Induction of Nitric Oxide Production Mediated by Tumor Necrosis Factor Alpha on Staphylococcal Enterotoxin C-Stimulated Bovine Mammary Gland Cells

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Mammary gland (MG) secretions (MGS) derived from secretory cows infected with coagulase-negative staphylococci (CoNS) showed somatic cell counts and lactoferrin similar to levels found in the MGS of secretory cows infected with Staphylococcus aureus. However, nitrate and nitrite (NOx) and staphylococcal enterotoxin C (SEC) were found in MGS infected with S. aureus at much higher levels than in cows infected with CoNS. These results suggested that NOx could be intimately correlated with the production of SEC in secretory cows infected with S. aureus. Therefore, we examined the production of NOx and the expression of proinflammatory cytokines and microsomal cytochrome P450 (CYP450) after injection of SEC into the MGS of secretory cows. We were able to detect NOx and the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) on MG cells of SEC-injected MGS. It was also found that CYP450 in the MG cells from SEC-injected MGS was down-regulated by approximately one-third in comparison with the cells from phosphate-buffered saline-injected MGS. This in vitro system also showed that NOx could be induced in the culture of bovine macrophage-lined cells (FBM-17) with the supernatants of SEC-stimulated bovine peripheral blood lymphocytes (BoPBLs) but not in the culture of peripheral mononuclear cells with SEC-stimulated BoPBLs. The expression of the mRNA for both inducible nitric oxide synthase and TNF-α in FBM-17 was enhanced by culturing with the supernatant of SEC-stimulated BoPBLs, although CYP450 was down-regulated. These results indicate that the down-regulation of CYP450 was caused by the production of TNF-α in SEC-stimulating MG cells containing macrophages and via NOx production. Therefore, we suggest that NOx released from activated MG cells via the superantigenic activity of SEC caused oxidative damage to the MG in S. aureus-induced mastitis.

Staphylococcus aureus is the major causative bacteria of bovine mastitis. S. aureus infections result in changes of T-cell subpopulations and proinflammatory cytokine and chemokine production (27, 35). Moreover, lymphocytes and leukocytes stimulated with staphylococcal enterotoxins produce proinflammatory cytokines (36) and nitric oxide (17), which show inflammatory effects. In ruminants, many strains of S. aureus isolated from mammary gland (MG) secretions (MGSs) produce staphylococcal enterotoxin C (SEC) (16, 20, 33). It was reported that SEC induced clinical signs of bovine mastitis such as an increase in the number of leukocytes in the MG (16). SEC binds to the specific Vβ chain of the T-cell receptor via the major histocompatibility complex class II molecules of antigen-presenting cells such as macrophages and activates bovine T lymphocytes (5, 18, 36). Then, the activated T lymphocytes and macrophages produce proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, gamma interferon, and tumor necrosis factor alpha (TNF-α) (35, 36). Therefore, it is suggested that the superantigenic activity of SEC induces bovine mastitis (5, 36). Thus, it is indicated that inflammatory cytokines such as IL-1, IL-6, and TNF-α are produced by activated leukocytes and lymphocytes in the early stages of staphylococcal mastitis.

Down-regulation of intracellular cytochrome P450 (CYP450) expression is caused by IL-1, IL-6, and TNF-α (24, 25), which are produced in response to certain toxins such as staphylococcal enterotoxin B (31), aflatoxin B1 (15), and Escherichia coli lipopolysaccharide (LPS) (19). These cytokines also induce the expression of the inducible isoform of nitric oxide (NO) synthetase (iNOS), which results in the production of NO in certain cells including hepatocytes, macrophages, endothelial cells, and leukocytes during the cellular response. Several studies have suggested that NO production is mediated by the down-regulation of CYP450 expression in cytokine and LPS models of inflammation, based on the ability of exogenously administered NO to down-regulate CYP450 gene expression and attenuate the down-regulation by NO inhibitors (13, 14). Then, the production of NO is regulated by the down-regulation of CYP450 and causes oxidative damage to tissues (7, 25).

In this study, we examined whether leukocytes stimulated...
with SEC induce the down-regulation of CYP450 expression mediated by TNF-α and production of NO.

MATERIALS AND METHODS

**Milk samples.** A total of 10 milk samples from 10 cows were collected: 3 from healthy MGs of 3 cows, 5 from chronic mastitic MGs of 5 cows, and 2 from acute mastitic MGs of 2 cows. For all samples, we examined the inflammatory signs of MGs with a PL tester (Nippon Zenyaku Kogyou, Fukushima, Japan) to perform the modified Californian mastitis test (MCMT) and detect clots. The somatic cell counts (SCC) of MGs were made with a flow cytometer (FACSCalibur; Becton Dickinson Co., Ltd., San Jose, Calif.) and based on a cell count due to the specific binding of propidium iodide to DNA (11). The concentration of lactoferrin (Lf) was measured by the single radial immunodiffusion method (Eco-Biosystem Institute, Co., Furukawa, Japan) (12). The causative staphylococci in mastitic MGs were isolated with staphylococcus no. 110 agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the staphylococcal counts were measured as described by Kai et al. (9, 10). Coagulate production was determined by tube test of rabbit plasma (Eiken Chemical Co., Ltd., Tokyo, Japan). Isolated staphylococci were identified with a commercial kit (Api staph system; bioMérieux sa., Marcy l’Etoile, France). The SEC concentration in MGs was measured by using the sandwich enzyme-linked immunosorbent assay with anti-SEC sheep immunoglobulin G (IgG) and horse-radish peroxidase-conjugated anti-SEC sheep IgG (Toxin Technology, Inc., Sarasota, Fla.) (16).

**SEC injection into the mammary glands of lactating cows.** The tested cows were bred in our laboratory, and 7 MGs of 3 Holstein cows were used. The cows were 3 to 5 years old and more than 6 months parturient. In this experiment, we used MGs for which there were no clinical signs of mastitis, the MCMT was negative, total bacteria counts in MGs were less than 10^3 CFU/ml, S. aureus and E. coli were not detected, and SCC in MGs were less than 3 × 10^6/ml. One hundred micrograms of SEC (Toxin Technology, Inc.) was dissolved in 10 ml of phosphate-buffered saline (PBS) and sterilized by filtration through a membrane filter (pore size, 0.45 μm). Before the experiment, these cows were injected with antibiotics (cefazolin sodium; Mitaka Pharmaceutical Co., Ltd., Tokyo, Japan) every day for 3 days. After the antibiotic treatment, SEC (4 udders) and/or PBS (3 udders) was injected into each MG after milking by using a cannula (9, 10, 16).

**Measurement of MGs.** We examined the MG for inflammatory signs by using a PL tester to perform the MCMT and SCC and detect clots in MGs. Milk samples were obtained aseptically from each injected MG. Polymorphonuclear cells (PMNs) and mononuclear cells (MNs) were collected with a cytopsin (Shandon Scientific Ltd., Runcorn, Cheshire, England) and stained with May-Grunwald-Giemsa reagent. After drying, a minimum of 300 cells were identified and the proportions of various cell types were determined. MG cells were classified as PMNs, MNs, and/or other type cells. The proportions of PMNs and MNs were calculated by division by the total cell count (11).

**Cells.** Cells of SEC-injected MGs were separated as described previously (9). MGs were centrifuged at 1,000 × g for 20 min at 4°C to remove fat and whey, and then washed three times with PBS containing 20 mM EDTA. After the wash, MG cells were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 5% fetal calf serum (FCS; GIBCO BRL, Life Technology, Inc., Gaithersburg, Md.). The bovine macrophage cell line used was FBM-17 (obtained from K. Yoshihara, National Institute of Animal Health) (37). The FBM-17 line was established from a fetal bovine thymus and expresses major histocompatibility complex class I and II antigens on the cell surface. The culture medium used for the FBM-17 cells was RPMI 1640 supplemented with 15% FCS including 10^{-5} M 2-mercaptoethanol. Both bovine peripheral blood lymphocytes (BoPBLS) and PMNs were collected from the peripheral blood of healthy Holstein cows. Heparinized peripheral blood was separated with Lympholight H (Sotocan, Hornby, Canada), and BoPBLS were collected as the lymphocyte fraction. The precipitated cells were incubated at 37°C for 4 h, and PMNs were collected as cells that floated on the culture medium. The culture medium for PMNs was RPMI 1640 containing 10% FCS. BoPBLS (2 × 10^6 cells/ml) were incubated with SEC (1 μg/ml) at 37°C for 18 h. FBM-17 cells and PMNs were cultured with the culture supernatants of SEC-stimulated BoPBLS for 18 h, and the concentration of NO in the supernatants was measured. The expression of iNOS and TNF-α was stimulated in both FBM-17 cells and PMNs with the supernatants of SEC-stimulated BoPBLS in RPMI 1640 medium containing 1% FCS, and samples were collected at 4 h after cultivation. For the measurement of CYP450 content, FMB-17 was incubated with the supernatant of SEC-stimulated BoPBLS for 3 days at 37°C in a CO2 incubator.

**NOs measurement of MGs and culture supernatant.** MGs were treated with cold 0.1 M sodium acetate solutions (pH 4.0) to precipitate caseins (2). The sodium acetate-treated MGs were centrifuged at 4,000 × g for 10 min at 4°C to

**TABLE 1. Concentrations of SEC and NOx in staphylococcal mastitic MGs.**

<table>
<thead>
<tr>
<th>Isolated species</th>
<th>Staphylococcus koenigii (n = 3)</th>
<th>Staphylococcus xylosus (n = 2)</th>
<th>Staphylococcus warneri (n = 2)</th>
<th>Staphylococcus sciuri (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus enteritidis</td>
<td>(6.7 ± 4.8) × 10^5</td>
<td>(7.8 ± 0.4) × 10^5</td>
<td>(2.5 ± 6.2) × 10^5</td>
<td>(1.2 ± 0.9) × 10^5</td>
</tr>
<tr>
<td>Staphylococcus capitis</td>
<td>(5.1 ± 0.1) × 10^5</td>
<td>(3.6 ± 2.8) × 10^5</td>
<td>(6.7 ± 1.7) × 10^5</td>
<td>(6.7 ± 1.6) × 10^5</td>
</tr>
<tr>
<td>Concentration (CFU/ml)</td>
<td>131.8</td>
<td>121.5</td>
<td>2.02</td>
<td>2.02</td>
</tr>
<tr>
<td>SCC (cells/ml)</td>
<td>801.9</td>
<td>799.1</td>
<td>110.0</td>
<td>119.7</td>
</tr>
<tr>
<td>SEC (ng/ml)</td>
<td>968.3</td>
<td>968.3</td>
<td>1,120</td>
<td>1,120</td>
</tr>
<tr>
<td>NOx (Lf/10^6)</td>
<td>558.1</td>
<td>558.1</td>
<td>11.4</td>
<td>11.4</td>
</tr>
</tbody>
</table>

Results are means ± standard deviations. Significant differences were calculated with the paired t-test (P < 0.05).
remove casein and fat, and the supernatants were collected. FBM-17 cells and/or PMNs were incubated with the supernatants of SEC (1 µg/ml)-stimulated BoPBLs for 18 h at 37°C in a CO₂ incubator. The cultures of both cells were centrifuged at 1,000 × g for 15 min, and the supernatants were collected. The nitrate and nitrite (NOx) concentrations of the supernatants of sodium acetate-treated MGSs and culture supernatants of both FBM-17 cells and PMNs were measured by adding 100 µl of Griess reagent [2% sulfanilamide in 5% phosphoric acid mixed with 0.2% N-(1-naphthy1)-ethylenediamine (1:1, vol/vol)] and monitoring the absorbance at 550 nm (2, 3).

Immunohistochemistry. Mammary gland parenchymal tissue was taken from the central area of the upper body of the gland and from the area surrounding the gland cistern. Tissue samples were cut into cubes of less than 1 cm³, quickly frozen in acetone dry ice at −70°C, and stored below −70°C. Cryostat sections, 5 µm thick, were prepared from the frozen tissues. The sections were incubated with monoclonal antibody to ovine IL-1β, IL-6, and TNF-α (Chemicon International, Inc., Temecula, Calif.) at a 1:100 dilution for 14 h at 4°C. After three washes with PBS, fluorescein isothiocyanate-conjugated goat F(ab')₂ anti-mouse IgG1 and/or goat F(ab')₂ anti-mouse IgG2a diluted at 1:500 was added and the sections were incubated at room temperature (20°C) for 45 min. The immunoreactive cells were observed by using a confocal laser microscope (MRC-1024; Bio-Rad, Richmond, Calif.) (34).

Analysis of mRNA of IL-6, TNF-α, and iNOS by RT-PCR. In MG cells, PMNs, and FBM-17 cells, the mRNA expression of IL-6, TNF-α, iNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by reverse transcription-PCR (RT-PCR). Total RNA was extracted from the cells of SEC- and/or PBS-injected MGs, PMNs, and FBM-17 cells by using guanidinium thiocyanate and isolated with oligo(dT)-cellulose (QuickPrep micro mRNA purification kit; Amersham Biosciences UK Ltd., Little Chalfont, England). The mRNA sample (20 µl) was heated at 65°C for 10 min and cooled on ice. The first standard cDNA synthesis was performed with a first standard cDNA synthesis kit (Amersham Biosciences UK Ltd.). The samples were heated at 90°C for 5 min. For each PCR, 0.75 µl of input first standard cDNA was used in a final reaction volume of 50 µl containing 200 µM deoxynucleoside triphosphate, 0.4 M specific forward primer, 0.4 µM specific reverse primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 U of Taq DNA polymerase (Takara, Kyoto, Japan). The specific forward and reverse primers for GAPDH, IL-6, TNF-α, and iNOS were based on previous reports (3, 4). Thermocycling was accomplished by using a program with an initial denaturing step of 95°C for 10 min followed by 40 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 60 s in a Perkin Elmer 9600 thermocycler (Perkin Elmer, Palo Alto, Calif.). The mRNA expression levels for IL-6, TNF-α, and iNOS are presented as relative units after normalization to the observed GAPDH level.

Measurement of microsomal CYP450 concentration. SEC-exposed MG cells and FBM-17 cells incubated with the culture supernatant of SEC (1 µg/ml)-stimulated BoPBLs for 15 h at 37°C were removed and homogenized in 10 volumes of 0.15% KCl containing 1 mM EDTA (pH 7.4). The homogenates were centrifuged at 10,000 × g for 20 min. The supernatants were again centrifuged at 105,000 × g for 90 min. Pellets were resuspended in 10 mM phosphate buffer containing 1.15% KCl and 1 mM EDTA, pH 7.4 (26). Concentrations of CYP450 were obtained from the molar absorptivity, 1.20 × 10⁵ M⁻¹ cm⁻¹ at 450 nm, of the CO-reduced form (8).

Statistical analysis. The results are shown as the means ± standard deviations for these experiments. Data were compared by using the two-tailed Student's t test to determine significance.

RESULTS

NOx and SEC concentrations in staphylococcal mastitic MGSs. We isolated coagulase-negative staphylococci (CoNS) in clinically normal MGSs, and these MGSs showed low levels of staphylococcal counts, concentrations of SEC and NOx, and other clinical mastitic markers such as SCC and the LF concentration in MGSs. Chronic mastitic MGSs were classified into two groups: CoNS-infected MGSs and S. aureus-infected MGSs. In acute mastitic MGSs, we found that only S. aureus-infected MGSs were confirmed. In CoNS-infected chronic MGSs, we detected the mastitis value of staphylococcus counts (6.7 × 10⁴ ± 4.8 × 10⁴ CFU/ml), SCC (801.9 × 10⁴ ± 799.1 × 10⁴/ml), and LF concentration (793.5 ± 479.3 µg/ml) as the same as those in S. aureus-infected chronic (staphylococcus counts, 7.8 × 10⁵ ± 1.2 × 10⁵ CFU/ml; SCC, 1,195.0 × 10⁴ ± 505 × 10⁴/ml; LF concentration, 908.3 ± 119.7 µg/ml) and acute (staphylococcus counts: 2.5 × 10⁶ ± 0.4 × 10⁶ CFU/ml; SCC, 1,025.0 × 10⁴ ± 345.0 × 10⁴/ml; LF concentration, 558.1 ± 230.5 µg/ml) mastitic MGSs. However, the concentrations of both SEC (chronic mastitic MGSs, 11.20 ± 2.29 ng/ml; acute mastitic MGSs, 11.14 ± 2.02 ng/ml) and NOx (chronic mastitic MGSs, 11.72 ± 1.81 µM/ml; acute mastitic MGSs, 11.15 ± 1.02
(A) SCC of each quarter were also measured by flow cytometry with propidium iodide (10). Both PMNs and MNs of MGs were made to adhere to slides by using cyto spun. Then the slides were stained with May-Grünwald-Giemsa reagents, and cells were classified as PMNs (B), MNs (C), and other type cells (D). ●, cell counts of SEC-injected MGs; ○, cell counts of PBS-injected MGs; *, significant difference compared with PBS-injected MGs. All cell counts are given as 10^4 cells per milliliter.

FIG. 2. Changes of cell counts in MGs from SEC-injected MGs. SCC (A) of each quarter were also measured by flow cytometry with propidium iodide (10). Both PMNs and MNs of MGs were made to adhere to slides by using cyto spun. Then the slides were stained with May-Grünwald-Giemsa reagents, and cells were classified as PMNs (B), MNs (C), and other type cells (D). ●, cell counts of SEC-injected MGs; ○, cell counts of PBS-injected MGs; *, significant difference compared with PBS-injected MGs. All cell counts are given as 10^4 cells per milliliter.

μM/ml) in S. aureus-infected MGs were higher than those in CoNS-infected chronic mastitic MGs (SEC concentration, 0.077 ± 0.06 ng/ml; NOx concentration, 4.31 ± 1.53 μM/ml) (Table 1). The differences in the concentrations of SEC and NOx in MGs between CoNS-infected MGs and S. aureus-infected MGs was significant (P < 0.01).

Clinical signs and cell concentrations in SEC-injected MGs. A positive result for the MCMT and the presence of clots in MGs from SEC-injected MGs were confirmed at 17 h postinjection (hpi). However, we could not detect clinical signs in PBS-injected MGs (Fig. 1). SCC increased to 1,735.0 × 10^4 ± 994.6 × 10^4/ml in MGs of SEC-injected MGs at 17 hpi (Fig. 2A), and PMNs accounted for approximately 80% (1,465.6 × 10^4 ± 753.8 × 10^4/ml) of the SCC of SEC-injected MGs (Fig. 2B). Moreover, the number of MNs from SEC-injected MGs (236.0 × 10^4 ± 216.2 × 10^4/ml) had increased over 15-fold in comparison with MNs from PBS-injected MGs at 17 hpi (Fig. 2C). The difference in the number of these cells in MGs between SEC-injected and PBS-injected MGs was significant (P < 0.01).

NOx of MGs and culture supernatant. In MGs from SEC-injected MGs, the concentration of NOx increased to 13.52 ± 3.45 μM/ml at 15 hpi. However, NO production did not occur in MGs from PBS-injected MGs (Fig. 3A). We were able to confirm that the NOx concentration in the MG differed significantly between SEC-treated cows and PBS-treated cows (P < 0.01). Moreover, we found that supernatants of the culture of FBM-17 cells with the supernatants of SEC-stimulated BoPBLs showed higher levels (2.10 ± 0.51 μM/ml) than the culture supernatants of unstimulated FBM-17 and the culture of PMNs with the supernatants of SEC-stimulated BoPBLs (P < 0.01) (Fig. 3B).
injected MGs than in PBS-injected MGs. However, the reaction for both IL-1β and IL-6 did not differ between SEC and PBS injection (Fig. 4). The expression of mRNA for TNF-α (4.2 ± 0.85 U) and iNOS (15.4 ± 3.21 U) was confirmed on SEC-injected MG cells. However, the mRNA of TNF-α and iNOS could not be detected in PBS-injected MG cells. No expression of mRNA for IL-6 was detected on either SEC- or PBS-injected MG cells. In the culture of FBM-17 cells with the supernatants of SEC-stimulated BoPBLs, the mRNA expressions of IL-6 (41.9 ± 3.16 U), TNF-α (29.4 ± 0.58 U), and iNOS (7.2 ± 1.24 U) were stronger than that in unstimulated FBM-17 cells (Fig. 5). We were able to confirm that the mRNA expressions of TNF-α and iNOS differed significantly (P < 0.01).

CYP450 concentrations in SEC-stimulated MGs and FBM-17 cells. Microsomal CYP450 concentrations in MG cells from SEC-injected MGs (average, 0.38 ± 0.31 nmol/10⁶ cells) were approximately one-third times lower than those in MG cells of PBS-injected MGs (1.55 ± 0.31 nmol/10⁶ cells) at 15 hpi. Moreover, microsomal CYP450 concentrations of the culture of FBM-17 cells with SEC-stimulated BoPBLs (0.24 ± 0.09 nmol/10⁶ cells) were approximately half of those in unstimulated FBM-17 cells (0.56 ± 0.16 nmol/10⁶ cells) and cells of SEC-injected MGs (Fig. 6). In the case of the MG cells and FBM-17 cells, we confirmed the significance of the difference between SEC stimulation and no stimulation (P < 0.01).

**DISCUSSION**

In staphylococcal mastitic MGs, some inflammatory markers, including SCC and Lf concentration in CoNS-infected chronic mastitic MGs, were confirmed at the same levels of *S. aureus*-infected MGs. However, the NOx and SEC concentrations in *S. aureus*-infected MGs were significantly (P < 0.01) higher than those in CoNS-infected chronic mastitic MGs. These results suggest that the NOx concentration in mastitic MGs correlates with the SEC concentration in MGs. Thus, we examined the production of NO in MGs via SEC injection into healthy bovine MGs. Kuroishi et al. (16) reported that the injection of SEC into healthy mammary glands resulted in symptoms of mastitis and an increase in SCC. In this study, the same results were obtained for the kinetics of SCC. The PMNs among MG cells increased after SEC injection to over 90%. Moreover, MNs had increased approximately 20-fold at 15 hpi, with a peak increase of approximately 120-fold after SEC injection in comparison with MN counts from PBS-injected MGs. In addition, NO was detected in the MGs of FBM-17 cells with SEC-stimulated cows at 15 hpi. Moreover, we detected the production of NO in the culture of FBM-17 cells with the supernatants of SEC-stimulated BoPBLs but not in the culture of PMNs with the supernatants of SEC-stimulated BoPBLs. In addition, NO production in the SEC-stimulated FBM-17 cells was less than that in the culture of FBM-17 cells with the supernatants of SEC-stimulated BoPBLs (data not shown). These results suggest that SEC caused the symptoms of mastitis and oxidative damage by NO from activated MNs, including macrophages, via superantigen activity.

We detected an increase in the mRNA expression for TNF-α and iNOS in MG cells from SEC-injected MGs in comparison with MG cells from PBS-injected MGs. Moreover, we confirmed the expression of TNF-α, but not IL-6, in the cistern area of the SEC-injected mammary gland. NO production was detected in the culture of FBM-17 cells with the supernatants of SEC-stimulated BoPBLs. In bovine mastitis, Bouchard et al. (2) reported that NO is released from MG cells during endotoxin-induced mastitis. However, neutrophils, the major component in mastitic SCC, express the mRNA of iNOS and cannot produce NO (3). Therefore, it is suggested that NO in MGs from SEC-injected cows was produced by activated macrophages via the superantigen activity of SEC.

In acute mastitis with *E. coli*, TNF-α is a major mediator of endotoxin shock, and elevated TNF-α concentrations in milk were found in cows that had died from acute *E. coli* mastitis during the periparturient period (32). In addition, Blum et al. (1) reported intramammary production of TNF-α and NOx in quarters challenged by *E. coli* and LPS administration but not...
in unchallenged quarters. The enhanced TNF-α production was likely responsible for the increased intramammary NOx production and probably caused enhanced systemic NOx formation, which may contribute to the severity of clinical signs. In a recent report, staphylococcal enterotoxin B stimulation caused a down-regulation of CYP450 expression (31). The CYP450 gene is down-regulated by proinflammatory cytokines, including TNF-α. In the acute-phase reaction of an inflamma-

FIG. 4. Expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in SEC-injected bovine MGs. Cryosections of the cistern area of PBS-injected MGs and SEC-injected MGs are shown. Tissues were stained with anti-ovine IL-1β, IL-6, and TNF-α (green). Cell nuclei were counterstained with propidium iodide (red).

FIG. 5. mRNA expression of IL-6, TNF-α, and iNOS in both SEC-stimulated MG cells and the culture supernatant (Sup) of SEC-stimulated BoPBLs stimulated with FBM-17 cells. MG cells were collected from PBS- and SEC-injected MG 15 h after SEC injection. The culture of FBM-17 cells with the supernatant of SEC (2 μg/ml)-stimulated BoPBLs was incubated at 37°C for 4 h. The mRNA expression of GAPDH, IL-6, TNF-α, and iNOS was analyzed by RT-PCR. The number under each band represents the expression level relative to the gene for GAPDH. ND, not detected.
tory disease, previous reports indicated that down-regulation of CYP450 induced the production of NO and superoxide (7, 23, 29) and caused oxidative damage to tissues (25). Several studies have suggested that NO production is mediated by the down-regulation of CYP450 expression in cytokine and LPS models of inflammation, based on the ability of exogenously administered NO to down-regulate CYP450 gene expression and an attenuation of the down-regulation by NO inhibitors (13, 14). In addition, Minamiyama et al. reported that NO dose-dependently inhibits the hydroxylation activity of CYP450 (22). In this study, the microsomal CYP450 concentration of MG cells and FBM-17 cells decreased after stimulation with the supernatants of SEC-stimulated BoPBLs. Moreover, we detected the expression of iNOS in SEC-stimulated MG cells and FBM-17, a macrophage cell line. Therefore, it is suggested that SEC stimulation caused the expression of TNF-α and iNOS on MG cells, and this reaction induced the down-regulation of NO inhibitors mediated by the down-regulation of CYP450. These results indicate that SEC stimulation caused the production of NO mediated by TNF-α expression and down-regulation of CYP450 in bovine MG cells. The expression of TNF-α activates the transcriptional pathway of nuclear factor-κB (NF-κB), and this activation induces the expression of iNOS, which results in the production of NO in leukocytes (24). Therefore, TNF-α expression is a marker of NF-κB activation. The results of the present study suggest that after stimulation with SEC, mononuclear cells and macrophages expressed iNOS mRNA and activated the NF-κB pathway. In inflammatory disease, some intracellular enzymes and factors of the transcriptional pathway show down-regulation and/or activation, including CYP450 and NF-κB. CYP450 and the transcriptional pathway of NF-κB play an important role in the MG. CYP450 has aromatase activity for the differentiation of adipose fibroblasts into mature adipocytes mediated by peroxisome proliferator-activated receptor gamma. TNF-α, which is expressed in adipose, also inhibits adipocyte differentiation (21, 28). NF-κB activation is a negative regulator of β-casein gene expression in normal mouse MGs (6), and NF-κB activation and down-regulation of β-casein expression are mediated by TNF-α (30). Thus, it is suggested that SEC inhibits the differentiation of adipose fibroblasts into mature adipocytes and causes the down-regulation of milk protein production.

These results indicate that increases in these oxidative factors correlated with the down-regulation of CYP450 and were caused by TNF-α, and NO induced the oxidative damage to MGs of cows with mastitis. Moreover, it is suggested that SEC is an important virulence factor in staphylococcal bovine mastitis.

REFERENCES