Original Paper

Sgk1 Sensitive Pendrin Expression in Murine Platelets

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Key Words

Slc26a4 • Mineralocorticoids • Deoxycorticosterone (DOCA) • Anion exchanger • Platelets • Serum and glucocorticoid inducible kinase 1 (SGK1)

Abstract

Background: The anion exchanger pendrin (SLC26A4) is required for proper development of the inner ear, and contributes to iodide organification in thyroid glands as well as anion transport in various epithelia, such as airways and renal tubules. SLC26A4 deficiency leads to Pendred syndrome, which is characterized by hearing loss with enlarged vestibular aqueducts and variable hypothyroidism and goiter. Pendrin expression in kidney, heart, lung and thyroid is up-regulated by the mineralocorticoid deoxycorticosterone (DOCA). Platelets express anion exchangers but virtually nothing is known about the molecular identity and regulation of those carriers. Other carriers such as the Na⁺/H⁺ exchanger are regulated by the mineralocorticoid-sensitive serum and glucocorticoid inducible kinase SGK1. **Methods:** The present study utilized i) quantitative reverse transcription polymerase chain reaction (RT-qPCR) to quantify the transcript levels of *Slc26a4* as compared to *Gapdh* and ii) western blotting to assess Slc26a4 protein abundance in murine platelets from gene-targeted mice lacking *Sgk1* (*sgk1*^{-/-}) and respective wild type animals (*sgk1*^{+/+}) treated without or with a subcutaneous injection of 2.5 mg DOCA for 3 h, or in *sgk1*^{+/+} platelets with or without *in vitro* treatment for 1 h with 10 µg/ml DOCA. **Results:** Slc26a4 was expressed in platelets, and *in vitro* DOCA treatment increased Slc26a4 mRNA levels in platelets isolated from *sgk1*^{+/+} mice. Moreover, *in vivo* DOCA treatment significantly up-regulated Slc26a4 mRNA levels in platelets isolated from *sgk1*^{+/+} but not *sgk1*^{-/-} mice. An increase in *Sgk1* mRNA levels paralleled that of Slc26a4 mRNA levels in platelets of *sgk1*^{+/+} mice. In addition, DOCA treatment further increased Slc26a4 protein abundance in platelets isolated from *sgk1*^{+/+} mice. **Conclusions:** Pendrin is expressed in platelets and is presumably regulated by SGK1 and mineralocorticoids.

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Introduction

Pendrin (SLC26A4), an exchanger transporting anions, such as chloride, bicarbonate and iodide [1-3], is expressed in a wide variety of tissues, including thyroid gland, inner ear, kidney, lung, liver and heart [4-6]. SLC26A4 is required for adequate development of the inner ear [7, 8] and it has been suggested that the carrier is involved in thyroid iodide transport [5, 9, 10]. Pendrin mediates anion transport in airways [11, 12] and contributes to renal tubular anion transport [6, 13, 14]. In the kidney, SLC26A4 influences expression and activity of the epithelial Na⁺ channel ENaC, thus influencing blood pressure regulation [14-18]. Moreover, SLC26A4 has been proposed to participate in cell volume homeostasis [19].

Loss or decrease of function mutations in the pendrin protein [20-25] result in autosomal-recessive Pendred syndrome (PS), a rare disorder invariably leading to sensorineural hearing loss with enlarged vestibular aqueducts [3]. Lack of functional pendrin further compromises iodide organification thus enhancing the risk of developing goiter and hypothyroidism [3, 26]. The development of clinically relevant goiter and hypothyroidism in PS may depend on nutritional iodide intake or other individual factors [5, 26].

Stimulators of SLC26A4 expression and function include acidification, aldosterone, intestinal natriuretic hormone, angiotensin II, interleukin-4 and interleukin-13 [27]. Mineralocorticoids have been shown to upregulate pendrin expression not only in kidney [14, 18], but in heart, lung and thyroid as well [28]. Mineralocorticoid receptors are expressed in many tissues [29], such as kidney, colon, heart, lung, blood vessels, adipose tissue, thyroid and hippocampus [30-35], and mineralocorticoids participate in the regulation of diverse functions, such as renal and colonic Na⁺ and K⁺ transport [34], salt appetite [36], blood pressure [37], cardiac remodelling and fibrosis [38-41], endothelial stiffness [42, 43], vascular stiffness [44], tissue calcification [45, 46], as well as apoptosis in hippocampal neurons [47]. Along those lines, aldosterone influences expression of a wide variety of genes [38, 39, 44, 46, 48-50].

Cells known to express anion exchangers include platelets [51-55], which are critically important for primary haemostasis [56] and decisively contribute to acute thrombotic occlusion in myocardial infarction [57-60] and ischemic stroke [61].

However, nothing is known about pendrin expression in platelets. The present study thus explored whether platelets express pendrin and, if so, whether the pendrin expression in platelets is sensitive to the mineralocorticoid deoxycorticosterone (DOCA).

Materials and Methods

Animal experimentation and isolation of mouse platelets

Experiments were performed with platelets isolated from 10-12 week old gene-targeted mice lacking functional serum and glucocorticoid inducible kinase Sgk1 (*sgk1*^{-/-}) and age- as well as sex-matched wild type mice (*sgk1*^{+/+}). Generation, breeding and genotyping of the mice have been described earlier [62]. All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities. The animals had free access to food (C1310, Altromin, Heidenau, Germany) and tap water. Where indicated, the animals were treated with a single subcutaneous injection (2.5 mg) of deoxycorticosterone (DOCA, Sigma, Taufkirchen, Germany), dissolved in soy bean oil and ethanol (1:1), 3 hours prior to isolation of platelets and subsequent determination of SLC26A4 transcript and protein levels. Platelets have been isolated as described previously [63, 64]. The mice were anesthetized and blood was drawn from the retroorbital plexus into heparinized tubes. Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards, PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. In order to prevent platelet aggregation, apyrase (0.02 U/ml, Sigma-Aldrich) and prostaglandin I₂ (0.5 μM, Calbiochem) were added to the PRP. After two washing steps, the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl₂). In a separate series, platelets were isolated from untreated wild type mice and exposed for 1 h to 10 μg/ml DOCA or solvent prior to the measurements.

RT-PCR analysis

To determine *Slc26a4* mRNA abundance, total RNA was extracted using Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Reverse transcription of 1 µg RNA was performed using oligo(dT)₁₂₋₁₈ primers (Invitrogen, Karlsruhe, Germany) and SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA samples were treated with RNase H (Invitrogen, Karlsruhe, Germany). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed with the iCycler iQ™ RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and iTaq™ Sybr Green Supermix with ROX passive reference dye (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

The following primers were used (5'→3' orientation): *Slc26a4* s: TTCGGTCTCTACTCTGCCTTT; *Slc26a4* as: CCCACCATTAAGTACCACG; *Gapdh* s: AGGTCGGTGTGAACGGATTG; *Gapdh* as: TGTAGACCATGTAGTTGAGGTCA. The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the $\Delta\Delta$ Ct method [65] using *Gapdh* as an internal reference.

Membrane preparation and western blot analysis

For determination of *Slc26a4* protein abundance, platelets were homogenized in an ice-cold K-HEPES buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) containing a protease inhibitor mix (Complete Mini, Roche Diagnostics, Germany; 1 tablet in a volume of 10 ml solution). Samples were centrifuged at 1500 g for 10 min at 4 °C. Subsequently, the supernatant was transferred to a new tube and centrifuged at 12000 g for 1 h at 4 °C. The resultant pellet was resuspended in K-HEPES buffer containing protease inhibitors. After measurement of the total protein concentration (Bio-Rad D_c Protein Assay; Bio-Rad, Hercules, CA, USA), 100 µg of crude membrane proteins were solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 8% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 (TBS-T) for 60 min, the blots were incubated with the respective primary antibodies (rabbit anti-pendrin 1:1000 [66] and rabbit monoclonal anti-*Gapdh* antibody (37 kDa; Cell Signaling Technology) 1:2000, diluted in 1% milk/TBS-T) either for 2 h at room temperature or overnight at 4 °C. After washing and subsequent blocking, the membranes were incubated for 1 h at room temperature with the secondary antibody conjugated with horseradish peroxidase (HRP) (1:2000, Cell Signaling). After washing, antibody binding was detected with the ECL detection reagent (Amersham). All bands were analyzed with Quantity One Software (Biorad).

Statistical analysis

As indicated, data are provided as means $\pm$ SEM; *n* represents the number of animals studied. In each animal, RT-PCR has been performed in duplicate. All data were tested for significance using Student's unpaired two-tailed t-test where applicable. Only differences with *p* < 0.05 were considered statistically significant.

Results

Platelets were isolated from either untreated mice or mice treated 3 h prior to isolation of the platelets with a single subcutaneous administration of 2.5 mg DOCA. In one series of experiments, platelets from untreated mice were treated *in vitro* for 1 h prior to measurements with either 10 µg/ml DOCA or solvent. *Slc26a4* transcript levels were determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) as well as *Slc26a4* protein abundance by Western blotting. As illustrated in Fig. 1A, *Slc26a4* transcripts were detected in both untreated and DOCA-treated platelets. *In vitro* treatment with DOCA significantly increased the *Slc26a4* transcript levels (Fig. 1B).

In order to determine whether mineralocorticoids similarly regulate the expression of the serum and glucocorticoid inducible kinase SGK1, the *Sgk1* mRNA levels were determined in platelets isolated from untreated mice or from mice treated by a single subcutaneous

Fig. 1. *Slc26a4* mRNA abundance in platelets without and with *in vitro* DOCA treatment. A. Original 2 % agarose gel showing specific amplification of pendrin cDNA at 105 bp and Gapdh cDNA at 120 bp following *in vitro* treatment of platelets without (CTL) and with desoxycorticosterone acetate (DOCA, 10 μ g/ml) for 1 h. B. Arithmetic means $\pm$ SEM ($n = 5$) of *Slc26a4* mRNA abundance in platelets isolated from untreated wild type mice following *in vitro* treatment without (control) and with desoxycorticosterone acetate (DOCA, 10 μ g/ml) for 1 h. The data are expressed as fold increase relative to the mean values of untreated platelets. * ($p < 0.05$) indicates statistically significant differences from value of untreated platelets.

Fig. 2. *Sgk1* mRNA abundance in platelets without and with DOCA treatment. Arithmetic means $\pm$ SEM ($n = 6$) of *Sgk1* mRNA abundance in platelets from wild type animals without treatment (white bar) and 3 hours following subcutaneous injection of 2.5 mg DOCA (black bar). The data are expressed as fold increase relative to the mean values of untreated animals. * ($p < 0.05$) indicates a statistically significant difference from untreated animals.

Fig. 3. *Slc26a4* mRNA abundance in *sgk1*^{-/-} and *sgk1*^{+/+} platelets without and with DOCA treatment. A. Original 2 % agarose gel showing specific amplification of pendrin cDNA at 105 bp and Gapdh cDNA at 120 bp in wild type mice without treatment (CTL) and 3 hours following subcutaneous injection of 2.5 mg DOCA (DOCA). B. Arithmetic means $\pm$ SEM ($n = 4$) of *Slc26a4* mRNA abundance in platelets from gene targeted mice lacking functional *Sgk1* (*sgk1*^{-/-}, white bars) and their wild type littermates (*sgk1*^{+/+}, black bars) without treatment (left bars) and 3 hours following subcutaneous injection of 2.5 mg DOCA (right bars). The data are expressed as fold increase relative to the mean values of untreated animals. * ($p < 0.05$) indicates a statistically significant difference from untreated animals.

administration of 2.5 mg DOCA. As shown in Fig. 2, DOCA treatment significantly increased *Sgk1* mRNA levels.

Fig. 4. Slc26a4 protein abundance in *sgk1*^{+/+} platelets without and with DOCA treatment. A. Original western blot showing pendrin and Gapdh protein abundance. B. Arithmetic means $\pm$ SEM ($n = 5$) of Slc26a4 protein abundance in platelets from wild type mice without treatment (white bar) and 3 hours following subcutaneous injection of 2.5 mg DOCA (black bar). The data are expressed as fold increase relative to the mean values of untreated animals. * ($p < 0.05$) indicates a statistically significant difference from untreated animals.

In order to define the role of Sgk1 in the regulation of Slc26a4 expression, experiments were performed in gene targeted mice lacking Sgk1 (*sgk1*^{-/-}) and their respective wild type littermates (*sgk1*^{+/+}). As illustrated in Fig. 3, DOCA treatment significantly increased *Slc26a4* mRNA levels in platelets from *sgk1*^{+/+} mice but not in platelets from *sgk1*^{-/-} mice.

Further experiments were performed to elucidate whether DOCA influences Slc26a4 protein abundance. As illustrated in Fig. 4A and B, Slc26a4 protein was detected in platelets from *sgk1*^{+/+} mice. DOCA treatment was followed by a significant increase of Slc26a4 protein abundance in platelets from *sgk1*^{+/+} mice (Fig. 4B).

Discussion

The present study demonstrates for the first time that platelets harbor pendrin mRNA and express pendrin protein. Moreover, the present observations reveal that both pendrin transcript levels and pendrin protein abundance are increased by treatment with the mineralocorticoid deoxycorticosterone (DOCA). DOCA is effective following *in vitro* treatment of platelets, indicating that DOCA has a direct influence on platelets. The present observations reveal that the effect of DOCA treatment on pendrin mRNA levels is paralleled by an increase and dependent on the presence of serum and glucocorticoid inducible kinase SGK1.

SGK1 has previously been shown to be strongly upregulated by mineralocorticoids in a variety of tissues [67]. Importantly, SGK1 is a powerful regulator of a variety of channels and transporters [67]. SGK1 regulates the expression of channels partially by up-regulating the transcription factor NF κ B [68], which is up-regulated by mineralocorticoids and participates in the signaling of inflammation and fibrosis [69]. As recently shown, SGK1 is expressed in platelets and critically involved in platelet function by regulating NF κ B-dependent transcription of Ca²⁺ channel moiety Orai1 in megakaryocytes [60, 63].

The observed upregulation of pendrin protein may be surprising in view of the lack of nuclei in blood platelets. However, blood platelets harbor pre-mRNA and mRNA and are capable to splice the intron-rich pre-mRNA into mature mRNA with subsequent translation into protein [70-72]. Previously, platelets have been shown to express interleukin-1 β (IL-1 β),

[73] tissue factor [74] and Orai1 [75]. Translation is stimulated by phosphatidylinositol 3 kinase (PI3K) [72] and by cytoskeletal reorganization [76]. PI3K dependent signaling includes SGK1 [67]. In the absence of stimulators of translation, mRNA is bound by the cytoskeletal core. The eukaryotic initiating factor eIF-4E localizes to the membrane skeleton [73] and interacts with the inhibitory 4E-BP1 molecule, which prevents initiation of translation [77]. Upon stimulation of translation, PI3K associates with the membrane skeleton [76] and triggers phosphorylation of 4E-BP1 [72, 77] with subsequent dissociation of the inhibitory binding molecules, redistribution of the translation initiation factors close to mRNA [73] and translation of mRNA [75]. Mineralocorticoids may thus influence platelet protein expression by nongenomic mechanisms [78, 79, 79-89], including PI3K [90, 91].

The present paper did not address the putative impact of pendrin on platelet function. In theory, $\text{Cl}^-/\text{HCO}_3^-$ exchangers such as SLC26A4 may contribute to cell volume regulation. Osmotic cell shrinkage is followed by parallel activation of Na^+/H^+ exchangers and $\text{Cl}^-/\text{HCO}_3^-$ exchangers leading to cellular NaCl uptake [92, 93]. The parallel extrusion of H^+ by the Na^+/H^+ exchanger and of HCO_3^- by the $\text{Cl}^-/\text{HCO}_3^-$ exchanger is osmotically not relevant, as H^+ and HCO_3^- are replenished in the cell from CO_2 via H_2CO_3 [92, 94]. Aldosterone is known to upregulate the Na^+/H^+ exchanger in the kidney [95, 96], heart [97-99], and several additional tissues [100-110]. Whether or not pendrin participates in platelet cell volume regulation, remains, however, to be shown.

In platelets, anion exchangers participate in the regulation of cytosolic pH [52, 54]. By mediating the extrusion of HCO_3^- in exchange of Cl^- , the carriers acidify the cells [52, 53]. The carriers are stimulated by thrombin [53]. They are inhibited by the stilbene derivatives 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulfonic acid (SITS) [53] and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) [55]. Inhibition of the carriers with DIDS blunts the stimulation of platelet aggregation by the thromboxane A2 agonist U-46619, collagen, ADP or thrombin [55]. However, whether the DIDS sensitive anion exchanger in platelets reflects pendrin remains uncertain and additional experiments are required to test whether those functions are mediated by pendrin.

The DIDS and SITS sensitive anion exchangers mediate the transport of peroxynitrite (ONOO^-), a reactive oxidant resulting from the reaction between nitric oxide and superoxide [51]. Prior treatment with ONOO^- inhibits the formation of cyclooxygenase (COX) products thromboxane A(2) and 12-hydroxyheptadecatrienoic acid, and arachidonic acid-induced platelet aggregation [51]. ONOO^- is presumably effective by tyrosine nitration [51]. Additional experiments are warranted on the contribution of pendrin to regulation of ONOO^- formation in platelets.

Given the role of platelets in vascular inflammation and thrombosis [111], the regulation of pendrin in platelets may contribute to the cardiovascular complications of mineralocorticoid excess such as thrombosis or inflammation [112]. However, additional experimental effort is needed to define the role of pendrin in the physiology and pathophysiology of blood platelets.

In conclusion, *Slc26a4* mRNA and protein were observed in platelets, where they were upregulated by DOCA, an effect requiring the presence of the serum and glucocorticoid inducible kinase SGK1.

Acknowledgements

This study has been supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University to F. Lang and M. Gawaz. This work was further supported by the FWF and the FP-7 to M.P. (P18608; PIRSES-GA-2008-230661).

Conflict of Interests

No conflict of interest.

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