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Influence of milk cytokines on mammary production of nitric oxide

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ABSTRACT

Cytokines such as interferon-gamma (IFN–γ) can stimulate the production of nitric oxide (NO) from mammary epithelial cells, which in turn may play a role in intramammary defence of the mammary gland. In this study, comparisons were made with the production of IFN–γ stimulated NO from Comma-D cells (murine mammary epithelial cell line) and explants of mammary tissue collected from both pregnant (day 12-14 of gestation) and lactating (day 12-14 postpartum) rats. Response to IFN–γ was measured as the production of nitrate and nitrite (NOx) in the culture medium. Comma-D cells and explants of mammary tissue from pregnant and lactating rats responded to treatment with 250 U/ml IFN–γ with an increase in NOx of 5231, 300 and 373% respectively. The response to IFN–γ was lower (P<0.001) in explants than in Comma-D cells. Inclusion of nitric oxide synthase (NOS) inhibitors aminoguanidine (100 μM) and N^{ω}-nitro-L-arginine (1 mM) in the culture medium reduced NOx production by the Comma-D cells to 31 and 19% of stimulated activity respectively. This suggests that Comma-D cells are a good model for investigating effects of cytokines on mammary epithelial cell production of NO, and may also provide a useful means of screening other milk bioactives for their potential role in intramammary defence.

Keywords: Comma-D; mammary explants; nitric oxide; milk; cytokines.

INTRODUCTION

A wide range of biologically active proteins and peptides, including a number of cytokines are found in both colostrum and milk (Fox & Flynn, 1992; Goto *et al.*, 1997; Schanbacher *et al.*, 1997; Hagiwara *et al.*, 2000). Concentrations of these molecules often increase during mastitis, for example, the concentrations of the cytokine tumor necrosis factor–α (TNF–α; Blum *et al.*, 2000) and the antimicrobial protein lactoferrin (Harmon *et al.*, 1976) are increased, following *Escherichia coli* infection of the bovine mammary gland.

Recent studies have shown that mammary epithelial cells respond to cytokines such as interferon-gamma (IFN–γ) with the production of nitric oxide (NO), an inorganic gaseous radical that is produced when the enzyme nitric oxide synthase (NOS) converts arginine to citrulline. Further, co-treatment of Comma-D cells with IFN–γ and TNF–α doubles the production of NO in comparison with that produced when the cells are treated with IFN–γ alone (Low *et al.*, 1997). NO production is also increased when explants of mammary tissue collected from estradiol and progesterone-primed female rats are treated with lipopolysaccharide (LPS; a component of bacterial cell walls; Onoda & Inano, 1998).

NO plays a role in many aspects of physiology such as neutrotransmission, blood flow regulation, cellular defence and platelet adhesion/aggregation. In the mammary gland, it is suggested that NO release plays a role in the control of mammary blood flow (Fleet *et al.*, 1993; Lacasse *et al.*, 1995; Lacasse *et al.*, 1996) and thus substrate supply to the gland. Nitrate and nitrite (NOx), the spontaneous oxidation products of NO (Palmer *et al.*, 1987) are found to increase in the milk of cows that have either spontaneous or induced mastitis (Lacasse *et al.*, 1997; Bouchard *et al.*, 1999), thereby suggesting that NO may play a role in the intramammary defence of the gland. Thus, determining the factors that control the production of NO may provide a means of being able to manipulate either mammary blood flow or intramammary defence mechanisms.

NO has a very short half-life and therefore production of NO by biological tissues is often measured indirectly. Various methods are available including measuring the activity of NOS, the enzyme responsible for the production of NO (Bredt & Snyder, 1989; Pollock *et al.*, 1991). NO production can also be determined by measuring the production of NOx. When NO reacts with O_2 it produces NO_2 which then spontaneously forms nitrate and nitrite (Palmer *et al.*, 1987). The measurement of NOx in culture medium is a commonly reported method for the indirect measurement of NO production and has been used for many tissue types including the mammary gland (Low *et al.*, 1997; Onoda & Inano, 1998).

In this study, the production of NO following stimulation of mammary cells and explants of mammary tissue with IFN–γ was investigated to confirm that normal mammary tissue was capable of the production of NO. Comparisons between the cells and explants were also investigated to determine if they responded in a similar manner.

MATERIALS AND METHODS

Cell and explant culture

The Comma-D cells (generous gift from Dr D. Medina, Baylor College of Medicine, Houston, TX, USA) used in this study were all between passages 17 and 27 and were cultured as described by Low *et al.* (1997) but with 100 U/ml penicillin (GibcoBRL/Life Technologies) and 100 μg/ml streptomycin (GibcoBRL/Life Technologies) in the culture medium.

In 13 independent experiments, cells were treated with 250 U/ml mouse recombinant IFN–γ (mIFN–γ; Roche Molecular Biosciences, Mannheim, Germany), 250 U/ml mIFN–γ + 1 mM N^{ω}-nitro-L-arginine (L-NNA; Sigma Chemical Co.), or 250 U/ml mIFN–γ + 100 μM aminoguanidine (AG; Sigma Chemical Co.). Treatments

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were applied to triplicate wells. Following 24 hours in culture containing the treatments, the medium was collected and stored at -20°C for subsequent analysis of NOx. The cells were collected by sonicating in 500 ml PBS (phosphate-buffered saline Dulbecco A tablets, Oxoid Ltd, Hampshire, England) to detach the cells and lyse the cell membranes. The resulting suspension was stored at -20 °C for subsequent analysis of DNA content.

Following euthanasia by the administration of 500 µl of 60 mg/ml sodium pentabarbitone (Sagital, May and Baker, Lower Hutt, USA), the right abdominal mammary gland of four pregnant (day 12-14 of gestation) and seven lactating (day 12-14 postpartum) rats were excised and diced into approximately 1 mm^3 pieces. Four explants were placed onto siliconized lens paper rafts, which were prepared following the method of Topper *et al.* (1975). Explants from each rat were cultured in independent experiments, in 24-well tissue culture plates (Nunclon™, Nalge Nunc International), containing 1 ml/well culture medium (DMEM:F12 [Dulbecco's modified Eagle's medium:Nutrient mixture F12] with 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg amphotericin B/ml [all GibcoBRL/Life Technologies] and 3.7 g/l $NaHCO_3$, pH 7.4). Following 24 hours at 37°C in humidified 5% CO_2:95% air environments the media were replaced with fresh medium containing 250 U/ml rat recombinant IFN–γ (rIFN–γ; GibcoBRL/Life Technologies). Treatments were applied to triplicate wells. Following a further 24 hours in culture the media were collected and stored at -20°C for subsequent analysis of NOx. Explants were collected and pooled across the treatment triplicates, weighed, placed into cryovials, snap frozen in liquid nitrogen and stored at -80°C.

All experimental animals were used with the approval of the Ruakura Animal Ethics Committee (DDS 0032/98 approval # 2991 and #3287).

Sample analyses

The production of NO by Comma-D cells and mammary explants was determined indirectly by the detection of NOx. Briefly, NOx in the culture media was measured using a modification of the methods of Misko *et al.* (1993) and Verdon *et al.* (1995) with the following changes. The media samples were mixed with NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase and incubated for 1.5 hours at room temperature. Each sample then received 10 µl of 50 µg/ml 2,3-diaminonapthalene in 0.62 M HCl. Following a 10-minute incubation in the dark, the reaction was stopped with the addition of 10 µl 2.8 M NaOH to each sample. The absorbance of the samples was read on a FL500 Bio-Tek fluorescent plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA) at 360 nm excitation and 460 nm emission. For Comma-D culture, NOx in the medium is expressed as pmol NOx per µg DNA. For explant culture, NOx in the medium is expressed as pmol NOx per mg tissue.

DNA content of the wells from which the Comma-D cells were harvested was analysed using a method based upon that of Labarca & Paigen (1980) with the following change. Cellular solution (37.5 µl) was combined with 37.5 µl PBS and 75 ml 2X PBSE (0.1 M sodium phosphate; 4 M NaCl and 0.004 M EDTA, pH 7.4) prior to the addition of bisBenzamide (Hoechst No. 33258; 0.1 µg/ml bisBenzamide in PBSE [0.05 M sodium phosphate; 2 M NaCl and 0.002 M EDTA, pH 7.4]).

Statistical analyses

Analyses of differences in DNA content or explant weight between treatments and experiments was done by ANOVA using SAS (SAS System for Windows, Release 6.12, 1996; SAS Institute Inc; Cary, NC, USA). Differences in the production of NOx between treated and non-treated cells and explants were also analysed by ANOVA. Means are expressed ± S.E.M. Direct comparison of output of NOx between Comma-D and explants could not be made due to the different units of measurement (DNA vs weight).

RESULTS

There was no significant effect of treatment on the DNA content of the cultures (results not shown). However, the treatment of Comma-D cells with 250 U/ml mIFN–γ resulted in a significant ($P<0.001$) increase in the production of NOx in the medium. Concentrations rose to 5231 ± 1853% of the amount detected in the medium of untreated cells. Co-treatment of Comma-D cells with 250 U/ml mIFN–γ and either 100 µM AG or 1 mM L-NNA decreased the amount of NOx in the medium to 31 ± 8% and 19 ± 2% respectively of the cells treated with mIFN–γ alone (Table 1).

Explant weight did not vary between treatments (data not shown). Treatment of mammary explants from both pregnant and lactating rats with 250 U/ml rIFN–γ also significantly ($P<0.05$) increased the NOx concentrations in the medium to 300 ± 219% and 373 ± 191% respectively of the amount detected in the medium of

TABLE 1: Nitric oxide production by Comma-D cells and explants of mammary tissue. Comma-D cells were treated with 250 U/ml mouse recombinant IFN–γ (mIFN–γ), 250 U/ml IFN–γ + 100 µM aminoguanidine (mIFN–γ + AG) or 250 U/ml IFN–γ + 1 mM N^{ω}-nitro-L-arginine (mIFN–γ + LNNA). Nitrate plus nitrite (NOx) measured in the medium of IFN–γ and inhibitor treated cells is presented as a % of the NOx produced by the IFN–γ treated cells. Data are presented as the mean ± S.E.M. for 5 independent experiments. The response of Comma-D cells and explants of mammary tissue from pregnant (day 12-14 of gestation) and lactating (day 12-14 postpartum) rats to treatment with 250 U/ml mIFN–γ or 250 U/ml rat recombinant IFN–γ (rIFN–γ) respectively, was compared. Response is defined as the amount of NOx produced by IFN–γ-treated cells and explants as a % of the amount produced by untreated cells or explants. Data are presented as the mean ± S.E.M. for 13 (Comma-D), 4 (explants from pregnant rats) and 7 (explants from lactating rats) independent experiments respectively.

		Treatment	NOx Production	
			% of IFN-g treated	% of non-treated
Comma-D		mIFN–γ	100[a]	5231±1853[a]
		mIFN–γ + AG	31±8[b]	
		mIFN–γ + LNNA	19±2[b]	
Explants	Pregnant	rIFN–γ		300±219[b]
	Lactating	rIFN-γ		373±191[b]

[ab] Means within columns having superscript letters in common are not significantly different ($P<0.01$).

untreated explants. There was no significant difference between the amounts of NOx, following treatment with IFN–γ, in the medium of explants from pregnant and lactating rats (Table 1).

The relative response to stimulation with 250 U/ml IFN–γ by the explants from either pregnant or lactating rats was significantly (P<0.001) lower than that of the Comma-D cells (Table 1).

DISCUSSION

Comma-D cells respond to treatment with IFN–γ with an increase in NO production, measured as the increase in nitrite and nitrate (NOx) in the medium. This finding is supported by the results of Low *et al.* (1997) who reported a six-fold increase in nitrite in the medium of IFN–γ treated Comma-D cells.

In this study, explants of mammary tissue collected from female rats during mid-pregnancy and mid-lactation were examined for their response to IFN–γ. Explants of mammary tissue responded to the treatment with significant increases in the amount of NOx in the medium. No difference in the response to IFN–γ was apparent between the explants collected from pregnant or lactating rats. Previous studies have shown that explants of mammary tissue, collected from female rats are responsive to LPS (Onoda & Inano, 1998; Turner *et al.*, 2000). Together these results confirm that the mammary gland is capable of the production of NO in response to various stimulators.

Initial aims of this study were to compare the production of NO by explants of mammary tissue, with that produced by Comma-D cells. Comma-D cells are derived from mammary tissue collected from a female mouse during mid-pregnancy (Danielson *et al.*, 1984). Although Comma-D cells exhibit many characteristics distinctive of normal mammary epithelial cells, they are an isolated system in that they lack many of the other cell types that exist within the normal mammary gland. The culture of explants of mammary tissue provides a system in which many of these other cell populations are still present. Although both Comma-D cells and explants responded to IFN–γ by increasing the production of NOx, there was a vast difference in the magnitude of the response that may result from the presence of these other cell types. Direct *in vivo* measurement of NO production within the mammary gland has not been described in the literature and thus it is difficult to determine whether the production of NOx by Comma-D cells or the explants is more reflective of physiological levels of production.

The results presented in this paper show that both Comma-D cells and explants of rat mammary tissue provide a good model with which to investigate the effect of cytokines on the mammary production of NO. Many of the bioactive proteins and peptides recently described in bovine milk have been shown to exert biological effects including antimicrobial behaviours (Fox & Flynn, 1992; Schanbacher *et al.*, 1997). The experiments reported in this paper describe tissue and explant culture systems that can be used to examine the effect of various biological compounds on NO, an enzyme with an apparent role in the defence of the mammary gland. They may also provide a useful means of screening other milk bioactives for potential roles in the defence of the mammary gland, through the stimulation or inhibition of NOS, the enzyme responsible for the production of NO. However, the work also highlights the potential problems with the use of cell-lines, in that the results obtained may not accurately reflect the relative magnitude of the responses *in vivo*, especially when other cell types/populations contribute to the response. Further work is required to fully elucidate the mechanisms behind the different responses of Comma-D cells and mammary explants.

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