

## IGF-I enhances cortisol secretion from guinea-pig adrenal gland: *In vivo* and *in vitro* study

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**Abstract.** Insulin-like growth factor (IGF)-I is a ubiquitously synthesized peptide that, along with IGF-II, acts via the IGF-R type I receptor. IGF-I and its receptor are expressed in the adrenal gland of humans and bovines, the secretion of which they seem to stimulate. As in humans and cows, the main glucocorticoid hormone secreted by guinea-pig adrenals is cortisol, and hence we have studied the adrenocortical effects of IGF-I in this species. *In vivo* experiments showed that prolonged IGF-I administration raised the plasma concentration of cortisol in both normal and dexamethasone/captopril-treated guinea pigs, thereby ruling out the possibility that IGF-I may act by activating the hypothalamic-pituitary-adrenal axis and the renin-angiotensin system. *In vitro* experiments demonstrated that IGF-I enhanced basal, but not maximally agonist [ACTH and angiotensin-II (Ang-II)]-stimulated, cortisol secretion from freshly dispersed guinea-pig inner adrenocortical cells. The IGF-I immuno-neutralization suppressed the IGF-I secretagogue effect, without altering the cortisol response to both ACTH and Ang-II. IGF-I raised cyclic-AMP and inositol triphosphate release from dispersed guinea-pig cells, and the effect was reversed by the adenylate cyclase inhibitor SQ-22536 and the phospholipase-C (PLC) inhibitor U-73122. SQ-22536, U-73122, the protein kinase (PK) A inhibitor H-89 and the PKC inhibitor calphostin-C decreased by approximately 50% the cortisol response of dispersed cells to IGF-I, and the combined exposure to SQ-22536 and U-73122 abolished it. We conclude that IGF-I stimulates glucocorticoid secretion from guinea-pig adrenocortical cells, acting via selective receptors coupled to both

the adenylate cyclase/PKA- and PLC/PKC-dependent signaling cascades.

### Introduction

Insulin-like growth factor (IGF)-I, also named somatomedin-C, once was thought to be exclusively produced by liver under the effect of the hypophyseal growth hormone, of which it mediates the growth promoting action (reviewed in refs. 1,2). Subsequent studies clearly demonstrated the ubiquitous synthesis by stromal cells of IGF-I and its possible nature as autocrine-paracrine mediator. IGF-I and the companion IGF-II act via a common receptor, named IGF-R type I, which, like its ligands, is widely distributed in tissues and organs (2,3).

Many lines of evidence indicate that IGF-I and IGF-R type I are expressed in the mammalian adrenals (4-8), where the main adrenal agonists ACTH and angiotensin-II (Ang-II) up-regulate their expression and enhance IGF-I secretion (9-13). Moreover, the interrelationships between adrenal agonists and IGF-I are reciprocal, inasmuch as the latter has been found to up-regulate the expression of ACTH and Ang-II receptors in adrenals (14-17), making it likely that IGF-I may promote adrenal steroid secretion.

In keeping with this contention, several investigations clearly demonstrated that IGF-I raises basal and agonist-stimulated glucocorticoid (cortisol) and androgen secretion from cultured human, bovine and sheep adrenocortical cells, an effect coupled with the up-regulation of expression of some enzymes of steroid synthesis, including 17 $\alpha$ -hydroxylase, 3 $\beta$ -hydroxysteroid dehydrogenase and C21-hydroxylase, but not cholesterol side-chain cleaving enzyme and 11 $\beta$ -hydroxylase (14-25). However, Naseeruddin and Hornsby (26) reported that IGF-I enhances 11 $\beta$ -hydroxylase mRNA, without altering 17 $\alpha$ -hydroxylase expression in bovine adrenocortical cells, and Fujii *et al* (27) described a clear-cut inhibitory effect of IGF-I on glucocorticoid and cyclic-AMP response of dispersed rat adrenocortical cells to ACTH.

Therefore, it seemed worthwhile to investigate the *in vivo* and *in vitro* effects of IGF-I on glucocorticoid secretion of the guinea-pig adrenals. Guinea pig was chosen because its main glucocorticoid hormone is cortisol, as in humans, cows and sheep.

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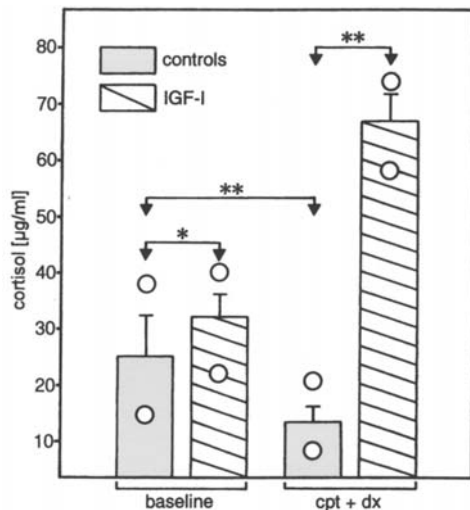


Figure 1. Effect of the prolonged administration of IGF-I on the blood concentration of cortisol in normal and dexamethasone (dx)/captopril (cpt)-treated guinea pigs. Bars are means  $\pm$  SD (n=12). Open circles indicate the range. \*P<0.05 and \*\*P<0.01.

## Materials and methods

**Animals and reagents.** Adult male guinea pigs, either bred in our laboratory facilities (*in vivo* experiments) or purchased from Charles-River (Como, Italy) (*in vitro* experiments), were kept under a 12/12-h light/dark cycle (illumination onset at 8:00 a.m.) at 23°C, and maintained on a standard diet and tap water *ad libitum*. The study protocol was approved by the local Ethics Committees for Animal Studies. The angiotensin-converting enzyme inhibitor captopril (Aceten) was obtained from Workhardt India (New Delhi, India) and dexamethasone (Dexona) from Cadilla India (New Delhi, India). The signaling cascade antagonists SQ-22536, U-73122, H-89 and calphostin-C (28 and refs. therein) were purchased from Biomol Research Laboratories (Milan, Italy). IGF-I, anti-IGF-I antibody (ab), ACTH, angiotensin-II (Ang-II), bovine serum albumin (BSA), phosphate-buffered saline (BSA), 3'-isobutyl-1-methylxanthine (IBMX), and all other chemicals and laboratory reagents were provided by Sigma-Aldrich Corporation (St. Louis, MO).

**In vivo experiments.** Animals were divided into two groups (n=24). One group was subcutaneously injected for 14 days with dexamethasone (2.5 mg/kg) and captopril (2.5 mg/kg). The other group was given daily injections of 0.9% NaCl. On the 6th day, half the animals in each group received IGF-I (1 mg/kg). At the end of the treatment, blood samples were collected from the retro-orbital vein (29), and stored at -20°C until cortisol assay.

**In vitro experiments.** Dispersed guinea-pig zona fasciculata-reticularis (inner) adrenocortical cells were obtained as previously described (29). Cells obtained from 6 guinea pigs were pooled to obtain a single cell suspension, and 4 or 6 cell suspensions for each incubation experiment were employed. Aliquots of each cell suspension ( $10^4$  cells in Krebs-Ringer bicarbonate buffer with 0.3% glucose and 0.2% BSA) were incubated as follows: i) IGF-I (from  $10^{-12}$  to  $10^{-6}$  M); ii) ACTH

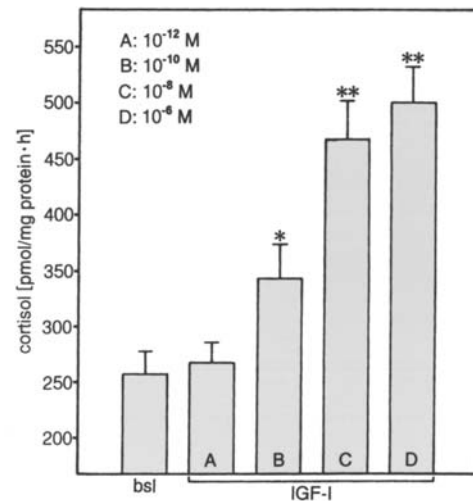


Figure 2. Effect of IGF-I on cortisol secretion from dispersed guinea-pig inner adrenocortical cells. Bars are means  $\pm$  SEM (n=4). \*P<0.05 and \*\*P<0.01 from baseline (bsl) value.

( $10^{-9}$  M) or Ang-II ( $10^{-8}$  M) alone or in the presence of IGF-I ( $10^{-8}$  M); iii) anti-IGF-I ab (100 ng/ml) alone or in the presence of IGF-I ( $10^{-8}$  M), ACTH ( $10^{-9}$  M) and Ang-II ( $10^{-8}$  M); iv) SQ-22536 ( $10^{-8}$  M) alone and in the presence of IGF-I ( $10^{-8}$  M) or ACTH ( $10^{-9}$  M) [cyclic-AMP (cAMP) assay]; v) U-73122 ( $10^{-5}$  M) alone and in the presence of IGF-I ( $10^{-8}$  M) or Ang-II ( $10^{-9}$  M) [inositol triphosphate (IP<sub>3</sub>) assay]; vi) SQ-22536 ( $10^{-4}$  M), U-73122 ( $10^{-5}$  M), H-89 ( $10^{-5}$  M) or calphostin-C ( $10^{-5}$  M) alone and in the presence of IGF-I ( $10^{-8}$  M); and vii) SQ-22536 ( $10^{-4}$  M) plus U-73122 ( $10^{-5}$  M) alone or in the presence of IGF-I ( $10^{-8}$  M). The incubation was carried out in a shaking bath at 37°C for 60 min (cortisol secretion) or 10 min (cAMP and IP<sub>3</sub> production), in an atmosphere of 95% air-5% CO<sub>2</sub>. Supernatants were stored at -80°C until assay, and protein concentration of dispersed cells was measured by the BCA protein assay kit (Sigma-Aldrich Corporation).

**Cortisol assay.** Cortisol blood concentration was measured by enzyme immuno-assay (EIA), as previously detailed (30). Cortisol was extracted from supernatants and purified by high pressure liquid chromatography (HPLC) (31-33), and its concentration was estimated by radio-immunoassay (RIA), using a commercial kit purchased from IRE-Sorin (Vercelli, Italy). Sensitivity, 90 pmol/l; intra- and inter-assay CVs, 6% and 8%, respectively.

**cAMP and IP<sub>3</sub> assays.** In the case of cAMP assay, the phosphodiesterase inhibitor IBMX ( $10^{-4}$  M) was added to prevent cAMP metabolism (28). cAMP and IP<sub>3</sub> were extracted from the incubation media (34), and their concentrations were measured by RIA, using the following commercial kits purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). cAMP Biotrak TRK 432 kit: sensitivity, 1 pmol/l; intra- and inter-assay CVs 5% and 7%, respectively. IP<sub>3</sub> Biotrak TRK 1000 kit: sensitivity, 2 pmol/l; intra- and inter-assay CVs, 6% and 8%, respectively.

**Statistics.** Data were expressed as means  $\pm$  SD or SEM, and their statistical comparison was performed by the paired sample

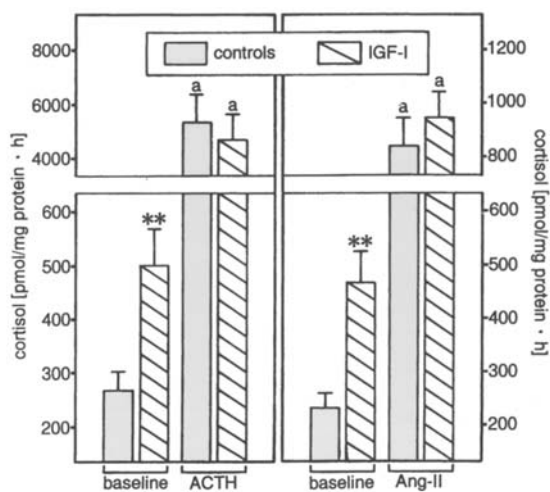


Figure 3. Effects of IGF-I ( $10^{-8}$  M) on basal and agonist-stimulated cortisol secretion from dispersed guinea-pig inner adrenocortical cells. Bars are means  $\pm$  SEM (n=6). \*\*P<0.01 from the respective control value; <sup>a</sup>P<0.01 from the respective baseline value.

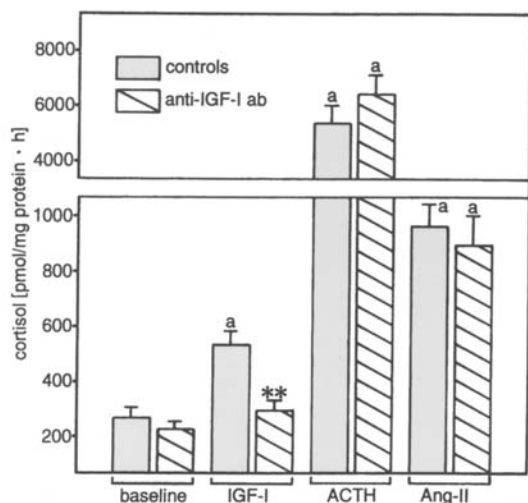


Figure 4. Effects of anti-IGF-I ab (100 ng/ml) on basal, IGF-I ( $10^{-8}$  M)-, ACTH ( $10^{-9}$  M)- and Ang-II ( $10^{-8}$  M)-stimulated cortisol secretion from dispersed guinea-pig inner adrenocortical cells. Bars are means  $\pm$  SEM (n=6). \*\*P<0.01 from the respective control value; <sup>a</sup>P<0.01 from the respective baseline value.

t-test (cortisol blood concentration) or by ANOVA, followed by Duncan's multiple range test.

## Results

The prolonged dexamethasone/captopril administration decreased by 47% the blood concentration of cortisol in guinea pigs. IGF-I treatment for 6 days raised the plasma level of cortisol by 26% in normal guinea pigs and by approximately 5-fold in dexamethasone/captopril-administered animals. Noteworthy, the cortisol response of the latter group of guinea pigs was approximately two-fold higher than that of normal animals (32 versus 68  $\mu$ g/ml) (Fig. 1).

IGF-I concentration-dependently enhanced cortisol secretion from dispersed guinea-pig inner adrenocortical cells, minimal and maximal effective concentrations being

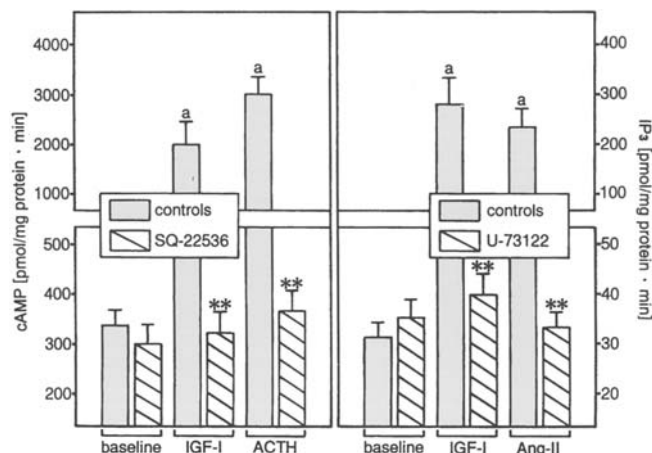


Figure 5. Effects of IGF-I ( $10^{-8}$  M) on cAMP (left panel) and IP<sub>3</sub> release (right panel) from dispersed guinea-pig inner adrenocortical cells. cAMP response to IGF-I and ACTH is suppressed by SQ-22536 ( $10^{-4}$  M), and IP<sub>3</sub> response to IGF-I and Ang-II by U-73122 ( $10^{-5}$  M). Bars are means  $\pm$  SEM (n=4). \*\*P<0.01 from the respective control value; <sup>a</sup>P<0.01 from the respective baseline value.

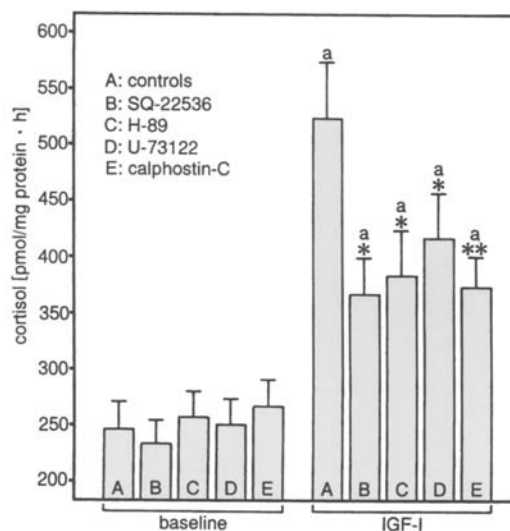


Figure 6. Effects of the signaling cascade inhibitors SQ-22536 ( $10^{-4}$  M), H-89 ( $10^{-5}$  M), U-73122 ( $10^{-5}$  M) and calphostin-C ( $10^{-5}$  M) on basal and IGF-I ( $10^{-8}$  M)-stimulated cortisol secretion from dispersed guinea-pig inner adrenocortical cells. Bars are means  $\pm$  SEM (n=6). <sup>a</sup>P<0.05 from the respective control value; \*\*P<0.01 from the respective baseline value.

$10^{-10}$  and  $10^{-8}/10^{-6}$  M, respectively (Fig. 2). IGF-I ( $10^{-8}$  M) did not affect the cortisol response of dispersed cells to either ACTH or Ang-II (Fig. 3). Anti-IGF-I ab suppressed the cortisol response to IGF-I ( $10^{-8}$  M), without altering either basal or ACTH- and Ang-II-stimulated cortisol secretion (Fig. 4).

Dispersed inner adrenocortical cells displayed marked cAMP and IP<sub>3</sub> responses to ACTH and Ang-II, respectively. These responses were abolished by the adenylate cyclase inhibitor SQ-22536 ( $10^{-4}$  M) and the phospholipase-C (PLC) inhibitor U-73122 ( $10^{-5}$  M), respectively. IGF-I ( $10^{-8}$  M) elicited a significant rise in cAMP and IP<sub>3</sub> release from dispersed cells, and again the effects were annulled by the two inhibitors (Fig. 5).

The cortisol response of dispersed inner adrenocortical cells to IGF-I ( $10^{-8}$  M) was significantly lowered (but not

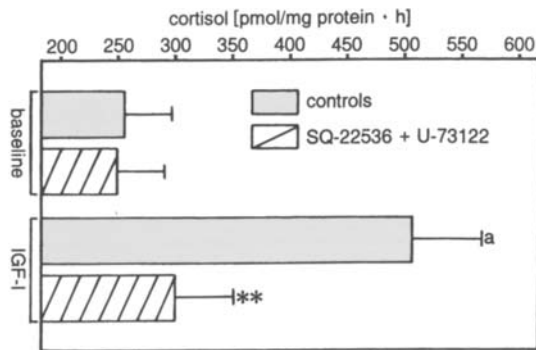


Figure 7. Effects of SQ-22536 ( $10^{-4}$  M) plus U-73122 ( $10^{-5}$  M) on basal and IGF-I ( $10^{-8}$  M)-stimulated cortisol secretion from dispersed guinea-pig inner adrenocortical cells. Bars are means  $\pm$  SEM (n=6). \*\*P<0.01 from the respective control value; \*P<0.01 from the respective baseline value.

abrogated) by SQ-22536 ( $10^{-4}$  M), the protein kinase (PK) A inhibitor H-89 ( $10^{-5}$  M), U-73122 ( $10^{-5}$  M) and the PKC inhibitor calphostin-C ( $10^{-5}$  M) (Fig. 6). The combined exposure to SQ-22536 and U-73122 completely suppressed the cortisol response to IGF-I (Fig. 7). Basal cortisol secretion was unaffected by the inhibitors (Figs. 6 and 7).

## Discussion

Our present *in vivo* findings clearly show that IGF-I is able to raise the blood level of cortisol not only in intact guinea pigs, but also in animals where the hypothalamic-pituitary-adrenal axis and renin-angiotensin system had been pharmacologically blocked by dexamethasone and captopril. These observations make it unlikely that the reciprocal interrelationships occurring between IGF-I and ACTH or Ang-II (see Introduction) may underlie the *in vivo* adrenal secretagogue action of IGF-I, and strongly suggest that this peptide acts directly on guinea-pig adrenocortical cells.

According to this contention, our *in vitro* experiments provide evidence that IGF-I specifically stimulates secretion of dispersed guinea-pig inner adrenocortical cells, without interfering with the ACTH and Ang-II receptors. In fact, IGF-I raises only basal secretion of cortisol, but not that enhanced by ACTH or Ang-II. Moreover, the immuno-neutralization of IGF-I with a selective ab annuls the secretagogue action of IGF-I, but does not alter the secretory response of dispersed cells to ACTH or Ang-II.

The lack of effect of IGF-I on agonist-stimulated cortisol secretion from guinea-pig adrenocortical cells is at variance with that occurring in human, bovine and sheep adrenocortical cells (14-25). Apart from inter-species differences, it is likely that this discrepancy may ensue from the different experimental approaches. In fact, previous findings were obtained in cultured adrenocortical cells exposed for 24-72 h to IGF-I, while our data enlighten the acute (60 min) effect of IGF-I. Evidence has been provided that the prolonged exposure to IGF-I up-regulates the expression of ACTH and Ang-II receptors in adrenocortical cells (14-17), thereby explaining their increased secretory response to the two agonists.

Our observations may suggest that the acute secretagogue action of IGF-I is mediated by receptors (IGF-R type I ?) that share with ACTH and Ang-II a common signaling mechanism.

This could obviously prevent any additivity between the secretory responses elicited by maximal effective concentrations of IGF-I ( $10^{-8}$  M) and ACTH ( $10^{-9}$  M) or Ang-II ( $10^{-8}$  M). Compelling evidence indicates that the main signaling mechanisms mediating the secretagogue action of ACTH and Ang-II on adrenocortical cells involve the activation of adenylate cyclase and PLC-dependent cascades (35-37 and refs. therein), and our present findings show that the same occurs for the acute secretagogue action of IGF-I.

The following pieces of evidence support this view: i) IGF-I enhances cAMP and IP<sub>3</sub> production from dispersed guinea-pig inner adrenocortical cells; ii) the adenylate cyclase inhibitor SQ-22536 and the PLC inhibitor U-73122, at a concentration able to abrogate cAMP response to ACTH and IP<sub>3</sub> response to Ang-II, respectively, suppress cAMP and IP<sub>3</sub> responses of guinea-pig adrenocortical cells to IGF-I; iii) SQ-22536 and U-73122, as well as the PKA and PKC inhibitors H-89 and calphostin-C, at concentrations that were previously found to block the PK activation-evoked adrenocortical-cell responses (32,34,38), cause an approximate 50% inhibition of the IGF-I-induced cortisol secretion from guinea-pig adrenocortical cells; iv) the simultaneous exposure to SQ-22536 and U-73122 abolished cortisol response to IGF-I; and finally v) no signaling cascade inhibitor *per se* affects the basal cortisol secretion over 60 min of static incubation, thereby ruling out the possibility that their effect was due to a nonspecific toxic lesion of the steroidogenic machinery of guinea-pig inner adrenocortical cells.

Taken together, our findings allow us to conclude that IGF-I stimulates glucocorticoid secretion from guinea-pig adrenals, probably acting via specific receptors coupled to both the adenylate cyclase/PKA- and PLC/PKC-dependent cascades. Further studies are needed to ascertain the physiological relevance of our *in vivo* and *in vitro* findings, in light of the hypothesis that locally synthesized IGF-I may mediate in a paracrine manner some of the adrenal chronic effects of ACTH and Ang-II (2,16,39).

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