Tamoxifen Improves Glucose Tolerance in a Delivery-, Sex-, and Strain-Dependent Manner in Mice

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Tamoxifen, a selective estrogen-receptor modulator, is widely used in mouse models to temporally control gene expression but is also known to affect body composition. We report that tamoxifen has significant and sustained effects on glucose tolerance, independent of effects on insulin sensitivity, in mice. IP, but not oral, tamoxifen delivery improved glucose tolerance in three inbred mouse strains. The extent and persistence of tamoxifen-induced effects were sex and strain dependent. These findings highlight the need to revise commonly used tamoxifen-based protocols for gene manipulation in mice by including longer chase periods after injection, oral delivery, and the use of tamoxifen-treated littermate controls. (Endocrinology 160: 782–790, 2019)

Materials and Methods
Animals

All procedures relating to animal care and treatment conformed to The Johns Hopkins University Animal Care and Use Committee and National Institutes of Health guidelines. Animals were group housed under a standard 12:12 light-dark cycle. Animals of both sexes were used for all analyses, and the used mouse strains, BALB/cJ, C57BL/6J, and 129S1/SvImJ, although the extent and persistence of enhanced glucose tolerance varied in a sex- and strain-dependent manner. In C57BL/6J mice, IP tamoxifen delivery enhanced glucose-stimulated insulin secretion (GSIS) in males, but not females. In addition, acute tamoxifen treatment enhanced basal insulin secretion in isolated islets from male C57BL/6J mice. Tamoxifen administration had no effect on insulin sensitivity. Intriguingly, oral administration of tamoxifen through diet did not affect glucose tolerance. Together, these results highlight the need for caution in interpreting results of metabolic changes when using IP tamoxifen delivery for temporal control of gene expression in mice, and suggest revised measures, such as extended chase periods after injection, oral delivery, and comparison with tamoxifen-treated littermate controls.
Male mice were individually housed with food overnight before tamoxifen injection. At 6 weeks of age, mice were injected IP with 100 mg/kg tamoxifen in corn oil, or corn oil alone, daily for 5 days. Metabolic assays were performed 1 to 3 weeks after the final injection.

**Oral administration**

Beginning at 5 weeks of age, mice were switched from standard chow to either control diet (TD.07570; Envigo) or tamoxifen diet (TD.130856; Envigo) for 10 days with a 2-day break on standard chow after the fifth day. Because oral tamoxifen administration has been reported to reduce food intake and body weight (6), mice were given 6 days to recover on standard chow before glucose tolerance testing.

**Glucose tolerance and in vivo insulin secretion**

Mice were individually housed and fasted overnight (16 hours) before glucose administration. For IP glucose tolerance and insulin secretion tests, mice were administered 2 g/kg glucose and 3 g/kg glucose, respectively. For oral glucose tolerance tests and insulin secretion tests, mice were administered 3 g/kg glucose by gavage feeding needle. Blood glucose levels were measured with a OneTouch Ultra 2 glucometer (LifeScan), and plasma insulin levels were measured with an Ultrasensitive Insulin ELISA (Crystal Chem) (7). The area under the curve was determined for glucose tolerance for each animal, using GraphPad Prism, version 8.

**Insulin tolerance**

Mice were individually housed with food overnight before 0.75U/kg IP insulin injection (Novolin-R; Novo Nordisk). Blood glucose levels were measured with a OneTouch Ultra 2 glucometer.

**Mouse islet isolations and in vitro insulin secretion**

Islets were isolated from male C57BL/6J mice as previously described (8). Briefly, islets were isolated by collagenase dispersion through the bile duct (Collagenase P, 0.375 to 0.4 mg/mL in Hanks balanced salt solution) and digestion at 37°C. Digested pancreata were washed with Hanks balanced salt solution plus 0.1% BSA and subjected to discontinuous density gradient using Histopaque (6:5 Histopaque 1119 to Histopaque 1077; Sigma). The islet layer (found at the interface) was collected, and islets were handpicked under an inverted microscope and cultured overnight in RPMI 1640 (5% FBS, 5 U/L penicillin-streptomycin). Islets were then handpicked to be size matched, and 5 to 10 islets per mouse were washed and pre- incubated for 30 minutes in Krebs-Ringer HEPES buffer containing 2.8 mM glucose. Tamoxifen (10 μM) or vehicle (dimethyl sulfoxide) was added and islets were incubated for 30 minutes. Supernatant was sampled and islets were then stimulated with 16.7 mM glucose for another 30 minutes. Supernatant fractions were removed and islets lysed in acid-ethanol overnight and subsequently neutralized in Tris buffer (0.885 M). Cellular and supernatant fractions were subjected to insulin ELISA (7).

**Results**

**IP tamoxifen injections improved glucose tolerance in mice**

To assess the effects of tamoxifen on glucose metabolism in mice, we used a common paradigm for tamoxifen-inducible gene inactivation in CreER/LoxP mouse models via IP administration of tamoxifen (100 mg/kg body weight in corn oil) for 5 consecutive days (9). Control animals were administered equivalent volumes of corn oil alone. We assessed glucose tolerance, insulin tolerance, and GSIS in vivo in three inbred mouse strains: BALB/cJ, C57BL/6J, and 129S1/SvImJ. Body weights were measured weekly and were unaffected in both sexes of all strains tested after tamoxifen injection.

After tamoxifen injections, we performed glucose tolerance tests on male and female mice from all three strains 1 week after the final administration. Tamoxifen injections significantly improved glucose tolerance in male and female BALB/cJ and C57BL/6J mice (Fig. 1A–1F), and in female, but not male, 129S1/SvImJ mice (Fig. 1G–1I). Of note, tamoxifen-injected male BALB/cJ mice also had significantly lower fasted blood glucose levels compared with controls (Fig. 1A). Together, these results suggest that IP tamoxifen administration enhances glucose tolerance in mice.

**Persistence of improved glucose tolerance in tamoxifen-injected mice was sex and strain dependent**

To determine if the effects of tamoxifen injection on glucose tolerance persisted, we extended the recovery period to 21 days after the final tamoxifen injection. Three weeks after tamoxifen delivery, male and female BALB/cJ mice and male C57BL/6J mice still had improved glucose tolerance (Fig. 2A–2D and 2F), albeit to a lower extent than the tamoxifen-induced effects observed 1 week after injections (Fig. 1A–1F). However, in female C57BL/6J mice, tamoxifen-induced enhancement of glucose tolerance was corrected by 3 weeks (Fig. 2E and 2F). Female 129S1/SvImJ mice still showed a modest improvement in glucose tolerance 3 weeks after injections (Fig. 2H and 2I). Glucose tolerance in male 129S1/SvImJ mice was still unaffected 3 weeks after injection (Fig. 2G and 2I). Together, these results suggest that the effects of IP tamoxifen delivery on improving glucose tolerance persist in a sex- and strain-dependent manner at 3 weeks after injections, with male mice tending to exhibit more long-lasting effects. Overall, the effects of tamoxifen on enhancing...
glucose tolerance 3 weeks postinjection were modest compared with 1-week postinjection, suggesting that extended recovery periods after tamoxifen delivery in CreER mouse lines may be sufficient to avoid tamoxifen-induced effects on glucose tolerance.

**IP tamoxifen administration enhanced insulin secretion in male C57BL/6J mice, but insulin sensitivity was unaffected**

Improved glucose tolerance can be caused by a variety of factors, including enhanced insulin secretion from...
pancreatic islets or improved insulin sensitivity in peripheral tissues. Estrogen-receptor signaling promotes GSIS in isolated islets, and tamoxifen can bind to pancreatic estrogen receptors (10,11). Furthermore, tamoxifen-induced Cre-mediated recombination persists up to 4 weeks after tamoxifen injection in pancreatic islets (12). Therefore, we asked if tamoxifen injection improves glucose tolerance by influencing insulin secretion. We assessed GSIS in vivo in male and female C57BL/6J mice, the most commonly used inbred mouse strain (13), 1 week after final IP tamoxifen injection. Tamoxifen injections enhanced GSIS in male, but not female, C57BL/6J mice.

Figure 2. Persistence of improved glucose tolerance in tamoxifen-injected mice was sex and strain dependent. Data are presented as mean ± SEM unless otherwise indicated. (A) Male and (B) female BALB/cJ mice (n = 5 to 8 mice per sex per condition) retained significantly improved glucose tolerance 3 weeks after IP tamoxifen (TMX) administration. (C) Area under the curve for BALB/cJ glucose tolerance. (D) Male, but not (E) female, C57BL/6J mice (n = 6 to 7 mice per sex per condition) retained significantly improved glucose tolerance 3 weeks after IP TMX administration. (F) Area under the curve for C57BL/6J mice glucose tolerance. (G) Glucose tolerance did not change in male 129S1/SvImJ mice (n = 4 per condition) in response to IP TMX administration. (H) Glucose tolerance in TMX-injected female 129S1/SvImJ mice (n = 4 per condition) was modestly improved 3 weeks after final injection. (I) Area under the curve remained significantly lower for female 129S1/SvImJ mice. Differences determined by t tests. *P < 0.05; **P < 0.01. Veh, vehicle.
suggesting sex-specific effects of tamoxifen on insulin secretion. Insulin tolerance tests revealed that tamoxifen injections had no effect on insulin sensitivity in male and female C57BL/6J mice (Fig. 3C and 3D). To assess if tamoxifen directly acts on β cells to influence insulin secretion, we isolated islets from male C57BL/6J mice and stimulated them with 10 μM tamoxifen before assessing insulin secretion. At low-glucose concentration (2.8 mM), tamoxifen treatment significantly increased insulin secretion (Fig. 3E). However, tamoxifen treatment did not augment insulin secretion in response to high-glucose concentration (16.7 mM; data not shown). Together, these results suggest that although tamoxifen can induce insulin secretion, tamoxifen-induced improvement of glucose tolerance in male C57BL/6J mice arises from islet-specific and islet-extrinsic factors. Furthermore, enhanced insulin sensitivity is not a contributing factor to the tamoxifen-induced improved glucose tolerance.

Release of intestinal incretin hormones modulates GSIS in vivo. Incretin release is stimulated by oral, but not IP, glucose delivery in mice (14). We assessed the effects of tamoxifen on glucose tolerance and insulin secretion in vivo in response to an oral glucose challenge via gavage. Male tamoxifen-injected C57BL/6J mice had enhanced glucose tolerance and insulin secretion in response to oral glucose compared with controls, and the tamoxifen effects were similar in magnitude to that seen with IP glucose delivery (Fig. 3F–3H). These results suggest that effects of tamoxifen on glucose tolerance are likely independent of incretin signaling.

**Oral tamoxifen delivery did not affect glucose tolerance**

Recent studies have described the use of tamoxifen-supplemented chow as an effective and noninvasive means for spatiotemporal gene activation in CreER mouse lines (15, 16). Furthermore, plasma tamoxifen levels are lower after oral tamoxifen delivery vs injection (17). Thus, we asked if oral tamoxifen delivery would also affect glucose tolerance in mice.

We fed mice commercially available tamoxifen-supplemented or control diets for 10 days with a 2-daybreak after the fifth day. This paradigm was based on the assumption that mice consume an average of 3 to 4 g of food daily and weigh 20 to 25 g, corresponding to ~40 mg of tamoxifen per kilogram of body weight per day (18). Approximate tamoxifen consumption was estimated by weighing tamoxifen-supplemented chow before and after the treatment period and calculating the milligrams of tamoxifen consumed per animal (based on 250 mg tamoxifen per kilogram of food).

These analyses suggested that comparable amounts of tamoxifen were delivered between IP injection and diet delivery (~500 mg/kg total from injection vs 440 ± 77 mg/kg for males and 432 ± 73 mg/kg for females from diet [values are mean ± SD for 10 males and 11 females from all three strains]). Interestingly, glucose tolerance was unaffected by oral tamoxifen administration in males and females in all three mouse strains tested 6 days after treatment (Fig. 4A–4I). These results suggest that the mode of tamoxifen administration significantly influences its effects on glycemic control.

**Discussion**

Here, we report that IP, but not oral, tamoxifen administration to three common inbred mouse strains induced a significant and often sustained improvement of glucose tolerance without affecting insulin sensitivity. Interestingly, male, but not female, C57BL/6J mice had increased GSIS in vivo. In isolated islets from male C57BL/6J mice, acute tamoxifen stimulation increased insulin secretion under basal conditions (2.8 mM glucose) but did not augment insulin secretion in response to high (16.7 mM) glucose concentration. These results suggest that although tamoxifen can promote islet insulin secretion, the enhanced GSIS in vivo is likely due to a combination of islet-dependent and islet-independent effects of tamoxifen.

Estrogens and estrogen-receptor signaling play important roles in β-cell function as well as protection of β-cell mass (11, 19–23). β cells express the estrogen receptors ERα, ERβ, and GPER, and all three have been implicated in aspects of β-cell biology. Specifically, ERα primarily promotes β-cell survival as well as glucose-stimulated insulin biosynthesis (11, 19–21). ERβ promotes insulin secretion via membrane depolarization, and GPER promotes β-cell survival and insulin secretion (19, 22). The actions of these estrogen receptors, combined with our results, suggest tamoxifen may act as an agonist for β-cell estrogen receptor(s) to increase insulin secretion in low-glucose conditions. This mechanism is consistent with previous studies demonstrating the role of estrogen signaling in depolarizing β-cell membranes to induce insulin secretion in low, but not high, glucose conditions in a mouse β-cell line (MIN6) and human islets (23). Our observations that GSIS in vivo was increased after IP tamoxifen delivery suggests that although tamoxifen can increase basal insulin secretion in isolated islets, islet-extrinsic factors may contribute to tamoxifen effects on glucose-stimulated insulin secretion and glucose tolerance in vivo. Our results suggest that incretin release, which is known to augment GSIS in vivo, is not a
Figure 3. IP tamoxifen (TMX) administration enhanced insulin secretion in male C57BL/6J mice, but insulin sensitivity was unaffected. Data are presented as mean ± SEM unless otherwise indicated. Differences were determined by t tests. (A) GSIS was improved after IP TMX administration in male C57BL/6J mice (n = 5 to 11 mice per condition). (B) GSIS was unchanged by IP TMX administration in female C57BL/6J mice (n = 5 to 8 mice per condition). Insulin sensitivity was unaffected by IP TMX administration in (C) male and (D) female C57BL/6J mice (n = 5 to 6 mice per condition per sex). (E) TMX stimulation significantly increased islet insulin secretion in low-glucose (2.8 mM) conditions (n = 6 vehicle-treated islet cultures and n = 5 TMX-treated islet cultures from six mice). One-sample t test; *P < 0.05. (F) Male TMX-injected C57BL/6J mice (n = 3 to 4 mice per condition) had improved glucose tolerance in response to an oral glucose challenge 1 week after the final TMX injection. (G) Area under the curve for oral glucose tolerance was significantly lower in TMX-injected male C57BL/6J mice. (H) Oral GSIS was improved after IP TMX administration in male C57BL/6J mice (n = 3 to 5 mice per condition). *P < 0.05. Veh, vehicle.
contributing factor to the effects of tamoxifen; we found similar effects of tamoxifen on improving glucose tolerance and insulin secretion with IP and oral glucose delivery. In addition to the role of estrogens in promoting β-cell insulin secretion, estrogen signaling promotes neural-dependent insulin secretion in male mice (24), and neuronal signaling might underlie the tamoxifen-induced increase in GSIS in male C57BL/6J mice. We found that tamoxifen administration had no effect on insulin sensitivity. However, glucose-clamp experiments are required to more precisely dissect the effects of tamoxifen on insulin sensitivity.

Enhanced glucose tolerance in female C57BL/6J mice upon tamoxifen treatment was not coupled with

Figure 4. Oral tamoxifen (TMX) administration did not affect glucose tolerance. Data are presented as mean ± SEM unless otherwise indicated. Glucose tolerance did not change in (A) male and (B) female BALB/cJ mice (n = 4 to 5 mice per sex per condition) in response to TMX diet. (C) Area under the curve for BALB/cJ mice glucose tolerance. Glucose tolerance did not change in (D) male or (E) female C57BL/6J mice (n = 4 to 5 mice per sex per condition) in response to TMX diet. (F) Area under the curve for C57BL/6J mice glucose tolerance. Glucose tolerance did not change in (G) male or (H) female 129S1/SvImJ mice (n = 3 to 5 mice per sex per condition) in response to TMX diet. (I) Area under the curve for 129S1/SvImJ mice glucose tolerance. Veh, vehicle.
increased in vivo GSIS, as was the case in males. Enhanced glucose tolerance in female C57BL/6J mice could arise from changes in insulin-independent glucose uptake mechanisms. Results of previous studies suggest insulin-independent glucose uptake is prominent during hyperglycemia in tissues such as skeletal muscle (25). Notably, estrogen-receptor signaling in skeletal muscle promotes membrane localization of the glucose transporter GLUT4 in an insulin-independent manner (26). These findings, together with our observations, suggest that tamoxifen may regulate glucose tolerance in female C57BL/6J mice by promoting insulin-independent glucose uptake.

Studies have reported on sex and strain differences in glucose metabolism in mice (27). Our results indicate that the magnitude and persistence of tamoxifen-induced enhancement of glucose tolerance were sex and strain dependent. The persistence of tamoxifen in the body has reported to be age dependent, with older animals being unable to effectively clear tamoxifen (28). We used young animals (2 months old), and tamoxifen-injected animals were always compared with age-matched, vehicle-injected controls. Therefore, age is unlikely to be a factor in the observed sex- and strain-dependent effects of tamoxifen on glucose tolerance. Tamoxifen persists differentially in different tissues. For example, tamoxifen has acute effects in the brain (28) and more long-lasting effects in pancreatic islets, with tamoxifen-induced Cre activity persisting for as long as 4 weeks after IP injections (12). Therefore, tamoxifen may act differently in distinct tissues in a sex- and strain-specific manner to influence glucose tolerance. C57BL/6J mice carry a genomic mutation resulting in diminished levels of the mitochondrial pump nicotinamide nucleotide transhydrogenase (Nnt), and NNT levels are thought to positively correlate with insulin secretion (29, 30). We found that BALB/cJ and female 129S1/SvImJ mice, which have not been reported to carry the NNT mutation, have improved glucose tolerance upon tamoxifen treatment, similar to C57BL/6J mice that carry the mutation. Therefore, the NNT mutation in C57BL/6J mice is unlikely to be a critical factor in the effects of tamoxifen on glucose tolerance and insulin secretion. However, a rigorous comparison of C57BL/6J and C57BL/6N mice, which do not carry the Nnt mutation (30), would shed light on this possibility.

Intriguingly, oral administration of tamoxifen did not affect glucose tolerance. Compared with injection-based tamoxifen administration, oral tamoxifen delivery results in lower plasma tamoxifen levels but higher circulating levels of tamoxifen metabolites, such as 4-hydroxytamoxifen (17). Improved glucose tolerance with tamoxifen injection might be due to the higher and persistent circulating tamoxifen levels compared with oral administration. This suggests that the effects of tamoxifen may be dose dependent; low doses of tamoxifen, even delivered IP, may not affect glucose tolerance. However, in the case of CreER lines, such low doses may not be effective for inducing gene recombination (31). In addition, this suggests that enhanced glucose tolerance after IP tamoxifen administration is likely due to tamoxifen itself, not one of its metabolites. If this is the case, then injection of 4-hydroxytamoxifen, a tamoxifen metabolite that is a potent activator of CreER, may bypass the tamoxifen-dependent effects, similarly to oral tamoxifen administration (2). Our findings highlight the need to revise commonly used tamoxifen-based protocols for gene manipulation in mice, including considering oral delivery, allowing longer chase periods after injection, and the use of tamoxifen-treated littermate controls.

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