Original Paper

1 α ,25(OH)₂D₃ Sensitive Cytosolic pH Regulation and Glycolytic Flux in Human Endometrial Ishikawa Cells

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Key Words

Vitamin D • Glycolysis • Cytosolic pH regulation • Na⁺/H⁺ exchanger • NHE1 • Monocarboxylate transporter • MCT4 • Tumor cells

Abstract

Background/Aims: Tumor cell proliferation is modified by 1,25-Dihydroxy-Vitamin D₃ (1,25(OH)₂D₃), a steroid hormone predominantly known for its role in calcium and phosphorus metabolism. 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 and lactate, which is in part accomplished by Na⁺/H⁺ exchangers, such as NHE1 and monocarboxylate transporters, such as MCT4. An effect of 1,25(OH)₂D₃ on those transport processes has, however, never been reported. As cytosolic pH impacts on apoptosis, the study further explored the effect of 1,25(OH)₂D₃ on apoptosis and on the apoptosis regulating kinase AKT, transcription factor Forkhead box O-3 (FOXO3A) and B-cell lymphoma protein BCL-2. **Methods:** In human endometrial adenocarcinoma (Ishikawa) cells, cytosolic pH (pHi) was determined utilizing (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence, Na⁺/H⁺ exchanger activity from Na⁺ dependent realkalinization after an ammonium pulse, NHE1 and MCT4 transcript levels using qRT-PCR, NHE1, MCT4, total & phospho AKT, total & phospho-FOXO3A and BCL-2 protein abundance by Western blotting, lactate concentration in the supernatant utilizing a colorimetric enzyme assay and cell death quantification using CytoTox 96®, Annexin V and Propidium Iodide staining. **Results:** A 24 hours treatment with 1,25(OH)₂D₃ (100 nM) significantly increased cytosolic pH (pHi), significantly decreased Na⁺/H⁺ exchanger activity, NHE1 and MCT4 transcript levels as well as protein abundance and significantly increased lactate concentration in the supernatant. Treatment of Ishikawa cells with 1,25(OH)₂D₃ (100 nM) further triggered apoptosis, an effect paralleled by decreased phosphorylation of AKT and FOXO3A as well as decreased abundance of BCL-2. **Conclusions:** In Ishikawa cells 1,25(OH)₂D₃ is a powerful stimulator of glycolysis, an effect presumably due to cytosolic alkalinization.

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Despite stimulation of glycolysis, $1,25(\text{OH})_2\text{D}_3$ stimulates slightly but significantly suicidal cell death, an effect presumably in part due to decreased activation of AKT with decreased inhibition of pro-apoptotic transcription factor FOXO3A and downregulation of the anti-apoptotic protein BCL-2.

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Introduction

The biologically active fat-soluble steroid vitamin D is the precursor of the active hormone $1\alpha,25$ -dihydroxy-vitamin D₃ ($1,25(\text{OH})_2\text{D}_3$) that plays a pivotal role in mineral homeostasis and skeletal health [1, 2]. $1,25(\text{OH})_2\text{D}_3$ further modifies the physiology of nonskeletal tissues and, thus, regulates a wide array of biological functions [3, 4]. $1,25(\text{OH})_2\text{D}_3$ activates various signaling cascades and is a powerful regulator of diverse cellular functions such as cell proliferation and differentiation, membrane transport, redox balance, adhesion, hemostasis and apoptosis [3, 4]. Formation of $1,25(\text{OH})_2\text{D}_3$ is down-regulated by Fibroblast Growth Factor 23 (FGF23) which, in turn, requires the membrane protein Klotho as a coreceptor [5]. As part of a negative feedback loop, both FGF23 and Klotho are stimulated by $1,25(\text{OH})_2\text{D}_3$ [5, 6]. Restriction of dietary vitamin D reverses accelerated aging in mice deficient in either FGF23 or Klotho [7].

The biological actions of $1,25(\text{OH})_2\text{D}_3$ are achieved by binding to the Vitamin D receptor (VDR) [8]. VDR expression has been identified in a variety of tissue types, including adipose tissue, brain, breast cancer cells, colon, liver, lung, muscle, ovary, pancreas, prostate, skin, stomach, and thyroid [9]. Expression of parathyroid hormone (PTH) and calcium insensitive 1α -hydroxylase has been identified in cancer and non-cancer cells, such as prostate, colon, lung, endothelial, brain, pancreatic β -cells, and monocytes [1]. Vitamin D and its metabolites may reduce progression of many types of cancers [2, 10]. Recent epidemiological data showed a strong correlation between poor vitamin D status and higher risk for chronic illnesses of various etiologies and some deadly cancers [11-13]. The anticancer property of $1,25(\text{OH})_2\text{D}_3$ has been studied in a wide variety of commonly occurring cancers (both *in vitro* and *in vivo*) [12, 14-19]. The anticancer activity of vitamin D results at least in part from stimulation of apoptosis by $1,25(\text{OH})_2\text{D}_3$ [20]. Pleiotropic signaling pathways regulating cell survival and apoptosis include the antiapoptotic serine/threonine protein kinase AKT which inhibits the proapoptotic transcription factor Forkhead box O-3 (FOXO3A) [21]. Apoptosis is further inhibited by the B-cell lymphoma 2 (BCL-2) [22].

Carriers particularly active in tumor cells include the Na^+/H^+ exchanger (NHE1) [23, 24], the most common isoform of the Na^+/H^+ exchanger family that is ubiquitously expressed in all mammalian cells [25]. Increased proliferation and energy consumption of tumor cells results in a tumor metabolic microenvironment that is hypoxic, acidic, and deprived of serum [26]. Energy supply of tumor cells depends on glycolysis with the respective formation of lactate [27, 28]. Glycolytic flux is disrupted by cytosolic acidification, which inhibits the rate limiting enzymes of glycolysis [29]. Maintenance of glycolysis and tumor cell survival thus requires cellular H^+ extrusion, which is accomplished by several carriers including Na^+/H^+ exchanger NHE1 [27, 30], Na^+ coupled bicarbonate cotransporter [30] and lactate or monocarboxylate transporter 4 (MCT4) [27, 30], extruding both, lactate and H^+ [31]. NHE1 is further instrumental in cell migration, which, in cancer, is necessary for tumors to invade and metastasize at sites distant from the primary tumor [32].

The present study explored whether $1,25(\text{OH})_2\text{D}_3$ affects cytosolic pH, NHE1 and MCT4 expression, Na^+/H^+ exchanger activity and glycolytic flux in human endometrial adenocarcinoma (Ishikawa) cells. In addition, the effect of $1,25(\text{OH})_2\text{D}_3$ on apoptosis was analysed.

Materials and Methods

Cell Culture

Ishikawa cells (well differentiated endometrial carcinoma cell model; ECACC-99040201) were cultured in DMEM/F12 without phenol red media, containing 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (ThermoFischer Scientific, Karlsruhe, Germany) were cultured as previously described [33]. Cells were treated with 1,25(OH)₂D₃ for 24 hours (100 nM, Sigma, Darmstadt, Germany).

Quantitative Real-time PCR

Total RNA was extracted from Ishikawa cultures using Trizol (ThermoFischer Scientific) based on a phenol-chloroform extraction protocol. Equal amounts of total RNA (2 μ g) were reverse transcribed by using the Superscript III First-Strand synthesis system for RT-PCR (ThermoFischer Scientific) using an oligo dT primer (Table 1). The resulting first-strand cDNA was diluted 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 KapaFast-SYBR Green (Peqlab, Germany) and Quantitative RT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The transcript levels 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

Whole cell protein extracts were obtained by direct lysis in Lammelli's Buffer and boiled at 100°C for 10 min. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, Little Chalfont, UK). Nonspecific binding sites were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBS-T. Individual membranes were incubated overnight at 4 °C with anti-NHE1 (PRS4377, Sigma, Germany), anti-SLC16A4 (MCT4, #ab180699, Abcam, Cambridge, UK), anti-AKT (#9272, Cell Signaling, Leiden, the Netherlands), anti-phospho-AKT (Thr308) (#13038, Cell Signaling), anti-FoxO3a (#2497, Cell Signaling), anti-phospho-FoxO3a (Ser253) (#9466, Cell Signaling), anti-BCL-2, (#2819, Cell Signaling) and anti-GAPDH (#2118, Cell Signaling). All primary antibodies were used at 1:1000. Membranes were then washed 3 times with TBS-T, followed by incubation with HRP-conjugated anti-rabbit secondary antibodies (#7074, 1:1000, Cell Signaling). Protein complexes were visualized with a chemiluminescent detection kit (WesternBright ECL Advanta, BioZym, Hessisch Oldendorf, Germany). Blots were not stripped and re-probed. Instead two different blots were run for the total and phospho proteins. Bands were quantified using ImageJ software.

Intracellular pH

For digital imaging of cytosolic pH (pH_i) the cells were incubated in a HEPES-buffered Ringer solution containing 10 μ M BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C [34]. 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 (Zeiss Axiovert 135, Carl Zeiss, Esslingen, Germany), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan). 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, Bensheim, Germany) and specialized computer software (Metafluor, Biberach an der Riss, Germany). Approximately, some 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 [35]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10 μ g/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted

Table 1.

Primer	Sequence
<i>NHE1</i>	forward (5'-3'): ACCTGGTTCATCAACAAGTTCCG reverse (5'-3'): TTCACAGCCAACAGGTCTACCA
<i>MCT4</i>	forward (5'-3'): AAATCGTCTGAGCCCTCCTT reverse (5'-3'): GCAGAAGATCCCTTTGATGTGG
<i>L19</i>	forward (5'-3'): GCAGCCGGCGCAAA reverse (5'-3'): GCGGAAGGGTACAGCCAAT

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 alkalization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [34]. Subsequent removal of NH_4Cl leads to cytosolic acidification due to NH_3 exit with dissociation of NH_4^+ and cellular retention of H^+ . The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [36]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3 :

$$\beta = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[\text{NH}_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[\text{NH}_4^+]_i$ immediately before the removal of ammonia. The pK for $\text{NH}_4^+/\text{NH}_3$ is 8.9 [37] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[\text{NH}_4^+]_o$) is 19.37 $[20/(1+10^{\text{pH}_o-\text{pK}})]$. The intracellular NH_4^+ concentration ($[\text{NH}_4^+]_i$) was calculated from:

$$[\text{NH}_4^+]_i = 19.37 \cdot 10^{\text{pH}_o-\text{pH}_i}$$

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. All chemicals were purchased from Sigma.

Cell Cycle and Apoptosis Analysis

After 24 h treatment, cells and medium were collected into a 15 ml centrifuge tube, spun down at 600 $\times$ g for 5 min. The supernatant was discarded and 1 ml of -20 °C cold ethanol:PBS mix (3:1) was added to the pellet during vortexing. The mixture was kept at -20 °C for overnight, and the next day washed with PBS again, added 200 μl Propidium Iodide (PI) mix containing 50 $\mu\text{g}/\text{ml}$ PI (Sigma, Germany) and 100 $\mu\text{g}/\text{ml}$ RNase A (Sigma, Germany), then incubated for 30 min at 37 °C before application of flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Germany), as well as cell cycle and death analysis.

The quantification of apoptotic cells was estimated by flow cytometry using the Annexin V apoptosis detection kit FITC (eBioscience; ThermoFischer Scientific) in accordance with the manufacturer's instructions. Briefly, cells were collected and washed with PBS, then suspended in 1 $\times$ binding buffer containing annexin V-FITC solution. After incubation at room temperature in the dark for 15 min, cells were washed with binding buffer and PI added. Again, the cells were incubated at room temperature in the dark for 15 min before application of flow cytometry for cell apoptosis analysis. Data was analyzed with Flowjo software (Oregon, USA)

Lactate concentration in supernatant

Lactate concentrations in supernatant were determined utilizing Lactate Assay Kit (Sigma, Germany) an enzymatic, colorimetric (570 nm)/ fluorometric ($\lambda_{\text{ex}} = 535 \text{ nm}/\lambda_{\text{em}} = 587 \text{ nm}$) assay using the manufacturer's protocol.

Cytotoxic assay

About 1×10^4 Ishikawa cells were seeded into 96 well plates, then treated with or without 1,25(OH) $_2$ D $_3$ (100 nM) for 24 hours and prepared for viability analysis using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany) following the protocol recommended by the manufacturer. A negative no-cell control and maximum LDH release control were also included. Firstly, 10 μl of 10 $\times$ Lysis Solution to maximum LDH release control well was added 45 minutes before adding CytoTox 96® Reagent. Then, 50 μl of supernatant was transferred to a fresh ELISA plate and 50 μl substrate was added to each

well. The plates were incubated at room temperature in the dark for 30 minutes. After that, 50 μl of stop solution were added to each well. The absorbance at 490nm was recorded and the percentage cytotoxicity was calculated using the manufacturer's protocol.

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed t -test or Two-way ANOVA using Microsoft Excel (Washington, USA) or Graphpad Prism (California, USA). Only results with $P < 0.05$ were considered statistically significant.

Results

The present study explored whether $1,25(\text{OH})_2\text{D}_3$ influences cytosolic pH (pHi) regulation and glycolytic flux in endometrial carcinoma Ishikawa cells.

In order to test, whether $1,25(\text{OH})_2\text{D}_3$ modified cytosolic pH (pHi), pHi was estimated from (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence. As illustrated in Fig. 1A&B, treatment of Ishikawa cells with $1,25(\text{OH})_2\text{D}_3$ (100 nM) for 24 hours was followed by a significant increase of cytosolic pH (pHi). In order to test, whether $1,25(\text{OH})_2\text{D}_3$ modified Na^+/H^+ exchanger activity, the ammonium pulse technique was applied (Fig. 1A&C). Addition of 20 mM NH_4Cl replacing NaCl in the superfusate was followed by NH_3 entry into the cells with subsequent transient cytosolic alkalization due to binding of H^+ to NH_3 with formation of NH_4^+ (Fig. 1A&C). 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. 1A&C). 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 $1,25(\text{OH})_2\text{D}_3$, the subsequent addition of Na^+ was followed by rapid cytosolic realkalinization, an observation pointing to Na^+/H^+ exchanger activity. As illustrated in Fig. 1C, the application of $1,25(\text{OH})_2\text{D}_3$ was followed by a significant decline of Na^+/H^+ exchanger activity. $1,25(\text{OH})_2\text{D}_3$ did not affect the buffer capacity of the cells (data not shown). Accordingly, the blunted realkalinization following $1,25(\text{OH})_2\text{D}_3$ treatment was due

Fig. 1. Effect of $1,25(\text{OH})_2\text{D}_3$ on pHi and NHE1 activity in Ishikawa cells. A. Original tracings of pHi and NHE1 activity, alterations of cytosolic pH (pHi) 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 $\pm$ SEM ($n = 3$ independent experiments) of cytosolic pH prior to the ammonium pulse (pHi) in Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). C. Arithmetic means $\pm$ SEM ($n = 3$ independent experiments) of Na^+ -dependent recovery of cytosolic pH ($\Delta\text{pH}/\text{min}$) in Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). * ($P < 0.05$) and **** ($P < 0.0001$) indicates statistically significant difference.

Fig. 2. Effect of $1,25(\text{OH})_2\text{D}_3$ on NHE1 isoform transcript levels and protein abundance in Ishikawa cells. A. Arithmetic means $\pm$ SEM ($n = 4$) of NHE1 transcript levels from Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). L19 was used as a housekeeping control. B. Original Western blot of NHE1 and GAPDH protein in cell lysates from Ishikawa cells with or without 24 hours treatment with 100 nM $1,25(\text{OH})_2\text{D}_3$. GAPDH was used as a loading control. C. Arithmetic means $\pm$ SEM ($n = 6$) of the NHE1/GAPDH protein abundance ratios in cell lysate from Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). * ($P < 0.05$) indicates statistically significant difference from untreated cells.

Fig. 3. Effect of $1,25(\text{OH})_2\text{D}_3$ on MCT4 isoform transcript levels and protein levels. A. Arithmetic means $\pm$ SEM ($n = 4$) of MCT4 transcript levels from Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). L19 was used as a housekeeping control. B. Original Western blot of MCT4 and GAPDH protein in cell lysates from Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). GAPDH was used as a loading control. C. Arithmetic means $\pm$ SEM ($n = 7$) of the MCT4/GAPDH protein abundance ratios in cell lysate from Ishikawa cells without or following treatment with 100 nM $1,25(\text{OH})_2\text{D}_3$. ** ($P < 0.01$), **** ($P < 0.0001$) indicates statistically significant difference from untreated cells.

Fig. 4. Effect of $1,25(\text{OH})_2\text{D}_3$ on lactate concentration in the supernatant of Ishikawa cells. Arithmetic means $\pm$ SEM ($n = 4$) of the lactate concentrations in the supernatant of Ishikawa cells without or with prior $1,25(\text{OH})_2\text{D}_3$ treatment (100 nM, 24 hours). * ($P < 0.05$) indicates statistically significant difference from untreated cells.

to decreased H^+ transport across the cell membrane and not due to increased buffer capacity.

To elucidate whether the observed decrease of Na^+/H^+ exchanger activity was paralleled by the respective alterations of Na^+/H^+ exchanger expression, the effect of $1,25(\text{OH})_2\text{D}_3$ on the transcript levels of the Na^+/H^+ exchanger (NHE1) isoform was determined utilizing qRT-PCR. Transcript levels were normalized to housekeeping gene L19. As illustrated in Fig. 2A, treatment of Ishikawa cells with $1,25(\text{OH})_2\text{D}_3$ (100 nM) for 24 hours was followed by a significant decrease of the transcript levels encoding NHE1. To investigate whether $1,25(\text{OH})_2\text{D}_3$ similarly influences the protein expression of NHE1, Ishikawa cells were again

Fig. 5. Effect of $1,25(\text{OH})_2\text{D}_3$ on Ishikawa cell apoptosis. A,B. (A) Representative FACS plot and (B) arithmetic means $\pm$ SEM ($n=12$) of cell cycle analysis following a 24 hours incubation without (black bars) and with (white bars) $1,25(\text{OH})_2\text{D}_3$ (100 nM). (C). Arithmetic means $\pm$ SEM ($n=12$) of dead cells following a 24 hours incubation without and with $1,25(\text{OH})_2\text{D}_3$ (100 nM). D,E. (D) Representative FACS plots and (E) arithmetic means $\pm$ SEM ($n=9$, right) of Annexin V and Propidium Iodide (PI) staining following a 24 hours incubation without (black bars) and with (white bars) the presence of 100 nM $1,25(\text{OH})_2\text{D}_3$. F. Arithmetic means $\pm$ SEM ($n=20$) of the percentage cytotoxic cells following a 24 hours incubation without and with $1,25(\text{OH})_2\text{D}_3$ (100 nM). G. Representative Western Blots of total AKT, phospho-AKT, total FOXO3A, phospho-FOXO3A, BCL-2 and GAPDH. H. Arithmetic means $\pm$ SEM ($n=4$) of total AKT, phospho-AKT, total FOXO3A, phospho-FOXO3A and BCL-2 ratio normalized to GAPDH in $1,25(\text{OH})_2\text{D}_3$ (100 nM) treated cells expressed in fractions of untreated cells (dotted line). I. Arithmetic means $\pm$ SEM ($n=4$) of phospho-FOXO3A relative to total FOXO3A in $1,25(\text{OH})_2\text{D}_3$ (100 nM) treated cells expressed in fractions of untreated cells. J. Arithmetic means $\pm$ SEM ($n=4$) of phospho-AKT relative to total AKT in $1,25(\text{OH})_2\text{D}_3$ (100 nM) treated cells expressed in fractions of untreated cells. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$ indicate statistically significant difference from untreated cells using two-way ANOVA followed by Sidak's multiple comparisons test and Student's t-test as appropriate.

treated with 100 nM $1,25(\text{OH})_2\text{D}_3$ for 24 hours, whole cell lysates collected and the lysates subjected to Western blotting. As illustrated in Fig. 2B&C, treatment of Ishikawa cells with $1,25(\text{OH})_2\text{D}_3$ was followed by a significant decline of NHE1 protein abundance.

Additional experiments explored whether $1,25(\text{OH})_2\text{D}_3$ affected transcription of the lactate extruding monocarboxyate transporter, MCT4. The MCT4 transcript levels were determined utilizing qRT-PCR. Transcript levels were normalized to housekeeping gene L19. Prior to measurements, Ishikawa cells were treated with or without $1,25(\text{OH})_2\text{D}_3$ (100 nM) for 24 hours. As illustrated in Fig. 3A, treatment of Ishikawa cells with $1,25(\text{OH})_2\text{D}_3$ was followed by a significant decrease of the transcript levels encoding MCT4. To investigate whether $1,25(\text{OH})_2\text{D}_3$ similarly influences the protein expression of MCT4, whole cell lysates were collected and the lysates subjected to Western blotting. As illustrated in Fig. 3B&C, treatment of Ishikawa cells with $1,25(\text{OH})_2\text{D}_3$ was followed by a highly significant decline of MCT4 protein abundance.

The cytosolic alkalinization following $1,25(\text{OH})_2\text{D}_3$ treatment was expected to impact on glycolysis. In order to quantify glycolytic flux, lactate concentration was determined in the supernatant of Ishikawa cells following 24 hours incubation in the absence or presence of $1,25(\text{OH})_2\text{D}_3$ (100 nM). As illustrated in Fig. 4, the incubation of Ishikawa cells was followed by a significant increase of lactate concentration in the supernatant, an effect significantly enhanced in the presence of $1,25(\text{OH})_2\text{D}_3$.

A final series of experiments determined whether treatment with $1,25(\text{OH})_2\text{D}_3$ influences suicidal cell death. We examined the cell cycle phase distributions in the different cultures using flow cytometry analysis after PI staining of cells. As shown in Fig. 5A&B, there was a significant decrease of $1,25(\text{OH})_2\text{D}_3$ treated cells in G0/G1 phase. $1,25(\text{OH})_2\text{D}_3$ treatment further increased the number of dead or dying cells containing $<2\text{ n DNA}$ content (Fig. 5C). To discriminate between apoptotic and necrotic cell death, the cells were stained with Annexin V-FITC and PI, and subjected to flow cytometry (Fig. 5D&E). These observations suggest that $1,25(\text{OH})_2\text{D}_3$ treated cells undergo early apoptosis. As illustrated in Fig. 5F, $1\alpha,25(\text{OH})_2\text{D}_3$ treatment was followed by a 20% increase in cell death.

A further series of experiments addressed the influence of $1,25(\text{OH})_2\text{D}_3$ on a signaling pathway known to regulate cell survival, i.e. the AKT/FOXO3A/BCL-2 signaling pathway [21, 22, 38]. Ishikawa cells were treated with or without $1,25(\text{OH})_2\text{D}_3$ (100 nM) for 24 hours and subjected to Western blotting. As illustrated in Fig. 5G-J, $1,25(\text{OH})_2\text{D}_3$ treatment decreased the levels of total & phospho-AKT and of total & phospho FOXO3A. Decreased phosphorylation of FOXO3A allows the proapoptotic transcription factor to enter the nucleus and modify gene expression. As shown in Fig. 5G, $1,25(\text{OH})_2\text{D}_3$ treatment decreased the expression of the anti-apoptotic protein BCL-2.

Discussion

The present study uncovers a novel and powerful effect of $1,25(\text{OH})_2\text{D}_3$ on cytosolic pH regulation and glycolytic flux of Ishikawa cells. Treatment with $1,25(\text{OH})_2\text{D}_3$ is followed by significant cytosolic alkalinization as well as decline of NHE1 transcript levels, NHE1 protein abundance, and Na^+/H^+ exchanger activity. Moreover, $1,25(\text{OH})_2\text{D}_3$ increases lactate production reflecting glycolytic flux and by the same token downregulates transcript levels and protein of the lactate extruding monocarboxylate transporter isoform MCT4.

The down-regulation of Na^+/H^+ exchanger activity following $1,25(\text{OH})_2\text{D}_3$ treatment is at least in part the result of cytosolic alkalinization, as Na^+/H^+ exchanger activity is highly sensitive to cytosolic pH and is turned off upon cytosolic alkalinization [39]. Possibly, cytosolic alkalinization similarly contributes to or even accounts for the downregulation of MCT4 transcription.

Downregulation of Na^+/H^+ exchanger activity and MCT4 should impair the cellular extrusion of H^+ and should thus lead to cytosolic acidification, the opposite of what is observed. The cytosolic alkalinization following $1,25(\text{OH})_2\text{D}_3$ treatment points to decreased cellular generation of H^+ or up-regulation of another H^+ extruding transport protein. For instance, $1,25(\text{OH})_2\text{D}_3$ may downregulate the protein expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit gp91phox and thus H^+ generating NADPH oxidase activity [40]. Whether $1,25(\text{OH})_2\text{D}_3$ modifies NADPH oxidase activity and/or additional carriers in Ishikawa cells, remains, however, elusive.

The cytosolic alkalinisation could contribute to or even account for the stimulation of glycolysis by $1,25(\text{OH})_2\text{D}_3$ treatment. As the pK of lactate is below 4 [41], 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 [27, 28] 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 [29]. The sensitivity of glycolysis to cytosolic acidification is an important safeguard of cells [29], as it avoids continued formation of lactate and H^+ if the cells are unable to dispose the H^+ produced by glycolysis.

Inhibition of glycolysis by cytosolic acidification disrupts the major energy source of tumor cells [27, 28]. Conversely, cytosolic alkalinization may foster survival of tumor cells [42-44]. Inhibition of Na $^+$ /H $^+$ exchanger and cytosolic acidification parallels apoptosis [45], whereby cytosolic acidification fosters the activation of caspases [46].

Despite cytosolic alkalinization 1,25(OH) $_2$ D $_3$ stimulates slightly but significantly apoptosis of Ishikawa cells. 1,25(OH) $_2$ D $_3$ has been shown to stimulate apoptosis of diverse tumor cells [3, 4]. The effect has been attributed in part to sustained increase of cytosolic Ca $^{2+}$ activity [47]. The present observation point to 1,25(OH) $_2$ D $_3$ sensitivity of the serine/threonine protein kinase AKT which supports cell survival and affects a variety of cellular processes including proliferation, survival, and protein translation [48]. Overexpression of AKT has been correlated with the development and metastasis of various cancers [38]. Activated AKT phosphorylates the pro-apoptotic transcription factor FOXO3A [21]. Phosphorylated FOXO3A associates with 14-3-3 protein which retains the transcription factor in the cytoplasm. In the absence of phosphorylation by AKT, FOXO3A can translocate to the nucleus and trigger cell death [49]. Apoptosis is inhibited by the anti-apoptotic protein BCL-2 [22]. The present study reveals that 1,25(OH) $_2$ D $_3$ treatment decreases phosphorylation of both, AKT and FOXO3A and leads to a decrease of BCL-2 protein abundance. Those effects could contribute to the stimulation of apoptosis by 1,25(OH) $_2$ D $_3$. The present observations, do, however, not rule out further 1,25(OH) $_2$ D $_3$ sensitive signaling mechanisms stimulating suicidal cell death. The stimulating effect of 1,25(OH) $_2$ D $_3$ on apoptosis is limited. The magnitude of the 1,25(OH) $_2$ D $_3$ induced apoptosis presumably depends on the presence or absence of antiapoptotic growth factors and of further anti- and pro-apoptotic mediators. It must further be kept in mind that 1,25(OH) $_2$ D $_3$ is not primarily a powerful stimulator of apoptosis but may, besides its stimulating effect on apoptosis of a wide variety of cells [3, 4, 50-54], inhibit apoptosis [55] and stimulate differentiation [50, 56].

In conclusion, the present study demonstrates that 1,25(OH) $_2$ D $_3$ treatment of human endometrial adenocarcinoma (Ishikawa) cells for 24 hours leads to cytosolic alkalinization with down-regulation of expression and function of the Na $^+$ /H $^+$ exchanger, downregulation of MCT4, and augmentation of glycolytic flux. In addition, 1,25(OH) $_2$ D $_3$ triggers apoptosis, an effect paralleled by downregulation of anti-apoptotic AKT/FOXO3A/BCL-2 signaling.

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Disclosure Statement

All authors declare that there are no conflicts of interest.

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