Upregulation of Store Operated Ca\(^{2+}\) Channel Orai1, Stimulation of Ca\(^{2+}\) Entry and Triggering of Cell Membrane Scrambling in Platelets by Mineralocorticoid DOCA

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Abstract
Background/Aims: Mineralocorticoid excess leads to vascular injury, which is partially due to hypertension but in addition involves increased concentration of cytosolic Ca\(^{2+}\) concentration in platelets, key players in the pathophysiology of occlusive vascular disease. Mineralocorticoids are in part effective by rapid nongenomic mechanisms including phosphatidylinositide-3-kinase (PI3K) signaling, which involves activation of the serum & glucocorticoid inducible kinase (SGK) isoforms. SGK1 has in turn been shown to participate in the regulation of the pore forming Ca\(^{2+}\) channel protein Orai1 in platelets. Orai1 accomplishes entry of Ca\(^{2+}\), which is in turn known to trigger cell membrane scrambling. Platelets lack nuclei but are able to express protein by translation, which is stimulated by PI3K signaling. The present study explored whether the mineralocorticoid desoxycorticosterone acetate (DOCA) influences platelet Orai1 protein abundance, cytosolic Ca\(^{2+}\)-activity ([Ca\(^{2+}\)]\(_i\)), phosphatidylserine abundance at the cell surface and/or cell volume. Methods: Orai1 protein abundance was estimated utilizing CF\(^{TM}\)488A conjugated antibodies, [Ca\(^{2+}\)]\(_i\) utilizing Fluo3-fluorescence, phosphatidylserine abundance utilizing FITC-labelled annexin V, and cell volume utilizing forward scatter in flow cytometry. Results: DOCA (10 \(\mu\)g/ml) treatment of murine platelets was followed by a significant increase of Orai1 protein abundance, cytosolic Ca\(^{2+}\)-activity ([Ca\(^{2+}\)]\(_i\)), phosphatidylserine abundance at the cell surface and/or cell volume. The effect on [Ca\(^{2+}\)]\(_i\), phosphatidylserine abundance and cell volume were completely abrogated by addition of the specific SGK inhibitor EMD638683 (50 \(\mu\)M). Conclusions: The mineralocorticoid...
DOCA upregulates Orai1 protein abundance in the cell membrane, thus increasing [Ca\(^{2+}\)]\(_i\), and triggering phosphatidylserine abundance, effects paralleled by platelet swelling.

**Introduction**

Mineralocorticoids regulate a wide variety of functions, such as epithelial transport [1, 2], salt appetite [3, 4], blood pressure [5, 6], cardiac remodeling and fibrosis [7-12], endothelial [13-17] and vascular [7, 18] stiffness, tissue calcification [19, 20], as well as neuronal function and survival [3, 21-23]. The mineralocorticoids are at least partially effective by regulating gene expression [9, 10, 18, 20, 24-26]. Beyond that, mineralocorticoids could be effective through nongenomic pathways [3, 7, 11, 22, 23, 27-33]. Signaling involved in nongenomic actions of mineralocorticoids include activation of phosphatidylinositol-3-kinase (PI3K) [34, 35].

Excessive mineralocorticoid action is associated with enhanced risk of vascular disease [7]. At least in theory, cardiovascular disease in mineralocorticoid excess could in part result from altered platelet function. As a matter of fact, increased cytosolic Ca\(^{2+}\) levels were found in platelets from patients with hyperaldosteronism [36]. Platelets are required for primary hemostasis following vascular injury but are decisive for the pathophysiology of acute thrombotic occlusion following atherosclerotic plaque rupture [37, 38]. Accordingly, they contribute to ischemic cardiovascular events such as myocardial infarction or ischemic stroke [39]. Beyond that platelets participate in the orchestration of vascular inflammation and atherogenesis [37, 40].

Platelets are activated by an increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) [41, 42], resulting from either activation of TRPC-channels [43] or from activation of ryanodine-receptors in the endoplasmic reticulum (ER) by inositol-1,4,5-trisphosphate (IP\(_3\)) thus leading to Ca\(^{2+}\)-release from intracellular stores into the cytosol [44]. The emptying of the intracellular Ca\(^{2+}\) stores activates the stromal interaction molecule 1 (STIM1) [45], which interacts with and activates the pore forming unit of the Ca\(^{2+}\)-release-activated channel (CRAC) Orai1 (CRACM1) in the plasma membrane [46]. The activation of STIM1/Orai1 thus leads to store operated calcium entry (SOCE) [47, 48], which plays a decisive role in platelet activation [41, 44, 49], cytoskeletal reorganization resulting in shape changes of activated platelets [50] and in phosphatidylserine translocation to the outer platelet membrane surface [51]. Cell membrane scrambling may further be triggered by Na\(^+\)/H\(^+\) exchanger activity [52].

Whether or not enhanced Orai1 activity contributes to the increase of cytosolic Ca\(^{2+}\) activity in mineralocorticoid excess remained elusive. Orai1 protein abundance and thus platelet SOCE are upregulated by the serum- and glucocorticoid-inducible kinase isoforms SGK1 [38, 53] and SGK3 [54]. Mineralocorticoids are strong stimulators of SGK1 expression [55]. SGK1 stimulates Orai1 transcription in megakaryocytes by upregulating the transcription factor NF-kB, which in turn stimulates Orai1 expression [38]. Circulating platelets are lacking nuclei and are thus unable to transcribe novel proteins [56]. Platelets harbour pre-mRNA and mRNA, however, and are able to splice the intronic rich pre-mRNA into mature mRNA with following translation into mature proteins [57-59]. As a result, platelets are capable to synthesize proteins such as interleukin-1β (IL-1β) [60], human tissue factor [61] and Orai1 [62].

The regulation of translation involves PI3K [59], which is known to be critically important for platelet activation [63]. Translation is further governed by cytoskeletal reorganization [64]. Prior to stimulation of translation, mRNA is bound to the cytoskeletal core and the eukaryotic initiating factor eIF-4E localizes to the membrane skeleton and the soluble fraction of platelets [60]. The eIF-4E further interacts with the inhibitory 4E-BP1...
molecule, which prevents initiation of translation [65]. Following activation of platelets, PI3K associates with the membrane skeleton [64] leading to PI3K dependent phosphorylation of 4E-BP1 [59, 65], dissociation of the inhibitory binding molecules with redistribution of the translation initiation factors close to mRNA and translation of mRNA [60]. The translation of Orai1 protein is thus sensitive to activation of PI3K [62].

The present study explored whether mineralocorticoids participate in the regulation of Orai1 protein abundance at the platelet surface and whether this effect impacts on Ca\(^{2+}\) entry and cell membrane scrambling.

**Materials and Methods**

**Mice**

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Experiments were performed in 10- to 12-week-old wild type mice of either sex. The mice had free access to water and control food (Ssniff, Soest, Germany).

**Preparation of mouse platelets**

Platelets were obtained from 10- to 12-week-old mice of either sex. The mice were anesthetized and blood was drawn from the retroorbital plexus into tubes with 300 µl acid-citrate-dextrose buffer. Blood parameters were analyzed with pocH-100iv automatic hematology analyzer (Sysmex). 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. Where necessary apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin I\(_2\) (0.5 µM; Calbiochem) were added to the PRP to prevent activation of platelets during isolation. After two washing steps the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl\(_2\)). For DOCA treatment, DOCA (10 µg/ml, Merck GmbH, Germany) was dissolved in ethanol (99.9%). As control the platelets were treated with ethanol only.

**Orai1 surface protein abundance**

Orai1 surface expression was analysed by flow cytometry. Washed platelets were incubated for 60 minutes (37°C) with Orai1 rabbit anti–mouse antibody (Abcam), washed once in Tyrode buffer, and stained in 1:250 diluted CF™ 488A-labeled anti–rabbit secondary antibody (Sigma, USA) for 30 minutes (37°C). Samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Phosphatidylserine exposure**

In order to determine phosphatidylserine exposure the platelet preparation was centrifuged at 660g for 5 minutes followed by washing once with Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\), staining with 3 µM Fluo-3AM (Biotium, USA) in the same buffer and incubating at 37°C for 30 minutes. The fluorescence was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm utilizing a BD FacsCalibur (BD Biosciences, CA, USA).

**Calcium measurements**

For measurement of intracellular Ca\(^{2+}\) concentration the platelet preparation was washed once in Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\), stained with 3 µM Fluo-3AM (Biotium, USA) in the same buffer and incubated at 37°C for 30 minutes. The fluorescence was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm utilizing a BD FacsCalibur (BD Biosciences, CA, USA).

**Statistical analysis**

Data are provided as means ± SEM; \(n\) represents the number of independent experiments. All data were tested for significance using ANOVA Tukey test. Results with \(p<0.05\) were considered statistically significant.
Results

The present study addresses nongenomic effects of the mineralocorticoid desoxycorticosterone acetate (DOCA) on Orai1 protein abundance in the cell membrane and Ca\(^{2+}\) entry into murine blood platelets. To this end, murine platelets were isolated from untreated mice and subsequently treated in vitro with DOCA (10 µg/ml) for 60 minutes. Orai1 protein abundance was determined using CF\(^{TM}\) 488A-labeled antibodies in flow cytometry. As illustrated in Fig. 1, Orai1 protein abundance within the platelet membrane was significantly increased by prior DOCA treatment.

Increased Orai1 function would be expected to enhance Ca\(^{2+}\) entry and thus cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Fluo3 fluorescence was thus employed to estimate [Ca\(^{2+}\)]\(_i\) in platelets. As illustrated in Fig. 2, in vitro treatment of platelets isolated from untreated mice with 10 µg/ml DOCA was followed by a significant increase of Fluo3 fluorescence. In order to test, whether the effect of DOCA was mediated by the serum & glucocorticoid inducible kinase SGK1, the experiments were...
repeated in the presence of the specific SGK inhibitor EMD638683 (50 µM). As illustrated in Fig. 2, the effect of DOCA was completely abrogated in the presence of EMD638683. In the absence of DOCA, EMD638683 did not significantly modify the Fluo3 fluorescence of platelets.

An increase of [Ca^{2+}], in platelets is expected to stimulate cell membrane scrambling with phosphatidyserine translocation to the platelet surface. Annexin V binding was employed in order to identify phosphatidyserine exposing platelets. As illustrated in Fig. 3, prior in vitro treatment of platelets isolated from untreated mice with 10 µg/ml DOCA was followed by a significant increase of the percentage annexin V binding platelets. Similar to what has been observed with Fluo3 fluorescence, the effect of DOCA on annexin V binding was completely abrogated in the presence of EMD638683. In the absence of DOCA, EMD638683 did not significantly modify the percentage of annexin V binding platelets.

In order to estimate cell volume, platelet forward scatter was determined in flow cytometry. As illustrated in Fig. 4, prior in vitro treatment of platelets isolated from untreated mice with 10 µg/ml DOCA was followed by a significant increase of the platelet forward scatter. Similar to what
has been observed with Fluo3 fluorescence and cell membrane scrambling, the effect of DOCA on platelet forward scatter was completely abrogated in the presence of EMD638683. In the absence of DOCA, EMD638683 did not significantly modify the platelet forward scatter.

Discussion

The present observations reveal a novel function of mineralocorticoids, i.e. the up-regulation of the pore forming Ca\(^{2+}\) release-activated channel (CRAC) Orai1 and subsequent increase of the store operated Ca\(^{2+}\) entry (SOCE) [44]. SOCE results from stimulation of Orai1 [66, 67] by STIM1 [68], which senses the Ca\(^{2+}\) content of the sarcoplasmatic reticulum [69]. The upregulation of Orai1 contributes to or even accounts for the observed increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).

Orai1 translation is presumably accomplished by the translation initiation factors eIF-4E and elf-2alpha eIF-4E [60, 70] as well as the inhibitory 4E binding protein 4E-BP1 [60]. Mineralocorticoids are known to activate nongenomically PI3K [34, 35]. PI3K phosphorylates 4E-BP1 [65], which binds eIF-4E and thus inhibits translation [65]. Translation is prevented as long as eIF-4E localizes to the membrane skeleton and the soluble fraction of platelets [60]. Following treatment with mineralocorticoids and subsequent activation of PI3K the translation initiation factors may redistribute to the proximity of mRNA [60] As shown earlier, both actin polymerization and PI3K activation are required for the triggering of Orai1 translation [62]. In this paper we demonstrate that the effect of DOCA on [Ca\(^{2+}\)]\(_i\) and cell membrane scrambling is abrogated in the presence of the selective [71] SGK inhibitor EMD638683 (50 µM). SGK isoforms are activated by PI3K signaling and it is tempting to speculate that PI3K signaling to protein translation involves SGK.

Both, Orai1 and STIM1 are critically important for full platelet activation [49, 69, 72] and the up-regulation of Orai1 is expected to boost the capacity of platelets to accomplish hemostasis [73]. Orai1 deficient mice suffer from severe bleeding [49]. Beyond that Ca\(^{2+}\) entry may be mediated by TMEM16F, a Ca\(^{2+}\)-activated, Ca\(^{2+}\)-permeable channel with inbuilt scramblase activity [74]. Moreover, Na\(^{+}\) entry following activation of transient receptor potential (TRP) nonselective cation channels TRPC3 and TRPC6 may increase cytosolic Na\(^{+}\) activity with subsequent reversal of the gradients for the Na\(^{+}\)/Ca\(^{2+}\) exchangers [74].

An increase in [Ca\(^{2+}\)]\(_i\) is known to trigger platelet degranulation, integrin \(\alpha_{IIb}\beta_3\) activation and adhesion of platelets with eventual thrombus formation [41]. The increase in [Ca\(^{2+}\)]\(_i\) further contributes to or even accounts for the observed stimulation of cell membrane phospholipid scrambling with translocation of phosphatidylserine to the platelet surface [40, 74-76]. Phosphatidylserine at the platelet surface enhances in turn the rate of thrombin formation and platelet procoagulant activity [74-77]. Phosphatidylserine exposure further fosters the binding and phagocytosis of the affected platelets by macrophages [78].

Conclusion

Activation of platelets by the mineralocorticoid DOCA is followed by up-regulation of Orai1 protein abundance in the cell membrane with subsequent increase of cytosolic Ca\(^{2+}\)-concentration and cell membrane scrambling.

Conflict of Interests

The authors of this manuscript state that they have not any conflicts to declare.
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