Video Article

Primary Culture of Rat Adrenocortical Cells and Assays of Steroidogenic Functions

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URL: https://www.jove.com/video/59016
DOI: doi:10.3791/59016

Keywords: adrenal gland, adrenocortical cell, steroidogenesis, endocrine, mineralocorticoid, glucocorticoid

Date Published: 11/30/2018

Citation: Chen, Y.C., Huang, B.M. Primary Culture of Rat Adrenocortical Cells and Assays of Steroidogenic Functions. *J. Vis. Exp.* (), e59016, doi:10.3791/59016 (2018).

Abstract

The hormone which the adrenal cortex secretes is vital for animals against stress and diseases. The method described here is the procedure of primary cultured rat adrenal cells and related functional assays (immunofluorescence staining of lipid droplet surface protein, as well as corticosterone analysis). Unlike an in vivo model, the variation of interexperiments in adrenal monolayer cultures is less and the experimental condition is easy to control. Besides, the source of rats is also more stable than other animals, like bovine ones. There are also several human adrenal cell lines (NCI-H295, NCI-H295R, SW13, etc.) that can be used in adrenal studies. However, the steroid production of these lines will still be influenced by numerous factors, which include serum lot number, passage number, mutant/loss of distinct genes, etc. Except for lacking 17α-hydroxylase, the primary culture of rat adrenocortical cells is a better and more convenient technique for studying adrenal physiology. In summary, primary rat adrenal cultures could be a good in vitro platform for researchers to investigate the mechanisms of the reagent of interest in the adrenal gland system.

Introduction

The endocrine system is responsible for regulating physiological activities and homeostasis[1]. Adrenal glands located at the cranial pole of the kidneys are one of the major endocrine organs which secrete mineralocorticoids, glucocorticoids, and androgen[2,3]. There are two distinct parts of the adrenal gland: the cortex and the medulla. The adrenal cortex consists of three layers: the outer glomerulosa, the intermediate fasciculata, and the inner reticularis[3]. The zona glomerulosa is the original secreting site of aldosterone, a mineralocorticoid, which aids in reabsorbing sodium ion and water in the kidneys[3]. The zona fasciculata primarily produces a basal level of glucocorticoids in normal physiological conditions[3]. Corticosteroids in rodents and cortisol in humans act to assist the body in coping with stress by regulating blood glucose[3]. To some extent, they can inhibit inflammatory responses and regulate the immune system[4,5]. In contrast to other mammals, mice and rats do not have a functional zona reticularis due to the lack of a 17α-hydroxylase expression in the adrenal gland[6,7]. Thus, adrenals from mice and rats are devoid of the secretion of adrenal C-19 steroids (cortisol and adrenal androgens).

Primary cultured adrenocortical cells have been shown to be useful for investigating mechanisms controlling the adrenal physiology[8]. Like other steroidogenic tissues, each adrenal zone synthesizes its steroids from free cholesterol[9]. Upon adrenocorticotropic hormone (ACTH) stimulation, the free cholesterol is released from breakdown lipid droplets and, thus, enhances steroid production in the fasciculata and reticularis[10].

Many groups have attempted to establish stable cell lines from adrenocortical carcinomas. However, several concerns have limited the use of adrenocortical cell lines as in vitro models[8]. The steroid responses of cell lines may differ between passages, the lot number of serum, and the quality of the serum, etc. Moreover, commercial adrenal cell lines (Y1 and SW13) do not secrete corticosterone, making it more difficult to study adrenal steroidogenesis[8]. Using bovine and equine adrenals as ex vivo models may be a good choice, even though tissues from these markets may obscure some unknown risks. Compared to them, managing rat adrenal glands is easier and the source is relatively more stable and pathogen-free. Taken together, the present method described here could be used to investigate the related mechanism of corticosterone synthesis in rat adrenal fasciculata cells.

Protocol

All the procedures including animal subjects have been approved by Institutional Animal Care and Use Committee of Kaohsiung Medical University.

1. Experimental Procedure

1. Sacrifice the adult (8- to 12-week-old) female/male SD rats by CO_2 euthanasia.
2. Apply 70% ethanol and let it be absorbed into the skin, and then wipe the skin with a tissue paper.

3. Put the rat on a clean plastic plate in the supine position.
4. Lift the skin with “external” curved forceps at the midline and perform a blunt dissection by creating a “**Y**” shape (from the level of the thigh to the subcostal angle).
    1. Rinse scissors with 70% ethanol and, then, cut the muscle, also creating a “**Y**” shape.
5. Find the left adrenal gland behind the stomach.
   NOTE: The right adrenal can be found deeply behind the liver.
    1. Use one pair of forceps to lift the stomach or liver and isolate the adrenals with another curved forceps.
6. After putting the adrenal glands into a sterile dish, carefully remove the adrenal capsule with fine forceps.
7. With a delicate pair of scissors, cut the adrenals into small pieces with a little bit of serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium.
8. Prepare three different pore-sized Pasteur pipets and use the first Pasteur pipet to aspirate all the adrenal pieces into a 50 mL conical tube containing medium with collagenase II (~0.5–0.8 mg/mL).
    1. Gently triturate the tissue pieces about 10x. Use the rest of the pipets and repeat the above steps until all the pieces become smaller.
       NOTE: The different pore sizes of the Pasteur pipets can be created by heating them with fire. The first pipet is the original size (about 1 mm), the pore size of the second one is about 0.8 mm, and the third Pasteur pipet is about 0.5 mm. During digestion, the pieces of adrenal will become smaller. Thus, via the mechanical procedure, the adrenals can be digested more completed. Incubate the tube in a water bath (at 37 °C) and shake it every 5 min, 4x in total.
9. After the 20 min incubation, add fresh and cool medium (sixfold volume; for example, 5 mL of tissue + 25 mL of cool DMEM/F-12 medium) containing 5% fetal bovine serum (FBS) and 2.5% horse serum to stop the enzyme effect.
10. Collect the cell pellets by centrifugation at 800 x *g*, 2x for 10 min.
11. Add an appropriate volume of medium to suspend the cells.
    NOTE: Each gland can make about ~3 x 10^5 to ~4 x 10^5 cells.
12. Plate the cells in desired plates or dishes with growth medium (1:1 [v/v] mixture of DMEM and Ham’s F-12 medium, supplemented with 25 mM HEPES and 1% penicillin and streptomycin) overnight (day 0) at 37 °C, in a humidified environment of 95% air and 5% CO_2.
    NOTE: Usually, we plate cells in 24-well plates (four glands/plate) for the corticosterone assay and in 35 mm dishes (one gland/approximately one to two dishes) for western blot analysis.
13. Carefully wash the cells 2x with serum-free warm DMEM/F-12 medium (day 1). Replace the new growth medium.
14. Maintain the cells at 37 °C in a humidified environment of 95% air and 5% CO_2 until day 3.

Representative Results

Using the procedure described here, primary cultured adrenal cells could be distinguished under a phase-contrast microscope (**Figure 1A**). To further confirm that the vesicles within the cytoplasm are lipid droplets, the immunofluorescence staining of adipose differentiation-related protein (ADRP) could be conducted on day-3 cultures (**Figure 1B**). Cells were grown on the glass coverslips. The cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. After washing three times with PBS, the cells were blocked and permeabilized with 0.1% Triton X-100 in 5% nonfat milk. The primary ADRP antibody was diluted (1:50) in 5% nonfat milk and was incubated overnight. After three times of PBS washing, the cells were incubated with goat anti-rabbit Alexa Fluor 488 secondary antibodies (1:100). After repeating the washing steps, the cells were mounted with ProLong Gold Antifade mounting medium and were examined under the fluorescence microscope. Moreover, the hormone-producing ability of the rat adrenal cells could be determined by corticosterone assay. Stimulation of ACTH on rat adrenal cells could serve as a positive control (**Figure 1C**). The collected media were diluted and assayed for corticosterone content, according to the procedure described by Chen et al.[11].

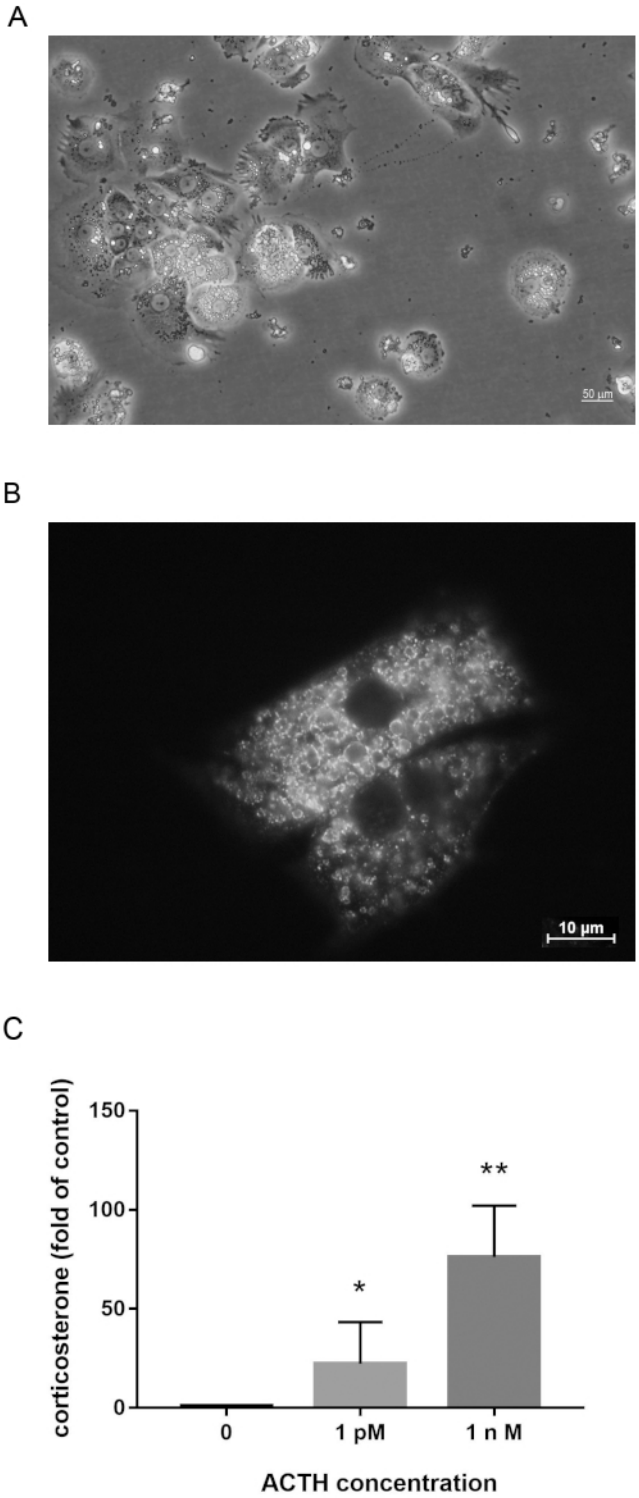

Figure 1: The characterization of adrenal cells and the corticosterone response to ACTH treatment. (A) Phase-contrast picture of day-3 primary cultured rat adrenal cells. (B) ADRP staining is shown. (C) Day-3 cultured rat adrenal cells were treated with ACTH (1 pM and 1 nM) for 6 h. The media were collected and stored in a -80 °C refrigerator until use. Corticosterone levels were measured by enzyme-linked immunosorbent assay (ELISA). Please click here to view a larger version of this figure.

Discussion

Adrenal glands play a key role in environmental adaptation[3]. The hormone by which the adrenal cortex secretes can regulate and adjust physiological functions and homeostasis[3]. The in vivo model reflects the real physiological effects in the body. However, it is still influenced by

many complex factors, causing unstable effects in experiments. In contrast, the *in vitro* cell model has advantages which makes it incomparable to the animal model. First, the environmental condition is easy to control in the in vitro system. Hormones are likely to be affected by any stimulus. In vivo experiments usually result in a nonspecific reaction because animals are prone to distress. Second, the substances are not easy to monitor in the body but are liable in the cultured system. Third, the in vitro system reduces the variances between each independent experiment. Many scientific reports show a culture media of adrenocortical cells containing 10%-20% FBS[12,13,14,15], whereas the protocol presented here uses 5% FBS and 2.5% horse serum to maintain the growth of adrenal cells for up to one week.

Steroidogenesis is started from the cleavage of free cholesterol by cytochrome P450 side-chain cleavage enzymes. As shown in **Figure 1A,B**, adrenal cells exhibit numerous and different sizes of lipid droplets, suggesting it is a good tool for the investigation of steroid biosynthesis and/or lipid biology (fasciculata cells have prominent and larger lipid droplets)[1,3]. The ADRP, also known as perilipin 2, is a lipid droplet surface protein which assists in the storage of neutral lipids within the lipid droplets[16]. Due to adrenocortical cells exhibiting an amount of lipid, the ADRP (or other lipid droplet surface proteins, like perilipin) can be used to examine whether the tested agents affect hormone synthesis[16].

The growth and function of adrenal glands are tightly controlled by hypothalamic corticotropic-releasing hormone and pituitary ACTH, neuropeptides, neurotransmitters, and growth factors, etc.[17]. **Figure 1C** reveals that the corticosterone production was significantly induced by ACTH treatment in a concentration-dependent fashion. Moreover, ACTH at 1 pM and 1 nM stimulated the corticosterone production about 30- and 70-fold, respectively. It has been demonstrated that day-3 cultured primary adrenal cells exhibit a better response to ACTH stimulation[15]. This is also the reason why day-3 culture was used in the present study. In addition, the measurement of corticosterone by ELISA kit is also a simple and easy tool for research. Therefore, the primary cultured rat adrenal cells not only provide the platform for studying adrenal steroidogenesis but may also apply to the investigation of inflammatory-related diseases.

The critical steps in the present protocol are the digestion of the adrenals and control of contamination. Therefore, control of the digestion time, physical suction force, and observation of the tissue digestion level are very important for cell survival. Furthermore, the method of cutting the skin and isolating the adrenals is also significant. Always immerse all the instruments in 70% ethanol to prevent any contamination by animal fur.In order to obtain more cells, the suction frequency should be controlled less than ten times during each suction. Also, if the condition of the tissues is not good for further steps, the incubation in collagenase-containing medium should not be longer than 30 min.

Because the adrenal glands of rats are small, the glomerulosa layer might be lost during the peeling of the adrenal capsule. It has been demonstrated that decapsulation resulted in the removal of the connective tissue capsule and the entire zona glomerulosa[18]. The adrenal glands of male rats are smaller than those of female rats. Additionally, the lipid droplets appear to be larger in 11-week-old female or male mice[6]. Regarding the available cells, female rats may be a good choice for experiments.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by a grant from the National Science Council Taiwan (NSC102-2320-B-037-008-MY3) and a National Cheng-Kung University Hospital Research Grant (NCKUH-10102045).

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