Hypertension

ORIGINAL ARTICLE

Alterations of Placental Sodium in Preeclampsia: Trophoblast Responses

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BACKGROUND: Evidence suggests that increasing salt intake in pregnancy lowers blood pressure, protecting against preeclampsia. We hypothesized that sodium (Na^+) evokes beneficial placental signals that are disrupted in preeclampsia.

METHODS: Blood and urine were collected from nonpregnant women of reproductive age (n=26) and pregnant women with (n=50) and without (n=55) preeclampsia, along with placental biopsies. Human trophoblast cell lines and primary human trophoblasts were cultured with varying Na^+ concentrations.

RESULTS: Women with preeclampsia had reduced placental and urinary Na^+ concentrations, yet increased urinary angiotensinogen and reduced active renin, aldosterone concentrations, and osmotic response signal TonEBP (tonicity-responsive enhancer binding protein) expression. In trophoblast cell cultures, TonEBP was consistently increased upon augmented Na^+ exposure. Mechanistically, inhibiting Na^+/K^+-ATPase or adding mannitol evoked the TonEBP response, whereas inhibition of cytoskeletal signaling abolished it.

CONCLUSIONS: Enhanced Na^+ availability induced osmotic gradient-dependent cytoskeletal signals in trophoblasts, resulting in proangiogenic responses. As placental salt availability is compromised in preeclampsia, adverse systemic responses are thus conceivable. ***(Hypertension.* 2024;81:00–00. DOI: 10.1161/HYPERTENSIONAHA.124.23001.)** • **Supplement Material**.

Key Words: adenosine triphosphatases ■ biopsy ■ preeclampsia ■ sodium ■ trophoblasts

Preeclampsia is a leading cause of maternal and fetal morbidity and mortality worldwide.[1] It is associated with lifelong consequences for both mother and her child, including increased risks of cardiovascular and metabolic diseases.[2]

During pregnancy, women retain a total of 500 to 900 mmol sodium (Na^+), while the plasma volume increases by 30% to 50%.[3] Counterintuitively, this does not translate into a rise in blood pressure,[4] due to vasodilatation related to angiogenic factors.[5] In contrast, lower plasma volume in preeclampsia is associated with hypertension, suggesting an inverse linkage between blood pressure and plasma volume.[6] This is further supported by the vain attempts to prevent preeclampsia by lowering dietary Na^+ intake or increasing Na^+ excretion via diuretics.[7,8]

Renal Na^+ retention raising the plasma volume is considered secondary to a renin-angiotensin system dependent, as well as a renin-angiotensin system independent VEGF vascular endothelial growth factor)-augmented aldosterone synthesis, previously described by our group.[9,10] This is further amplified by a direct action of angiotensin II on the proximal tubule and by an activating posttranslational cleavage of subunits of the epithelial Na^+ channel in the cortical collecting duct, as we previously reported.[11,12]

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ORIGINAL ARTICLE

NOVELTY AND RELEVANCE

What Is New?

These findings highlight the placenta's role as a previously unrecognized sensor of salt levels, complementing the role of the kidneys.

What Is Relevant?

The breakdown in the mechanism responsible for retaining salt indicates potential abnormalities in Na^+-related signals within the placenta.

Clinical/Pathophysiological Implications?

Maintaining an appropriate level of Na^+ exposure in the placenta, such as through increased dietary salt intake, could be explored as a potential early preventive or therapeutic approach for preeclampsia.

Nonstandard Abbreviations and Acronyms

AGT	angiotensinogen
DC	dendritic cell
EGFR	epidermal growth factor receptor
ITGB1	integrin β1
MAPK	mitogen-activated protein kinase
NFAT5	nuclear factor of activated T-cells 5
PlGF	placental growth factor
SMIT	sodium myo-inositol cotransporter
TH-Aldo	tetrahydroaldosterone
TonEBP	tonicity-responsive enhancer binding protein
VEGF	vascular endothelial growth factor

The relevance of appropriate Na^+ availability is based on animal models and human diseases with aldosterone synthase deficiency. These data suggest that Na^+ supplementation can restore an aldosterone-replete phenotype.[13,14] Furthermore, increasing salt intake in pregnancy lowered blood pressure and protected against preeclampsia[14,15]; similar data by our group is observed in the first trimester of human pregnancy[12] and in pregnant animal models.[13,16,17] However, both, most susceptible individuals and potential effector mechanisms, are yet insufficiently defined.

Large amounts of Na^+ are stored in the skin interstitium, leading to functional consequences,[5,18,19] although it is appreciated that Na^+ is also stored in other tissues (eg, muscle). TonEBP (Tonicity-responsive enhancer binding protein), also known as the NFAT5 (nuclear factor of activated T-cells 5), is a signal transcription factor activated upon osmotic changes. Na^+ accumulation is sensed by dendritic cells (DCs), activating TonEBP, which then stimulates vascular endothelial growth factor-C (VEGF-C).[20,21] Disruption of this TonEBP-VEGF-C axis in rats increased blood pressure.[5] TonEBP is abundantly expressed in the human term placenta in physiological pregnancies,[22] yet its function is unknown.

TonEBP is required for the maturation and function of DCs and is involved in the pathogenesis of autoimmune diseases and inflammation.[23] The placenta exhibits a DC-like phenotype,[24] and the breakdown of its immune tolerance may contribute to preeclampsia.[25]

Given the functional observations and the DC-like phenotype of trophoblasts, we hypothesized that Na^+ evokes placental/trophoblastic signals, which are beneficial for a healthy pregnancy and that maintained placental Na^+ availability is essential to pregnancy.

Thus, we aimed to measure placental Na^+ availability and to identify Na^+-induced changes in trophoblast signaling, as modeled by exposing trophoblast cell lines and primary human trophoblasts to different Na^+ concentrations.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Details of participant, recruitment, sample collection, and methodology used are provided in the Supplemental Material. Briefly, urinary and placental Na^+ concentrations were measured using inductively coupled mass spectrometry.[26] Blood and urine renin-angiotensin system concentrations were measured using ELISA kits. Urinary tetrahydroaldosterone was measured using gas chromatography–mass spectrometry. All urinary concentrations were corrected for creatinine concentrations. Cell culture experiments were completed on isolated primary trophoblast cells, BeWo (CCL94), JEG-3 (HTB36), and HTR-8/SVneo cell lines.[27] Expression of mRNA and protein was measured using quantitative RT-PCR and Western blot, respectively.

Statistical Analysis

All graphs and data are presented as mean±SD or median interquartile range (IQR), as appropriate for the data distribution. Data from human placentae were analyzed by Mann-Whitney *U* test. Data from the cell lines were analyzed by repeated measures 2-way ANOVA and Tukey multiple comparisons test to compare ≥3 means, and Sidak multiple comparisons test to compare only 2 means. Data from primary CTBs were analyzed

by repeated measures 1-way ANOVA and Tukey multiple comparisons test. The null hypothesis was rejected at $P<0.05$. All statistical analyses were performed using SPSS (version 26; IBM) and GraphPad Prism (version 8; GraphPad Software).

RESULTS

Clinical Samples

The Table summarizes the demographic data and pregnancy outcome of the women recruited into this study. Throughout, controls refer to normotensive pregnant women.

Urinary Na^+ Concentrations

Urinary Na^+ concentrations differed between all groups ($P<0.05$) and were higher in normotensive pregnant controls compared with nonpregnant women ($P<0.01$; Figure 1A). All women with preeclampsia had urinary Na^+ concentrations lower than normotensive pregnant controls ($P<0.0001$). When further subgrouped, urinary Na^+ concentrations in early- and late-onset preeclampsia were lower than normotensive controls ($P<0.05$ for both; Figure 1A).

Lower Na^+ Content in Term Human Placentae From Women With Preeclampsia

Differences in placental Na^+ concentrations were observed between all groups ($P<0.05$). Placentae from all women with preeclampsia had markedly lower Na^+ content ($P<0.05$ for all; Figure 1B) compared with those of normotensive women.

Blood and Urine Renin-Angiotensin System Concentrations

Plasma active renin concentrations were different between groups ($P<0.05$), with the highest in nonpregnant women ($P<0.05$; Figure 1C), when compared with normotensive pregnancy; women with preeclampsia had even lower plasma active renin concentrations ($P<0.0001$), a difference that was maintained when subgrouped by early-onset preeclampsia only (Figure 1C).

Maternal plasma AGT (angiotensinogen) concentrations differed between groups ($P<0.05$) with all pregnancy groups having higher concentrations than nonpregnant women ($P<0.0001$ for all; Figure 1D).

Tetrahydroaldosterone:creatinine ratios were different between groups ($P<0.05$), with an increase in normotensive controls when compared with the nonpregnant group ($P<0.001$; Figure 1E). Aldo excretion was lower in the preeclampsia group as compared with normotensive controls ($P<0.0001$) and independent of the higher BMI in women with preeclampsia.

Cell Viability

To assess the impact of different cell culture treatments on cell viability, we used the MTT assay. More than 70% viability at the end of the experiment was considered acceptable, and this was achieved in all experiments performed, with 2 exceptions: after 24 hours of incubation with 170 mmol/L Na+, only 65% of BeWo cells were viable, and after inhibition of Na+/K+-ATPase by ouabain, only 41% of BeWo cells were viable (Table S4).

Table. Clinical and Obstetric Data of Subject Groups*

Parameter	NP (n=30)	NC (n=55)	EO-PE (n=21)	LO-PE (n=29)
Age, y	27±7.6	30±5.5	31±5.6	29±5.8
Booking body mass index, kg/m²	24.7±4.7†	27.6±6.3‡	32.0±7.7	29.0±7.7
Nonsmoker	27 (90)	51 (93)	18 (86)	26 (90)
Nulliparous	NA	13 (24)	14 (67)	18 (62)
Maximum systolic blood pressure, mm Hg	125±13†	128±12‡§	164±9	158±12
Maximum diastolic blood pressure, mm Hg	78±9†	80±8‡§	102±6	101±6
PCR, g/mmol	NA	NA	185 (62–361)	115 (58–253)
Gestational age at delivery, wk	NA	39.2±0.9‡	32.9±3.0¶	38.6±1.7
Mean birthweight, g	NA	3545±57‡§	1826±749¶	3069±620
Fetal gender male	NA	30 (55)	11 (52)	14 (48)
Cesarean section	NA	38 (69)	14 (67)	15 (52)

*Data represented as mean±SD, median (interquartile range) as appropriate, except for smoking status, parity and Cesarean sections, baby gender, and early-onset PE, which are shown as number (percentage). Booking was at time of first trimester antenatal visit. EO-PE indicates early-onset preeclampsia; LO-PE, late-onset preeclampsia; NA, not applicable; NC, normotensive control; NP, nonpregnant; and PCR, Proteinuria:Creatinine Ratio.

†$P<0.05$ between NP and EO-/LO-PE.

‡Between NC and EO-PE.

§Between NC and LO-PE.

¶Between EO-PE and LO-PE.

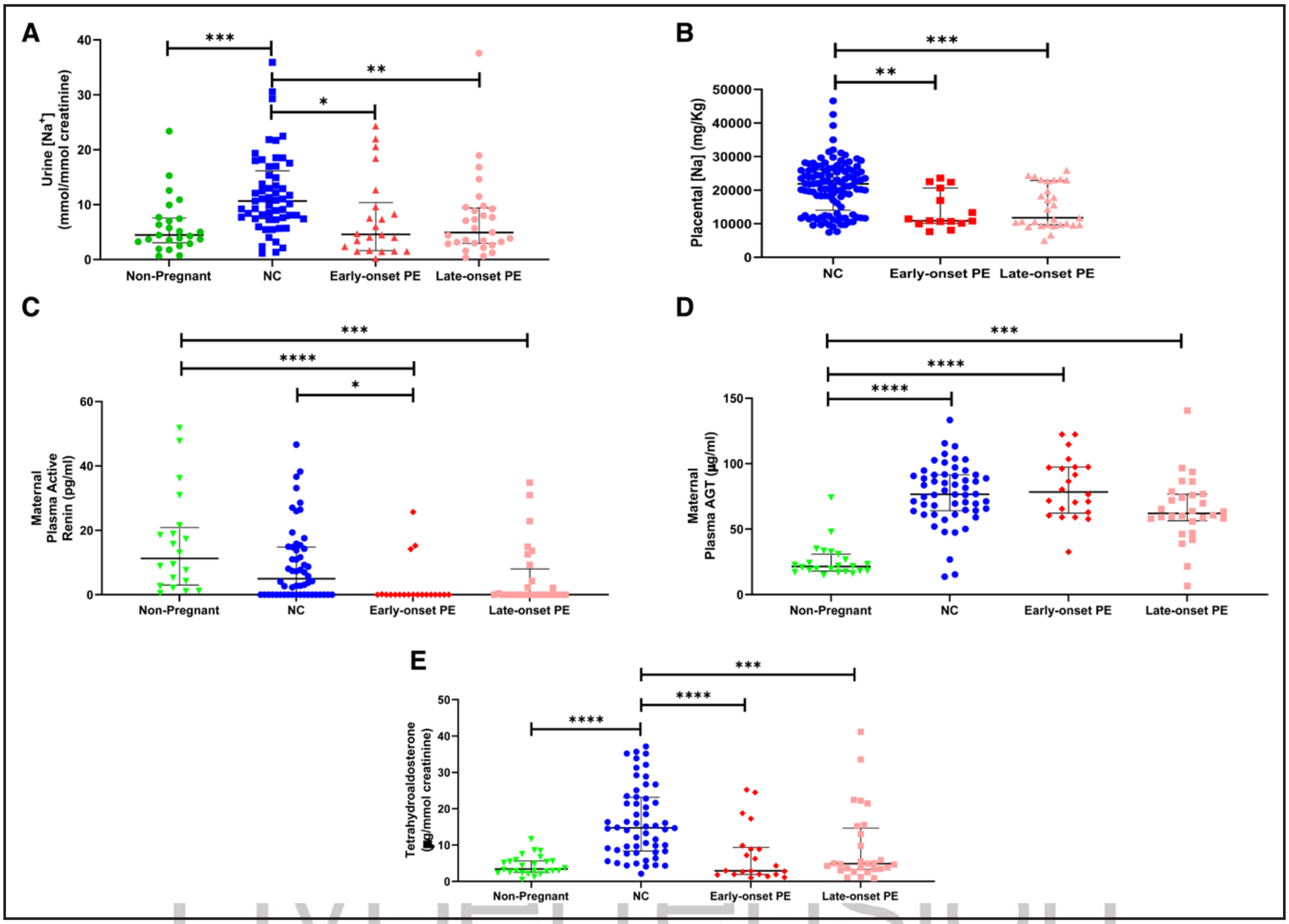

Figure 1. Urinary and placental sodium and renin-angiotensin-aldosterone concentrations in nonpregnant women; normotensive control (NC) and preeclampsia (PE).

Data are shown separately for early (diagnosis ≤34 weeks) and late (diagnosis >34 weeks) onset PE. **A**, Urinary sodium corrected for creatinine (nonpregnant, n=26; NC, n=55; early-onset PE, n=21; late-onset PE, n=29); (**B**) human placental sodium (NC, n=55; early-onset PE, n=15; late-onset PE, n=28); (**C**) maternal plasma active renin (nonpregnant, n=26; NC, n=55; early-onset PE, n=21; late-onset PE, n=29); (**D**) maternal plasma (angiotensinogen; nonpregnant, n=26; NC, n=55; early-onset PE, n=21; late-onset PE, n=29); and (**E**) urine tetrahydroaldosterone/creatinine (nonpregnant, n=26; NC, n=55; early-onset PE, n=21; late-onset PE, n=29). Data presented as median (interquartile range)], $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

Effect of NaCl on TonEBP, SMIT, VEGF-C, Flt-1, and PlGF mRNA Expression in Human Trophoblast Cell Lines and in Human Primary Term Cytotrophoblasts

The human trophoblast cell lines HTR-8/SVneo, JEG-3, and BeWo were exposed to different NaCl levels (103–120 mmol/L) or supraphysiological NaCl concentrations (up to 170 mmol/L) for 1 to 24 hours. In all cell lines, NaCl dose dependently increased *TonEBP* mRNA expression as compared with physiological NaCl concentrations, peaking between 3 and 6 hours of exposure (Figure 2A). In HTR-8/SVneo and BeWo cells, *TonEBP* mRNA expression returned to baseline levels after 24 hours, whereas transcripts remained elevated in JEG-3 at 24 hours (Figure 2A). TonEBP protein expression was in line with the mRNA data in all 3 cell lines (Figure S1). *SMIT* (*Sodium myo-inositol cotransporter*) mRNA expression dose dependently increased with rising NaCl concentrations and peaked a few hours later than *TonEBP* expression (Figure 2B). Furthermore, TonEBP and *SMIT* expression also increased in BeWo stimulated with forskolin (data not shown).

BeWo and JEG-3 cells only marginally expressed VEGF-C, a downstream signal of TonEBP (Ct>35; Table S2). In contrast, HTR-8/SVneo cells highly expressed basal *VEGF-C* levels (Ct=23), which were upregulated 2-fold upon high NaCl exposure (Figure 2C).

As Flt-1 is to some extent alternatively spliced, thus leading to soluble forms pathogenically relevant to preeclampsia, we assessed its expression in response to NaCl. High NaCl concentrations initially increased *Flt-1* mRNA expression, with only moderate changes beyond 8 hours in HTR-8/SVneo, whereas the response in JEG-3 cells was at later times, pointing toward a late suppression by high NaCl levels (Figure S2A). Flt-1 mRNA expression was missing in BeWo (Table S2).

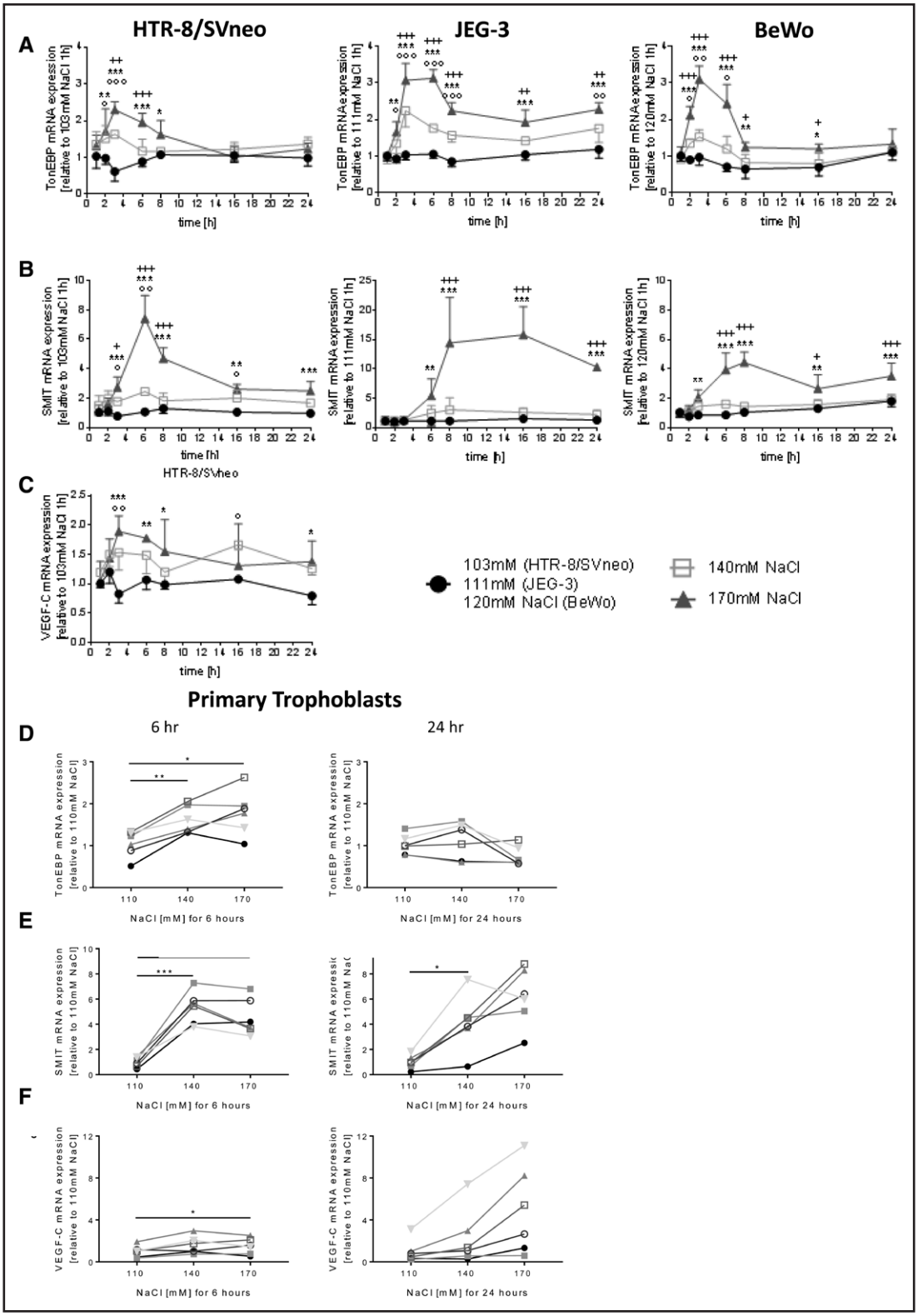

Figure 2. XXX.

Effect of different NaCl concentrations on mRNA expression of TonEBP (tonicity-responsive enhancer binding protein), SMIT (sodium myo-inositol cotransporter), VEGF-C (vascular endothelial growth factor-C), in human trophoblast cell lines (A–C), and isolated primary term cytotrophoblasts (D–F). The human trophoblast cell lines HTR-8/SVneo (first trimester trophoblasts), JEG-3, and BeWo (trophoblasts, derived from choriocarcinoma cell lines) were incubated with normal (103 mmol/L for HTR-8/SVneo, 111 mmol/L for JEG-3, 120 mmol/L for BeWo) or high NaCl concentrations (140–170 mmol/L) for 1 to 24 hours. The mRNA expression of TonEBP, SMIT, and VEGF-C was measured by qPCR. **A** and **B**, High NaCl increased TonEBP and its downstream gene *SMIT* in all cell lines; (**C**) VEGF-C could only be quantified in HTR-8/SVneo and increased with high NaCl. Human primary term cytotrophoblasts were incubated with normal (110 mmol/L) or (*Continued*)

Supraphysiological NaCl concentrations stimulated PlGF (placental growth factor) mRNA expression in HTR-8/SVneo and JEG-3 cells. In contrast, BeWo did not respond to increasing NaCl concentrations (Figure S2B).

Similar experiments in 6 independent isolations of primary human term cytotrophoblasts confirmed the results above. High NaCl concentrations stimulated *TonEBP* expression as early as 6 hours with no late response at 24 hours (Figure 2D). The *SMIT* transcript levels were also significantly increased at 6 hours and remained elevated for 24 hours (Figure 2E). Increasing NaCl concentrations stimulated *VEGF-C* mRNA expression after 24 hours in primary human term cytotrophoblasts (Figure 2F).

All third trimester trophoblast samples unambiguously indicated a suppression of Flt-1 transcripts at 24 hours (Figure S2C), a finding which was also present in most isolates for *PlGF* (Figure S2D).

In summary, secondary signals of trophoblast Na^+ exposure included the upregulation of TonEBP, SMIT, and VEGF-C.

Mechanism of TonEBP Activation in Response to High NaCl

As it is not yet known which factors play a major role in TonEBP activation in the placenta, we first addressed the guanine nucleotide exchange factor Brx, an important TonEBP regulator in lymphocytes.[28] Brx knockdown by siRNA did not reduce the expression of *TonEBP* or *SMIT* (Figure S3) upon exposure to increased NaCl levels.

As numerous intracellular signals are induced by Na^+ fluxes, the role of several NaCl transporters in TonEBP activation and inhibition in BeWo cells was investigated: Na^+-influx via the epithelial Na^+ channel,[29] the Na^+-K^+-$2Cl^-$ cotransporter,[30] the Na^+-Cl^- cotransporter,[31] Cl^- channels,[32] and the Na^+-H^+ exchanger[33] in medium equilibrated to normal (120 mmol/L) or high NaCl (170 mmol/L). Transcript levels of *TonEBP* and *SMIT*, induced in response to high NaCl, were not affected by the specific Na^+-influx inhibitors (Figure 3A and 3B). However, blocking Na^+-efflux via Na^+/K^+-ATPase inhibition (ouabain) resulted in enhanced and abolished *TonEBP* responses upon incubation with low and high NaCl levels, respectively (Figure 3A), consistent with altered intracellular osmolality. In low NaCl conditions, the *TonEBP* increase was not translated into an *SMIT* response, despite high TonEBP mRNA expression upon inhibition with ouabain (Figure 3B).

In summary, TonEBP was not regulated by Brx or altered by individual Na^+ channels, though severe alterations of intracellular Na^+ availability by interfering with Na^+/K^+-ATPase did result in altered TonEBP expression.

To differentiate whether hypertonicity or hyperosmolarity activates TonEBP in trophoblasts, diffusible and nondiffusable osmotic stimuli were tested. NaCl and D-mannitol are effective osmoles leading to extracellular hypertonicity and cell shrinkage, whereas urea is a highly diffusible osmolyte.[34] HTR-8/SVneo cells were incubated with different concentrations of NaCl, D-mannitol, or urea. High NaCl (140–170 mmol/L NaCl) clearly upregulated *TonEBP* mRNA expression up to 3-fold at 6 hours, whereas the application of D-mannitol at low levels (74 mosmol/L) upregulated *TonEBP* mRNA expression up to 2-fold, even though this did not reach significance. High D-mannitol (134 mosmol/L added; P=0.071) and high urea (134 mosmol/L added; P=0.246) did not further upregulate *TonEBP* expression (Figure 4A). At 24 hours, no cellular responses were observed upon NaCl, D-mannitol, or urea stimulation in HTR-8/SV neo (Figure 4A). Hypertonicity, caused by high NaCl or D-mannitol, upregulated *SMIT* mRNA expression up to 10-fold after 6 hours. Conversely, hyperosmolarity mimicked by urea showed no effect on *SMIT* mRNA expression after 6 hours (Figure 4B). In contrast to *TonEBP* (Figure 4A), the elevated *SMIT* expression upon high NaCl, high D-mannitol, and high urea was significant at 24 hours (Figure 4B).

In summary, TonEBP was enhanced after osmotic challenge with NaCl, D-mannitol, and urea, but only translated into an SMIT signal in NaCl and D-mannitol, but not in the freely diffusible urea.

Consequently, factors activated by cytoskeleton signals were assessed, such as ITGB1 (integrin β1),[35] focal adhesion kinase,[36] Src-family kinases,[37] p38α/β MAPK (mitogen-activated protein kinase),[38] and EGFR (epidermal growth factor receptor).[39] Inhibition or knockdown of these factors has been shown to prevent hypertonicity-induced TonEBP activation in other cell types. As these factors have not been tested for their relative impact in a pregnancy-specific cell type, HTR-8/SVneo cells were treated with ITGB1 siRNA, and inhibitors for focal adhesion kinase, Src-family kinase, p38α/β MAPK, and EGFR alone or in combination with normal (103 mmol/L) or high NaCl (170 mmol/L) conditions. As G-protein coupled receptors have been proposed to activate TonEBP,[40] the inhibition of G-protein coupled receptors was additionally tested. Individual inhibition of these factors diminished

Figure 2 Continued. high NaCl concentrations (140–170 mmol/L) for 6 (**left**) or 24 hours (**right**). **D**, High NaCl increased TonEBP, (**E**) its downstream genes *SMIT* and (**F**) *VEGF-C*. **A**–**C**, Data are presented as mean±SD (n=3 biological replicates). Error bars are shown only in 1 direction for better readability. For some points, the error bars would be shorter than the height of the symbol and are not displayed. **D** through **F**, Each symbol and shade of gray represents a different trophoblast isolation from an individual placenta (n=6). *, ○, or ^+P<0.05; **, ○○, or ^{++}P<0.01; ***, ○○○, or ^{+++}P<0.001. * shows significances between 170 mmol/L NaCl and normal NaCl (103 mmol/L for HTR-8/SVneo, 111 mmol/L for JEG-3, 120 mmol/L for BeWo). ○ shows significances between 140 mmol/L NaCl and normal NaCl. $^+$ shows significances between 170 mmol/L and 140 mmol/L NaCl. *P<0.05, **P<0.01, ***P<0.001.

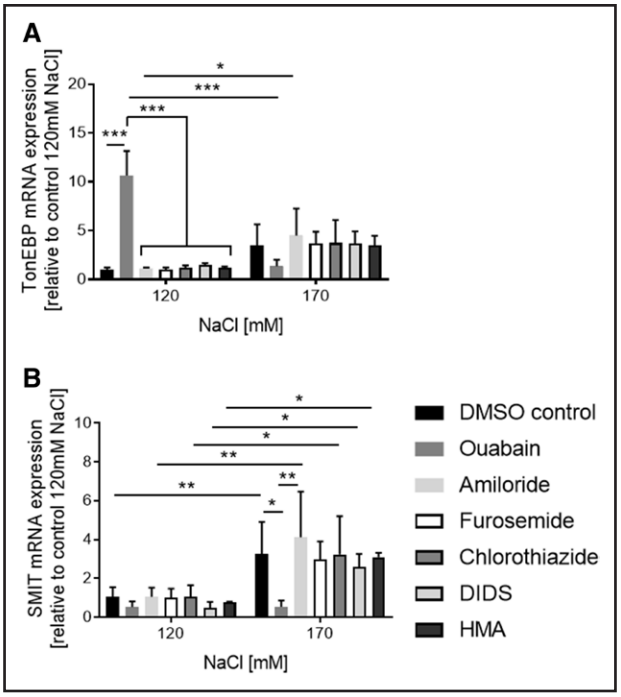

Figure 3. Mechanism of TonEBP (tonicity-responsive enhancer binding protein) activation in response to high NaCl.

BeWo cells were incubated with inhibitors of NaCl transporters in normal (120 mmol/L) and high NaCl(170 mmol/L) for 6 hours. Na/K-ATPase was inhibited by 10^{-4} M ouabain, epithelial sodium channel by 10^{-5} M amiloride, Na-K-2Cl cotransporter by 10^{-4} M furosemide, NaCl cotransporter by 10^{-4} M chlorothiazide, anion exchanger by 10^{-4} M 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid, and sodium-proton exchanger by 10^{-6} M 5-(*N,N*-hexamethylene)amiloride. The mRNA expression of (**A**) TonEBP and (**B**) SMIT (sodium myo-inositol cotransporter) was measured by qPCR. None of the NaCl-influx-transporter inhibitors (amiloride, furosemide, chlorothiazide, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, 5-(*N,N*-hexamethylene)amiloride) prevented the TonEBP and SMIT increase at high NaCl. Only inhibition of the **sodium**-efflux (ouabain) via the essential Na/K-ATPase affected the TonEBP and SMIT expression. Data are presented as mean±SD (n=3 biological replicates). **P*<0.05, ***P*<0.01, ****P*<0.001.

the *TonEBP* increase at high NaCl exposure (Figure 4C). Either alone or in combination, the lowest *TonEBP* activation was observed in the presence of the p38α/β MAPK inhibitor (SB202190), suggesting this to be a key signaling step toward *TonEBP* expression upon osmotic challenge (Figure 4C). *SMIT* expression was not affected by inhibiting ITGB1 or the GPER1, whereas a strong and consistent reduction in *SMIT* expression was again observed upon inhibiting the p38α/β MAPK pathway (Figure 4D).

In summary, signaling related to cytoskeleton responses was involved in TonEBP and SMIT expression.

DISCUSSION

In pregnancy, blood pressure is low despite plasma volume expansion and Na^+ retention. In contrast, in preeclampsia, blood pressure is increased though intravascular volume is low.[41] Likewise, Na^+ retention is compromised in individuals destined to later develop preeclampsia.[42]

Increasing Na^+ in pregnant women does not cause a rise in blood pressure.[12] Nevertheless, uncertainty exists as to the potential active role of Na^+ in promoting vasodilatory signals. Our novel data now show that during pregnancy a substantial placental Na^+ retention occurs, which is reduced in preeclampsia. By simulating alterations in Na^+ homeostatic conditions in trophoblasts, high Na^+ exposure clearly augmented the tonicity signal TonEBP, resulting in enhanced proangiogenic signals (PlGF and VEGF-C). This response was driven by an altered transcellular osmotic gradient, as simulated by different osmolytes, via cytoskeletal-based signaling.

Consistent with earlier findings,[43] maternal plasma renin and tetrahydroaldosterone concentrations are decreased in women with preeclampsia. As aldosterone synthesis in pregnancy is strongly driven by angiogenic signaling,[9,10] antiangiogenesis in preeclampsia compromises aldosterone availability and thus Na^+ retention.[44]

The placental Na^+ content as assessed previously,[45] was within a similar concentration range to those we observed in normal human placentae, yet the median content was lower in both early- and late-onset preeclampsia than in normotensive pregnancy. As either the renal Na^+ retention, placental intracellular Na^+ stores, and potentially to a much larger extent, the extracellular storage in compartments such as the cellular glycocalyx are conceivable, several mechanisms could be involved. A limitation was that we were not able to distinguish whether the Na^+ concentrations were from intracellular pools or not, and future work is required to elucidate this. Moreover, our Na^+/creatinine ratios were on spot urines and not 24-hour urines without control or assessment of patients' dietary sodium intake and medication use, and treatments may have differed between patient groups before isolation of placentae. Moreover, when controlling for the use of antihypertensives, no differences in Na+ data were found. In line with the proposed Na^+ availability in the placenta being critical, we demonstrated that both placenta and trophoblasts express markers of DCs characterizing tissues and cells responsive to Na^+-induced signals.[5] We confirm DC marker expression in our placental tissues (median [IQR], 9399 [2828–22 426] normalized copy number). TonEBP and SMIT expression has been described in the human placenta,[22] being progressively upregulated throughout gestation, with the limitation that true physiological concentrations of NaCl were not used in these experiments. Accordingly, TonEBP was also dose dependently upregulated in trophoblasts upon Na^+ exposure.

Of interest, placental expression of several Na^+ channels was reported to be reduced in preeclampsia, such as

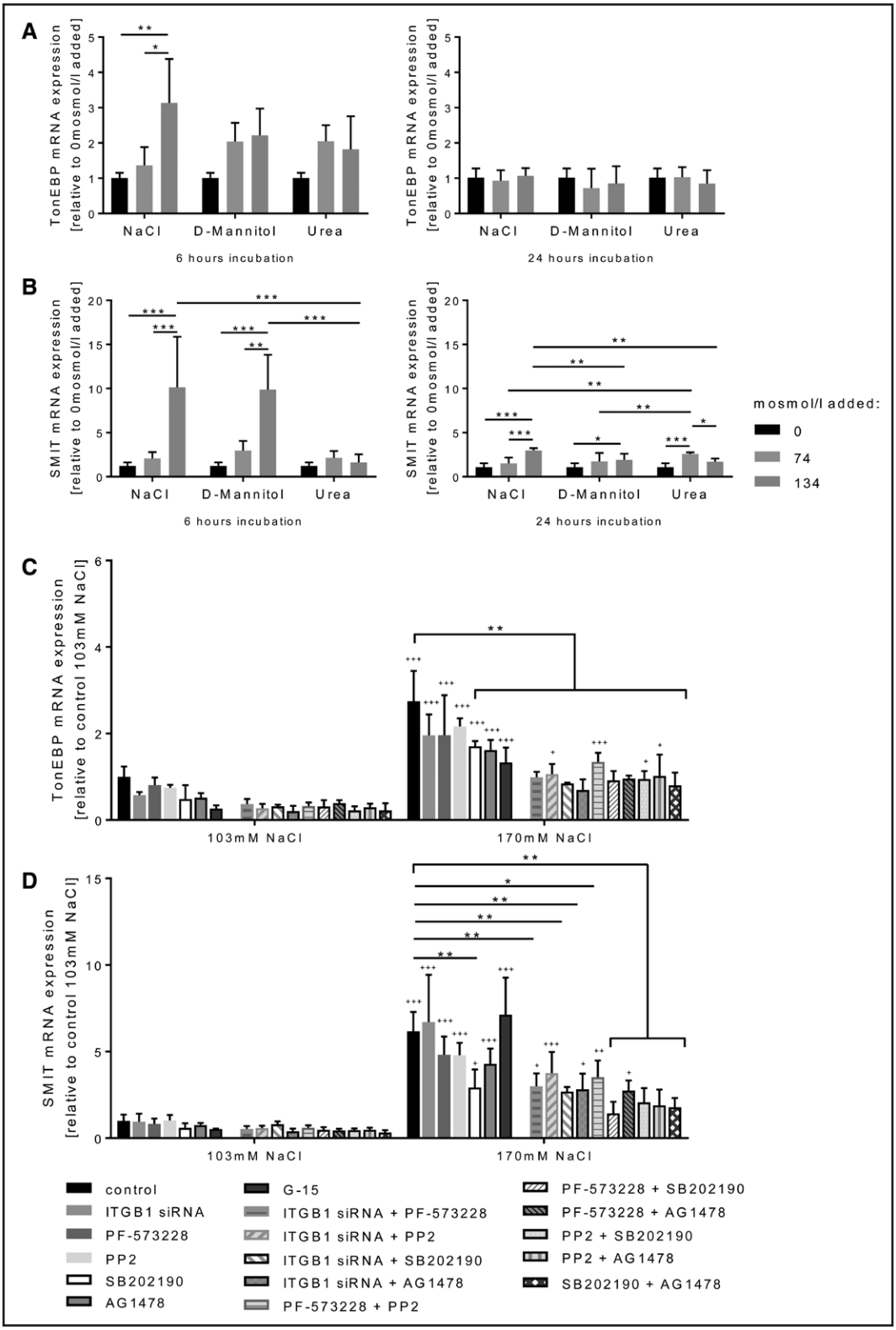

Figure 4. Mechanism of TonEBP (tonicity-responsive enhancer binding protein) activation in response to high NaCl. HTR-8/SVneo cells were incubated with different concentrations of NaCl, D-mannitol or urea (0, 74, or 134 mosmol/L added) for 6 (**left**) or 24 hours (**right**). In this experiment, concentrations are given as osmotic concentrations (mosmol/L) and not as molar concentrations (mmol/L). If no NaCl was added, the osmotic concentration corresponded to 103 mmol/L NaCl, 74 mosmol/L NaCl added to 140 mmol/L NaCl, and 134 mosmol/L NaCl added to 170 mmol/L NaCl. The mRNA expression of TonEBP and SMIT (sodium myo-inositol cotransporter) was measured by qPCR. NaCl and D-mannitol, which cause hypertonicity and cell shrinkage, upregulate TonEBP (**A**) and its downstream gene *SMIT* (**B**) more efficiently than urea, which causes hyperosmolarity but not hypertonicity and cell shrinkage. **C** and **D**, Different factors were (*Continued*)

epithelial Na^+ channel and NHE-3, a finding in line with the compromised aldosterone availability of preeclampsia.[46–48] Strong redundancy of Na^+-induced TonEBP activation was present in trophoblasts unaffected by inhibiting single Na^+ channels, including epithelial Na^+ channel. However, the activity of Na^+/K^+-ATPase was found to be critical for the maintenance of osmotic equilibrium, as inhibition of Na^+/K^+-ATPase in hypotonic conditions initially increased TonEBP but did not result in an SMIT signal.

While TonEBP regulation induced by changes in ambient tonicity has been known for some time in the kidney,[49] other cell types, such as macrophages, have also been identified as contributing to the regulation of Na^+ handling.[5] In our study, in both established trophoblast cell lines and primary human term trophoblasts, hypertonic TonEBP regulation was paralleled by SMIT, via VEGF-C transcript expression. The regulation of the placental vascular endothelial growth factor homolog PlGF and Flt-1 showed upregulation on NaCl exposure in the cell lines but downregulation in the primary cells. In the latter, isolates with low VEGF-C expression demonstrated high PlGF levels and vice versa, suggesting a compensatory mechanism. However, we have previously reported that aldosterone, which is likely to enhance Na^+ exposure via various mechanisms, upregulated PlGF.[50]

In contrast to observations of ouabain resistance of Na^+ transport in the placenta,[51] we have shown that TonEBP signaling was altered when Na^+/K^+-ATPase was inhibited. This is consistent with retention of intracellular Na^+ as was proposed by Orlov and Hamet.[52]

The importance of a transmembrane osmotic gradient was underlined by an osmotic response to the nondiffusible osmolyte, D-mannitol, but not to diffusible urea. Consistent with a transmembrane osmotic difference affecting cytoskeletal changes by consecutive Ca^{++}-activated p38α/β MAPK activation, its inhibition suppressed the osmotic signal, supporting the hypothesis that this pathway is central. Cytoskeletal signaling involved ITGB1 and transmembrane spanning receptors such as the epidermal growth factor receptor via Src-family and focal adhesion kinases. In line with other observations, our data suggest that TonEBP is regulated by receptor and integrin interaction such as the epithelial growth factor receptor.[53] Of interest, our finding that ITGB1 enhances other osmotic stimuli in trophoblasts concurs with the original findings in the renal medulla.[35]

Tonicity changes affect the cytoskeleton in other cell types, and NaCl, mannitol, and urea have been implicated in those responses.[54] We have now shown in trophoblasts that, while NaCl clearly enhanced TonEBP, the response was weaker with the other osmolytes. The nondiffusible mannitol elicited a vast early SMIT response, while the diffusible urea required long exposure to respond, contrasting with observations in renal MDCK cells.[54]

The first observation of abnormal placental Na^+ handling in preeclampsia was made ≈70 years ago. Using ^{24}Na normal saline as a tracer, a diminished rate of transfer of sodium across the placenta was observed in (toxemic) cases.[55] We have now shown, for the first time, that the placental Na^+ content is drastically lower in placentae from women with preeclampsia and that the Na^+ environment is important for the regulation of trophoblast signaling relevant to maintain maternal endothelial integrity.

Overall, these observations open intriguing avenues, and we speculate that instead of restricting NaCl intake in pregnancies considered to be at risk of preeclampsia, a carefully monitored dietary increase of NaCl may possibly be considered as a potential early preventive strategy. However, further work is required before any such strategies are clinically introduced.

PERSPECTIVES

In preeclampsia, women experience a depletion of placental Na^+ reserves alongside decreased aldosterone levels and urinary sodium. This suggests a breakdown in the mechanism responsible for retaining salt and indicates potential abnormalities in Na^+-related signals within the placenta. Additionally, when trophoblasts are exposed to higher levels of Na^+, they exhibit increased expression of VEGF-C, a molecule known for its protective and vasodilatory effects on endothelial cells, mediated through TonEBP signaling. These findings highlight the placenta's role as a previously unrecognized sensor of salt levels, complementing the role of the kidneys. It raises the possibility that maintaining an appropriate level of Na^+ exposure in the placenta could be explored as a potential early preventive or therapeutic approach for preeclampsia.

Figure 4 Continued. knocked down or inhibited alone or combined in normal (103 mmol/L) and high NaCl (170 mmol/L) conditions in HTR-8/SVneo cells. Integrin β1 was knocked down by siRNA, focal adhesion kinase was inhibited by 10^{-6} M PF-573228, Src-family kinase by 10^{-6} M PP2, p38α/β by 10^{-6} M SB202190, EGFR (epidermal growth factor receptor) by 10^{-6} M AG1478 and G-protein coupled receptor 30 by 10^{-6} M G-15. The mRNA expression of TonEBP and SMIT was measured by qPCR. Inhibition of p38α/β diminished the SMIT increase at high NaCl more pronounced than ITGB1 (integrin β1) knockdown or inhibition of focal adhesion kinase, Src-family kinase, EGFR, or G-protein coupled receptor 30. Any combination of 2 inhibitors prevented the TonEBP and SMIT increase at high NaCl similar or even more than inhibition of p38α/β alone. Data are presented as mean±SD (n=3 biological replicates). In graphs **C** and **D** the symbol + written directly above the 170 mmol/L NaCl bars shows that 170 mmol/L compared with 103 mmol/L NaCl is significant. * or $^{+}P<0.05$, ** or $^{++}P<0.01$, *** or $^{+++}P<0.001$.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Expanded Materials & Methods
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References

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