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Stress-induced Changes in Plasma Concentrations of Immunoreactive β-Endorphin and Cortisol in Response to Routine Surgical Procedures in Lambs

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Abstract

Following four different surgical procedures in lambs 3–5 weeks old, plasma immunoreactive β-endorphin (β-EP) and cortisol were assayed at 15 min and 24 h as determinants of post-operative stress. A threefold increase in mean plasma β-EP levels occurred 15 min after tail docking, and a maximal eight- to tenfold increase occurred in response to castration and/or mulesing with tail docking. Significant increments in mean plasma cortisol levels followed these surgical procedures with the maximal response 15 min after mulesing plus castration with tail docking. The physiologically active 'free' cortisol in plasma represents about 25% of the cortisol, as measured, and the two are highly correlated. At 24 h, β-EP levels in all treated groups were similar to controls, although a small elevation in cortisol levels was still present in the lambs subjected to mulesing. Ultrafiltration of plasma extracts showed that peak β-EP levels contained about 40% immunoreactivity from low molecular weight species (mol. wt $<10\,000$). By specific radioimmunoassay and reverse-phase high-performance liquid chromatography this comprised about 75% $\beta\text{-EP}_{1-31}$, the most potent analgesic endorphin, 10% $\beta\text{-EP}_{1-27}$, and 15% α-*N*-acetyl-β-EP. Increased $\beta\text{-EP}_{1-31}$ levels may modulate post-operative pain in lambs.

Introduction

It is common practice in Australia to carry out certain routine surgical procedures with lambs, without anaesthetic, before weaning. These include tail docking, castration of male lambs, and mulesing (removal of skin from the breech and the tail) to reduce blowfly strike (Dun and Donnelly 1965). Surgery has been shown to increase the plasma levels of the pro-opiomelanocortin-derived peptides β-endorphin (β-EP), β-lipotrophin (β-LPH), and adrenocorticotrophin (ACTH) in the human (Smith *et al.* 1985). In the rat the concomitant release of these peptides from the pituitary in response to acute stress (Guillemin *et al.* 1977) further stimulates the adrenocortical release of corticosteroids.

In response to a need for re-evaluation of stress in farm animals (Dantzer and Mormede 1983), a study has now been carried out using the pituitary and adrenocortical release of β-EP and cortisol, respectively, to assess the relative post-operative stress of some routine surgical procedures in lambs.

Materials and Methods

Fifty crossbred (Border–Leicester × Merino) lambs 3–5 weeks old weighing 9·4 ± 0·3 (s.e.m.) kg were used. They were suckling on ewes grazing in a small paddock prior to treatment. The lambs, matched for weight, were divided into five groups of 10. Group 1 was used as controls and included

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male and female lambs which underwent similar handling procedures in the holding cradles used in the operations, group 2 (female lambs) received tail docking, group 3 (male lambs) were castrated and then tail docked, group 4 (female lambs) were mulesed and then tail docked, and group 5 (male lambs) were mulesed, castrated and then tail docked. All operative procedures were carried out in less than 1 min. Jugular vein blood samples (10 ml) were collected into heparinized vacutainers (*a*) pretreatment, (*b*) 15 min post-operative, and (*c*) 24 h after surgery. After immediate centrifugation at 4°C, the plasma was removed and stored frozen until assayed.

Surgical Procedures

The lambs were placed into a cradle which used simple hind-leg restraints to hold the lambs at an inclined position on their backs. The mules treatment incorporated standard breech cuts commencing level with and next to the base of the tail. Wool-bearing skin was removed adjacent to the natural bare area beneath the tail with the cuts terminating approximately half way from the anus to the hock. Cuts were also made on the tail so a V of wool-bearing skin remained. The apex of the V terminated about one-third of the way down the docked tail to protect the tail from ultraviolet light. Surgical castration was carried out by cutting off about one-third of the bottom part of the scrotum, and then extracting the testicles one at a time with a 'serrated clamp-type, lamb-marking knife'. Tails were docked with a knife immediately below the third palpable joint which is about level with the vulva of the ewes. All wounds were dressed with activated charcoal, (Crown Wound Powder, Heriot Agencies Pty Ltd, Victoria), to aid healing and reduce bacterial activity. As in normal animal husbandry practice, no anaesthetic was used.

Radioimmunoassay

The heterologous double-antibody radioimmunoassay (RIA) described by Lim *et al.* (1982*a*) was used for the assay of β-EP in plasma extracts. In addition to β-EP_{1-31}, β-LPH, β-EP_{1-27}, α-*N*-acetyl-β-EP_{1-31}, and α-*N*-acetyl-β-EP_{1-27} have been found to cross-react 100% on a molar basis with this antiserum (R56) while α-EP_{1-16} and α-EP_{1-17} have negligible cross-reactivity. The antigenic site is considered to be in the region of amino acids 20–27 (Guillemin *et al.* 1977). The α-*N*-acetyl-β-EP was assayed in some ultrafiltrates of plasma extracts using an antiserum (R92) which specifically recognizes the α-acetylated *n*-terminus of endorphins, as described by Cheng *et al.* (1985). Sensitivity of these peptide RIA's was 4 pg/tube, and the within-assay coefficient of variation (CV) was 8%; between-assay CV was 14%. Plasma (1 or 2 ml) was extracted by the vycor glass method essentially as described by Copolov *et al.* (1983) prior to assay for total extractable β-EP. The extract was taken up in assay buffer for RIA of β-EP, or in 0·5 ml 0·1% (w/v) gelatine in 10 mM acetic acid for centrifugal ultrafiltration to remove β-LPH or other larger molecular weight precursors of β-EP. Recovery of 500 pg β-endorphin added to plasma and then extracted was 80%, and 60% after ultrafiltration. Recovery of 1–5 ng ovine β-LPH (C. H. Li) added to plasma was 60% after extraction, and undetectable after ultrafiltration.

Ultrafiltration was carried out at room temperature at 5000 *g* for 45 min in a fixed-angle rotor in microconcentrators (Centricon 10, Amicon Scientific, Australia). The concentrates containing peptides (mol. wt >10 000) were then washed twice with 0·5 ml 0·1% (w/v) gelatine–10 mM acetic acid, and centrifuged as before. The ultrafiltrates containing peptides (mol. wt <10 000) were then assayed for β-EP and, in some 15 min post-operative samples, for α-*N*-acetyl-β-EP also. In addition some ultrafiltrates from 15 min post-operative samples were pooled after lyophilization, and taken up in 50% (v/v) acetic acid–10^{-3} M sodium periodate to obtain a molecular profile of immunoreactive β-EP by reverse-phase high-performance liquid chromatography (HPLC) using a radially compressed Nova-Pak C-18 column (Smith and McDermott 1984; Smith *et al.* 1986).

Direct RIA of cortisol ('free' plus protein-bound) in plasma was as described by Fell *et al.* (1985). The assay has a sensitivity of 1·0 nmol/l, a within-assay CV of 2% and a between-assay CV of 8%. Centrifugal ultrafiltration of plasma prior to determination of 'free' cortisol was as in Fell *et al.* (1985).

Results

In comparison with mean plasma β-EP levels (83 ± 23 pg/ml) in controls, an eight- to tenfold increase in mean levels of total extractable immunoreactive β-EP occurred (680–797 pg/ml) in three of the surgically treated groups of lambs following mulesing and/or castration with tail docking (groups 3, 4 and 5,

Table 1) at 15 min post-operation. A smaller rise in plasma β-EP (266 ± 80 pg/ml) occurred in response to tail docking (group 2) although the latter was not significantly different at the 1% level from the controls. No significant differences in any of the groups relative to the controls were observed at 24 h post-operation.

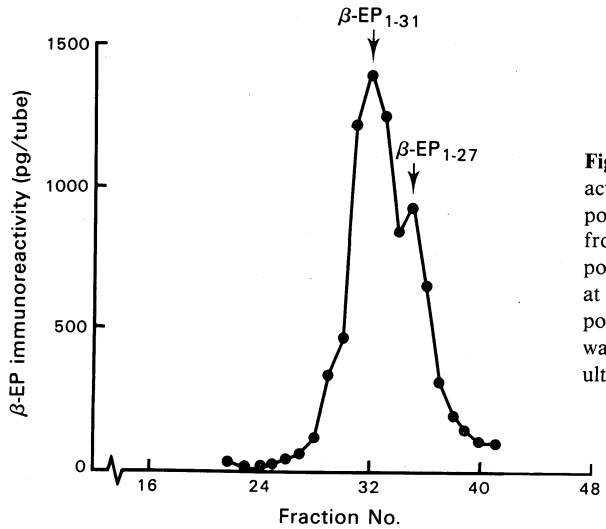

Fig. 1. Profile of β-EP immunoactivity after reverse-phase HPLC of pooled ultrafiltrates of plasma obtained from surgically treated lambs, 15 min post-operation. Fractions were collected at 0·5-min intervals; the elution position of synthetic standards (arrows) was determined in separate runs by ultraviolet absorbance at 214 nm.

High mean values (947 ± 228 pg/ml) of total extractable immunoreactive β-EP in six plasma pools from 15 min post-operation plasma samples, were found to contain 44 ± 7% immunoreactive β-EP (374 ± 71 pg/ml) with molecular weight <10 000, after ultrafiltration to remove β-LPH and other high molecular weight (<10 000) precursors of β-EP. The β-EP in these ultrafiltrates was further partitioned into approximately 75% $\beta\text{-EP}_{1\text{-}31}$ and 10% $\beta\text{-EP}_{1\text{-}27}$ by reverse-phase HPLC (Fig. 1), and 15% α-*N*-acetyl-β-EP as determined by specific RIA for α-*N*-acetyl-β-EP.

Table 1. Effect of surgery on plasma concentrations of immunoreactive β-endorphin (β-EP) and cortisol

Using one-way ANOVA, means (±s.e.m.) in each column with different superscripts are significantly different ($P < 0{\cdot}01$). There were 10 lambs in each group

Group No.	Treatment[A]	Pretreatment levels of:		Post-operative levels after:			
				15 min		24 h	
		β-EP (pg/ml)	Cortisol (nmol/l)	β-EP (pg/ml)	Cortisol (nmol/l)	β-EP (pg/ml)	Cortisol (nmol/l)
1	Controls	93 ± 15^{a}	54 ± 9^{a}	83 ± 23^{a}	87 ± 9^{a}	173 ± 47^{a}	68 ± 10^{a}
2	T	65 ± 11^{a}	38 ± 9^{a}	266 ± 80^{a}	136 ± 16^{b}	104 ± 21^{a}	44 ± 6^{a}
3	C+T	81 ± 14^{a}	32 ± 5^{a}	797 ± 186^{b}	171 ± 11^{bc}	166 ± 32^{a}	44 ± 8^{a}
4	M+T	51 ± 6^{a}	36 ± 3^{a}	707 ± 204^{b}	187 ± 21^{c}	119 ± 27^{a}	120 ± 23^{b}
5	M+C+T	87 ± 25^{a}	40 ± 8^{a}	680 ± 95^{b}	232 ± 17^{d}	138 ± 32^{a}	121 ± 10^{b}

[A] T, tail docking; C, castration; M, mulesing.

With mean plasma cortisol as a stress indicator, significant ($P < 0{\cdot}01$), post-operative increases in cortisol occurred relative to control levels (87 ± 9 nmol/l) at 15 min following tail docking with or without castration and/or mulesing

(Table 1). Increments in cortisol were associated with increased surgical treatment; thus cortisol levels were highest (232 ± 17 nmol/l) following mulesing plus castration and tail docking (group 5), but lower with mulesing or castration plus tail docking (187 ± 21 or 171 ± 11 nmol/l), or tail docking alone (136 ± 16 nmol/l). Groups 3 and 4 were not significantly different in terms of cortisol response. In addition at 24 h post-operation mean plasma cortisol levels in lambs subjected to mulesing (groups 4 and 5) were still nearly twofold higher ($P < 0{\cdot}01$) than control levels (Table 1).

When total ('free' plus protein bound) assayable cortisol was compared with the physiologically active 'free' cortisol in plasma from lambs 15 min post-operation, as shown in Fig. 2, a very good correlation ($r = 0{\cdot}94$) was obtained.

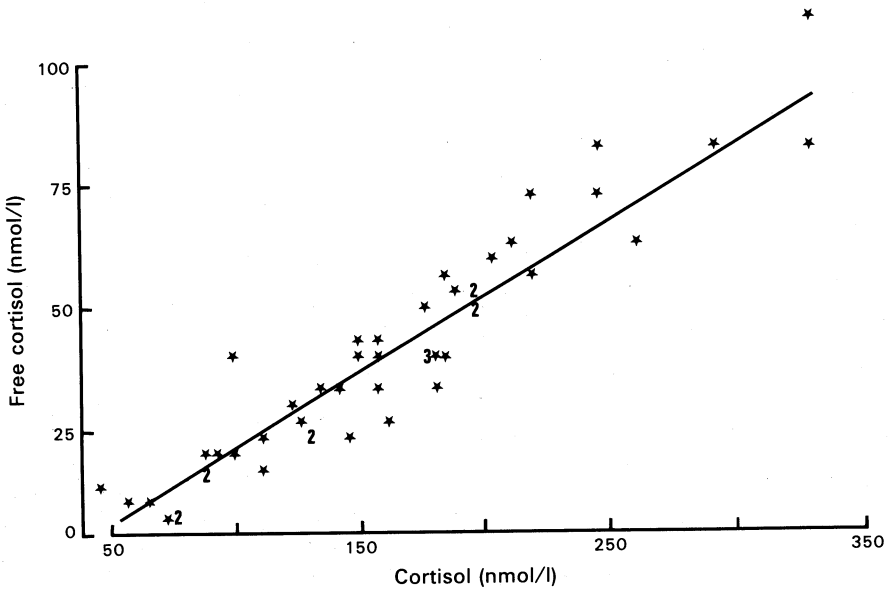

Fig. 2. Correlation between plasma 'free' cortisol (assayed after centrifugal ultrafiltration), and total assayable plasma cortisol concentrations in 15-min post-operative blood samples from 50 lambs. The regression equation is $y = -15 + 0{\cdot}32x$, $r = 0{\cdot}94$.

Discussion

The experimental protocol was designed to assess the relative stress in lambs of different combinations of routine surgical procedures, conducted with minimal handling response. Only three blood samples were taken from each lamb, thus allowing only the acute response 15 min post-operation, and any residual physiological stress at 24 h to be compared with controls and pretreatment samples.

Within the constraints of this protocol it was possible to show that significant increases in immunoreactive β-EP and cortisol occurred in response to surgery at 15 min post-operation. The possibilities of sex differences in response were not considered in this study. Some assessment was made, however, of the relative physiological stress imposed by the treatments. Thus, using mean plasma β-EP as the hormonal indicator of stress or pain, it was shown that castration or mulesing along with tail docking stimulated a maximal 8–10-fold increase in β-EP levels.

When the lambs were exposed to all three surgical treatments, the mean response was similar, although there was less individual variation. A smaller, but not statistically significant, rise in β-EP levels occurred after tail docking.

β-EP_{1-31} is known to be a more potent analgesic than β-LPH and β-EP_{1-27}, and α-*N*-acetyl forms of β-EP_{1-31} and β-EP_{1-27} have negligible opiate activity (Feldberg and Smyth 1977; Deakin *et al.* 1980). On a molar basis, however, all these forms are equally immunoreactive with the antibody used in the RIA. In order to assess the percentage of β-EP_{1-31} in the peak plasma levels of immunoreactive β-EP, ultrafiltration was first carried out to remove β-LPH and other high molecular weight precursors of β-EP. These were found to account for about 60% of the extractable β-EP immunoreactivity. Of the 40% β-EP immunoreactivity in the ultrafiltrate, specific RIA for the α-*N*-acetyl forms of β-EP showed that only 15% was present as α-*N*-acetyl forms. Of the remainder, reverse-phase HPLC showed that the predominant molecular species in the ultrafiltrate was β-EP_{1-31} (75%) with a small proportion present as β-EP_{1-27} (10%). This indicates that the potent analgesic form, β-EP_{1-31}, increases to levels of the order of 200–300 pg/ml in peripheral plasma in response to surgical stress or pain.

The degree of stress-induced analgesia (Wolfle and Liebeskind 1983) that might be associated with such increased peripheral plasma β-EP_{1-31} levels in the lamb was not assessed in these preliminary experiments. Increased levels of β-EP may not always be correlated with the degree of induced analgesia, for example, after footshock in rats (Lim *et al.* 1982*b*) or after acupuncture in horses (Bossut *et al.* 1983), although β-EP_{1-31} has been shown to be a potent analgesic by intravenous injection (Tseng *et al.* 1976; Feldberg and Smyth 1977).

It is generally agreed that the anterior pituitary is the main source of peripheral β-EP_{1-31} (Guillemin *et al.* 1977; Smyth and Zakarian 1980), but to what degree peripheral β-EP_{1-31} modulates pain perception (Melzack and Wall 1965), or under what conditions the anterior pituitary contributes to concentrations in the brain (Bergland and Page 1979), or cerebrospinal fluid after electro-acupuncture (Sjolund *et al.* 1977; Clement-Jones *et al.* 1980), is still controversial (Rossier *et al.* 1977; Lim *et al.* 1983).

In contrast to β-EP, using plasma cortisol as the hormonal indicator of stress at 15 min post-operation, the results showed that the most significant increase in mean cortisol levels occurred following all three surgical treatments (mulesing plus castration with tail docking), with somewhat less response to mulesing or castration with tail docking. As with plasma β-EP a smaller rise in cortisol levels occurred after tail docking alone. Of interest, at 24 h, there was evidence that in lambs subjected to mulesing the levels of cortisol were still significantly elevated, indicating some residual physiological stress associated with this treatment, whilst β-EP levels in all groups at 24 h were similar to the controls. It was apparent that the lambs experienced some discomfort when walking and lying at 24 h after the surgery.

With the much studied footshock-induced analgesia, a role for glucocorticoids in mediating this phenomenon has been found (MacLennan *et al.* 1982; Lim *et al.* 1983), in that analgesia is restored in adrenalectomized rats by corticosterone or dexamethasone. In the present studies there was a marked increase in the physiologically active plasma 'free' cortisol that was highly correlated with total assayable cortisol. The 'free' cortisol represented about 25% of the total cortisol as determined by centrifugal ultrafiltration, and agreed with values in the adult

sheep. Peak values of 'free' cortisol in lambs at 15 min post-operation were comparable with maximal values in the sheep after an intramuscular administration of Synacthen (Fell *et al.* 1985), and were considerably higher than those found in sheep following yarding or transport, or in calves in response to surgical castration (Fell *et al.* 1986). In the present experiments therefore, post-operation pain following surgery in lambs may be modulated by the marked rise in β-EP_{1-31}, that was in evidence 15 min after surgery, and this could be assisted by the associated increased plasma 'free' cortisol levels. These findings could have important implications for animal welfare, but further work will be required in order to confirm these implications.

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