Original Paper

Stimulation of Suicidal Erythrocyte Death by Ipratropium Bromide

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Key Words

Phosphatidylserine • Ipratropium bromide • Calcium • Cell volume • Eryptosis

Abstract

Background/Aims: Ipratropium bromide, an anticholinergic agent widely used in obstructive lung disease, has previously been shown to trigger suicidal death of nucleated cells or apoptosis. Despite their lack of mitochondria and nuclei, key organelles in the execution of apoptosis, erythrocytes may similarly undergo suicidal cell death, which is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine-exposure at the cell surface. Triggers of eryptosis include increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$). The present study explored whether ipratropium bromide triggers eryptosis. **Methods:** $[\text{Ca}^{2+}]_i$ was estimated utilizing Fluo3 fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from annexin-V-binding, and hemolysis from hemoglobin release. **Results:** A 48 h exposure to ipratropium bromide (1 nM) significantly increased $[\text{Ca}^{2+}]_i$, decreased forward scatter and increased annexin-V-binding. Ipratropium bromide treatment was followed by slight but significant increase of hemolysis. Removal of extracellular Ca^{2+} or inhibition of Ca^{2+} permeable cation channels with amiloride (1 mM) virtually abolished cell membrane scrambling. Ca^{2+} ionophore ionomycin (1 μM , 30 min) increased the percentage of phosphatidylserine exposing erythrocytes to similarly high levels in the absence and presence of ipratropium bromide (1 nM). **Conclusions:** Ipratropium bromide triggers suicidal erythrocyte death or eryptosis, an effect mainly due to stimulation of Ca^{2+} -entry.

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Introduction

Ipratropium bromide, a nonselective antagonist of the muscarinic receptors located on airway smooth muscle [1], is widely used to counteract rhinorrhea of common cold [2], asthma and chronic obstructive pulmonary disease (COPD) [3-7] as well as prevention of

the Arthus reaction [8]. When applied locally, the drug is well tolerated [2]. Advantages of the drug include its limited systemic absorption from the lungs when given as an inhaled preparation [4].

In order to gain some insight into cellular toxicity of ipratropium bromide, the present study explored, whether the drug triggers suicidal erythrocyte death or eryptosis. Similar to apoptosis of nucleated cells, suicidal erythrocyte death or eryptosis is characterized by cell membrane scrambling and cell shrinkage [9]. Eryptosis is triggered by a wide variety of xenobiotics [10-21]. They are in part effective by stimulating Ca^{2+} entry through Ca^{2+} -permeable cation channels [22, 23] thus increasing cytosolic Ca^{2+} concentration. Ca^{2+} activates Ca^{2+} -sensitive K^{+} channels [24] resulting in K^{+} exit, hyperpolarization, Cl^{-} exit, cellular loss of KCl together with osmotically obliged water and thus cell shrinkage [25]. Ca^{2+} further triggers cell membrane scrambling with phosphatidylserine exposure at the cell surface [26]. Erythrocytes are sensitized to the cell membrane scrambling effect of Ca^{2+} by ceramide, which thus similarly stimulates eryptosis [27]. Eryptosis is further stimulated by energy depletion [28] and activation of caspases [18, 29-32]. Cell membrane scrambling is further influenced by AMP activated kinase AMPK [23], cGMP-dependent protein kinase [33] and Janus-activated kinase JAK3 [10].

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V). Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to ipratropium bromide (Tocris, Bristol, U.K.) at the indicated concentrations. In Ca^{2+} -free Ringer solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 μl cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Apoptosis assay

CaCo cells were grown in DMEM medium containing 10% fetal calf serum under standard culture conditions (37°C, 5% CO_2). 10^5 cells were seeded in 6 well plates and cultivated with fresh culture medium

Fig. 1. Effect of ipratropium bromide on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 1 nM ipratropium bromide. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) ipratropium bromide (0.1 - 1 nM). * ($p < 0.05$) indicates significant difference from the absence of ipratropium bromide (ANOVA).

for 24 h. The cells were incubated in the absence or presence of 1 nM ipratropium for 24 hours. Then, apoptosis was determined by fixing the cells for 30 minutes in 70% ice-cold ethanol followed by staining the fixed cells with a combination of 1:200 Annexin V-FITC 1:200 dilution (ImmunoTools, Friesoythe, Germany) and 50 μ g/ml propidium iodide (PI) (Mabtag, Friesoythe, Germany) in the dark on ice for 60 minutes. The cells were washed once in PBS and analyzed immediately in FL-1/FL-2 in BD FACS Calibur. Annexin-V FITC and PI double positive cells were considered to be apoptotic.

Statistics

For FACS analysis each single value represents the average of 10^4 cells. Arithmetic means $\pm$ standard error of the mean (SEM) were calculated from the average values of different erythrocyte batches. SEM was taken to provide an estimate of the statistical certainty of the arithmetic means. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and t test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored, whether ipratropium bromide triggers suicidal erythrocyte death. A hallmark of eryptosis is cell shrinkage. Thus, forward scatter has been determined in FACS analysis to estimate cell volume. As shown in Fig. 1, ipratropium bromide treatment decreased forward scatter, an effect statistically significant at 0.4 nM ipratropium bromide.

Another hallmark of eryptosis is cell membrane scrambling with phosphatidylserine exposure at the cell surface. In order to test whether ipratropium bromide treatment results in cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by FACS analysis. As shown in Fig. 2, a 48 h exposure to ipratropium bromide increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 0.4 nM ipratropium bromide.

Additional experiments were performed to explore whether ipratropium bromide triggered hemolysis. To this end, hemolysis was estimated from hemoglobin release into the supernatant. As shown in Fig. 2, exposure of erythrocytes for 48 h to ipratropium bromide was followed by an increase of hemoglobin concentration in the supernatant, an effect reaching statistical significance at 0.8 nM ipratropium bromide concentration (Fig. 2B). The percentage of hemolysed erythrocytes was, however, almost one order of magnitude smaller than the percentage of phosphatidylserine exposing cells.

Fig. 2. Effect of ipratropium bromide on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 1 nM ipratropium bromide. B. Arithmetic means $\pm$ SEM of erythrocyte annexin-V-binding ($n = 8$) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of ipratropium bromide (0.1 - 1 nM). For comparison, arithmetic means $\pm$ SEM ($n = 4$) of the percentage of hemolysis is shown as grey bars. * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.001$) indicates significant difference from the absence of ipratropium bromide (ANOVA).

Fig. 3. Effect of ipratropium bromide on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 1 nM ipratropium bromide. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized histogram in arbitrary units of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) ipratropium bromide (0.1 - 1 nM). * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.001$) indicates significant difference from the absence of ipratropium bromide (ANOVA).

As both, cell shrinkage and cell membrane scrambling could result from increase of cytosolic Ca^{2+} activity, additional experiments explored the effect of ipratropium bromide on cytosolic Ca^{2+} activity. To this end, cytosolic Ca^{2+} was estimated by Fluo3 fluorescence. As illustrated in Fig. 3, treatment with ipratropium bromide was followed by a slight increase of Fluo3 fluorescence, an effect reaching statistical significance at 0.8 nM.

A further series of experiments explored whether the observed ipratropium bromide induced cell membrane scrambling required Ca^{2+} entry. To this end, erythrocytes were exposed to 1 nM ipratropium bromide in the presence or in the nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, the effect of ipratropium bromide on annexin-V-binding was virtually abolished in the nominal absence of Ca^{2+} .

Ca^{2+} entry into erythrocytes could be accomplished by opening of amiloride sensitive cation channels. In order to test whether those channels were involved in the effect of

Fig. 4. Effect of Ca^{2+} withdrawal on ipratropium bromide-induced annexin-V-binding. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 1 nM ipratropium bromide in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of calcium. *** ($p < 0.001$) indicates significant difference from the absence of ipratropium bromide (ANOVA). ### ($p < 0.001$) indicates significant difference from the respective values in the presence of Ca^{2+} .

Fig. 5. Effect of cation channel blocker amiloride on ipratropium bromide-induced annexin-V-binding. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 1 nM ipratropium bromide in the absence (left bars, - amiloride) and presence (right bars, + amiloride) of amiloride (1 mM). *** ($p < 0.001$) indicates significant difference from the absence of ipratropium bromide (ANOVA). ### ($p < 0.001$) indicates significant difference from the respective values in the absence of amiloride.

Fig. 6. Effect of ipratropium bromide on ionomycin-induced annexin-V-binding. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 30 min treatment with Ringer solution without (white bar) or with (black bars) 1 nM ipratropium bromide in the absence (left bars, -ionomycin) and presence (right bars, +ionomycin) of Ca^{2+} ionophore ionomycin (1 μM). * ($p < 0.05$) indicates significant difference from the absence of ipratropium bromide (ANOVA). ### ($p < 0.001$) indicates significant difference from the respective values in the absence of ionomycin.

ipratropium bromide on cell membrane scrambling, erythrocytes were treated with 1 nM ipratropium bromide in the absence or presence of amiloride (1 nM). As illustrated in Fig. 5, the effect of ipratropium bromide on annexin-V-binding was virtually abrogated in the presence of amiloride.

An additional series of experiments explored whether the difference in cell membrane scrambling between presence or absence of ipratropium bromide could be attenuated by treatment of the erythrocytes with the Ca^{2+} ionophore ionomycin (1 μM). As illustrated in Fig. 6, the exposure of erythrocytes to ionomycin was followed by a marked increase of the percentage phosphatidylserine exposing erythrocytes to similarly high levels in the presence or absence of 1 nM ipratropium bromide. This observation suggests that ipratropium bromide is mainly effective through increase of cytosolic Ca^{2+} activity.

To elucidate whether 1 nM ipratropium bromide is similarly effective in nucleated cells, additional experiments were performed in CaCo cells. As a result, the exposure to 1 nM ipratropium bromide for 24 hours was followed by an increase of annexin-V-binding in $3.4 \pm 0.8 \%$ ($n = 11$) of CaCo cells, a value significantly ($p < 0.05$) higher as in CaCo cells without ipratropium bromide treatment ($1.4 \pm 0.4 \%$, $n = 11$). The forward scatter tended to be lower in ipratropium bromide treated CaCo cells ($614 \pm 7 \%$, $n = 11$) than in untreated CaCo cells ($633 \pm 7 \%$, $n = 11$), an effect, however, not reaching statistical significance ($p = 0.06$). The percentage of CaCo cells entering late apoptosis approached $0.8 \pm 0.1 \%$ ($n = 4$) without and $3.5 \pm 0.7 \%$ ($n = 5$) with prior ipratropium bromide treatment for 24 hours, values significantly ($p < 0.05$) different.

Discussion

The present observations show for the first time that ipratropium bromide triggers eryptosis, the suicidal death of erythrocytes. Ipratropium bromide exposure elicits erythrocyte membrane scrambling and leads to erythrocyte shrinkage.

According to the present observations, ipratropium bromide increases cytosolic Ca^{2+} activity, an effect presumably resulting from activation of non-selective cation channels. It is noteworthy that the erythrocyte cation channels are activated by oxidative stress [34]. The molecular identity of the Ca^{2+} permeable erythrocyte cation channels remained ill-defined but has been shown to somehow involve the transient receptor potential channel TRPC6 [22].

The increase of cytosolic Ca^{2+} activity presumably accounts for the effect of ipratropium bromide on forward scatter. The increase of cytosolic Ca^{2+} concentration leads to activation of Ca^{2+} sensitive K^+ channels [24, 35] with subsequent exit of K^+ following its chemical gradient, hyperpolarization of the cell membrane, electrically driven exit of Cl^- and thus cellular loss of KCl followed by osmotically obliged water [25]. The exit of water leads to cell shrinkage, which is apparent from the decrease of forward scatter.

An increase of cytosolic Ca^{2+} activity further stimulates cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface [26, 36, 37]. The scrambling effect of ipratropium bromide was virtually abrogated in the nominal absence of extracellular Ca^{2+} , indicating that ipratropium bromide was mainly effective by increasing cytosolic Ca^{2+} activity. The scrambling effect of ipratropium bromide was further blunted in the presence of the cation channel blocker amiloride, suggesting that ipratropium bromide activated the unspecific, Ca^{2+} permeable cation channels of erythrocytes.

Apparently, ipratropium bromide did not modify Ca^{2+} sensitivity of cell membrane scrambling, as the Ca^{2+} ionophore ionomycin was similarly effective in the presence and absence of ipratropium bromide.

As observed following stimulation with other challenges [27, 29], only a small percentage of erythrocytes entered eryptosis following treatment with ipratropium bromide ($\approx 6\%$) which was approximately twice the percentage of eryptotic erythrocytes in the absence of ipratropium bromide ($\approx 2.5\text{--}3.8\%$). Without the respective increase of erythropoiesis this effect would substantially decrease the number of circulating erythrocytes. The sensitivity of erythrocytes to stimulators of eryptosis may depend on erythrocyte age [38].

The concentration required for the effects of ipratropium bromide on cell membrane scrambling is in the range of the ipratropium bromide concentrations observed *in vivo* [39]. However, the substance is mainly applied locally by inhalation, which limits its concentrations in circulating blood [2, 4]. The observed stimulation of eryptosis may thus occur only at inadequate use of the drug. Nevertheless eryptosis may be relevant in individuals with diseases triggering of eryptosis. Enhanced eryptosis has been observed in a variety of clinical disorders [9], including diabetes [32, 40, 41], renal insufficiency [42], hemolytic uremic

syndrome [43], sepsis [44], sickle cell disease [45], malaria [46-50], Wilson's disease [50], iron deficiency [51], phosphate depletion [52] and presumably metabolic syndrome [53].

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and excessive eryptosis may thus lead to anemia [9]. Moreover, phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO [54] with subsequent impairment of microcirculation [54-59]. Phosphatidylserine exposing erythrocytes further stimulate blood clotting [55, 60, 61]. Thus, excessive eryptosis may lead to thrombosis.

At least in theory, ipratropium bromide could similarly trigger Ca^{2+} entry and cell membrane scrambling of nucleated cells. Owing to the limited systemic absorption from the lungs when given as an inhaled preparation [4], ipratropium bromide does, presumably, rarely approach cytotoxic concentrations in blood.

Conclusion

In conclusion, ipratropium bromide stimulates amiloride sensitive Ca^{2+} entry with subsequent increase of cytosolic Ca^{2+} activity, stimulation of Ca^{2+} sensitive K^+ channels and Ca^{2+} dependent triggering of cell membrane scrambling. Thus, ipratropium bromide elicits eryptosis, the suicidal death of erythrocytes.

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