvist et al 2003). The maternal uterine environment establishes fetal ambient temperature, and excess fetal heat is dissipated through a fetal/maternal temperature gradient (Lotgering et al 1983). During exercise, an increased maternal core temperature may compromise or possibly reverse this gradient (Lotgering et al 1983). In effect, the fetus may become a partial recipient of the thermal energy generated during maternal exercise.

Ample literature defines excessive heat as an established teratogen (Arora et al 1979; Geoff et al 1988). In the rat, a maternal body core temperature increase of 2.5°C was established as a threshold after which 9.5-day-old fetal rats developed abnormally (Germain et al 1985).

The duration of the exposure required to induce fetal malformations declined as temperatures increased above this threshold. Temperature-induced fetal defects have been experimentally produced in chickens, rats, hamsters, guinea pigs, rabbits, sheep, pigs, and monkeys (for complete review, see Edwards et al 2003). However, the type and severity of the fetal defects were dependent upon the species, stage of development, and intensity of the heat stressor (Edwards et al 2003).

Without doubt the fetus is dependent upon the uterine environment for life, however, a certain level of autonomy is retained by the fetus. For example, heat shock proteins (HSPs), a highly conserved set of cellular proteins that are quickly synthesized in response to hyperthermia, amino acid analogues, transition metals, oxidizing agents, some poisons, anoxia, ethanol, and cellular damage (for reviews, see Lindquist 1986; Higo et al 1989) may be important as a fetoprotective mechanism (Bae et al 2003). Heat shock proteins are categorized into families by their relative molecular weights (M_r): large HSPs (HSP90 kilodaltons [kDa]), HSPs of 68–74 000 kDa (re-

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ferred collectively as HSP70s), and small HSPs of 15–30 000 kDa. Most cells and organisms synthesize HSP70 proteins during heat shock or other environmental stress (Hahnel et al 1986), and cells that harbor these proteins appear to possess the ability to survive an otherwise lethal heat exposure (Riabowol et al 1988). Hsp73c is an abundant protein of unstressed cells and appears to play an essential function in the normal cellular life cycle. Conversely, Hsp72i is often the most pronounced translation product when a cell is stressed (Velazquez et al 1980).

At the present time, it remains unclear whether a chronic maternal exercise program alters the maternal/fetal heat gradient to a degree that would induce a fetal heat shock protein response, especially in animals working at 60–70% aerobic capacity ($\dot{V}O_{2\max}$). This moderate exercise protocol for rats was chosen because it represents the intensity at which many individuals train (Wolfe and Mottola 2002). As such, the purpose of the present study was to determine if exercise (60–70% $\dot{V}O_{2\max}$) in trained animals, elevates maternal body core temperature to a degree that would induce a fetal heat shock protein response.

RESULTS AND DISCUSSION

Maternal weight gain

Anabolic fat storage appears to be an essential maternal adaptation during pregnancy because the caloric needs of the fetoplacental unit increase with advancing gestation (Savard et al 1986). However, maternal weight gain differed significantly between prepregnancy trained animals ([PR], 97.3 ± 5.3 g) and nonexercising pregnant control, ([PC], 128.4 ± 7.2 g, $P < 0.01$; Table 1) groups at day 20 of gestation. This result is consistent with previous investigations, which have speculated that decreased weight gain in pregnant exercising animals may be attributed to a smaller litter size (Treadway et al 1986), diminished fetal weight, and a decrease in maternal fat stores (Mottola et al 1986). The additional metabolic cost of endurance exercise during pregnancy may selectively reduce adiposity in a regionally specific manner due to a decrease in fat storage that occurs when trained pregnant rats exercise throughout pregnancy (Savard et al 1986). Our PR animals ate the same amount as the PC animals (unpublished observations), and food and water were removed from the PC group while the PR group exercised. The PR animals expended more energy due to exercise and yet did not compensate by eating more. The smaller weight gain in the PR animals may be due to the lighter fetal body and placenta weights, partially explaining the difference in maternal weight gain detected between the groups. Adequate maternal caloric ingestion may be more

Table 1 A summary comparing the pregnant control and the pregnant running animals

	Pregnant control	Pregnant runner
Prepregnancy body weight (g)	256.5 ± 4.1 ($n = 10$)	245.5 ± 4.2 ($n = 10$)
Maternal weight gain (g)	128.4 ± 7.2 ($n = 10$)	$97.3 \pm 5.3^*$ ($n = 10$)
Fetal body weights (g) (All fetuses)	2.00 ± 0.02 ($n = 130$)	$1.90 \pm 0.01^*$ ($n = 133$)
Fetal body weights (g) (Random sample)	2.05 ± 0.04 ($n = 30$)	$1.94 \pm 0.03^*$ ($n = 30$)
Placental weights (g) (All fetuses)	0.50 ± 0.01 ($n = 130$)	$0.48 \pm 0.01^*$ ($n = 133$)
Placental weights (g) (Random sample)	0.52 ± 0.01 ($n = 30$)	0.48 ± 0.01 ($n = 30$)
Placental/fetal body weight ratio (%) (All fetuses)	25.3 ± 0.5 ($n = 130$)	25.0 ± 0.4 ($n = 133$)
Placental/fetal body weight ratio (%) (Random sample)	25.3 ± 0.7 ($n = 30$)	25.0 ± 0.5 ($n = 30$)
Number/litter	14.9 ± 1.0 ($n = 10$)	13.4 ± 0.6 ($n = 10$)
Number of resorption sites	1.1 ± 0.3 ($n = 10$)	1.1 ± 0.3 ($n = 10$)

Forty-five virgin Sprague-Dawley rats aged 60–80 days (Harlan Sprague-Dawley, Ltd., IN, USA) were housed in a 12:12-hour light: dark controlled room, and provided with food (Purina Rodent Chow) and water ad libitum. Ambient temperature was regulated at $20 \pm 2^\circ\text{C}$ with a relative humidity between 45–55%. The animals were placed randomly into one of the following three groups: nonexercising pregnant control animals ([PC], $n = 15$), prepregnancy trained animals ([PR], $n = 15$) that were mated and continued exercising up to and including day 18 of gestation (term = 21 days; Baker et al 1979), and trained nonpregnant running animals ([NPR], $n = 15$) that exercised at the same intensity and duration as the PR group. All animal weights were recorded daily throughout the course of the study. On day –4, the rats destined to become pregnant were injected subcutaneously with luteinizing hormone-releasing hormone ([LHRH], Sigma Chemicals, 50 μg suspended in 0.1% bovine serum albumin), and 4.5 days later each female was placed with one male breeder for a duration of 12 hours. Impregnation was confirmed after the breeding period by the presence of a vaginal plug and this was designated as day +1 of gestation. A detailed explanation of this protocol has been published previously (Mottola and Christopher 1991).

* = Significant difference between groups ($P < 0.05$). Values presented are mean $\pm$ standard error of mean (SEM).

important for fetal growth than thermoregulatory responses to exercise.

Adult core temperature increases

No significant differences were detected between PR and nonpregnant running animal (NPR) adult body core pre-exercise and postexercise temperatures (Table 2A, B). On average, core temperatures of the PR animals increased by $0.61 \pm 0.2^\circ\text{C}$ and NPR increased by $0.53 \pm 0.1^\circ\text{C}$ during exercise ($P > 0.05$). Nonpregnant basal temperatures were similar to those reported in the literature (Wilson et al 1978; Shellock and Rubin 1984). Nevertheless, rat strain and depth of rectal probe insertion have produced variability (Sonne and Galbo 1980). Milne and Noble (2002)

Table 2A Mean pre- and postexercise temperatures (°C) of the nonpregnant and pregnant running animals. Data were recorded from day 12–18 of gestation for the pregnant animals and similar time frame for the nonpregnant group

	Pregnant runner	Nonpregnant runner
Pre-exercise Temperature (°C)	38.29 ± 0.04 (n = 10)	38.63 ± 0.05 (n = 15)
Postexercise Temperature (°C)	38.90 ± 0.05 (n = 10)	39.23 ± 0.04 (n = 15)

Nonpregnant running animal (NPR) and prepregnancy trained animal (PR) groups were trained on a rodent treadmill for a period of 3 weeks. Each animal exercised 5 days per week at an intensity approximating 60–70% aerobic capacity (VO_{2max}) (30 m/min, 10° incline) for 1 hour/day, with all exercise and animal handling being conducted during the dark cycle (Mottola and Christopher 1991). Food and water were removed from the cages of the nonexercising animals during this time period. Once pregnancy was established for the PR group, the PR and NPR rats exercised daily on the treadmill at 30 m/min, 10° incline for 1 hour/day. Colonic temperatures were recorded prior to and immediately following the exercise bout from day 12 to 18 of gestation for the PR animals and during the same time frame for the NPR group. Colonic temperature was determined by the insertion of an animal digital rectal probe (Yellowsprings Instrument, Yellowsprings OH), 4 cm past the anal sphincter. Values presented are mean ± standard error of mean (SEM).

suggested that the exercise-induced elevation of Hsp70 is intensity-dependent for acute exercise in untrained male rodents and elicited an average temperature increase of 1.7°C after exercise at 30 m/minute, 2% grade for 60 minutes. Mean postexercise temperatures of our trained exercising pregnant (38.9°C) and trained nonpregnant animals (39.2°C) did not differ significantly, with an average increase of $0.6 \pm 0.2^\circ\text{C}$ and $0.5 \pm 0.1^\circ\text{C}$, respectively, even though the intensity of exercise was higher than reported in the study of Milne and Noble (2002). The lack of increased core temperature with exercise may be a significant effect of training. Training also may produce a thermoregulatory response to protect the animal from heat-induced changes (McArdle and Jackson 2002). However, with the increased metabolic cost of supporting a gravid uterus during exercise, an interesting question is raised with regard to heat dissipation mechanisms utilized by the pregnant trained animal. Bell (1987) hypothesized that the increased body mass associated with advancing gestation possibly could act in a passive heat storage capacity. Others have reported that thermoregulation steadily improves over the course of pregnancy (Lindqvist et al 2003) and may be due to the increase in plasma volume expansion (Clapp 1991). Although the resolution of heat dissipation mechanisms in exercising trained pregnant rats was beyond the scope of the present investigation, future work should address this issue.

Heat shock response

Placental, fetal hind limb and heart Hsp72i signals in the PR group did not differ significantly from those of the

Table 2B Daily adult exercise-induced body core temperature (°C) changes from rest for both nonpregnant and pregnant running animals. Data were recorded from day 12–18 of gestation for the pregnant animals and similar time frame for the nonpregnant group

Day	Pregnant runner	Nonpregnant runner
12	0.39 ± 0.08	0.42 ± 0.22
13	0.55 ± 0.12	0.60 ± 0.13
14	0.68 ± 0.01	0.48 ± 0.09
15	0.72 ± 0.21	0.51 ± 0.09
16	0.72 ± 0.13	0.64 ± 0.13
17	0.85 ± 0.12	0.44 ± 0.14
18	0.41 ± 0.17	0.62 ± 0.12

Values presented are mean ± standard error of mean (SEM).

PC group (Table 3). Hsp72i levels also could be affected by factors other than temperature increase (Milne and Noble 2002). It is of interest to note that an Hsp72i signal could not be detected in fetal brain or kidney tissue from either group even when incremental volumes of fetal brain and kidney homogenate, ranging from 100–250 µg total protein were electrophoresed (Data not shown). The mean body core temperature of the pregnant exercising rats increased by 0.6°C, which is well below the teratogenic threshold of 2°C proposed by Edwards et al (2003). Previously, we have determined that pregnant swim-trained rats in water of $37.6 \pm 0.1^\circ\text{C}$ in the late stages of pregnancy produced an increase in maternal body core temperature of $2.3 \pm 0.1^\circ\text{C}$, which increased the number of fetal abnormalities (Mottola et al 1993), although fetal Hsp72i was not determined. It would appear, therefore, that an exercise intensity of 60–70% VO_{2max} in a thermoneutral environment does not induce developmental problems or a heat shock response in the fetuses of exercising trained pregnant rats, because maternal thermoregulatory mechanisms may be protective in the trained animal.

Fetal growth

Although no gross abnormalities were observed in fetuses of either group, fetuses from the PR group were significantly lighter (1.90 ± 0.01 g) than those of the PC group (2.00 ± 0.02 g, $P < 0.01$; Table 1). This result is supported by the findings of Terada (1974). However, several other investigations did not detect any group fetal weight variability (Mottola et al 1986; Savard et al 1986; Treadway et al 1986; Jones et al 1990). Conversely, a previous study in our lab (Mottola and Christopher 1991) showed an increase in the fetal body weights of trained exercising rats; both studies employed an intensity of 60–70% VO_{2max} . An important consideration during the present study was the fact that the pregnant animals exercised daily during days 12–18 of gestation, constituting 7 consecutive days of exercise with no rest. This contrasted our previous study (Mottola and Christopher 1991) in that the

Table 3 A comparison of densitometer results for Hsp72i in placental, fetal limb, and heart tissue

	Placenta	Limb	Heart
Pregnant control	54.4 ± 8.1	34.9 ± 4.3	9.9 ± 1.5
Pregnant runner	60.9 ± 8.9	35.7 ± 1.8	8.8 ± 1.3

On day 20 of gestation (48 hours after the last exercise bout because previous experimentation suggested that a 48-hour time period provided the optimal peak of Hsp 72i accumulation [Sim J., unpublished observations] for prepregnancy trained animal [PR] and nonpregnant running animal [NPR] groups), each adult animal was anaesthetized with sodium pentobarbital (intraperitoneal (IP) 45 mg/kg body weight; Mottola and Christopher 1991). All fetuses and placentas were excised, blotted, weighed, and scrutinized for gross morphological abnormalities. Lower limb, brain, heart, kidneys, and placental tissues from three randomly chosen fetuses per litter were removed, blotted, weighed, immediately immersed in liquid nitrogen, and stored at -80°C . In total, 60 fetuses were assessed for Hsp 72i accumulation. During analysis, homogenizing buffer (600 mM NaCl, 15 mM tris hydroxymethyl aminomethane, pH 8.1) was added to thawed tissues and samples were homogenized for 20–30 seconds using a Tekmar, Ultra-Turrax homogenizer. All heart tissue was solubilized by a Fisher sonic dismembrator. Cellular debris was pelleted by centrifugation at 16 000 rpm for 3 min in a Beckman Microfuge E centrifuge and the pellet discarded. Sample protein concentration was determined by the method outlined by Lowry et al (1951). The remaining supernatant was added to sample buffer (1:4 5× buffer sample), then denatured by boiling (Laemmli 1970). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). A 4% stacking gel and a 12% separating gel were used to attain optimal protein separation. All gels contained a lane of 25 μg of a maternal soleus homogenate (positive control for Hsp 72i) and a lane for molecular weight standards (Rainbow standards, Amersham). The remaining 8 lanes contained a random placement of 100 μg of each fetal sample alternating between nonexercising pregnant control animal (PC) and prepregnancy trained animal (PR) groups using similar tissues on individual gels. Protein was transferred from each gel onto nitrocellulose membrane (Towbin et al 1979; 40 mA for a period of 2.5 hours). Membranes were blocked (Gershoni and Palade 1983) by washing with phosphate buffered saline (PBS) for 3 min, bathed in a 5% nonfat dry milk (NFDm) in PBS solution for 1 hour. Blots then were washed in PBS for 3 min, followed by immersion in primary (1°) antibody (#799, specific for the inducible Hsp71 referred to here as Hsp 72i; Tanguay et al 1993) mixed with 2% NFDm in PBS (1:2400) for a period of 4 hours. Blots were washed again 3 times in PBS (3 min each) and immersed with goat, anti-rabbit peroxidase conjugated secondary (2°) antibody (Amersham; 2% NFDm; 1:1500) for 1 hour at room temperature. Once again, blots were washed 3 times (3 min each) and reacted with a 4-chloro-1-naphthol solution (2 min) to visualize protein bands, followed by immersion in PBS. All band signals were scanned by a LKB laser densitometer and individual band intensities were expressed as a percentage of the maternal soleus homogenate. Values presented are mean $\pm$ standard error of mean (SEM). These results are expressed as a percentage of the maternal soleus homogenate.

animals exercised for 5 consecutive days, followed by 2 days of rest. The additional exercise stress without rest days in the present study may have impeded fetal growth, which resulted in a lower fetal body weight. In a case-control design study of 529 women, we investigated the impact of frequency, intensity, and duration of recreational activity during pregnancy on low birth weight, and found that women who exercised 5 or more times per week during the third trimester, were 4.6 times more likely to give birth to a low birth weight baby

(Campbell and Mottola 2001). It may be that frequency of maternal activity is a determining factor in fetal growth.

A significant group difference (0.48 ± 0.01 g vs 0.50 ± 0.01 g, $P < 0.01$) was recorded for placental tissue weights from PR and PC groups, respectively. However, no significant differences between groups could be detected in placental/fetal body weight ratios, litter size, or number of resorption sites (Table 1). This finding may indicate that the fetuses of the PR group did not appear to experience hypoxic uterine conditions, which would have produced disproportional growth between placenta and fetus (Rahima and Bruce 1987). In addition, the accumulation of Hsp72i was not changed in the placental tissue of the PR group, which would indicate that the placenta was not unduly stressed. Furthermore, chronic maternal hypoxia exposure in late gestational rats has been shown to suppress the expression of Hsp70 in fetal hearts (Bae et al 2003), which also was not seen in the fetuses of our trained pregnant animals. Thus, it seems that, even though the fetus and placenta were smaller in the exercising animals, this was not due to exercise-induced hypoxic uterine conditions.

Based on the results of the present study, we suggest that a smaller fetoplacental unit may not necessarily signal developmental difficulties. It would seem that, from visual scrutinization, Hsp72i analysis, placental/fetal body weight ratio, and fetal outcome determination, that a smaller fetoplacental unit in the rat may not necessarily indicate developmental distress. However, adequate maternal caloric ingestion during pregnancy to compensate for energy expended while exercising may be an important determinant of fetal growth and deserves further investigation.

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