

# Stimulatory Effect of Food Restriction on the Steroidogenesis of Aldosterone in Ovariectomized Rats

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## Abstract

Food or calorie restriction (FR or CR) induces several physiological changes including weight loss, metabolic adaptations, mineral and hormonal changes. However, the effects of FR on aldosterone steroidogenesis in zona glomerulosa (ZG) cells have not been elucidated. Therefore, the present study was designed to investigate the effects of FR on aldosterone secretion and the involved mechanisms in ovariectomized (Ovx) rats. Ovx rats were divided into *ad libitum* fed (control) and FR groups. The FR rats exhibited decreased body weight, water intake, urine flow, sodium excretion and increased plasma aldosterone in comparison with control rats. FR elevated the basal and angiotensin II-stimulated aldosterone secretion from ZG cells. The conversions of 25-hydroxy-cholesterol to pregnenolone or corticosterone to aldosterone in ZG cells of FR group were greater than that in control group. FR group had a higher protein expression of steroidogenic acute regulatory (StAR) protein in ZG cells. However, there was no different protein expression of cytochrome P450 side-chain cleavage enzyme (P450scc) in ZG cells between control and FR groups. In summary, the increased activities of P450scc and aldosterone synthase as well as the protein expression of StAR protein in ZG cells are involved in the effects of FR on aldosterone steroidogenesis in Ovx rats. We also suggest that the increase of aldosterone might be associated with anti-diuresis and anti-natriuresis in FR group. These results are helpful for understanding the role of aldosterone in physiological adaptation and renal sodium conservation during FR.

**Key Words:** aldosterone, food restriction, P450scc, StAR protein, zona glomerulosa cells

## Introduction

Food or calorie restriction (FR or CR), defined as reduced food or energy intake without malnutrition, has been shown to extend lifespan by slowing physiological decline and delaying age-associated

diseases, such as obesity, cardiovascular disease, type II diabetes, and cancer, in different animal species (9, 27). However, most of these studies were conducted to investigate the long-term effects of the FR. The studies of long-term FR in rodent are usually associated with lifespan research and the period of

FR lasting for a year or several years. Short-term and/or strict FR, ranges from days to months, is carried out in studies of body weight management or combination with different experimental goals or clinical conditions. Short-term FR or fasting has been shown to induce several beneficial effects on different clinical condition, including surgical stress, inflammation, chemotherapy and insulin resistance. For example, 3 days of 100% FR or 2-4 weeks of 30% FR resulted in similar protection against renal ischemia reperfusion injury in mice (28). This protection correlated with improved insulin sensitivity, reduced expression of markers of inflammation and insulin/insulin-like growth factor-1 signaling, and increased expression of markers of antioxidant defense (28). In a study of stroke-prone spontaneously hypertensive rat (SHRSP), 2 weeks of 50% FR significantly delayed the onset of stroke and extended survival time (8). The mechanisms of the FR-induced delay of stroke onset might be mediated by suppression of inflammation. FR rats displayed decreased plasma levels of interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and monocyte chemoattractant protein-1 (MCP-1) and their mRNA expressions in adipose tissue. Four weeks of up to 50% FR also attenuated lipopolysaccharide (LPS)-induced sickness behavior (fever, loss of appetite and body weight) in a dose-dependent manner in part by attenuating proinflammatory hypothalamic mRNA levels of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) and increasing anti-inflammatory gene expression of suppressor of cytokine signaling 3 (SOCS3) and interleukin-10 (IL-10) (24). Case studies of cancer treatment showed that fasting in combination with chemotherapy is feasible, safe, and has the potential to ameliorate side effects (fatigue, weakness, and gastrointestinal side effects) caused by chemotherapies, but did not interfere with chemotherapeutic potency (32).

The effects of fasting or FR on the body also include weight loss, metabolic adaptations, mineral changes, hormonal changes and psychological effects. It is well known that aldosterone plays an important role in regulating sodium and water homeostasis. Theoretically, fasting or FR may lead to reduce sodium intake, therefore alter aldosterone secretion, and then adjust the balance of sodium and water. But the effects of fasting or FR on sodium excretion and renin-angiotensin-aldosterone system (RAAS) were conflicting (5, 10, 30). Rapoport *et al.* (30) found that urinary excretion of sodium decreased and the aldosterone secretion increased after 7 days of starvation in patients. Boulter *et al.* (5) reported a natriuresis during the first week of fasting and followed by an anti-natriuresis (sodium conservation phase)

if fasting was prolonged in the study of obese and healthy men. During natriuretic phase of fasting the aldosterone secretion elevated while plasma renin activity (PRA) declined. The natriuresis induced by fasting may be due to renal tubular insensitivity to aldosterone whereas this response was restored during the later phase of fasting. Dessi-Fulgheri *et al.* (10) reported that no significant difference of PRA, plasma aldosterone and urinary sodium excretion were observed after 4 days of low calorie diet in obese hypertensive patients. The reasons for these controversial results are unknown. However, there were several obvious differences in conditions among these studies, including sodium intake, fasting duration and FR levels, gender, body weight, and health status.

Therefore, the present study was designed to investigate the effects of short-term and strict FR on [1] urinary sodium excretion, [2] plasma aldosterone level, and [3] aldosterone steroidogenesis in zona glomerulosa (ZG) cells of control and FR rats. Since the production of aldosterone is stimulated by estradiol (18), the ovariectomized (Ovx) rats were applied in the present study.

## Materials and Methods

### Animals

Sprague-Dawley female rats of 3 months old were housed in a temperature-controlled room (22  $\pm$  1°C) with 14 h of artificial illumination daily (0600-2000) and given water *ad libitum*.

In order to avoid the influence of estrogens, female rats were Ovx for four days. The Ovx rats were placed into metabolic cage for 6 days and allowed *ad libitum* access to food. The average daily food intake is 16.8 g/day. Then the Ovx rats were divided into three groups: control (standard rat chow diet *ad libitum*), FR 50% and FR 75% groups (50% and 75% reduction of the food intake *ad libitum*) for 6 days. The changes of body weight before and after FR, and every 24 h of water intake and urine volume were measured. To investigate whether FR will affect urinary sodium excretion, the urine of day 6 was collected and the concentration of sodium in urine was detected.

### Effect of FR on Plasma Aldosterone and Corticosterone

After 6 days, the Ovx rats of control, FR 50% and FR 75% were decapitated. The adrenal glands from control and FR 75% groups were rapidly removed and stored in 0.9% (w/v) NaCl in an ice bath. The trunk blood was obtained and the plasma was separated by centrifugation at 10,000  $\times$  g for

30 min. The plasma sodium, ghrelin and glucose were determined. A flame photometer (EFOX 5053; Eppendorf, Hamburg, Germany) was used to determine the levels of plasma and urinary sodium. Plasma samples were mixed with diethyl ether (10-fold volume), shaken for 30 min, centrifuged at  $1,000 \times g$  for 5 min, and then quick-frozen in a mixture of acetone and dry ice. The organic phase was collected, dried and reconstituted by a 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) in borate buffer (pH 7.8) before measurement of aldosterone or corticosterone by radioimmunoassay (RIA).

*Effect of FR on Basal and Ang II-Stimulated Aldosterone Secretion and the Activities of Cytochrome P450 Side-Chain Cleavage Enzyme (P450scc) and Aldosterone Synthase in ZG Cells*

The ZG cells of rat adrenal glands were prepared by a method as described elsewhere (18). After pre-incubation of ZG cells with Krebs-Ringer bicarbonate buffer containing 3.6 mM potassium, 11.1 mM glucose and 0.2% BSA (KRBGA medium) for 1 h at 37°C in a shaker bath (100 cycles per min) aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, ZG cells ( $4 \times 10^4$  cells) from control or FR rats was incubated with 0.3 ml KRBGA medium (basal release), angiotensin II (Ang II,  $10^{-6}$  M), corticosterone ( $10^{-5}$  M, a precursor of aldosterone synthase) or 25-hydroxy-cholesterol ( $10^{-5}$  M, a membrane-permeable cholesterol, substrate of P450scc) combined with trilostane ( $10^{-5}$  M, an inhibitor of 3 $\beta$ -hydroxysteroid dehydrogenase, 3 $\beta$ -HSD). After 1 h incubation, 0.2 ml ice-cold KRBGA medium was added to stop the incubation under ice-bath. The medium was centrifuged at  $200 \times g$ , collected, and measured for aldosterone or pregnenolone by RIA, respectively.

*Effects of FR on the Expressions of Steroidogenic Acute Regulatory (StAR) Protein and P450scc in ZG Cells*

Further to assess the effects of FR on the expressions of StAR protein (30 kDa) and P450scc (54 kDa) in ZG cells, gel electrophoresis and Western blot analysis were used to identify the expressions of P450scc and StAR protein in ZG cells of control and FR Ovx rats.

The protein extracts of the ZG cells from control and FR Ovx rats were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Nen Life Science Products, Inc., Boston, MA, USA) using a Trans-Blot SD semi-dry transfer cell (170-3940, Bio-Rad, Hercules, CA, USA). Then the membranes were incubated with anti- $\beta$ -actin antibodies (1:8000, for loading control)

combined with anti-P450scc antibodies (1: 2000, rabbit) or anti-StAR antibodies (1: 8000, rabbit). The anti-P450scc antibody and anti-StAR antibody were kindly provided by Dr. B. C. Chung (16) and Dr. D. M. Stocco (22), respectively. The specific protein bands were detected by chemiluminescence using the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham International plc., Buckinghamshire, UK), exposed to x-ray film and scanned by a scanner (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA).

*RIAs of Hormones*

The aldosterone concentration in plasma and medium samples was measured by RIA as described previously (18). The anti-aldosterone antiserum (No. 088) was provided by the National Institute of Health (NIH, USA). The sensitivity of aldosterone RIA was 4 pg per assay tube. The intra- and inter-assay coefficients of variation were 7.5% (n = 5) and 8.1% (n = 5), respectively.

The pregnenolone concentration in medium samples was measured by RIA as described previously (17). The antipregnenolone antiserum was purchased from Biogenesis Inc (Sandown, NH, USA). The sensitivity of the pregnenolone RIA was 4 pg per assay tube. Intra- and inter-assay coefficients of variation were 2.3% (n = 6) and 3.7% (n = 4), respectively.

The concentration of corticosterone in plasma samples was determined by RIA as described elsewhere with anticorticosterone PSW# 4-9 (23). The sensitivity of the corticosterone RIA was 5 pg per assay tube. The intra- and inter-assay coefficients of variation were 3.3% (n = 5) and 9.2% (n = 4), respectively.

The concentration of ghrelin in plasma samples was determined by RIA as described elsewhere with anti-r-ghrelin antiserum (YJC 13-31) (6). The sensitivity of the ghrelin RIA was 5 pg per assay tube. The intra- and inter-assay coefficients of variation were 7.1% (n = 5) and 12.3% (n = 4), respectively.

*Statistical Analysis*

The data are expressed as mean  $\pm$  standard error of the mean (SEM). The treatment means were tested for homogeneity using analysis of variance (ANOVA), and the difference between specific means was tested for significance by using Duncan's multiple-range test or Student's *t*-test (33). A difference between two means was considered to be statistically significant when *P* was less than 0.05.

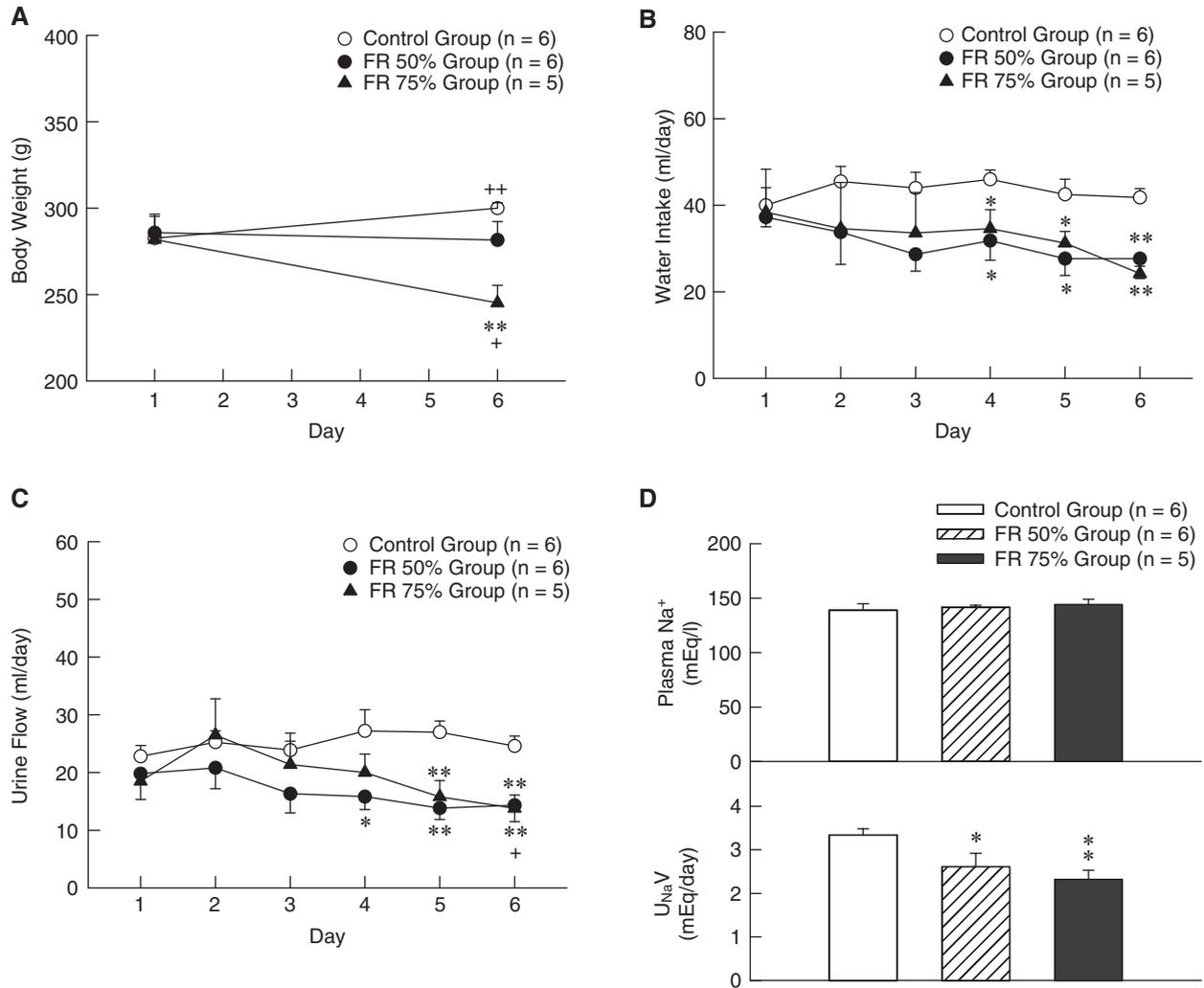


Fig. 1. Effect of FR on body weight, water intake, urine flow, plasma sodium, and urinary sodium excretion in Ovx rats. ANOVA with Duncan's test was used to compare the differences among the groups of control, FR 50% and FR 75% or differences of water intake or urine flow among 6 days in each group. Comparison of body weight before and after FR was performed with paired *t*-test. \*, \*\*,  $P < 0.05$ ,  $P < 0.01$  as compared with control group, respectively. +, ++,  $P < 0.05$  and  $P < 0.01$  as compared with the day 1, respectively. Each value represents the mean  $\pm$  SEM.

## Results

### Effect of FR on Body Weight, Water Intake, Urine Flow, Plasma and Urinary Sodium in Ovx Rats

In control rats, body weight was increased  $17.3 \pm 3.4$  g under 6 days standard rat chow diet *ad libitum*. FR 50% or 75% resulted in  $11.3 \pm 2.0$  or  $25.0 \pm 3.5$  g loss of body weight (Fig. 1A). On the day 6, body weight in FR 75% group was lighter than that in control and FR 50% groups ( $P < 0.01$ ). The FR rats exhibited decreased water intake, urine flow, and urinary sodium excretion after 6 days ( $P < 0.05$  or  $P < 0.01$ , Fig. 1B ~ Fig. 1D). However, there was no significant difference of the level of plasma sodium among control and FR groups.

These results show that FR leads to a decrease in sodium intake, therefore decreases urinary sodium excretion and maintains the balance of plasma sodium.

### Effect of FR on Plasma Aldosterone, Corticosterone, Ghrelin and Glucose in Ovx Rats

The levels of plasma aldosterone in control and FR rats are demonstrated in Fig. 2. FR 50% and FR 75% for 6 days resulted in a significant increase ( $P < 0.01$ ) on the level of plasma aldosterone in comparison with control rats. Treatment of FR 75% for 6 days elevated plasma corticosterone, however, there was no significant difference between control and FR 50% groups (Fig. 2). Regarding the

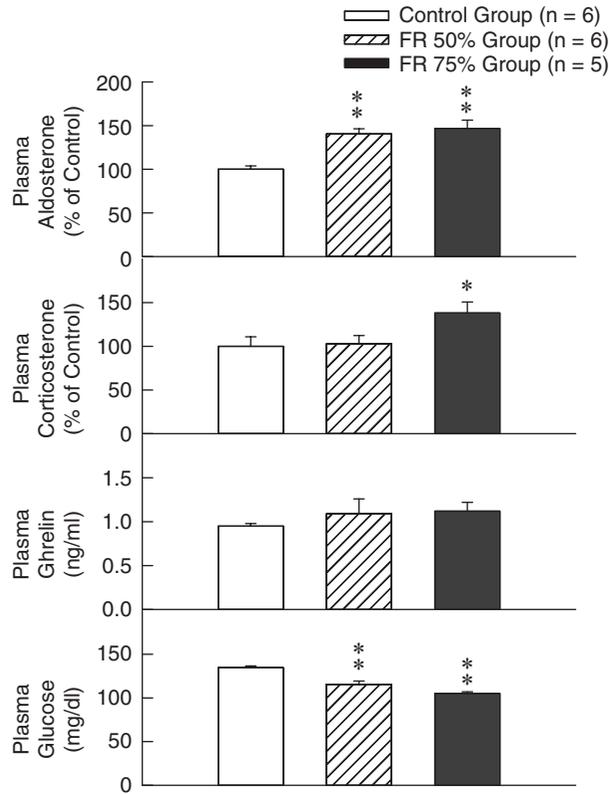


Fig. 2. Effect of FR on plasma aldosterone, corticosterone, ghrelin, and glucose in Ovx rats. ANOVA with Duncan's test was used to compare the differences among the groups of control, FR 50% and FR 75%. \*, \*\*,  $P < 0.05$ ,  $P < 0.01$  as compared with control group, respectively. Each value represents the mean  $\pm$  SEM.

levels of plasma ghrelin, there was no significant difference among control and FR rats (Fig. 2). FR induced a significant decrease in plasma glucose, however, the level of plasma glucose is still in normal range (Fig. 2).

#### Effect of FR on Basal and Ang II-Stimulated Aldosterone Release in ZG Cells

Figure 3 shows the results of basal (vehicle, upper panel) and Ang II ( $10^{-6}$  M)-stimulated (lower panel) aldosterone secretion in ZG cells of control and FR 75% rats. Ang II induced the significant increase in aldosterone secretion in control and FR 75% groups as compared with basal release ( $P < 0.05$ ,  $P < 0.01$ ). There was a significant differences in basal and Ang II-stimulated aldosterone secretion between control and FR 75% groups ( $P < 0.01$ ).

#### Effect of FR on Aldosterone Steroidogenesis in ZG Cells

Five biosynthetic enzymes are responsible for the steroidogenesis of aldosterone in ZG cells, *i.e.*,

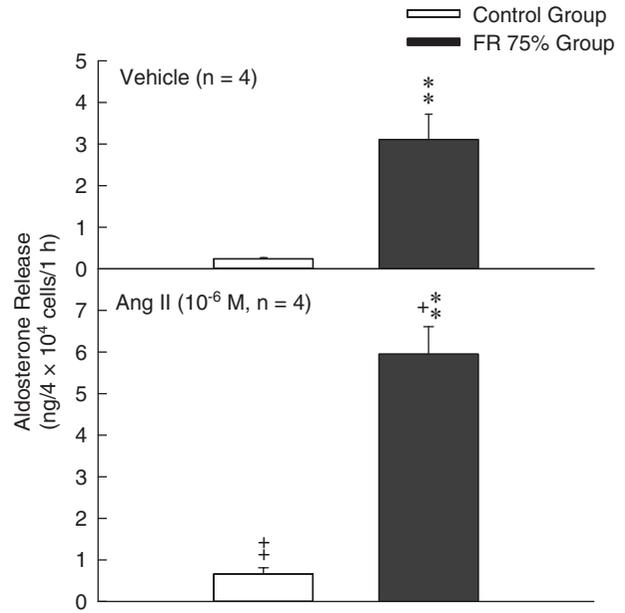


Fig. 3. Effect of FR on basal (vehicle) and Ang II ( $10^{-6}$  M)-stimulated aldosterone secretion in ZG cells. Unpaired *t*-test was used to compare the differences between the groups of control and FR 75% in the presence or absence of Ang II. +, ++,  $P < 0.05$  and  $P < 0.01$  as compared with the basal release, respectively. \*\*,  $P < 0.01$  as compared with control group. Each value represents the mean  $\pm$  SEM.

P450scc,  $3\beta$ -HSD, 21-hydroxylase,  $11\beta$ -hydroxylase, and aldosterone synthase (37). Traditionally, the rate-limiting step of steroid synthesis in steroidogenic tissues is the conversion of cholesterol to pregnenolone induced by P450scc. It has been indicated that the steroidogenic rate of P450scc in different steroidogenic tissues is determined by StAR protein (34).

The effects of FR 75% on the activity of P450scc in ZG cells are shown in Fig. 4A. In the presence of trilostane ( $10^{-5}$  M, an inhibitor of  $3\beta$ -HSD), the FR 75% group had greater basal pregnenolone release from ZG cells than that in control group ( $P < 0.01$ , upper panel of Fig. 4A). Incubation of trilostane combined with 25-hydroxy-cholesterol ( $10^{-5}$  M) produced a significant ( $P < 0.05$ ,  $P < 0.01$ , lower panel of Fig. 4A) increase in the secretion of pregnenolone in both control and FR 75% groups as compared with trilostane alone (upper panel). Moreover, there was a higher pregnenolone production ( $P < 0.05$ , lower panel of Fig. 4A) in FR 75% group than in control group. Corticosterone at  $10^{-5}$  M produced a significant ( $P < 0.01$ , lower panel of Fig. 4B) elevation in aldosterone secretion in both control and FR groups. Furthermore, there were significant increases in aldosterone release in FR 75% group ( $P < 0.05$ , Fig. 4B). According to these results, we

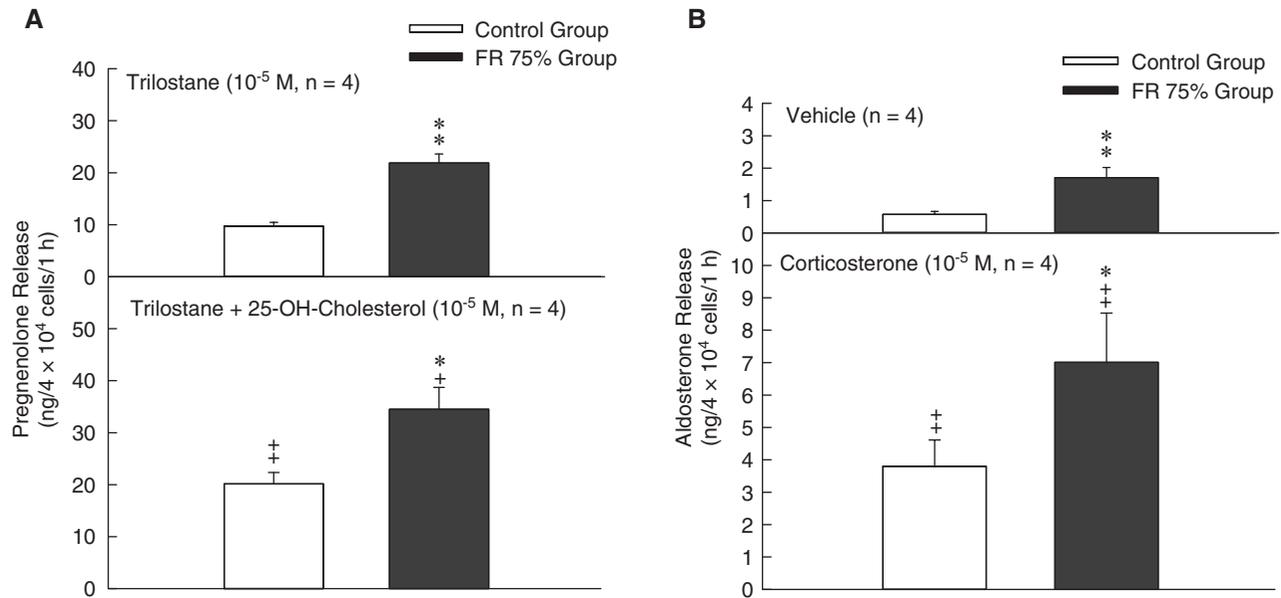


Fig. 4. Effect of FR on the activities of P450scc and aldosterone synthase in ZG cells. Unpaired *t*-test was used to compare the differences between the groups of control and FR 75% in the presence or absence of 25-hydroxy-cholesterol or corticosterone. +, ++,  $P < 0.05$  and  $P < 0.01$  as compared with the basal release, respectively. \*, \*\*,  $P < 0.05$ ,  $P < 0.01$  as compared with control group, respectively. Each value represents the mean  $\pm$  SEM.

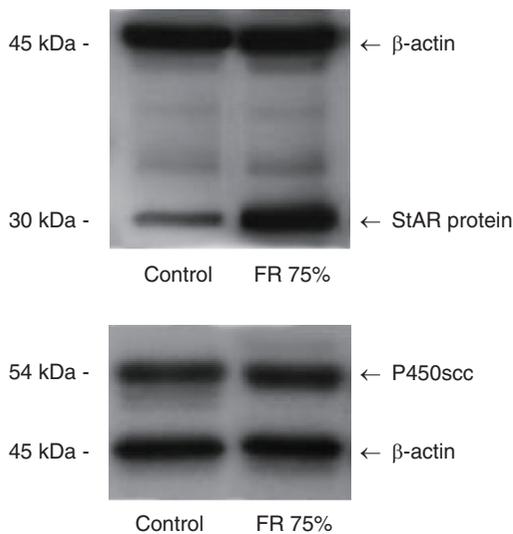


Fig. 5. Effects of FR on the protein expression of StAR protein and P450scc in ZG Cells. Protein extracts were subjected to 10% SDS-PAGE and blotted into the transfer membrane. The membrane was incubated with the anti-StAR antibodies, anti-P450scc antibodies, and anti- $\beta$ -actin antibodies (for loading control) overnight at 4°C. Then, incubated with anti-rabbit IgG and anti-mouse IgG for 1 h. The bands for StAR protein, P450scc, and  $\beta$ -actin were visualized by ECL reaction. This experiment was repeated twice with the similar results.

suggest that the activities of P450scc (conversion of cholesterol to pregnenolone) and aldosterone syn-

thase (conversion of corticosterone to aldosterone) are higher in FR 75% group than in control group.

Western blot analysis showed that there was no different expression of P450scc in ZG cells between control and FR 75% rats (Fig. 5). However, FR 75% group had higher expression of StAR protein than that in control group.

## Discussion

The present study demonstrated that short-term and strict FR induces an increased level of plasma aldosterone in Ovx rats. There was higher basal and Ang II-stimulated aldosterone secretion from ZG cells in FR group than that in control group. The conversions of 25-hydroxy-cholesterol to pregnenolone and corticosterone to aldosterone were greater in FR group than in control group. FR resulted in an increased protein expression of StAR protein in ZG cells but did not affect the expression of P450scc in ZG cells. Taken together, the stimulatory effect of FR on aldosterone is associated with the increased activities of P450scc and aldosterone synthase, as well as an increased expression of StAR protein in ZG cells. These findings provide a helpful explanation for the mechanism of FR-induced anti-natriuresis (Fig. 1) and the adaptation of the body fluid and electrolytes during FR.

Prolonged exposure to reactive oxygen species (ROS) is thought to be one of the major causes of aging (15). ROS has also been shown to inhibit steroido-

genesis in MA-10 tumor Leydig cells at the level of cholesterol transfer (35) and has been suggested to be related to the degeneration of Leydig cell during aging (7). Oxidative damage is a consequence of excessive oxidative stress and/or insufficient antioxidant potential. Diemer *et al.* (11) showed that cAMP-stimulated progesterone production or StAR protein expression is inhibited by exposure to ROS. Abidi *et al.* (1) indicated that exposure of mouse adrenocortical cell line to oxidants leads to a significant reduction in steroid secretion. They also provided a direct evidence that oxidative stress negatively impacts adrenal steroidogenesis. Aging is characterized by an increase of oxidative damage, and FR has been found to downregulate the gene expression of oxidative stress and alleviate oxidative damage in several different tissues (19, 21, 26, 29, 36). Taken together of these results, the role of oxidative stress and/or antioxidant capacity in FR-stimulated aldosterone steroidogenesis in ZG cells should be interesting and worthy for further exploration topic.

Ghrelin is produced primarily in the gastrointestinal organs in response to starvation, and serves as a peripheral signal notifying the brain to stimulate feeding (3). Gualillo *et al.* (14) found a significant increase of plasma ghrelin and gastric ghrelin mRNA in normal female rats treated with 30% FR only after 16 days (long latency) and maintained to day 21. Abou Heif (2) reported that 30% FR for 8, 16, 21 days produced a significant increase of serum ghrelin which is directly related to the duration of fasting. Martin *et al.* (25) found that 20-40% chronic FR maintained for 6 months did not affect plasma ghrelin level in adult male rats but female rats had decreased levels. In our study, FR 50% or 75% for 6 days showed an increasing tendency in circulating ghrelin but no significant difference. The reasons for these inconsistent results are not clear, and we suggest they may be related to various factors such as the duration or level of FR/fasting or gender differences.

Glucocorticoids from adrenal cortex, one of the glucose-counterregulatory hormones, help to maintain glucose level for glucose-dependent tissues such as central nervous system during fasting state by promoting hepatic gluconeogenesis, peripheral resistance to insulin and lipolysis in adipose tissue. Glucocorticoids protect the body against the self-injury of uncontrolled inflammatory and immune responses. It has been established that human ghrelin exerts a potent stimulating effects on the hypothalamic-pituitary-adrenal (HPA) axis, including corticotropin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and cortisol secretion (13, 20). Rucinski *et al.* (31) demonstrated a direct stimulating effect of ghrelin on corticosterone output by cultured rat

adrenocortical cells accompanied by an increase in expression of P450<sub>scc</sub>. Recently, Fontana *et al.* (12) investigated the effects of 2-year FR (25%) on circulating levels of IGF-1, IGF-binding proteins and cortisol in nonobese men and women and found that long-term FR results in mild and transient increase in serum cortisol level. In the present study, FR 75% for 6 days resulted in an increased plasma corticosterone and a decreased plasma glucose ( $105.1 \pm 1.9$  mg/dl, Fig. 2). However, plasma glucose is still in the normal range (70 ~ 110 mg/dl overnight fasting) (4). In the FR state, the mechanism of maintaining blood glucose may be associated with increased levels of glucocorticoid through the activation of the HPA axis. Ghrelin and glucocorticoids may play a cooperative or synergistic role in energy homeostasis. It is well known that elevated glucocorticoids possess anti-inflammatory effects. In the present study, FR 75% induced an increased secretion of corticosterone. Therefore, we hypothesize that the beneficial effects of short-term and strict FR on some clinical conditions, such as the renal ischemia reperfusion injury (28), chemotherapy (32), and stroke (8) may be associated with the increased glucocorticoids through improving excessive and inappropriate inflammation.

In summary, the present data suggests that FR increases aldosterone steroidogenesis through a mechanism involving an activation of P450<sub>scc</sub> and aldosterone synthase, as well as an increase of StAR protein expression in ZG cells. Our results provide an insight into the role of aldosterone as a hormone signal for the adaptation to FR and permit adequate renal sodium conservation during starvation in rats.

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