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17 β -ESTRADIOL ATTENUATES CYTOKINE-INDUCED NITRIC OXIDE PRODUCTION IN RAT HEPATOCYTE

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Abstract

Objective—Nitric Oxide (NO) regulation during shock and sepsis is complex. NO production by endothelial NO synthase (eNOS) maintains microvascular perfusion and prevents shock-induced organ injury. However, overproduction of NO by inducible NO synthase (iNOS) contributes to liver dysfunction after shock, and is associated with increased tissue damage and mortality. Estrogen improves organ function and outcome after shock and sepsis but the mechanism is unknown. We hypothesized that 17 β -Estradiol would improve organ function by regulating the production of hepatocyte nitric oxide.

Methods—Isolated rat hepatocytes were stimulated in-vitro with pro-inflammatory cytokines to induce NO synthesis with or without estrogen. Nitrite was detected after 24hours. INOS protein was determined using western blot.

Results—Cytokine stimulation increased nitrite and iNOS protein in a dose-dependent manner. The cytokine-induced nitrite increase was significantly decreased by estrogen. Inducible nitric oxide synthase expression was also diminished in cultures with the higher doses of estrogen.

Conclusion—17 β -Estradiol decreases cytokine-stimulated iNOS expression and NO production. Downregulation of iNOS expression may account for the beneficial role of estrogens in models of sepsis and shock.

BACKGROUND

The number of new cases of severe sepsis has been on the rise over the last two decades^{1,2}. As a result, mortality remains high, at about 30% for the most severe cases despite advances in critical care management². Death from complications of sepsis is still the leading cause of mortality in the surgical ICU³. Gender has been reported to influence mortality after sepsis. Weinstein and his colleagues were able to demonstrate a statistically significant difference in mortality between male and female patients hospitalized with sepsis⁴. Several studies also indicate that women may be immunologically better positioned to tolerate septic challenges compared to men^{5, 6}. This has been largely attributed to the differential cell-mediated and humoral immune reactivity to infection between both sexes^{7, 8}. Clinical studies demonstrate a more favorable disposition in women of reproductive age after severe sepsis and shock. There is a decreased incidence of severe sepsis of 36% in women compared to 64% in men⁹, women also have a 14% survival advantage after severe sepsis, and are better compensated hemodynamically after shock^{10, 11}. These age groups of women have a reduced length of ICU and hospital stay^{12, 13}, and are 40% less likely to progress onto multiple organ dysfunction syndrome (MODS) after sepsis and shock^{7, 13, 14}. Trials also show that men are

30% more likely to develop sepsis compared to women². Additionally, after trauma, women with a higher injury severity score have been demonstrated to fare better than their male counterparts¹⁴. Also worthy of note is that women have been shown to have better outcomes in other disease entities such as cirrhosis, hepatocellular cancers and after cerebrovascular events.

Laboratory findings have shown that female rats tolerate sepsis and trauma better than male rats but the specific mechanisms responsible for these findings are still being investigated. A persistent observation has been that these advantages seen in female rats are more pronounced during the estrogenic phase of their reproductive cycles when estrogen in circulation peaks^{15, 16}. Some notable findings include that, female rats have a survival advantage after caecal ligation and puncture¹⁷, an immune advantage has also been reported in female mice in hemorrhage - resuscitation models¹⁸. There is however, loss of any survival advantage after ovariectomy in these female rats^{15, 19}. Additionally, in trauma-hemorrhage models, estrogen administration to rats improved cardiac and hepatocellular function and protected against pulmonary injury^{16, 19 - 22}.

Excessive nitric oxide (NO) production during sepsis plays a significant role in the pathophysiologic sequelae of sepsis and hemorrhagic shock²³. The production of NO by inducible nitric oxide synthase (iNOS) has deleterious effects on tissues and organs in sepsis, shock and other inflammatory conditions^{24, 25}. Estrogen administration improves shock induced hepatocellular injury and has been suggested to alter hepatic dysfunction through modulation of NO production²⁶ but whether estrogen regulates iNOS production by hepatocytes has not been proven. We hypothesized that 17 β -Estradiol will attenuate pro-inflammatory cytokine-induced nitric oxide production in rat hepatocyte.

METHODS

Animals and Hepatocyte Isolation

Adult male Sprague Dawley rats weighing 200 to 300 g (Harlan Sprague-Dawley Madison, WI) were used in all experiments. All experiments were performed in adherence to National Institutes of Guide for the Care and Use of Laboratory Animals and approved by University of Louisville Institutional Animal Care and Use Committee. Hepatocytes were harvested using a modification of the Seglen in-situ perfusion technique, as previously described^{27,28}. Briefly, the liver was perfused with a 0.05% collagenase solution (Type IV, Sigma, St. Louis, MO) for 20 minutes. The liver capsule was dissected out after perfusion and the parenchyma homogenized to produce a cell suspension. Hepatocytes were then separated from non-parenchymal cells by repeated centrifugation, filtering and resuspension. Cells were enumerated and assessed by trypan blue exclusion and ensured to be consistently more than 90% viable.

Hepatocyte Culture and Treatment

Hepatocyte were plated at a concentration of 1×10^6 cells/mL in 6-well plates (Corning) in Williams medium E (0.24mmol/L L-arginine) supplemented with 15 mmol/L HEPES, 2 mmol/L L-glutamine, 10^5 U/L penicillin, 100mg/L streptomycin (Life Technologies, Grand Island NY), and 10% heat-inactivated, low endotoxin calf serum (Hyclone Laboratories, Logan, UT) . The cells were incubated at 37°C in a 5% CO₂ incubator, medium was replaced 3 hours after plating and the cells allowed to recover overnight. After an 18-hour incubation, the medium was aspirated and the cells were treated with a cytokine mix (cm) consisting of recombinant rat interferon gamma (rrIFN γ) at 100U/mL and recombinant human interleukin 1 beta (rrIL-1b) at 200U/mL (Life Technologies, Grand Island NY) in serum-free antibiotic-free Williams medium E. Where indicated, different concentrations of

17 β -estradiol (Sigma, St. Louis, MO) were added to the culture medium at the same time. Dimethyl sulfoxide (DMSO) or ethanol (Sigma, St. Louis, MO) was used as the vehicle control.

In further experiments, cells were separately treated with the estrogen alpha receptor (ER- α) agonist, propyl pyrazole triol (PPT, 10 μ M) and ER- α antagonist methyl-piperidino-pyrazole (MMP, 10 μ M) purchased from Tocris Bioscience, Ellisville, MO.

Nitrite (NO⁻²) Determination

Twenty-four hours after exposure of hepatocytes to recombinant cytokines and estrogen, production of NO⁻², which is a stable and measurable metabolite of NO was assessed. The concentration of NO⁻² in culture supernatant was measured using an automated colorimetric procedure based on the Griess reaction.

Western blot analysis

Western blots were performed to measure hepatocyte iNOS protein levels. Samples were separated using the BioRad Mini-PROTEAN Tetra electrophoresis system on 8% polyacrylamide gels prepared in the lab with reagents also from BioRad (Hercules, CA). Proteins were transferred onto nitrocellulose membranes. The membranes were incubated with iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz CA) overnight at 4°C followed by horseradish peroxidase-conjugated secondary anti-rabbit antibody for 1 hour at room temperature. Membranes were stripped and then reblotted with β -actin using mouse monoclonal antibody (Millipore, Billerica, MA) to confirm equal protein loading in each lane. Signal densities were evaluated by NIH ImageJ medical imaging software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, <http://imagej.nih.gov/ij/>). Protein levels were quantified by densitometric evaluation and were corrected to the corresponding β -actin densities.

INOS mRNA Expression

INOS mRNA was determined by real time PCR using Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City CA). Total RNA was isolated from hepatocytes using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was generated from the total RNA samples using TaqMan Reverse Transcription Reagents (Applied Biosystems). TaqMan Gene Expression Assays (Applied Biosystems) for iNOS genes was purchased as probe and primer sets. Samples were tested in triplicate for each different set of experiment, and the average values were used for quantification. 18S rRNA was used as endogenous control. The comparative CT method ($\Delta\Delta$ CT, cycle threshold) was used for quantification of gene expression. Analysis was performed using the StepOne software (Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

Each set of experiments was performed separately and independently using livers from at least three different rats, with each rat constituting a separate experiment. All conditions in each of these separate rats were tested in triplicates. Data are presented as mean + SEM. Statistical difference between treatment groups was determined using two-tailed student's t-test. Level of statistical significant difference was set at $p < 0.05$.

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RESULTS

To evaluate the effect of estrogen on hepatocyte NO production, cultured hepatocytes were stimulated to produce NO in the presence of increasing concentrations of 17 β -Estradiol (E2). Simultaneous incubation with E2 inhibited cytokine-stimulated hepatocyte NO production in a dose dependent manner (Fig. 1A). The MTT assay was performed to measure cell viability. This demonstrated that there were no cytotoxic effects of E2 on the hepatocytes, with no significant difference between groups treated with E2 and controls (data not shown). Western blot analysis of iNOS protein demonstrated that E2 dose-dependently suppressed cytokine-induced iNOS protein expression (Fig. 1B). Significant attenuation was observed at the 25 μ M dose of E2.

Hepatocytes are normally cultured in media containing 5-10% low endotoxin calf serum but calf serum may contain endogenous hormones. Therefore, we compared hepatocytes cultured in serum-containing and serum-free media for their ability to produce NO in the presence of E2 and found that smaller doses of E2 were required to produce a comparable lowering of NO production when hepatocytes were cultured in serum-free media (Fig. 2). Phenols have also been shown to have estrogenic effects on breast cancer cells²⁹ and Williams media E contains phenol red as a dye. We therefore, tested the effect of E2 on NO production by cytokine-stimulated hepatocytes cultured in phenol red-free vs phenol red-containing media. We observed that there was reduced suppressive effect of E2 on NO production when using phenol red-free media, however, this was not significantly different from cells cultured in phenol red-containing media (data not shown). Thus, for further experiments we used serum-free Williams medium E containing phenol red and selected E2 concentrations of 10 μ M and 25 μ M.

In a time course experiment, exposure of hepatocytes to E2 significantly diminished cytokine-stimulated NO production, starting at 8 hours (Fig. 3). This suppression of NO production by E2 persisted up to 36 hours of culture.

To evaluate the mechanism by which E2 regulates cytokine-stimulated hepatocyte NO production, we examined iNOS mRNA as an index of iNOS gene expression. Cytokines increased iNOS mRNA levels starting at about 2hrs, peaking at 8hrs and declining at later time points, corresponding with previous studies^{30, 31}. E2 decreased iNOS mRNA expression at each time point studied (Fig. 4).

Hepatocytes contain both estrogen receptors alpha and beta (ER- α and ER- β), which are the primary estrogen receptors mediating the physiological effects of estradiol³². ER- α has been shown to regulate iNOS expression in the liver²⁶. We therefore tested the role of ER- α in mediating the effect of E2 on hepatocyte iNOS activation. MPP, a specific ER- α antagonist had no effect on cytokine-induced NO production. PPT, a selective ER- α agonist, suppressed cytokine-induced hepatocyte NO production but with lower efficacy than estradiol (Fig. 5), suggesting that the effects of E2 may be partially but not completely mediated through ER- α .

DISCUSSION

Excessive NO production has damaging effects during inflammatory processes and significantly contributes to acute liver dysfunction in severe sepsis and shock²³. The present study demonstrates that E2 attenuates pro-inflammatory cytokine-induced NO production in hepatocytes. This effect was most apparent at the higher doses of E2 used. This finding may represent one mechanism through which estrogens influence gender based differences in liver damage and other organ injury after sepsis²². Hepatocytes are capable of sustained iNOS expression, producing excessive amounts of NO in the process²⁴. Our study

demonstrates that E2 regulates hepatocyte NO production by decreasing iNOS mRNA levels and iNOS protein expression in cytokine stimulated cells. Hepatocyte NO production is primarily regulated at the level of iNOS gene expression³³⁻³⁵ and we demonstrated decreased iNOS mRNA and protein levels in E2-cultured hepatocytes. Whether E2 regulates iNOS mRNA production, mRNA stability, post-transcriptional modification or post-translational protein stability or degradation in hepatocytes will require further investigation.

A recent study showed that following hepatic injury after trauma-hemorrhage, ER- α activation was able to mediate a reduction in iNOS expression²⁶. However, our results demonstrate that in this in-vitro model, ER- α did not account for the full extent of estradiol's action. The reasons for the differences in these studies are not clear. Several tissue and cell types may be affected by E2 administration in an in-vivo model of inflammation that would not play a role in our in-vitro hepatocyte culture model. These factors and the mechanisms involved will need to be elucidated in further studies to give a complete picture of estradiol's effect on iNOS regulation.

As with any experimental finding, this study has limitations. The doses of E2 used in this study were higher than doses of E2 used in other studies of E2 effects on cellular function³⁶. However, these doses approximate the doses used in several in-vivo studies when E2 is used as a pharmacologic agent^{15, 37}. Additionally, it is known that estrogen undergoes rapid metabolism in the liver by the cytochrome P-450 system³⁸. The proportion of the E2 administered that might be rapidly metabolized and therefore inactivated by the hepatocytes in these experiments is unknown. Using NO production as an index of ongoing cellular injury may not best represent the several alterations in cellular function that occur after noxious insults. Other markers of cell injury may need to be assessed, validated and shown to reflect the global picture of overall cell health. Finally, results from in-vitro cell culture studies may be difficult to translate into clinical effects, without the necessary transition to in-vivo models. Clearly, evidence supports a role of sex steroids in influencing progression of organ dysfunction, but a link between the mechanisms of these effects and outcome is obscure^{10, 39}. The process by which sex hormones and their receptors modulate organ function remains the focus of considerable research studies, as this is yet to be fully elucidated. Further experimental studies will need to be carried out using older rats and/or female rats to determine how this will impact the results.

In conclusion, these data demonstrate that E2 dose-dependently decreases cytokine-stimulated NO production in hepatocytes and does so, at least in part, by decreasing iNOS mRNA and protein levels. The specific mechanism for the E2-mediated effect on hepatocyte NO production will require further studies to identify. E2 mediated effects on hepatocyte iNOS may represent one mechanism for the beneficial effects of estrogens in in-vivo models of shock and sepsis.

Acknowledgments

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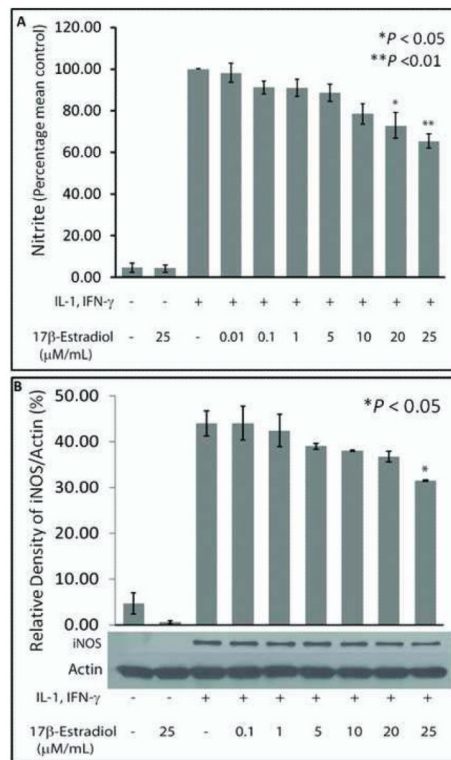


Figure 1.

Nitrite (NO^-_2) production of hepatocytes simultaneously treated with rhIL-1 β , rrIFN- γ , and different concentrations of E2 in serum-containing medium. Results are the mean + SEM of 3 independent rat experiments performed separately in which NO^-_2 was measured from supernatant of hepatocytes cultured in triplicates for each rat experiment. (A) NO^-_2 measured from cells stimulated with the cytokines alone minus E2 (3rd column from Y-axis) was used as positive control and all other treatments expressed as a percentage of the control. Asterisk represents a significant ($p < 0.05$) difference from control cells. (B) Western blot analysis of iNOS protein. A dose-dependent suppression of cytokine-stimulated iNOS protein expression by E2 is demonstrated.

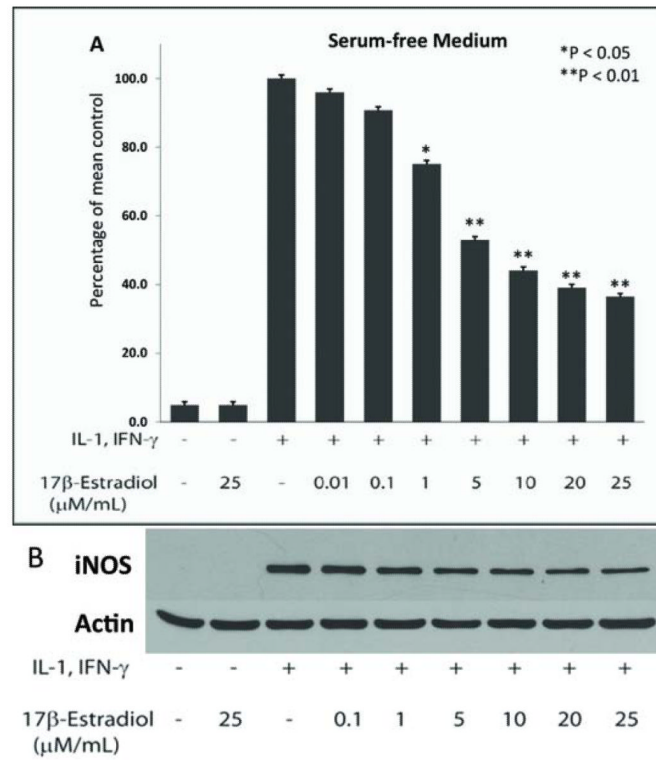


Figure 2.

NO $_2^-$ production in cytokine-stimulated hepatocytes cultured in serum-free antibiotic-free medium: (A) Statistically significant suppression of NO $_2^-$ by estradiol begins to occur at the 1 μ M ($p < 0.05$) in cells cultured in serum-free, antibiotic-free medium. B. Western blot demonstrating iNOS expression in hepatocytes cultured in serum-free medium. Significant decrease in iNOS protein was noted at 10-25 μ M dose of E2 ($p < 0.05$).

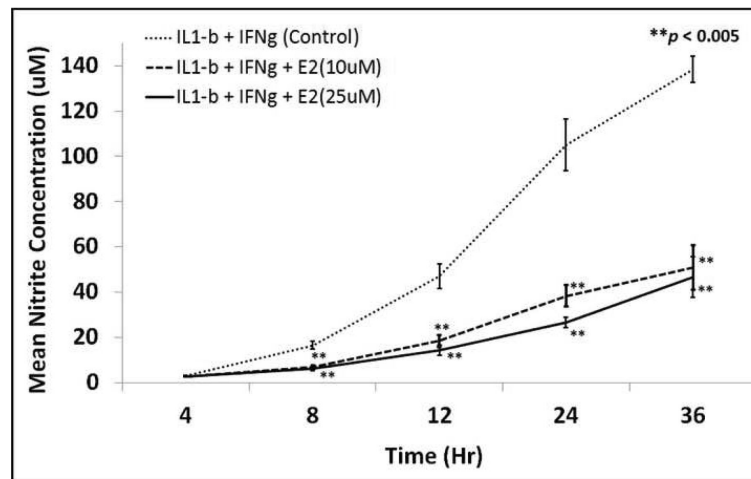


Figure 3.

Time-course relationship of cytokine-induced NO_2^- production in hepatocyte exposed to two different concentrations of E2 (the two bottom line graphs) *versus* hepatocytes cultured without E2. Results are the mean + SEM of 3 different rats in which NO_2^- was measured from supernatant of hepatocytes cultured in triplicates. The NO_2^- measured are absolute values in uM.

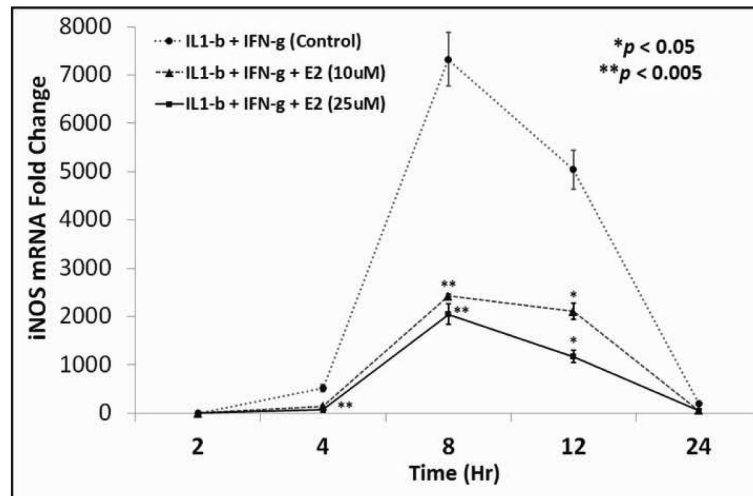


Figure 4. Line graph demonstrating iNOS mRNA expression in cytokine-stimulated hepatocytes exposed to two different doses of E2 (the two bottom line graphs) versus hepatocytes not exposed to E2 (upper line graph).

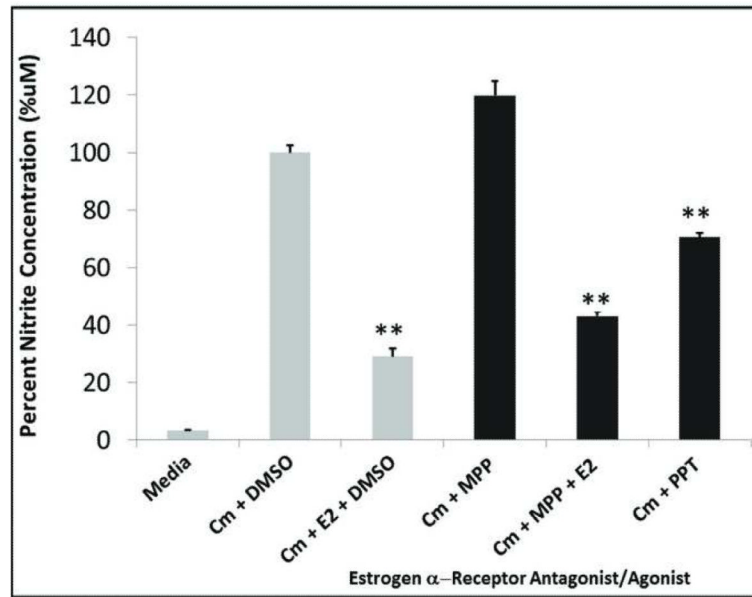


Figure 5. Suppression of NO production involves ER- α . Hepatocytes were treated as described in Fig. 1. Concentrations of ER- α ligands were 10uM E2, 10uM PTT, a selective ER- α agonist and 10uM MMP, a specific ER- α antagonist. CM= Cytokine mix of rhIL-1b plus rrIFN-g. DMSO = Vehicle used as negative control.