Salt Transiently Inhibits Mitochondrial Energetics in Mononuclear Phagocytes

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BACKGROUND: Dietary high salt (HS) is a leading risk factor for mortality and morbidity. Serum sodium transiently increases postprandially but can also accumulate at sites of inflammation affecting differentiation and function of innate and adaptive immune cells. Here, we focus on how changes in extracellular sodium, mimicking alterations in the circulation and tissues, affect the early metabolic, transcriptional, and functional adaption of human and murine mononuclear phagocytes.

METHODS: Using Seahorse technology, pulsed stable isotope-resolved metabolomics, and enzyme activity assays, we characterize the central carbon metabolism and mitochondrial function of human and murine mononuclear phagocytes under HS in vitro. HS as well as pharmacological uncoupling of the electron transport chain under normal salt is used to analyze mitochondrial function on immune cell activation and function (as determined by Escherichia coli killing and CD4+ T cell migration capacity). In 2 independent clinical studies, we analyze the effect of a HS diet during 2 weeks (URL: http://www.clinicaltrials.gov. Unique identifier: NCT02509962) and short-term salt challenge by a single meal (URL: http://www.clinicaltrials.gov. Unique identifier: NCT04175249) on mitochondrial function of human monocytes in vivo.

RESULTS: Extracellular sodium was taken up into the intracellular compartment, followed by the inhibition of mitochondrial respiration in murine and human macrophages. Mechanistically, HS reduces mitochondrial membrane potential, electron transport chain complex II activity, oxygen consumption, and ATP production independently of the polarization status of macrophages. Subsequently, cell activation is altered with improved bactericidal function in HS-treated M1-like macrophages and diminished CD4+ T cell migration in HS-treated M2-like macrophages. Pharmacological uncoupling of the electron transport chain under normal salt phenocopies HS-induced transcriptional changes and bactericidal function of human and murine mononuclear phagocytes. Clinically, also in vivo, rise in plasma sodium concentration within the physiological range reversibly reduces mitochondrial function in human monocytes. In both a 14-day and single meal HS challenge, healthy volunteers displayed a plasma sodium increase of $x = 2\text{mM}$ and $x = 2.3\text{mM}$, respectively, that correlated with decreased monocytic mitochondrial oxygen consumption.

CONCLUSIONS: Our data identify the disturbance of mitochondrial respiration as the initial step by which HS mechanistically influences immune cell function. Although these functional changes might help to resolve bacterial infections, a shift toward proinflammation could accelerate inflammatory cardiovascular disease.

Key Words: bacterial killing, humans complex II macrophages metabolism mitochondrial respiration monocytes salt
Western lifestyle is associated with an increased risk for hypertension and cardiovascular disease.¹ In modern societies, the consumption of processed foods is on the rise. Unfortunately, they generally contain a high salt (HS) content, leading to a salt intake often greater than the recommendations of processed foods. High salt intake and local inflammation can lead to tissue Na⁺ accumulation in skin and muscle.²,³ Furthermore, chronic HS intake and local inflammation can lead to tissue Na⁺ accumulation in skin and muscle.⁴,⁵ In contrast, recent data demonstrate that Na⁺ can accumulate in various tissues and may even reach hypertonic concentrations in the interstitial fluid. Na⁺ magnetic resonance imaging measurements in humans and absolute Na⁺ quantification in experimental models demonstrate that both chronic HS intake and local inflammation can lead to tissue Na⁺ accumulation in skin and muscle.⁶,⁷ Furthermore, tissue Na⁺ accumulation in humans on aging,³ in patients with hypertension,⁸ primary hyperaldosteronism,⁹ heart failure,¹⁰ and end-stage renal disease,¹¹ but also in noncardiovascular disease, such as autoimmune disease.¹² Of note, tissue Na⁺ can be removed by sodium-glucose cotransporter 2 inhibition (SGLT2i) in patients with type 2 diabetes,¹³ by diuretics in patients with heart failure,¹⁴ and after surgery of aldosterone-producing tumors.¹⁵ Furthermore, macrophages (MΦ) possess a homeostatic function, by which they sense and regulate peripheral salt and water balance.¹⁶ Conversely, HS boosts in vitro and in vivo proinflammatory M1 MΦ,¹⁷–¹⁹ and helper T type 17 cell responses, whereas it inhibits anti-inflammatory regulatory T cells²⁰–²² and M2 MΦ.²³,²⁴ These findings indicate that Na⁺ is an important factor in tissue microenvironment, probably acting also at the cellular level of immune cells, and thereby impacts disease.

MΦ play a pivotal role in the pathogenesis of atherosclerosis, acute myocardial infarction, and heart failure, highlighting the importance of myeloid cells for cardiovascular health and cardiovascular disease.²⁵ MΦ display a wide range of phenotypes. They sense signals from the environment, followed by profound energetic and transcriptional reprogramming.²⁶ This reprogramming determines MΦ functions, such as immune responses, homeostatic tissue function, phagocytosis, and clearance of apoptotic cells, and defense against pathogens and malignancies.²⁷ The programming of MΦ is directly linked to changes in central metabolic pathways. Mitochondria synthesize ATP by oxidative phosphorylation. For efficient oxidative phosphorylation, mitochondria create a membrane potential (ψ) across their inner membrane, determined by the proton (H⁺) gradient generated by the electron transport chain (ETC) to drive the synthesis of ATP.²⁸ In vitro analyses of “classic” lipopolysaccharide (LPS)-activated macrophages (M[ILPS]) have shown that the latter increase glycolysis and pentose phosphate pathways, and exhibit a truncated tricarboxylic acid cycle that effectively redirects their mitochondria from ATP to reactive oxygen species production.²⁹ On the other hand, late-phase M2-like MΦ upregulate fatty acid oxidation and oxidative phosphorylation.³⁰ These experiments are mostly performed 24 hours after activation and therefore reflect an advanced state of phenotypic adaptation. By contrast, little is known about early metabolic alterations that occur within the first hours of activation and environmental adaptation. Here we examine how changes in Na⁺, mimicking alterations in the circulation (+4 mM) and tissues (+40 mM), might affect the early metabolism of monocytes and MΦ, respectively. We hypothesize that mononuclear phagocytes challenged by an altered Na⁺ microenvironment immediately adapt their cellular metabolism, activation status, and function.

**METHODS**

The authors declare that all supporting data and analytical methods are available within the article and its Data Supplement. All materials used for this study are listed with their respective

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**Nonstandard Abbreviations and Acronyms**

| Symbol | Definition |
|--------|------------|
| ψ      | mitochondrial membrane potential |
| BMDM   | bone marrow–derived macrophage |
| ETC    | electron transport chain |
| HS     | high salt |
| MΦ     | macrophage |
| NS     | normal salt |
| OCR    | oxygen consumption rate |
| PBMC   | peripheral blood mononuclear cell |
Human Material

Human monocytes were derived from venous whole blood, drawn from healthy volunteers in accordance with the ethical standards of the institutional review board of the Charité-Universitätsmedizin Berlin (ethical approval EA2/046/17), and written informed consent was obtained from all participants before study entry. Subjects were recruited locally at the Experimental and Clinical Research Center Berlin-Buch. The long-term salt intervention study reevaluated here was previously performed in our laboratory (URL: http://www.clinicaltrials.gov. Unique identifier: NCT02509962). The short-term salt intervention study (pizza meal challenge) can be found at Clinical Trials (URL: http://www.clinicaltrials.gov. Unique identifier: NCT04175249).

Volunteers and study participants recruited during this study were between 24 and 56 years old. Study participants from the long-term salt intervention study (URL: http://www.clinicaltrials.gov. Unique identifier: NCT02509962) were all male. Study participants from the short-term salt intervention study (URL: http://www.clinicaltrials.gov. Unique identifier: NCT04175249), as well as volunteers for the in vitro +2 and +4 mM NaCl treatment, were equally distributed (10 male, 10 female). Volunteers for the human monocytes and human *Escherichia coli* killing studies were healthy donors.

Human peripheral blood mononucleated cells (PBMCs) used for functional killing experiments were drawn from healthy volunteers with the ethical approval 20-1707-101 from the institutional review board of the University of Regensburg. In addition, PBMCs were obtained from the peripheral blood of healthy subjects or isolated fromuffy coats of healthy donors purchased from the Belgian Red Cross in compliance with institutional guidelines. Murine MΦ were generated from the bone marrow of male, 9- to 12-week-old, wild type C57BL/6J mice. Animals were purchased from Harlan Laboratories and Charles River and bred at the animal facility from the Max Delbrück Center, Berlin, and from the University of Regensburg, respectively. Mice were housed under standard light-dark cycles (12 h/12 h) and specific pathogen-free conditions. They were fed a normal chow diet (V1124-300, Ssniff Spezialdiäten GmbH) and tap water ad libitum. Bone marrow cells were differentiated for 9 to 10 days in DMEM (41966-029, Gibco by Thermo Fisher Scientific), supplemented with 10% (v/v) fetal bovine serum (S0615, Biochrom by Merck), 5% (v/v) adult horse serum (S-HEU03-I, Cell Concepts), 1% (v/v) nonessential amino acids (M7145, Sigma by Merck), 50 μM β-mercaptoethanol (M3148, Sigma by Merck), and 20% (v/v) L929 cell conditioned medium in in-house–sealed, hydrophobic Teflon-coated bags (FT FEP 100C, DuPont/American Duralife) at 37°C and 10% CO₂. Purity of BMDMs was regularly determined by flow-cytometric analysis and consistently at ≥90% CD11b⁺ F4/80⁺ double-positive cells (553310, BD Biosciences and 48-4801-80, eBioscience by Thermo Fisher Scientific, respectively).

Generation of Human Macrophages

Monocytes were sorted from PBMCs by using the EasySep CD14 Positive Selection kit II (17858, Stemcell Technologies) according to the manufacturer’s protocol. Sorted monocytes were further differentiated in X-VIVO15 medium (BE02-060F, Lonza), supplemented with 10% FCS (S1400, BioWest) as well as 100 ng/mL macrophage colony-stimulating factor (300-25, Peprotech) for 7 days.

Generation of Murine BMDMs

Animal care and use followed the regulations of the German Animal Welfare Act. The procedures followed were approved by the local State authorities (X9009/18) or by Umweltamt der Stadt Regensburg and performed in accordance with institutional guidelines. Murine MΦ were generated from the bone marrow of male, 9- to 12-week-old, wild type C57BL/6J mice. Animals were purchased from Harlan Laboratories and Charles River and bred at the animal facility from the Max Delbrück Center, Berlin, and from the University of Regensburg, respectively. Mice were housed under standard light-dark cycles (12 h/12 h) and specific pathogen-free conditions. They were fed a normal chow diet (V1124-300, Ssniff Spezialdiäten GmbH) and tap water ad libitum. Bone marrow cells were differentiated for 9 to 10 days in DMEM (41966-029, Gibco by Thermo Fisher Scientific), supplemented with 10% (v/v) fetal bovine serum (S0615, Biochrom by Merck), 5% (v/v) adult horse serum (S-HEU03-I, Cell Concepts), 1% (v/v) nonessential amino acids (M7145, Sigma by Merck), 50 μM β-mercaptoethanol (M3148, Sigma by Merck), and 20% (v/v) L929 cell conditioned medium in in-house–sealed, hydrophobic Teflon-coated bags (FT FEP 100C, DuPont/American Duralife) at 37°C and 10% CO₂. Purity of BMDMs was regularly determined by flow-cytometric analysis and consistently at ≥90% CD11b⁺ F4/80⁺ double-positive cells (553310, BD Biosciences and 48-4801-80, eBioscience by Thermo Fisher Scientific, respectively).

Macrophage Activation and Stimulation

BMDMs as well as human blood-derived monocytes were cultivated in DMEM (11966-025, Gibco by Thermo Fisher Scientific), supplemented with 10 mM glucose (X9972, Carl Roth), 10% (v/v) fetal bovine serum (S0615, Biochrom by Merck), 1% (v/v) penicillin-streptomycin (15140-122, Gibco by Thermo Fisher Scientific), 1% (v/v) HEPES (15630-056, Gibco by Thermo Fisher Scientific), and 50 μM 9-mercaptoethanol (M3148, Sigma by Merck). BMDMs were activated with 10 ng/mL LPS (L3024, Sigma by Merck) or 10 ng/mL interleukin (IL) 4 (404-ML-010, R&D Systems by bio-technne) and 10 ng/mL IL-13 (PMC0134, Invitrogen by Thermo Fisher Scientific). Human MΦ were activated with 100 ng/mL macrophage colony-stimulating factor (300-25, Peprotech), 100 ng/mL ultrapure LPS (tlr3-3eps, Invivogen) and 20 ng/mL interferon (IFN)γ (300-02, Peprotech) or with 100 ng/mL...
macrophage colony-stimulating factor (300-25, Peprotech) and 20 ng/mL IL-4 (113040045, Immunotoools). For the activation under HS conditions, respective concentrations of sodium chloride (NaCl, 9265.1, Carl Roth) were added to the medium. Pharmacological inhibition of mitochondrial function was performed with Oligomycin A (75351, Sigma by Merck), N5,N6-bis(2-fluorophenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine (BAM15, SML1700-5MG, Sigma by Merck), rotenone (R8875, Sigma by Merck) or antimycin A (A8674, Sigma by Merck) at concentrations of 10 μM, respectively. DMSO (D4540, Sigma by Merck) was included as solvent control if necessary. Dimethyl malonate (136441, Sigma by Merck) was used at a concentration of 10 mM.

Bacterial Infection

E. coli infection experiments are described in the Supplemental Methods in the Data Supplement.

Seahorse

Real-time measurement of oxygen consumption rate (OCR) was performed using the Seahorse XFe96 FluxPak (102416-100, Agilent) and the Seahorse XFe Analyzer (Agilent). BMDMs and human monocytes were cultivated (and stimulated) in XFe96 microplates as described under Macrophage Activation and Stimulation. Before the analysis, the cells were washed with unbuffered, DMEM-formulated Seahorse base medium (102363-000, Sigma by Merck) and 2 mM L-glutamine (25030-081, Gibco by Thermo Fisher Scientific) and incubated for 30 minutes at 37°C, without CO2. For the analysis, cells were then loaded onto the Seahorse XFe Analyzer together with a precalibrated disposable cartridge. A mitochondrial stress test was performed. The assay was designed to repeat 5 cycles (of 3 min mixing followed by 3 min measuring) per phase. During the assay, 2 μM Oligomycin A (75351, Sigma by Merck), 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (C2920, Sigma by Merck), 1 μM rotenone (R8875, Sigma by Merck), and 1 μM antimycin A (A8674, Sigma by Merck) were injected. After the assay, a nuclear staining with Hoechst (B2261, Sigma by Merck) was performed (10 μg/mL in cell culture medium for 45 minutes at 37°C). Cells were then washed with PBS and detached with 2 mM EDTA in PBS for 10 min on ice. Cell number was then determined on a BD LSRFortessa with high-throughput sampler (BD) using FACSDiva and FlowJo to normalize OCR to cell count per well. Human blood-derived monocytes were treated equally. However, as monocytes are nonadherent, cells needed to be centrifuged between washes (for 5 min at 300 g), and detaching was not necessary.

Pulsed Stable Isotope-Resolved Metabolomics

BMDMs were cultivated in 10-cm cell culture dishes and stimulated as indicated above. One hour before the stimulatory end point (i.e., after 2 hours and 23 hours), medium was changed to U-13C-glucose (CLM-1396-PK, Cambridge Isotope Laboratories) or U-13C-glutamine (CLM-1822-H-PK, Cambridge Isotope Laboratories) containing DMEM (A14430-01, Gibco by Thermo Fisher Scientific), supplemented with 10% (v/v) fetal bovine serum (S0615, Biochrom by Merck), 1% (v/v) penicillin-streptomycin (15140-122, Gibco by Thermo Fisher Scientific), 1% (v/v) HEPES (15630-056, Gibco by Thermo Fisher Scientific), 50 μM 8-mercaptopethanol (M3148, Sigma by Merck), 10 mM glucose, and 2 mM glutamine (unlabeled or U-13C-labeled, depending on the experiment). After 1 hour of U-13C-labeling (including the respective stimulants), cells were washed with a buffer containing 140 mM NaCl, 5 mM HEPES, and 4 mM KCl and lysed in methanol-chloroform-water (5:2:1 v/v/v), containing 2 μg/ml cinnamic acid (133760, Sigma by Merck) as internal control. For metabolite extraction, cell lysates were shaken for 60 min at 4°C and centrifuged for 15 min at 4°C and 10,000 g. Polar phase was collected and dried under vacuum. Extracts were resuspended in 20% methanol, shaken for 60 min at 4°C and polar phase was again dried under vacuum. Sample derivatization was performed as described elsewhere.35 In parallel to the samples, a set of Ident and Quant mixes were extracted, derivatized, and measured.35 These allow a more reliable identification of isomeric metabolites as well as quantification of 63 compounds, respectively.

The samples and Ident/Quant standards were injected in split mode (split 1:5, injection volume 1 μL) in a temperature-controlled injector (CAS54, Gerstel) with a baffled glass liner (Gerstel). The following temperature program was applied during sample injection: initial temperature of 80°C for 30 s followed by a ramp with 12°C/min to 120°C and a second ramp with 7°C/min to 300°C and final hold for 2 minutes. Gas chromatographic separation was performed on an Agilent 6890 N, equipped with a VF-5 ms column. Helium was used as carrier gas with a flow rate of 1.2 mL/min. Gas chromatography was performed with the following temperature gradient: 2-minute heating at 67.5°C, first temperature gradient with 5°C/min up to 120°C; subsequently, a second temperature increase of 7°C/min up to 200°C, 12°C/min up to 320°C and a hold of 6 minutes. The spectra were recorded in a mass range of 60 to 600 mass units with 10 spectra/s at a detector voltage of 1650 V. The gas chromatography–mass spectrometry chromatograms were initially preprocessed with ChromaTOF (Leco). Metabolite identification and quantification were performed with MAULISILVIA.36 Metabolite quantities were normalized to protein content (determined in parallel), the internal control cinnamic acid, and the sum of all peak areas. Data processing was performed with R (MTXQC).

Intracellular Na+ Quantification

Human monocytes (10⁶ cells) were stimulated with HS for 60 minutes, harvested, and washed with iso-osmolar sucrose solution. The pellet was lysed, and total Na+ was determined by atomic absorption spectrometry (iCE 3000 Series, Thermo Scientific).37

Statistical Analyses

No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to the sample allocation during experiments and outcome assessment. Statistical analyses were performed with GraphPad Prism Version 8.3.0. Normality of the data were tested by the Kolmogorov-Smirnov test. Significance between 2 groups was analyzed by t test (when normally distributed), Mann-Whitney test (when nonnormally distributed) or Wilcoxon matched-pairs signed-rank test (for nonnormally distributed, matched data). In Na+ quantifications, outliers were identified
with ROUT and excluded from analysis. For >2 groups with 1 variable only, 1-way ANOVA with a Tukey post hoc test (for normally distributed data) or Kruskal-Wallis test with a Dunn post hoc test (for nonnormally distributed data) were used. Matched data with more than 2 groups (or time points) were analyzed by Friedman test, and false discovery rate (FDR) correction was performed using the Benjamini-Hochberg procedure. The relationship between normal distributed data sets with single time point comparison was determined by Pearson correlation and linear regression analysis. Comparison between multiple time points was performed by linear mixed-effects model applied to rank-transformed between–time point difference data. Exact statistical tests used are described in the respective figure legends. Respective P values were depicted in the figures. After false discovery rate correction, q values were reported. For the statistical analysis of the gene expression data, the Mann-Whitney U test comparing M(LPS) and M(IL4+IL13) at each time point to untreated M0, as well as comparing normal salt (NS) to HS at each time point for each activation group, was performed. False discovery rate correction was done across genes using the Benjamini-Hochberg procedure.

**RESULTS**

**HS Inhibits Mitochondrial Respiration in Murine and Human Mononuclear Phagocytes**

The importance of cellular metabolism for immune cell function in the advanced phase has been extensively studied. However, little is known about how murine and human MΦ adapt their cellular metabolism in the first hours after sensing a change in a microenvironment. Therefore, we performed real-time measurement of glycolysis and mitochondrial respiration after activating murine BMDMs (Figure 1A, Figure IA in the Data Supplement) for 3 hours under NS or HS (addition of 40 mM NaCl) conditions. It is interesting that although the extracellular acidification rate was not affected (Figure 1B in the Data Supplement), basal and maximal OCR were decreased under HS (Figure 1B, Figure IC and ID in the Data Supplement). Accordingly, HS induced a significant reduction in ATP levels (Figure 1C) and ψ, determined by tetramethylrhodamine ethyl ester flow cytometry (Figure 1D, Figure IE in the Data Supplement). Recently, we showed that an additional 40 mM of extracellular Na+ increases intracellular Na+ within minutes by approximately 20%, accompanied by an enhanced antibacterial defense. To mimic the effect of raised intracellular Na+ and to demonstrate that Na+ directly affects mitochondrial function, we treated isolated mitochondria from unactivated BMDM with increasing concentrations of NaCl and found the ψ to decrease dose-dependently (Figure 1E). In line with our data, Ip and Medzhitov previously reported an inhibition of mitochondrial respiration in nonactivated MΦ under HS, although at much higher concentrations (100 mM). To verify our findings, we next sought to determine whether HS diminishes mitochondrial function in human mononuclear phagocytes. We treated freshly isolated CD14+ human monocytes (Figure 1F in the Data Supplement) as well as monocyte-derived MΦ (Figure 1F) with HS. In human monocytes, HS led to a significant increase in intracellular Na+ content (Figure 1G). As for murine MΦ, we found significantly decreased OCR (Figure 1H and 1I), ATP levels (Figure 1J), and ψ (Figure 1K) compared with NS, independent of the activation status. Furthermore and in agreement with our previous findings, short-term HS challenge significantly accelerated E. coli killing in murine MΦ (Figure 1L) and human CD14+ monocytes (Figure 1M). We next tested whether HS would not only alter M1-like killing capacity but also affect M2-like MΦ functionality. As a surrogate assay, we measured T cell migration, which is promoted by M2-like MΦ function. Indeed, HS-treated M(IL-4) less potently promoted CD4+ T cell and CD4+ regulatory T cell migration (Figure 1N). Taken together, these data demonstrate that a change in the sodium microenvironment results in a rapid loss of mitochondrial function consistently in all M1- and M2-like murine and human MΦ, highlighting a species-overlapping regulatory mechanism. The functional outcome, however, was different in M1- and M2-like MΦ.

**HS Inhibits Mitochondrial ETC at Complex II**

To dissect the molecular mechanism underlying the HS-induced mitochondrial dysfunction, we performed pulsed stable isotope-resolved metabolomics in murine BMDM. Using pulsed isotope labeling with U-13C6-glucose or U-13C5-glutamine (Figure 2A), we analyzed the metabolic turnover within a defined time window during activation, in contrast to classic cumulative labeling experiments that cover the entire course of activation. We performed these analyses in the third and 24th hours of M(LPS) and M(IL4+IL13) activation, labeling during the last hour only (Figure 2A in the Data Supplement). Surprisingly, we found a significant reduction in the succinate to fumarate conversion under HS after only 3 hours of activation (Figure 2B, Figure IIB through IIIE in the Data Supplement), which continued to persist at 24 hours (Figure 2C, Figure IIIF through IIII in the Data Supplement). Of note, this finding was independent of the activating stimulus. Furthermore, glycolysis was only minimally induced after 3 hours of LPS-treatment compared with 24 hours, and in line with our previous extracellular acidification rate results, HS had no effect either, independent of LPS or IL4+IL13 treatment (Figure IIJ and IIK in the Data Supplement). During the oxidation of succinate to fumarate, reduced flavin adenine dinucleotide (FADH2) is formed and serves as a first electron acceptor. However, FADH2 is covalently bound to complex II. Therefore, we measured levels of nonprotein-bound flavin adenine dinucleotide (FAD) in murine M(LPS) and M(IL4+IL13) and found them to be decreased after 3
Figure 1. High salt inhibits mitochondrial respiration in murine and human mononuclear phagocytes.

A. Pictogram of the generation and activation of murine bone marrow–derived macrophages (BMDMs). B through D, BMDMs were treated for 3 hours with lipopolysaccharide (LPS) or interleukin (IL) 4+IL13, under normal salt (NS) or high salt (HS) conditions. (B) Basal oxygen consumption rate (OCR) (pooled data n=10 from 3 independent experiments). Data are depicted as box and whisker with minimum to maximum. Significance was analyzed by unpaired, 2-tailed t test. C, Relative ATP content, with Oligomycin A (Oligo, 10 μM) treatment as positive control for ATP decrease (pooled data n=12 from 2 independent experiments). Data are depicted as box and whisker with minimum to maximum. Significance was analyzed by 1-way ANOVA with Tukey’s post hoc test. D, Relative TMRE mean fluorescence intensity (MFI), with mitochondrial uncoupler BAM15 (10 μM) as positive control for mitochondrial depolarization (pooled data n=10 from 2 independent experiments). (Continued)
Figure 1 Continued. Data are depicted as box and whisker with minimum to maximum. Significance was analyzed by Kruskal-Wallis test with Dunn’s post hoc test (LPS groups) or by one-way ANOVA with Tukey’s post hoc test (IL4+IL13 groups). E, Relative TMRE MFI in isolated unactivated BMDM mitochondria, treated with increasing concentrations of NaCl (serial dilutions from 64 mM to 1 mM NaCl) for 3 hours (n=4). F, Pictogram of the generation and activation of human monocyte-derived macrophages. G, Relative intracellular Na+ content in human monocytes after 1 hour of HS treatment (n=13). Significance was analyzed by unpaired, two-tailed t test. H, Basal OCR in freshly isolated human monocytes (n=8 male donors), treated for 3 hours with 40 mM of NaCl (HS). Data are shown as donor-paired mean and were analyzed by paired, 2-tailed t test. I through K, Human monocyte-derived macrophages (MΦ) activated for 6 hours with LPS+ interferon (IFN) γ or IL4, under NS or HS conditions. I, Basal OCR (n=14 for M(LPS+IFNγ) NS and HS, n=15 for M(IL4) NS, and n=12 for M(IL4) HS); J, ATP level in relation to NS (n=11 for NS, n=12 for HS for both M(LPS+IFNγ) and M(IL4), respectively); K, TMRE fluorescence intensity in relation to NS (n=14 for M(LPS+IFNγ) NS and HS, n=15 for M(IL4) NS and HS). I through K, Data are shown as box and whisker with minimum to maximum and were analyzed by unpaired, 2-tailed t test. Relative E. coli colony-forming units in murine BMDMs (L) (pooled data n=9 from 3 independent experiments) and human monocytes (M) (n=8), treated under NS or HS conditions, infected with E. coli for 3 hours. Data are shown as box and whisker with minimum to maximum in relation to NS and were analyzed by unpaired, 2-tailed t test. N, Effect of human M2 macrophages treated with NS and HS on CD4+ T cell and CD4+ regulatory T cell (Treg) migration (n=8 each). Data are shown as donor-paired mean and were analyzed by Wilcoxon matched-pairs signed-rank 2-tailed t test.

Coupled ETC Is Crucial for Early MΦ Activation and Function

To highlight the importance of complex II inhibition and mitochondrial ψ depolarization on MΦ function, we evaluated MΦ activation on HS, as well as on dimethyl malonate, antimycin A, and BAM15 treatment, respectively, under NS conditions. Indeed, pharmacological inhibition of complex II with dimethyl malonate (Figure 4A), inhibition of complex III with antimycin A (Figure 4B), and mitochondrial depolarization with BAM15 (Figure 4C) phenocopied the HS effect on the expression profile of representative marker genes Nos2 and Retnla1 after only 3 hours. Functionally, mitochondrial uncoupling under NS conditions with BAM15 accelerated defenses against E. coli in murine MΦ and human monocytes similarly as under HS conditions (Figure 4D and 4E). Of note, BAM15 neither affected E. coli growth directly (Figure III in the Data Supplement), nor compromised cell viability, as determined by lactate dehydrogenase secretion (Figure IIE and IIIF in the Data Supplement). It is interesting that, whereas after 3 hours of activation, HS inhibited the expression of several proinflammatory genes.
Figure 2. High salt inhibits mitochondrial electron transport chain at complex II.

A. Pictogram of the central carbon metabolism and ETC. Glucose-derived 13C-label is represented as red dots. Glutamine-derived 13C-label is represented as blue dots. Mitochondrial membrane potential is ψ.

B. Bone marrow–derived macrophages (BMDMs) treated for 3 h (B) or 24 h (C) with lipopolysaccharide (LPS) or interleukin (IL) 4+IL13 under normal salt (NS) or high salt (HS) conditions. B and C. 13C-glutamine-derived 13C-fumarate to 13C-succinate ratio as surrogate for succinate dehydrogenase/complex II activity (n=5). Data are depicted as box and whisker with minimum to maximum. Significance was analyzed by unpaired 2-tailed t test.

D. Relative (deproteinized) FAD content (n=4). Data are depicted as box and whisker with minimum to maximum. Significance was analyzed by unpaired 2-tailed t test.

E. Relative (deproteinized) FAD content in freshly isolated monocytes (n=6 donors), cultivated for 3 hours under NS or HS conditions. Data are depicted in relation to the donor-paired NS control. Significance was analyzed by Wilcoxon matched-pairs signed-rank 2-tailed test.

F. Relative complex II activity after purification of complex II from BMDMs under increasing concentrations of additional NaCl (0 mM to 4 mM) (n=3 or 4). Data are depicted as mean in relation to the NS control. Significance was analyzed by 1-way ANOVA with Tukey's post hoc test.

G. Relative complex II+III activity in bovine (Continued)
in mouse M(LPS+IFNγ) MΦ (Figure 4A–4C, Figure IIIA and IIIB in the Data Supplement), over time there was a switch toward enhanced proinflammatory signature at 24 hours (Figure 4F and 4H, Figure IIIA and IIIB in the Data Supplement) and nitric oxide production (Figure 4G), as described previously.8,17,37 Considering anti-inflammatory activation, HS reduced the overall early and late marker gene expression in mouse M(IL4+IL13) MΦ (Figure IIIA and IIIB in the Data Supplement), as reported previously for 24 hours.26 Similarly, 24 hours of HS boosted the expression of proinflammatory genes in LPS+IFNγ-treated human MΦ (Figure 4I), whereas it inhibited anti-inflammatory gene expression in IL4-treated human MΦ after 24 hours (Figure IIIC in the Data Supplement).

Taken together, our data demonstrate that a change in the Na+ microenvironment results in a rapid loss of mitochondrial respiration. This mitochondrial dysfunction, in line with previous reports,45 has profound consequences on murine MΦ activation and function. We recently demonstrated that HS boosts Bnip3 expression.17 Bnip3 is linked to mitochondrial dysfunction46 and thus could contribute to our antibacterial phenotype.

High Dietary Salt Intake Transiently Inhibits Mitochondrial Respiration in Human Monocytes

In contrast to tissue MΦ, which might sense substantially higher local Na+ levels, especially in inflammatory areas,8 circulating monocytes are exposed to the more subtle Na+ changes in the bloodstream. To directly assess how physiologically relevant changes in plasma Na+ affect monocyte mitochondrial function, we analyzed...
Figure 4. Coupled electron transport chain is crucial for early macrophage activation and function.

Relative expression of Nos2 and Retnla1 in untreated, lipopolysaccharide (LPS)- or interleukin (IL) 4+IL13-treated bone marrow–derived macrophages (BMDMs) activated for 3 hours under normal salt (NS) or high salt (HS) or NS+dimethyl malonate (DMM) (A) (pooled data n=16 from 2 independent experiments), NS+antimycin A (AA) (B) (pooled data n=8 from 2 independent experiments), or NS+BAM15 (C) (pooled data n=6 from 2 independent experiments). D and E, Relative Escherichia coli (E. coli) colony-forming units (CFUs) in BMDMs (D) (pooled data n=18 from 3 independent experiments) and freshly isolated human monocytes (E) (pooled data n=10 from 3 independent experiments), 3 hours after infection under NS, HS, or NS+BAM15 (10 μM) treatment. Relative gene expression of Nos2 (F) and Il6 (H), as well as nitrite in the supernatant (G) in untreated or LPS-treated BMDMs activated for 24 hours under NS or HS. F through H, Data (n=6 each) are depicted as box and whisker with minimum to maximum. Significance was analyzed by 1-way ANOVA with Tukey’s post hoc test (A through C, E through H) and Kruskal-Wallis test with Dunn’s post hoc test for D. I, Relative gene expression of Il6, Il8, Tnf, and Il23 in human macrophages activated for 24 hours with LPS+interferon (IFN)γ under NS or HS conditions (n=6 each for Il6, Il8, and Il23, n=5 each for Tnf). For I, data are shown as donor-paired mean and were analyzed by paired, 2-tailed t test (Il6, Il8, Il23) or Wilcoxon matched-pairs signed-rank 2-tailed test (Tnf).
plasma Na⁺ changes and oxidative respiration in monocytes from healthy humans challenged with 6 g of salt (administered as NaCl tablets) in addition to their accustomed diets over the course of 14 days, followed by a 14-day washout phase (NCT02509962). We found a significant increase in plasma Na⁺ levels during the HS intervention compared with baseline. Fourteen days after cessation of HS intake, plasma Na⁺ returned to baseline (Figure 5A). Of note, changes in basal OCR in human monocytes over the course of the study displayed a significant negative correlation with changes in plasma Na⁺ concentrations (Figure 5B). These findings were independent and not driven by differences in monocyte subpopulations, as these were unchanged during the HS intervention (Figure IVA and IVB in the Data Supplement). We thus analyzed in vitro whether the observed 2

**Figure 5.** Minimal sodium (Na⁺) changes inhibit monocytic mitochondrial function. **A**, Plasma Na⁺ (n=8) as well as (B) fold-change in monocytic basal oxygen consumption rate (OCR) in relation to difference in plasma Na⁺ compared with baseline (n=5 for day 3, n=4 for day 14, n=6 for day 28) from a chronic salt intervention study in male volunteers. **C**, Basal OCR, (D) ATP level, and (E) tetramethylrhodamine ethyl ester (TMRE) mean fluorescence intensity (MFI) in freshly isolated monocytes from 10 male and 10 female volunteers, treated in vitro for 3 hours with +2 mM and +4 mM NaCl. **A**, Significance of plasma Na⁺ was analyzed by Friedman test, and false discovery rate (FDR) correction was performed via Benjamini-Hochberg procedure. **B**, Relationship between the change in plasma Na⁺ and the change in basal OCR (of days 3, 14, and 28 compared with baseline) was determined by linear mixed-effects model applied to rank-transformed between–time point difference data, accounting for donor. **C** through **E**, Data were analyzed by Friedman test with Benjamini-Hochberg FDR correction. NS indicates normal salt; and TMRE, tetramethylrhodamine ethyl ester.
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**DISCUSSION**

One major finding of our study is that circulating monocytes respond to moderate changes in plasma Na⁺ within the reference range, whereas MΦ sense higher Na⁺ concentrations with subsequent changes on mitochondrial energetics, MΦ activation, and function. Furthermore, we have found a strong inhibition of complex II+III and ATP production in vitro under HS. Of note, HS actions on gene expression and bactericidal function could be recapitulated under NS using a mitochondrial uncoupler. Intracellular electrolyte and energy homeostasis are tightly regulated processes. From the physiological perspective, the early reduced mitochondrial ATP production induced by HS will provoke adaptive responses, by which the cell will reach again a more favorable energetic homeostasis. Our data provide the first evidence that disturbances in this critical phase might have long-term functional consequences. These findings raise the intriguing questions of whether and to what extent changes in intracellular Na⁺ affect oxidative phosphorylation and function of other cell types, such as dendritic, T, B, and intestinal cells or cardiomyocytes, equally subjective to dietary sodium changes. We did not investigate functional consequences in vivo. Thus, future HS intervention studies will be needed to identify whether immune and other cells modulate their function on different diets. Especially in the context of cardiovascular disease, functional downstream effects of HS will be of great interest, considering salt-sensitive and -insensitive patients. Therapy could in the future be accompanied by dietary recommendations, dependent on the personal responsiveness of cells on HS. Including diseased patients will therefore be of major interest in future studies, as well as the investigation if salt sensitivity is correlated or even partially caused by mitochondrial dysfunction in certain cell types.

Recently, we found that Slc8a1/NCX1 senses high extracellular Na⁺. Pharmacological and genetic inhibition of Slc8a1/NCX1 provided mechanistic insights on Na⁺ influx in mononuclear phagocyte cells, although we cannot exclude that other channels or mechanisms also promote the redistribution of Na⁺ from the extracellular NaCl could also trigger mitochondrial dysfunction in human monocytes acutely. Strikingly, our measurements showed that mitochondrial function in monocytes obtained from healthy study participants was reduced, as indicated by significantly decreased OCR, ATP, and ψ levels, when treating them in vitro for 3 hours with only +2 and +4 mM of NaCl (Figure 5C–5E). All parameters showed a dose-dependent reduction independent of sex (Figure 5C–5E). We aimed to mimic these acute and relatively mild Na⁺ changes in vivo with a meal challenge, highly representative of a real-life situation. Here, 20 healthy donors (10 men and 10 women) ate a single pizza meal with almost 10 g of salt per serving (NCT04175249) (Figure 6A). Three hours after the meal challenge, plasma Na⁺ increased significantly by 1 to 6 mM, returning to baseline by 8 hours after the meal challenge (Figure 6B). It is striking that basal OCR (Figure 6C) and ψ (Figure 6E) decreased significantly 3 hours after the meal intake. One limitation related to our study design is the fact that we cannot fully exclude the caloric challenge as a potential confounder. However, the significant negative correlation of OCR and plasma Na⁺ differences between 3 hours after the meal and baseline speak against it (Figure 6D). These data demonstrate that dietary sodium intake has immediate effects on mononuclear mitochondrial respiration, and this in turn might affect their activation and function, further highlighting the significance of dietary sodium intake on monocyte/macrophage immunometabolism and function in humans.

**Figure 6. Single meal inhibits monocytic mitochondrial function.**

A, Scheme of the study protocol with a picture of a representative pizza containing 9.8±0.3 g NaCl. Before, 3 hours and 8 hours after the meal challenge, monocytes were isolated from 10 male and 10 female volunteers (n=20 per time point). B, Plasma Na⁺, C, monocytic basal oxygen consumption rate (OCR) in relation to baseline, and E, TMREʰ high monocytes in relation to baseline at baseline, 3 hours, and 8 hours after pizza. D, Relationship between change in plasma Na⁺ and the change in basal OCR (of 3 hours compared with baseline) was determined by Pearson correlation and linear regression analysis. TMRE indicates tetramethylrhodamine ethyl ester.
cellular to intracellular compartment. Interestingly, mitochondrial mitochondrial sodium calcium exchanger has been shown to be crucial for mitochondrial salinization on isotonic hypoxia, with subsequent deficiency in electron transfer from complex II to complex III and superoxide burst. Increased intracellular Na⁺ levels not only occur on extracellular hypertonic conditions in immune cells, but also occur under ischemic NS conditions, eg, in the heart. Excitation coupling of healthy cardiac myocytes consumes large amounts of ATP, which is replenished by mitochondrial oxidative phosphorylation. During cardiac ischemia, intracellular and mitochondrial Na⁺ levels increase along with a reduction in mitochondrial respiratory function with reduced ATP, which might be a reason for the imbalance of the energy demand and thus further contribute to heart failure. SGLT2i is a new promising class that reduces mortality in patients with cardiovascular risk. Experimentally, SGLT2i has direct cardiovascular effects by decreasing cardiac intracellular Na⁺ and Ca²⁺ and increasing mitochondrial Ca²⁺. Therefore, it is tempting to speculate that SGLT2i might also affect mitochondrial respiratory function with reduced ATP, which might be a reason for the imbalance of the energy demand and further contribute to heart failure. SGLT2i is a new promising class that reduces mortality in patients with cardiovascular risk. Experimentally, SGLT2i has direct cardiovascular effects by decreasing cardiac intracellular Na⁺ and Ca²⁺ and increasing mitochondrial Ca²⁺. Therefore, it is tempting to speculate that SGLT2i might also affect mitochondrial respiratory function with reduced ATP, which might translate into long-term functional changes. Although these functional changes might help to resolve bacterial infections, a shift toward an inflammatory phenotype might accelerate cardiomycopathy.

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Acknowledgments

The authors thank Jana Czyż, Gabriele Nⁱdíaey, Juliane Anders, Ute Gerhardt, MD, Jens Köhler, and Fardad Ramezaní for assistance. S.G. led and conceived the project, designed and performed most experiments, and analyzed and interpreted the data. H.B. conducted the clinical study, and performed PBMC isolation and flow-cytometric analysis together with S.G. PN. performed murine BMDM and human peripheral blood monocyte bacterial killing and growth experiments. R.W. DS, and A.G. performed analyses of ATP, tetramethylrhodamine ethyl ester, gene expression, and T cell migration in human macrophages. C.Z. together with S.G. measured and analyzed pulsed stable isotope-resolved metabolomics experiments. T.B. and E.T. performed sectioned transmission electron microscopy imaging and 3-dimensional reconstruction. VMP. performed mitochondrial isolation and ETC complex assays together with S.G. L.K. performed peripheral blood monocyte bacterial killing and cell culture experiments in human monocytes for intracellular Na⁺ measurement. M.V. performed intracellular Na⁺ quantifications. A. Maifeld, A. Mährer, and N.W. performed the clinical study, which was reanalyzed by S.G. and H.B. S.K.F. performed statistical analyses. K.B., J.S., and R.D. gave major conceptual input. D.N.M., S.K., J.J., and M.K. supervised the experiments and interpreted the data. S.G. and D.N.M. wrote the article with key editing by S.K., H.B., and M.K. and further input from all authors.

Disclosures

None.

Supplemental Materials

Expanded Methods
Data Supplement Figures I–IV
Data Supplement Table I

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ARTICLE INFORMATION

Received November 17, 2020; accepted March 27, 2021.

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