Induction of Erythrocyte Shrinkage by Omeprazole

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Abstract

Omeprazole, a proton pump inhibitor blocks the H⁺/K⁺-ATPase channels of gastric parietal cells. It is used for the treatment of peptic ulcer. Prolonged use of omeprazole may involve in inducing anemia. The key marker of epyptosis includes membrane blebbing, cell shrinkage and phosphatidylserine (PS) exposure at the cell surface. In current study, the epyptotic, oxidative as well as hemolytic effects of therapeutical doses (0.5, 1 and 1.5 µM) of omeprazole were investigated after exposing erythrocytes for 48 hours. Investigation of epyptosis was done by cell size measurement, PS exposure determination and calcium channel inhibition. As a possible mechanism of omeprazole induced epyptosis, oxidative stress was investigated by determining the catalase, glutathione peroxidase and superoxide dismutase activities. Similarly, necrotic effect of omeprazole on erythrocytes was also evaluated through hemolysis measurement. Results of our study illustrated that 1.5 µM of omeprazole may induce significant decrease in superoxide dismutase, glutathione peroxidase and catalase activities as well as triggered the erythrocytes shrinkage, PS exposure and hemolysis. Role of calcium was also confirmed in inducing erythrocyte shrinkage. It is concluded that the exposure of erythrocytes with 1.5 µM omeprazole may enhance the rate of epyptosis and hemolysis by inducing oxidative stress.

Keywords

oxidative stress, calcium, cell size, epyptosis

Introduction

A proton pump inhibitor omeprazole⁴ has a broad spectrum use especially in the treatment of gastroesophageal reflux disease and Zollinger-Ellison syndrome. Peptic ulcers caused by stress, nonsteroidal anti-inflammatory drugs and infection of Helicobacter pylori have been treated by using omeprazole.²-⁴ Previous studies related to the adverse effects of omeprazole reflects that its excessive use results in production of oxidative stress which leads to the stimulation of apoptosis in jurkat cells⁵ and in the induction of iron deficiency anemia in patients.⁶,⁷

Characteristics of epyptosis mainly include the shrinkage of cells⁸ and cell membrane scrambling which leads toward phosphatidylserine translocation.⁹ Splenic macrophages recognize and engulf erythrocytes exposed with phosphatidylserine.¹⁰ Stress condition including oxidation, osmotic shock and energy depletion activates Ca²⁺ [Ca²⁺]i, permeable cation channels. The increased activity of cytosolic Ca²⁺ [Ca²⁺]c, results in Ca²⁺ sensitive K⁺ channels activation leading toward cell shrinkage¹¹ by subsequent KCl and water loss from the cell.¹² Phosphatidylserine translocation due to breakdown of phosphatidylserine asymmetry of erythrocyte’s cell membrane is also the result of increased [Ca²⁺]i.¹³ In addition, phosphatidylserine-exposed erythrocytes may adhere to the vascular wall and can therefore play a vital role in the
pathogenesis of thrombosis and thrombo-occlusive diseases. Several xenobiotic compounds are reported as stimulators of eryptosis. Uncontrolled eryptosis also contributes in the pathophysiology of several clinical conditions mainly by the induction of anemia in patients.

The current study explores the eryptotic effect of the therapeutical doses of omeprazole possibly through oxidative stress induction.

**Material and Method**

For experimental work screened blood samples were collected from different blood banks of Faisalabad city. The work has been conducted with the approval of directorate of graduate studies and IBC, University of Agriculture Faisalabad, Pakistan.

Leukocyte depleted erythrocytes were prepared by following the protocol described by Rana et al., 2019. To isolate the cells, the anti-coagulated whole blood was centrifuged at 500 g for 10 min at 4°C. Ringer solution was added to the erythrocyte pellet after removing the supernatant plasma followed by centrifugation at 500 g at 4°C for 10 min. Again removed the supernatant and ringer solution was added to erythrocyte pellet making the volume of the solution double to the volume of erythrocyte pellet alone. The solution was again centrifuged at same speed for 10 min followed by removing the supernatant. This washing step was repeated 3 times. This washing procedure is adequate to get 70 to 80% hematocrit of human erythrocytes. Isolated erythrocytes were stored in separate micro-centrifuge tubes. *In vitro* incubation of erythrocytes was performed at a hematocrit of 0.4% in ringer solution that contain (in mM) MgSO4 1, NaCl 125, KCl 5, glucose 5, CaCl2 1, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 32 at 37°C for 48 h. Isolated erythrocytes were then exposed to lower concentrations (0.5, 1 and 1.5 μM) of omeprazole (Sigma-Aldrich, USA) while the control cells were completely untreated. Dimethyl sulfoxide (DMSO) was used as solvent for omeprazole.

**Oxidative Stress Measurement**

To determine the oxidative stress in omeprazole exposed erythrocytes, antioxidant enzyme’s (catalase, glutathione peroxidase, superoxide dismutase) assays were performed.

**Superoxide Dismutase**

Superoxide dismutase activity was measured by following the protocol of Rana et al. (2019). The reaction solution contained methionine 0.222 g in 15 ml H2O, NBT 0.015 g in 17.5 ml H2O, Triton-X100 (0.0375 ml) in 17.5 ml H2O, riboflavin (0.0132 g) in 17.5 ml H2O and buffer 0.2 M.

**Catalase**

Catalase activity was determined following the protocol of Ullah et al. (2018). Phosphate buffer (pH 7) 50 mM, H2O2 5.9 mM and enzyme extract 0.1 ml was added in reaction mixture and absorbance was read at 240 nm.

**Glutathione Peroxidase**

Phosphate buffer (pH 5.0) 50 mM, guaiacol 20 mM, H2O2 40 mM and enzyme extract 0.1 ml were added in reaction mixture by following the protocol of Ullah et al. (2018) and the enzyme activity was measured at 470 nm after every 20 Sec.

**Cell Size Measurement**

Mean cell volume (MCV) was measured to determine the size of control and treated cells with the help of automated hematology analyzer.

**FACS Analysis of Annexin-V-Binding**

After 48 hr incubation with 1.5 μM omeprazole, 50 µl cell suspension was washed in ringer solution with 5 mM CaCl2 and stained by using Annexin-V-FITC (ImmunoTools, Friesoythe, Germany) for 20 min under protection from light. Annexin-V fluorescence intensity was measured in FL-1 with an excitation wave-length of 488 nm and an emission wavelength of 530 nm on an FACS Calibur (BD, Germany).

**Confirmation of Ca2+ Role**

Amlodipine is a calcium channel blocker. Omeprazole exposed erythrocytes were treated with 10 μM amlodipine to confirm the calcium role in triggering of eryptosis. The inhibition of eryptosis was confirmed by MCV measurement.

**Hemolysis Measurement**

After incubation, samples were centrifuged (3 min at 400 g at room temperature) and supernatant was collected for the determination of hemolysis. Hemoglobin concentration was measured at 405 nm. The absorption of the supernatant of erythrocytes lysed in dH2O was defined as 100% hemolysis.

**Statistical Analysis**

All data is expressed as arithmetic means ± SEM. Statistical analysis was made by using ANOVA with Tukey’s test as post test or t test, as appropriate.

**Results**

The current study was designed to explore primarily the eryptotic effect of omeprazole by adopting the mechanism of oxidative stress induction. To confirm this, antioxidant’s enzymatic activities, erythrocyte’s size, PS exposure at cell surface, hemolytic activity and confirmation of calcium role
in the induction of suicidal death of erythrocytes were determined. The omeprazole concentrations used in the study were lower than the concentrations already reported to treat *S. cerevisiae* and S180 cells (18). Figure 1 is illustrating that 48 hour exposure of erythrocytes to omeprazole (0.5, 1 & 1.5 μM) resulted in significant decrease in activities of superoxide dismutase at 1.5 μM with respect to control. Figure 2 is demonstrating the catalase activities after 48 hour omeprazole (0.5 -1 & 1.5 μM) exposure to human erythrocytes. Results of this experiment showed moderate but significant decrease in enzyme’s activity at 1.5 μM concentration of omeprazole. Figure 3 is indicating a significant reduction in the activities of glutathione peroxidase after 48 hour exposure of erythrocytes with omeprazole (0.5 -1 & 1.5 μM). The variation in anti oxidative enzymes activities in erythrocytes after omeprazole treatment under *in vitro* condition confirmed the generation of oxidative stress which may be due to generation of reactive oxygen species.

Cell shrinkage is an important marker of cryptosis. The 48-hour exposure of omeprazole (1.5 μM) to erythrocytes resulted in lowering the mean cell volume, which may be due to erythrocytes shrinkage (Table 1). For additional confirmation of omeprazole induced cryptosis, PS-exposure in erythrocytes was determined after 48 hr. exposure of 1.5 μM omeprazole. The original histogram in Figure 4 depicting the clear increase of PS exposure in treated cells as compared to the untreated cells. As PS exposure is among the main features of cryptosis,

**Table 1. Effect of Omeprazole Exposure on Mean Cell Volume (MCV) and Hemolysis of Erythrocytes.**

| Parameters       | 0 μM     | 1.5 μM   | p value   |
|------------------|----------|----------|-----------|
| MCV (fl)         | 90 ± 1.69| 74 ± 0.77| <0.001****|
| Hemolysis (%)    | 1.35 ± 0.13| 3.22 ± 0.38| <0.05*    |

Values are arithmetic mean ± SEM, where SEM: Standard error of mean. MCV: Mean cell volume. **** highly significant at p < 0.001, * significant at p < 0.05.
so an increased % of PS-exposed cells is the confirmation of stimulated eryptosis. To rule out the hemolytic role of omeprazole, the results of % hemolysis in omeprazole treated cells are shown in Table 1. The 48-hour exposure of erythrocytes with omeprazole (1.5 μM) resulted in negligible but statistically significant increase in hemolysis % as compared to control cells.

For the confirmation of Ca^{2+} role in the stimulation of oxidative stress induced eryptosis, calcium channel blocker amlodipine was used. Figure 5 is illustrating the cell size measurement of erythrocytes after 48-hour exposure to omeprazole (1.5 μM) and amlodipine (10 μM). The results showed significant increase in mean cell volume in amlodipine treated cells that is surely due to the inhibition of calcium entry and subsequent prevention of cell shrinkage. Non-selective cation channels are triggered by oxidative stress. The result of this experiment showed no shrinkage, so confirming the role of calcium in the omeprazole induced shrinkage of erythrocytes.

Discussion

Oxidative stress is a reported mechanism of eryptosis and variation among anti-oxidative enzymes in an important indicator of oxidative stress. Superoxide dismutase enzyme catalyzes the dismutation of O_2 free radicals that on accumulation may result in lowering the superoxide dismutase’s level and mitochondrial dysfunction resulting in oxidative stress. Catalase is an iron-dependent enzyme that may act peroxidatively and catalytically and considered as a major antioxidant enzyme. Hydrogen peroxide’s accumulation leads to the reduced catalase activity and oxidative stress. Catalase showed a protective effect against oxidants in cells with its overproduction. Similarly, the inhibition of glutathione peroxidase activity may leads to the accumulation of H_2O_2 as it prevents the accumulation of oxidized lipids and promotes the decomposition of hydrogen peroxide into water and oxygen. Previous study reported that omeprazole may induce oxidative stress through the generation of ROS. In current in vitro studies, the reduction of all 3 enzyme activities after omeprazole treatment is the confirmation of oxidative stress induction.

Erythrocyte shrinkage and PS exposure on erythrocyte membrane is among the marker of eryptosis. In current studies, the lowering of mean cell volume and increased % of PS-exposed cells is the confirmation of stimulated eryptosis. Disposing of defective erythrocytes before hemolysis is an important physiological role of eryptosis. Hemoglobin is released through hemolyzed erythrocytes that may be filtered through kidney or may precipitate in acidic lumen of renal tubules. In performed studies, it is noted that omeprazole treatment slightly but significantly increased the hemolysis %. Non-selective cation channels are triggered by oxidative stress. Amlodipine inhibits non-selective cation channels and blocks the Ca^{2+} entry. As reported before, cell shrinkage is a characteristic of eryptosis. The result of our experiment showed no shrinkage, so confirming of calcium role in the omeprazole induced shrinkage of erythrocytes. By removing intracellular and extracellular Ca^{2+}, similar effects would be observed. As for as, the limitation of this research work is concerned, the used concentration of omeprazole are therapeutic, so the future prospect related to this work is to find out the eryptotic effects of physiological doses of omeprazole.

Conclusion

The performed in vitro studies are confirming the oxidative, eryptotic and hemolytic effects of used therapeutical doses of omeprazole. 1.5 μM omeprazole may generate oxidative stress by lowering the antioxidative enzymes activities, enhance the rate of eryptosis by promoting cell shrinkage and PS exposure. Slight increase in hemolysis % was also observed.

Authors’ Note

Ayesha Naveed and Kashif Jilani contributed equally and thus shares first authorship.

Declaration of Conflicting Interests

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