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Estrogen receptors in mast cells from arterial walls

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ABSTRACT: We examined the presence of estrogen receptors (ER) in vascular mast cells and a possible genomic effect of estrogens on the expression of mast cell (MC) mediators such as chymase, TNFα, NOS and IL-10, which are known to affect the course of atherosclerosis. Immunocytochemical detection of mast cell tryptase and the co-localization of ERs in MCs from abdominal aortic vessels from 10 fertile woman, 10 postmenopausal women and 15 men was performed. The genomic expression of IL-10, TNFα, and NOS was analyzed by RT-PCR and chymase activity by spectrophotometry after 24 h incubation with 17-β estradiol (0.2-0.5 ng/mL) in rat purified peritoneal MCs.

A similar number of MCs were found in both intima and adventitia layers from men, and fertile and post-menopausal women, while ERs were detected only in the arterial walls from fertile women. The mRNA expressions of IL-10 and TNFα, as well as chymase activity, were not affected. A moderate increment of NO and both NOS, and a reduction in TNFα cytotoxicity was observed after incubating peritoneal MCs with 17-β estradiol at a concentration of 0.5 ng/mL. Taken together, these results indicate that vascular MCs express ERs. The data demonstrate that estrogens can directly modify vascular MC activity. This is a novel mechanism of synergistic cooperation for the protective role of estrogens in the genesis of atherosclerosis.

Introduction

The development of postmenopausal atherosclerosis has been convincingly linked to estrogen withdrawal caused by ovarian insufficiency. Estrogen replacement therapy can prevent heart disease in postmenopausal women through a mechanism not completely understood (Kannel *et al.,* 1976; Hayashi *et al.,* 1995). The benefits of estrogens include the ability to favorably alter the lipoprotein profile, i.e. to increase high-density lipoproteins and to decrease low-density lipoproteins (LDL) and prevent oxidative modification of LDL (Haarbo *et al.,* 1991; Tang *et al.,* 1996). Estrogens also decrease the expression of adhesion molecules involved in monocyte attachment to vascular endothelium and of certain chemokines that favor monocyte migration into the subendothelial space (Goldstein *et al.,* 1979; Frazier-Jessen and Kovacs, 1995).

MCs are strategically positioned in the human arterial intima. The effect that MC mediators have on the vasculature suggests that they could be key cells in inducing vascular changes during physiological as well as pathological processes. MCs have been found in the intima of large and small blood vessels (Kubes and Granger, 1996) and in close vicinity to cholesterol-loaded macrophage foam cells in fatty streaks. They produce chymase, a specific MC serine protease that modifies LDL causing cholesteryl ester accumulation in macrophages. Therefore, MCs may play an active role in the intracellular deposition of these lipids into the atherosclerotic lesions through an increase in the delivery of chymase (Kovanen, 1993).

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ERs regulate the expression of MC mediators in bladder and uterine tissue (Theoharides, 1996; Cocchiara *et al.,* 1992). Therefore, the presence of estrogens may determine whether MCs will have a pro- or an anti-inflammatory action (Hunt *et al.,* 1997; Jeriorska *et al.,* 1995; Spanos *et al.,* 1996). Together with a number of other pro-inflammatory cytokines, TNFα has been demonstrated to be involved in the early stages of atherosclerosis. TNFα induces upregulation of VCAM-1 and selectins in endothelial cells and also to activate their receptors located on leukocytes, which permit adhesion to endothelium and entry of monocytes and T lymphocytes into the intima to form the initial lesions of atherosclerosis (McHale *et al*., 1999). TNFα is also involved in the chemotaxis of smooth muscle cells from the underlying media into the intima of the artery, thereby favoring atherosclerosis (Sherry and Cerami, 1988). Activated monocytes and tissue macrophages were originally thought to be the principal source of TNFα. However, some studies have shown that only MCs store significant amounts of TNFα and are thus able to release this mediator immediately upon stimulation (Gordon and Galli, 1990). Moreover, the amount of mRNA for TNFα is a thousand times higher in unstimulated MCs as compared to macrophages, which makes MCs the most important source for rapid release of this cytokine (Dery *et al.,* 2000).

Nitric oxide (NO) is a prominent feature of many physiological vascular events. NO produced in the endothelium participates in maintaining homeostasis in the normal artery, and plays a major role in the maintenance of vascular tone. NO modulates the early events in the development of atherosclerosis. It provides an anti-atherosclerotic effect by producing vascular dilatation and inhibiting monocyte adhesion to the endothelium (Gerrity, 1981). It also enhances Th-2 cell function, which may shift the balance of cytokine profiles towards a Th-2 type-reactivity (Kolb and Kolb-Bachofen, 1998). Little is known about the nitric oxide synthase (NOS) isoforms in MCs and further characterization is necessary. Molecular and biochemical studies have demonstrated that MCs express both NOS-2 and NOS-3 (Bissonette *et al.,* 1991). 17-β estradiol increases the activity of NOS-3 in endothelial cells *in vitro* but inhibits NOS-2 in macrophages (Hayashi *et al.,* 1997). Therefore, it seemed relevant to analyze the action of estrogens on both NOS expression in the model of rat peritoneal MCs *in vitro*.

IL-10 is produced by multiple cell types playing an important role as a natural damper of immune and inflammatory reactions. It modulates the expression of soluble mediators and cell surface molecules on cells of myeloid origin. It is known as an anti-inflammatory cytokine, since it strongly inhibits production of pro-inflammatory cytokines and chemokines by activated monocytes/macrophages (Fiorentino *et al.,* 1991; de Waal *et al.,* 1991). In addition, IL-10 upregulates the expression of soluble p55 and p75 TNFα receptors inhibiting the production of NO and TNFα by mouse macrophages and stabilizing MCs (Hart *et al.,* 1996).

ERs were detected in human arterial MCs by immunocytochemistry. The possible action of estrogens in MCs was studied through the expression of two early atherogenic mediators, chymase and TNFα (Lopez-Virella and Virella, 1992; Kovanen, 1993). Expression of IL-10 both NOS and NO production, which may be involved in minimizing the risks of atherosclerosis, were also examined (Marietta *et al.,* 1996; Pinderski *et al.,* 1999).

MCs are found in small numbers within the vessels thereby complicating their purification for further *in vitro* studies. Some studies involving MCs in atherogenesis have been made *in vitro* using rat serosal MCs, mouse macrophages and isolated human LDL. In that model, MCs induced the formation of macrophage foam cells resembling those typical of atherosclerotic lesions (Kovanen, 1991; 1993). In our study, we used rat peritoneal MCs, the rodent model of a connective tissue-type MC that corresponds to human MCs containing both tryptase and chymase (Kaartinen *et al*., 1994). It is known that rat peritoneal fluids have a large number of MCs that express ER, therefore both genomic and non-genomic effects of estrogens can be observed (Cocchiara *et al.,* 1992).

No studies have been done regarding the presence of ERs in arterial MCs, or how estrogens may affect the expression or release of some mediators that affect the atherosclerotic process. This work was undertaken to analyze if human arterial MCs express ERs, and if MCs are somehow involved in the protective role that estrogens display in atherosclerosis.

Materials and Methods

Autopsy material

Abdominal aortic arteries of 35 patients from 10 fertile women, 10 postmenopausal women and 15 men were used. The autopsy material was fixed in Carnoy`s fluid (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 24 h and then cut into 4 mm pieces which were embedded in paraffin.

Animals

Sprague-Dawley rats weighing 250-300 g were used. They were maintained under a dark/light cycle (12 h dark/ 12 h light) in a controlled temperature room (24-25ºC) with access to drinking water and laboratory food.

Immunocytochemistry

Four μm paraffin sections were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 10 min. The sections were then treated with proteases and incubated with the primary antibody. One serial section was incubated with an anti-tryptase monoclonal antibody AA1 (1:25) to identify MCs, while the other serial section (sequential tissue) was incubated with a specific monoclonal antibody 1D 5 (1:50) to identify ERs (both from Dako Corporation, CA, USA). Staining was achieved according to the indirect immunoperoxidase method. The slides were incubated in an avidin-biotin-peroxidase complex (Dako Corporation, CA, USA) for 30 min and the reaction was developed with 0.5 mg/mL 3-3` diaminobenzidine tetrahydrochloride (Sigma, St Louis, USA) and 0.1 mL of 0.3% H_2O_2 in 50 mM Tris-HCl, pH 7.6. Sections were counter-stained with hematoxylin, dehydrated and mounted.

Mast Cells

MCs were obtained by peritoneal lavage of both male and female rats with a phosphate-buffered saline (PBS) solution containing 130 mM NaCl, 5 mM NaH_2PO_4 and 5 mM Na_2HPO_4, pH 7.4. MCs were sedimented by centrifugation at 150 g for 5 min and then purified by recovering the pellet fraction after a 72.5% isotonic sterile Percoll (Pharmacia, Uppsala, Sweden) centrifugation. MCs comprised approximately 1% of unfractioned cells and ≥ 99% of cells after density gradient fractionation, as determined by metachromatic staining in 0.1% acidic toluidine blue. Purified peritoneal MCs were resuspended in Dulbecco's Modified Eagle's Medium containing 0.1% BSA without phenol red and serum. The cells were cultured for 24 h (37ºC, 5% CO_2 and 95% O_2) either in the presence or in the absence of 17-β estradiol (0.2-1.0 ng/mL). MC viability was not affected after culturing the cells with estradiol for 24 h at concentrations up to 0.8 ng/mL, as assessed by staining with 0.4% trypan blue.

Mast Cell Counting and Histamine Measurement

In order to determine the number of MCs, aliquots of the peritoneal MC primary culture were stained with 0.1% toluidine blue disolved in 0.7 N HCl. Histamine concentration from the supernatants was assayed by high performance liquid chromatography coupled with fluorometry as already described (Yamatodani *et al*., 1982; Rudolph *et al*., 1997).

Isolation of mast cell granules

MC granules were isolated and degranulated at 37°C with 1 ug/ml Compound 48/80. After 15 min, they were centrifuged at 12,000 g for 15 minutes to sediment the remnant granules.

Chymase solution assays

Chymase-like activity was analyzed by the addition of 50 μl of sample aliquots to an assay buffer containing 1 mM of the substrate succinyl-L-Ala-Ala-Pro-Phe-4-nitroanilide (Sigma), 1.8 M NaCl, and 9% dimethylsulfoxide in 0.45 M Tris-HCl, pH 8.0. Changes in the absorbance at 410 nm were monitored spectrophotometrically at 37°C (Coussens *et al.,* 1999).

Measurement of NO production

The concentration of nitrite, a metabolite of NO and marker of NO production was determined spectrophotometrically after reduction of nitrate with hydrazine (Green *et al*., 1982). Samples of 500 μl aliquots deproteinized with 1M NaOH were incubated for 15 min at 25°C with 1 mL of Griess's reagent (1% sulfanilamide in 2.5% H_3PO_4), and the absorbance was read at 543 nm according to a calibration curve with $NaNO_2$ (1-100 μM) (Okhawa *et al.,* 1979).

Detection of cytokine and NOS-2 mRNAs by RT-PCR analysis

Total RNA was extracted by the guanidinium-phenol-chloroform method (Chomczynski and Sachi, 1987). The yield and RNA purity were estimated spectrophotometrically at 260 and 280 nm. Total RNA (3 μg) was used for the generation of cDNA using a deoxythymidine primer of 20 nucleotides and 45 U avian myoblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). A preliminary RT-PCR

experiment, in which RNA concentrations from 100 ng to 1 μg were used, revealed that within this range of concentrations the intensity of the band was linearly related to the amount of the starting RNA. Routine protocols were followed to avoid RNAse contamination of glassware and chemicals. PCR amplification reactions for TNFα, NOS and IL-10 mRNA were performed on aliquots of cDNA (equivalent to 350 ng of starting RNA).

For TNFα target, the primers were designed from a rat TNFα cDNA sequence (Williams and Coleman, 1995). The sequences were 5` - CCA CGT CGT AGC AAA CCA CCA AG - 3` and 5`-CAG GTA CAT GGG CTC ATA CC-3`, which amplified a 316 bp fragment. NOS-2 primers were designed from the mouse cDNA sequence reported by Xie *et al.* (1992), and their sequences were sense primer 5`-AGG AGG ATG CCT TCC GCA GCT G-3` and antisense primer 5`-CTG GGA GCT GAT GGA GTA GT-3`, which amplified a 700 bp fragment. For IL-10 target, the primers were 5´-TGC CAA CCC TTG TCA GAA ATG ATC AAG -3´ and 5´-GTA TCC AGA GGG TCT TCA GCT TCT CTC -3´, which amplified a 127 bp fragment and were derived from a partial cDNA sequence for rat IL-10 (Noble *et al.,* 1993). β-actin was used as an invariant control sequence in the PCR reactions to correct for reaction-to-reaction variation in amplification efficiency. For β-actin target, the primers were 5`- AGA AGA GCT ATG AGC TGC CTG ACG -3` and 5`-CTT CTG CAT CCT GTC AGC CTA CG -3`, which amplified a 236 bp fragment and was derived from the rat beta-actin sequence (Nudel *et al.,* 1983).

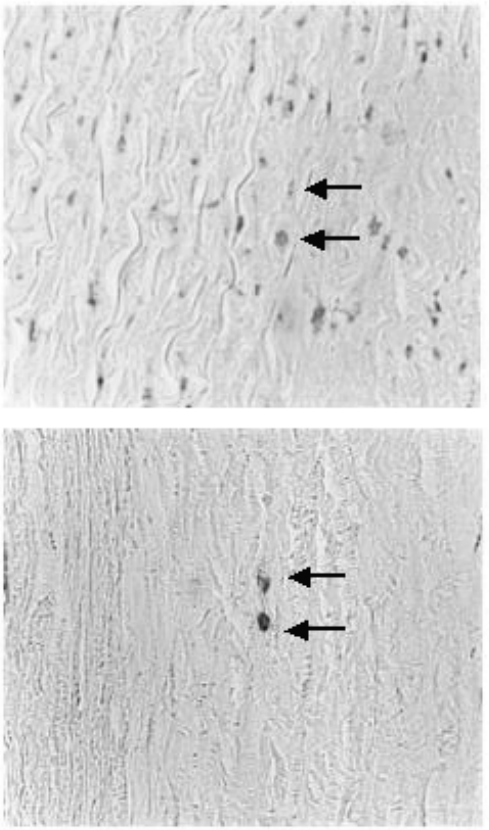

FIGURE 1. Immunohistochemical staining of ER (A) and tryptase activity (B) in MCs present in aortic vessels. ERs were detected in the nucleus of most cells, including MCs (arrows) in sections of aortic vessels from fertile women. (B) A sequential section that was stained for tryptase (arrows) confirmed the presence of ERs in MCs (X 40).

All PCR reactions were performed in a final volume of 50 μl using a PCR buffer containing 50 mM KCl, 50 mM Tris-HCl, pH 8.4 (Promega, Madison, WI, USA) that contained 5 μg/mL of each primer, 1 μl of 10 mM dNTPs, 2.5 U Taq polymerase (Gibco BRL, NY, USA) and 1.5 mM $MgCl_2$. For TNFα, the PCR profile consisted of one denaturation cycle at 94ºC for 2 min followed by 35 denaturation cycles at 94ºC (45s), annealing at 60ºC (45s), and extension at 72ºC (30s). For NOS-2, an initial denaturation was performed at 94ºC for 1 min followed by 29 denaturation cycles at 94ºC (45s), annealing at 50ºC (45s), and extension at 72ºC (30s). For IL-10 PCRs an annealing temperature of 70ºC was used instead of 60ºC. Aliquots of reactions were run on 1.5% agarose gels in TAE buffer (50 mM Tris-HCl, 0.1% acetic acid, 0.1 mM EDTA, pH 8.0) stained with ethidium bromide. Photographs were taken under UV illumination on Polaroid 667 film.

TNF-α Analysis

Peritoneal MCs incubated for 1 h in RPMI 1640 medium without phenol red were gently washed, resuspended in fresh medium and treated with 0.5 ng/mL 17-β estradiol for 24 h. The cell free supernatants were collected at different times from independent wells. The amount of TNFα released was quantified by an ELISA assay (Gordon and Galli, 1990). Cytotoxic activity in L929-TNFα sensitive cells was measured in the following way: L929 cells were added to different sample dilutions of the supernatant and incubated for 18 h. Crystal violet 0.5%, which stains remnant surviving cells, was then applied for 15 minutes. At the end of the incubation, colorimetric intensity at 570 nm was measured on an ELISA reader (Bio-Tek, Winooski, Vermont).

Assay for Lipid peroxidation

Malondialdehyde, a product of lipid peroxidation, was determined. Briefly, homogenized peritoneal MCs were combined with a solution containing thiobarbituric acid, which reacts with malondialdehyde giving thiobarbituric reactive substances (TBARS) that were measured spectrophotometrically at 532 nm (Vega *et al.,* 1999).

Western blot analysis of NOS-2 and NOS-3

Specificity of the rabbit antibody to NOS-2 and NOS-3 was verified using Western blot analysis. Proteins were obtained from rat peritoneal MC pellets incubated for 24 h in medium that contained 0.5 ng/mL 17-β estradiol. Cells were solubilized in a loading buffer containing 15% glycerol, 2% SDS and 1% 2-mercaptoethanol in 125 mM Tris-HCl, pH 6.8. Proteins were separated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis using 7.5% resolving gels and blotted on nitrocellulose membranes (Scheicher and Shuell, Kenne, NH). Blots were blocked for 1 h with 1% non-fat dry milk in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.6 (TBS) at room temperature. They were then incubated for 2 h at room temperature with the mouse monoclonal antibody against either NOS-2 or NOS-3 (Santa Cruz Biotechnology, Inc., CA, USA, 1:200 and 1:1000, respectively) in TBS containing 0.5% gelatin for NOS-2 and 0.2% BSA. Bound antibody was detected using a rabbit IgG mouse antibody conjugated with alkaline phosphatase. The blots were washed in TBS-BSA and immunoreactive proteins were visualized with BCIP/NBT according to the manufacturer's instructions. Pre-stained protein markers were used for molecular mass determinations. The nitrocellulose membrane was scanned by an optical scanner (Storm; Molecular Dynamics), and the signals were quantified using digital image analyzing software (ImageQuaNT; Molecular Dynamics).

Data Analysis

The data are expressed as the mean ± SD for individual experiment. One- and two-way ANOVA was performed followed by multiple comparison analysis as appropriate. The results of analysis were considered to be significant when $P \leq 0.05$.

Results

Immunocytochemical analysis in human aortic vessels revealed that MCs were mainly distributed in the arterial intima and adventitia. The cells were comparatively large, with a centrally located nucleus and prominent intracellular granules. A characteristic trait was their location in the shoulders of the atheromas, as previously described by Kaartinen *et al*. (1994). No changes in MC concentration or distribution were observed in men, fertile or menopausal women. Nevertheless, ERs were only found in sections of aortic tissue from fertile aged women and detected in the nucleus of most cells, including MCs (Fig.1).

At concentrations up to 1.0 ng/mL, 17-β estradiol did not activate peritoneal MCs, as monitored by measuring histamine release from these cells, which re-

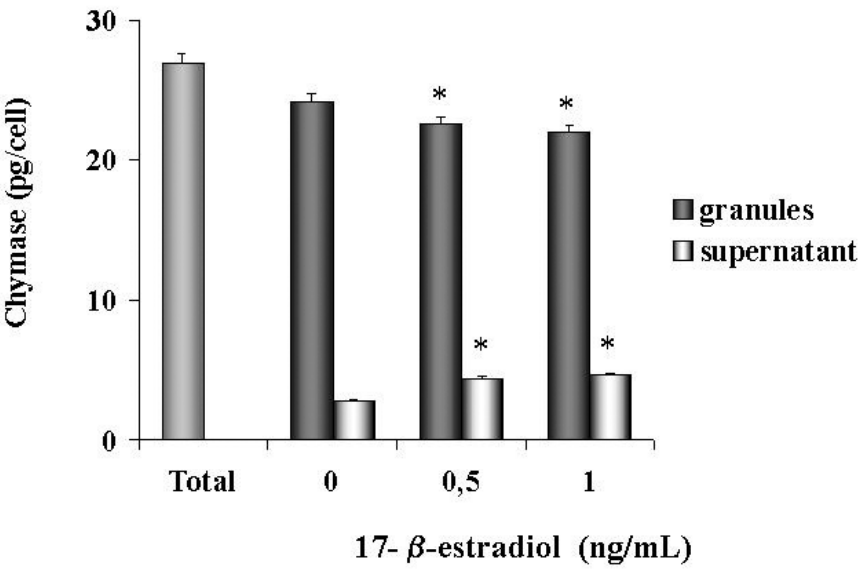

FIGURE 2. Effect of 17-β estradiol on the distribution of chymase activity in rat peritoneal mast cells. Cells were exposed for 24 h to either RPMI 1640 medium alone (control) or to medium containing different concentrations of 17-β estradiol (0.5 and 1.0 ng/mL). Total chymase activity remained constant (approx. 27.1 ± 0.61 pg/cell). Results are the means ± SD of six independent experiments. *$P \leq 0.05$.

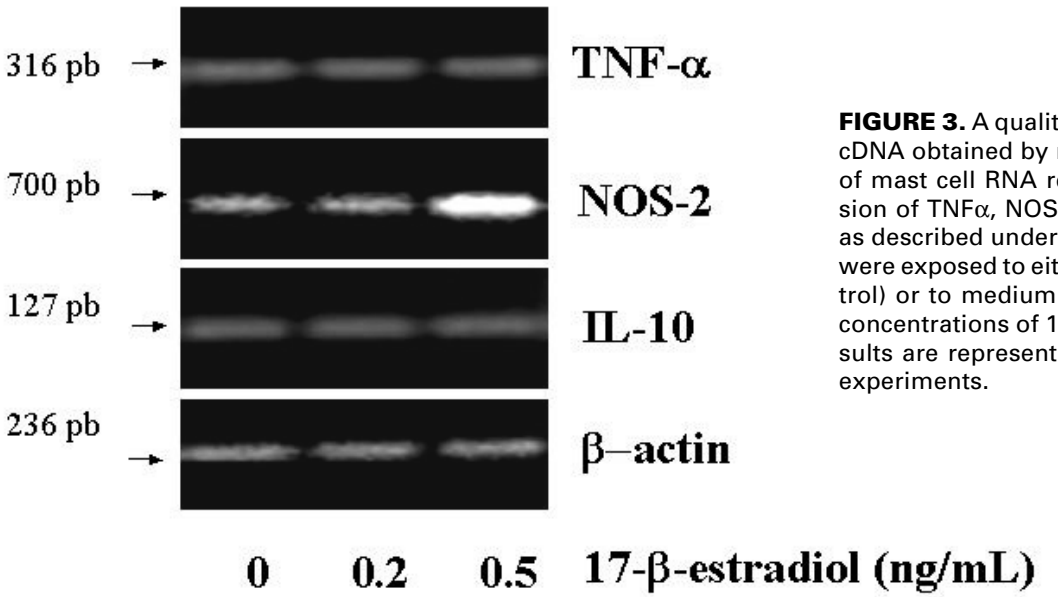

FIGURE 3. A qualitative PCR analysis of cDNA obtained by reverse transcription of mast cell RNA reflecting the expression of TNFα, NOS-2, IL-10 and β-actin, as described under Methods. Mast cells were exposed to either RPMI alone (control) or to medium containing different concentrations of 17-β estradiol. The results are representative of six separate experiments.

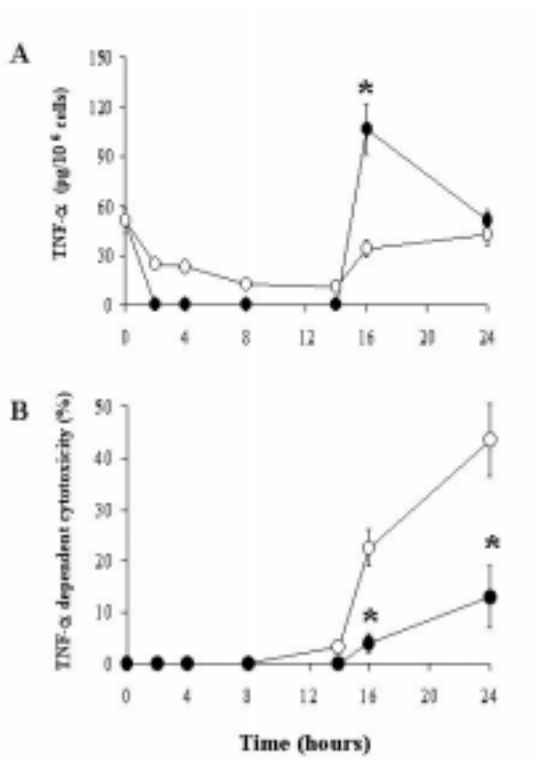

FIGURE 4. Time course of 17-β estradiol-induced TNFα in rat peritoneal mast cells. Cells were exposed at different times to either RPMI 1640 medium alone (open circles) or to medium containing 0.5 ng/mL 17-β estradiol (closed circles). Supernatants from different wells were harvested and analyzed either for protein (pg/10^6 cells) by ELISA (A), or for bioactivity (B) by measuring cytotoxic activity in L929-TNFα sensitive cells, (see materials and methods). Results are representative of six separate experiments. * $P \leq 0.05$.

mained constant throughout the experiments (1,300 ± 500 ng/10^6 cells). Likewise, chymase activity was not affected either. Both the incubation medium and total MC content remained constant (2.1 ± 0.1 pg/mL pg/mL and 27.1 ± 0.6 pg/mL, respectively). Nevertheless, isolated granules showed a decrease capacity to store the enzyme. As shown in Figure 2, chymase activity was reduced in the particulate fraction and increased in the same proportion in the soluble fraction. This response was not affected by 5 μg/ml cycloheximide.

As determined by RT-PCR and shown in Figure 3, mRNA encoding for TNFα, NOS-2 and IL-10 were successfully detected in MCs before the addition of 17-β estradiol. An increase in mRNA for NOS-2 was observed after 24 h incubation with 0.5 ng/mL 17-β estradiol, however mRNAs for TNFα and IL-10 did not change.

ELISA determinations of cell free supernatants showed that TNFα decreased from 50.8 ± 7.3 pg/10^6 cells to undetectable levels after 2 h incubation with 0.5 ng/mL of 17-β estradiol. After 14 h, a significant increment was observed (101.0 ± 11.1 pg of TNFα /10^6 cells) which could be attributed to a genomic action of 17-β estradiol, since this increase was not observed in the absence of the hormone (42.0 ± 6.0 pg of TNFα/10^6 cells). Cycloheximide (5 μg/mL) incorporated 3 h before the end of the incubation period inhibited the effect of 17-β estradiol on the synthesis of TNFα by 50% (data not shown). Despite the greater production of the cytokine, bioactive TNFα was significantly reduced in

the supernatants of 17-β estradiol treated MCs as compared to controls (Fig. 4).

A significant increase in NOS protein expression, due to an increment in both NOS-2 and NOS-3, was observed after incubation with 0.5 ng/mL 17-β estradiol (1.4 and 1.2 times respectively) (Fig. 5A). The time-course analysis of the amount of NO released from MCs showed that NO production remained constant during the first 4 h and then gradually increased during the next 10 hrs (from 0.40 ± 0.07 μmoles/mg protein to 1.26 ± 0.19 μmoles/ mg protein) (Fig. 5B). The increase in NO production did not affect MC viability, as shown by trypan blue exclusion. Furthermore, the degree of lipid peroxidation was not modified, since there was no significant differences in TBARS levels from MCs incubated in the presence or absence of estradiol (0.95 ± 0.03 and 0.91 ± 0.05 nmoles TBARS/mg of protein, respectively). No differences were observed between MCs prepared from males or female rats.

Discussion

Kubes and Granger (1996) showed that MCs found in the arterial intima of large and small vessels could have a key role in the development of atherosclerosis. This study complements the above results showing that MCs from fertile women, but not from men or menopausal women, express ER. Since the activation of ER can generate differences in the functional state of MCs, it is suggested that intimal MCs may affect the development of atherosclerosis depending on ER activation.

Depending on the functional state, MCs release specific mediators, some of them acting as atherogenic or antiatherogenic. As described before, serine proteases with chymase activity are classified as pro-atherogenic enzymes because of their ability to support foam cell formation (Wang *et al*., 1995). They are a family of specific MC proteases stored in secretory granules in an enzymatically active state. Chromosomal mapping stud-

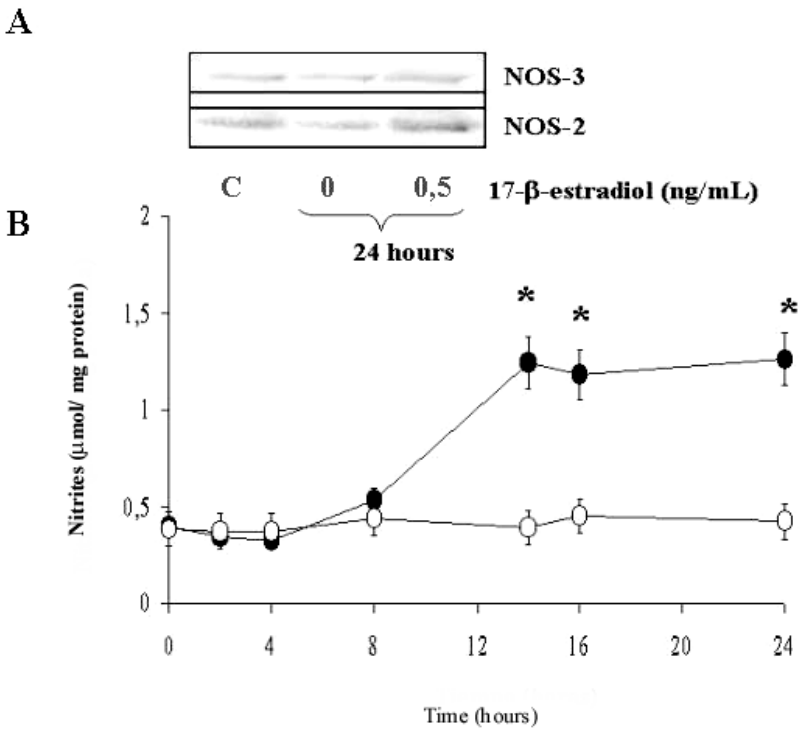

FIGURE 5. NOS protein expression and NO release. (A) Western blot analyses for NOS protein in rat peritoneal mast cells. **C**: control before incubation; **0**: control after 24 h incubation; **0.5**: 17-β estradiol (0.5 ng/mL). Whole cell lysate was isolated from untreated mast cells (open circles) and mast cells treated for 24 h with 0.5 ng/mL 17-β estradiol (closed circles). Cell lysate protein (6 μg) was loaded in each lane, resolved on SDS-PAGE gel and probed with a monoclonal antibody against NOS-3 and NOS-2. (B) Time course of nitrite production in supernatants from untreated peritoneal mast cells (open circles) and treated with 0.5 ng/mL 17-β estradiol (closed circles). Results are representative of six separate experiments. * $P \leq 0.05$.

ies have revealed that the genes that encode them reside at a complex in the mouse chromosome 14 (Gurish *et al*., 1993) being regulated by IL-10 (Ghildyal *et al*., 1993). Our results showed that 17-β estradiol (0.5 ng/mL) did not affect the synthesis of the enzyme as total chymase activity was not modified after 24 h incubation with the hormone. Nevertheless, a redistribution of the enzyme not dependent on MC degranulation was evident.

Although there is some controversy on whether TNFα may become pro-atherogenic or anti-atherogenic, the effects as a pro-inflammatory cytokine promoting early stages of atherogenesis are well documented (Neumann *et al*., 1996; Rutledge *et al*., 1997). Regarding the effect of estrogens on TNFα expression, results are controversial depending on the tissue analyzed. An increase in the expression of the TNFα gene has been reported in mouse uterine MCs *in vivo* (Roby and Hunt, 1995), but a decrease in its production has been reported in bone (An *et al*. 1999). In our experiments, we did not find any change on TNFα mRNA expression by incubating peritoneal MCs with 17-β estradiol at a concentration of 0.5 ng/mL.

Regarding the TNFα cytotoxic effect, it is worthwhile to point out that in spite of the increase in TNFα release there was a significant reduction in the supernatant cytotoxicity from MCs treated with 17-β estradiol. This reduced cytotoxicity could be attributed to a simultaneous release of soluble TNFα receptors that could neutralize the TNFα cytotoxic action (Tracy and Cerami, 1993). Furthermore, TNFα inhibits histamine release which can be considered as a novel autocrine regulatory function of this cytokine on MCs (Brzezinska-Blaszczyk *et al*., 2000).

NO has become an important regulatory molecule in the vascular and immune system. Targets of NO reactivity are abundant in all cells. Immune cells readily sense NO at levels below their cytotoxic effects exhibiting an immune-regulatory activity that might limit tissue damage during atherosclerosis. The time course analysis of nitrite concentration in MC supernatants showed a significant but moderate increase in NO after 14 h incubation with 17-β estradiol. It is interesting to note that at least two NOS isoforms (NOS-2 and NOS-3) have been reported in MCs. Rat peritoneal MCs expressed both NOS isoforms but when incubated with 17-β estradiol a moderate increment was observed, as determined by western blot analysis (Fig. 5 A). NO production was also stimulated by the hormone. This local NO production did not seemed to be toxic, since lipid peroxidation was not increased under these experimental conditions. Therefore, we postulate that NO production, evoked by the genomic action of estrogens in MCs, may have a regulatory paracrine action on endothelium favoring an anti-atherosclerotic effect through the activation of Th 2 associated molecules. The fact that we did not find an increase in IL-10 in MCs agrees with previous findings showing that NO might upregulate IL-4 but not IL-10 production (Kallmann *et al*., 1999). Furthermore, it is known that MCs are highly reactive in an ambient lacking NO (Gaboury *et al*., 1996). Therefore, the NO produced by the action of estrogens could also exert an autocrine modulatory effect inhibiting MC degranulation.

In summary, it has been demonstrated that ERs are detected primarily in arterial MCs of fertile women. MCs respond to 17-β estradiol mainly by a moderate increment in NOS mRNA and NO and a reduction in TNFα cytotoxicity, two mechanisms that may reduce the risks of atherosclerosis. The current study extends the concept of a link between MCs and the protective role of estrogens in the development of atherosclerosis, possibly through non-genomic and genomic effects.

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