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Blood plasma response and urinary excretion of nitrite and nitrate in milk-fed calves after oral nitrite and nitrate administration

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Abstract

There is marked endogenous production of nitrate in young calves. Here we have studied the contribution of exogenous nitrate and nitrite to plasma concentrations and urinary excretion of nitrite and nitrate in milk-fed calves. In experiment 1, calves were fed 0 or 200 μmol nitrate or nitrite/kg$^{0.75}$ or 100 μmol nitrite *plus* 100 μmol nitrate/kg$^{0.75}$ with milk for 3 d. In experiment 2, calves were fed 400 μmol nitrate or nitrite/kg$^{0.75}$ with milk for 1 d. Plasma nitrate rapidly and comparably increased after feeding nitrite, nitrate or nitrite *plus* nitrate. The rise of plasma nitrate was greater if 400 than 200 μmol nitrate or nitrite/kg$^{0.75}$ were fed. Plasma nitrate decreased slowly after the 3-d administration of 200 μmol nitrate or nitrite/kg$^{0.75}$ and reached pre-experimental concentrations 4 d later. Urinary nitrate excretions nearly identically increased if nitrate, nitrite or nitrite *plus* nitrate were administered and excreted amounts were greater if 400 than 200 μmol nitrate or nitrite/kg$^{0.75}$ were fed. After nitrite ingestion plasma nitrite only transiently increased after 2 and 4 h and urinary excretion rates remained unchanged. Plasma nitrate concentration remained unchanged if milk was not supplemented with nitrite or nitrate. Nitrate concentrations were stable for 24 h after addition of nitrite to full blood in vitro, whereas nitrite concentrations decreased within 2 h. In conclusion, plasma nitrate concentrations and urinary nitrate excretions are enhanced dose-dependently by feeding low amounts of nitrate and nitrite, whereas after ingested nitrite only a transient and small rise of plasma nitrite is observed because of rapid conversion to nitrate.

Keywords: Nitrite; Nitrate; Calf

1. Introduction

Nitrate (NO_3^-) and nitrite (NO_2^-) can be ingested with food and water [1]. Furthermore, NO_3^- and NO_2^- can be endogenously synthesized from nitric oxide (NO), whereby the endogenous production of NO can be stimulated by three NO synthases (NOS) [2–6]. Inducible NOS (iNOS) is expressed in cells like monocytes and Kupffer cells of the liver. Thus, iNOS production is well known to be enhanced and followed by increased NO_3^- formation in various types of infections or after the administration of endotoxin and cytokines [7]. This is also the case for cattle [8–10], as reviewed by Jungi [11] and Elsasser et al. [12]. In addition, two of the enzymes (endothelial and neuronal NOS) are continuously expressed, are termed constitutive NOS, and are responsible for the production of relatively stable amounts of NO, NO_2^- and NO_3^-. Furthermore, there is NOS-independent NO production [13–15].

In blood plasma particularly high NO_3^- concentrations were measured in healthy (premature) calves born 2 wk before the normal term (mean: 470 μmol/L; range: 230–830 μmol/L) and in neonatal calves before their first meal (mean: 830 μmol/L; range 290–1050 μmol/L), somewhat lower concentrations in 1 to 4 months old milk-fed calves (mean: 50 μmol/L; range: 30–70 μmol/L), whereas plasma concentrations in heifers were very low (3–5 μmol/L) and in dairy cows were below the detection limit [8,10,16; Blum, unpublished observations]. Thus, plasma levels in

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premature, neonatal and veal calves were about 50-, 90- and 6-fold higher, respectively, than the maximal NO_3^- concentrations measured (9 μmol/L) when endotoxin challenges were combined with arginine infusions in heifers to enhance NO formation [8]. In neonatal and veal calves the high plasma levels were paralleled by corresponding urinary and salivary NO_3^- excretion, although animals were strictly fed milk which did not contain measurable amounts of NO_3^- or NO_2^-. These data indicated that NO_3^- in premature, neonatal and young calves was endogenously produced and that the high circulating amounts of NO_3^- were primarily of constitutive nature [16; Blum, unpublished observations]. The question remained, however, what amounts of exogenous (orally administered) NO_3^- and NO_2^- are needed to influence the NO_2^- or NO_3^- status in milk-fed calves. Effects of ingested NO_2^- or NO_3^- on the NO_2^- and NO_3^- status were expected to be much greater in milk-fed calves than in cattle with functioning forestomachs, in which microbes metabolize NO_3^- primarily to NO_2^- [1] and to hydroxylamine and ammonia. Although blood plasma NO_3^- concentrations closely correlate with NO_3^- ingestion in mature ruminants [17], it is the absorbed NO_2^- which is of major concern because it is about 10 times more toxic than NO_3^- [1]. In milk-fed pre-ruminant calves, in which the foregut development and digestive function is depressed, almost only NO_3^- and practically no NO_2^- was measurable in blood plasma, saliva and urine, suggesting that NO_2^- is rapidly converted to NO_3^- [16]. Based on these premisses we have studied changes in blood plasma concentration and urinary excretion of NO_2^- and NO_3^- after oral administration of NO_3^- and NO_2^- in milk-fed calves. The goal was to study the sensitivity of changes in blood plasma and urine to small amounts of exogenous NO_2^- and NO_3^- and whether the NO_2^- and NO_3^- status can be modified by ingested NO_2^- and NO_3^- in preruminant calves. This is of importance because young calves, as young animals of other species, are well known to be particularly susceptible to NO_2^- and NO_3^- intoxications [1].

2. Materials and methods

2.1. Animals, management and feeds

Experimental protocols were approved by the Committee for the Permission of Animal Experiments of the Canton of Freiburg, Granges-Paccot, Switzerland.

Six male veal calves (Simmental × Red Holstein) were studied in experiment 1 and another 12 male calves in experiment 2. They were bought at the age of 5 to 6 wk and housed at the Research Station in Posieux, Switzerland. Calves were weighed weekly and examined daily with respect to health status. They were kept in loose housing systems on straw litter and fed whole milk twice daily (at 0800 and 1600) in amounts of 100–150 g/kg body weight (BW). The experiments started at the age of 8 wk, i.e., when calves reached a BW of 80 ± 2 kg. At 1 d before the start of experiments calves were moved into boxes which allowed optimal feeding, blood sampling and urine collection. Ambient temperature during experiments was between 12–16°C.

Whole milk was from the milk pool of 60–70 cows of the research station and its NO_3^- and NO_2^- concentrations were below the detection level.

2.2. Drugs

The NO_3^- and NO_2^- were purchased as sodium salts from Fluka Chemie AG, Buchs, Switzerland.

2.3. Experimental procedures

2.3a. Experiment 1. Calves were administered once daily for 3 d (on d 1, 2, and 3) either no NO_2^- or NO_3^- (control group, Gr0), 200 μmol NO_3^-/kg$^{0.75}$ BW (group $GrNO_3$), 200 μmol NO_2^-/kg$^{0.75}$ BW (group $GrNO_2$), or 100 μmol NO_3^- *plus* 100 μmol NO_2^-/kg$^{0.75}$ BW (group $GrNO_2NO_3$). For this purpose NO_2^- and/or NO_3^- were added to whole milk of the morning meal. During the ensuing 4 d they received no NO_3^- or NO_2^- to study respective clearance rates from blood plasma. The experiment was designed as crossover:

Calves	Control Period	NO_3^-	NO_2^-	NO_2^- + NO_3^-
1, 2	wk 1	wk 2	wk 3	wk 4
3, 4	wk 1	wk 3	wk 4	wk 2
5, 6	wk 1	wk 4	wk 2	wk 3

Blood samples were taken from jugular veins with evacuated tubes containing anticoagulants (1.8 g dipotassium-EDTA/L blood). On d 1 blood samples were obtained before and at 2, 4 and 6 h after the morning meal (together with NO_2^- and/or NO_3^- administrations), on days 2 and 3 samples were taken 6 h after the morning meal (combined with NO_2^- and/or NO_3^- administrations), and on d 4 preprandially and 6 h postprandially. Tubes were cooled on ice until centrifuged at 1000 × g for 20 min at 4°C. Supernatants (plasma) were frozen at −20°C for later analyses.

Urine was sampled using an urinal, formed of elastic silicone (Rhodorsil RTV 585, Aseol AG, Berne, Switzerland) and fixed on the calf around the abdomen at 1 d before the experiments started. Urine was collected in bottles which were cooled on ice. Eight fractions were collected. Fraction 1: on d 1 from 0800 to 1400; fraction 2: on d 1 from 1400 to d 2 at 0800; fraction 3: on d 2 from 0800 to 1400; fraction 4: on d 2 from 1400 to d 3 at 0800; fraction 5: on d 3 from 0800 to 1400; fraction 6: on d 3 from 1400 to d 4 at 0800; fraction 7: on d 4 from 0800 to 1400; fraction 8: on d 4 from 1400 to d 5 at 0800. After measuring the volume of the fractions the samples were partitioned into aliquots and stored at −20°C until analyzed.

Table 1
Plasma NO_3^- and NO_2^- concentrations in the control group (GrO), and after oral administration on d 1, d 2 and d 3 of 200 μmol NO_3^-/$kg^{0.75}$ (group $GrNO_3$), 200 μmol NO_2^-/$kg^{0.75}$ (group $GrNO_2$) or 100 μmol NO_2^- *plus* 100 μmol NO_3^-/$kg^{0.75}$ (group $GrNO_2NO_3$) with the morning meal (at 0800)

Trait	Group	Day 1 preprandial	2 h postprandial	4 h postprandial	6 h postprandial	Day 2 6 h postprandial	Day 3 6 h postprandial	Day 4 preprandial	preprandial
(μmol/L)									
NO_3^-	GrO	42 ± 8^{AB}	41 ± 8^{C}	41 ± 8^{B}	45 ± 7^{B}	36 ± 5^{B}	32 ± 5^{B}	31 ± 5^{B}	30 ± 4^{B}
	$GrNO_3$	23 ± 2^{B}	814^{*B}	$114 \pm 6^{*A}$	$124 \pm 7^{*A}$	$158 \pm 8^{*A}$	$179 \pm 10^{*A}$	114 ± 14^{A}	61 ± 13^{A}
	$GrNO_2$	51 ± 13^{A}	$117 \pm 13^{*A}$	$126 \pm 13^{*A}$	$130 \pm 15^{*A}$	$152 \pm 14^{*A}$	$164 \pm 17^{*A}$	103 ± 17^{A}	57 ± 14^{AB}
	$GrNO_2NO_3$	29 ± 3^{AB}	$94 \pm 3^{*AB}$	$122 \pm 4^{*A}$	$126 \pm 3^{*A}$	$155 \pm 5^{*A}$	$176 \pm 4^{*A}$	115 ± 6^{A}	67 ± 5^{A}
NO_2^-	GrO	5.4 ± 2.4	4.4 ± 0.9^{B}	5.4 ± 1.5^{AB}	2.2 ± 0.3^{B}	4.0 ± 1.0	4.6 ± 1.3	3.2 ± 0.5	2.7 ± 0.3
	$GrNO_3$	5.4 ± 1.2	2.7 ± 0.6^{B}	3.4 ± 0.7^{B}	3.8 ± 0.7^{AB}	4.0 ± 0.7	3.6 ± 0.8	4.4 ± 1.3	2.2 ± 0.2
	$GrNO_2$	2.3 ± 0.4	$7.8 \pm 1.9^{*A}$	$7.1 \pm 0.8^{*A}$	$5.5 \pm 1.3^{*A}$	3.2 ± 0.7	$6.0 \pm 1.2^{*}$	4.6 ± 1.7	4.7 ± 1.5
	$GrNO_2NO_3$	3.5 ± 1.0	5.0 ± 1.1^{AB}	3.2 ± 0.9^{B}	5.4 ± 0.9^{AB}	3.4 ± 0.7	4.0 ± 5.3	3.6 ± 1.1	4.3 ± 0.8

^{ABC} Values are means ± SEM, $n = 6$ calves per group. Means with different capital superscript letters (A, B, C) are significantly different ($P < 0.05$) between groups. * Means are significantly different ($P < 0.05$) from preprandial values on d 1, d 2, and d 3 within groups.

2.3b. Experiment 2. Six calves received 400 μmol NO_2^-/$kg^{0.75}$ BW ($GrHNO_2$) and the other six calves were administered 400 μmol NO_3^-/$kg^{0.75}$ BW ($GrHNO_3$) with the morning meal at 0800.

Blood samples were taken before (at 0800) and at 2, 4, 6, 8, and 24 h after NO_2^- or NO_3^- administrations. Procedures of blood sampling were as in experiment 1.

Urine samples were collected in bottles, which were cooled on ice, in 3 fractions. Collection of fraction 1 started at 1400 on the d before the experiment and lasted up to 0800 (time of NO_2^- or NO_3^- administrations) on the experimental day; fraction 2 was collected from 0800 (time of NO_2^- or NO_3^- administrations) to 1400; and fraction 3 was collected from 1400 up to 0800 on the next day (i.e., 24 h after NO_2^- or NO_3^- administrations). After the volume of the fractions was measured aliquots were frozen at −20°C until analyzed.

2.3c. Experiment 3. NO_2^- was dissolved in amounts to reach final concentrations of up to about 50 μmol/L of whole blood. Blood was obtained from a veal calf and stored at 18°C for 24 h. The NO_2^- and NO_3^- concentrations were measured before (control) and immediately after the NO_2^- addition to blood and at 2, 4, 6, and 24 h afterwards to study the stability of NO_2^- in vitro.

2.4. Laboratory analyses

Plasma NO_2^- and NO_3^- concentrations were measured as described by Kahl et al. [8] and Blum et al. [20]. In milk prior to determination of NO_2^- or NO_3^- the samples were centrifuged twice at 10,000 × g at 4°C for 15 min, followed by removal of the whey fraction between the supernatant (fat layer) and the infranatant (precipitates) and storage at −20°C until assayed. This excluded inhibitory matrix effects on biochemical reactions of the assays [10]. The NO_3^- was converted by added NO_3^- reductase to NO_2^-, which was then measured by the Griess reaction. The NO_2^- was measured in the absence of NO_3^- reductase. Standards ranged from 3.25 to 100 μmol/L. The sensitivity of the assay was ≤3.25 μmol/L. Recovery of added NO_2^- and NO_3^- to blood plasma or whey was between 97 and 102%, respectively. Coefficients of variation within and between assays were ≤2 and ≤3%, respectively.

2.5. Statistical analyses

Values of NO_3^- and NO_2^- in blood plasma and of urinary excretion of NO_3^- and NO_2^- (evaluated from concentrations of NO_3^- and NO_2^- in urine and the quantity of fractions) are expressed as means ± SEM.

Time and treatment differences of pre- and postprandial values in plasma and urine were evaluated by mixed procedures using the repeated measure analysis [18]. The model used was $Y_{ijkl} = \mu + group_i + time_j + animal_k + e_{ijkl}$, where Y_{ijkl} = measured value, μ = general mean, $group_i$ = effects of different NO_2^-, or NO_3^-, or NO_2^- + NO_3^- administration, $time_j$ = effect of time within group, $animal_k$ = effects of calves within group, and e_{ijkl} = residual error. Effects were significant if $P < 0.05$.

The half-life of NO_3^- in blood plasma after the last administration of NO_2^-, NO_3^-, and NO_2^- *plus* NO_3^- was determined according to Wartak [19].

3. Results

3.1. Experiment 1

Plasma NO_2^- and NO_3^- concentrations in calves administered 200 μmol NO_2^-, NO_3^-, or NO_2^- plus NO_3^-/$kg^{0.75}$ for 3 d (Table 1). Preprandial NO_3^- concentrations on d 1 in $GrNO_2$ were slightly higher ($P < 0.05$) than in $GrNO_3^-$. In GrO the NO_3^- concentrations did not change significantly during the whole experimental period. However, NO_3^- concentrations increased ($P < 0.001$) after feeding on d 1

Table 2
Urinary excretion of NO_3^- and NO_2^- in control group (GrO), and after oral administration on d 1, d 2 and d 3 of 200 μmol $NO_3^-/kg^{0.75}$ (group $GrNO_3$), 200 μmol $NO_2^-/kg_{0.75}$ (group GrNO2) or 100 μmol NO_2^- *plus* 100 μmol $NO_3^-/kg^{0.75}$ (group $GrNO_2NO_3$) with the morning meal (at 0800)

Trait	Group	Fraction 1 Day 1 0800–1400	Fraction 2 Day 1 1400–Day 2 0800	Fraction 3 Day 2 0800–1400	Fraction 4 Day 2 1400–Day 3 0800	Fraction 5 Day 3 0800–1400	Fraction 6 Day 3 1400–Day 4 0800	Fraction 7 Day 4 0800–1400	Fraction 8 Day 4 1400–Day 5 0800
(μmol/L)									
NO_3^-	GrO	35 ± 8^B	26 ± 4^B	36 ± 8^B	23 ± 4^B	32 ± 3^B	24 ± 2^B	20 ± 2^B	15 ± 4
	$GrNO_3$	84 ± 12^A	78 ± 12^A	129 ± 11^A	74 ± 11^A	119 ± 18^A	91 ± 21^A	80 ± 14^A	31 ± 12*
	$GrNO_2$	111 ± 20^A	74 ± 7^A	108 ± 14^A	85 ± 7^A	125 ± 21^A	79 ± 9^A	99 ± 16^A	21 ± 4*
	$GrNO_2NO_3$	93 ± 14^A	81 ± 13^A	144 ± 19^A	74 ± 12^A	144 ± 21^A	70 ± 9^A	86 ± 14^A	27 ± 12*
NO_2^-	GrO	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1^C	0.3 ± 0.1	0.3 ± 0.0^C	0.3 ± 0.1^B	0.3 ± 0.1^B
	$GrNO_3$	1.4 ± 0.7	2.7 ± 1.7	2.8 ± 1.2	5.8 ± 2.4^B	4.5 ± 2.1	4.1 ± 2.4^{BC}	2.7 ± 1.8^{AB}	2.5 ± 1.2^{AB}
	$GrNO_2$	1.2 ± 0.5	2.0 ± 1.4	0.9 ± 0.3	3.8 ± 1.8^{BC}	1.3 ± 0.5	6.5 ± 3.6^B	1.6 ± 0.9^{AB}	6.1 ± 2.7^A
	$GrNO_2NO_3$	0.3 ± 0.1	2.2 ± 1.2	1.4 ± 0.4	10.9 ± 4.7^A	3.4 ± 1.1	18.0 ± 5.3^A	6.0 ± 2.4^A	6.1 ± 3.3^A

^{ABC} Values are means ± SEM, n = 6 calves per group. Means with different capital superscript letters (A, B, C) are significantly different ($P < 0.05$) between groups. * means are significantly different ($P < 0.05$) from fractions 1 to 7.

(within 2 h) and on d 2 and d 3 (after 6 h) when NO_3^-, NO_2^- or NO_2^- plus NO_3^- were administered. The increments of NO_3^- at 6 h after NO_3^-, NO_2^- or NO_2^- *plus* NO_3^- intakes in the 3 groups were similar. However, in all three NO_3^- and/or NO_2^- supplemented groups NO_3^- concentrations after 6 h on d 2 and 3 were higher ($P < 0.001$) than on d 1 and on d 3 were higher ($P < 0.001$) than on d 2. The NO_3^- concentrations decreased with a $t_{1/2}$ of 22.5 h after the last NO_2^-, NO_3^- or NO_2^- *plus* NO_3^- administration. Preprandial concentrations on d 4 and on d 5 (not shown) were still higher ($P < 0.001$) than preprandial concentrations on d 1, but reached basal concentrations on d 7, i.e., 4 d after the last NO_3^- and/or NO_2^- administration.

The NO_2^- concentrations slightly increased ($P < 0.05$) in $GrNO_2$ on d 1 (at 2, 4 and 6 h after NO_2^- feeding) and on d 3 (at 6 h after NO_2^- feeding), whereas NO_2^- concentrations did not significantly change in the other groups. Concentrations in $GrNO_2$ on d 1 were higher ($P < 0.05$) than in GrO at 2 and 6 h than in $GrNO_3$ at 2 and 4 h, and than in $GrNO_2NO_3$ at 4 h.

Urinary excretion of NO_2^- and NO_3^-, excretion in calves administered 200 μmol NO_2^-, NO_3^-, or NO_2^- plus $NO_3^-/kg^{0.75}$ (Table 2). The excreted amounts of NO_3^- in fractions 1 to 7 in $GrNO_3$, $GrNO_2NO_3$ and $GrNO_2$ were greater ($P < 0.01$) than in GrO, and then decreased ($P < 0.01$) in fraction 8 to values in GrO. Within GrO there were no significant differences between fractions. In $GrNO_3$ and $GrNO_2NO_3$ the excretions of NO_3^- in fractions 3 and 5 were greater ($P < 0.01$) than in fraction 1, whereas in fraction 8 of $GrNO_3$, $GrNO_2$ and $GrNO_2NO_3$ were lower ($P < 0.05$) than in fraction 1. There were no significant differences of NO_3^- excretion between $GrNO_3$, $GrNO_2NO_3$, and $GrNO_2$.

The NO_2^- excretion was very small in all groups and more than 10 times lower than NO_3^- excretions. The NO_2^- excretion was slightly, but significantly ($P < 0.05$) greater than in GrO in $GrNO_3$ in fraction 4, in $GrNO_2$ in fractions 6 and 8, and in $GrNO_2NO_3$ in fractions 4, 6, 7 and 8.

3.2. Experiment 2

Plasma NO_2^- and NO_3^- concentrations in calves administered 400 μmol NO_2^- or $NO_3^-/kg^{0.75}$ (Fig. 1). The NO_3^- concentrations increased ($P < 0.001$) after the administra-

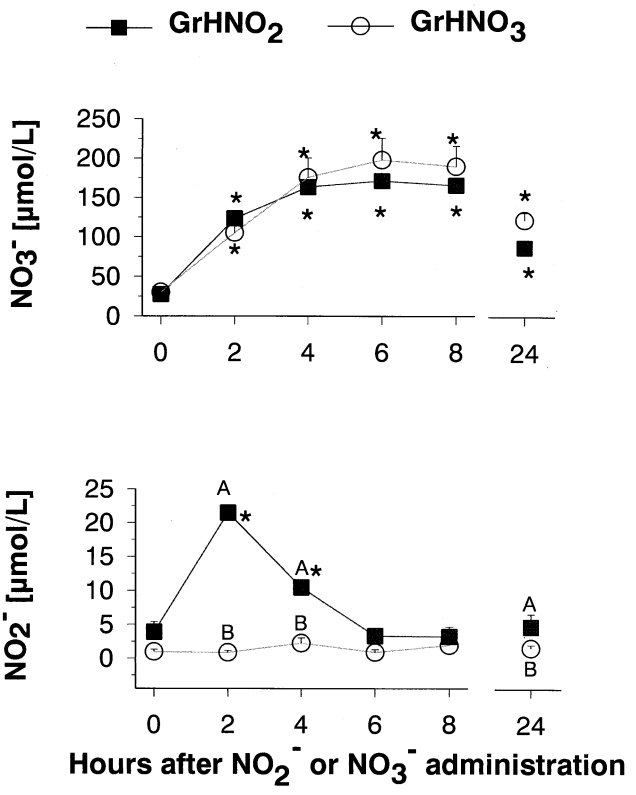

Fig. 1. Pre- and postprandial plasma NO_3^- and NO_2^- concentrations of calves fed 400 μmol $NO_2^-/kg^{0.75}$ ($GrHNO_2$), and 400 μmol $NO_3^-/kg^{0.75}$ ($GrHNO_3$) at the morning meal with whole milk. Values are means ± SEM, $n = 6$ *per* group. *, Means are significantly different ($P < 0.05$) from values at time 0. Means with different capital superscript letters (A, B) are significantly different ($P < 0.05$) between groups.

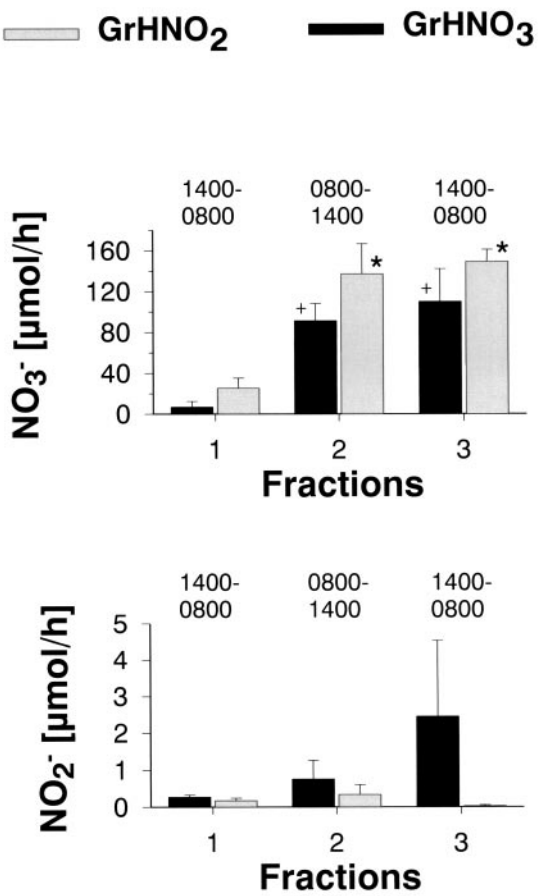

Fig. 2. Urinary excretion of NO_3^- and NO_2^- of calves fed fed 400 μmol $NO_2^-/kg^{0.75}$ ($GrHNO_2$) or 400 μmol $NO_3^-/kg^{0.75}$ ($GrHNO_3$). Values are means ± SEM, $n = 6$ per group. The NO_2^- or NO_3^- were administered on d 1 at the morning meal with milk. Urine was collected in 3 fractions; fraction 1: from d 0 (d before supplementation) 1400 to d 1 at 0800; fraction 2: d 1 from 0800 to 1400; fraction 3: from d 1 at 1400 to d 2 at 0800. *, means are significantly different ($P < 0.05$) from values of fraction 1 within $GrHNO_2$. +, means are significantly different ($P < 0.05$) from values of fraction 1 within $GrHNO_3$.

tion of NO_2^- or NO_3^- in both groups. At 24 h after the NO_2^- or NO_3^- administrations concentrations of NO_3^- were decreased, but values of both groups were still higher ($P < 0.05$) than at time 0. There were no significant group differences.

Concentrations of NO_2^- increased ($P < 0.01$) and reached a peak at 2 h after the administration of NO_2^-, but at 6 h after feeding returned to basal concentrations. After the NO_3^- administration plasma NO_2^- concentrations did not increase. Concentrations at 2, 4, and 24 h were higher ($P < 0.05$) in $GrHNO_2$ than in $GrHNO_3$.

Urinary excretion of NO_2^- and NO_3^-, in calves administered 400 μmol NO_2^- or $NO_3^-/kg^{0.75}$ (Fig. 2). The excretion of NO_3^- increased ($P < 0.05$) after NO_3^- or NO_2^- intakes and was greater ($P < 0.01$) in fractions 2 and 3 than in fraction 1. Values in fractions 2 and 3 were similar and there were no significant group differences. Urinary NO_2^- excretion did not change significantly in both groups.

3.3. Experiment 3

Stability of NO_2^- and NO_3^- in vitro (Table 3). The concentration of NO_3^- remained stable after addition of NO_2^- to full blood, whereas the NO_2^- concentration decreased within 2 h and at 6 h addition reached nearly basal values.

4. Discussion

Basal plasma NO_3^- and NO_2^- concentrations in the 8 wk old veal calves were in the range of 40 ± 6 and 4 ± 1 μmol/L, respectively. These values were much higher than in heifers and dairy cows, in which NO_3^- and NO_2^- are barely measurable or even absent from plasma, but were lower than in premature and neonatal calves [8,16; Blum, unpublished observations]. Calves in this study were strictly fed whole milk obtained from cows without mastitis which did not contain measurable amounts of NO_3^- or NO_2^-, in accordance with other studies [10; Blum, unpublished observations]. This also supports previous findings [16] that NO_3^- present in plasma in young calves is primarily endogenously produced.

Amounts of supplemented NO_2^- or NO_3^- were much below the toxic level [1] and there was in fact no evidence of any clinical sign of intoxication. Plasma concentrations of NO_3^- increased rapidly and dose-dependently after the oral intake of only 200 or 400 μmol NO_2^- or $NO_3^-/kg^{0.75}$. Maximal plasma values reached were at the low range of values measured in newborn calves [16]. Because the plasma NO_2^- concentrations were very low, NO_3^- was the main component in plasma.

The NO_3^- clearance rates of NO_3^- with a $t_{1/2}$ of about 22 h were relatively low. Thus, 24 h after the last intake of

Table 3
Blood plasma concentrations of NO_3 and NO_2 before and after addition of 50 μmol NO_2/L full blood

Trait	Before NO_2^- addition	Immediately after NO_2^- addition	2 h after NO_2^- addition	4 h after NO_2^- addition	6 h after NO_2^- addition	24 h after NO_2^- addition
NO_3^- (μmol/L)	55	105	99	104	105	100
NO_2^- (μmol/L)	0	40	10	9	3	9

Values are derived from one experiment and means of duplicate determinations.

200 μmol NO_2^- or NO_3^- per $kg^{0.75}$ the plasma NO_3^- concentrations were still higher than on d 1. Ingested NO_2^- or NO_3^- therefore remain for relatively long in the circulation and can therefore exert possibly harmful effects for a prolonged time, if present in too high amounts.

While there was no evidence for NO_3^- conversion to NO_2^-, NO_2^- was obviously very rapidly converted to NO_3^- in vivo because in calves fed NO_3^-, NO_2^- or NO_2^- *plus* NO_3^-, the behaviour of plasma NO_3^- concentrations was nearly identical. After oral NO_2^- administration only a transient and small increase of plasma NO_2^- concentrations could be measured, which was slightly greater when 400 than 200 μmol $NO_2^-/kg^{0.75}$ were administered. This is in accordance with Granger et al. [20], who stated that intestinally absorbed NO_2^- is oxidized to NO_3^- almost completely during the first passage through the liver. Our additional experiment with full blood to which NO_2^- was added suggested that the oxidation of NO_2^- to NO_3^- occurs rapidly in vitro, too, whereas NO_3^- remains quite stable.

Both NO_2^- and NO_3^- in young calves are excreted by salivary glands and kidneys [16]. This study shows that ingested NO_2^- and NO_3^- were at least in part excreted through urine in the form of NO_3^- and that urinary NO_3^- excretion mirrored ingested NO_2^- and NO_3^-. On the other hand, urinary NO_2^- excretion was not enhanced even when plasma NO_2^- concentrations increased, likely because the transient rise in blood plasma was too small and because NO_2^- is converted to NO_3^-.

In conclusion, this study confirms previous data that 8 wk old milk-fed calves, which ingest no or but trace amounts of NO_3^- or NO_2^- with milk, have relatively high preprandial plasma NO_3^- and very low plasma NO_2^- concentrations. However, it can be demonstrated that plasma NO_3^- concentrations and urinary excretions rapidly increase depending on the administered dose by oral administration of very small amounts of NO_3^- and NO_2^- with milk. An NO_3^- to NO_2^- conversion barely occurs. However, the data also show that ingested NO_2^- is rapidly converted to NO_3^-. Only NO_3^- was excreted by urine in significant amounts. Thus, small amounts of exogenous NO_3^- or NO_2^- can significantly add to endogenously produced NO_3^- in milk-fed calves.

References

[1] H.-J. Hapke, Toxikologie für Veterinärmediziner. 2nd edn., Verlag F. Enke, Stuttgart, Germany, 1988.

[2] L.C. Green, S.R. Tannenbaum, Nitrate synthesis in the germfree and conventional rat. Science 212 (1981a) 56–58.

[3] L.C. Green, K.R. De Luzuriaga, D.A. Wagner, W. Rand, N. Istfan, V.R. Young, S.R. Tannenbaum. Nitrate biosynthesis in man. Proc Natl Acad Sci USA 78 (1981b) 7764–7768.

[4] S. Moncada, R.M.J. Palmer, E.A. Higgs. Nitric oxide: Physiology, Pathophysology, and Pharmacology. Pharm Rev 43 (1991) 109–142.

[5] E. Anggard. Nitric oxide: mediator, murderer, and medicine. The Lancet 343 (1992) 1199–1206.

[6] J.R. Lancaster. Nitric oxide in cells. American Scientist 80 (1992) 248–259.

[7] D.A. Geller, P.D. Freeswick, D., Nguyen, A.K. Nussler, M. Di Silvio, R.A. Shapiro, S.C. Wang, R.L. Simmons, T.R. Billiar. Differential induction of nitric oxide synthase in hepatocytes during endotoxemia and the acute-phase response. Arch Surg 129 (1994) 165–171.

[8] S. Kahl, T.H. Elsasser, J.W. Blum. Nutritional regulation of plasma tumor necrosis factor-α and plasma and urinary nitrite/nitrate response to endotoxin in cattle. Proc Soc Exp Biol Med 215 (1997) 370–376.

[9] V. Rajaraman, B.J. Nonnecke, S.T. Franklin, D.C. Hammell, R.L. Horst. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J Dairy Sci 81 (1998) 3278–3285.

[10] J.W. Blum, H. Dosogne, D. Hoeben, F. Vangroenweghe, H.M. Hammon, R.M. Bruckmaier, C. Burvenich. Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. Domest Anim Endocrinol 19 (2000) 223–235.

[11] T.W. Jungi. Nitric oxide synthesis by macrophages: a lesson learned from ruminants. Current Trends Immunol 1 (1998) 1–10.

[12] T.H. Elsasser, S. Kahl, T.S. Rumsey, J.W. Blum. Modulation of growth performance in disease: reactive nitrogen compounds and their impact on cell proteins. Domest Anim Endocrinol 19 (2000) 75–84.

[13] D.J. Meyer. Enzymatic/nonenzymatic formation of nitric oxide. Nature Medicine 1 (1995) 1103–1120.

[14] J.L. Zweier, P.H. Wang, A. Samouilov, P. Kuppusamy. Enzyme-independent formation of nitric oxide in biological tissues. Nature Medicine 1 (19950 804–809.

[15] E. Weitzberg, J.O.N. Lundberg. Nonenzymatic nitric oxide production in humans. Nitric Oxide 2 (1998) 1–7.

[16] J.W. Blum, B.R. Hüsler, C. Morel, H. Hammon, R.M. Bruckmaier. Blood plasma concentrations and salivary and urinary excretion of nitrite/nitrate in calves: dependency on age and nutritional effects. Proc Joint Meeting American Dairy Science Association and American Society of Animal Science, Denver, CO, USA, July 1998, Abstr. 509, p. 132.

[17] R. Hambitzer, H. Velke, K. Ramm, H. Sommer. Bewertung der Nitratbelastung von laktierenden und trockenstehenden Milchkühen anhand der Nitratwerte im Blutplasma. Tierärztl Umschau 42 (1987) 775–780.

[18] SAS Institute Inc. Introductory comparison of the GLM and mixed procedures. SAS Inst Inc, Cary, NC, 1994.

[19] J. Wartak. Clinical pharmacokinetics. Praeger Publishers, New York, 1983.

[20] D.L. Granger, J.B. Hibbs, L.M. Broadnax. Urinary nitrate excretion in relation to murine macrophage activation. J Immunol 146 (1991) 1294–1302.