

INFLUENCE OF STEROIDS ON OXIDANT GENERATION IN ACTIVATED HUMAN GRANULOCYTES AND MONONUCLEAR LEUKOCYTES

Richard A. Cassidy

U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas 78234–6315

Received 5 Dec 2002; first review completed 23 Dec 2002; accepted in final form 4 Feb 2003

ABSTRACT—Steroids, in particular, 17 β -estradiol (E_2), have been reported to improve the response to trauma in animal models. In these models, the leukocyte plays a critical role in the inflammatory cascade. We examined the affects of E_2 , hydrocortisone (H), progesterone (P_4), and E_2 with P_4 on oxidant production in human granulocytes (PMNs) and mononuclear leukocytes (MNCs). Each cell type was loaded with 2,7-dichlorodihydrofluorescein and then simultaneously activated with human cytokines (tumor necrosis factor α , interleukin-1 β , and interferon γ) and hemoglobin and inhibited with and without equimolar concentrations of each steroid treatment or nitric oxide (NO) synthesis inhibitors. After incubations of 1 or 5 h, intracellular oxidants were quantified by flow cytometry. Activation by cytokines combined with hemoglobin, resulted in a 450–575% increase in oxidant production that was synergistically greater than the sum of either component alone. Pharmacological levels of E_2 decreased oxidants in MNCs at 1 h. In contrast at 5 h, H decreased oxidants more than E_2 . The addition of P_4 to E_2 concentrations almost eliminated oxidants from 1 h-activated MNCs. None of the steroids significantly reduced oxidants in PMNs, suggesting that the E_2 effect on MNCs was not caused by its nonreceptor-mediated antioxidant properties. Because L-NMMA inhibited at least 55% of the total oxidants, part of E_2 dampening effects would be attributed to NO. These results suggest that steroid-attenuated MNC-derived NO may reduce autocrine and paracrine effects on inflammation if appropriate doses of steroids are given soon after injury.

KEYWORDS—Estradiol, cortisol, progesterone, monocytes, neutrophil, nitric oxide, flow cytometry, inflammation

INTRODUCTION

Traumatic injury induces alterations in immune function that may lead to the systemic inflammatory response syndrome and ultimately to multiple organ dysfunction syndrome and death (1). Whether this response can be manipulated to improve outcome from injury is unknown, but recent advances in understanding gender (2, 3) and age (4, 5) differences in humans and in a variety of animal injury models (6–10) suggest that certain steroids, especially 17 β -estradiol (E_2), may ameliorate the inflammatory response to trauma. Recently, the oxidant, nitric oxide, which is produced by leukocytes, has been reported to mediate early events in the inflammatory cascade (11–13). With the objective of improving outcome after trauma, we tested the hypothesis that certain steroids would ameliorate intracellular oxidant generation after activation of human mononuclear leukocytes (MNCs) or polymorphonuclear leukocytes (PMNs).

We modified a previously described isolated human leukocyte model (14) to simulate extravasated leukocytes as they might be activated in damaged tissue. To activate the leukocytes we used a “two-hit” model of a cytokine cocktail of human inflammatory cytokines (tumor necrosis factor [TNF]- α , interleukin [IL]-1 β , and interferon [IFN]- γ) combined with human hemoglobin. To approximate early intervention, leukocytes were exposed to stimulants and

steroids simultaneously. We measured the effect of 10 concentrations (10^{-9} to 10^{-5} M) of estradiol (E_2), hydrocortisone (H), progesterone (P_4), and a combination of E_2 and P_4 on intracellular oxidant levels and cell viability in both PMNs and MNCs by flow cytometry after 1 and 5 h of stimulation and treatment.

MATERIALS AND METHODS

PMNs and MNCs isolation

This protocol was approved by the U.S. Army Institute of Surgical Research Human Use Committee for research involving the drawing of blood samples from human subjects subsequent to informed consent. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Human PMNs and MNCs were obtained from sodium citrate (0.105 M) anticoagulated venous blood from nonsmoking, drug-free, healthy male volunteers. PMNs and MNCs were harvested from blood by centrifugation of an equal volume of blood layered on to Polymorphprep, (GIBCO, Grand Island, NY) at 550 g for 35 min at 20°C. Separated PMNs and MNCs layers were aspirated with a pipette and washed twice in Hank's Balanced Salt Solution (HBSS; GIBCO, Grand Island, NY). Only plasticware made from polypropylene was used in these experiments. Two hypotonic lyses were performed to remove residual red blood cells. The purity of isolated PMNs and MNCs was approximately 98% and 92%, respectively, as determined by an automated cell count of 5 replicates of isolated leukocytes from individual donors subsequent to the establishment of the isolation procedures (ABX Pentra 120, Montpellier, France). During the study, the cell number of isolated MNCs and PMNs were determined with a hemocytometer (Fisher Scientific, Pittsburgh, PA) for dilutions to correct final concentrations so that equal numbers of cells were compared in each experiment.

Intracellular oxidants and cell viability

PMNs (1.0×10^7 /mL) and MNCs (2.0×10^7 /mL) isolated within 2 h of blood donation were loaded with 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA; 2 μ M; Molecular Probes, Eugene, OR) in HBSS for 30 min at 37°C in a water bath (14). DCFH-loaded PMNs (5.0×10^5) or MNCs (1.0×10^6) in 50 μ L of HBSS were combined in tubes (12 $\times$ 75 mm) containing 50 μ L of either no stimulant, a cytokine cocktail of human TNF α (2500 U/mL), IL-1 β (500 U/mL), and IFN- γ (1250 U/mL; all R&D Systems, Minneapolis, MN), human hemoglobin (100 μ g/mL), combination of cytokine cocktail and hemoglobin, or phorbol myristate acetate (50 nM; Calbiochem, San Diego, CA) used as a positive control. PMNs or MNCs, stimulated with a combination of cytokine cocktail and hemoglobin, were also simultaneously treated with and without ethanol (molecular biology grade; 0.05%; Calbiochem, San Diego, CA) used to solubilize steroids in supplemented HBSS; nitric oxide synthase (NOS) inhibitor, *N*-methyl-L-arginine (L-NMMA; 10 mM); its inactive stereoisomer

Address reprint requests to Richard A. Cassidy, U.S. Army Institute of Surgical Research, 3400 Rowley E. Chambers Ave., Fort Sam Houston, TX 78234–6315. E-mail: rcassidy@dccl.com.

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DOI: 10.1097/01.shk.0000070740.34700.cd

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 01 JUL 2003		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Influence of steroids on oxidant generation in activated human granulocytes and mononuclear leukocytes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Cassidy R. A.,				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78009				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

mer, *N*-methyl-D-arginine (D-NMMA; 10 mM); or a selective inhibitor of iNOS, *L*-*N*⁶-(1-Iminoethyl)lysine (L-NIL; 10 μ M; all, Calbiochem, San Diego, CA); or 10 concentrations of either E₂, P₄, or H (10⁻⁹ to 10⁻⁵ M); or 5 E₂ concentrations (6 $\times$ 10⁻⁷ to 10⁻⁵ M) combined with a single concentration of P₄ (6 $\times$ 10⁻⁷ M). Activated PMNs or MNCs were incubated for 1 or 5 h with the agents described above in HBSS supplemented with physiological concentrations of Ca²⁺ (1.5 mM), Mg²⁺ (900 μ M), glutamine (1.0 mM; GIBCO, Grand Island, NY), arginine (100 μ M), amino acids (1 $\times$ MEM; GIBCO), and tetrahydrobiopterin (1.0 μ M). Flow cytometric analyses were performed with an argon laser (488 nm) and emission light measured behind a filter transmitting 530/30 nm light on a FACCalibur (Becton Dickinson, San Jose, CA) with CELLQuest data acquisition and analysis software. PMNs or MNCs were gated by forward- and side-scatter. For each sample, 10,000 PMNs or MNCs were collected. The mean channel fluorescence was determined on a linear scale from a single-parameter histogram.

Viability of activated PMNs and MNCs was assessed by propidium iodide (PI) uptake as previously described (14). Flow cytometric analysis of intracellular PI levels was measured after a 5-min treatment with PI (1.0 μ g/mL) at room temperature.

Design and data analysis

The effect of various concentrations of each steroid or combination of steroids on intracellular oxidant and cell viability was assessed at 1 and 5 h in MNCs or PMNs isolated from one of six possible donors on a given day. Replicates of experiments from three to five different donors for each steroid were pooled for statistical analysis. To compare the effect of steroids or L-NMMA within each experiment, all treatment results had the background fluorescence equal to that in the nonstimulated MNCs or PMNs subtracted and then compared, as a percentage, to the level of fluorescent DCF in MNCs or PMNs activated with a combination of cytokines and hemoglobin. Intracellular oxidant measurements in cells incubated with the three highest steroid concentrations or the L-NMMA provided the basis for Bonferroni-correction for the four comparisons of greatest interest. For a given replicated steroid treatment, a two-tailed one-group Bonferroni-corrected *t* test was performed against cells activated with cytokine and hemoglobin (reference value equal 100%).

RESULTS

Compared with mean background fluorescence in unstimulated MNCs or PMNs, the mean intracellular levels of oxidized

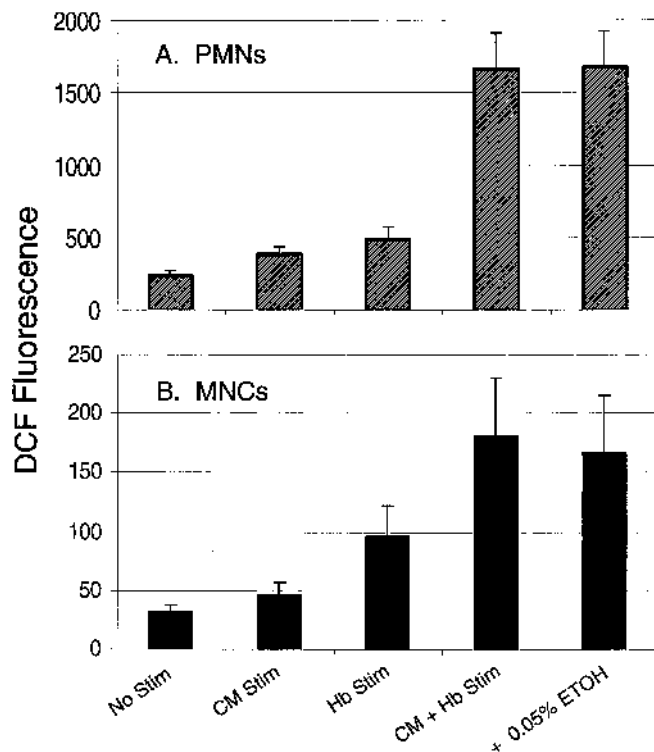


FIG. 1. Comparison of intracellular oxidants at 5 h in PMNs (A) and MNCs (B) not stimulated and stimulated with cytokines (CM), hemoglobin (Hb), CM plus Hb, or CM plus Hb with ethanol (0.05% ETOH) used to solubilize steroids (mean $\pm$ SEM; *n* = 14 incubations performed in conjunction with steroid treatment experiments).

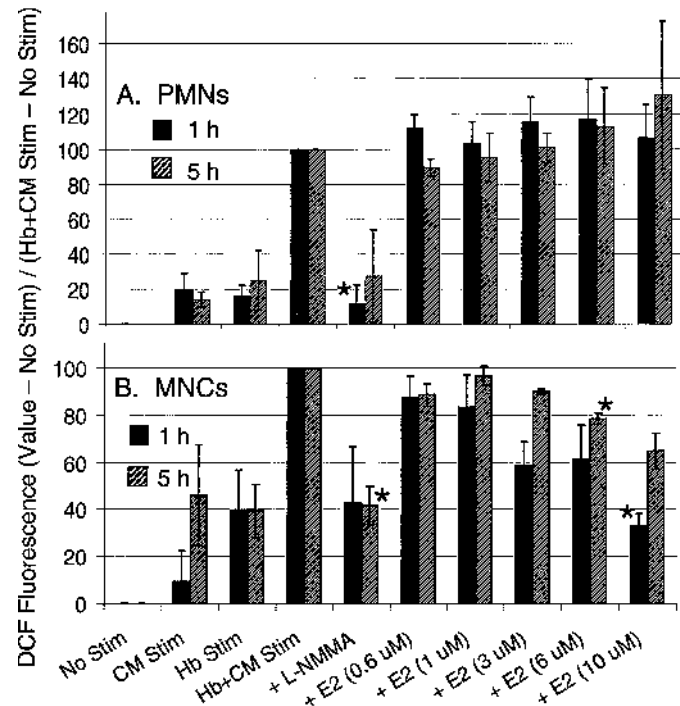


FIG. 2. Effects of E₂ or a NO synthase inhibitor on intracellular oxidants in cytokine- and hemoglobin-stimulated PMNs (A) and MNCs (B) incubated for 1 or 5 h. **P* < 0.05 vs. CM- plus Hb-stimulated MNCs or PMNs (mean $\pm$ SEM; *n* = 3).

DCF increased 44% and 46% with cytokine stimulation, 201% and 159% with hemoglobin stimulation, and 461% and 572% with a combination of hemoglobin and cytokine stimulation after 5 h in MNCs and PMNs, respectively (Fig. 1). Ethanol at the highest concentration used to solubilize the steroids (0.05%) did not alter oxidant levels. After 1 h of activation, the relative increases in mean oxidized DCF were lower than at 5 h with increases of 9% and 94% for cytokine stimulation, 42% and 176% for hemoglobin stimulation, and 97% and 363% for a combination of hemoglobin and cytokine stimulation in MNCs and PMNs, respectively (data not shown). At both 1 and 5 h, the mean level of intracellular oxidants produced by the combination of cytokines and hemoglobin was greater than the sum of the mean for leukocytes treated separately with cytokines or hemoglobin.

The effects of E₂, H, P₄, or a combination of E₂ and P₄ on levels of intracellular oxidants in MNCs and PMNs after 1 or 5 h of cytokine and hemoglobin activation are depicted in Figures 2–5 as a percentage of oxidants in activated leukocytes. Only the highest five of the 10 concentrations are displayed. The lower five concentrations (1 to 300 nM) of each steroid had negligible effects on oxidant levels in activated leukocytes. As compared with leukocytes activated with cytokines and hemoglobin for 1 and 5 h, some steroids had a concentration-dependent dampening effect on oxidant levels in MNCs and little effect on oxidants in PMNs, except a consistent, although modest trend for H to reduce oxidants in PMNs at 5 h. Of the three steroids tested in activated MNCs, E₂ (10 μ M) had the greatest dampening effect (65%) on oxidant production after 1 h, as compared with 20% for H and 30% for P₄. Combining P₄ (0.6 μ M) with E₂ concentrations further reduced intracellular

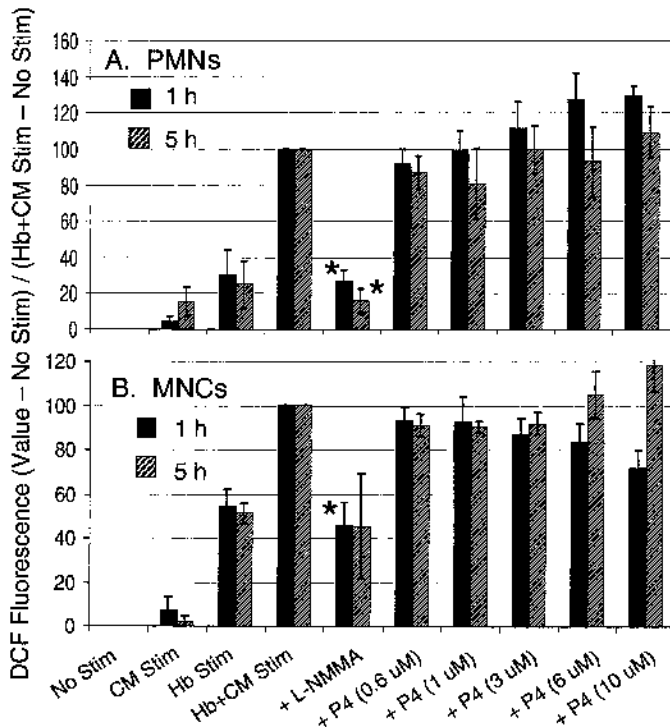


FIG. 3. Effects of P_4 or an NO synthase inhibitor on intracellular oxidants in cytokine- and hemoglobin-stimulated PMNs (A) and MNCs (B) incubated for 1 or 5 h. * $P < 0.05$ vs. CM- plus Hb-stimulated MNCs or PMNs (mean $\pm$ SEM; $n = 5$).

oxidants by 10–20% over values for E_2 alone, with a 75% reduction at the highest E_2 (10 μ M) concentration. In contrast to E_2 , H appears to reduce oxidant levels in MNCs to a greater extent after 5 h as compared with 1 h of stimulation.

Cell membrane damage (index by intracellular propidium iodide) in cytokine- and hemoglobin-activated MNCs and PMNs did not differ from unstimulated leukocytes, or any steroid or NOS inhibitor treatments (data not shown). These results support the idea that the treatment effects were not due to cell membrane damage allowing the DCF fluorescent probe to leak from the leukocyte. Overall, L-NMMA inhibited 80% of the cytokine- and hemoglobin-induced oxidant production in PMNs and 55% in MNCs. The specific inhibitor of inducible NO synthase, L-NIL, had negligible effects on activated MNCs and PMNs (data not shown). These results suggest that the predominant oxidant inhibited by the steroids is NO, probably derived from constitutive NOS. In only MNCs activated for 5 h, the supposedly inactive stereoisomer D-NMMA appeared to reduce intracellular oxidant levels (data not shown).

DISCUSSION

Human PMNs and MNCs were activated with cytokines and hemoglobin and treated with and without equimolar concentrations of E_2 , H, P_4 , or the combination of E_2 and P_4 to assess steroid effects on intracellular oxidant production (14). DCFH loaded into MNCs and PMNs is oxidized to fluorescent DCF by $NO\cdot$, superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$; Ref. 20). We and others have previously shown that this approach is sensitive, reproducible, and capable of measuring a wide concentration range of intracel-

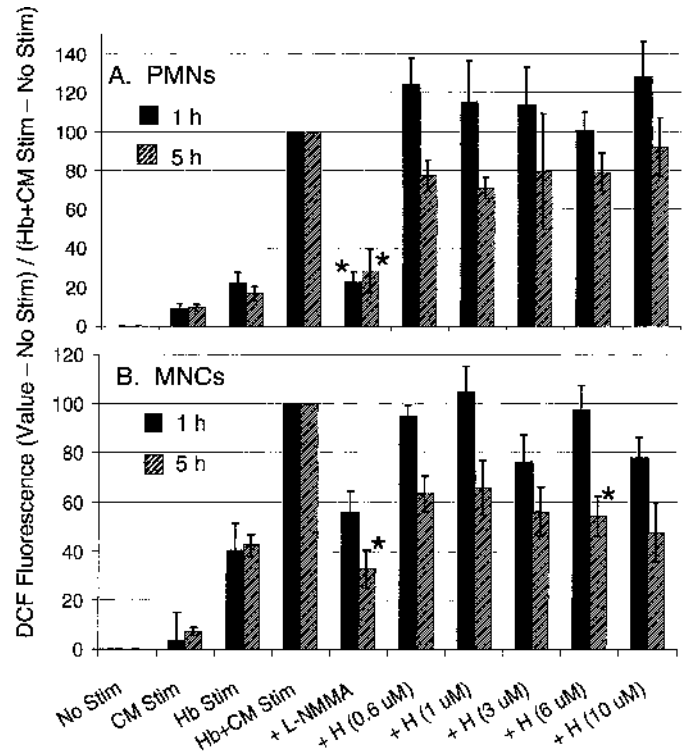


FIG. 4. Effects hydrocortisone (H) or a NO synthase inhibitor on intracellular oxidants in cytokine- and hemoglobin-stimulated PMNs (A) and MNCs (B) incubated for 1 or 5 h. * $P < 0.05$ vs. CM- plus Hb-stimulated MNCs or PMNs (mean $\pm$ SEM; $n = 4$).

lular oxidants (14, 15). Human PMNs and MNCs contain constitutive neuronal NO synthase or endothelial NO synthase, respectively; inducible NO synthase; and NADPH oxidase that synthesize $NO\cdot$ and $O_2^{\cdot-}$ (14–17). The constitutive NO synthase and NADPH oxidase activities increase with free intracellular calcium (18), whereas, iNOS expression is upregulated by inflammatory cytokines (19). In this study, L-NMMA inhibited approximately 80% of the cytokine- and hemoglobin-induced oxidant production in PMNs and 55% in MNCs, indicating that NO was the predominant oxidant produced by activated MNCs and PMNs. The NO produced was derived from constitutive NO synthase because incubation of leukocytes with L-NIL, the specific inhibitor of inducible NO synthase, did not reduce total intracellular oxidant levels. These results suggest that the inflammatory cytokines and hemoglobin used in this study increase NO production by cNOS.

The current two-hit hypothesis (20) or the multiple hit paradigm (9) of severe inflammation leading to systemic inflammatory response syndrome or multiple organ dysfunction syndrome consists of an initial insult, such as hemorrhage, which primes the immune system for an amplified response to a second or subsequent inflammatory mediator(s). To simulate the multiple inflammatory mediators released after severe trauma, we used a cocktail of human inflammatory cytokines (TFN- α , IL-1 β , and IFN- γ), previously shown to be released from injured tissues, in combination with human hemoglobin as the second hit. Free hemoglobin is found in blood after trauma (21) and transfusions (22). As supported by this study results, hemoglobin and its breakdown product, heme, are

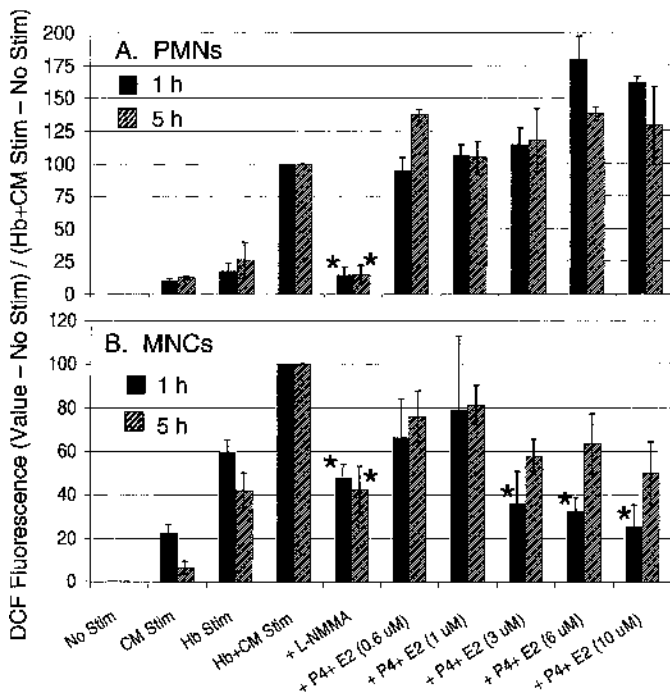


FIG. 5. Effects of E_2 and P_4 or a NO synthase inhibitor on intracellular oxidants in cytokine- and hemoglobin-stimulated PMNs (A) and MNCs (B) incubated for 1 or 5 h. * $P < 0.05$ vs. CM- plus Hb-stimulated MNCs or PMNs (mean $\pm$ SEM; $n = 4$).

proinflammatory mediators that can further augment oxidant production in leukocytes primed by other activating molecules (14). We found in both MNCs and PMNs that the combined effect of the cytokine mix and hemoglobin on intracellular oxidants was greater than the sum of oxidants induced by either cytokine mix or hemoglobin, suggesting that this multiple hit would amplify the inflammatory cascade. This suggests that blood substitutes containing hemoglobin may activate leukocytes to a greater extent in trauma patients than in nontrauma patients. Here we showed that steroids E_2 , H, and E_2 plus P_4 provided at the time of activation reduced oxidant production in MNCs derived from male donors stimulated with a mixture of inflammatory cytokines and hemoglobin. Whether such treatments would exert such an affect in humans experiencing traumatic injury is currently unknown but suggest that such an approach might improve outcome.

Studies in animal models and in humans suggest that steroids derived from the ovaries have a protective effect that results in decreased mortality and improved immune function compared with injury- and age-matched males (23). Women have a reduced risk of sepsis after trauma (2, 3). Survival after severe trauma is also age dependent and biphasic, peaking in young adults and decreasing in prepubescent and older adult patients (4, 5). The biphasic, age-dependent survival in trauma patients mirrors plasma levels of E_2 , testosterone (T), and dehydroepiandrosterone (DHEA) in women, and of T and DHEA in men (24–26). DHEA and T are converted to E_2 by aromatase, whose expression and activity in tissues are increased by inflammatory cytokines (27) and glucocorticoids (28) after trauma. Higher DHEA levels have been linked to an improved outcome following injury. Interestingly, concentrations of DHEA (0.2 μ M) and conjugated DHEA sulfate (0.1–

10 μ M), the most abundant steroid in circulation, of young adults are sufficient, if converted, to generate pharmacological levels of E_2 in injured tissue (25, 29). The aromatase recently described in leukocytes may facilitate the conversion of precursors to micromolar levels of E_2 in injured tissues. Combining H with E_2 for the early treatment of severe injury may provide not only dampening intracellular oxidant production in leukocytes but also increase the aromatization of DHEA and of deleterious androgens to E_2 in injured tissues.

Steroids have been reported to improve outcome after injury. In animal models, E_2 treatment within 1–2 h of injury improves outcome following ischemic-reperfusion injury (6), carotid artery injury (7), pulmonary inflammatory response to foreign material (8), and response to trauma-hemorrhage (9). In each of these injury models, the beneficial effect of E_2 may be receptor mediated since these effects can be blocked by E_2 receptor antagonists (ICI 182,780 or tamoxifen; Refs. 7–10) and may not be mediated by vascular effects (10).

Extravasated leukocytes and oxidants derived from leukocytes play a critical role in the inflammatory cascade and the resultant injury. Depletion of neutrophils and monocytes or the blocking of leukocyte adhesion molecules with antibodies ameliorate lethal hemorrhagic shock (30) and hemorrhage-induced lung injury (31), and lung injury induced by hind limb ischemia (11). Administering scavengers of reactive oxygen species or inhibitors of nitric oxide synthase also reduced endothelial injury to similar low levels, suggesting that reactive species, such as NO, are major mediators in the inflammatory cascade (11). NO has been reported to induce the production of inflammatory cytokines (12, 13) that may further induce the migration and activation of leukocytes. E_2 have been reported to dampen injury by potentially reducing NO production by leukocytes (8).

Multiple studies have found E_2 receptors in/on MNCs (32) whereas, E_2 receptors were found on PMNs only after activation (33). Hydrocortisone receptors in both MNCs and PMNs were approximately equal and present without activation (34). No reports of P_4 receptors in/on leukocytes were found. Interestingly, steroid effects on intracellular levels of oxidants in this study appear to coincide with the reported levels of steroid receptors in/on MNCs and PMNs. For MNCs, pharmacological levels of E_2 or H reduced oxidant levels whereas P_4 had little discernible effects. E_2 had a greater effect at 1 h than at 5 h, whereas the converse was true for H. These different time courses suggest E_2 is altering pathways not involving protein synthesis, whereas H may be altering protein synthesis. For PMNs, E_2 or P_4 had no effect on oxidants, whereas all hydrocortisone concentrations showed a modest trend for reducing oxidant levels after 5 h. In this study, pharmacological concentrations of E_2 markedly reduced intracellular levels of oxidants in MNCs, but not in PMNs, further supporting the concept that E_2 effects in MNCs were not due to its nonreceptor-mediated antioxidant properties. Other nonsteroidal treatments that dampen PMNs oxidant production may complement E_2 -activity previously reported in a variety of injury models (6–10). In contrast, physiological levels of E_2 (1–100 nM) have been reported to increase NO production by an E_2 receptor-mediated

process in non-activated MNCs, indicating a major difference between activated and non-activated MNCs (35).

Similarly, activated macrophages derived from lungs or a macrophage cell line (J774) treated with pharmacological levels of E_2 reduced NO production (36, 37). NO, generated by cNOS or iNOS, has been demonstrated to act as an early proinflammatory mediator in both MNCs and PMNs by increasing inflammatory transcription factors (nuclear factor- κB) and cytokine expression (TNF- α and IL-1 β) that subsequently activate inflammatory cascades resulting in lung and liver injury (12, 13). One of the mechanisms by which pharmacological levels of E_2 may reduce tissue injury is by inhibiting NO-induced cytokine (38) and chemokine (39) synthesis in monocytes and macrophages that amplify the early events in the inflammatory cascade. Because NO is cell membrane permeant and is known to bind to regulatory proteins and alter redox potential in neighboring cells, E_2 may also dampen NO paracrine and toxic effects on neighboring cells (12–14).

The early dampening by E_2 of the adhesion of leukocytes to vascular endothelium and subsequent extravasation of leukocytes into injured tissues has been proposed as a mechanism for E_2 salutary effects (6, 8). This mechanism is consistent with the requirement of E_2 treatment within 0.5 h of injury for optimal beneficial effects (40). In contrast with treatment with NO synthase inhibitors that have been reported to worsen certain types of injuries (41), E_2 reduces injury while concomitantly increasing endothelial NO synthase levels and perfusion of ischemic tissues (42). Results of this study suggest that E_2 may limit NO production in monocytic cells that initiate early inflammatory events. These attributes support the use of E_2 in the treatment of the severely injured patients potentially providing benefits similar to that already demonstrated in a variety of animal injury models. Though our results do not address combining E_2 and H, the combined use of H and E_2 for the early treatment of severe injury could conceivably provide not only a modest dampening effect on oxidant production by PMNs and lower oxidants in MNCs for a longer period of time (up to 5 h), but also increase the aromatization of DHEA and of deleterious androgens to E_2 in injured tissues. Future studies using the same experimental procedures could assess how the interaction of MNCs and PMNs, or longer incubations, as found in the clinical scenario, affect steroid dampening of induced intracellular oxidant levels.

The approach of using isolated human leukocytes activated with relevant human inflammatory mediators as a model also provides insights into inflammatory mechanisms and enables the selection of drug or combination of drugs that will dampen major processes of the inflammatory cascade. Intracellular oxidant production, expression of adhesion molecules, and expression of cytokines and chemokines in/on leukocytes are major cellular adaptations to injury that contribute to the inflammatory cascade. These adaptations by leukocytes can be evaluated with cytofluorimetric procedures and can be used as endpoints for selecting treatments that dampen specific inflammatory processes following injury. The selected drug or combination of drugs derived from these cost-effective, proximal *in vitro* procedures can then be further validated in appropriate animal models.

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