Low abundance of sweat duct Cl\textsuperscript{−} channel CFTR in both healthy and cystic fibrosis athletes with exceptionally salty sweat during exercise

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Brown MB, Haack KK, Pollack BP, Millard-Stafford M, McCarty NA. Low abundance of sweat duct Cl\textsuperscript{−} channel CFTR in both healthy and cystic fibrosis athletes with exceptionally salty sweat during exercise. Am J Physiol Regul Integr Comp Physiol 300: R605–R615, 2011. First published January 12, 2011; doi:10.1152/ajpregu.00660.2010.—To understand potential mechanisms explaining interindividual variability observed in human sweat sodium concentration ([Na\textsuperscript{+}]), we investigated the relationship among [Na\textsuperscript{+}] of thermoregulatory sweat, plasma membrane expression of Na\textsuperscript{+} and Cl\textsuperscript{−} transport proteins in biopsied human eccrine sweat ducts, and basal levels of vasopressin (AVP) and aldosterone. Lower ductal luminal membrane expression of the Cl\textsuperscript{−} channel cystic fibrosis transmembrane conductance regulator (CFTR) was observed in immunofluorescent staining of sweat glands from healthy young adults identified as exceptionally “salty sweaters” (SS) (n = 6, P < 0.05) and from patients with cystic fibrosis (CF) (n = 6, P < 0.005) compared with ducts from healthy young adults with “typical” sweat [Na\textsuperscript{+}] (control, n = 6). Genetic testing of healthy subjects did not reveal any heterozygotes (“carriers”) for any of the 39 most common CFTR and/or ENaC mechanistically underlies the observation of high sweat [Na\textsuperscript{+}] in these apparently healthy “salty sweaters,” potentially linked to a genetic cause, such as being heterozygous for a CF mutation (44). A mutation of the CF gene occurs with high frequency in humans, 1 in 25 Caucasians of European descent (20, 56). Some reports have shown a greater incidence of heterozygocity for a CF mutation in non-CF persons prone to chronic CF-like conditions, such as sweat stimulation technique (41, 65, 82), and collection technique (38, 74), the excreted sweat can vary greatly between individuals (50, 70, 76). Since primary (initial) sweat appearing within the sweat gland is always isotonic to plasma, mechanisms underlying the interindividual “physiological” variability are presumably due to differences in electrolyte reabsorption within the sweat duct (66). A principal source of Cl\textsuperscript{−} conductance in the ductal reabsorption process (62), the cystic fibrosis transmembrane conductance regulator (CFTR) is highly expressed (18, 19) and constitutively active (60) in the apical membrane of the normal sweat duct. In addition to serving as a cAMP-activated Cl\textsuperscript{−} channel, CFTR plays a critical role in transepithelial absorption through its influence on other ion transport proteins. This is particularly illustrated by the observation that epithelial sodium channel (ENaC) is functionally dependent on CFTR in several absorbing tissues, including those of the airways, intestine, and sweat glands. The exact mechanism of the CFTR-ENaC interaction is not understood, but it is clear that in the sweat gland, CFTR activity regulates luminal Na\textsuperscript{+} entry into the ductal cell via ENaC (61). Misregulation of CFTR-dependent ENaC conductance is known to contribute to the faulty electrolyte transport of the inherited autosomal recessive disease cystic fibrosis (CF) (23, 53, 54). Most CF patients in the United States bear the ΔF508 mutation in the CFTR gene, which results in the nearly complete loss of CFTR protein at the plasma membrane (14, 64). In the CF sweat gland, such a loss or malfunction of plasma membrane CFTR results in excretion of sweat with a three- to five-fold higher-sodium chloride concentration ([NaCl]) than sweat from healthy individuals (57, 75). The secreted primary sweat in CF is not different than in non-CF; thus, it is the failure of NaCl reabsorption across the CFTR-deficient sweat duct membrane [calculated to be ~20% of normal on the basis of the Thayesen/Schwartz model of isotonic secretion (7)] that results in the excessively salty sweat of patients (72).

Studies of pharmacologically and thermally induced sweat have identified that some non-CF subjects have sweat Na\textsuperscript{+} levels that approach those of CF subjects (68). It is possible that reduced expression of ductal membrane transport proteins CFTR and/or ENaC mechanistically underlies the observation of high sweat [Na\textsuperscript{+}] in these apparently healthy “salty sweaters,” potentially linked to a genetic cause, such as being heterozygous for a CF mutation (44). A mutation of the CF gene occurs with high frequency in humans, ~1 in 25 Caucasians of European descent (20, 56). Some reports have shown a greater incidence of heterozygocity for a CF mutation in non-CF persons prone to chronic CF-like conditions, such as sodium; sweat gland; heat; thermoregulation; eccrine; epithelial sodium channel

FLUID EXCRETION BY THE ECCrine SWEAT gland is accomplished by a two-step process common to all mammalian fluid secretory systems. First, upon typically cholinergic stimulation, the coiled secretory portion of the gland produces and secretes an isotonic primary fluid. Second, Na\textsuperscript{+} and Cl\textsuperscript{−} are partially reabsorbed from the primary fluid during passage through the subsequent ductal portion of the gland, resulting in a final excreted sweat that, in most humans, is hypotonic to the plasma (54, 66). Despite controlling for factors known to influence sweat electrolyte concentration such as acclimation status (3),...
idiopathic pancreatitis (17, 73), bronchiectasis, nontuberculous mycobacterial infections (85), and chronic rhinosinusitis (58, 83). Whether the sweat electrolyte composition of CF heterozygotes (carriers) differs from noncarriers is debated, mostly because of the difficulty sorting out the major confounding factor of sweat rate (54). With pilocarpine (cholinergic)-induced sweat testing, sweat Cl− and Na+ levels for CF heterozygotes have been measured significantly higher than those for noncarriers but lower than for CF patients (homozygotes) (39). Further, in pilocarpine-induced sweat tests performed on CF heterozygotes and noncarriers with chronic pancreatitis (n = 134), noncarriers had the lowest sweat [Na+] and [Cl−], and heterozygotes had the highest concentrations (73). Thus, it is plausible that apparently healthy individuals with greater salt loss in sweat may carry CF gene mutations present in CF patients.

Another potential mechanism underlying interindividual variability in salt lost in sweat is the influence of hormones that regulate salt and fluid retention. Absorption of water and Na+ across the renal epithelium is dictated by AVP and aldosterone (ALDO), mainly via effects on aquaporin channels and ENaC (9, 24, 47, 71), but it is not clear whether these hormones can directly influence water and Na+ absorption across the epithelium of the sweat gland. While there is electrophysiological evidence for ENaC in the human sweat duct (55, 59), there is limited and conflicting evidence concerning the presence of aquaporins (33, 46, 78). Interindividual differences in urine concentration and renal handling of fluid and Na+ via ENaC and aquaporins have been attributed to differences in blood [AVP] and [ALDO] (13, 25, 71), but no study has investigated a relationship between these hormones and interindividual variability in the saltiness of sweat.

The purpose of the present study was to investigate mechanisms underlying exceptionally salty sweat: expression of sweat duct Na+ and Cl− channels CFTR and ENaC, and plasma concentrations of ALDO and AVP. Subjects were apparently healthy individuals with high sweat [Na+] CF patients with phenotypically very high sweat [Na+], and, serving as control, healthy individuals with “typical” sweat [Na+]. Our a priori hypothesis was that an inverse association between ductal CFTR and/or ENaC abundance and sweat [Na+] exists where, consequently, exceptionally salty sweaters (including CF and some healthy subjects) would exhibit decreased CFTR and/or ENaC abundance at the ductal luminal membrane compared with individuals with typical sweat [Na+]. Because a genetic link to CF has been theorized for individuals with high sweat [Na+] (26), but never directly investigated, incidence of CF mutations within the healthy subjects exhibiting large variability in saltiness of sweat was investigated. Upon finding higher basal [AVP] in the exceptionally salty sweaters, sweat gland aquaporin-5 (AQP5) expression was additionally examined in skin biopsies of a subset of subjects.

METHODS

Preliminary screening and subject selection. Initial subjects were recreationally active young adults (aged 18–40 yr) not known to have cystic fibrosis. During preliminary sweat collection sessions (i.e., 30–60 min of cycling or running at self-selected pace until 1.5–2 ml of sweat was obtained), six exceptionally “salty sweaters” (SS) with sweat [Na+] >70 mmol/l, and six individuals with typical sweat [Na+] (<60 mmol/l) (control) were identified. The cut-point of >70 mmol/l used for selecting SS is ~2 SD higher than the mean recently reported for regional sweat [Na+] collected under similar conditions with a similar technique (42).

In addition, six young adults with cystic fibrosis (CF) were recruited through the Emory University Cystic Fibrosis Center and the local community to participate as volunteers. All CF had sweat [Cl−] values from previous diagnostic pilocarpine testing of >75 mmol/l. One CF subject was ΔF508/R1162X, one was ΔF508/1717–1G→A, and the remaining four were homozygous for ΔF508 mutations. All CF subjects were in stable clinical status with an FEV1 >75% of predicted value, performed aerobic exercise for a minimum of 4 h/wk, and were cleared by their physician for participation. None of the control subjects and only one of the SS subjects had a family history of CF. Informed written consent was obtained from both CF and non-CF subjects as approved by the Institutional Review Boards at the Georgia Institute of Technology and Emory University.

Study design and subject characteristics. A cross-sectional research design was used with the three subject groups selected on the basis of sweat [Na+]. Of primary interest was the relationship between abundance of CFTR and ENaC at the sweat duct and [NaCl] of regional sweat samples collected during prolonged exercise. Matching of each SS subject with a control subject was based on the criterion that control have lower sweat [Na+] by ~50% (means ± SD % difference in sweat [Na+] for control compared with matched SS = −50.7 ± 10.0%), Non-CF pairs were also matched by age, sex, training history, anthropometry, body composition, and maximal aerobic capacity (V̇O2 max). Body composition was assessed using dual-energy X-ray absorptiometry (DEXA) (Lunar Prodigy, GE Medical Systems, Madison, WI), and maximal aerobic capacity was determined by measurement of oxygen consumption (V̇O2) (Parvo Medics, Salt Lake City, UT) during a graded, incremental, maximal cycling protocol performed 1 to 2 wk prior to testing.

Pretesting controls. Paired control and SS subjects were tested in the same month, and between the months of December through May, to control for natural heat acclimation [a modifier of sweat composition (3)]. CF subjects were not necessarily tested within the same month as their non-CF counterparts; however, this was not a major study limitation, as CF sweat composition does not change with heat acclimation (49). All testing was performed at approximately the same time of day to minimize potential differences in sweat duct membrane transport proteins under circadian influence (28, 52). To avoid potential influence of estrogen and progesterone on ductal channel expression during the luteal phase (29), all female subjects were tested in the early follicular phase of the menstrual cycle.

Subjects abstained from caffeine at least 12 h prior to the experiment, and they abstained from alcohol and physical exercise at least 24 h prior to reporting for testing. Twenty-four-hour food logs indicated that subjects complied with instructions to consume a standardized breakfast meal (bagel, toast, or English muffin with cream cheese, butter, and/or peanut butter, and orange juice) on the morning of testing. There was no difference (P > 0.05) among groups in sodium intake relative to body weight (13.4 ± 8.0 mg/kg) for the morning of testing, and for the average of 3 days prior to testing (62.6 ± 24.4 mg/kg). To minimize variation in pretesting hydration, subjects followed a euhydration protocol consisting of 12 ml water/kg body wt the evening before and also the morning of testing. Euhydration was confirmed with urine-specific gravity (USG) values <1.021 (5), 1 h prior to and immediately prior to testing, and with measurement of serum osmolality <290 mOsm/kg H2O (70). Subjects began with similar (P < 0.05) means ± SD serum osmolality (control 280.1 ± 3.3, SS 282.7 ± 2.8, and CF 284.0 ± 3.2 mOsm/kg H2O), and USG (control 1.006 ± 0.003, SS 1.006 ± 0.001, and CF 1.009 ± 0.004).

Skin biopsies. Skin biopsies provided sweat gland tissue for quantification of ductal membrane CFTR and ENaC by immunofluorescence. Biopsies were performed immediately prior to the prolonged exercise protocol to circumvent potential differences in ductal channel
expression due to exercise and/or sweating responses. Two full-thickness 4-mm diameter punch biopsies were removed by a dermatologist under local anesthetic (67) from the skin overlying the scapula contralateral to the regional sweat collection site. Biopsied tissue was immediately rinsed in ice-cold lactated Ringer solution and maintained in a second vessel of ice-cold lactated Ringer solution with glucose for tissue transport until placed in OCT embedding medium (Miles, Elkhart, IN) and frozen in isopentane cooled in liquid nitrogen. Frozen biopsies were stored at −80°C until sectioned for immunohistochemistry experiments.

Sweat collection during prolonged exercise in the heat. Sweat electrolyte concentration was obtained immediately following biopsy procedures during a prolonged exercise protocol in the heat at similar relative exercise intensity and dehydration. Subjects performed cycle ergometry in a heated environmental chamber (32–33°C and 35% relative humidity) in 20-min bouts, separated by 5-min rest periods, until 3% body weight loss was achieved. VO2 was measured every 20 min by open-circuit spirometry (Parvo Medics, Salt Lake City, UT) to maintain a workload previously determined per subject to correspond to an exercise intensity of 50% of VO2max. Sweat was collected with the modified Brisson method (8) using a collection pouch constructed with impermeable Parafilm (7 cm × 8 cm) (American Can, Greenwich, CT) and OpSite wound dressing (10 cm × 14 cm) (Smith and Nephew, Largo, FL). The sweat collection site was the scapular region, an accessible area that correlates well to whole-body sweat concentrations for [Na+] and [Cl−] (51). The skin of the scapula was cleaned with alcohol, deionized water, and sterile gauze, and air dried before application of the collection pouch. Sweat was aspirated from the collection pouch every 20 min during cycling. Sweat [Na+], [Cl−], and [K+] were measured in triplicate using a chemist analyzer (Nova 5, Nova Biomedical, Waltham, MA). Frequent removal of accumulated sweat minimized electrolyte leaching from the analyzer (Nova 5, Nova Biomedical, Waltham, MA). VO2 was measured at every 20 min by open-circuit spirometry (Parvo Medics, Salt Lake City, UT) to maintain a workload previously determined per subject to correspond to an exercise intensity of 50% of VO2max. Sweat was collected with the modified Brisson method (8) using a collection pouch constructed with impermeable Parafilm (7 cm × 8 cm) (American Can, Greenwich, CT) and OpSite wound dressing (10 cm × 14 cm) (Smith and Nephew, Largo, FL). The sweat collection site was the scapular region, an accessible area that correlates well to whole-body sweat concentrations for [Na+] and [Cl−] (51). The skin of the scapula was cleaned with alcohol, deionized water, and sterile gauze, and air dried before application of the collection pouch. Sweat was aspirated from the collection pouch every 20 min during cycling. Sweat [Na+], [Cl−], and [K+] were measured in triplicate using a chemist analyzer (Nova 5, Nova Biomedical, Waltham, MA). Frequent removal of accumulated sweat minimized electrolyte leaching from the analyzer (Nova 5, Nova Biomedical, Waltham, MA).

Sectioning and immunostaining. Skin biopsy sections at a thickness of 6 μm were cut using a cryostat (Leica CM3050-S; Bannockburn, IL) at a chamber temperature of −20°C, beginning at the epidermis and advancing into the dermis. To confirm that sections for immunostaining contained ductal portions of the gland, initial sections at each cut depth were treated with the nuclear stain hematoxylin and examined under light microscopy for ductal characteristics. Sweat ducts were distinguished from the secretory coil portion of the sweat gland by their two-cell thickness and well-defined lumen (Fig. 1). To obtain representation from as much of the entire length of the sweat duct as possible for immunofluorescence assay, sections were inspected for presence of ducts at cut depths spaced every 90 to 150 μm. Sections cut in immediate succession from those found to contain adequate ducts were picked up onto silane-coated slides (Histobond, Mienfeld, Lauda-Königshofen, Germany), air dried, and fixed in a −20°C solution of acetone-methanol (50:50) for 10 min. Following rinsing with PBS, plated sections were permeabilized with 0.25% Triton in PBS for 10 min. After blocking in 1% BSA in PBS, sections were incubated with primary antibodies overnight at 4°C. M3A7 (sc-58615; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal antibody (Ab) raised against recombinant human CFTR NBD 2 domain, was used to detect CFTR at a dilution of 1:10. Of the eight main CFTR antibodies established in the literature, M3A7 has been used to distinguish healthy and ΔF508 sweat ducts by CFTR immunostaining (16, 34, 35) and has an epitope outside of the ΔF508 deletion. ENaC was detected with a dilution of 1:200 the rabbit polyclonal Ab G-19 (sc-9890; Santa Cruz Biotechnology), which targets an epitope near the C terminus of human AQPS. In these sections, containing with a monoclonal anti-mouse Ab against the tight junction protein ZO-1 (33–9100; Invitrogen, Life Technologies, Carlsbad, CA) at a dilution of 1:300 was performed to delineate the apical membrane of the inner luminal ductal cell. After thorough washes to remove excess primary antibody, sections were incubated for 60 min at room temperature with the appropriate AlexaFluor 488, and AlexaFluor 594-conjugated secondary antibodies at a 1:500 dilution (Invitrogen). To confirm lack of nonspecific binding by secondary antibodies, omission of primary antibodies was performed on some sections from each subject. After thorough washes, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI; C. epifluorescence image original magnification ×40). The apical membrane of a ductal lumen (arrowhead) is indicated in B.

Fig. 1. Eccrine sweat gland tubules in biopsied human skin. To demonstrate morphology of secretory coil (S) and reabsorptive duct (arrows), 6-μm cryosections were stained with hematoxylin (A; B, bright field image original magnifications ×20) and 4',6-diamidino-2-phenylindole (DAPI; C. epifluorescence image original magnification ×40). The apical membrane of a ductal lumen (arrowhead) is indicated in B.

Genetic testing. To investigate a potential relationship between high sweat [Na+] and a CF genotype, a venous blood sample was collected for genetic testing from non-CF subjects immediately prior to the prolonged exercise protocol. Genomic DNA (gDNA) was isolated from whole blood and tested by an outside laboratory (Emory Genetics, Atlanta, GA) using an allele-specific primer extension assay (Tag-It Cystic Fibrosis Kit, Luminox, Toronto, Canada). This assay...

Hormone assays. Venous blood samples were obtained immediately following the biopsy and prior to the prolonged exercise protocol from all subjects for assessment of basal [ALDO] and [AVP]. Following quiet sitting for 12 min and removal of a ~1.5-ml waste sample, venous blood was drawn into an EDTA-treated Vacutainer, centrifuged at 3,000 rpm for 10 min to separate plasma and stored at −20°C. An additional resting venous blood sample was collected into a serum separator tube, allowed to clot prior to centrifugation, and used to measure serum osmolality via the freezing point depression method (MicroOsmette Precision Systems, Natick, MA). Resting plasma hormone levels were determined by an outside laboratory (Yerkes Biomarkers Core Laboratory, Emory University, Atlanta, GA) using a commercially available radioimmunoassay kit for ALDO (Diagnostic Systems Laboratories, Beckman Coulter, Webster, TX), and a commercially available enzyme immunoassay kit for AVP (Assay Designs, Ann Arbor, MI).

Statistical analysis. For each subject, the overall mean pixel intensity per area for CFTR and for ENaC was computed as an average of all imaged ducts’ mean pixel intensity per area values. This consolidated value was used to determine between group differences in CFTR and ENaC expression. Immunostaining procedures for matched subjects were always concurrently performed. Differences in subject characteristics, sweat electrolytes, resting plasma [hormones], and expression of membrane channels CFTR and ENaC among subject groups Control, SS, and CF were determined using a one-way ANOVA. Post hoc testing (Tukey) was performed to determine between-group differences. For the six paired non-CF subjects for whom repeated immunostaining was performed, the coefficient of variation for relative expression, calculated as mean pixel intensity per area divided by the total mean pixel intensity per area of both subjects within a matched pair, was determined with Pearson-Product-Moment Correlation. The relationship for mean pixel intensity per area of CFTR staining and ENaC staining with [Na⁺] of sweat was analyzed using Pearson-Product Moment Correlation. All statistical testing was conducted using SPSS (ver. 17.0, Chicago, IL). An α level of 0.05 was used to indicate statistical significance. All values are presented as means ± SD.

RESULTS

Subjects. Physical characteristics of the study subjects are presented in Table 1. The two groups of non-CF subjects (those with high and typical sweat [Na⁺]) were similar in their physical characteristics and exercise training volume. As expected, given the nature of the disease, CF subjects were younger, and had lower maximal aerobic capacity and weekly training volume compared with non-CF subjects.

Sweat electrolytes and sweat rate. Sweat electrolytes summarized in Table 2 are calculated for each subject as their average across all collections (from 0.5 to 3% dehydration). As expected, given the procedure for the subject selection, sweat [Na⁺] and [Cl⁻] were higher for SS and CF compared with control (P < 0.001) and also higher for CF compared with SS (P < 0.001). Consistent with previous reports (68, 76), CF sweat [K⁺] tended to be higher than non-CF groups (P = 0.09) and control and SS had similar sweat [K⁺] (P = 0.858). Sweat [Na⁺] and [Cl⁻] increased with progressive dehydration in all three groups as expected (45). As designed, control, SS, and CF achieved similar percent body weight loss (% dehydration) at the termination of exercise (3.0 ± 0.2% for control, 2.9 ± 0.2% for SS, and 2.9 ± 0.2% for CF). There also was no difference in sweat rate expressed relative to body weight (Table 2).

CFTR and ENaC abundance. CFTR and ENaC immunostaining was quantified only in images identified clearly as a sweat duct. Ductal portions of the sweat gland were distinguished morphologically from secretory coil portions of the gland by nuclear staining. Ducts (coiled and straight portion) have a two-cell-layered wall and appear in cross section as two layers of nuclei surrounding a clearly defined lumen (examples shown in Fig. 1B, and arrows in Fig. 1A and C). In contrast, the secretory coil is only one cell thick, has a more poorly defined lumen, and is usually larger in diameter with larger nuclei (marked “S” in Fig. 1, A and C). Representative epifluorescent images of immunostaining for control (row 1), SS (row 2), and CF (row 3) are presented in Fig. 2. As expected, CFTR (stained green) was localized primarily to the apical membrane of the ductal lumen. eENaC (stained red) also was located primarily at the ductal lumen, but additionally, it was distributed throughout the cytoplasm, consistent with regulation by membrane trafficking (12).

A primary objective of this investigation was to determine whether lower abundance of the sweat duct membrane transport proteins responsible for the reabsorption of NaCl was related to the exceptionally salty sweat of some apparently healthy individuals as it does for patients with CF. A key finding in support of this hypothesis is that CFTR expression was indeed lower (P < 0.05) in SS than in control (Figs. 2 and 3). CFTR expression in CF was lower than in control (P < 0.005) as expected, but, not lower than SS (P = 0.241) (Figs. 2 and 3). eENaC expression was not different (P = 0.957) among groups (Figs. 2 and 3). Immunofluorescent staining for CFTR and

Table 1. Physical characteristics, exercise training volume, and aerobic fitness (V̇O₂max) of control, non-CF salty sweaters, and cystic fibrosis subjects

<table>
<thead>
<tr>
<th>Physical Characteristics</th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>31.2 ± 4.7</td>
<td>31.2 ± 7.3</td>
<td>22.2 ± 2.5*†</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>68.4 ± 13.9</td>
<td>73.2 ± 15.7</td>
<td>64.0 ± 13.9</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>15.3 ± 8.0</td>
<td>18.0 ± 8.6</td>
<td>15.3 ± 5.6</td>
</tr>
<tr>
<td>Training volume, h/wk</td>
<td>12.2 ± 4.9</td>
<td>11.8 ± 4.3</td>
<td>5.1 ± 1.6*†</td>
</tr>
<tr>
<td>V̇O₂max, ml·kg⁻¹·min⁻¹</td>
<td>53.9 ± 5.8</td>
<td>49.5 ± 6.9</td>
<td>39.9 ± 4.5*†</td>
</tr>
</tbody>
</table>

Values are express as means ± SD. SS, salty sweaters; CF, cystic fibrosis. *CF significantly < control; †CF significantly < SS. P < 0.05; n = 18, 4 males and 2 females per group.

Table 2. Sweat electrolytes concentration and sweat rate relative to body weight of control, SS, and CF subjects

<table>
<thead>
<tr>
<th>Sweat Characteristics</th>
<th>Control</th>
<th>SS</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweat [Na⁺], mmol/l</td>
<td>46.2 ± 10.3</td>
<td>94.9 ± 15.2*</td>
<td>132.6 ± 6.4†</td>
</tr>
<tr>
<td>Sweat [Cl⁻], mmol/l</td>
<td>47.8 ± 8.5</td>
<td>87.1 ± 17.4*</td>
<td>127.0 ± 12.1†</td>
</tr>
<tr>
<td>Sweat [K⁺], mmol/l</td>
<td>4.9 ± 0.4</td>
<td>4.3 ± 0.3</td>
<td>7.4 ± 3.2†</td>
</tr>
<tr>
<td>Sweat rate, ml·kg⁻¹·h⁻¹</td>
<td>13.1 ± 2.1</td>
<td>12.4 ± 3.5</td>
<td>11.0 ± 1.7</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD; n = 18. *Significantly > control (P < 0.005). †CF significantly > SS (P < 0.05).
ENaC was absent on negative control sections not incubated with primary antibodies (Fig. 2, 4th column). In the three subject pairs for which repeat sectioning and immunostaining were performed, the coefficients of variation (CV) between initial and repeat relative expression of CFTR were 7.2%, 4.0%, and 4.3% (mean 5.5 ± 1.8%). The relative expression of ENaC in these repeated pairs had CVs of 1.7%, 19.1%, and 0.4% (mean 7.1 ± 10.4%).

**Relationship between channel abundance and sweat electrolytes.** Consistent with the observation that abundance of luminal CFTR was lower in CF and non-CF subjects with exceptionally salty sweat compared with control, significant inverse relationships were observed between luminal CFTR abundance and sweat [Na⁺] ($r = -0.639, P < 0.005$) (Fig. 4) across all three groups, and for luminal CFTR and sweat [Cl⁻] ($r = -0.594, P < 0.010$; not shown); namely, greater sweat [Na⁺] and [Cl⁻] was associated with lower sweat duct CFTR. There was no relationship observed between CFTR abundance and sweat electrolytes when examined for just the six CF subjects ($r = 0.143, P = 0.788$ for Na⁺; and $r = 0.304, P = 0.558$ for Cl⁻).

Consistent with the finding of similar ENaC expression among groups, there was no significant relationship between luminal ENaC expression and sweat [Na⁺] ($r = 0.211, P = 0.401$) (Fig. 4) or sweat [Cl⁻] ($r = -0.307, P = 0.216$; not shown). When examined for just the six CF subjects, however, there was a significant positive relationship for luminal ENaC abundance and sweat [Cl⁻] ($r = 0.846, P = 0.034$), and a trend for luminal ENaC abundance to correlate with sweat [Na⁺] ($r = 0.741, P = 0.092$).

**Genotyping.** None of the healthy subjects were heterozygous for any of the 39 most common disease-causing CFTR mutations in the United States. While the test panel used in this study is considered to accurately predict 99% of all non-Hispanic Caucasian carriers of a disease-causing CFTR mutation in the United States (1), it does not detect CFTR mutations with milder or unknown phenotypes.

**Basal plasma aldosterone and vasopressin concentration.** There was no difference ($P = 0.585$) among groups in basal [ALDO] (control = 159.8 ± 63.0, SS = 125.7 ± 46.8, CF = 160.5 ± 68.8 pg/ml). Basal [AVP] was higher ($P = 0.029$) in SS (89.6 ± 34.9 pg/ml) compared with control (49.9 ± 13.2 pg/ml) but in CF (72.0 ± 33.9 pg/ml), basal [AVP] was not different ($P > 0.05$) than either non-CF group. A significant positive relationship was determined for sweat [Na⁺] and plasma [AVP] ($r = 0.702, P = 0.016$) in non-CF healthy subjects, indicating higher sweat [Na⁺] was associated with higher [AVP].
Aquaporin-5 in the sweat gland. Since higher plasma [AVP] was observed in SS compared with control, additional cryosectioning and immunostaining were performed to investigate a potential relationship to a transport protein whose membrane expression is known to be regulated by AVP in other reabsorptive epithelium, the water channel AQP5 (Fig. 5). In these sections, costaining for tight junction protein ZO-1 was performed to specifically demarcate the apical membrane of the inner luminal ductal cells (green, Fig. 5C). Immunoreactivity corresponding to AQP5 (red) was observed intensely along the basolateral membrane of the dark cells of the secretory coil (Fig. 5, labeled “S”). AQP5 staining was less abundant in the ductal segments of the sweat gland (arrows) and was observed to vary greatly by location along the duct. In general, ductal AQP5 was observed to be localized to the apical membrane of the more distal tubule segments (Fig. 5A arrowhead, and 5F) and minimal or absent in the more proximal coiled ductal segments (Fig. 5, A–C arrows, D–E). While this pattern of AQP5 staining appeared quite similar across groups, image analysis (to rule out between-group differences in ductal AQP5 abundance) was not performed due to the potential for error introduced by the variable, location-dependent expression observed.

DISCUSSION

This study investigated factors that may account for known interindividual variability of salt loss in humans during thermoregulatory sweating. Given that primary sweat produced by the secretory coil of the sweat gland is isotonic to extracellular fluid (66) and that extracellular fluid [NaCl] is maintained in a tight physiological range across humans, we examined the reabsorptive duct of the sweat gland for differences in the abundance of membrane transport machinery that may explain variable electrolyte reabsorption. To accomplish this, measurements of sweat electrolytes (during exercise in the heat eliciting moderate dehydration) and abundance of luminal membrane CFTR and ENaC were performed in healthy subjects with exceptionally salty sweat compared with those with “typical” sweat [Na⁺], and compared with subjects with CF. Immunoreactivity to CFTR was localized primarily to the apical membrane of the inner luminal ductal cells, the principal site of NaCl reabsorption (66). Immunoreactivity to αENaC was also greatest at the apical membrane of the inner luminal ductal cells, but it was additionally distributed throughout the inner and outer luminal cells, consistent with a channel protein regulated in large part by dynamic trafficking (12). Intriguingly, a significant association was demonstrated for ductal luminal membrane CFTR abundance and thermoregulatory sweat [NaCl]. Further, CFTR abundance in sweat ducts of healthy salty sweaters was found to be less than in control and not significantly different than in CF subjects.

In light of this finding of altered CFTR abundance in SS, it is somewhat surprising that none of these subjects were identified as carriers for CF. This suggests that 1) either the lower CFTR abundance in the SS group was not related to CFTR
gene mutation status, or 2) the testing panel used was not complete and thus failed to detect all other potential CFTR gene mutations. The test panel used in this study, and the most commonly used for CF genetic testing, detects the most frequently occurring mutations of the CFTR gene that are associated with development of the classical form of the disease. Approximately 4% of individuals of European Caucasian descent are carriers of one of these disease-causing CFTR mutations (20, 56), which are associated with higher prevalence of chronic CF-like conditions (17, 58, 73, 83, 85). SS were all well-trained athletes who reported no chronic respiratory or digestive conditions, so perhaps it should not be surprising that they failed to genotype positively for any of the disease-causing CFTR mutations in the testing panel. More than 1,600 mutations and more than 300 polymorphisms of the CFTR gene have been identified (21), however, and most have not been associated with clinical abnormalities (44). The prevalence of carriers in the population for one of the many other non-disease-causing CFTR variants is not known. Thus, it is still possible that a less-common CFTR gene mutation associated with a non-disease-causing phenotype, perhaps only manifesting in the sweat gland, is responsible for lower CFTR abundance that may play a mechanistic role in exceptional salting sweat of healthy individuals and can explain the remaining variability. The most obvious is channel function.

While CFTR abundance was reduced in SS and CF, ENaC abundance was remarkably similar among groups. In addition to serving as a cAMP-activated Cl⁻ channel, CFTR plays a critical role in transepithelial absorption through its influence on other ion transport proteins, including ENaC (37, 81). Na⁺ conductance through ENaC is functionally dependent on CFTR in several absorbing tissues, including those of the sweat gland, where ENaC-mediated transport of Na⁺ requires Cl⁻ conductance through CFTR in the same direction, supporting absorption (59, 61). Therefore, it is reasonable that [Na⁺] in sweat was markedly higher in SS and CF, despite ENaC abundance equivalent to control and that no association was determined between sweat [Na⁺] and luminal expression of ENaC.

Our findings indicate that reduced CFTR abundance at the luminal membrane of the sweat duct is related to the high sweat [Na⁺] found in some apparently healthy individuals. However, noting that reduced CFTR abundance accounted for only 21% of the variance in sweat [Na⁺] within non-CF subjects, and that sweat [Na⁺] in SS was approximately twice that of control, while CFTR expression was only 25% less, we acknowledge that methodological limitations and/or other unidentified mechanistic factors may be implicated. In terms of methodology, evaluation of channel abundance via immunostaining in sections obtained from variable locations along the sweat duct may not have completely captured relative deficiencies in a total number of ductal channels in the sweat glands of each subject biopsy. A technique for stringent quantification of total ductal channel protein, such as immunoblotting, may be preferred to immunohistochemistry for this reason. However, it was not possible to obtain sufficient protein in the four to six sweat glands homogenized from a single subject’s 4-mm punch biopsy to perform immunoblotting experiments, and increasing the size of tissue biopsied was not an ethically reasonable option.

There are a number of other possible factors in addition to CFTR abundance that may play a mechanistic role in exceptionally salty sweat of healthy individuals and can explain the remaining variability. The most obvious is channel function.
Because it is not possible with these data to assess whether CFTR and ENaC channel “function” is different for individuals with exceptionally salty sweat compared with control, functional impairment of these or other transepithelial transport channels as a contributing mechanism remains to be investigated.

Functional impairment of CFTR was almost certainly an additional factor in the high sweat [Na⁺] for CF subjects, all six of whom had ΔF508 mutations on at least one allele, and four of whom were homozygous for ΔF508. In cells expressing ΔF508, CFTR protein is produced, but misprocessing within the endoplasmic reticulum results in most of the protein being directed toward degradation instead of insertion into the plasma membrane (14). Therefore, it was expected that luminal CFTR immunostaining would be largely absent in CF as other investigators have demonstrated for ΔF508 sweat ducts (34, 35). Interestingly, there was not a complete absence of luminal CFTR staining in CF tissue. While many ductal sections showed scarce immunoreactivity to anti-CFTR, some CF ducts were found to stain for CFTR similar to that in non-CF SS tissue, and overall, mean CFTR abundance in CF was still approximately half that of control, although it is not clear that this ΔF508-CFTR protein was in the plasma membrane itself. This finding is unexpected as immunostaining with this same antibody at similar dilutions has previously been characterized by other investigators to be minimal in the ΔF508 sweat duct (16, 34); however, these studies did not report statistical analyses of staining intensity compared with that in healthy control tissue, so it is difficult to compare their findings with the values reported here. Nonetheless, ductal CFTR abundance in homozygous ΔF508 and other CF subjects of this study appears qualitatively to be greater compared with that reported for ΔF508 CF over a decade ago and may represent an effect due to concomitant patient therapeutics at the time of study.

In contrast to the relationship observed for non-CF subjects (Fig. 4A, square symbols), for CF subjects, there was no association between sweat duct CFTR abundance and sweat [Na⁺] (Fig. 4A, circles), or [Cl⁻] (not shown). Despite the apparent presence of some luminal CFTR (albeit reduced) in CF ducts sampled, NaCl in sweat was still almost three-fold higher for CF than control. This is consistent with the understanding that for the few ΔF508 CFTR channels that escape degradation and are trafficked to the luminal membrane, channel function is compromised, possibly because of reduced channel activity (22) and/or stability (40).

Investigation of a relationship between sweat [Na⁺] and circulating levels of the chief sodium and fluid regulatory hormones, ALDO and AVP, led to a second major finding of this study. Unexpectedly higher basal plasma [AVP] was observed in SS compared with control. Elevated AVP has been suggested as a potential contributing mechanism to exercise-induced hyponatremia based primarily on retrospective investigation of postexercise blood [AVP] in some symptomatic endurance athletes (77, 80). Interestingly, exceptionally salty sweat is also believed to be a risk factor in the development of this potentially fatal condition (43, 44, 43). The question is raised, then, whether the elevated postexercise [AVP] measured in some athletes suffering hyponatremia is truly a failure to appropriately suppress AVP, as has been suggested (48, 77, 80), or, in fact, could merely reflect higher overall basal [AVP] as observed in SS of the present study. This is an area ripe for further investigation.

The curious finding of higher plasma [AVP] in SS prompted additional experiments to examine the expression of a membrane protein known to be regulated by AVP in other epithelia, the water channel aquaporin. Interindividual differences in renal collecting duct aquaporins have been implicated in pathology related to fluid and sodium management such as hypertension (13, 24), and it has been suggested that aquaporins might play a role in the saltiness of sweat, particularly, as a possible point of control for acute regulation of the sweat gland by vasopressin (31, 45). We surmised that greater basal [AVP] might serve to increase membrane aquaporin expression in the sweat duct, thereby increasing ductal water reabsorption and thus might suggest another potential mechanism for the variability in healthy athletes’ sweat salt content. Despite speculation of aquaporins as modifiers of sweat tonicity (31, 45), their mere presence in the human eccrine sweat duct had not been systematically examined prior to this study. Expression of AQP5, previously demonstrated in rodent paw sweat glands (46, 78), was examined with additional immunostaining in a subset of our study subjects. In contrast to the intense immunostaining for AQP5 observed in secretory coil segments, ductal staining was sparse and varied greatly by location along the duct (Fig. 5). Proximal coiled ductal segments showed little to no immunoreactivity corresponding to AQP5. In contrast, the more distal straight duct was observed to stain for AQP5 along the inner luminal cell apical membrane, consistent with the single immunofluorescence image of human tissue shown by Nejsum and coworkers in their report of AQP5 expression in rodent sweat glands (46). This location-dependent expression for AQP5 along the sweat duct is similar to that found in renal tubules, where principal cells at the more distal collecting duct have greater expression than the more proximal ascending and descending loops of the nephron. A similar pattern of variable AQP5 distribution has been noted in the ductal system of the human pancreas (11) and lung epithelium (36). While the finding of intense secretory coil staining for AQP5 in this human tissue is consistent with that demonstrated for rodent (46, 78) and human (33) sweat glands, as well as in other glandular epithelium (10, 30, 79), our observation of a location-dependent expression pattern for ductal AQP5 likely explains the conflicting findings in the literature for its presence (46) vs. absence (33, 78) in rodent and human sweat duct. Furthermore, our finding that AQP5 is expressed in both the secretory coil and distal duct helps to explain an early report in the literature that subcutaneous injection of AVP simultaneously decreases sweat rate and increases sweat [Na⁺] (27). AQP5 appeared to be expressed similarly for all subject groups; however, no quantitative image analysis was performed because of the potential for error in making cross-sectional comparisons of immunofluorescence intensity for channels that apparently have site-specific expression in ductal segments captured at unavoidably imprecise and undeterminable locations along the duct. Future work exploring a potential relationship between AQP5 expression and sweat tonicity will need to restrict analysis to specified locations along the duct and will likely require pooling of numerous isolated whole duct segments to obtain sufficient channel protein for quantification.

Since it is not possible to know for certain from these and other data whether aquaporins actually do play a role in reabsorption
at the sweat duct, it is important to consider other possible mechanisms for the curiously higher plasma [AVP] in SS to relate to greater sweat [Na⁺]. such as via hydroxysteroid dehydrogenase type II (HSD2). The activity of HSD2, an enzyme that confers specificity on mineralocorticoid receptors (MR) (e.g., aldosterone receptor), transiently increased with the addition of AVP to rat collecting duct tubules in vitro (2). While this effect has not been investigated in eccrine sweat ducts, MR expression has been demonstrated on basolateral membranes and HSD2 expression has been demonstrated on luminal membranes of sweat ducts (32).

An alternative interpretation of the elevated plasma [AVP] in SS is not as a mechanism per se, but rather as an adaptive response to the high sweat Na⁺ loss, for greater renal conservation of salt. Chronic elevated plasma [AVP] increased ENaC subunit protein production (47, 69) and ENaC-mediated Na⁺ transport (47) in renal collecting duct, and antinatriuretic effects of AVP have been demonstrated in normovolemic rats and humans (4, 6, 15). Since subjects in this study were regular exercisers, it is even more plausible that the elevated basal [AVP] observed in SS is simply a consequence (compensatory natriferic adaptation) of frequent and profound salt loss. Whether a higher AVP linked to higher sweat sodium is causal or consequential, this interesting observation merits further investigation.

In conclusion, we have demonstrated that reduced abundance of the Cl⁻ channel CFTR at the apical membrane of the sweat gland’s reabsorptive duct is linked to greater salt loss in some apparently healthy individuals during thermoregulatory sweating. CF subjects and non-CF subjects with exceptionally salty sweat had significantly lower ductal CFTR abundance compared with their control counterparts with “typical” sweat [Na⁺]. Differences in CFTR-mediated Cl⁻ and Na⁺ transport in ducal NaCl reabsorption could be a potential mechanism underlying variability in human sweat [Na⁺] (i.e., promoting a CF-like phenotype). Genetic testing failed to establish a link between healthy salty sweaters and any of the 39 most common disease-causing CFTR mutations; nonetheless, a relationship to other untested CFTR variants cannot be entirely ruled out. Further research should investigate how other epithelial transport proteins, such as aquaporins, and elevated AVP levels in the blood, may potentially relate to the variability in human eccrine sweat electrolyte composition.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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MECHANISMS UNDERLYING VARIABILITY IN HUMAN SWEAT Na⁺


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