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Original Paper

Negative Effect of Ellagic Acid on Cytosolic pH Regulation and Glycolytic Flux in Human Endometrial Cancer Cells

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Key Words
Glycolysis • Cytosolic pH regulation • Na^+/H^+ exchanger • NHE1 • Tumor cells

Abstract
Background/Aims: Key properties of tumor cells include enhanced glycolytic flux with excessive consumption of glucose and formation of lactate. As glycolysis is highly sensitive to cytosolic pH, maintenance of glycolysis requires export of H^+ ions, which is in part accomplished by Na^+/H^+ exchangers, such as NHE1. The carrier is sensitive to oxidative stress. Growth of tumor cells could be suppressed by the polyphenol Ellagic acid, which is found in various fruits and vegetables. An effect of Ellagic acid on transport processes has, however, never been reported. The present study thus elucidated an effect of Ellagic acid on cytosolic pH (pHi), NHE1 transcript levels, NHE1 protein abundance, Na^+/H^+ exchanger activity, and lactate release. ***Methods:*** Experiments were performed in Ishikawa cells without or with prior Ellagic acid (20 μM) treatment. NHE1 transcript levels were determined by qRT-PCR, NHE1 protein abundance by Western blotting, pH_i utilizing (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence, Na^+/H^+ exchanger activity from Na^+ dependent realkalinization after an ammonium pulse, cell volume from forward scatter in flow cytometry, reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein fluorescence, glucose uptake utilizing 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose, and lactate concentration in the supernatant utilizing a colorimetric (570 nm)/ fluorometric enzymatic assay. ***Results:*** A 48 hour treatment with Ellagic acid (20 μM) significantly decreased *NHE1* transcript levels by 75%, NHE1 protein abundance by 95%, pHi from 7.24 ± 0.01 to 7.02 ± 0.01, Na^+/H^+ exchanger activity by 77%, forward scatter by 10%, ROS by 82%, glucose uptake by 58%, and lactate release by 15%. ***Conclusion:*** Ellagic acid (20μM) markedly down-regulates ROS formation and NHE1 expression leading to decreased Na^+/H^+ exchanger activity, pHi, glucose uptake and lactate release in endometrial cancer cells. Those effects presumably contribute to reprogramming and growth inhibition of tumor cells.

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Introduction

Endometrial cancer is a leading cause of cancer-related death in women and the incidence is rising steadily in almost all countries [1]. Development and progression of endometrial cancer is influenced by genetic mutations and epigenetic changes in various cancer-related genes as well as disordered hormone levels [2]. Prophyllaxis and treatment of cancer could be accomplished by Ellagic acid (EA) [3-5], a naturally occurring phenolic constituent of ellagitannins in grapes, nuts, strawberries, blackcurrent, raspberries, green tea, and pomegranate [6]. EA treatment is a new and highly effective strategy in reducing carcinogenesis [7, 8]. EA exerts anticancer effects through its antiproliferative and pro-apoptotic actions [5]. EA further provides neuroprotection in Parkinson's Disease [9] and favourably influences the clinical course of Hepatitis C virus infection [10] and several chronic diseases, such as diabetes [11].

EA can act through multiple pathways and can be used as a dietary agent for preventing and treating many common forms of cancer [7, 8]. Through the action of human colonic microflora, EA is partially converted into metabolites, including hydroxy-6H-benzopyran-6-one derivatives, primarily urolithin A (UA) [12]. Both, EA and urolithins enter the circulation. Recent studies based on *in vitro* testing have shown preliminary evidence on the anti-inflammatory, anticarcinogenic, antiglycolytic, antioxidant, and antimicrobial effects of urolithins [5, 6, 12].

Typical properties of cancer cells include strong stimulation of glycolysis with the respective formation of lactate [13, 14]. A prerequisite for unhindered glycolytic flux is avoidance of cytosolic acidification, which inhibits the rate limiting enzymes of glycolysis [15]. Mechanisms counteracting cytosolic acidification of tumor cells include activation of Na^+/H^+ exchangers such as NHE1 [13, 16]. NHE1 expression is sensitive to oxidative stress [17]. Besides its impact on glycolysis, the carrier participates in cell volume regulation [18].

The present study explored whether Ellagic acid (20 μM; EA) affects cytosolic pH, Na^+/H^+ exchanger NHE1 expression, Na^+/H^+ exchanger activity, oxidative stress, cell volume, cellular glucose uptake and glycolytic flux in human endometrial Ishikawa cells.

Materials and Methods

Cell Culture

Ishikawa cells (well differentiated endometrial carcinoma cell model) were cultured in DMEM/F12 without phenol red media, containing 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (Invitrogen, Germany). Cells were treated as described with *Ellagic acid* (#E2250 20 μM Sigma, Germany) for 48h.

Quantitative Real-time PCR

Total RNA was extracted from Ishikawa cultures 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 1:20 with distilled water and used as template in qRT-PCR analysis. L19 was used to normalize for variances in input cDNA. Detection of gene expression was performed with KappaFast -SYBR Green (VWR-Peqlab, Germany) and Quantitative RT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Germany). The expression levels of the samples are provided 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.

Primer	Sequence
NHE1	forward (5'-3'):ACCTGGTTCATCAACAAGTTCCG
	reverse (5'-3'): TTCACAGCCAACAGGTCTACCA
L19	forward (5'-3'): GCAGCCGGCGCAAA
	reverse (5'-3'): GCGGAAGGGTACAGCCAAT

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Western blotting

For determination of NHE1 protein abundance, whole cell protein extracts were prepared by lysing cells in RIPA buffer. Protein yield was quantified using the Bio-Rad DC protein assay kit (Bio-Rad, USA). Equal amounts of proteins (30 µg) were separated on 10% sodium dodecyl sulfate–polyacrylamide (SDS) gel before electrotransfer onto the PVDF membrane (Amersham Biosciences, Germany). Nonspecific binding sites were blocked by 1 hour incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS; 130 mmol/L NaCl, 20 mmol/L Tris, pH7.6 and 1% Tween). NHE1 was identified by primary antibodies against human NHE1 (1:1000, #gtx85047, Genetex) and antibody against GAPDH (1:1000, #8884, Cell Signalling, The Netherlands) served as a loading control. For detection, a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (1:2000, Cell Signaling, The Netherlands) or secondary anti-mouse IgG antibody conjugated with HRP (1:2000, GE Healthcare,UK) was used. Protein complexes were visualized with a chemiluminescent detection kit (GE Healthcare). All experiments were performed in 3 or more cell cultures. Bands were quantified with ImageJ Software.

Intracellular pH

For digital imaging of cytosolic pH (pH_i), the cells were incubated in a HEPES-buffered Ringer solution containing 10 µM BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C [19-22]. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Axiovert 135, Zeiss, Germany), which was used in the epifluorescence mode with a 40 x oil immersion objective (Neoplan, Zeiss). BCECF was successively excited at 490/10 nm and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 10-20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH_i values using the high-K^+/nigericin calibration technique [23]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K^+/nigericin (10 µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [19]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (ß) of the cells [24]. Assuming that NH_4^+ and NH_3 are in equilibrium between cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$ß = \Delta[NH_4^+]_i/\Delta pH_i$,

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [25] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 $[20/(1+10^{pHo-pK})]$. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi}$.

The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+, indicating that there was no relevant further exit of NH_4^+.

NHE1 activity was measured as the slope of the first 5 minutes of recovery from acidification and was expressed as ΔpH_i per minute, which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG Cl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration: 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 µM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C.

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Lactate concentration in supernatant

Lactate concentration in the supernatant was determined utilizing a Lactate Assay Kit (Sigma #MAK064), an enzymatic assay involving colorimetric (570 nm)/fluorometric (λex = 535 nm/λem = 587 nm) measurements according to the manufacturer's protocol.

Determination of cellular glucose uptake and forward scatter in flow cytometry

After incubation under the respective experimental conditions, the cells were first washed with PBS and then detached with warm 0.25% trypsin (#25200-056, Invitrogen, Germany). Cells were collected after a brief centrifugation. The fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (#N13195, 2-NBD-glucose; Invitrogen, Darmstadt, Germany) was used to measure the relative uptake of glucose by flow cytometry in a FACS Calibur (BD Biosciences, Germany). Cells (1×10^6) were incubated with 2-NBD-glucose (10 μM) in phosphate buffered saline for 1 hour at 37°C, subsequently washed twice in cold PBS and analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Changes in glucose uptake after inhibitor treatment were calculated as differences in geometric mean of the fluorescence. In parallel forward scatter was determined as a measure of cell volume.

Determination of reactive oxygen species in flow cytometry

After incubation under the respective experimental conditions, the cells were detached by 0.25% trypsin (as described above). Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, #D6883, Sigma). The cell suspension was washed in Ringer solution and stained with DCFDA in Ringer solution containing DCFDA at a final concentration of 10 μM. The cells were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded cells were resuspended in 200 μl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD Biosciences). Subsequently, the geometric mean of the DCFDA dependent fluorescence was determined.

Statistics

Data are provided as arithmetic means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test and only results with $P < 0.05$ were considered statistically significant.

Results

The present study explored whether Ellagic acid (EA) influences cytosolic pH (pHi) regulation and glycolytic flux in Ishikawa cells. In a first step, the effect of a 48 hours exposure to EA (20 μM) on the transcript levels of the Na^+/H^+ exchanger isoform NHE1 was determined utilizing qRT-PCR. As illustrated in Fig. 1A, treatment of Ishikawa cells with EA (20 μM, 48 hours) was followed by a significant decrease of the transcript levels encoding NHE1.

EA (20 μM) similarly influences the protein expression of NHE1 in Ishikawa cells. As illustrated in Fig. 1B,C, treatment of Ishikawa cells with EA (20 μM) for 48 hours was followed by a significant decline of NHE1 protein abundance.

In order to test, whether the down-regulation of NHE1 transcription and protein expression was paralleled by alterations of Na^+/H^+ exchanger activity and cytosolic pH (pHi), pHi was estimated from BCECF fluorescence, and Na^+/H^+ exchanger activity utilizing the ammonium pulse technique. As illustrated in Fig. 2A,B, EA treatment for 48 hours significantly decreased pHi. Addition of 20 mM NH_4Cl replacing NaCl in the superfusate was followed by NH_3 entry into the cells with subsequent transient cytosolic alkalinization due to binding of H^+ to NH_3 to form NH_4^+ (Fig. 2A). Subsequent removal of NH_4Cl was followed by cytosolic acidification due to NH_3 exit with dissociation of NH_4^+ and cellular retention of H^+ (Fig. 2A). In the absence of Na^+ the cytosolic pH remained acidic, an observation pointing to the absence of appreciable Na^+-independent H^+ extruding transport systems. In the absence of EA, the subsequent addition of Na^+ was followed by rapid cytosolic realkalinization, an

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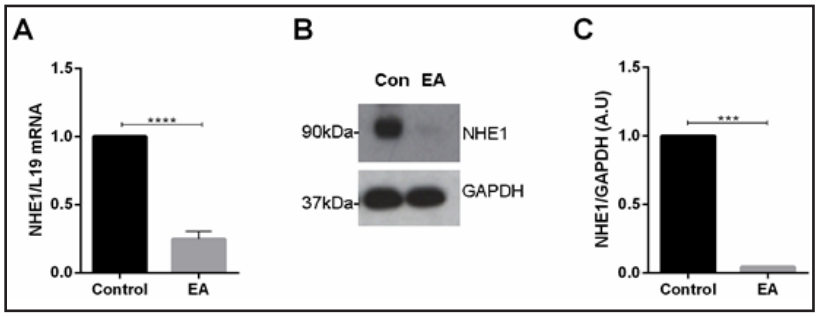

Fig. 1. Effect of Ellagic acid (EA) on NHE1 isoform transcript levels and protein levels. A. Arithmetic means ± SEM (n = 8) of NHE1 transcript levels from Ishikawa cells exposed for 48 hours without (control, black bar) or with EA (grey bar; 20 μM). L19 was used as a housekeeping control. **** (P<0.0001) indicates statistically significant difference from untreated cells. B. Original Western blot of NHE1 and GAPDH protein levels from Ishikawa cells with or without a 48 hour treatment with EA (20 μM). GAPDH was used as a loading control. C. Arithmetic means ± SEM (n = 3) of the NHE1/GAPDH protein abundance ratios in cell lysate from Ishikawa cells exposed for 48 hours without (control, black bar) or with EA (grey bar; 20 μM).*** (P<0.001) indicates statistically significant difference from untreated cells.

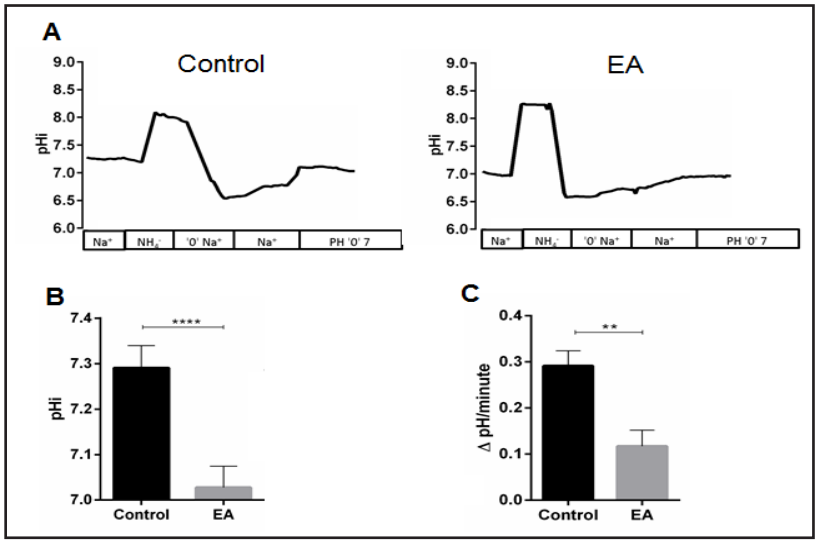

Fig. 2. Effect of Ellagic acid (EA) on pHi and Na^+/H^+ exchanger activity in Ishikawa cells. A. Original tracings showing alterations of cytosolic pH (pH_i) in Ishikawa cells prior to, during and following an ammonium pulse. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step (see bars below each original tracing), NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_o 7.0) applied in a fourth step to calibrate each individual experiment. B. Arithmetic means ± SEM (n = 8 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in Ishikawa cells exposed for 48 hours without (control, black bar) or with EA, (grey bar; 20 μM). **** indicates statistically significant difference (P<0.0001). C. Arithmetic means ± SEM (n = 8 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) reflecting Na^+/H^+ exchanger activity in Ishikawa cells exposed for 48 hours without (control, black bar) or with EA (grey bar; 20 μM). ** indicates statistically significant difference (P<0.01).

observation pointing to Na^+/H^+ exchanger activity. As illustrated in Fig. 2C, the treatment with EA was followed by a significant decrease of Na^+/H^+ exchanger activity.

According to previous observations oxidative stress may affect expression of NHE1 [17]. Thus, we measured ROS abundance using flow cytometry. As illustrated in Fig. 3A,B ROS was significantly down-regulated in EA treated cells.

Na^+/H^+ exchanger activity participates in the orchestration of cell volume regulation [18]. Thus, cell volume was estimated from forward scatter determined by flow cytometry. According to the forward scatter, cell volume was reduced in EA treated cells (Fig. 3C,D)

The cytosolic acidification following EA (20 μM) treatment was expected to impact on glycolysis. As alterations of glucose consumption by glycolysis were expected to be paralleled by the respective alterations of glucose uptake, the cellular glucose uptake was estimated from 2-NBD-glucose uptake determined by flow cytometry. As shown in Fig. 4A, a 48 hours

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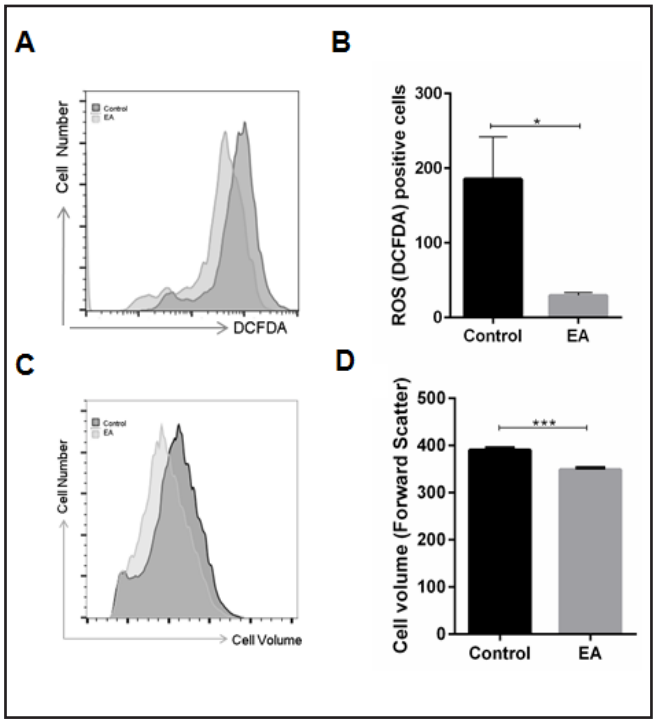

Fig. 3. Effect of Ellagic acid (EA) on ROS production and cell volume in Ishikawa cells. A. Representative original FACS histograms of ROS in Ishikawa cells exposed for 48 hours without (control, dark grey) or with EA (light grey; 20 μM). B. Arithmetic means ± SEM (n = 12 independent experiments) of ROS (DCFDA) (Geometric Mean Fluorescence [MFI]) of Ishikawa cells exposed for 48 hours without (control, black bar) or with (EA, grey bar) EA (20 μM). C. Representative original FACS histograms of cell volume in Ishikawa cells exposed for 48 hours without (control, dark grey) or with EA (light grey; 20 μM). D. Arithmetic means ± SEM (n = 12 independent experiments) of forward scatter (Geometric Mean Fluorescence [MFI] intensity of Ishikawa cells) exposed for 48 hours without (control, black bar) or with EA (grey bar; 20 μM). * (P<0.05), ***(p<0.001) indicates statistically significant difference from untreated cells

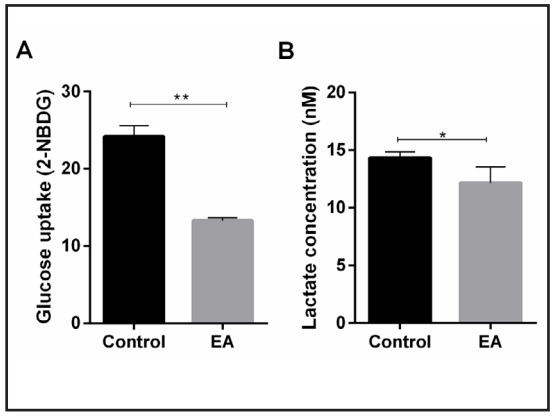

Fig. 4. Effect of Ellagic acid (EA) on glucose uptake and lactate concentration in the supernatant of Ishikawa cells. A. Arithmetic means ± SEM (n = 8) of the glucose uptake (Geometric Mean Fluorescence [MFI] intensity measured by 2-NBDG dye by FACS) without (control, black bar) or with prior 48 hour EA (20 μM) treatment (grey bar). **(P<0.01) indicates statistically significant difference from untreated cells. B. Arithmetic means ± SEM (n = 4) of the lactate concentrations in the supernatant of Ishikawa cells exposed for 48 hours without (control, black bar) or with EA (grey bar; 20 μM). * (P<0.05) indicates statistically significant difference from untreated cells.

incubation of Ishikawa cells in the presence of EA (20 μM) was followed by a significant decrease of cellular glucose uptake.

Alterations of glycolytic flux are further expected to impact on alterations of lactate concentrations determined in the supernatant of Ishikawa cells following a 48 hour incubation in the absence or presence of EA. As illustrated in Fig. 4B, a 48 hour incubation of Ishikawa cells in the presence of EA (20 μM) was followed by a significant decrease of lactate concentration in the supernatant.

Discussion

The present study uncovers a novel and powerful effect of Ellagic acid (EA) on Na^+/H^+ exchanger NHE1 expression and activity, which in turn impacts on cytosolic pH regulation,

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cell volume and glycolytic flux of Ishikawa cells. Treatment with EA is followed by a significant decline of NHE1 transcript levels, NHE1 protein abundance, and Na^+/H^+ exchanger activity.

The down-regulation of Na^+/H^+ exchanger activity following EA treatment prevented Na^+/H^+ exchanger mediated extrusion of H^+ following cytosolic acidification and thus contributed to the maintenance of an acidic cytosolic pHi. The present observations do, however, not rule out that cytosolic acidification is due to dysregulation of additional H^+ extruding transport proteins following EA treatment. Additional H^+ extruding carriers in tumor cells include Na^+ coupled bicarbonate cotransporters [16] and lactate or monocarboxylate transporters [13, 16], the latter extruding both, lactate and H^+ [26]. The Na^+/H^+ exchanger activity is, however, highly sensitive to cytosolic pH and is strongly stimulated by cytosolic acidification [27]. The down-regulation of Na^+/H^+ exchanger activity precludes stimulation of Na^+/H^+ exchanger activity following cytosolic acidification and is thus a prerequisite for the down-regulation of cytosolic pH following EA treatment.

The present observations reveal that EA decreases reactive oxygen species. NHE1 expression and Na^+/H^+ exchanger activity are highly sensitive to oxidative stress [17, 21]. Na^+/H^+ exchanger activity in turn impacts on NADPH oxidase activation and generation of reactive oxygen species [17].

Cellular functions highly sensitive to cytosolic pH further include glycolysis [15]. As the pK of lactate is below 4 [28], the acid is fully dissociated at the prevailing cytosolic pH and thus glycolysis is paralleled by cellular formation of H^+. In order to accomplish their high rates of glycolysis [13, 14] tumor cells up-regulate H^+ extrusion. Cytosolic acidification due to malfunction of Na^+/H^+ exchangers and/or other H^+ extruding transport proteins turns off glycolysis [15]. The sensitivity of glycolysis to cytosolic acidification is an important safeguard of cells [15], as it avoids continued formation of lactate and H^+ if the cells are unable to dispose the H^+ produced by glycolysis. By the same token the inhibition of glycolysis by cytosolic acidification disrupts the major energy source of tumor cells [13, 14].

Maintenance of alkaline cytosolic pH is thus decisive for the survival of tumor cells [29-31]. Inhibition of Na^+/H^+ exchanger and cytosolic acidification parallels apoptosis [32], whereby cytosolic acidification fosters the activation of caspases [33]. Accordingly, the observed cytosolic acidification may contribute to the known stimulation of apoptosis by EA [34, 35].

Beyond its effect on cytosolic pH, Na^+/H^+ exchanger activity participates in the regulation of cell volume, which involves parallel activity of Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger [36, 37]. The carriers mediate the entry of NaCl in exchange for H^+ and HCO_3^-, which are replenished from CO_2 and are thus not osmotically relevant [36, 37]. Timely increase of cell volume is a prerequisite for cell proliferation [36, 37] and stimulation of cell proliferation is paralleled by a shift of the Na^+/H^+ exchanger cell volume regulatory set point to larger volumes [37].

In conclusion, the present study demonstrates that EA down-regulates expression and function of the Na^+/H^+ exchanger, decreases cytosolic acidification with subsequent impairment of glycolysis. Those effects may well contribute to the anti-proliferative effects of EA.

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Disclosure Statement

The authors have no competing interests to declare.

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