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Original Paper

LEFTYA Activates the Epithelial Na^+ Channel (ENaC) in Endometrial Cells *via* Serum and Glucocorticoid Inducible Kinase SGK1

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Key Words
Na^+ channels • Endometrium • Amiloride • Infertility • SGK1

Abstract
Background: Serum & glucocorticoid inducible kinase (SGK1) regulates several ion channels, including amiloride sensitive epithelial Na^+ channel (ENaC). SGK1 and ENaC in the luminal endometrium epithelium, are critically involved in embryo implantation, although little is known about their regulation. The present study explored whether SGK1 and ENaC are modulated by LEFTYA, a negative regulator of uterine receptivity. ***Methods:*** Expression levels were determined by qRT-PCR and Western blotting, ENaC channel activity by whole cell patch clamp and transepithelial current by Ussing chamber experiments. ***Results:*** Treatment of Ishikawa cells, an endometrial adenocarcinoma model cell line of endometrial epithelial cells, with LEFTYA rapidly up-regulated *SGK1* and *ENaC* transcript and protein levels. Induction of *ENaC* in response to LEFTYA was blunted upon co-treatment with the SGK1 inhibitor EMD638683. ENaC levels also significantly upregulated upon expression of a constitutively active, but not a kinase dead, SGK1 mutant in Ishikawa cells. LEFTYA increased amiloride sensitive Na^+-currents in Ishikawa cells and amiloride sensitive transepithelial current across the murine endometrium. Furthermore, LEFTYA induced the expression of *ENaC* in the endometrium of wild-type but not of *Sgk1*-deficient mice. ***Conclusions:*** LEFTYA regulates the expression and activity of ENaC in endometrial epithelial cells *via* SGK1. Aberrant regulation of SGK1 and ENaC by LEFTYA could contribute to the pathogenesis of unexplained infertility.

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Introduction

A successful pregnancy requires the endometrium to first engage with a competent embryo, embed the conceptus into the decidua (stroma), and then support invasion of extra-embryonic trophoblast leading to the formation of the placenta [1, 2]. These carefully orchestrated events require a specialized uterine microenvironment, commonly referred to as the window of implantation. Failure of the endometrium to express this phenotype is thought to be a major cause of subfertility and IVF treatment failure [3, 4].

Unexplained infertility has been associated with impaired endometrial expression of *LEFTY2*, encoding LEFTYA (originally designated as Endometrial Bleeding-Associated Factor EBAF), a member of the Transforming Growth Factor-β superfamily that antagonizes Nodal signaling [5]. Induction of LEFTYA in the endometrium is associated with a reciprocal decrease in NODAL expression during the mid- to late-luteal phase of the menstrual cycle [6]. Transition of NODAL to LEFTYA dominance appears to be essential for implantation, as loss of NODAL or overexpression of LEFTYA severely compromises fertility in mice [7].

LEFTYA is highly induced by decidualizing stromal cells and levels rise sharply following closure of the window of implantation during the late luteal phase of the cycle [8-10]. It is expressed as a polypeptide that requires processing by proprotein convertase (PC5) for its activation [11]. In response to falling progesterone levels, LEFTYA is activated [12] and increases expression of matrix metalloproteinases (MMP)-1, MMP3 and MMP9, which are enzymes that proteolytically degrade the stratum functionalis prior to menstruation. In the murine endometrium, LEFTYA decreases during the peri-implantation period and transient overexpression during this time results in implantation failure [13, 14]. Similarly, LEFTYA secretion is markedly reduced during the implantation window in the endometria and sera of normal fertile women, whereas elevated LEFTYA levels are associated with infertility [13]. Taken together, these observations strongly implicate LEFTYA in the regulation of endometrial receptivity, although its mechanism of action remains unclear.

We previously reported that aberrant levels of endometrial Serum & Glucocorticoid inducible Kinase (SGK1) was also linked to unexplained infertility in both humans and mice [15, 16]. SGK1 is a serine/threonine protein kinase with considerable homology to AKT. Targets of SGK1 include the ubiquitin ligase NEDD4-2, a key hormone-dependent regulator of sodium (Na^+) transport in mammalian epithelia. NEDD4-2 binds to and ubiquitinates amiloride-sensitive epithelial sodium channel (ENaC) at the cell surface, which targets surface ENaC for degradation and thus, reduces epithelial Na^+ transport [17]. SGK1 regulates Na^+ transport by inhibiting the ubiquitin ligase NEDD4-2, thereby enhancing the expression of ENaC [18-22]. Recently, ion channels in the endometrium have emerged as important players in regulating endometrial receptivity. Abnormal expression or function of ion channels in the endometrium may lead to impaired endometrial receptivity and implantation failure [20, 21]. ENaC is localized at the apical membrane in a wide variety of epithelia, including endometrial epithelium. ENaC is essential to sodium and water homeostasis in the body [23, 24]. Up-regulation of ENaC leads to absorption of luminal fluid and uterine 'closure', which likely facilitates apposition of the blastocyst to the luminal epithelium [25]. A previous study demonstrated that deregulated endometrial ENaC expression was associated with failure to conceive [19, 25]. Furthermore, secreted TGFβ participates in the crosstalk of endometrial cells and the preimplantation embryo [26].

Our previous study demonstrated that expression of constitutively active SGK1 prevented expression of key endometrial receptivity genes, perturbed uterine fluid handling *via* ENaC and abrogated embryo implantation [16]. Little is known about regulation of endometrial SGK1 expression. The present study explored whether LEFTYA (which antagonizes TGFβ signalling) modifies SGK1 expression as well as the expression and function of ENaC. To this end, qRT-PCR and Western blotting were employed to quantify SGK1 and ENaC expression. Whole cell patch clamp as well as Ussing chamber experiments were performed to quantify ENaC activity.

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Materials and Methods

Animal experiments

All animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act under Project Licences granted by the Home Office, United Kingdom and according to the German law for the welfare of animals approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium). Prior to the experiments mice had access to food and water *ad libitum*, and were kept under constant humidity (55 ± 10%), temperature (22 ± 2°C) and 12h light-dark cycle conditions. Experiments were performed using 6-8 week old C57BL/6 wild type female mice (Charles River Ltd, Margate, UK), wild type (*Sgk1*$^{+/+}$) or Sgk1 knockout (*Sgk1*$^{-/-}$) female mice [27] at the estrus stage. The mice were sacrificed and the uterus removed. The excised uterus (*ex vivo*) was washed and the horns flushed with (100 µl) of either PBS or LEFTYA (500 ng ml^{-1}; in PBS). After 24h the uterus was harvested for further analysis.

Cell culture

Ishikawa cells, a human endometrial adenocarcinoma cell line widely used as a model of luminal endometrial epithelial cells [28, 29], were maintained in Dulbecco's modified Eagle's medium/F12-phenol free media (Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-Glutamine and 100 U ml^{-1} penicillin/streptomycin (Invitrogen) and incubated at 37°C in a humid atmosphere maintained at 5% (v/v) CO_2, and routinely tested for mycoplasma infection. Where indicated, the cells were treated with LEFTYA as described previously [30] (R&D Systems, Wiesbaden, Germany) in the absence and presence of SGK1 inhibitor EMD 638683 (50 µM; Tocris, Cologne, Germany) for the indicated periods and with the indicated concentrations. Ishikawa cells were further transiently transfected with the constitutively active SGK1 mutant ^{S422D}SGK1 [hSGK1^{SD} in pIRES-EGFP or in pcDNA 3.1(+)] or the inactive mutant ^{K127N}SGK1 [hSGK1^{KN} in pIRES-EGFP or pcDNA 3.1(+)] [28] using Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Quantitative Real-time PCR (qRT-PCR).

Total RNA was extracted from Ishikawa cultures or from snap frozen whole uteri using Trizol (Invitrogen) based on a phenol-chloroform extraction protocol. Equal amounts of total RNA (2 µg) were reverse transcribed by using the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) using an oligo dT primer. The resulting first-strand cDNA was diluted and used as a template in qRT-PCR analysis. Primers were designed with PrimerBlast. L19 and Cyclophilin (Cyclo) represent non-regulated (housekeeping) human and murine genes, respectively, and their expression was used to normalize for variances in input cDNA. Detection of gene expression was performed with KappaFast-SYBR Green (Peqlab, Erlangen Germany) and quantitative RT-PCR (qRT-PCR) was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany). The expression levels of the samples were expressed as arbitrary units defined by the $\Delta\Delta C_t$ method. All measurements were performed in triplicate. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity.

Western blotting

For determination of protein abundance, total protein lysates from Ishikawa cells were prepared by lysing cells in RIPA buffer. Protein yield was quantified using the Bio-Rad DC protein assay kit (Bio-Rad). Equal amounts of proteins (30 µg) were separated on 10% sodium dodecyl sulfate–polyacrylamide (SDS) gel before electrotransfer onto the PVDF membrane (Amersham Biosciences, Freiburg Germany). Nonspecific binding sites were blocked by overnight incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS-T; 130 mmol/L NaCl, 20 mmol/L Tris (pH7.6) and 1% Tween). Primary antibodies used were αENaC (1:500, #E4653,Sigma, Taufkirchen, Germany), phospho-SGK1 (1:1000, ♯5599, Cell Signaling, Erlangen, Germany), phospho-NEDD4-2 (1:1000 #ab168349, Abcam, Cambridge, UK), Total-NEDD4-2 (1:1000, #40135, Cell Signaling), Total-SGK1(1:1000 #3272, Cell Signaling) and β-actin (1:1000, #4967, Cell Signaling), which was used as a loading control. For detection, a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (1:2000, #7074, Cell Signaling) or secondary anti-mouse IgG antibody conjugated with HRP (1:2000, #7076, Cell Signaling) was used. Protein complexes were visualized with a chemiluminescent detection kit (Invitrogen). All experiments were performed in 3 or more cell cultures. Bands were quantified with ImageJ Software.

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Flow cytometry and ELISA

Cyclooxygenase-2 (COX-2) expression was analyzed by flow cytometry. Cultured cells were detached, washed three times with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde for 15 min on ice. Then the cells were incubated for 60 min (37 °C) with anti-COX-2 primary antibody (1:200, #ab23672, Abcam), washed once in PBS, and stained in 1:250 diluted CF™ 488A-labeled anti–rabbit secondary antibody (Sigma) for 30 min (37 °C). Samples were immediately analyzed on a FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany). In parallel cultures, the supernatants were also collected and soluble 6-keto-PGF1α levels (#ab141709; Abcam) were measured using an Enzyme-linked immunosorbent assay (ELISA). Soluble levels in the cell culture media were determined according to the manufacturer's protocol.

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode as described previously [31]. Ishikawa cells were continuously superfused through a flow system inserted into the dish [32]. The bath was grounded *via* a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (2-4 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Currents were elicited by 200 ms square wave voltage pulses from -160 to +40 mV in 20 mV steps delivered from a holding potential of -50 mV with an acquisition frequency of 10 and 3 kHz low-pass filtered [33]. The liquid junction potential ΔE between the pipette and the bath solutions and between the salt bridge and the bath solutions was estimated as described previously [34]. Data were corrected for the estimated ΔE values.

Ishikawa cells were superfused with a bath solution containing: 145 mM/l Na-gluconate, 2.7 mM/l KCl, 1.8 mM/l $CaCl_2$, 2 mM/l $MgCl_2$, 5.5 mM/l glucose, 10 mM/l HEPES/NaOH, pH 7.4.The patch clamp pipettes were filled with: 135 mM/l K-gluconate, 6 mM/l NaCl, 2 mM/l $MgCl_2$, 10 mM/l HEPES/KOH, pH 7.2 [19].

Ussing chamber experiments

ENaC activity was estimated from the amiloride-sensitive potential difference and current across the murine endometrial epithelium. After removing the outer serosal and the muscular layer under a microscope, tissues were mounted onto a custom-made mini-Ussing chamber with an opening diameter of 0.99 mm and an opening area of 0.00769 cm^2. Transepithelial potential difference (V_{te}) was determined continuously and transepithelial resistance (R_{te}) estimated from the voltage deflections (ΔV_{te}) elicited by imposing rectangular test currents of 1 μA and 1.2 s duration at a rate of 8/min. R_{te} was calculated according to Ohm's law [35]. The serosal and luminal perfusate contained (in mM): 145 NaCl, 1 $MgCl_2$, 2.6 Ca-gluconate, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 glucose. To assess ENaC induced current, 50 μM amiloride (Sigma, in DMSO) was added to the luminal perfusate [36]. Colonic epithelium was used as a positive control.

Statistical analysis

Data are provided as means ± SEM, n represents the number of replicate experiments investigated. Data were tested for significance using ANOVA or Students *t*-Test as appropriate. $P < 0.05$ were considered statistically significant.

Results

The present study explored whether LEFTYA modifies endometrial expression of SGK1 and endometrial expression as well as activity of ENaC. In a first series of experiments qRT-PCR was employed to quantify the effect of LEFTYA on *SGK1* and *ENaC* transcript levels. As illustrated in Fig. 1A-D, a 5-120 min. treatment of Ishikawa cells with LEFTYA (25 ng/ml) increased the transcript levels of *SGK1* and of all three ENaC subunits *αENaC*, *βENaC*, and *γENaC*. As shown by Western blotting for phosphorylated SGK1 and αENaC (Fig. 1E-G), the increase of transcript levels was paralleled by an increase of protein abundance,

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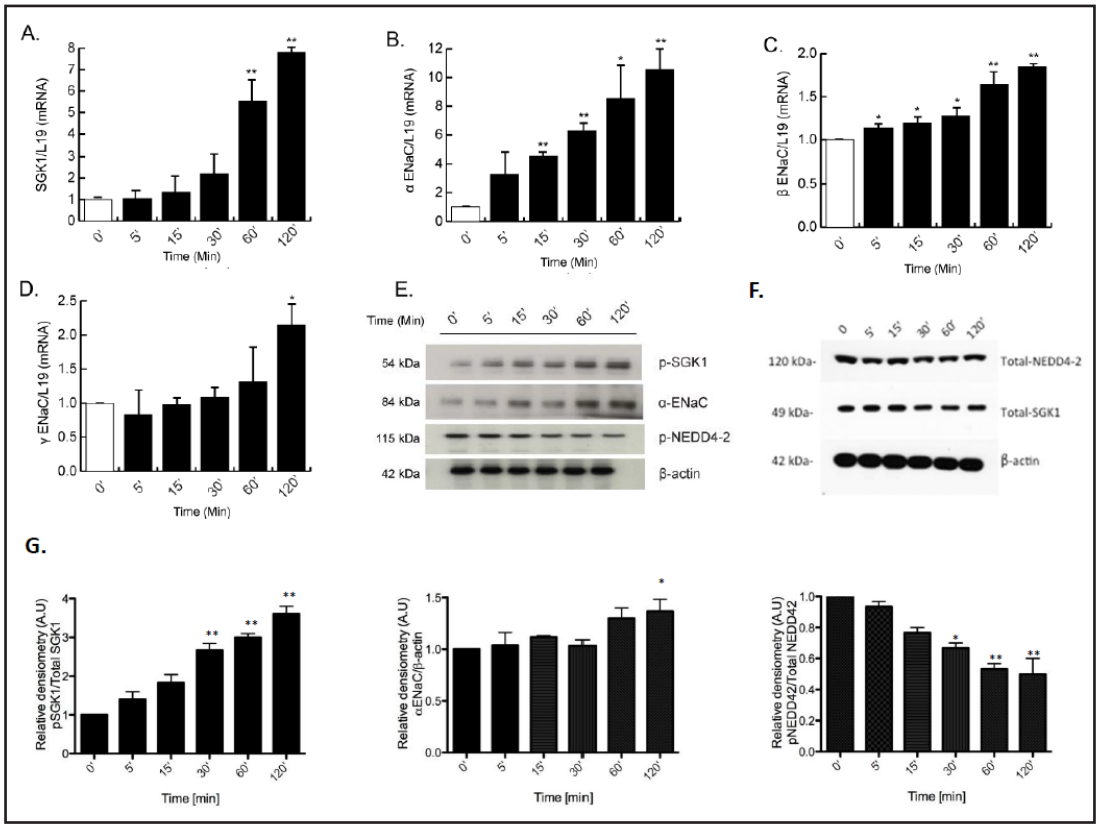

Fig. 1. Effect of LEFTYA on *SGK1* and *ENaC* subunit transcript levels as well as SGK1, NEDD4-2, and αENAC protein abundance in Ishikawa cells. A-D. Arithmetic means ± SEM (n = 4) of the (A) *SGK1*, (B) *αENaC*, (C) *βENaC*, (D) *γENaC* over *L19* transcript levels in Ishikawa cells prior to (0) and 5-120 min following treatment with 25 ng/ml LEFTYA. Statistically significant difference was observed when *p<0.05, **p<0.001 using one way ANOVA. E. Original Western blot of phosphorylated SGK1 (p-SGK1), αENAC, phosphorylated NEDD4-2 (pNEDD4-2) and β-actin protein cell lysates from Ishikawa cells prior to (0) and 5-120 min following treatment with 25 ng/ml LEFTYA. β-actin was used as a loading control. F. Represent the original Western blot of Total-SGK1 and Total-NEDD4-2 from cell lysates prior (0) to and 5-120 min following treatment with 25 ng/ml LEFTYA. β-actin was used as a loading control. G. Arithmetic means ± SEM (n = 5) of phosho-SGK1/Total-SGK1, αENAC/β-actin and phospho-NEDD4-2/Total-NEDD4-2 protein abundance ratios in cell lysates from Ishikawa cells. Statistically significant difference was observed when *p<0.05, ***p<0.01 using Student's *t*-Test.

reflecting activated kinase activity. Conversely levels of phospho-NEDD4-2 decreased. No change was seen in total NEDD4-2 or SGK1 levels (Fig. 1F&G). To test whether LEFTYA could regulate COX-2, a key implantation gene, Ishikawa cells were treated with LEFTYA or remained untreated (Control). As illustrated in Fig. 2, LEFTYA significantly decreased COX-2 expression, both at transcript and protein level. In keeping with previously published findings, that LEFTYA can block decidualization-specific genes, we also observed a statistical decrease in Prolactin (PRL) and Bone morphogenetic protein 2 (BMP2) (Fig. 3). No change was seen in Wingless-type MMTV integration site family, member 4 (WNT4) (Fig. 3).

In order to quantify the contribution of SGK1 to the regulation of αENaC by LEFTYA, Ishikawa cells were treated in the absence and presence of SGK1 inhibitor EMD638683 (50 μM; EMD) with or without LEFTYA. As illustrated in Fig. 4, LEFTYA again increased the *αENaC* levels. The administration of EMD638683 alone slightly but significantly increased *αENaC* transcript levels, an effect presumably unrelated to SGK1 inhibition. In the presence of both EMD638683 and LEFTYA, *αENaC* levels in Ishikawa cells did not change when compared to

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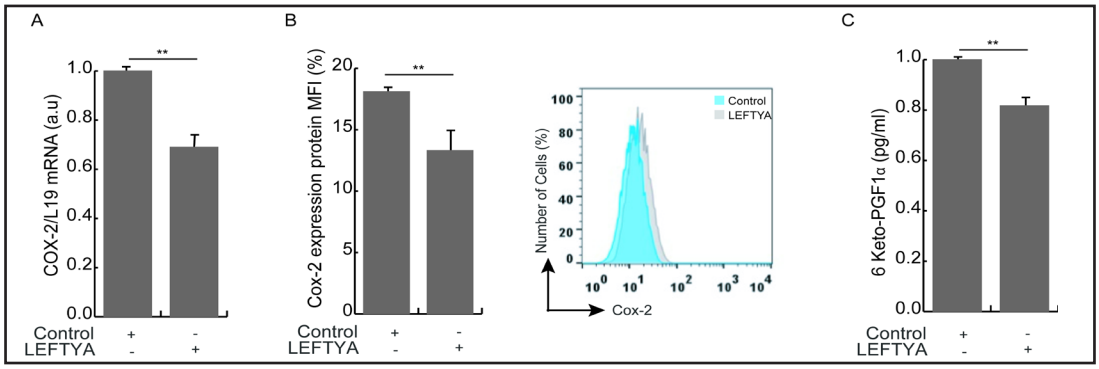

Fig. 2. Effect of LEFTYA on COX-2 transcripts and protein levels. Ishikawa cells were treated with 25 ng/ml LEFTYA for 120 min or remained untreated (Control). A. Arithmetic means ± SEM (n = 4) of COX-2 over L19 transcript levels in Ishikawa cells. B. Cells were stained with COX-2 antibody and subjected to FACS. Left, the median fluorescence intensity (MFI) was quantified and Right, original FACS histogram for Control (grey) and LEFTYA (blue). C. In parallel cultures 6-Keto PGF1-α levels were measured using ELISA. Statistically significant difference was observed when $^{**}p<0.001$ using Student's *t*-Test.

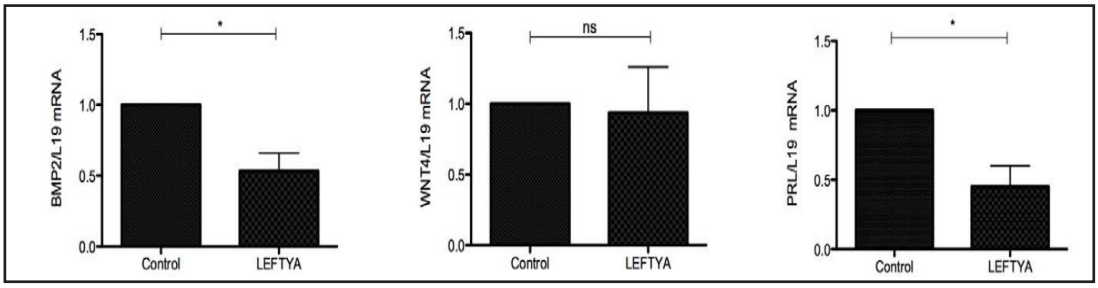

Fig. 3. Expression of key uterine implantation genes. Expression genes coding Bone Morphogenetic Protein 2 (*Bmp2*), Wingless-Type MMTV Integration Site Family, Member 4 (*Wnt4*), and Prolactin (*PRL*) was examined by qRT-PCR in Ishikawa cells treated with or without LEFTYA (n=6). Data are presented as arithmetic means ± SEM. * indicates $p< 0.05$ or ** $p< 0.01$ (Student's *t*-test).

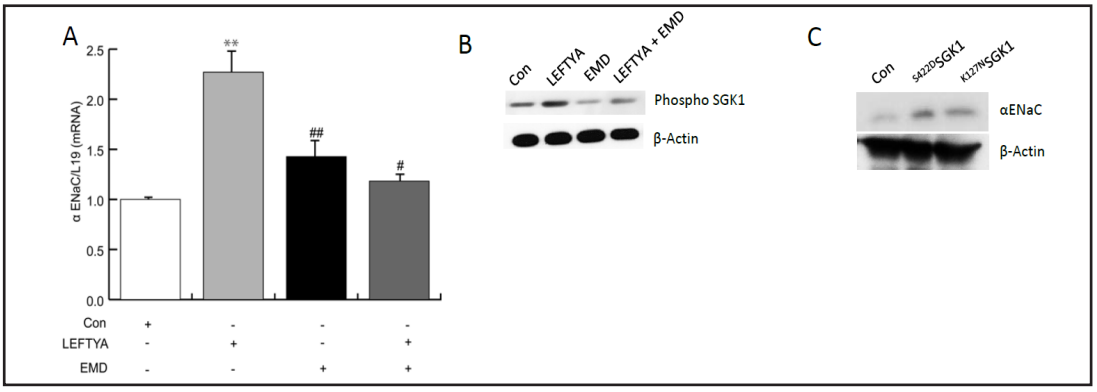

Fig. 4. αENaC transcript levels in Ishikawa cells following LEFTYA treatment in absence and presence of SGK1 inhibitor EMD638683 and following transfection with active or inactive SGK1. A. Arithmetic means ± SEM (n = 3) of the αENaC over L19 transcript levels from untreated (white), LEFTYA treated (grey), EMD638683 treated alone (50 μM; black) or in combination of both LEFTYA and EMD638683 (dark grey). Statistically significant difference was observed between untreated and LEFTYA treated cells (**p<0.01), or EMD638683 treated cells (*p<0.05) as well as between LEFTYA treated cells and LEFTYA+EMD638683 treated cells (#p<0.05) using one way ANOVA. B.Original Western blot of αENAC and β-actin. Protein cell lysates from untreated Ishikawa cells or cells treated with LEFTYA, EMD638683 treated alone (50 μM; EMD; black) or in combination of both LEFTYA+EMD638683. C. Cell lysates from Ishikawa cells after transfection with empty vector, constitutively active ^{S422D}SGK1 and kinase dead ^{K127N}SGK1. Original Western blot of αENAC and β-actin protein.

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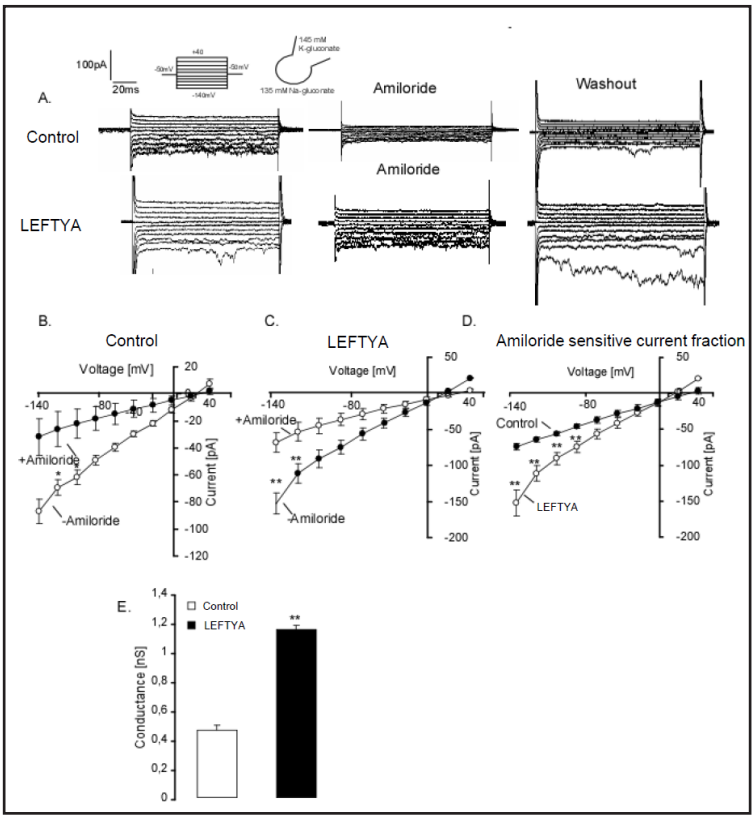

Fig. 5. Effect of LEFTYA on amiloride sensitive Na^+ currents in Ishikawa cells. A. Original tracings of whole cell currents in untreated (Control; upper panels) and LEFTYA treated (lower panels; 25 ng/ml, 120 min) Ishikawa cells. Without (left panels) and with (middle panels) presence of amiloride (50 µM) and after washout of amiloride (right panels). B,C,D. Arithmetic means (± SEM, n = 6-7) of whole cell currents as a function of potential difference across the cell membrane in (B) Control and (C) LEFTYA treated (25 ng/ml, 120 min) (D) Amiloride senstive current fraction of LEFTYA treated cells in Ishikawa cells. E. Arithmetic means (± SEM, n = 6-7) of inward conductance (nS) of the cell membrane calculated by linear fit of I/V-curves (in D) from -80 mV to -140mV in untreated (Control, white bar) and LEFTYA treated (25 ng/ml, 120 min, black bar) Ishikawa cells. Statistically significant difference from untreated cells compared with LEFTYA (**p<0.01) using Student's t-Test.

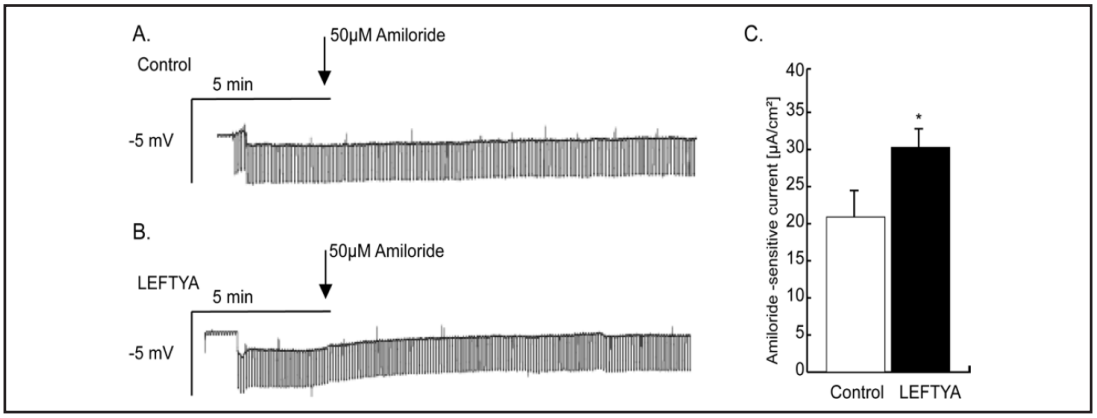

Fig. 6. Effect of LEFTYA on amiloride induced transepithelial current across murine endometrial epithelium. A,B. Representative original tracings showing the effect of amiloride (50 µM) on the transepithelial potential difference across (A) Control and (B) LEFTYA (500 ng/ml) treated murine endometrium. The voltage deflections result from injection of 1 µA current pulses and reflect the transepithelial resistance. Arrow indicates addition of amiloride (50 µM). C. Arithmetic means ± SEM (n = 6) of the amiloride (50 µM) induced equivalent short-circuit current across (µA/cm^2) Control (PBS; white bar) and LEFTYA (500 ng/ml; black bar) treated murine endometrial epithelium. Statistically significant difference between Control and LEFTYA treated mice (*p<0.05) using Student's t-Test.

the control. However, when compared to LEFTYA alone, treatment with both EMD638683 and LEFTYA significantly reduced *αENaC* transcript levels.

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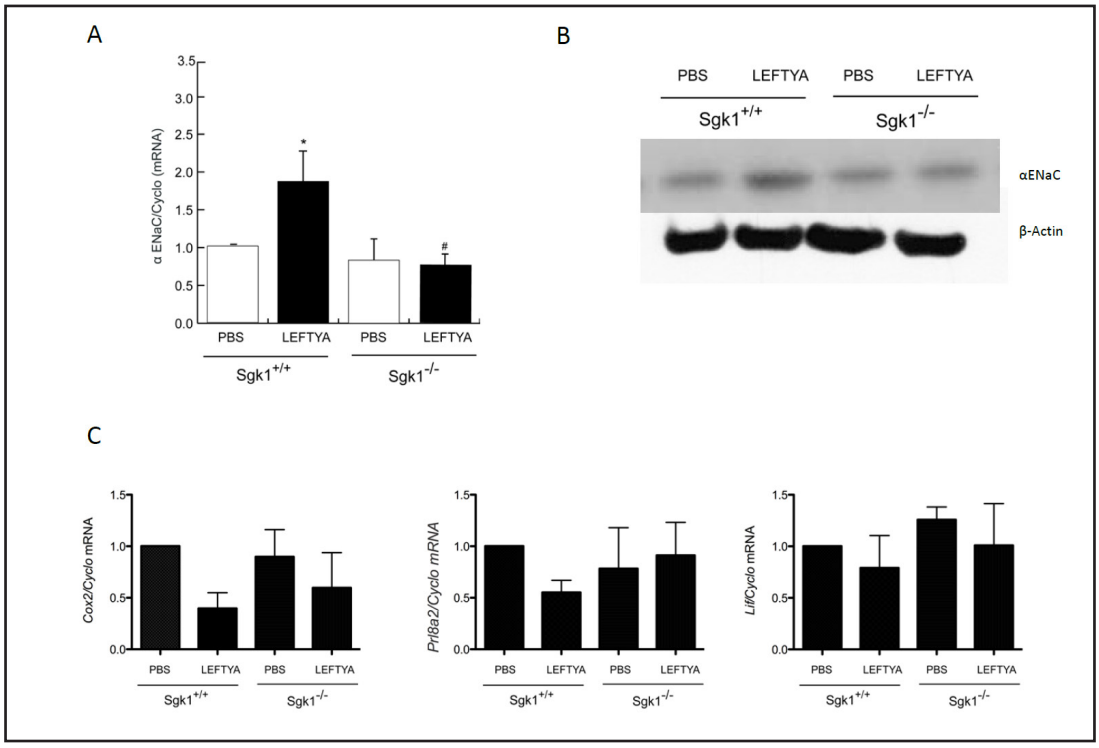

Fig. 7. SGK1 sensitivity of *αENaC* and key murine implantation genes. A. Arithmetic means ± SEM (n=5) of the αENaC over Cyclo transcript levels from endometrium isolated from wild type mice (left bars, *sgk1*$^{+/+}$) or gene targeted mice lacking SGK1 (right bars *sgk1*$^{-/-}$) 24 hours after flushing without (white bars) and with (black bars) LEFTYA (500 ng/ml). Statistically significant difference was observed from PBS-treated *sgk1*$^{+/+}$ compared with LEFTYA (*sgk1*$^{+/+}$) treated mice (*p<0.05). Further, we observed statistical difference between LEFTYA (*sgk1*$^{+/+}$) and LEFTYA *sgk1*$^{-/-}$ treated mice (#p<0.05) using one-way ANOVA. B. Original Western blot of phosphorylated αENAC and β-actin protein cell lysates from murine experiments. C.Expression genes coding Cyclooxygenase (*Cox2*), Prolactin family 8, subfamily a, member 2 (*Prl8a2*) and Leukemia inhibitory factor (*Lif*) was examined by qRT-PCR endometrium isolated from wild type mice (left bars, *sgk1*$^{+/+}$) or gene targeted mice lacking SGK1 (right bars *sgk1*$^{-/-}$) 24 hours after flushing without (white bars) and with (black bars) LEFTYA (500 ng/ml). Data are presented as arithmetic means ± SEM. * indicates $p< 0.05$ or ** $p< 0.01$ (Student's *t*-test).

In order to test, whether induction of SGK1 is sufficient for up-regulation of *αENaC* expression, a further series of experiments explored whether *αENaC* protein levels in endometrial cells are modified by SGK1 transfection. As shown in Fig. 4C, *αENaC* levels were significantly enhanced following transfection of Ishikawa cells with the constitutively active ^{S422D}SGK1 and significantly down-regulated upon transfection of a kinase dead mutant (^{K127N}SGK1). Thus, *αENaC* protein levels in Ishikawa cells are up-regulated by SGK1 even in the absence of LEFTYA. The negative effect of ^{K127N}SGK1 points to competitive displacement of endogeneous SGK1 from the target protein by the inactive mutant [37].

Patch clamp experiments were performed to test whether the increase of ENaC protein corresponded to enhanced ENaC activity. To this end, amiloride sensitive Na^+ currents in Ishikawa cells were determined utilizing whole cell patch clamp. As illustrated in Fig. 5A-D, the amiloride-sensitive whole cell currents were significantly increased by LEFTYA treatment (25 ng/ml, 120 min). Fig. 5B&C display the whole cell currents as a function of potential difference across the cell membrane in untreated and LEFTYA treated Ishikawa cells both, in the absence and presence of amiloride (50 μM). Fig. 5D displays the amiloride sensitive current fraction as a function of potential difference across the cell membrane. The effect of LEFTYA on the whole cell current was paralleled by the respective effect on inward conductance calculated

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from the individual I-V relations by linear regression of inward current between -80 and -140 mV (Fig. 5E). The reversal potential of the currents under control conditions and after treatment with LEFTYA were not statistically significant.

Ussing chamber experiments were performed to quantify the amiloride-sensitive transepithelial current (electrogenic Na^+ transport) across murine endometrial epithelium *in situ*. As illustrated in Fig. 6, the amiloride (50 μM) sensitive transepithelial potential difference and the amiloride-sensitive equivalent short-circuit current across the murine endometrial epithelium were significantly increased by LEFTYA treatment (500 ng/ml). At lower concentrations no discernable effects were seen.

A final series of experiments explored whether LEFTYA influenced endometrial *αENaC* transcript levels and whether this effect was modulated by SGK1. To test this conjecture, uteri from wild type mice (*Sgk1*$^{+/+}$) and Sgk1-deficient mice (*Sgk1*$^{-/-}$) were excised and flushed with LEFTYA (500ng/ml) or with PBS. The uteri were then cultured for 24 hours and the expression of *αENaC* was examined by qRT-PCR. As illustrated in Fig. 7A&B, LEFTYA treatment (500 ng/ml) was followed by a significant increase of *αENaC* transcript and protein levels in endometrium from *Sgk1*$^{+/+}$ mice but not from *Sgk1*$^{-/-}$ mice. Implantation genes Cox2, Leukemia inhibitory factor (Lif) and Prolactin family 8, subfamily a, member 2 (Prl8a2) tended to be lower in the *Sgk1*$^{-/-}$ mice and the LEFTYA flushed *Sgk1*$^{+/+}$ mice than in control mice, a difference, however, not reaching statistical significance (Fig. 7C).

Discussion

Gene ablation studies in mice have been pivotal in identifying critical implantation regulators. Within this network of genes, many encode secreted factors, including growth factors (e.g.heparin-binding EGF-like growth factor), cytokines (e.g. leukemia inhibitory factor) and various morphogens (e.g. bone morphogenetic protein 2) [2]. These establish paracrine gradients that control a distinct temporal-spatial pattern in order to enable the embryo to breach the luminal epithelium and embed in the decidualizing stroma .

The present study demonstrates that LEFTYA is a major regulator of the expression and activity of SGK1 and ENaC in the endometrium. Further, we reveal that LEFTYA participates in the regulation of fluid transport across the endometrial epithelium.

In keeping with previous findings, treatment of Ishikawa cells with LEFTYA (25 ng/ml) rapdily increased the transcript levels of *SGK1* and *αENaC*, *βENaC* and *γENaC* subunits within 60 mins [16, 38]. Like TGFβ1 [26], LEFTYA was shown to be a strong stimulator of SGK1 expression. Further, the effect of LEFTYA on *αENaC* levels was abolished by the SGK1 inhibitor EMD638683. Transfection of Ishikawa cells with the constitutively active ^{S422D}SGK1 mutant was sufficient to increase *αENaC* protein levels. By contrast, transfection with ^{K127N}SGK1, a kinase dead mutant, significantly decreased *αENaC* levels, an observation pointing to the transdominant inhibitory effect of the mutant. The role of SGK1 in regulating endometrial *αENaC* expression was further illustrated by comparing wild type mice (*Sgk1*$^{+/+}$) and Sgk1-deficient (*Sgk1*$^{-/-}$) mice. Interestingly, even in the absence of LEFTYA treatment, *αENaC* transcript levels were significantly lower in *Sgk1* knockout mice. Furthermore, LEFTYA significantly increased *αENaC* transcript levels in wild type but not *Sgk1*$^{-/-}$ mice, demonstrating that LEFTYA-dependent regulation of ENaC in the endometrium is largely if not exclusively dependent on SGK1.

Up-regulation of ENaC activity is expected to stimulate endometrial salt and fluid absorption. Failure to down-regulate LEFTYA secretion during the mid-luteal phase of the cycle may lead to premature 'closure' of the uterine lumen *via* ENaC-mediated fluid absorption, resulting in implantation failure [20, 39]. The present observations, however, do not provide insight into the purported inhibitory effects of LEFTYA on decidualization of the endometrial stroma [40, 41]. A previous study reported that induction of ENaC activity in response to embryonic proteases promotes decidualization by depolarizing the cell membrane of epithelial cells, leading to activation of voltage gated Ca^{2+} channels, induction of COX-2 in

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response to Ca^{2+} signaling and enhanced prostaglandin E2 (PGE2) production [19]. As PGE2 stimulates differentiation of endometrial stromal cells, up-regulation of ENaC by LEFTYA is unlikely to account for the negative effect of LEFTYA on decidualization [41]. The possibility must be considered that LEFTYA upregulates ENaC but by the same token disrupts the link between ENaC and decidualization. On the other hand, at least in theory, activation of ENaC prior to implantation may compromize fertility [20, 39], whereas activation of ENaC may be required during embryo implantation. It must further be kept in mind that, besides SGK1 [42], several other kinases regulate ENaC, including; SGK2&3, Protein Kinase A, Casein Kinase II, G protein-coupled receptor kinase 2, Inhibitor of Nuclear Factor Kappa-B kinase subunit beta, Phosphoinositide-Dependent Kinase 1, Protein Kinase C, Extracellular Signal-Regulated Protein Kinases 1 and 2 and AMP-Activated Protein Kinase [43].

Besides the effect on endometrial fluid transport, ENaC accomplishes Na^+ transport in a wide variety of tissues and contributes to the physiology and pathophysiology of diverse functions, including renal salt excretion [42], blood pressure [44], cell volume [45], pulmonary fluid transport [46, 47] and endothelial function [48]. It is tempting to speculate that LEFTYA may influence ENaC activity in non-uterine tissues. Moreover, SGK1 and ENaC may also participate in the known effects of LEFTYA on embryonic morphogenesis [49].

In conclusion, LEFTYA is a powerful regulator of SGK1 and ENaC in the endometrium and contributes to the complex regulatory network that controls embryo implantation. Our findings provide new insight on how deregulation of SGK1, ENaC and LEFTYA contribute to unexplained infertility and implantation failure in IVF.

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Disclosure Statement

All authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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