Plasma Cortisol and Cortisone Concentrations in Normal Subjects and Patients With Adrenocortical Disorders

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Two isozymes of the 11β-hydroxysteroid dehydrogenase (11-HSD) are responsible for the interconversion of cortisol (F) and cortisone (E). The type 1 isozyme, 11-HSD1, acts mainly as a reductase in vivo, activating E to F, whereas the type 2, 11-HSD2, acts as a dehydrogenase, inactivating F to E. 11-HSD1 is the most abundant in the liver and 11-HSD2 in the kidney. In this study, we attempted to determine which isozyme and organs primarily contribute to equilibrium of plasma F and E concentrations in the peripheral circulation and to clarify differences in 11-HSD activities among adrenocortical disorders. Upon selective catheterizations for adrenocortical and renovascular disorders, plasma F and E concentrations in the femoral vein were closer to those in the renal vein than those in the hepatic vein. Values for mean plasma F/E ratios in the peripheral vein were in-between those of the adrenal and renal veins. A double reciprocal plot between peripheral plasma F and E concentrations in patients with various adrenocortical tumors was almost identical to that in normal subjects. Mean plasma F/E ratio in peripheral blood was higher in patients with Cushing's syndrome and was lower in patients with primary aldosteronism and nonfunctioning adrenocortical adenoma than that in normal subjects. These results suggest that renal 11-HSD2 is a main factor controlling the equilibrium of plasma F and E concentrations in the periphery and that cortisol and aldosterone excess do not change the equilibrium of plasma F and E concentrations in the peripheral circulation, but may alter expression of 11-HSD2. Alternation of 11-HSD2 activities as well as corticosteroid levels may be important in the pathophysiology of adrenocortical disorders.

CORTISOL (F) is the primary glucocorticoid in humans. It is synthesized and released into the circulation from the zona fasciculata of the adrenal cortex. Cortisol enters cells where it binds the cytosolic glucocorticoid receptor (GR) or mineralocorticoid receptor (MR). The affinity of cortisol for the MR is greater than that for the GR. The steroid:receptor complex is translocated into the nucleus where it exerts receptor-specific transcriptional activation of genes responsible for glucocorticoid or mineralocorticoid effects. Cortisone (E) is the 11-keto form of cortisol and is inactive as a glucocorticoid. Two isozymes with 11β-hydroxysteroid dehydrogenase activity (11-HSD) are involved in the interconversion between F and E.[1,2] Type 1 isozyme, 11-HSD1, primarily acts in vivo as an nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase, activating E by converting it to F. 11-HSD1 is expressed in glucocorticoid target tissues, such as the liver, gonad, pituitary, cerebellum, and vessels[3-5] where it may serve to regulate local cortisol levels in these tissues. In contrast, the type 2 isozyme, 11-HSD2, acts as an NAD^+-dependent dehydrogenase, converting F to inactive E, and is found in mineralocorticoid target tissues, such as the kidney, colon, lung, and salivary gland.[6-9] The 11-HSD2 isozyme provides specificity of the MR for aldosterone by inactivating F at the prereceptor level. This is crucial because plasma concentration of F is approximately 1,000-fold higher than that of aldosterone.[10,11] In congenital or acquired 11-HSD2 deficiency, cortisol binds the MR and produces mineralocorticoid effects.[12]

11-HSD1 is most abundant in the liver[3] and 11-HSD2 in the kidney.[13] The 2 isozymes catalyze the interconversion of F and E in opposite directions, so plasma concentrations of F and E in the peripheral circulation should be able to be determined by the relative activities of these isozymes. We have attempted to understand how much these organs interconvert F and E by measuring plasma F and E concentrations in the adrenal, right renal, hepatic, and femoral (peripheral) veins obtained by selective venous sampling and to clarify which isozyme or organ primarily contributes to the equilibrium of these concentrations in the peripheral blood. Individual differences in equilibrium in the peripheral plasma between F and E concentrations are determined by the relative strength of activities of the 2 isozymes in the whole body. Comparisons have been made between normal subjects and patients with various adrenocortical disorders to evaluate the effect of glucocorticoid or mineralocorticoid excess on the opposing 11-HSD activities.

MATERIALS AND METHODS

Materials

F-peroxidase, bovine serum albumin (BSA), Tween 80, and 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) were purchased from Sigma Chemical (St Louis, MO). Goat affinity purified antirabbit IgG Fc antibody, horseradish peroxidase-avidin D, and urea peroxide were purchased from Rockland (Gilbertsville, PA), Vector Laboratories (Burlingame, CA), and Calbiochem (La Jolla, CA), respectively. [1,2,6,7-^{3}H]F (specific activity, 74 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). [1,2,6,7-^{3}H]E was obtained by converting [1,2,6,7-^{3}H]F incubated for 60 minutes with rat renal cytosol from adrenalectomized rats followed by

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purification using thin layer chromatography (Whatman, Clifton, NJ) developed with 25% acetone in methylene chloride. A 96-well plate for enzyme-linked immunosorbent assay (ELISA) was obtained from Nunc-Immuno Plates (Nalge Nunc International, Rochester, NY). E-3-carboxymethoxylamine (CMO)-biotinamidecaproylhydrazide (E-biotin) was prepared as described previously.[14] Anti-F or anti-E antiserum was developed by immunization of F-3-CMO-BSA or E-3-CMO-BSA in New Zealand white rabbits. Cross reactivity of anti-F and anti-E antiserum were 4.2% and 3.4% against E and F, respectively.

Subjects and Selective Venous Catheterization

Selective venous catheterizations were performed in 24 patients suspected of having adrenocortical tumors or renovascular stenosis. Mean patients' age was 45 ± 13, with 6 men and 18 women. The diagnoses were: 2 idiopathic hyperaldosteronism (IHA), 12 aldosterone-producing adenoma (APA), 1 preclinical Cushing's syndrome due to an adrenal adenoma (PCS), 2 Cushing's syndrome(CS) due to an adrenal adenoma, 2 ganglioneuroma, 1 renovascular hypertension, and 4 high renin essential hypertension. The diagnoses of adrenal tumor were verified by surgery in all patients. A written informed consent was obtained from each patient. The catheterization was performed between 2 PM and 5 PM without corticotropin (ACTH) stimulation. For patients with adrenocortical disorders, the hepatic, right renal, adrenal, and femoral venous blood was sampled. Only the renal and femoral venous blood was obtained in patients with renovascular hypertension or high renin essential hypertension. Blood was drawn as slow as possible to avoid contamination of the inferior vena cava, and adequate positioning of the catheter was confirmed by venography after the sampling. The plasma obtained from the left renal vein was not chosen for measurement of F or E because of a possible contamination of the left adrenal effluent. The blood was collected in tubes containing EDTA-Na_2 and centrifuged at 2,000 rpm for 10 minutes. The plasma was stored at −20°C until analysis.

Basal Plasma Concentrations of F and E in Normal Subjects and Patients With Adrenocortical Disorders

We measured basal plasma F and E concentrations between 8 AM and 9 AM in 98 outpatient normal subjects as controls (90 men and 8 women, aged 50 ± 10 years) during their visit to Matsunami General Hospital for medical check-ups. They had normal blood pressure and had not taken any medication. We also examined plasma F and E concentrations in peripheral venous blood obtained at 8 AM after 30 minutes of bed rest in 60 patients with various adrenocortical disorders (18 men and 42 women, aged 48 ± 16 years). The diagnoses of these patients were: 11 nonfunctioning adrenocortical adenoma (NFA), 4 IHA, 16 APA, 6 PCS, 11 CS, and 12 Cushing's disease (CD). These diagnoses were based on both hormonal and radiographic findings. Patients with NFA exhibited no signs or symptoms of hormonal excess and plasma cortisol concentration was suppressible below 1 μg/dL after 1 mg oral dexamethasone.[15] The NFA lesion by computed tomography (CT) had a relatively lower density compared with the liver scan and had a diameter between 2 and 4 cm. Patients with PCS exhibited no symptoms or signs of CS and normal basal plasma cortisol concentration with low normal or suppressed plasma ACTH concentration, but had plasma cortisol that was not completely suppressed by 1 mg dexamethasone.[16,17] In all patients with APA and PCS, the diagnosis was verified histologically after surgery. Four adrenal glands of the 11 patients with NFA were surgically removed. In addition, in 4 patients with NFA, 15 with APA, 4 with CS, and 8 with CD, venous E and F concentrations were measured after surgery as well. In patients with NFA and APA, plasma 1 and 2 months after surgery was also measured. In patients with CS and CD, plasma, 1 year after surgery, was measured while not receiving glucocorticoid. A written informed consent was obtained from each individual.

Measurement of Plasma Cortisol and Cortisone Concentrations

We measured plasma F and E concentrations by their respective ELISA. A total of 100 μL plasma and 50 μL phosphate-buffered saline (PBS, pH 7.4) containing 4,000 dpm of [1,2,6,7-^{3}H]F was extracted with 3 mL methylene chloride. After centrifugation and aspiration of the aqueous layer, the organic layer was evaporated to dryness and then reconstituted in 1 mL PBS. Radioactivity of the 200-μL out of 1 mL was counted for correcting the recovery. Mean recovery rate for [1,2,6,7-^{3}H]F was 84.2% ± 11.2%. Mean recovery rate for [1,2,6,7-^{3}H]E, 82.9% ± 5.1%, was almost the same as that for [1,2,6,7-^{3}H]F, therefore the recovery rate of [1,2,6,7-^{3}H]F was used for that of E as well. For F, a 96-well ELISA plate was coated with rabbit anti-F antiserum in 200 μL 0.1 mol/L sodium carbonate buffer (pH 9.0) per well at 4°C overnight. The plate was washed 4 times with washing buffer (PBS with 0.1% Tween 80) using an automatic microplate washer (Bio-Rad Laboratories, Hercules, CA). Triplicate 10-μL samples and F standards were incubated with 0.2 μg/mL F-peroxidase in 200 μL assay buffer (PBS with 0.5% BSA and 0.1% Tween 80) per well at 4°C overnight. After washing, the plate was developed for 1 hour with 200 μL 0.1 mol/L sodium citrate buffer (pH 4.0) containing 0.1 mg/mL urea peroxide and 0.4 mmol/L ABTS. It was read at 405 nm by a microplate reader (Model 550, Bio-Rad Laboratories), and the data were analyzed with computer software. For E, an ELISA plate was coated overnight with 1 μg goat antirabbit IgG Fc antibody in 200 μL of the carbonate buffer per well. After washing, triplicate 10-μL samples and E standards were incubated with biotin-conjugated cortisone and rabbit anti-E antiserum in 200 μL assay buffer per well at 4°C overnight. After washing the plate, it was incubated with 10 pg horseradish peroxidase avidin D in 200 μL assay buffer per well for 45 minutes at room temperature. It was then developed and read as described above. Intra-assay and interassay coefficients of variations in the ELISA were 8.7% and 12.5% for F and 6.9% and 14.9% for E, respectively.

Statistical Analysis

All data were expressed as the mean ± SD. Statistical analysis was performed by using Wilcoxon signed rank test for paired comparisons or Dunnett's test for comparisons among multiple groups. *P* value less than .05 was considered significant.

RESULTS

Plasma F and E Concentrations and F/E Ratio in the Adrenal, Right Renal, Hepatic, and Peripheral Veins

Plasma samples from the right adrenal vein were available from only 5 patients. In 24 patients, plasma in 20 adrenal veins (bilaterally obtained in 3 patients), 22 right renal veins, 14 hepatic veins, and 24 femoral veins was available. Mean plasma F and E concentrations and mean plasma F/E ratios are shown in Fig 1. Mean plasma F and E concentrations in the adrenal vein were higher than that in the femoral vein. In contrast, plasma F/E ratio was lower in the renal and higher in the adrenal and hepatic veins than that in the femoral veins. Therefore, the ratio in the femoral vein was in-between that of the renal and adrenal veins.

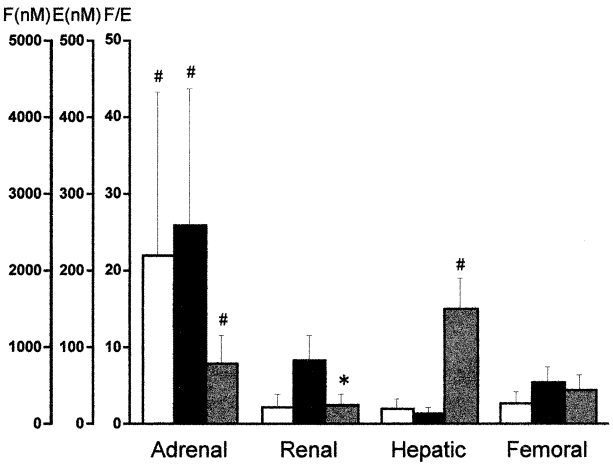

Fig 1. Mean plasma F and E concentrations and F/E ratios in the adrenal, renal, hepatic, and peripheral veins obtained by selective venous sampling. Open, closed, and hatched bars are indicated as plasma F, E, and F/E ratio, respectively. *$P < .05$; #$P < .01$ compared with concentrations in the femoral vein.

Correlations Between Plasma F and E Concentrations in the Veins

There was a significant correlation between plasma concentrations of F in the periphery and E in the femoral ($r = .95$, $P < .0001$), right renal ($r = .88$, $P < .0001$), and hepatic veins ($r = .90$, $P < .0001$). Double reciprocal plots are presented in Fig 2. The correlation between plasma concentrations of F and E in the femoral veins was quite similar to that between plasma concentration of F in the femoral vein and that of E in the right renal vein.

Correlation Between Basal Plasma F and E Concentrations in Normal Subjects and Patients With Various Adrenocortical Disorders

As revealed by double reciprocal plots (Fig 3), there were significant correlations between plasma concentrations of F and E in the peripheral vein both in normal subjects ($r = .66$, $P < .0001$) and in patients with various adrenocortical disorders ($r = .75$, $P < .0001$). The linear regressions in normal subjects and in patients with adrenocortical disorders were almost identical, suggesting that equilibrium between plasma F and E in the peripheral vein in patients with various adrenal disorders was similar to those in normal subjects.

Basal Plasma F and E Concentrations and F/E Ratios in Peripheral Veins in Normal Subjects and Patients With Various Adrenocortical Disorders

Mean plasma F and E concentrations and F/E ratio in the peripheral vein in normal subjects and patients with various adrenocortical disorders are shown in Fig 4. Mean plasma F concentration was higher in those with CS and CD than that in normal subjects. Mean plasma E concentration was higher in patients with PCS and CD than that in normal subjects. Mean F/E ratio was lower in patients with NFA (2.8 ± 0.6) and APA (3.0 ± 0.8) and higher in those with CS (5.0 ± 1.3) and CD (5.1 ± 1.6) than in normal subjects (4.0 ± 1.2). In normal subjects, there was no significant difference between the sexes (men, 4.0 ± 1.2; women, 4.3 ± 1.0).

Correlation Between Basal Plasma F/E Ratio and Serum Potassium Concentration and Blood Pressure in Patients With Adrenocortical Disorders

A significantly negative correlation was found between basal plasma F/E ratio and serum potassium concentration only in patients with APA ($R = -.581$, $P < .05$) as shown in Fig 5. No significant correlation was observed between the ratio and blood pressure in any adrenocortical disorders.

Change in Plasma F/E Ratio Before and After Surgery in Patients With Various Adrenocortical Disorders

Mean plasma F/E ratio increased and became closer to that in normal subjects after surgery in patients with NFA and APA (2.9 ± 0.8 to 4.0 ± 0.4 in NFA, n = 4, P = not significant [NS]; 3.3 ± 0.9 to 3.8 ± 1.4 in APA, n = 14, $P < .05$). In patients with CS and CD, mean basal plasma F/E ratio decreased after surgery (5.6 ± 1.4 to 2.5 ± 0.8 for CS, n = 4, P = NS; 5.3 ± 1.8 to 2.4 ± 1.6 for CD, n = 8, $P < .05$). The value after surgery was lower than that in normal subjects, whereas the mean value before surgery was higher than in normal subjects.

DISCUSSION

A previous study has measured plasma concentrations of F, E, and F/F ratio in the hepatic, renal, and femoral veins.[18] We have verified the findings and attempted to clarify which 11-HSD isozyme primarily contributes to the equilibrium of these concentrations in peripheral blood. Plasma F and E concentrations in the femoral vein were closer to those in the renal vein than those in the hepatic vein obtained by selective venous sampling, as shown in Fig 1. Values for mean plasma F/E ratios in the peripheral vein were in-between those of the adrenal and

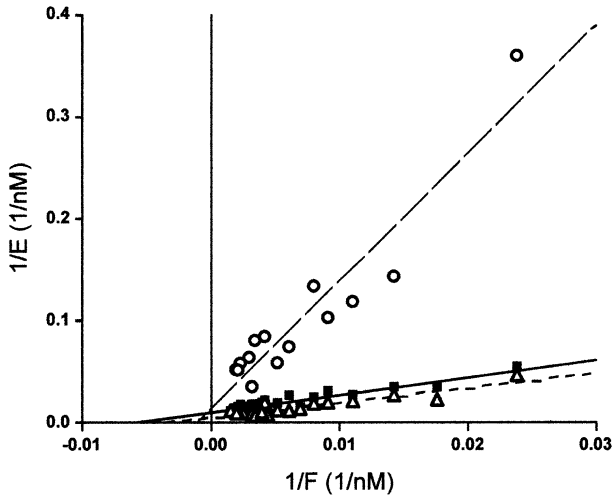

Fig 2. Linear correlations between plasma F in the femoral vein and plasma E in the femoral (■ and a solid line), renal (△ and a dotted line), or hepatic vein (○ and a broken line) in double reciprocal plots.

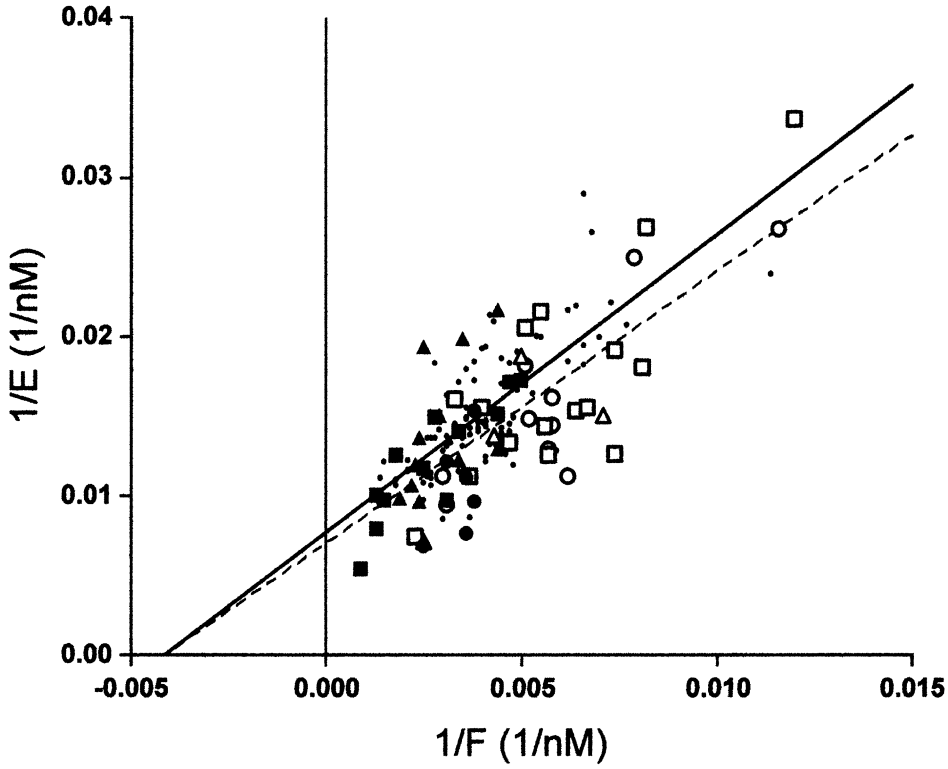

Fig 3. Linear correlations between plasma F and E concentrations in the peripheral vein in double reciprocal plots in 98 normal subjects (● and a solid line) and 60 patients with various adrenocortical disorders (○, NFA; △, IHA; □, APA; ●, PCS; ▲, CS; ■, CD and a dotted line).

renal veins. Significant linear correlations were found in double reciprocal plots between plasma F in the femoral vein and plasma E in the hepatic, renal, or femoral veins, as shown in Fig 2. The slope of the curves was very similar for femoral and renal plasma, but was considerably steeper for hepatic vein plasma. These results indicate that the interconversion between plasma F and E in the liver and kidneys correlates with the predominant type of enzyme in these organs. Renal and femoral (peripheral) correlations were similar, suggesting that the enzyme controlling the equilibrium between plasma F and E concentrations in the peripheral vein is the same one as in the kidney, which is 11-HSD2. If several enzymes are involved in a reaction, the enzyme that most efficiently catalyzes the substrate conversion is the one with a Km value that is as low as

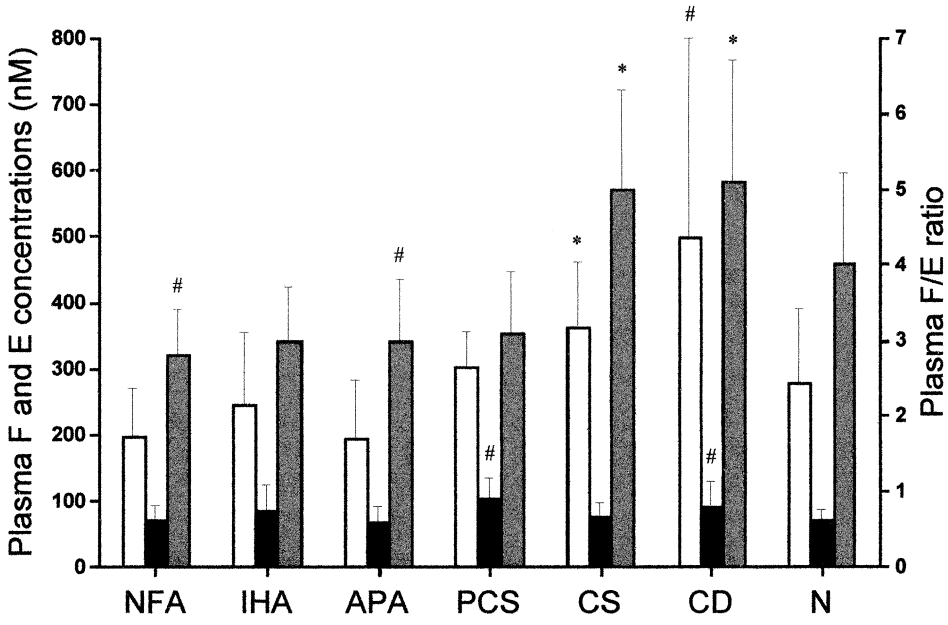

Fig 4. Plasma F and E concentrations and F/E ratio in the peripheral vein in normal subjects and various adrenocortical disorders. Open, closed, and hatched bars are indicated as F, E, and F/E ratio, respectively. *$P < .05$; #$P < .01$ compared with the ratio in normal subjects (N).

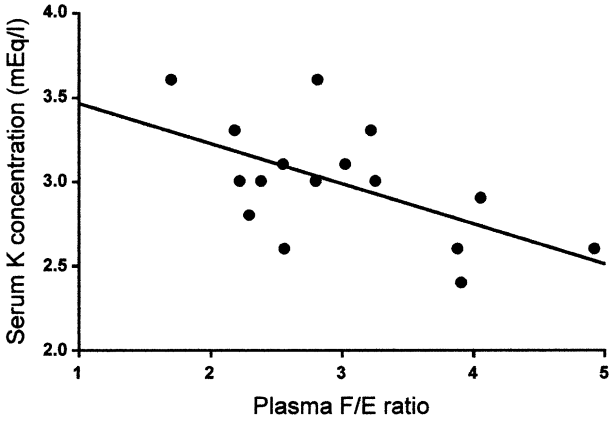

Fig 5. A negative correlation between basal plasma F/E ratios and serum potassium concentrations in patients with APA.

the substrate concentration. The Km for F for the 11-HSD2 is in the nanomolar range comparable to the physiologic plasma free F concentration, while the Km of 11-HSD1 is in the micromolar range.[2]

Our catheterization study revealed that approximately 10% of F in plasma was converted to E (F: 264 ± 156 to 230 ± 154 nmol/L; E: 55 ± 19 to 83 ± 32 nmol/L) when passing through the kidney. That 11-HSD2 catalyzes such a conversion is predominantly expressed in the kidneys should not be surprising. There are 2 reasons that the situation is different for liver effluent. The liver has a dual blood supply, receiving approximately 20% from the hepatic artery and 80% from the portal vein.[19] Plasma F and E concentrations in the portal vein are probably different from those in the hepatic artery, because 11-HSD1 exists in abdominal fat.[4,20,21] and 11-HSD2 in the colon.[7] However, no measurements of plasma F and E concentrations in the portal vein are available in our studies. Second, metabolic enzymes for F and E other than 11-HSD exist in the liver. The amounts of tetrahydrocortisol (THF) and tetrahydrocortisone (THE), metabolites of F and E produced in the liver, excreted in the urine over 24 hours are almost equal.[22] In our studies, both plasma F and E concentrations decreased to a similar extent in the hepatic vein in comparison to those in the femoral vein (F: 264 ± 156 to 209 ± 108 nmol/L; E: 55 ± 19 to 14 ± 7 nmol/L). Considering that 11-HSD1 is predominant in the liver,[23] at least some E in the plasma is converted to F in the liver, as shown by an increase in the F/E ratio. The lower concentration of F and E and the increased ratio of F/E suggest that not only plasma E is activated to F, but also F and E in the plasma are converted to ring A reduced metabolites when passing through the liver.

Plasma F and E concentrations in the peripheral vein were significantly correlated in the double reciprocal plots in normal subjects as well as in patients with various adrenocortical disorders. The correlations were almost identical in normal subjects and in patients with adrenocortical disorders, suggesting that equilibrium between plasma F and E in the peripheral vein is constant regardless of the variation in plasma F or aldosterone concentration. Plasma F/E ratio are affected by both 11-HSD1 and 11-HSD2.[24] The ratios in the peripheral vein appear to reflect a predominance of 11-HSD2 activity over that of 11-HSD1; the lower ratio reflecting more 11-HSD2 activity. Mean plasma F/E ratio in our normal subjects was approximately 4.0, a value similar to that determined in another group of subjects by high-performance liquid chromatography (HPLC).[25]

Higher concentrations of plasma F and ratios of plasma F/E were observed in patients with CD and CS, with the ratios becoming lower than normal after surgery. Previous studies also reported higher plasma F/E ratios in patients with CD and CS compared with normal subjects, while plasma E concentrations were not different among the groups.[18,26] In an in vitro study, cortisol-secreting adrenal adenomas expressed the 11-HSD2 gene, but the activity of the enzyme was suppressed in adenomas when compared with the normal adrenal cortex.[27] We have also demonstrated that expression of 11-HSD2 mRNA in Cushing's adenoma was 3 times higher than that in normal adrenals.[28] In the present study, however, higher plasma F/E ratio in patients with CD and CS was suggestive of lower activity of 11-HSD2. This discrepancy may be explained by substrate inhibition of the 11-HSD2 produced by excessive F, reducing its activity and/or overwhelming the enzyme capability.[18,29] Lower plasma F/E ratios observed in the postoperative period in the present study may represent 11-HSD2 overexpression in the kidneys or contralateral adrenal gland. One can speculate that this phenomenon explains why patients with CS often need relatively large doses of F for replacement immediately after surgery.

We have been interested in mineralocorticoid excess being able to affect 11-HSD2 activity in humans, which may regulate mineralocorticoid action of F. Lower plasma F/E ratios were observed in patients with APA with normalization of the ratios after surgery, suggesting that 11-HSD2 activity may be increased in these disorders. In IHA patients, low plasma F/E ratios were also found, but it was not significant because of the small number of the patients. In patients with primary aldosteronism, mineralocorticoid excess causes hypertension and hypokalemia. We have reported that 11-HSD2 mRNA levels in aldosterone-producing adenomas were 2.5 times higher than those in normal adrenals.[28] 11-HSD2 activity may be upregulated to minimize mineralocorticoid activity of circulating F in these disorders. In the present study, we also found a negative correlation between plasma F/E ratios and serum potassium concentrations in APA patients, which may suggest that F, which failed to be inactivated in the kidney, was at least partly involved in pathogenesis of hypokalemia as well as in those patients.

Lower plasma F/E ratios were also observed in patients with NFA. It has been shown that in NFA, the adenomas express all of the steroidogenic enzymes to produce F.[30] NFA is a clinical diagnosis; the adenoma is thought to be nonfunctional because there is no net F or aldosterone excess. It is possible that excessive F is produced, but higher 11-HSD2 activity promptly inactivates the F to E in the adenoma. This hypothesis could be applied to patients with PCS, because plasma F/E ratio was relatively low with higher plasma E concentration. We have shown that 11-HSD2 mRNA levels were high in NFA and preclinical Cushing's adenomas compared with those in control adrenals or in adenomas causing overt CS.[28]

In summary, 11-HSD2 rather than 11-HSD1 primarily contributes to the equilibrium of the interconversion between plasma F and E in the peripheral circulation. Plasma F/E ratios were increased with glucocorticoid excess and decreased with mineralocorticoid excess and NFA. Alteration in 11-HSD2 activities as well as in corticosteroid levels may be important in the pathophysiology of adrenocortical disorders. The mechanisms by which mineralocorticoids and glucocorticoids regulate 11-HSD2 expressions need to be elucidated.

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