SUPPLEMENTARY FILE

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METHODS:

Preparation of ZG incubates

In brief, rats were sacrificed following 5-7 minutes of isoflurane anesthesia, after which adrenals were rapidly excised and placed in ice-cold Krebs-Ringer bicarbonate solution. These adrenals were then harvested, and the outermost capsulated portion, containing predominately ZG cells, was carefully peeled back to separate it from the Zona Fasciculata (ZF)/medulla interior. Tissues were subsequently digested with collagenase and DNAse in Krebs-Ringer bicarbonate solution (KREBS) (Worthington Biochemical, Freehold, NJ, USA). Cells were then incubated for 60 minutes at 37°C, with 95% O_2 and 5% CO_2. Following incubation, cells were resuspended in Krebs solution, then filtered and centrifuged at 2000 rpm for 10 minutes. Cells were washed and re-suspended in KREBS, then cell count, viability, and purity were evaluated under the microscope. Only ZG cell samples with <10% ZF contamination were studied. After a batch of cells are prepared and assessed for purity and responsiveness, several aliquots are obtained depending on the specific experimental protocols. Only two aliquots were used from the batch of cells studied in this report (**Figure 1**). Then cells were diluted to an optimal concentration (5×10^4), as large concentrations of ZG cells are known to inhibit ALDO secretion *in vitro*. Within 150 minutes of sacrifice, diluted cells were plated for further studies. Thus, an **incubate** consisted of a pool of ZG cells from several male or female rats. The ZG cells experiments were performed on 3-10 independent incubates (biologically distinct replicates). Each incubate had its own technical internal replicate that varied from 2-8 as defined in **Protocol**. Thus, the ZG cell results are reported as per incubate.

RESULTS:

Corticosterone levels basally and following 3β-HSD2 blockade and in response to ANGII were higher in incubates of female rat ZG cells than from incubates of male rat ZG cells.

Our pilot results showed that corticosterone is a more efficient substrate than 11-deoxycorticosterone. Hence, this study only measured the corticosterone levels in **Experiment 1 and 2,** and in response to ANGII acute stimulation in **Experiment 3A** because exogenous corticosterone has been added prior to any other studies with trilostane pre-treatment. Females had higher corticosterone than males in both **Experiments 1 and 2**, which indicates that blocking the conversion of pregnenolone to progesterone by trilostane did not change the effect of sex (**Supplementary Figure 1A-B**). A similar trend was observed in the ANGII stimulation condition (**Experiment 3A**), at which females had a more robust response to this acute stimulation (**Supplementary Figure 1C**).

In response to FLUDRO female and male ZG cell incubates had similar levels of ALDO and corticosterone (Experiments 4A and 4B).

In general, the percent change in corticosterone levels with FLUDRO increased in ZG incubates from female and male mice. The ALDO levels were not as consistent, but usually were reduced. These directional effects were similar whether or not the incubates were pre-treated with trilostane or not (**Supplementary Figures 2** and **3**).

Supplementary Figure 1. Effect of biologic sex on corticosterone levels.

Experiment 1 and 2: Corticosterone levels were significantly greater in 3 unique incubates from female rats in both **Experiment 1** (9 incubates from male rats) (*A*) and **Experiment 2** (8 incubates from male rats) (*B*).

Experiment 3A: Six incubates from female rats stimulated with ANGII had significantly higher corticosterone levels than ten incubates from male rats (*C*).

The data are presented as mean ± SEM. Because the data are not normally distributed, they are first log-transformation before analyses: t-tests with Welch correction.

ANGII = angiotensin II

Supplementary Figure 2. The effect of sex on steroid responses to FLUDRO: Experiment 4A

Following pre-incubation of all incubates with FLUDRO for 15 minutes, ½ of the incubates were treated with ANGII for 1 hour and media was collected for steroid measurements. Each bar represents the response of an individual ZG cell incubate. The open bars represent baseline responses to FLUDRO, and the filled bars represent responses to ANGII. Comparison of

results from ZG cell incubates from male versus female rats (A versus B for CORT and C versus D for ALDO) were not significant (p = 0.20 and 0.71 respectively).

The data are presented as mean ± SEM. Because the data are not normally distributed, they are first log-transformed before analyses: t-tests with Welch correction.

FLUDRO = fludrocortisone; ALDO = aldosterone; CORT = corticosterone; ANGII = angiotensin II; ZG= Zona Glomerulosa

Supplementary Figure 3. <u>The effect of sex on steroid responses to FLUDRO: Experiment 4B</u>

Following pre-incubation of all incubates with trilostane for 1 hour and FLUDRO for 15 minutes, ½ of the incubates were treated with ANGII for 1 hour and media was collected for steroid measurements. Each bar represents the response of an individual ZG cell incubate. The open bars represent baseline responses to trilostane and FLUDRO only, and the filled bars represent responses to ANGII. Comparison of results from ZG cell incubates from male versus female rats (A versus B for CORT and C versus D for ALDO) were not significant (p = 0.11 and 0.87 respectively).

The data are presented as mean ± SEM. Because the data are not normally distributed, they are first log-transformed before analyses: t-tests with Welch correction.

FLUDRO = fludrocortisone; ALDO = aldosterone; CORT = corticosterone; ANGII = angiotensin II; ZG= Zona Glomerulosa