# Supplemental Material

# Fast Feedback Inhibition of ACTH Secretion by Endogenous Cortisol in Humans

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## Abstract

BACKGROUND: Using high-frequency blood sampling, we demonstrate glucocorticoid fast feedback (FF) mediated by endogenous cortisol in 6 normal humans. METHODS: We stimulated ACTH secretion by oCRH with the experimental paradigm in which a high frequency blood sampling was designed for plasma ACTH and cortisol determinations. RESULTS: We saw previously unrecognized variability in the timing of key events such as onsets of ACTH and cortisol secretion, onset and offset of FF, and in FF duration. This variability mandated analyses referenced to casewise event times rather than referenced simply to time since oCRH administration. The mean time of FF onset was 4.0 (range 0-9; median 3) minutes after cortisol secretion began, and mean FF duration was 7.5 (range 3-18; median 6.0) minutes. The FF effect was rate-sensitive and does not reflect level-sensitive cortisol feedback. In agreement with previous estimates using hydrocortisone infusions, the rate of rise of cortisol that triggered FF was approximately 44 nmol/L/minute or 1.6 µg/dL/minute. FF onset followed the trigger cortisol slope with an average lag of 1 (range 0-3; median 0) minute. Unexpectedly, this trigger cortisol slope quickly declined within the FF period. CONCLUSIONS: This experimental design may enable new physiological studies of human FF that is mediated by endogenous cortisol, including mechanisms, reproducibility, and generalizability to other activating stimuli.

# Supplementary Information

## Supplementary Methods

*Procedures*

The PLATEAU condition involved a short term pre-infusion of hydrocortisone and was designed to examine rapid feedback of recently elevated but currently plateaued plasma cortisol levels on the ACTH response to oCRH. The RAMP condition was designed to test for instantaneous feedback when hydrocortisone was administered intravenously at the same time as oCRH.

*Laboratory Methods*

Plasma ACTH was measured by a two-site immunoradiometric assay obtained from Nichols Institute (San Juan Capistrano, California). The lower limit of working sensitivity is 220 fmol/L (1 pg/mL). The assay is highly specific and exhibits no cross-reactivity with related physiologic peptides. In our laboratory, the inter-assay coefficient of variation (CV) is 9% and the intra-assay CV is 4% at a plasma ACTH concentration of 6600 fmol/L (30 pg/mL). Total plasma cortisol was assayed using a fluorescence polarization immunoassay (TDx$^{TM}$) obtained from Abbott Laboratories. The lower limit of working sensitivity is 22 nmol/L (0.8 µg/dL). The assay is highly specific and exhibits no cross-reactivity with related physiologic steroids. In our laboratory, the interassay CV is 7.7% and the intra-assay CV is 6.5% at a plasma cortisol concentration of 110 nmol/L (4 µg/dL).

In evaluating changes of plasma hormone concentrations from one sample to the next, we required that the consecutive measurements differ by at least 2 standard deviations based on the intra-assay CV in order for the two concentrations to be considered reliably different.

This criterion was applied casewise and event-wise to identify key events such as time of first ACTH response (A-ON), time of first cortisol response (C-ON), time of FF onset (FF-ON), and time of FF offset (FF-OFF). We calculated rates of change (slopes) of plasma hormone concentrations casewise for the relevant periods in each event.

*Statistics*

The statistical criteria for identifying significant hormone concentration differences between blood samples were stated in Methods. Plasma hormone concentrations at different times, such as cortisol at FF-ON and at FF-OFF, were compared by paired t-tests. Recognizing the small sample, for comparisons involving multiple sampling epochs we used 1-way repeated measures analysis of variance (ANOVA) only when data from all 6 cases were available (see Supplementary Table S1, where some data were missing because of short FF duration). Exploratory, hypothesis-generating analyses of possible age and sex effects were performed on a *post hoc* basis after the data were inspected.

## Supplementary Results

*ACTH Slopes*

Consistently with the definitions of FF-ON and FF-OFF, the ACTH concentration slopes differed significantly before, during and after the FF period. We first compared 3 macro-level time intervals: (a) before FF began (the casewise period from A-ON to FF-ON); (b) the casewise FF period itself from FF-ON to FF-OFF; and (c) after FF ended (the casewise period from FF-OFF to Amax) (Table 3 and Fig. 3a). As noted, these periods had varying casewise durations. The mean plasma ACTH concentration slopes in these intervals were (a) 666 (median 648; range 282-1070) fmol/L/min, (b) -202 (median -226; -497 to +193)

fmol/L/min, and (c) 282 (median 291; range 131-410) fmol/L/min (Table 3 and Fig. 3a). By repeated measures analysis of variance these plasma ACTH concentration slopes were significantly different ($F = 14.5$, $p < 0.001$). On post hoc pairwise comparisons by the Student-Newman-Keuls method, the mean plasma ACTH concentration slope for each epoch was significantly different from each of the others ($p < 0.05$).

On closer inspection, the mean plasma ACTH concentration slope in the 3 minutes before FF-ON was 1004 (median 756; range 380-2320) fmol/L/min (Supplementary Table S1). In the first 3 minutes of FF this mean slope fell to -278 (median -390; range -853 to 280) fmol/L/min ($p = 0.039$ by paired t-test). The maximum observed change of ACTH slope occurred across those two periods in every subject, and averaged -1282 (median -1089; range -3173 to -160) fmol/L/min.

*Cortisol Slopes*

We compared the rates of change (slopes) of plasma cortisol concentrations as just described for the plasma ACTH concentration slopes. We first compared the casewise time intervals: (a) before FF began (the casewise period from C-ON to FF-ON); (b) the casewise FF period itself from FF-ON to FF-OFF; and (c) after FF ended (the casewise period from FF-OFF to Cmax). As noted, these three measures had variable casewise time periods (Table 3). The mean slope of plasma cortisol concentration during the FF period was 30.2 nmol/L/minute (1.08 µg/dL/min), which was 58% greater than the mean slope of plasma cortisol before FF, and over 3 times greater than the mean slope in the post-FF period (Table 3 and Fig. 3b). By repeated measures analysis of variance these values were significantly different ($F = 8.6$, $p = 0.007$). On post hoc testing by the Student-Newman-Keuls method, the slope of plasma cortisol concentration during the FF period differed significantly from the slope after FF ($p = 0.005$). Thus, termination of the FF period was associated with a

significant reduction of the plasma cortisol concentration slope. The plasma cortisol concentration slope in FF did not differ significantly from the slope pre-FF ($p = 0.064$). The cortisol slopes before FF and after FF did not differ significantly from each other ($p = 0.066$).

Closer inspection of the data confirmed that the highest plasma cortisol concentration slopes occurred in close proximity to FF-ON. We compared 6 successive 3-minute casewise time periods (Supplementary Table S1 and Supplementary Fig. S1), spanning the FF-ON event. Because of short FF duration, a reduced number of cases was available for the final 2 time periods. The mean plasma cortisol concentration slope rose progressively from 9 minutes before FF-ON through 6 minutes before FF-ON through 3 minutes before FF-ON until the first 3 minutes coincident with FF-ON, then declined in the next 6 minutes. The highest mean cortisol slope was seen in the 3 minutes coincident with FF-ON. A substantial increase of mean cortisol slope was observed also in the preceding and in the following 3-minute intervals.

We conducted a 1-way repeated measures ANOVA on the first 4 plasma cortisol slopes leading up to and including FF-ON and for which complete data were available (see Supplementary Table S1). This analysis confirmed significant differences among slopes ($F = 12.6$; $p = 0.001$). By pairwise multiple comparisons using the Student-Newman-Keuls method, the mean plasma cortisol slope coincident with FF-ON (0-3 minutes) was significantly greater than all other slopes preceding FF-ON.

Trigger Cortisol Slopes: The mean trigger cortisol slope was 41.3 (median 44.1; range 22.5-51.6) nmol/L/minute or 1.5 (median 1.6; range 0.81-1.87) µg/dL/minute (Supplementary Table S1). In the data grouped by time since oCRH administration (Fig. 2) the trigger slope of plasma cortisol was 48 nmol/L/min or 1.74 µg/dL/min. Casewise, the mean delay between the trigger slope and FF-ON was 1 minute (median 0) (Supplementary Table S1). In 4 cases, the trigger slope was coincident with FF-ON, and it preceded FF-ON by 3 minutes in 2 cases.

In an exploratory analysis, we found no significant correlation between the trigger slope of plasma cortisol and the maximal change of slope of plasma ACTH at FF-ON (r = -0.17). Likewise, when we used logarithmic transformation of the trigger cortisol slope, following Fehm and colleagues [21], we found no significant correlation (r = 0.41).

Maximal Cortisol Slopes: In 5 of the 6 cases, the maximal observed plasma cortisol slope was identical to the trigger slope. The maximal observed plasma cortisol slope occurred coincidently with the onset of FF in 3 subjects, three minutes earlier in 2 subjects, and three minutes later in 1 subject. Thus, the mean delay between onset of the steepest plasma cortisol concentration slope and FF-ON was 0.5 minutes (actually 0-3 minutes because of the lagging of event times as described in Methods). The mean maximal plasma cortisol concentration slope was 46.8 (median 46.2; range 38.4-55.6) nmol/L/minute or 1.70 (median 1.67; range 1.39-2.01) μg/dL/minute.

**Cortisol Slopes within FF:** Inspection of the casewise serial cortisol slopes in Supplementary Table S1 indicates that, within the FF period, the cortisol slopes quickly dropped well below the trigger/peak slopes seen at FF-ON (0-3 minutes). In case #2, for instance, the C slope dropped by 58% below the trigger C slope after 3 minutes, and by 83% after 6 minutes, even though the FF duration was 18 minutes. In Case #1 the C slope dropped by 84% below the trigger C slope after 3 minutes, and in case #5 the decline was 80% after 3 minutes. We present these casewise examples of the rapidly declining plasma cortisol slopes during FF while recognizing that the overall data are too sparse to allow formal statistical analysis. These data suggest that the major stimulus for the FF inhibition of ACTH release was the trigger/peak slope of plasma cortisol concentration and that the FF effect could carry over for 3-15 minutes after the trigger surge of slope ended.

## Supplementary Fig. Legend

**Supplementary Fig. S1.**

Box and whisker plot comparison of cortisol slopes (nmol/L/min) in casewise sampling periods spanning FF onset and preceding FF-OFF. FF-ON occurs in the 0-3 minute period. The number of cases declines after FF-ON because of short duration of FF in some subjects (Supplementary Table S1; Results). Each box displays the median, 75$^{th}$ percentile, and 25$^{th}$ percentile values. The vertical lines indicate highest and lowest observed values, and the dot indicates an outlier value.