WOOL FOLLICLE FUNCTION IS INFLUENCED BY AN INTERACTION BETWEEN POLYAMINES AND CYSTEINE

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SUMMARY

Ornithine decarboxylase (ODC), the key polyamine biosynthetic enzyme, was assayed in follicle-rich sheep skin extracts in the presence and absence of cysteine in a series of *in vitro* and *in vivo* experiments. Inclusion of 20mM cysteine in the assay reagents resulted in a 17% reduction in enzyme activity ($P<0.04$), presumably through post-translational modification of the enzyme. Infusion of cysteine into sheep also reduced skin ODC activity to a similar extent (39%) ($P<0.05$). Associated with the reductions in skin ODC activity were similar changes in ODC mRNA and changes in the composition of the fibres produced. The effects of cysteine infusion on fibre composition were similar to those seen during infusion of the specific inhibitor of ODC, difluoromethylomithine (DFMO) (Nancarrow *et al.* 1992). Given that both cysteine and DFMO inhibit ODC *in vitro* and *in vivo* and result in similar changes in fibre shape and composition when infused systemically, the possibility of a common mechanism is introduced whereby cysteine acts, at least in part, via effects on polyamine metabolism.

Keywords: polyamines, ornithine decarboxylase, cysteine, follicle function, wool

INTRODUCTION

Amino acids, particularly the sulphur amino acids, methionine and cyst(e)ine, are essential for wool fibre synthesis (Reis 1979; Reis 1989). However, the precise role of these amino acids in fibre synthesis has not been determined. Reis (1989) suggested that methionine may be a contributor to many biochemical pathways, such as polyamine synthesis, and consequently may have a role in the regulation of fibre synthesis in addition to its role as a substrate for keratin synthesis. This hypothesis seems likely given that 60% of the requirement for methionine by cultured wool follicles can be attributed to spermidine synthesis (Hynd and Nancarrow 1995). In a recent study, systemic infusion of difluoromethylomithine (DFMO), the specific inhibitor of omithine decarboxylase (ODC), the rate-limiting polyamine biosynthetic enzyme, was shown to have a profound effect on wool fibre shape and composition (Nancarrow *et al.* 1992) implying an important role for polyamines in the regulation of fibre synthesis. This finding was supported by wool follicle culture experiments where polyamines, particularly spermidine, were shown to be essential for DNA and fibre synthesis (Hynd and Nancarrow 1995). Interestingly, the changes in fibre composition during infusion of DFMO resembled those obtained by Fratini *et al.* (1994) and Rogers *et al.* (199 1) during systemic infusion of cysteine; however the changes in fibre morphology were slightly different. While cysteine infusion caused an increase in both fibre elongation and diameter, DFMO increased diameter but decreased fibre length.

The hypothesis that cysteine may exert some of its effects on follicle function by influencing polyamine synthesis was examined in a series of *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

Sheep

Four l-year old Corriedale wethers weighing approximately 50kg were housed in individual pens and offered a low-protein diet of 600g pellets (7.4MJ metabolisable energy/kg dry matter, 7.8% crude protein) and 600g oaten chaff (6.8MJ metabolisable energy/kg dry matter, 4% crude protein). Water was supplied *ad libitum.*

Inhibition of omithine decarboxylase by cysteine in vitro

ODC activity was measured in follicle-rich skin extracts from the sheep, as previously described by Hynd and Nancarrow (1995). Briefly, skin biopsies were taken from the midside of the sheep under local anaesthetic, follicle-rich skin homogenates were prepared and the activity of ODC was assayed in duplicates (Seely *et al.* 1982). Samples were incubated at 37°C for 1 hour in the presence and absence of 20mM L-cysteine hydrochloride (Sigma) in the incubation reagents. Heat-killed homogenate blanks were used.

Soluble protein concentrations of the supernatant extracts were determined (Bradford 1976) and enzyme activities expressed as pmoles radiolabelled carbon dioxide produced per hour per mg protein.

Effects of systemic cysteine on follicular ornithine decarboxylase and fibre composition

Sheep were fitted with indwelling jugular catheters and infused with cysteine (4g/day in 700mL sterile saline) for 8 days as described by Fratini *et al.* (1994). ODC activity was measured in skin extracts 1 week pre-infusion, following 2 and 8 days of cysteine infusion, and 1 week post-infusion as described by Hynd and Nancarrow (1995).

Skin biopsies were collected on the day prior to the sampling for ODC assays for determination of the proportion of the fibre cross-sectional area occupied by paracortical cells. Transverse skin sections (8μm) were cut at the sebaceous gland level, stained with methylene blue (Clarke and Maddocks 1965) and the area of paracortex measured using image analysis (n=100 follicles/sheep/treatment period). Area of paracortex was expressed as a percentage of the fibre cross-sectional area.

Wool follicle patches were simultaneously collected for Northern analysis of ODC mRNA levels. Northern blots of wool follicle poly (A)+ mRNA were prepared as described by Fratini *et al.* (1994). The blots were hybridised with a [^{32}P]-oligolabelled cDNA probe for mouse ODC (Gupta and Coffino 1985) made from a 700bp Pst I fragment of the pOD48 cDNA insert, then washed twice in 2x SSC with 0.1% SDS at 40°C and once in 2x SSC with 0.1% SDS at 65°C for 30 minutes. Blots were exposed in a PhosphorImager cassette, then scanned and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amount of RNA bound to the membranes was determined using a highly-conserved [^{32}P]-oligolabelled probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.* 1985). The whole 1.3kb cDNA insert coding for GAPDH was excised with Pst I and used as a probe. Northern blots previously hybridised with the mouse ODC probe were stripped and re-hybridised with the GAPDH probe, then washed as before. Hybridisation signals were scanned and quantified using a Phosphorimager and the ODC signals were expressed as a proportion of the amount of RNA as determined by GAPDH.

Statistics

Data were statistically tested by analysis of variance and differences between the means were tested using Duncan's New Multiple Range test (SuperANOVA, Abacus Concepts Inc., Berkeley, CA).

RESULTS

Inhibition of omithine decarboxylase activity by cysteine in vitro

Mean ODC activity in follicle-rich sheep skin extracts was significantly reduced by 17% from 198±30.5 to 165±26.2 pmoles CO_2/hr.mg protein (means&E) (P<0.04) by the inclusion of 20mM cysteine in the assay reagents.

Effects of systemic cysteine on follicular omithine decarboxylase and fibre composition

Orthocortical and paracortical cell areas Fibre area was increased significantly after 7 days of infusion with cysteine (P<0.05) then recovered rapidly to pre-infusion levels in the recovery period (Table 1). Coincident with the increase in fibre area was a 4-fold increase in the area of paracortex after 7 days of cysteine infusion (P<0.0001). The elevation in the cross-sectional area occupied by paracortex was disproportionate to the increase in the area of orthocortex, resulting in a higher proportion of paracortical cells comprising the fibre cross-sectional area (P<0.0006). This increase in the proportion of paracortical cells in the fibre in response to cysteine was significant following 1 day of infusion, and further increased after 7 days, whereafter rapid recovery to the basal level was observed following cessation of the infusion. In addition to the elevated paracortex percentage, the intensity of the staining of the cells increased.

Table 1. Fibre cross-sectional area (μm^2), area of paracortex (μm^2) and the proportion of the fibre cross-sectional area occupied by paracortex (%) prior to, during and post-infusion with cysteine

Values are the means ± SE of the 4 sheep.

Parameter	Pre-Infusion	1d Cysteine	7d Cysteine	Post-Infusion
Fibre Area	439 ± 50.1^{aA}	512 ± 26.8^{ab}	629 ± 54.7^{b}	448 ± 36.1^{a}
Paracortex Area	65 ± 12.5^{a}	145 ± 21.6^{b}	259 ± 9.6^{c}	67 ± 9.2^{a}
Paracortex (%)	16 ± 2.3^{a}	29 ± 4.5^{b}	44 ± 4.2^{c}	16 ± 1.6^{a}

[A] Means within rows with different superscripts are significantly different (P<0.05).

Ornithine decarboxylase activity ODC activity in follicle-rich skin extracts was affected ($P<0.05$) by intravenous infusion of cysteine (Table 2). Mean ODC activity was reduced by 39% after 2 days infusion whereafter ODC activity recovered to the control level by day 8 of the infusion. Post-infusion, the level of activity was increased to a level 19% higher than the mean basal activity ($P>0.05$).

Table 2. Skin ODC activity (pmoles CO_2/hr.mg protein) prior to, during and post-infusion of cysteine

Sheep	Pre-Infusion	2d Cysteine	8d Cysteine	Post-Infusion
10	199.6	182.8	291.0	306.8
18	238.1	98.6	134.8	208.1
91	127.3	56.5	214.9	217.7
95	238.3	153.7	234.3	228.6
Mean ± SE	201±26.1^{aA}	123±28.2^{b}	219±32.3^{a}	240±22.6^{a}

^{A} Means with different superscripts are significantly different ($P<0.05$).

Northern blot analysis Infusion of cysteine resulted in a small but non-significant reduction of mean ODC mRNA levels after 1 day of infusion ($P<0.16$) to 71% of the basal levels (Table 3). ODC mRNA levels then recovered to 82% of the basal level by day 7 of the infusion and rose to 23% higher than the control levels in the recovery period. Although not significant, these trends are similar to those observed for ODC activity.

Table 3. Northern analysis of ODC mRNA expression (arbitrary units) in wool follicles prior to, during and post-infusion with cysteine

Sheep	Pre-Infusion	1d Cysteine	7d Cysteine	Post-Infusion
10	179	166	176	197
18	248	147	237	389
95	259	180	147	257
Mean ± SE	229±25.0^{aA}	164±9.6^{a}	187±26.5^{a}	281±56.7^{a}

^{A} Means with different superscripts are significantly different ($P<0.05$).

ODC hybridisation signals were normalised for the amount of poly (A)' RNA using levels of GAPDH. Insufficient RNA was extracted from the post-infusion sample for sheep 91 and this animal was omitted from the analysis in table 3.

DISCUSSION

These results indicate that the key enzyme for polyamine synthesis, ODC, is present in the follicle fraction of sheep skin and that its activity is responsive to the supply of cysteine *in vitro* and *in vivo*. Inclusion of 20mM cysteine in the assay reagents resulted in a 17% reduction in enzyme activity ($P<0.04$). Intravenous infusion of cysteine also inhibited ODC activity in the skin ($P<0.05$), and had profound effects on fibre composition. Post-infusion, ODC mRNA and enzyme activity were slightly higher than the basal levels, probably as a by-product of feedback mechanisms induced during the infusion. The reduction in ODC activity during cysteine infusion was accompanied by a reduction in ODC mRNA of similar magnitude which suggests transcriptional regulation of ODC by cysteine. Post-translational modification of ODC by cyst(e)ine has also been reported (Mitchell 1981) and is supported by the *in vitro* results presented here. Although not significant, trends indicate that follicle ODC mRNA is depressed by cysteine which represents the first time an amino acid has been shown to regulate ODC expression through both transcriptional and post-translational control.

Given that both cysteine and DFMO (Hynd and Nancarrow 1995) inhibit ODC *in vitro* and *in vivo* and result in similar changes in fibre shape and composition when infused systemically (Nancarrow *et al.* 1992), it is possible that cysteine acts, at least in part, via effects on polyamine metabolism. If this is the case, the different effects of cysteine and DFMO on fibre shape (Nancarrow *et al.* 1992) may indicate that DFMO is a more potent inhibitor of ODC than cysteine. These observations introduce the possibility that the effects of various factors on follicle function are mediated via changes in ODC levels and, by association, intracellular polyamine concentrations. Without measurement of the polyamines *per se* this hypothesis is

merely conjecture; however, the large number of factors that affect follicle activity and fibre synthesis which have effects on ODC in other tissues (Scalabrino and Lorenzini 199 1; Scalabrino *et al.* 199 1) lend strong support to this notion.

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