Original Paper

Regulation of Na⁺/H⁺ Exchanger in Dendritic Cells by Akt1

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Key Words

LPS • Oxidative stress • Cytosolic pH • Na⁺/H⁺ exchanger • ROS • Cell volume

Abstract

Background/Aims: Dendritic cells (DCs), antigen-presenting cells critically important for primary immune response and establishment of immunological memory, are activated by bacterial lipopolysaccharides (LPS) resulting in stimulation of Na⁺/H⁺ exchanger, ROS formation and migration. The effects are dependent on phosphoinositide 3 (PI3) kinase and paralleled by Akt phosphorylation. The present study explored the contribution of the Akt isoform Akt1. **Methods:** Cytosolic pH (pH_i) (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence), Na⁺/H⁺ exchanger activity (Na⁺ dependent realkalinization after an ammonium pulse), cell volume (forward scatter in FACS analysis), and ROS production (2',7'-dichlorodihydrofluorescein diacetate [DCFDA] fluorescence) were determined in DCs isolated from bone marrow of mice lacking functional Akt1/PKBα (*akt1*^{-/-}) and their wild type littermates (*akt1*^{+/+}). **Results:** Forward scatter was lower in *akt1*^{-/-} than in *akt1*^{+/+} DCs, whereas pH_i, Na⁺/H⁺ exchanger activity and ROS formation were less in untreated *akt1*^{-/-} and *akt1*^{+/+} DCs. Exposure of DCs to LPS was followed by increase of forward scatter and ROS formation to a similar extent in *akt1*^{-/-} and in *akt1*^{+/+} DCs. A 4 hours treatment with either LPS (1 μg/ml) or tert-butylhydroperoxide (tBOOH, 5 μM) significantly stimulated Na⁺/H⁺ exchanger activity in both genotypes, effects, however, significantly blunted in *akt1*^{-/-} DCs. **Conclusion:** The present observations demonstrate that Akt1 is required for the full stimulation of Na⁺/H⁺ exchanger activity by LPS or oxidative stress in dendritic cells.

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Introduction

The antigen-presenting dendritic cells (DCs) are critically involved in primary immune responses, establishment of T cell immunity and generation of immunological memory [1, 2]. Toll-like receptor triggering by bacterial lipopolysaccharides (LPS) fosters DCs maturation associated with expression of costimulatory molecules and MHC class II [3-6] and production

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of proinflammatory chemokines/cytokines [7-9]. Upon LPS exposure DCs form reactive oxygen species (ROS) [10-12] which in turn contributes to the killing of pathogens [13].

Similar to what has been shown for other cell types [14] including macrophages [15], ROS production is paralleled by generation of H^+ , which in turn influences NADPH oxidase [16, 17]. Thus, LPS-induced ROS production is in DCs paralleled by activation of Na^+/H^+ exchangers [18]. The Na^+/H^+ exchangers further participate in the regulation of cell volume [15, 19-21].

Regulation of DC function involves the phosphoinositide 3 kinase (PI3 kinase) [22-25], which suppresses the IL-12 production and limits the Th1 polarization [26] and IFN- β synthesis [27]. PI3 kinase participates in the regulation of Na^+/H^+ exchanger, cell volume and ROS formation in DCs [28]. Downstream signalling of the PI3 kinase includes protein kinase PKB/Akt isoforms [29], which have been shown to be phosphorylated and thus presumably activated by LPS [28]. Akt/PKB regulates the transport across the cell membrane of diverse substances including glucose [30-32], amino acids [33-35], Ca^{2+} [36], Na^+ [37], K^+ [38] and H^+ [39]. However, whether Akt1 activation is required for the effects of LPS on cell volume, ROS formation and Na^+/H^+ exchanger activity, remained elusive.

The isoform Akt1 has in DCs been shown to participate in the signalling leading to upregulation of interleukin 2 and interferon $INF\gamma$ [40]. It is stabilized by LPS exposure and is decisive for proinflammatory signal-mediated DC survival and maturation [41]. Moreover, Akt1 fosters B cell maturation and survival [42]. Akt fosters survival of further cell types [43, 44] and participates in the generation of reactive oxygen species [45] as well as nitric oxide [46]. Akt1 contributes to the regulation of angiogenesis [47] and has previously been shown to participate in the regulation of migration [48], which in other cells has been shown to require the activation of Na^+/H^+ exchangers [15, 49-51].

The present study thus explored the impact of Akt1 in the stimulation of Na^+/H^+ exchanger activity and Na^+/H^+ exchanger dependent cellular functions, such as cell volume and ROS formation. To this end experiments have been performed in DCs from Akt deficient mice (*akt1^{-/-}*) and their wild type littermates (*akt1^{+/+}*). The mice have previously been shown to suffer from reduced muscle mass [52] and severe atherosclerosis [53].

Materials and Methods

Animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Dendritic cells (DCs) were cultured from bone marrow [54, 55] of 7-11 weeks old female mice lacking functional Akt1/PKB α (*akt1^{-/-}*) and their female wild type littermates (*akt1^{+/+}*) [56, 57]. The mice were kindly provided by Morris J. Birnbaum, Division of Endocrinology, Diabetes and Metabolism at the University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. The mice had free access to tap drinking water and control diet (C 1314, Altromin Heidenau, Germany).

Cell Culture

Dendritic cells (DCs) were cultured from bone marrow according to a standard protocol with slight modifications as previously described [58-61]. Briefly, bone marrow-derived cells were flushed out of the cavities from the femur and tibia with PBS. Cells were then washed twice with RPMI1640 and seeded out at a density of 2×10^6 cells per 60-mm dish. Cells were cultured for 6 days at 37° C in a 5 % CO_2 incubator. The cell culture medium consisted of RPMI 1640 (GIBCO, Carlsbad) containing: 10 % fetal calf serum (FCS), 1 % penicillin/streptomycin, 1 % glutamine, 1 % non-essential amino acids (NEAA) and 0.05 % β -mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, Preprotech Tebu) and fed with fresh medium containing GM-CSF on days 3 and 6. At day 7, $\geq 80\%$ of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed at days 7-9 of DCs culture.

Immunostaining and flow cytometry

Cells (4×10^5) were incubated in 100 μ l FACS buffer (phosphate buffered saline (PBS) plus 0.1% FCS) containing fluorochrome-conjugated antibodies at a concentration of 10 μ g/ml. A total of 2×10^4 cells were

analyzed. Staining with FITC-conjugated anti-mouse CD11c (BD Pharmingen, Heidelberg, Germany) was used as a positive marker for dendritic cells. After incubating with the antibody for 60 minutes at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometric analysis.

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) [62-66] for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any de-esterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10nm 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 [67]. 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 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) [68].

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^+ [69]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [69]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3 :

$$\beta = \Delta[NH_4^+]_i / \Delta pH_i$$

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 [70] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 $[20/(1+10^{pH_o-pK})]$. The intracellular NH_4^+ concentration ($[NH_4^+]_i$) was calculated from:

$$[NH_4^+]_i = 19.37 \cdot 10^{pH_o-pH_i}$$

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^+ .

To calculate the $\Delta pH/min$ during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) 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 μ M 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.

ROS production

ROS production in DCs was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) [71, 72]. Briefly, 2×10^5 cells were taken in a culture dish and treated with LPS. After the treatment, cells were collected and DCFDA (Sigma, Schnelldorf, Germany) was added to the cell suspension at a final concentration of 10 μ M. After 30 minutes of incubation in the dark at 37°C in a 5 % CO_2 incubator, cells were centrifuged and the pellet was washed twice with ice-cold PBS. The pellet was then resuspended in FACS buffer and the fluorescence was analysed with a flow cytometer (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). DCFDA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

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 and only results with $p < 0.05$ were considered statistically significant.

Results

To possibly disclose an effect of Akt1 on the volume of dendritic cells (DCs), forward scatter was determined utilizing FACS analysis. As illustrated in Fig. 1, forward scatter was significantly lower in *akt1*^{-/-} DCs than in *akt1*^{+/+} DCs. Treatment with lipopolysaccharide (LPS, 1 μ g/ml) was within 4 hours followed by a significant increase in forward scatter of both *akt1*^{-/-} and *akt1*^{+/+} DCs. LPS treatment did, however, not abrogate the difference between genotypes, i.e. after LPS treatment forward scatter was again significantly lower in *akt1*^{-/-} than in *akt1*^{+/+} DCs.

To determine the formation of reactive oxygen species (ROS) DCFDA fluorescence was determined in FACS analysis. As shown in Fig. 2, ROS formation was significantly less in *akt1*^{-/-} than in *akt1*^{+/+} DCs. LPS treatment led within 4 hours to a significant increase of ROS formation in both, *akt1*^{-/-} and *akt1*^{+/+} DCs. The LPS effect was attenuated by treatment of catalase. The effect of LPS on ROS formation was higher in *akt1*^{+/+} than in *akt1*^{-/-} DCs.

Cytosolic pH (pH_i) was similar in untreated *akt1*^{-/-} and *akt1*^{+/+} DCs. Exposure of DCs to LPS was followed by a significant decrease of pH_i in *akt1*^{-/-} DCs but not *akt1*^{+/+} DCs. Following LPS treatment pH_i was significantly lower in *akt1*^{-/-} than in *akt1*^{+/+} DCs (Fig. 3).

Fig. 1. Effect of LPS on forward scatter in *akt1*^{+/+} and *akt1*^{-/-} DCs.

a. Representative FACS histograms depicting the forward scatter of DCs from Akt1 knockout mice (*akt1*^{-/-}, right panel) and their wild type littermates (*akt1*^{+/+}, left panel) following incubation for 4 hours without (control, red line) or with LPS (1 μ g/ml, blue line).

b. Arithmetic means $\pm$ SEM ($n = 3$ -5 independent experiments) of the forward scatter of *akt1*^{+/+} DCs (white bars, SD = 4.7 (Ctrl) and 6.8 (LPS)) or *akt1*^{-/-} DCs (black bars, SD = 5.5 (Ctrl) and 3.1 (LPS)) following a four hours incubation without (left bars) or with (right bars) LPS (1 μ g/ml) # indicates statistically significant difference ($p < 0.05$) from control, * indicates statistically significant difference ($p < 0.05$) from *akt1*^{+/+} mice.

Fig. 2. Effect of LPS on reactive oxygen species (ROS) production in *akt1*^{+/+} and *akt1*^{-/-} DCs. a. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in murine DCs from Akt1 knockout mice (*akt1*^{-/-}, right panel) and their wild type littermates (*akt1*^{+/+}, left panel) following incubation for 4 hours without (control, red line) or with (blue line) lipopolysaccharide (LPS, 1 µg/ml), LPS+catalase (10µM) (brown line) and catalase (green line) (10µM) only. b. Arithmetic means ± SEM (n = 3-5 independent experiments) of the percentage of ROS Geometric Mean Fluorescence (MFI) intensity positive

akt1^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) following a four hours incubation without (left bars) or with (right bars) LPS (1 µg/ml), LPS+ catalase (10µM) and catalase (10µM) # indicates statistically significant difference (p<0.05) from control.

In a further series of experiments Na⁺/H⁺ exchanger activity was determined utilizing the ammonium pulse technique (Fig. 3). The addition of NH₄Cl in the extracellular bath was followed by NH₃ entry into the cells, subsequent binding of H⁺ to form NH₄⁺ and thus transient cytosolic alkalinization (Fig. 3). The removal of NH₄Cl in a second step was followed by NH₃ exit and cellular retention of H⁺ thus leading to cytosolic acidification (Fig. 3). In the absence of Na⁺ the acidification was not followed by a pH recovery, indicating that the cells did not express appreciable Na⁺-independent H⁺ extruding transport systems. The addition of Na⁺ was followed by a rapid cytosolic realkalinization in both genotypes, an observation pointing to Na⁺/H⁺ exchanger activity. As shown in Fig. 3, prior to LPS treatment, the increase of Na⁺-dependent pH recovery was again similar in *akt1*^{+/+} DCs and *akt1*^{-/-} DCs.

Treatment of the DCs with lipopolysaccharide (LPS, 1 µg/ml) was followed by a marked and statistically significant increase in Na⁺-dependent realkalinization of *akt1*^{+/+} DCs (Fig. 3). The increase in Na⁺-dependent pH recovery following LPS treatment was significantly blunted in *akt1*^{-/-} DCs. The ROS degrading enzyme catalase abrogated the stimulating effect of LPS in both genotypes (Fig. 3).

A further series of experiments studied the effect of oxidative stress on Na⁺/H⁺ exchanger activity. Oxidative stress by exposure of DCs to tert-butylhydroperoxide (tBOOH, 5 µM) was followed by marked acidification of both *akt1*^{-/-} and *akt1*^{+/+} DCs (Fig. 4). Similar to what was observed following treatment with LPS, exposure of DCs to tBOOH was followed by an increase in Na⁺ dependent realkalinization in *akt1*^{+/+} DCs but not *akt1*^{-/-} DCs. Accordingly, following oxidative stress, the Na⁺ dependent realkalinization was significantly higher in *akt1*^{+/+} DCs than in *akt1*^{-/-} DCs. The effect of tBOOH was significantly blunted by catalase treatment in both *akt1*^{-/-} and *akt1*^{+/+} DCs (Fig.5).

Fig. 3. NHE activity in *akt1*^{+/+} and *akt1*^{-/-} DCs with or without LPS treatment. Alterations of cytosolic pH (pH_i) in bone marrow derived DCs from Akt1 knockout mice (*akt1*^{-/-}) and their wild type littermates (*akt1*^{+/+}) following an ammonium pulse. To load the cells with H⁺, 20 mM NH₄Cl was added and Na⁺ removed (replaced by NMDG) in a first step (see bars below each original tracing), NH₄Cl 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. a. Time dependent changes (± SEMs) of cytosolic pH in typical experiments

following 4 hours treatment without or with 1 μg/ml LPS, LPS+ catalase (10 μM) and catalase (10 μM) (left panels) in *akt1*^{+/+} DCs (upper panels) and similarly in *akt1*^{-/-} DCs (right panels). b. Arithmetic means ± SEM (n = 3-6 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) 4 hours after treatment without (control, left bars) or with 1 μg/ml LPS (LPS, right bars), LPS+ catalase (10 μM) and catalase (10 μM). # indicates statistically significant difference (p<0.05) from control, * indicates statistically significant difference (p<0.05) from *akt1*^{+/+} mice. c. Arithmetic means ± SEM (n = 3-6 independent experiments) of Na⁺-dependent recovery of cytosolic pH (ΔpH/min) in *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) following an ammonium pulse 4 hours after treatment without or with 1 μg/ml LPS, LPS+ catalase (10 μM) and catalase (10 μM). # indicates statistically significant difference (0.05) from control, * indicates statistically significant difference (0.05) from *akt1*^{+/+} mice.

Fig. 4. NHE activity in *akt1*^{+/+} and *akt1*^{-/-} DCs with or without tBOOH treatment. Alterations of cytosolic pH (pH_i) in bone marrow derived DCs from Akt1 knockout mice (*akt1*^{-/-}) and their wild type littermates (*akt1*^{+/+}) as described in Fig. 3. a. Time dependent changes ($\pm$ SEMs) of cytosolic pH in typical experiments following 4 hours treatment without (left panels) or with 5 μ M tert-butylhydroperoxide (tBOOH, right panels) in *akt1*^{+/+} DCs (upper panels) and *akt1*^{-/-} DCs (lower panels). b. Arithmetic means $\pm$ SEM (n = 3-4 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) 4 hours after treatment without (control, left

bars) or with 5 μ M tBOOH (tBOOH, right bars). # indicates statistically significant difference from control, * indicates statistically significant difference from *akt1*^{+/+} mice. c. Arithmetic means $\pm$ SEM (n = 3-4 independent experiments) of Na⁺-dependent recovery of cytosolic pH (ΔpH /min) in *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) following an ammonium pulse 4 hours after treatment without (control, left bars) or with 5 μ M tBOOH (tBOOH, right bars). # indicates statistically significant difference (p<0.05) from control, * indicates statistically significant difference (p<0.05) from *akt1*^{+/+} mice.

Discussion

The present study reveals that Akt1 contributes to the signalling of LPS in dendritic cells (DCs). Specifically, Akt1 is required for the full effect of LPS on Na⁺/H⁺ exchanger activity. TLR4 activation by LPS leads to activation of phosphatidylinoside (PI) 3 kinase (PI3 Kinase)/AKT pathway thus fostering activation of inflammatory events with release of various chemokines and cytokines as well as generation of ROS and NO [73]. Previous studies [28] revealed that stimulation of the Na⁺/H⁺ exchanger activity by LPS required the PI3 kinase and was disrupted by PI3K inhibitors wortmannin and LY294002.

The present study did not elucidate the mechanism linking Akt1 to Na⁺/H⁺- exchanger activity. It is noteworthy, however, that the related serum & glucocorticoid inducible kinase

Fig. 5. Impact of catalase on NHE activity in *akt1*^{+/+} and *akt1*^{-/-} DCs following tBOOH treatment. Alterations of cytosolic pH (pH_i) in bone marrow derived DCs from Akt1 knockout mice (*akt1*^{-/-}) and their wild type littermates (*akt1*^{+/+}) as described in Fig. 4 with t-BOOH+ Catalase. a. Time dependent changes ($\pm$ SEM) of cytosolic pH in typical experiments following 4 hours treatment without (control, left panels) or with 5 μ M tert-butylhydroperoxide (tBOOH, right panels) + catalase in *akt1*^{+/+} DCs (upper panels) and *akt1*^{-/-} DCs (lower panels). b. Arithmetic means $\pm$ SEM (n = 3-4 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) 4 hours after treatment without (control, left bars) or with 5 μ M tBOOH+ catalase (10 μ M) (tBOOH, right bars). c. Arithmetic means $\pm$ SEM (n = 3-4 independent experiments) of Na⁺-dependent recovery of cytosolic pH ($\Delta pH_i/min$) in *akt1*^{+/+} DCs (white

bars) or *akt1*^{-/-} DCs (black bars) following an ammonium pulse 4 hours after treatment without (control, left bars) or with 5 μ M tBOOH+ catalase (10 μ M) (tBOOH, right bars). d. Left hand side shows representative FACS plot and right hand side shows respective arithmetic means $\pm$ SEM (n = 3-5 independent experiments) of the percentage of ROS Geometric Mean Fluorescence (MFI) intensity positive *akt1*^{+/+} DCs (white bars) or *akt1*^{-/-} DCs (black bars) following a four hours incubation without (left bars) or with (right bars) t-BOOH (5 μ M), t-BOOH (5 μ M)+ catalase (10 μ M) and catalase (10 μ M). # indicates statistically significant difference (p<0.05) from control, ** indicates statistically significant difference (p<0.01) from *akt1*^{+/+} mice.

1 (SGK1) with virtually identical phosphorylation sites as Akt [74] up-regulates NHE1 presumably by direct phosphorylation of the carrier protein [75].

Inhibition of PI3 kinase further disrupted the effect of LPS on the formation of Reactive Oxygen Species (ROS). Moreover, Akt1 is required for the upregulation of Na⁺/H⁺ exchanger activity by oxidative stress. Mechanisms activating Akt following oxidative stress include inactivation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an enzyme dephosphorylating phosphatidylinositol phosphates and thus disrupting PI3 Kinase signaling [76].

According to the present study, the effect of LPS significantly enhances ROS formation in *akt1*^{-/-} DCs indicating that ROS formation does not critically depend on the presence of Akt1. Therefore, the effect of LPS on ROS formation obviously involves PI3K dependent signaling other than Akt1.

ROS formation has previously been shown to be sensitive to cytosolic pH [16, 17] and were thus similarly expected to be sensitive to Na⁺/H⁺ exchanger activity. According to the present observations Na⁺/H⁺ exchanger activity is similar prior to LPS treatment but is significantly more sensitive to stimulation by LPS in *akt1*^{+/+} DCs than in *akt1*^{-/-} DCs. Moreover, ROS formation is also higher in *akt1*^{+/+} DCs than in *akt1*^{-/-} DCs, an observation suggesting that Akt1 could be regulating the ROS formation in LPS stimulated and unstimulated cells. Nevertheless, the effect of ROS on Na⁺/H⁺ exchanger activity appears to be significantly blunted in Akt deficient mice (*akt1*^{-/-}). Along those lines, induction of oxidative stress by administration of tBOOH similarly stimulates the Na⁺/H⁺ exchanger activity, an effect again significantly blunted in *akt1*^{-/-} DCs.

Differences in Na⁺/H⁺ exchanger activity are expected to impact on cell volume, which is regulated in a wide variety of cells by parallel activity of Na⁺/H⁺ exchanger and Cl⁻/HCO₃⁻ exchanger [20, 21]. The carriers mediate the entry of NaCl in exchange for H⁺ and HCO₃⁻, which are replenished from CO₂ and are thus not osmotically relevant [20, 21]. Cell volume is indeed larger in *akt1*^{+/+} DCs than in *akt1*^{-/-} DCs. The increase of cell volume is expected to inhibit in turn Na⁺/H⁺ exchanger activity. Accordingly, the Na⁺/H⁺ exchanger activity is not significantly different between *akt1*^{+/+} DCs and *akt1*^{-/-} DCs prior to LPS treatment. In addition to the impact of Na⁺/H⁺ exchangers on cell volume, they do regulate cytosolic pH, which is known to participate in the regulation of diverse further functions of dendritic cells, macrophages and/or monocytes [30, 77-82].

In conclusion, the present study demonstrates that Akt1 participates in the signaling mediating the stimulation of Na⁺/H⁺ exchanger activity by LPS and oxidative stress. The present observations suggest that LPS triggers ROS formation by at least partially Akt1 independent signaling and that ROS leads to subsequent Akt1 sensitive activation of Na⁺/H⁺ exchanger activity.

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Disclosure Statement

No conflict of interest.

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