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# Estradiol activates mast cells via a non-genomic estrogen receptor-α and calcium influx

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## Abstract

**Background—**Allergic airway diseases are more common in females than in males during early adulthood. A relationship between female hormones and asthma prevalence and severity has been suggested, but the cellular and molecular mechanisms are not understood.

**Objective—**To elucidate the mechanism(s) by which estrogens enhance the synthesis and release of mediators of acute hypersensitivity.

**Methods—**Two mast cell/basophil cell lines (RBL-2H3 and HMC-1) and primary cultures of bone marrow derived mast cells, all of which naturally express estrogen receptor-α, were examined. Cells were incubated with physiological concentrations of 17-β-estradiol with and without IgE and allergens. Intracellular $Ca^{2+}$ concentrations and the release of β-hexosaminidase and leukotriene $C_4$ were quantified.

**Results—**Estradiol alone induced partial release of the preformed, granular protein β-hexosaminidase from RBL-2H3, BMMC and HMC-1, but not from BMMC derived from estrogen receptor-α knock-out mice. The newly synthesized $LTC_4$ was also released from RBL-2H3. Estradiol also enhanced IgE-induced degranulation and potentiated $LTC_4$ production. Intracellular $Ca^{2+}$ concentration increased prior to and in parallel with mediator release. Estrogen receptor antagonists or $Ca^{2+}$ chelation inhibited these estrogenic effects.

**Conclusion—**Binding of physiological concentrations of estradiol to a membrane estrogen receptor-α initiates a rapid onset and progressive influx of extracellular $Ca^{2+}$, which supports the synthesis and release of allergic mediators. Estradiol also enhances IgE-dependent mast cell activation, resulting in a shift of the allergen dose response.

### Keywords

Estrogen; Estrogen receptor-α; Human; Rodent; Mast cells/basophils; Allergy

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## 1. Introduction

Asthma and other allergic diseases of the airways are up to three times more common in women than in men during early to middle adulthood (De Marco et al., 2002; Mannino et al., 2002; Schatz and Camargo, 2003). A number of clinical and epidemiological studies suggest that female hormones contribute to these differences. A recent study found that women taking hormone replacement therapy had a higher risk of new onset asthma (Barr et al., 2004). Further, 30–40% of women who have asthma, experience worsening of their symptoms during the perimenstrual phase (perimenstrual asthma, PMA), when estrogen and progesterone concentrations are changing rapidly (Vrieze et al., 2003). Also, a recent study shows estrogen receptor (ER) polymorphisms are associated with airway hyperresponsiveness and lung function decline, particularly in females with asthma (Dijkstra et al., 2006).

However, very little is known about the mechanism(s) by which physiological concentrations of female hormones might promote the development of or increase the morbidity of asthma in women during their peak reproductive years (De Marco et al., 2002; Mannino et al., 2002; Schatz and Camargo, 2003; Skobeloff et al., 1992).

Serum concentrations of mast cell-derived leukotriene (LT) $C_4$ are significantly higher during the perimenstrual phase inwomen with PMA, compared with those during the midcycle week. Further, LT receptor antagonists have been reported to reduce symptom scores and improve pulmonary function in PMA (Nakasato et al., 1999). These findings suggest a relationship between female hormones, mast cell-derived mediators of acute hypersensitivity, and asthma severity. However, the cellular and molecular mechanisms by which female hormones might alter mast cell and basophil function have not been elucidated.

Mast cells, in various tissues, express ERs (Harnish et al., 2004; Jiang et al., 2002; Nicovani and Rudolph, 2002; Zhao et al., 2001). Super-physiological concentrations of estradiol ($E_2$, 1–10 μM) has been shown to induce (Spanos et al., 1996; Vliagoftis et al., 1992) or inhibit (Harnish et al., 2004) mast cell degranulation. Further, preincubating basophils or mast cells with physiological concentrations of $E_2$ has been shown to increase the subsequent histamine release induced by cross-linking surface-bound IgE with antibodies (Cocchiara et al., 1990; Cocchiara et al., 1992). The effects of hysiological concentrations of $E_2$ alone and the cellular and molecular mechanism(s) underlying estrogenic effects on mediator release from mast cells require further investigations.

In this study, we provide the first evidence that mast cells exposed to physiological concentrations of $E_2$ undergo partial degranulation. While $E_2$ alone had only a limited effect on LT synthesis, simultaneous exposure to $E_2$ and allergen cross-linking of surface IgE had a synergistic effect on the synthesis and release of $LTC_4$. The mechanisms underlying these independent and synergistic effects of $E_2$ were elucidated by demonstrating the requirement for extracellular $Ca^{2+}$ and non-genomic activity of ER-α.

## 2. Materials and methods

### 2.1. Cells and cell culture

RBL-2H3, a rat basophilic leukemia cell line (Morita and Siraganian, 1981), was obtained from the American type culture collection (Manassas, VA) and HMC-1, human mast cell line, from Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN) (Butterfield et al., 1988). RBL-2H3 were grown in DMEM supplemented with 10% FCS (Hyclone, South Logan, UT) and HMC-1 in IMDM (Cellgro, Kansas City, MO) with 10% iron-supplemented calf serum (Hyclone, South Logan, UT). To avoid exposure to estrogens in our culture system, we used dextran/charcoal-

treated FCS and phenol-red free media for the last 48 h of culture and throughout the experiments, as described (Lambert et al., 2005).

The primary cultures of bone marrow-derived mast cells (BMMC) were developed from the marrow of the femurs of C57B6 mice, as described (Odom et al., 2004). Wild type (WT) C57B6 mice were purchased from the Jackson Laboratory (Houston, TX) and ER-α (knockout) KO mice were obtained by breeding previously generated heterozygous ER-α KO mice (Lambert et al., 2005). After 4 weeks, the cultures contained 98% pure mast cells, as assessed by toluidine blue staining. BMMCs were used to confirm that the effects of $E_2$ occurred through ER-α, by comparing the cells from WT and ER KO mice. All animal experimental protocols were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

### 2.2. Detection of mRNA for ERs in cultured cells

Mast cells and basophils have been reported to express ERs (Zhao et al., 2001). To confirm and type the ERs expressed by RBL-2H3, BMMC and HMC-1 cells, we used RT-PCR. RNA from these cells were purified using TRIzol Reagent (Invitrogen, Carlsbad, CA,) and cDNAs were synthesized with Superscript RT (Gibco BRL), using oligo dT (Pharmacia, Piscataway, NJ). PCR for RBL-2H3 and BMMC were performed with primers: 5′-GGGCTTTCCCCCAGCTCAAC-3′ and 5′-GCACACGGCACAGTAGCGAG-3′ for ER-α, and 5′-AACTACAGTGTTCCCAGCAGC-3′ and 5′-AGGACCAGACACCGTAATGA-3′ for ER-β, and 5′-GGAGAAACCTGCCAAGTATG-3′ and 5′-AGAGTGGGAGTTGCTGTTGA-3′ for the housekeeping gene, G3PDH, as described (Lambert et al., 2004). PCR for ER expression in HMC-1 was performed with primers: 5′-TGCCCTACTACCTGGAGAACG-3′ and 5′-GTCCTTCTCTTCCAGAGAC-3′ for ER-α and 5′-TCACATCTGTATGCGGAACCT-3′ and 5′-TAGCGATCTTGCTTCACACCA-3′, 5′-TCACATCTGTATGCGGAACCT-3′ and 5′-TAGCGATCTTGCTTCACACCA-3′, and 5′,5′-CGCTAGAACACACCTTACCTG-3′ and CTGTGACCAGAGGGTACAT-3′ for ER-β.

### 2.3. Mast cell activation experiments

The RBL-2H3 cells harvested by trypsinization, and HMC and BMMC cells by centrifugation were distributed into 96- or 24-well flat bottom plates, cultured for 2 days in estrogen-free medium to allow damaged membrane receptors to be resynthesized, and stimulated with various concentrations of $E_2$ (Sigma–Aldrich) for various times. To examine the effects of cross-linking cell surface IgE on the release of mediators, the RBL-2H3 and BMMC were sensitized for 1 h with 100 ng/ml mouse monoclonal anti-DNP IgE antibody (Sigma–Aldrich) and HMC-1 cells with serum from a patient with house dust mite sensitivity. After washing away unbound IgE, the cells were stimulated with DNP–BSA (10 haptenes/1 carrier molecule) complexes (10 ng/ml, Biosearch Technologies Inc., Novato, CA) or dust mite allergen extract (DM, *Dermatophagoides farinae*, Hollister-Stier, Spokane, WA) in the presence or absence of $E_2$. Patients' sera were obtained according to a protocol approved by the Institutional Review Board of the University of Texas Medical Branch.

### 2.4. Assessment of degranulation by release of the granular protein β-hexosaminidase (β-hex)

Enzymatic assays for β-hex have been used extensively to assess the extracellular release of mast cell and basophil granule contents (Dastych et al., 1999). Cells ($2 \times 10^4$) that had been cultured for 2 days in estrogen-free medium were stimulated by changing the media to 200 μl of Tyrode's buffer (Dastych et al., 1999) containing various stimuli. β-hex release was quantified as previously described, (Dastych et al., 1999) using *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (8 nM, Sigma–Aldrich) as the substrate. The β-hex release into media was expressed as the percentage of the total amount of β-hex originally in the cells as determined

by detergent lysis with Triton X-100. (% specific release = 100 × [experimental β-hex release − spontaneous β-hex release]/total cellular β-hex).

### 2.5. Measurement of $LTC_4$ synthesis and release

Cells ($1 \times 10^5$) were cultured for 2 days prior to stimulation. The LT concentration of each supernatant was quantified using an enzyme immunoassay kit, with anti-$LTC_4$ and $LTC_4$-acetylcholinesterase conjugate, according to the manufacturer's recommendations (Cayman Chemical, Ann Arbor, MI).

### 2.6. Assessing the change in intracellular $Ca^{2+}$ content by confocal microscopy

Imaging of intracellular $Ca^{2+}$ fluxes was performed as previously described (Suzuki et al., 2003). Briefly, RBL-2H3 cells ($5 \times 10^4$) were plated in the wells of chamber slides (Nalge Nunc Int, Naperville, IL) and cultured for 2 days. After pretreatment with 3 μM Fluo3-AM (Molecular Probes, Eugene, OR) for 30 min, the cells were washed to remove free Fluo3-AM and then stimulated. $Ca^{2+}$ mobility in the cells was measured every 30 s by imaging under a Zeiss LSM laser scanning confocal microscope, using a Zeiss-40 lens (Zeiss, Thornwood, NY). We evaluated the fluorescence of 20 cells from the digital images of three separate experiments saved from each time point, using an LSM 5 Image Examiner (Zeiss).

### 2.7. Use of chemical inhibitors and stimulators

We also examined the effects of ER antagonists tamoxifen (Sigma–Aldrich) and ICI 182,780 (Tocris, Ellisville, MO), $Ca^{2+}$ ionophore A23187 (Sigma–Aldrich), and EGTA (Sigma–Aldrich), an agent which chelates extracellular $Ca^{2+}$, on the degranulation and release of newly formed $LTC_4$ and the changes in intracellular $Ca^{2+}$ concentration.

### 2.8. Statistical analyses

Data were expressed as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance. Where differences between groups were present, they were identified by Scheffe's test. A $p$ value of $<0.05$ was defined as statistically significant. A repeated measures analysis, utilizing restricted maximum likelihood estimation (REML) was used to obtain parameter estimates, using the MIXED procedure in SAS® (Cary, 2000). Each set of measurements from the same batch were considered a correlated cluster of observations. Compound symmetry structures were used when possible for the covariance structure. Between-group comparisons were made using differences of least squares means.

## 3. Results

### 3.1. RBL-2H3, HMC-1 and BMMC cells express mRNA for ER-α, but not ER-β

The amplicons from RT-PCR assays of mRNA from RBL-2H3, HMC-1 and BMMC cells were analyzed by gel electrophoresis (Fig. 1). The results indicate that these cells express mRNA for ER-α, but not ER-β. The negative results for ER-β were confirmed, using multiple sets of primers that amplify segments of the known alternate splicing variants of the ER-β transcripts (results not shown).

### 3.2. Exposure to physiological doses of $E_2$ alone induces the release of substantial amounts of a preformed granular protein β-hex and weakly induces $LTC_4$ synthesis by mast cells

A series of experiments were performed to elucidate the effects of $E_2$ alone and in combination with allergen cross-linking of surface IgE antibodies on mast cells. Synthesis and release of mediators of acute hypersensitivity by RBL-2H3 were assessed. All mediator measurements were performed in duplicate or triplicate and each figure presents the combined data from three independent experiments. A set of repeated measures mixed model fits of the time course data

(Figs. 2A and B, 3A and 6A–C) showed significant group, time and (group × time) interaction effects (all $p < 0.01$), indicating group differences were dependant upon time, and the need to make comparisons between-groups across the time course. The significance of between-group differences, calculated using differences of least square means from the mixed models, are indicated in the figure legends.

Incubating estrogen-starved RBL-2H3 cells with as little as 10 pM $E_2$ induced a statistically significant release of β-hex, within 5 min (Fig. 2A). The time course and dose response of new synthesis and release of $LTC_4$ from RBL-2H3 cells is shown in Figs. 2B and D, respectively. As little as 100 pM $E_2$ induced a significant release of $LTC_4$ by 10 min. The $E_2$-induced β-hex release represented approximately 20% of that released by $Ca^{2+}$ ionophore A23187. While $E_2$ significantly enhanced $LTC_4$ production, compared to control cells, this only represented approximately 1–2% of that induced by $Ca^{2+}$ ionophore (data not shown). Fig. 2E and F shows that the ER antagonist tamoxifen inhibits $E_2$-driven β-hex release.

### 3.3. IgE-mediated β-hex release and $LTC_4$ synthesis are enhanced by $E_2$

Cross-linking IgE anti-DNP antibodies on the surface of RBL-2H3 cells with DNP–BSA complexes (nominal allergen) rapidly induced β-hex release (Fig. 3A). Simultaneous addition of 100 pM $E_2$ with the allergen had an additive effect on the degranulation after 30 min of incubation (Fig. 3A). Despite the small effect of $E_2$ alone on $LTC_4$ synthesis, $E_2$ substantially potentiated the allergen-induced $LTC_4$ synthesis (Fig. 3B).

To elucidate the nature of the $E_2$ effects on allergen-induced degranulation, an allergen dose-response curves were generated on RBL-2H3 cells in the presence and absence of an optimal concentration of $E_2$. The results indicated that the addition of $E_2$ caused a statistically significant shift in the dose–response to DNA–BSA (Fig. 3C).

To confirm these effects of $E_2$ in humans and in primary (non-transformed) mast cells, similar experiments were preformed on HMC-1 cells and BMMCs. The results, shown in Fig. 4A, indicate that physiological concentrations of $E_2$ induced significant release of β-hex from the human mast cell line HMC-1. As in the case of RBL-2H3 cells, simultaneous introduction of $E_2$ and allergen to IgE sensitized HMC-1 cells significantly enhanced the β-hex release (Fig. 4B). Fig. 4C shows that $E_2$ alone induces a dose-dependent release of β-hex from WT BMMC cells.

### 3.4. $E_2$ stimulation of β-hex release and $LTC_4$ synthesis is mediated through an ER-α

Addition of 100 nM of the ER antagonist tamoxifen, 15 min prior to the addition of $E_2$ to RBL-2H3 cells inhibited most of the β-hex release (Fig. 2E) and essentially all of the $LTC_4$ synthesis (Fig. 2F). Similarly, ICI (100 nM), another ER antagonist, blocked $E_2$-induced degranulation of HMC-1 cells (Fig. 4A).

The expression of mRNA for ER-α but not ER-β, in mast cells (Fig. 1) suggested that ER-α might be the isoform involved in these estrogenic effects. We therefore produced BMMCs from ER-α KO mice and tested the ability of $E_2$ to release preformed mediators from these cells. The results, shown in Fig. 4C, indicate that ER-α is required for the $E_2$-induced mediator release, while the responses of the KO BMMCs to $Ca^{2+}$ ionophore and allergen were similar to those of the BMMCs from WT animals (data not shown).

### 3.5. $E_2$ enhances $Ca^{2+}$ ionophore-mediated degranulation and $LTC_4$ synthesis

One mechanism that is common to the release of preformed mediators from mast cell granules and the activation of LT synthesis is an increase in intracellular $Ca^{2+}$ concentration. In addition, estrogens have been shown to rapidly increase intracellular $Ca^{2+}$ in other cells types via a

membrane-associated, specific ER (Bulayeva et al., 2005; Wozniak et al., 2005). Thus, we evaluated the effects of $E_2$ on the activation of RBL-2H3 cells by several different concentrations of the $Ca^{2+}$ ionophore A23187. $E_2$ significantly enhanced the effect of a sub-optimal concentration ($10^{-7}$ M) of A23187 on the release of β-hex and $LTC_4$ (Fig. 5). As anticipated, $E_2$ did not enhance the effects of an optimal concentration ($10^{-5}$ M) of A23187 (data not shown). These results support the concept that intracellular $Ca^{2+}$ may be an important intermediate in the estrogenic effects on mediator release by mast cells.

### 3.6. $E_2$ alone increases intracellular $Ca^{2+}$ in RBL cells and potentiates the effects of IgE cross-linking on intracellular $Ca^{2+}$ concentrations

To directly evaluate the effects of $E_2$ on intracellular $Ca^{2+}$ concentration, RBL-2H3 cells were incubated with 100 pM $E_2$ and the fluorescence of the intracellular $Ca^{2+}$ dye Fluo 3-AM was monitored in individual cells. As shown in Fig. 6A, $E_2$ alone significantly increased the intracellular $Ca^{2+}$ after as little as 2.5 min incubation. The $Ca^{2+}$ concentration increased progressively for at least 30 min in the experiments shown (Fig. 6B) and continued for at least 60 min (data not shown). Preincubation of cells with tamoxifen (100 nM) completely prevented the increase in intracellular $Ca^{2+}$ (Fig. 6A), indicating that this prolonged effect of $E_2$ was mediated by an ER.

The effects of IgE cross-linking by allergen on intracellular $Ca^{2+}$ are shown in Fig. 6B. In contrast to the effect of $E_2$, IgE cross-linking alone caused a more rapid increase in intracellular $Ca^{2+}$, which returned to basal levels within 10 min, as previously described by others (Ishizaka et al., 1980;Smith et al., 1996). The combination of 100 pM of $E_2$ and IgE cross-linking increased both the early transient and prolonged increase in intracellular $Ca^{2+}$ concentration. However, the combination of these stimuli had more than an additive effect on the progressive increase in $Ca^{2+}$. Fig. 6B shows the first 30 min of this progression. $E_2$ enhanced the early phase of the $Ca^{2+}$ rise after 3 min and IgE cross-linking enhanced the prolonged rise in $Ca^{2+}$ seen in cells treated with the $E_2$ alone. The gradual rise in intracellular $Ca^{2+}$ concentrations continued for at least 60 min (data not shown).

### 3.7. Extracellular $Ca^{2+}$ is required for $E_2$ to induce and potentiate degranulation and $LTC_4$ synthesis

To distinguish between intracellular and extracellular pools as the source of $Ca^{2+}$ for the $E_2$-induced increase in intracellular free $Ca^{2+}$, EGTA a chelator of extracellular $Ca^{2+}$ was added to the extracellular media. The data shown in Fig. 6C indicate that EGTA completely abrogated the $E_2$-induced slow rise in intracellular $Ca^{2+}$, but not the early, rapid IgE-mediated $Ca^{2+}$ flux. This finding allowed us to determine whether the slow increase in intracellular free $Ca^{2+}$ is required for $E_2$ to induce the synthesis and release of the mast cell mediators. The addition of EGTA to the culture media containing $E_2$ reduced the estrogenic stimulation of β-hex release and $LTC_4$ synthesis to the level of unstimulated cells, as shown in Fig. 7A and B. EGTA also significantly decreased IgE-mediated β-hex release and $LTC_4$ synthesis (Fig. 7C and D). However, the residual release with IgE cross-linking was still significantly higher than that from unstimulated controls. Thus, the data shown in Figs. 6C, 7C and 7D are consistent with the current concept that IgE cross-linking causes a rapid release of $Ca^{2+}$ from an intracellular pool, followed by recruitment of extracellular $Ca^{2+}$ channels, leading to influx of extracellular $Ca^{2+}$. $Ca^{2+}$ from both pools is required for full mediator release. In contrast, $E_2$'s effects on mediator release seem to be completely dependent on an influx of extracellular $Ca^{2+}$.

## 4. Discussion

The results of our experiments demonstrate that physiological concentrations of $E_2$ rapidly stimulate estrogen-starved, murine and human mast cell lines (RBL-2H3 and HMC-1) and

primary cultures of bone marrow mast cells (BMMCs) to release β-hex, a marker for the granules that contain preformed allergic mediators. The synthesis and release of $LTC_4$ by RBL-2H3 cells was also stimulated by exposure to $E_2$. The finding that tamoxifen inhibited these effects suggested that they were mediated through specific α or β ERs. Since we found mRNA for ER-α, but not ER-β in all of these mast cells, it was likely that ER-α was involved in this estrogenic effect on mast cells. This proposition was substantiated by the demonstration that BMMC from ER-α KO mice did not degranulate in response to $E_2$. In addition to these direct effects, $E_2$ also enhanced IgE-dependent synthesis and release of β-hex and $LTC_4$. However, the interaction of these two stimuli on the release of these two types of mediators seemed to differ quantitatively. $E_2$ alone induced the release of a substantial proportion of preformed mediators and was additive with the IgE-dependent release. In contrast, $E_2$ alone had a statistically significant, but small effect on the synthesis and release of $LTC_4$. However, $E_2$ strongly potentiated the effects of IgE cross-linking on $LTC_4$ release.

Our study also provides insight into the mechanisms of the estrogenic effects on the synthesis and release of allergic mediators from mast cells. While our results demonstrate that the effects of $E_2$ on mast cells require the specific ER-α, the rapid onset of $E_2$'s effects on mast cell activation indicates that it does not function through the classical (genomic) mechanisms, which require enhanced mRNA and protein synthesis over a 2 h or longer period (Simoncini et al., 2004). Rather our findings suggest that $E_2$'s effects on mast cell mediator release are through a membrane-associated (non-genomic) form of ER-α. This type of ER stimulation has been shown to induce rapid cellular responses through G-protein activation, (Bulayeva et al., 2004; Collins and Webb, 1999; Doolan et al., 2000; Improta-Brears et al., 1999; Stefano et al., 1999; Watson et al., 1999) which can include various signaling pathways involving $Ca^{2+}$ fluxes, and modulation of mitogen-activated protein kinases and adenyl cyclase (Bulayeva et al., 2004; Collins and Webb, 1999; Doolan and Harvey, 2003; Nadal et al., 2000; Song et al., 2004; Zivadinovic et al., 2005). Indeed, we found that intracellular $Ca^{2+}$ was increased within the first 2.5 min after estrogenic stimulation of RBL. However, in contrast to the rapid rise of intracellular $Ca^{2+}$ induced by IgE cross-linking, the $E_2$-induced rise was completely inhibited by the extracellular $Ca^{2+}$ chelator EGTA. EGTA also reduced the release of preformed mediators and substantially reduced new synthesis of mediators, indicating that the effects of $E_2$ we observed depend on the influx of extracellular $Ca^{2+}$.

We were also interested in understanding the mechanisms underlying the additive and synergistic effects of estrogens on the IgE-mediated release of preformed mediators and synthesis and release of LTs, respectively. Our results suggest that intracellular $Ca^{2+}$ concentrations may explain, at least in part, these observations. The combination of $E_2$ and IgE cross-linking not only increased the amplitude and duration of the early $Ca^{2+}$ peak, but also caused a progressive rise in intracellular $Ca^{2+}$ (not seen with IgE cross-linking alone). Addition of EGTA to the media abrogated the estrogenic effects on the early rise and resulted in a progressive fall in intracellular $Ca^{2+}$ thereafter (data not shown). While the differential effects of intracellular $Ca^{2+}$ on preformed (β-hex) and newly synthesized ($LTC_4$) mediators are not explained directly by our results, it is interesting to speculate that the greater and more prolonged increase in intracellular $Ca^{2+}$ is more effective in supporting the translocation of cytosolic phospholipase $A_2$ ($cPLA_2$) to the nuclear membrane (Durstin et al., 1994; Pouliot et al., 1996). This translocation has been shown to increase $PLA_2$ activity and subsequent arachidonic acid turn-over after estrogenic stimulation (Fiorini et al., 2003; Wang et al., 2001). However, these same conditions may also enhance the lipoxygenase activity required to convert arachidonic acid to LTs. In addition, the other steps involved in the exocytosis of preformed peptides (movement of vesicles to the membrane, docking of the vesicles at membrane sites) may involve estrogenic effects other than $Ca^{2+}$ elevation.

This study is the first to show that: (1) physiological concentrations of $E_2$ alone induce mast cells to release a substantial component of their preformed mediators and potentiate the IgE-dependent synthesis and secretion of a potent LT, (2) $E_2$'s effect on mast cells is mediated by a non-genomic effect of ER-α and (3) these $E_2$ effects require influx of extracellular $Ca^{2+}$. Perhaps, our finding with the greatest potential clinical relevance was that the addition of $E_2$ significantly shifted the antigen dose response curve for mediator release. These findings may help to explain why women during their peak reproductive years have an increased incidence of asthma, and more specifically why women with PMA have increased concentrations of $LTC_4$ in their blood during this phase of their menstrual cycle, when estrogens (and progesterone) concentrations are rapidly changing. Interestingly, the most active concentration of $E_2$ (100 pM) in our cell culture media was similar to those in the plasma of women during the perimenstrual phase (Skobeloff et al., 1996).

Given the strict requirement for extracellular $Ca^{2+}$ for the estrogenic effects on mediator release, blocking one or more types of $Ca^{2+}$ channels on basophils and mast cells might abrogate estrogen's effects on the release of allergic mediators. This approach might improve the management of asthma in women. However, a more complete understanding of the effects of $E_2$, other estrogenic compounds (e.g. dietary estrogens and environmental estrogens) and other female hormones on each step of mast cell activation may suggest other therapeutic approaches for allergic diseases.

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## Abbreviations

**β-hex**
: β-hexosaminidase

**BMMC**
: bone marrow derived mouse mast cells

**DM**
: dust mite

**$E_2$**
: estradiol

**ER**
: estrogen receptor

**HMC**
: human mast cell line

**KO**
: knockout

**LT**
: leukotriene

**PBS**
phosphate buffered saline

**PMA**
perimenstrual asthma

**RBL-2H3**
rat basophilic cell line-2H3

**WT**
wild type

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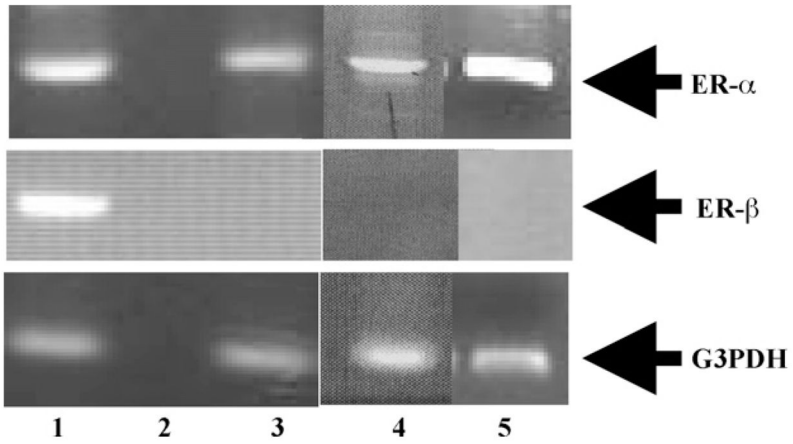


**Fig. 1.**

Expression of mRNA for ER-α in RBL-2H3, HMC-1 and BMMC cells; RT-PCR analysis of total RNA isolated from each of the cells. Lane 1: rat ovary = positive control; lane 2: no RNA = negative control; lane 3: RBL-2H3, lane 4: HMC-1 and lane 5: BMMCs.

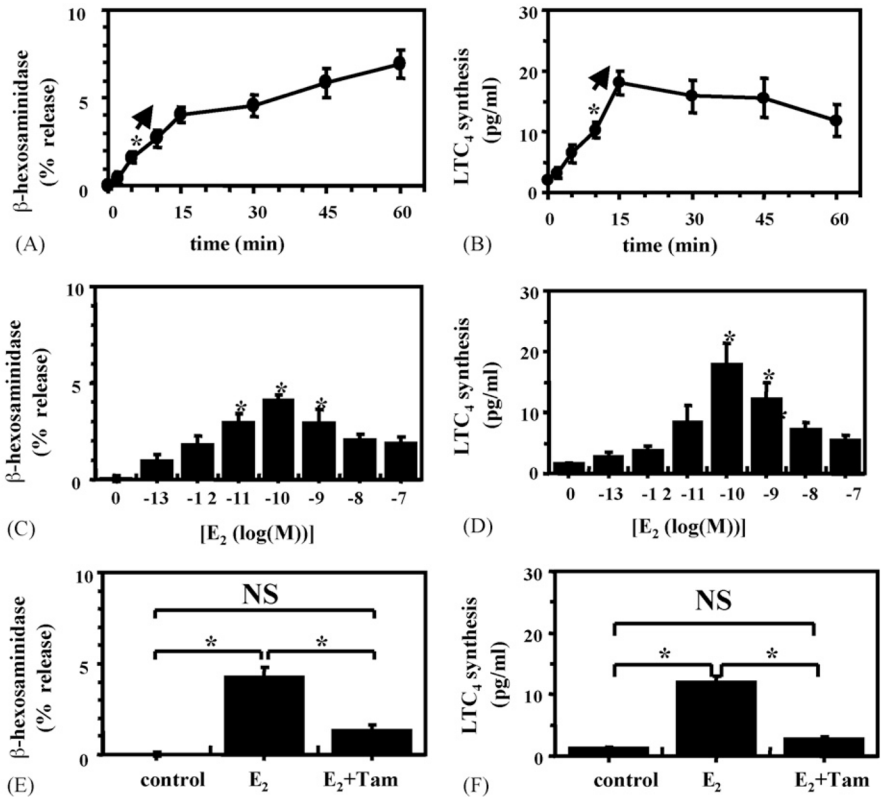


**Fig. 2.**

$E_2$ promotes rapid β-hex release and $LTC_4$ synthesis on RBL-2H3 cells: (A, C and E) represent β-hex release and (B, D and F) $LTC_4$ release; (A and B) show time course after the addition of 100 pM $E_2$; (C and D) dose responses 15 min after $E_2$ addition; (E and F) effects of tamoxifen pretreatment. *$p < 0.05$ vs. control. Tam = tamoxifen, NS = not significant.

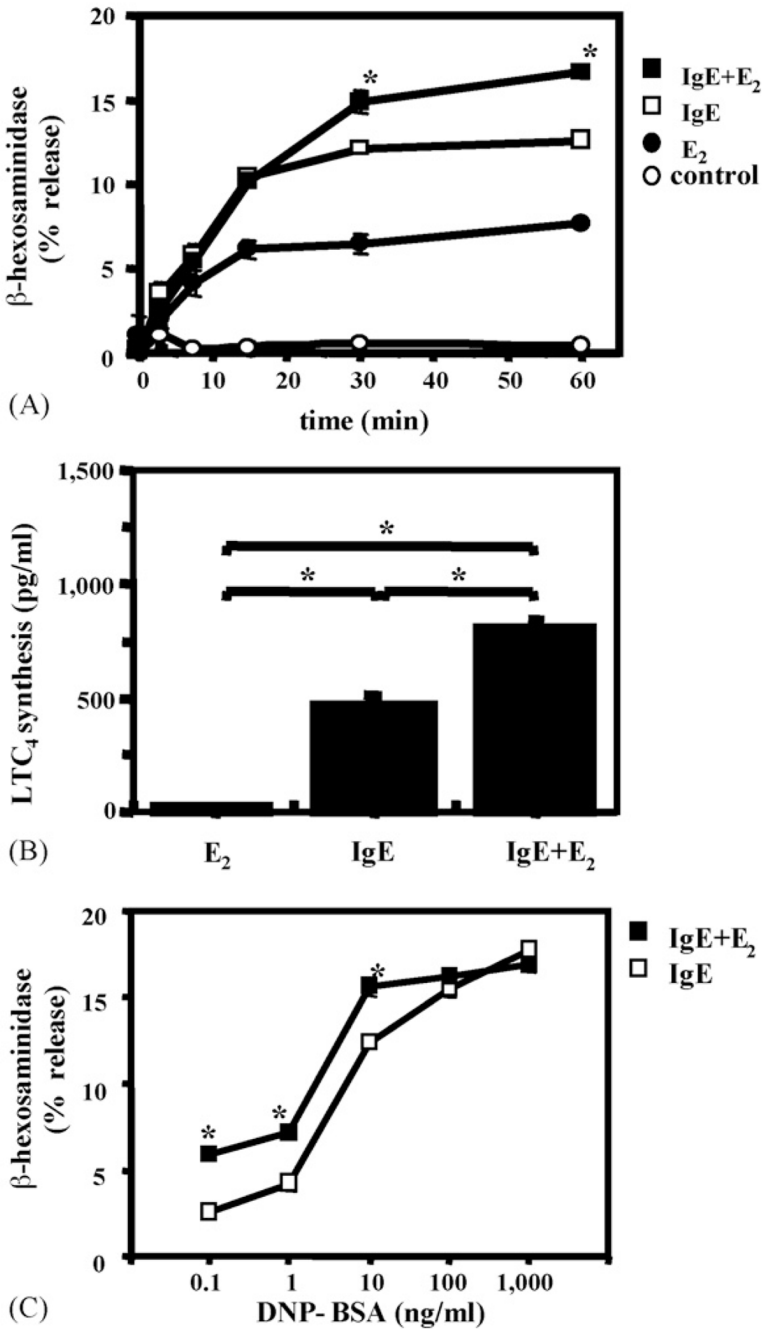


**Fig. 3.**

$E_2$ enhances IgE-mediated β-hex release and potentiates $LTC_4$ synthesis from RBL-2H3 cells: (A) time course of the effect of 100 pM $E_2$ on β-hex release by IgE + allergen. $^{*}p < 0.05$ for the effect of $E_2$ at time points indicated; (B) effect of $E_2$ on $LTC_4$ synthesis. $*p < 0.05$ for the $E_2$ effects. IgE = IgE anti-DNP + DNP–BSA; (C) dose response of $E_2$ effects on β-hex release. $^{*}p < 0.05$.

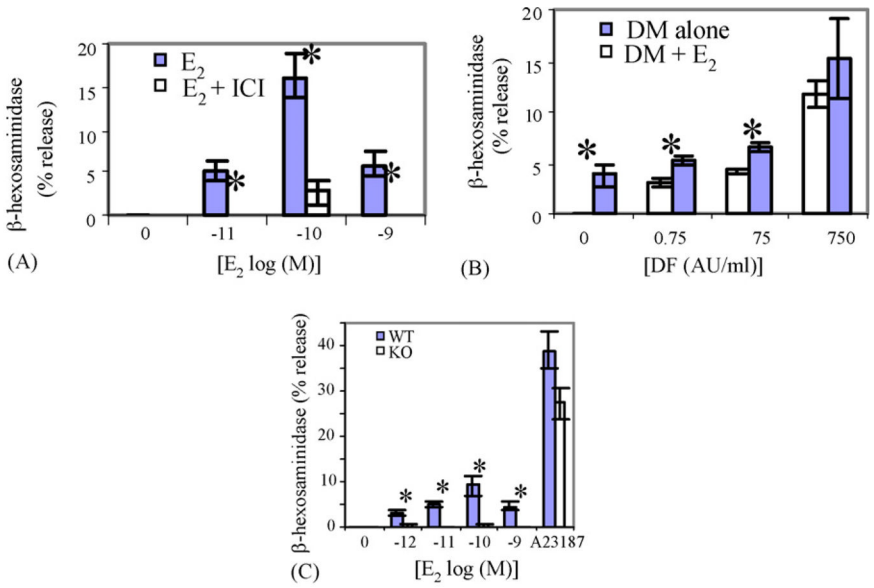


**Fig. 4.**

$E_2$ promotes release of β-hex from HMC-1 and BMMC: (A) dose response for $E_2$ on β-hex release from HMC-1 and inhibition by ICI182,780. *$p < 0.05$ vs. no $E_2$; (B) dose response of DM-induced β-hex release from HMC-1, with and without $E_2$. *$p < 0.05$ with and without $E_2$; (C) dose response of $E_2$ on β-hex release from WT and ER-α KO BMMCs. *$p < 0.05$ between WT and KO.

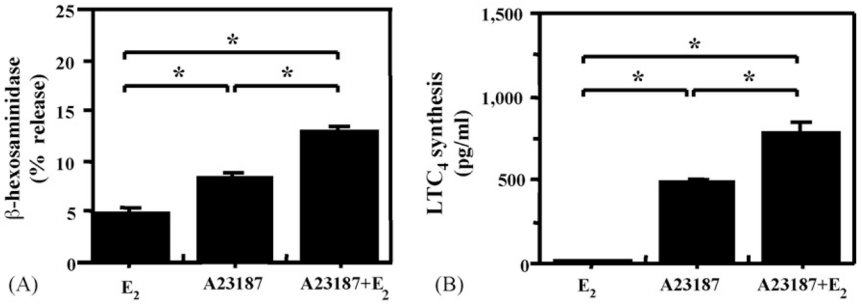


**Fig. 5.**

$E_2$ potentiates the effects of sub-optimal concentrations of A23187 on the release of-β-hex and $LTC_4$ synthesis; RBL-2H3 cells were incubated with 100 pM $E_2$ alone, $10^{-7}$ M A23187 alone or the combination of the two followed by measurement of: (A) β-hex release; (B) $LTC_4$ release in the supernatant 15 min later. $^{s}p < 0.05$ between indicated groups.

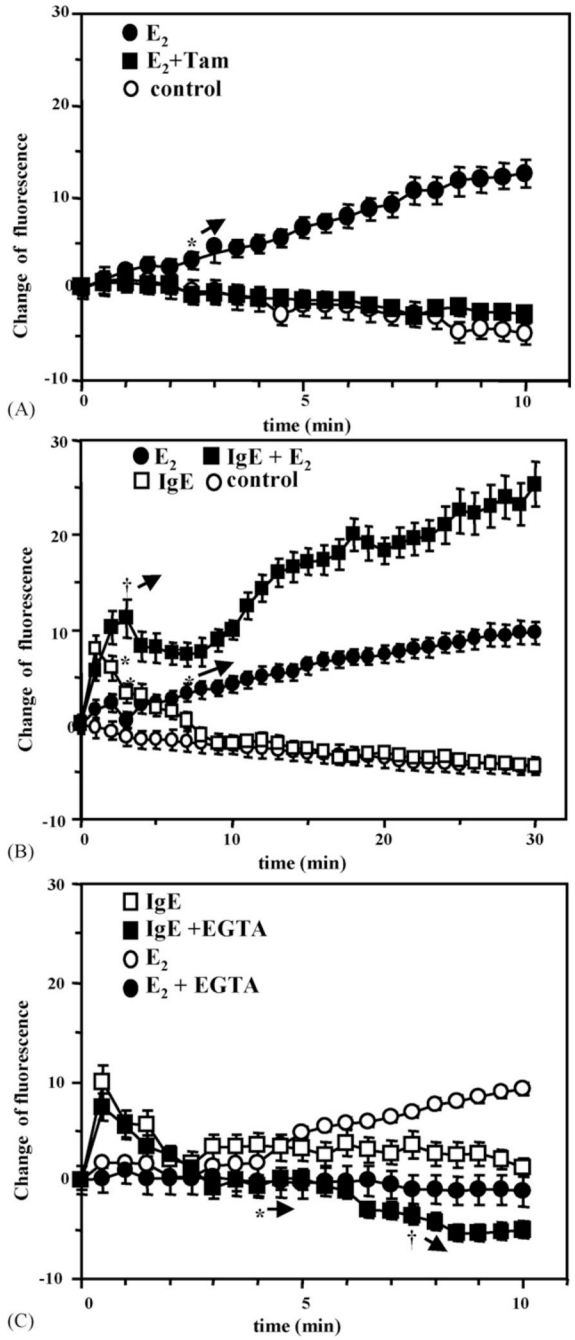


**Fig. 6.**

$E_2$ increases intracellular $Ca^{2+}$ and potentiates the effects of IgE cross-linking on mobilization of $Ca^{2+}$ from RBL-2H3 cells: (A) $E_2$ ± tamoxifen (Tam) on intracellular $Ca^{2+}$ influx. $^{*}\rightarrow$: this and subsequent time points were different from the controls ($p < 0.05$); (B) $E_2$ on allergen cross-linking of IgE. $^{*}p < 0.05$ $E_2$ vs. control. $^{\dagger}p < 0.05$. IgE vs. IgE + $E_2$; (C) EGTA on $E_2$-induced influx of intracellular $Ca^{2+}$ after IgE cross-linking. $^{*}p < 0.05$ between $E_2$ vs. $E_2$ + EGTA, $^{\dagger}p < 0.05$ between IgE and IgE + EGTA.

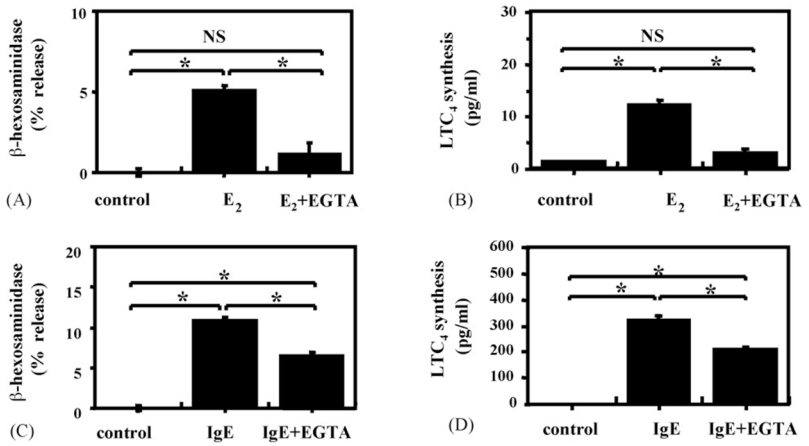


**Fig. 7.**

Addition of EGTA inhibits $E_2$-stimulated β-hex release and $LTC_4$ synthesis from RBL-2H3 cells: (A and B) effects of EGTA on RBL-2H3 stimulated with $E_2$; (C and D) EGTA effects on cells incubated with anti-DNP-IgE, followed by DNP–BSA; (A and C) β-hex release; (B and D) $LTC_4$ synthesis were measured in the supernatants. $^{*}p < 0.05$ between indicated groups. IgE = IgE anti-DNP + DNP−BSA, NS = not significant.