Glucocorticoid inhibits expression of V-1, a catecholamine biosynthesis regulatory protein, in cultured adrenal medullary cells

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Abstract V-1 acts as a positive and coordinate regulator of gene expression of catecholamine biosynthetic enzymes in PC12D cells. The present study was conducted to investigate the mechanism controlling expression of V-1 in the adrenal gland. Immunohistochemical analysis demonstrated that noradrenergic chromaffin cells more highly expressed V-1 than adrenergic chromaffin cells preferentially expressing the glucocorticoid receptor in rat adrenal glands. Western blotting showed that in cultured bovine adrenal medullary cells, dexamethasone, a synthetic glucocorticoid, inhibited expression of V-1, and that this inhibition was prevented by RU-486, a glucocorticoid receptor antagonist. These results suggest that adrenal expression of V-1 is differentially controlled by glucocorticoids through the specific receptor, and that thereby V-1 regulates catecholamine biosynthesis in a catecholaminergic phenotype-dependent manner.

Key words: V-1 protein; Catecholamine biosynthesis; Adrenal chromaffin cells; Glucocorticoid

1. Introduction

Catecholamines (dopamine, noradrenaline and adrenaline) play multiple crucial roles as neurotransmitters and hormones in the nervous and endocrine systems, respectively [1]. Catecholamines are synthesized by the cooperative actions of four enzymes for catecholamine biosynthesis: tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), dopamine β-hydroxylase (DBH), and phenylethanolamine *N*-methyltransferase (PNMT) [1]. However, the molecular mechanism which controls the coordinate expression of four catecholamine biosynthetic enzymes in a transmitter phenotype-dependent manner remains to be elucidated.

V-1 is a novel soluble protein consisting of 117 amino acids that contains 2.5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat [2,3]. We have recently shown that overexpression of V-1 elicits the coordinate up-regulation of TH, AADC, and DBH mRNA in the neuronal cell line PC12D, and consequently, dopamine and noradrenaline levels are increased [4,5], suggesting the possible involvement of V-1 in phenotype-dependent transcriptional regulation of the catecholamine biosynthetic enzymes in vivo.

V-1 is expressed in the chromaffin cells of rat adrenal medulla [4], which consists of two major subpopulations: adrenergic and noradrenergic chromaffin cells. We more recently found that expression of V-1 in noradrenergic chromaffin cells was higher than that in adrenergic chromaffin cells of rat adrenal gland by immunohistochemistry. It is well-known that the adrenal medulla PNMT activity requires the high levels of glucocorticoids received from the cortex [6]. Glucocorticoid receptor appears to be to a large extent confined to PNMT-positive adrenergic cells in the adrenal medulla [7]. In the present study, to examine whether glucocorticoid affects expression of V-1 in the adrenal chromaffin cells, we analyzed the effect of glucocorticoid on expression of V-1 in cultured bovine adrenal medullary cells. Other intrinsic factors controlling the adrenal expression of V-1 will be also discussed. Here we describe the first evidence that glucocorticoid acts as a negative regulator of expression of V-1 in the cultured adrenal medullary cells.

2. Materials and methods

2.1. Immunohistochemistry

Adult Wistar rats were deeply anesthetized with pentobarbiturate and fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), and the adrenal glands were removed and immersed in the same solution for an additional 6 h. After dipping overnight with PBS containing 30% sucrose, tissues were quickly frozen in liquid nitrogen, and 20-μm-thick sections were made on a cryostat. Sections were mounted on glass slides and first observed under a fluorescence microscope for detection of catecholamines as previously described [8,9,10]. Following taking microphotographs of the fluorescent cells, immunohistochemistry using anti-V-1 antibody was performed as described previously [4], except that sections were stained by a successive incubation with the primary antibody at a concentration of 0.5 μg/ml and the biotinylated anti-rabbit IgG and streptoavidin-peroxidase (Histofine kit, Nichirei).

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2.2. Primary culture of bovine adrenal medullary cells

Bovine adrenal cells were isolated as previously reported [11,12]. For Western blotting of the time course for expression of V-1 and catecholamine biosynthetic enzymes in the cells in culture, cells were cultured for one week, as previously described [11,12]. For analysis of the effect of glucocorticoids on expression of V-1, cells were cultured in the presence or absence of 1 μM dexamethasone and/or 1 and 10 μM RU-486 for 2 or 3 days.

2.3. Cell extract preparation

Cells were lysed in RIPA buffer (0.15 M NaCl, 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0), including protease inhibitors and phosphatase inhibitors, and centrifuged at 14 000 rpm for 20 min. The resultant supernatants were used as cell extracts for Western blotting. Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad).

2.4. Western blotting

Cell extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (Bio-Rad) or nitrocellulose membranes (Schleicher and Schuell). The extracts were analyzed by Western blotting with anti-V-1 antibody, anti-TH antibody (Eugene Tech International), anti-AADC antibody (Signal Transduction) and anti-DBH antibody (Signal Transduction). The blotted membrane was blocked in 5% or 10% skim milk in TBT buffer (0.15 M NaCl, 0.05% or 0.1% (v/v) Tween 20, 10 mM Tris–HCl pH 7.6) for 1 h at room temperature. The membrane was thereafter incubated successively with primary antibodies in 5% or 10% skim milk/TBT overnight at 4°C and horseradish peroxidase conjugated protein A or anti-sheep IgG for 1 h at room temperature. Following stripping the antibody from the blots, the blots were reprobed with anti-14-3-3β protein antibody (sc-628, Santa Cruz Biotechnology). Immunoreactivity was visualized with enhanced chemiluminescence detection reagents (Pierce) and the immunoreactive signals were quantified using a densitometer (ATTO Densitograph).

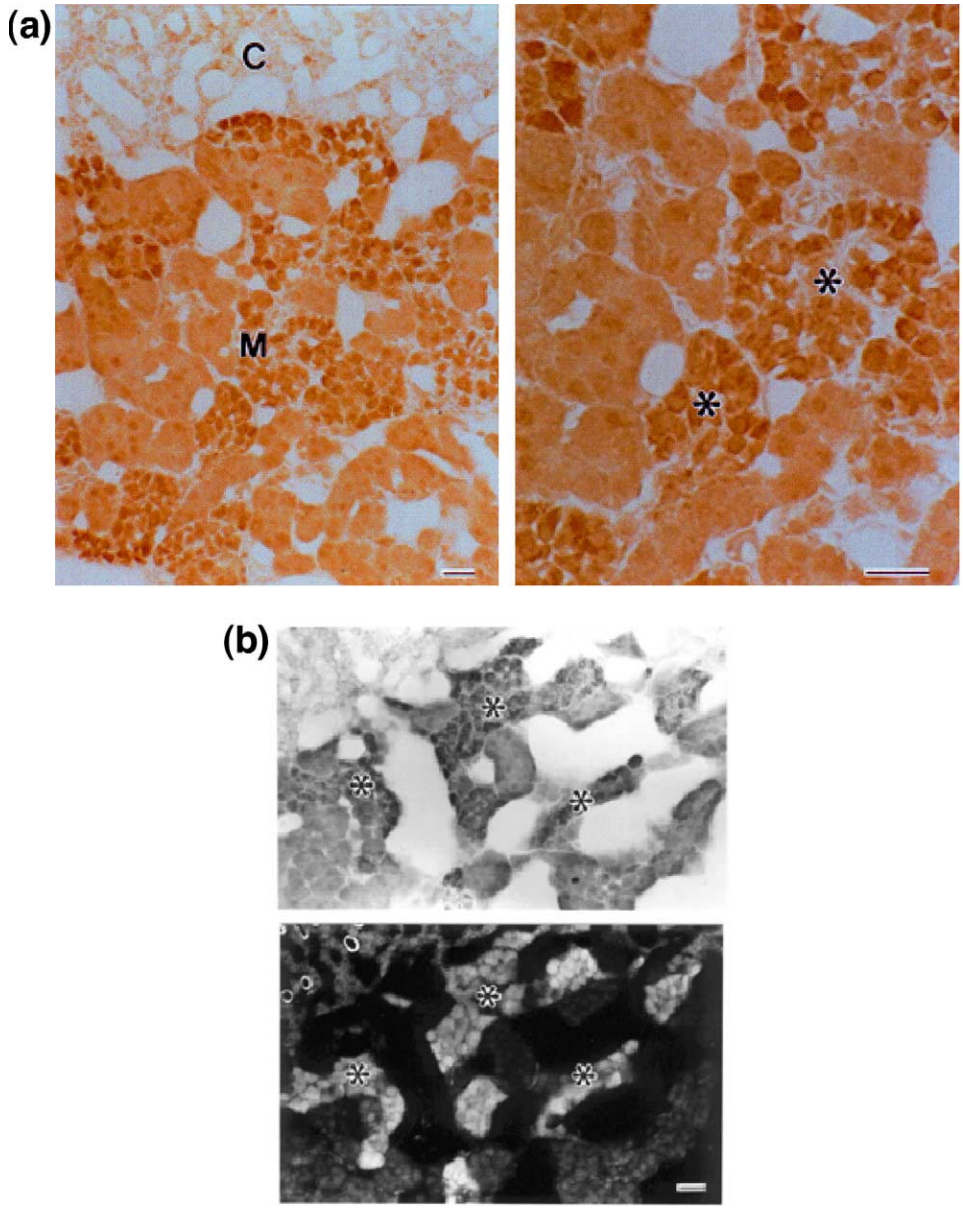

Fig. 1. Differential expression of V-1 in adrenergic and noradrenergic chromaffin cells of rat adrenal medulla revealed by immunohistochemistry. a: Left, the sections of rat adrenal glands stained by anti-V-1 antibody; right, higher magnification of cells in the same section. Small-sized chromaffin cells [23] indicated by asterisks are more intensely immunopositive. C: cortex, M: medulla. b: Chromaffin cells exhibiting more intense immunoreactivity for V-1 (asterisks in upper panel) are identified as fluorescent noradrenergic cells (lower panel) under a fluorescence microscope. Scale bar = 20 μm.

3. Results

3.1. Differential expression of V-1 in adrenergic and noradrenergic chromaffin cells of rat adrenal medulla

We have recently reported the localization of expression of V-1 in rat adrenal medullary chromaffin cells [4]. We analyzed in more detail expression of V-1 in adrenergic and noradrenergic chromaffin cells of the adrenal gland by immunohistochemistry combined with the procedure for detection of catecholamines. As shown in Fig. 1, immunohistochemical analyses revealed that both the adrenergic and noradrenergic chromaffin cells expressed V-1, but fluorescent noradrenergic chromaffin cells expressed V-1 more intensely than the adrenergic cells.

3.2. Down-regulated expression of V-1 followed by a decrease in expression of TH and DBH in bovine adrenal medullary cells in culture

Anti-V-1 antibody utilized for the present study specifically recognized a single 12 kDa protein band in the extract of cultured bovine medullary cells, of which the molecular size corresponded to that of rat V-1 protein as previously reported (data not shown). We thus concluded that this immunoreactive protein with 12 kDa was bovine V-1 protein. To examine the functional role of V-1 in the regulation of expression of catecholamine biosynthetic enzymes, we first analyzed expression of V-1, TH, AADC and DBH proteins in bovine adrenal medullary cells in culture by Western blotting. When the cells were cultured for one week, TH and DBH proteins were markedly decreased between 3 and 5 days in culture, while expression of AADC protein appeared to change little (Fig. 2). On the other hand, expression of V-1 steeply decreased

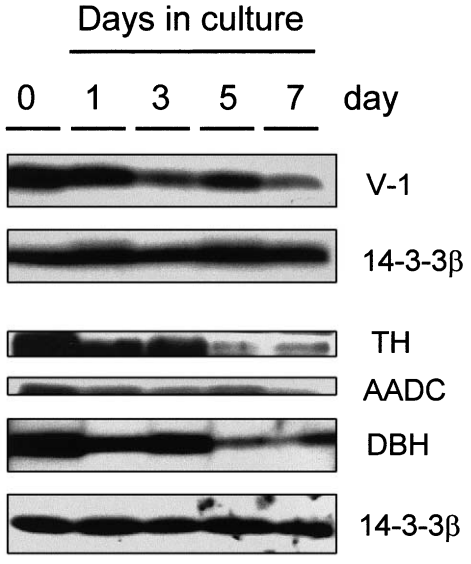

Fig. 2. Changes in expression of V-1 and the catecholamine biosynthetic enzymes in cultured bovine adrenal medullary cells. The isolated adrenal medulla cells were cultured for the indicated times. Proteins of cell extracts were resolved on 18% and 10% SDS–polyacrylamide gels for analyses of expression of V-1, TH, AADC, DBH and 14-3-3β (internal control) proteins by Western blotting. Immunoreactive bands of TH, AADC and DBH proteins expectedly showed molecular weight of 60 kDa, 56 kDa and 75 kDa, respectively. Similar results were obtained in two independent experiments.

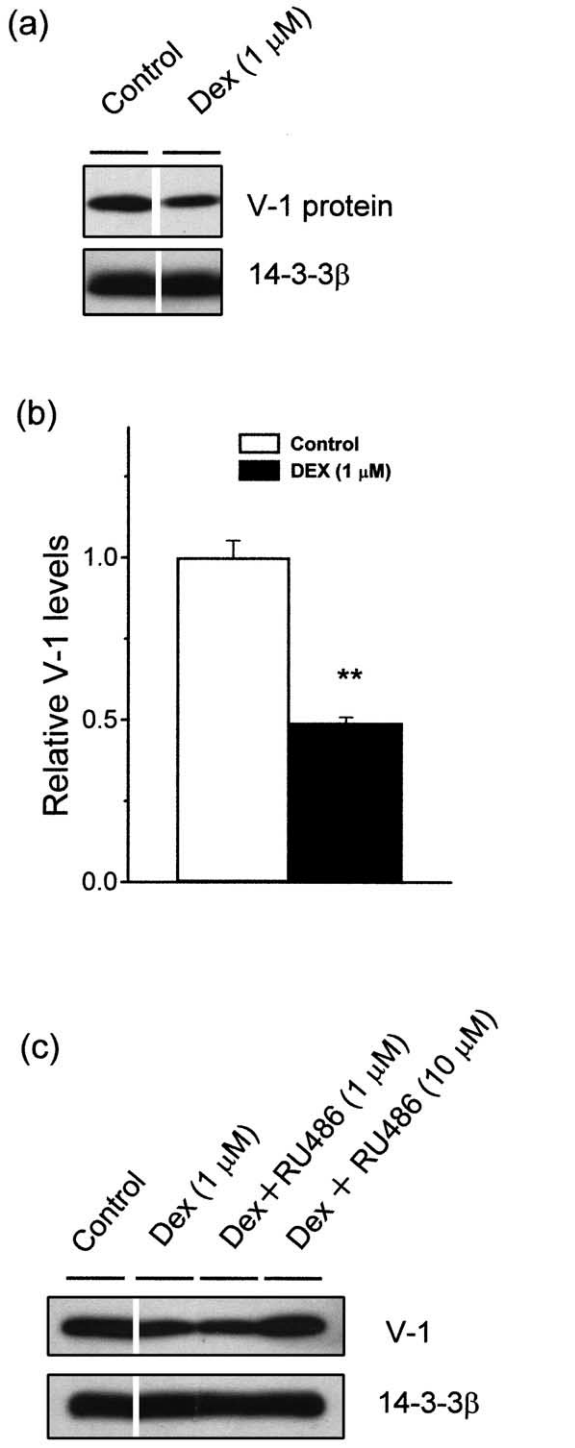

Fig. 3. Effect of dexamethasone (Dex) on expression of V-1 in the cultured cells. a: Cells were untreated (Control) or treated with 1 μM Dex for 2–3 days. Proteins of cell extracts were separated on 18% SDS–polyacrylamide gels for Western blotting of expression of V-1 and 14-3-3β (internal control) proteins. b: The histogram indicates the protein levels of V-1. Values denote the means ± S.E.M. ($n = 3$). Statistical analyses were performed by unpaired t-test. Significant differences from control are indicated at $^{**}P < 0.01$. c: Changes in expression of V-1 in the cells cultured in the absence or presence of Dex and/or with RU-486. The cells were treated with the indicated concentrations of these drugs for 2–3 days. Cell extracts were separated on 18% SDS–polyacrylamide gels for Western blotting. Shown are representative expression patterns ($n = 2$).

between 1 and 3 days in culture, and thereafter the expression level was almost sustained except for a slight increase at 5 days in culture.

3.3. Inhibition of V-1 expression by glucocorticoid

The observations obtained by immunohistochemistry raises the possibility that glucocorticoids negatively regulate expression of V-1. Next we tested whether glucocorticoids inhibit expression of V-1 in cultured bovine adrenal medullary cells. The cultured cells were treated with 1 μM dexamethasone, a synthetic glucocorticoid, and/or 1 μM or 10 μM of RU-486, a glucocorticoid receptor antagonist. Western blotting showed that expression of V-1 in the cells treated with 1 μM dexamethasone decreased to approximately 50% of that of the untreated cells (Fig. 3a,b). This decrease in expression of V-1 by 1 μM dexamethasone was blocked by 10 μM RU-486, while at 1 μM, this inhibitor did not effectively antagonize the inhibitory action of dexamethasone (Fig. 3c).

4. Discussion

This study is the first to demonstrate that glucocorticoid inhibits expression of V-1, an ankyrin repeat protein having a catecholamine biosynthesis-promoting activity, in bovine adrenal medullary cells in culture. Consistent with the data on the inhibitory action of dexamethasone on expression of V-1, the present immunohistochemistry revealed that in rat adrenal gland, V-1 was more intensely expressed in the noradrenergic chromaffin cells than in the adrenergic chromaffin cells, to which glucocorticoid receptor appeared to be to a large extent restricted [7]. Furthermore, we evidently showed that an apparent decrease in expression of V-1 preceded a remarkable reduction in those of TH and DBH proteins in the adrenal medullary cells in culture.

The present immunohistochemical data showed that, in rat adrenal gland, noradrenergic but not adrenergic chromaffin cells preferentially expressed V-1. Expression of GAP-43, a neuron-specific gene product, has been shown to be negatively regulated by glucocorticoids in PC12 cell [13]. This protein has been also reported to be exclusively localized in noradrenergic chromaffin cells in the postnanal rat adrenal gland [14]. Unlike GAP-43, V-1 is localized in both noradrenergic and adrenergic chromaffin cell subpopulations in rat adrenal gland, but the noradrenergic cell subpopulation preferentially expresses V-1. Like rat, mouse exhibited such a preferential expression of V-1 in the adrenal gland (unpublished observations). Therefore it is fairly plausible to consider that V-1 acts in both the catecholaminergic cell subpopulations. Taken together with our previous data obtained from the overexpression experiment of V-1 gene in PC12D cells [4], the findings of the present immunohistochemical study suggest that V-1 controls the regulation of catecholamine biosynthesis in both noradrenergic and adrenergic chromaffin cells of the adrenal gland.

It has previously been shown that bovine adrenal medullary cells show progressive losses of catecholamine content and the biosynthetic enzyme activities in culture [15]. In this study, our results consistently revealed that bovine adrenal medullary cells exhibited a dramatic reduction in expression of TH and DBH proteins between 3 and 5 days in culture after these cells remarkably decreased expression of V-1 between 1 and 3 days in culture. It was also observed that the total catecholamine content of the cells was apparently decreased between 3 and 5 days in culture (unpublished data). From the fact that the time course for the reduction in expression of V-1 was earlier than that of expression of TH and DBH proteins in the cultured cell, it is conceivable that V-1 may act upstream of the regulatory mechanism of expression of these enzymes, and that the down-regulation of their expression may be a consequence of the reduction in expression of V-1. It is therefore possible to interpret that V-1 participates in the positive regulation of the expression of genes encoding TH and DBH to promote catecholamine biosynthesis. In support of this interpretation, we have recently found that stable overexpression of V-1 stimulates expression of mRNA for TH, AADC and DBH in PC12D cells to increase the contents of dopamine and noradrenaline [4]. These results of the present study strengthen the notion that V-1 up-regulates expression of genes encoding catecholamine biosynthetic enzymes, although how the expression of these genes is regulated by V-1 remains to be clarified.

Glucocorticoids are secreted in the adrenal medulla to regulate PNMT mRNA level [16], and expression of PNMT is induced in cultured chromaffin cells treated with glucocorticoids [17]. A quite important finding in this study is that expression of V-1 is markedly decreased in the cultured cells treated with dexamethasone as compared with untreated cells, and that the inhibition of expression of V-1 by dexamethasone is blocked by treatment with a glucocorticoid receptor antagonist, RU-486. The glucocorticoid receptor has been reported to participate in negative regulation of action of transcription factors such as AP-1 and NF-κB [18,19,20]. Although the mechanism by which glucocorticoids inhibit expression of the V-1 gene in the cultured adrenal medullary cells has not been yet elucidated, these findings indicate that expression of V-1 in the cultured cells is inhibited by glucocorticoid via the specific receptor. It is thus suggested that expression of V-1 in the medullary chromaffin cells is under negative control by glucocorticoids originating from the cortex in the adrenal gland.

It is of interest that despite no addition of glucocorticoid to culture medium, expression of V-1 in the adrenal medullary cells was decreased during cell culture for 1 to 3 days (Fig. 2). This result is inconsistent with that glucocorticoids regulate negatively expression of V-1 via the specific receptor (Fig. 3), because adrenal medulla is usually influenced by endogenous glucocorticoids from adrenal cortex in vivo. Therefore, besides a negative regulation of expression of V-1 gene by glucocorticoids, this gene may be positively controlled by other intrinsic factors in adrenal chromaffin cells, i.e. splanchnic nerves and/or peptidergic neurons that release acetylcholine and several neuropeptides such as vasoactive intestinal polypeptide and pituitary adenylate cyclase activating polypeptide [21]. Indeed, these neurons exert a positive effect on cAMP production in the chromaffin cells via activation of nicotinic acetylcholine receptors in vivo [22]. Forskolin, a stimulator of adenylate cyclase responsible for cAMP production, elevated the expression of V-1 in PC12D cells (unpublished data). Therefore, an attractive interpretation of a reduction in expression of V-1 in adrenal medullary cells cultured in the absence of glucocorticoid is that this reduction in expression of V-1 in the cultured cells is due to a lack of a transsynaptic positive regulation of V-1 gene by splanchnic or peptidergic neurons.

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