J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1-3, pp. 399-404, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

IGF1 AND 2 IN TWO MODELS OF ADRENAL GROWTH

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Summary—Insulin-like growth factors (IGFs) 1 and 2 were measured in the adrenal glands of rats undergoing either compensatory growth following left unilateral adrenalectomy or adrenal regeneration following bilateral adrenal enucleation. In normal rat adrenal gland, the tissue concentration of IGF2 (7.45 ± 0.99 pg/μg protein) was higher than IGF1 (1.26 ± 0.23 pg/μg protein), both peptides being more abundant in the inner zones of the adrenal gland compared to the capsule-glomerulosa. During compensatory growth of the right adrenal gland, IGF1 and 2 increased significantly compared with control right adrenal glands at 24 h following left unilateral adrenalectomy ($P < 0.001$). At 68 h, the increase remained significant for IGF1 ($P = 0.012$). The two peptides were measured in the regenerating adrenal gland at 7, 14 and 21 days following bilateral enucleation. Whilst there was a trend towards an increase in the IGF1 and 2 content of regenerating adrenal glands, the increase was significant only for IGF2 in the left adrenal gland at 21 days following enucleation. Plasma IGF1 and 2 did not increase compared to controls during the experiments (110.97 ± 1.95 and 46.33 ng/ml, respectively), suggesting that the changes in tissue IGF reflect increased local production during rapid growth of the adrenal gland.

INTRODUCTION

A considerable amount of evidence exists to question the role of ACTH as an important adrenal mitogen. Whilst hypophysectomy [1] and dexamethasone treatment [2] lead to adrenal atrophy, pointing to a corticotrophic factor as the adrenal growth regulator, it would appear that the mitogenic product of the corticotroph is distinct from ACTH. Passive immunization of rats with ACTH antisera sufficient to reduce circulating corticosterone levels neither results in adrenal atrophy [3, 4] nor inhibits compensatory growth following unilateral adrenalectomy [4, 5]. The regeneration of the adrenal cortex following bilateral adrenal enucleation similarly is not affected by ACTH antiserum treatment [6]. In fact, ACTH has been shown to inhibit the rapid proliferative response of the remaining adrenal gland after unilateral adrenalectomy [2] and its anti-mitotic effect on adrenocortical mitogenesis *in vitro* is a well-known phenomenon [7, 8].

Since the elucidation of the sequence of pro-opiomelanocortin (POMC) [9] and the purification of its N-terminal glycopeptide (N-POMC) [10], attention has focused on N-POMC as a contender for the non-ACTH corticotroph-derived adrenal mitogen. Immunoneutralization of circulating N-POMC inhibited normal adrenal growth in young rats [3] and prevented compensatory adrenal growth [5] and adrenal regeneration following bilateral adrenal enucleation [6] in adult rats. The hypothesis that a cleavage product of N-POMC (1–76) may be the adrenal mitogen was formulated upon the observation that trypsinization of the peptide was necessary before expression of its mitogenic activity [3]. Extreme N-terminal POMC peptides [N-POMC (2–59) and N-POMC (1–28)] were indeed found to have potent mitogenic effects both *in vivo* and *in vitro* [3, 11], N-POMC (1–28) partially reversing the adrenal atrophy in enucleated rats after hypophysectomy [11], leading to the proposal that the naturally occurring cleavage product, N-POMC (1–48/9) may be the true pituitary-derived adrenal mitogen. In the light of evidence that excluded the pituitary intermediate lobe as the source of this peptide [5, 6], it was proposed that the mitogenic peptide was generated post-secretionally by a neurally mediated cleavage of N-POMC (1–74) at the adrenal gland to initiate compensatory adrenal growth [5] and by a different mode of N-POMC processing in the pituitary

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands, 16–21 September 1990.

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anterior lobe corticotroph during adrenal regeneration [6].

It would be of interest to know whether the N-terminal POMC peptide has a direct mitogenic action on the adrenocortical cell itself or whether it serves to mobilize a local growth factor which is in turn responsible for the mitogenic activity. The insulin-like growth factors (IGFs) are one class of tissue mitogens which may fulfil this role. Both IGF1 and 2 exert potent proliferative effects on adrenocortical cells *in vitro* [12–14]; possibly via interactions with high affinity IGF binding sites which have been described in the adrenal gland [15–19]. Local synthesis of IGF1 is likely to occur in adult adrenal tissue in view of the relative abundance of IGF1 mRNA [20] and the immunohistochemical localization of the peptide [21] in the mature rat adrenal gland. Whilst the abundance of IGF2 mRNA in most tissues including the adrenal gland is greatest in the foetus and declines in the postnatal period [22–24], IGF2 mRNA nevertheless is expressed in the adult human adrenal gland [25] and in a variety of rat tissues IGF2 mRNA persists into adulthood sometimes at higher levels than IGF1 mRNAs [22, 26].

In order to investigate the importance of the IGFs in the regulation of adrenal growth, we have studied the tissue levels of IGF1 and 2 in adrenal glands undergoing rapid growth stimulated by either unilateral adrenalectomy or bilateral adrenal enucleation. We report significant increases in the adrenal concentrations of both peptides during compensatory adrenal growth.

EXPERIMENTAL

Animals and experimental procedures

Female Sprague–Dawley rats (140–160 g body wt) were housed at 20°C in a 12 h light–dark cycle with water and chow available *ad libitum*. Enucleated rats were given 0.9% NaCl as drinking water for the first 3 days after the operation. Operations were performed under halothane–oxygen anaesthesia and the animals housed separately after the surgical procedures. Bilateral adrenal enucleation was performed as described previously [11, 27] and animals killed by decapitation at 7, 14 and 21 days after the operation. In the compensatory adrenal growth experiments, animals were killed 24 and 68 h following left unilateral adrenalectomy. Blood was collected in chilled tubes containing 10 mg EDTA, centrifuged, and plasma immediately frozen. Adrenal glands were freed of surrounding fat and frozen immediately on dry ice prior to storage at −70°C until extraction.

Tissue extraction

Frozen whole adrenal glands were extracted in 0.5 ml ice-cold IM acetic acid using glass–glass homogenizers. In 1 series of experiments the capsule with adherent zona glomerulosa and remaining inner zone were homogenized separately. After centrifugation (12,000 ***g***, 4°C, 30 min) an aliquot was removed for protein estimation and the supernatant frozen and lyophilized. The extract was reconstituted in 0.5 ml assay buffer for measurement in the IGF1 and 2 radioimmunoassays (RIAs).

IGF RIAs

The IGF1 RIA has been described elsewhere [28]. The antiserum was raised in rabbits to recombinant human N-Met^{1}IGF1 conjugated to egg albumin, and displays 100% cross-reactivity with human, ovine and rat IGF1. Recombinant human authentic sequence IGF1 was used as a radio-ligand and standard in this assay and separation of bound and free fractions was by second antibody. Crossreactivity with IGF2 is $< 0.5\%$. The IGF2 RIA uses a mouse monoclonal antibody (Amano Pharmaceutical Co. Ltd, Nagoya, Japan) raised by Tanaka *et al.* (unpublished data) against a preparation of rat IGF2 isolated from culture medium obtained from a rat cell line [29]. Using ovine IGF2 as a standard and [^{125}I]–ovine IGF2 as a tracer, the antibody crossreacts $< 1\%$ with ovine and human IGF1 (much less than the 10% crossreaction with these peptides suggested by the manufacturer). Measurement of IGF1 and 2 in plasma was performed after dissociation from their plasma binding proteins by extraction in acid–ethanol for IGF1 [28] and C18 Sep-Pak extraction for IGF2. Recoveries of [^{125}I]–IGF1 and [^{125}I]–IGF2 added to plasma samples before extraction were $> 90\%$ for the appropriate extraction method. Whilst individual plasma samples were assayed separately for IGF1, plasmas from each treatment group were pooled for the measurement of IGF2.

Protein was measured using Coomassie protein assay reagent (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as standard.

Table 1. Plasma concentrations of IGF1 and 2 in rats from each treatment group

Treatment group	IGF1 (ng/ml plasma)	IGF2 (ng/ml plasma)
Control	110.97 ± 1.95 (18)	46.33
24 h ADX	109.69 ± 1.12 (12)	38.7
68 h ADX	116.84 ± 2.38 (11)	47.6
7 d Enucl.	110.07 ± 1.78 (17)	32.0
14 d Enucl.	101.69 ± 3.00[a] (15)	26.4
21 d Enucl.	92.15 ± 3.06[b] (11)	29.1

Plasma concentrations of IGF1 and 2 in control rats; in rats at 24 or 68 h after left unilateral adrenalectomy (24 and 68 h ADX, respectively); and in rats at 7, 14 or 21 days after bilateral adrenal enucleation (7, 14 or 21 d Enucl., respectively). Values for IGF1 are means ± SEM (n in parentheses for each group). Values for IGF2 are single measurements of a pool comprising every sample from each group.
[a]Significantly different from control ($P = 0.012$); [b]significantly different from control ($P = 0.000$).

Statistics were performed using the student's unpaired t-test. Values are means ± SEM for each group.

RESULTS

Both IGF1 and 2 were found to be present in extracts of normal rat adrenal gland, the IGF2 content (7.45 ± 0.99 pg/μg protein, $n = 9$) being considerably higher than the content of IGF1 (1.26 ± 0.23 pg/μg protein, $n = 9$). There were no significant differences in tissue levels of IGF1 or 2 between control left and right adrenal glands ($P > 0.48$). Both peptides were more abundant in the inner zones of the adrenal gland (IGF1, 1.25 ± 0.20 pg/μg protein, $n = 10$; IGF2, 6.58 ± 0.80 pg/μg protein, $n = 10$), compared to the capsule-glomerulosa preparations where the concentration of IGF1 was 0.50 ± 0.05 pg/μg protein, $n = 12$, and IGF2 was 3.99 ± 0.22 pg/μg protein, $n = 9$.

During compensatory growth of the right adrenal gland the tissue content of IGF1 and 2 increased in comparison with right adrenal glands from control rats (Fig. 1). The adrenal IGF1 content was significantly higher than control glands 24 h (5.28 ± 0.45 pg/μg protein, $n = 4$, $P = 0.000$) and 68 h (3.48 ± 0.58 pg/μg protein, $n = 6$, $P = 0.012$) following left unilateral adrenalectomy. Whilst the IGF2 content of right adrenal glands at 24 h was significantly greater than controls (17.14 ± 1.37 pg/μg protein, $n = 5$, $P = 0.001$) this increase did not reach significance at 68 h (13.44 ± 2.32 pg/μg protein, $n = 5$, $P = 0.061$). Plasma IGF1 concentrations at 24 and 68 h following unilateral adrenalectomy were not significantly different from the circulating IGF1 levels measured in control rats. Likewise the plasma IGF2 concentration did not appear to differ from the controls at the same time points following unilateral adrenalectomy (Table 1).

Turning to the IGF tissue levels during adrenal regeneration, the results would indicate a discrepancy between the IGF2 concentrations of left and right glands at each time point after adrenal enucleation, the IGF2 content of the left gland being greater than that of the right gland (Table 2). The differences were significant for IGF2 at 7 ($P = 0.024$) and 14 days ($P = 0.007$) following enucleation. In view of these observations, left and right glands are treated separately and compared to their respective left or right controls for each peptide (Table 2). Whilst there was a trend towards an increase in the IGF1 and 2 content of regenerating adrenal

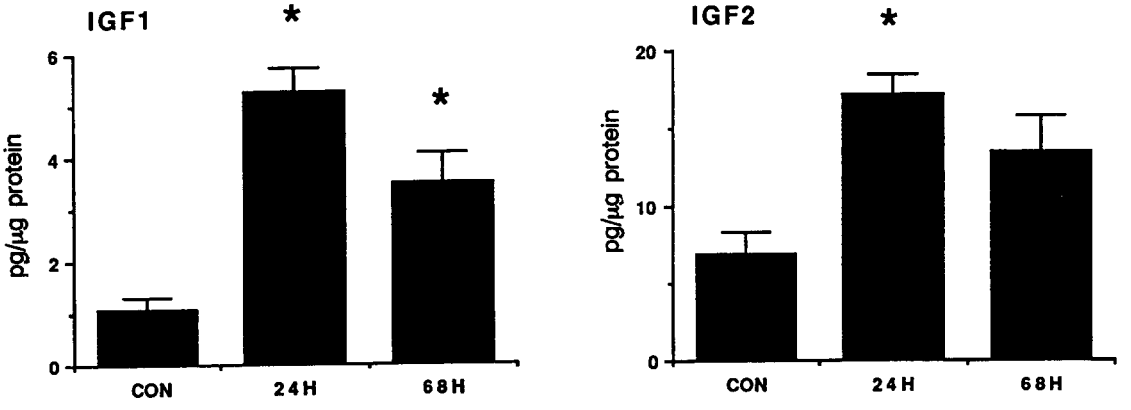

Fig. 1. IGF1 (left panel) and IGF2 (right panel) content of acid extracts of right adrenal glands taken from control rats (CON) and from rats 24 h (24H) and 68 h (68H) after left unilateral adrenalectomy. Values are means ± SEM. *Significantly different from control ($P < 0.012$).

Table 2. Tissue concentrations of IGF1 and 2 in regenerating rat adrenal glands

	IGF1 (pg/μg protein)		IGF2 (pg/μg protein)	
	Left	Right	Left	Right
Control	1.42 ± 0.37 (5)	1.06 ± 0.26 (4)	7.89 ± 1.48 (5)	6.90 ± 1.45 (4)
7 d Enucl.	1.05 ± 0.33 (4)	0.93 ± 0.39 (5)	6.48 ± 1.19[a] (5)	2.96 ± 0.67[b] (6)
14 d Enucl.	2.36 ± 0.77 (6)	1.33 ± 0.69 (7)	12.12 ± 2.15[c] (6)	4.59 ± 1.08 (7)
21 d Enucl.	2.87 ± 0.85 (4)	2.36 ± 0.93 (5)	17.70 ± 2.96[d] (4)	11.81 ± 2.37 (5)

Concentrations of IGF1 and 2 in extracts of left and right adrenal glands from control rats, and from rats at 7, 14 and 21 days following bilateral adrenal enucleation (7, 14 or 21 d Enucl., respectively). Values are means ± SEM (*n* in parentheses for each group).

[a]Significantly different from right gland at 7 days ($P = 0.024$); [b]Significantly different from right control gland ($P = 0.024$); [c]Significantly different from right gland at 14 days ($P = 0.007$); [d]Significantly different from left control gland ($P = 0.016$).

glands after enucleation, the increase was significant only for the IGF2 content of the left adrenal gland at 21 days compared with control glands ($P = 0.016$, Table 2). The results would suggest that the rise in tissue concentrations of IGF1 and 2 occurred earlier in the left adrenal gland (at 14 days) than in the right gland (at 21 days). The right adrenal IGF2 content is in fact significantly less than the control gland 7 days after enucleation ($P = 0.024$, Table 2). There was no change in the plasma concentration of IGF1 at 7 days after adrenal enucleation compared with control rats ($P = 0.736$). However plasma IGF1 levels decreased significantly compared to controls at 14 ($P = 0.012$) and 21 days ($P = 0.000$) following enucleation. Plasma IGF2 concentrations appeared to be lower than control levels at each time point after enucleation (Table 1).

DISCUSSION

A previous report of the simultaneous measurement of IGF1 and 2 in adult adrenal tissue recorded a higher concentration of IGF2 than 1 in human adrenal medulla [30]. The present study reaffirms this observation in whole rat adrenal gland where the concentration of IGF2 was 5.08-fold greater than IGF1. The majority of this immunoreactivity was located in the inner zone of the adrenal gland. It would appear from an immunocytochemical study [21] that the chromaffin cells of the adrenal medulla may be the richest source of IGF1 in the adrenal gland, suggesting that the majority of the IGF1 immunoreactivity measured in the inner zone may originate from the medulla. Since it has been reported that adrenocortical cells synthesize and release a peptide which is indistinguishable from authentic IGF1 [31], adrenocortical tissue may also contribute to the IGF1 immunoreactivity in the inner zone extracts. The distribution of IGF2 in the adrenal gland has yet to be determined by immunocytochemistry.

Several studies have reported a decline in detectable IGF2 mRNA in most tissues except brain and skeletal muscle during post-natal development [22–24], implicating IGF2 as a foetal growth factor. The adrenal gland is no exception and the high concentration of IGF2 mRNA in the foetal adrenal gland [23] declines dramatically so that barely detectable levels of IGF2 mRNA have been reported in the adult adrenal gland in relation to an abundance of IGF1 mRNA [20]. Whilst this data is not in complete agreement with the relative concentrations of adrenal IGF1 and 2 reported here, there are several reasons why tissue mRNA levels may not correlate with peptide concentration, and caution must be exercised before extrapolating mRNA tissue concentrations with levels of peptide expression.

The control of compensatory adrenal growth by a reflex arc which is stimulated by removal or pinching of one adrenal gland is well established [32]. A previous study in our laboratory has shown the requirement for a small N-terminal N-POMC peptide in the control of compensatory adrenal growth, the peptide being generated by a neurally mediated cleavage of the mitogenic precursor, N-POMC (1–76), at the adrenal gland [5]. The compensatory growth seen in the remaining adrenal gland in the present study was accompanied by a rapid and highly significant increase in both IGF1 and 2 at 24 and 68 h following left unilateral adrenalectomy. The time course of this increase

in peptide levels paralleled the rapid rise in weight, DNA and RNA that typifies compensatory growth of the gland [5].

Like compensatory adrenal growth, regeneration of the adrenal cortex following bilateral adrenal enucleation is controlled by an N-terminal N-POMC peptide [6, 11]. Whilst a 2-fold increase in the content of IGF1 and 2 occurred at 21 days following enucleation, this increase was significant only for IGF2 in the left adrenal gland. Further experimentation with larger treatment groups is clearly necessary before the involvement of IGF1 and 2 in adrenal regeneration can be assessed. The reasons for the discrepancy in IGF content of left and right regenerating adrenal glands and the delayed increase in the IGF content of the right adrenal gland are not understood. It is interesting to note however that the rise in IGF1 and 2 in the left adrenal gland parallels the time course of the increase in N-POMC and ACTH-immunoreactive peptides in the anterior pituitary glands of rats after adrenal enucleation [6].

In summary, the results have shown that both compensatory adrenal growth and adrenal regeneration are accompanied by an increase in adrenal tissue concentrations of IGF1 and 2. Since the plasma concentrations of IGF1 and 2 during compensatory adrenal growth were unchanged compared to control plasma and indeed were lower than the control levels during adrenal regeneration, it is unlikely that the increased tissue levels were due to contamination of adrenal extracts with plasma IGF. This would suggest that the raised IGF tissue concentrations are a result of increased IGF synthesis within the adrenal gland. The cell types responsible for the increase in tissue IGF remain to be established by immunocytochemistry; whilst the peptides may originate from chromaffin cells of the adrenal medulla in compensatory adrenal growth, cells within the regenerating adrenal cortex must be the source of IGF in this growth model. Further experimentation is required, using specific N-POMC peptide antibodies to neutralize the growth-promoting effect of the mitogenic peptide, to ascertain whether the rise in adrenal IGF concentrations during compensatory growth and regeneration is stimulated by N-POMC. Moreover in view of the reported presence of growth hormone receptors on adrenocortical cells [31] it is necessary to establish whether the stimulation of adrenal IGF1 is growth hormone dependent. Indeed, previous experiments indicate that a pituitary factor in addition to N-terminal N-POMC, is necessary for complete restoration of the regeneration process in hypophysectomized rats [11]. The possible involvement of IGF1 in the growth and regeneration of the adrenal gland is not without precedent; raised levels of IGF1 and/or IGF1 mRNA have been reported in compensatory renal growth [33, 34], angiogenesis [35] and regeneration of sciatic nerve [36] and muscle [37]. However, in the light of substantial data demonstrating an important role for IGF1 in the induction and maintenance of steroidogenic function of adrenocortical cells [15, 38] it is possible that the IGFs may also play a role in stimulating adrenal steroidogenesis during compensatory growth and regeneration and in regulating the differentiation of the regenerating adrenal cortex after enucleation.

Acknowledgements—This work was funded by the Wellcome Trust, U.K. and Maff Tech, New Zealand. We are grateful to Mr B. Edgeley for excellent technical expertise.

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