Estrogen prevents increased hepatic aquaporin-9 expression and glycerol uptake during starvation

Janne Lebeck,1 Patrizia Gena,2 Heidi O’Neill,1,3 Mariusz T. Skowronski,4 Sten Lund,5 Giuseppe Calamita,2 and Jeppe Praetorius1
1Department of Biomedicine, the Water and Salt Research Center, Aarhus University, 5The Medical Research Laboratories, Clinical Institute and Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus, Denmark; 2Department of General and Environmental Physiology, University of Bari Aldo Moro, Bari, Italy; 3Department of Biochemistry, RCSI, Dublin, Ireland; 4Department of Animal Physiology, University of Warmia and Mazury, Olsztyn, Poland
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Lebeck J, Gena P, O’Neill H, Skowronski MT, Lund S, Calamita G, Praetorius J. Estrogen prevents increased hepatic aquaporin-9 expression and glycerol uptake during starvation. Am J Physiol Gastrointest Liver Physiol 302: G365–G374, 2012. First published November 23, 2011; doi:10.1152/ajpgi.00437.2011.—In starvation, glycerol is released from adipose tissue and serves as an important precursor for hepatic gluconeogenesis. By unknown sex-specific mechanisms, women suppress the endogenous glucose production better than men and respond to metabolic stress with higher plasma glycerol levels. Hepatic glycerol uptake is facilitated by aquaporin-9 (AQP9), a broad-selectivity neutral solute channel, and represents an insulin-regulated step in supplying gluconeogenesis with glycerol. In the present study, hepatic AQP9 abundance was increased 2.6-fold in starved male rats as assessed by immunoblotting and immunohistochemistry. By contrast, starvation had no significant effect on hepatic AQP9 expression in female rats. Coordinates, plasma glycerol levels remained unchanged with starvation in male rats, whereas it was increased in female rats. The different responses to starvation were paralleled by higher glycerol permeability in basolateral hepatocyte membranes from starved male rats compared with starved females. Ovariectomy led to a starvation-response pattern identical to that observed in male rats with increased hepatic AQP9 expression and unchanged plasma glycerol levels. In cultured hepatocytes, 17β-estradiol and the selective estrogen receptor α-agonist, propyl pyrazole triol, caused a decrease in AQP9 expression. Our results support that a sex-specific regulation of the hepatic glycerol channel AQP9 during starvation contributes to the higher plasma glycerol levels observed in women during fasting and possibly results in a lower cytosolic availability of glycerol. Furthermore, the sexual dimorphism in the hepatic handling of glycerol during starvation might be explained by 17β-estradiol preventing the starvation-induced increase in hepatic AQP9 abundance.

AQP9; orchiectomy; ovariectomy; estradiol; gluconeogenesis

IN STATES OF LOW ENERGY SUPPLY, hepatic generation and release of glucose from glycogenolysis and gluconeogenesis is crucial for maintaining the function of many organs. Glycerol is an important gluconeogenic precursor accounting for ~25% of hepatic glucose de novo synthesis in starved rats (31). Sex differences in the metabolic response to starvation exist in both humans and rodents. In general, females appear to preserve the lean body mass better than males during fasting by utilizing relatively more fat than muscle mass for energy needs (6, 16, 42). In humans, females suppress their endogenous glucose production to a greater extent than males during starvation (5) and respond to starvation with lower blood glucose levels (24). Furthermore several observations have been made of females responding to both fasting and moderate-intensity exercise with higher plasma glycerol levels than males (5, 6, 14, 25).

Aquaporin-9 (AQP9) is a broad-selectivity neutral solute channel that facilitates the hepatic uptake of glycerol (33, 37, 38). In the liver, AQP9 protein expression is predominantly confined to the basolateral plasma membrane domain in perivenous hepatocytes (7, 33). The importance of AQP9 in controlling hepatic uptake of glycerol is illustrated by the increased plasma glycerol levels found in AQP9 knockout (KO) mice (33). Previous studies in male animals have demonstrated that hepatic AQP9 expression is increased in states with low plasma insulin levels, such as starvation and streptozotocin-induced diabetes mellitus as well as in insulin-resistant obese (db/db) mice (4, 19). These findings led to the notion that AQP9 plays an important role in supplying glycerol for the increased gluconeogenesis in these states (23). In support, AQP9-deficient Leprdb/Leprd mice that become obese and develop type 2 diabetes had lower blood glucose levels than Leprd/Leprd, suggesting that the obese AQP9 KO mice has a reduced capacity to generate glucose (33).

The expression of AQP9 protein has been demonstrated to be ~20% higher in male than in female rats in the postprandial state (26). Therefore, we speculated that sex-specific regulation of hepatic AQP9 during starvation might contribute to explaining the higher plasma glycerol level observed in females during metabolic stress as well as the lower plasma glucose during starvation in females. In the present study, we found that the increase in hepatic AQP9 expression previously observed in starved males does not occur in females. In addition, the higher expression of AQP9 in starved males is paralleled by higher glycerol permeability of the basolateral hepatocyte membrane and increased plasma glycerol level only in starved females. This supports the notion that sex-specific regulation of AQP9 during starvation contributes to the higher plasma glycerol level observed in females. However, despite a possibly lower availability of glycerol as a gluconeogenic substrate in females, blood glucose levels did not differ between male and female rats in the starved state. Ovariectomy enabled a starvation-induced increase in hepatic AQP9 expression in females without a concurrent increase in plasma glycerol levels just as observed in starved males. Studies on hepatocytes support the idea that the sexual dimorphism in hepatic AQP9 regulation might be ascribed to 17β-estradiol suppressing hepatic AQP9 expression.
G366 SEX DIFFERENCES IN HEPATIC GLYCEROL METABOLISM

MATERIALS AND METHODS

Animal experiments. Wistar rats (Taconic Europe, Lille Skensved, Denmark; and Harlan, San Pietro al Natisone, Italy) were kept at 21°C with a 12-h:12-h artificial light/dark cycle and 55% humidity. All rats had free access to tap water and were maintained on a standard rodent diet (Altromin, Lage, Germany; or Harlan). All experimental protocols were approved by the Danish Ministry of Justice or similar authorities in Italy.

Protocol 1: 96-h starvation. Male and female rats were age matched. Male rats weighed 302 ± 3 g (n = 13), and female rats weighed 204 ± 3 g (n = 13). In the starved groups (n = 7 each), the food was removed 96 h before the experiment was terminated. The rats were anesthetized with isoflurane; after laparotomy, a blood sample was taken from the abdominal aorta, and liver samples were collected.

Protocol 2: streptozotocin-induced diabetes mellitus. Male (n = 9, 205 ± 1 g body wt) or female rats (n = 20, 182 ± 2 g body wt) were rendered diabetic by intravenous injection of streptozotocin (55 mg/kg body wt) (Sigma, St. Louis, MO) as previously described (29). Fourteen days after the injection, the animals were anesthetized with halothane, and the experiment was terminated as described in protocol 1.

Protocol 3: orchiectomy. Male rats (n = 14, 300 ± 2 g body wt) were anesthetized with isoflurane and injected with buprenorphine subcutaneously (6 µg/250 g). The testes and epididymis were exposed, and, in the castrated group (n = 6), the rete testis and the testicular blood vessels were ligated and the testes removed. In the sham-operated group (n = 7), the scrotal incision was closed after ligation. In the starved group (n = 6), the exposed ovaries were replaced in the abdomen. For the starved ovarietomized group, the food was removed 72 h before terminating the experiment on the 25th postoperative day as described in protocol 1.

Cell culture. The WIF-B9 hepatocytes were grown at 7% CO2 at 37°C in F12 Coon’s modification medium (Sigma) supplemented with L-glutamine (Invitrogen, Carlsbad, CA), antibiotic antimycotic solution (Sigma), sodium hypoxanthine, aminopterin and thymidine supplement (Invitrogen), and 5% FBS (Invitrogen), where the total estradiol (E2) level was ~10^{-10} M. Before experiments the cells were kept in control medium containing 0.5% FBS for 20 h. In the experiments with propyl pyrazole triol (PPT) (Tocris Bioscience) the control medium contained either 0% or 0.5% FBS as indicated. Gel samples were prepared after 24-h exposure to the experimental medium indicated in the figure. The presented data were obtained from two or three independent experiments, and n indicates the total number of wells.

Semiquantitative immunoblotting. SDS-PAGE and immunoblotting were performed as previously described (35). The bands detected with Hyperform (Amersham Bioscience, Uppsala, Sweden) were semiquantified within the linear range using ImageJ software after background subtraction, and each lane was adjusted to the band intensity of actin from the same membrane.

Immunohistochemistry. The animals were perfusion fixed with 3% paraformaldehyde in 0.01 M (pH 7.4) through the abdominal aorta. Paraffin tissue sections (2 µm) were obtained, and immunofluorescence labeling for AQP9 was performed as previously described (35). Microscopy was performed using a Leica TCS SL (SP2) laser scanning confocal microscope with the HCX PL APO 40 objective. Quantitative analysis was performed with Image-Pro Analyzer 6.2 software. For each image, a binary mask was created to define the total area of all hepatocytes in the image (Fig. 1E). A second mask defined the AQP9 immunofluorescence signal above background (Fig. 1F). The total immunofluorescence signal within the area of the second mask was divided by the total hepatocyte area from the area

Fig. 1. Immunohistochemical analysis of aquaporin-9 (AQP9) abundance in livers from male and female rats exposed to either 96-h starvation or streptozotocin (STZ) injection. Representative images of immunofluorescence labeling with anti-AQP9 antibody (Alpha Diagnostics, San Antonio, TX) in the centrilobular region from control (A or C) and starved (B or D) male and female rats and control (H or J) and STZ-diabetes mellitus (DM) male and female rats, as indicated. Arrows indicate AQP9 labeling in the basolateral plasma membrane domain facing the sinusoids. CV: central vein. E and F: examples of masks used for the semiquantitative analysis of hepatic AQP9 abundance relative to total cell area in the immunofluorescence images. E: mask of the total cell area from B. F: mask of the fluorescence labeling representing AQP9 in B. E: summarized values of the AQP9 immunofluorescence signal/cell area, n = 4 in each group. Means ± SE were normalized to the control male rat level. *Denotes statistically significant difference.
mask of the same original image. Mean values ± SE from four control rats and four starved rats were computed for both male and female rats and normalized to the level of the control male value.

PCR. Total RNA was extracted from rat liver using TRizol Reagent Solution (Ambion, Austin, TX). Two micrograms of total RNA from each sample were transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time qPCR reactions had the following final concentrations: 1× mAgg9 Target Primers and probe and 1× TaqMan Universal PCR Master mix (Applied Biosystems) and 20 ng of each cDNA sample. qPCR was carried out using the StepOne Real-Time PCR System (Applied Biosystems) and the following cycling conditions: incubation required for optimal AmpErase UNG enzyme activity (50°C for 10 min), and 40 cycles of annealing (95°C for 15 s) and extension (60°C for 1 min). A negative control without cDNA was run in parallel with each assay, and data were normalized to β-actin. For RT-PCR, total RNA was extracted from rat liver or WIF-B9 hepatocytes using the RiboPure Kit (Ambion). To generate cDNA, RT-PCR was performed after DNase treatment using 10 U/μL of SuperScript II Reverse Transcriptase (Invitrogen). PCR from the obtained cDNA was performed with HotStarTaq Master Mix (Qiagen, Valencia, CA) and 10% cDNA and 0.5 pmol of each primer (Table 1).

Stopped-flow light-scattering measurement of membrane glycerol and water permeability. Liver samples were homogenized and vesicles of basolateral plasma membranes were prepared by differential centrifugation as devised previously (3). The enriched basolateral plasma membranes were collected, and the protein concentration was assayed by the Lowry method. Volume changes were monitored by stopped-flow light-scattering analyzer (BioLogic, Claix, France) permeabilized with HotStarTaq Master Mix (Qiagen, Valencia, CA) and 10% cDNA and 0.5 pmol of each primer (Table 1).

Presentation of data and statistical analyses. Data are presented as mean values ± SE. Statistical analysis was accomplished by unpaired Student’s t-test or ANOVA unless otherwise stated. Nonparametric tests were used when indicated in figure legends. P values <0.05 were considered statistically significant.

RESULTS

Starvation increases hepatic AQP9 expression only in male rats. Male and female rats were subjected to 96-h starvation to assess the influence of sex on regulation of hepatic AQP9 in a state with increased gluconeogenesis. The expression of AQP9 in response to starvation was examined by immunohistochemistry. Representative images are shown in Fig. 1. As previously described, labeling for AQP9 in the liver is primarily localized to the basolateral plasma membrane domain of hepatocytes surrounding the central veins (Fig. 1, A–D and H–K), with decreased labeling toward the portal triad. When quantifying the AQP9 immunofluorescence signal in the perivenous region of control and starved rats, we found a 3.4-fold increase in starved male rats compared with controls (Fig. 1G). By contrast, starvation had no significant effect on hepatic AQP9 abundance in female rats. In the postprandial state, no difference in hepatic AQP9 expression was observed between males and females. After starvation, however, the hepatic AQP9 abundance was 1.8-fold higher in males compared with females. A similar sex-specific pattern of hepatic AQP9 expression was observed when examining the effect of streptozotocin-induced diabetes in male (Fig. 1, H and I) and female rats (Fig. 1, J and K) by immunohistochemistry. The observed sex-specific regulation of hepatic AQP9 in response to starvation was confirmed by immunoblotting, thus demonstrating a 2.6-fold increase in total AQP9 protein abundance in starved male rats (Fig. 2, A and C) and no increase in AQP9 expression in starved females (Fig. 2, B and C).

Higher membrane permeability to glycerol and water in hepatocytes from starved male rats. A separate 96-h starvation experiment was conducted to evaluate the biophysical impact of the sex-specific regulation of hepatic AQP9 abundance in starvation. First, the observed sex difference in hepatic AQP9 expression after starvation was confirmed by both qPCR and immunoblotting (Fig. 3A). Stopped-flow light-scattering analysis was carried out using basolateral membrane vesicles prepared from livers of starved male and female rats. Glycerol permeability (\(P_{\text{gly}}\)) was measured by light scattering following a 150 mM inwardly directed gradient of glycerol. Figure 3B shows representative biphasic light-scattering traces. The initial shrinkage of vesicles until osmotic equilibrium is reached

Table 1. Primer sequences

<table>
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<th>Transcript</th>
<th>Direction</th>
<th>Sequence</th>
<th>Product Size, bp</th>
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<tr>
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<td>Reverse</td>
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<tr>
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<td></td>
<td>Reverse</td>
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<td></td>
<td>Reverse</td>
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<tr>
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<td>Forward</td>
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<td>585</td>
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<tr>
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<td>272</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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AQP9, aquaporin-9; INSR, rat insulin receptor; ESR1, rat estrogen receptor-α; PGR, rat progesterone receptor; ESR2, rat estrogen receptor-β.
is followed by a slower decrease reflecting swelling of the vesicles caused by diffusional entry of glycerol and osmotic water influx. The $P_{\text{gly}}$ value of the vesicles from the starved males was significantly higher than the one obtained with the female counterpart (Fig. 3D). Furthermore, the basolateral membrane $P_{\text{gly}}$ values of male and female hepatocytes decreased to a comparable level, when the vesicles were challenged with a 10-min treatment with 0.5 mM phloretin, a compound known to inhibit the AQP9-mediated diffusion of glycerol (38).

Water permeability in vesicles of basolateral hepatocyte membranes from male and male rats were measured in response to a 140 mM inwardly directed gradient of mannitol. Figure 3C shows representative light-scattering data. In line with the glycerol permeability experiments, the coefficient of osmotic water permeability ($P_{\text{w}}$) of vesicles from starved males was significantly higher than that of the starved females. Again, this sex-dependent difference was diminished when exposing the vesicles for 5 min to 0.3 mM HgCl$_2$, a blocker of the AQP9 water conductance (Fig. 3E). Hence, the sex difference in hepatic AQP9 abundance was followed by a matching sex difference in basolateral hepatocyte membrane permeability for both glycerol and water, suggesting a lower hepatic uptake capacity of glycerol in females during starvation.

**Hepatic GlyK protein is more abundant in male rats.** Once glycerol is in the cytosol, glycerol kinase (GlyK) phosphorylates it into glycerol-3-phosphate (G-3-P). The hepatic GlyK abundance was analyzed to evaluate whether the initial hepatic glycerol metabolism was regulated in a sex-specific manner. In the postprandial state, male rats demonstrated a twofold higher abundance of GlyK compared with females (Fig. 4, A and D). However, the hepatic GlyK abundance was unaffected by starvation in both males and females (Fig. 4, B–D), thus suggesting a persisting sex difference in hepatic GlyK abundance during starvation.

**Starvation increases plasma glycerol levels only in female rats.** Compared with fed controls, p-insulin was decreased by 78% and 80% in response to starvation in male and female rats, respectively (Table 2). Starvation caused increased p-NEFA levels in both male and female rats, whereas only starved females displayed a significant increase in p-glycerol (Fig. 4E), thus giving a crude indication of the fact that the lipolytic rate and thereby release of NEFA is increased in both females and males, whereas the sex-specific increase in hepatic AQP9 could explain the lack of a significant increase in p-glycerol in starved males.

**Starvation increases hepatic AQP9 expression female rats after ovariectomy.** The observed sex differences in the initial hepatic handling of glycerol led us to hypothesize that either male sex would have a stimulatory effect or female sex would have an inhibitory effect on its regulation. Ovariectomy of male rats caused a 1.5-fold increase in hepatic AQP9 abundance (Fig. 5, A and B) with a correlated drop in plasma glycerol (Fig. 5C). By contrast, ovariectomy alone had no effect on the hepatic expression of AQP9 and GlyK (Fig. 6, A, B, and E), and p-glycerol remained unchanged (Fig. 6G). However, similar to starved males, starvation in ovarioctomized females resulted in a 1.9-fold increase in hepatic AQP9 abundance, whereas no effect on hepatic GlyK abundance was observed (Fig. 6, C, D, and F). Also in similarity to starved males and contrasting the changes in starved intact females, plasma glycerol remained unchanged in the starved ovarioctomized rats. Starved ovarioctomized rats displayed increased plasma NEFA concentration, whereas ovariectomy alone had no effect on the plasma level of neither glycerol nor NEFA (Fig. 6G). Throughout the experiment, the ovarioctomized rats gained more weight than the sham-operated controls, whereas starvation of ovarioctomized rats resulted in a final weight similar to the controls (Table 3). Both plasma insulin and blood glucose levels were decreased in the starved ovarioctomized rat, whereas no statistically significant difference was observed between control and ovariectomized females (Table 3). In all, ovariectomy caused a male response pattern of the hepatic AQP9 expression and plasma glycerol levels upon starvation.

**Effects of insulin, 17β-estradiol, and PPT in WIF-B9 hepatocytes.** The observed obliteration of the female response to starvation in terms of hepatic AQP9 expression and p-glycerol level in ovarioctomized rats led to the hypothesis that female sex hormones plays a suppressive role in the regulation of hepatic AQP9 expression. An in vitro system was chosen to test this hypothesis directly, as it would be difficult to accomplish by in vivo experiments with its many confounding factors. As previously described, WIF-B9 hepatocytes are a rat hepatoma and human fibroblast hybrid cell line that expresses rat AQP9 mRNA and protein (Fig. 7, A and B) (12). Here, we find that human AQP9 mRNA is also expressed in the WIF-B9 cells (Fig. 7A). Furthermore, the rat insulin receptor as well as the transcript for estrogen receptor-α (ERα, ESR1) was detected in both male rat liver and WIF-B9 cells. By contrast, the transcript for estrogen receptor-β (ERβ, ESR2) was only detected in the WIF-B9 cells, and the progesterone receptor was absent from both rat liver and WIF-B9 cells (Fig. 7A). To determine the effect of insulin exposure on the AQP9 abundance in...
WIF-B9, the hepatocytes were subjected to different physiological relevant concentrations of insulin. AQP9 protein expression was regulated in a concentration-dependent manner with highest protein abundance at low insulin levels (Fig. 7B), demonstrating that the regulation of AQP9 in WIF-B9 hepatocytes is comparable with the in vivo regulation observed in males. Similarly, we found that 17β-estradiol (E2) decreased WIF-B9 AQP9 expression in a concentration-dependent manner with a 2.3-fold difference between 10⁻¹⁰ and 10⁻¹¹M of E₂ (Fig. 7C). Because rat liver and the WIF-B9 cell line differ in ER subtype expression, we used the highly selective ERα agonist PPT to evaluate the role of signaling through the more liver-relevant ERα upon the expression of AQP9. As shown in Fig. 7D, PPT also caused a decreased abundance of AQP9, demonstrating that signaling through ERα alone also decreases the abundance of AQP9. However, a decreased expression of AQP9 was only observed in the presence of 0.5% FBS, suggesting that additional factors need to be present in order for PPT to suppress the abundance of AQP9. Finally, coadministering insulin and E2 significantly reduced AQP9 abundance compared with insulin alone (P < 0.05, Fig. 7E). Thus E2 seems to suppress the effect of low insulin on AQP9 expression by directly affecting the hepatocytes.

DISCUSSION

The overall metabolism of fuel molecules displays sex differences, and in this context many hepatic proteins are

Fig. 3. Glycerol and water permeability of basolateral membrane vesicles from hepatocytes of male and female rats after 96-h starvation. A: qPCR and immunoblotting for hepatic AQP9 expression in the basolateral membrane vesicle specimens from starved male and female rats used for the stopped-flow light-scattering studies. Means ± SE values were normalized to male expression levels. *Represents statistically significant difference. B: basolateral glycerol permeability: representative traces of stopped-flow light-scattering in response to a 150 mM inwardly directed concentration gradient of glycerol in males and females, as indicated. The initial increase in light-scattering (timing of 0.1 s) results from osmotic water efflux (vesicle shrinkage) followed by a slower decrease (timing of seconds) caused by glycerol entry triggering influx of water (vesicle swelling). Trend lines exemplify the slopes used for the calculations behind the summarized data in D. C: basolateral water permeability: representative traces of stopped-flow light scattering in response to a 140 mM inwardly directed hypertonic mannitol gradient in males and females, as indicated. Trend lines exemplify the slopes used for the calculations behind the summarized data in E. D: coefficients of basolateral membrane glycerol permeability (P₉ₒ) from starved male and female rats. The P₉ₒ values were calculated in the presence and absence of 0.5 mM phloretin for 10 min. Data are presented as means ± SE; n = 5. E: calculated coefficients of osmotic water permeability (Pₒ) of basolateral membrane vesicles from starved male and female rats. The Pₒ values are calculated in the presence and absence of 0.3 mM HgCl₂ for 5 min. Data are means ± SE; n = 4. *#, #Represent statistically significant differences.
expressed in a sexually dimorphic fashion (13, 17, 39). With relevance to hepatic glycerol metabolism, the expression of AQP9 protein is 20% higher in male than in female rats in the postprandial state (26). The hepatic abundance of AQP9 is inversely regulated in response to variations in plasma insulin levels, possibly through a negative insulin response element (IRE) in the promoter region of AQP9 (19). However, the evidence supporting insulin as a key regulator of hepatic AQP9 expression has been generated only in male rodents. In the present study, we found that the insulin-related regulation of hepatic AQP9 during starvation is blunted in female rats, most likely through the action of E2.

In the starved state, the hepatic expression of AQP9 was found to be approximately twofold higher in males than in females, and by far exceeds the 20% higher abundance of hepatic AQP9 observed in males in the postprandial state (26). The hepatic abundance of AQP9 is inversely regulated in response to variations in plasma insulin levels, possibly through a negative insulin response element (IRE) in the promoter region of AQP9 (19). However, the evidence supporting insulin as a key regulator of hepatic AQP9 expression has been generated only in male rodents. In the present study, we found that the insulin-related regulation of hepatic AQP9 during starvation is blunted in female rats, most likely through the action of E2.

In the starved state, the hepatic expression of AQP9 was found to be approximately twofold higher in males than in females, and by far exceeds the 20% higher abundance of hepatic AQP9 observed in males in the postprandial state (26). It could be speculated that this sex-specific regulation of AQP9 could be due to variations in the plasma insulin level between sexes. Here, the relative decrease in plasma insulin levels in response to starvation was similar in male and female rats although the absolute values tended to be higher in males. This could potentially influence the regulation of hepatic AQP9 abundance, but in contrast to the observed changes the trend toward lower insulin levels in female rats would predict an even more pronounced increase in the expression of AQP9 in females.

Glycerol is known to permeate AQP9 in heterologous expression systems. To verify this in the native tissue, we found it necessary to ascertain whether the difference in hepatic AQP9 abundance was sufficient to cause a biologically relevant difference in glycerol permeation. Here, we show that the sexual dimorphism in hepatic AQP9 abundance in starved rats is correlated with higher permeability for both glycerol and water in the basolateral plasma membranes of hepatocytes from males. These findings are consistent with a biophysical study by Calamita and coworkers reporting 1) positive correlation between AQP9 protein abundance and glycerol permeability, 2) a significantly lower (50%) $P_{gly}$ in the basolateral hepatocyte plasma membrane of AQP9 KO mice than that of wild-type mice, 3) unlike AQP9 wild-type mice no increase in $P_{gly}$ occurs in AQP9 KO mice during fasting, and 4) the existence of facilitated pathways other than AQP9 providing minor contribution to the transport of glycerol into the hepatocyte (P. Gena, A. Rojek, D. Ferri, E. Fanelli, M. Svelto, G.

Table 2. Biological parameters in fed controls and starved female and male rats

<table>
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<tr>
<th>Biological parameter</th>
<th>Female</th>
<th>Starved</th>
<th>Male</th>
<th>Starved</th>
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<tr>
<td></td>
<td>Controls</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NEFA/glycerol ratio, mmol/l</td>
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<td>2.8 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>4.6 ± 0.2*</td>
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<tr>
<td>Blood glucose, mmol/l</td>
<td>5.8 ± 0.2</td>
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<td>5.0 ± 0.1</td>
<td>3.5 ± 0.2*</td>
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<td>Plasma insulin, pmol/l</td>
<td>51.2 ± 1.5</td>
<td>10.5 ± 2.3*</td>
<td>130.8 ± 39.7</td>
<td>28.7 ± 10.1*</td>
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<tr>
<td>Start body weight, g</td>
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<td>201.4 ± 4.8</td>
<td>300.3 ± 4.8</td>
<td>304.6 ± 3.0</td>
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<tr>
<td>Final body weight, g</td>
<td>209.7 ± 2.0</td>
<td>175.4 ± 4.2*</td>
<td>310.2 ± 6.8</td>
<td>274.9 ± 3.1*</td>
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</table>

Values are means ± SE; $n$, number of rats. For p-insulin, $n = 3$ for female controls. *Represents statistically significant difference between control and starved males or between control and starved females. NEFA, nonesterified fatty acids.
plasma levels of NEFA were increased in both sexes, whereas the plasma glycerol levels were only significantly increased in starved females. These findings indicate that the lipolytic rate is increased in both male and female rats and suggest that males metabolize glycerol more efficiently than females. The observed plasma levels of glycerol are within range of previous observations (11, 22). In contrast to our findings based on aortic blood, increased plasma glycerol levels in starved male rodents were reported elsewhere after blood sampling from the prehepatic portal vein (19, 34). To our knowledge, there is no general consensus regarding sex differences in plasma glycerol levels in humans. However, several groups have reported higher plasma glycerol levels in women than in men in response to both fasting and moderate-intensity exercise (5, 6, 14, 25). This sex-specific difference has also been observed in exercising type 1 diabetics (10). In some of the studies, the higher plasma glycerol levels were accompanied by the plasma levels of NEFA and were therefore thought to arise solely from a higher lipolytic rate in women. However, the AQP9 expression pattern observed in the present study suggests that sexual dimorphism in hepatic handling of glycerol contributes to the higher plasma glycerol levels observed in women in response to fasting. Furthermore, the lower intracellular hepatic availability of glycerol as a gluconeogenic substrate in starved females would be in line with the notion of women suppressing their glucose de novo synthesis to a greater extent than men during starvation (5) and with the lower blood glucose levels in women during prolonged fasting (24).

In response to starvation, lipolysis is initiated and glycerol and NEFA are released for further metabolism. In this study, the GlyK protein level was not affected by starvation in males compared with females in the postprandial state. This is in accordance with a higher hepatic GlyK activity in the postprandial state in male rats in previous studies (8, 40). In our study, the GlyK protein level was not affected by starvation in neither males nor females, indicating that the sex differences in abundance persist during starvation. The existing data on the relationship between fasting and hepatic GlyK appear inconsistent. Fasting of male mice has been reported to increase the hepatic GlyK mRNA expression (19, 19, 30), whereas a decreased activity has been observed in starved male rats (36).

We next speculated that the sexual dimorphism could be mediated by sex hormones and in particular by testosterone or estrogen. The expression pattern of AQP9 and the sex difference in AQP9 abundance persist during starvation. The existing data on the relationship between fasting and hepatic GlyK appear inconsistent. Fasting of male mice has been reported to increase the hepatic GlyK mRNA expression (19, 19, 30), whereas a decreased activity has been observed in starved male rats (36).

In a recent study, both AQP3 and AQP7 expression was decreased in starved male rats (36). Immunohistochemical analysis did not suggest that the expression of these proteins overlap with that of AQP9 and their functional roles within the liver remains to be elucidated. To our knowledge none of the water-selective aquaporins expressed in the liver (AQP0, AQP1, AQP4, AQP8, and AQP11) colocalize with AQP9 in the basolateral plasma membrane domain (20). Thus a functional overlap with AQP9 in these membranes seems most unlikely. Moreover, evidence for a sex-specific regulation of any of these aquaporins has not been reported thus far. Finally, the HgCl2-insensitive water permeability and the phloretin-resistant glycerol permeability in hepatocytes do not change with sex in our study. Thus AQP9 remains the best candidate for explaining the sex difference in permeability after starvation.

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estrogens. Decreasing the testosterone levels by orchiectomy caused a small but significant increase in hepatic AQP9 expression, suggesting that male testosterone levels are not essential for an increase in hepatic AQP9 abundance to occur. Decreasing the estrogen levels by ovariectomy had no effect on hepatic AQP9 abundance. The higher weight gain observed in the ovariectomized rats is a well-described phenomenon ascribed to increased food intake (reviewed in Ref. 2). The weight gain was accompanied by a trend for higher plasma levels of insulin, which could influence the regulation of hepatic AQP9 abundance and thereby mask the effect of ovariectomy. However, the increased hepatic AQP9 abundance found in starved ovariectomized rats suggests that ovary-derived factors exert an inhibitory effect on regulation of hepatic AQP9 abundance during starvation. This is in accordance with neonatal exposure to diethylstilbestrol causing lower hepatic AQP9 abundance in 20-day-old male rats (41). Furthermore, ovariectomy resulted in an elimination of the increased plasma glycerol level found in response to starvation in intact females. This could either be ascribed to increased hepatic glycerol uptake through AQP9 or decreased glycerol production from lipolysis. An attenuated lipolytic response is, however, contradicted by the increased plasma NEFA levels in the starved ovariectomized rats. Thus ovariectomy leads to a male response pattern to starvation regarding AQP9 expression and plasma glycerol levels.

Table 3. Biological parameters from control, ovariectomized, and starved ovariectomized rats

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Ovariectomized</th>
<th>Starved Ovariectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>NEFA/glycerol ratio</td>
<td>3.5 ± 0.2</td>
<td>3.3 ± 0.4</td>
<td>5.5 ± 0.4†</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>5.7 ± 0.1</td>
<td>5.8 ± 0.4</td>
<td>3.7 ± 0.3†</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>72.2 ± 14.7</td>
<td>189.9 ± 49.9</td>
<td>21.7 ± 4.1†</td>
</tr>
<tr>
<td>Start body weight, g</td>
<td>207.1 ± 3.3</td>
<td>200.8 ± 3.3</td>
<td>206.3 ± 4.3</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>242.2 ± 4.4</td>
<td>279.6 ± 7.7*</td>
<td>234.9 ± 6.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. *Represents statistically significant difference when compared to control rats, and †represents statistically significant difference when compared to ovariectomized rats.
Estrogens are recognized as important modulators of metabolic homeostasis with effects on both glucose and lipid metabolism (reviewed in Refs. 9 and 27), and in normal rat liver ERα is the predominant estrogen receptor (18). ERα KO mice have increased body weight and display decreased whole body insulin sensitivity with impaired suppression of the endogenous glucose production by insulin (1, 15). In the present study, we found that AQP9 protein abundance is suppressed by E2 in a concentration-dependent manner in cultured hepatocytes. In addition, the ERα-selective agonist PPT also caused a decrease in hepatocyte AQP9 expression when administered in media containing 0.5% serum. This suggests that E2 through ERα could be at least partially responsible for the observed sex difference in the regulation of hepatic AQP9 during starvation. Furthermore, the inhibition of AQP9 induction during starvation by E2 is in line with the previously described suppression of hepatic glucose production by estradiol (9).

Classical genomic effects of ERs are conducted by binding to estrogen response elements (EREs). In addition, ERs are known to regulate gene transcription by binding to other DNA-bound transcription factors, such as AP1, NF-κB and some SP-1 binding sites (9). Previous analysis of the 5′ flanking region of human AQP9 found consensus recognition sites for AP-1 and NF-κB (38), and in mouse putative sites for AP1, NF-κB, and SP-1 were identified (19). Thus there seems to be several candidate sequences for interaction with E2 in the promoter region of AQP9, even though no putative EREs have been identified. Another possible mechanism could be E2 modulating other signaling pathways such as the insulin signaling pathway itself. In rat uterus, E2 acutely increased the phosphorylation status of Akt and the downstream target FoxO1 (21), the transcription factor known to interact with the IRE in the promoter region of AQP9 (28). We here found that ovariectomy alone had no apparent effect on the initial hepatic glycerol handling, suggesting that E2 per se has no effect on hepatic AQP9 abundance. Along the same line, PPT treatment of hepatocytes only caused a decrease in AQP9 abundance in the presence of FBS.

In conclusion, we demonstrate that the induction of hepatic AQP9 expression and hepatic glycerol permeability during starvation/low-insulin levels is blunted in female rats. The higher expression of hepatic AQP9 observed in starved male rats is paralleled by higher glycerol permeability in hepatocytes from males and a concomitant lack of increase in plasma glycerol. Ovariectomy obliterated both the starvation-induced sex-specific regulation of AQP9 and the increase in plasma glycerol observed in starved intact females. Our studies on hepatocytes supported that the sexual dimorphism in hepatic AQP9 regulation may be ascribed to E2 suppressing the expression of hepatic AQP9. This E2-mediated regulation of hepatic AQP9 expression might contribute to the reported sex differences in glycerol metabolism, such as the higher plasma glycerol level reported in fasting and exercising women.

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REFERENCES
SEX DIFFERENCES IN HEPATIC GLYCEROL METABOLISM


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