Flutamide protects against trauma-hemorrhage-induced liver injury via attenuation of the inflammatory response, oxidative stress, and apoptosis

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The onset of resuscitation, and animals were killed 2 h thereafter. Flutamide (25 mg/kg) or vehicle was administered subcutaneously at were also evaluated. Cytokines (TNF-α, IL-6) and chemokines (ke...globally. Effects remain largely unknown. To study this, male C3H/HeN mice were subjected to a midline laparotomy and hemorrhagic shock (35±5 mmHg for ~90 min), followed by resuscitation with Ringer lactate. Flutamide (25 mg/kg) or vehicle was administered subcutaneously at the onset of resuscitation, and animals were killed 2 h thereafter. Hepatic injury was assessed by plasma α-glutathione S-transferase concentration, liver myeloperoxidase activity, and nitrotyrosine formation. Hepatic malondialdehyde and 4-hydroxyalkenals (lipid peroxidation indicators), cellular DNA fragmentation, and the expression of inducible nitric oxide synthase and hypoxia-inducible factor-1α levels were determined by cytometric bead array. The results indicate that flutamide administration after trauma-hemorrhage reduced liver injury, which was associated with decreased levels of α-glutathione S-transferase, myeloperoxidase activity, nitrotyrosine formation, lipid peroxidation, and cytokines/chemokines (systemic, liver tissue, and intracellular cytokines/chemokines). Cellular apoptosis, hepatocyte hypoxia-inducible factor-1α, and inducible nitric oxide synthase expression were also decreased under such conditions. Thus administration of flutamide following trauma-hemorrhage protects against liver injury via reduced inflammation, cellular oxidative stress, and apoptosis.

hypoxia-inducible factor-1α; inflammation; inflammatory cytokines; Kupffer cells

TRAUMA-HEMORRHAGIC SHOCK IS a multifactorial injury that includes hypoxemia and reoxygenation injury, leading to multiple organ dysfunction and failure (26). Sex has been found to be a major determinant in the outcome from trauma-hemorrhage (4). Studies have shown that the female sex steroid estrogen protects organ and immune cell functions following trauma-hemorrhagic shock (2, 10, 11, 13, 25). In contrast, the male hormone testosterone has been found to be related to many of the deleterious effects observed following hemorrhagic shock (2, 10). Studies have shown that depletion of testosterone levels by castration before trauma-hemorrhage prevented the depression in immune and cardiovascular function (24, 32). Since functional androgen receptors (AR) are found in male rat livers (19), testosterone may play an important role in hepatic dysfunction induced by trauma-hemorrhage. Flutamide, a nonsteroidal AR antagonist, has been shown to normalize immune functions in male animals after trauma-hemorrhage (10, 36, 37). Furthermore, studies have shown that treatment of male rats with flutamide following trauma-hemorrhage improved depressed cardiac, hepatic, adrenal, and endothelial functions (2, 10, 14, 36, 37). Flutamide potentially mediates its salutary effects by blocking activation of AR. Studies have also indicated that administration of flutamide in male rodents produced an increase in plasma estrogen levels and estrogen receptor (ER) expression on T-lymphocytes and cardiomyocytes following trauma-hemorrhagic shock (14, 34). Thus multiple mechanisms appear to exist, producing the salutary effects of flutamide. Several studies support the concept that maintenance of hepatic circulation is essential for prevention of hepatocellular dysfunction induced by trauma-hemorrhage (39). Nitric oxide (NO) generated by NO synthases (NOS) plays a role in maintaining hepatic circulation following hemorrhagic shock (36). At least two isoforms of NOS are found in the liver: endothelial NOS (eNOS) and inducible NOS (iNOS). NO generated by eNOS plays a vital physiological role in maintaining appropriate microvascular tone and blood flow and exhibits protective effects (29). In contrast, iNOS activation may promote hepatocyte damage and death following trauma-hemorrhage (41). Furthermore, ischemic injury in the liver affects hepatocytes, Kupffer cells, and endothelial cells. Interestingly, these three cell types show different responses to ischemia: hepatocytes and Kupffer cells are more sensitive to warm ischemia-reperfusion (I/R), and endothelial cells are more sensitive to cold ischemia. In this regard, trauma-hemorrhage shares some similarities with warm I/R (31). The primary insult of trauma-hemorrhage is the induction of cellular hypoxia. The adaptive response to hypoxia is orchestrated through the induction of hypoxia-inducible factor 1 (HIF-1), a heterodimer formed from α- and β-subunits (42). Studies have shown that 17β-estradiol can attenuate the induction of HIF-1α in Hep3B cells by hypoxia (28). However, it remains unknown whether flutamide has any similar effect on HIF-1α induction in hepatocytes. The aim of this study, therefore, was to determine whether flutamide administration following trauma-hemorrhage would attenuate systemic and hepatic inflammation, liver injury, hepatocellular oxidative stress/apoptosis, and protein expression of iNOS and HIF-1α.

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Methods

Animal model of trauma-hemorrhage. After 1-wk acclimatization in the animal facility, male C3H/HeN mice (8–12 wk, 19–25 g, Charles River Laboratories, Wilmington, MA) were fasted overnight, but allowed water ad libitum. The animals were anesthetized using 2% isoflurane (Attane, Minrad, Bethlehem, PA) inhalation, and a 2-cm midline laparotomy was performed to induce soft tissue trauma, which was closed in layers with sutures (Ethilon 6/0, Ethicon, Somerville, NJ). Both femoral arteries and the right femoral vein were cannulated with polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD). Blood pressure was measured via one of the arteries using a blood pressure analyzer (Micro-Med, Louisville, KY). Within 10 min after awakening, animals were rapidly bled through the other arterial catheter to a mean arterial blood pressure (MBP) of 35.0 ± 5.0 mmHg. Hypotension was maintained until the animals could no longer keep an MBP of 35–40 mmHg, unless additional fluid in the form of Ringer lactate (RL) was administered. This time was defined as the trauma-hemorrhage mice.

Sham mice received the same volume of flutamide or vehicle (propanediol; Sigma) in 1 ml of lysis buffer (pH 7.4) containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma) on ice and centrifuged at 12,000 g for 20 min at 4°C. To measure nitrotyrosine, supernatant (100 μl) was analyzed according to the manufacturer’s instruction.

Analysis of cytokines and chemokine levels. The concentration of cytokines (TNF-α, IL-6) and chemokines [keratinocyte-derived chemokine (KC), monocyte chemotactrant protein (MCP)-1] in plasma and liver tissue was determined by flow cytometry using Cytometric Bead Array, according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA). Intracellular cytokines/chemokines were also measured in hepatocytes and Kupffer cells. Briefly, 50 μl of mixed capture beads were incubated with 50 μl sample for 1 h at 25°C, following which 50 μl of mixed phycoerythrin detection reagent was added. After incubation for 1 h at 25°C in the dark, the complexes were washed twice and analyzed using the LSR flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was carried out using the accompanying FACSDiva and FCAP Array software (BD Biosciences). Tissue and intercellular cytokine and chemokine content were normalized to protein concentration.

Western blot analysis of HIF-1α and iNOS. Approximately 0.05 g of snap-frozen liver tissue and hepatocytes were homogenized in 0.5 ml of lysis buffer containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail. The lysates were centrifuged at 14,000 g for 20 min at 4°C, and the protein concentration of supernatant was determined with the Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA). Extracts containing equal amounts of protein were denatured by boiling for 5 min in LDS sample buffer (Invitrogen, Carlsbad, CA). Samples were separated on 4–12% SDS-polyacrylamide gels (Invitrogen) and then electrophoretically transferred onto nitrocellulose membrane (Invitrogen) at 35 V for 60 min. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline-Tween for 1 h at room temperature and then incubated with the primary antibodies against iNOS (1:1000), β-actin (Cell Signaling Technology, Beverly, MA), or HIF-1α (Abcam, Cambridge, MA) (1:1000) overnight at 4°C. After washing with Tris-buffered saline-Tween three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody for 1 h at room temperature and developed by enhanced chemiluminescence (Amersham, Piscataway, NJ). Rabbit monoclonal β-actin was used as the loading control. Quantification of immunoblot was performed with ChemiImager 5500 imaging software (Alpha Innotech, San Leandro, CA), and density values were obtained from six rats/group and were pooled and presented as means ± SE.
Analysis of hepatic lipid peroxidation. Lipid peroxidation is a well-established indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds, including malonaldehyde (MDA) and 4-hydroxyalkenals (HAE), upon decomposition, and the measurement of MDA and HAE has been used as an indicator of lipid peroxidation. The MDA and HAE in liver tissue were determined using the Lipid Peroxidation Microplate Assay Kit (cat. no. FR 22, Oxford Biomedical Research, Oxford, MI). Absorbance of the stable chromophore yielded was measured with ELISA reader and KC4 software (Power wave X, Bio-Tek Instruments, Winooski, VT) at 586 nm. One milligram of total protein was used to standardize the lipid peroxidation formation.

DNA fragmentation in hepatocyte and Kupffer cells. DNA fragmentation (apoptosis) was determined using an assay that is based on measurement of the amount of mono- and oligonucleosomes in the cytoplasmic fraction of tissue extracts. The commercially available assay kit (Roche Diagnostics) was used according to the manufacturer’s instructions.

Statistical analysis. Statistical analysis was performed using SigmaStat software. The data are presented as means ± SE (n = 6 animals/group). *P < 0.05 vs. sham groups or trauma-hemorrhage animals treated with flutamide; #P < 0.05 vs. sham groups.

RESULTS

Plasma α-GST level, hepatic MPO activity, and nitrotyrosine levels. To determine whether flutamide administration affected hepatic injury following trauma-hemorrhage, selected markers of hepatic injury (i.e., plasma α-GST, hepatic MPO
activity, and nitrotyrosine levels) were assessed. As shown in Fig. 1, no significant difference in plasma α-GST level (A), MPO activity (B), and nitrotyrosine formation (C) was found between vehicle- and flutamide-treated sham animals. In contrast, trauma-hemorrhage induced a marked increase in plasma α-GST levels, hepatic MPO activity, and tissue nitrotyrosine protein compared with shams. While treatment of rats with flutamide attenuated the increased hepatic injury markers, plasma α-GST levels remained elevated compared with shams.

**Cytokine and chemokine concentrations.** Following trauma-hemorrhage, cytokine (TNF-α, IL-6) and chemokine (KC and MCP-1) levels in plasma (Fig. 2), hepatic tissue (Fig. 3), hepatocytes (Fig. 4), and Kupffer cells (Fig. 5) were increased compared with shams. Although flutamide administration following trauma-hemorrhage attenuated the increase in cytokine and chemokine content at a systemic, tissue, and cellular level, the values remained significantly higher than those observed in shams. Flutamide administration did not alter cytokine or chemokine content in sham animals.

**Hepatic lipid peroxidation.** The degree of hepatic lipid peroxidation between vehicle- and flutamide-treated sham animals was not statistically different (Fig. 6). However, there was a significant increase in lipid peroxidation (i.e., MDA and HAE levels) of liver tissue following trauma-hemorrhage compared with sham values. Treatment of animals with flutamide prevented the increase in hepatic lipid peroxidation levels following trauma-hemorrhage.

**Cellular DNA fragmentation.** As shown in Fig. 7, there was no difference in DNA fragmentation in hepatocytes (A) and Kupffer cells (B) in sham animals treated with vehicle or flutamide. Following trauma-hemorrhage, hepatocyte and Kupffer cell DNA fragmentation was significantly increased. However,
treatment of rats with flutamide normalized these levels to sham values.

**Hepatocyte iNOS and HIF-1α expression.** The expression of iNOS (Fig. 8A) and HIF-1α (Fig. 8B) in hepatocytes was significantly increased in the trauma-hemorrhage group treated with vehicle compared with shams. Administration of flutamide following trauma-hemorrhage prevented the increase in iNOS and HIF-1α expression. No difference in iNOS and HIF-1α protein levels in sham animals treated with vehicle or flutamide was detectable.

**DISCUSSION**

Hemorrhagic shock is a systemic insult that initiates a cascade of events, leading to organ dysfunction and damage (4, 10, 12). Interestingly, a sex-dimorphic response has been observed in immune and organ functions following hemorrhagic shock (1, 4). More specifically, androgens have been shown to be responsible for producing immune and organ dysfunction, whereas female sex hormones (such as estrogen) exhibit protective properties following trauma-hemorrhage (1, 4). Previous results have shown that flutamide, an AR antagonist, administration following trauma-hemorrhage improves immune and organ functions in males under those conditions (2, 10, 11, 14). Although flutamide produces salutary effects, the precise molecular and cellular mechanism(s) by which flutamide produces those effects remains unclear. Our present discussion will focus on the role of iNOS and HIF-1α in the context of hemorrhagic shock.

**Fig. 5.** Effects of flutamide administration on intracellular TNF-α (A), IL-6 (B), KC (C), and MCP-1 (D) concentration of Kupffer cells after sham operation or trauma-hemorrhage. Values are means ± SE (n = 6 animals/group). *P < 0.05 vs. sham groups or trauma-hemorrhage animals treated with flutamide; #P < 0.05 vs. sham groups.

**Fig. 6.** Effects of flutamide on lipid peroxidation (malonaldehyde (MDA) + 4-hydroxyalkenals (HAE)) of liver tissue after sham operation or trauma-hemorrhage. Values are means ± SE of 6 animals in each group. *P < 0.05 vs. sham groups or trauma-hemorrhage animals treated with flutamide.

**Fig. 7.** Effects of flutamide on cellular DNA fragmentation of hepatocytes (A) and Kupffer cells (B) after sham operation or trauma-hemorrhage. Values are means ± SE (n = 6 animals/group). *P < 0.05 vs. sham groups or trauma-hemorrhage animals treated with flutamide.
A

![Graph A](image)

**Fig. 8.** Effects of flutamide on hepatic expression of inducible nitric oxide synthase (iNOS; A) and hypoxia-inducible factor (HIF-1α) (B) after sham operation or trauma-hemorrhage. Blots obtained from several experiments were analyzed using densitometry, and densitometric values were pooled from 4–6 animals in each group. β-Actin was used as a loading control. Values are means ± SE of 4–6 animals in each group. *P < 0.05 vs. sham groups or trauma-hemorrhage animals treated with flutamide.

results indicate that administration of a single dose of flutamide following hemorrhagic shock attenuated hepatic injury, cytokine/chemokine production, protein expression (HIF-1α and iNOS), lipid peroxidation, oxidative stress, and hepatocyte apoptosis. These findings further corroborate our laboratory’s previous studies, suggesting that flutamide or estrogen treatment prevents the depression of organ function following hemorrhagic shock (10, 11). The signaling events contributing to local hepatocellular damage are diverse and involve complex interactions between hepatocytes, sinusoidal endothelial cells, Kupffer cells, and infiltrating inflammatory cells. Of particular importance is the interaction between activated Kupffer cells and hepatocytes, which is an early key event in the induction of postischemic oxidative stress in the liver (5).

Our laboratory’s previous studies have focused primarily on cytokine/chemokine production by different types of cells following trauma-hemorrhage (9–11). The present study examined not only plasma and hepatic tissue proinflammatory mediators, but also measured intracellular levels (hepatocytes and Kupffer cells) of cytokines and chemokines. Additionally, we examined apoptosis in those cells. These measurements collectively reflect the direct effects of hemorrhage on cellular functions. The findings from our present studies are in agreement with our laboratory’s previous results (15) and indicate that flutamide administration following trauma-hemorrhage attenuates the increase in IL-6, TNF-α, KC, and MCP-1 contents under those conditions. However, their levels remain elevated compared with shams. Along with the elevations in the cytokines and chemokines, we observed an increase in neutrophil infiltration in the liver. Although the precise mechanism responsible for neutrophil recruitment is not known, studies have shown increase in adhesion molecules, and chemokines/chemokines accelerate neutrophil recruitment in the liver, which produces hepatic injury following trauma-hemorrhage (4, 10, 15).

It is possible that an increase in IL-6 and TNF-α, together with chemokines (KC and MCP-1), may help in the recruitment of the neutrophils to the liver. Activated neutrophils may release free radicals (such as NO, superoxide), thereby perpetuating and amplifying the hepatic injury (6). NO derived from iNOS can react with superoxide to form peroxynitrite, an intermediate that, in the presence of hydroxyl radicals, can cause nitration of proteins, predominantly on tyrosine residues. Nitrotyrosine formation can result in the malfunction of those proteins and produce subsequent liver injury (7). Under normal conditions, reactive oxygen species (ROS) are neutralized via diverse antioxidant mechanisms (23). However, in stress conditions, the balance between ROS and antioxidant processes shifts toward the former, resulting in oxidative stress and cellular cytotoxicity (30). Within the liver, the cytotoxic effects of ROS result in nitrosylation of iron-sulfur groups and tyrosine residues, inactivation of the heme group, and lipid peroxidation (40). Furthermore, elevated ROS production is associated with a significant decrease in endogenous antioxidant SOD, catalase, glutathione peroxidase, and increased levels of MDA and HAE (indexes of lipid peroxidation) in the liver during reperfusion (8). Therefore, administration of agents such as flutamide or estrogen, particularly in the early stages of resuscitation, can significantly decrease oxidative stress and subsequent hepatic damage following trauma-hemorrhage.

Recent studies have indicated apoptosis to be a major mode of cell death following an insult to the liver (17). In line with this, different studies have demonstrated apoptotic death in hepatocytes and/or Kupffer cells after cold or warm ischemia in the rat liver (35). Trauma-hemorrhagic shock shares some similarities with warm I/R in organ and cell injury. During reperfusion, TNF-α and other mediators activate many of the proteins involved in apoptosis, along with mitochondria cytochrome c release into the cytoplasm, which can lead to DNA destruction and cell death (27). Our results demonstrated that flutamide administration following trauma-hemorrhage attenuates trauma-hemorrhage-induced DNA fragmentation in both hepatocytes and Kupffer cells. Although the precise mechanism(s) of the salutary effect of flutamide on hepatocellular apoptosis remains unclear, previous findings from our laboratory provide evidence that flutamide administration following trauma-hemorrhage modulated cardiac ER protein expression and increased plasma estrogen levels in male rats (10, 14). Another study indicated that AR antagonism with flutamide led to an upregulation of stromal ER expression in human prostate.
Estrogen has the ability to markedly reduce activation of caspase-3-like activity and decrease DNA strand breaks in the nuclei of cardiomyocytes (27). Our previous results indicated that estrogen and/or flutamide improves immune and other organ functions following trauma-hemorrhage. As trauma-hemorrhage produces hypoxic insult, it is likely that hypoxia becomes the major cause of organ dysfunction under those conditions (2, 4, 10, 11, 14, 15). It has been suggested that HIF-1α is constantly produced, but is rapidly degraded under normoxia. However, under hypoxic conditions, the level of HIF-1α is increased. We found that the increase in HIF-1α was attenuated after flutamide administration following trauma-hemorrhage. Thus flutamide may also mediate its salutary effects by protecting organs from hypoxic insult. The results also showed that, following iNOS, protein was increased and so was HIF-1α following trauma-hemorrhage. Although these results suggest that there might be a relationship between iNOS and HIF-1α, we did not measure NO production, and thus it remains to be established whether or not there is a direct correlation between iNOS and HIF-1α. Nonetheless, it appears that flutamide administration may block AR as well as upregulate ER, and both events should be helpful in protection against trauma-hemorrhage-induced tissue injury and cellular apoptosis.

Plutamide can cause hepatotoxic effects in some patients, and it could be argued that the long-term use of flutamide might induce hepatic toxicity (33). However, a prospective study in a large number of patients has shown that the incidence of flutamide-induced liver toxicity is quite low (22). Another in vitro study has demonstrated that high concentration of flutamide attenuates the activation of neutrophils and decreases the neutrophil-mediated injury in isolated hepatocytes (38). Nonetheless, our findings suggest that administration of a single dose (25 mg/kg intraperitoneally) of flutamide as an adjunct to resuscitation following trauma-hemorrhage is effective in attenuating organ injury and cytokine production under those conditions (10).

Although the present results indicate salutary effects of flutamide at a single time point, 2 h after the end of resuscitation, it remains unclear whether these effects are sustained for longer periods of time after treatment. However, previous studies have shown that the salutary effects of such agents as estradiol, flutamide, and dehydroepiandrosterone are sustained for prolonged intervals following their administration (4, 16, 18, 20). Furthermore, if the improvement in organ functions under those conditions (10).

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**GRANTS**

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**REFERENCES**


