Inhibitory Effect of Thymol on Suicidal Erythrocyte Death

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Key Words

Cell volume • Eryptosis • Phosphatidylserine • Calcium • Cell death

Abstract

The antibacterial plant component thymol has antioxidant activity. Oxidative stress is known to activate Ca^{2+} -permeable cation channels with subsequent Ca^{2+} entry, activation of Ca^{2+} -sensitive K^+ channels, cell shrinkage, cell membrane scrambling and phosphatidylserine exposure at the erythrocyte surface. Cell shrinkage and phosphatidylserine exposure are typical features of suicidal erythrocyte death or eryptosis. Eryptotic cells are cleared from circulating blood thus causing anemia and may adhere to the vascular wall thus interfering with the microcirculation. The present experiments explored whether thymol interacts with eryptosis. Annexin V-binding was utilized to determine phosphatidylserine exposure, forward scatter to detect alterations of cell volume and Fluo3 fluorescence to depict changes of the cytosolic Ca^{2+} activity. Oxidative stress (30 min. 0.3 mM tert-butylhydroperoxide), energy depletion (48 h glucose removal) and isotonic cell shrinkage (48 h replacement of extracellular Cl^- with gluconate)

significantly increased annexin V-binding and decreased the forward scatter, effects significantly blunted in the presence of thymol 2.5 – 20 $\mu\text{g/ml}$. Thymol is a potent inhibitor of suicidal erythrocyte death particularly following oxidative stress. In conclusion, thymol may be useful in counteracting anemia and impairment of microcirculation.

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Introduction

Phosphatidylserine-exposing erythrocytes are engulfed by macrophages and thus rapidly eliminated from circulating blood [1]. Erythrocyte phosphatidylserine is exposed following phospholipid scrambling of the cell membrane [2, 3], which is triggered by increase in the cytosolic Ca^{2+} activity [4]. Cytosolic Ca^{2+} activity may increase due to activation of non-selective cation channels with subsequent increase in the cytosolic Ca^{2+} concentration [4-9] e.g. following isoosmotic cell shrinkage

(chloride replacement by gluconate) [4], oxidative stress [9] and energy depletion [4, 6-8]. The cation channels are in part TRPC6 [10]. Ca^{2+} further activates Ca^{2+} -sensitive K^{+} channels [11, 12]. As erythrocytes express Cl^{-} channels [13], the activation of K^{+} channels leads to subsequent exit of KCl with osmotically obliged water and thus to cell shrinkage [14]. The Ca^{2+} sensitivity of phospholipid scrambling is enhanced by ceramide [15]. Several clinical conditions associated with anemia have been shown to be paralleled by accelerated eryptosis [16], such as iron deficiency [1], phosphate depletion [17], Hemolytic Uremic Syndrome [18], sepsis [19], malaria [20-22], or Wilson's disease [23]. Moreover, eryptosis may be triggered by a wide variety of endogenous substances and xenobiotics, such as antiA IgG antibodies [24], cordycepin [25], hemin [26], amiodarone [27], methylglyoxal [28], amyloid peptides [29], anandamide [30], lipopeptides [31] retinoic acid [32], paclitaxel [33], amantadine [34], chlorpromazine [35], ciglitazone [36], cyclosporine [37], Bay-5884 [38], curcumin [39], valinomycin [40], listeriolysin [41], aluminium [42], bismuth [43], mercury [44], copper [23], cadmium [45], selenium [46], vanadate [47], gold [48] and arsenic [49]. Inhibitors of eryptosis include caffeine [50].

Thymol, a component of several plants, is well known for its antimicrobial potency [51-55]. In mammalian cells, it has been shown to counteract cell proliferation [56]. On the other hand, thymol may influence generation of reactive oxygen species [57, 58] and decrease the cytosolic Ca^{2+} activity [59].

The present study explored the influence of thymol on eryptosis following oxidative stress, energy depletion and chloride removal. As a matter of fact, thymol indeed significantly blunted the eryptosis following those challenges.

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with banked erythrocytes 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 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 the indicated time periods. Where stated, chloride was replaced by gluconate, glucose deleted from the medium or tert-butylhydroperoxide (0.3 mM, Sigma, Schnelldorf, Germany) added. Thymol (Sigma) was used at concentrations between 2.5 and 20 µg/ml.

Fig. 1. Effect of oxidative stress on cytosolic Ca^{2+} activity in the presence and absence of thymol. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 30 minutes with 0.3 mM tert-butylhydroperoxide in the absence (-, black line) and presence (+, red line) of 20 µg/ml thymol. B. Arithmetic means $\pm$ SEM ($n = 17-18$; 9 different erythrocyte specimens were investigated) of the normalized Fluo3 fluorescence in erythrocytes following incubation for 30 minutes in the absence (open bars) or presence (closed bars) of tert-butylhydroperoxide (0.3 mM) in the absence (0) or presence of 2.5 – 20 µg/ml thymol. *** ($p < 0.001$) indicates significant difference from absence of tert-butylhydroperoxide, ### ($p < 0.001$) indicates significant difference from the absence of thymol (ANOVA).

FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described previously [60]. After incubation under the respective experimental condition, cells were washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-Fluos (Roche, Mannheim, Germany) in this solution for 20 min under protection from light. In the following, forward scatter 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 (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 20 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.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made using ANOVA with Tukey's test as post-test, as appropriate.

Fig. 2. Effect of oxidative stress on phosphatidylserine exposure in the presence and absence of thymol. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 30 minutes with 0.3 mM tert-butylhydroperoxide in the absence (-, black line) and presence (+, red line) of 20 µg/ml thymol. B. Arithmetic means $\pm$ SEM (n = 9 different blood specimens) of annexin V-binding in erythrocytes following incubation for 30 minutes in the absence (open bars) or presence (closed bars) of tert-butylhydroperoxide (0.3 mM) in the absence (0) or presence of 2.5 – 20 µg/ml thymol. *** (p<0.001) indicates significant difference from absence of tert-butylhydroperoxide, #### (p<0.001) indicates significant difference from the absence of thymol (ANOVA).

Fig. 3. Effect of oxidative stress on erythrocyte forward scatter in the presence and absence of thymol. A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 30 minutes with 0.3 mM tert-butylhydroperoxide in the absence (-, black line) and presence (+, red line) of 20 µg/ml thymol. B. Arithmetic means $\pm$ SEM (n = 17-18; 9 different erythrocyte specimens were investigated) of normalized erythrocyte forward scatter following incubation for 30 minutes in the absence (open bars) or presence (closed bars) of tert-butylhydroperoxide (0.3 mM) in the absence (0) or presence of 2.5 – 20 µg/ml thymol. *** (p<0.001) indicates significant difference from absence of tert-butylhydroperoxide (ANOVA).

Fig. 4. Effect of thymol on eryptosis following oxidative stress in the presence and absence of extracellular Ca^{2+} . A. Arithmetic means $\pm$ SEM (n = 4 different erythrocyte specimens were investigated) of the percentage of PS-exposing erythrocytes exposed for 30 min. to Ringer solution with or without 10 µg/ml thymol with or without 0.3 mM tert-butylhydroperoxide in the absence or presence of extracellular Ca^{2+} . B. Arithmetic means $\pm$ SEM (n = 4 different erythrocyte specimens were investigated) of the forward scatter of erythrocytes exposed for 30 min. to Ringer solution with or without 10 µg/ml thymol with or without 0.3 mM tert-butylhydroperoxide in the absence or presence of extracellular Ca^{2+} .

Results

To elucidate the effect of oxidative stress on cytosolic Ca^{2+} activity in the absence and presence of thymol, Fluo3 fluorescence was determined prior to and following addition of tert-butylhydroperoxide (0.3 mM). The exposure of erythrocytes for 30 minutes to 0.3 mM tert-butylhydroperoxide was followed by an increase in Fluo3 fluorescence (Fig. 1B). The addition of thymol (2.5-30 µg/ml) decreased the Fluo3 fluorescence in the presence of 0.3 mM tert-butylhydroperoxide. For a statistically significant effect 2.5 µg/ml thymol were required. Thus,

oxidative stress increased the cytosolic Ca^{2+} activity, an effect blunted by thymol (= 2.5 µg/ml) (Fig. 1A,B).

As increases in the cytosolic Ca^{2+} activity stimulate erythrocyte cell membrane scrambling and thus phosphatidylserine exposure at the erythrocyte surface [4], annexin V-binding was employed to determine the percentage of phosphatidylserine-exposing erythrocytes. Similar to what was observed with Ca^{2+} activity, 0.3 mM tert-butylhydroperoxide markedly increased annexin V-binding (Fig. 2A,B). The presence of thymol (= 2.5 µg/ml) significantly blunted the effect of 0.3 mM tert-butylhydroperoxide on the percentage of annexin V-binding cells. Accordingly, 0.3 mM tert-butylhydro-

Fig. 5. Effect of glucose depletion on cytosolic Ca^{2+} activity in the presence and absence of thymol. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without glucose in the absence (-, black line) and presence (+, red line) of 20 $\mu\text{g}/\text{ml}$ thymol. B. Arithmetic means $\pm$ SEM ($n = 20$; 10 erythrocyte specimens were studied) of the normalized Fluo3 fluorescence in erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the absence (0) or presence of 2.5 – 20 $\mu\text{g}/\text{ml}$ thymol. *** ($p < 0.001$) indicates significant difference from the presence of glucose.

Fig. 6. Stimulation of phosphatidylserine exposure by glucose depletion in the presence and absence of thymol. A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without glucose in the absence (-, black line) and presence (+, red line) of 20 $\mu\text{g}/\text{ml}$ thymol. B. Arithmetic means $\pm$ SEM ($n = 5$ erythrocyte specimens) of the percentage of phosphatidylserine-exposing erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the absence (0) or presence of 2.5 – 20 $\mu\text{g}/\text{ml}$ thymol. *** ($p < 0.001$) indicates significant difference from the presence of glucose, ## ($p < 0.01$) indicates significant difference from the absence of thymol (ANOVA). C. Arithmetic means $\pm$ SEM ($n = 5$ erythrocyte specimens) of the percentage of phosphatidylserine-exposing erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of extracellular chloride in the absence (0) or presence of 2.5 – 20 $\mu\text{g}/\text{ml}$ thymol. *** ($p < 0.001$) indicates significant difference from the presence of chloride, ## ($p < 0.01$) indicates significant difference from the absence of thymol (ANOVA).

peroxide stimulated cell membrane scrambling, an effect significantly blunted by thymol ($= 2.5 \mu\text{g}/\text{ml}$).

Cytosolic Ca^{2+} further shrinks erythrocytes by activation of Ca^{2+} -sensitive K^{+} channels [14]. Accordingly, forward scatter in FACS analysis was employed to detect alterations of cell volume. Tert-butylhydroperoxide (0.1 mM and 0.3 mM) significantly decreased erythrocyte forward scatter, an effect not modified by the presence of thymol (Fig. 3).

To investigate whether thymol influences eryptosis following oxidative stress independently of Ca^{2+} erythrocytes were exposed to 0.3 mM tert-butylhydroperoxide in the presence or absence of extracellular Ca^{2+} .

As shown in Fig. 4A, eryptosis following oxidative stress was inhibited by 10 $\mu\text{g}/\text{ml}$ thymol to a similar extent in the presence and in the absence of extracellular Ca^{2+} . Furthermore, oxidative stress caused cell shrinkage both, in the presence and absence of extracellular Ca^{2+} , an effect not influenced by thymol (Fig. 4B). Thus, despite attenuating an increase in the cytosolic Ca^{2+} concentration following oxidative stress, thymol appears to exert an inhibitory effect on eryptosis which is independent of Ca^{2+} .

Eryptosis could further be triggered by energy depletion [61], which was accomplished by removal of glucose for 48 hours. As illustrated in Fig. 5, a 48 hours incubation of erythrocytes in the absence of glucose indeed

Fig. 7. Forward scatter prior to and following glucose depletion in the presence and absence of thymol. A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without glucose in the absence (-, black line) and presence (+, red line) of 20 $\mu\text{g/ml}$ thymol. B. Arithmetic means $\pm$ SEM ($n = 20$; 10 erythrocyte specimens were studied) of the normalized forward scatter of erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the absence (0) or presence of 2.5–20 $\mu\text{g/ml}$ thymol. *** ($p < 0.001$) indicates significant difference from the presence of glucose, ## ($p < 0.01$) indicates significant difference from the absence of thymol (ANOVA).

significantly increased the Fluo3 fluorescence. The addition of thymol tended to slightly blunt the increase in Fluo3 fluorescence following glucose depletion, an effect, however, not reaching statistical significance. Thus, glucose depletion increased the cytosolic Ca^{2+} activity, an effect only slightly modified by the presence of thymol.

In a next series of experiments, the effect of glucose depletion on phosphatidylserine-exposure in the presence and absence of thymol was determined. As shown in Fig. 6, glucose depletion increased annexin V-binding. Thymol decreased the percentage of annexin V-binding erythrocytes, an effect reaching statistical significance at 20 $\mu\text{g/ml}$ thymol (Fig. 6A,B). Thus, energy depletion stimulates cell membrane scrambling, an effect blunted by thymol. Isotonic cell shrinkage (replacement of extracellular Cl^- by gluconate) similarly increased the percentage of annexin V-binding erythrocytes, an effect again significantly blunted at higher concentrations of thymol (Fig. 6C).

Glucose depletion further decreased the erythrocyte forward scatter in FACS analysis (Fig. 7). The effect was again blunted in the presence of thymol. Statistically significant effects of thymol were observed at 20 $\mu\text{g/ml}$ thymol. Accordingly, thymol blunted the cell shrinkage following energy depletion.

Discussion

The present study confirms previous observations [16] demonstrating that both, oxidative stress and glucose depletion lead to increase in cytosolic Ca^{2+} activity with subsequent stimulation of cell membrane scrambling and cell shrinkage. More importantly, the present observations disclose that those effects are significantly blunted by thymol. Accordingly, thymol is a potent inhibitor of

suicidal erythrocyte death similar to resveratrol [62]. Thymol is particularly effective against the eryptotic effect of oxidative stress, which presumably relates to its antioxidant effect [57].

The antieryptotic effect of thymol during oxidative stress is at least partially due to inhibition of Ca^{2+} entry. However, thymol effectively blunted the eryptosis following oxidative stress in the absence of extracellular Ca^{2+} . Moreover, thymol had only a weak effect on the increase in cytosolic Ca^{2+} activity, yet still inhibited eryptosis following energy depletion. Thus, thymol apparently blunts the eryptosis even under conditions, when Ca^{2+} entry persists. Accordingly, thymol is presumably in part effective by influencing mechanisms downstream of Ca^{2+} .

Ca^{2+} entry during oxidative stress probably accounts for the subsequent cell shrinkage. Surprisingly, though, thymol did not abrogate the cell shrinkage despite its inhibitory effect on Ca^{2+} entry. In theory, the Ca^{2+} sensitivity of cell membrane scrambling and cell shrinkage could be different and the blunting of Ca^{2+} entry by thymol in oxidative stress may be sufficient to abrogate cell membrane scrambling but not cell shrinkage. The seeming discrepancy may further be due to the involvement of additional mechanisms.

The effect of thymol may be of biological relevance. Eryptotic cells bind to phosphatidylserine receptors, which are expressed by macrophages [63]. The macrophages engulf and degrade the phosphatidylserine-exposing cells [64]. Thus, eryptotic erythrocytes are rapidly cleared from circulating blood [1, 65, 66]. Accordingly, stimulation of eryptosis leads to disappearance of the affected erythrocytes and thus to anemia. As pointed out in the introduction, a wide variety of clinical conditions and toxic substances causing anemia lead to enhanced eryptosis [16]. Thymol may prove effective in counteracting the anemia in those clinical conditions and under the respective toxic effects.

Eryptosis may further lead to impairment of micro-circulation, as eryptotic erythrocytes adhere to the vascular wall [67-71], and phosphatidylserine exposure stimulates blood clotting [67, 72, 73]. Suicidal erythrocytes have thus been suggested to participate in the vascular injury of metabolic syndrome [74]. In conclusion, thymol may exert a powerful protective effect against suicidal erythrocyte death. Specifically, thymol blunts the eryptotic effect of oxidative stress,

isotonic cell shrinkage and energy depletion.

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