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RESEARCH ARTICLE | *Translational Physiology*

Kcne4 deletion sex dependently inhibits the RISK pathway response and exacerbates hepatic ischemia-reperfusion injury in mice

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Hu Z, Jepps TA, Zhou L, Liu J, Li M, Abbott GW. *Kcne4* deletion sex dependently inhibits the RISK pathway response and exacerbates hepatic ischemia-reperfusion injury in mice. *Am J Physiol Regul Integr Comp Physiol* 316: R552–R562, 2019. First published February 13, 2019; doi:10.1152/ajpregu.00251.2018.—Activation of antiapoptotic signaling cascades, such as the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways, is protective in a variety of tissues in the context of ischemia-reperfusion (IR) injury. Hepatic IR injury causes clinically significant hepatocellular damage in surgical procedures, including liver transplantation and hepatic resection, increasing associated morbidity and mortality. We previously found that the cardiovascular-expressed K^+ voltage-gated channel ancillary subunit KCNE4 sex specifically influences the cardiac RISK/SAFE pathway response to IR and that *Kcne4* deletion testosterone dependently exacerbates cardiac IR injury. Here, we discovered that germline *Kcne4* deletion exacerbates hepatic IR injury damage in 13-mo-old male mice, despite a lack of *Kcne4* expression in male mouse liver. Examining RISK/SAFE pathway induction, we found that *Kcne4* deletion prevents the hepatic ERK1/2 phosphorylation response to IR injury. Conversely, in 13-mo-old female mice, *Kcne4* deletion increased both baseline and post-IR GSK-3β inhibitory phosphorylation, and pharmacological GSK-3β inhibition was hepatoprotective. Finally, castration of male mice restored normal hepatic RISK/SAFE pathway responses in *Kcne4*$^{-/-}$ mice, eliminated *Kcne4* deletion-dependent serum alanine aminotransferase elevation, and genotype independently augmented the hepatic post-IR GSK-3β phosphorylation response. These findings support a role for KCNE4 as a systemic modulator of IR injury response and uncover hormonally influenced, sex-specific, KCNE4-dependent and -independent RISK/SAFE pathway induction.

ischemia; KCNE; MinK-related peptide; potassium channel; RISK/SAFE

INTRODUCTION

Liver ischemia-reperfusion (IR) injury is an important cause of hepatocellular damage during or after surgical procedures such as liver transplantation and hepatic resection. Restoration of blood flow to the ischemic liver can cause injury, including hepatic inflammation, liver failure, and multiple organ dysfunction, all of which are associated with increased morbidity or mortality. However, the underlying cellular and molecular mechanisms of hepatic IR injury are not thoroughly understood. Therefore, identification of therapeutic targets to enhance hepatic tolerance to IR injury and, therefore, minimize its adverse consequences is of great importance for patients with liver disease.

Various reports indicate that ion channels can influence susceptibility to reperfusion-induced hepatic injury (21). Thus, activation of transient receptor potential vanilloid 1 channels (21) or store-operated Ca^{2+} channels (13) may raise tolerance to liver IR injury. In addition, Ca^{2+}-activated K^+ channels, including large-conductance K^+ (BK) channels, have been implicated in various aspects of liver function and disease. For example, BK channels are overexpressed in liver cirrhosis and have been shown to regulate vascular tone in this condition (22). Furthermore, inhibition of hepatic mitochondrial BK channels by Bax has been shown to activate the mitochondrial permeability transition pore (mPTP) (3), especially in Ca^{2+}-loaded mitochondria (15). mPTP opening is associated with apoptosis during IR injury (7, 20). The activation of prosurvival signaling cascades, including the reperfusion injury salvage kinase (RISK) and the survivor activating factor enhancement (SAFE) pathways, has been shown to exert strong organ protection against reperfusion injury, at least in part, by regulating mPTP activity (9, 25).

Members of the KCNE family (also known as MinK-related peptides) of single-transmembrane-domain ancillary subunits form complexes with voltage-gated K^+ (K_v) channel α-subunits and alter essential aspects of channel biology, including trafficking, gating kinetics, conductance, and pharmacology. Coassembly with different KCNEs can permit specific K_v channel α-subunits to perform diverse functions in a range of cell types and locations. Recently, we discovered that KCNE2 and KCNE4 influence the extent of damage induced by IR injury in the heart, where both subunits are expressed in humans and mice. *Kcne4* is upregulated by dihydroxytestosterone (DHT) in mouse heart, such that it is more highly expressed in young adult male than female cardiac tissue; following menopause, when DHT levels rise in female mice, cardiac *Kcne4* expression increases. Additionally, in aging female mice, *Kcne4* deletion constitutively activates cardioprotective pathways, attenuating arrhythmogenesis resulting from

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stringent cardiac IR injury. In contrast, *Kcne4* deletion in aging male mice fails to precondition and also impairs postischemic cardioprotective RISK/SAFE pathway induction, predisposing to arrhythmogenesis. Supporting the role of DHT in this disparity, castration eliminated the sex-specific difference in *Kcne4*-linked RISK/SAFE pathway induction (11).

The majority of prior research on KCNE4 has been focused on its roles in the heart, kidney, vasculature, and blood cells. Here, given the clinical importance of hepatic IR injury, we investigated whether KCNE4 influences IR-related tissue damage in the liver and, again, studied the effects of sex in this process. Our findings reveal that KCNE4 is an important and sex-specific modulator of hepatic IR injury in male mice and delineate some of the intracellular signaling pathways involved. Strikingly, this occurs despite negligible hepatic expression of KCNE4 in males, suggesting that KCNE4 is a master regulator of processes affecting IR injury response.

MATERIALS AND METHODS

Animals. Animal studies are reported in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Animal Experiments) guidelines (14). We generated $Kcne4^{+/+}$ and $Kcne4^{-/-}$ C57BL/6 mice as previously described (5) and housed and used them according to the recommendations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (8th ed., 2011). The Institutional Animal Care and Use Committees of the University of California, Irvine, and Sichuan University (permit no. 2015033A) approved the studies. For the majority of the study, 120 adult male and female mice generated from $Kcne4^{+/+} \times Kcne4^{-/-}$ crosses were used at an average age of 13 mo. In addition, for studies on the hepatic artery and portal vein, three male and three female 8-mo-old C57BL/6 mice were used. Mice for the latter experiment were euthanized by cervical dislocation, and the experiments were performed in accordance with the European Union legislation for the protection of animals used for scientific purposes and approved by the Danish National Animal Experiments Inspectorate. All mice were housed in a constant-temperature (25°C) room with a 12:12-h light-dark cycle. Mice were given ad libitum access to water and standard mouse chow.

Liver IR surgical procedures. Pentobarbital sodium (50 mg/kg ip) was used for mouse anesthesia. Loss of the corneal reflex, lack of response to toe pinching, and heart rate were indicators for adequacy of anesthesia. After anesthesia, mice ($n = 5–8$ per group) were placed in a supine position. After laparotomy, the portal vein, hepatic artery, and venous trunk were isolated and clamped with an atraumatic microvascular clip to induce ischemia (30 min). Liver ischemia was confirmed by a change in color (to a paler shade) of the liver. The clip was loosened after 30 min of ischemia, and the ischemic liver was reperfused for 3 h. Throughout the experiment, body temperature was maintained with a heating blanket. The GSK-3β inhibitor SB216763 (2 mg/kg iv; Sigma, St. Louis, MO) was administered after the hepatic ischemia, 5 min before commencement of reperfusion. Male $Kcne4^{+/+}$ and $Kcne4^{-/-}$mice (8–10 mo old) were castrated to establish a testosterone deficiency model. Mice were euthanized 3 h after reperfusion. Blood samples were taken immediately from the heart after reperfusion, and fresh liver samples were collected for histology and Western blot analysis.

Hematoxylin-and-eosin staining. At the end of 3 h of reperfusion, right hepatic lobes were taken, rinsed in saline, and dried before immersion in 10% phosphate-buffered formalin solution overnight at room temperature. Liver samples were then fixed and embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin ($n = 4–5$ mice per group).

Histological evaluation of liver damage. Histopathological examinations were performed under a light microscope in a double-blind manner. A modified Suzuki numerical scoring system (24) was used for evaluation. Briefly, hepatic damage, including congestion and hepatocyte cytoplasm vacuolization, was graded according to severity (0: no damage, 1: mild damage, 2: moderate damage, and 3: marked damage) and distribution (0: no damage, 1: focal damage, 2: multifocal damage, and 3: diffuse damage). A mean score for each variable was determined for each liver.

Serum analyses. Whole blood was removed and centrifuged (4,000 rpm for 10 min at 4°C) to obtain plasma. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified using a chemistry analyzer (model BS-120, Mindray Medical, Shenzhen, China). All samples were measured in duplicate ($n = 8–15$ mice per group). Serum cholesterol/cholesteryl ester was quantified in duplicate by ELISA (Abcam, Cambridge, UK).

Real-time quantitative PCR. RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples of absorbance ratios (A_{260}/A_{280}) between 1.90 and 2.10 were used for synthesis. RNA (20–200 μg) was used for cDNA synthesis (Vazyme Biotech, Nanjing, Jiangsu, China). A fast quantitative PCR (qPCR) kit (Vazyme Biotech) was used for reverse transcription. Real-time qPCR was performed in a PCR system (product no. CFX 96, Bio-Rad, Hercules, CA). The primer sequences were as follows (5): 5′-CTTTGCTCGATGGAAGGGGAC-3′ (forward) and 5′-GCTGTCGTTGAGAGGCGTC-3′ (reverse) for *Kcne4* and 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse) for *Gapdh*. Samples were run in triplicate. Relative expression levels of *Kcne4* were normalized to *Gapdh*.

RNA was extracted from mouse hepatic arteries and portal veins ($n = 3$ males and 3 females for each vessel) using the RNeasy Micro Kit (Qiagen, Vedbaek, Denmark). Reverse transcription was performed with nanoScript2 (Primerdesign, Eastleigh, UK) according to the manufacturer's instructions. For qPCR, we used 15 ng of cDNA per well and Precision-iC SYBR green master mix (Primerdesign) with the real-time PCR detection system (catalog no. CFX96, Bio-Rad). The most stable reference genes were determined by geNorm analysis (Biogazelle qbase+) and found to be *β-actin* and *Gapdh*. The mean quantitation cycle values of these reference genes were used to determine the relative abundance of *Kcne4* and *Kcnq4*. Primers for *Kcne4* and *Kcnq4* were designed and synthesized by Primerdesign with the following sequences: 5′-GGAGGAGGGGGCTGATGA-3′ (forward) and CTGGTGGATGTTCTCGGAAGA-3′ (reverse) for *Kcne4* and 5′-GTGGTCTTTGGCTTGGAGTATAT-3′ (forward) and 5′-CGATGACACAGAAGGGTTTCC-3′ (reverse) for *Kcnq4*. The following cycling conditions were used: initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min; data were collected during each cycling phase. Melt curve analysis, to ensure that each primer set amplified a single, specific product, completed the protocol.

Western blot analyses. Liver tissues ($n = 4–5$ mice per group) were homogenized in radioimmunoprecipitation assay buffer and then centrifuged (10 min, 12,000 rpm) to eliminate debris. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL). Proteins (15 μg/lane) were separated on 10% SDS-polyacrylamide gels and then transferred onto nitrocellulose (VWR, Batavia, IL) or polyvinylidene difluoride (Bio-Rad) membranes. The blots were blocked for 45 min with 5% nonfat milk in Tris-buffered saline-Tween 20 at room temperature. Primary antibodies (rabbit, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA) raised against phosphorylated (Thr^{202}/Tyr^{204}) ERK1/2, total ERK1/2, phosphorylated (Ser^{473}) AKT, total AKT, phosphorylated (Ser^{9}) GSK-3β, total GSK-3β, and phosphorylated (Tyr^{705}) STAT3 and total STAT3 were used to analyze signaling pathways. All samples were run in technical duplicates on separate gels in addition to biological replicates. For detection of Kcne4 protein, a custom rabbit polyclonal antibody (a kind gift from Drs. Daniel Levy and Steve A. N. Goldstein) raised to a peptide within the COOH-terminal domain (residues 136–150 human numbering), was used, as previously reported (17).

Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody (Bio-Rad). Immunoreactive bands were detected with a chemiluminescence kit (ECL, Millipore, Billerica, MA). An Amersham Imager 600 system (GE Healthcare, Little Chalfont, UK) and a G:Box system (Syngene, Cambridge, UK) were used for image visualization. Band densities were determined using ImageJ data acquisition software (National Institutes of Health, Bethesda, MD). Phosphorylation signal densities were normalized to total protein signal densities.

Statistical analysis. Data are presented as means ± SE. Comparisons between two groups were made using unpaired two-tailed Student's *t*-tests. For multiple comparisons over three groups, ANOVA was applied followed by Newman-Keuls test or Dunnett's T3 test, depending on the equality of homogeneity of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Kcne4 expression in mouse liver is negligible. KCNE4 protein exists as a "long" form in human tissues, expressed by a recently discovered transcript with three exons; in *Mus musculus*, a shorter form is expressed, because the exon 1 start site is absent (1). Although some previous studies suggested *KCNE4* transcript expression in human liver (18, 26), we did not detect *KCNE4* transcript in male human liver, but we previously detected *KCNE4* transcript and protein in male human kidneys (1). Therefore, we examined *Kcne4* transcript and Kcne4 protein in male and female mouse liver and kidney, with the advantage of being able to utilize knockout tissue as a negative control. Using real-time qPCR, we detected *Kcne4*

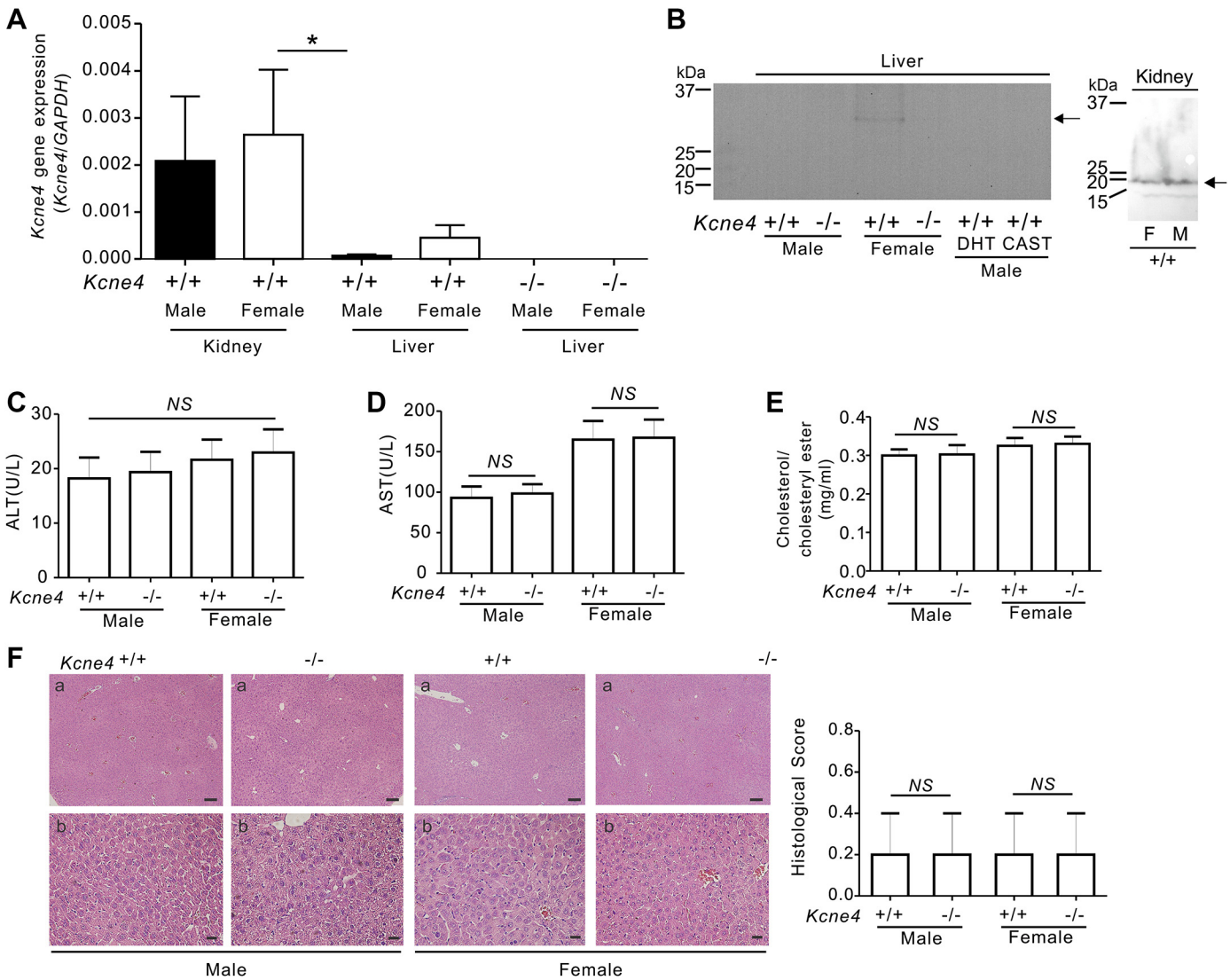


Fig. 1. Deletion *Kcne4* does not change baseline hepatic function. *A*: real-time quantitative PCR of *Kcne4* transcript expression in liver and kidney tissue from male and female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice (n = 3–6). *B*: Western blot of Kcne4 protein expression in liver tissue from male [normal, castrated (CAST), and castrated and dihydroxytestosterone (DHT)-treated] and female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice (*left*) and Western blot of Kcne4 protein expression in female (F) and male (M) mouse kidney tissue. Each blot is representative of results from 2 experiments. *C* and *D*: serum levels of baseline alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice of either sex (n = 9 male and 13–15 female). *E*: serum cholesterol/cholesteryl ester content in $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice of either sex (n = 6–8 male and 6–7 female). *F*: histological micrographs of hematoxylin-eosin-stained liver sections from male and female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice, representative of 4–5 mice per genotype (*left*), and histological scores of both genotypes at baseline (*right*; n = 4–5 mice per genotype). Scale bars = 100 mm (*a*) and 20 mm (*b*). Values are means ± SE. *$P < 0.05$; NS, no significant difference between genotypes.

transcript expression in both male and female mouse kidney lysates. In contrast, *Kcne4* transcript expression was barely detectable in male mouse liver lysate but was present in female mouse liver at ~20% of the level we measured in female mouse kidney (Fig. 1*A*). In accord with these data, we observed a triplet of faint bands at 30–35 kDa corresponding to Kcne4 protein [and similar to our previous observation for higher-molecular-weight forms of "short" KCNE4 expressed in Chinese hamster ovary cells (1)] in female $Kcne4^{+/+}$ liver lysate, but no corresponding signal in male $Kcne4^{+/+}$, male $Kcne4^{-/-}$, or female $Kcne4^{-/-}$ liver lysate. Nor did we detect Kcne4 protein in livers of castrated male mice or castrated mice supplemented with DHT (Fig. 1*B*, *left*). We detected robust Kcne4 protein bands in both male and female mouse kidney, consistent with the qPCR data (Fig. 1*B*, *right*), at a lower molecular weight (~20 kDa), consistent with our previous observation of the lower-molecular-weight form of cloned "short" KCNE4 (1, 5). We therefore conclude that Kcne4 is expressed at very low levels in female mouse liver (compared with kidney) and is essentially absent from male mouse liver at 13 mo of age.

Kcne4 deletion does not alter baseline liver function. We next assessed the effects of *Kcne4* deletion on serum levels of ALT and AST. We found that *Kcne4* deletion did not alter baseline ALT or AST in either sex ($P > 0.05$; Fig. 1, *C* and *D*). We previously found that mice with germline deletion of the Kcne2 K^+ channel β-subunit exhibited nonalcoholic fatty liver disease with disturbance of cholesterol homeostasis, despite the fact that Kcne2 is also not expressed in mouse liver (16). In contrast, *Kcne4* deletion did not alter serum cholesterol/cholesteryl ester content ($P > 0.05$; Fig. 1*E*). Consistent with these findings, we observed no hepatic pathology at baseline in male or female $Kcne4^{-/-}$ livers, similar to the wild-type mouse livers ($P > 0.05$; Fig. 1*F*). Thus, *Kcne4* deletion does not alter baseline liver function in male or female mice.

Kcne4 deletion sex dependently predisposes to liver IR injury. The functional importance of *Kcne4* deletion in hepatic IR injury is completely unknown. Therefore, we measured serum levels of ALT and AST in $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice of either sex subjected to 30 min of ischemia and 3 h of reperfusion. We found that *Kcne4* deletion markedly exacerbated liver injury following IR in male, but not in female, mice. Thus, male $Kcne4^{-/-}$ mice exhibited 1.6-fold higher serum ALT and 2.2-fold higher serum AST than $Kcne4^{+/+}$ mice postreperfusion ($P < 0.001$; Fig. 2, *A* and *B*). Using hematoxylin-and-eosin staining, we also quantified liver pathological alterations in male mice of either genotype following 3 h of reperfusion. The degree of liver injury was less severe in $Kcne4^{+/+}$ than $Kcne4^{-/-}$ mice, which exhibited various hepatic pathological changes, such as steatosis, edema, and vacuolization ($P < 0.05$; Fig. 2, *C*).

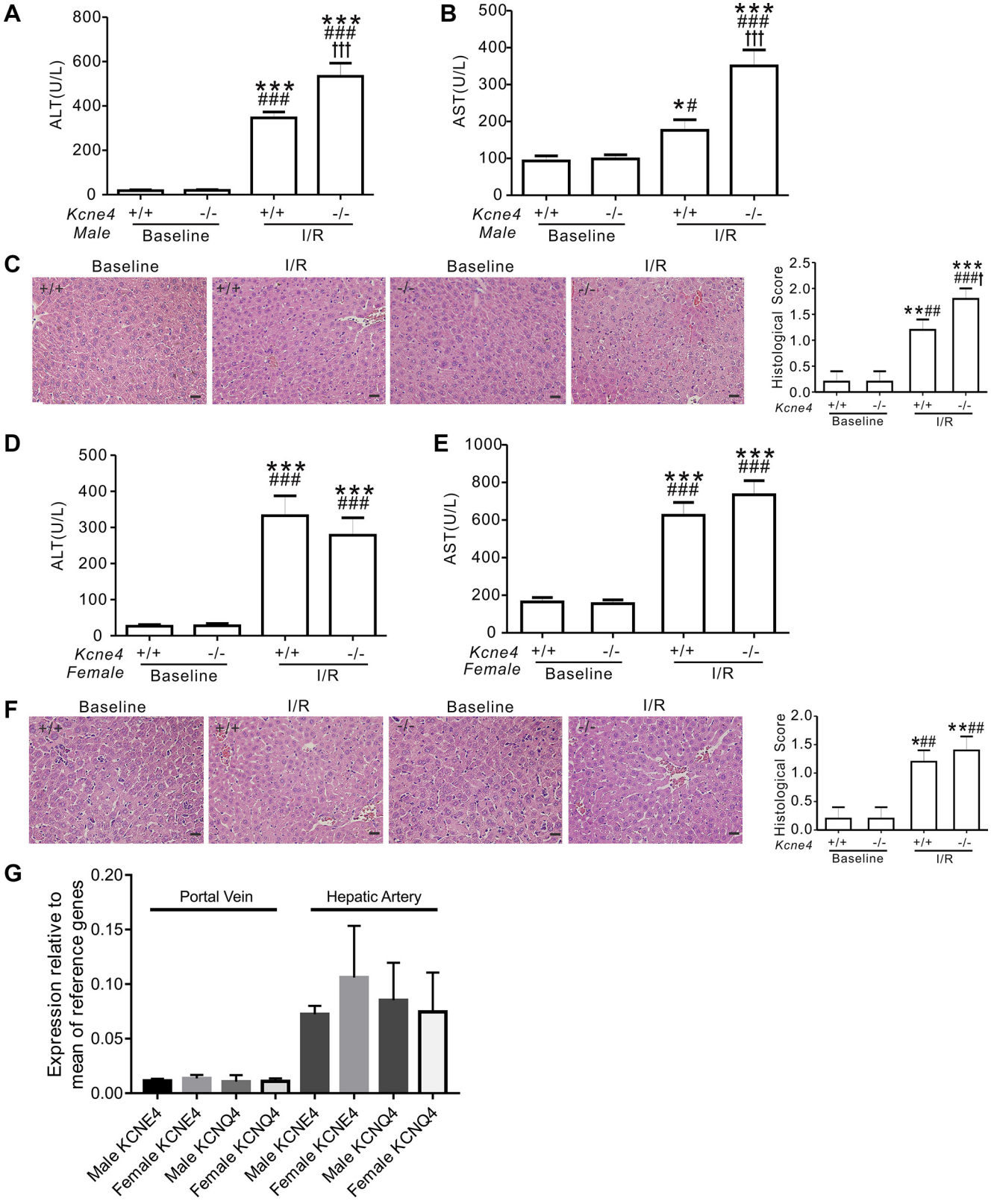
Reperfusion caused significant liver injury in female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice as determined by concentrations of plasma ALT and AST ($P < 0.001$ vs. $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice at baseline; Fig. 2, *D* and *E*). However, there was no difference in post-IR injury serum ALT and AST levels between the two genotypes ($P > 0.05$). Meanwhile, although reperfusion resulted in hepatic congestion and cytoplasmic vacuolization ($P < 0.05$ and $P < 0.01$ vs. $Kcne4^{+/+}$ *and* $Kcne4^{-/-}$ mice at baseline), no histopathological differences were observed between female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice post-IR ($P > 0.05$; Fig. 2*F*). The male-only *Kcne4* deletion-associated predisposition to hepatic IR injury was notable, given that we detected no *Kcne4* transcript or Kcne4 protein in male mouse livers (Fig. 1, *A* and *B*). Next, we quantified transcript expression of *Kcne4* and its known α-subunit partner *Kcnq4* (2, 12) in mouse portal vein and hepatic artery. Expression of either transcript was higher in hepatic artery than portal vein, but neither showed sex-specific expression (Fig. 2*G*). Therefore, the sex dependence of *Kcne4* deletion-linked hepatic IR injury predisposition does not appear to arise from sex-dependent differences in *Kcne4* expression in the liver or the primary hepatic blood vessels.

Kcne4 deletion causes baseline GSK-3β elevation in female mice. Current evidence suggests that the activation of two major intrinsic prosurvival signaling pathways, the RISK and SAFE pathways, confers strong cardioprotection in the context of IR injury. The RISK pathway includes protein kinase B (AKT), ERK1/2, and the downstream target GSK-3β; the SAFE pathway includes STAT-3. Of note, activation (via phosphorylation) of AKT and ERK1/2, which are major molecules in the RISK pathway, results in inhibition of the mPTP and subsequent inhibition of downstream GSK-3β. Phosphorylation (Ser^9) of GSK-3β results in inhibition of GSK-3β activity (4), which enhances cardiac myocyte survival against myocardial IR injury in local or remotely ischemic preconditioned hearts (10, 25).

We did not detect a difference in total ERK1/2, AKT, GSK-3β, or STAT-3 protein levels among livers isolated after 180 min of reperfusion in either genotype. In males, *Kcne4* deletion did not alter baseline phosphorylation of any of the four proteins (Fig. 3, *A–D*). In contrast, phosphorylation of

Fig. 2. Deletion of *Kcne4* aggravates hepatic damage after liver ischemia-reperfusion (IR) injury in male mice. *A* and *B*: serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in male $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice ($n = 8$ per genotype) subjected to 30 min of liver ischemia followed by 3 h of reperfusion. *$P < 0.05$, ***$P < 0.001$ compared with baseline male $Kcne4^{+/+}$ mice; #$P < 0.05$, ###$P < 0.001$ compared with baseline male $Kcne4^{-/-}$ mice; †††$P < 0.001$ compared with male $Kcne4^{+/+}$ mice post-IR (by 1-way ANOVA). Values for male $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice at baseline from Fig. 1 are shown for comparison. *C*: histological micrographs of hematoxylin-eosin-stained liver sections from male $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice at baseline and post-IR injury, representative of $n = 4$–5 mice per genotype (*left*), and morphological evaluation of liver damage after reperfusion in livers from male mice of both genotypes (*right*; $n = 4$–5 mice per genotype). Scale bars = 20 mm. **$P < 0.01$, ***$P < 0.001$ compared with baseline male $Kcne4^{+/+}$ mice; ##$P < 0.01$, ###$P < 0.001$ compared with baseline male $Kcne4^{-/-}$ mice; †$P < 0.05$ compared with male $Kcne4^{+/+}$ mice post-IR (by 1-way ANOVA). *D* and *E*: serum levels of ALT and AST in female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice ($n = 8$ per genotype) subjected to 30 min of liver ischemia followed by 3 h of reperfusion. ***$P < 0.001$ compared with baseline female $Kcne4^{+/+}$ mice; ###$P < 0.001$ compared with baseline female $Kcne4^{-/-}$ mice (by 1-way ANOVA). Values for female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice at baseline from Fig. 1 are shown for comparison. *F*: histological micrographs of hematoxylin-eosin-stained liver sections from female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice at baseline and post-IR injury, representative of $n = 4$–5 mice per genotype (*left*), and morphological evaluation of liver damage after reperfusion in livers from female mice of both genotypes (*right*; $n = 4$–5 mice per genotype). Scale bars = 20 mm. *$P < 0.05$, **$P < 0.01$ compared with baseline female $Kcne4^{+/+}$ mice; ##$P < 0.01$ compared with baseline female $Kcne4^{-/-}$ mice (by 1-way ANOVA). *G*: real-time quantitative PCR of *Kcne4* and *Kcnq4* transcript expression in portal vein and hepatic artery from male and female mice ($n = 3$). Values are means ± SE.

liver GSK-3β was dramatically increased only in female *Kcne4*$^{-/-}$ mice ($P < 0.05$) compared with livers from *Kcne4*$^{+/+}$ mice at baseline (Fig. 3). *Kcne4* deletion did not alter baseline phosphorylation of ERK1/2, AKT, or STAT-3 (Fig. 3, *A–D*). Given that GSK-3β phosphorylation was enhanced in female *Kcne4*$^{-/-}$ mice at baseline and female mice were protected from the *Kcne4*-deletion-linked predisposition to greater IR-related damage observed in male mice (Fig. 2),



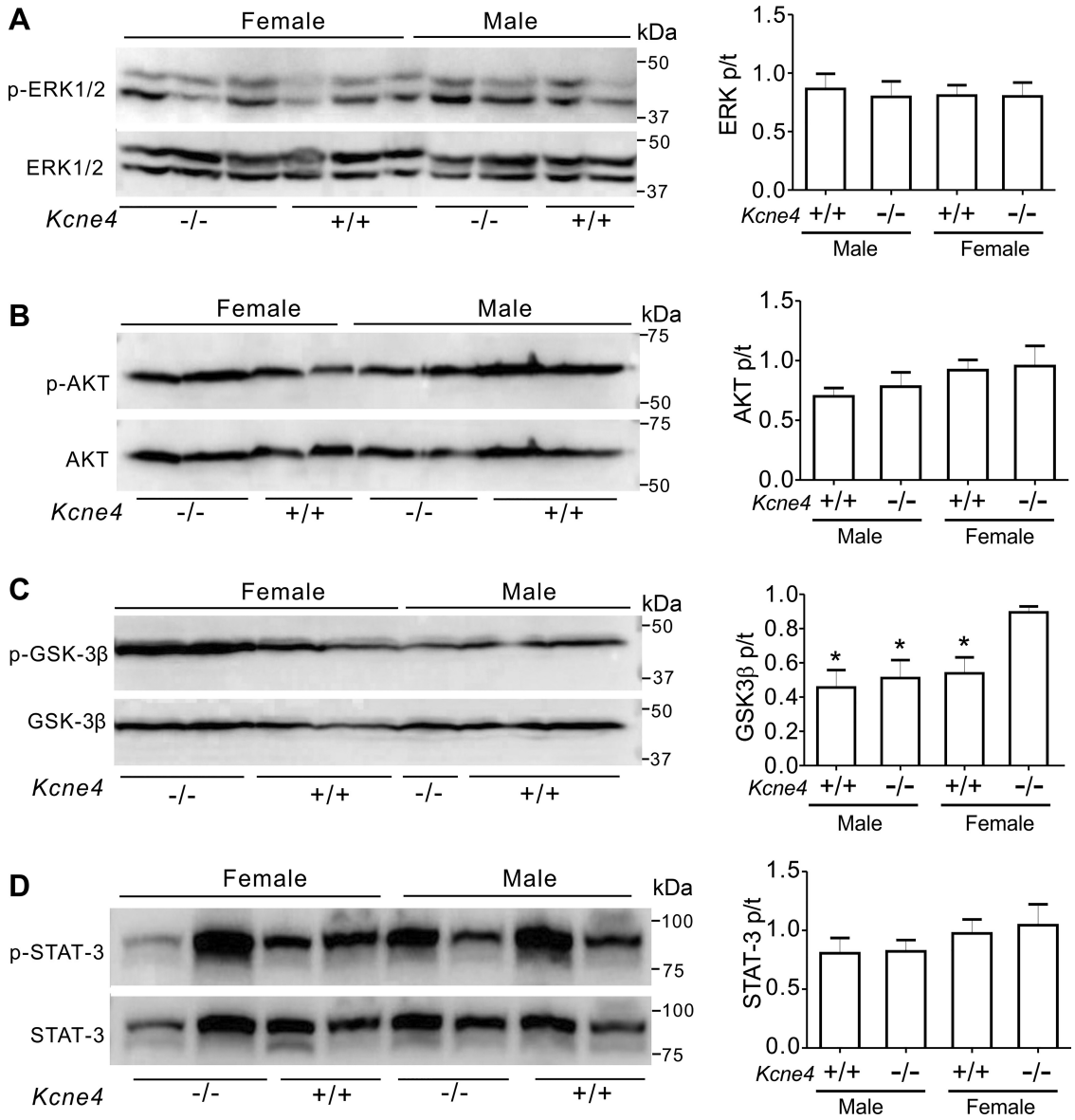


Fig. 3. Deletion of *Kcne4* increases liver GSK-3β phosphorylation at baseline in female mice. *Left*: representative Western blots showing baseline hepatic phosphorylated (p) and total (t) ERK1/2 (*A*), phosphorylated and total AKT (*B*), phosphorylated and total GSK-3β (*C*), and phosphorylated and total STAT-3 (*D*) expression in male and female $Kcne4^{+/+}$ and $Kcne4^{-/-}$ mice (1 mouse per lane). *Right*: ratio of phosphorylated to total ERK ($n = 4$–5), phosphorylated to total AKT ($n = 4$–5), phosphorylated to total GSK-3β ($n = 4$–5), and phosphorylated to total STAT-3 ($n = 4$–5) band densities from blots at *left*. Values are means ± SE.*$P < 0.05$ compared with female $Kcne4^{-/-}$ mice (by 1-way ANOVA).

one can speculate that baseline GSK-3β phosphorylation contributes to this protection in female mice.

Kcne4 deletion impairs hepatic ERK1/2 phosphorylation in male mice but enhances GSK-3β phosphorylation in female mice after liver IR. The signaling proteins in RISK and SAFE pathways were previously shown to be phosphorylated during ischemic episodes in myocardial or liver samples, and this elevation was greater if preceded by protective stimuli, such as ischemic preconditioning (8). Here, examining RISK/SAFE pathway protein phosphorylation following IR injury, we found that ERK1/2 phosphorylation was increased in livers from male $Kcne4^{+/+}$ mice after IR injury compared with livers from $Kcne4^{+/+}$ or $Kcne4^{-/-}$ mice at baseline. In contrast, the ratio of phosphorylated to total ERK1/2 was not elevated post-IR in male $Kcne4^{-/-}$ mice, being equivalent to the levels we observed in male $Kcne4^{-/-}$ mice at baseline ($P < 0.001$ vs. livers from $Kcne4^{+/+}$ mice post-IR; Fig. 4*A*). Phosphorylation of AKT and STAT-3 was genotype independently enhanced post-IR in males, while GSK-3β phosphorylation was not statistically significantly increased, after 180 min of reperfusion in either genotype (Fig. 4, *B–D*). Thus, *Kcne4* deletion impaired the hepatic ERK1/2 response to IR injury in male mice.

In female mice, phosphorylation of all four RISK/SAFE pathway proteins studied was increased in the liver after 180 min of reperfusion in either genotype compared with baseline (Fig. 5, *A–D*). In addition, the ratio of phosphorylated to total GSK-3β in female $Kcne4^{-/-}$ mice post-IR injury was almost double that in female $Kcne4^{+/+}$ mice post-IR injury ($P < 0.05$ vs. female $Kcne4^{+/+}$ mice post-IR; Fig. 5*C*). Thus, *Kcne4*

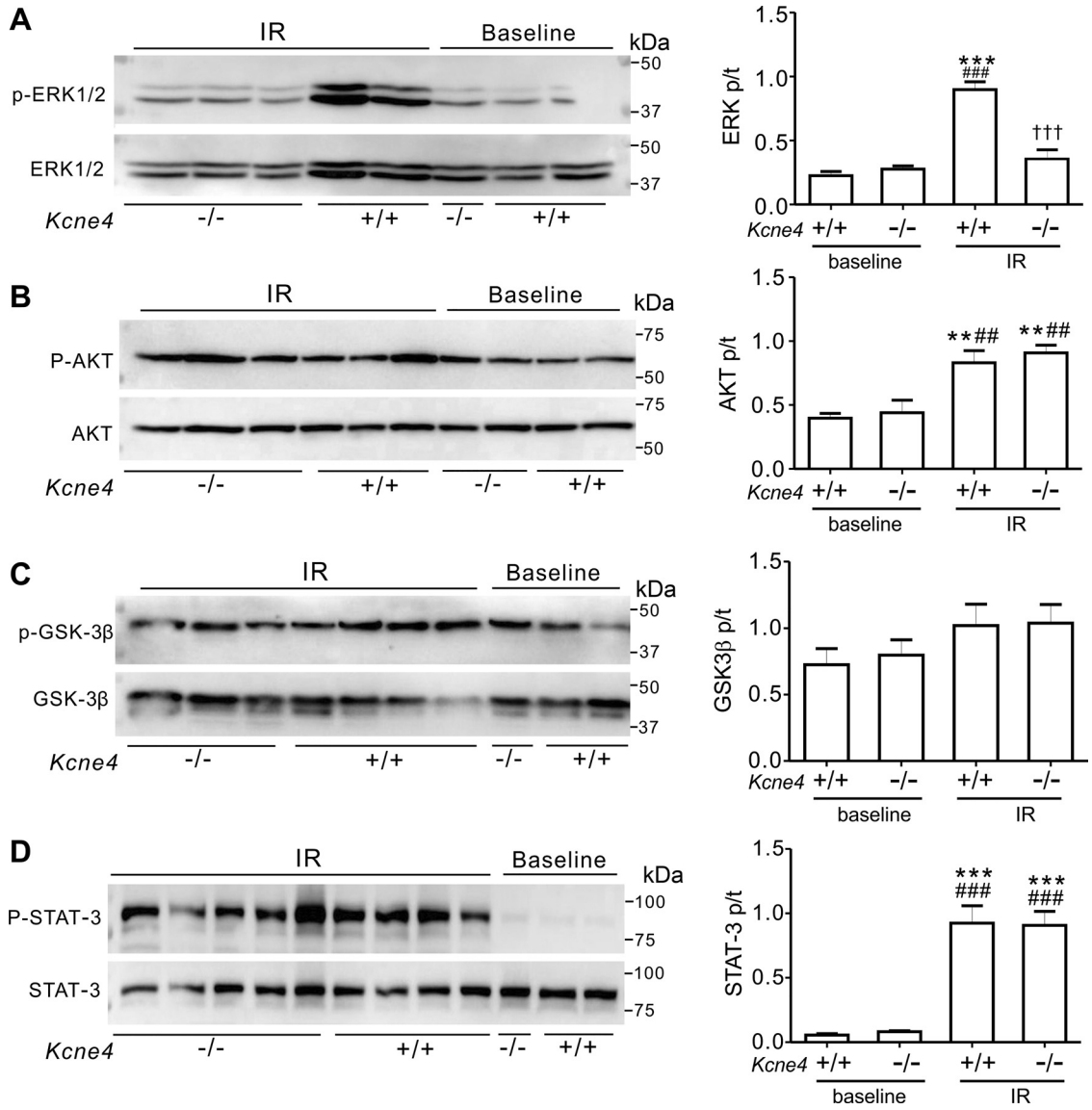


Fig. 4. Deletion of *Kcne4* impairs post-ischemia-reperfusion (IR) hepatic reperfusion injury salvage kinase (RISK) pathway induction in male mice. *Left*: representative Western blots showing male baseline and post-IR injury hepatic phosphorylated (p) and total (t) ERK1/2 (*A*), phosphorylated and total AKT (*B*), phosphorylated and total GSK-3β (*C*), and phosphorylated and total STAT-3 (*D*) expression for 1 mouse per lane. *Right*: ratio of phosphorylated to total ERK (*A*, $n = 4$–5), phosphorylated to total AKT (*B*, $n = 4$–5), phosphorylated to total GSK-3β (*C*, $n = 4$–5), and phosphorylated to total STAT-3 (*D*, $n = 4$–5) band densities from blots at *left*. Values are means ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline $Kcne4^{+/+}$ mice; $\#\#P < 0.01$, $\#\#\#P < 0.001$ compared with baseline male $Kcne4^{-/-}$ mice; †††$P < 0.001$ compared with male $Kcne4^{+/+}$mice post-IR (by 1-way ANOVA).

deletion results in increased post-IR hepatic GSK-3β phosphorylation.

Prereperfusion pharmacological inhibition of GSK-3β further protects female mice from hepatic IR injury. Inhibition of GSK-3β (such as occurs when GSK-3β is phosphorylated) is known to exert cardiac or hepatic protection against IR injury (6, 23). We found that *Kcne4* deletion in female (but not male) mice upregulates GSK-3β inhibitory phosphorylation at baseline and that *Kcne4* deletion exacerbates hepatic IR injury in male (but not female) mice. These findings suggest that GSK-3β inhibition is protective in hepatic IR injury in our model. We next administered the GSK-3β inhibitor SB216763 after the hepatic ischemia 5 min before commencement of reperfusion. Inhibition of GSK-3β conferred hepatic protection, mimicking the effect of liver ischemic conditioning; i.e., independent of genotype, inhibitor-treated female *Kcne4* mice had similar reductions in serum concentrations of ALT ($P <$ 0.001 vs. $Kcne4^{+/+}$ or $Kcne4^{-/-}$ mice without inhibitor post-IR; Fig. 6*A*) and AST ($P < 0.001$; Fig. 6*B*). SB216763 brought GSK-3β phosphorylation levels in livers from $Kcne4^{+/+}$ mice to a level equivalent to that in $Kcne4^{-/-}$ mice and did not further increase GSK-3β phosphorylation levels after reperfusion in livers from $Kcne4^{-/-}$ mice [which, in the absence of SB216763, were higher than in wild-type mice (Fig. 5)]. This, in combination with the failure of the increased GSK-3β phosphorylation in livers from $Kcne4^{-/-}$ mice after reperfusion to protect from hepatic IR injury beyond limiting it to that in equivalent livers from wild-type mice, suggests that the timing and/or mode of GSK-3β phosphorylation/inhibition is a deciding factor in the efficacy of protection from hepatic IR

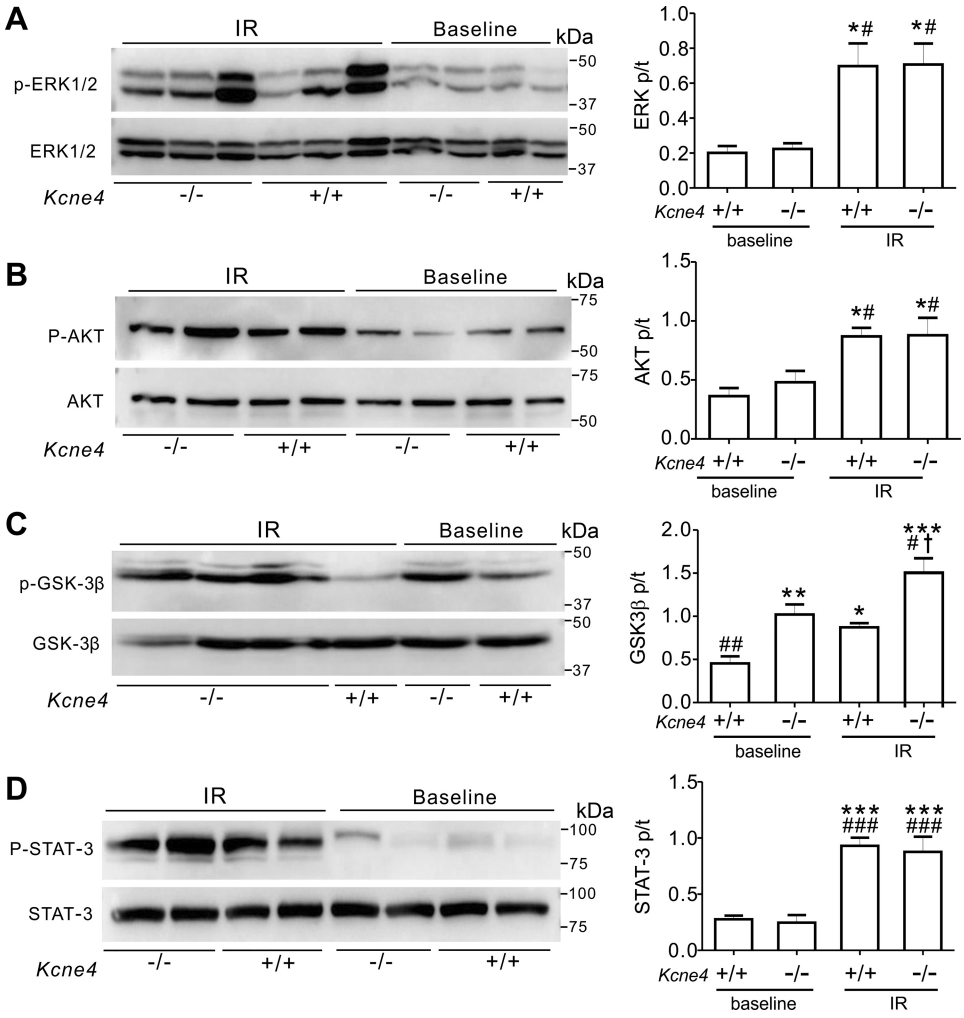


Fig. 5. Deletion of *Kcne4* increases post-ischemia-reperfusion (IR) hepatic GSK-3β phosphorylation in female mice. *Left*: representative Western blots showing female baseline and post-IR injury hepatic phosphorylated (p) and total (t) ERK1/2 (*A*), phosphorylated and total AKT (*B*), phosphorylated and total GSK-3β (*C*), and phosphorylated and total STAT-3 (*D*) expression of 1 mouse per lane. *Right*: ratio of phosphorylated to total ERK ($n = 4–5$), phosphorylated to total AKT ($n = 4–5$), phosphorylated to total GSK-3β ($n = 4–5$), and phosphorylated to total STAT-3 ($n = 4–5$) band densities from blots at *left*. Values are means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline female *Kcne4*$^{+/+}$ mice; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ compared with baseline female *Kcne4*$^{-/-}$ mice; $\dagger P < 0.05$ compared with female *Kcne4*$^{+/+}$mice post-IR (by 1-way ANOVA).

injury (in livers from *Kcne4*$^{-/-}$ mice, GSK-3β phosphorylation is already elevated at baseline, while we administer SB216763 between ischemia and reperfusion).

Kcne4 deletion DHT dependently impairs RISK/SAFE pathway induction and liver function in male mice. To further explore the mechanism of sex-dependent predisposition to liver IR injury in *Kcne4* deletion, we castrated male *Kcne4*$^{-/-}$ mice to evaluate if the *Kcne4* deletion-exacerbated hepatic IR injury in male mice is DHT-dependent and how DHT regulates the RISK or SAFE pathway. Strikingly, castration produced an effect similar to that observed in female (but not noncastrated male) mice, i.e., elevated baseline GSK-3β phosphorylation arising from *Kcne4* deletion ($P < 0.01$; Fig. 7, *A–D*). Notably, castration also abolished *Kcne4* deletion-dependent susceptibility of male mice to liver IR injury, such that there was no difference in serum ALT or AST concentration between male *Kcne4*$^{+/+}$and *Kcne4*$^{-/-}$mice after 180 min of reperfusion.

Throughout the study, we observed higher AST levels in female than male mice at baseline, regardless of genotype, and post-IR, regardless of genotype, even though female mice post-IR showed equivalent or less liver damage (and none at baseline in either sex/genotype) according to histological score (Figs. 1 and 2). In healthy human subjects, serum AST was previously found to be lower in women than men, and the same was observed for patients with hyperbilirubinemia. Yet serum AST-to-ALT ratio was higher in women in either group than in men (19), a pattern we also observed in the mice in this study, because ALT levels were less sex-dependent but AST levels were higher in female than male mice. Strikingly, here we observed that castration eliminated the *Kcne4*-deletion-dependent increase in post-IR serum ALT; furthermore, castration eliminated genotype-dependent differences in post-IR serum AST, bringing it to levels equivalent to those in female mice ($P > 0.05$; Fig. 7, *E* and *F*).

Additionally, while *Kcne4* deletion prevented the post-IR increase in ERK1/2 phosphorylation that we observed in livers from male *Kcne4*$^{+/+}$ mice (Fig. 4*A*), castration restored the post-IR ERK1/2 phosphorylation response to levels in wild-type mice (Fig. 7*G*). Importantly, post-IR phosphorylation (Ser^9) of GSK-3β was also enhanced by castration, which tripled the GSK-3β phosphorylation (Ser^9) level compared with intact *Kcne4*$^{-/-}$ mice, elevating it beyond that in intact or castrated wild-type mice (Fig. 7*H*). Interestingly, castration also doubled the level of post-IR GSK-3β phosphorylation (Ser^9) in wild-type mice compared with intact wild-type mice (Fig. 7*H*). In contrast to these changes, AKT and STAT-3 phosphorylation levels were not affected by castration in either genotype post-IR ($P > 0.05$ vs. intact mice post-IR; Fig. 7, *I* and *J*).

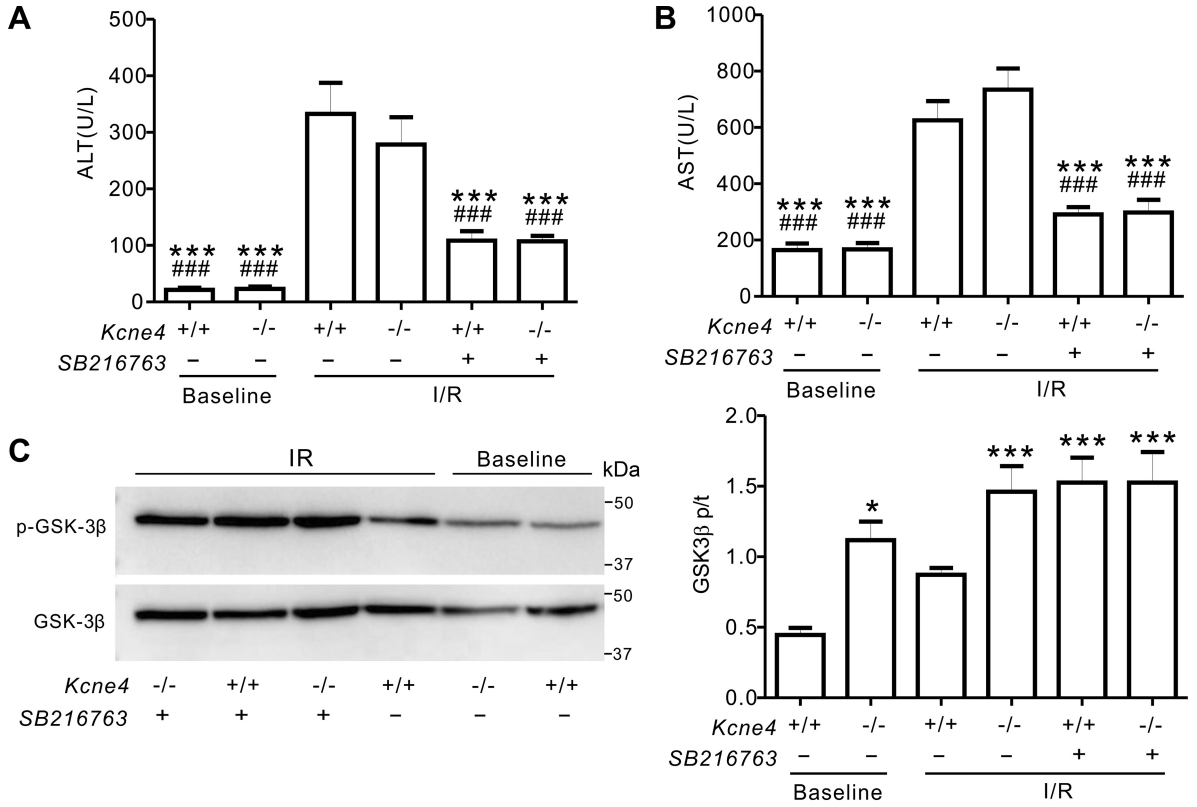


Fig. 6. Pharmacological inhibition of GSK-3β is hepatoprotective in female mice. *A* and *B*: serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice subjected to 30 min of liver ischemia followed by 3 h of reperfusion in the presence (+) or absence (−) of the pharmacological inhibitor SB216763. Data for female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice at baseline or post-ischemia-reperfusion (IR) from Figs. 1 and 2 are shown for comparison. Values are means ± SE; $n = 8$ per genotype. ***$P < 0.001$ compared with female *Kcne4*$^{+/+}$ mice post-IR without SB216763; ###$P < 0.001$ compared with female *Kcne4*$^{-/-}$ mice post-IR without SB216763 (by 1-way ANOVA). *C*: representative Western blots of phosphorylated (p) and total (t) GSK-3β isolated from livers of female *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice in the presence (+) or absence (−) of SB216763 at baseline and post-IR injury (*left*) and ratio of phosphorylated to total GSK-3β protein band density (*right*). Values are means ± SE; $n = 4$–5 per genotype. *$P < 0.05$, ***$P < 0.0001$ compared with baseline female *Kcne4*$^{+/+}$ mice (by 1-way ANOVA).

DISCUSSION

Our primary finding is that *Kcne4* deletion sex dependently exacerbates hepatic IR injury, a phenomenon we observe only in male mice. Examining the RISK/SAFE pathways to elucidate molecular mechanisms underlying this disparity, we found that *Kcne4* deletion is associated with a loss of the post-IR ERK1/2 phosphorylation response only in male mice. Conversely, in female mice only, GSK-3β phosphorylation (Ser^9) is enhanced at baseline and post-IR by *Kcne4* deletion. Finally, mimicking GSK-3β phosphorylation (Ser^9) by pharmacological inhibition of GSK-3β was highly protective against hepatic IR injury.

The activation of prosurvival signaling cascades, including the RISK and SAFE pathways, has been shown to exert strong organ protection against reperfusion injury (9, 25). IR injury is a multifactorial process involving a variety of intracellular signaling pathways, and more thorough understanding of the role of proteins such as Kcne4 and its interactions with multiple effectors and the underlying signaling mechanisms may lead to improvements in the management of ischemia and/or reperfusion injury.

We previously showed that germline deletion of the widely expressed *Kcne2* gene in mice preconditions the heart, attenuating the acute cardiac myocyte damage caused by an imposed myocardial IR injury (9). This study indicated that, besides its function in regulating cardiac electrical excitability, KCNE2 plays a pivotal role in susceptibility to IR injury. More recently, we found that *Kcne4* deletion dictates predisposition of the heart to IR injury. Moreover, this occurs sex specifically, with males being more prone to the effects of *Kcne4* deletion with respect to IR injury, a disparity we found to be mediated by DHT (11). In the current study we observed similar effects in mouse liver. The striking difference is that we did not detect hepatic *Kcne4* transcript or Kcne4 protein expression in the male mice used in this study; however, in female mice, we detected very low levels of both transcript and protein (compared with the kidney). In contrast, *Kcne4* is detectable in hearts (both atria and ventricles) from male and female mice at 13 mo of age (and, in young adults, is more highly expressed in males, because of the higher DHT levels) (5). From the results of these studies, we conclude that Kcne4 is a general, systemic regulator of the response to IR injury; otherwise, it would not be feasible for *Kcne4* deletion to influence hepatic IR injury in male mice, as it is not expressed in the liver. Thus, although the BK channel, the activity of which is inhibited by formation of complexes with KCNE4, is expressed in the liver and is thought to regulate mPTP activity, our data do not suggest that loss of KCNE4 from hepatic BK channel complexes can explain our results. Nor can the sex specificity of our data be explained by differences in KCNE4 expression in the hepatic

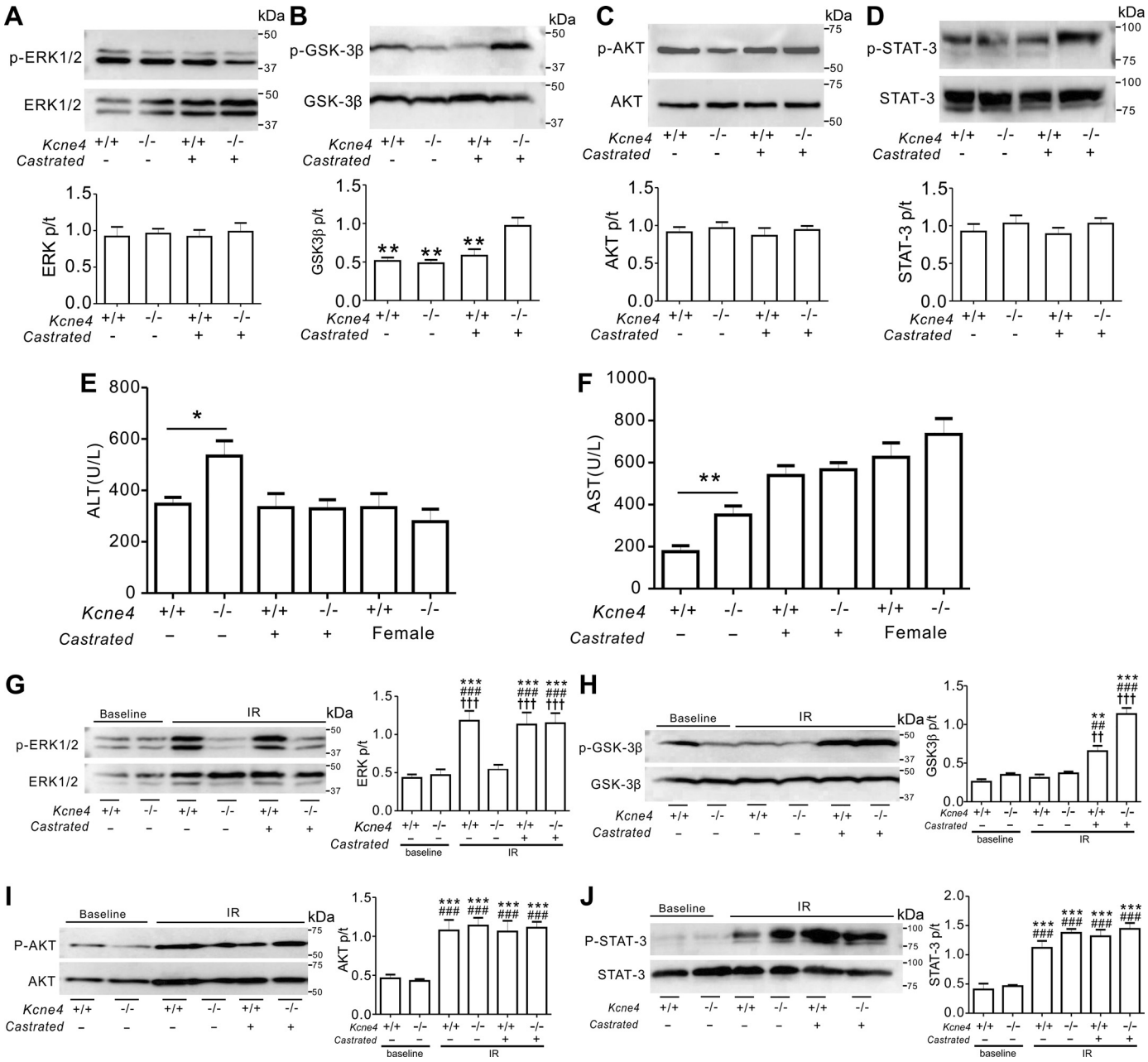


Fig. 7. Effects of castration on liver ischemia-reperfusion (IR) injury and reperfusion injury salvage kinase/survivor activating factor enhancement (RISK/SAFE) pathway response. *A–D*: representative Western blots of phosphorylated (p) and total (t) ERK1/2, phosphorylated and total GSK-3β, phosphorylated and total AKT, and phosphorylated and total STAT-3 in livers from castrated (+) and noncastrated (−) male *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice (*top*) and ratios of phosphorylated to total ERK, phosphorylated to total GSK-3β, phosphorylated to total AKT, and phosphorylated to total STAT-3 band densities from blots above (*bottom*). Values are means ± SE; $n = 4$–5 for each genotype. $^{**}P < 0.01$ compared with castrated male *Kcne4*$^{-/-}$ mice (by 1-way ANOVA). *E* and *F*: serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in female and castrated (+) and noncastrated (−) male *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice post-IR ($n = 8$–11 per genotype). Data for female and noncastrated male *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice from Fig. 2 are shown for comparison. Values are means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with noncastrated male *Kcne4*$^{+/+}$ mice (by 1-way ANOVA). *G–J*: representative Western blots of phosphorylated and total ERK1/2, phosphorylated and total GSK-3β, phosphorylated and total AKT, and phosphorylated and total STAT-3 in livers from castrated and noncastrated male *Kcne4*$^{+/+}$ and *Kcne4*$^{-/-}$ mice at baseline and post-IR injury (*left*) and ratio of phosphorylated to total ERK, phosphorylated to total GSK-3β, phosphorylated to total AKT, and phosphorylated to total STAT-3 band densities from blots at *left* (*right*). Values are means ± SE; $n = 4$–5 per genotype. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline noncastrated male *Kcne4*$^{+/+}$ mice; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with noncastrated male *Kcne4*$^{-/-}$mice; $^{\dagger\dagger}P < 0.001$, $^{\dagger\dagger\dagger}P < 0.001$ compared with noncastrated male *Kcne4*$^{-/-}$ mice post-IR (by 1-way ANOVA).

vasculature, as we found sex-independent expression of KCNE4 in the portal vein and hepatic artery. One possibility is that the differential expression and functionality of Kcne4 in other components of the systemic vasculature dictate its influence on IR injury; we previously found that *Kcne4* deletion altered α-adrenergic vascular reactivity in male, but not female, mice (2). Alternatively, the sex-specific differences we previously observed with respect to the greater requirement for Kcne4 in male than female mouse heart (5) may dictate the sex specificity of predisposition to Kcne4-dependent IR injury in the liver.

Perspectives and Significance

In addition to our finding that *Kcne4* deletion sex dependently exacerbates hepatic IR injury in male mice, our study includes some novel and potentially important observations independent of *Kcne4* genotype. First, we found that baseline and serum AST levels are higher in 13-mo-old female mice than in their age-matched male counterparts, despite similar liver histology. As many studies of mice do not include groups from both sexes, it is important to note that it is inappropriate to compare AST levels, or AST-to-ALT ratios, between different studies if different sexes were used between studies. Second, our data strongly suggest that this sex-specific difference in serum AST arises from DHT, as baseline and post-IR AST levels were similar in castrated male and age-matched female mice. Finally, castration doubled the level of post-IR hepatic phosphorylation (Ser^9) of GSK-3β in wild-type male mice compared with intact wild-type mice post-IR. This suggests that hormonal status (specifically, DHT, in this case) dictates the capacity of the liver (and, likely, other tissues) to initiate a protective response to IR injury, a finding that warrants further investigation, as it could provide potential therapeutic avenues to enhance IR injury prevention or identify particularly vulnerable populations (if the results can be extrapolated to human IR injury).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.H., T.A.J., and G.W.A. conceived and designed research; Z.H., T.A.J., M.L., and G.W.A. performed experiments; Z.H., M.L., and G.W.A. analyzed data; Z.H., J.L., and G.W.A. interpreted results of experiments; Z.H., T.A.J., M.L., and G.W.A. prepared figures; Z.H. and G.W.A. drafted manuscript; Z.H., T.A.J., and G.W.A. edited and revised manuscript; Z.H., T.A.J., L.Z., J.L., M.L., and G.W.A. approved final version of manuscript.

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