Short communication

Cobalt-deficiency-induced hyperhomocysteinaemia and oxidative status of cattle

G. I. Stangl*, F. J. Schwarz, B. Jahn and M. Kirchgessner

Institute of Nutrition Sciences, Technical University of Munich, 85350 Freising-Weihenstephan, Germany

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In ruminants, Co is required for the synthesis of vitamin B_{12}, which in turn is needed for the resynthesis of methionine by methylation of homocysteine and thus, cobalamin deficiency may induce hyperhomocysteinaemia which is brought into context with perturbations of the anti-oxidative–prooxidative balance. The present study was conducted to explore whether Co deficiency in cattle is also associated with homocysteine-induced disturbances of oxidative status. Co deficiency was induced in cattle by feeding two groups of animals on either a basal maize-silage-based diet that was moderately low in Co (83 μg Co/kg DM), or the same diet supplemented with Co to a total of 200 μg Co/kg DM, for 43 weeks. Co deficiency was apparent from a reduced vitamin B_{12} status in serum and liver and an accumulation of homocysteine in plasma which was in excess of 4·8 times higher in Co-deprived cattle than in controls. The much increased level of circulating homocysteine did not indicate severe disturbances in antioxidant–prooxidant balance as measured by individual markers of lipid peroxidation, protein oxidation, and the antioxidative defence system. There were no quantitative difference in plasma thiol groups, nor were there significant changes in concentrations of α-tocopherol, microsomal thiobarbituric acid-reactive substances and carbonyl groups in liver. However, there was a trend toward increased plasma carbonyl levels indicating a slight degradation of plasma proteins in the hyperhomocysteinaemic cattle. Analysis of the hepatic catalase (*EC* 1.11.1.6) activity revealed an 11 % reduction in Co-deficient cattle relative to the controls. These results indicate that long-term moderate Co deficiency may induce a severe accumulation of plasma homocysteine in cattle, but considerable abnormalities in oxidative status failed to appear.

Cobalt deficiency: Homocysteine: Oxidative status: Cattle

Ruminants normally do not have any dietary source of vitamin B_{12}, and therefore rely entirely on rumen bacteria for their supply of this vitamin. Synthesis of vitamin B_{12} in the rumen is dependent on a continuous supply of dietary Co. Vitamin B_{12} deficiency in ruminants can, therefore, be induced by long-term consumption of Co-inadequate diets. Among other functions, vitamin B_{12} is needed for the resynthesis of methionine by methylation of homocysteine via methionine synthase (*EC* 2.1.1.13). Thus, vitamin B_{12} deficiency is characterized by excessive levels of plasma homocysteine as are seen in vitamin B_{12}-deficient human subjects (Guttormsen *et al.* 1996), fruit bats (Van der Westhuyzen *et al.* 1985), pigs (Young *et al.* 1997) and lambs (Kennedy *et al.* 1994). During the last few years, hyperhomocysteinaemia has frequently been associated with oxidative alterations of lipids and proteins (e.g. Kennedy *et al.* 1994; Mele & Meucci, 1996; Young *et al.* 1997), although there are a lot of conflicting results in this field.

Although Co-responsive disorders of cattle have been reported in different parts of the world (Musewe & Gombe, 1980; Duncan *et al.* 1986), we are not aware of any previous investigation that has dealt with Co-deficiency-induced hyperhomocysteinaemia and the oxidative–antioxidative balance of these animals. The present study was undertaken to investigate (1) the occurrence of hyperhomocysteinaemia in cattle moderately depleted of Co, because moderate Co deficiency is more widespread than severe Co deficiency, and (2) the possible role of homocysteine in the development of

Abbreviations: DNPH, 2,4 dinitrophenylhydrazine; TBARS, thiobarbituric acid-reactive substances.

* **Corresponding author:** Dr Gabriele I. Stangl, fax +49 8161 715367, email stangl@weihenstephan.de

oxidative stress. The variables used to assess oxidative–antioxidative balance were: thiobarbituric acid-reactive substances (TBARS), as a putative marker of tissue lipid peroxidation; thiol groups and carbonyl formation, as markers for protein oxidation; and α-tocopherol and catalase (*EC* 1.11.1.6) activity, as factors with antioxidative defence potential.

Materials and methods

Animals and diets

Twenty-one male cattle of the German Simmental breed with an average body weight of 207 (SD 4) kg were randomly allocated to two groups, and were fed on a maize-silage-based diet which was either Co-sufficient (200 μg/kg DM) or Co-deficient (83 μg/kg DM). Co analysis of the feed was carried out as described recently (Stangl *et al.* 1999). The basal diet, which consisted of maize silage and a concentrate, was supplemented with sufficient amounts of minerals and vitamins according to recommended guidelines (National Research Council, 1996). The animals were individually fed and were allowed free access to water and maize silage (26 μg Co/kg DM). The concentrate was fed in amounts of 2·4 kg/d, and consisted of (g/kg): 375 soyabean meal, 275 ground maize, 292 ground peas, 41·7 vitamin–mineral mixture, and 16·3 limestone. The basal Co concentration of the concentrate used for the deficiency group was 190 μg Co/kg DM. Concentrate used for the Co-sufficient group was fortified with $CoSO_4.7H_2O$ to a final concentration of 640 μg Co/kg DM. The Co-sufficient and Co-deficient groups consisted of eleven and ten animals respectively. The duration of the experiment was 43 weeks. All cattle were treated in accordance with normal animal husbandry practices.

Sample collection and analysis

At week 43, 18–20 h after the last feed, all cattle were slaughtered, and blood and liver were excised. Serum and plasma samples were obtained by centrifugation at 4° for 10 min at 1100 ***g***. From each animal, liver samples were collected from the same region of the liver and stored at –80° before analysis.

Serum and liver concentrations of vitamin B_{12} were determined using a competitive binding radioimmunoassay kit (ICN, Costa Mesa, CA, USA) that worked with an extracting reagent (containing 1 M-NaOH and an organic extracting enhancer) to release vitamin B_{12} from transcobalamines. In the test kit used in the present study, the non-specific vitamin B_{12}-binding R-protein was removed by affinity chromatography. Before radioimmunoassay quantification of liver vitamin B_{12}, a tissue homogenate with borate buffer (pH 9·2, containing 10 g bovine serum albumin/l) was prepared.

Plasma levels of total homocysteine (free or bound to proteins) in blood were determined by HPLC according to the method of Cornwell *et al.* (1993). Plasma samples were prepared for derivatization according to the method of Ubbink *et al.* (1991) using 7-fluorbenzo-2-oxa-1,3-diazole-4-sulfonamide as derivatization reagent. Homocysteine was separated using a reversed-phase column (Nucleosil 120–5 C_{18}, 250 mm × 4·6 mm i.d., 5 μm film thickness; Machery & Nagel, Düren, Germany). The fluorescence spectrophotometer was operated at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The mobile phase, pumped at 1·5 ml/min, consisted of 0·1 M-KH_2PO_4 (adjusted to pH 2·1 with orthophosphoric acid, containing 100 ml acetonitrile/l).

The concentration of α-tocopherol was determined by HPLC (Balz *et al.* 1993). Tissue homogenate was saponified with NaOH in the presence of pyrogallol as an antioxidant. δ-Tocopherol was added as internal standard. Tocopherols extracted with *n*-hexane were separated on a LiChrosorb Si 60 column (5 μm particle size, 250 mm length, 4 mm i.d.; Merck, Darmstadt, Germany) with *n*-hexane–1,4 dioxane (94 : 6, v/v) as an eluent (isocratically) and detected by fluorescence (excitation wavelength 295 nm, emission wavelength 320 nm).

For measurement of catalase in tissue, portions of the liver were prepared by the method of Cohen *et al.* (1970). The activity of catalase was measured spectrophotometrically using the method of Aebi (1970). The determination of the enzyme activity was based on the measurement of the rate of conversion of H_2O_2 at 240 nm (25°) in the presence of the enzyme.

The determination of total thiol groups in plasma (from protein and glutathione) was done by the spectrophotometric method of Hu (1994). The normalization of total thiol groups for total protein was done to even out possible differences in plasma protein content.

TBARS in liver microsomes were determined by HPLC (Fukunaga *et al.* 1993). Liver microsomes were obtained by centrifugation at 105 000 ***g*** for 1 h at 4°, and the precipitate was used for the TBARS assay. TBARS were separated using a LiChrosorb RP-18 Cartridge (5 μm particle size, 250 mm length, 4 mm i.d., Merck). A water–acetonitrile mixture (80 : 20, v/v) was used as mobile phase (isocratically); the fluorescence was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. 1,1,3,3 Tetramethoxypropane was used as internal standard.

Measurement of carbonyl formation was carried out by a modification of the method of Reznick & Packer (1994) which is based on the spectrophotometric detection of the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyl to form protein hydrazones. Liver was prepared for analysis according to the method described recently (Cao & Cutler, 1995). The main difficulty within the estimation of protein carbonyl groups has been the finding that the nucleic acids in plasma, and particularly in liver, may erroneously contribute to higher estimation of carbonyls. However, the removal of nucleic acid with streptomycin sulfate (Reznick & Packer, 1994) was unsatisfactory. We found that an additional sample centrifugation at 41 000 ***g*** for 45 min offered the best protein : nuclei acid ratio. For reaction of the carbonyl groups to 1 ml samples of extracted proteins 4 ml 10 mM-DNPH in 2·5 M-HCl was added. The removal of free DNPH was the most critical analytical step in the assay of carbonyls. Most of the free DNPH was removed by a repeated washing procedure (at least four washing steps) with ethanol–ethyl acetate (1 : 1, v/v). The carbonyl content was

then calculated by obtaining the spectra at 355–390 nm of the DNPH-treated samples. We assessed each liver sample in six replicate runs and each plasma sample in five replicate runs. The CV for the multiple analysis was found to be about 10 %.

Protein concentrations in liver homogenate, microsomes and the supernatant fraction were measured by the method of Smith *et al.* (1975) using bicinchoninic acid. Protein levels in plasma were determined by the Biuret method.

Statistics

Single classification ANOVA was used for analysis of vitamin B_{12} levels, homocysteine and markers of oxidative stress. Differences between means were considered significant at $P < 0{\cdot}05$. Data are presented as means and standard deviations.

Results and discussion

The effects of Co deficiency on vitamin B_{12} status, homocysteine and oxidative–antioxidative balance are shown in Table 1. Analysis of serum and liver demonstrated that vitamin B_{12} concentrations were clearly diminished after prolonged Co deficiency. Measurement of vitamin B_{12}, which was done by a competitive binding radioimmunoassay developed for human diagnostic purposes, provided similar values to those reported by Price *et al.* (1993) who used a specific approach for estimation of bovine plasma vitamin B_{12}. We suggest that the current analytical procedure, using an extracting reagent, guaranteed the complete release of vitamin B_{12} from the specific bovine transcobalamins in serum. Although the Co-deficient cattle were only moderately depleted of Co, because their diet contained 83 μg Co/kg DM and about 100 μg Co/kg diet is considered adequate for cattle (National Research Council, 1996), their homocysteine level in plasma was in excess of 4·8 times higher than that of their controls, and it was remarkable that the homocysteine accumulation was about twice as high as that observed with severely Co-deficient lambs (Kennedy *et al.* 1994). The much increased level of circulating total homocysteine therefore supports the use of homocysteine as a sensitive index for the diagnosis of Co deficiency in cattle.

Hyperhomocysteinaemia in Co-deprived cattle was not accompanied by distinct abnormalities of the antioxidant–prooxidant balance as measured by TBARS, α-tocopherol, thiols and carbonyl groups. Minor changes occurred only with the plasma carbonyl groups which tended to be higher in Co-deficient cattle than in the controls ($P = 0{\cdot}08$) and with the catalase which was slightly but statistically significant reduced by Co deficiency. The reduced activity of the haem-enzyme catalase may possibly result from a decreased formation of succinyl-CoA, necessary for haem synthesis, via the cobalamine-dependent methylmalonyl-CoA mutase (*EC* 5.4.99.2) pathway rather than from hyperhomocysteinaemia. The trend toward increased formation of carbonyl compounds in plasma may possibly indicate slight radical-mediated protein damage. In contrast, the liver carbonyls did not show any trend toward increased values, leading to the assumption that liver tissue may exhibit stronger antioxidative defence potential. However, our findings are in contrast to some previous studies with man and animals that have found associations between hyperhomocysteinaemia and oxidative alterations of lipids and proteins as measured by rapid changes in plasma redox thiol status, elevated levels of cardiac TBARS and elevated levels of cardiac malondialdehyde (e.g. Brown & Strain, 1990; Preibisch *et al.* 1993; Ueland *et al.* 1996; Young *et al.* 1997). However, Preibisch *et al.* (1993) have demonstrated that homocysteine exhibits an expressed prooxidative effect only in the presence of either Fe or Cu as transition-metal ions. In contrast, other studies did not report any hyperhomocysteinaemia-induced changes of plasma lipid hydroperoxides (Dudman *et al.* 1993), vitamin E concentrations of LDL or TBARS (Blom *et al.* 1995). In addition, Mele & Meucci (1996) who examined the oxidative status of plasma

Table 1. Vitamin B_{12} status, homocysteine concentration in blood plasma and oxidative status of cobalt-sufficient and cobalt-deficient cattle

(Mean values and standard deviations for samples from eleven animals in the Co-sufficient group and ten animals in the Co-deficient group)

	Co-sufficient group		Co-deficient group	
	Mean	SD	Mean	SD
Serum				
Vitamin B_{12} (pmol/l)	905	338	218***	101
Plasma				
Homocysteine (μmol/l)	7·39	1·82	35·5***	6·4
Thiols (mol/g protein)	3·93	0·36	4·31	0·76
Carbonyl (nmol/mg protein)	0·38	0·04	0·42	0·05
Liver				
Vitamin B_{12} (nmol/kg)	259	49	43·4***	22·0
α-Tocopherol (μmol/g protein)	8·75	3·41	10·53	3·42
Microsomal TBARS (nmol/g protein)	160	41	173	56
Carbonyl (nmol/mg protein)	0·92	0·20	0·96	0·17
Catalase activity (U/mg protein)†	536	58	478*	50

TBARS, thiobarbituric acid-reactive substances.
Mean values were significantly different from those for the Co-sufficient group: * $P < 0{\cdot}05$, *** $P < 0{\cdot}001$.
† 1U catalase is defined as 1 μmol H_2O_2 substrate decomposed/min at 25°.

proteins after incubation with elevated homocysteine levels found neither any loss of plasma thiol groups nor any enhancement of plasma protein carbonyl formation.

It can be concluded from the foregoing observations that Co-deficiency-induced hyperhomocysteinaemia, along with a slight reduction of hepatic catalase activity did not induce a distinct prooxidative situation in cattle. However, additional studies in this area are warranted to delineate the linkage between homocysteine and oxidative reactions *in vivo*. The much reduced levels of vitamin B_{12}, together with the distinct accumulation of homocysteine in cattle given 83 μg Co/kg diet DM lead one to assume that the currently recommended Co levels in diet for cattle (National Research Council, 1996) seem distinctly to underestimate the Co requirement of these animals.

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