Regulation of Na⁺/H⁺ exchanger in dendritic cells by Akt2

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Abstract Dendritic cells (DCs) are antigen-presenting cells decisive in primary immune responses and establishment of immunological memory. They are activated by bacterial lipopolysaccharides (LPS), which lead to activation of Na⁺/H⁺ exchanger activity, cell swelling, reactive oxygen species (ROS) formation, and migration. The effects require functional phosphoinositide 3 kinase and are paralleled by Akt phosphorylation. The present study explored the putative involvement of the Akt isoform Akt2. To this end, experiments were performed in DCs isolated from bone marrow of mice lacking functional Akt2/PKBß (akt2−/−) and respective wild-type animals (akt2+/+). Based on BCECF fluorescence, cytosolic pH (pHi) was significantly lower in akt2−/− than in akt2+/+ DCs. Transient exposure to NH₄Cl was followed by profound cytosolic acidification in both genotypes. Subsequent re-alkalinization was largely dependent on Na⁺ thus reflecting Na⁺/H⁺ exchanger activity and was significantly lower in akt2−/− than in akt2+/+ DCs. According to forward scatter in FACS analysis, cell volume was significantly lower in akt2−/− than in akt2+/+ DCs. Exposure of DCs to LPS led within 4 h to significant increases of Na+/H+ exchanger activity, cell volume, ROS production, and migration in akt2+/+ mice, and its effects were significantly blunted in akt2−/− DCs. The present observations disclose a role of Akt2 in the regulation of pHi, cell volume, ROS production, and migration in dendritic cells.

Keywords LPS · Toll-like receptors · Cytosolic pH · ROS · Cell volume · Akt2/PKBß · Migration

Introduction

Dendritic cells (DCs), antigen-presenting cells, are critically involved in primary immune responses, development of T cell immunity, and establishment of immunological memory [3, 9]. Stimulation of Toll-like receptors by bacterial lipopolysaccharides (LPS) is followed by DCs maturation, expression of costimulatory molecules, and MHC class II [15] as well as production of proinflammatory chemokines/cytokines [5]. DCs further generate reactive oxygen species (ROS) [4] which participates in the killing of pathogens [25].

As shown in other cell types [11] including macrophages [8], ROS production is paralleled by production of H⁺, which in turn influences ROS-generating NADPH oxidase [38]. Along those lines, ROS production is paralleled by the operation of Na⁺/H⁺ exchangers [30]. The isoform most abundantly expressed in DCs is NHE1 [31]. Na⁺/H⁺ exchangers further play a decisive role in the regulation of cell volume [8, 20], which in turn influences a wide variety of further cellular functions including ROS formation and migration [20].

Dendritic cell function is regulated by phosphoinositide 3 kinase [34]. Most recently, DC Na⁺/H⁺ exchanger activation, cell swelling, and ROS formation have been shown to depend on PI3 kinase [31]. PI3 kinase-dependent signaling includes the protein kinase PKB/Akt isoforms, which are activated by the phosphoinositide-dependent kinase PDK1 [10]. PKB/Akt has been shown to be phosphorylated and thus presumably activated by LPS [31]. Three PKB/Akt isoforms have been identified, i.e., Akt1, Akt2, and Akt3 or PKBα, PKBβ, and PKBγ, respectively.
DCs (2 × 10^6 cells) were left unpulsed (control) or pulsed with LPS for 4 h and then snap frozen in dry ice–ethanol bath. Cell pellets were thawed on ice and washed twice with PBS then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at −80°C until use for Western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat milk in triethanolamine-buffered saline and 0.1% Tween 20. Then the blots were probed overnight with anti-p-Akt, anti-Akt, and GAPDH antibodies (1:1,000, cell signaling) diluted in 5% milk in PBS and 0.1% Tween 20, washed five times, probed with secondary antibodies conjugated with horseradish peroxidase (1:2,000) for 1 h at room temperature, and washed finally five times. Antibody binding was detected with the enhanced chemiluminescence kit (Amersham, Freiburg, Germany). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany).

Immunostaining and flow cytometry

Cells (4 × 10^5) were incubated in 100-μl FACS buffer (PBS plus 0.1% FCS) containing fluorochrome-conjugated antibodies at a concentration of 10 μg/ml. A total of 4 × 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 min 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 (pHi), the cells were incubated in a HEPES-buffered Ringer’s solution containing 10 μM BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C [26]. After loading, the chamber was flushed for 5 min with Ringer’s 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 oil immersion objective (Zeiss Neoplan, Germany) [27–31]. BCECF was successively excited at 490/10 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 (Metaflour, USA). Between 10 and 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 (490/204 nm) was plotted versus time for each cell.

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. DCs were cultured from bone marrow [1, 23] of 7–11 weeks old mice lacking functional Akt2/PKBβ (akt2−/−) and respective wild-type animals (akt2+/+). Prior to the experiments, mice had free access to control diet (1314, Altromin, Heidenau, Germany) and tap drinking water.

Cell culture

DCs were cultured from bone marrow according to a standard protocol with slight modifications as previously described [17, 22, 39]. Briefly, bone marrow-derived cells were flushed out of the cavities from the femur and tibia with phosphate-buffered saline (PBS). Cells were then washed twice with RPMI 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% CO₂ 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, 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, ≥80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed at days 7–9 of DCs culture.

Western blotting

DCs (2 × 10^6 cells) were left unpulsed (control) or pulsed with LPS for 4 h and then snap frozen in dry ice–ethanol solution.
Briefly, 4×10^5/ml cells were taken in a culture dish and dichlorodihydrofluorescein diacetate (DCFDA) [35]. Assuming that NH_4^+ the cell suspension at a final concentration of 10^−5 M and DCFDA (Sigma, Schnelldorf, Germany) was added to treated with LPS. After the treatment, cells were collected and analyzed with a flow cytometer (FACSCalibur 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.

**Determination of cell volume**

Cell volume was estimated from forward scatter in flow cytometric analysis. Briefly, 4×10^5 cells/ml were taken in a culture dish and treated with LPS. After the treatment, cells were collected, centrifuged, the pellet was resuspended in FACS buffer, and analyzed with flow cytometry (FACSCalibur from Becton Dickinson; Heidelberg, Germany).

**Migration**

For migration assays, transwell inserts (BD Falcon 353097) were employed with a pore diameter size of 8 µm. The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 µl) with CCL21 (250 ng/ml, Peprotech) in the lower chamber. The upper chambers were filled with 500-µl cell culture medium containing DCs in a concentration of 1×10^5 cells/ml. The chamber was placed in a 5% CO_2 at 37°C incubator for 4 h. In the following, the non-migrated cells were removed by scrubbing with a cotton-tipped swab for two times and washing with PBS. The membrane was removed with a scalpel and fixed in 4% PFA for 15 min. The migrated cells were then analyzed by staining the membrane with the pores and counting the migrated cells with DAPI.

**Statistics**

Data are provided as means±SEM; n represents the number of independent experiments. Where indicated, the data were additionally normalized and expressed as percentage of akt2^{+/+} control. All data were tested for significance using Student’s unpaired two-tailed t test or ANOVA and only results with p<0.05 were considered statistically significant.

**Results**

In a first series of experiments, Western blot analysis was employed to study the expression of Akt2 in DCs. As
illustrated in Fig. 1, Akt2 was expressed in akt2+/+ DCs but not in akt2−/− DCs. Additional experiments were performed to study the phosphorylation of Akt after treatment with bacterial LPS. Unspecific anti-Akt antibodies and anti-phospho-Akt antibodies revealed that the total protein abundance of Akt was not different between untreated and LPS treated DCs, but the phosphorylated Akt protein was significantly higher in LPS treated akt2+/+ DCs than in respective untreated DCs pointing to Akt activation. The abundance of phosphorylated Akt protein following LPS treatment was lower in akt2−/− DCs than in akt2+/+ DCs (Fig. 1).

A second series of experiments addressed the role of Akt2 in the regulation of Na+/H+ exchanger activity in DCs. To this end, BCECF fluorescence was employed to determine cytosolic pH (pHi). As a result, cytosolic pH (pHi) was significantly lower in akt2−/− DCs than in akt2+/+ DCs (Fig. 2b; Table 1).

Na+/H+ exchanger activity in DCs was determined utilizing the ammonium pulse technique (Fig. 2). To this end, NH4Cl was added to the perfusate leading to NH3 entry into the cells, binding of H+ to form NH4+ and thus transient cytosolic alkalization (Fig. 2a). Subsequent removal of NH4Cl was followed by NH3 exit and retention of H+ within the cell resulting in cytosolic acidification (Fig. 2a). No pH recovery was observed in the absence of Na+, indicating that the cells did not express appreciable Na+-independent H+ extrusion mechanisms (Table 1). The addition of Na+ resulted in a rapid pH recovery reflecting Na+/H+ exchanger activity. As illustrated in Fig. 2a, the Na+-dependent pH recovery was significantly lower (by 26±8%) in akt2−/− DCs than in akt2+/+ DCs.

LPS treatment did not significantly affect the buffer capacity of the cells (Table 1). Thus, the relation between H+ transport across the cell membrane and cytosolic pH was comparable in the presence and absence of LPS. As evident from the ammonium pulse experiments, a 4-h LPS treatment was followed by a marked and statistically significant increase in Na+-dependent re-alkalinization (Table 1; Fig. 2c). As illustrated in Fig. 2c, the increase of Na+-dependent pH recovery following LPS treatment was significantly higher in akt2+/+ DCs (81±22%) than in akt2−/− DCs (11±19%).

Na+/H+ exchangers are among the major transport systems regulating cell volume. Thus, forward scatter was determined utilizing FACS analysis to estimate cell volume. As shown in Fig. 3, forward scatter was significantly higher in akt2+/+ DCs than in akt2−/− DCs. LPS treatment was followed within 4 h by a significant increase of forward scatter in both genotypes, reflecting cell swelling. Forward scatter was after LPS treatment again significantly higher in akt2+/+ DCs than in akt2−/− DCs.

Na+/H+ exchanger activity is further critically important for the ROS formation in the DCs. Therefore, DCFDA fluorescence was employed to determine ROS formation...
(Fig. 4). Prior to LPS treatment, ROS formation tended to be higher in akt2+/+ DCs than in akt2−/− DCs, a difference, however, not reaching statistical significance. As illustrated in Fig. 4, LPS treatment was followed within 4 h by an increase of ROS production following LPS treatment was significantly higher in akt2+/+ DCs (32±4%) than in akt2−/− DCs (13±4%).

Na+/H+ exchangers further participate in the machinery of migration. Thus, a transwells migration assay was performed with akt2+/+ DCs and akt2−/− DCs. As shown in Fig. 5, migratory activity was significantly larger in akt2+/+ DCs than in akt2−/− DCs and NHE1 inhibitor cariporide (10 μM), which virtually abolishes the LPS induced Na+/H+ exchanger activity [30], decreases the migratory activity of akt2+/+ DCs by some 50%. Cariporide did not significantly modify the migratory activity of akt2−/− DCs.

Discussion

The present study reveals a novel function of Akt2, i.e., the regulation of Na+/H+ exchanger activity, cell volume, ROS formation, and migration in DCs. Akt has previously been reported to stimulate [32] or to inhibit [36] Na+/H+ exchanger activity. Moreover, Na+/H+ exchangers have
been suggested to regulate Akt [18]. To the best of our knowledge, the isoform of Akt regulating NHE1 has never been identified.

As shown earlier [31], the Na+/H+ exchanger rather than Na+-independent H+ pumps are decisive for pH regulation in DCs. Stimulation of the Na+/H+ exchanger activity by LPS was disrupted by pharmacological inhibition of the PI3 kinase by wortmannin or LY294002 and is thus dependent on PI3 kinase signaling [31]. Inhibition of PI3 kinase further prevented the effect of LPS on the formation of ROS, which is important for the killing of pathogens and thus for the innate immune response [25]. ROS formation is pH sensitive and thus requires H+ extrusion [13, 38].

The activation of the Na+/H+ exchanger by LPS is further followed by cell swelling, an effect again requiring functional PI3 kinase [31]. In a wide variety of cells, the carrier is activated during regulatory cell volume increase and, together with the Cl−/HCO3− exchanger, accomplishes cell volume regulatory NaCl uptake [16, 20]. The H+ and HCO3− extruded by the two carriers in exchange for NaCl are regenerated from CO2, which readily enters the cell across the cell membrane [16, 20]. Cell swelling further influences ROS formation and modifies cellular antioxidant defense [12]. In HeLa cells, the regulatory cell volume increase following osmotic cell shrinkage, was paralleled by Akt phosphorylation, and inhibited by pharmacological blockage of Akt, by expression of a dominant negative Akt mutant, or by silencing with small interfering RNA of Akt1 but not Akt2 [37]. The present study did not address whether Na+/H+ exchanger activity and cell volume are a

|                | Cytosolic pH (units) | Buffer capacity (mM/pH unit) | Sodium-independent pH recovery (Δ pH units/min) | Sodium-dependent pH recovery (NHE activity, Δ pH units/min) | Number of cells |
|----------------|----------------------|------------------------------|---------------------------------------------|----------------------------------------------------------|----------------|
| control akt2+/+| 7.29±0.01            | 15.4±0.9                     | -0.064±0.005                                | 0.139±0.009                                              | 237            |
| control akt2−/−| 7.21±0.01b           | 16.1±1.7                     | -0.076±0.007                                | 0.101±0.007b                                             | 242            |
| LPS akt2+/+    | 7.32±0.01            | 15.4±2.4                     | -0.062±0.005                                | 0.275±0.017a                                             | 221            |
| LPS akt2−/−    | 7.23±0.01b           | 16.4±1.2                     | -0.072±0.008                                | 0.183±0.011a, b                                          | 230            |

aSignificant difference from the respective control value

bSignificant difference from the respective value in akt+/+ DCs

![Fig. 3](image)

**Fig. 3** Effect of LPS on forward scatter in akt2+/+ and akt2−/− DCs. a Representative FACS histograms depicting the forward scatter of DCs from Akt2 knockout mice (akt2−/−, right panel) and respective wild-type mice (akt2+/+, left panel) following incubation for 4 h without (control, black line) or with LPS (1 μg/ml, green line). b Arithmetic means±SEM (n=6 independent experiments) of the forward scatter of akt2+/+ DCs (white bars) or akt2−/− DCs (black bars) following a 4-h incubation without (left bars) or with (right bars) LPS (1 μg/ml). Number sign indicates statistically significant difference from respective control. Asterisk indicates statistically significant difference from akt2+/+ mice.
function of Akt1 in DCs. Possibly, both kinase isoforms regulate Na\(^+\)/H\(^+\) exchanger activity, but osmotic cell shrinkage stimulates only Akt1. Clearly, additional experimental effort is required to define the role of Akt isoforms in the regulation of Na\(^+\)/H\(^+\) exchanger activity and cell volume.

Cytosolic pH and Na\(^+\)/H\(^+\) exchanger further modify migration [8], which is important for the translocation of DCs to peripheral tissues and from there to lymphoid tissues [33]. Migration of DCs is again dependent on PI3 kinase activity [31]. Ample evidence suggests a role of Akt isoforms including Akt2 in the regulation of migratory activity in a wide variety of cells [6]. Migration of DCs was reported to be abrogated by genetic knockdown of Akt2 but not of Akt1 [21]. Involvement of the Na\(^+\)/H\(^+\) exchanger has not been shown in that paper.

Cytosolic pH modifies a variety of further functions of macrophages and/or monocytes [24]. Inhibition of PI3 kinase in DCs has previously been shown to influence K\(^+\) channel activity and release of IL-12 [34]. Whether or not those functions depend on the activation of the Na\(^+\)/H\(^+\) exchanger remains to be shown.

PI3 kinase and thus Akt are activated by IGF1 and insulin [2], which are thus expected to influence regulation of cytosolic pH, cell volume, and ROS formation in dendritic cells.

In conclusion, the present study demonstrates a role of Akt2 in the regulation of cytosolic pH in bone marrow-derived DCs.

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