Acute Cholesterol-induced Anti-natriuretic Effects

ROLE OF EPITHELIAL Na⁺ CHANNEL ACTIVITY, PROTEIN LEVELS, AND PROCESSING

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The epithelial Na⁺ channel (ENaC) is modulated by membrane lipid composition. However, the effect of an in vivo change of membrane composition is unknown. We examined the effect of a 70-day enhanced cholesterol diet (ECD) on ENaC and renal Na⁺ handling. Rats were fed a standard chow or one supplemented with 1% cholesterol and 0.5% cholic acid (ECD). ECD animals exhibited marked anti-diuresis and antinatriuresis (40 and 47%), which peaked at 1–3 weeks. Secondary compensation returned urine output and urinary Na⁺ excretion to control levels by week 10. During these initial changes, there were no accompanying effects on systolic blood pressure, serum creatinine, or urinary creatinine excretion, indicating that the these effects of ECD preceded those which modify renal filtration and blood pressure. The effects of ECD on ENaC were evaluated by measuring the relative protein content of α, β, and γ subunits. α and γ blots were further examined for subunit cleavage (a process that activates ENaC). No significant changes were observed in α and β levels throughout the study. However, levels of cleaved γ were elevated, suggesting that ENaC was activated. The changes of γ persisted at week 10 and were accompanied by additional subunit fragments, indicating potential changes of γ-cleaving proteases. Enhanced protease activity, and specifically that which could act on the second identified cleavage site in γ, was verified in a newly developed urinary protease assay. These results predict enhanced ENaC activity, an effect that was confirmed in patch clamp experiments of principal cells of split open collecting ducts, where ENaC open probability was increased by 40% in the ECD group. These data demonstrate a complex series of events and a new regulatory paradigm that is initiated by ECD prior to the onset of elevated blood pressure. These events lead to changes of renal Na⁺ handling, which occur in part by effects on extracellular γ-ENaC cleavage.

Hypercholesterolemia is an established contributor to atherosclerosis and cardiovascular diseases. Increased cholesterol is also known to cause or contribute to renal injury by impairing the filtration permeability barrier and podocyte permeability (1). However, little is known regarding the effects of elevated dietary cholesterol on membrane cholesterol and renal sodium transport. We have recently demonstrated that short term membrane cholesterol enrichment modifies renal sodium transport, leading to increased absorption and antinatriuresis (2). These short term effects were observed within minutes of enrichment and were mediated, in part, by effects on sodium absorption in the loop of Henle. No other reports have examined the effects of membrane cholesterol enrichment on renal Na⁺ transport in vivo and especially so under mild enrichment conditions that precede the vasculature effects of hypercholesterolemia.

It is established that membrane cholesterol content can modulate the activity of integral membrane proteins and that experimental depletion or enrichment of cholesterol modifies channel and transporter activity (1, 3–8). These effects have been attributed to either direct interaction between cholesterol and the membrane channels and transporters (8) or changes of membrane order and fluidity brought about by changes of lipid composition (9). A contributor to both mechanisms is the presence of cholesterol-rich “raft” membrane microdomains, where integral proteins can preferentially partition and where partitioning into such domains can modify protein activity and/or stability (10–14).

The epithelial Na⁺ channel (ENaC) has also been shown to partition into such membrane domains (5, 13–15) with downstream effects of cholesterol enrichment or depletion on channel activation by kinases (5) and channel internalization (14). Membrane cholesterol enrichment was also recently shown to enhance channel activity or NP₆ₒ (channel number × open probability) by ~55% in response to membrane stretch (15). Given that channel activity is rate-limiting to sodium absorption in the cortical collecting duct and that it is a critical determinant of urinary sodium composition and excretion, such effects could translate to enhanced in vivo Na⁺ retention by the kidneys.

Direct or indirect effects of cholesterol and/or rafts on ENaC have been limited to single cells or to cultured epithelial cells. ENaC has been shown to be modified by changes of...
membrane order or fluidity, with membrane rigidification causing stimulation (9). Rigidification is similar to the effect caused by membrane cholesterol enrichment and the partitioning of the channel into lipid rafts. Thus, this effect is consistent with channel stimulation by cholesterol enrichment which is also observed in cultured Xenopus A6 epithelia (15, 16) and Xenopus oocytes expressing ENaC.3

Despite the established cellular effects of modifying membrane cholesterol and/or fluidity on ENaC activity, little is known of the physiological significance of this phenomenon in whole kidneys or in intact animals. We have begun addressing this question by examining the acute effects of membrane cholesterol enrichment on renal function in the rat and have demonstrated enhanced sodium reabsorption via effects on the activity of the furosemide-sensitive Na+/K+ 2Cl− (NKCC) transporter (2). To further advance this work, we examined the in vivo effects of a longer, diet-induced effect of renal membrane cholesterol enrichment on Na+ handling. We also examined the effects on ENaC activity and subunit processing. To carry out these experiments, we developed new tools to examine channel subunit processing and to measure the activity of urinary proteases capable of activating this channel.

We demonstrate marked changes of renal sodium clearance observed within 3 weeks of the dietary cholesterol change. These effects were accompanied by increased cleaved γ-ENaC protein levels and increased activity of urinary proteases capable of this cleavage. These changes were accompanied by enhanced ENaC activity as measured in patch clamp experiments of split open collecting duct principal cells. At times between 3 and 10 weeks, the effects of ECD on sodium excretion (UNa+/V) rebounded despite the sustained elevation of channel-activating urinary proteases, indicating additional compensatory mechanisms. Altogether, our data provide evidence for a physiologically novel and complex regulatory process that targets ENaC activity and involves effects of membrane cholesterol content. We predict that disruption of such regulatory and compensatory processes may lead to non-genetic changes of Na+ and water balance, leading to effects on blood volume and pressure.

EXPERIMENTAL PROCEDURES

Procedures involving animals were approved by the Institutional Animal Care and Use Committees of the University at Buffalo and the University of Texas Health Sciences Center at San Antonio.

Antibody Development and Western Blotting—Subunit-specific antibodies were generated against peptides with sequences from predicted antigenic sites in the ENaC subunits. This utilized the services of Genscript Corp. (Piscataway NJ). Briefly, two rabbits per immunogen were used. Animals were immunized with keyhole limpet hemocyanin-coupled peptides. Coupling was carried out via existing or added terminal cysteines. After three boosts, animals were euthanized, and collected serum was antigen-purified, lyophilized, and stored for later usage. The immunogenic sequences were derived from the human ENaC subunits (α, β, and γ) in antigenic segments that were either identical or highly conserved with rats (supplemental Fig. S1). The sequences for the α-subunit were 4EEALIEFHRSYREC57 and 355GDPEAFMDDGFFNC668 for the N-terminal and loop antibodies, respectively. The sequence for the β and γ subunit C-terminal antigens were 619CNYDSLRLQPLDYESDSEGAI640 and 628CNTLRLER-AFSNQLTDTDQMLEL649, respectively. The β-subunit C-terminal antibody was provided by L. G. Palmer (Weil Medical School of Cornell University) and used the same antigenic sequence as our β-subunit antibody (17). Antibodies were verified to recognize ENaC subunits by comparison with signals from epitope-tagged MDCK cells (supplemental Fig. S1).

Kidney homogenates were probed with antibodies to β and γ and two antibodies to α-ENaC. The distinct antigenic sites for these antibodies allowed the detection of cleaved fragments of α. To our knowledge, this has not been done before for α-ENaC because of the absence of an antibody that recognizes the cleaved and therefore activated fragment in native tissue. A monoclonal mouse anti-GAPDH antibody (Assay Design International, Ann Arbor, MI) was used to assure equal protein loading and to normalize ENaC expression.

Kidneys were harvested and snap frozen on dry ice. Kidney pieces were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) with a mixture of protease inhibitors (Halt Protease Inhibitor, Pierce) using a Polytron homogenizer (Brinkmann Instruments). Homogenates were frozen overnight and thawed on ice, followed by sonication. The detergent-solubilized fractions were separated by centrifugation at 4000 × g for 10 min. Total protein concentration was measured using the BCA protein assay (Pierce). Samples (30 μg of protein) were mixed with SDS-sample buffer containing b-mercaptoethanol, heated for 10 min at 70 °C, and resolved by SDS-PAGE on 4–20% bis-Tris gels (Invitrogen). Proteins were transferred electrophoretically to nitrocellulose membranes. Membranes were blocked for 40 min at room temperature with 5% nonfat milk in TTBS (0.1% Tween, pH 7.4). After blocking, membranes were incubated overnight with primary antibodies (1:1000, final concentration 0.3–0.4 μg/ml) at 4 °C. A secondary goat HRP-conjugated anti-rabbit antibody (Thermo Scientific) was used at 1:10,000 (0.1 ng/ml final concentration) for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence (Super Signal West Dura, Thermo Scientific). All data were digitally acquired using an Alpha Innotech imager (Santa Clara, CA) or Bio-Rad gel documentation station. All intensities were verified to be subsaturating and were quantitated using NIH Image or Bio-Rad Quantity One software.

Animals, Animal Care and Housing—Male Sprague-Dawley rats (100–120 g, Charles River Laboratories, Kingston NY) were fed a control rodent diet (2016 Teklad Global 16% protein rodent diet, Harlan Laboratories, Indianapolis IN) or the same diet containing 1% cholesterol and 0.5% cholic acid (ECD). A total of 20 male rats were divided into equal control and ECD groups. A subset of animals was sacrificed 2–3 weeks after the diet change (4 animals/group), whereas the remaining animals were sacrificed at 10 weeks (6 animals/group). Animals were also implanted with wireless pressure telemeters (see below). Animals were allowed to acclimate to

3 M. S. Awayda, unpublished observations.
the metabolic cages (Tecniplast USA, Exton, PA) for up to 2 weeks. Animals were maintained on a 12-h day/night cycle throughout the study. After the acclimation period and a control period of 1–2 weeks, half of the animals were switched to the ECD. Urine was collected daily, and feces were collected at 2–4-day intervals. Samples were frozen until further analyses. Body weight was monitored twice a week. Rats in both groups had free access to food and water. Food and water intake was determined every 1–3 days throughout the study. At the end of the 2–3-week and 10-week periods, organs were collected and snap frozen on dry ice. Blood was collected into heparinized tubes by cardiac puncture, and plasma was separated immediately and frozen for later analyses.

Blood Pressure Measurements—Rats from the control and ECD diets were implanted with wireless pressure biotelemeters (PA-C40, DSI (St. Paul, MN)) as described previously (18). Briefly, a biotelemeter was implanted in the abdominal cavity at the start of the study. The pressure catheter lead of the transmitter was inserted in the descending aorta to allow measurement of systolic/diastolic pressures as well as heart rate. Animals were allowed to recover for 3–4 days from the surgical implantation before being transferred back to the metabolic cages. Pressure was measured using a wireless receiver placed outside the cages at 1-h intervals for a period of 10–15 s. Values from a single hour were averaged and used as a single point (18).

Analytic Methods—Urine as well as plasma Na⁺ were measured using flame photometry (model 943, Instrumentation Laboratories, Lexington, MA). Osmolarity was measured using a micro-osmometer (model 5004, Precision Systems Inc., Natick, MA). Aldosterone was measured from heparinized plasma by radioimmunoassay (Siemens, Los Angeles, CA). Urine and plasma creatinine were measured on a clinical autoanalyzer (Roche Applied Science). Plasma insulin was measured with a radioimmunoassay (Siemens, Los Angeles, CA). Urine and plasma total protein was measured by the Bradford assay (Bio-Rad). Fecal water content was measured as the difference in weight before and after desiccation for 2 days. Total fecal Na⁺ content was measured by resuspending an entire single sample of dried feces (usually collected from 2–3 days) with a fixed amount of water, followed by centrifugation to remove particulates, followed by analysis using a flame photometer.

Cholesterol was measured using the Amplex Red cholesterol assay (Invitrogen). Tissues were homogenized on ice in Ringer’s buffer using a Polytron homogenizer and then sonicated. Lipids were extracted from homogenates using the method of Bligh and Dyer (19). Samples were dried, resuspended in assay reaction buffer, and processed for measurement according to the manufacturer’s instructions. Fluorescence was measured on a Bio-Tek (Winooski, VT) fluorescence microplate reader using excitation/emission wavelengths of 530 and 590 nm. Data were normalized to total protein measured using the BCA assay.

Patch Clamp Analysis—Cell-attached patch clamp was carried out on isolated split open rat cortical collecting ducts as described previously (20). These experiments used the same rats and diet as those with metabolic cages with 6 rats/group. After 2–3 weeks of the experimental diet, animals were sacrificed, and the kidneys removed and sliced into 1-mm slices and placed on ice-cold saline. Collecting ducts were manually isolated and allowed to adhere onto poly-l-lysine coated slides. Ducts were opened by sharpened micropipettes and used for patch clamping using an Axon Instruments amplifier (Axonclamp 200B, Axon Instruments, Sunnyvale CA). Only patches with GigaOhm seals were used. Recordings were carried out with an applied pipette potential of ~60 mV (intracellular relative to ground), resulting in inward Na⁺ currents through ENaC. Kidneys from older animals could not be successfully patched, and this limited channel analysis to those after 3 weeks of the cholesterol diet.

Amidolytic Assay—To examine the urinary activity of γ-ENaC-activating proteases, we focused on the two establish cleavage sites in the extracellular loop of this subunit (21, 22). Peptides containing the minimal cleavage sequences (RKRR or RKRR) were synthesized by Genscript Corp. and were coupled to aminomethylcoumarin at the C-terminal end. These peptides provide a reporter assay for examining proteolytic cleavage after R4K3R2R1 or R4K3R2R1K1 (where position number indicates proximity to the cleaved peptide bond) as described previously (23). The presence of an intact bond between the terminal amino acid (P1) and coumarin leads to the self-quenching of coumarin’s fluorescence. After cleavage, free aminomethylcoumarin was measured fluorometrically at excitation/emission wavelengths of 360/460 nm. To determine cleavage rates, we measured the appearance of fluorescence in reactions containing 10 µl of urine, 5 µl of peptide (50 µM final concentration), and 85 µl of Tris buffer at pH 7.9. Timed fluorescence was measured at 10–15-min intervals using a Bio-Tek microplate reader. Slopes derived from the initial linear part of the reaction (first 30–60 min) were used to calculate cleavage rates. The samples for these experiments were obtained from urine collected on ice to prevent degradation. In these experiments, the metabolic cages were modified to allow continuous cooling of collected urine to 2 °C. Statistical significance was determined at the p < 0.05 level using Student’s t test.

RESULTS

Elevated Cholesterol Intake Acutely Impairs Urinary Na⁺ Excretion—No differences were observed in the measured urinary parameters between the two animal groups during the acclimation period, indicating appropriate equilibration in the metabolic cages. As mentioned above, the ECD containing 1% cholesterol and 0.5% cholic acid was chosen because it represents an appreciable but not overwhelming increase of cholesterol. Moreover, because rats can compensate for enhanced dietary cholesterol by reducing endogenous synthesis (24), this diet and this animal model are expected to result in only mild hypercholesterolemia.

Within the initial time period of 1–3 weeks, the ECD caused Na⁺ retention, resulting in marked anti-natriuresis with a 40% decrease of UNaV (Fig. 1A). This decrease was evident between weeks 1 and 3, and UNaV returned to control by...
**Cholesterol Activates ENaC by Stimulating Urinary Proteases**

At the end of the 10-week period, indicating the presence of secondary compensatory mechanisms that counteracted the initial acute effects of ECD on renal electrolyte transport. This effect is explored in more detail below by examining ENaC activity and processing.

As is evident in Table 1, the Na\(^+\) intake of both groups of animals, as determined by food intake, was unchanged throughout the study, indicating that the effect on \(U_{\text{Na}}V\) was probably a renal effect of ECD leading to stimulation of the Na\(^+\) reabsorption machinery. Accompanying the enhanced Na\(^+\) reabsorption, ECD increased urinary osmolarity (\(U_{\text{Osm}}\)) accompanied by a decrease of urinary volume (\(U_V\); Fig. 1, B and C). These effects were sustained throughout the study, indicating that they probably represent an effect of ECD on water handling and balance separate from those resulting in anti-natriuresis.

The water intake and losses are summarized in Table 1. Intakes of Na\(^+\) and water were unchanged between the two groups throughout the study, further demonstrating the specificity of the effects of ECD on \(U_{\text{Na}}V\). Animals on the ECD diet exhibited increased water loss through the feces. These additional losses averaged ~3 ml of H\(_2\)O/day and remained constant throughout the study. These effects were separate from those of the ECD causing the anti-natriuresis, which were of renal origins (see below).

Total fecal Na\(^+\) content was unchanged in the ECD group. Na\(^+\) content was measured in both groups at weeks 2 and 10 and normalized to the control group. Na\(^+\) content was 0.97 ± 0.11 and 1.02 ± 0.13 (\(n = 3\)) in the ECD group at weeks 2 and 10, respectively. These results indicate that the changes of Na\(^+\) excretion are of renal origin, as predicted from the absence of a correlation between the time course of changes of water and Na\(^+\) excretion. Further ruling out an effect of renal Na\(^+\) handling secondary to decreased GI Na\(^+\) absorption is the observation that ECD did not affect aldosterone levels (see below), which would have been elevated in response to such a decrease.

Animal weights as shown in Table 1 were slightly elevated in the ECD group at week 10 (~5% increase). To better delineate any potential effects of ECD on weight, we examined the time-dependent changes of weight, where each animal serves as its own control. As shown in Fig. 2, the daily change of animal weight was numerically higher in the ECD within the initial 2–3-week period. However, this difference did not reach statistical significance (\(p > 0.05\)).

Given the above data, we conclude that the effects of ECD on \(U_{\text{Na}}V\) are either of renal (e.g. ion transport) or extrarenal (pressure or filtration) origins, leading to effects on urine formation. To rule out the extrarenal effects, we examined the urinary content of creatinine and total protein. Both \(U_{\text{TP}}V\) and \(U_{\text{CR}}V\) were unchanged. Plasma creatinine values were

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**TABLE 1**

General metabolic cage data from control and ECD animals

|                      | Control | ECD | Control | ECD | Control | ECD |
|----------------------|---------|-----|---------|-----|---------|-----|
| Food intake (g)      | 20.0 ± 1.0 | 19.3 ± 0.7 | 21.1 ± 0.5 | 22.3 ± 1.0 | 20.9 ± 0.7 | 20.9 ± 1.2 |
| Water intake (g)     | 23.8 ± 1.4 | 26.2 ± 1.8 | 28.6 ± 1.0 | 27.4 ± 1.0 | 31.9 ± 1.2 | 32.9 ± 1.1 |
| Feces (wet) (g)      | 6.4 ± 0.4 | 7.6 ± 0.5 | 7.2 ± 0.3 | 12.2 ± 0.5 | 7.2 ± 0.2 | 10.5 ± 0.5 |
| Feces (dry) (g)      | 3.6 ± 0.2 | 4.4 ± 0.1 | 2.9 ± 0.3 | 4.8 ± 0.6 | 3.1 ± 0.5 | 4.4 ± 0.6 |
| Water loss feces (g) | 0    | 0.4 | 0       | 3.1 | 0       | 3.0 |
| Mean body mass (g)   | 205 ± 7 | 205 ± 8 | 290 ± 10 | 298 ± 12 | 377 ± 13 | 400 ± 19 |
also unchanged and averaged 0.75 ± 0.03 and 0.72 ± 0.03 mg/dl (n = 8) in the control and ECD groups, respectively. Given that animal weight was also unchanged, this indicates that glomerular filtration was probably unchanged because this would have caused changes to $U_{\text{CRH}}$. Moreover, the absence of change of $U_{\text{TPH}}$ indicates that filtration across the renal corpuscle, which is determined by the endothelial/epithelial permeability, was also unchanged. These results support an effect of ECD on renal Na$^+$ transport.

The ECD caused an increase of plasma cholesterol content at both early and end time points (not shown); however, these measurements were highly variable and depended largely on the amount of food consumed by these animals prior to being fasted and sacrificed. In lieu of such measurements, we demonstrate an enrichment of renal cholesterol content by measuring urinary cholesterol excretion and renal membrane cholesterol content. As shown in Fig. 3, the ECD led to a significant increase of urinary cholesterol excretion ($U_{\text{CHOL}}$; Fig. 3C) at both weeks 3 and 10. Thus, our ECD was sufficient in delivering additional cholesterol to enhance that which is filtered from plasma into urine despite the fact that rats can partially compensate for their cholesterol intake by decreasing endogenous synthesis (24).

The effects of ECD on renal membrane cholesterol content are shown in Fig. 3D. These values represent total renal membrane cholesterol content, which is the sum of cholesterol in the apical, basolateral, and intracellular membranes. At week 2/3, ECD increased cholesterol content by ~16%; however, these differences did not reach statistical significance. At week 10, ECD increased membrane cholesterol by a statistically significant 36%. These small changes are not surprising because cholesterol is an integral component of the plasma membrane and because cholesterol enrichment of membranes varies considerably between cells types and the methods used for cholesterol delivery (see “Discussion”).

**Cholesterol Activates ENaC by Stimulating Urinary Proteases**

To further rule out changes of renal perfusion as a contributor to the observed anti-natriuresis, we measured blood pressure in conscious, unrestrained animals via implanted telemeters. As shown in Fig. 4, ECD did not
affect diurnal or nocturnal systolic blood pressure (SBP) and mean arterial pressure in the initial 3 weeks of the study. However, these animals exhibited a tendency toward increased pressure by week 10. These data are summarized in Table 2 and indicate no changes within the first 3 weeks and a modest elevation of SBP and DBP with no effect on animals’ heart rate or activity at week 10. This mild effect is consistent with the mild increase of cholesterol with our ECD.

Our data indicate that the observed anti-natriuresis preceded the effects of cholesterol on blood pressure and, as proposed above, was due to a renal effect of the ECD on Na⁺ transport. As noted, the small increase of pressure observed at week 10 occurred with no change of heart rate, activity, and nocturnal behavior, further ruling out increased stress or a major change of blood volume as underlying mechanisms of the effects of ECD. To rule out hormonal changes, we examined the levels of insulin and aldosterone. Both hormones are known to cause prolonged changes to ENaC activity in vivo and in vitro. Moreover, cholesterol is a precursor of the aldosterone synthetic pathway, and prolonged hypercholesterolemia in rats has been shown to depress insulin levels (25). As summarized in Table 3, ECD was without effect on both insulin and aldosterone levels, further supporting an effect of ECD on renal Na⁺ transport.

Absence of Renal Mass Changes with ECD—Body mass increased in both groups of animals with age. No major difference in body weight was observed between these two groups of animals. Organ weight was similarly unchanged at both

| TABLE 2 |
| Non-invasive telemetric data from control and ECD animals |
| Daily values were computed from 24 hourly samples and included day and night values. See Table I legend and “Experimental Procedures” for more details. |

|                            | Start | Week 2/3 | Week 10 |
|---------------------------|-------|----------|---------|
|                            | Control  | ECD     | Control  | ECD     | Control  | ECD     |
| Diastolic blood pressure (mm Hg) | 86 ± 1.2 | 85 ± 1.0 | 86 ± 1.1 | 84 ± 1.7 | 85 ± 1.0 | 89 ± 1.0* |
| Systolic blood pressure (mm Hg)  | 117 ± 1.1 | 119 ± 1.0 | 117 ± 1.1 | 117 ± 1.6 | 117 ± 1.2 | 123 ± 1.2* |
| Heart rate (beats/min)         | 446 ± 5.1 | 449 ± 5.6 | 418 ± 4.7 | 415 ± 5.0 | 366 ± 5.0 | 373 ± 5.4 |
| Activity (arbitrary units)      | 4.0 ± 0.3 | 4.4 ± 0.4 | 2.4 ± 0.2 | 2.3 ± 0.2 | 1.9 ± 0.2 | 2.1 ± 0.2 |

* Significantly different from control.

| TABLE 3 |
| Activity of ENaC-modifying hormones |
| Serum was analyzed as described under “Experimental Procedures.” n = 4–10 in each group. See the legend to Table 1 for more details. Both aldosterone and insulin values were not statistically significantly different from control. |

|                          | Week 2/3      | Week 10       |
|--------------------------|---------------|---------------|
|                          | Control  | ECD     | Control  | ECD     |
| Aldosterone (ng/ml)       | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.8 ± 0.3 | 0.5 ± 0.2 |
| Insulin (ng/ml)           | 0.25 ± 0.19 | 0.16 ± 0.05 | 0.51 ± 0.19 | 0.44 ± 0.15 |
early and late time points with the exception of the liver (Fig. 5). The absence of gross changes in the weight of both the heart and kidneys further indicates the absence of major pressure or perfusion effects in the ECD group.

The above data indicate that the anti-natriuresis observed in the initial 3 weeks after the ECD was probably due to effects of cholesterol enrichment on renal Na$^+$ transport proteins. Given our interest in ENaC and our preliminary data indicating an effect of membrane cholesterol enrichment on ENaC activity in heterologous expression systems, we examined the effects of ECD on channel subunit expression with emphasis on channel subunit cleavage, a process recently shown to increase channel activity (26–29).

**Effects of ECD on α-ENaC**—Expression and processing of this subunit was examined using semiquantitative Western blotting with subunit-specific antibodies. As shown in supplemental Fig. S1, these antibodies were verified to detect ENaC stably expressed in MDCK cells. Full-length, unprocessed α-ENaC was faint but was detected with the N-terminal epitope antibody and migrated at ~100 kDa (Fig. 6A). Decreased dietary Na$^+$ intake from a diet containing 1.25% Na$^+$ has been previously shown to enhance rat αENaC processing, leading to lower levels of uncleaved protein (30). Therefore, the lower level of full-length αENaC protein in our blots was probably due to the 0.25% Na$^+$ content of our diet.

At week 3, full-length αENaC expression was unchanged between the control and cholesterol groups (Fig. 6A). Cleaved or proteolytically processed α subunit was observed when probed with the loop antibody with two detectable products, which migrated at ~65 and ~37 kDa. The 65-kDa fragment was of the size expected and observed in tagged α with proteolytic processing at the extracellular loop near the first transmembrane domain. The ~37-kDa fragment was also observed in MDCK cells (supplemental Fig. S1). These fragments were not detected with the loop antibody or with secondary antibody alone, indicating that they could be due to novel cleavage sites past the identified furin cleavage sites or partial intracellular degradation of this subunit, although we cannot completely rule out nonspecific detection. The levels of these fragments increased by ~2.5-fold in the ECD group; however, this occurred with no changes of full-length or cleaved α (at least for α cleaved at or near established sites (21, 29, 31)), and the significance of this effect remains unclear.

α-ENaC protein levels at week 10 are shown in Fig. 6B. Further changes were observed from the results obtained at week 3, and the levels of full-length and 65-kDa cleaved α remained unchanged between the control and ECD groups (Fig. 6B, panels 2 and 4).

**Enhanced Cholesterol Diet Increases γ-ENaC Levels and Processing**—γ-ENaC processing at the extracellular loop near the first transmembrane domain was also recently shown to activate ENaC (22, 28). This processing was further dependent on dietary Na$^+$ intake (30). As evident in Fig. 8, γ was mostly in the processed form, consistent with that observed with α. Processed γ migrated as a doublet at ~60 kDa, although in control animals, the signal from the lower molecular weight protein was faint and much lower than that of the upper protein. At 3 weeks, ECD increased processed γ levels by ~1.8-fold (Fig. 8A). At 10 weeks, this increase persisted, with the ECD group exhibiting ~2-fold higher γ levels (Fig. 8B).

An interesting observation with processed γ was the appearance of additional cleavage products at week 10, where γ migrated as a clear triplet. To a certain extent, this was observed in both groups of animals; however, the changes were more marked in the ECD group. The additional γ product exhibited a molecular mass shift of 2–5 kDa and migrated near 50 kDa, resulting in two proteins in the 50–55 kDa range. When compared with control, the levels of these two smaller γ proteins were ~3-fold higher in the ECD at week 10. Given the shift in molecular weight, we predict that these additional fragments may represent further proteolytic processing of γ at the second cleavage site (KRKR) or slightly downstream of this site, thereby explaining the presence of additional products. Thus, the 60 kDa band may represent cleavage at the first site at KRKR, resulting in the higher molecular weight. This is consistent with a recent report demonstrating cleavage of γ by elastase and plasmin at sequences near the second cleavage site of KRKR (32). In this case, the levels of additional γ forms of 50–55 kDa may represent higher activity of proteases that can target the second site on this subunit. This hypothesis is tested below.
Elevated Cholesterol Intake Increases γ-ENaC Cleaving Urinary Protease Activity—The changes to γ-ENaC levels and processing indicate that ECD enhanced γ protein levels as well as subunit proteolysis. However, our data, like nearly all kidney Western blots, cannot differentiate between changes to plasma membrane- and intracellular membrane-bound ENaC subunits. Moreover, these data cannot differentiate between increased proteolysis in intracellular membranes (e.g. Golgi) or at the plasma membrane (e.g. urinary proteases). Given recent data linking enhanced extracellular protease activity to conditions that favor Na⁺ retention and hypertension (33), we developed an assay to

FIGURE 6. ECD does not affect α-ENaC expression or processing. Western blot of renal homogenates probed with two α-ENaC antibodies. Effects at weeks 3 and 10 are summarized in A and B, respectively. Full-length α subunit migrated at ~100 kDa and was detected with the N-terminal antigen antibody at both time points (A1 and B1) and the loop antigen antibody at week 10. The level of this subunit was low in contrast to that of other fragments and was unchanged by the ECD. Proteolytically cleaved α was the dominant observed protein and was only detected with the loop antibody (A2 and B2). This cleaved subunit migrated at 65 kDa, with a second protein detected at 37 kDa (see “Experimental Procedures”). ECD did not significantly affect levels of processed 65-kDa α at either 3 or 10 weeks. Data are normalized to GAPDH expression. n = 4–6 in each group. CONT, control; Error bars, S.E.
examine the activity of urinary proteases that can target γ-ENaC.

The protease targets on γ-ENaC are well established and highly conserved between species, which simplifies the interpretation of any observed effects. Urinary protease cleavage rates of peptides reflecting the two γ extracellular cleavage sites, after RKRR and RKRK, are shown in Fig. 9. These represent the first and second identified cleavage sites on γ. It is expected that cleavage after the first site would result in a larger fragment than that after the second, a difference that is
likely to translate to a shift in the apparent molecular mass of 2–5 kDa.

At the start of the study, the cleavage rates of both peptides were similar to each other and between the two animal groups, indicating assay reproducibility. To examine the time course of these changes, data were summarized in weekly intervals between weeks 1 and 4 and at the end point. The cleavage rate after the first γ site increased in the ECD group within the first experimental week. This difference returned toward control by the second week and remained minimally elevated until the end of the study. On the other hand, cleavage after the second γ site was not maximally elevated until the second week of the study and remained highly elevated throughout the study. These data demonstrate assay specificity because activity was markedly different between these two peptides. These data also indicate that the ECD group may have undergone early enhanced cleavage of γ at the first site, followed by delayed and sustained cleavage at the second site. These changes are consistent with the shift in apparent size observed in the Western blots above and indicate that activation of γ-ENaC-targeting urinary proteases by ECD is a likely mechanism that explains the anti-natriuresis. Given these effects, we predicted that the ECD would increase ENaC open probability ($P_o$), a parameter known to be activated by channel cleavage (26, 27, 34). This is tested below.

_Elevated Cholesterol Intake Increases ENaC Open Probability_—To test the effects of elevated cholesterol on ENaC $P_o$, cell-attached patch clamp of principal cells in split open collecting ducts was used to measure channel activity. Experiments were limited to younger animals, which allowed the manual dissection of collecting ducts. This limited our analysis to the effect of 3 weeks of ECD. ENaC activity was enhanced in animals on the ECD (Fig. 10). These animals exhibited a 40% increase of apparent $P_o$ without a significant effect on the number of active channels per patch. These data and the results above indicate that the effects of ECD on ENaC at week 3 were mediated by urinary protease activation of the channel.

At week 10, $U_{Na}V$ returned toward control. However, any further changes of channel $P_o$ could not be measured because patch clamp measurements could be made in these older animals. However, based on the results of the protease assay and the observed fragmentation of γ-ENaC, we predict that elevated ENaC activity was sustained and that renal compensation occurred by other unidentified mechanisms to restore Na$^+$ balance.

**DISCUSSION**

We describe for the first time acute and rapid effects of enhanced dietary cholesterol on renal function. These changes occurred prior to any observed increase of blood pressure and were manifested as rapid (within a time frame of days) anti-natriuresis. We also demonstrate effects of the ECD and, by extension, changes of renal membrane cholesterol content on ENaC activity in isolated split open collecting ducts. We further develop an antibody that recognizes the
cleaved form of the α subunit of ENaC and a protease assay that examines the activity of urinary proteases that can target the γ subunit of this channel. Using these surrogates of channel activation, we further demonstrate enhanced γ subunit cleavage by ECD and the coordinated enhanced activity of proteases that can target the first followed by those that can target the second cleavage sites on γ. We propose that changes of activity of these extracellular proteases provide a mechanism for the observed Na⁺ retention. Secondary compensatory mechanisms were also observed that normalized Na⁺ excretion in the ECD animals. This compensation was observed despite the sustained increase of ENaC-activating proteases and probably represents regulatory processes in upstream segments of the nephron. Altogether, these results represent a cascade of events that modify renal Na⁺ handling, specifically in the CCD, in response to changes in membrane cholesterol composition. Despite the initiation of compensatory mechanisms to correct the impaired U₄₆Vₓ, if left unchecked, chronically elevated cholesterol intake can potentially contribute to sodium retention and volume-dependent hypertension and may underlie physiological or pathophysiological changes of renal function by changes of membrane composition.

Membrane Cholesterol and ENaC Activity—Mammalian plasma membranes consist of ~50% cholesterol. Rat renal membrane cholesterol content is in the range of 0.3–0.5 μM/mg of protein when normalized to plasma membrane protein and 2–3 μg/100 μg when normalized to total cellular protein (35). Our data are consistent with these values. In the present study, ECD elevated renal membrane cholesterol content by 16 and 36% over control at weeks 3 and 10, although the change at 3 weeks was not statistically significant. These changes are in the range of those observed in vitro. For example, Christian et al. (36) have found that even under the harshest and prolonged (7-h) treatment with cholesterol-enriching agents (e.g. cholesterol cyclodextrin mixture) and under ideal conditions obtained by using cultured cells, membrane cholesterol increases in a variable range of 40–300%, depending on cell type and enrichment method. When they examined enrichment after 1 h, a commonly used in vitro maneuver, the changes were only in the 25–70% range, similar to those we find in vivo.

Using these short enriching conditions and in vitro cultured cells, others have reported activation of ENaC in the range of ~55% (15). Our own data in Xenopus oocytes indicate an increase of ENaC activity in the range of 50–200% with a 1-h stimulation of membrane cholesterol content with a cholesterol/cyclodextrin mixture. Therefore, the current changes of renal membrane cholesterol content are in the range of those capable of causing marked in vitro changes and also represent the first reported in vitro effects on ENaC.

Notwithstanding the exact magnitude of activation, our data demonstrate that dietary cholesterol modifications can and do affect renal Na⁺ transport via ENaC and that these changes would precede the changes of blood pressure observed with prolonged or marked hypercholesterolemia. We also predict, given differences between cholesterol synthesis in rats and humans, that these changes may be exaggerated in humans, where there is a lack of a compensatory decrease of endogenous synthesis (24).

The observed anti-natriuretic effects of cholesterol are probably multifaceted. We cannot rule out effects on upstream segments of the nephron, and indeed such effects are likely, given the compensation observed. However, we can determine that ENaC activity was enhanced by the ECD without an effect on the two main hormones that could cause long term changes of channel activity, insulin and aldosterone. In this case, the ensuing effect is probably due to either a combination of potential direct effects of cholesterol on the channel mediated by modification of the lipid bilayer or secondary effects of ECD on ENaC by modifying the activity of ENaC-acting proteases. In this respect, both mechanisms have been shown to affect ENaC activity, where it was shown in cell systems that membrane cholesterol content modifies the activity of channels and transporters, including ENaC (5, 13, 15, 37, 38) and that α- and γ-ENaC cleavage enhances channel activity (21, 22, 29). Interestingly, recent data further indicate that it is cleavage of γ in vivo that is more important to channel activation rather than α (28, 30, 32), a result consistent with the current findings.

ENaC Activation, Expression, Processing, and Cleavage—The CCD is accepted to be the final regulated step in Na⁺ reabsorption in the nephron. The CCD reabsorbs 2–3% of the initial filtered Na⁺ load and, by doing so, determines the exact magnitude of excreted Na⁺ (usually 1–2%, depending on Na⁺ balance). Therefore, under most conditions, the CCD reabsorbs 50–75% of the Na⁺ leaving the distal tubule. ENaC activity is rate-limiting to Na⁺ reabsorption in the CCD; therefore, the activity of this channel determines the portion of Na⁺ reabsorbed in the CCD and that which is excreted. We find that ECD increased activity by nearly 40%, which would be expected to cause a similar, if not larger, decrease of U₄₆Vₓ, given the argument above. This is exactly the range observed (Fig. 1) and demonstrates the importance of the effect of a small but significant increase of ENaC Pₒ.

α- and γ-ENaC have been identified to be endogenously processed by intracellular serine proteases, namely furin (31, 39). In rats on a low Na⁺ diet, nearly all surface γ and the majority of the total cellular pool of γ is in the shorter processed form (30). Our data are consistent with this because the majority of γ is processed in animals that were fed a relatively low Na⁺ diet (0.25%). In the present study, enhanced processing by low Na⁺ was also extended to the α subunit. These data suggest the existence of a feedback loop, which modifies subunit processing based on Na⁺ balance and intake.

It is important to keep in mind that the plasma membrane ENaC protein pool is a small portion of total cellular ENaC. Further, a large ENaC pool also exists at the membrane in an inactive or electrically silent form (27, 34, 40). Therefore, changes of ENaC protein levels at the entire cell content could understate changes at the plasma membrane, and large changes at the plasma membrane may only lead to small changes at the total cellular pool. A better determinant of ac-

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4 M. S. Awayda, unpublished observations.
tivated ENaC would be to examine the levels of processed subunits. This has not been possible until now, with the development of a new α-ENaC antibody that recognizes the processed form of the subunit. Examining the processed levels of ENaC subunits represents an improvement in the ability to determine ENaC activation as compared with examining total ENaC protein and especially unprocessed ENaC. However, this alone is not a complete surrogate of active channels because the proteolytically processed ENaC pool also includes ENaC processed in the Golgi and other intracellular membrane by proteases, such as furin. Thus, a better surrogate of renal ENaC activation is only obtained after examining processed and subunit protein levels as well as luminal channel-activating proteases. This is now made feasible by the development of the protease assay. Collectively, these data, along with measurement of channel activity or NPo, indicate that ECD activates ENaC by activating luminal proteases.

**Protease Activity: Candidate Proteases**—Collectively, our data indicate increased urinary protease activity after the ECD. They also indicate that different extracellular proteases are acting on the γ subunit. The time course of these changes was largely delineated from our novel amidolytic assay. These assays are commonly used to examine protease activity. In most cases, a peptide of a specific sequence is associated as a substrate of a known protease, and biological activity toward a specific peptide is interpreted as the presence of this specific protease. However, proteases have cross-reactivity with many substrates and vice versa. For example, the peptide PFR (proline-phenylalanine-arginine) has been used to examine the activity of prostatin (41). However, PFR is a target of many proteases, including kallikreins and trypsin. To circumvent these problems, we developed this assay using peptides with sequences specific to ENaC. Assuming that these sites are solution-accessible in the channel, a finding verified experimentally by our laboratory and by other investigators (21, 22, 29), it is likely that the reported activity is a reasonable representation of the activity of proteases that can target these sites. This approach sacrifices the ability to identify a specific proteases, probably a complicated issue because many proteases may act on these ENaC sequences, especially given the number of proteases that have been shown to cleave the ENaC subunits (see discussion in Ref. 29). However, this approach results in a better understanding of the overall activity of proteases targeting these sequences and therefore provides a better representation or index of real-time channel activation. Moreover, it is important to keep in mind that these peptides continue to exhibit a remarkable degree of specificity because virtually no overlap in the changes of activity was observed between these two homologous peptides (Fig. 9).

A different temporal response was observed for proteases that targeted the first and second sites in γ, suggesting that they represent two distinct extracellular proteases that are stimulated by hypercholesterolemia. Candidate extracellular proteases that can act on this subunit include elastase, prostatin, and plasmin/plasminogen (27, 32, 42, 43). The latter two proteases are present in urine. Plasmin was also recently shown to cleave γ-ENaC at a site slightly downstream of RKRR (the second cleavage site on γ), and in this case, such cleavage may explain the presence of two additional products in cleaved γ that are shifted from the main cleaved form at 60 kDa (32). Moreover, hypercholesterolemia has been shown to increase elastase and plasmin activity in the rat (44). A similar effect was also observed in humans, where the activity of renin, a known protease, is enhanced in hypercholesterolemia (45). The activities of many lipases and sterol regulatory elements are also regulated by proteolytic cleavage, many by serine proteases (46). Thus, hypercholesterolemia induced by the ECD probably activated urinary proteases, which in turn increased ENaC activity manifested as an increase of P_0, leading to increased Na^+ retention.

**Dietary Changes**—Although the contribution of hypercholesterolemia to atherosclerosis and cardiovascular disease is well established, the acute effects of elevated dietary cholesterol on arterial blood pressure secondary to renal solute regulation are not well described. The high prevalence of obesity associated with hypercholesterolemia and hypertension suggests that elevated cholesterol contributes to the manifestation of elevated blood pressure. Our data are the first to demonstrate that an ECD can cause sufficient changes of circulating cholesterol and cause changes of ENaC activity, leading to an acute impairment of renal excretion. These effects occurred prior to any significant changes of SBP, and our data suggest that elevated dietary cholesterol can induce renal dysfunction before an increase in SBP can be diagnosed and that chronic hypercholesterolemia can exacerbate arterial pressure changes by confounding renal effects that favor Na^+ retention, which are further confounded by the abundance of salt in Western diets.

**Conclusions**—The present study demonstrates that ECD can acutely impair renal function by inducing anti-natriuresis and by sustained changes to ENaC processing, activity, and the activity of ENaC-activating urinary proteases. Our data further demonstrate the presence of compensatory mechanisms that act to restore Na^+ balance. These mechanisms may include changes in the activity of a Na^+ transporter in a segment upstream of the collecting duct or alternatively small compensation in the density of plasma membrane-bound ENaC protein. In either case, our data collectively demonstrate a new paradigm of channel regulation that may account for changes in renal sodium handling induced by diet and may underlie a component that contributes to essential hypertension.

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