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ESTROGEN SUPPRESSES CARDIAC IL-6 AFTER TRAUMA- HEMORRHAGE VIA A HYPOXIA-INDUCIBLE FACTOR 1 α - MEDIATED PATHWAY

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Abstract

Cardiac dysfunction is a major concern after trauma-hemorrhage, and increased IL-6 is one of the underlying causes for producing the dysfunction. Studies have shown that administration of 17 β -estradiol (estrogen) after trauma-hemorrhage normalized cardiac IL-6 levels and restored cardiac functions under those conditions. Because hypoxia-inducible factor (HIF) 1 α is expressed during hypoxia and cellular stress and up-regulates the expression of IL-6, we hypothesized that HIF-1 α induces the increased cardiac IL-6 after trauma-hemorrhage and that estrogen suppresses this induction. To examine this, C3H/HeN mice were subjected to trauma-hemorrhage or sham operation. Vehicle, the HIF- α inhibitor YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, a novel activator of platelet guanylate cyclase], or estrogen was administered to trauma-hemorrhage and sham groups during resuscitation. Mice were killed at 2 h after resuscitation, and cardiac IL-6, HIF-1 α , and nuclear factor (NF) κ B activities were measured. IL-6, NF- κ B, and HIF-1 α levels were markedly elevated after trauma-hemorrhage; all of these parameters were normalized by estrogen as well as YC-1 administration after trauma-hemorrhage. Because elevated IL-6 levels after trauma-hemorrhage were decreased with YC-1 treatment, it indicates that IL-6 expression in cardiomyocytes is induced via HIF-1 α . In addition, estrogen decreased the elevated HIF-1 α , NF- κ B, and IL-6 levels after trauma-hemorrhage. These results indicate that the beneficial effects of estrogen on cardiac function after trauma-hemorrhage seem to be mediated by the inhibition of HIF-1 α expression and activity.

Keywords

Cardiac function; HIF-1 α inhibitor; NF- κ B; cytokine

INTRODUCTION

Numerous studies have shown that traumatic injury, with or without severe blood loss, induces immune/organ dysfunction which increases susceptibility to sepsis and multiple organ failure (1–9). Such conditions account for the high mortality of those patients and remain the leading cause of death despite the advances in medical management over the past decades (10). Trauma-hemorrhage exerts a negative impact on the immune system as it induces alterations in many immune functions including cytokine production (11–14). Several cell types show

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enhanced proinflammatory cytokine production, including cardiomyocytes, which have recently been identified to contribute to increased IL-6 production after trauma-hemorrhage (15–18).

Trauma-hemorrhage not only contributes to changes in immune function, but also produces an adverse effect on organ performance such as hepatic, renal, and cardiac functions. In rodent models, the association between increased cardiac IL-6 levels after burn, sepsis, or trauma-hemorrhage and cardiac dysfunction combined with poor outcome has been identified (15, 18). Furthermore, blockade of IL-6 activity after trauma-hemorrhage counteracts IL-6 effects, thus leading to normalization of cardiac function (19). In addition, Yang et al. (19) demonstrated an increase of cardiac nuclear factor (NF) κ B, which is related to proinflammatory cytokine transcription and release by cardiomyocytes (20,21). Therefore, an increase in IL-6 per se and/or NF- κ B activation may induce higher cardiac IL-6 levels and contribute to the loss of normal cardiac function after trauma-hemorrhage.

Studies have shown that administration of 17 β -estradiol (estrogen) in male rodents after trauma-hemorrhage normalized the enhanced proinflammatory cytokine release from several cell types such as Kupffer cells and cardiomyocytes (15). Estrogen treatment after trauma-hemorrhage was also able to restore cardiac function, presumably because of the normalization of IL-6 release (15). Nonetheless, despite ongoing efforts to clarify the estrogen's actions after trauma-hemorrhage, the precise mechanism responsible for producing the salutary effects of estrogen under those conditions remains unclear.

Hypoxia-inducible factor (HIF) 1 is a protein frequently referred to as the O₂-sensing transcriptional factor. It is ubiquitously expressed and presents as a heterodimeric protein which consists of HIF-1 α and HIF-1 β subunits. In the presence of O₂, HIF-1 α is hydroxylated and quickly degraded. Under hypoxic conditions, HIF-1 α is stabilized, binds to HIF-1 β , and translocates into the nucleus, where it induces the transcription of various hypoxia-related elements (HREs). These include the expression of NF- κ B and inflammatory cytokines such as IL-6 (22,23). Studies have also shown an induction of HIF-1 under nonhypoxic conditions, such as stimulation with LPS, hormones, cytokines, and after T-cell receptor activation (24).

Because trauma-hemorrhage encompasses several stimuli that are capable of inducing HIF-1 production and activation, and HIF-1 may be responsible for releasing the proinflammatory cytokines such as IL-6, either directly or through activation of NF- κ B, we hypothesized that the trauma-hemorrhage-induced increase in cardiac IL-6 levels is mediated via activation of the HIF-1 α pathway. Furthermore, because estrogen administration after trauma-hemorrhage normalizes cardiomyocyte IL-6 release under those conditions, we also hypothesized that the beneficial effects of estrogen after trauma-hemorrhage are mediated via inhibition of HIF-1 α expression.

MATERIALS AND METHODS

Animals and experimental groups

All animal studies were carried out in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male C3H/HeN mice, 8 to 12 weeks old and weighing 19 to 23 g, were obtained from Charles River Laboratories (Wilmington, Mass). Mice were randomly assigned to 1 of the 2 procedural groups (trauma-hemorrhage or sham) and to 1 of the 3 treatment groups (vehicle, estrogen, or YC-1). The dosages of the experimental drugs were as calculated to result in approximately 100 μ L/mouse and prepared as follows: estrogen (Sigma, St Louis, Mo) 1 mg/kg body weight (BW) dissolved in normal saline; YC-1 (AG Scientific, San Diego, Calif) 5 mg/kg BW dissolved in 1 part ethanol, 1 part Cremophor EL

(Fluka, St Louis, Mo), and 4 parts normal saline. To block the expression of HIF-1 α before the onset of trauma-hemorrhage, each corresponding mouse received an i.v. injection of the YC-1 or vehicle 30 min before trauma-hemorrhage or sham procedure. To inhibit HIF-1, various authors have used dosages of YC-1 ranging from 2 to 30 mg/kg BW. We chose the dose of 5 mg/kg BW based on the work of Hsiao et al. (25). Estrogen was given at the beginning of resuscitation.

Trauma-hemorrhage procedure

Mice in the trauma-hemorrhage groups were anesthetized with isoflurane (Minrad, Bethlehem, Pa) and restrained in a supine position. A 2-cm midline laparotomy was performed, which was closed in 2 layers with sutures (Ethilon 6/0; Ethicon, Somerville, NJ) and moistened with bupivacaine. Both femoral arteries and a femoral vein were cannulated with 0.011-inch polyethylene tubing (Becton Dickinson, Sparks, Md). Blood pressure was measured via 1 of the arterial lines using a blood pressure analyzer (Micro-Med, Louisville, Ky); the other arterial catheter was used to withdraw blood. Within 10 min after awakening, animals were rapidly bled to a mean arterial blood pressure of 35 ± 5 mmHg, which was maintained for 90 min. At the end of the procedure, the animals were resuscitated with 4 times the shed blood volume using Ringer's lactate through the venous line. The catheters were then removed and the vessels ligated; the incisions were moistened with bupivacaine and closed with sutures (Ethilon 6/0; Ethicon). Sham-operated animals underwent the same surgical procedures, but were neither hemorrhaged nor resuscitated.

Tissue harvesting, sample preparation

Two hours after the end of resuscitation, the animals were killed, and the hearts were removed. The hearts were rinsed with phosphate-buffered saline to remove blood, separated into 2 parts and snap frozen in liquid nitrogen for mRNA or protein analysis.

Sample preparation was carried out on ice. For protein analysis, the heart was suspended in 500 μ L phosphate-buffered saline containing protease inhibitors (Complete Mini Kit; Roche, Basel, Switzerland). The tissue was then minced and sonicated to extract proteins. After centrifugation at 16,000g, clarified supernatants were collected and stored for further analysis. mRNA analysis was carried out according to the manufacturer's guidelines. Briefly, the heart was lysed in 800- μ L TriZOL reagent (Invitrogen, Carlsbad, Calif), and mRNA was extracted and purified using the Mini-to-Midi RNA Kit (Invitrogen). Subsequently, cDNA was prepared using the Superscript-III Kit (Invitrogen).

Analysis of mRNA-levels

Reverse transcriptase-polymerase chain reaction was carried out on an HT7900RT real-time polymerase chain reaction machine (Applied Biosystems, Foster City, Calif) using a 20- μ L reaction containing 2.5- μ L cDNA. Primers and probes for IL-6 and HIF-1 α were obtained, prepared, and premixed from Applied Biosystems (TaqMan Gene Expression Assays; Applied Biosystems). Color development was recorded, and analyses were carried out using SDS Software 2.2.1 (Applied Biosystems).

Analysis of protein levels

The levels of IL-6 in whole-heart protein extracts were determined using the cytometric bead array according to the manufacturer's instructions (FlexSet; BD Biosciences, San Jose, Carlsbad, Calif). Fluorescence intensity was recorded using a BD LSRII flow cytometer, and cytokine levels were calculated using FCAP-Array software (BD Biosciences).

Hypoxia-inducible factor 1 α exists in 2 forms. The total HIF-1 α content represents the amount of HIF-1 α produced and stabilized in the cell. The active HIF-1 α represents the amount of HIF-1 α which is bound to its HIF-1 β counterpart and forms an active form which is capable of binding to specific DNA sequences referred to as HRE. To determine total HIF-1 α and active HIF-1 α , 2 enzyme-linked immunosorbent assay (ELISA)-based assays were carried out. Total HIF-1 α was analyzed using the Total HIF-1 α Kit (R&D Systems, Minneapolis, Minn). This kit uses an HIF-1 α antibody to evaluate HIF-1 α levels. Active HIF-1 α was analyzed using the active HIF-1 α kit (R&D Systems). This kit uses HRE-specific nucleotide sequences to evaluate the levels of HIF-1 α capable of binding to DNA HRE sequences. All testing was performed according to the manufacturer's guidelines. The same test technique applies to NF- κ B. Activated NF- κ B binds to specific nucleotide sequences, which are immobilized to an ELISA plate. Thus, using an ELISA-based test setup for p65 (Active Motif, Carlsbad, Calif), the levels of NF- κ B were determined. Color development was recorded for 450 nm with a 655-nm correction using a PowerWaveX ELISA reader (Bio-Tek Instruments, Winooski, Vt).

Total protein levels were determined using BioRad total protein reagents (BioRad). Cytokine, HIF-1 α , and NF- κ B levels were normalized to total protein content.

Statistical analysis

All statistical analyses were calculated using SigmaStat 2.03. Significance ($P < 0.05$) was determined using 1-way ANOVA and Tukey test.

RESULTS

Protein levels of IL-6, NF- κ B, and HIF-1 α

Cardiac IL-6 levels increased dramatically after trauma-hemorrhage (Fig. 1). Although sham-animal IL-6 levels were near the lower detection limits, a nearly 40-fold increase in cardiac IL-6 levels was observed after trauma-hemorrhage. Treatment with either estrogen or YC-1 significantly decreased IL-6 levels in the trauma-hemorrhage group to a similar degree. However, the trauma-hemorrhage mice treated with estrogen or YC-1 still had higher levels of cardiac IL-6 compared with their sham counterparts.

Similar to IL-6, protein levels of total HIF-1 α (Fig. 2A) and active HIF-1 α (Fig. 2B) were increased after trauma-hemorrhage. Total HIF-1 α increased approximately 3-fold, whereas active HIF-1 α increased by approximately 60%. Treatment of mice with either estrogen or YC-1 normalized HIF-1 α levels to levels observed in sham counterparts. Although the amount of active HIF-1 α remained higher in YC-1-treated animals compared with untreated shams, the values were not significantly different from sham YC-1-treated animals. After trauma-hemorrhage, an almost 2-fold induction in NF- κ B activity was observed (Fig. 3), which was also normalized to sham levels after estrogen or YC-1 treatment.

mRNA expression of IL-6 and HIF-1 α

No differences were observed in cardiac IL-6 mRNA expression in all sham groups (vehicle/estrogen/YC-1). In contrast, IL-6 mRNA expression was significantly increased after trauma-hemorrhage. Treatment of trauma-hemorrhage mice with estrogen or YC-1 normalized the expression of IL-6 mRNA to sham levels (Fig. 4A). The mRNA expression of HIF-1 α by cardiac tissue was markedly up-regulated in both trauma-hemorrhage groups treated with vehicle and YC-1 compared with their respective shams. Trauma-hemorrhage animals treated with estrogen showed no increase in HIF-1 α mRNA expression after trauma-hemorrhage (Fig. 4B).

DISCUSSION

Traumatic injury with hemorrhage remains a common cause of mortality. Despite advances in medical treatment and improvements in intensive care unit therapy, patients still experience a deranged immunoinflammatory response and organ dysfunction under such conditions. These derangements can lead to higher susceptibility to sepsis, subsequent multiple organ failure, and death (11,26–28). Ongoing efforts have been directed at understanding the mechanistic underpinnings for the development of organ dysfunction after trauma-hemorrhage. With regard to the heart, IL-6 has been shown to be an important factor responsible for producing postinjury cardiac dysfunction (15). Cardiomyocytes exposed to such insults release high amounts of IL-6 (15); however, cardiac function was normalized in rats subjected to trauma-hemorrhage which were administered IL-6–neutralizing antibodies (19). Nonetheless, because IL-6 was demonstrated to be harmful and neutralization of IL-6 proved beneficial, the underlying mechanisms for aberrant cardiac IL-6 production remain unclear. Our findings here suggest that up-regulation of HIF-1 α in cardiac tissue is critical for the increased IL-6 production by the heart.

Hypoxia-inducible factor 1 is a heterodimeric protein which consists of the subunits HIF-1 α and HIF-1 β . It was originally described as the oxygen sensor of the cell. This concept relates to the HIF-1 α subunit, which in the presence of oxygen is prolyl hydroxylated by the HIF prolyl-hydroxylase. The “marked” HIF-1 α is quickly degraded by the E3 ubiquitin ligase complex and removed by proteasome. Accordingly, under hypoxic conditions, HIF-1 α is stabilized and translocates to the nucleus where it can bind to HIF-related elements. Several genes have been identified as being up-regulated by HIF-1, which include cell survival and cell function under hypoxic conditions (29,30). However, recent studies have shown a much wider regulatory role for HIF-1, suggesting the necessity of HIF-1 for several intracellular functions not only in the hypoxic environment but also during cellular stress under normoxic conditions and even for normal cell function. Importantly, among the different genes up-regulated by HIF-1 is the induction of NF- κ B (31,32). Nuclear factor κ B is a transcriptional factor, which has a wide range of effects, including regulating the release of various pro-inflammatory cytokines (23). Studies have demonstrated that the expression of IL-6 is directly linked to the activation of the NF- κ B pathway. Furthermore, the beneficial effects of estrogen on the immunoinflammatory responses seem to be related in part to the regulation of the NF- κ B pathway (33).

In this study, we demonstrate the connection between HIF-1 α , IL-6, and estrogen. Mice subjected to trauma-hemorrhage showed a significant increase in IL-6 expression at the level of mRNA and protein. This release of IL-6 by cardiomyocytes is believed to be responsible for producing cardiac dysfunction that occurs after trauma-hemorrhage. Along with the increase in IL-6, an increase in HIF-1 α was observed, both total protein quantity and active HIF-1 α capable of binding to the HREs. Similarly, HIF-1 α mRNA was also increased. Inhibition of HIF-1 with YC-1 was effective in suppressing both the trauma-hemorrhage–mediated increase in HIF-1 α and IL-6, suggesting a causative relationship between HIF-1 α activation and IL-6 expression after trauma-hemorrhage. Although we used YC-1 as an inhibitor of HIF-1 α in our experiments, it was originally developed as an anticlotting drug which induced soluble guanylyl cyclase (sGC), thereby increasing cyclic guanosine 3'5' monophosphate levels. Several studies using YC-1 for this purpose showed confusing results; however, eventually, a powerful inhibitory effect on HIF-1 α was identified. The exact mechanism of HIF-1 α inhibition by YC-1 is still unclear, but studies have provided evidence for an intracellular degradation of stabilized HIF-1 α (34). Although there are other drugs that inhibit HIF-1 α , they either have serious side effects (e.g., inhibiting topoisomerase I) or their mechanisms of inhibition remain completely unknown (e.g., PX-478). Accordingly, YC-1 is now broadly used as a potent HIF-1 α inhibitor (35). Importantly, in our study, IL-6 was

decreased in YC-1–treated mice; however, after an increase in cyclic guanosine 3'5' monophosphate after sGC induction, one would expect an increase in IL-6. In this regard, studies have shown that HIF-1 α increases NF- κ B, and NF- κ B subsequently increases IL-6 along with other cytokines (19,22,23).

Previous studies have clearly demonstrated sex differences in immune function, susceptibility to sepsis, and organ function after trauma-hemorrhage. Those studies have shown that females have an advantage over their male counterparts in tolerating the deleterious consequences of trauma-hemorrhage (11). Ongoing research has revealed that the female hormone estrogen is central to this beneficial effect and that treatment of trauma-hemorrhaged and septic male animals with estrogen provides a protective effect on a number of immunological, inflammatory, and physiological levels (36). Interestingly, administration of estrogen resulted in a decrease in both HIF-1 α and IL-6 levels. Because HIF-1 α induces NF- κ B, NF- κ B increases IL-6, and estrogen decreases NF- κ B levels, we propose this as the underlying link between the observed expression of HIF-1 α after trauma-hemorrhage, the subsequent increase in IL-6 and the beneficial effects of estrogen in decreasing HIF-1 α which in turn decreases NF- κ B and consequently IL-6.

In this study, we investigated only a single time point after trauma-hemorrhage and resuscitation. In principle, it could be argued that investigating a single time point is not sufficient to conclude a potential role of HIF-1 α in the observed effects on NF- κ B and IL-6 after trauma-hemorrhage. However, studies have shown that if a pharmacological agent was effective in improving cell and organ function early after the insult, that agent also improved cell and organ function at later time points. Furthermore, it also improved the survival of animals subjected to trauma-hemorrhage and induction of subsequent sepsis (11,37,38). Thus, based on those studies, it would seem that the salutary effects of estrogen on HIF-1 α expression after 2 h would persist even if one examined those effects at 24 or 48 h after estrogen treatment.

In summary, our study shows salutary effects of YC-1 in trauma-hemorrhage because its administration was able to normalize cardiac IL-6 levels which are considered to be responsible for producing cardiac dysfunction after trauma-hemorrhage. Thus, YC-1 may be a useful adjunct for the treatment of organ and immune dysfunction because it produces alterations in proinflammatory pathways. A potential limitation of its use, however, may be the possible anticoagulation effects because YC-1 was developed as an anticoagulant agent. Although no increased rate of bleeding was observed in our experiments, caution must be exercised in the use of this drug in the clinical setting of severe hemorrhage, for example, polytrauma. Nonetheless, drugs for anticoagulation such as drotrecogin alfa are used in patients with sepsis and multiple organ failure, and a potential anticoagulatory effect of YC-1 may thus be beneficial in those settings. In addition, we found that estrogen exerts its beneficial effects through HIF-1 α pathway. Nonetheless, further studies are needed to investigate any potential synergistic effects of YC-1 and estrogen.

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Fig. 1. Cardiac IL-6 levels, normalized per milligram of total protein

Data are presented as mean $\pm$ SEM. $n = 5-8$ animals/group; $*P < 0.05$ versus other groups and $\#P < 0.05$ versus sham as determined by 1-way ANOVA and Tukey test. T-H indicates trauma-hemorrhage; E2, estrogen.

Fig. 2. Cardiac HIF-1 α levels

A, Total HIF-1 α content, normalized per milligram of total protein; B shows active HIF-1 α content, normalized to optical density of total protein. Data are presented as mean $\pm$ SEM. $n = 4-8$ animals/group; * $P < 0.05$ as determined by 1-way ANOVA and Tukey test.

Fig. 3. Cardiac NF-κB activation

Values normalized per milligram of total protein and calculated as fold increase compared with sham. Data are presented as mean $\pm$ SEM. $n = 4-8$ animals/group; $*P < 0.05$ as determined by 1-way ANOVA and Tukey test.

Fig. 4. Cardiac mRNA-levels of (A) IL-6 or (B) HIF-1 α

Values are presented as $\Delta\Delta C_t$ of target mRNA to control mRNA and normalized to sham levels. Data are presented as mean $\pm$ SEM. $n = 6$ animals/group; * $P < 0.05$ versus other groups and # $P < 0.05$ versus sham and trauma-hemorrhage (T-H)-E2 as determined by 1-way ANOVA and Tukey test; ** $P < 0.05$ versus sham as determined by 1-way ANOVA and Tukey test.