

High-Salt Diet Causes Osmotic Gradients and Hyperosmolality in Skin Without Affecting Interstitial Fluid and Lymph

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Abstract—The common notion is that the body Na^+ is maintained within narrow limits for fluid and blood pressure homeostasis. Several studies have, however, shown that considerable amounts of Na^+ can be retained or removed from the body without commensurate water loss and that the skin can serve as a major salt reservoir. Our own data from rats have suggested that the skin is hypertonic compared with plasma on salt storage and that this also applies to skin interstitial fluid. Even small electrolyte gradients between plasma and interstitial fluid would represent strong edema-generating forces. Because the water accumulation has been shown to be modest, we decided to reexamine with alternative methods in rats whether interstitial fluid is hypertonic during salt accumulation induced by high-salt diet (8% NaCl and 1% saline to drink) or deoxycorticosterone pellet implantation. These treatments resulted both in increased systemic blood pressure, skin salt, and water accumulation and in skin hyperosmolality. Interstitial fluid isolated from implanted wicks and lymph draining the skin was, however, isosmotic, and Na^+ concentration in fluid isolated by centrifugation and in lymph was not different from plasma. Interestingly, by eluting layers of the skin, we could show that there was an osmolality and urea gradient from epidermis to dermis. Collectively, our data suggest that fluid leaving the skin as lymph is isosmotic to plasma but also that the skin can differentially control its own electrolyte microenvironment by creating local gradients that may be functionally important. (*Hypertension*. 2017;69:660-668. DOI: 10.1161/HYPERTENSIONAHA.116.08539.)

• **Online Data Supplement**

Key Words: blood pressure ■ body water ■ extracellular fluid ■ extracellular matrix ■ hypertension

A commonly accepted core mechanism in fluid volume and blood pressure regulation is the parallel relationship between body Na^+ and extracellular fluid content.¹ It is assumed that Na^+ readily equilibrates between the intravascular and interstitial compartments that together constitute the extracellular fluid and that Na^+ concentrations are not remarkably different between the interstitial and intravascular volume. This idea is based on the relatively simple physicochemical concept of passive body fluid equilibrium in closed systems.² To maintain blood pressure homeostasis, body fluid volume and thereby body Na^+ content has to be maintained within narrow limits.

In long-term observations in humans, however, several studies have shown that considerable amounts of Na^+ are retained or removed from the subjects' bodies without commensurate water retention or loss.³⁻⁶ This finding suggests that Na^+ could be stored somewhere in the body without commensurate water retention and thereby be inactive from a fluid balance viewpoint.⁷ Previously unidentified extrarenal, tissue-specific regulatory mechanisms that control the release and storage of Na^+ from a kidney-independent reservoir are

a requirement in this scenario, thereby questioning the usual notion of a 2-compartment model.² Later studies have indicated that skin might serve as major Na^+ reservoir.⁸⁻¹⁰ An implication of these observations might be that there is not a strict isotonicity of all body fluids and that skin electrolyte concentrations do not necessarily equilibrate with blood electrolytes.

One consequence of such electrolyte accumulation in excess of water would be that it might cause local hypertonicity. Indeed, using vapor pressure osmometry, we recently demonstrated that Na^+ accumulation in skin as a consequence of feeding the rats a high-salt diet (HSD) results in a tissue that is hyperosmotic relative to plasma.¹¹ Supporting this notion, interstitial fluid (IF) sampled by microdialysis was found to be ≈ 10 mosmol/kg, and the Na^+ concentration in tissue regions presumed to be lymphatics ≈ 20 mmol/L higher than plasma.¹¹

Such electrolyte gradients would, even with a low capillary reflection coefficient, represent formidable transcapillary forces that would favor massive edema formation. Because the fluid accumulation in rats where salt accumulation has been induced by HSD and deoxycorticosterone acetate (DOCA) combined

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with 1% saline drinking water has been shown to be modest,^{10,11} we asked whether the gradients reported by us were representative and decided to isolate IF using alternative approaches. The present results suggest that there is no osmotic gradient between IF in deeper dermis and plasma, but rather local gradients in skin such that the most superficial layer next to the keratinocytes contains more osmolytes than the deeper dermis. Herein, we thereby underpin the recently proposed hypothesis that the skin may act as a functional counter current system and thus capable of differentially controlling its own microenvironment.

Methods

Detailed Methods are available in the [online-only Data Supplement](#).

Animal Protocols

All animal experiments were conducted in accordance with the regulations of the Norwegian State Commission for Laboratory Animals, and with approval from the AAALAC International accredited Animal Care and Use Program at University of Bergen. Male Sprague-Dawley rats ($n=61$), 8 to 10 weeks old, and with a body weight of 250–300 g were randomly assigned to either low salt diet (LSD) ($<0.1\%$ NaCl and tap water; $n=20$) or HSD (8% NaCl and 1% saline water to drink; $n=21$) for 2 weeks. Another group received DOCA (50 or 100 mg/pellet; Innovative Research of America; $n=20$) implanted subcutaneously and were given 1% saline water to drink for 3 consecutive weeks. Rats were anesthetized with 2% isoflurane in combination with O_2 or with pentobarbital sodium, 50 mg/kg body weight, given IP, in terminal experiments.

Blood pressure was measured noninvasively with a tail cuff system (CODA-6; Kent Scientific, Torrington, CT).

Isolation of IF and Lymph

IF was collected by implantation of multifilamentous nylon wicks¹² presoaked in mock IF (50% plasma diluted in Ringer acetate) for 90 minutes or by centrifugation of skin at 100g.¹³ Lymph (10–15 μ L) was sampled after cannulating a collecting lymphatic of ≈ 200 μ m running laterally on the tail.

Analytic Procedures

Osmolality in IF, lymph, skin samples, and plasma was measured in a vapor pressure osmometer as described in detail previously.¹¹ The size distribution of macromolecules in plasma and lymph was determined by size-exclusion chromatography, and tissue electrolytes and water content were analyzed as described previously.¹¹ Tissues were ashed and Na^+ and K^+ concentrations measured by atomic adsorption spectrometry (Model 3100; Perkin Elmer).

From skin discs made with a cork bore, we cut samples containing epidermis–upper dermis and lower dermis that were eluted overnight to isolate osmolytes that were assessed with the vapor pressure osmometer. Urea was measured in plasma and skin discs using a colorimetric assay kit (BioVision, Milpitas, CA), and the protocol described by the manufacturer.

Data Analysis and Statistics

Data and statistical analysis was performed using GraphPad PRISM version 6.0. Results were compared using 2-tailed t tests. Differences between groups were assessed by ANOVA followed by Tukey multiple comparisons test unless otherwise specified. Values are given as mean \pm SD, and $P<0.05\%$ was considered statistically significant.

Results

Establishment of Models of Salt-Sensitive Hypertension

A major question that we wanted to address was whether there were electrolyte gradients between IF and plasma in situations

where salt accumulated in the tissue. We, therefore, decided to use the same models as those used previously to demonstrate salt accumulation, namely feeding a HSD¹⁰ and the DOCA-salt model without uninephrectomy.¹⁴

Blood pressure was measured using the tail cuff method at the initiation of HSD or DOCA treatment (day 0) and was not different between rats assigned to LSD, HSD, and DOCA-salt groups. As shown in Figure 1A, HSD and DOCA salt resulted in an increase in mean blood pressure that averaged 18 and 51 mmHg compared with LSD, respectively, in line with what have been reported previously where blood pressure was measured with a catheter in awake animals.^{10,14}

To investigate whether the HSD and DOCA-salt treatment resulted in salt accumulation, we measured the skin water content by drying the skin until stable weight and found that there was a modest increase in water content of 7% in HSD compared with LSD, a difference that was more pronounced, averaging 18% in the DOCA-salt rats (Figure 1B). The HSD and DOCA-salt treatment also resulted in an increase in Na^+ content relative to water (Figure 1C) of 17% and 25%, respectively, and to Na^+ content relative to dry weight of 37% and 43% (Figure 1D). The more pronounced Na^+ than water accumulation in HSD and DOCA salt than that in LSD rats suggests that some is bound as inactive in tissue glycosaminoglycans.¹⁰ Collectively, the blood pressure increase and salt and water accumulation in skin corresponds well to what has been found in previous experiments with these models.^{10,14}

Tissue Osmolality

We recently demonstrated that HSD results in skin hyperosmolality relative to plasma¹¹ and wanted to verify this finding in the present series. Whereas the osmolality in dermis was not different from plasma in LSD rats (Figure 2), the average osmolality of dermis was 12 and 13 mosmol/kg higher than that in plasma in HSD rats and DOCA-salt rats (Figure 2), respectively, thereby verifying that skin salt accumulation leads to tissue hyperosmolality.

Isolation of IF Using Wicks

Having shown that the diet induced salt accumulation and tissue hyperosmolality, we asked whether the increased tissue salt was reflected in the IF and implanted nylon wicks¹⁵ that had been primed by mock IF, that is, a 1:1 mixture of rat plasma and Ringer solution, subcutaneously for IF isolation. The wick technique was chosen as an alternative to microdialysis applied in a previous study.¹¹ In an attempt to verify that the wick fluid would be capable of reflecting changes in surrounding IF, we in preliminary experiments used a crossover method,¹⁶ in which wicks were soaked in solutions that were made hyperosmolal (340 mosmol/kg) by adding NaCl or hypoosmolal (250 mosmol/kg) by diluting the Ringer solution with distilled water. The rationale for this method is that the hyperosmotic wick fluid will be diluted and the hypoosmotic will be concentrated and that the true IF concentration is found where the line connecting the points cross the identity line for observed and primer solution values. We observed that after 90 minutes of implantation, the osmolality in fluids isolated from wicks presoaked in hypo- and hypertonic priming

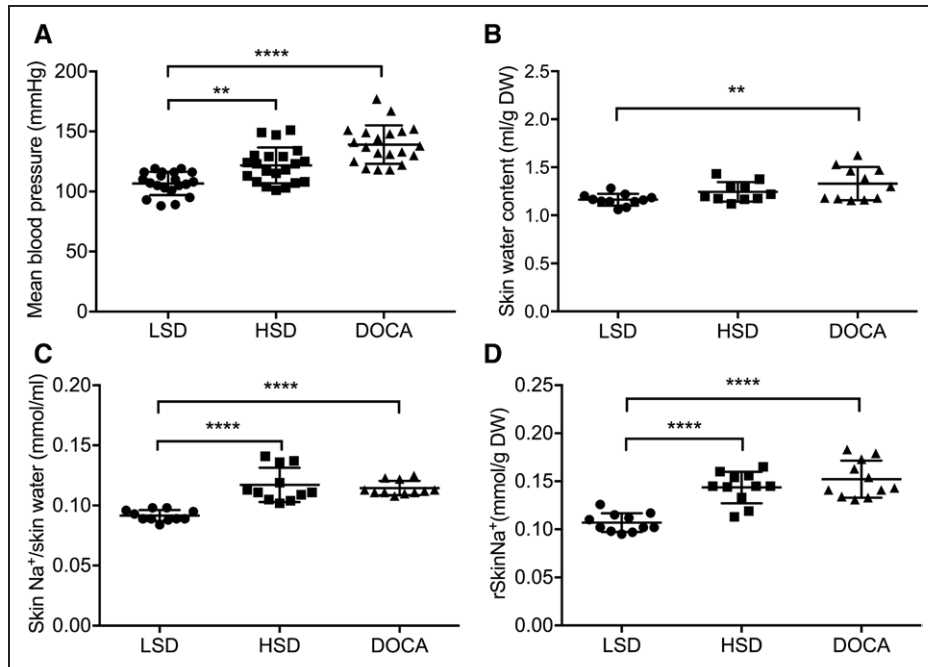


Figure 1. **A**, Individual values of mean blood pressure measured with tail cuff plethysmography in awake rats in low-salt diet (LSD; $n=20$), high-salt diet (HSD; $n=21$), and deoxycorticosterone acetate (DOCA)-salt diet ($n=20$). Also shown are mean and SD. $**P<0.01$ and $****P<0.0001$ when compared with LSD. **B**, Individual values of skin water content in LSD ($n=11$), HSD ($n=11$), and DOCA-salt diet ($n=11$). Also shown are mean and SD. $**P<0.01$ when compared with control. **C**, Individual values of skin Na^+ content relative to skin water in LSD, HSD, and DOCA-salt diet ($n=11$ for all diets). Also shown are mean and SD. $****P<0.0001$ when compared with control. **D**, Individual values of skin Na^+ content relative to dry weight in LSD, HSD, and DOCA-salt diet ($n=11$ for all diets). Also shown are mean and SD. $****P<0.0001$ when compared with control.

solutions was identical and, thus, that this was a sufficient equilibration period and that the wick fluid would be able to reflect the surrounding IF.

We then implanted wicks subcutaneously that had been presoaked in plasma and Ringer solution with an osmolality averaging that of plasma (306 mosmol/kg). As shown in Figure S1 in the [online-only Data Supplement](#), the osmolality in wick fluid was similar to that of plasma in LSD and

HSD rats, suggesting that there is no gradient between IF and plasma even in HSD rats with hyperosmolar skin.

Isolation of IF by Skin Centrifugation

We wanted to use an alternative method for IF isolation and chose tissue centrifugation that has been shown to yield samples representative for IF, at least with respect to proteins.¹³ In an attempt to reduce the potential contamination from intracellular fluid, we applied a lower g-force (100g) to isolate fluid than in the initial method evaluation study. In contrast to wick fluid, the mean osmolality in IF isolated by centrifugation was 13 mosmol/kg higher than that in plasma (Figure 3A) in LSD rats. A slightly, but not significantly, higher difference between centrifugate and plasma of 17 mosmol/kg was found in HSD and DOCA-salt rats, the latter having an even higher salt accumulation in skin than the HSD rats.

Because we suspected that the higher osmolality in skin IF than in plasma irrespective of salt intake was a result of leakage of intracellular ions (notably K^+) during centrifugation, we measured the K^+ and Na^+ concentration in both fluids. We found that the K^+ concentration in IF was ≈ 20 mmol/L higher in HSD and LSD than in plasma (Figure 3B). In contrast, IF Na^+ concentration was 10 to 15 mmol/L lower than plasma (Figure 3C), suggesting that the IF isolation procedure led to diffusion of K^+ down a steep concentration gradient combined with a slight extrusion of intracellular fluid rich in K^+ diluting the extracellular Na^+ . This observation explains the measured difference in osmolality electrolyte concentration and suggest that the method is unsuitable for IF electrolyte studies.

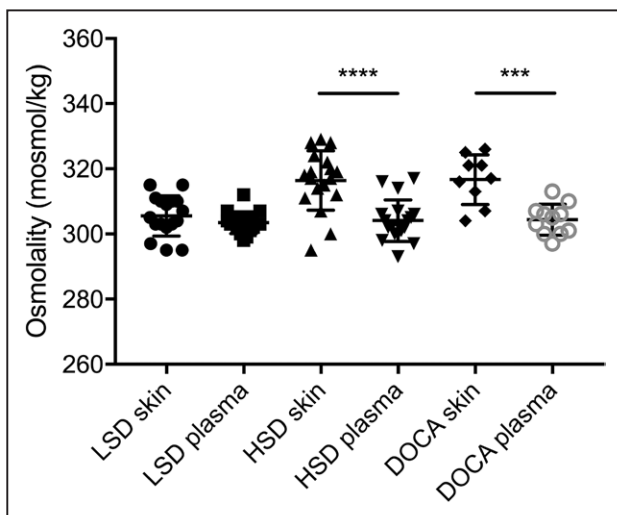


Figure 2. Individual osmolality values of skin ($n=17$) and plasma ($n=17$) in low-salt diet (LSD), high-salt diet (HSD; $n=18$, skin and plasma), and deoxycorticosterone acetate (DOCA)-salt diet ($n=9$ for skin and $n=11$ for plasma). Also shown are mean and SD. $***P<0.001$ and $****P<0.0001$ when compared with plasma.

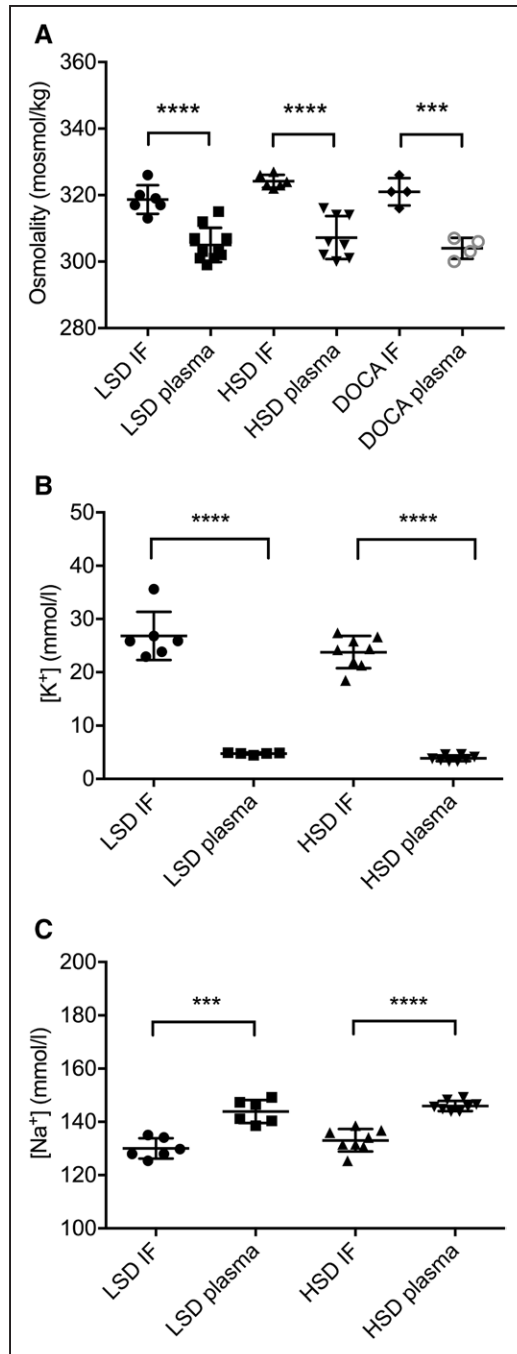


Figure 3. **A**, Individual osmolality values of skin interstitial fluid (IF) isolated by centrifugation ($n=6$) and plasma ($n=10$) in low-salt diet (LSD), high-salt diet (HSD; $n=6$ for IF, $n=8$ for plasma), and deoxycorticosterone acetate (DOCA)-salt diet ($n=4$ for IF and plasma). Also shown are mean and SD. *** $P<0.001$, **** $P<0.0001$ when compared with respective plasma. **B**, Individual values of K⁺ concentration in skin interstitial fluid (IF; $n=6$) and plasma ($n=5$) in LSD and HSD ($n=8$ for IF and plasma). Also shown are mean and SD. **** $P<0.0001$ when compared with respective plasma. **C**, Individual values of Na⁺ concentration in skin interstitial fluid (IF) and plasma in LSD ($n=6$ for IF and plasma) and HSD ($n=8$ for IF and plasma). Also shown are mean and SD. *** $P<0.001$, **** $P<0.0001$ when compared with respective plasma.

Osmolality in Lymph Is Similar to Plasma

It is generally agreed that in a steady-state situation, lymph is representative for IF¹⁷ and can, thus, serve as a reference fluid

in our context. After some trial and error, we managed to cannulate collecting lymphatics draining the tail skin and were, with intermittent gentle massage of the tail, able to isolate 10 to 15 μL in 10 to 20 minutes. All the included samples were clear and without any blood contamination when inspected in situ with the stereomicroscope used during cannulation.

In preliminary experiments, we found that there may be evaporation during the sampling period as shown by an increase in osmolality of $\leq 5\%$ of plasma contained in capillaries exposed to conditions similar to the harvesting situation. Harvesting into mineral oil-filled capillaries might have reduced this problem but was not feasible because of loss of capillary force necessary to sample lymph. In each experiment, we, therefore, corrected for evaporation during harvesting by the increase in osmolality in an equal volume of plasma isolated from a distal cut of the tail vein. The duration of the exposure and the conditions were identical to those prevailing during lymph sampling.

The osmolality of lymph and corresponding tail plasma is shown in Figure 4. We observe that there was no difference in osmolality between lymph and tail plasma (used as reference in this series) neither in LSD and HSD, nor in DOCA-salt rats (Figure 4). In this series, we also measured Na⁺ concentration in lymph recovered from the osmometer after measurement, and even though we had rather high scatter in the data, we found no difference in concentration between lymph and plasma for any of the groups (data not shown).

Because the tail lymph originated from skin that is different from that of the back, we measured Na⁺ concentration in tail skin after ashing. As for back skin, we found a modest increase in water content in DOCA-salt rats (Figure S2A) and a marked increase in skin Na⁺ relative to water (Figure S2B) and dry weight (Figure S2C). Collectively, these data suggest that the tail skin behaved as back skin and that there is no electrolyte gradient between plasma and IF, even in situations where the tissue osmolality is increased.

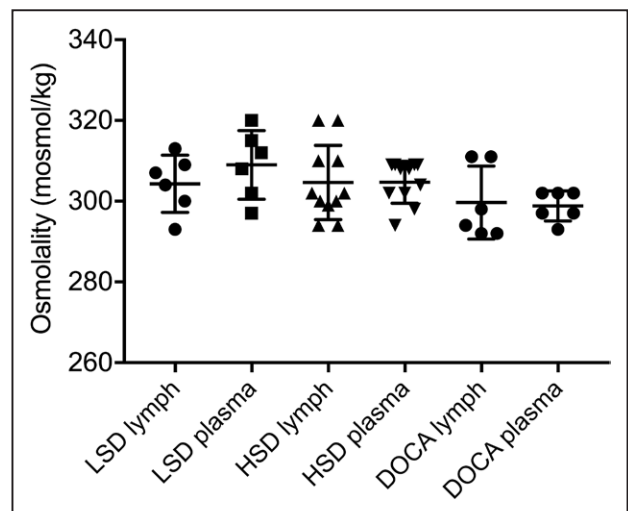


Figure 4. Individual osmolality values of skin lymph and plasma in low-salt diet (LSD; $n=6$ for lymph and plasma; **A**), high-salt diet (HSD; $n=11$ for lymph and plasma; **B**), and deoxycorticosterone acetate (DOCA)-salt diet ($n=6$ for lymph and plasma; **C**). Also shown are mean and SD. None of the lymph osmolality values were different from the corresponding plasma.

No Difference in Capillary Sieving Properties

Having lymph available enabled us to assess the sieving properties of the capillary wall that may be affected if the high-salt load results in a low-grade inflammation^{14,18} by size-exclusion chromatography (Figure 5). We first assessed the concentration of albumin in plasma and lymph in all groups and found that plasma albumin was 33.0 and 28.0 mg/mL in the LSD and HSD groups, respectively ($P>0.05$), with corresponding albumin lymph to plasma (L/P) ratios of 0.31 and 0.29 ($P>0.05$; Figure 5A). In DOCA salt, plasma albumin averaged 18.8 mg/mL ($P<0.01$ and $P=0.056$ when compared with LSD and HSD, respectively), with a L/P of 0.31 ($P<0.05$ for plasmas for both of the other groups; Figure 5A).

In the chromatograms, we were able to separate protein peaks that we have earlier identified as $\alpha 2$ macroglobulin, fibrinogen, murinoglobulin, haptoglobin, IgG, and $\alpha 2$ antitrypsin by mass spectrometry.¹⁹ We related their respective UV signals to that of albumin in lymph and plasma to investigate whether there were increased L/P ratios relative to their respective albumin concentration (Figure 5B). There were no indications of such reduced size selectivity because none of the respective relative concentrations differed significantly between the groups (Figure 5B). Collectively, the unchanged sieving properties suggest that there was no difference in capillary size selectivity during high-salt conditions.

Osmolyte and Electrolyte Gradients in Skin

Because the lymph osmolality and Na^+ concentration in lymph were not different from plasma, we asked whether there could be electrolyte gradients within the skin as suggested in a recent article.²⁰ We, therefore, eluted, in distilled H_2O , skin samples separated into epidermis–upper dermis (upper 0.5 mm) and lower dermis (subsequent 0.5 mm) with the tissue slicer as described for the punch biopsies. That the latter sample type did not include subcutaneous fat was shown

by regular hematoxylin-eosin–stained histological sections of back skin from 2 rats.

In samples reconstituted from eluate that had been evaporated to dryness, the osmolality per gram dry weight was 24%, 36%, and 111% higher in epidermis–dermis than in dermis for LSD ($P<0.01$), HSD ($P<0.01$), and DOCA salt ($P<0.0001$; Figure 6A). Moreover, in these samples, the osmolality in epidermis–dermis was significantly higher in HSD and DOCA-salt rats than that in LSD rats ($P<0.05$ and $P<0.01$, respectively). These experiments suggest that there is an osmolyte gradient from epidermis to dermis in skin and that this gradient is increased during salt accumulation.

To determine whether the elutable fraction reflected the salt retained in the skin, we eluted tissue slices of skin in distilled H_2O . By ashing the tissue after elution, this fraction was measured by relating the mass of Na^+/K^+ in eluate to that remaining in eluted skin. We found that $21\pm 7\%$ and $14\pm 3\%$ of Na^+ and $24\pm 4\%$ and $20\pm 4\%$ of K^+ ($n=6$ for all groups) remained in the tissue after elution. Although there was a tendency to a higher eluted fraction of Na^+ in HSD, none of these fractions differed significantly between HSD and LSD diets. Elution should thereby reflect a given fraction of the tissue level of these electrolytes.

When searching for osmolytes that contributed to the observed gradient, we assessed Na^+ and K^+ in reconstituted eluate and found no concentration differences in these electrolytes in LSD skin (Figure 6B and 6C). There was, however, a Na^+ concentration gradient opposite that for osmolality. Na^+ in dermis exceeded the concentration in epidermis–dermis with 16% ($P<0.05$) and 27% ($P<0.01$) in HSD and DOCA-salt rats, respectively (Figure 6B). For K^+ , a concentration gradient after that of osmolytes was found for DOCA salt only (Figure 6C). These experiments suggested that Na and K^+ could not explain the observed osmolyte gradient.

Because urea is an important contributor to the osmotic gradient found in the kidney medulla, we determined urea in

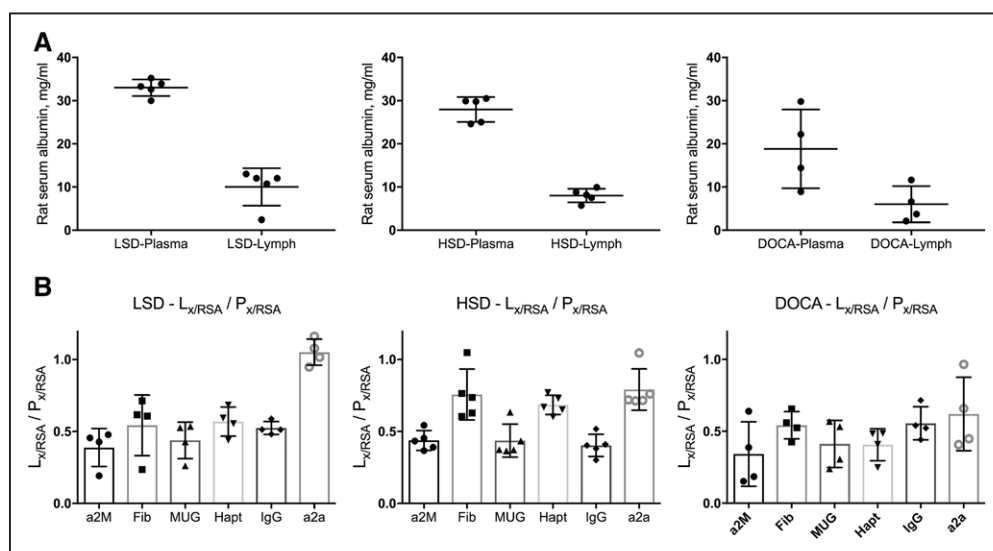


Figure 5. **A**, Individual values of concentration of rat serum albumin in plasma and lymph as determined by high-performance liquid chromatography in low-salt diet (LSD; $n=5$ for lymph and plasma; **left**), high-salt diet (HSD; $n=5$ for lymph and plasma; **middle**), and deoxycorticosterone acetate (DOCA)–salt ($n=4$ for lymph and plasma; **right**). Also shown are mean and SD. **B**, Individual values for sieving of plasma proteins relative to the corresponding sieving of albumin in lymph and plasma in LSD ($n=4$; **left**), HSD ($n=5$; **middle**), and DOCA salt ($n=4$; **right**). Also shown are mean and SD. $\alpha 2a$ indicates $\alpha 2$ antitrypsin; $\alpha 2M$, $\alpha 2$ macroglobulin; Fib, fibrinogen; Hapt, haptoglobin; and MUG, murinoglobulin.

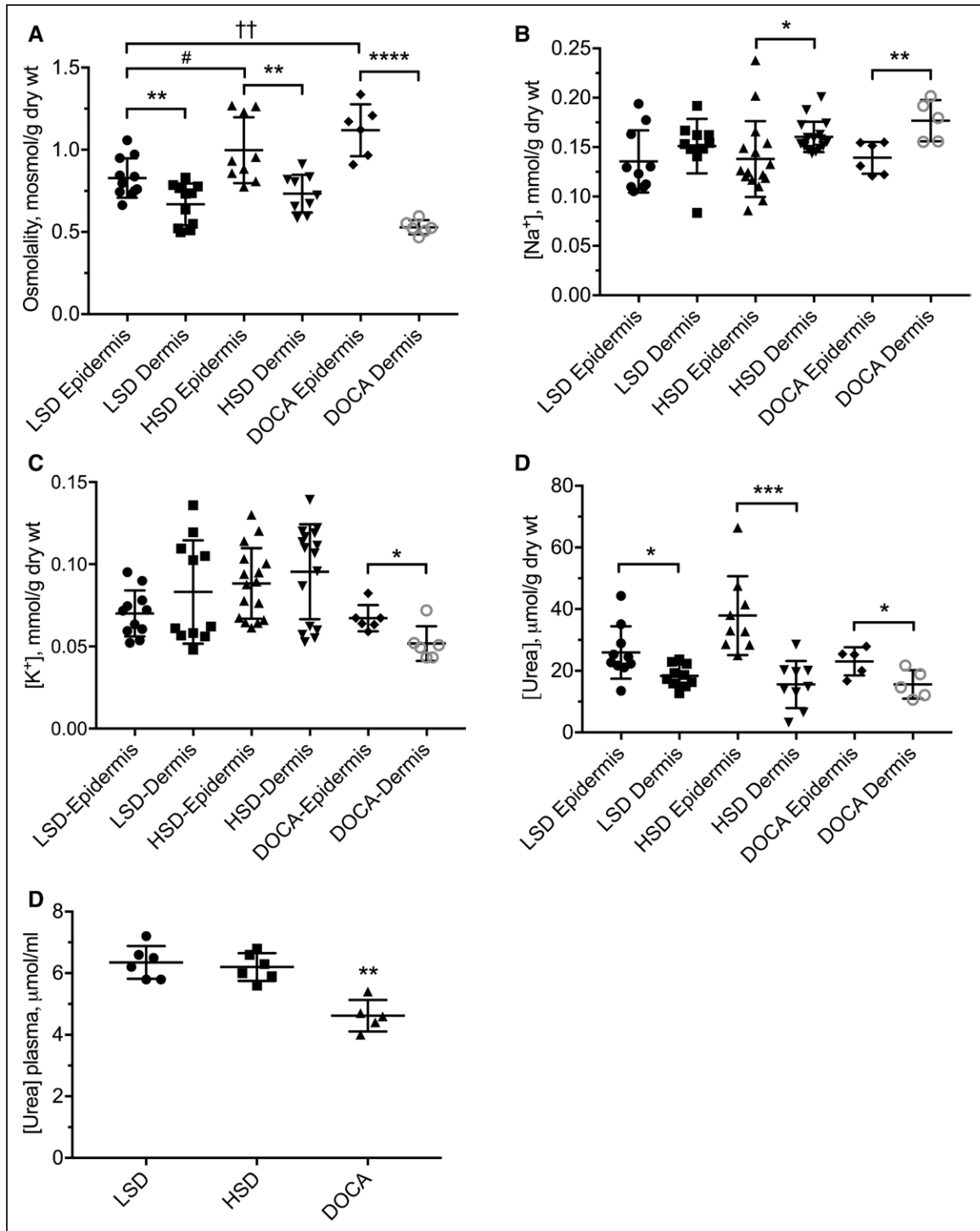


Figure 6. Individual values of osmolal content after elution in distilled H₂O of epidermis and associated upper dermis (epidermis) and corresponding lower dermis (dermis) isolated from rats on low-salt diet (LSD; n=11), high-salt diet (HSD; n=9), and deoxycorticosterone acetate (DOCA)-salt diet (n=6). (#*P*<0.05 and ††*P*<0.01 vs LSD epidermis, ANOVA followed by Dunnett test). **B.** Individual values of Na⁺ content of epidermis and associated upper dermis (epidermis) and corresponding lower dermis (dermis) isolated from rats on LSD (n=10), HSD (n=17), and DOCA-salt diet (n=6). **C.** Individual values of K⁺ content of epidermis and associated upper dermis (epidermis) and corresponding lower dermis (dermis) isolated from rats on LSD (n=11), HSD (n=16), and DOCA-salt diet (n=6). **D.** Individual values of urea content of epidermis and associated upper dermis (epidermis) and corresponding lower dermis (dermis) isolated from rats on LSD (n=10), HSD (n=9), and DOCA-salt diet (n=5). **E.** Individual values of urea concentration in plasma isolated from rats on LSD (n=6), HSD (n=6), and DOCA-salt diet (n=5). (***P*<0.01, ANOVA). All panels also show mean and SD. For (A–D), **P*<0.05, ***P*<0.01, *****P*<0.0001, when comparing epidermis–dermis and dermis with Student *t* tests in corresponding tissues.

epidermis–dermis and lower dermis after homogenization and elution of excised skin. Interestingly, the urea concentration was higher in epidermis–dermis than in lower dermis in LSD, HSD, and DOCA-salt rats, being most pronounced in HSD (Figure 6D), suggesting that urea apparently contributed to the observed osmolal gradient, and supporting the idea of countercurrent exchange in the skin.²⁰ Plasma urea was equivalent in LSD and HSD, both exceeding the concentration in DOCA-salt rats ($P < 0.01$ for both comparisons; Figure 6E).

Discussion

Several recent studies have suggested that the skin may serve as reservoir for Na^+ and thereby be involved in fluid homeostasis and blood pressure regulation. Herein, we investigated whether Na^+ accumulation induced either by a HSD or DOCA combined with 1% saline to drink was reflected in skin IF. Both experimental interventions resulted in increased blood pressure and moreover in elevated skin tissue osmolality compared with plasma. The osmolality of skin IF isolated from implanted wicks or from tail skin lymph was, however, similar to that of plasma, suggesting that fluid drained from the interstitium is isosmotic to plasma. Notwithstanding this finding, by elution, we were able to demonstrate that there was an osmolality and urea gradient from epidermis through dermis to subcutis. This gradient may reflect a proposed countercurrent mechanism for electrolyte homeostasis²⁰ and calls for a more refined analysis of osmolyte and electrolyte distribution in the various layers of skin.

Evaluation of Methods and Limitations

We used wick implantation, tissue centrifugation, and lymph vessel cannulation to access the IF in skin, all having their own limitations that have to be taken into consideration when interpreting the data. They have all been evaluated and shown to reflect protein concentration in IF²¹, not so with electrolytes. Clearly, as shown here, the centrifugation method is unsuitable for studies of IF electrolytes, likely because of K^+ leakage from the cells during fluid isolation.

Wick implantation results in an acute inflammatory response and protein extravasation that subsides within 30 minutes.²² To reduce the potential influence of inflammation that may affect ion homeostasis across cell membranes and thus wick fluid osmolality, we harvested wicks after 90-minute implantation time. That ion gradients are equilibrated quickly between surrounding interstitium and wick fluid was shown by the crossover experiments with hypo- and hyperosmolal priming solutions that produced identical wick fluid osmolality after a 30-minute equilibration period. Provided that 90-minute implantation time made the influence of the inflammatory reaction minimal, wick fluid should reflect osmolality in the subcutaneous space. This assumption is supported by our finding of similar osmolality in wick fluid and lymph that may be considered as reference IF (see below).

Lymph can be harvested involving minimal trauma and represents the gold standard for IF, provided steady-state conditions²¹ that were prevailing in our experiments. Because the other major component of the tail, tendon, does not contain lymphatics,²³ the harvested tail lymph will be representative for skin. Moreover, tail skin accumulated salt to a similar

extent as back skin and should therefore be representative for skin in general. Accordingly, the lymph and wick data suggest that IF from skin is isosmotic with plasma in situations where the tissue is hyperosmotic as a result of salt accumulation. Another implication of this observation is that we, by tissue osmometry, are able to detect the inactive sodium stored in negatively charged glycosaminoglycans in the tissue⁸ that is not reflected in the IF leaving skin.

Comparison to Previous Lymph and IF Data

The question whether there is an electrolyte gradient between IF and plasma has been addressed in several previous studies, and the results are diverging and inconclusive. Haljamäe²⁴ developed a liquid-paraffin cavity technique where he could sample nanoliter samples from subcutis. Together with collaborators, he found higher concentrations of Na^+ and K^+ in subcutis than in plasma and in IF harvested from subcutaneously implanted capsules.²⁵ They ascribed these deviations from the expected Gibbs–Donnan distribution to charged anionic macromolecules in the extracellular matrix. In support of these data are the finding of ion gradients between IF sampled by wicks and lymph from subcutis and plasma.²⁶ In contrast, Gilányi et al²⁷ sampled IF with the liquid-paraffin and capsule technique and found no difference in Na^+ , K^+ , and Cl^- concentration between IF and plasma, in agreement with capsule data from subcutis obtained by Gullino et al.²⁸ The reason for this discrepancy is not evident, but as pointed out by Szabó and Magyar,²⁶ the small sample sizes make the IF samples prone to evaporation and other handling errors²⁷ that will increase the ion concentration.

If we accept that there is no gradient between IF and lymph, both sampled from subcutis in control situation, then the question is whether there is a gradient when NaCl accumulates and the tissue gets hyperosmotic relative to plasma. We addressed this issue in a recent article using energy-dispersive x-ray electron microprobe analysis on lymph vessels and microdialysis and found significantly higher Na^+ concentration with both techniques.¹¹ The microdialysis probes were placed intracutaneously, and although the dialysate had higher Na^+ concentration and osmolality than arterial plasma, there was no difference between LSD and HSD rats. The explanation for this finding is not readily evident, but these data might question the ability of the method to reflect IF osmolality shown to be similar in HSD and LSD or tissue osmolality shown here to be increased in HSD and DOCA-salt rat skin. The significantly higher Na^+ concentration in lymphatics than in plasma observed with the x-ray electron microprobe analysis may reflect intracutaneous electrolyte gradients discussed in more detail below.

Osmolyte and Electrolyte Gradients in Skin and Implications of the Data

Even though our data suggest that IF in subcutis and lymph draining from the skin are isosmotic to plasma, the elution experiments clearly show that more osmolytes can be mobilized from superficial skin, including the epidermis, than deeper dermal parts. These observations agree well with previous x-ray dispersive studies, where a gradient was demonstrated from epidermis to subcutis in human²⁹ and in guinea

pig skin.³⁰ In line with these observations, by application of 7T ²³Na⁺ magnetic resonance imaging, it was shown that there was a substantial accumulation of Na⁺ in or directly under epidermis.³¹ Interestingly, this Na⁺ accumulation was not associated with a significant volume increase in the same area as would have been expected if the Na⁺ exerted at least some of its crystalloid osmotic effect. The apparent conundrum of skin Na⁺ gradients and lack of corresponding fluid accumulation led Hofmeister et al²⁰ to suggest that there is a functional countercurrent mechanism that enables the skin to differentially control its own microenvironment. Our data showing an epidermal to dermal osmolyte gradient that also includes urea and is increased in salt accumulation supports the hypothesis. Apparently, the lower content of Na⁺ in epidermis–dermis than in the deeper dermis suggests that Na⁺ does not contribute to this gradient. We should, however, bear in mind that Na⁺ will predominantly distribute in the extracellular fluid space that will likely be higher in dermis than in the more cell-rich epidermis. Na⁺ concentration in the available space in epidermis may, therefore, be higher than in dermis and contribute to the osmotic gradient in vivo, calling for additional experiments where electrolyte gradients and distribution volumes are determined at higher resolution at the tissue level.

Perspectives

Here, we have investigated whether salt accumulation in skin results in an IF that is hypertonic relative to plasma. Wick fluid and lymph, which may both represent IF during steady-state conditions and thus representative for IF returning to the general circulation, were isosmotic to plasma. Elution experiments of epidermis together with upper dermis and lower dermis suggested that there is an osmotic gradient from superficial to deeper layers of skin. This finding indicates that the skin may differentially control its own microenvironment and together with the kidney actively participate in fluid volume regulation. A more detailed assessment of this gradient in higher resolution may lead to a better understanding of the functional implications of this observation.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Through isolation of skin lymph from rats and tissue elution, we could address whether there are osmotic gradients between interstitial fluid and plasma and within the skin during salt accumulation.

What Is Relevant?

- Recently, it has been shown that considerable amounts of Na^+ can be retained or removed from the body without commensurate water loss and that the skin can serve as a major salt reservoir.

Summary

Skin lymph is isosmotic to plasma, and the skin can differentially control its own electrolyte microenvironment by creating local gradients.

High-Salt Diet Causes Osmotic Gradients and Hyperosmolality in Skin Without Affecting Interstitial Fluid and Lymph

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Supplemental materials

High salt diet causes osmotic gradients and hyperosmolality in skin without affecting interstitial fluid and lymph

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Short title: Interstitial fluid and lymph in hyperosmolal skin

Detailed Methods

Ethical approval. All animal experiments were conducted in accordance with the regulations of the Norwegian State Commission for Laboratory Animals, harmonized to be in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and Council of Europe (ETS 123) conforming with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited Animal Care and Use Program at University of Bergen.

Animals. The experiments were performed in male Sprague-Dawley rats (n=61) from Møllegaard Breeding Colony (Skensved, Denmark), 8-10 weeks old and with a body weight of 250-300g. All rats were exposed to light on a 12:12-h cycle in a humidity- and temperature controlled environment. The rats were hosted in cages with constant temperature (25°C) and had unrestricted access to tap water. They were randomly assigned to either low salt (LSD) (<0.1% NaCl and tap water) (n=20) or high salt diet (HSD), (8% NaCl and 1% saline water to drink) for 2 weeks (n=21). Another randomly assigned group received deoxycorticosterone acetate (DOCA) (50 or 100 mg/pellet, Innovative Research of America) (n=20) that were implanted subcutaneously on their back and were given 1% saline water to drink for 3 consecutive weeks. All chows were obtained from Altromin, Lippe, Germany.

Blood pressure was measured noninvasively with a tail cuff system (CODA-6, Kent Scientific, Torrington, CT) before start and at the day of terminating the experiment.

Anesthesia and surgery. During DOCA tablet implantation or sham experiments, rats were anesthetized with 2% isoflurane in combination with O₂. In the terminal experiments, rats were anesthetized with pentobarbital sodium, 50 mg/kg body weight, given intraperitoneally unless otherwise specified. While anesthetized, the body temperature was maintained at 37 ± 1°C using a heating pad and lamp. Further anesthesia was supplied during the experiment when needed. Upon termination of the acute experiment, a final blood sample was obtained by cardiac puncture, and the rat was killed by excising the heart while still under anesthesia.

Interstitial fluid collection using wicks. Interstitial fluid was collected from anesthetized rats using a method slightly modified from that described by Aukland and Fadnes.¹ After shaving the back, multifilamentous nylon wicks (diameter ~1 mm), prewashed in acetone and ethanol,² were sewn into the subcutis for ~5 cm transverse to the spinal cord. Before implantation, the wicks were soaked in 50% plasma diluted with Ringer's solution for ~30 minutes. Five wicks were implanted in each anesthetized rat for 90 min and then removed before sacrifice. Bleeding at the entrance or exit site of the wick was rarely observed, and such wicks were excluded. To avoid potential blood contamination, both ends of each wick, along with any blood-tinged portions, were cut off and the remaining sections were immediately transferred to pre-weighed vials provided with a funnel, and centrifuged at 2000 g for 5 minutes. Isolated fluid was either analyzed immediately or frozen at -80°C for later analysis. Next, a blood sample was taken and the animal killed as described above.

Isolation of interstitial fluid from skin by tissue centrifugation. Skin interstitial fluid (IF) was isolated as described in detail previously.³ Immediately after euthanasia, the rats were

transferred to an incubator with 100% relative humidity, and excised skin samples were placed in a nylon mesh basket (pore size $\sim 15 \times 20 \mu\text{m}$) and put into a 1.5 ml microcentrifuge tube and centrifuged at $\sim 100 g$ (1000 rpm) for 10 min in an Eppendorf 5417R centrifuge. The skin IF was collected from the centrifugation tubes inside the humidity chamber, and were either analyzed directly or stored at -80°C for later analysis.

Sampling of tail lymph. For lymph sampling we used a modified procedure of that described by Aukland et al.⁴ The rat tail has two major collecting lymphatics on each side that run dorsally and ventrally to the tail vein but are usually several hundred microns separated from the vein by loose connective tissue with frequent communicating branches crossing the vein. A ~ 2 cm skin incision was made on the lateral aspect of the tail a 3-4 cm from the tail base, usually exposing the lateral tail vein. The skin was then loosened on both sides of the incision by blunt dissection, and 5 μl of Evans blue (4.5 mg/ml, USP, Harvey Labs, Philadelphia, PA), was injected subcutaneously 3-4 cm distally to the incision to visualize the lymphatics. Dye appeared almost immediately in the exposed lymphatics after light massage at the injection site, and loose connective tissue covering the vessel was dissected away under the stereomicroscope to ease cannula insertion. One lymphatic was ligated at the proximal end of the incision, resulting in a rapid expansion of the obstructed lymph vessels. An incision was made with a pair of fine scissors, and a polyethylene cannula pulled out to an outer tip diameter of $\sim 200 \mu\text{m}$ was introduced into the vessel and secured with 8-0 silk. The free end of the cannula was inserted into a 20 μl glass capillary coated with heparin in the sampling end. The amount of lymph sampled was determined with a Vernier caliper. Lymph was either analyzed directly (in the osmometer, see below), or stored at -80°C for later analysis.

Measurement of osmolality. Osmolality in IF, lymph and plasma was measured in a vapor pressure osmometer (5500 Wescor vapor pressure osmometer, Wescor Inc.) that had been calibrated with standards 100–1000 mOsmol/l. Osmolality in skin samples was measured as described in detail previously,⁵ using a method slightly modified from that described by Tornheim⁶ and Knepper.⁷ Briefly, immediately after terminating the experiment, the rat back skin was closely clipped, excised, and snap frozen in liquid nitrogen, and stored at -20°C until further analysis. Skin was kept on dry ice, and discs were prepared with a cork bore, 7.5 mm, and stored on dry ice. From these, 0.5-mm-thick discs were cut using a chilled tissue slicer (Thomas Stadie-Riggs Tissue Slicer, Thomas Scientific); immediately after cutting, they were transferred to the sample chamber of the osmometer. Reported values are the average of 3 to 5 individual samples, and the calibration was checked before and after each reading.

Size exclusion chromatography. The size distribution of macromolecules in plasma and lymph was determined by high resolution size exclusion chromatography using a 4.6 mm ID x 30.0 cm TSK gel Super SW3000 column (Tosoh Biosciences, Stuttgart, Germany) with an optimal separation range for globular proteins of 10–500 kDa on an Ettan LC System (GE Healthcare). The eluted proteins were detected by UV at 220 nm and the mobile phase was 0.1 M Na_2SO_4 in 0.1 M phosphate buffer (pH 6.7).

Albumin was quantified using a size-exclusion column (Super SW2000, 4.6 x 300 mm, 18674, Tosoh Bioscience, Tokyo, Japan) and reversed phase column (Proswift Rp4H 1 x 50 mm, Dionex, Sunnyvale, CA) coupled in series as described previously.⁸ Albumin was separated from IgG and other larger plasma proteins in the first dimension. Subsequently,

the sample that eluted in the retention time of albumin was loaded onto the second column by an inline switch at a flow rate of 0.35 ml/min and with an eight-minute acetonitrile gradient (5 – 60 %) for separation from proteins with similar molecular weight and quantification of the albumin peak. The albumin concentration was determined based on the area under the curve for standards and samples.⁹

Chemical analysis of electrolytes and water content. Chemical analysis of tissue electrolytes and water content was performed as described previously.⁵ Carcasses were completely skinned, and carcasses and skin were weighed separately to obtain wet weight. Samples were then desiccated at 90°C for 72 hours to obtain dry weight (DW). Because weights were unchanged with further drying, the difference between wet weight and DW was considered as tissue water content. Tissues were then ashed at 190°C and 450°C for 24 hours at each temperature level, and then at 600°C for 48 hours. Thereafter the ash was dissolved in 5% or 10% HNO₃. Na⁺ and K⁺ concentrations in dissolved ash and fluids were measured by atomic adsorption spectrometry (Model 3100, Perkin Elmer).

Extraction of skin electrolytes by elution. Skin discs from HSD and LSD groups were made using a cork bore with diameter 7.5 mm, and kept on dry ice. From the same disc we cut one sample containing epidermis and upper layer of dermis and another containing deeper layer of dermis with the tissue slicer as described above under “Measurement of osmolality”. The individual samples were weighed and transferred to Eppendorf tubes containing 400 µl of distilled water that were left on a shaker over night at room temperature. The following day, the skin was removed and dried until stable weight in the incubator at 35°C. The Eppendorf tube containing the fluid was placed in a vacuum evaporator to vaporize completely. Thereafter 20 µl of distilled water was added to reconstitute the sample. After vortexing, the osmolality, and Na⁺ and K⁺ concentrations, were measured as described above.

Measurement of urea. Urea was measured in plasma and skin discs (see above) using a colorimetric assay kit (BioVision, Milpitas, CA, USA) and the protocol described by the manufacturer. Skin was homogenized in 500 µl assay buffer, left over night on a shaker at room temperature, then centrifuged at 15 000 g, before samples of supernatant were added to the assay plate.

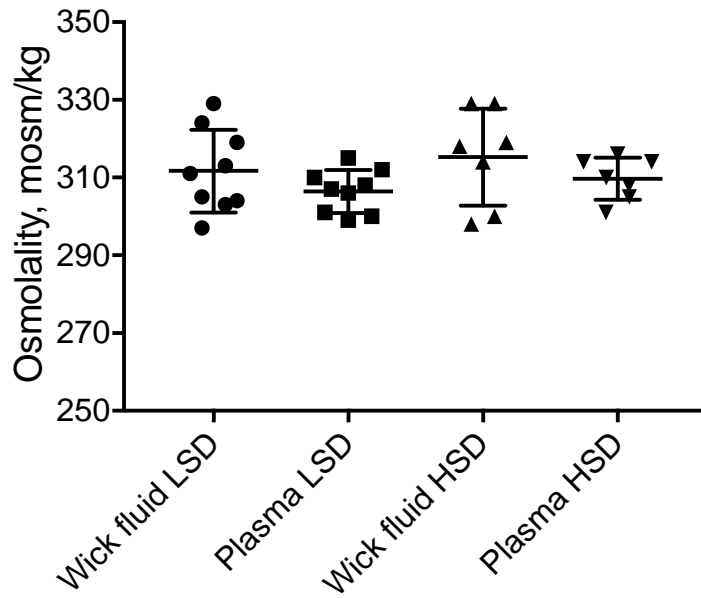
Data analysis and statistics. Data and statistical analysis was performed using GraphPad PRISM Version 6.0. Results were compared using two-tailed t-tests. Differences between groups were assessed by analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. Values are given as mean ± SD, and p < 0,05 % was considered statistically significant.

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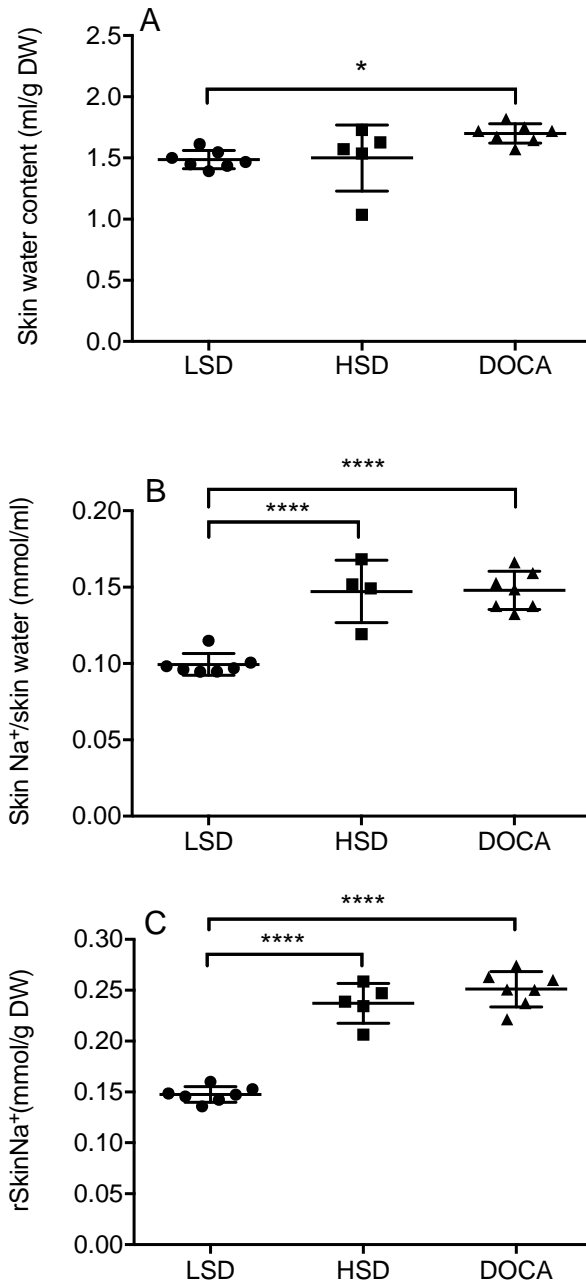
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Supplemental Figures



Supplemental Figure S1. Individual osmolality values of wick fluid isolated from skin and plasma in rats on low salt diet (LSD) (n=9 for wick fluid and plasma) and high salt diet (HSD) (n=7 for wick fluid and plasma). Also shown mean and SD.



Supplemental Figure S2.

A. Individual values of tail skin water content in low salt diet (LSD) (n=7), high salt diet (HSD) (n=5) and DOCA salt diet (n=7). B. Individual values of tail skin Na⁺ content relative to skin water in LSD (n=7), HSD (n=4) and DOCA salt diet (n=7). C. Individual values of tail skin Na⁺ content relative to dry weight in LSD (n=7), HSD (n=5) and DOCA salt diet (n=7). All panels also show mean and SD. * $p < 0.05$, ****: $p < 0.0001$, when compared with LSD.