G Protein-Coupled Receptor 30-Dependent Protein Kinase A Pathway Is Critical in Nongenomic Effects of Estrogen in Attenuating Liver Injury after Trauma-Hemorrhage

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Although nongenomic effects of 17 β -estradiol (E2) are mediated via the estrogen receptor α (ER- α), the existence of another novel ER, G protein-coupled receptor 30 (GPR30), has been suggested as a candidate for triggering a broad range of E2-mediated signaling. GPR30 also acts independently of the ER to promote activation of the protein kinase A (PKA) pathway, which protects cells from apoptosis through Bcl-2. In this study, we examined whether the salutary effects of E2 in attenuating hepatic injury after trauma-hemorrhage are mediated via GPR30- or ER- α -regulated activation of PKA-dependent signaling. At 2 hours after trauma-hemorrhage, administration of E2-conjugated to bovine serum albumin (E2-BSA, membrane impermeable) or E2 induced the up-regulation of ER- α and GPR30 and attenuated hepatic injury. This was accompanied by increases in PKA activity and Bcl-2 expression. Inhibition of PKA in E2-BSA-treated trauma-hemorrhage rats by PKA inhibitor H89 prevented the E2-BSA attenuation of hepatic injury. Isolated hepatocytes were transfected with small interfering RNA to suppress GPR30 or ER. We found that suppression of GPR30 but not ER- α prevented E2-BSA- or E2-induced PKA activation and Bcl-2 expression. These results suggest that the nongenomic salutary effect of E2 in reducing hepatic injury after trauma-hemorrhage is mediated through the PKA-dependent pathway via GPR30 but not ER- α . (*Am J Pathol* 2007, 170:1210–1218; DOI: 10.2353/ajpath.2007.060883)

Despite numerous advances in intensive care medicine, sepsis and organ dysfunction remain the major causes of death after trauma.^{1–5} Previous studies have shown that administration of 17 β -estradiol (E2) after trauma-hemorrhage improved hepatic function.^{6–8} This effect was suggested to be regulated by two different mechanisms, genomic and nongenomic. The ability of E2 conjugated to bovine serum albumin (E2-BSA, putatively a membrane-impermeable compound) to mimic the effects of E2 have been used to distinguish nongenomic from genomic events.^{9–11} Studies have reported that nongenomic effects occur through estrogen receptors (ER- α and ER- β) targeted to the plasma membrane⁹ or through a recently described novel membrane-associated ER.^{12,13} ER- α has been demonstrated to be predominantly present in rat liver.¹⁴ Our recent study has also shown that ER- α but not ER- β mediated E2-attenuated hepatic injury after trauma-hemorrhage.¹⁵ Nonetheless, whether this beneficial effect of E2 in attenuating liver injury after trauma-hemorrhage is mediated via the novel membrane-associated ER or classic ER- α remains unknown.

G protein-coupled receptor 30 (GPR30) is activated by E2 and acts independently of the ER to promote activation of the adenylyl cyclase/cAMP-dependent protein kinase A (PKA) pathway.^{16,17} PKA-dependent signaling mediates anti-apoptotic effects after ischemic liver injury¹⁸ and regulates cell survival through the anti-apoptotic protein Bcl-2.¹⁹ In view of this, we hypothesized that the salutary effect of the nongenomic pathway of E2 on he-

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patic injury after trauma-hemorrhage is mediated through the PKA-dependent Bcl-2 pathway via up-regulation of GPR30 or ER- α . Plasma α -glutathione S-transferase (α -GST) is a more sensitive and specific marker of hepatocellular damage than is aminotransferase activity and correlates better with hepatic histology.^{20–22} α -GST has also been advocated as a superior marker of hepatocellular damage compared with the aminotransferase or bilirubin concentration in clinical patients.²³ In this study, we examined the effects of E2-BSA and E2 after trauma-hemorrhage on α -GST levels, GPR30 and ER- α levels, PKA activity, and Bcl-2 levels in the liver. A group of E2-BSA-treated trauma-hemorrhage rats was co-treated with the PKA inhibitor H89 to determine the role of PKA in nongenomic effects of E2 on protection against hepatic injury. Moreover, inhibition of GPR30 or ER- α by transfection of small interfering RNA (siRNA) in isolated hepatocytes was performed to determine whether GPR30 or ER- α is critical in E2-mediated activation of the PKA pathway.

Materials and Methods

Rat Trauma-Hemorrhagic Shock Model

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were fasted overnight but were allowed free access to water before the experiments. Trauma-hemorrhage and resuscitation was then performed as described previously.²⁴ In brief, rats were anesthetized by isoflurane inhalation, and a 5-cm midline laparotomy was performed to induce soft-tissue trauma. The abdominal wound was then closed in layers, and polyethylene (PE-50; Becton Dickinson & Co., Sparks, MD) catheters were placed in both femoral arteries and the right femoral vein, subsequently tunneled through the dorsal skin, and the incision sites were then closed. The rats were then placed into a Plexiglas chamber (21 $\times$ 9 $\times$ 5 cm) in a prone position and allowed to awaken, after which they were bled rapidly within 10 minutes to a mean arterial pressure of 35 to 40 mm Hg. This level of hypotension was maintained until the animals could no longer maintain a mean arterial pressure of 35 mm Hg unless some fluid in the form of Ringer's lactate solution was administered. This time was defined as maximal bleed-out. After the maximal bleed-out, mean arterial pressure was maintained between 35 and 40 mm Hg until 40% of the maximal bleed-out volume was returned in the form of Ringer's lactate solution ($\sim$ 90 minutes from the onset of bleeding). The rats were then resuscitated with four times the volume of maximal bleed-out with Ringer's lactate for 60 minutes. After resuscitation, the catheters were removed; the incisions were flushed with lidocaine and were closed with sutures. Sham-operated animals underwent all surgical procedures, but neither hemorrhage nor resuscitation was performed.

The animals were sacrificed at 2 hours after the end of resuscitation or sham operation. In the treatment group, E2-BSA (equivalent to 1 mg/kg E2; Sigma, St. Louis, MO), E2 (1 mg/kg; Sigma), or vehicle (BSA) was administered

intravenously during resuscitation. In another group, E2-BSA was administered along with 0.4 mg/kg PKA antagonist H89 (10 minutes before E2-BSA, subcutaneously; Sigma) or H89 alone. This dosage of H-89 has been shown to be capable of reducing the myocardial PKA activity by $\sim$ 55% *in vivo*.²⁵ All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. The present study used measurement at a single time point, ie, at 2 hours after treatment, and thus it remains unclear whether the salutary effects of E2 in attenuating hepatic injury are sustained for longer periods of time, ie, 24 hours after treatment. In this regard, our previous studies have shown that if improvement in cell and organ functions by any pharmacological agent is evident early after treatment, those salutary effects are sustained for prolonged intervals, and they also improve the survival of animals.^{26,27} Thus, although a time point other than 2 hours was not examined in this study, based on our previous studies,^{26,27} it would seem that the salutary effects of E2 would be evident even if one measured those effects at another time point after trauma-hemorrhage and resuscitation.

Measurement of Hepatic Injury

At 2 hours after trauma-hemorrhage or sham operation, blood samples with heparin were obtained and plasma was separated by centrifugation (3000 rpm, 10 minutes), immediately frozen, and stored at -80°C . Hepatic injury was determined by measuring plasma levels of α -GST using a commercially available enzyme immunoassay kit according to the manufacturer's instructions (Biotrin International Ltd., Dublin, UK).

Western Blot Analysis

Protein aliquots were used to determine protein concentration (Bio-Rad DC Protein Assay; Bio-Rad Laboratories, Hercules, CA). Samples were mixed with 4 $\times$ lithium dodecyl sulfate sample buffer and were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred electrophoretically onto nitrocellulose paper. The membranes were immunoblotted with anti-rabbit GPR30 (Abcam Inc., Cambridge, MA), anti-rabbit ER- α (Upstate, Lake Placid, NY), anti-mouse Bcl-2 (Transduction Laboratory, Lexington, KY), and anti-mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Abcam Inc.) antibodies. This was followed by the addition of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody. After the final wash, membranes were probed using enhanced chemiluminescence (Amersham, Piscataway, NJ) and autoradiographed.

Measurement of PKA Activity

Liver tissues or hepatocytes were lysed by probe sonication for 10 seconds. The sample (10 μg of total pro-

Table 1. Effects of E2-BSA and E2 on Plasma α -Glutathione *S*-Transferase (α -GST) Levels after Sham Operation or Trauma-Hemorrhage

Sham		Trauma-hemorrhage (μ g/L)		
Veh	E2-BSA	Veh	E2-BSA	E2
86.7 $\pm$ 7.0	85.8 $\pm$ 8.9	1879.1 $\pm$ 378.2*	760.5 $\pm$ 209.8	536.8 $\pm$ 166.7

Values are mean $\pm$ SEM of six to eight animals in each group. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle.
 * $P < 0.05$ versus sham Veh or sham E2-BSA.

teins) was then assayed immediately, and PKA activity was measured using fluorescent-labeled kemptide²⁸ according to the manufacturer's instructions (Pep Tag assay; Promega, Madison, WI). Phosphorylated kemptide was separated from unphosphorylated substrate by agarose gel electrophoresis. In this assay, enzyme-induced phosphorylation is assessed after electrophoretic separation on agarose gels of the phosphorylated peptide from the nonphosphorylated peptide. Both forms of the peptide were visualized with UV light.

Preparation of Hepatocytes

Normal rat hepatocytes were isolated as previously described.²⁹ In brief, the portal vein was catheterized with an 18-gauge needle, and the liver was perfused with Hanks' balanced salt solution without calcium and magnesium (Life Technologies, Inc., Grand Island, NY) at 37°C and 40 ml/minute for 5 minutes, which was immediately followed by perfusion with 0.04% collagenase IV (Sigma) in Hanks' balanced salt solution containing 1.25 mmol/L CaCl₂ at 37°C and 14 ml/minute for 5 minutes. The liver was then removed and transferred to a Petri dish containing the above-mentioned collagenase IV solution. The liver was minced, incubated for 15 minutes at 37°C, and passed through a sterile mesh stainless steel screen into a beaker containing 10 ml of ice-cold complete WEM (William's E medium, 10% fetal calf serum, 2 mmol/L L-glutamine, 0.1 mg of streptomycin/ml, and 100 U of penicillin/ml) (Life Technologies, Inc.). The cell suspension was washed two times by centrifugation at 50 $\times$ *g* for 3 minutes at 4°C in complete WEM to remove cell debris and nonparenchymal liver cells. The residual cell suspension was washed twice by centrifugation at 800 $\times$ *g* for 10 minutes at 4°C in complete WEM. The final cell pellet was resuspended in complete WEM and plated in a 0.01% collagen I (Sigma)-coated 60-mm plate at a cell density of 2 $\times$ 10⁶ cells/ml. After 3 hours of incubation (37°C, 95% humidity, and 5% CO₂), nonadherent cells were removed, and medium was changed to OPTI-MEM medium (Invitrogen, Carlsbad, CA) for siRNA transfection.

siRNA Synthesis and Transfection

Templates for siRNA synthesis were designed on the basis of the GPR30 and ER- α sequences such that the total length was 21 bases, which comprised the 19 nucleotides after an AA doublet, and the relative %GC of the template was 35 to 55%. The sequences of GPR30 used were anti-sense 5'-AACATGTACAGCAGCGTCTTCCCTGTCTC-3' and sense 5'-AAGAAGACGCTGCTGTACAT-

GCCTGTCTC-3'. The sequences of ER- α used were anti-sense 5'-AAGCTCCTGTTTGCTCCTAACCCCTGTCTC-3' and sense 5'-AAGTTAGGAGCAAACAGGAGCCCTGTCTC-3'. These target sequences were selected by following the Tuschl method.³⁰ The specificity of each siRNA sequence was checked by BLAST search. Synthesis was performed using the Ambion Silencer siRNA Construction Kit (Ambion Inc., Austin, TX). After synthesis, sense and anti-sense oligos were annealed to generate siRNAs. Transfection of 200 nmol/L siRNA in OPTI-MEM medium containing oligofectamine was added into the cells. After 6 hours of incubation, the siRNA/oligofectamine mixtures were replaced by phenol red-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 2 mmol/L L-glutamine, 0.1 mg of streptomycin/ml, 100 U of penicillin/ml, and 10% CD-FBS (charcoal-dextran stripping; Hyclone, Logan, UT). Twenty-four hours after the transfection, cells were treated with either E2-BSA (10 nmol/L E2), E2 (10 nmol/L), or vehicle (BSA). The expression levels of the mRNA and protein of the target molecule were examined at 48 hours after transfection.

Statistical Analysis

All data are presented as mean $\pm$ SEM. One-way analysis of variance and Tukey's test were used for the comparison among groups. Paired comparisons were performed using the one-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Effect of E2-BSA or E2 on Plasma α -GST Levels

As shown in Table 1, no significant difference in plasma α -GST levels was observed between vehicle- and E2-BSA-treated sham groups. Trauma-hemorrhage increased ($P < 0.05$) plasma α -GST levels greater than 20-fold. E2-BSA or E2 treatment attenuated the trauma-hemorrhage-induced increase in plasma α -GST. The levels in E2-BSA-treated rats were similar to those observed in E2-treated animals after trauma-hemorrhage.

Effect of E2-BSA or E2 on GPR30 and ER- α Protein Levels

GPR30 (Figure 1) and ER- α (Figure 2) protein levels did not change between trauma-hemorrhage and sham-operated animals receiving vehicle. E2-BSA or E2 adminis-

Figure 1. Effects of E2-BSA and E2 on protein levels of GPR30 in the liver after sham operation or trauma-hemorrhage. GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from six animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle. * $P < 0.05$ versus sham-Veh, sham-E2-BSA, or trauma-hemorrhage-Veh.

tration after trauma-hemorrhage increased ($P < 0.05$) GPR30 and ER- α protein levels compared with the vehicle-treated trauma-hemorrhage group.

Effect of E2-BSA or E2 on PKA Activity

The result showed that there was no difference in PKA activity (phosphorylated band) between vehicle- and E2-BSA-treated sham groups (Figure 3). After trauma-hemorrhage, PKA activity was decreased ($P < 0.05$) in vehi-

Figure 2. Effects of E2-BSA and E2 on protein levels of ER- α in the liver after sham operation or trauma-hemorrhage. GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from six animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle. * $P < 0.05$ versus sham-Veh, sham-E2-BSA, or trauma-hemorrhage-Veh.

Figure 3. Effects of E2-BSA and E2 on PKA activity in the liver after sham operation or trauma-hemorrhage. The lower band represents phosphorylated fluorescent-labeled PKA substrate (kemptide), and the upper band represents the remaining unphosphorylated peptide. The densitometric values pooled from six animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle. * $P < 0.05$ versus other groups.

cle-treated rats compared with sham-operated animals. E2-BSA or E2 administration after trauma-hemorrhage restored PKA activity to levels similar to those of sham-operated group.

Effect of E2-BSA or E2 on Bcl-2 Protein Levels

There was no significant difference in Bcl-2 levels between vehicle and E2-BSA-treated sham groups (Figure 4). Trauma-hemorrhage decreased ($P < 0.05$) Bcl-2 protein levels in vehicle-treated rats compared with sham-operated animals, which was normalized by E2-BSA or E2 treatment.

Figure 4. Effects of E2-BSA and E2 on protein levels of Bcl-2 in the liver after sham operation or trauma-hemorrhage. GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from six animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle. * $P < 0.05$ versus other groups.

Figure 5. Effect of PKA inhibitor H89 on plasma α -GST levels after sham operation or trauma-hemorrhage. Values are mean $\pm$ SEM of four to six animals in each group. Data were compared by one-way analysis of variance and Tukey's test. Veh, vehicle. * $P < 0.05$ versus all treatments in sham-operated group.

Effect of PKA Inhibitor H89 on Plasma α -GST Levels

To evaluate whether the nongenomic effect of E2 in attenuating hepatic injury after trauma-hemorrhage is mediated via activation of PKA, a group of E2-BSA-treated trauma-hemorrhage rats was pretreated with the PKA inhibitor H89. As shown in Figure 5, trauma-hemorrhage increased ($P < 0.05$) plasma α -GST levels. However, E2-BSA treatment attenuated the trauma-hemorrhage-induced increase in plasma α -GST. Administration of PKA inhibitor H89 prevented the E2-BSA attenuation of α -GST levels after trauma-hemorrhage. No significant difference was observed in α -GST in sham-operated animals treated with E2-BSA and H89 or H89 alone.

Suppression of GPR30 or ER- α Expression by Transfection with GPR30 or ER- α siRNA

To differentiate whether the salutary effect of E2 in attenuating hepatic injury after trauma-hemorrhage is mediated by GPR30 or ER- α , the expression of GPR30 or ER- α was silenced by interfering RNA in hepatocytes isolated from normal rats. Expressions of GPR30 mRNA (Figure 6A) and protein (Figure 6B) in hepatocytes transfected with GPR30 siRNA were lower than that in hepatocytes transfected with negative siRNA in vehicle-treated hepatocytes ($P < 0.05$). Moreover, expressions of ER- α mRNA (Figure 7A) and protein (Figure 7B) in hepatocytes transfected with ER- α siRNA were lower than that in hepatocytes transfected with negative siRNA in vehicle-treated hepatocytes ($P < 0.05$). Expressions of GPR30 and ER- α were increased ($P < 0.05$) in E2-BSA- or E2-treated cells as compared with vehicle-treated hepatocytes, which was prevented by transfection with GPR30 or ER- α siRNA (Figures 6 and 7).

Suppression of GPR30 but Not ER- α Prevented E2-BSA or E2-Up-Regulated PKA Activity and Bcl-2 Protein Levels

Suppression of GPR30 by transfection with GPR30 siRNA decreased ($P < 0.05$) PKA activity (Figure 8A) and Bcl-2

Figure 6. Effect of GPR30 siRNA on GPR30 mRNA expression (A) and protein levels (B) in isolated hepatocytes. Cells were treated with E2-BSA or E2 after transfection with negative or GPR30 siRNA. GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from three animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-tailed Student's *t*-test. Veh, vehicle. * $P < 0.05$ versus negative siRNA; # $P < 0.05$ versus vehicle-treated cell transfected with negative siRNA.

protein levels (Figure 8B) in vehicle-treated hepatocytes as compared with transfection with negative siRNA. PKA activity and Bcl-2 protein levels were increased ($P < 0.05$) in E2-BSA- or E2-treated hepatocytes as compared with vehicle-treated hepatocytes, which were prevented by transfection with GPR30 siRNA. Suppression of ER- α by transfection with ER- α siRNA did not affect PKA activity in vehicle-, E2-BSA-treated, or E2-treated hepatocytes as compared with transfection with negative siRNA (Figure 8C).

Discussion

The findings of this study reveal that plasma α -GST levels were markedly increased, and PKA activity and Bcl-2 levels were significantly decreased at 2 hours after trauma-hemorrhage. Administration of E2-BSA or E2 after trauma-hemorrhage decreased plasma α -GST levels and induced the increase in GPR30 and ER- α levels that was accompanied by the normalization of PKA activity and Bcl-2 levels. Inhibition of PKA in E2-BSA-treated trauma-

Figure 7. Effect of ER- α siRNA on ER- α mRNA expression (A) and protein levels (B) in isolated hepatocytes. Cells were treated with E2-BSA or E2 after transfection with negative or ER- α . GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from three animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-tailed Student's *t*-test. **P* < 0.05 versus negative siRNA; #*P* < 0.05 versus vehicle-treated cell transfected with negative siRNA.

hemorrhage rats abrogated the beneficial effect of E2-BSA in attenuating hepatic injury after trauma-hemorrhage. Furthermore, suppression of GPR30 by transfection with GPR30 siRNA prevented E2-BSA- or E2-up-regulated PKA activity and Bcl-2 levels, whereas suppression of ER- α did not affect PKA activity *in vitro*. These *in vivo* and *in vitro* results collectively suggest that the salutary effect of the nongenomic pathway of E2 on hepatic injury after trauma-hemorrhage is mediated through PKA activation via GPR30 but not ER- α (Figure 9).

It has been reported that E2 acts through soluble intracellular receptors. Once activated, these receptors translocate to the nucleus, where they function as ligand-dependent transcription factors.³¹ This mode of action of two such estrogen-binding receptors, ER- α and ER- β , is well understood.^{32–34} However, the existence of functional ER in the plasma membrane has also been described.^{12,35} It has been suggested that such membrane receptors mediate rapid nongenomic signaling events, widely observed after stimulation of cells and tissues with E2, including the generation of second messengers as

Figure 8. GPR30 and ER- α siRNA on PKA activity (A and C) and Bcl-2 levels (B) in isolated hepatocytes. Cells were treated with E2-BSA or E2 after transfection with siRNA. The lower band represents phosphorylated fluorescent-labeled PKA substrate (kemptide), and the upper band represents the remaining unphosphorylated peptide. GAPDH was used as a loading control. Blots obtained from several experiments were analyzed using densitometry, and the densitometric values pooled from three animals in each group are shown as mean $\pm$ SEM in the bar graph. Data were compared by one-tailed Student's *t*-test. **P* < 0.05 versus negative siRNA; #*P* < 0.05 versus vehicle-treated cell transfected with negative siRNA.

Figure 9. The mechanism of nongenomic effect of E2 on attenuation of hepatic injury after trauma-hemorrhage. The membrane-impermeable BSA-conjugated E2 (E2-BSA) or 17 β -estradiol (E2) attenuates hepatic injury after trauma-hemorrhage through activation of the PKA-dependent anti-apoptotic pathway via GPR30 but not ER- α .

well as the activation of receptor tyrosine kinase and protein-lipid kinase pathways.^{35–37} Novel receptors and novel forms of ER have been postulated to mediate many of these signal transduction events.³⁵ We have previously found that the expression of ER- α is predominant in the liver and that treatment of ER- α but not ER- β agonist attenuated hepatic injury and decreased neutrophil infiltration in the liver after trauma-hemorrhage.^{15,38} However, whether the salutary effect of the nongenomic pathway of E2 on hepatic injury after trauma-hemorrhage is mediated via a novel estrogen-associated receptor or the traditional ER- α remains unknown.

The membrane-located GPR30, an alternative to the classic ERs, is involved in the rapid nongenomic estrogen-mediated signaling through direct binding of E2.¹² It has been reported that E2 activates adenylate cyclase and induces cAMP/PKA signaling pathway via GPR30 in human keratinocytes³⁹ and breast cancer cells.⁴⁰ The PKA-dependent signaling pathway plays a key role in the development of hepatic tolerance to hypoxia/reperfusion⁴¹ and mediates anti-apoptotic effects after ischemic liver injury.¹⁸ Moreover, PKA can phosphorylate the pro-survival transcription factor CREB (cAMP response element-binding protein).¹⁹ This consequently induces Bcl-2 expression and apoptosis resistance in a variety of cell types.^{19,42–44} E2 has also been reported to induce Bcl-2 transcription via cAMP signaling in MCF-7 breast

cancer cells.⁴⁵ In addition, inhibition of GPR30 suppressed the E2-induced increase in cAMP signaling.⁴⁶ The present study demonstrated that administration of E2-BSA or E2 after trauma-hemorrhage attenuated hepatic injury and up-regulated GPR30 and ER- α levels that was accompanied by normalization of downstream signaling of PKA and Bcl-2. These results indicate that administration of E2-BSA and E2 after trauma-hemorrhage are equally effective and suggest that salutary effects of E2 on hepatic injury are mediated via the nongenomic pathway. Furthermore, inhibition of PKA in E2-BSA-treated trauma-hemorrhage rats abolished the beneficial effect of E2-BSA in attenuating hepatic injury. Based on these findings, we propose that E2-BSA mediates its nongenomic effect on protection against hepatic injury via GPR30 or ER- α -signaling pathway in which PKA plays an important role in nongenomic-mediated pathway after trauma-hemorrhage.

GPR30 acts independently of ER- α and ER- β to promote rapid estrogen action.⁴⁰ Overexpression of GPR30 in MDA-MB-231 breast cancer cells restores the activation of adenylyl cyclase by E2,⁴⁰ and suppression of GPR30 expression with anti-sense oligonucleotides or siRNA prevents E2-mediated cAMP-dependent signaling in keratinocytes³⁹ and in SKBR3 breast cancer cells that lack ER- α and ER- β .¹⁷ In the current study, we found that inhibition of GPR30 by transfection with GPR30 siRNA prevented E2-BSA- or E2-mediated increases in PKA activity and Bcl-2 levels in isolated hepatocytes, whereas suppression of ER- α did not affect E2-BSA- or E2-up-regulated PKA activity. Because many studies have reported that PKA regulates survival signaling through Bcl-2,^{19,42–44} we did not measure Bcl-2 levels in the ER- α -suppressed hepatocytes. These results, therefore, suggest that the nongenomic effect of E2 on attenuation of hepatic injury after trauma-hemorrhage is mediated through PKA and Bcl-2 pathway via GPR30 but not ER- α . However, we cannot exclude the possibility that ER- α somehow contributes to E2-BSA- or E2-mediated protection of hepatic injury after trauma-hemorrhage. The activation of PKA pathway independent of ER- α suggests the existence of two separate E2 response mechanisms in E2-mediated attenuation of hepatic injury. Other investigators have reported that ER- α activates anti-apoptotic p38 MAPK and PI3K-Akt pathways.³⁵ Thus, it can be suggested that ER- α also regulates p38 MAPK and PI3K-AKT pathways in E2-mediated protection against hepatic injury after trauma-hemorrhage. Nonetheless, additional studies are needed to clarify the signaling pathway underlying E2 action on ER- α after trauma-hemorrhage.

In summary, our results indicate that administration of E2-BSA or E2 after trauma-hemorrhage attenuated trauma-hemorrhage-induced hepatic injury and increased both GPR30 and ER- α levels and normalized PKA activity and Bcl-2 levels. Additional treatment with a PKA inhibitor in E2-BSA-treated trauma-hemorrhage rats prevented the salutary effect of E2-BSA on hepatic injury. Furthermore, suppression of GPR30 but not ER- α prevented E2-BSA- or E2-mediated up-regulation of PKA activity and Bcl-2 levels *in vitro*. These results suggest that the nongenomic effect of E2 in attenuating hepatic injury after trauma-

hemorrhage is mediated through the PKA-dependent pathway via GPR30. These findings suggest a novel therapeutic strategy for the treatment of hepatic injury after trauma and should be useful in designing innovative therapeutic approaches for the treatment of trauma patients.

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