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Thymoquinone-Induced Platelet Apoptosis

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ABSTRACT

Thymoquinone (TQ) is a nutrient with anticarcinogenic activity that stimulates suicidal death of tumor cells. Moreover, TQ triggers suicidal death of erythrocytes or eryptosis, an effect at least partially due to increase in cytosolic Ca^{2+} activity and ceramide formation. The present experiments explored whether TQ influences apoptosis of blood platelets. Cell membrane scrambling was determined utilizing Annexin V binding to phosphatidylserine exposing platelets, cytosolic Ca^{2+} activity utilizing Fluo 3-AM fluorescence, caspase activity utilizing immunofluorescence and Western blotting of active caspase-3 and inactive procaspase-3, mitochondrial potential utilizing $DiOC_6$ fluorescence and ceramide by FACS analysis of ceramide-binding antibodies. A 30 min exposure to TQ ($\geq$5 μM) was followed by Annexin V binding, paralleled by caspase activation, increase of cytosolic Ca^{2+} activity, mitochondrial depolarization, and ceramide formation. P-selectin exposure and integrin $\alpha_{IIb}\beta_3$ activation did not increase in response to TQ. Nominal absence of extracellular Ca^{2+} blunted but did not fully abolish the TQ-induced activation of caspase-3. The effects of TQ on platelets are significantly abolished with phosphoinositide-3 kinase (PI3K) inhibitor wortmannin and G-protein coupled receptor (GPCR) inhibitor pertussis toxin treatment prior to TQ stimulation. In conclusion, TQ triggers suicidal death of blood platelets in a PI3K-dependent manner, possibly through a GPCR family receptor; an effect paralleled by increase of cytosolic Ca^{2+} activity, ceramide formation, mitochondrial depolarization, and caspase-3 activation. J. Cell. Biochem. 112: 3112–3121, 2011.

KEY WORDS: THYMOQUINONE; PLATELETS; PHOSPHATIDYLSERINE; CASPASE-3; APOPTOSIS

Thymoquinone (TQ) is a component of *Nigella sativa* [Badary et al., 2007; Khader et al., 2009] which exerts anticarcinogenic activity [Ali and Blunden, 2003; Salem, 2005; Gali-Muhtasib et al., 2006, 2008; Aggarwal et al., 2008], an effect at least partially due to stimulation of tumor cell apoptosis [Shoieb et al., 2003; Gali-Muhtasib et al., 2004b; Rooney and Ryan, 2005a; Roepke et al., 2007].

The stimulation of apoptosis by TQ involves p53 [Gali-Muhtasib et al., 2004a, 2008; Roepke et al., 2007], NFκB [Sethi et al., 2008], suppression of Akt (proteinkinase B) and extracellular signal-regulated kinase [Yi et al., 2008; Xuan et al., 2010], decrease of reduced glutathion [Rooney and Ryan, 2005b] and caspase activation [El Mahdy et al., 2005; Rooney and Ryan, 2005b; Xuan et al., 2010].

Platelets are anucleated blood cells that originate from the cytoplasm of megakaryocytes and that are mainly involved in hemostasis and clot formation after tissue trauma [Ruggeri, 2002]. Non-activation of platelets cause uncontrolled bleeding and improper activation causes thrombotic disorders [Jackson, 2007; Ombrello et al., 2010]. Platelets are very sensitive to dietary and therapeutic components in blood [Pandey et al., 1991; McCloskey et al., 2008; Antovic, 2010; Krone, 2010], which often requires clinical attention.

The present study explored the effect of TQ on platelet apoptosis. It is shown that exposure of platelets to TQ indeed stimulates phosphatidylserine exposure, an effect at least partially due to increase of cytosolic Ca^{2+} activity, depolarization of the mitochondrial potential, ceramide formation, and caspase activation.

Abbreviations: EGTA, glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; NFκB, nuclear factor κB; PBS, phosphate-buffered saline; DiOC6, 3,3′-dihexyloxacarbocyanine iodide.

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MATERIALS AND METHODS

ISOLATION AND STIMULATION OF HUMAN PLATELETS

Fresh EDTA-anticoagulated blood was obtained from the blood bank of Eberhard-Karls-Universität Tübingen, Germany, according to the Ethics Committee of the University (184/2003V). The blood was centrifuged at 160*g* for 20 min at 20°C. The platelet rich plasma was separated and centrifuged at 2,000*g* at 20°C for 2 min and washed twice in 900 μl platelet buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, pH 7.4) without calcium and 100 μl acid citrate dextrose solution (80 mM trisodium citrate, 52 mM citric acid, 180 mM glucose). The isolated platelets were suspended in platelet buffer with 0.1% BSA. Care was taken not to expose platelets to excess shear or oxygen.

The stimulation of platelets was done in platelet buffer with 5 mM $CaCl_2$ with 1×10^6 platelets in each reaction so that the total volume came to 1 ml. The platelets were added with 1, 5, 10, and 50 μM TQ (Sigma–Aldrich) and incubated for 30 minutes at 37°C. A positive control with 1 U/ml thrombin (Calbiochem) and a negative control without TQ were compared with each set of experiment.

PHOSPHATIDYLSERINE EXPOSURE

Phosphatidylserine exposure was measured with 1:100 dilution of Annexin V Fluos (Roche, Mannheim, Germany) and incubated at 37°C for 30 min. The fluorescence was measured in FL-1 in BD FacsCalibur (BD Biosciences, CA).

INTRACELLULAR CA^{2+}

Stimulated platelets were incubated for 30 min with 5 μM Fluo 3-AM (Biotium, CA) and measured in FL-1.

CASPASE-3 ACTIVITY

Formation of active caspase-3 was detected according to the manufacturer's instruction of CaspGlow Fluorescein Active Caspase-3 staining kit from BioVision (CA). Two sets of experiments were done to check the effect of calcium on caspase-3 formation. One set contained platelet buffer with 2 mM EGTA and another set contained platelet buffer with 2 mM $CaCl_2$. Both contained 1×10^6 platelets and were stimulated with the same concentration of TQ and thrombin as mentioned before. Active caspase-3 produced in the cells was measured by CaspGlow Fluorescein Active Caspase-3 staining kit from BioVision (CA). It should be pointed out that flow cytometry is a more sensitive method than Western blotting.

CASPASE-3 PROTEIN ABUNDANCE

1×10^8 platelets were stimulated with the defined concentrations of TQ and washed twice in platelet buffer, lysed with 200 μl of 1× RIPA lysis buffer (Cell Signaling Technology). The cell lysate was centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was taken for Western blot.

For detection of caspase-3, 40 μg protein was loaded for electrophoresis, followed by blotting onto Protran nitrocellulose membrane (Whatman, Dassel, Germany), blocked with TBS-T (NaCl 80 g/L, Tris–HCl 24.2 g/L, Tween 20 0.1%, pH 7.6) containing 5% BSA and stained overnight with 1:1,000 dilution of caspase-3 rabbit monoclonal antibody (Cell Signaling Technology). The blots were incubated for 1 h with 1:5,000 dilution of the HRP-conjugated anti-rabbit Ig (Cell Signaling Technology). The binding of antibodies was detected by Enhanced Chemiluminescence Kit (Amersham Biosciences, Freiburg, Germany).

MITOCHONDRIAL MEMBRANE POTENTIAL

Platelets were first stimulated with TQ as described before and 1×10^7 platelets were dissolved in Phosphate buffered saline (PBS; Invitrogen, CA) supplemented with 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA, and 10 mM HEPES (pH 7.4) in a total volume of 1 ml and stained with 10 nM $DiOC_6$ (Invitrogen) for 10 min. The stained cells were centrifuged at 1,000*g* for 5 min at 20°C, resuspended in PBS and measured in FL-1 [Leytin et al., 2006a,b].

CERAMIDE FORMATION

For detection of ceramide formation, 1×10^8 platelets were stimulated as described before and centrifuged at 2,000*g* for 2 min and the pellet was incubated with 50 μl of 1:5 dilution of mice antibody to human ceramide (Alexis) in PBS with 1% BSA for 1 h at 37°C and 5% CO_2. Then primary stained cells were centrifuged and the pellet was stained with 50 μl of 1:50 dilution secondary goat anti-mouse IgG (BD Pharmingen, Hamburg, Germany) for 20 min. The geomean of the FITC-labeled secondary Ig was measured.

PLATELET DEGRANULATION AND INTEGRIN $\alpha_{IIB}\beta_3$ ACTIVATION

Platelets were stimulated as described before. Stimulated platelets were stained with FITC-conjugated antibody to human CD62P or PAC1 antibody (BD Biosciences) according to manufacturer's instruction for 30 min and analyzed by FACS.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Fresh isolated platelets were adhered to a fibrinogen surface (20 μg/ml) on chamber slides and fixed with 2% paraformaldehyde. The platelets were washed and blocked with 2% bovine serum albumin for 30 min, followed by an Triton X-100 treatment for permeabilization. For primary antibody treatment 1:100 dilution of Annexin V Fluos (Roche) or 1:50 Caspase-3 rabbit monoclonal antibody (Cell Signaling Technology) was incubated for 2 h at RT. Chamber slides were washed and incubated with secondary antibody labeled with FITC (Santa Cruz) in the case of the caspase-3 staining. The actin cytoskeleton was stained with rhodamine–phalloidin (Invitrogen). Confocal microscopy was performed using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging, Jena, Germany) with a A-Plan 63 × ocular.

INHIBITION OF PI3K SIGNALING

Isolated platelets were suspended in platelet buffer with 5 mM $CaCl_2$ in a concentration of 1×10^6/ml and three sets of experiments were done. Platelets were pretreated with wortmannin (100 nM; Calbiochem) or DMSO for 15 min and then stimulated with TQ (10 and 50 μM) or thrombin (1 U/ml) for 30 min at 37°C. Samples were stained with Annexin V Fluos, Fluo 3-AM, anti-active Caspase-3-FITC, or DiOC6 and measured in the FACS as mentioned before.

INHIBITION OF G-PROTEIN COUPLED RECEPTOR (GPCR)

1×10^6 platelets were suspended in platelet buffer containing 5 mM $CaCl_2$ and one set of platelets were pretreated with 1 ng/ml pertussis toxin (PTX) for 30 min. Both sets were then added with 1 U/ml thrombin or TQ (10 or 50 μM). All the samples were stained with Annexin V Fluos or anti-active caspase-3-FITC and analyzed with FACS.

STATISTICAL ANALYSIS

The statistical significance of the results was determined by one-way ANOVA from the raw data. The statistics for calcium-dependence of caspase-3, PI3K inhibition, and GPCR inhibition was determined by Student's paired *t*-test on the raw data of the experiments from platelet stimulation with and without $CaCl_2$.

RESULTS

A key event of suicidal death of nucleated cells and erythrocytes is cell membrane phospholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface. The phosphatidylserine exposing cells can be identified utilizing Annexin V binding. To explore, whether TQ stimulates suicidal platelet death, Annexin V binding has been determined prior to and following treatment with TQ. As illustrated in Figure 1, a 30 min exposure to TQ was indeed followed by a marked increase of the percentage Annexin V binding platelets. The effect reached statistical significance at ≥5 μM TQ (Fig. 1).

In a wide variety of cells, cell membrane scrambling is triggered by the executor caspase-3. Thus, a second series of experiments was

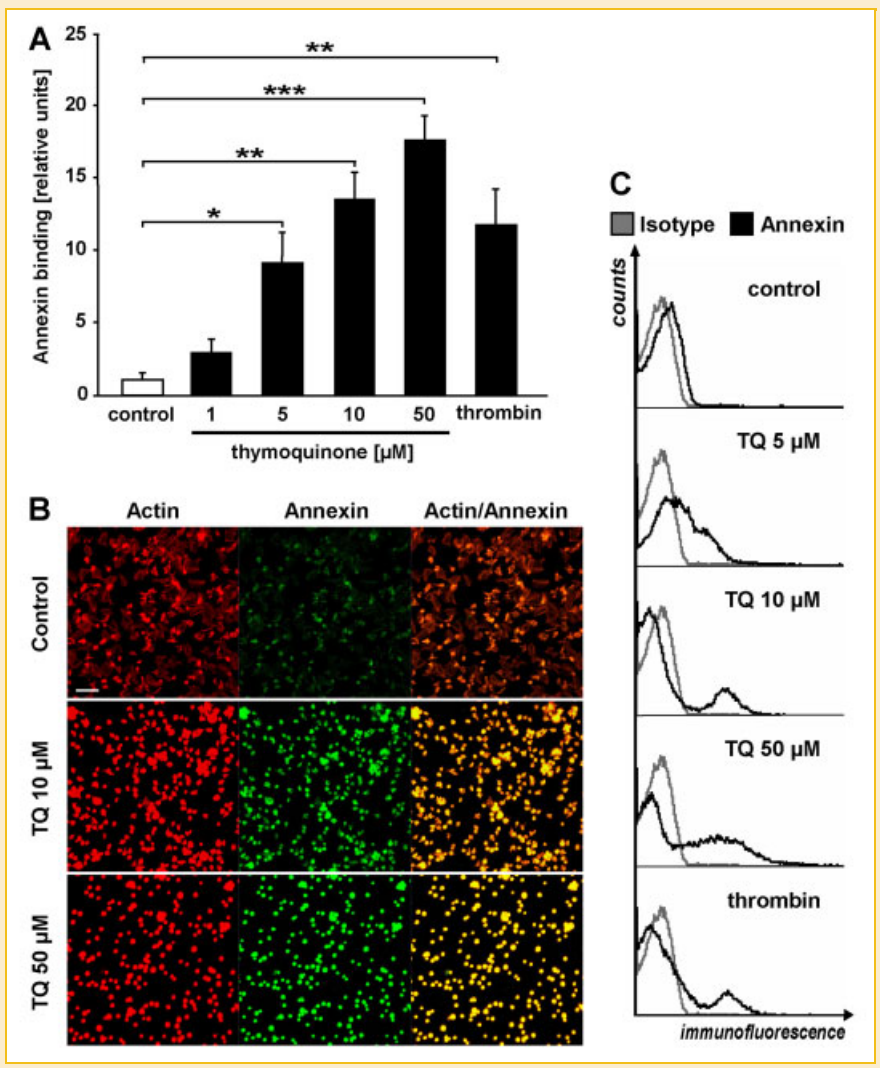

Fig. 1. Effect of thymoquinone (TQ) on phosphatidylserine exposure in human platelets. A: Arithmetic mean ± SEM (n = 8) of the relative units of human platelets binding Annexin V Fluos following a 30 min exposure to platelet buffer in the absence (control, open bar) and presence of thymoquinone (TQ; 1–50 μM) or of thrombin (1 U/ml closed bars) as positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant difference to value in the absence of TQ. B: Representative immunofluorescence Annexin V staining following a 30 min exposure to platelet buffer in the absence (control) or presence (10 and 50 μM) of TQ. Red—rhodamine phalloidine, green—annexin V. Magnification bar represents 5 μm. C: Representative histograms of Annexin V binding of platelets exposed for 30 min to (from above) 0 (control), 1–50 μM TQ or 1 U/ml thrombin.

performed to explore whether TQ stimulates caspase-3 in platelets. As illustrated in Figure 2, caspase-3 activity was indeed increased by a 30 min exposure to TQ, an effect reaching statistical significance at $\geq$1 μM TQ. The activation of caspase-3 was paralleled by a decline of procaspase-3 (Fig. 2), an effect reaching statistical significance at $\geq$5 μM TQ.

Cell membrane scrambling can further be activated by increased cytosolic Ca^{2+} activity. Thus, an additional series of experiments explored whether TQ modifies intracellular Ca^{2+} activity. As shown in Figure 3, a 30 min exposure to TQ was indeed followed by an increase of Fluo 3-AM fluorescence, reflecting enhanced cytosolic Ca^{2+} activity. A next series of experiments explored whether the increase of cytosolic Ca^{2+} activity was required for the stimulation of caspase-3. To this end, experiments were performed in the presence and nominal absence of extracellular Ca^{2+}. As depicted in Figure 3, the increase of caspase activity was significantly blunted in

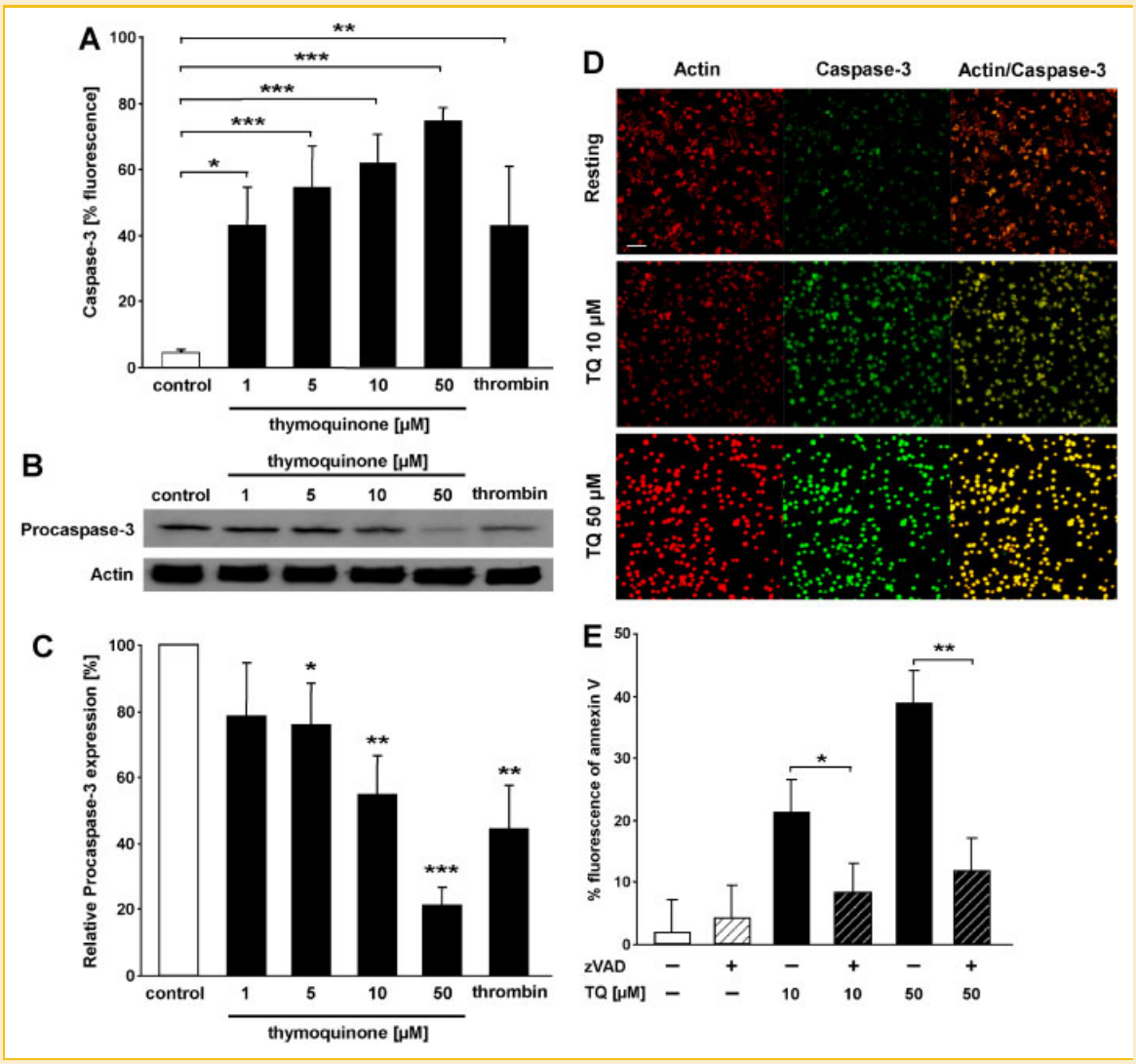

Fig. 2. Effect of thymoquinone (TQ) on caspase-3 activation in human platelets. A: Arithmetic mean ± SEM (n = 8) of the % human platelets expressing active caspase-3 following a 30 min exposure to platelet buffer 5 mM $CaCl_2$ in the absence (open bar) and presence (closed bars) of thymoquinone (TQ, 1–50 μM) or of thrombin (1 U/ml) as positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant difference to value in the absence of TQ. B: Original Western blot of procaspase-3 abundance in human platelets following a 30-min exposure to platelet buffer 5 mM $CaCl_2$ in the absence (open bar) and presence (closed bars) of TQ (1–50 μM) or of thrombin (1 U/ml) as positive control. Platelet extracts were blotted with Ig against procaspase-3 (34 kDa). Protein loading was controlled by determination of β-actin. C: Arithmetic mean ± SD (n = 4) of the ratio of procaspase-3 and β-actin following a 30 min exposure to platelet buffer 5mM $CaCl_2$ in the absence (control) or presence (1, 5, 10, and 50 μM) of TQ or of thrombin (1 U/ml) as positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant difference to value in the absence of TQ. D: Representative immunofluorescence staining of caspase-3 staining following a 30 min exposure to platelet buffer 5 mM $CaCl_2$ in the absence (control) or presence (10 and 50 μM) TQ. Red—rhodamine phalloidine, green—caspase-3. Magnification bar represents 5 μm. E: Arithmetic mean ± SEM (n = 8) of the % human platelets binding Annexin V Fluos following a 30 min exposure to platelet buffer without (white bar) or with (black bar) thymoquinone (TQ) in the absence (not dashed) and presence (dashed) of pancaspase inhibitor zVAD (1 μl/ml). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant difference to value in the presence of thymoquinone but absence of zVAD.

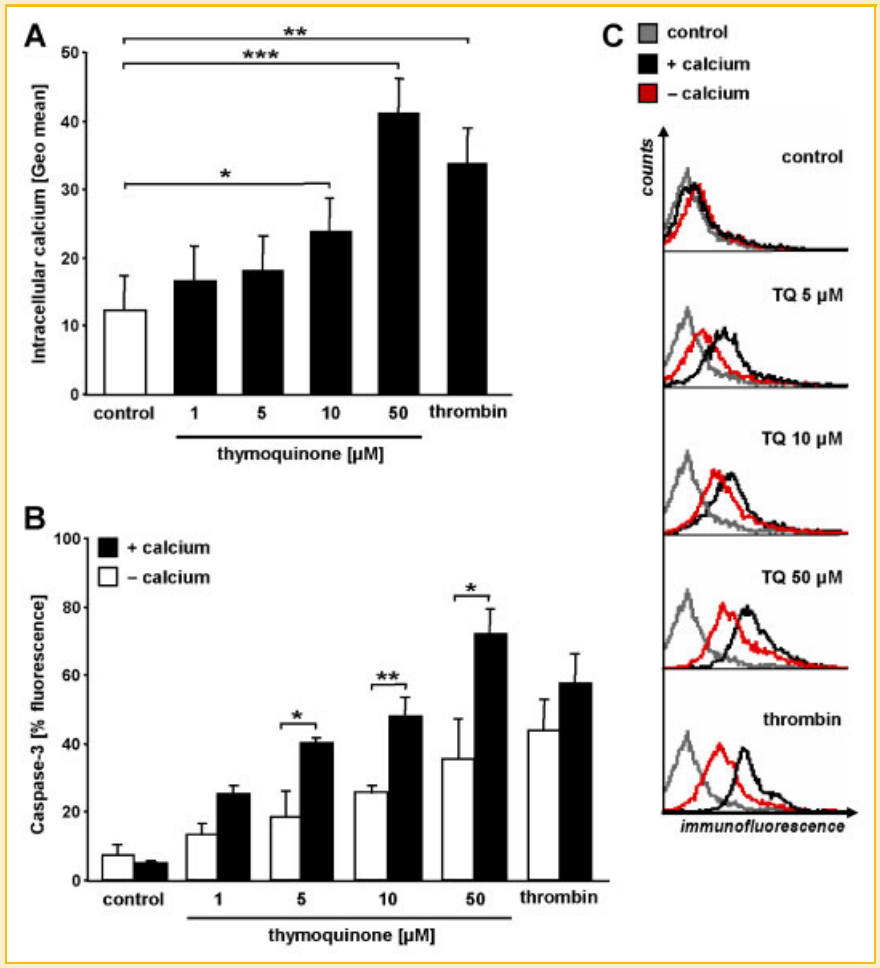

Fig. 3. Effect of thymoquinone on increase of intracellular calcium and calcium dependence of caspase-3 activity in thymoquinone (TQ)-treated human platelets. A: Arithmetic mean ± SEM (n = 8) of Fluo 3-AM fluorescence in human platelets following a 30 min exposure to platelet buffer 5 mM $CaCl_2$ in the absence (control, open bar) and presence (closed bars) of thymoquinone (TQ, 1–50 μM) or of thrombin (1 U/ml) as positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant difference to value in the absence of thymoquinone. B: Arithmetic mean ± SEM (n = 8) of % of active caspase-3 (17/19 kDa) induction in TQ-treated (1–50 μM) human platelets. $^{*}P < 0.05$ and $^{**}P < 0.01$ represent significant difference between active caspase-3 induced in platelets stimulated in 0 mM $CaCl_2$ and 2 mM $CaCl_2$ by Student's paired *t*-test. (C) Representative histograms of caspase-3 fluorescence reflecting caspase-3 activity in platelets exposed for 30 min to platelet buffer 5 mM $CaCl_2$ without (control, gray lines) or with (from above) 0 (control), 1–50 μM TQ or 1 U/ml thrombin as positive control in the presence (black lines) or absence (red lines) of Ca^{2+}.

the nominal absence of extracellular Ca^{2+}. However, the complete removal of extracellular Ca^{2+} did not fully prevent the TQ-induced stimulation of caspase-3.

In search for an additional mechanism stimulating caspase activation, we explored whether TQ stimulates the formation of ceramide. As shown in Figure 4, a 30 min exposure to TQ was followed by an increase of ceramide abundance at the cell surface. The effect reached statistical significance at ≥10 μM TQ (Fig. 4B).

Caspases are further activated following mitochondrial depolarization. Accordingly, the mitochondrial potential was determined prior to and following a 30 min exposure to TQ. As shown in Figure 4A, TQ treatment was followed by a decline of mitochondrial potential, an effect reaching statistical significance at ≥10 μM TQ.

TQ has not been found to increase the activation-dependent markers of degranulation or integrin $\alpha_{IIb}\beta_3$ activation in platelets, namely CD62P and PAC-1. Additional experiments were performed to determine the signaling pathway required for the TQ induced caspase activation. A first series of experiments addressed the putative involvement of the phosphoinositide-3-kinase (PI3K) pathway, which is known to impact on apoptosis [Zimmermann et al., 2001; Duronio, 2008]. As a result, the inhibition of PI3K with 100 nM wortmannin [Chakravarty, 1993] significantly downregulated phosphatidylserine (PS) exposure, intracellular calcium influx, caspase-3 activation and abrogated the depolarization of mitochondrial membrane following TQ and thrombin treatment. Accordingly, PI3K is apparently involved in the triggering of platelet apoptosis by TQ. PI3K is acivated by GPCR [Fan and Weiss, 2010; Hawkins et al., 2010]. Pretreatment of platelets with 1 ng/ml of the GPCR blocker PTX [Chakravarty, 1993; Carbonetti, 2010] significantly blunted the PS exposure and caspase-3 activation

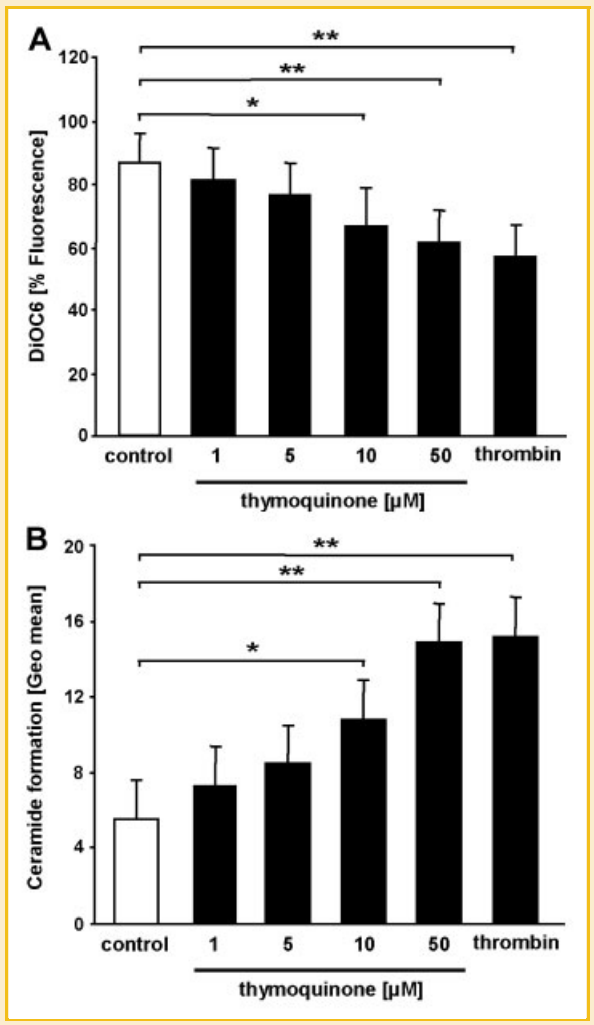

Fig. 4. Depolarization of mitochondrial membrane and ceramide formation in thymoquinone (TQ)-treated human platelets. A: Depolarization of mitochondrial membrane after thymoquinone (TQ) treatment. Here the graph is constructed from arithmetic mean ± SEM (n = 11) with FACS analysis of TQ (1–50 μM) stimulated platelet stained with 10 nM DiOC6. $^{*}P < 0.05$ and $^{**}P < 0.01$ show significant decrease in mitochondrial membrane potential. The statistical analysis is done with one-way ANOVA. B: Formation of ceramide in TQ (1–50 μM) stimulated platelets. Arithmetic mean ± SEM (n = 11) for fluorescence of anti-ceramide antibodies. $^{*}P < 0.05$ and $^{**}P < 0.01$ show significant increase in ceramide formation with increasing dose of TQ. The statistical analysis is done with one-way ANOVA.

by TQ. Thus, TQ triggering of caspases involves PTX sensitive GPCR.

DISCUSSION

The present study demonstrates that exposure of human platelets to TQ leads to scrambling of the platelet cell membrane. The effect is paralleled by increase of cytosolic Ca^{2+} activity, depolarization of the mitochondrial potential, ceramide formation, and caspase activation.

TQ has previously been shown to decrease intracellular calcium in mast cells by inhibiting its uptake and stimulating its efflux, effects mediated by inhibition of PKC [Chakravarty, 1993]. In other cells, the effect of TQ was shown to be inhibited by staurosporine, a substance known to inhibit protein kinase C [Tamaoki and Nakano, 1990]. However, staurosporine has been shown to inhibit other kinases [Toledo and Lydon, 1997] and to induce apoptosis of several

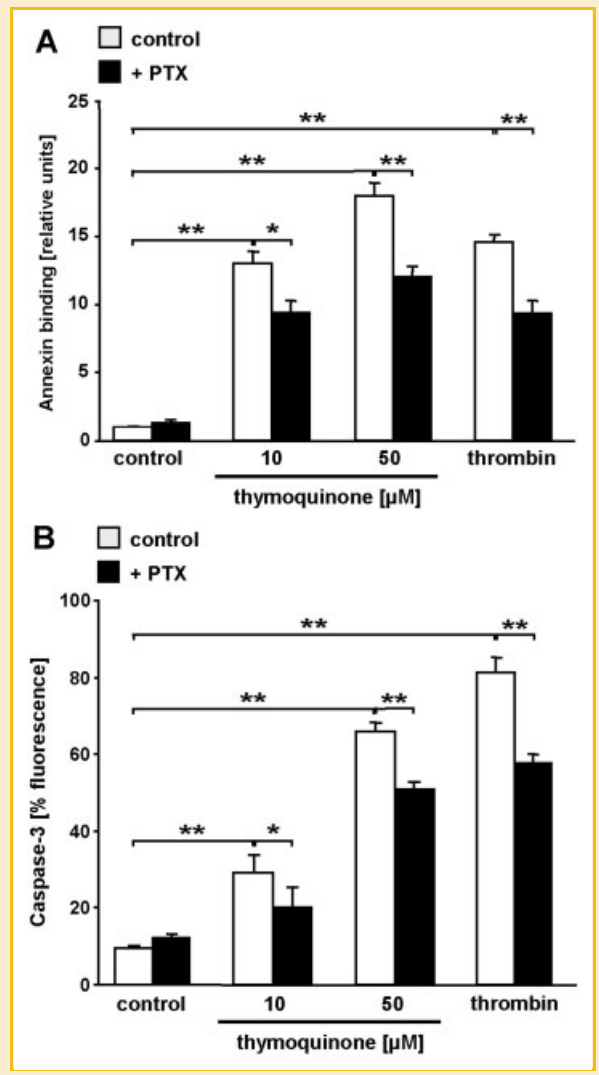

Fig. 5. Effect of the GPCR inhibitor pertussis toxin (PTX) on thymoquinone (TQ)-stimulated human platelets. A: Arithmetic mean ± SEM (n = 5) of Annexin V FITC fluorescence from human platelets preincubated with (black bar) and without (white bar) GPCR inhibitor PTX followed by stimulation with TQ (10 and 50 μM). Thrombin (1 U/ml) stimulation was used as positive control, PBS as negative control. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant difference between PTX-inhibited and non-inhibited platelets under TQ stimulation determined by Student's *t*-test. B: Arithmetic mean ± SEM (n = 5) of anti-active caspase-3-FITC fluorescence from human platelets preincubated with (black bar) and without (white bar) PTX followed by TQ stimulation (10 and 50 μM). Thrombin (1 U/ml) stimulation was used as positive control, PBS as negative control. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant difference between PTX-inhibited and non-inhibited platelets under TQ stimulation determined by Student's *t*-test.

nucleated cells [Bondzio et al., 2008; Leskiewicz et al., 2008; Liu et al., 2008; Vereninov et al., 2008; Lau et al., 2009; Nicolier et al., 2009; Rehm et al., 2009]. TQ has further been shown to inhibit PKB/AKT and extracellular signal-regulated kinase, [Yi et al., 2008], glutathion depletion [Rooney and Ryan, 2005b], and/or caspase activation [El Mahdy et al., 2005; Rooney and Ryan, 2005b]. In this study, pretreatment with GPCR inhibitor PTX [Chakravarty, 1993] or phosphoinositide-3 kinase (PI3K) inhibitor Wortmannin [Chakravarty, 1993] blunted TQ-induced phosphatidylserine exposure and active caspase-3 induction (Figs. 5 and 6). TQ interacts with μ- and κ-opioid receptors [Abdel-Fattah et al., 2000], which are expressed in human platelets [Mehrishi and Mills, 1983]. Since μ- and κ-opioid receptors are coupled to Gαi/o [Clark et al., 2006; Bruchas and Chavkin, 2010], TQ may be effective via Gαi/o-associated GPCR.

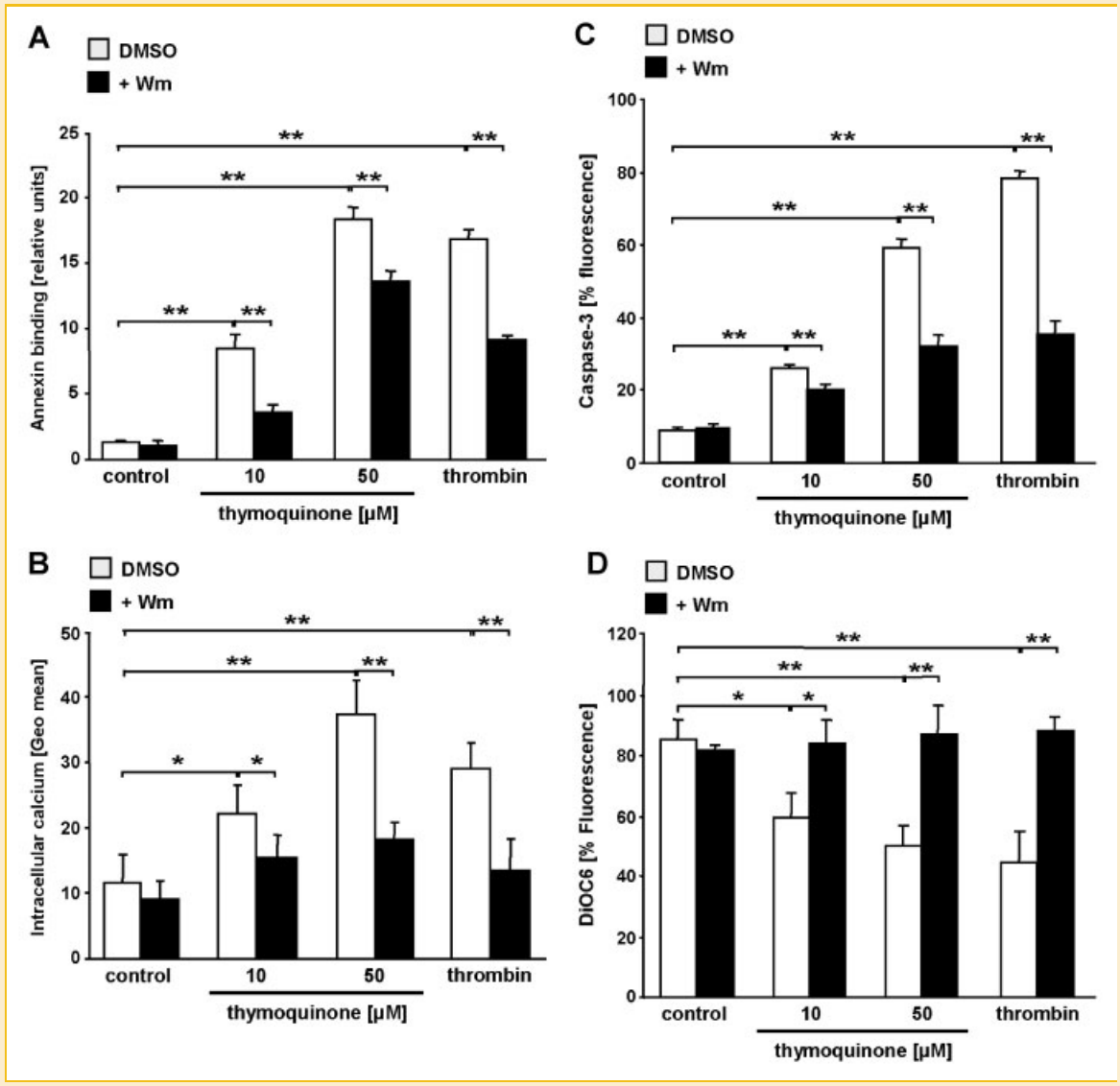

Fig. 6. Effect of PI3K inhibition on thymoquinone (TQ)-stimulated human platelets. A: Arithmetic mean ± SEM (n = 8) of the relative units of Annexin V FITC bound to human platelets following 15 min preincubation with phosphoinositide-3-kinase (PI3K) inhibitor wortmannin (100 nM, black bar) and 30 min incubation with thymoquinone (10 or 50 μM). Platelets not inhibited (DMSO as solvent control) are represented as white bar. Platelets treated with thrombin (1 U/ml) was taken as positive control, platelets without any treatment were taken as negative control. Student's *t*-test between inhibited and non-inhibited platelets exhibited significant difference with $^{*}P < 0.05$ and $^{**}P < 0.01$. B: Arithmetic mean ± SEM (n = 5) from fluorescence of Fluo 3-AM in human platelets treated with (100 nM, black bar) or without wortmannin (DMSO, white bar) and 30-min incubation with TQ (10 and 50 μM) (black bar). Thrombin (1 U/ml) was used as positive control. Student's *t*-test between inhibited and non-inhibited platelets exhibited significant difference with $^{*}P < 0.05$ and $^{**}P < 0.01$. C: Arithmetic mean ± SEM (n = 8) of active caspase-3 in human platelets following 15-min preincubation with (black bar, 100 nM) or without (DMSO as solvent control) PI3K inhibitor wortmannin and 30 min incubation with TQ (10 and 50 μM). Thrombin (1 U/ml) was used as positive control. Student's *t*-test between inhibited and non-inhibited platelets exhibited significant difference with $^{*}P < 0.05$ and $^{**}P < 0.01$. D: Arithmetic mean ± SEM (n = 7) from fluorescence of DiOC6 in human platelets treated with PI3K inhibitor wortmannin (Wm, black bar) or DMSO solvent control (white bar). Student's *t*-test between inhibited and non-inhibited platelets exhibited significant difference with $^{*}P < 0.05$ and $^{**}P < 0.01$.

The thymoquinione concentrations needed for the activation of caspases by TQ are similar to those proven effective in other cells [Mansour and Tornhamre, 2004; Kaseb et al., 2007; Vaillancourt et al., 2011]. In vivo, TQ has proven effective at a daily dosage of 5–10 mg/kg (30–60 μmol/kg) [Mahgoub, 2003; Vaillancourt et al., 2011]. Whether or not this dosage yields plasma concentrations affecting platelet function and/or survival, remains to be shown. Thrombocytemia may be expected to foster bleeding and inadequate platelet activation may result in thrombotic disorders [Jackson, 2007; Ombrello et al., 2010].

TQ may in part be effective through modification of transcription factors, such as p53 [Gali-Muhtasib et al., 2004a, 2008; Roepke et al., 2007] and NFκB [Sethi et al., 2008]. However, TQ has been shown to stimulate cell membrane scrambling in erythrocytes [Qadri et al., 2009], cells devoid of nuclei and unable to modify gene expression.

Two simultaneous events might occur in the stimulated platelets: activation and induction of apoptosis with phosphatidylserine exposure. Activation might be characterized by degranulation-dependent P-selectin exposure on the membrane whereas apoptosis in the non-nucleate cell fragments can be assessed from phosphatidylserine exposure, caspase-3 activation, depolarization of mitochondria, and release of microparticles from platelets [Qadri et al., 2009]. Triggering of Ca^{2+} entry by store depletion and Ca^{2+} ionophore treatment leads in platelets to caspase 3 activation [Ben Amor et al., 2006]. However, thrombin-induced caspase activation and translocation was not abrogated by removal of Ca^{2+} [Ben Amor et al., 2006]. Instead, the effects of thrombin on caspase translocation involves PKC and actin filament polymerization [Amor et al., 2006]. Thrombin is a strong stimulator of platelet activation and death because it has multiple receptors in platelet surface, PAR-1, PAR-4, and GP Ib/IX/V being the reported ones till date [Martorell et al., 2008; Rivera et al., 2009; Li et al., 2010]. Contact between thrombin and its receptors triggers numerous pathways involving different types of G-proteins, intracellular protein kinases and signaling molecules such as diacyl glycerol and guanylyl cyclase [Leytin et al., 2007; Martorell et al., 2008; Stefanini et al., 2009]. The ultimate effect is platelet shape change, activation of PI3K/Akt pathway, calcium-dependent expression of integrin $\alpha_{IIb}\beta_3$ on the membrane and secretion of granular contents [Chakravarty, 1993]. At concentrations of 1–10 U/ml thrombin, generated during blood coagulation, activates caspase-3, depolarizes platelet mitochondria and upregulates pro-apoptotic proteins Bax and Bad [Leytin et al., 2006a], at concentrations of 0.05–0.1 U/ml thrombin triggers only activation [Leytin et al., 2007].

In contrast to the antiapoptotic effect of the PI3K/Akt pathway in most nucleated cells [Duronio, 2008; Hernandez-Aya and Gonzalez-Angulo, 2011] inhibition of PI3K counteracted platelet apoptosis. PI3K is involved in platelet activation through integrin signaling [Kovacsovics et al., 1995]. Agonist-activated platelets are dying [Kulkarni et al., 2007] because platelet activation by strong agonists at high doses such as thrombin and collagen are irreversible, resulting in platelet cytoskeleton destruction and death [Wolf et al., 1999; Brown et al., 2000].

In contrast TQ promotes only platelet apoptosis presumably since degranulation and integrin $\alpha_{IIb}\beta_3$ activation was not upregulated.

In conclusion, exposure of platelets to TQ triggers phospholipid scrambling with cell shrinkage and phosphatidylserine exposure at the platelet surface. The effects are expected to accelerate the clearance of platelets from circulating blood and thus predispose to the development of thrombocytopenia.

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REFERENCES

Abdel-Fattah AM, Matsumoto K, Watanabe H. 2000. Antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, in mice. Eur J Pharmacol 400:89–97.

Aggarwal BB, Kunnumakkara AB, Harikumar KB, Tharakan ST, Sung B, Anand P. 2008. Potential of spice-derived phytochemicals for cancer prevention. Planta Med 74:1560–1569.

Ali BH, Blunden G. 2003. Pharmacological and toxicological properties of *Nigella sativa*. Phytother Res 17:299–305.

Amor NB, Pariente JA, Salido GM, Rosado JA, Bartegi A. 2006. Thrombin-induced caspases 3 and 9 translocation to the cytoskeleton is independent of changes in cytosolic calcium in human platelets. Blood Cells Mol Dis 36:392–401.

Antovic A. 2010. The overall hemostasis potential: A laboratory tool for the investigation of global hemostasis. Semin Thromb Hemost 36:772–779.

Badary OA, Abd-Ellah MF, El Mahdy MA, Salama SA, Hamada FM. 2007. Anticlastogenic activity of thymoquinone against benzo(a)pyrene in mice. Food Chem Toxicol 45:88–92.

Ben Amor N, Pariente JA, Salido GM, Rosado JA, Bartegi A. 2006. Thrombin-induced caspases 3 and 9 translocation to the cytoskeleton is independent of changes in cytosolic calcium in human platelets. Blood Cells Mol Dis 36:392–401.

Bondzio A, Stumpff F, Schon J, Martens H, Einspanier R. 2008. Impact of *Bacillus thuringiensis* toxin Cry1Ab on rumen epithelial cells (REC)—A new in vitro model for safety assessment of recombinant food compounds. Food Chem Toxicol 46:1976–1984.

Brown SB, Clarke MC, Magowan L, Sanderson H, Savill J. 2000. Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis. A caspase-independent cell clearance program. J Biol Chem 275:5987–5996.

Bruchas MR, Chavkin C. 2010. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. Psychopharmacology (Berl) 210:137–147.

Carbonetti NH. 2010. Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools. Future Microbiol 5:455–469.

Chakravarty N. 1993. Inhibition of histamine release from mast cells by nigellone. Ann Allergy 70:237–242.

Clark MJ, Furman CA, Gilson TD, Traynor JR. 2006. Comparison of the relative efficacy and potency of mu-opioid agonists to activate Galpha(i/o) proteins containing a pertussis toxin-insensitive mutation. J Pharmacol Exp Ther 317:858–864.

Duronio V. 2008. The life of a cell: Apoptosis regulation by the PI3K/PKB pathway. Biochem J 415:333–344.

El Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA. 2005. Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells. Int J Cancer 117:409–417.

Fan QW, Weiss WA. 2010. Targeting the RTK-PI3K-mTOR axis in malignant glioma: Overcoming resistance. Curr Top Microbiol Immunol 347:279–296.

Gali-Muhtasib H, Diab-Assaf M, Boltze C, Al Hmaira J, Hartig R, Roessner A, Schneider-Stock R. 2004a. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. Int J Oncol 25:857–866.

Gali-Muhtasib HU, Abou Kheir WG, Kheir LA, Darwiche N, Crooks PA. 2004b. Molecular pathway for thymoquinone-induced cell-cycle arrest and apoptosis in neoplastic keratinocytes. Anticancer Drugs 15:389–399.

Gali-Muhtasib H, Roessner A, Schneider-Stock R. 2006. Thymoquinone: A promising anti-cancer drug from natural sources. Int J Biochem Cell Biol 38:1249–1253.

Gali-Muhtasib H, Ocker M, Kuester D, Krueger S, El Hajj Z, Diestel A, Evert M, El Najjar N, Peters B, Jurjus A, Roessner A, Schneider-Stock R. 2008. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. J Cell Mol Med 12:330–342.

Hawkins PT, Stephens LR, Suire S, Wilson M. 2010. PI3K signaling in neutrophils. Curr Top Microbiol Immunol 346:183–202.

Hernandez-Aya LF, Gonzalez-Angulo AM. 2011. Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer. Oncologist 16:404–414.

Jackson SP. 2007. The growing complexity of platelet aggregation. Blood 109:5087–5095.

Kaseb AO, Chinnakannu K, Chen D, Sivanandam A, Tejwani S, Menon M, Dou QP, Reddy GP. 2007. Androgen receptor and E2F-1 targeted thymoquinone therapy for hormone-refractory prostate cancer. Cancer Res 67:7782–7788.

Khader M, Bresgen N, Eckl PM. 2009. In vitro toxicological properties of thymoquinone. Food Chem Toxicol 47:129–133.

Kovacsovics TJ, Bachelot C, Toker A, Vlahos CJ, Duckworth B, Cantley LC, Hartwig JH. 1995. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. J Biol Chem 270:11358–11366.

Krone RJ. 2010. Managing coronary artery disease in the cancer patient. Prog Cardiovasc Dis 53:149–156.

Kulkarni S, Woollard KJ, Thomas S, Oxley D, Jackson SP. 2007. Conversion of platelets from a proaggregatory to a proinflammatory adhesive phenotype: role of PAF in spatially regulating neutrophil adhesion and spreading. Blood 110:1879–1886.

Lau PN, Chow KB, Chan CB, Cheng CH, Wise H. 2009. The constitutive activity of the ghrelin receptor attenuates apoptosis via a protein kinase C-dependent pathway. Mol Cell Endocrinol 299:232–239.

Leskiewicz M, Regulska M, Budziszewska B, Jantas D, Jaworska-Feil L, Basta-Kaim A, Kubera M, Lason W. 2008. Neurosteroids enhance the viability of staurosporine and doxorubicin treated differentiated human neuroblastoma SH-SY5Y cells. Pharmacol Rep 60:685–691.

Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. 2006a. Thrombin-triggered platelet apoptosis. J Thromb Haemost 4:2656–2663.

Leytin V, Mykhaylov S, Starkey AF, Allen DJ, Lau H, Ni H, Semple JW, Lazarus AH, Freedman J. 2006b. Intravenous immunoglobulin inhibits anti-glycoprotein IIb-induced platelet apoptosis in a murine model of immune thrombocytopenia. Br J Haematol 133:78–82.

Leytin V, Allen DJ, Lyubimov E, Freedman J. 2007. Higher thrombin concentrations are required to induce platelet apoptosis than to induce platelet activation. Br J Haematol 136:762–764.

Li Z, Delaney MK, O'Brien KA, Du X. 2010. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol 30:2341–2349.

Liu AH, Cao YN, Liu HT, Zhang WW, Liu Y, Shi TW, Jia GL, Wang XM. 2008. DIDS attenuates staurosporine-induced cardiomyocyte apoptosis by PI3K/Akt signaling pathway: Activation of eNOS/NO and inhibition of Bax translocation. Cell Physiol Biochem 22:177–186.

Mahgoub AA. 2003. Thymoquinone protects against experimental colitis in rats. Toxicol Lett 143:133–143.

Mansour M, Tornhamre S. 2004. Inhibition of 5-lipoxygenase and leukotriene C4 synthase in human blood cells by thymoquinone. J Enzyme Inhib Med Chem 19:431–436.

Martorell L, Martinez-Gonzalez J, Rodriguez C, Gentile M, Calvayrac O, Badimon L. 2008. Thrombin and protease-activated receptors (PARs) in atherothrombosis. Thromb Haemost 99:305–315.

McCloskey DJ, Postolache TT, Vittone BJ, Nghiem KL, Monsale JL, Wesley RA, Rick ME. 2008. Selective serotonin reuptake inhibitors: Measurement of effect on platelet function. Transl Res 151:168–172.

Mehrishi JN, Mills IH. 1983. Opiate receptors on lymphocytes and platelets in man. Clin Immunol Immunopathol 27:240–249.

Nicolier M, Decrion-Barthod AZ, Launay S, Pretet JL, Mougin C. 2009. Spatiotemporal activation of caspase-dependent and -independent pathways in staurosporine-induced apoptosis of p53wt and p53mt human cervical carcinoma cells. Biol Cell 101:455–467.

Ombrello C, Block RC, Morrell CN. 2010. Our expanding view of platelet functions and its clinical implications. J Cardiovasc Transl Res 3:538–546.

Pandey SC, Davis JM, Schwertz DW, Pandey GN. 1991. Effect of antidepressants and neuroleptics on phosphoinositide metabolism in human platelets. J Pharmacol Exp Ther 256:1010–1018.

Qadri SM, Mahmud H, Foller M, Lang F. 2009. Thymoquinone-induced suicidal erythrocyte death. Food Chem Toxicol 47:1545–1549.

Rehm M, Huber HJ, Hellwig CT, Anguissola S, Dussmann H, Prehn JH. 2009. Dynamics of outer mitochondrial membrane permeabilization during apoptosis. Cell Death Differ 16:613–623.

Rivera J, Lozano ML, Navarro-Nunez L, Vicente V. 2009. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica 94:700–711.

Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, Schneider-Stock R, Gali-Muhtasib H. 2007. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. Cancer Biol Ther 6:160–169.

Rooney S, Ryan MF. 2005a. Effects of alpha-hederin and thymoquinone, constituents of *Nigella sativa*, on human cancer cell lines. Anticancer Res 25:2199–2204.

Rooney S, Ryan MF. 2005b. Modes of action of alpha-hederin and thymoquinone, active constituents of *Nigella sativa*, against HEp-2 cancer cells. Anticancer Res 25:4255–4259.

Ruggeri ZM. 2002. Platelets in atherothrombosis. Nat Med 8:1227–1234.

Salem ML. 2005. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. Int Immunopharmacol 5:1749–1770.

Sethi G, Ahn KS, Aggarwal BB. 2008. Targeting nuclear factor-kappa B activation pathway by thymoquinone: Role in suppression of antiapoptotic gene products and enhancement of apoptosis. Mol Cancer Res 6:1059–1070.

Shoieb AM, Elgayyar M, Dudrick PS, Bell JL, Tithof PK. 2003. In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. Int J Oncol 22:107–113.

Stefanini L, Roden RC, Bergmeier W. 2009. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. Blood 114:2506–2514.

Tamaoki T, Nakano H. 1990. Potent and specific inhibitors of protein kinase C of microbial origin. Biotechnology (NY) 8:732–735.

Toledo LM, Lydon NB. 1997. Structures of staurosporine bound to CDK2 and cAPK—New tools for structure-based design of protein kinase inhibitors. Structure 5:1551–1556.

Vaillancourt F, Silva P, Shi Q, Fahmi H, Fernandes JC, Benderdour M. 2011. Elucidation of molecular mechanisms underlying the protective effects of thymoquinone against rheumatoid arthritis. J Cell Biochem 112:107–117.

Vereninov AA, Rubashkin AA, Goryachaya TS, Moshkov AV, Rozanov YM, Shirokova AV, Strelkova EG, Lang F, Yurinskaya VE. 2008. Pump and channel K (Rb+) fluxes in apoptosis of human lymphoid cell line U937. Cell Physiol Biochem 22:187–194.

Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, Green DR. 1999. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood 94:1683–1692.

Xuan NT, Shumilina E, Qadri SM, Gotz F, Lang F. 2010. Effect of thymoquinone on mouse dendritic cells. Cell Physiol Biochem 25:307–314.

Yi T, Cho SG, Yi Z, Pang X, Rodriguez M, Wang Y, Sethi G, Aggarwal BB, Liu M. 2008. Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways. Mol Cancer Ther 7:1789–1796.

Zimmermann KC, Bonzon C, Green DR. 2001. The machinery of programmed cell death. Pharmacol Ther 92:57–70.