Effect of Vitamins A and E on Nitric Oxide Production by Blood Mononuclear Leukocytes from Neonatal Calves Fed Milk Replacer\textsuperscript{1,2,3}

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ABSTRACT

This study evaluated the effects of dietary vitamin A and E on the in vitro capacity of blood mononuclear leukocytes from calves to produce nitric oxide. Calves fed milk replacer received 100 IU/d of vitamin E as RRR-\textalpha-tocopherol or RRR-\textalpha-tocopheryl acetate and 0, 1700, 34,000, or 68,000 IU of vitamin A as retinyl acetate. Leukocytes from calves produced greater amounts of nitric oxide relative to leukocytes from adult cattle. The greater production of nitric oxide by calf leukocytes may be typical of the immature neonatal immune system. Nitric oxide production by calves fed RRR-\textalpha-tocopherol and either 1700 or 34,000 IU of vitamin A was less than that of calves in other groups and was more typical of production by leukocytes from cows. Our data suggest that optimal amounts of dietary vitamins A and E prompt the maturation of this response toward one that is more typical of adult cattle. Leukocytes from 1-wk-old calves produced less nitric oxide and were less responsive to stimuli than were leukocytes from older calves, a possible consequence of suppressive factors that were present in the ingested colostrum or in the circulation at birth.

(\textit{Key words}: calf, nitric oxide, vitamin A, vitamin E)

Abbreviation key: IFN-\greekgamma = interferon-\greekgamma, iNOS = inducible NOS, L-NMMA = N\textsuperscript{G}-monomethyl-L-arginine, MNL = mononuclear leukocytes, NO = nitric oxide, NOS = NO synthase, PWM = pokeweed mitogen, RA = retinoic acid.

INTRODUCTION

Nitric oxide (NO) production is a component of the innate immune system that has not been well studied in neonates. Nitric oxide synthases (NOS) produce NO by the oxidation of the 1-guanido nitrogen of L-arginine. Isoforms of this enzyme can be broadly divided into two categories: constitutive NOS and inducible NOS (iNOS). Constitutive NOS is normally expressed in cells and generates small amounts of NO for short periods in response to increases in intracellular calcium. Absent in resting cells, expression of iNOS can be induced in a variety of cell types, including monocytes, macrophages, keratinocytes, hepatocytes, and kidney cells by stimuli such as bacteria, cytokines, hormones, and lipoproteins (8,22). Once expressed, iNOS can generate large amounts of NO for extended periods. Nitric oxide acts as an intracellular signaling molecule or as a neurotransmitter when produced in low quantities. When produced in higher quantities for extended periods, NO is involved in the killing of microorganisms and tumor cells (23) and in hematopoiesis (27). Chronic production of NO in association with superoxide anion generates toxic radicals, which can damage cell membranes, cause inflammation, and induce apoptosis (8).

Expression and activity of iNOS in mononuclear phagocytes is regulated in a species-specific manner. In rodents, but not in humans or cattle, iNOS is strongly induced by interferon-\greekgamma (IFN-\greekgamma), lipopolysaccharides, or tumor necrosis factor-\textalpha (8). The crosslinking of the Fc receptor for IgE induces production of NO by human monocytes (10). In bovine macrophages, iNOS is induced by heat-killed Gram-positive or Gram-negative bacteria and by combinations of endotoxin and cytokines (1, 20, 33).
Vitamins A (28) and E (6) are essential to ensure normal immune function and resistance to infectious diseases. Newborn calves have very low concentrations of vitamin A metabolites and α-tocopherol in their circulation (15). Concentrations of these compounds increase as the calves age, and adult concentrations are attained within a few months after birth (15). Colostrum, milk, formulated diets, and injections containing retinoids are major sources of these vitamins for the neonatal calf (30). Supplements are usually in the form of retinyl and tocopheryl esters, which are hydrolyzed by intestinal hydrolases prior to absorption by enterocytes.

Retinoic acids (RA) are vitamin A metabolites that inhibit the in vitro production of NO from L-arginine by murine macrophages (21) and human keratinocytes (4). In contrast, combinations of RA and vitamin D induce iNOS and NO production in a promonocytic cell line. Inhibition of NO production prevents differentiation of this cell line as induced by these compounds (9). Thus, regulation of NO production by RA might be influenced by stage of differentiation and other compounds in the intracellular environment.

Antioxidants, such as vitamin E, protect cells from the toxic effects of NO by scavenging reactive nitrogen radicals (5, 17). Higher oxides of NO, such as nitrogen dioxide, are more efficiently detoxified by RRR-γ-tocopherol than by RRR-α-tocopherol (7).

The objective of this study was to evaluate the capacity of mononuclear leukocytes (MNL) from neonatal calves to produce NO in vitro and to determine whether the amount of dietary vitamin A affects NO production. In the US, young calves are frequently fed milk replacers with 10- to 20-fold higher levels of vitamin A than the daily allowance recommended by the NRC (24). Therefore, calves were fed a milk replacer that was supplemented to provide 0, 1700, 34,000, or 68,000 IU/d of vitamin A (as retinyl acetate) and vitamin E (100 IU/d) in the form of RRR-α-tocopherol or RRR-α-tocopheryl acetate. Six or 7 calves were assigned to each group. All procedures related to the calves were approved by the Institutional Animal Care and Use Committee of the South Dakota State University (Brookings).

Blood Collection and Mononuclear Leukocyte Isolation

Calves were bled by jugular venipuncture at 4 ± 3 d of age (designated wk 1) and weekly thereafter until 1 mo of age. Blood was collected into 10% (vol/vol) 2× acid-citrate-dextrose and shipped overnight at ambient temperature to the National Animal Disease Center (ARS, USDA, Ames, IA).

Blood MNL were isolated and enriched by density gradient centrifugation as described previously (26). Erythrocytes were eliminated by hypotonic lysis prior to density gradient centrifugation of buffy coat cells. The MNL were resuspended in RPMI 1640 medium containing 25 mM HEPES buffer (Gibco Laboratories, Grand Island, NY) that was supplemented with 2 mM L-glutamine, antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin), and antimycotics (0.25 μg/ml of amphotericin B) (all from Sigma Chemical Co., St. Louis, MO).

Blood from 10 adult nonlactating Holstein cows was collected and similarly processed. The average age of these cows was 21 (± 5) mo.

In Vitro Production and Measurement of NO

Production of NO by MNL was assayed in flat-bottomed, 96-well polystyrene tissue culture plates inoculated with 2.0 × 10⁶ cells/ml in a final volume of 200 μl of RPMI containing 5% (vol/vol) of heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT).

Cell cultures were either unstimulated or were stimulated with 4 μg/ml of pokeweed mitogen (PWM;
Sigma Chemical Co.), or 75 ng/ml of sonicated Cryptosporidium parvum oocysts in PBS (provided by J. A. Harp, National Animal Disease Center, ARS, USDA), or 200 μg/ml (wet weight) of heat-killed Salmonella typhimurium (provided by T. J. Stabel, National Animal Disease Center, ARS, USDA). The plates were incubated at 39°C in a humidified atmosphere with 5% CO₂. Culture supernatants were harvested after the plates were centrifuged (620 × g at 21°C for 5 min). Replicate cultures were established so that supernatants could be harvested after 24, 48, and 96 h of incubation. Duplicate wells for each treatment were prepared.

Nitrite is the stable oxidation product of NO, which correlates with the amount of NO produced in culture supernatants (11). The amount of stable nitrite was determined by the method of Green et al. (11). The NO assay was performed using microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA). The culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide; Sigma Chemical Co.) in 2.5% phosphoric acid (Mal- linckrodt Chemicals, Inc., Paris, KY) and 0.05% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma Chemical Co.). The mixture was incubated at 21°C for 10 min. Absorbances of test and standard wells were measured at 570 nm using an automated ELISA plate washer and reader (Dynatech MR7000; Dynatech Laboratories Inc.). The amount of nitrite in test samples was determined from a standard curve of absorbances regressed on the NO concentrations for the dilution of the test sample that yielded absorbance readings that fell in the linear portion of the curve. The concentration of NO in culture supernatants was calculated by multiplying the value from the standard curve by the dilution factor and was expressed as nanograms per milliliter.

### Statistical Analysis

Data were analyzed as a split-plot with repeated measures ANOVA using the general linear models procedure of SAS (29). The dietary supplementation of vitamins A and E and their interactions constituted the main plot, and the age of the calves was the repeated measure or the split plot. At the second level, the age of the calves was the main plot, and the in vitro treatment of the MNL was the split plot. At the third level, the in vitro treatment of the MNL was the main plot, and the time of harvest was the split plot. The model was also used to evaluate the various interactions. Statistical significance was declared at P < 0.05. When the main effects or interactions were significant, indicated significant differences were taken from the matrices of the Student's two sample t test that accompanied the least squares means from the ANOVA. Responses of cows were compared against responses of calves using the ANOVA procedure of SAS (29). Effects of model included physiological status (cow vs. calf and cow vs. calf by individual treatment and by age) and status versus time in culture.

### RESULTS

#### In Vitro Production of NO

The addition of L-NMMA reduced nitrite production by 95 to 100% in all cultures, which indicated that nitrite production was due to the activity of NOS specifically (data not shown). Therefore, the nitrite was an indicator of NO produced in the leukocyte cultures.

Production of NO by cow leukocytes was lower (P = 0.0001) than that by calf leukocytes (Figure 1). The kinetics of the response of leukocytes from calves...
differed from those of cows, although this trend was not statistically significant (P > 0.05). Calf leukocytes responded to stimuli (either PWM or heat-killed bacteria) within 24 h in culture; however, responses of adult leukocytes to these stimuli were apparent only at 48 h (Figure 1). At 48 and 96 h, leukocytes stimulated with either PWM or heat-killed bacteria produced 1.5- to 2-fold more NO than did parallel cultures of unstimulated leukocytes (P = 0.0001; Figure 1). The amount of NO produced by leukocytes stimulated with C. parvum oocysts was similar (P > 0.05) to that produced by unstimulated leukocytes. Production of NO by older calves was greater (P = 0.02) than was production of NO by 1-wk-old calves. Leukocytes from 2- to 5-wk-old calves were more (P ≤ 0.0005) responsive to stimuli (PWM or heat-killed S. typhimurium) than were leukocytes from 1-wk-old calves (Figure 1, a, b, and c).

Leukocytes from calves fed RRR-α-tocopherol produced less (P = 0.03) NO (11.4 ± 0.4 μM) than leukocytes from calves that were fed RRR-α-tocopheryl acetate (15.2 ± 0.5 μM). The effect of vitamin A was found within the group of calves that were fed RRR-α-tocopherol (P = 0.03). Unstimulated leukocytes or leukocytes stimulated with S. typhimurium from calves that were fed RRR-α-tocopherol as

![Figure 1](image1.png)

**Figure 1.** Mean (± SEM) production of nitrite by blood mononuclear leukocytes from calves (open bars) or cows (solid bars). Leukocytes were unstimulated (a) or stimulated with pokeweed mitogen (b), Cryptosporidium parvum oocysts (c), or heat-killed Salmonella typhimurium (d) Asterisks represent differences (P ≤ 0.05) from corresponding unstimulated cultures presented in panel a.

![Figure 2](image2.png)

**Figure 2.** Mean (±SEM) production of nitrite by calf blood mononuclear leukocytes that were unstimulated (○), stimulated with pokeweed mitogen (△), Cryptosporidium parvum oocysts (x), or heat-killed Salmonella typhimurium (●) for 24 (a), 48 (b), or 96 (c) h in vitro.

Table 1. Nitric oxide (micromolar concentration of nitrite) produced by unstimulated blood mononuclear leukocytes from calves and cows.

<table>
<thead>
<tr>
<th>Animal type</th>
<th>n</th>
<th>Vitamin A</th>
<th>Vitamin E</th>
<th>Time in culture</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Alcohol</td>
<td>24 h</td>
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<td></td>
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<td></td>
<td>Ester</td>
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<td></td>
<td></td>
<td></td>
<td>(IU/d)</td>
<td></td>
</tr>
<tr>
<td>Cows</td>
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<td>0</td>
<td>0</td>
<td>2.0</td>
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<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0.7</td>
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<td>0.7</td>
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<tr>
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<tr>
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<td></td>
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*Higher than corresponding responses of cows, (P < 0.05).
1Calves at 21 to 32 d of age.

In Vitro Production of IFN-γ

Production of IFN-γ by calf leukocytes was unaffected by age or type of dietary treatment (P > 0.05; data not shown). Leukocytes from adult cattle that were stimulated with PWM produced 2 to 2.5 times more IFN-γ than did leukocytes from calves (P = 0.0001; Figure 4). The secretion of IFN-γ by leukocytes that were stimulated with C. parvum did not exceed (P > 0.05) the basal secretion of IFN-γ by unstimulated leukocytes. Leukocytes that were stimulated with PWM or heat-killed S. typhimurium produced similar amounts (P > 0.05) of NO; the latter leukocytes produced approximately three times less IFN-γ than did the former (Figures 3 and 4), indicating that IFN-γ secretion and NO production were not related in this system.

**DISCUSSION**

In the present study, the baseline production of NO by calf leukocytes exceeded that of cow leukocytes. Recent research by Alvarez et al. (2) also suggested that age might affect NO production by leukocytes. Alvarez et al. (2) found that production of NO, as well as superoxide anion, by resident (not elicited) and activated peritoneal macrophages from young (3-mo-old) rats was substantially greater than production by comparable cell populations from intermediate (12-mo-old) and older (24-mo-old) rats. The researchers (2) suggested that differences in NO production between young, intermediate, and older rats were not different (P > 0.05) from that of cows (Table 1). However, leukocytes from these calves showed a trend toward higher NO production after 48 or 96 h in culture. The production of NO by unstimulated leukocytes from 4- to 5-wk-old calves from the other groups exceeded (P ≤ 0.02) production by leukocytes from cows.
production might be linked to changes in glucose utilization that are linked to age. Leukocytes from calves also responded to stimuli earlier than did leukocytes from adults. Production of high amounts of NO in the absence of stimulation may be a characteristic feature of the physiologically immature immune system of the newborn calf. As the calves age, the baseline NO produced by unstimulated MNL may decrease to concentrations that are more typical of the baseline NO produced by unstimulated MNL from adults. Uncontrolled and chronically high concentrations of NO in vivo may be detrimental and may contribute to the enhanced susceptibility of the neonate to infectious diseases (8) or exacerbate clinical manifestations of inflammatory reactions (31).

Secretion of NO plays a role in the decrease of fecal shedding of C. parvum oocysts in nude mice (19). Sonicated C. parvum oocysts did not induce nitrite production in calves or cows but has been shown to induce in vitro blastogenesis of splenocytes (32) and in vitro IFN-γ secretion by CD4+ T cells (14) from mice previously exposed to C. parvum. In the present study, sonicated oocysts did not induce IFN-γ secretion by calf MNL. Conceivably, repeated exposure of calves to C. parvum may be necessary to induce both IFN-γ and NO in MNL in vitro.

Leukocytes from 1-wk-old calves produced less NO than did leukocytes from older calves. Responses of these calves might have been suppressed by compounds such as prostaglandins or transforming growth factor-β in the colostrum (12) or by glucocorticoids in the circulation (18).

Leukocytes from calves that were fed RRR-α-tocopherol produced less NO than did leukocytes from calves that were fed RRR-α-tocopheryl acetate. No correlations between the concentrations of retinol, RRR-α-tocopherol, or RRR-γ-tocopherol in plasma and the production of NO by MNL from calves or cows were detected (data not shown). Horwitt et al. (16) showed that serum RRR-α-tocopherol concentrations after ingestion of equivalent amounts RRR-α-tocopherol or RRR-α-tocopheryl acetate are similar within 24 h after ingestion. However, the rise in serum RRR-α-tocopherol is more rapid when RRR-α-tocopherol is ingested. In the present study, calves fed the RRR-α-tocopherol might have experienced a more rapid rise in serum RRR-α-tocopherol than calves that were fed RRR-α-tocopheryl acetate. It is unknown whether the form of vitamin E ingested influences conversion to RRR-γ-tocopherol, a compound which is more efficient than RRR-α-tocopherol in reducing oxidation products of NO (7). It can be hypothesized that, in calves that were fed RRR-α-tocopherol, the oxidation of NO to nitrite was reduced because of the antioxidant activity of RRR-α-tocopherol or RRR-γ-tocopherol. Although nitrite does not contribute to microbial killing within the cell, nitrite may be reduced to NO within lysosomes (23), thus contributing to the antimicrobial capacity of the leukocyte. Other oxidized forms of NO, such as peroxynitrite, mediate microbial killing at optimal concentrations and cause cell damage at higher concentrations. Vitamin E protects cells from the toxic effects of oxidized forms of NO such as peroxynitryl radicals (17) and nitrogen dioxide (7). Thus, cells with greater concentrations of vitamin E might have had decreased efficiency of intracellular killing or lesser damage caused by oxidized products of NO.

Among calves fed RRR-α-tocopherol, those that received no vitamin A had leukocytes that produced the most NO, even in the absence of stimuli such as PWM or heat-killed bacteria. In a recent study (31) of the effects of vitamin A deficiency on immediate and delayed-type hypersensitivity, spontaneous release of NO from peritoneal macrophages was five times higher in rats that were deficient in vitamin A than in the control group of rats. Immunized rats that were deficient in vitamin A also displayed a consistently stronger immediate skin reaction after intracutaneous antigen injection than did immunized control rats. The injection of olive oil into the footpads of rats that were deficient in vitamin A induced more severe inflammatory responses mediated by granulocytes compared with controls. The possibility that
vitamin A deficiency not only promotes excessive production of NO but also aggravates inflammatory reactions in general (31) must be investigated in cattle.

Preliminary examination of the concentrations of several different isomers of RA in plasma of calves in the present study indicated that the concentrations of 9,13-di-cis-RA, 9-cis-RA, and 13-cis-RA were greatest for calves fed 68,000 IU/d of vitamin A and were lowest for calves not given vitamin A (25). Studies with chickens indicate that the RA content of blood leukocytes gradually increases with vitamin A intake (13). Halevy et al. (13) showed that expression of mRNA for RA receptor-α was correlated positively with intracellular concentrations of RA up to a point but then declined as the concentration of RA continued to increase (13). Thus, at very low or very high concentrations of RA, as is found at very low or very high vitamin A intake, respectively, reactivity to RA might have been reduced. Retinoic acid inhibits NO production in rodent macrophages (21) and human keratinocytes (4). The higher concentrations of NO that were found for calves with no vitamin A intake or high vitamin A intake could have been due to lack of inhibition of iNOS by RA via its receptor. No differences in NO production by leukocytes from calves fed RRR-α-tocopheryl acetate and the different concentrations of vitamin A were detected. Further studies are needed to elucidate the mechanism by which the form of dietary vitamin E influences NO production by blood MNL.

The production of NO by leukocytes from calves that were fed RRR-α-tocopherol and vitamin A at either 1700 or 34,000 IU/d was more similar to that of cows than the production of NO by calves in the other treatment groups. Conceivably, optimal amounts of RA, acting at the level of transcription of iNOS, as well as either RRR-α-tocopherol or RRR-γ-tocopherol, reacting with products of NO, are necessary to maintain the low NO production observed in unstimulated leukocytes from adult cattle.

CONCLUSIONS

Although production of NO by leukocytes that are stimulated by pathogens is an important component of the innate immune system, excess production of this molecule can be damaging to the tissues of the host. Leukocytes from calves produced unusually high concentrations of NO when compared with those produced by cows, a possible indicator of the immaturity of the immune system of the neonatal calf. The data suggest that optimal amounts of dietary vitamins A and E may be necessary for the maturation of the NO response of neonatal calves toward an adult phenotype. Whether such maturation in NO production would increase the competency of the immune system of the calf has yet to be determined.

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