Anti-inflammatory effects of retinoids and carotenoid derivatives on caspase-3–dependent apoptosis and efferocytosis of bovine neutrophils

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Objective—To evaluate immunomodulatory properties of all-trans retinoic acid and a fully oxidized β-carotene dietary product in calves with *Mannheimia haemolytica*–induced pneumonia.

Animals—Twenty-five 6- to 10-week-old male Holstein calves for experimental inoculations and three 8- to 30-week-old Angus heifers for blood donations.

Procedures—In vitro, neutrophils and monocyte-derived macrophages isolated from blood of healthy Angus heifers were treated with all-trans retinoic acid (1μM) or fully oxidized β-carotene (8.3 μg/mL) for various times and assessed for markers of cellular death, antimicrobial function, and production of proinflammatory leukotriene B_4. Following 28 days of dietary supplementation with fully oxidized β-carotene, Holstein calves were experimentally inoculated with *M haemolytica*. Bronchoalveolar lavage fluid was collected at 3 and 24 hours after challenge inoculation and analyzed for markers of apoptosis.

Results—In vitro, all-trans retinoic acid and fully oxidized β-carotene induced cell-selective, caspase-3–dependent apoptosis in neutrophils, which subsequently enhanced efferocytosis in macrophages. Conversely, neither treatment altered phorbol 12-myristate 13-acetate–induced oxidative burst, phagocytosis of nonopsonized zymosan (complement or antibody independent), or *M haemolytica*–induced leukotriene B_4 production in bovine neutrophils. In vivo, fully oxidized β-carotene enhanced leukocyte apoptosis in bronchoalveolar lavage fluid as well as subsequent efferocytosis by macrophages without altering numbers of circulating leukocytes.

Conclusions and Clinical Relevance—Neutrophil apoptosis and subsequent efferocytosis by macrophages are key mechanisms in the resolution of inflammation. Findings for the present study indicated that all-trans retinoic acid and fully oxidized β-carotene could be novel nutraceutical strategies that may confer anti-inflammatory benefits for cattle with respiratory tract disease. (*Am J Vet Res* 2014;75:1064–1075)

Excessive extravasation and activation of neutrophils is a primary component of airway inflammation and the pathogenesis of inflammatory disease.[1] Neutrophils become activated and migrate to the tissues in response to bacterial products and locally secreted proinflammatory mediators, including IL-8 (CXCL8) and LTB_4.[2–4] In inflammatory disease states, excessive recruitment and activation of neutrophils at the site of inflammation can result in local tissue damage that exacerbates inflammation.[1] Infiltrating neutrophils that are not efficiently cleared from the tissues undergo

Abbreviations

BAL	Bronchoalveolar lavage
EBTr	Bovine embryonic tracheal
FITC	Fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
HETE	Hydroxyeicosatetraenoic acid
HI-FBS	Heat-inactivated fetal bovine serum
IL	Interleukin
IMDM	Iscove modified Dulbecco medium
LT	Leukotriene
MDBK	Madin-Darby bovine kidney
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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necrosis, which releases proteolytic and histotoxic cellular contents, triggers a proinflammatory state in surrounding cells, and enhances tissue damage that leads to self-perpetuating chronic inflammation.[5,6]

In homeostatic conditions, infiltrating cells are disarmed and removed through apoptosis.[7] During apoptosis, caspases contribute to disassembling and packaging cellular components into membrane-bound apoptotic bodies.[8] The formation of these apoptotic bodies prevents spillage of cytotoxic cellular contents into the surrounding milieu. Apoptotic neutrophils lose their functional capacities and become hyporesponsive to external stimuli,[9,10] which further reduces their proinflammatory capacities and facilitates their nonphlogistic removal from a site of inflammation. Apoptotic cells also produce signals required for their detection and removal by phagocytes.[11,12] The phagocytic clearance of apoptotic cells, a process known as efferocytosis, protects surrounding tissues from spillage of harmful cellular contents and triggers an anti-inflammatory phenotype within phagocytes, which stimulates the production of anti-inflammatory mediators (transforming growth factor-β and IL-10) that promote resolution of inflammation.[13,14] Hence, neutrophil apoptosis and subsequent efferocytosis are critical mechanisms in the resolution of inflammation. Novel treatments that promote apoptosis and facilitate the nonphlogistic removal of inflammatory cells from a site of inflammation could be beneficial in the context of inflammatory disease and may offer possible avenues for drug development.

Mannheimia haemolytica, a primary bacterial agent in bovine respiratory disease complex, causes severe inflammatory tissue damage that can ultimately result in respiratory failure and death in many animals. Bovine respiratory disease complex is the most costly disease of the North American cattle industry,[15] with a mean annual incidence rate ranging from 14% to 20% and a mortality rate of 4%.[16] Much of the tissue damage associated with *M haemolytica* is attributed to the excessive infiltration and activation of neutrophils.[5,17] *Mannheimia haemolytica* secretes leukotoxin, which promotes the release of proinflammatory mediators such as IL-8 and LTB_4 from neutrophils and exacerbates the host inflammatory response.[3,18,19] Moderate concentrations of *M haemolytica* leukotoxin can induce apoptosis in bovine leukocytes.[20] Higher concentrations of *M haemolytica* leukotoxin cause leukocyte necrosis via the formation of cellular membrane pores,[21,22] which contribute to the release of histotoxic cellular contents and ultimately lead to the self-perpetuating inflammation characteristic of inflammatory disease.

Retinoids are a class of compounds related to vitamin A that have potential as anti-inflammatory treatments but whose mechanisms of action remain unclear. Retinoic acid, the active metabolite of vitamin A, has anti-inflammatory properties when used in the treatment of inflammatory skin diseases.[23] Dietary supplementation with vitamin A for rodents with experimentally induced pulmonary inflammation can reduce pulmonary infiltrate[24,25] and attenuate damage following inflammatory stimuli.[26] Furthermore, in vitro studies have revealed that retinoic acid can inhibit the production of proinflammatory LTB_4 from stimulated rat neutrophils[27] and reduce the production of reactive oxygen species.[28] Moreover, retinoids have proapoptotic effects in immune cells, including T lymphocytes[29] and dendritic cells.[30] However, retinoids also can have antiapoptotic properties in pulmonary epithelial cells[31] and eosinophils.[32] The effects of retinoids in bovine neutrophils are currently unknown.

Fully oxidized β-carotene is a carotenoid derivative produced by the complete autocatalytic radical oxidation of β-carotene,[33] and it is commercially available. Although retinoic acid and fully oxidized β-carotene are products of the oxidation of β-carotene (provitamin A), they differ chemically. Retinoic acid is a single compound generated in vivo via a highly specific, enzymic, oxidative cleavage of the central double bond of β-carotene. By contrast, fully oxidized β-carotene is an oxygen-rich mixture of products resulting from spontaneous, nonspecific action of oxygen at multiple double-bond sites, which leads principally to a novel class of newly discovered β-carotene–oxygen copolymers and minor amounts of non–vitamin A cleavage compounds. Results of a study[33] on in vitro gene expression suggest that fully oxidized β-carotene has the potential to modulate several facets of innate immunity, including the ability to limit inflammation. Because fully oxidized β-carotene does not have the provitamin or antioxidant activities of β-carotene, it affords a possible new veterinary nutraceutical product with novel modes of action that can be used to test the benefits of a dietary, carotenoid-derived product in vivo. We hypothesized that both all-trans retinoic acid and fully oxidized β-carotene would have proapoptotic effects in bovine neutrophils and that this would facilitate the phagocytic removal of these neutrophils by macrophages. Fully oxidized β-carotene may represent a novel nutraceutical to aid in the treatment of inflammatory diseases, such as *M haemolytica*–induced pneumonia in cattle. The purpose of the study reported here was to evaluate the immunomodulatory properties of all-trans retinoic acid and the carotenoid-derived dietary product fully oxidized β-carotene in cattle with *M haemolytica*–induced pneumonia, which is characterized by severe inflammation.

Materials and Methods

Animals—Two groups of calves were used for the experiments. Three healthy 8- to 30-week-old Angus heifers were used as blood donors for in vitro experiments; these heifers were housed outdoors at a veterinary science research station.[a] Twenty-five Holstein steers (age, 1 to 3 weeks; mean ± SD body weight, 56.5 ± 5.8 kg) were used in all in vivo experiments. Steers were housed individually indoors at the veterinary sciences research station,[a] fed antimicrobial-free milk replacer[b] twice daily, and provided ad libitum access to water. A photoperiod of 12 hours of light to 12 hours of darkness was maintained. Room temperature was maintained between 17°C and 23°C with a relative humidity of 40%. Animal care and experimental procedures, including routine blood collection for in vitro experiments, were conducted in accordance with the standards of the Canadian Council on Animal Care and were approved by the University

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of Calgary Life and Environmental Sciences Animal Care Committee.

Neutrophil purification—Whole blood was collected from each of the 3 Angus heifers. Blood samples were collected from a jugular vein into evacuated tubes containing 1.5 mL of anticoagulant acid citrate dextrose solution A,[c] as described elsewhere.[34] Blood samples then were pooled and centrifuged for 20 minutes at 1,200 × *g* and 4°C. Plasma and the buffy coat were removed, and the remaining cells were washed with 20 mL of HBSS and centrifuged for 10 minutes at 1,200 × *g* and 4°C. Contaminating erythrocytes were eliminated by 3 sequential lysis steps involving the addition of 20 mL of cold filter–sterilized hypotonic lysis solution (10.6mM Na_2HPO_4 and 2.7mM NaH_2PO_4) and the restoration of isotonicity with 10 mL of cold 3× hypertonic restoring solution (10.6mM Na_2HPO_4, 2.7mM NaH_2PO_4, and 462mM NaCl). The cell mixture was centrifuged at 1,200 × *g* for 10 minutes, and the resulting cell pellet was resuspended in warm (37°C) HBSS containing 10% HI-FBS[d] to optimize the cell environment. The concentration of neutrophils and percentage of viable cells were determined by microscopic examination and enumeration of cells that excluded 0.4% trypan blue stain[e] with a hemacytometer.[f] Differential cell counts were performed on stained[g] cytocentrifuged preparations[h] to assess neutrophil purity. Neutrophil populations used for all in vitro experiments were > 90% pure and > 90% viable.

Monocyte isolation, macrophage differentiation, and cell line culture—Isolation of circulating monocytes was performed by differential centrifugation for 20 minutes at 1,200 × *g* and 4°C, as described elsewhere.[34] Plasma was removed, and the buffy coat was isolated and diluted 1:1 with filter-sterilized saline (0.9% NaCl) solution. The mixture was overlaid onto sterile-filtered polysucrose and a sodium diatrizoate gradient[i] to facilitate monocyte isolation and was then centrifuged for 40 minutes at 1,200 × *g* and 4°C. Supernatant was discarded; monocytes were isolated from the opaque interface and washed with 20 mL of HBSS. Contaminating erythrocytes were eliminated by lysis with a hypotonic solution. Purified monocytes were resuspended in IMDM[j] containing 10% HI-FBS and cultured for macrophage differentiation, as described in another study.[35] Differential counts were performed on stained[g] cytocentrifuged preparations to assess monocyte purity. Cells were incubated at 37°C and 5% CO_2 in 48-well plates for coculture experiments. Nonadherent cells were removed by 3 sequential washes with warm (37°C) HBSS; adherent monocytes were incubated in a humidified chamber at 37°C and 5% CO_2 in IMDM containing 10% HI-FBS, penicillin (100 U/mL), streptomycin (100 U/mL), and tylosin (80 µg/mL). Monocytes were incubated in a humidified chamber at 37°C and 5% CO_2 for 7 days to allow for macrophage differentiation (first day of culture was defined as day 1). Cell culture medium was replenished every 2 to 3 days. Experiments were performed with IMDM containing 10% HI-FBS with no antimicrobials. Macrophage differentiation was confirmed by morphology evaluation of stained cells (Romanowski stain[g]) via light microscopy; esterase staining was performed on days 2, 5, and 7 to assess macrophage maturation, as described previously.[34] On day 7, > 95% of the macrophages had differentiated into esterase-positive adhering macrophages. Circulating monocytes spontaneously differentiate into esterase-positive macrophages, and this approach to confirm macrophage differentiation has been verified.[34] Viability of cells isolated for macrophage differentiation was > 95% pure throughout the study. Macrophages were > 95% mature on day 7.

The MDBK epithelial cell line NBL-1[k] was grown in Dulbecco modified Eagle medium. The EBTr fibroblast cell line NBL-4[k] was grown in minimum essential medium Eagle[i] (HEPES modification) containing Earle's salts, 25mM HEPES and sodium bicarbonate (1.5 g/L), 2mM L-glutamine, 1.0mM sodium pyruvate, penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% HI-FBS. Cells were used between passages 126 and 136 (MDBK epithelial cell line NBL-1) or 38 and 40 (EBTr fibroblast cell line NBL-4). All cell lines were cultured in a humidified chamber at 37°C and 5% CO_2.

In vitro treatments—The day of experiments, cell culture medium was replaced with medium containing no antimicrobials. Cells then were incubated for 24 hours with or without treatment. Purified bovine neutrophils or bovine cell lines were treated with 1µM all-trans retinoic acid,[i] fully oxidized β-carotene (8.3 µg/mL),[l] 1µM staurosporine, or 10% HI-FBS, with the appropriate cell culture medium as a control sample. For inhibitor experiments, purified bovine neutrophils were pretreated by incubation for 1 hour with a caspase-3–selective inhibitor[m] (20µM) or vehicle (dimethyl sulfoxide) at 37°C and 5% CO_2 prior to the treatment period. All in vitro experiments were performed at 37°C and 5% CO_2, as described in another study.[35]

In vivo experiment—All steers were allowed to acclimate for a period of at least 7 days prior to group assignment. Calves were assigned into 1 of 3 groups on the basis of equalized body weight distribution. Inoculated-treated calves (n = 10) received a single daily dose (10 mg/kg) of a fully oxidized β-carotene dietary product[l] for 28 days and were inoculated intratracheally with 2 × 10^8 CFUs of *M haemolytica* in 10 mL of endotoxin-free PBS solution at the end of that period (ie, day 28). The dietary product was administered daily within the milk replacer during the evening feeding. Inoculated-untreated calves (n = 7) did not receive the dietary product and were inoculated intratracheally with 2 × 10^8 CFUs of *M haemolytica* in 10 mL of endotoxin-free PBS solution on day 28. Control calves (n = 8) did not receive the dietary product and were inoculated intratracheally with 10 mL of endotoxin-free sterile PBS solution on day 28.

In vivo bacterial challenge exposure—On day 28, lidocaine[n] was infused into the tissues overlying the trachea. An incision was made in the skin and trachea, and a sterile 16-gauge trocar was inserted through the incision into the trachea of each calf. A sterile 1.7-mm catheter[o] was then inserted through the trocar, with the catheter tip positioned at the tracheal bifurcation. Inoculation consisted of 10 mL of endotoxin-free PBS solution without (sham) or with (challenge exposure) 2 × 10^8 CFUs of *M haemolytica* injected through the

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catheter and into the lungs, as described elsewhere.[35,36] The catheter and trocar were removed after inoculation, and the incision site was wiped with sterile gauze and sealed with tissue adhesive.[p]

BAL—Samples of BAL fluid were collected 3 and 24 hours after inoculation. Samples were obtained by 3 sequential washes with 20 mL of endotoxin-free sterile HBSS.[36] After BAL samples were collected at 24 hours, calves were euthanized by IV injection of sodium pentobarbital. Microscope slides were prepared with BAL fluid (100 µL/slide) by use of cytocentrifugation[h] (10 minutes at 113 × *g*). Cells were then stained[g] or fixed with freshly prepared 4% paraformaldehyde in pyrogen-free PBS solution (0.15M NaCl; pH, 7.2) for cell identification and apoptosis detection. Infiltration of neutrophils was measured by enumeration of neutrophils as a percentage of the total cells in BAL fluid cytocentrifuge samples.

The remaining BAL fluid was centrifuged for 10 minutes at 1,500 × *g*. To confirm the presence of bacteria, a portion of the BAL fluid was serially diluted and plated on Columbia agar for colony enumeration of *M haemolytica*. The remainder of the supernatants was snap-frozen in liquid nitrogen and stored at –70°C for future analyses. Cell pellets were resuspended in HBSS for cell enumeration with a hemacytometer. Equal numbers of leukocytes were used for detection of apoptosis. Protein concentrations in remaining cell pellets were standardized for western blot analysis.

Apoptotic DNA fragmentation—Apoptosis was examined with a cell-death detection ELISA kit[q] used in accordance with the manufacturer's instructions.[35,36] Colorimetric development of the assay was measured at 405 nm with a microplate reader.[r] Apoptosis was expressed as absorbance ratios of each experimental group relative to unmanipulated control samples (values for control samples were arbitrarily set to 1.0). Bovine neutrophils (10^6 cells) were incubated with all-trans retinoic acid, fully oxidized β-carotene, or staurosporine at 37°C and 5% CO_2 for 0.25 to 0.5 hours. To assess whether the proapoptotic effects of all-trans retinoic acid and fully oxidized β-carotene were caspase-3 dependent, neutrophils were incubated in the presence of a caspase-3–selective inhibitor[m] for 1 hour at 37°C and 5% CO_2 prior to the treatment period.

Cellular death was also examined by means of fluorescent microscopy of in situ TUNEL staining, which labeled both apoptotic and necrotic cells. Cytocentrifuge preparations fixed in 4% paraformaldehyde were washed with PBS solution and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Slides then were washed with PBS solution and incubated in the dark with TUNEL reaction mixture for 1 hour at 37°C, in accordance with the manufacturer's instructions. Slides were counterstained with Hoechst nuclear stain at 37°C for 30 minutes, after which the slides were washed with PBS solution and mounted. Fluorescence was assessed at 488 nm (excitation) and 515 nm (emission) for TUNEL staining and 350 nm (excitation) and 461 nm (emission) for Hoechst nuclear staining. Detection of TUNEL-positive cells for all cytocentrifugation samples was assessed by enumeration of at least 100 cells in 3 randomly selected fields of view/sample at 400× magnification by use of a fluorescent microscope and fluorescent camera.[s]

Western blotting—Cleavage of caspase-3 was assessed by means of western blotting. Treated cells were washed with HBSS and lysed with a lysis buffer (1% detergent, 0.1% SDS, and 0.5% sodium deoxycholate diluted in PBS solution) containing a protease inhibitor cocktail.[q] Total protein concentrations were determined by means of a Bradford protein assay[t] performed in accordance with the manufacturer's instructions and standardized at 1 to 3 mg of protein/mL for each experiment. Whole-cell lysates were diluted 1:1 in 2× electrophoresis buffer (17% [vol/vol] glycerol, 8% [vol/vol] β-mercaptoethanol, 5% [wt/vol] SDS, 22% [vol/vol] 1M Tris-HCl [pH, 7.0], and 0.04% [wt/vol] bromophenol blue) and heated at 90°C for 3 minutes. Proteins were resolved on a 10% SDS-polyacrylamide gel by electrophoresis and then electrotransferred to nitrocellulose membranes. Membranes were blocked in 5% [wt/vol] bovine serum albumin in Tris-buffered saline solution with 0.1% Tween for 1 hour at 21°C. Membranes were then probed with rabbit polyclonal anti–cleaved caspase-3,[u] rabbit polyclonal anti–β-actin,[u] or rabbit polyclonal anti-myeloperoxidase[v] at a dilution of 1:500. After exposure to each primary antibody, blots were incubated with anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (final dilution, 1:1,000) for 1 hour at 21°C. Bands were developed with an enhanced chemiluminescence detection system,[w] in accordance with the manufacturer's instructions.

Detection of cytotoxicosis—To assess the cytotoxic effects of fully oxidized β-carotene and all-trans retinoic acid, cellular supernatants were isolated and analyzed for the presence of lactate dehydrogenase with a commercially available cytotoxicosis detection kit.[q] Photometric development was measured at 490 nm. Amount of necrosis was reported relative to that for cells treated with 5% Triton X-100.

LTB_4 assay—To examine the effects of retinoic acid and fully oxidized β-carotene on neutrophil LTB_4 production in vitro, the concentration of LTB_4 was measured in neutrophil supernatants. Protein precipitation and solid-phase extraction were performed as per manufacturer's instructions. The LTB_4 concentrations were quantified at 405 nm by means of a competitive ELISA kit,[x] in accordance with the manufacturer's instructions. Specificity of the assay was 100% for LTB_4, 6.6% for 5(*S*)-HETE, 3.7% for 5(*R*)-HETE, and < 1% for 15(*R*)-HETE, 15(*S*)-HETE, LTC_4, LTD_4, and LTE_4. Detection limit of the assay was 7 pg/mL.

Neutrophil function assays—Nitro blue tetrazolium reduction was assessed in treated neutrophils as a marker for oxidative burst function.[37,38] For these experiments, treated neutrophils were stimulated by incubation with phorbol 12-myristate 13-acetate (0.6 µg/mL) in the presence of nitro blue tetrazolium substrate[i] for 1 hour at 37°C and 5% CO_2. Formazan deposits were solubilized from cellular lysates and detected at 620 nm. Data were reported as absorbance ratios for each experimental group relative to the absorbance for unstimulated control cells.

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For phagocytosis experiments, purified bovine granulocytes (4×10^6) were cultured in complete Dulbecco modified Eagle medium.[i] Cells were treated with fully oxidized β-carotene for 24 hours, then incubated with FITC-labeled nonopsonized zymosan for 2 hours at 37°C and 5% CO_2 to stimulate pathogen-driven immune activation and phagocytosis. Briefly, unlabeled nonopsonized zymosan particles[j] were labeled by incubation overnight with FITC[i] (250 ng/mL) and continuous shaking at 4°C in carbonate buffer (0.1M sodium carbonate and 0.1M sodium bicarbonate; pH, 9.6). After staining, zymosan-FITC was washed twice with 1× PBS solution. For examination of binding and internalization events, cells were harvested and analyzed on an imaging multispectral flow cytometer,[y] as described in another study.[39] To distinguish between bound and internalized particles, a gating mask was created with software.[y] This gating mask allowed differentiation between internalized (degree of internalization > 0) and bound (degree of internalization < 0) zymosan particles in the x-y plane. Furthermore, analysis of the degree of focus of each zymosan particle relative to the cell nucleus identified internalized particles for the z-axis. The impact of fully oxidized β-carotene on binding and internalization capacity was assessed separately on the basis of differential capacity to induce downstream antimicrobial effects. At least 10,000 cells/sample were acquired.

Efferocytosis detection assays—For detection of in vitro efferocytosis, myeloperoxidase activity was assessed in neutrophil-macrophage cocultures. For these experiments, treated neutrophils were cocultured with monocyte-derived bovine macrophages in a humidified chamber at 37°C and 5% CO_2. Myeloperoxidase activity was measured in coculture supernatants and monolayers by means of a kinetic assay.[40] Photometric development of the assay was measured at 460 nm at multiple time points to yield data on enzyme activity, which was defined as the change in optical density over time. Data were reported as the fold change relative to values for control cocultures (values of control cocultures were arbitrarily set to 1.0). In vivo efferocytosis was assessed by direct microscopic enumeration of efferocytosis-positive macrophages within cytocentrifuged samples of BAL fluid, which was defined as macrophages containing at least 1 apoptotic neutrophil within phagocytic vacuoles. Data were reported as the percentage of the total macrophages.

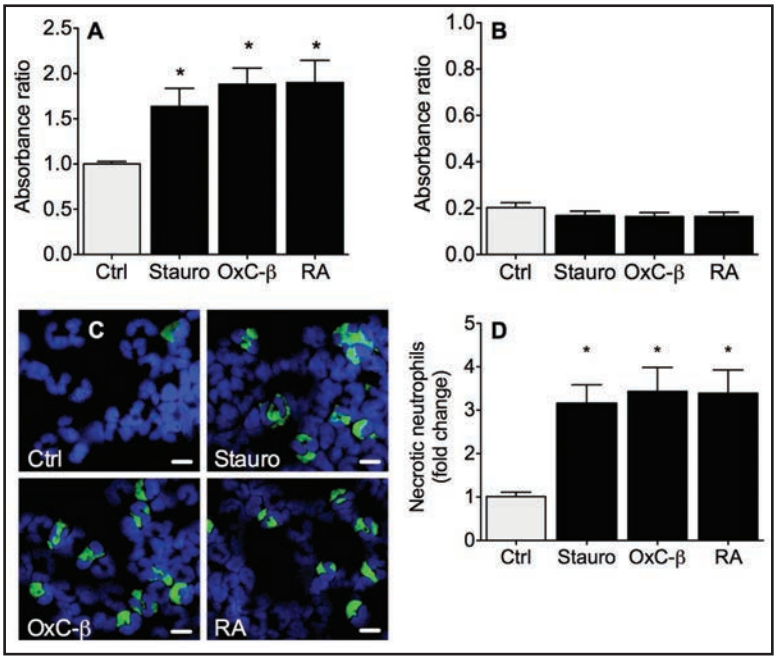

Figure 1—Amounts of apoptotic mononucleosomes and oligonucleosomes in bovine neutrophils (A), release of lactate dehydrogenase from bovine neutrophils (B), photomicrographs of apoptotic bovine neutrophils after fluorescent staining (C), and enumeration of apoptotic or necrotic bovine neutrophils (D). A—Mean ± SEM values for neutrophils incubated with all-trans retinoic acid (1μM [RA]), fully oxidized β-carotene (8.3 μg/mL [OxC-β]), staurosporine (1μM [Stauro]), or control medium (Ctrl) for 0.5 hours (n = 6 cultures/group). Values are calculated as ratios of the absorbance for each treatment versus that measured for neutrophils incubated in control medium (10% HI-FBS in HBSS), with the absorbance for the control medium arbitrarily set to 1.0. B—Mean ± SEM values of a cell-death ELISA for neutrophils incubated for 0.5 hours with the 3 treatments in panel A and another control medium (n = 6 cultures/group). Values are calculated as ratios of the absorbance for each treatment versus that measured for neutrophils incubated in control medium (5% Triton X-100). C—Photomicrographs of apoptotic neutrophils after incubation in various treatments and fluorescent staining to reveal viable (blue) and apoptotic and necrotic (green) neutrophils. TUNEL stain and Hoechst nuclear counterstain; bar = 10 μm. D—Mean ± SEM fold change for each treatment, compared with results for the control medium, as determined by use of fluorescent staining. *Value differs significantly ($P < 0.05$) from the value for the control treatment.

Statistical analysis—Data for all in vitro experiments were analyzed by means of a 1-way ANOVA. Multicomparison post hoc analysis was performed with the Tukey test. For in vivo analyses where data were not normally distributed, the nonparametric Kruskal-Wallis test was used followed by a Dunn post hoc analysis. Values were considered significant at $P < 0.05$. Multiple replicates of a minimum of 3 separate experiments were performed. Values were reported as mean ± SEM.

Results

Incubation with all-trans retinoic acid and fully oxidized β-carotene for 0.5 hours both significantly increased neutrophil apoptosis relative to results for control cocultures, as determined by ELISA results (**Figure 1**). Release of lactate dehydrogenase was also measured as an indication of cellular necrosis and remained < 20% of total lactate dehydrogenase in all experiments, which indicated a low amount of necrosis. Staining with TUNEL corroborated these findings, which indicated that treatment with all-trans retinoic acid or fully oxidized β-carotene significantly increased numbers of TUNEL-positive neutrophils, compared with results for control cultures. Staurosporine (1μM), which was used as a positive control treatment in all experiments, also induced apoptosis in neutrophils.

Consistent with caspase-3 activation, there was a significant increase in the appearance of cleaved caspase-3 fragments in neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene, relative

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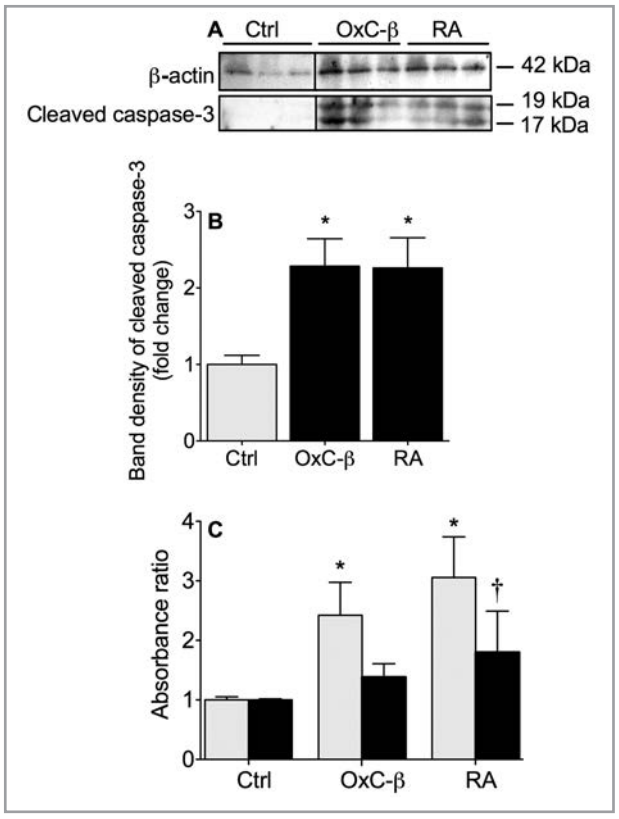

Figure 2—Western blot of cleaved caspase-3 in bovine neutrophils incubated for 0.25 hours in control medium (10% FBS-HBSS), fully oxidized β-carotene (18.3 μg/mL), or all-trans retinoic acid (1μM; A) and mean ± SEM values for densitometric analysis (B) and cell death (C) of bovine neutrophils (n = 3 or 4 cultures/group) incubated with (black bars) and without (gray bars) caspase-3 inhibitor (20μM) for 1 hour, followed by incubation in control medium, fully oxidized β-carotene, or all-trans retinoic acid for 0.5 hours. For panel B, values are normalized to results for β-actin and represent the fold change for each treatment versus that for the control medium, with the value for the control medium arbitrarily set to 1.0. For panel C, values are the ratios of the absorbance for each treatment versus that measured for neutrophils incubated in control medium, with the absorbance for the control medium arbitrarily set to 1.0. *Value differs significantly ($P < 0.05$) from the value for the control medium without inhibitor. †Within a treatment, value differs significantly ($P < 0.05$) from the value for neutrophils incubated without the inhibitor.

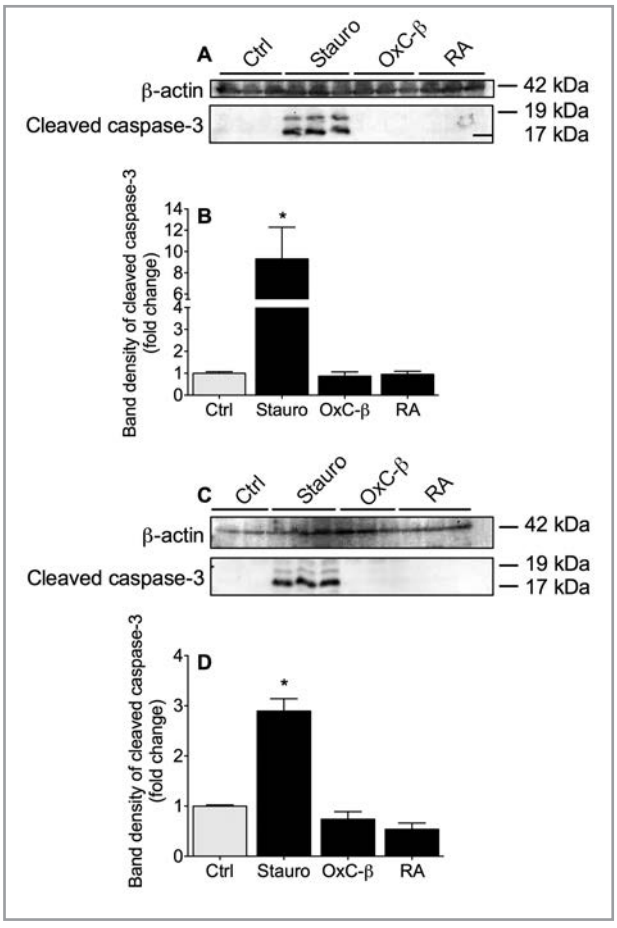

Figure 3—Western blot of cleaved caspase-3 (A and C) and mean ± SEM amount of caspase-3 fragmentation (B and D) in bovine epithelial cells (A and B) and bovine fibroblasts (C and D) incubated for 24 hours in all-trans retinoic acid, fully oxidized β-carotene, staurosporine, or control medium (n = 3 cultures/group). For panels B and D, densitometry values are normalized to results for β-actin and represent the ratio of cleaved caspase-3 for each treatment versus that measured for the control medium, with the value for the control medium arbitrarily set to 1.0. *See* Figure 1 for remainder of key.

to results for control cultures (**Figure 2**). Furthermore, pretreatment of bovine neutrophils with the selective caspase-3 inhibitor reduced the proapoptotic effects of all-trans retinoic acid and fully oxidized β-carotene.

Incubation with staurosporine for 24 hours induced caspase-3 cleavage in MDBK epithelial cells and EBTr fibroblasts, relative to results for control cultures, whereas all-trans retinoic acid and fully oxidized β-carotene failed to induce caspase-3 cleavage (**Figure 3**). Moreover, lactate dehydrogenase release remained < 30% for all experiments (data not shown).

Bovine monocyte-derived macrophages were cocultured with treated neutrophils to determine whether apoptotic neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene could be readily efferocytosed by macrophages. Myeloperoxidase activity was measured in coculture lysates and supernatants as a marker for efferocytosis. Findings indicated that bovine neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene were readily efferocytosed by macrophages in that intracellular myeloperoxidase activity was significantly higher in cellular lysates of macrophages cocultured with neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene, relative to myeloperoxidase activity for control cocultures (**Figure 4**). Correspondingly, myeloperoxidase activity was significantly lower in coculture supernatants of macrophages incubated with bovine neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene, compared with myeloperoxidase activity for control cocultures. Mean ± SEM baseline myeloperoxidase activity in monocyte-derived bovine macrophages was 0.0037 ± 0.0003 mU/min, relative to results for control cocultures, which supported the hypothesis that myeloperoxidase activity was indicative of phagocytosed neutrophils.

Neutrophils cocultured with live *M haemolytica* for 1 hour released significantly higher amounts of LTB_4 into the extracellular milieu. Treatment with all-trans retinoic acid or fully oxidized β-carotene did not significantly alter LTB_4 concentrations in cell culture supernatants (**Figure 5**). Furthermore, in vitro experiments

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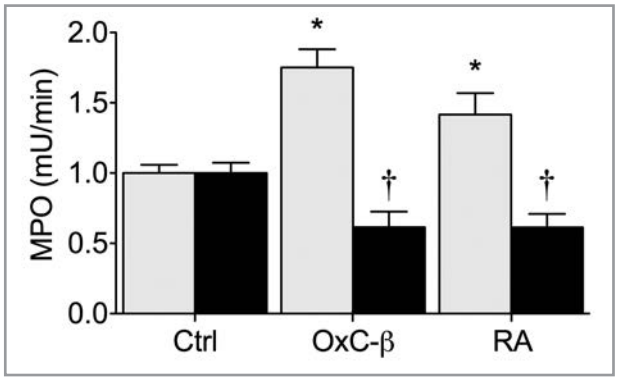

Figure 4—Mean ± SEM intracellular (gray bars) and extracellular (black bars) myeloperoxidase (MPO) activity in coculture lysates and supernatants, respectively, of purified bovine neutrophils incubated with all-trans retinoic acid, fully oxidized β-carotene, or control medium and cocultured with bovine monocyte-derived macrophages for 0.5 hours (n = 3 cultures/group). Values represent MPO activity for each treatment versus that for the control medium, with the value for the control medium arbitrarily set to 1.0 mU/min. *Value differs significantly ($P < 0.05$) from the value for the intracellular control medium. †Within a treatment, value differs significantly ($P < 0.05$) from the value for intracellular MPO activity. *See* Figure 2 for remainder of key.

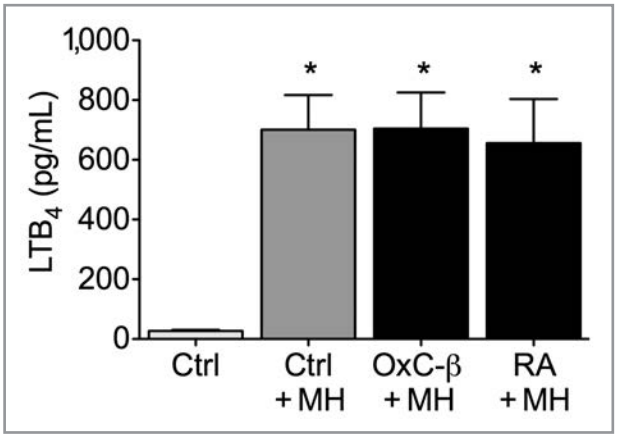

Figure 5—Mean ± SEM LTB_4 concentration in purified bovine neutrophils incubated with all-trans retinoic acid, fully oxidized β-carotene, or control medium in the presence of *Mannheimia haemolytica* (MH; multiplicity of infection, 10:1) or control medium without *M haemolytica* for 1 hour (n = 4 cultures/group). Leukotriene B_4 was quantified in cellular supernatants by means of a competitive LTB_4 ELISA. *Value differs significantly ($P < 0.05$) from the value for control medium without *M haemolytica*.

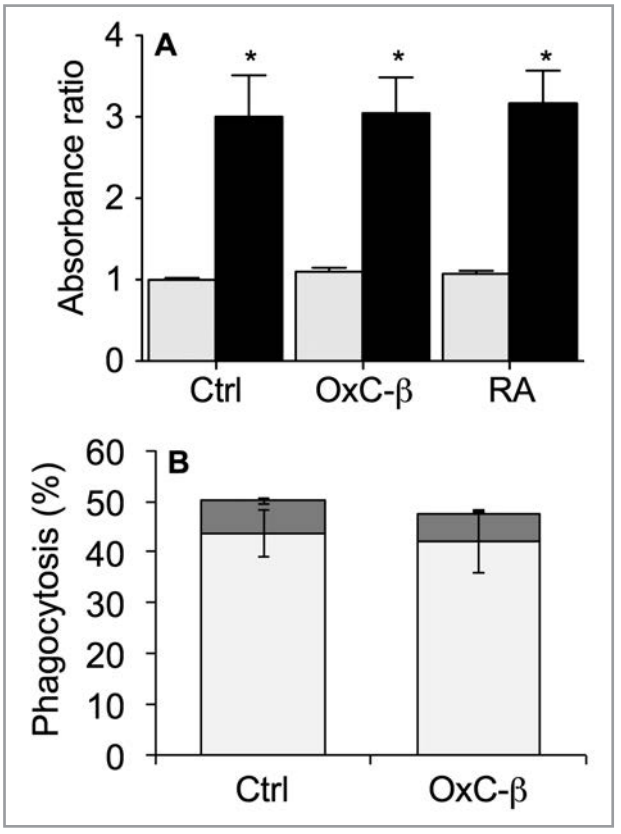

Figure 6—Amount of stimulation for purified neutrophils incubated with all-trans retinoic acid, fully oxidized β-carotene, or control medium with (black bars) and without (gray bars) subsequent stimulation with phorbol 12-myristate 13-acetate (0.6 μg/mL) in the presence of nitro blue tetrazolium (0.1 μg/mL) for 1 hour (A) and percentage of granulocytes with zymosan granules bound to the surface of (dark gray bars) or internalized in (light gray bars) those granulocytes, which were incubated with fully oxidized β-carotene (8.3 μg/mL) for 24 hours and subsequently stimulated with FITC-labeled zymosan for 2 hours before analysis on a multispectral flow cytometer (B). Values represent mean ± SEM (n = 3 cultures/group). In panel A, formazan deposits from cellular lysates were solubilized and quantified by absorbance at 620 nm, and data represent the ratio for the absorbance values for each treatment versus that for the control medium, with the value for the control medium arbitrarily set to 1.0. *Within a treatment, value differs significantly ($P < 0.05$) from the value for the unstimulated neutrophils.

were also performed to determine whether treatment with all-trans retinoic acid or fully oxidized β-carotene could alter neutrophil function. Analysis of nitro blue tetrazolium reduction revealed that all-trans retinoic acid or fully oxidized β-carotene did not significantly alter oxidative burst in unstimulated or phorbol 12-myristate 13-acetate–stimulated bovine neutrophils (**Figure 6**). In addition, analysis of granulocyte phagocytosis revealed no significant alteration in the percentage of bound or internalized zymosan following treatment with fully oxidized β-carotene for 24 hours.

Given the proapoptotic effects of fully oxidized β-carotene on bovine neutrophils in vitro, differential cell counts were performed on blood samples collected from calves at the start (day 0) and end (day 28) of the treatment phase to ensure that typical numbers of circulating leukocytes were maintained. Differential counts revealed no significant alterations in circulating leukocyte populations as a function of time or as a result of dietary inclusion of fully oxidized β-carotene (**Figure 7**). In addition, treatment with fully oxidized β-carotene did not alter the composition of leukocytes infiltrating the respiratory tract (data not shown).

In samples obtained 3 hours after challenge exposure, TUNEL staining of leukocytes in BAL fluid suggested an increase in leukocyte apoptosis in bacterial-inoculated calves treated for 28 days with fully oxidized β-carotene, relative to results for other groups in which there were fewer TUNEL-positive leukocytes (**Figure 8**). A cell-death detection ELISA confirmed that BAL fluid isolated from bacterial-inoculated calves treated for 28 days with fully oxidized β-carotene contained significantly higher numbers of apoptotic leukocytes than were contained in BAL fluid of untreated bacterial-inoculated calves at 3 hours but not at 24 hours after inoculation. Western blot data supported these findings, given that caspase-3 fragmenta-

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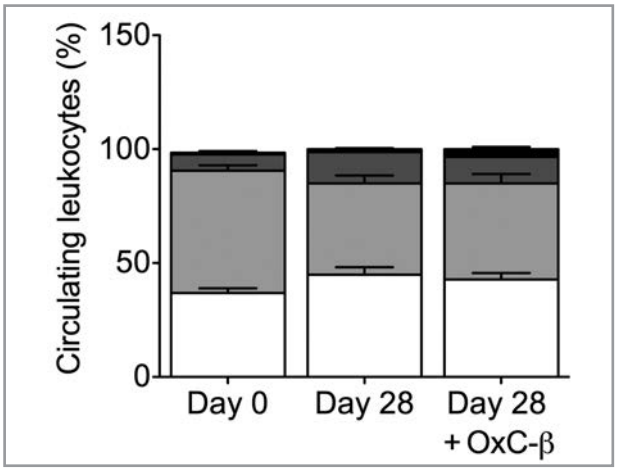

Figure 7—Mean ± SEM percentage of circulating neutrophils (white bars), lymphocytes (light gray bars), monocytes and macrophages (dark gray bars), and eosinophils (black bars) in blood smear preparations obtained from Holstein steers at the start (day 0) and end (day 28) of the treatment period (n = 7 to 10 cattle/group).

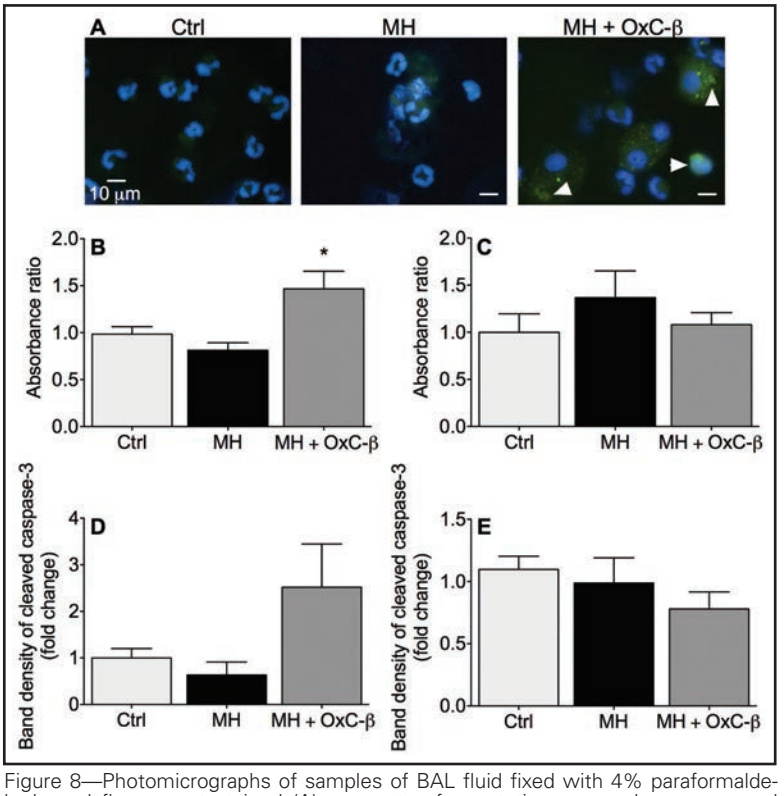

Figure 8—Photomicrographs of samples of BAL fluid fixed with 4% paraformaldehyde and fluorescent stained (A); amounts of apoptotic mononuclesosomes and oligonucleosomes measured by means of a cell-death ELISA in BAL fluid collected at 3 (B) and 24 (C) hours after inoculation from calves that did not receive fully oxidized β-carotene and were not (Ctrl) or were (MH) subsequently challenge inoculated with *M haemolytica* and calves that received fully oxidized β-carotene for 28 days and were challenge inoculated with *M haemolytica* (MH + OxC-β); and apoptotic caspase-3 fragmentation measured by use of western blotting techniques in samples of BAL fluid collected 3 (D) and 24 (E) hours after challenge inoculation with *M haemolytica* from the same 3 groups of calves. Values represent mean ± SEM (n = 7 to 10 BAL samples/group). In panel A, staining of apoptotic (green) and viable (blue) cells is evident. Notice the apoptotic leukocytes (arrowheads). TUNEL stain and Hoechst nuclear counterstain; bar = 10 μm. In panels B and C, values are the ratios of the absorbance for each treatment group versus that measured for the control group, with the absorbance for the control group arbitrarily set to 1.0. For panels D and E, values are normalized to results for MPO activity and represent the ratio of cleaved caspase-3 for each treatment group versus that measured for the control group, with the value for the control group arbitrarily set to 1.0. *Value differs significantly ($P < 0.05$) from the value for the MH group.

tion had a similar pattern in calves treated for 28 days with fully oxidized β-carotene at 3 hours but not at 24 hours after inoculation.

To determine whether apoptotic leukocytes within the BAL fluid were readily cleared by macrophages, enumeration of efferocytosis-positive macrophages in leukocyte populations of BAL fluid was performed (**Figure 9**). The data indicated enhanced efferocytosis in bacterial-inoculated calves treated for 28 days with fully oxidized β-carotene at 3 hours after inoculation, at which time neutrophil apoptosis was detected. At 24 hours after inoculation, when neutrophil apoptosis was no longer detectable, there was no observable effect of treatment with fully oxidized β-carotene on efferocytosis, relative to results for control calves.

Discussion

The study reported here represented a proof-of-concept study that used calves to evaluate the effects of fully oxidized β-carotene in bovine respiratory disease. Selective induction of neutrophil apoptosis offers a potential avenue for the development of novel anti-inflammatory treatments, including nutraceuticals.[21,41] Results from the present study indicated the mechanisms by which all-trans retinoic acid and the oxygen-rich carotenoid derivative fully oxidized β-carotene conferred immune-modulatory effects. Findings indicated that all-trans retinoic acid and fully oxidized β-carotene promoted apoptosis in bovine neutrophils in vitro as determined by TUNEL staining and quantification of apoptotic mononucleosomes and oligonucleosomes. Moreover, this effect was observed in the absence of necrosis, as determined by the release of intracellular lactate dehydrogenase. Proapoptotic effects of all-trans retinoic acid and fully oxidized β-carotene were associated with increased fragmentation of caspase-3 and were caspase-3 dependent. Furthermore, all-trans retinoic acid and fully oxidized β-carotene did not induce apoptotic caspase-3 fragmentation in bovine epithelial cells or fibroblasts, which suggested that the proapoptotic effects observed in this study were, at least in part, cell selective. The apparent selective effects of all-trans retinoic acid observed in this study are consistent with other results that indicated retinoic acid can have proapoptotic effects in some cell lines and antiapoptotic effects in others.[42] Apoptotic cells must be cleared from tissues for the resolution of inflammation. Similarly, neutrophils treated with all-trans retinoic acid and fully oxidized β-carotene were readily taken up by monocyte-derived bovine macrophages. Importantly, treatment with these retinoid and carotenoid derivatives did not impair cellular antimicrobial mechanisms. All-trans retinoic acid and fully oxidized β-carotene did not alter neutrophil oxidative

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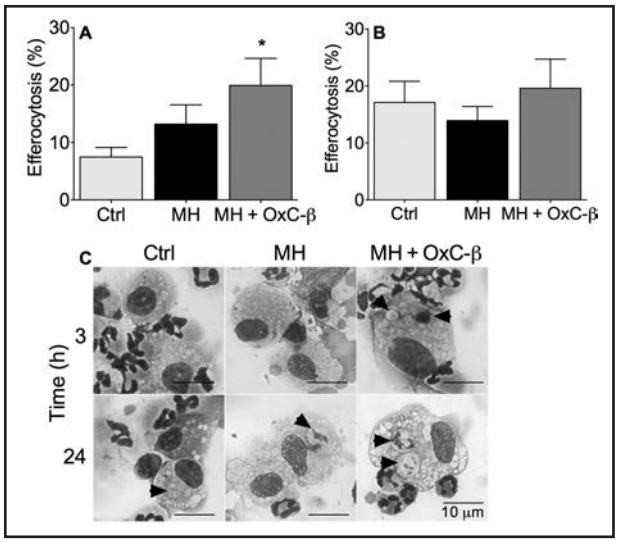

Figure 9—Percentage of efferocytosis-positive macrophages within BAL fluid collected at 3 (A) and 24 (B) hours after inoculation from calves that did not receive fully oxidized β-carotene and were not (Ctrl) or were (MH) subsequently challenge inoculated with *M haemolytica* and calves that received fully oxidized β-carotene for 28 days and were challenge inoculated with *M haemolytica* (MH + OxC-β) and representative micrographs of cells obtained from each of those groups of calves (C). In panels A and B, values represent mean ± SEM (n = 7 to 10 BAL samples/group). In panel C, notice the efferocytosis-positive macrophages (arrowheads). Romanowski stain; bar = 10 μm. *Value differs significantly ($P < 0.05$) from the value for the Ctrl group.

burst function activated by phorbol 12-myristate 13-acetate. It is important to mention that the use of this stimulant may prevent detection of signaling through intracellular or extracellular calcium mobilization and signal events associated with assembly of NADPH oxidase. Moreover, fully oxidized β-carotene did not alter granulocyte binding and attachment of nonopsonized zymosan. Finally, in contrast with findings for antimicrobials with proresolution properties, the retinoid- or carotenoid-derived compounds used in the study reported here did not affect the production of LTB_4, which indicated that the products likely differ in their mode of action relative to proresolution antimicrobials.[35,36] These observations prompted investigations of a clinical model of bovine respiratory disease to confirm findings in cattle with pneumonia. In calves with *M haemolytica*–challenge-exposed lungs, dietary supplementation with fully oxidized β-carotene enhanced leukocyte apoptosis, as determined by TUNEL staining and quantification of mononucleosomes and oligonucleosomes in BAL fluid. This was observed in the absence of any detectable effect on circulating leukocyte populations. Furthermore, the proapoptotic effects of fully oxidized β-carotene were observed in combination with enhanced efferocytic uptake of neutrophils in BAL fluid. The present findings suggested novel mechanisms through which all-trans retinoic acid and fully oxidized β-carotene possess anti-inflammatory properties by inducing apoptosis in neutrophils without altering neutrophil functional properties. Of further interest was the finding that retinoic acid and fully oxidized β-carotene, 2 extremely dissimilar chemical entities, caused similar effects at the level of neutrophil apoptosis. Fully oxidized β-carotene is devoid of vitamin A, related retinoid compounds, or higher-molecular-weight norisoprenoids[33] and has yielded negative results when tested for the ability to induce expression of a retinoic acid receptor responsive gene in an in vitro bioassay (unpublished data). This suggests that retinoic acid and fully oxidized β-carotene may act via distinct mechanisms with convergent effects on neutrophil apoptosis. In addition, fully oxidized β-carotene does not possess the provitamin or antioxidant activity of β-carotene; thus, its mode of action is also distinct from those traditionally ascribed to the carotenoid class.[33]

Much of the severe tissue damage associated with *M haemolytica*–induced pneumonia is host mediated.[5] *Mannheimia haemolytica* infection is characterized by severe pulmonary inflammation in which local necrosis and the self-perpetuating recruitment of neutrophils are responsible for causing inflammatory injury that can lead to respiratory failure and death within 48 to 72 hours.[19–21] In bovine lungs at the peak of infection, *M haemolytica* leukotoxin induces necrosis in neutrophils and alveolar macrophages.[19] This necrosis disarms the host innate immune barrier by impairing antibacterial defenses and also causes collateral damage to surrounding cells.[19] The resulting release of toxic compounds and proinflammatory mediators from necrotic leukocytes promotes a proinflammatory phenotype in surrounding leukocytes that exacerbates the inflammatory injury and contributes to disease pathogenesis.[43] Findings from the present study suggested that fully oxidized β-carotene may help resolve inflammation by promoting apoptosis in neutrophils and thereby attenuate pulmonary tissue damage associated with *M haemolytica* infection. In vitro findings provided further support to this hypothesis by identifying the mechanisms through which fully oxidized β-carotene and all-trans retinoic acid induced apoptosis in neutrophils. Further studies are needed to investigate the degree to which biological activity of all-trans retinoic acid and fully oxidized β-carotene is maintained after digestion through a functional rumen of adult cattle. Moreover, studies are warranted to evaluate longer periods of infection to determine the manner by which the use of nutraceuticals may modulate clinical respiratory disease in cattle.

Retinoids are associated with numerous health benefits and are involved in the regulation of diverse biological processes, including embryonic development, immune function, cellular differentiation, and apoptosis.[44–46] Research in rodents with experimentally induced pulmonary inflammation has revealed that provision of vitamin A can reduce the amount of pulmonary infiltrate[24,25] and attenuate damage[26] following inflammatory stimuli, which suggests an anti-inflammatory role for these compounds. Retinoids and retinoid-related compounds have both proapoptotic[26–30,47] and antiapoptotic effects,[32,48,49] depending on the cell type and local stimuli. To the authors' knowledge, the results reported here provided the first evidence of the proapoptotic effects of all-trans retinoic acid and fully oxidized β-carotene in neutrophils. Apoptosis can be initiated by 1 of 2 mechanisms, namely the intrinsic mitochondrial pathway or the extrinsic death receptor–mediated pathway.[50,51] In an attempt to shed light on the mechanisms through which all-trans retinoic

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acid and fully oxidized β-carotene promote neutrophil apoptosis, the present study revealed that the proapoptotic effects of both these compounds were caspase-3 dependent. However, further investigation is needed to determine whether these effects resulted from extrinsic activation or via the intrinsic mitochondrial pathway. Retinoid compounds are hydrophobic and readily taken up by cells via diffusion across the plasma membrane. Future studies will be needed to determine whether the mechanism of cellular uptake impacts or possibly initiates the intrinsic apoptotic pathway or whether activation of nuclear retinoic acid receptors influences paracrine signaling of FasL and thereby acts through the extrinsic pathway.[29]

Some retinoids can modulate neutrophil function and alter circulating leukocyte counts.[52] In contrast, data from the present study indicated that all-trans retinoic acid and fully oxidized β-carotene did not alter neutrophil oxidative burst or binding of nonopsonized zymosan in vitro. Furthermore, differential counts revealed that long-term dietary supplementation with fully oxidized β-carotene did not significantly alter circulating leukocyte proportions. These findings indicated that long-term treatment with fully oxidized β-carotene did not negatively impact circulating leukocytes, despite the compound's proapoptotic properties. Future studies will be needed to assess the mechanisms by which fully oxidized β-carotene affects the molecular basis of phagocytosis, such as effects on IgG, complement receptor binding, and internalization functions.

Leukocyte apoptosis plays a critical role in the resolution of inflammation. After an animal develops an infection, proinflammatory processes must be downregulated and infiltrating leukocytes must be cleared from tissues to return tissue homeostasis; neutrophil apoptosis is a key mechanism in this process.[14,53] Cells undergo distinct morphological changes during apoptosis, including nuclear condensation, DNA fragmentation, cellular shrinkage, and budding.[54,55] Apoptotic neutrophils also lose many of their proinflammatory capacities owing to functional changes that render the cells unresponsive to external stimuli.[9] These changes facilitate the nonphlogistic removal of dying cells from tissues. Retinoid compounds can attenuate LTB_4 production in rat neutrophils[27]; LTB_4 is a proinflammatory lipid mediator that plays an essential role in neutrophil migration and activation.[2] Interestingly, no noteworthy changes in production of LTB_4 by bovine neutrophils were observed after treatment with all-trans retinoic acid or fully oxidized β-carotene in vitro. Whether these differences reflected a host species–dependent response to these retinoid- and carotenoid-derived compounds requires further investigation.

Apoptotic neutrophils are removed from tissues by macrophages in a process known as efferocytosis.[56] Prompt efferocytosis of apoptotic cells protects surrounding tissues from harmful cellular contents that would otherwise be released into the extracellular milieu as a result of postapoptotic necrosis. Results of the present study indicated that increases in leukocyte apoptosis observed in the lungs of calves treated with fully oxidized β-carotene were associated with enhanced efferocytosis at 3 hours after inoculation. These findings were consistent with in vitro data for neutrophils treated with both fully oxidized β-carotene and all-trans retinoic acid, which suggested that retinoid-treated apoptotic neutrophils were readily cleared by macrophages. The recognition of apoptotic cells also triggers an anti-inflammatory phenotype in macrophages that promotes the resolution of inflammation. Within this phenotype, macrophages curtail the production of proinflammatory mediators and switch to the preferential production of anti-inflammatory and proresolution mediators, including transforming growth factor-β, IL-10, and prostaglandin E_2, that suppress the inflammatory response.[10,57] Benefits of an apparent short-lived induction of efferocytosis are consistent with those listed in a number of prior studies[22,34,35] on the immunomodulatory benefits of some antimicrobials. Indeed, promotion of efferocytosis, even for a short time, appears sufficient to activate the resolution of inflammation in a disease such as pneumonia in calves, which often kills the host in 48 to 72 hours if inflammation is not resolved. Future studies should investigate whether the recognition of neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene impacts the production of anti-inflammatory mediators (eg, IL-10) by macrophages and clarify the specific signals produced by neutrophils treated with all-trans retinoic acid or fully oxidized β-carotene necessary for uptake and clearance of those neutrophils.

On the basis of use of a clinically relevant model of *M haemolytica* infection in calves and complementary in vitro experiments with isolated bovine neutrophils, all-trans retinoic acid and fully oxidized β-carotene promoted selective, local apoptosis of neutrophils. These effects were caspase-3 dependent and occurred in the absence of detectable effects on neutrophil function, LTB_4 production, and circulating leukocyte proportions. Furthermore, analysis of the data indicated that the apoptosis of cells subsequently enhanced their efferocytic clearance by macrophages. Findings in other studies[22,35,41] have indicated the ability of treatments with proapoptotic properties to facilitate resolution of inflammation after infection. Findings of the present study indicated that the proapoptotic effects of all-trans retinoic acid and fully oxidized β-carotene may confer anti-inflammatory properties in calves with respiratory tract disease. Perhaps most importantly, analysis of the data indicated novel effects of all-trans retinoic acid and fully oxidized β-carotene in bovine neutrophils, and together these compounds may provide new mechanisms through which retinoid- or carotenoid-derived nutraceuticals can promote the resolution of inflammation. These findings should pave the way toward in vivo experiments to test the effects of fully oxidized β-carotene in feedlot cattle.

a. Spy Hill Campus, University of Calgary, Calgary, AB, Canada.
b. Pro-Gro, Grober Nutrition, Cambridge, ON, Canada.
c. ACD solution A, BD Vacutainer Systems, Franklin Lakes, NJ.
d. PAA Laboratories Inc, Dartmouth, Mass.
e. Gibco, Grand Island, NY.
f. VWR Scientific, Edmonton, AB, Canada.
g. Diff-Quik stain set, Siemens Healthcare Diagnostics, Tarrytown, NY.

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h. CytoSpin 4 cytocentrifuge, Thermo Scientific, Pittsburgh, Pa.
i. Sigma-Aldrich, Oakville, ON, Canada.
j. Life Technologies, Burlington, ON, Canada.
k. ATCC, Manassas, Va.
l. Avivagen Inc, Ottawa, ON, Canada.
m. Ac-DMQD-CHO, Calbiochem, Kankakee, Ill.
n. 2% Lidocaine HCl with epinephrine injectable solution, Ayerst Laboratories, Madison, NJ.
o. Kendall Sovereign, Tyco Health Care Group Inc, Mansfield, Mass.
p. Vet-Bond, 3M Animal Care Products, Saint Paul, Minn.
q. Roche Diagnostics, Laval, QC, Canada.
r. SpectraMAX M2e microplate reader, Molecular Devices, Menlo Park, Calif.
s. Retiga 2000X with Q Capture Suite software, Q Imaging, Surrey, BC, Canada.
t. Bio-Rad Laboratories, Mississauga, ON, Canada.
u. Cell Signaling, Beverly, Mass.
v. Biorbyt Ltd, San Francisco, Calif.
w. Amersham ECL Prime western blotting detection reagent, GE Healthcare Life Sciences, Pittsburgh, Pa.
x. LTB_4 EIA kit, Cayman Chemical, Ann Arbor, Mich.
y. IDEAS, ImageStream, Amnis, Seattle, Wash.

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