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Estradiol impairs hypothalamic molecular responses to hypoglycemia

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Abstract

In rats and humans estradiol attenuates neuroendocrine responses to hypoglycemia. Since neuroendocrine responses to hypoglycemia are mediated by hypothalamic neurons, we assessed if estradiol attenuates hypoglycemia-induced gene expression in the hypothalamus in female ovariectomized mice. As expected, estradiol-implanted ovariectomized mice exhibited increased plasma estradiol, increased uterine weight, decreased body weight, decreased visceral adiposity, and enhanced glucose tolerance with decreased plasma insulin. Estradiol-implanted mice exhibited attenuated hypoglycemia-induced gene expression of both glucose transporter 1 (Glut1) and inhibitor of kappa beta signaling (IκB) in the hypothalamus but not in liver. Estradiol also attenuated hypoglycemia-induced plasma glucagon, pituitary proopiomelanocortin (POMC), and adrenal c-fos, consistent with impaired counterregulatory responses to hypoglycemia. In addition, estradiol inhibited hypothalamic expression of carnitine palmitoyltransferase (CPT1a and CPT1c) and pyruvate dehydrogenase kinase 4 (PDK4), effects that would be expected to enhance the accumulation of long-chain fatty acids and glycolysis. Taken together, these findings suggest hypothalamic mechanisms mediating attenuation of hypoglycemia-induced neuroendocrine responses.

Keywords

estradiol; hypothalamus; hypoglycemia; gene expression

Introduction

Clinical studies indicate that the steroid hormone estradiol appears to play a major role in the sexual dimorphism present in counterregulatory responses to hypoglycemia (Davis et al., 2000; Sandoval et al., 2003). Estradiol treatment blunted neuroendocrine, metabolic and autonomic responses to insulin-induced hypoglycemia in healthy humans (Sandoval et al., 2003) and in rats (Adams et al., 2005; Sandoval et al., 2007). On the other hand,

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counterregulatory responses to insulin-induced hypoglycemia are known to be largely mediated by glucose-sensing neurons in hypothalamus (Borg et al., 1994; Borg et al., 1995; Borg et al., 1997). Since the hypothalamus is also a key site mediating central regulation of metabolism by estradiol (Tritos et al., 2004; Musatov et al., 2007), we hypothesized that the attenuation of counterregulatory responses to estradiol is mediated by attenuated hypothalamic responses to hypoglycemia.

We have previously reported the induction of several hypothalamic genes by hypoglycemia, and hypothesized that at least some of these molecular responses are associated with, and may mediate, counterregulatory responses to hypoglycemia (Mastaitis et al., 2005). One way to assess this hypothesis would be to assess the hypothalamic induction of these genes under circumstances in which counterregulatory responses are impaired. Therefore in the present study we assessed if hypothalamic induction of these marker genes would be impaired by estradiol. As described below, estradiol attenuated the induction of a subset of these genes by hypoglycemia in hypothalamus but not in liver. Furthermore, estradiol produced a hypothalamic profile of gene expression that would be expected to suppress hypothalamic lipid oxidation and enhance hypothalamic glucose metabolism, suggesting a possible mechanism mediating the attenuation of hypothalamic and neuroendocrine responses to estradiol.

Results

Estradiol implants increase plasma estradiol and uterine weight and decrease body weight gain, adiposity, and plasma leptin

All mice in the present study were ovariectomized, and either implanted with a placebo (OVX-P) or estradiol (OVX-E). As expected, estradiol implants increased plasma estradiol by about 3-fold, and increased uterine weight by almost 10-fold (Table 1), while reducing adiposity (Table 1) and plasma leptin (Table 2) by over 50%. Consistent with reduced adiposity, estradiol attenuated weight gain over the 7-week period (Fig. 1A), apparently at least partially independent of estradiol-induced anorexia over the entire 7-week period (Fig. 1B). Nevertheless, we were unable to demonstrate a statistically significant effect of estradiol on total heat production (Fig. 2A), even normalized to body weight (data not shown). Furthermore, estradiol did not produce a significant effect on horizontal activity (Fig. 2B, $p=0.24$ by two-way ANOVA). Interestingly, estradiol increased respiratory quotient (RQ) across the light/dark cycle (representative data from the dark period were shown in Fig. 2C, $p=0.02$), indicating an increase in whole-body carbohydrate utilization. Consistent with effects of estradiol to reduce adiposity and increase carbohydrate utilization, estradiol significantly reduced excursion of glucose (Fig. 3A) and insulin (Fig. 3B) after a glucose challenge, although estradiol did not significantly influence baseline levels of plasma glucose or insulin.

Estradiol implants attenuate the induction of gene expression in hypothalamus by insulin-induced hypoglycemia

As we previously reported for intact male mice (Mastaitis et al., 2005), in ovariectomized and placebo-implanted female mice, hypoglycemia induced expression of glucose transporter 1 (Glut1) in both liver and hypothalamus, and inhibitor of kappa beta signaling (I κ B) in hypothalamus (Figure 4). In contrast estradiol attenuated the induction of both Glut1 and I κ B in the hypothalamus (Fig. 4) (significant interaction between estradiol and hypoglycemia by two-way ANOVA, $p<0.05$). However, hypoglycemia-induced expression of Glut1 and I κ B in the liver was not attenuated by estradiol, nor was hypoglycemia-induced expression of angiotensinogen and inhibitor of DNA-binding isoform 1 (ID1) in hypothalamus (data not shown) (main effect of hypoglycemia but no interaction of estradiol with hypoglycemia, nor a main effect of estradiol by two-way ANOVA). Nevertheless, estradiol attenuated the induction of glucagon, pituitary POMC, and adrenal c-fos by hypoglycemia (Table 2),

consistent with reports that estradiol attenuated counterregulatory neuroendocrine responses to hypoglycemia (Sandoval et al., 2003; Adams et al., 2005; Sandoval et al., 2007).

Estradiol treatment produces pro-glycolytic hypothalamic gene expression

Since hypoglycemic counterregulation is largely mediated by glucose-sensing neurons in hypothalamus (Borg et al., 1994; Borg et al., 1995; Borg et al., 1997), and hypothalamic neurons sense glucose through its metabolism, especially glycolysis (Yang et al., 1999; Cheng et al., 2008), we assessed the effect of estradiol on hypothalamic expression of genes which regulate metabolic fates of glucose (Mobbs et al., 2007), including phosphofructokinase (PFK), glucokinase (GK), pyruvate dehydrogenase kinase 4 (PDK4), enzymes that control glycolysis, and carnitine palmitoyl transferase-1 isoforms a and c (CPT1a-c), enzymes that control fatty acid oxidation. By two-way ANOVA, there was not a significant interaction between insulin-induced hypoglycemia or estradiol, nor significant effects of insulin-induced hypoglycemia, on the expression of any of these metabolic genes (except for a main effect of insulin-induced hypoglycemia to induce hepatic GK [induction of hypothalamic GK narrowly missed significance, $p=0.078$] and hypothalamic CPT1c), nor an effect of estradiol on PFK or GK (not shown). However, by two-way ANOVA estradiol did significantly inhibit expression of CPT1a ($p=0.005$), CPT1c ($p=0.026$) and PDK4 ($p=0.0495$) in the hypothalamus, but not the liver (Fig. 5).

Estradiol treatment increases hypothalamic expression of AgRP and NPY

We further assessed the effect of estradiol on hypothalamic expression of several feeding-related neuropeptides such as agouti-related peptide (AgRP), neuropeptide Y (NPY) and proopiomelanocortin (POMC). As with genes regulating metabolic capacity, by two-way ANOVA there was no main effect of hypoglycemia, nor an interaction between estradiol and hypoglycemia, on hypothalamic expression of these genes (Fig.6), whereas estradiol treatment significantly induced expression of both AgRP ($p=0.009$) and NPY ($p=0.004$), and inhibited expression of hypothalamic POMC (though this effect narrowly failed to achieve statistical significance, $p=0.07$).

Discussion

Since several reports have indicated that estradiol attenuates neuroendocrine responses to hypoglycemia in rats and humans (Sandoval et al., 2003; Adams et al., 2005; Sandoval et al., 2007), and these neuroendocrine responses to hypoglycemia appear to be mediated by neurons in the hypothalamus (Borg et al., 1994; Borg et al., 1995; Borg et al., 1997), and that inhibition of hypothalamic estradiol receptors enhances responses to glucopenia (Tritos et al., 2004; Musatov et al., 2007), we hypothesized that estradiol attenuates hypothalamic responses to hypoglycemia. To test this hypothesis we assessed if estradiol would attenuate hypothalamic expression of genes we have previously shown to be induced by hypoglycemia (Mastaitis et al., 2005). The present study demonstrated that as hypothesized estradiol does attenuate the induction of GLUT-1 and I κ B in the hypothalamus, but not in liver (Fig.4). Consistent with previous reports that estradiol attenuates neuroendocrine responses to hypoglycemia, we also observed that estradiol attenuated the effect of hypoglycemia on plasma glucagon, pituitary POMC, and adrenal c-fos (Table 2). To the extent that the induction of pituitary POMC may be associated with hypoglycemia-induced secretion of ACTH and glucocorticoid secretion, and the induction of adrenal c-fos may associated with hypoglycemia-induced secretion of adrenal epinephrine, which, due to acute stress effects from sacrifice procedures were not measured in the present study, these markers also corroborate impaired neuroendocrine responses to hypoglycemia as previously reported (Sandoval et al., 2003; Adams et al., 2005; Sandoval et al., 2007).

Since hypothalamic responses to glucose are mediated by glucose metabolism (Yang et al., 1999; Cheng et al., 2008), we examined effects of estradiol on hypothalamic expression of genes regulating glucose metabolism (Mobbs et al., 2007). This analysis indicated that estradiol inhibited hypothalamic (but not hepatic) expression of CPT1a and PDK4 (Fig.5) both of which normally act to reduce glucose metabolism (Mobbs et al., 2007). These results suggest that at least one mechanism by which estradiol attenuates hypothalamic responses to hypoglycemia is by increasing hypothalamic glucose metabolism. It should be noted that although estradiol also inhibited the expression of CPT1c, this isoform does not catalyze fatty acid oxidation (Wolfgang et al., 2008), so the significance of its down-regulation in influencing responses to hypoglycemia is unknown. Furthermore, since the present studies do not distinguish between direct and indirect effects of estradiol or hypoglycemia on hypothalamic gene expression, this hypothesis will require further analysis using direct hypothalamic infusion of estradiol and/or glucose. Finally, since not all responses to hypoglycemia, for example the induction of angiotensinogen and inhibitor of DNA-binding isoform 1, were attenuated by estradiol, it is likely that effects of estradiol on metabolic gene expression were not uniform throughout all areas of the hypothalamus. A more detailed analysis of the effects of hypoglycemia and estradiol on individual hypothalamic nuclei will be a necessary follow-up study to resolve this issue.

Effects of estradiol on expression of genes influencing hypothalamic metabolism might also be relevant to other metabolic effects of estradiol, including reduced adiposity (Wade et al., 1985) and improved glucose tolerance (Dubuc, 1985; Heine et al., 2000). For example, the inhibition of brain CPT1a (Obici et al., 2003) and whole-body CPT1c (Wolfgang et al., 2006) has been reported to reduce food intake and adiposity, consistent with effects of estradiol. Similarly, inhibition of hypothalamic pyruvate dehydrogenase kinase improves peripheral glucose homeostasis (Lam et al., 2005). However, other reports of the effects of brain CPT1a on energy balance suggest opposite results (Aja et al., 2008), possibly reflecting hypothalamic nucleus-specific mechanisms (Lopez et al., 2006; Gao et al., 2007; Lopez et al., 2008). Furthermore, although in the present study estradiol treatment influenced hypothalamic expression of genes known to influence energy balance and glucose homeostasis (NPY, AgRP, and POMC), the effect was in a direction that should increase adiposity and impair glucose homeostasis, as previously reported (Baskin et al., 1995; Tritos et al., 2004), suggesting these effects are indirect and compensatory. Thus, as indicated above, further analysis of the role of metabolic genes in specific hypothalamic nuclei may clarify mechanisms mediating effects of estradiol on metabolic functions.

Experimental Procedure

Animals

Female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and individually housed in an AAALAC-approved animal facility with 12-h light/dark cycles (lights on at 7am), given free access to water and standard rodent chow. All studies were approved by the appropriate institutional animal review board (Institutional Animal Care and Use Committee). On day 1, bilateral ovariectomy was performed on 8-week-old mice under Avertin (Tribromoethanol) anesthesia; meanwhile, mice were randomized to implantation of either placebo pellets (OVX-P) or estradiol pellets (OVX-E) containing 0.72mg of 17 β -estradiol released over 60 days (Innovative Research of America, Sarasota, FL). After recovery for 7 days, food intake and body weight were measured every week.

Measurement of metabolic rate and physical activity

Metabolic rate and physical activity were determined using an indirect calorimetry system as previously described (Makimura et al., 2001). Mice were placed into metabolic cages

(Accuscan, Columbus, OH) at week 4 and allowed to acclimate for at least 24 hours to exclude any interference caused by stress or the new environment. V_{O_2} (volume of oxygen consumption), V_{CO_2} (volume of carbon dioxide production), heat production, respiratory quotient ($RQ = V_{CO_2}/V_{O_2}$), and horizontal activity were calculated per 10-min period over at least one light/dark cycle.

Glucose tolerance test

Glucose tolerance test was performed at week 5. Mice were fasted overnight (16 h) by removing food before lights out (18:30) and injecting intraperitoneally (i.p.) with glucose (2 g/kg body wt) at 10:30. A drop of blood was taken from the tail vein immediately before glucose injection and 15, 30, 60, and 120 min after injection. Blood glucose concentration was measured immediately by using Contour blood glucose meter (Bayer). Plasma insulin concentrations during the glucose tolerance test were also measured, using a rat insulin ELISA kit with mouse insulin standard (Crystal Chem Inc., Downers Grove, IL).

Insulin-induced hypoglycemia

After week 7, OVX-P and OVX-E mice were injected with human insulin (Sigma-Aldrich; i.p., ~2.2U/kg body wt) or saline to produce hypoglycemia or euglycemia, respectively. As described previously (Mastaitis et al., 2005), this protocol produced blood glucose <2.0 mmol/l from 30 min after injection of insulin until the time of sacrifice at 3 h after injection, without producing unconsciousness, seizures, or death. Mice were killed by decapitation after a brief exposure to carbon dioxide. Blood samples were collected, from which serum was separated and stored at -20°C; hypothalamic and other brain areas, along with peripheral tissues, were quickly removed, weighed, frozen on dry ice, and stored at -70°C until RNA extraction.

Hormone assays

Blood was collected upon sacrifice and hormone levels were measured in isolated serum samples. Serum leptin was assayed by ELISA (Crystal Chem, Downers Grove, IL), serum glucagon was assayed by ELISA (Waco Chemicals, Richmond, VA), and serum estradiol was measured by EIA (Cayman Chemical, Ann Arbor, MI).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). A previously described protocol was used for qRT-PCR (Mastaitis et al., 2005). Briefly, 5 µg of total RNA was converted into cDNA, with 100 pg cDNA utilized for each individual reaction in a 40-cycle three-step PCR using the ABI Prism 7900 thermocycler (Applied Biosystems, Foster City, CA). The PCR master mix contained 1x PCR buffer (20 mM Tris, pH 8.4, 50 mM KCl), 5 mM MgCl₂, 200 µM dNTPs, 0.5x SYBR green (Molecular Probes), 200 nM each primer pair, and 0.25 units of Platinum Taq (Invitrogen). Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Cyclophilin (CYC), α -tubulin and RPS11 were used as housekeeping genes for normalization. The primer sequences for the genes examined in the present study will be provided upon request. Data were obtained as Ct (threshold cycle) values obtained from ABI SDS software package, and the relative quantification of mRNA level (fold regulation) was determined by standard $\Delta\Delta$ Ct methods.

Statistical analysis

Data are presented as mean $\pm$ SEM (n=5-8 mice/group). Statistical significance level was set at 0.05. For the analysis of body weight, food intake and glucose tolerance test, student's t-test was performed to compare two groups at each independent time point. Two-way ANOVA analyses followed by post-hoc Tukey-Kramer HSD tests (metabolic cage data and gene expression data in Fig 4-6) were carried using JMP software.

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Fig.1. Estradiol treatment decreases body weight gain but not daily food intake
Mice were ovariectomized and implanted with either placebo (OVX-P) or estradiol (OVX-E). Body weight (**A**) and food intake (**B**) were monitored weekly. Data are presented as mean $\pm$ SEM (n=12-14/group) and statistical difference was determined by Student's t-test (* $p < 0.05$).

Fig.2. Estradiol treatment has no effect on heat production or physical activity but increases respiratory quotient

Parameters of metabolic rate and physical activity were determined by indirect calorimetry over at least one light/dark cycle. Estradiol had no significant effect on (A) heat production or (B) horizontal activity (number of beam breaks due to horizontal movement per 10-min period), but (C) increased respiratory quotient (RQ). Statistical difference was determined by two-way ANOVA followed by Tukey HSD test ($n=8$; $p<0.05$)

Fig.3. Estradiol treatment enhances glucose tolerance

Mice were challenged with glucose (at 0min) after overnight fasting. Blood samples were collected to measure blood glucose (**A**) and plasma insulin (**B**) concentrations. Data are presented as mean $\pm$ SEM (n=12-14/group) and statistical difference was determined by Student's t-test (*p<0.05)

Fig.4. Estradiol treatment attenuates the induction of hypothalamic gene expression by insulin-induced hypoglycemia

Hypoglycemia was induced by i.p. insulin injection alone, as described in Materials and Methods. Mice were sacrificed 3 hours after insulin injection and tissue samples were collected for gene expression analysis. Total RNA was extracted to measure relative mRNA level by quantitative real-time PCR (qRT-PCR). Data are calculated as fold regulation and presented as mean $\pm$ SEM (n=5-8). Groups with different letters are statistically different ($p < 0.05$) by two-way ANOVA followed by Tukey HSD test.

Fig.5. Estradiol treatment produces pro-glycolytic hypothalamic gene expression

Hepatic and hypothalamic CPT1a (A), CPT1c (B) and PDK4 (C) mRNA levels were measured by qRT-PCR. Data are calculated as fold regulation and presented as mean $\pm$ SEM (n=5-8). Statistical differences were determined by two-way ANOVA. There was not a significant interaction between estradiol and hypoglycemia, nor a main effect of hypoglycemia on the expression of these genes except hepatic GK (not shown) and hypothalamic CPT1c (p=0.021), whereas estradiol significantly inhibited the expression of CPT1a (p=0.005), CPT1c (p=0.026) and PDK4 (p=0.0495) in hypothalamus but not in liver.

Fig.6. Estradiol treatment induces hypothalamic expression of AgRP and NPY

Hypothalamic AgRP, NPY, and POMC mRNA levels were measured by qRT-PCR. Data are presented as mean $\pm$ SEM (n=5-8). Statistical difference was determined by two-way ANOVA. There was no main effect of hypoglycemia, nor an interaction between estradiol and hypoglycemia, whereas estradiol significantly induced expression of both AgRP (p=0.009) and NPY (p=0.004), and inhibited expression of hypothalamic POMC (though this effect narrowly failed to achieve statistical significance, p=0.07).

Table 1

Measurements at the end of the Study

	OVX-P (n=10-12)	OVX-E (n=13-14)
Fat (g)	0.50±0.052	0.18±0.008(* p<10 ⁻⁵)
Uterus (g)	0.02±0.005	0.18±0.014(* p<10 ⁻⁸)
Estradiol (pg/ml)	10.6±1.11	34.1±7.66 (* p<0.05)

Upon sacrificem intra-abdominal fat and uterus were isolated and weighed; blood samples were collected and serum estradiol levels were measured. Data are means±SEM and statistical difference was determined by Student's t-test.

Table 2
OVX-E and OVX-P subgroups and measurements

Group	P-Sal(n=5)	P-Ins(n=7)	E-Sal(n=5)	E-Ins(n=8)
BW (g)	23.7±0.3	24.8±0.3	22.7±0.5	23.3±0.3
BW gain (g)	5.1±0.4	5.5±0.4	3.9±0.5	4.3±0.4
Leptin (pg/ml)	4894.4±1180.0	9592.8±1327.3	1192.9±210.4 *	2797.8±213.0 #
Glucagon (pg/ml)	590.4±226.5	917.9±239.9	622.9±190.5	472.4±50.9
POMC (A.P.) (FR of mRNA)	1.00±0.27 (n=3)	1.25±0.19	0.95±0.27	0.67±0.12 \$
c-fos (A.G) (FR of mRNA)	1.00±0.33	3.63±0.73 *	2.10±1.30	3.43±0.74

Serum leptin and glucagon levels were measured as described in Materials and Methods. mRNA expression of POMC in anterior pituitary (A.P.) and c-fos in adrenal gland (A.G) was measured by qRT-PCR. Data are means±SEM. Statistical difference was determined by Student's t-test (p<0.05,

* vs. P-Sal

vs. E-Sal

\$ vs. P-Ins).