Systemic PPARγ Deletion Causes Severe Disturbance in Fluid Homeostasis in Mice

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Abstract

The pharmacological action of PPARγ in promoting sodium and water retention is well documented as highlighted by the major side effect of body weight gain and edema associated with thiazolidinedione (TZD) use. However, a possible physiological role of PPARγ in regulation of fluid metabolism has not been reported by previous studies. Here we analyzed fluid metabolism in inducible whole-body PPARγ knockout mice. The null mice developed severe polydipsia and polyuria, reduced urine osmolality, and modest hyperphagia. The phenomenon persisted during 3 days of pair feeding and pair drinking, accompanied with progressive weight loss. After 24-h water deprivation (WD), the null mice had a lower urine osmolality, a higher urine volume, a greater weight loss, and a greater rise in hematocrit than the floxed control. Urinary vasopressin (AVP) excretion was not different between the genotypes under basal condition or after WD. The response of urine osmolality to acute and chronic DDAVP treatment was attenuated in the null mice but the total abundance or phosphorylation of aquaporin 2 (AQP2) in the kidney or AVP-induced cAMP production in IMCD suspensions was unaffected. Overall, PPARγ participates in physiological control of fluid homeostasis through an unknown mechanism involving cAMP/AQP2-independent enhancement of AVP response.

Key words: peroxisome proliferator-activated receptor, vasopressin, kidney
Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that heterodimerizes with the retinoid X receptor and binds to PPAR-responsive elements in the regulatory region of target genes. This region is involved in various aspects of metabolism, including lipid uptake, fatty acid metabolism and glucose homeostasis. Therefore, the modulation of PPARγ action is of intense interest in the treatment of insulin resistance and related metabolic disorders (1). Expression of PPARγ is highest in adipose tissue, where it controls adipocyte differentiation and lipid storage (30). In addition, it has high expression in macrophages and plays a role in modulating inflammation (5, 25, 26, 28). PPARγ has four isoforms that are widely expressed in various other tissues, including brain, colon, muscles, kidney, spleen and pancreas (29). It has been implicated in the pathology of numerous diseases such as obesity, diabetes, atherosclerosis and cancer, and its agonists have been used in the treatment of hyperlipidemia and hyperglycemia (29).

Emerging evidence suggests a potential role of PPARγ in regulation of fluid metabolism. The fluid regulatory action of PPARγ is highlighted by the well-documented side effect of body weight gain and edema associated with thiazolidinedione (TZD) use. TZDs are synthetic PPARγ agonists including rosiglitazone and pioglitazone, both of which are highly effective in managing hyperglycemia in patients with type 2 diabetes but are limited by fluid retention. The mechanism of TZD-induced fluid retention appears multifactorial involving altered
tubular transport function in both distal and proximal tubules and also the change in
capillary permeability in adipose tissues (20, 23, 33). These studies provide insights
into the pharmacological action of TZDs but none of them address the physiological
function of PPARγ in the setting of fluid metabolism. Mice lacking PPARγ in the
collecting duct showed resistance to TZD-induced fluid retention but no disturbance
in sodium or water balance under physiological condition (9, 34). Here, we report that
mice with inducible systemic deletion of PPARγ develop polydipsia and polyuria and
hypoosmotic urine, suggesting a novel physiological function of PPARγ in regulation
of fluid metabolism.

Materials and Methods

Transgenic mouse lines

PPARγ f/f mice and EsrCre/flox mice were originally generated by Yang et al. (32).
For in vivo experiments, 2- to 4-month old male mice were maintained under a 12:12
hour light-dark cycle. All procedures were in accordance with the guidelines approved
by the University of Utah Institutional Animal Care and Use Committee.

Tamoxifen administration

Tamoxifen stock solution was prepared as previously described with modifications
(14). Briefly, 100 mg tamoxifen (Sigma) was suspended in 150 µl of ethanol,
followed by the addition of 850 µl of peanut oil (Sigma) and dissolved at 55°C. Two-
mo-old PPARγ f/f mice and EsrCre/flox mice were administered 50 µl (5 mg) of
tamoxifen solution per day by oral gavage for 5 consecutive days (termed floxed and KO, respectively). All experiments were conducted at least 10 days after the last tamoxifen administration.

**Water studies**

Male floxed and KO mice had free access to water and standard food and were acclimated for 1 week to the metabolic cages (Hatteras Instruments). After 1 week, metabolic studies were performed. Daily food intake, water intake and body weights were measured, and urine was collected over a 24-h period. The excretion of urine electrolytes, osmolality of urine and plasma, and concentrations of plasma Na⁺ and K⁺ were measured.

For dehydration studies, all mice were subjected to 24 h of water deprivation (WD) by removing the water bottles from the metabolic cages (Hatteras Instruments). The control groups of animals had free access to water and standard food, while the WD groups were provided only with standard food. Urine was collected over a 24-h period. Urine volume, urine osmolality, body weight, hematocrit and urinary vasopressin (AVP) excretion were measured.

For pair feeding and pair drinking studies, all mice were acclimated for 1 week to the metabolic cages, and 2 days of baseline measurements were taken as described above. Subsequently, the food and water intake of KO mice was restricted to the average of
floxed mice, while floxed mice had free access to water and standard food. Daily food
intake, water intake and body weights were measured, and urine was collected over a
24-h period. Urine volume and osmolalities were measured.

1-desamino-8-D-arginine vasopressin (DDAVP) studies

For the acute DDAVP experiment, urine was emptied from mice by bladder massage
and urine osmolalities were measured as basal value, then the mice were
intraperitoneally injected with DDAVP (720 ng/kg body weight). Over a 2-h period in
which mice had no access to food or water, urine was collected by bladder massage
and urine osmolalities were measured.

For the chronic DDAVP experiment, mice were acclimatized to metabolic cages, and
3 days of baseline measurements were taken as described above. Subsequently, mice
were given DDAVP (0.25 ng/h) via subcutaneous osmotic minipump (Alzet model
1002, Alzet, Cupertino, CA) for 5 days, with free access to water and standard food.
At the end of the experiment, urine osmolalities were measured. Mice were killed by
halothane inhalation, and their kidneys were harvested for analysis of protein and
gene expression.

Enzyme immunoassay

Collecting duct segments were acutely isolated as previously described (13). Briefly,
the inner medulla was minced in Hank's balanced salt solution (HBSS; Sigma) and
incubated in collagenase-hyaluronidase solution at 37°C for 30–45 min. Inner
medullary collecting duct (IMCD) fragments were washed and incubated with HBSS.

IMCD fragments were incubated for 30 min with 1 mmol/L IBMX (Sigma) before addition of DDAVP (10 nmol/L; Sigma) for 10 min. Cells were extracted with 0.1 mol/L HCl, and cAMP levels were measured by enzyme-linked immunosorbent assay (ELISA; Cayman Chemical, Ann Arbor, MI). Total cell protein was measured by a Bradford assay. In WD model, mice urinary AVP was measured by ELISA (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions.

**Immunoblotting**

Lysates of the kidney medullary tissue were stored at −80°C until assayed. Protein concentrations were determined by using coomassie reagent. An equal amount of the whole tissue protein was denatured at 100°C for 5 min, separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation with rabbit polyclonal antibodies against aquaporin 2 (AQP2) (a gift from Dr. Mark A. Knepper), pAQP2-ser256 (Abcam, cat. no. ab109926), pAQP2-ser269 (Abnova, cat. no. PAB17203) and mouse monoclonal anti-β-actin (Sigma, cat. no. A1978). The blots were washed with TBS followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected by using enhanced chemiluminescence methods. The immunoreactive bands were quantified with the Gel and Graph Digitizing System (Silk Scientific).
Statistical analysis

All values are presented as mean ± SE. Statistical analysis was performed by using a Student t test or analysis of variance. For multiple comparisons, a two-way ANOVA with the Bonferroni correction was performed. Differences were considered to be significant when $P < 0.05$.

Results

Physiological data from systemic PPARγ null mice under basal condition

Before tamoxifen administration, PPARγ $\text{f/f}$ and EsrCre/flx mice had the same phenotype, as indicated by similar body weight, food intake, water intake and urine volume between the genotypes. After tamoxifen administration, PPARγ KO mice showed a phenotype characterized by polyphagia, polydipsia and polyuria, with reduced urine osmolality (Figure 1). However, there was no difference in urinary excretion of sodium, potassium and chloride; plasma osmolality; or plasma sodium and potassium concentration (Table 1).

Effect of systemic PPARγ deletion on urine concentrating capability during pair feeding and pair drinking

Because PPARγ KO mice showed polyphagia and polydipsia, we performed a pair feeding and pair drinking experiment to determine whether the polyuria in these mice resulted from polyphagia and polydipsia. During 3 days of restricting food and water intake to the same level as in floxed mice, the KO mice showed a gradual decrease of
urine volume associated with a progressive loss of body weight, without an obvious increase in urine osmolality (Figure 2). The lack of response of urine osmolality to restricted food and water intake in the KO mice suggests impairment in urine concentrating capability intrinsic to the kidney.

**Effect of systemic PPARγ deletion on urine concentrating ability after 24 h of WD**

To confirm urine concentrating defect in PPARγ KO mice, we performed a WD experiment. After 24-h WD, both floxed and KO mice had a reduced urine volume (floxed: WD 0.73 ± 0.09 vs. basal 1.01 ± 0.13 ml, \( P < 0.01 \); KO: WD 1.31 ± 0.22 vs. basal 2.32 ± 0.27 ml, \( P < 0.01 \)) and an elevated urine osmolality (floxed: WD 3478.1 ± 114.8 vs. basal 2538.4 ± 202.9 mOsmol/kg H₂O, \( P < 0.01 \); KO: WD 1945.4 ± 55.0 vs. basal 1526.4 ± 64.9 mOsmol/kg H₂O, \( P < 0.01 \)) (Figure 3). However, in response to WD, the urine osmolality of KO mice was still much lower than the normal level. The relative change in urine osmolality after WD was 48.6 ± 0.1% in floxed mice 24.7 ± 0.04% in the KO mice (\( n = 7 \) per group, \( P = 0.06 \)). Moreover, in response to WD, KO mice had a much greater decrease in body weight (floxed 7.65 ± 0.56% vs. KO 15.92 ± 0.99%, \( P < 0.01 \)) and a greater increase in hematocrit (floxed: WD 0.530 ± 0.012 vs. basal 0.521 ± 0.012, \( P > 0.05 \); KO: WD 0.549 ± 0.016 vs. basal 0.497 ± 0.008, \( P < 0.05 \)) (Figure 3). This result suggested that the KO mice had impaired ability to concentrate urine leading to plasma volume contraction and body weight loss during WD.
Effect of DDAVP on urinary concentration in systemic PPARγ null mice

AVP is crucial for urinary concentration. To determine whether systemic PPARγ deletion impaired AVP response, we performed a DDAVP experiment. For the acute DDAVP experiment, we administered DDAVP (by intraperitoneal application) before collecting urine for 2 h by bladder massage. Administration DDAVP significantly increased urine osmolality in floxed mice, but not in KO mice (shown in Figure 4A; floxed: DDAVP 3576.6 ± 156.0 vs. Basal 2803.7 ± 190.4 mOsmol/kg H2O, P < 0.05; KO: DDAVP 1709.6 ± 101.2 vs. Basal 1668.2 ± 180.7 mOsmol/kg H2O, P > 0.05).

We next performed a chronic DDAVP experiment. Mice were given DDAVP at 0.25 ng/h via subcutaneous osmotic minipump (Alzet model 1002) for 5 days, and urine volume and urine osmolalities were monitored. The results showed a significantly increased urine osmolality in floxed mice, but no response in KO mice (shown in Figure 4B and 4C). These results indicate that systemic knockout of PPARγ may impair AVP response.

Effect of systemic PPARγ deletion on urinary AVP excretion and AVP-stimulated cAMP accumulation

Urinary AVP and AVP-induced cAMP were analyzed in control and WD mice. Both floxed mice and KO mice increased urinary AVP in response to WD, with no difference between the genotypes (shown in Figure 5A; floxed: WD 2.16 ± 0.34 vs. basal 0.48 ± 0.09 pmol/24 h, P < 0.05; KO:WD 2.61 ± 0.33 vs. basal 0.32 ± 0.06
pmol/24 h, \( P < 0.05 \), suggesting that renal AVP content did not account for the

differences in urine osmolality between the groups. To evaluate the effect of PPAR\(\gamma\)
deletion on AVP-stimulated cAMP accumulation, we treated IMCD suspension with
10 nmol/L DDAVP for 15 min and measured cAMP levels by ELISA. The IMCD
fragments from both floxed mice and KO mice showed an increased cAMP level in
response to DDAVP, with a 3.5-fold increase in floxed mice and a 4.1-fold increase in
KO mice (shown in Figure 5B; floxed: DDAVP 328.4 ± 33.3 vs. vehicle 94.2 ± 10.6
pmol/mg, \( P < 0.05 \); KO: DDAVP 1400.6 ± 164.7 vs. vehicle 340.4 ± 22.8
pmol/mg, \( P < 0.05 \)).

Effect of systemic PPAR\(\gamma\) deletion on AQP2 expression in the inner medulla

The expression of AQP2 protein was evaluated in the inner medulla from animals
with chronic DDAVP infusion. No difference was observed in basal and DDAVP-
stimulated AQP2 expression between floxed and KO mice (Figure 6). Since there was
no difference in total AQP2 expression, we performed Western blot analysis to assess
the phosphorylation of AQP2 in the inner medulla in response to DDAVP. Both floxed
mice and KO mice increased pAQP2-Ser256 and pAQP2-Ser269 in response to
DDAVP. No difference in the expression of pAQP2-Ser256 was observed between
floxed mice and KO mice, and the expression of pAQP2-Ser269 in KO mice even
seemed to be higher (Figure 6). These data indicate that the AVP/cAMP/AQP2 axis is
remained intact in the KO mice.
In our study, we employed a mouse model of inducible PPARγ deficiency generated by using the tamoxifen system and found that systemic PPARγ null mice exhibited a polyuria phenotype and a defect in the AVP response. These results provide new insights into the physiology of PPARγ in the renal regulation of water balance as well as the mechanism of TZD-induced fluid retention. In the absence of PPARγ, KO mice ate more food, drank more water and excreted more urine during a 24-h period. After 24 h of WD, KO mice showed a slight increase in urine osmolality, although it was still much lower than in floxed mice, accompanied by a dramatic loss of body weight and a significant increase in hematocrit. In addition, we performed a pair feeding and pair drinking experiment to exclude the impact of food and water intake and found no change in urine osmolality in the KO mice but a persistent loss of body weight. These results indicate that the systemic knockout of PPARγ caused a urine concentrating defect. It is well-known that AVP plays a crucial role in determining urine concentrating capability through modulation of water permeability in the collecting duct. AVP regulates water permeability via targeting AQP2 to the apical membrane of the principal cells, allowing precise control of water excretion. After acute administration, AVP can directly increases the apical plasma membrane targeting of AQP2 protein in principal cells and stimulates AQP2 phosphorylation. Chronic infusion of AVP increases the protein abundance of AQP2 by transcriptional activation of the AQP2 gene as well as by increasing its apical membrane targeting in collecting duct principal cells. AVP-dependent upregulation of AQP2 is mediated by
the vasopressin type 2 receptor (V2R), the Gs-coupled protein receptor, which activates adenylyl cyclase type VI and increases cAMP levels (21). To explore the mechanism of the urinary concentration defect in PPARγ KO mice, we performed acute and chronic DDAVP experiments to compare the AVP response between the two genotypes. Interestingly, both acute and chronic DDAVP responses were impaired in the null mice. However, urinary AVP excretion at basal condition or after WD was unaffected. These results suggest that the KO mice developed nephrogenic diabetes insipidus but not central diabetes insipidus. AVP via V2R activates adenylyl cyclase and increases cAMP levels and protein kinase A (PKA), which phosphorylates the water channel aquaporin-2 (AQP2) in its COOH-terminal tail on serine residue 256 (S256) (8, 17). Other AQP2 phosphorylation sites include S261, S264, and S269 (11). Immunohistochemistry showed that pS269- AQP2 localizes exclusively in the apical plasma membrane of the connecting tubule and CD (16). Further more, PPARγ agonists have been shown to alter the expression of AQP2 (27). However, we found no suppression of DDAVP-induced cAMP production or expression of total AQP2 and phosphorylated AQP2 (pAQP2-ser256 and pAQP2-ser269) in the inner medulla of the null mice. And the cAMP response to DDAVP was similar in PPARγ deficient IMCD fragments. Notabley, though the increase ratio is similar between floxed and KO mice, the basal cAMP level seems higher in KO mice, likely reflecting the compensatory response of the attenuated antidiuretic response of AVP. These data suggest that systemic PPARγ deletion may cause urinary concentration defect through an unknown mechanism independently of the canonical AVP/cAMP/AQP2 axis. In
support of this notion, CD-specific deletion of PPARγ did not affect fluid metabolism under physiological condition. Besides the CD, AVP stimulates sodium reabsorption in the thick ascending limb (TAL), which contributes to urine concentrating capability. A possibility exists that PPARγ may target the TAL to modulate AVP action. The vascular and central mechanisms are unlikely since the polyuria phenotype is not seen in mice with conditional deletion of PPARγ in endothelium(12), smooth muscle(3) and neuron(15).

In recent years, the mechanism of TZD-induced fluid retention has been extensively investigated (18, 24, 33). In particular, CD-specific PPARγ null mouse models showed significant reductions in PPARγ-agonist-induced fluid retention compared with that of floxed littermates (9, 34). Additionally, PPARγ agonists inhibit AVP-mediated anion transport in the MDCK-C7 cell line, which is a well-characterized cell culture model of the principal cell type (19). Recently, emerging evidence suggests that increased proximal tubular fluid reabsorption may also play a contributory role. In primary cultures of human proximal tubular cells, pioglitazone and a structurally unrelated PPARγ agonist, L-805646, increased Na⁺/H⁺ exchanger 3, AQP1 and AQP7 expression at both the mRNA and the protein level (22). Moreover, the results of Endo et al. strongly suggest that TZDs transactivate epidermal growth factor receptor in a PPARγ-dependent manner in the proximal tubule by activating c-Src, ultimately leading to Na⁺/H⁺ exchanger 3 and sodium-bicarbonate cotransporter-driven sodium reabsorption (7).
Irrespective of the underlying mechanism of the fluid metabolism phenotype induced by systemic PPARγ deletion, the present study demonstrates a crucial role of the energy regulator PPARγ in the control of urine concentrating capability. The link of energy metabolism and fluid balance has been well recognized but the molecular basis of this phenomenon remains elusive. Obesity is associated with increased renal fluid reabsorption that contributes to fluid retention and hypertension(4, 6, 10).

Conversely, fasting is well known to cause fluid loss as a result of enhanced natriuretic/diuretic response(2, 31). Our results support PPARγ as a key mediator that integrates the status of energy metabolism with renal excretory function. A better understanding of this pathway may provide insights into the mechanism of disturbance of fluid metabolism associated with metabolic syndrome.

In summary, the present study showed that systemic PPARγ deletion causes polydipsia, polyuria, and hypoosmotic urine, accompanied with partially blunted AVP response. Despite the lack of increase in urine osmolality following DDAVP administration, the null mice had intact AVP/cAMP/AQP2 axis. Although the detailed underlying mechanism is unknown, these results for the first time demonstrate an essential physiological role of PPARγ in regulation of fluid homeostasis and also offer new insights into the mechanism of TZD-induced fluid retention.

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References


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**Figure Legends**

Fig. 1. Systemic PPARγ null mice and their floxed control mice were placed in metabolic cages and 24-h urine collection was performed under basal condition with free access to food and water. *A*: Food intake. *B*: Water intake. *C*: Urine volume. *D*: Urine osmolality. *N* = 5–7 per group. Values are means ± SE;

Fig. 2. Effect of systemic PPARγ deletion on urine concentrating ability during 3-d pair feeding and pair drinking. During this time period, the null mice were given the same amounts of food and water as the floxed control. Body weight (*A*), urine volume (*B*) and urine osmolality (*C*) were measured daily at 2 days prior to pair feeding and pair drinking. Values are means ± SE; *n* = 5–7 per group. (*P* < 0.05 floxed versus KO)

Fig. 3. Effect of systemic PPARγ deletion on urine concentrating ability after 24 h of WD. Urine osmolality (*A*), urine volume (*B*), body weight change (*C*) and hematocrit (*D*) in response to 24 h of WD in PPARγ floxed and KO mice. Values are means ± SE; *n* = 5–7 per group.

Fig. 4. Effect of DDAVP on urinary concentration in systemic PPARγ null mice. *A*: Urine was emptied from mice by bladder massage and urine osmolalities were measured as basal value, then the mice were intraperitoneally injected with DDAVP (720 ng/kg body weight). Over a 2-h period in which mice had no access to food or
water, urine was collected by bladder massage and urine osmolalities were measured. Values are means ± SE; n = 5–7 per group. B and C: Mice were given DDAVP (0.25 ng/h) via subcutaneous osmotic minipump, and urine osmolalities(B) and urine volume(C) were monitored for 5 days. Values are means ± SE; n = 5–7 per group. (* P < 0.05 floxed versus KO; # P < 0.05 basal versus DDAVP)

Fig. 5. Effect of systemic PPARγ deletion on urinary AVP excretion and AVP-stimulated cAMP accumulation. A: ELISA detection of urinary AVP excretion in PPARγ floxed and KO mice under basal condition and after 24-h WD. Values are means ± SE; n = 5–7 per group. B: AVP-stimulated cAMP accumulation in freshly IMCD fragments from PPARγ floxed and KO mice. The IMCD fragments were treated with vehicle or 10 nmol/L DDAVP for 15 min in the presence of IBMX for 15 min. Values are means ± SE; n = 5–7 per group.

Fig. 6. Effect of systemic PPARγ deletion on AQP2 expression in the inner medulla. Semiquantitative immunoblots of membrane AQP2 (A and B), pAQP2-Ser256 (A and C), and pAQP2-Ser269 (A and D) proteins in PPARγ floxed and KO mice under basal conditions or after 1 week of DDAVP infusion. Values are means ± SE; n = 5–7 per group.

Table 1. Urine and plasma electrolytes in PPARγ KO and floxed mice. Values are
given as average ± SE. Number of mice indicated in parentheses.
Fig. 1

A) Food intake (g)

B) Water intake (ml)

C) Urine volume (ml)

D) Urine osmolality (mOsm/kg H₂O)

*p < 0.05
Fig. 3

A. Bar chart showing urine osmolality (mOsm/kg H₂O) for floxed and KO groups. The black bars represent WD, and the white bars represent Basal. The p-values are indicated as p<0.05.

B. Bar chart showing urine volume (ml) for floxed and KO groups. The black bars represent floxed KO, and the white bars represent p<0.05.

C. Bar chart showing body weight change (%) for floxed and KO groups. The black bars represent floxed KO, and the p-value is indicated as p<0.05.

D. Bar chart showing hematocrit for floxed and KO groups. The black bars represent floxed KO, and the p-value is indicated as p<0.05.
Fig. 4

A

![Graph of Urine Osmolality](image)

- WT
- KO

p<0.05

B

![Graph of Urine Osmolality](image)

- floxed
- KO

C

![Graph of Urine Volume](image)

- floxed
- KO
Fig. 6

A

AQP2

pAQP2-ser256

β-actin

floxed/Vehicle  floxed/DDAVP  KO/Vehicle  KO/DDAVP

B

Vehicle

DDAVP

p<0.05

p<0.05

floxed  KO

C

pAQP2-ser256/β-actin

p<0.05

floxed  KO

D

pAQP2-ser269/β-actin

p<0.05

floxed  KO
Table 1
Urine and Plasma electrolytes in PPARγ floxed and KO mice.

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<th>KO</th>
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<td><strong>Urine</strong></td>
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<td>Na⁺ excretion(μmol/24h)</td>
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Values are given as average±SEM. Number of mice indicated in parentheses.