Endothelial function is impaired after a high-salt meal in healthy subjects¹⁻³

Kacie M Dickinson, Peter M Clifton, and Jennifer B Keogh

ABSTRACT
Background: Dietary salt is related to blood pressure (BP), and cardiovascular disease and increased sodium intakes have been shown to impair vascular function. The effect of salt on endothelial function postprandially is unknown.

Objective: The aim was to investigate the postprandial effect of dietary salt on endothelial function as measured by flow-mediated dilatation (FMD) and peripheral arterial tonometry in healthy subjects.

Design: Sixteen healthy, normotensive subjects received a meal with added salt (HSM; 65 mmol Na) and a control low-salt meal (LSM; 5 mmol Na) on 2 separate occasions in a randomized order. Endothelial function was measured while fasting and postprandially at 30, 60, 90, and 120 min by using FMD and reactive hyperemia peripheral arterial tonometry. BP was also measured.

Results: Baseline FMD, reactive hyperemia index (RHI), and BP values were similar across interventions. Overall FMD was reduced 2 h postprandially. FMD was significantly more impaired after the HSM than after the LSM at 30 min [HSM (mean ± SD): 3.39 ± 2.44%; LSM: 6.05 ± 3.21%; P < 0.01] and at 60 min (HSM: 2.20 ± 2.77%; LSM: 4.64 ± 2.48%; P < 0.01). No significant differences in BP or RHI were observed between meals.

Conclusions: An HSM, which reflects the typical amount of salt consumed in a commonly eaten meal, can significantly suppress brachial artery FMD within 30 min. These results suggest that high salt intakes have acute adverse effects on vascular dilatation in the postprandial state. This trial was registered at www.anzctr.org.au/trial_view.aspx?ID=335115 as ACTRN1261000124033.

INTRODUCTION

Compelling evidence shows links between salt intake, increased blood pressure (BP), and risk of cardiovascular disease (CVD). A meta-analysis has confirmed that higher salt intakes are associated with a greater incidence of strokes and cardiovascular events (1). Several other large meta-analyses have shown that modest reduction in dietary salt of 1–3 g/d (17–51 mmol Na/d) lower BP and reduce CVD risk in those with high or normal BP (2–5).

Endothelial dysfunction, considered to be an initial step in the development of atherosclerosis (6), has been shown with higher salt intakes (7, 8). Dietary salt reduction has been shown to improve endothelial function assessed by flow-mediated dilatation (FMD) in a chronic study (9). However, the mechanisms relating salt and endothelial dysfunction are still not clear.

Metabolic abnormalities in the postprandial state are known to contribute to endothelial dysfunction and atherosclerosis progression in healthy people (10). Increases in metabolic factors, such as glucose and triglycerides, have been shown to induce endothelial dysfunction postprandially by increasing oxidative stress and decreasing the bioavailability of nitric oxide (11). Short-term high salt intakes have been shown to similarly produce reductions in nitric oxide (12) and increase asymmetric dimethylarginine (ADMA) production (an endogenous nitric oxide inhibitor) (13), but whether salt impairs endothelial function postprandially and the mechanisms responsible have not been studied.

Current population salt intakes range from 9 to 12 g/d (14) (153–204 mmol Na/d), and a recent systematic review of the sodium content in foods indicates that it may not be uncommon to consume an amount of salt in excess of the current recommendations (ie, 6 g/d, or 102 mmol Na/d) in a single meal (15). It is known that this amount of salt can raise plasma sodium postprandially by as much as 3 mmol/L (16, 17), and recent in vitro evidence shows that a change in sodium of this magnitude mediates direct effects on vascular smooth muscle cells and endothelial cells (18, 19).

Determining whether a high salt meal has a detrimental effect on endothelial function in the postprandial state is of interest given the high levels of nondiscretionary salt consumed in foods and typical meals. Therefore, we undertook a study of the vascular responses of a group of healthy adults to a high-salt meal (HSM; 65 mmol Na) and a low-salt control meal (LSM; 5 mmol Na).
SUBJECTS AND METHODS

Subjects

Seventeen men and women aged 18–70 y were recruited by personal contact and public advertisement at the local university and hospital and from within Commonwealth Scientific and Industrial Research Organization (CSIRO) Food and Nutritional Sciences Adelaide. Inclusion criteria were body mass index (BMI; in kg/m²) ≥18 and ≤27, systolic BP (SBP) < 130 mm Hg, diastolic BP (DBP) < 90 mm Hg, weight stability in the preceding 3 mo, and no use of antihypertensive medication, systemic steroids, folate supplementation, or nonsteroidal antiinflammatory drugs. Participants were not excluded if they were taking other vitamin or mineral supplements provided their dosage and frequency remained unchanged for the duration of the study. Seventeen volunteers met the selection criteria; one participant withdrew before commencing for reasons unrelated to the study. The study was approved by the CSIRO Human Research Ethics Committee (HREC09/35) and the University of Adelaide Human Research Ethics Committee (H-172-2009). All participants gave written informed consent.

Study methods

In a randomized crossover design, participants completed the study protocol on 2 mornings and consumed an HSM (65 mmol Na) or an LSM (5 mmol Na) separated by a minimum 1-d washout period. The nutrient composition of the test meals is described in Table 1. The treatment order was completely randomized. The participants were assigned to treatment order by a numbered random-allocation sequence generated by using a computer program (CLINSTAT software; Martin Bland, York, United Kingdom) by a person independent to the study. After randomization, 9 participants received the LSM at visit 1, and 7 participants received the HSM at visit 1. Participants were required to fast from 2200 the night before (no food, water only) and refrain from alcohol, vigorous exercise, and caffeine in the 24 h before each study morning.

On arrival, body height was measured at baseline to the nearest 0.1 cm with a stadiometer (SECA, Hamburg, Germany) while the participants were barefoot. Body weight was measured to the nearest 0.05 kg with calibrated electronic digital scales (AMZ 14; Mercury, Tokyo, Japan) while the participants were wearing light clothing and no footwear. The study protocol lasted 2.5 h.

Blood pressure

Seated BP was measured with an automated sphygmomanometer (SureSigns V3; Philips, North Ryde, Australia) while fasting at visits 1 and 2. After 5 min of rest, 4 consecutive BP measurements were taken 1 min apart. The first reading was discarded, and the mean of the next 3 consecutive readings with SBP readings within 10 mm Hg and DBP readings within 5 mm Hg of each other were taken as the fasting measurement. Additional measurements were made if required.

Vascular function testing

All vascular measurements were performed by a single trained operator. Baseline measures of vascular function were performed in the morning after an overnight fast in a quiet temperature-controlled room. Subjects lay quietly for 5 min before the baseline endothelial function measurements were obtained.

Brachial artery FMD

Endothelium-dependent FMD of the right brachial artery was measured in the longitudinal plane above the antecubital fossa, with a 7.5-MHz linear array transducer (Accuson Aspen Duplex, Mountain View, CA) before and after forearm ischemia was caused by inflation of a sphygmomanometer cuff applied to the right forearm 2 cm below the olecranon process to 200 mm Hg for 5 min. Images were recorded at baseline (before compression), 30 s before cuff release, and then every 15 s after cuff release for 3 min. All images were stored for offline analysis. Arterial diameter was measured offline by a single observer using ultrasonic calipers at end diastole, incident with the R-wave on the electrocardiogram. The FMD response was calculated as the percentage change in the baseline diameter of the artery.

Peripheral arterial tonometry

Endothelial function was assessed with peripheral arterial tonometry—a novel previously validated method (21, 22) simultaneously with FMD assessment. Peripheral arterial tonometry (PAT) noninvasively measures changes in pulsatile arterial volume at rest and during reactive hyperemia recorded with a fingertip peripheral arterial tonometer (Endo-PAT2000; Itamar Medical Ltd, Caesarea, Israel). The pulse signal changes are amplified and fed to a personal computer and stored for offline analysis by computer algorithm. The PAT reactive hyperemia index (RHI) is defined as the ratio of the average pulse wave amplitude over a 1-min period of reactive hyperemia starting 1 min after cuff deflation compared with the average pulse wave amplitude over a 210-s preocclusion baseline period (22).

After fasting baseline endothelial function and BP measurements were obtained, the participants were provided with 250 mL of no-added-salt tomato soup or an identical tomato soup with added salt to be consumed within 5 min. The nutrient composition of the test meals differed only in their sodium content (Table 1). After consuming the meal, the participants remained in a supine position and the ultrasound transducer in a fixed position on the upper arm for the duration of the test (2 h). FMD and RHI were measured every 30 min after the completion of the meal for 2 h. Brachial BP on the left (opposite) arm was measured every 30 s before cuff release, and then every 15 s after cuff release for 3 min. All images were stored for offline analysis. Arterial diameter was measured offline by a single observer using ultrasonic calipers at end diastole, incident with the R-wave on the electrocardiogram. The FMD response was calculated as the percentage change in the baseline diameter of the artery.

**TABLE 1**

Nutrient composition of the test meals

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Low-salt meal</th>
<th>High-salt meal</th>
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</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>130</td>
<td>1494</td>
</tr>
<tr>
<td>(mmol)</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>130</td>
<td>130</td>
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</table>

A HIGH-SALT MEAL IMPAIRS FLOW-MEDIATED DILATATION
30 min immediately after the 3-min postocclusion period for FMD and RHI measurement. The endothelium-independent response to nitroglycerin was not assessed, and flow rates in response to ischemia were not measured. The operator who performed the FMD and RHI measurements and offline analysis was blinded to the intervention.

24-h Dietary recall

The participants were not asked to modify their diet in any way before or during the study. A 24-h dietary recall was conducted by a dietitian at visit 1, and the participants were provided with a copy of the recall in a meal plan format that they were instructed to consume the day before visit 2 to minimize the influence of diet constituents on fasting and postprandial vascular responses. Dietary data were analyzed by using Foodworks Professional Edition 2007 (version 5; Foodworks Professional Edition; Xyris Software, Highgate Hill, Australia).

Statistical analyses

On the basis of power calculations from our previous study (9) to detect a mean difference in FMD of 1.75% in a crossover design, 16 subjects were required to complete the study (α = 0.05; 80% power). Data were tested for normality of distribution and to ensure that residuals had an approximately constant SD (Kolmogorov-Smirnov test, Q-Q plots, and inspection of histograms). The results are expressed as means ± SDs or medians with ranges. Baseline comparisons between treatments were performed by using a paired t test or Wilcoxon’s signed-rank statistic as appropriate. Repeated-measures analysis of variance (ANOVA) with meal (ie, LSM or HSM) and time (0, 30, 60, 90, and 120 min) as within-subject factors was used to assess the effects of diet intervention on the dependent variables relative to baseline. Greenhouse-Geisser adjusted results are reported when sphericity was not met.

Preliminary analyses were performed to ensure no violation of assumptions of linearity. Pearson’s correlation was used to assess just adjusted results are reported when sphericity was not met.

RESULTS

Subjects

Sixteen volunteers completed the study, and their key characteristics are described in Table 2. No significant baseline differences were observed in any of the outcomes variables between interventions. No significant effect of age or BMI group was observed for any of the outcome variables assessed.

Vascular function

Brachial artery FMD

Precompression brachial artery diameter (LSM: 3.18 ± 0.52 mm; HSM: 3.19 ± 0.52 mm; P = 0.606) and baseline FMD

Table 2

Baseline characteristics of the participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>37.4 ± 18.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72.6 ± 13.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.8 ± 4.7</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>64 ± 11</td>
</tr>
</tbody>
</table>

All values are means ± SDs; n = 16 (10 women and 6 men). DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; SBP, systolic blood pressure.

Table 3

Measures of vascular function and blood pressure at the beginning of each intervention

<table>
<thead>
<tr>
<th></th>
<th>Low-salt meal</th>
<th>High-salt meal</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>72.5 ± 13.9</td>
<td>72.6 ± 14.0</td>
<td>0.586</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>109 ± 9</td>
<td>110 ± 9</td>
<td>0.554</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>67 ± 7</td>
<td>68 ± 9</td>
<td>0.730</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>80 ± 7</td>
<td>81 ± 9</td>
<td>0.532</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>64 (47–84)</td>
<td>64 (45–101)</td>
<td>0.306</td>
</tr>
<tr>
<td>Fasting BA diameter (mm)</td>
<td>3.18 (2.4–4.0)</td>
<td>3.25 (2.4–4.2)</td>
<td>0.472</td>
</tr>
<tr>
<td>Postrelease BA diameter (mm)</td>
<td>3.44 ± 0.55</td>
<td>3.43 ± 0.53</td>
<td>0.892</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>8.35 ± 2.14</td>
<td>7.61 ± 2.73</td>
<td>0.168</td>
</tr>
<tr>
<td>RHI</td>
<td>2.04 ± 0.60</td>
<td>2.03 ± 0.62</td>
<td>0.972</td>
</tr>
</tbody>
</table>

1 n = 16 (10 women and 6 men). BA, brachial artery; DBP, diastolic blood pressure; FMD, flow-mediated dilatation; HR, heart rate; MAP, mean arterial pressure; RHI, reactive hyperemia index; SBP, systolic blood pressure. 
2 Paired Student’s t test or Wilcoxon’s signed-rank test as appropriate. 
3 Mean ± SD (all such values). 
4 Median; range in parentheses (all such values).
Blood pressure

Premeal SBP, DBP, MAP, and heat rate were not significantly different between treatments (Figure 3). A significant effect of time was observed for SBP, DBP, and MAP (SBP: $P = 0.005$; DBP: $P = 0.026$; MAP: $P = 0.035$). BP decreased within 30 min, but had returned to premeal values by 120 min. No significant effect of diet or diet $\times$ time interaction was observed (Figure 3). Change in FMD was not significantly related to the change in SBP, DBP, or MAP at 30 and 60 min with either meal.

Sodium intake

Mean sodium intake assessed by 24-h dietary recall was $99.3 \pm 56.7$ mmol/d (5.8 g salt) before the LSM compared with $93.9 \pm 47.1$ mmol/d (5.5 g salt) before the HSM (NS). No significant relation between sodium intake and change in FMD was observed (data not shown). No significant differences in
other nutrients were observed in the 24 h before each intervention (NS).

DISCUSSION

This study showed that the amount of salt similar to that in a commonly eaten meal (15) impairs FMD in the postprandial phase compared with an LSM in a group of healthy normotensive adults. The maximum impairment in FMD observed with the HSM occurred at 60 min. This impairment in FMD in response to the HSM is of a similar magnitude to that shown in healthy subjects after a high-fat meal (11, 23–25).

These findings are consistent with previous reports in humans that salt intake impairs endothelial function in the short term (7, 26). We previously showed that FMD was impaired after 2 wk, when sodium intake increased from 50 mmol/d (2.9 g salt) to 150 mmol/d (8.8 g salt) (9), and a study in young healthy volunteers showed that salt loading of 250 mmol/d (14.7 g salt) for 5 d impaired the endothelium-dependent response to acetylcholine (8). Despite the differences in salt intake, we did not observe any differences in BP between the meals. In addition, we did not find any significant relation between change in FMD and change in BP with the HSM compared with the LSM at 30 and 60 min. We found no evidence that BP is involved in the mechanism by which salt impairs FMD.

We speculate that one of these possible mechanisms by which salt impairs endothelial function is via an alteration in plasma sodium. Two studies have reported responses to oral salt loading over a postprandial time period, which showed a rise in plasma sodium in response to 100 mmol Na (5.8 g salt) loading in healthy persons of 3 mmol/L, which occurred within 2–3 h of consuming the test meal (16, 17). It has been postulated that a high salt intake may acutely impair vascular function by raising plasma sodium by as much as 3 mmol/L. Oberleithner et al (19) have shown, in culture, that increasing plasma sodium concentrations within a narrow physiologic range (135–145 mmol/L) stiffens human endothelial cells and reduces nitric oxide production. Li et al (18) produced similar results in cultured bovine endothelial cells by increasing sodium concentration from 137 to 142 mmol/L and showing a subsequent 25% reduction in endothelial nitric oxide synthase (eNOS) activity, observed in a dose-dependent manner. Thus, it may be plausible that the 65-mmol sodium load (3.8 g salt) in the current study induced changes in endothelial function via alterations in plasma sodium. A repeat of the current study including plasma sodium samples and other parameters of endothelial function, such as nitric oxide and eNOS would confirm this.

The LSM also impaired postprandial FMD. This was not unexpected because it is known that FMD is sensitive to postprandial metabolites and is consistent with other meal studies investigating postprandial FMD responses to mixed meals (27).

The postprandial state is a complex phase, so it is possible that other metabolic or hormonal contributors are involved in the postprandial FMD response observed. For example, other normal cardiovascular responses to meal ingestion are sympathetic nervous system activation and increased plasma renin activity (28), which may increase peripheral vascular resistance and may mediate decreased vasodilatation in circulatory beds other than the gastrointestinal tract.

We found no significant differences in endothelial function, as measured by RHI, between treatments (Figure 2). The RHI response was not related to FMD. Reports of an association between FMD and RHI measurements are inconsistent in the literature in both healthy subjects and those with CVD risk factors (29, 30). The small sample size in our current study may have limited any observation of an association.

Lack of association between the 2 measures of endothelial function may also be explained by different mechanisms underlying the hyperemic response in different vascular beds. In conduit arteries, FMD is principally mediated by endothelium-derived nitric oxide (31), whereas the hyperemic response in the cutaneous microcirculation has been shown to be mainly nitric oxide (or endothelium) independent. Prostaglandins, adenosine, pH, PO₂, and myogenic influences have been reported as principal regulators (32, 33) of this response in these vascular beds. In contrast, one study reports that 50% of the hyperemic response in the microcirculation is NO-mediated (34). We suspect that the mechanism for the effect of dietary salt on endothelial function is primarily the downregulation of nitric oxide bioavailability. The differences in the influence of nitric oxide in microvascular compared with conduit artery vascular responses may reflect a difference in their susceptibility to the effects of dietary salt and may explain the differences we observed between the 2 responses.

The differential responses of FMD and RHI in the postprandial phase may be explained by resistance of the microcirculation to the effects of postischemic metabolites. For example, in a study by Gori et al (35), vascular function was assessed in young adults after ischemia and reperfusion, which showed blunted conduit artery FMD responses but no changes in microvascular reactive hyperemia.

A limitation of the current study was that biomarkers (plasma sodium, nitric oxide, and ADMA), which would provide information regarding the underlying mechanisms, were not measured. Studying the postprandial endothelial responses to meals high in salt as well as fat or carbohydrate would more closely reflect the way in which salt is consumed in real life.

In conclusion, we found that the amount of salt contained in an HSM significantly suppressed FMD within 30 min in a group of apparently healthy normotensive men and women. It appears that higher salt intakes have acute adverse effects on vascular dilatation in the postprandial phase. It is unclear whether this has any long-term pathophysiologic consequences, but these results contribute to the body of research showing the non-BP effects of high salt intakes. The mechanisms responsible for the dietary salt-induced endothelial dysfunction need to be investigated more intensively.

We acknowledge JLM-Acutek Health Care, which provided a time-limited loan of the Endo-PAT2000 device during the study, and Kylie Lange for assistance with statistical analyses.

The authors’ responsibilities were as follows—KMD, JBK, and PMC: performed the statistical analyses, interpretation of the data, and critical review of the manuscript; JBK and PMC: contributed to the statistical analyses, interpretation of the data, and critical review of the manuscript; and JBK: oversaw the study. None of the authors had any conflicts of interest in relation to this manuscript.
REFERENCES


There has been a marked increase in the incidence of autoimmune diseases in the past half-century. Although the underlying genetic basis of this class of diseases has recently been elucidated, implicating predominately immune-response genes, changes in environmental factors must ultimately be driving this increase. The newly identified population of interleukin (IL)-17-producing CD4+ helper T cells (TH17 cells) has a pivotal role in autoimmune diseases. Pathogenic IL-23-dependent TH17 cells have been shown to be critical for the development of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, and genetic risk factors associated with multiple sclerosis are related to the IL-23–TH17 pathway. However, little is known about the environmental factors that directly influence TH17 cells. Here we show that increased salt (sodium chloride, NaCl) concentrations found locally under physiological conditions in vivo markedly boost the induction of murine and human TH17 cells. High-salt conditions activate the p38/MAPK pathway involving nuclear factor of activated T cells 5 (NFAT5; also called TONEBP) and serum/glucocorticoid-regulated kinase 1 (SGK1) during cytokine-induced TH17 polarization. Gene silencing or chemical inhibition of p38/MAPK, NFAT5 or SGK1 abrogates the high-salt-induced TH17 cell development. The TH17 cells generated under high-salt conditions display a highly pathogenic and stable phenotype characterized by the upregulation of the pro-inflammatory cytokines GM-CSF, TNF-α and IL-2. Moreover, mice fed with a high-salt diet develop a more severe form of EAE, in line with augmented central nervous system infiltrating and peripherally induced antigen-specific TH17 cells. Thus, increased dietary salt intake might represent an environmental risk factor for the development of autoimmune diseases through the induction of pathogenic TH17 cells.

Although we have recently elucidated many of the genetic variants underlying the risk of developing autoimmune diseases, the significant increase in disease incidence, particularly of multiple sclerosis and type 1 diabetes, indicates that there have been fundamental changes in the environment that cannot be related to genetic factors. Diet has long been postulated as a potential environmental risk factor for this increasing incidence of autoimmune diseases in developed countries over recent decades. One such dietary factor, which rapidly changed along with the Western diet and increased consumption of processed foods or ‘fast foods’, is salt (NaCl). The salt content in processed foods can be more than 100 times higher in comparison to similar home-made meals. We have shown that excess NaCl uptake can affect the innate immune system. Macrophages residing in the skin interstitium modulate local electrolyte composition in response to NaCl-mediated extracellular hypertonicity, and their regulatory activity provides a buffering mechanism for salt-sensitive hypertension. Moreover, blockade of the renin-angiotensin system, can modulate immune responses and affect EAE. Thus, to investigate whether increased NaCl intake might have a direct effect on CD4+ T-cell populations and therefore represents a risk factor for autoimmune diseases, we investigated the effect of NaCl on the in vitro differentiation of human TH17 cells. We induced hypertonicity by increasing NaCl concentration by 10–40 mM (high-salt) in the culture medium and thus mimicked concentrations that could be found in the interstitium of animals fed a high-salt diet. As we previously reported, TH17-promoting conditions for naive CD4+ cells only induced a mild TH17 phenotype. Surprisingly, stimulation under increased NaCl concentrations markedly induced naive CD4+ cell expression of IL-17A as determined by flow cytometry.

Figure 1 | Sodium chloride promotes the stable induction of TH17 cells. a, Naive CD4+ cells were differentiated into TH17 cells in the presence (NaCl) or absence (None) of additional 40 mM NaCl and analysed by flow cytometry (FACS) for IL-17A (n = 20). b, IL-17A expression was measured by qRT–PCR (left panel, n = 10) and ELISA (right panel, n = 5). c, Cells were stimulated as in a under the indicated increased NaCl concentrations and analysed by FACS (one representative experiment of five is shown). d, Cells were stimulated as in a and were rested in the presence of IL-2. After 1 week, cells were re-stimulated as in a in the presence or absence of NaCl for another week and analysed by FACS (one representative experiment of five is shown). ***P < 0.001. qRT–PCR data are depicted as relative expression. For all figures, error bars show, unless indicated elsewhere, mean ± s.e.m.
We then examined the pathways whereby high-salt concentration induced this inflammatory phenotype. It has been shown that increased NaCl concentrations associated with augmented hypertonicity could induce immune system activation. Moreover, it is known that hypertonic stress in mammals is sensed through p38/MAPK, a homologue to HOG1, the ancient yeast hypertonic stress-response element. The key translator of this cascade is the osmosensitive transcription factor NFAT5. Analysis of the microarray data set indicated the stimulation of both inflammatory and classic hypertonicity induced pathways. The CD4+ cells expressed high levels of the NFAT5 targets SGK1 (ref. 22) and the sodium/myo-inositol co-transporter SLC5A3 (Fig. 2a, b and Supplementary Figs 7 and 8). Therefore, we proposed that increased NaCl concentration leads to phosphorylation of p38/MAPK that activates other downstream targets, including NFAT5. The phosphorylation of p38/MAPK was indeed increased in the presence of high-salt conditions (Fig. 3a, c and Supplementary Fig. 6). Our data indicate that NFAT5 is involved in this NaCl-induced inflammatory pathway. Because it has been shown previously that NFAT5 influences responses of immune cells under similar conditions, we silenced NFAT5 by a short hairpin RNA (shRNA) in naive CD4+ cells. As expected, NFAT5 silencing reduced SLC5A3 expression, but also decreased IL-17A and CCR6 expression (Fig. 3d). A direct downregulation of p38/MAPK-mediated knockdown of MAPK14 in CD4+ cells led to less IL-17A production (Supplementary Fig. 9b). High-salt concentration could also promote p38/MAPK activation via the release of ATP. However, by interfering with this pathway we could not observe significant changes on T17 differentiation (data not shown).

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**Figure 2** | High-salt-induced T17 cells display a pathogenic phenotype.

A. Microarray analysis of naive CD4+ cells differentiated into T17 cells in the presence (NaCl) or absence (none) of additional 40 mM NaCl. Depicted is a selection of 26 up- and downregulated genes (mean fold change of two independent experiments). B. qRT–PCR analysis of differentially expressed genes in the two groups (**P < 0.01, ***P < 0.001**. **Figure 2** | High-salt-induced T17 cells display a pathogenic phenotype.

A. Microarray analysis of naive CD4+ cells differentiated into T17 cells in the presence (NaCl) or absence (none) of additional 40 mM NaCl. Depicted is a selection of 26 up- and downregulated genes (mean fold change of two independent experiments). B. qRT–PCR analysis of differentially expressed genes in the two groups (**P < 0.01, ***P < 0.001**.**
and 12 and data not shown). High-salt-induced expression of IL-17A was dependent on p38/MAPK. Enhanced TH17 differentiation could be blocked by SB202190 (Fig. 3g).

In this investigation, we found that modest increases in NaCl concentration could stimulate an almost logarithmic in vitro induction of IL-17A in naive CD4+ cells mediated through p38/MAPK, NFAT5 and SGK1. Importantly, the addition of 40 mM of NaCl to Th17 differentiation cultures not only increased IL-17A expression but also led to a pathogenic phenotype of Th17 cells. In line with these findings, common salt added to the diet of mice led to severe worsening of EAE accompanied by increased numbers of Th17 cells.
Do these data indicate that increased salt intake is the long-sought-after environmental factor associated with the epidemic of autoimmune disease? Although these data present an attractive hypothesis, the direct causality of salt intake and incidence of autoimmune disease is yet to be demonstrated. That is, no in vitro observation can prove causality in humans; instead, our data indicate that clinical trials with severe curtailment of salt intake for individuals at risk for developing autoimmune disease are required. Clinical scenarios in which a dietary salt restriction protocol could be tested are multiple sclerosis or psoriasis, both autoimmune diseases with strong Th17 components. Additionally, excess salt content in diet should be investigated as a potential environmental risk factor for autoimmune diseases. However, this study would be difficult in Western cultures where the application of a true low-salt diet, representing the conditions in which Homo sapiens were environmentally selected in Africa, is difficult to achieve. Nevertheless, although there might be additional mechanisms contributing to the observed effects, the pathways identified in this study may offer new targets for the treatment of autoimmune diseases, with interference in the p38/MAPK, NFAT5 and SGK1 pathways aimed at blocking the generation of pathogenic T<sub>H</sub>17 cells.

**METHODS SUMMARY**

**Human cell sorting.** Peripheral blood mononuclear cells (PBMCs) were obtained from the peripheral blood of healthy subjects in compliance with institutional review board (IRB) protocols. CD<sup>−</sup> T cells were isolated by negative selection using magnetic beads (Miltenyi Biotec). Subsequently, naïve T cell were sorted as CD<sup>4</sup>CD<sup>25</sup>CD<sup>127</sup>−CD<sup>45RO</sup>−CD<sup>45RA</sup>− and memory cells were obtained by sorting for CD<sup>4</sup>CD<sup>25</sup>−CD<sup>127</sup>−CD<sup>45RO</sup>−CD<sup>45RA</sup>− on a FACS Aria (BD Biosciences).

**Human differentiation assays.** Naïve, memory or total CD<sup>4</sup> T cells were stimulated by plate-bound anti-CD3 and soluble anti-CD28 in serum-free X-VIVO15 medium (BioWhittaker) where indicated in the presence of various cytokines (IL-1β, IL-6, IL-21, IL-23, TGF-β1) and different concentrations of NaCl. Cells were analysed for cytokine expression by intracellular flow cytometry. Cytokine secretion was measured by ELISA (eBioscience). mRNA expression was determined by quantitative RT–PCR (Applied Biosystems).

**EAE induction and high-salt diet.** Male C57BL/6J mice (Harlan) were immunized with 200 μg MOG<sub>35-55</sub> in an equal amount of complete Freund’s adjuvant and received 200 ng pertussis toxin intraperitoneally on days 0 and 2 post induction. The clinical evaluation was performed daily on a 5 point scale ranging from 0 (no clinical sign) to 5 (moribund). Mice received normal chow and tap water ad libitum (control) or sodium-rich chow containing 4% NaCl and tap water containing 1% NaCl ad libitum (high-salt diet).

**Full Methods** and any associated references are available in the online version of the paper.

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3. Ascherio, A. & Munger, K. L. Environmental risk factors for multiple sclerosis. Part II: new targets for the treatment of autoimmune diseases, with interferon and high-salt diet. Male C57BL/6J mice (Harlan) were immunized with 200 μg MOG<sub>35-55</sub> in an equal amount of complete Freund’s adjuvant and received 200 ng pertussis toxin intraperitoneally on days 0 and 2 post induction. The clinical evaluation was performed daily on a 5 point scale ranging from 0 (no clinical sign) to 5 (moribund). Mice received normal chow and tap water ad libitum (control) or sodium-rich chow containing 4% NaCl and tap water containing 1% NaCl ad libitum (high-salt diet).

What might be the physiological role for the effect of high-salt on the induction of inflammatory T<sub>H</sub>17 cells? The concentration of Na<sup>+</sup> in plasma is approximately 140 mM, similar to standard cell culture media. Less well appreciated is that in the interstitium and lymphoid tissue, considerably higher Na<sup>+</sup> concentrations between 160 mM and even as high as 250 mM can be encountered—the ‘high-salt’ conditions that we found to induce inflammatory T<sub>H</sub>17 cells. Thus, this may be a mechanism for decreasing immune activation in the blood while favouring an inflammatory response in lymphoid tissues or with migration of cells into tissue. In this context it could be expected that other immune cells can react on high-salt conditions as well and potentially contribute to the effects observed in vivo.

**Supplementary Information** is available in the online version of the paper.

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**Author Information** The microarray data sets have been deposited in the Gene Expression Omnibus database under accession number GSE42569. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.K. (markus.kleinewietfeld@yale.edu) or D.A.H. (david.hafler@yale.edu).

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METHODS

Antibodies, recombinant cytokines and reagents. The following monoclonal antibodies and reagents were used as follows: for surface staining, anti-CD4 (RPA-T4), anti-CD45RO (UCHL1), anti-CD45RA (HI100), anti-CD25 (M-A251), anti-CD127 (hiL-7R-M21), anti-CCR6 (11A9) and AnnexinV all from BD Biosciences; for intracellular staining, anti-IL-17A (eBio64DEC17), anti-TNF-α (MAb1), anti-IFN-γ (4S.B3), anti-IL-2 (Miq-17H12), anti-RORC (AFK-9JS), anti-GATA3 (TWAI) and anti-Tbet (eBioB10) from eBioscience, and anti-pp38 (36/p38) (BD Biosciences) and anti-GM-CSF (BVD2-21C11) from Biolegend; and T cells were performed as described before12. In brief, monocytes were pulsed (Miltenyi Biotec). Cells were cultured in 96-well round-bottom plates (Costar) at cocktail (TGF-β1) and anti-CD3 (UCHT1) and anti-CD28 (28.2) from BD Biosciences. Recombinant human TGF-β1 was purchased from eBioscience, recombinant human IL-1β, IL-6, IL-12 and IL-23 and neutralizing anti-IFN-γ (257/8) and anti-IL-4 (3007) were purchased from R&D Systems, and recombinant human IL-21 was purchased from Cell Sciences. CFSE was obtained from Invitrogen.

Human cell isolation and stimulation. Peripheral blood was obtained from healthy control volunteers in compliance with Institutional Review Board protocols. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation. Untouched total CD4 + T cells were isolated from PBMCs by negative selection via the CD4 + T-cell isolation kit (Miltenyi Biotec). Naive (CD45RA + CD45RO – CD69 – HLA-DR –), central memory (CD45RA - CD45RO + CD69 – HLA-DR –), and memory (CD45RA – CD45RO – CD69 + HLA-DR +) T cells were sorted by high-speed flow cytometry with a FACS Aria (BD Biosciences) to a purity >98% as verified by post-sort analysis. Dead cells were excluded by propidium iodide (BD Biosciences). CD4 + monocytes were isolated by positive selection with CD14 microbeads (Miltenyi Biotec). Cells were cultured in 96-well round-bottom plates (Costar) at 5 x 10^4 cells per well in serum-free X-VIVO15 medium (BioWhittaker), and stimulated for 20 min before cells were fixed (Cytofix buffer, BD Biosciences). Cells were stained with the LIVE/DEAD Fixation Solution (Invitrogen) and stained with the respective antibodies for intracellular cytokine detection for 30–45 min. Before fixation, cells were stained with the Fix/Perm (eBioscience) according to the manufacturer’s instructions, and made permeable (Fix/Perm; eBioscience) in the presence of GolgiPlug (BD Biosciences), fixed with 4% methanol for 15 min and stained with the respective antibodies for intracellular cytokine detection for 30–45 min. Dead cells were excluded by propidium iodide (BD Biosciences). CD4 + cytokine producing cells were counted by flow cytometry and analyzed by Flowjo software. The values are represented as the difference of the four possible pair-wise comparisons were over a cutoff of 1.5-fold change were reported. A z-score was computed as additional filter by comparing the ratio of mean expression levels in the NaCl-treated samples to the expression levels in the control samples. Only cases with a corresponding P-value lower than 0.05 were reported.

Time PCR System (Applied Biosystems). The values were represented as the difference in Ct values normalized to β2-microglobulin for each sample as per the following formula: relative expression change = (2^(-ΔCt sample) × 10^3).

Lentiviral transduction of human T cells was carried out as described before31. In brief, 5 x 10^6 human naive CD4 + T cells per well were stimulated for 24 h prior to infection. Cells were then transduced with viral particles containing a vector expressing the indicated specific shRNA or as controls a vector expressing an unspecific shRNA or expressing GFP. Transduction was mediated at a multiplicity of infection (MOI) of 5 by centrifugation at 2,500 r.p.m. for 30 min at room temperature in the presence of 3 μg ml ^ -1 polybrene (Millipore). After 48 h the supernatant (Invitrogen) was added to the cultures at a concentration of 0.5 μg ml ^ -1 to select for successfully transduced cells and was controlled by flow cytometry for GFP and propidium iodide. The specific RNAi Consortium clones were TRCN0000020019 for NFAT5 and TRCN0000040175 for SGK1. For shRNA transfections, control shRNA (ON-TARGETplus non targeting 1) and a pool of four specific shRNAs for MAPK14 (ON-TARGETplus SMARTpool 1432) were obtained from Thermo Scientific Dharmacon. Cells were transfected by using Human T Cell Nucleofector kit and a Nucleofector II device as recommended by the manufacturer (Lonza/Amamax).

Microarray analysis. Cells for microarray analysis were collected at day 7 of culture and total RNA was isolated using Trizol reagent according to the manufacturer’s instructions. Expression data were generated by using GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) at the Yale Center for Genome Analysis (YCGA). For analysis, the data were normalized using the GenePattern software32 with the Robust Multi Array (RMA) algorithm33. The COMBAT software was used to remove batch effects. Fold change was computed between the average expression levels of each probe set in samples with the different conditions. To avoid spurious fold levels due to low expression values, a small constant (c = 50) was added to the expression values. Only cases where more than 50% of the four possible pair-wise comparisons were over a cutoff of 1.5-fold change were reported. A z-score was computed as additional filter by comparing the mean expression levels in the NaCl-treated samples to the expression levels in the control samples. Only cases with a corresponding P-value lower than 0.05 were reported.

Western blotting. Western blotting was performed as described before34. Phospho-p38 was detected by using anti-phospho-p38 (Cell Signaling Technology). Anti-β-actin and anti-SKG1 antibodies were obtained from Cell Signaling Technology and anti-NFAT5 antibodies were purchased from Pierce/Thermo Scientific. Primary antibodies were detected by peroxidase-conjugated streptavidin (Jackson Immuno Research), secondary anti-rabbit-HRP-conjugated (Cell Signalling Technology or Jackson Immuno Research) and secondary anti-mouse-HRP-conjugated (Bio-Rad) antibodies.

Mice. EAE induction, high-salt diet and blood pressure analysis. C57BL/6 mice were purchased from Harlan and housed at the in-house animal care facility of the University of Erlangen under standardized conditions. EAE induction was done as described before35. Briefly, male mice were immunized with 200 μg MOG35-55 (Charite) in an equal amount of complete Freund’s adjuvant and received 200 ng pertussis toxin (List Biochemicals) intraperitoneally on days 0 and 2 post induction. The clinical evaluation was performed on a daily basis by a 5-point scale ranging from 0, no clinical sign; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hindlimb paralysis, 4, hindlimb and forelimb paralysis, 5, moribund. Mice were not scored normally not water and ad libitum (control group) or sodium-rich chow containing 4% NaCl (SNIF) and tap water containing 1% NaCl ad libitum (high-salt group). Inhibition of p38/MAPK in vivo was done as described before36. In brief, mice were maintained on a control or high-salt diet and either received 1 mg kg ^ -1 SB202190 (Tocris) intraperitoneally or vehicle from day – 3 post induction of EAE. Brain leukocytes were isolated by percoll gradient centrifugation on day 17 post EAE induction, stimulated by PMA/ionomycin and analysed by flow cytometry for IL-17A and CD4 expression. Mx-Cre / p38<sup>-/-</sup> mice were maintained on a C57BL/6 background were a gift from J.-F. David. Mice were injected with 13 μg kg ^ -1 body weight recombinant-murine polyinosinic-polycytidylic acid (polyI:C, Sigma-Aldrich) on days 0, 2, 6 and were killed on day 8 for isolation of splenocytes. Blood pressure analysis was performed by the taft cuff method as described previously37. All animal experimentation was performed in accordance to the German animal protection law.

Histology. On day 20 post induction, mice were perfused with 4% paraformaldehyde and then the lumbar, thoracic and cervical part of their spinal cord was embedded in paraffin. Spinal cord cross-sections were stained with haematoxylin and eosin to assess inflammation. T cells were labelled by anti-CD3 (Serotec), macrophages/microglia by anti-Mac3 (BD Biosciences) and IL-17-positive cells by anti-IL-17 (Abcam).
Biosciences) for 48 h. For T<sub>H17</sub> cell differentiation, spleen and lymph node cells from 10-week-old 2D2 mice were pooled and CD4<sup>+</sup>CD62L<sup>-</sup> naive T cells were isolated by magnetic cell sorting (Miltenyi Biotec). Cells were cultured at 2 × 10<sup>6</sup> cells ml<sup>-1</sup> and stimulated for 4 days with 2 × 10<sup>5</sup> irradiated (30 Gy) syngenic splenocytes per ml and 1 µg ml<sup>-1</sup> anti-CD3 (2C11, BD Biosciences) in the presence of TGF-β1 (5 ng ml<sup>-1</sup>) and IL-6 (20 ng ml<sup>-1</sup>) and where indicated of additional 40 mM NaCl. For APC free TH17 differentiations, naive T cells were sorted as CD4<sup>+</sup>CD62L<sup>-</sup>CD44<sup>lo</sup>CD25<sup>-</sup> and stimulated by plate-bound anti-CD3 (2 µg ml<sup>-1</sup>) and anti-CD28 (2 µg ml<sup>-1</sup>) in the presence of IL-6 (40 ng ml<sup>-1</sup>) and TGF-β1 (1 ng ml<sup>-1</sup>) or IL-6 (40 ng ml<sup>-1</sup>) and IL-23 (10 ng ml<sup>-1</sup>) (all from R&D Systems) and were cultured for 4 days. In some experiments, 10 µM SB202190 (TOCRIS) was added to the cultures. For TH1 differentiation, naive CD4<sup>+</sup>T cells were cultured for 96 h with anti-CD3, anti-CD28, IL-12 (20 ng ml<sup>-1</sup>) (BioLegend) and anti-IL-4 (10 µg ml<sup>-1</sup>) (1B11, BioLegend). To monitor proliferation, cells were labelled with fixable proliferation dye (eBioscience) according to the manufacturer’s protocol. For intracellular flow cytometry, cells were stimulated for 4 h with PMA/ionomycin in the presence of monensin and stained for CD4 (RM4-5, eBioscience) and intracellular IL-17A (eBio17B7, eBioscience), IFN-γ (XMG1.2, eBioscience), Tbet (4B10, eBioscience) or RORC/ROR<sup>+</sup>c (AFKJS-9, eBioscience), excluding dead cells by a fixable viability dye (eBioscience). For murine gene expression analysis, mRNA was prepared using PeqLab Gold HP total RNA kit (PeqLab) and cDNA was prepared using superscript II reverse transcriptase (Invitrogen). RNA was isolated from EAE animals at day 14 post induction. Reactions were performed on a 7900 Sequence Detection System (Applied Biosystems). Primers were obtained from Applied Biosystems and target expression was normalized to β-actin expression. For cytokine secretion analysis, cells were stimulated as indicated and supernatants were collected after 3 days of culture. Monoclonal antibody pairs and recombinant cytokine standards were purchased from R&D systems (IL-17A, IFN-γ).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data were analysed by an unpaired t-test in case of two groups and by one-way ANOVA using Tukey’s post-hoc test in multiple groups. Data tested against a specified value were analysed by a one-sample t-test. EAE was analysed using a non-parametric Mann–Whitney U-test. Data were presented if not indicated elsewhere as mean ± s.e.m. P < 0.05 was considered to be statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).