Genome and Hormones: Gender Differences in Physiology
Selected Contribution: Effects of gender on reduced-size liver ischemia and reperfusion injury

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Harada, Hirohisa, Kevin P. Pavlick, Ian N. Hines, Jason M. Hoffman, Sulaiman Bharwani, Laura Gray, Robert E. Wolf, and Matthew B. Grisham. Selected Contribution: Effects of gender on reduced-size liver ischemia and reperfusion injury. J Appl Physiol 91: 2816–2822, 2001.—Hepatic resection with concomitant periods of ischemia and reperfusion (I/R) is a common occurrence in resectional surgery as well as reduced-size liver transplantation (e.g., split liver or living donor transplantation). However, the I/R induced by these types of surgical manipulations may impair liver regeneration, ultimately leading to liver failure. The objectives of the study were to develop a murine model of reduced-size liver I/R and assess the role of gender in this model of hepatocellular injury. We found that 100% of female mice survived the surgery indefinitely, whereas all male mice had greater initial liver injury and died within 5 days after surgery. The protective effect observed in females appeared to be due to ovarian 17β-estradiol, as ovariectomy of females or administration of a selective estrogen antagonist to female mice resulted in enhanced liver injury and greater mortality following reduced-size liver I/R. Conversely, 17β-estradiol-treated male mice exhibited less hepatocellular damage and survived indefinitely. Taken together, these data demonstrate an estrogen-mediated protective pathway(s) that limits or attenuates hepatocellular injury induced by reduced-size liver I/R.

inflammation; estrogen; ovariectomy; nitric oxide; reactive oxygen species

RSL transplantation, a novel surgical alternative to whole liver transplantation, is being applied to expand the depleted donor organ pool (31, 47). However, the hepatocellular injury induced by I/R is thought to impair liver regeneration and may lead to primary graft dysfunction and failure (47). The recent development of new surgical techniques and organ preservation methods has improved the outcome of RSL transplants for children and small adults, making them comparable to those who have undergone whole liver transplants (46). However, the growing disparity between the limited number of available donor organs and the expanding demand for transplantation has led to extending the application of RSL transplants to normal-sized adult recipients (47). Data obtained from some clinical reports have indicated a higher incidence of primary failure of the graft after such procedures due to the impaired ability of the liver to regenerate from I/R that was caused during the preservation process (3, 14). The mechanisms by which I/R after RSL (RSL + I/R) promotes liver injury are not clear at the present time. However, numerous experimental studies using warm, full-size livers with I/R indicate that certain proinflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-12], reactive oxygen species, and neutrophils may contribute to hepatocellular injury (21, 23, 36). Recent work by Selzner and co-workers (45) has shown that I/R impairs the regenerative capacity of the liver, although a protective strategy was not identified or proposed.

It is becoming increasingly appreciated that gender differences exist in susceptibility to and mortality from a variety of cardiovascular diseases, including vascular occlusive disease, stroke, hypertension, and atheroscler-
rosis (19). Gender dimorphism is also apparent in cardiovascular and hepatocellular dysfunctions observed after experimental trauma with hemorrhage (26, 27). In addition, data obtained from clinical studies suggest that female patients with hepatocellular carcinoma have a better chance of survival than males after surgical resection of the liver (41). Retrospective clinical studies suggest that gender of donors and/or recipients may influence the outcome of liver transplantation; however, these data are variable, and no clear consensus has been reached (4, 6, 25, 37). Finally, it should be noted that distinct gender differences have been reported in experimental studies using models of alcohol- or drug-induced liver injury (33, 42).

Because of the lack of a well-defined model of RSL + I/R and because nothing is known regarding how gender may affect liver injury in response to RSL + I/R, we wished to develop and characterize a murine model of RSL + I/R and to assess the effects of gender in this novel model. Data obtained in the present study suggest that female mice are protected to a much greater extent than males from the injurious effects of RSL + I/R; the physiological and clinical significance is also discussed.

MATERIALS AND METHODS

Animals. Age-matched (7- to 9-wk-old) male and female C57BL/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME). All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (revised 1996), approved by the Council of the American Physiological Society, and with federal and state regulations.

Animal model of RSL + I/R. Fasted (16–18 h) mice were anesthetized via a single intramuscular injection of ketamine (150 mg/kg) and xylazine (7.5 mg/kg). After we performed a midline laparotomy, the intestinal loops were gently exteriorized from the abdominal cavity to expose the liver. The membranes and ligaments surrounding each lobe were then dissected away. Approximately 70% liver ischemia was achieved by occluding the hepatic arterial and portal venous blood supply to the left lateral and median lobes using a microaneurysm clip; mice were given an intra-peritoneal dose of heparin (200 U/kg) to prevent blood coagulation. Intestinal congestion was avoided during clamping by the bypass portal flow through the nonischemic lobes (right lateral, caudate, and quadrate lobes). After 45 min of ischemia, the microaneurysm clip was removed. Immediately after the onset of reperfusion, the vessel branches leading to the nonischemic lobes were ligated with 6-0 chronic suture, and these nonischemic lobes were then tied with 4-0 silk suture at the pedicles and excised immediately, leaving the two reperfused lobes. We used the same protocol for control mice, which underwent partial hepatectomy but without vascular clamping. Sham control mice were treated in an identical fashion but without hepatectomy or vascular clamping. The abdominal cavity was closed with 4-0 silk suture, and the animals were allowed to recover and given free access to food and water. To examine the effects of ovariectomy after RSL + I/R in female mice, ovariectomies or sham ovariectomies were performed on female mice under ketamine-xylazine anesthesia 2 wk before RSL + I/R.

Liver injury, regeneration, and survival after RSL + I/R. To assess the impact of gender in this model, male (n = 10) and female (n = 9) mice were subjected to RSL + I/R and observed daily through day 7 postsurgery. All surviving animals were euthanized on day 8, and the degree of liver regeneration was quantified. In addition, seven ovariectomized female mice were subjected to RSL + I/R to examine the effect of ovariectomy on 7-day survival and hepatocellular injury. Ovariectomized mice were allowed to recover for 14 days before being subjected to RSL + I/R. To investigate the early phase of liver injury and regeneration, male (n = 56) and female (n = 63) mice were subjected to RSL + I/R (or RSL control or sham surgery) and killed at different time points (0, 1, 3, 6, 20, and 30 h) after surgery. Liver was harvested and weighed for assessment of the restoration of liver weight-to-body weight ratio as an indicator of regeneration.

Estrogen and antiestrogen administration. 17β-Estradiol (E2) (Sigma Chemical, St. Louis, MO) or ICI-182780 (Tocris Cookson, Ballwin, MO) was administered to assess the effects of estrogen or its receptor antagonist of RSL I/R. E2 and ICI-182780 were reconstituted in ethanol and diluted with PBS-corn oil immediately before administration. Either E2 (1 μg in 100 μl of corn oil with 0.1% ethanol) or oil-ethanol vehicle was injected subcutaneously into male mice (n = 32) 24 h before RSL I/R; this was followed by the same dose at the time of reperfusion. Similarly, ICI-182780 (25 μg in 100 μl of corn oil with 4% ethanol) or the oil-ethanol vehicle was injected into female mice (n = 32) in an identical fashion.

Blood and tissue analyses. At the reperfusion time points indicated, blood was obtained from the inferior vena cava at the time of death for analysis of serum alanine aminotransferase (ALT) as an indicator of hepatocellular injury using commercially available reagents (Sigma Chemical). Liver tissue was also removed, weighed, and stored in 10% PBS-buffered formalin for histopathological analysis. Formalin-fixed liver specimens were embedded in JB-4 plastic, and 5-μm-thick sections were cut and stained with hematoxylin and eosin.

Statistical analyses. All values are expressed as means ± SE. Statistical significance between two groups of parametric data was evaluated using an unpaired Student’s t-test. Survival rates for 7 days were analyzed using the generalized Wilcoxon’s test. Statistical significance was accepted at P < 0.05.

Fig. 1. Cumulative survival rates of male (○) and female (△) mice following reduced-size liver ischemia and reperfusion (RSL + I/R). Survival rates for 7 days were 0% in males (n = 10) and 100% in females (n = 9). *P < 0.001 compared with male mice.
RESULTS

Effect of gender on survival and liver injury following RSL + I/R. In our first series of experiments, a mouse model for RSL + I/R was developed, and the 7-day survival rates of both male and female mice were examined. We found that 100% of female mice survived indefinitely after surgery, whereas all male mice died within 5 days after RSL + I/R (Fig. 1). This protective effect in females correlated well with reduced liver injury compared with their male counterparts, as assessed by lower serum ALT levels in females vs. males observed 20 h after RSL + I/R (Fig. 2). We also observed that serum ALT levels were significantly higher in males both immediately after ischemia and 3 h after ischemia compared with that shown in females (data not shown). ALT levels of sham control animals were within the normal range, and no gender difference was apparent (76.6 ± 32.6 vs. 84.9 ± 19.5 IU/l for males vs. females, respectively; Fig. 2). In addition, no significant gender difference was observed in control mice subjected to RSL but not ischemia (598.2 ± 16.4 vs. 756.7 ± 46.0 IU/l; Fig. 2); however, ALT levels were significantly elevated in both male and female mice compared with sham control animals. In RSL + I/R animals, the ALT levels were significantly greater in male mice than in female mice (6,291 ± 1,091 vs. 2,361 ± 528 IU/l; P < 0.05; Fig. 2). Histopathological inspection at the 20-h time point agreed well with ALT determinations, revealing pyknotic nuclei and necrosis in male mice (Fig. 3A), whereas female mice displayed minimal changes (Fig. 3B). However, we did observe significant infiltration of mononuclear leukocytes (monocytes, lymphocytes) into the postischemic male and female livers (Fig. 3). In addition, using liver weight-to-body weight ratio as an indicator of liver regeneration, we found that female liver mass increased steadily beginning 3 h after surgery and was fully restored by day 8, whereas the liver weight-to-body weight ratio for male mice decreased throughout the course of the experiment (Fig. 4A). Administration

Fig. 2. Serum alanine aminotransferase (ALT) levels in male and female mice 20 h after sham operation, RSL control without ischemia, and RSL + I/R. No gender differences were observed in sham-operated or RSL control mice, whereas, in RSL + I/R mice, serum ALT level was significantly greater in male than in female mice; n = 5/group. *P < 0.05 compared with RSL + I/R females.

Fig. 3. Histopathological appearance of the liver 20 h after RSL + I/R. In male mice, hepatocyte necrosis and pyknosis with massive interstitial hemorrhage spread over a wide area (A), whereas, in female mice, these changes were minimal, although significant infiltration of lymphocytes and monocytes was observed (B). 17β-Estradiol (E2)-treated male mice display attenuated liver injury such that the histological findings appeared to be similar to those of female mice (C and B). However, ICI-182780-treated female mice showed enhanced liver injury similar to that of male mice (D and A). All sections (A–D) were stained with hematoxylin and eosin (×200). Bars = 100 μm.
of E2 to male mice resulted in a complete regeneration of liver mass by day 8 (Fig. 4B) and is comparable to the regeneration capability of female mice in this model (Fig. 4). Liver weight-to-body weight ratios were not determined in male mice after 30 h because most males did not survive past this point.

Role of estrogen on survival and liver injury following RSL + I/R. To address the mechanisms by which female mice were protected from the injurious effects of RSL + I/R, female mice were ovariectomized, allowed to recover for 14 days, and then subjected to RSL + I/R. Ovariectomized mice showed a significant reduction in the postsurgery 7-day survival rate compared with sham-operated controls (14% vs. 100%, \( P < 0.05 \); Fig. 5). To further address the role of ovarian hormones in this protective effect, we assessed the effects of the selective estrogen receptor antagonist ICI-182780 in female mice administered ICI-182780 had enhanced histopathological injury, similar to that of untreated male mice (Fig. 3D).

DISCUSSION

Data obtained in the present study clearly demonstrate that female mice are protected to a much greater
extent than male mice from the injurious effects of RSL + I/R. This protective effect appears to be due to ovarian E2 because ovariectomy of females or administration of a selective estrogen-receptor antagonist enhanced liver injury and increased mortality of females subjected to RSL + I/R. In addition, administration of E2 to male mice increased their survival rate and reduced liver injury. Taken together, these data demonstrate the existence of one or more estrogen-mediated protective pathways that limit or attenuate hepatocellular injury induced by RSL + I/R.

These data differ from numerous previously reported experimental studies that attempted to define the mechanisms by which warm I/R injures the liver (21, 36). In contrast to our studies, virtually all of these previous reports used male mice and full-size liver I/R as their models. Data obtained from these previous studies suggest that hepatocellular damage induced by I/R involves both acute hepatocellular injury and subacute or late inflammatory tissue damage (36). The acute phase is characterized by Kupffer cell activation and oxidative stress (21–23), which ultimately initiates the inflammatory cascade culminating in the accumulation of neutrophils that may injure the liver at later time points after reperfusion (24). In the present study, with our model of RSL + I/R, we obtained data involving not only the acute and subacute phases of I/R injury but also including hepatocyte regeneration initiated by partial hepatectomy (11). Interestingly, although we observed a gender difference in our model of RSL + I/R, previous studies have not reported this. In addition, no gender differences were reported in previous partial hepatectomy and regeneration studies, even though estrogen was shown to possibly play a role in the process of growth and differentiation of hepatocytes (9, 12). Thus gender dimorphism may only be observed when liver I/R and resection are combined. The reasons for this interesting observation deserve further investigation.

The precise mechanisms by which estrogen protects the liver in our model of RSL + I/R are not known; however, there are several possibilities. For example, data obtained from several different laboratories suggest that the acute posts ischemic hepatocyte injury may be mediated directly or indirectly by reactive oxygen species generated during the early reperfusion period (10, 21). It is also becoming widely appreciated that nitric oxide (NO) inhibits reactive oxygen species-mediated reactions and that ischemia is associated with a decrease in the bioavailability of NO (15, 35). In addition, changes in the production of NO have been implicated as one of the cellular biochemical-related pathways regulated by estrogen that may contribute to gender and hormonal differences in the progression of cardiovascular disease (18, 29, 39). In the present study, the protective effect of estrogen may involve its direct effect on the vascular endothelium by increasing the production of endothelial cell-derived NO. Estrogen is known to increase NO production primarily through the upregulation of endothelial cell NO synthase (eNOS) gene expression (28). It has been shown that a gender difference exists in basal NO release by rabbit aorta (16, 17), and this increase in eNOS is thought to significantly contribute to the cardiovascular protection exerted by estrogen (5).

Estrogen may also increase NO production through novel nongenomic pathways that directly enhance NOS activity. For instance, it has been shown that estrogen can increase NO release form bovine aortic endothelial cells through actions of estrogen receptor-α localized in plasmalemmal caveolae (32). Our laboratory has provided evidence that eNOS plays an important role in reducing or limiting hepatic I/R injury in vivo (30). Therefore, it is possible that estrogen may function by upregulating eNOS and/or enhancing production of eNOS-derived NO during RSL + I/R, which would increase the bioavailability of NO and decrease hepatic injury by promoting vasodilation and enhancing reperfusion of the remnant tissue. In addition, estrogen has been shown to have antioxidant properties, including inhibition of low-density lipoprotein oxidation (38, 48), decreased lipoprotein(a) levels (40).
and reduction in superoxide anion production (2, 7). Reduction in superoxide anion production would allow for an increase in NO bioavailability, since NO would not be converted to peroxynitrite. I/R injury induces pathological changes, including endothelial dysfunction.

In our RSL + I/R model, we show that female mice have a greater capacity to regenerate their remnant liver compared with male mice (Fig. 4). The mechanism(s) that permits this regeneration is not fully understood, but, in our model, administration of E2 to male mice or ICI-182780 to female mice reverses the gender difference, suggesting that E2 may play a role in the regeneration process. There has been considerable experimental evidence to suggest that estrogen may modulate liver regeneration (8, 9, 12, 13). After partial hepatectomy, the expression of estrogen receptors in hepatocytes increases and estrogen induces DNA synthesis and mitosis in hepatocytes (13). It has also been shown that estrogen stimulates cholangiocyte proliferation in rat liver (1). Initiation of liver regeneration requires expression of certain cytokines such as TNF-α and IL-6 and the activation of transcription factors such as nuclear factor-κB and signal transducer and activator of transcription-3 (STAT3) (11). TNF-α and IL-6 have been shown to promote hepatocyte viability and proliferation. Inhibition of either of these cytokines not only blocks hepatocyte DNA synthesis but also increases hepatocyte apoptosis (11). In addition, targeted disruption of the type II (inducible) NO synthase (iNOS) gene severely inhibits liver regeneration after partial hepatectomy, suggesting that iNOS-derived NO may directly or indirectly mediate liver regeneration (44). It has been shown that one of the early changes that occur immediately following partial hepatectomy is an induction of iNOS and the release of NO in vivo (20, 43). Therefore, it will be necessary to investigate whether protective effects of estrogen in this RSL + I/R model is possibly due to increases in the expression of genes involved in liver regeneration (e.g., TNF-α, IL-6, and iNOS). The potential induction of gene expression may result via an estrogen and/or estrogen-receptor complex alone or in synergy with additional factors (e.g., nuclear factor-κB and/or STAT3).

Our data may have important clinical implications related to surgical resection and/or liver transplantation. For example, it has been reported in some studies that female patients with hepatocellular carcinoma have better survival and lower tumor recurrence rates after surgical resection, although the underlying mechanism(s) remains to be elucidated (34, 41). In addition, it is thought that donor and/or recipient gender may affect the outcome of liver transplantation. A recent study dealing with the long-term survival outcomes of a large cohort of liver transplant recipients in the United States has shown that patient survival was significantly better in female recipients (25). However, other studies have shown that female donors were associated with a poorer outcome after liver transplants (6, 37). The reasons for these disparate results are not clear, but a variety of different factors may confound interpretation of these data. For example, age (postmenopausal), race, underlying diseases of the donor and/or recipient, and/or medications may all greatly influence the outcome of liver transplant. Failure to account and control for these different variables may make it very difficult for investigators to accurately assess the role of gender in reduced-size or full-size liver transplantation. Indeed, because we can precisely control for gender, age, and strain of the mouse, we can more accurately determine how gender affects the outcome of our model of RSL + I/R. Estrogen replacement therapies have been widely accepted as effective treatments for certain cardiovascular or osteoporosis diseases based on the well-established protective effects of estrogen on the cardiovascular system or bone cells (19). With further advances in our understanding of the mechanisms by which E2 protects the liver, it may be possible to exploit the estrogen-mediated protective pathways to limit or attenuate some of the complications of liver surgery.

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REFERENCES


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