Effects of Progesterone and Estradiol Sex Hormones on the Release of Microparticles by RAW 264.7 Macrophages Stimulated by Poly(I:C)²

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Microparticles (MPs) are small membrane-bound vesicles that display proinflammatory and prothrombotic properties. These particles can be released by macrophages stimulated by ligands of the Toll-like receptors (TLRs) in a process that depends on nitric oxide (NO) production. Since sex hormones can modulate macrophage responses, we investigated the effects of progesterone and estradiol on macrophage particle release in vitro, comparing the responses with those induced by the glucocorticoid dexamethasone. As a model system for particle release, RAW 264.7 cells were stimulated in vitro with poly(I:C), a ligand of TLR3. Microparticles were measured by flow cytometry, while NO was measured by the Griess reaction. As the results of these studies showed, progesterone but not estradiol can block particle release by RAW264.7 cells treated with poly(I:C); dexamethasone was also active. Furthermore, while progesterone and dexamethasone inhibited NO production under the same culture conditions, neither agent blocked the production of particles stimulated by the NO donors dipropylene triamine NONOate \{(2-aminoethyl)-(3-aminopropyl)amino\} diazen-1-ium-1,2-diolate and \{(2-aminoethyl)-(2-ammonioethyl)amino\} diazen-1-ium-1,2-diolate. Studies using RU486 to assess the role of hormone receptors indicated that while this agent blocked the inhibition of particle and NO production by dexamethasone, it did not affect the inhibition by progesterone. Together, these results indicate that progesterone but not estradiol can inhibit particle release by stimulated macrophages and suggest a mechanism that may contribute to the immunomodulatory effects of this sex hormone.

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since prior studies have demonstrated its effectiveness in inducing MP release by macrophages and since this compound can mimic the effects of viral double-stranded RNA (13). For a comparison of the effects of estradiol and progesterone, we have studied dexamethasone as a model for a glucocorticoid (5, 36). We have also investigated the potential role of receptors in these responses by characterizing the effects of RU486.

In studies assaying MPs by flow cytometry, we show herein that progesterone can inhibit MP release by stimulated RAW 264.7 cells, whereas estradiol is without effect; like progesterone, dexamethasone can block particle release. We have further investigated the role of NO in this response, including the effects of hormones on particle release induced by NO donors. Together, results of these studies indicate that progesterone and dexamethasone can modulate particle release by stimulated RAW 264.7 cells. These findings suggest that inhibition of particle release may be a mechanism for immunosuppression in the innate immune response and that, as a sex hormone, progesterone can impact a variety of clinical settings in which gender differences occur.

MATERIALS AND METHODS

Cell culture. RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 20 μg/ml gentamicin (Invitrogen) at 37°C in 5% CO2. Approximately every 2 to 3 days, cells were subcultured when they reached 80% confluence.

Cell stimulation and hormone treatments. Briefly, RAW 264.7 cells from a culture at 80% confluence were harvested and then plated at a density of 2.5 x 10⁶ per well in a 6-well plate in phenol red-free RPMI 1640 medium supplemented with 10% charcoal dextran-stripped fetal bovine serum (HyClone, Rockville, IL) and 20 μg/ml gentamicin. Cells were incubated for 20 h, at which time they were washed twice with phosphate-buffered saline (calcium free, magnesium free; Invitrogen) and incubated in phenol red-free RPMI 1640 medium supplemented with 2% charcoal dextran-stripped fetal bovine serum and 20 μg/ml gentamicin. The medium contained the following hormones, with concentrations indicated: water-soluble progesterone (0.001 to 10 μM), water-soluble estrogen (0.01 to 10 μM), and dexamethasone (0.001 to 1 μM) (all purchased from Sigma Aldrich, St. Louis, MO). At this point, high-molecular-weight poly(I:C) (Invivogen, San Diego, CA) was added to give a final concentration of 250 ng/ml. This concentration was determined on the basis of prior dose-response experiments and shown to induce production of both MPs and NO. After 22 h of incubation, the cell supernatants were removed by aspiration and centrifuged at 500 x g for 5 min. The resulting supernatants were transferred to new 1.5-ml tubes. The samples were immediately assayed for MP levels; samples for NO measurement were stored at –20°C until use.

FIG. 1. Flow cytometric measurement of MPs from stimulated RAW 264.7 macrophage cells. Following 22 h of culture of RAW 264.7 cells stimulated with 250 ng/ml poly(I:C), supernatants were harvested, centrifuged for 5 min at 500 x g, and then analyzed by flow cytometry. (A) Results of side scatter (SSC) analysis with a threshold setting established to reduce background events from medium alone. FSC, forward scatter. (B) MPs (gray fill) were analyzed for size by comparison with beads of defined sizes (0.1, 0.5, and 1.0 μm).

Effects of hormones on MP production by NO donors. Cells were prepared as described above, except that the cells were first incubated for 1 h with hormones in the presence of 2% charcoal dextran-stripped fetal bovine serum and 20 μg/ml gentamicin. At that time, various concentrations of the NO donor (3-1-(2-aminoethyl)-N-(2-aminoethoxy)amino) diazen-1-ium-1,2-diolate (DETA/NO) or dipropylencetriamine NONOate (1-1-(N-(3-aminopropyl)-N-(3-ammonio-propyl)amino) diazen-1-ium-1,2-diolate) (DPTA/NO) (both products of Enzo Life Sciences, Plymouth Meeting, PA) were added. Cells were then cultured for 22 h, and then the levels of MPs and NO were assessed.

Effect of RU486 treatment of hormonal modulation of MP and NO production stimulated by poly(I:C). Cells were treated as described above, except that cells were preincubated for 1 h with various concentrations of RU486 (Sigma Aldrich) in hormone-free, phenol red-free RPMI 1640 medium supplemented with 2% charcoal dextran-stripped fetal bovine serum and 20 μg/ml gentamicin. Poly(I:C) and hormones or controls were then added, and cells were cultured for 22 h for determination of microparticles and nitric oxide.

Assay of microparticles. Undiluted samples were analyzed by use of a side-scatter detection setting and a FACScan flow cytometer (Becton Dickinson, Mansfield, MA). By using predetermined parameters, events above a given size threshold were collected. Events were collected until 10,000 events were counted or 1 min of counting had elapsed. Background events for buffer alone were subtracted, and the counts/μl of sample were calculated. Particle size was determined by comparison with beads of defined sizes of 0.1, 0.5, and 1.0 μm.

Nitric oxide determination. One hundred microliters of supernatant was incubated with 100 μl of Griess reagent I (1% sulfanilamide, 2.5% phosphoric acid) and 100 μl Griess reagent II (0.1% naphthylenediamine, 2.5% phosphoric acid) for 5 min at room temperature. Plates were read at an optical density at 550 nm using an automatic plate reader (UV Max; Molecular Devices, Sunnyvale, CA).

Statistics. All experiments were performed in triplicate. Data were analyzed using the Student’s t test.

RESULTS

As shown previously, poly(I:C) is an effective stimulus of MP release by RAW 264.7 cells, inducing production of particles in a time- and dose-dependent manner (13). In the current studies, we tested poly(I:C) at a concentration of 250 ng/ml and measured MPs in the medium 22 h later by flow cytometry. For this purpose, we measured MP numbers by side scatter with the instrument at logarithmic gain, calculating particle numbers on the basis of the time for event accumulation given a known flow rate. Figure 1A illustrates a fluorescence-activated cell sorter analysis of the medium of stimulated cells following centrifugation to remove cells, while Fig. 1B shows the size range of the particles measured in comparison with the sizes of beads of defined size. These findings indicate that the particles detected have a size dis-
tribution consistent with MPs. In these studies, while there was variability in particle number depending on the experiment, the results of the effects of hormones were highly consistent among experiments.

Having confirmed the activity of poly(I:C) in stimulating particle release, we next assessed the effects of hormones, testing the effects of progesterone, estradiol, and dexamethasone. As shown in Fig. 2, progesterone inhibited particle production by RAW 264.7 cells stimulated by poly(I:C) in a dose-dependent way, whereas estradiol over a similar range of concentrations failed to inhibit particle release by the stimulated cells. These findings are consistent with those of prior studies indicating differences in the effects of hormones on macrophages (25, 38). As expected, dexamethasone potently inhibited particle release. For dexamethasone, inhibition was observed over a range of 0.001 to 1.0 μM, which was lower than the concentrations of progesterone for inhibition.

Since previous studies have demonstrated that particle release by RAW 264.7 cells can depend on the production of NO (13), we determined the effects of the hormones on NO generation. As shown in Fig. 3, progesterone showed inhibition of NO production at the highest concentrations tested, whereas estradiol did not affect NO production using concentrations as high as 10 μM. By comparison, dexamethasone potently blocked the production of NO stimulated by poly(I:C) over a dose range similar to that required to inhibit particle production. These results are consistent with the possibility that inhibition of NO production can reduce particle production, with the greater potency of dexamethasone compared to that of progesterone evident in both the inhibition of particle release and NO generation.

While hormones could affect particle release by inhibiting the production of NO (and potentially other mediators influencing particle release), a direct effect of either progesterone or dexamethasone on NO-induced particle release could also occur. To assess this possibility, we measured the effects of the hormones on particle release from RAW 264.7 cells incubated with the NO donors DETA/NO and DPTA/NO. Previous studies have shown that both DETA/NO and DPTA/NO release NO in culture and cause particle production in a time- and dose-dependent manner (13). As shown in Fig. 4, while DETA/NO and DPTA/NO caused particle release at levels much higher than those induced by poly(I:C), progesterone did not prevent this release. Similar results were obtained with dexamethasone, which also failed to block particle release by NO.

We next investigated the effects of hormone receptors in this response by determining whether RU486 could prevent the inhibitory effect of dexamethasone or progesterone on these responses. We tested dexamethasone first, since RU486 is known to be an effective inhibitor of the action of this agent. As shown in Fig. 5, RU486 could reduce the inhibition of particle release induced by dexamethasone. Similarly, RU486 could block the inhibitory effects of dexamethasone.
methasone on NO production induced by poly(I:C). In contrast, RU486 did not block either the ability of progesterone to inhibit MP release by RAW 264.7 cells stimulated by poly(I:C) or the production of NO. Together, these results suggest that while both dexamethasone and progesterone can block MP release by RAW 264.7 cells stimulated by poly(I:C), the responses differ in the effect of RU486 and therefore receptor involvement.

**DISCUSSION**

Results of these studies provide new insights into the immunomodulatory effects of sex hormones as well as glucocorticoids and suggest that effects on MP release from activated macrophages could contribute to their immunosuppressive activities. Thus, using RAW 264.7 macrophage cells as a model, we showed that progesterone and dexamethasone can block MP release from

![Graphs showing effects of sex hormones on NO production.](image)

**FIG. 3.** Effects of sex hormones on NO production by RAW 264.7 cells stimulated with poly(I:C). RAW 264.7 cells were incubated with various concentrations of progesterone (A), estradiol (B), or dexamethasone (C) and then stimulated with poly(I:C) (250 ng/ml) for 22 h. NO released into the supernatants was then measured. Data shown (means and SDs of triplicates) are representative of one of three repetitions. *, P < 0.05 by Student’s t test for poly(I:C) samples without hormone versus treatments with hormone. Treatments with poly(I:C) produced levels of NO which were statistically significant with respect to those of control samples for all conditions except dexamethasone at 0.1 μM and 1 μM.

![Graphs showing effects of hormones on MP production.](image)

**FIG. 4.** Effects of hormones on MP production induced by NO donors. RAW 264.7 cells were preincubated with or without progesterone at 10 μM (A) or dexamethasone at 0.05 μM (B) for 1 h and then treated with the NO donor DETA/NO or DPTA/NO at concentrations varying from 0 to 1,000 μM for 22 h. Data shown (means and SDs of triplicates) are representative of one of three repetitions. In these experiments, hormone treatments failed to produce any statistically significant (P < 0.05, Student’s t test) changes in MP production compared to corresponding hormone-free samples.
cells stimulated with poly(I:C) under conditions in which estradiol is without effect. We also showed that both progesterone and dexamethasone, while they are able to inhibit generation of NO, did not affect the release of MPs induced by NO itself. Furthermore, we showed that while RU486 could block the inhibitory effects of dexamethasone, it did not affect those of progesterone. Together, these findings suggest that while progesterone and dexamethasone can block a pathway(s) leading to NO production and particle release, they may differ in their mechanisms of action.

Microparticles are gaining increasing attention as important mediators in innate immunity as well as agents for intercellular communication (3, 9, 40). These particles can detach from cells by a budding process and carry a wide range of intracellular molecules (6, 7, 22). As products of activated and dying cells, MPs resemble alarmins in their immunological activities and potential role in immunopathogenesis. Alarmins are endogenous molecules that stimulate innate immune responses following release from dying cells or secretion from immune cells by nonconventional pathways. Alarmins are similar to death (or damage)-associated molecular patterns (DAMPs) which, like pathogen-associated molecular patterns (PAMPs), can stimulate Toll-like receptors and non-TLR receptors to induce innate immunity. Alarmins can be large or small molecules, with proteins and nucleic acids being members of this class of molecules; in some instances, alarmin activity results from degradation or posttranslational modification (4, 16, 30).

In contrast to alarmins, which can act as single molecular species, MPs are very large structures that encompass many components which can act as an ensemble. Not surprisingly, given their origin, MPs can include DAMPs as components, with the presence of these molecules in a large structure contributing to their overall activity. Indeed, as we showed previously, the release of high-mobility group box protein 1 (HMGB1) and MPs from RAW 264.7 cells as well as other murine cell lines or sources occurs under similar conditions, with poly(I:C) and LPS inducing the release of both (13, 19, 20). With HMGB1 release, alpha interferon plays an important role in the release process stimulated by poly(I:C), although with MP release, NO serves as an important inducer. Once in the extracellular milieu, both HMGB1 and microparticles can serve as downstream mediators of the actions of some but not all TLRs; MPs can also exert important prothrombotic activities likely because of the display of tissue factor.

While the factors leading to MP release have been well characterized, the inhibitory factors have received less attention, although the identity and function of these factors are important in elucidating regulatory interactions during disease pathogenesis as well as developing novel approaches to immunosuppressive therapy. Thus, our results indicate that both progesterone and dexamethasone can inhibit MP release by RAW 264.7 cells activated by a TLR ligand. In contrast, these agents did not affect the MP production induced by NO, suggesting that their effects occur at a step distal to TLR stimulation. The inhibitory action of dexamethasone was associated with inhibition of NO synthesis, suggesting that blockade of NO synthesis, perhaps in association with inhibition of the production of cytokines or other downstream mediators, accounts for its action. In contrast, with progesterone, while
inhibition of NO production occurred at high concentrations (10 \mu M), inhibition of particle release consistently occurred at lower concentrations. While these results suggest that progesterone affects MP release by a mechanism other than NO inhibition, it is nevertheless possible that the reductions of NO observed with progesterone are sufficient to account for the inhibitory activity.

Another difference between the effects of progesterone and dexamethasone concerns the potential role of hormone receptors. We investigated this issue by assessing the effects of the receptor antagonist RU486 on the inhibitory effects of progesterone and dexamethasone. As our results indicate, while RU486 abrogated the inhibitory effects of dexamethasone on both NO production and MP release, it did not comparably affect the inhibitory action of progesterone. These findings point to an important role of the glucocorticoid receptor in the effects of dexamethasone and are consistent with the findings of many studies on the mechanisms of glucocorticoid-induced immunosuppression. Even though the inhibitory actions of RU486 are complicated, the results of these studies are clear (5).

In contrast to results with dexamethasone, RU486 did not produce similar effects with progesterone, assessed in terms of either production of NO or MP release. As shown by studies of others, macrophages, including RAW 264.7 cells, do not have progesterone receptors. Nevertheless, these cells are able to respond to the effects of progesterone, although the effects of progesterone on NO production by macrophages have differed among studies (14, 25, 34, 38). Along with data presented herein, these studies suggest that progesterone mediates its actions on macrophages by a mechanism other than a classical hormone receptor; the nature of this mechanism has not yet been identified, although it may reflect a role of a membrane hormone receptor (10, 11). Operationally, however, the lack of effect of RU486 is important in using this agent to block sex hormone responses either in vivo or in vitro (8).

It is important to note that our conclusions on the mechanisms of MP release are derived from observations on the RAW 264.7 cell line. While this line has been commonly used to characterize the functional and phenotypic properties of macrophages, other macrophage cell lines as well as macrophages obtained from humans or animals may differ in their response to either stimulation of TLRs or the effects of hormonal manipulation; these differences could account for the disparate findings reported in the literature on the role of sex hormones in particular. Nevertheless, our previous study demonstrated that RAW 264.7 cells and murine bone marrow-derived macrophages showed many similarities in their release of HMGB1, supporting the use of this cell line to characterize murine responses (19, 20). Studies with other macrophage populations as well as in vivo models are in progress to investigate this issue further.

While the mechanisms for the immunosuppressive effects of progestational agents require further investigation, the current studies are nevertheless important in defining a potentially important action of progestational agents and glucocorticoids on the immune system. The immunosuppressive effects of glucocorticoids are well-known and form the foundation of their use in the therapy of immune-mediated diseases. Among sex hormones, the effects of estrogens have received significant attention as an important determinant of sex-related differences in the immune response, although as shown in our studies as well as those of others, progestins can exert important activities that can affect overall male-female differences in immune responses, changes in immune responses during estrus and pregnancy, and the effects of progestational agents as contraceptives (39). In this regard, the immune effects of progestational agents may be important in increasing susceptibility to virus infection as well as attenuating responses to vaccines (21, 26, 36).

In the context of immune-mediated diseases, the immunosuppressive effects of progestins may be important in developing new approaches to the prevention or treatment of diseases such as systemic lupus erythematosus or the complications of pregnancy such as preeclampsia (18, 33). As MPs have proinflammatory and prothrombotic activities, their reduction would be expected to benefit conditions where both inflammation and vascular abnormalities contribute to pathogenesis. In view of the extensive safety experience with progestational agents in contraception and prevention of preterm delivery, their use as immunosuppressive agents in other settings is reasonable. Since both NO production and blood MP levels can readily be measured in subjects in clinical studies, biomarkers are available to assess whether responses analogous to those that we have described in vivo can occur in vivo. Studies are therefore in progress to elucidate further the immunosuppressive properties of progesterone and its ability to attenuate production of MPs and their associated effects on immune cells, the clotting system, and the vasculature.

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