The effect of endotoxin on functional parameters of mammary CID-9 cells

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Abstract

The effect of endotoxin on mammary CID-9 cells, which differentiate in culture and express β -casein, was investigated. Cells in culture supplemented with lactogenic hormones and dripped with EMS-Matrix (EMS-drip), were treated daily with endotoxin (0.5–500 μ g/ml). Endotoxin at concentrations of less or equal to 10 μ g/ml did not affect cell growth and viability up to 5 days post endotoxin treatment. Endotoxin (0.01–10 μ g/ml) was added to the culture medium, upon confluence, and functional parameters were examined within 48 h post endotoxin treatment. Nuclear factor- κ B (NF- κ B) (p52) increased in nuclear extracts from endotoxin-stimulated cells within 1 h of treatment, while β -casein mRNA and protein expression decreased in a concentration-dependent manner at 24 and 48 h post treatment. Zymography showed that the 72 and 92 kDa gelatinase activity increased in cells at 24 and 48 h post endotoxin treatment at 10 and 50 μ g/ml. At the latter concentration, the active form of 72 kDa gelatinase was induced at 48 h. Interleukin-6 and tumor necrosis factor- α levels increased at 1–3 h post endotoxin treatment and peaked at 6 h in cells on plastic and EHS-drip. Nerve growth factor (NGF) levels increased in control and endotoxin-treated cells in a time-dependent manner, and endotoxin increased NGF levels in culture at 6 and 9 h post endotoxin treatment. This study shows that endotoxin activated NF- κ B, suppressed β -casein expression and upregulated gelatinases, cytokines and NGF. This model could be used to investigate the role of mammary cells in initiating and propagating inflammation and to test candidate molecules for potential anti-inflammatory properties.

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Introduction

Mastitis, inflammation of the mammary gland, is common in lactating dairy cows and causes major problems for the dairy industry throughout the world, where it affects milk production and causes substantial economical losses (Riollet *et al.* 2000, Waller 2000). In humans, mastitis manifests itself by symptoms such as breast tenderness, redness and heat, in addition to an increase in heart rate and temperature, and approximately 20–33% of women who breast-feed are diagnosed as having mastitis (Inch & Fisher 1995, Semba & Neville 1999). The impact of the above at both the medical and the economic level cannot be overlooked. Efforts to decipher the mechanism of this inflammation must address the role that both immune cells, recruited to the gland in response to inflammation, and mammary parenchymal cells play in this process.

Several studies have addressed the role of cytokines (Jackson *et al.* 1990, Shuster *et al.* 1993, 1997, Homaidan

et al. 1995, 1999), matrix metalloproteinases (MMPs) (Talhouk *et al.* 2000a), and cell–cell interaction (Von Andian *et al.* 1991, Martin *et al.* 1998a,b) in cell and/or tissue function during inflammation (Oliver & Smith 1982, Lengemann & Pitzrick 1987, Colditz *et al.* 1990). Moreover, the role of immune cells in inflammation has been extensively studied, but few studies have addressed the role that non-immune parenchymal cells play in mediating inflammatory reactions, especially in the mammary tissue (Okada *et al.* 1997, 1999, Boudjellab *et al.* 1998). In brief, these studies demonstrate the production of cytokines by mammary parenchymal cells *in vitro* and that endotoxin (ET) stimulates the synthesis and secretion of cytokines such as interleukin (IL)-6, IL-1, IL-8 and tumor necrosis factor (TNF)- α , suggesting that these cytokines may be involved in resolving mammary bacterial infections. The effect of ET on functional parameters of mammary cells such as production of β -casein and extracellular matrix (ECM) remodeling proteinases was not addressed.

ET-induced inflammation affects many cell types, such as lymphocytes, macrophages, epithelial and endothelial cells. One of the initial ET-induced signals is the activation of the transcription factor nuclear factor- κ B (NF- κ B), which in turn activates several other genes that are major players in the inflammatory process, such as nerve growth factor (NGF), cytokines including IL-1 β , TNF, IL-6, IL-8 and ECM-degrading proteases (Lee *et al.* 1993, Read *et al.* 1993, Boudjellab *et al.* 1998, Bondeson *et al.* 1999, Kim & Koh 2000, Tomita *et al.* 2000, Baldwin 2001, Tak & Firestein 2001). In addition, some studies have shown that gelatinases are able to regulate the secretion of cytokines, mainly TNF- α and IL-1 β , and vice versa (Gearing *et al.* 1994, Zhang *et al.* 1998).

The aim of this study was to determine the effect of ET on functional parameters of mammary gland non-immune cells and elucidate the role these cells play in the initiation and propagation of the inflammatory process during mastitis. To this effect, we used CID-9 cells, a heterogeneous population of cells, consisting of fibroblasts, epithelial and myoepithelial cells. The CID-9 cells differentiate in culture and express β -casein in response to ECM, cell-cell interaction and prolactin (Schimdhäuser *et al.* 1990, El-Sabban *et al.* 2003). Briefly, we demonstrate that ET activates NF- κ B, downregulates casein expression, and increases gelatinase and cytokine expression. Moreover, we suggest that this is a suitable *in vitro* model to study the role of non-immune cells in mammary gland inflammation and for investigating the effect of several anti-inflammatory agents on mammary cell function, during mastitis.

Materials and Methods

Materials

Highest grade materials were used. BSA, Coomassie blue R-250, insulin, ovine hydrocortisone, ovine prolactin and trypsin-EDTA, were obtained from Sigma Chemical Co. (St Louis, MO, USA). *Escherichia coli*, strain 055:B5, lipopolysaccharide, previously used to induce mastitis in lactating dairy cows (Shuster *et al.* 1993) was purchased from Difco Laboratories (Detroit, MI, USA). Complete Protease Inhibitors, poly(dI-dC), and polynucleotide kinase, were purchased from Boehringer, Mannheim, Germany. Hybond-N membrane, Rediprime kit, and [α - 32 P]dCTP were from Amersham Pharmacia Biotech. Immobilin-P membranes were from Millipore Continental Water Systems (Bedford, MA, USA). Enhanced chemiluminescence (ECL), horseradish peroxidase-conjugated anti-rabbit IgG and specific antibodies for NF- κ B (polyclonal rabbit p50, p52 or p60, anti-NF- κ B IgG) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell culture media and reagents were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Bio-Rad protein assay was from Bio-Rad (Hercules, CA, USA). EHS-Matrix, growth-factor-reduced Matrigel, was purchased from

Collaborative Biomedical Products (Two Oak Park, Bedford, MA, USA). CID-9 mammary cell strain, polyclonal rabbit anti-mouse milk antiserum, and β -casein c-DNA inserts were provided by Dr Mina Bissell, Lawrence Berkeley National Laboratory, Berkeley, CA, USA.

CID-9 cell cultures

Low passage numbers (17–21) of the CID-9 mouse mammary cell strain were used throughout. For maintenance and propagation, cells were grown in 'growth medium' consisting of Dulbecco's Modified Eagle's Medium Nutrient Mixture/F12 Ham (DMEM/F12) with 5% fetal bovine serum (FBS), insulin (5 μ g/ml) and gentamicin (50 μ g/ml) in a humidified incubator (95% air 5% CO₂) at 37 °C.

Depending on the experimental setup, as indicated for each experiment, CID-9 cells were seeded after trypsinization at 3.0×10^6 cells/75 cm² culture dish, alternatively at 5.0×10^6 cells/75 cm² culture dish, with diluted reconstituted basement membrane, growth-factor-reduced, Matrigel (1.5% v/v), in Hanks' balanced salt solution (HBSS) that was dripped (referred to here as EHS-drip) onto cells 24 h after plating (El-Sabban *et al.* 2003). Cells cultured on plastic or EHS-drip were first plated in growth medium for initial cell attachment and spreading. Twenty-four hours after plating, cells were washed three times with HBSS and growth medium was replaced with either differentiation or non-differentiation media. These consist of FBS-free DMEM/F12 media containing insulin (5 μ g/ml), hydrocortisone (1 μ g/ml) and either supplemented with or lacking ovine prolactin (3 μ g/ml) respectively, and supplemented with gentamicin (50 μ g/ml). Media were changed on daily basis.

ET treatment

Two modes of ET treatments were applied. For cell viability experiments, cells plated on EHS-drip were supplemented with 0.5, 5, 10, 50 and 500 μ g/ml ET on day 1 of culture. The medium supplemented with ET was changed on a daily basis for up to 6 days in culture (or 5 days post ET treatment). Cell counting was done on days 2, 4 and 6 of culture. Cells were washed twice with HBSS and then trypsin-EDTA was added at 37 °C, until all the cells detached. The dissociated viable cells were then counted using trypan blue staining (Tarraf *et al.* 2003). Triplicate wells were counted for each concentration of ET at each time point.

For all other experiments, the cells were allowed to grow in ET-free growth medium on EHS-drip or plastic until they reached confluence. Then, they were shifted to differentiation media, supplemented with 1% FBS, and were treated with either 0.01, 0.1, 1, 5, 10 or 50 μ g/ml ET, and samples were collected at 3, 6, 9, 12, 15, 18, 24 and 48 h depending on the experiment and as indicated in the text and respective figure legends.

NF- κ B electrophoretic mobility shift assay (EMSA)

CID-9 cells, cultured on EHS-drip, were washed twice in 5 ml ice-cold PBS, and the cells were collected (gently scraped by a rubber policeman) and centrifuged at 420 *g* for 5 min at 4°C. Cell membranes were lysed, and hence nuclei were released, by resuspending the pellet in 250 μ l buffer A (10 mM Tris-HCl pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, and one tablet Complete Protease Inhibitors/30 ml buffer). The suspension was left on ice for 10 min followed by a 45 s homogenization at a moderate speed (15 000 r.p.m.), using a Polytrone (Kinametica, Littau-Luzern, Switzerland). The nuclei were collected by centrifugation at 4500 *g* for 5 min at 4°C, and then lysed by resuspension in 100 μ l buffer B (20 mM Tris-HCl, pH 7.8, 420 mM KCl, 1.5 mM MgCl₂, 20% glycerol and one tablet Complete Protease Inhibitors/30 ml buffer), with gentle agitation at 4°C for 30 min. The debris was cleared by centrifugation at 10 000 *g* for an additional 30 min at 4°C, and the supernatant was stored at -70°C until used. On the day of the assay, protein quantification, using the microtiter Bradford assay, was performed for the samples, using BSA as a standard.

EMSAs were performed using a ³²P-radiolabeled deoxyoligonucleotide sequences (from Sigma-Genosys, Cambridge, UK) of NF- κ B-binding DNA: W-22, 5'-AGTTGAGGG-GACTTTCCAGGC-3' (consensus sequence italicized) and M-22 (1-pb missense control), 5'-AGTTGAGGCGACTTCCAGGC-3'.

After end labeling with polynucleotide kinase purifying and annealing probes, identical amounts of radioactivity (2 $\times$ 10⁴ counts/min) were added to the binding reactions containing 10 μ g cell nuclear extracts in a final volume of 40 μ l in DNA-binding buffer (20 mM Hepes, pH 7.9, 1 mM MgCl₂, and 4% Ficoll) containing 0.15 μ g poly(dI-dC) as a non-specific competitor. The mixtures were incubated for 30 min before separation on non-denaturing 4% polyacrylamide gels at room temperature by electrophoresis in Tris-borate-EDTA buffer.

As a control of the specificity of the band, non-labeled oligonucleotide competitors were added in 100-fold molar excess immediately before the addition of a radio-labeled probe of the same sequence. For supershift experiments, specific antibodies for NF- κ B (polyclonal rabbit p50, p52 or p60, anti-NF- κ B IgG) were added, at 2 μ g/reaction, to the samples, 30 min before the addition of the labeled probe.

Distribution of ³²P-labeled bands was visualized by autoradiography and the autoradiograms were scanned and quantified using NIH Image 1.62 software (<http://rsb.info.nih.gov/nih-image/>). The arbitrary values obtained from the scanning were plotted as percentage of control at each time point.

RNA extraction and Northern blotting

For RNA extraction, CID-9 cells were seeded on EHS-drip with differentiation media as described above, and treated

with different ET concentrations of 0.01, 1, 5 and 10 μ g/ml on day 4 of culture. Twenty-four and 48 h post ET treatment, total cellular RNA was extracted from ET-treated cultured CID-9 cells and control untreated CID cells according to Chomczynski & Sacchi (1987). Northern analysis for β -casein was performed as described by El-Sabban *et al.* (2003).

Protein extraction and Western blotting

CID-9 cells were cultured on EHS-drip, with differentiation media and treated with 0.01, 0.1, 1 and 5 μ g/ml ET on day 4 of culture. Protein extraction was done on day 6 of culture (48 h post ET treatment) by scraping the cells into lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate) and shearing them several times through a 21-gauge needle. Complete Protease Inhibitors were added in a concentration of 40 μ l (of one Complete Protease Inhibitor tablet dissolved in 2 ml water) per 1 ml lysis buffer, and the cell extracts were centrifuged at 15 000 *g*. The supernatants were resolved on an equal protein basis as determined by Bio-Rad assay with BSA as a standard, on 12% polyacrylamide gel under denaturing conditions. After electrophoresis, resolved proteins were transferred to Immobilon-P membranes using a wet blot apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) with a transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). Membranes were blocked overnight in a wash buffer (100 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.3% Tween 20) with 2% fatty acid-free BSA. The membranes were then incubated for 1 h in polyclonal rabbit anti-mouse milk antiserum (diluted 1:10 000 in blocking buffer) and washed three times, for 20 min each, to remove unbound antiserum, and then placed in the secondary antibody (horseradish peroxidase-conjugated anti-rabbit) at a dilution of 1:5000 for 1 h at room temperature while shaking. Finally, membranes were washed three times (20 min per wash). This step was followed by the use of enhanced chemiluminescence using the ECL system in conjunction with horseradish peroxidase-conjugated secondary antibodies. Membranes were then exposed to X-ray films for varying time periods. All washings and incubations were done at room temperature.

Substrate-gel electrophoresis

Media were sampled from cultures treated with different ET concentrations, and from control cultures, at different time points. The samples were stored at -70°C until the day of the assay. Gelatinase activity in medium samples was analyzed with minor modifications of the method described by Talhouk *et al.* (1991). Equal sample volumes were loaded and run on 7% polyacrylamide gels impregnated with gelatin (3 mg/ml). The gels were run in 1 $\times$ electrophoresis running buffer (0.0025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS). After electrophoresis, the gels were washed twice consecutively for 30 min,

at room temperature, in a 2.5% Triton X-100 solution in running buffer. After washing, the gels were incubated for 24 h in substrate buffer (50 mM Tris-HCl, 5 mM CaCl₂, 0.02% NaN₃, pH 8.0) at 37 °C. The gels were then stained for 2 h, at room temperature, in 0.05% Coomassie blue R-250, in 50% methanol and 10% acetic acid. The gels were then destained in water overnight. The gelatinases were visualized as clear white bands on darkly stained blue gels. Photographs of substrate gels are shown as negative images.

ELISA

Media were sampled from cultures treated with different ET concentrations, and from control cultures, at different time points. Complete Protease Inhibitors were added to the samples and were stored at -70 °C until assayed.

The concentration of IL-6 and TNF- α in the medium samples was measured using a modification of a two-site (sandwich) ELISA as described previously (Safieh-Garabedian *et al.* 1995). Immunoaffinity-purified polyclonal sheep anti-mouse IL-6 or TNF- α antibodies were used to coat high-binding 96-well microtiter plates (Immunoplate MaxiSorp; NUNC, Rockslide, Denmark). Recombinant mouse IL-6 or TNF- α was used as the standard, and a biotinylated immunoaffinity-purified polyclonal sheep anti-mouse IL-6 and TNF- α as a recognition antibody respectively. All wells were coated (100 μ l/well) with the coating antibody (2 μ g/ml in bicarbonate coating buffer) and were incubated overnight at 4 °C. The plates were then washed four times (300 μ l/well) with wash/dilution buffer. The plate wells were then blocked with blocking buffer (3% BSA in PBS) (300 μ l/well); the plate was then incubated at 37 °C for 1 h. While incubating the plate, the standard dilutions were prepared ranging from 1000 to 1.9 pg/ml in wash/dilution buffer. After washing as described the medium samples were used as such without diluting them. The standard dilutions and the samples were added in duplicates (100 μ l/well). The blank wells received wash/dilution buffer. The plates were incubated at 4 °C overnight and washed as previously described, and the biotinylated antibody was diluted 1:4000 in 1% normal sheep serum in wash/dilution buffer and added (100 μ l/well). The plates were then incubated at 4 °C overnight, washed, and 100 μ l/well streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:8000 in wash/dilution buffer were added. The plates were then incubated at room temperature, washed and the chromogen (500 μ l 3,3',5,5'-tetramethylbenzidine + 6 μ l 30% H₂O₂ + 5 ml acetate buffer in 45 ml water) was added to all wells (100 μ l/well). The plates were kept in the dark at room temperature for 10–15 min. The reaction was then stopped by the addition of 1 M H₂SO₄ (100 μ l/well). The optical density was read by a plate reader (Dynatech Medical Products Limited, Billingham, UK) using a 450 nm filter.

NGF was assayed using a Promega E_{max} Immunoassay System (Promega Corporation, Madison, WI, USA),

following the protocol provided in the kit's Technical Bulletin. Triplicate wells were assayed for each concentration of ET at each time point.

Data analysis

Cell numbers were analyzed in triplicate as a completely randomized design over days with six treatments: control, 0.5, 5, 10, 50 and 500 μ g/ml ET. Means were separated according to the least significant difference (LSD) test. Significant differences between average levels of cytokines and NGF were determined after appropriate ANOVA using Tukey's test. All analyses were performed using SPSS 11.5 (SPSS 2002).

Results

The effect of ET on CID-9 cell morphology and viability

To determine the effect of ET concentrations on CID-9 cell viability, different ET concentrations of 0.5, 5, 10, 50 and 500 μ g/ml were added daily to CID-9 cells plated on EHS-drip. Cell count was assessed daily over a period of 5 days post ET treatment. ET significantly affected the cell number within each day as indicated by ANOVA (data not shown). Cell count and viability were not significantly affected when cells were treated with 0.5, 5 and 10 μ g/ml ET, throughout the 5 day treatment of the cells with ET. This was in contrast to the effect of ET at the higher concentrations of 50 and 500 μ g/ml. At 50 μ g/ml, approximately 60% of the cells remained attached and were viable as noted by the decrease in cell count by day 5 post ET treatment, whereas at 500 μ g/ml, ET was highly toxic to the cells and only about 20% of the cells were attached and viable by day 5 post ET treatment (Fig. 1). Similar results were obtained when the cells were plated on plastic (data not shown). The data clearly demonstrated that low concentrations of ET, such as 0.5, 5 and 10 μ g/ml, do not affect the viability and growth morphology of CID-9 cells up to at least 5 days post ET treatment. Based on the above, cells were treated in all the following experiments upon confluence with a single dose of ET at concentrations of 10 μ g or less and for periods extending from 1 to 48 h, depending on the type of the assay used.

ET induces NF- κ B nuclear translocation and activation in CID-9 cells

One of the main markers for a cell response to ET is the activation and nuclear translocation of NF- κ B. EMSA was performed on nuclear extracts at 1 and 3 h post ET treatment with different ET concentrations of 0.01, 0.1, 1 and 10 μ g/ml. The activation of NF- κ B by ET in CID-9 cells was noted within 1 h of exposure. Maximal stimulation was reached at 1 h with 10 μ g/ml ET (Fig. 2A). The shifted bands were quantified using a densitometer, and the

Figure 1 Bar graphs showing viable counts of CID-9 cells cultured in differentiation medium on EHS-drip and treated with different ET concentrations ($\mu\text{g/ml}$). The values are the average of triplicate wells ($\pm$ S.D.). Means within the same day, with the same letter, are not significantly different according to the LSD test ($P \leq 0.05$).

arbitrary values resulting there from were represented as the percentage of activation of each sample relative to the bands obtained with the control at each time point (Fig. 2B). At 6, 9 and 12 h, no activation of NF- κ B was evident (data not shown).

In order to explore the type of NF- κ B dimers involved in the CID-9 cell activation process, supershift experiments were performed using specific antibodies for NF- κ B subunits p50, p52 or p60. These experiments

demonstrated that p52, and not p50 or p60, was activated in response to 10 $\mu\text{g/ml}$ ET treatment (Fig. 2C).

The effect of ET on β -casein and gelatinase expression CID-9 cells

ET inhibited in a dose-dependent manner β -casein expression by CID-9 cells plated on EHS-drip. The expression of β -casein was assessed in samples extracted from confluent CID-9 cells plated on EHS-drip and treated with different ET concentrations. Northern blot analysis was performed on total RNA, extracted on day 1 or 2 post ET treatment (Fig. 3A and B respectively). Western blot analysis was done on protein samples, extracted on day 2 post ET treatment (Fig. 3C). Both Northern blot analysis at days 1 and 2 post ET treatment and Western blots analysis at 2 days post ET treatment showed that ET decreased β -casein mRNA and protein levels respectively in CID-9 cells and in a dose-dependent manner.

In addition, substrate-gel electrophoresis showed that both the 72 and 92 kDa gelatinase activities increased in CID-9 cells cultured on EHS-drip by 24 and 48 h post ET treatment at 10 and 50 $\mu\text{g/ml}$. The gelatinase activity was higher in the 50 $\mu\text{g/ml}$ ET-treated CID-9 cells and the activation of the 72 kDa gelatinase, which is revealed by the appearance of a 64 kDa fragment, was noted 48 h post ET treatment (Fig. 4).

The effect of the ET on cytokine and NGF production in CID-9 cells

ET stimulatory effect on cytokine TNF- α and IL-6 was noted as early as 1 h post ET treatment, while an NGF increase due to ET was noted at 6 and 9 h. TNF- α ELISA revealed that ET treatment stimulated the production of this cytokine in cells cultured on plastic (Fig. 5Ai) and on EHS-drip (Fig. 5Aii). The secretion of TNF- α started to increase by 1–3 h post treatment, and reached a maximal level by 6 h. This increase was dose-dependent, showing maximum levels of TNF- α with 10 $\mu\text{g/ml}$ ET.

Figure 2 (A) A representative EMSA showing the activation of NF- κ B with increasing ET concentrations (0.01, 0.1, 1, 10 $\mu\text{g/ml}$), at 1 and 3 h. The maximum activity is observed at 1 h post exposure to ET. The signal of NF- κ B in the 10 $\mu\text{g/ml}$ ET-treated CID-9 cell nuclear extract, at 1 h, is inhibited by 100-fold addition of the unlabelled probe. (B) Graphic analysis of ET-induced NF- κ B activation, as determined by densitometric quantification of the shifted bands, plotted as percentage of control (the ET concentrations are in $\mu\text{g/ml}$). (C) Supershift analysis by selective antibodies raised against the NF- κ B subunits p50, p52 and p60 revealed the presence of the subunit p52 in the nucleus.

Figure 3 Northern blot analysis of β -casein mRNA in CID-9 cells on EHS-drip on day 1 (A) and day 2 (B) post ET treatment. (C) Western blot analysis of β -casein in CID-9 cells on day 2 post ET treatment. (Cdiff: control cells receiving differentiation medium only; Cfbs: control cells receiving differentiation medium supplemented with FBS; PI(+): cells on plastic with differentiation medium (negative control). Arabic numerals appearing above lanes indicate concentration (μ g/ml) of ET.

Figure 4 Gelatinase zymography of samples taken from CID-9 cell conditioned media 24 and 48 h post ET treatment of CID-9 cells on EHS-drip. Note increased gelatinase activity in 10 and 50 μ g/ml ET-treated cells compared with control non-treated cells.

Figure 5 TNF- α (A), IL-6 (B) and NGF (C) ELISA for CID-9 cells cultured on plastic (i) or on EHS-drip (ii) and treated with different concentrations of ET. The values are the average of triplicate samples ($\pm$ s.d.). Means within the same time post ET treatment, with the same letter, are not significantly different.

The effect of ET on the levels of IL-6, in CID-9 cells, was similar to that of TNF- α . IL-6 assays demonstrated that different ET concentrations induced the production of this cytokine by CID-9 cells cultured on plastic (Fig. 5Bi) and on EHS-drip (Fig. 5Bii) at 1–3 h post ET treatment. In fact, by 6 h post treatment with 1, 5 and 10 $\mu\text{g/ml}$ ET, IL-6 levels reached about 600 pg/ml, which is around 8-fold higher than the levels observed in controls. The control untreated cells maintained a constant basal level less than 100 pg/ml in both plastic and EHS-drip cultures.

As detected by NGF assays, non-treated control CID-9 cells increased NGF production in a time-dependent manner. ET enhanced NGF levels in cells cultured on plastic (Fig. 5Ci) and on EHS-drip (Fig. 5Cii), by 6 and 9 h post ET treatment.

Discussion

Numerous work has focused on the role immune cells and cytokines play in the initiation and propagation of mammary inflammation (reviewed by Outteridge & Lee 1981, Riollot *et al.* 2000). However, few studies aimed to decipher the involvement of non-immune cells, mainly endothelial and epithelial cells, in the inflammatory process (Pugin *et al.* 1993, Read *et al.* 1993, Okada *et al.* 1997, 1999, Yao *et al.* 1997, Boudjellab *et al.* 1998, Martin *et al.* 1998a,b, Rahman 2000). Our study aimed to determine the effect of ET on functional parameters of mammary gland non-immune cells. This may elucidate the role that these cells play in propagating inflammatory signals to the immune system during bacterial infections of the mammary gland, mastitis.

ET is reported to simulate inflammation-like conditions in several *in vitro* and *in vivo* models. *In vitro*, ET was shown to activate primary cultures of alveolar epithelial cells (Haddad *et al.* 2001), umbilical vein endothelial cells (Pugin *et al.* 1993, Read *et al.* 1993), and bovine mammary epithelial cells (Okada *et al.* 1997, 1999, Boudjellab *et al.* 1998), in addition to stimulating macrophages (Morris *et al.* 1992, Morrison *et al.* 1994), T cells (Mattern *et al.* 1994) and B cells (Sibley *et al.* 1988) from different species. *In vivo*, when introduced in the mammary gland, ET induced mastitis in several animal models, such as sheep (Colditz 1987), goat (Dhondt *et al.* 1977, Lengemann & Pitzrick 1987) and cows (Oliver & Smith 1982, Shuster *et al.* 1993).

CID-9 cells acquire a differentiated phenotype in response to cell–ECM, and cell–cell interaction and to lactogenic hormones (Schmidhauser *et al.* 1990, El-Sabban *et al.* 2003). This makes them a suitable model to address the effect of soluble mediators and ET on mammary cell function (Talhouk *et al.* 2000b 2001). In contrast to other studies on either primary bovine mammary cells or MAC-T bovine epithelial cell line (Boudjellab *et al.* 1998, Okada *et al.* 1999) that were limited to monitoring the effect of ET on cytokine production, in this study, we monitored, in addition to cytokine and NGF production,

the effect of ET on NF- κ B activation and on the differentiation phenotype as indicated by β -casein and gelatinase expression. The data presented in this study showed that daily exposure of confluent CID-9 cells on EHS-drip to ET concentrations up to 10 $\mu\text{g/ml}$ did not alter the apparent morphology (data not shown) and the viability of CID-9 cells. Moreover, single exposure of CID-9 cells to ET induced NF- κ B activation, increased gelatinase activity, inhibited β -casein expression and stimulated the production of inflammatory cytokines, such as IL-6 and TNF- α , and altered NGF production in these cells in a dose-dependent manner.

The optimal ET concentrations that were used to stimulate cells *in vitro* and the level of cytokines produced vary according to the cell type in question. In other words, distinct cell types are responsive to different ranges of ET concentrations. For instance, immune cells, such as macrophages and lymphocytes, are stimulated by low ET concentrations, in the range of nanograms (Sibley *et al.* 1988, Morris *et al.* 1992, Mattern *et al.* 1994), and produce several-fold the level of cytokines, especially TNF- α and IL-6 (Mullarkey *et al.* 2003) compared with those produced by epithelial cells. Epithelial cells (Epstein *et al.* 1990, Wille *et al.* 1992), on the other hand, including mammary epithelial cells (Boudjellab *et al.* 1998, Okada *et al.* 1999) as in this study, require relatively higher concentrations, in the microgram range, to elicit a response that typically does not exceed a few hundred picograms.

The transcription factor NF- κ B is clearly one of the most important regulators of proinflammatory gene expression. It is considered to be a marker for cell activation by ET (Read *et al.* 1993). Translocation of NF- κ B to the nucleus leads to subsequent activation of several genes involved in the inflammatory process including cytokines TNF- α , IL-1 β , IL-6 and IL-8, cytokine receptors and MMPs (Baldwin 1996, Yokoo & Kitamura 1996, Han *et al.* 1998, Mengshol *et al.* 2000). In addition, as shown in this study, it also leads to the downregulation of β -casein expression at both the mRNA and protein level. We suggest this occurs by either of two pathways. Previous studies on the mammary gland reported that NF- κ B is involved in regulating β -casein expression (Doppler *et al.* 2000). NF- κ B activation inhibits the prolactin-induced β -casein expression by either inhibiting STAT5 tyrosine phosphorylation, which is an essential step for its activation (Yang *et al.* 2000), or NF- κ B can inhibit STAT5 from binding to the β -casein gene promoter region by allosteric hindrance due to overlapping binding sites for STAT5 and NF- κ B in the β -casein gene promoter (Doppler *et al.* 2000, Geymayer & Doppler 2000). In other words, by inhibiting β -casein expression, ET puts the CID-9 cells in a de-differentiated state. This occurs in parallel to an increased cytokine and gelatinase production by these cells.

Alternatively, the expression of the MMPs by mammary epithelial cells was previously reported to directly affect casein expression and the differentiation of the mammary gland. In fact, a coordinated balance between MMPs and

their inhibitors is crucial to ensure normal development and differentiation of the mammary gland. The increased expression of stromelysin and type IV collagenase during involution of the mammary gland leads to degradation of the basement membranes and loss of β -casein expression (Talhok et al. 1992, Sympton et al. 1994) and triggers apoptotic events (Strange et al. 1992, Boudreau et al. 1995). Whether the ET-induced gelatinase expression and loss of β -casein expression are independent events induced by NF- κ B activation or whether the increased gelatinase leads to loss of β -casein expression and apoptosis was not clear from our studies.

Okada et al. (1997) reported that bovine mammary primary cells sustain IL-6 and IL-1 production until day 14 in culture. Recently, it has been shown that ET increased the production of these cytokines in culture (Okada et al. 1999). In our study neither IL-1 β , nor IL-4, IL-10 or IL-18 were detected in ET-treated or non-treated cultures (data not shown), while only IL-6, TNF- α and NGF production were enhanced upon ET treatment. The discrepancy could be due to species differences, or due to the fact that the primary culture preparation of Okada et al. (1997, 1999) was not devoid of immune cells, which contributed to the reported IL-1 levels. Alternatively, the levels of IL-1 cytokines, and for that matter, the levels of IL-4, IL-10 or IL-18, in our culture system were below the detection limit of the assays used in this study. No previous reports have demonstrated NGF production by mammary cells. However, the role of NGF in ET-induced inflammation and hyperalgesia has been well documented. Our laboratory reported earlier that intra-plantar injections of ET induced local inflammation in rats and increased NGF levels. The levels of NGF were reduced upon treatment of the rats with analgesics or anti-inflammatory drugs (Safieh-Garabedian et al. 1996, 1997). Based on that we speculate that the role of increased NGF levels by mammary cells upon exposure to ET, as in the case during mastitis, may be involved in nociception. Boudjellab et al. (1998) showed that a bovine mammary epithelial cell line (MAC-T) expresses IL-8 upon ET stimulation. No attempts to assay for IL-8 were undertaken in our study.

Due to the fact that CID-9 mammary cells are a heterogeneous population, the cellular source of the cytokines and NGF in culture, as assessed by ELISA, cannot be determined. However, mammary SCp2 cells (epithelial sub-clones of CID-9 mammary cells (Desprez et al. 1993)) grown in culture under the same conditions as CID-9 cells produce cytokines in comparable levels and patterns in response to ET stimulation (data not shown); thus we speculate that the cytokines produced in culture in response to ET may be products of the epithelial cell component of CID-9 cells.

In conclusion, ET-treated CID-9 cells suppress β -casein expression and upregulate markers of de-differentiation, such as gelatinases and cytokines, which are involved in stimulating and recruiting immune cells to the site of infection *in vivo* (Shuster et al. 1993). This study suggests that

mammary cells may participate in the immune reaction elicited by mammary gland, *in vivo*, in response to an ET challenge, mastitis, or during involution (Oliver & Smith 1982, Colditz 1987, Persson et al. 1992, Schanbacher et al. 1993, 1997). This model could be used to further decipher how ET modulates casein expression and markers of de-differentiation of mammary cells in culture and to investigate the effect of candidate molecules for their potential anti-inflammatory properties, during mastitis.

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