Original Paper

Sulphate and Chloride-Dependent Potassium Transport in Human Erythrocytes are Affected by Crude Venom from Nematocysts of the Jellyfish Pelagia noctiluca

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Key Words
Erythrocytes • Oxidative stress • Band 3 protein • GSH • Crude venom • Pelagia noctiluca

Abstract
Background: It has been reported that biologically active compounds extracted from Cnidaria venom may induce damage by oxidative stress. Erythrocytes are constantly exposed to oxidative stresses, which can contribute to sulphydryl (SH-) group oxidation and cell membrane deformability accompanied with activation of K-Cl co-transport and inhibition of anion transport. In this regard, Band 3 protein is responsible for mediating the electroneutral exchange of chloride (Cl-) for bicarbonate (HCO3-), particularly in erythrocytes, where it is the most abundant membrane protein. The aim of this study was to elucidate the effect of crude venom extracted from Pelagia noctiluca nematocysts on Band 3 -mediated anion transport in human erythrocytes.

Methods: Erythrocytes were tested for SO42- uptake, K+ efflux, glutathione (GSH) levels and concentration of SH- groups. Results: The rate constant of SO42- uptake decreased progressively to 58% of control with increasing venom doses, and showed a 28% decrease after 2 mM NEM treatment. These effects can be explained by oxidative stress, which was reflected by decreased GSH levels in venom-treated erythrocytes. Hence, the decreased efficiency of anion transport may be due to changes in Band 3 structure caused by SH-group oxidation and reduced GSH concentration. In addition, an increased Cl- -dependent K+ efflux was observed in venom-treated erythrocytes.

Conclusion: Our results suggest that crude venom from Pelagia noctiluca alters cell membrane transport in human erythrocytes.
Introduction

The Cnidaria members, Aiptasia mutabilis (Anthozoa) and Pelagia noctiluca (Scyphozoa), indigenous to the Strait of Messina (Italy) have become increasingly abundant in the recent years with no seasonality [1]. The accidental contact of humans with some Cnidaria specimens can produce severe local systemic pathologies and, in some cases, may lead to death [2, 3]. The toxicity of these animals is manifested in part through delivery of toxins from nematocysts, specialized stinging organoids characteristic of the Phylum Cnidaria [4, 5]. Nematocysts have a three-layered capsule wall contained within highly specialized cells, termed nematocytes, which are located in tentacles, acontia and acrorhagi. The nematocyst occupies most of the nematocyte cytoplasm and is composed of an inverted tubule immersed in a capsular fluid in which toxins are stored [6, 7]. The tubule is an extension of the wall structure, originating first outside the capsule and then invaginating within the wall. When both mechanical and chemical stimuli are applied, the nematocyst tubule rapidly everts to penetrate prey integuments, and then injects toxins. This response, termed discharge, represents a rapid form of exocytosis, occurs within 3 msec [8, 9] and is controlled by a complex mechanism that has still not been completely described. Compounds of protein nature have been isolated from Cnidaria venom, including pore-forming toxins (also called cytolysins), phospholipases, Na\(^+\) and K\(^+\) channel inhibitors, other neurotoxins and proteinase inhibitors [7, 10-19].

Erythrocytes are especially sensitive to oxidative stresses and it has been reported that biologically active compounds extracted from Cnidaria venom may cause cellular damage by inducing oxidative stress [7]. The oxidation of sulphhydril (SH-) groups can result in conformational changes in Band 3, a protein mediating anion exchange (with specific regard to Cl\(^-/\)HCO\(_3\)\(^-\)) and whose structural integrity has a close relationship with K-Cl co-transport [20]. Band 3 is composed of two functionally distinct domains: a C-terminal region responsible for catalyzing a one-to-one exchange of anions across the plasma membrane and an N-terminal cytoplasmic domain anchored to the cytoskeleton [21].

In the present study, we investigated K\(^+\) efflux, SO\(_4\)\(^{2-}\) transport, the cellular redox state, as well as gross morphological changes in human erythrocytes elicited by treatment with P. noctiluca crude venom in the context of Band 3 and the KCC.

Materials and Methods

Nematocyst isolation

Specimens of the Scyphozoan Pelagia noctiluca were collected from the Strait of Messina (Sicily, Italy) during Spring/Summer 2012. Nematocysts were isolated from oral arms according to Salleo et al. [22]. Briefly, oral arms were excised from each specimen and nematocysts were isolated from the nematocytes via osmotic lysis in 4 °C distilled water. The resulting suspension was repeatedly filtered through plankton nets (100 µm, 60 µm and 40 µm mesh) and spun (for 5 min at 4000 x g at 4 °C) to discard debris. Once isolated, nematocysts were counted in a Burker chamber and processed for venom extraction or stored at -20 °C for later use.

Venom extraction

Samples containing 90-100 nematocysts/µl were re-suspended in physiological solution (145 mM NaCl, 10 mM phosphate, pH 7.4, 300mOsm/Kg H\(_2\)O) and sonicated 30 times for 20 sec at 70MHz on ice. Nematocyst debris was separated by centrifugation (for 10 min at 4000 x g at 4 °C) to discard debris. Once isolated, nematocysts were counted in a Burker chamber and processed for venom extraction or stored at -20 °C for later use.

Erythrocyte preparation and sulphate transport measurement

Human blood obtained after informed consent from 10 healthy volunteers was collected in heparinized tubes and centrifuged (5 min at 1000 x g) in 150 mM NaCl, 20 mM HEPES, pH 7.4. After centrifugation, theuffy coat was carefully removed. The sample was then re-suspended to 3% (for SO\(_4\)\(^{2-}\) transport
measurement) or 20% (for K⁺ efflux measurement, see below) haematocrit with isotonic medium (in mM: 118 NaSO₄, 20 HEPES, 15 glucose, 300mOsm/Kg) each time following 3, 5 min centrifugations at 1000 x g. The osmotic pressure of all solutions was measured by a freezing point depression osmometer (Fiske, OS). After re-suspension, erythrocytes were treated with 0.05, 0.1 or 0.2% v/v crude venom at 25 °C, pH 7.4. At specified intervals, 5 ml samples of the suspensions were removed and added to a test tube containing 5 μM 4,4’-diisothiocyanato-stilbene-2,2’-disulfonate (DIDS) stopping medium (10 ml) and kept on ice. The presence of DIDS, a compound that binds irreversibly and specifically to the extracellular moiety of the integral membrane Band 3 protein, inhibits SO₄²⁻ transport in erythrocytes [23].

After the last sample withdrawal, erythrocytes were washed three times in sulphate-free medium (in mM: 150 NaCl, 20 HEPES, pH 7.4) at 0 °C and then hemolysed with 70% v/v thricloroacetic acid (TCA). The membranes were then discarded by pelleting them with a 10 min, 4000 x g at 4 °C centrifugation. Sulphate ions in the supernatant were precipitated by sequentially adding and mixing 15 ml 50% glycerol, 20 ml 4 mM NaCl and HCl (hydrochloric acid 37%) solution (12:1) and 15 ml 124 mM BaCl₂H₂O to obtain a homogeneous barium sulphate precipitate. The intracellular sulphate concentration was measured by atomic absorption spectrophotometry at 425 nm. Using a calibrated standard curve, the absorption was converted to mM of intracellular SO₄²⁻, which is necessary for calculating the rate constant from the following equation: Cₜ = C₀ (1-e⁻kt) + C∞, where C₀, Cₜ and C∞ represent the intracellular sulphate concentrations measured at times t, t∞ and 0, respectively. e indicates the Eulero number (2.7182818), k is a constant accounting for specific velocity of the process and t is time at which intracellular SO₄²⁻ concentration is measured [24, 25]. This method is reproducible and the results do not vary more than 3%. Sulfate uptake was measured as [SO₄²⁻]x10⁻³/cells.

K⁺ efflux and cellular volume measurements

For K⁺ efflux measurements, according to Crupi et al. [26] with slight modifications, erythrocytes were re-suspended to 20% hematocrit with either isotonic media A (150 mM choline chloride, 20 mM HEPES, 15 mM glucose, 0.1 mM ouabain, 307 mOsm/Kg) at pH 7.4) or B (150 mM choline nitrate, 20 mM HEPES, 15 mM glucose, 0.1 mM ouabain, 307 mOsm/Kg) at pH 7.4) containing or not containing varying concentrations of venom or 2 mM N-ethylmaleimide (NEM) for 1 h at 25 °C. The suspensions were spun at 4 °C for 5 min at 3000 x g and 50 µl of the supernatants were placed in 10 ml 5 mM LiNO₃.

Erythrocyte cell volume was measured by an ABX Pentra 120 hematology analyzer before (t=0) and after incubation (t=1h) with either medium A or B, with or without venom or 2mM NEM and expressed in femtoliters (fl). Prior to incubation (t=0), cellular volume ranged between 80-100 fl and was not altered by venom or NEM treatment (t=1h).

K⁺ content of the medium was measured by a flame photometer and was corrected for the initial cell volume and expressed in mM/L red blood cells (RBCs) per h (mEq/L RBC/h). K⁺ content was corrected since the cell suspensions were incubated for 1 h and possible volume changes were unable to be measured during that incubation time.

Glutathione (GSH) measurement

GSH concentration was measured in whole blood re-suspended to 3% hematocrit before and after incubation with or without 0.05, 0.1 or 0.2% v/v crude venom or 2 mM NEM for 2 h at 25 °C. After incubation, GSH levels were determined with Cayman’s GSH assay kit that utilizes a carefully optimized enzymatic recycling method with glutathione reductase, according to the manufacturer’s instructions and using the end point method [25]. The amount of oxidized glutathione (GSGG) was calculated with the following formula: ½ GSSG = GSHtot − GSHredox.

Isolation of erythrocyte membrane proteins and determination of sulphydryl groups

Intact erythrocytes (treated with or without 0.05, 0.1 or 0.2% v/v crude venom for 2 h at 25 °C) were washed with isotonic phosphate buffered saline (PBS, 5 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) and hemolysed with 20 volumes of cold hypotonic buffer (5mM Na₂HPO₄, 50 mM NaCl, pH 7.4) to obtain a final concentration of 5% (cells/water). Membranes were pelleted by centrifugation at 20,000 x g for 20 min at 0 °C. The process was repeated with the same hypotonic buffer to remove hemoglobin [24]. One volume of membranes was then incubated with nine volumes of 0.1 M NaOH for 30 min at 0 °C in the presence of 0.2 mM dithiothreitol (DTT) and 20 µg/ml phenylmethylsulfonil fluoride (PMSF). The samples were then centrifuged at 56,000 x g.
for 30 min at 0 °C. The pellet was washed thrice with 5 mM Na₂HPO₄, pH 8.0. Successively, 0.2 ml of the pellet was solubilized for 30 min at 37 °C with 0.3 ml 20% sodium dodecyl sulphate (SDS) and 3 ml Na₂HPO₄ pH 8.0. Next, 0.1 ml 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 100 mM Na₂HPO₄, pH 8.0 was added and incubated for 20 min at 37 °C. The absorbance of the solution at 405 nm was then measured. Using a standard curve obtained from at least five samples of known GSH concentrations (dissolved in physiological saline), the absorbance measured from the experimental samples was converted to µg/ml of thiol groups.

**Erythrocytes staining with Giemsa**

The erythrocytes underwent Giemsa staining for detecting possible morphological alterations to cell membrane. Giemsa stain is a classic blood film stain using Giemsa’s solution (Sigma-Aldrich, Milan, Italy), a mixture containing methylene blue, eosin and Azure B. A slide with blood smear, fixed with pure methanol for 30 seconds, was immersed in 5% Giemsa stain solution for 20–30 minutes. Stained erythrocytes were observed under a Zeiss Axio Imager Z1 connected to a video camera and then counted. Percentage of damaged cells (with altered shape compared to control cells, see arrows in Fig. 4) was calculated by correlating their number to the total number of cells.

**Statistics**

Data are expressed as mean values ± SEM. Statistical analyses were performed using paired Student’s t test or a one-way ANOVA where appropriate. p<0.05 was considered statistically significant.

**Results**

*P. noctiluca* crude venom inhibits sulphate uptake in human red blood cells

A time course of SO₄²⁻ uptake (as a measure for the level of anion transport) in human erythrocytes treated with or without 0.05, 0.1, or 0.2% v/v crude venom isolated from *P. noctiluca* nematocysts, NEM (2 mM) or DIDS (5 µM). Data are presented as means ± SEM of at least 5 experiments where *p* < 0.05 versus control (untreated) erythrocytes, as determined by one way ANOVA followed by Dunett’s post hoc test.

**Table 1.** Rate constants x (min⁻¹) of SO₄²⁻ uptake in human erythrocytes in the absence (control) or presence of 0.05, 0.1, 0.2 % v/v crude venom isolated from *P. noctiluca* nematocysts, NEM (2 mM), or DIDS (5 µM). Data are presented as means ± SEM of at least 5 experiments where *p* < 0.05 versus control (untreated) erythrocytes, as determined by one way ANOVA followed by Dunett’s post hoc test.

![Fig. 1. Time course of SO₄²⁻ uptake in human erythrocytes treated with or without 0.05, 0.1, or 0.2% v/v crude venom isolated from *P. noctiluca* nematocysts, NEM (2 mM) or DIDS (5 µM). Bars represent the mean ± SEM from at least 5 experiments.](image)
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*P. noctiluca* crude venom decreases GSH and sulphydryl group levels in human red blood cells

GSH functions as an antioxidant with free radical scavenging activity, resulting in the oxidation of GSH to glutathione disulfide (GSSG). Accordingly, the GSH/GSSG ratio acts as a gauge for the intracellular redox state, where decreases in the GSH/GSSG ratio are indicative of oxidative stress. Treatment of erythrocytes with 0.1% and 0.2% v/v crude venom, as well as NEM, significantly decreased the GSH/GSSG ratio (Fig. 2 and Table 2). These data suggest that the crude venom isolated from *P. noctiluca* nematocysts induces oxidative stress.

The concentration of SH– groups, as an indicator of possible alterations in the tertiary structure of integral membrane proteins, is depicted in Fig 3. These data show a significant reduction in the concentration of SH– groups (µg/ml) in integral membrane proteins after

**Table 2.** Reduced (GSH) and oxidized (GSSG) glutathione (µmol/L of cells), as well as the GSH/GSSG ratios in human erythrocytes treated with or without 0.05, 0.1, 0.2% v/v crude venom isolated from *P. noctiluca* nematocysts or NEM (2 mM) for 2 h. Values represent means ± SEM of at least 5 experiments where *p < 0.05 versus control (untreated) erythrocytes, # p < 0.05 versus 0.05 % v/v venom-treated erythrocytes, as determined by one way ANOVA followed by Dunnett’s post hoc test.

| Metabolites   | control    | 0.05         | 0.1         | 0.2         | NEM 2 mM |
|---------------|------------|--------------|-------------|-------------|----------|
| GSH (µmol/L)  | 744.31±37  | 698±26      | 651±22*#    | 611±19*#    | 595±30**#|
| GSSG (µmol/L) | 281.35±13  | 353±15      | 370±11*#    | 377±12*#    | 325±22**#|
| GSH/GSSG      | 2.645±0.05 | 1.977±0.03  | 1.759±0.01*#| 1.62±0.03*# | 1.83±0.01*#|

**Table 3.** K+ efflux measured in human erythrocytes in the absence (control) or presence of 0.2% v/v crude venom from *P. noctiluca* nematocysts or NEM (2mM) for 1 h in either Cl– containing or Cl– free medium. Data are presented as means ± SEM of at least 5 experiments where *p < 0.05 versus control (untreated) erythrocytes, as determined by one way ANOVA followed by Dunnett’s post hoc test.

|                | control | 0.2% v/v venom | NEM (2mM) |
|----------------|---------|----------------|------------|
| Cl–            | 6.1±0.1 | 37.3±0.2*      | 38.5±0.1*  |
| NO₃            | 4.2±0.1 | 8.2±0.1*       | 8.4±0.1*   |
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Treatment with increasing concentrations of crude venom. A significant decrease in the concentration of SH– groups was also seen following NEM treatment, suggesting that the crude venom may induce damage to erythrocyte integral membrane proteins similarly to NEM.

*P. noctiluca* crude venom elicits a Cl– dependent K+ efflux in human erythrocytes

Table 3 shows the K+ efflux (mEq/l RBC/h) measured in human erythrocytes treated with or without 0.2% v/v crude venom isolated from *P. noctiluca* nematocysts for 100 min. Both treatments significantly increased K+ efflux compared to the control, so that, according to what was previously observed by Adragna and Laff [30] on NEM-treated erythrocytes, a possible oxidative effect of crude venom on a K+ transport system, putatively KCC, can be suggested.

To better verify the Cl– dependence of K+ efflux altered by treatment of erythrocytes with crude venom, K+ efflux was measured in either Cl– containing or in Cl– free medium, by replacing Cl– with NO3–, and compared with that measured after NEM treatment (Table 3). In Cl– free conditions, K+ efflux in venom-treated cells was significantly reduced from 37.3 to 8.2 mEq/l RBC/h and, in NEM– treated cells, was significantly reduced from 38.5 to 8.4 mEq/l RBC/h (inhibited by 80%, p<0.05, Student’s t-test).

*P. noctiluca* crude venom alters the morphological state of human erythrocytes

Upon light microscope observations, 0.2%v/v crude venom-treated erythrocytes exhibited morphological alterations, like increased cell membrane deformability, compared to the control (Fig. 4). A similar effect was observed in erythrocytes exposed to 0.1% v/v venom (data not shown). NEM treatment, as already shown [30], induced cell shrinkage, while the exposure of erythrocytes to 0.05% v/v crude venom did not induce any morphological change (data not shown).

After treatment with different concentrations of crude venom, damaged erythrocytes were counted (Fig. 5). No increase in damaged cells was detected with the lowest venom dose (0.05%v/v), whereas higher doses (0.1 and 0.2%v/v) resulted in a significant increase in damaged erythrocytes with respect to the control (30% ± 5 and 80% ± 6 respectively, p<0.05).
Discussion

NEM (0.5–2 mM) was previously shown to activate K-Cl cotransport in human, rabbit and sheep erythrocytes [31-33], activate the tetramer-to-dimer transition of spectrin (attributed to a weakened interaction between ankyrin and AE1/Band 3) [34], and alter the activity of protein phosphatase 1 and 2A (serine phosphatases) [35], calpain-1 [36] and the tyrosine phosphatase SHP1 (Src homology phosphatase-1) [37]. Band 3 is mainly phosphorylated at three tyrosine residues (8, 21, and 46) in the cytoplasmic domain [38]. In particular, Band 3 phosphorylation has been associated with membrane deformability, suggesting that the visco-elastic properties of human erythrocytes may be regulated by Band 3 tyrosine phosphorylation [39]. Other studies using erythrocytes suggested that swelling-activated transport via KCC and shrinkage-activated transport via NKCC are virtually inactive in isosmotic artificial medium since they are co-ordinately controlled by a common mechanism termed “volume set point”. Treatment with oxidant agents reduces the “volume set point” and activates KCC [32]. The decreased efficiency of anion transport observed following NEM treatment could either be due to alterations in the structural state of Band 3 (by SH-group oxidation, for example) or to cell shrinkage (by increased K⁺ efflux). The latter hypothesis is substantiated by the fact that NEM, while activating a K⁺ efflux (Table 3), also reduced SO₄²⁻ uptake (Fig. 1). Regarding the former aspect, the decrease in GSH levels (Table 2) detected in cells treated either with NEM or different venom concentrations is indicative of oxidative stress. A decrease in SH-groups in integral membrane proteins (Fig. 3), consistent with decreased GSH concentrations (Fig. 2), was also detected in venom-treated cells. Interestingly, oxidative stress is known to cross-link integral membrane proteins and hemoglobin with Band 3, leading to the formation of high molecular-mass aggregates [30,40-41] and accompanying decreases in GSH levels and oxidation of membrane thiols [42].

The finding that low concentrations of crude venom from *P. noctiluca* may provoke oxidative stress on erythrocyte membrane proteins provides novel information about its mechanism of action. As far as marine toxins are concerned, some of them are defined as cytolytic or neurotoxic, and target different animals, such as insects, crustaceans and vertebrates, at the level of membrane transport systems, including voltage-gated Na⁺ and K⁺ channels and acid-sensing ion channels [7, 43]. In this respect, palytoxin-group toxins (PITX) extracted from the tropical microalga *Ostreopsis ovata*, exert their potent biological activity by altering ion homeostasis mechanisms in excitable and non-excitable tissues [44]. Specifically, PITX induces a massive intracellular Na⁺ influx via modulation of the Na⁺/K⁺ ATPase [45]. These authors suggested that such Na⁺ overload is the crucial step in mediating overproduction of ROS and cell death in human HaCaT keratinocytes. It has also been described that oxidative stress promoted by different drugs, through a direct interaction with hemoglobin, may lead to the activation of caspase 3, which, in turn, influences anion flux via Band 3, as well as glucose metabolism [46]. In addition, it was recently described that non-lytic doses of *P. noctiluca* crude venom directly induces mitochondrial trans-membrane potential collapse and generation of reactive oxygen species (ROS) in SH-SY5Y cells derived from human neuroblastoma [47].

Our results show that both crude venom- and NEM-treated erythrocytes exhibit, in addition to a reduction in SH-groups, an increased Cl⁻-dependent K⁺ efflux (Table 3). In this regard, it has been demonstrated that treatment with thiol-oxidizing and alkylating agents, such as diamide, NEM or orthovanadate lead to alterations in both the erythrocyte redox state and in the activation of KCC, thus resulting in cell shrinkage [30]. On this basis, the hypothesis that non-hemolytic doses of *P. noctiluca* crude venom may interfere with membrane transport is reinforced, since it can provoke oxidative stress by reducing SH-groups, as well as activate Cl⁻-dependent K⁺ efflux, similarly to NEM. Moreover, an increase in K⁺ efflux is further supported by the changes in erythrocyte morphology observed after exposure to either crude venom or NEM. In both cases, erythrocyte shrinkage was detected. Oxidative damage to erythrocyte membranes may increase the permeability to K⁺ ions via increased activity of KCC, as reported by Crupi et al. [26].
Conclusion

In conclusion, the present study provides novel findings about the biological activity of crude venom isolated from *P. noctiluca* nematocysts. Our findings may contribute to uncovering the mechanism of action of biologically active compounds extracted from marine animal venoms—a theme that has been under much debate [7].

Conflict of Interests

The Authors have no conflicts of interest to disclose.

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