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Mitochondria-targeted triphenylphosphonium-based compounds inhibit FcεRI-dependent degranulation of mast cells by preventing mitochondrial dysfunction through Erk1/2

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ABSTRACT

Aims: FcεRI-dependent activation and degranulation of mast cells (MC) play an important role in allergic diseases. We have previously demonstrated that triphenylphosphonium (TPP)-based antioxidant SkQ1 inhibits mast cell degranulation, but the exact mechanism of this inhibition is still unknown. This study focused on investigating the influence of TPP-based compounds SkQ1 and C_{12}TPP on FcεRI-dependent mitochondrial dysfunction and signaling during MC degranulation.

Main methods: MC were sensitized by anti-dinitrophenyl IgE and stimulated by BSA-conjugated dinitrophenyl. The degranulation of MC was estimated by β-hexosaminidase release. The effect of TPP-based compounds on FcεRI-dependent signaling was determined by Western blot analysis for adapter molecule LAT, kinases Syk, PI3K, Erk1/2, and p38. Fluorescent microscopy was used to evaluate mitochondrial parameters such as morphology, membrane potential, reactive oxygen species and ATP level.

Key findings: Pretreatment with TPP-based compounds significantly decreased FcεRI-dependent degranulation of MC. TPP-based compounds also prevented mitochondrial dysfunction (drop in mitochondrial ATP level and mitochondrial fission), and decreased Erk1/2 kinase phosphorylation. Selective Erk1/2 inhibition by U0126 also reduced β-hexosaminidase release and prevented mitochondrial fragmentation during FcεRI-dependent degranulation of MC.

Significance: These findings expand the fundamental understanding of the role of mitochondria in the activation of MC. It also contributes to the rationale for the development of mitochondrial-targeted drugs for the treatment of allergic diseases.

1. Introduction

Mast cells (MC) are involved in many inflammatory and allergic diseases, including mastocytosis, bronchial asthma, atopic dermatitis, psoriasis, neuroinflammation and brain disorders [1]. The signaling through the high-affinity IgE receptor FcεRI is studied in detail. FcεRI-dependent signaling results in the release of pre-formed mediators via calcium-dependent cell degranulation and subsequent synthesis of eicosanoids and cytokines. This leads to the inflammation of surrounding tissues [2,3].

There is growing evidence of mitochondrial involvement in FcεRI-dependent activation. MC degranulation is accompanied by mitochondrial fragmentation mediated by GTPase Drp1, and subsequent translocation of the mitochondria to the plasma membrane. Inhibition of Drp1 suppresses mitochondrial fragmentation and their movement to the plasma membrane, reduces degranulation of MC [4]. Mitochondrial

Abbreviations: ATP, adenosine triphosphate; C_{12}TPP, dodecyltriphenylphosphonium bromide; DNP-BSA, dinitrophenyl hapten molecules, conjugated to Bovine Serum Albumin; Drp1, dynamin related protein 1; Erk1/2, extracellular signal-regulated kinase; ETC, electron transport chain; FBS, fetal bovine serum; FcεRI, high-affinity IgE receptor; HBSS, Hanks' balanced salt solution; IgE, immunoglobulin E; MC, mast cells; OXPHOS, oxidative phosphorylation; PBS, phosphate buffered saline; RBL-2H3, rat basophilic leukemia; ROS, reactive oxygen species; SkQ1, 10-(6′-plastoquinonyl)decyltriphenylphosphonium; STAT3, signal transducer and activator of transcription 3; TPP, triphenylphosphonium; ΔΨm, mitochondrial membrane potential.

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fission is a tightly regulated process resulting in the division of one mitochondrion into two daughter organelles [5]. Fragmented mitochondria may be transported to the cell compartments according to their demands, or, alternatively, be subjected to elimination by mitophagy. Mitochondrial fragmentation happens in response to multiple stimuli. It can facilitate the metabolic adaptation of the cell, mitochondrial distribution and quality control [6].

Little is known about the interconnection between mitochondrial function, mitochondrial fragmentation, and degranulation of MC. It was found that mitochondrial UCP2 protein regulating mitochondrial ROS production suppresses histamine production and MC degranulation [7]. The degranulation of stimulated MC is also inhibited by some uncouplers of respiration and oxidative phosphorylation (OXPHOS) [8–10].

Recently, many mitochondria-targeted compounds started to be used as intracellular sensors and promising anti-inflammatory drugs. These compounds were shown to affect redox status, mitochondrial electron transport chain (ETC) activity, ATP synthesis, etc. [11]. Many of these compounds are chemically bound to the lipophilic cation triphenylphosphonium (TPP) that specifically accumulates in negatively charged mitochondria. TPP-based compounds, such as the membrane-penetrating cation dodecyltriphenylphosphonium bromide (C_{12}TPP) and its conjugates with antioxidant plastoquinone SkQ1 and antioxidant ubiquinone MitoQ, prevent mitochondrial fragmentation in various cell cultures [12–14]. Our previous data demonstrate that SkQ1 inhibits MC degranulation, but the mechanism is still unknown [15]. Thus, we hypothesized that mitochondria-targeted TPP-based compounds may decrease MC activation by preventing mitochondrial fragmentation.

2. Material and methods

2.1. Cell culture

RBL-2H3 cells (purchased from ATCC) were cultivated in the medium containing 70% α-MEM, 20% RPMI-1640, 10% fetal bovine serum (FBS), 2 mM L-glutamine (PanEco, Russia) at 37 °C and 5% CO_2. The cells were passaged every 3 days at 1:4 to 1:8 dilution.

2.2. Cytotoxicity assay

Cytotoxic activity of C_{12}TPP (Sigma, USA) was estimated in the MTT test. RBL-2H3 cells were plated in a 96-well plate (10,000 cells/well) and treated with 0.2–400 nM of C_{12}TPP for 72 h at 37 °C and 5% CO_2. On day 4, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Chem Impex Intl Inc., USA)) was added to 5 mg/ml, and the cells were incubated for 3 h at 37 °C and 5% CO_2. The precipitate was dissolved in 100 μl DMSO. The absorbance of the samples was determined with the iMARK Microplate Reader (BioRad, USA) at 540 nm.

2.3. Beta-hexosaminidase release

RBL-2H3 cells were plated on 24-well plates (100,000 cells/well) and treated with 0.2 and 2 nM of SkQ1 (kindly provided by the Institute of Mitoengineering, Lomonosov Moscow State University, Russia) and C_{12}TPP for 72 h or with 10 μM of U0126 (Cell Signalling, USA) for 2 h. After treatment, cells were incubated for 16 h with 0.2 μg/ml monoclonal anti-dinitrophenyl antibody (anti-DNP IgE) (Sigma, USA) and washed in Hanks' Balanced Salt Solution (HBSS) (PanEco, Russia). Degranulation was activated by incubating cells with 1 μg/ml dinitrophenyl hapten molecules, conjugated to Bovine Serum Albumin (DNP-BSA) (Molecular Probes, USA) in HBSS for 15 min.

The activity of β-hexosaminidase, a marker of RBL-2H3 cells degranulation, was measured in the supernatant of centrifuged conditioned medium (4390g, 7 min, 4 °C) and in the cell lysate (0.2% Triton X-100 in HBSS, 10 min, 37 °C) by adding 25 μl of samples to 100 μl of 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (1.8 mM solution in 40 mM citrate buffer, pH 4.5) (Chem Impex Intl Inc., USA) for 30 min at 37 °C. The reaction was terminated by adding 175 μl of glycine/NaOH buffer (200 mM, pH 10.7). The absorbance was measured at 460 nm (excitation wavelength 355 nm) with a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, USA). The release level of β-hexosaminidase (%) in the sample was determined using the formula A / (A + B) × 100%, where A is fluorescence intensity of the conditioned medium, and B is fluorescence intensity of the cell lysate.

2.4. Mitochondria visualization with MitoTracker Green

Cells were plated on 35-mm^2 confocal dishes (200,000 cells/dish) and treated with 0.2 nM of SkQ1 and C_{12}TPP for 72 h or with 10 μM of U0126 for 2 h. After treatment, cells were sensitized by anti-DNP IgE for 16 h, washed with medium and incubated with 500 nM MitoTracker Green (Invitrogen, USA) for 30 min at 37 °C and 5% CO_2. After washing in the HBSS, cells were observed using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Germany) equipped with an AxioCAM HRM camera at a magnification of ×1000. Then cells were stimulated by DNP-BSA for 15 min at 37 °C and 5% CO_2 to induce FcεRI-dependent degranulation and then observed under a microscope.

2.5. Immunofluorescence

Cells were grown on glass coverslips and treated with 0.2 nM of SkQ1 and C_{12}TPP for 72 h. After treatment cells were sensitized by anti-DNP IgE for 16 h and stimulated by DNP-BSA for 15 min. After washing in medium cells were fixed with 2% paraformaldehyde in PBS/medium mix 1:1 for 10 min at 37 °C and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Then cells were stained with anti-cytochrome C monoclonal antibodies (BD Pharmingen, USA) with phalloidin-TRITC (Sigma, USA), and Hoechst 33342 (Biotium, USA).

2.6. Reactive oxygen species detection assay

Mitochondrial reactive oxygen species (ROS) were assayed with MitoTracker Orange CM-H_2TMRos (Thermo Scientific, USA). Cells were plated in a 96-well plate (10,000 cells/well) and treated with 0.2 nM of SkQ1 and C_{12}TPP for 72 h. After treatment, cells were incubated with 500 nM MitoTracker Orange CM-H_2TMRos for 30 min at 37 °C and 5% CO_2. Then cell degranulation was induced. After washing in HBSS, fluorescence was monitored at 560 nm and 590 nm emission with a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, USA).

2.7. Mitochondrial membrane potential assay

Mitochondrial membrane potential (ΔΨm) was assayed with TMRM (Thermo Scientific, USA). Cells were plated in a 96-well plate (10,000 cells/well) and treated with 0.2 nM of SkQ1 and C_{12}TPP for 72 h. After treatment, cell degranulation was induced. Then cells were incubated with 50 nM TMRM for 30 min at 37 °C and 5% CO_2. After washing in HBSS, fluorescence was monitored at 560 nm and 590 nm emission with a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, USA).

2.8. Plasmid cloning for mitochondrial ATP imaging

A duplex of mitochondrial targeting signal of cytochrome *c* oxidase subunit VIII from mitAT1.03 plasmid [16] was amplified by PCR using the following primers: Forward - 5′-TGCATGGGCCCCCCCTCTAGACTCGAG-3′ and Reverse - 5′-TCGTCAGATCTCTTGGGATCGCCGAGA-3′. The amplified product (224 bp) was digested with *Apa*I-*Bgl*II and cloned into the cyto-iATPSnFR1.0 plasmid [17] at the same restriction sites. The plasmid cyto-iATPSnFR1.0 was a gift from Baljit Khakh (Addgene plasmid # 102550). The N-terminal HA-tag prevented the translocation of the ATP-sensor into the mitochondria and was

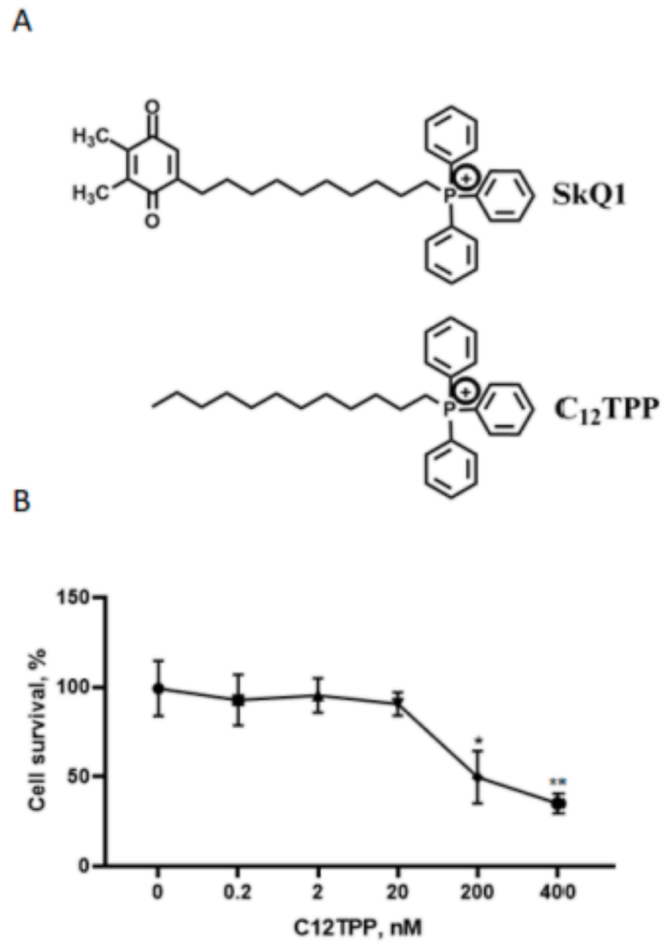

Fig. 1. (A) Chemical structure of SkQ1 and C_{12}TPP; (B) the effect of C_{12}TPP on the viability of RBL-2H3 cells. Cells were incubated with 0.2–400 nM of C_{12}TPP for 72 h and viability was measured by MTT test. Results are expressed as mean ± SD (n = 4). *p ≤ 0.05, **p ≤ 0.01 as compared to the untreated control cells calculated by one-way ANOVA, Dunnett test.

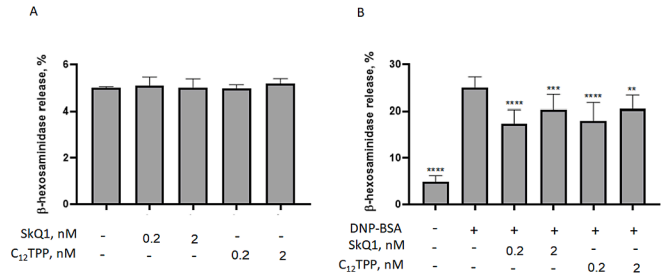

Fig. 2. The effect of TPP-based compounds on spontaneous (A) and FcεRI-dependent (B) degranulation of RBL-2H3 cells. Cells were incubated with SkQ1 and C_{12}TPP at concentrations of 0.2 and 2 nM for 72 h. To induce FcεRI-dependent degranulation RBL-2H3 cells were sensitized by anti-DNP IgE and stimulated by DNP-BSA for 15 min. The level of degranulation was estimated by beta-hexosaminidase release. Results are expressed as mean ± SD (n ≥ 6). **p ≤ 0.01, ***p ≤ 0.001, **** ≤ 0.0001 as compared to the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test.

excised by *Eco*RI-*Xba*I digestion, followed by fill-in and self-ligation. The nucleotide sequence of the resulting plasmid mito-iATPSnFR1.0 was verified by Sanger sequencing. Thus we obtained a single-wavelength sensor for mitochondrial ATP imaging.

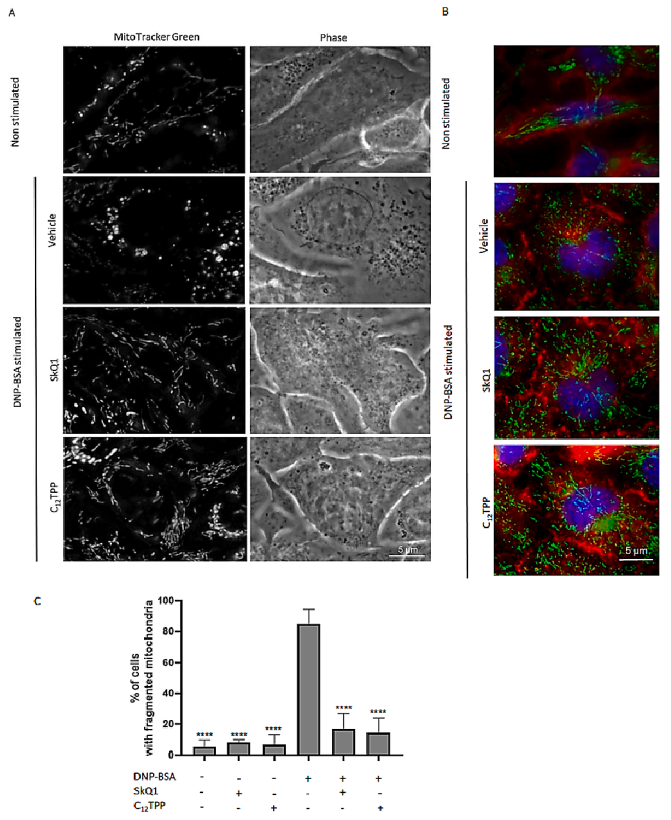

Fig. 3. The effect of TPP-based compounds on mitochondrial fragmentation during FcεRI-dependent degranulation of RBL-2H3 cells. (A) Fluorescence microscopy of the mitochondria (labeled with MitoTracker Green) in the RBL-2H3 cells (left) and phase-contrast microscopy (right). Cells were treated with 0.2 nM of SkQ1 and C_{12}TPP for 72 h. After treatment, cells were sensitized by anti-DNP IgE, washed with medium and incubated with 500 nM MitoTracker Green for 30 min. After washing in the HBSS, cells were stimulated by DNP-BSA for 15 min to induce FcεRI-dependent degranulation. (B) Immunofluorescence of the mitochondria (green), actin cytoskeleton (red) and nuclei (blue) in the RBL-2H3 cells. After treatment with 0.2 nM of SkQ1 and C_{12}TPP for 72 h cells were sensitized by anti-DNP IgE for 16 h and stimulated by DNP-BSA for 15 min. After washing in medium cells were fixed with 2% paraformaldehyde and stained with anti-cytochrome C monoclonal antibodies (mitochondria) with phalloidin-TRITC (actin cytoskeleton), and Hoechst 33342 (nuclei). (C) The percentage of cells with mainly fragmented mitochondria. From 100 to 200 cells were analyzed in four independent experiments. Results are expressed as mean ± SD (n ≥ 6). ****p ≤ 0.0001 as compared with the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Mitochondrial ATP visualization

2,000,000 RBL cells were transfected with 10 μg mito-iATPSnFR1.0 plasmid in 400 μl ice-cold culture medium by electroporation (1000 μF, 250 V, 0.4 cm-cuvettes) using Gene Pulser Xcell Electroporation System (BioRad, USA) and plated on 35-mm^2 confocal dishes. The next day, cells were exposed to 0.2 nM of SkQ1 and 0.2 μg/ml anti-DNP IgE for 18 h. After washing in the HBSS, fluorescence was monitored using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Germany) equipped with an AxioCAM HRM camera at a magnification of ×1000. Then cells were stimulated with 1 μg/ml DNP-BSA for 15 min and fluorescence was detected. Fluorescence intensities of mitochondria were calculated with FIJI software.

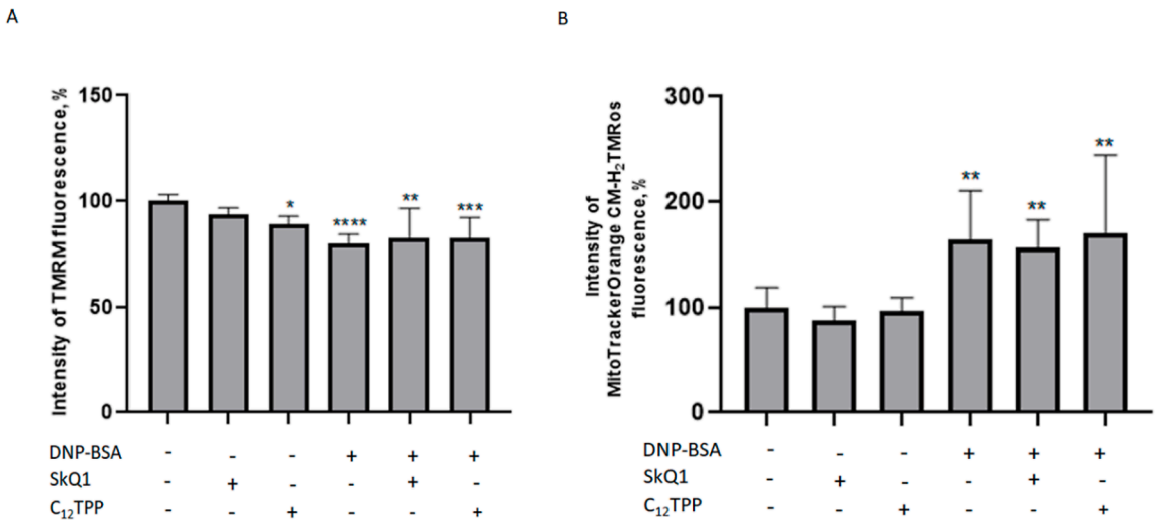

Fig. 4. The effect of TPP-based compounds on mitochondrial membrane potential (A) and ROS production (B) during FcεRI-dependent degranulation of RBL-2H3 cells. Cells were treated with SkQ1 and C_{12}TPP for 72 h. After treatment, cells were incubated with 500 nM MitoTracker Orange CM-H_2TMRos for 30 min to detect ROS. cell degranulation was induced. Then cell degranulation was induced. After washing in medium, cells were incubated with 50 nM TMRM for 30 min to detect mitochondrial membrane potential. Fluorescence was monitored at 560 nm and 590 nm emission with a Fluoroskan Ascent microplate fluorometer. Results are expressed as mean ± SD (n = 4). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 as compared with the untreated non-stimulated cells calculated by one-way ANOVA, Dunnett test.

2.10. Western immunoblotting

Cells were washed with cold PBS then lysed in sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 50 mM DTT; 0,01% bromophenol blue) and heated at 95 °C for 3 min. Proteins were separated on 12% polyacrylamide gel and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, USA). The membranes were incubated with the primary antibodies against LAT, phospho-LAT(Tyr191), Syk, phospho-Syk (Tyr525/526), PI3K p85, phospho-PI3K p85 (Tyr458)/p55 (Tyr199), p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) (Cell Signalling, USA), STAT3 and phospho-STAT3 (Ser727) (Cell Applications Inc., USA). As secondary antibodies, HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG (Sigma, USA) were used. The membranes were developed by BioRad ChemiDoc™ XR+ System using extended duration substrate Super-Signal West Dura (Thermo Scientific, USA). Densitometric analysis of the blots was done by Image Lab software.

2.11. Data analysis

The data were analyzed statistically and graphs were plotted using the GraphPad Prism6 software. Data were analyzed using one-way analysis of variance (ANOVA) with a Dunnett test. Data are presented as mean ± SD.

Image processing was performed using FIJI software.

3. Results

3.1. Effect of TPP-based compounds on the FcεRI-dependent degranulation of RBL-2H3 cells

According to our previously published data, SkQ1 had no influence on cell viability of RBL-2H3 cells up to a concentration of 800 nM as determined by MTT-test for up to 72 h of incubation [15], while C_{12}TPP decreased cell viability at concentrations higher than 200 nM (Fig. 1).

We have previously demonstrated that long-term incubation of RBL-2H3 cells with SkQ1 at low concentrations (0.2 and 2 nM) significantly decreased antigen-stimulated degranulation of cells [15]. In the current study, we used the long-term pretreatment (18–72 h) of RBL-2H3 cells with 0.2–2 nM of SkQ1 and C_{12}TPP. These compounds had no effect on spontaneous degranulation level measured in the absence of the antigen (Fig. 2A).

Meanwhile, both compounds decreased antigen-induced release of β-hexosaminidase by 30% at a concentration of 0.2 nM and by 20% at a concentration of 2 nM (Fig. 2B). Thus, we have observed the inhibitory effect of TPP-based compounds on FcεRI-dependent degranulation of RBL-2H3 cells.

3.2. TPP-based compounds decrease mitochondrial fragmentation during FcεRI-dependent degranulation of RBL-2H3 cells

To detect whether the inhibitory effect of TTP-based compounds on MC degranulation is associated with the change in mitochondrial morphology, we visualized mitochondria using MitoTracker Green fluorescent dye and by detecting cytochrome *c* immunofluorescence (Fig. 3). In unstimulated RBL-2H3 cells, most mitochondria had a tubular shape. Cells stimulated by an antigen for 15 min underwent mitochondrial fragmentation and actin cytoskeleton reorganization. Pretreatment of RBL-2H3 cells with TPP-based compounds prevented antigen-induced mitochondrial fission but did not affect actin rearrangement (Fig. 3).

3.3. TPP-based compounds do not change mitochondrial membrane potential and ROS production during FcεRI-dependent degranulation of RBL-2H3 cells

In general, mitochondrial fragmentation is accompanied by decreased mitochondrial membrane potential (ΔΨm) and increased mitochondrial ROS production. These characteristics are considered to be hallmarks of mitochondrial dysfunction [18–21]. To measure ΔΨm and mtROS we have used fluorescent probes TMRM, and MitoTracker Orange CM-H2TMRos, respectively. Antigen stimulation of cells for 15 min resulted in a decrease in ΔΨm (by 20%) and an increase in mitochondrial ROS level ~50% (Fig. 4). The TPP-based compounds had no effect on these parameters (Fig. 4).

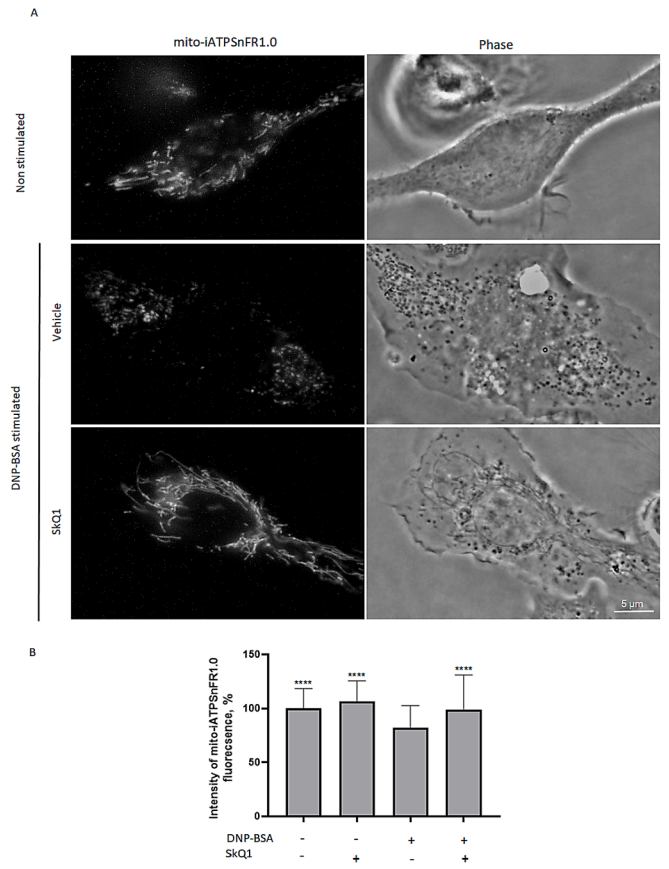

Fig. 5. The effect of SkQ1 on mitochondrial ATP level during FcεRI-dependent degranulation of RBL-2H3 cells. (A) Fluorescence microscopy of the mitochondrial ATP in RBL-2H3 cells transfected with mito-iATPSnFR1.0 (left) in the RBL-2H3 cells and phase-contrast microscopy (right). Cells were transfected with mito-iATPSnFR1.0 by electroporation, then treated 0.2 nM SkQ1 and sensitized by anti-DNP IgE for 18 h. After washing in the HBSS, fluorescence was monitored using an Axiovert 200 M fluorescence microscope. Then cells were stimulated with DNP-BSA for 15 min and fluorescence was detected. (B) Fluorescence intensities of mito-iATPSnFR1.0 were calculated in cell mitochondria in four independent experiments. Results are expressed as mean ± SD (n = 4). ****p ≤ 0.0001 as compared with the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test.

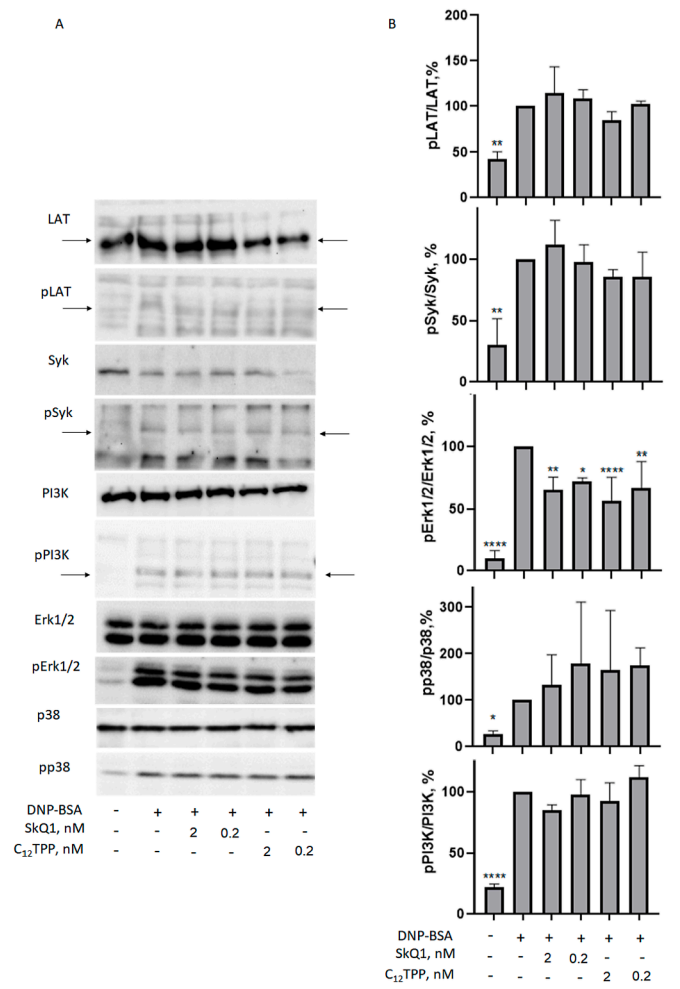

Fig. 6. The effect of TPP-based compounds on FcεRI-induced phosphorylation of signaling molecules in RBL-2H3 cells. Cells were treated as shown in Fig. 3 and used for Western blot analysis. (A) Representative Western blots of cell lysates; (B) histograms denoting the relative amounts of phospho-proteins. Results are expressed as mean ± SD (n ≥ 6). *p ≤ 0.05, **p ≤ 0.01, **** ≤ 0.0001 as compared with the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test.

3.4. SkQ1 prevents the decrease in the mitochondrial ATP production during FcεRI-dependent degranulation of RBL-2H3 cells

We assumed that antigen-induced mitochondrial fragmentation is accompanied by a decrease in OXPHOS activity and ATP level. To measure the mitochondrial ATP level we constructed a single-wavelength sensor mito-iATPSnFR1.0 and transfected RBL-2H3 cells. Antigen-stimulated mast cells demonstrated a ~20% decrease in the level of mitochondrial ATP compared to the control unstimulated cells (Fig. 5). SkQ1 prevented the decrease in mitochondrial ATP level (Fig. 5).

3.5. Effect of TPP-based compounds on the component of FcεRI-dependent signaling

To study signaling pathways involved in the inhibitory effects of TPP-based compounds we investigated the activation of several key components of FcεRI-dependent signaling by Western blotting. Among them are molecules such as adapter molecule LAT, kinases Syk, PI3K, Erk1/2, and p38 (Fig. 6).

Pretreatment of RBL-2H3 cells with TPP-based compounds resulted in a decrease in the Erk1/2 kinase activity (by 30–40%) and did not affect other components of FcεRI-dependent signaling (Fig. 6). To test whether the inhibitory effect of SkQ1 and C_{12}TPP on the antigen-dependent mitochondrial fragmentation of RBL-2H3 cells is associated with Erk1/2 activity we used U0126, an inhibitor of MEK1/2 kinase. Incubation of RBL-2H3 cells with 10 μM U0126 for 2 h before antigen stimulation reduced the release of β-hexosaminidase by 24% (Fig. 7A) and prevented mitochondrial fragmentation (Fig. 7B, C).

Mitochondrial fragmentation during MC degranulation is reported to be mediated by GTPase Drp1 phosphorylated at serine 616 [4]. Erk1/2-dependent mitochondrial fission also relies on Drp1 [22]. Thus we measured relative Drp1 serine 616 phosphorylation by Western blot analysis. Unexpectedly, FcεRI-dependent degranulation of RBL-2H3 did not influence the relative Drp1 phosphorylation level (Fig. 8).

Antigen-dependent activation of MC may be accompanied by Erk1/2-dependent phosphorylation of mitochondrial STAT3, which leads to an increase in OXPHOS activity [23]. We checked whether the inhibitory effect of SkQ1 and C_{12}TPP is associated with Erk1/2-dependent phosphorylation of mitochondrial STAT3. Using Western blotting, we showed that pretreatment of RBL-2H3 cells with SkQ1 and C_{12}TPP did not affect the change in the level of STAT3 phosphorylation at serine 727 after antigen-dependent stimulation (Fig. 8).

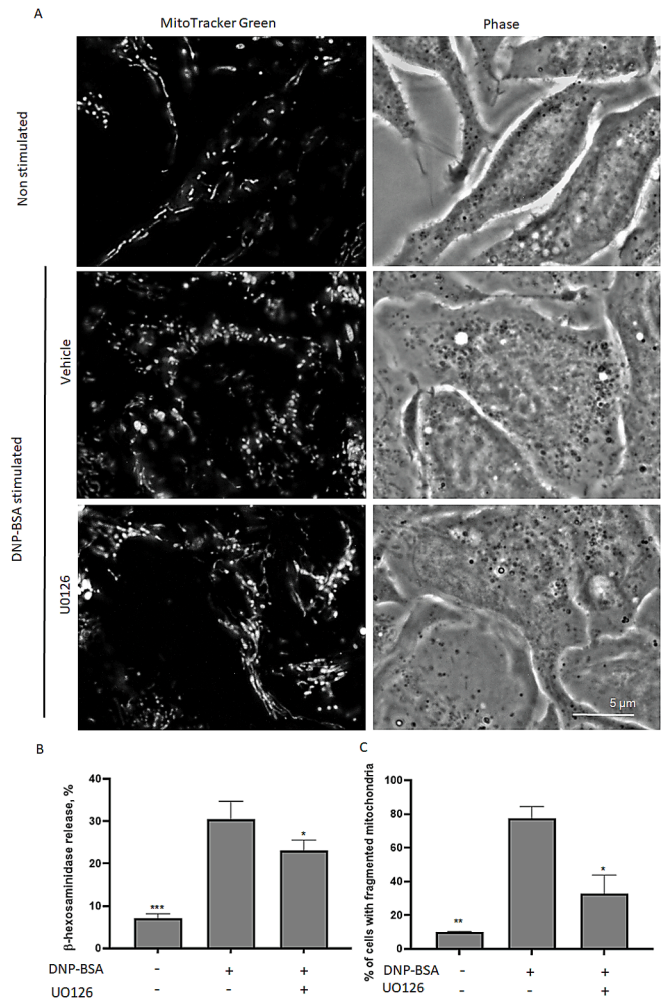

Fig. 7. The effect of MEK inhibitor U0126 on FcεRI-dependent degranulation of RBL-2H3 cells and mitochondrial fragmentation. (A) Fluorescence microscopy of the mitochondria (labeled with MitoTracker Green) in the RBL-2H3 cells (left) and phase-contrast microscopy (right). Cells were treated with 10 μM of U0126 for 2 h, sensitized by anti-DNP IgE, and incubated with 500 nM MitoTracker Green for 30 min. After washing in the HBSS, cells were stimulated by DNP-BSA for 15 min to induce FcεRI-dependent degranulation. (B) Beta-hexosaminidase assay for degranulation. (C) The percentage of cells with fragmented mitochondria. From 100 to 200 cells were analyzed in four independent experiments. Results are expressed as mean ± SD (n = 4). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as compared with the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test.

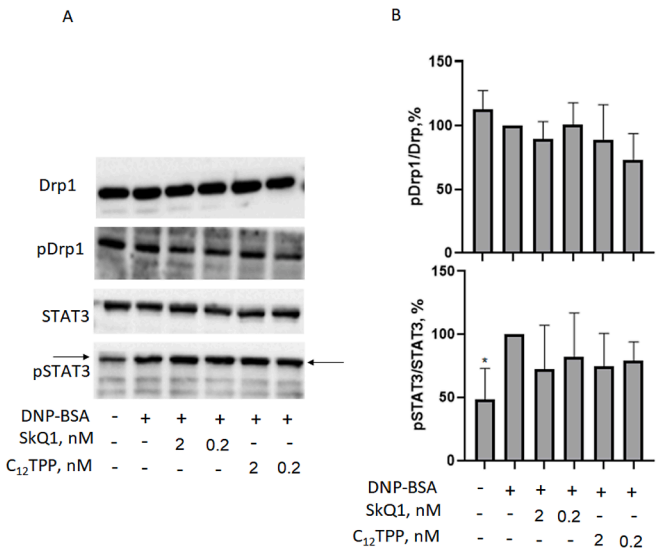

Fig. 8. The effect of TPP-based compounds on FcεRI-induced phosphorylation of Drp1 and STAT3 in RBL-2H3 cells. Cells were treated as shown in Fig. 3 and used for Western blot analysis. (A) Representative Western blots of cell lysates; (B) histograms denoting the relative amounts of phospho-proteins. Results are expressed as mean ± SD (n = 4). *p ≤ 0.05 as compared with the untreated DNP-BSA stimulated cells calculated by one-way ANOVA, Dunnett test.

4. Discussion

FcεRI-dependent signaling is studied well and it plays an important role in the pathogenesis of allergic diseases [24,25]. Here, we provide evidence that TPP-based compounds SkQ1 and C_{12}TPP can inhibit FcεRI-dependent degranulation of RBL-2H3 cells by preventing mitochondrial fragmentation through Erk1/2 kinase.

There is growing evidence of mitochondrial involvement in the FcεRI-dependent activation of MC. It is considered that MC degranulation is accompanied by mitochondrial fragmentation mediated by GTPase Drp1, and subsequent translocation of the mitochondria to the plasma membrane. Inhibition of Drp1 reduces degranulation of MC and suppresses mitochondrial fragmentation and translocation [4]. Mitochondrial fission is observed for some types of immune cells. For example, the differentiation of effector T cells is accompanied by mitochondrial fission and reducing OXPHOS activity [26]. During antigen-specific T-cell activation mitochondria undergo fragmentation, and move to the sites of immune synapse formation [27]. The functions of M1 macrophages rely on glycolysis and their pro-inflammatory activation results in mitochondrial fission. Activation of $CD1c^{+}$ myeloid dendritic cells is required for mitochondrial fission and mitophagy. Probably, fragmentation of mitochondria is needed for the transport of these organelles [28,29]. The change in mitochondrial morphology is often associated with altered OXPHOS activity. Elongated mitochondria possess high OXPHOS activity which is decreased in fragmented mitochondria [30,31].

In the present study, we demonstrated that antigen-dependent degranulation of MC is accompanied by mitochondrial fragmentation, decreased ΔΨm and ATP levels (Figs. 3–5). This probably points at a reduced OXPHOS activity in degranulating MCs. These data suggest that OXPHOS is not the main source of energy for MC degranulation. In agreement with our observation, antigen-induced activation of murine bone marrow-derived MC resulted in the increased glycolysis rate [32]. TPP-based compounds prevented mitochondrial fragmentation and the decrease in mitochondrial ATP during degranulation of RBL-2H3, but did not affect the change in ΔΨm measured by the accumulation of TMRM (Figs. 3–5). It is interesting to note that inhibition of mitochondrial fragmentation does not result in full inhibition of MC degranulation (Figs. 2, 3). This indicates that the degranulation is not dependent solely on mitochondrial fission. The limited role of mitochondrial function in mast cell degranulation was demonstrated earlier [33]. Inhibition of mitochondrial pyruvate dehydrogenase completely abolished cytokines secretion but decreased MC degranulation only by 50%. Thus, cytokine secretion is more dependent on mitochondrial bioenergetic function than degranulation.

A lot of evidence indicates that mitochondrial fragmentation is associated with ROS increase [34–36]. We showed that antigen-dependent stimulation of RBL-2H3 cells is accompanied by the elevation of mitochondrial ROS, in agreement with previously published data [37]. However, the TPP-based compounds did not reduce ROS production (Fig. 4B). The inhibitory effect of TPP-based compounds on MC degranulation was accompanied by a moderate inhibition of Erk1/2 kinase activation (Fig. 6). It should be noted that SkQ1 decreases Erk1/2 activation in certain cell lines [38,39]. One possible mechanism is that SkQ1 can increase the level of DUSP6 phosphatase, a negative regulator of Erk1/2 [38]. However, we found no influence of TPP-based

compounds on the DUSP6 level in RBL-2H3 cells (data not shown).

Erk1/2 is known to play a critical role in antigen-dependent degranulation of MC [40–42]. It is interesting to note that Erk1/2 can be localized in mitochondria [43] and even regulate mitochondrial dynamics. Migration of T cells is dependent on Erk1/2-phosphorylation of Drp1 at serine 616 and the subsequent mitochondrial fragmentation [27,44]. Erk1/2-dependent phosphorylation of Drp1 on serine 579 and mitochondrial fragmentation is a necessary step in the reprogramming of induced pluripotent stem cells [22]. Also, Erk1/2 can suppress the mitochondrial fusion by phosphorylating Mfn1 at threonine 562 [45].

We have shown that chemical inhibition of Erk1/2 in RBL-2H3 cells decreases degranulation and prevents mitochondrial fragmentation (Fig. 7). However, mitochondrial fragmentation was not associated with Drp1 phosphorylation on serine 616 (Fig. 8). In addition to the canonical Drp1-dependent mitochondrial fragmentation, Drp1-independent mitochondrial fission was shown in mammalian cells [46]. Moreover, the changes in mitochondrial morphology during RBL-2H3 antigen-dependent degranulation may be possibly explained by the formation of indented mitochondrial spheroids in a fission-independent manner [18].

Antigen-dependent stimulation of MC is known to be accompanied by Erk1/2-dependent phosphorylation of mitochondrial STAT3, which is involved in ATP production by regulating ETC [23]. However, the TPP-based compounds did not affect the Erk1/2-dependent activation of mitochondrial STAT3 (Fig. 8). Furthermore, Erk1/2 can regulate MC degranulation by the regulation of microtubules stability [40], by direct phosphorylation of the exocyst component Exo70 [47,48], or by reversing the inhibitory effect of AMPK [41].

The present study has the following limitations: first, we have used tumor RBL-2H3 cells as a model of MC and thus our knowledge is limited to only in vitro data. Second, the fluorescent dyes TMRM and MitoTracker Orange, used in our experiments for measurement of mitochondrial ΔΨm and ROS, respectively, may be prone to artifacts. It is known that C_{12}TPP in yeast cells upregulates expression of pleiotropic drug resistance transporters and as result prevents the accumulation of the PDR transporter substrate Nile red [49]. TMRM is also a substrate for multidrug resistance transporter [50]. These data suggest that TPP-based compounds may affect cellular TMRM accumulation, so it is possible that this cationic dye is not suitable for studying the effects of TPP-based compounds. MitoTracker dyes also can be influenced by such factors as the loss of ΔΨm [51]. Unfortunately, currently, there are no reliable methods for the detection of mitochondrial ΔΨm and ROS in a living cell.

5. Conclusion

The results of the current study indicate that the inhibitory effect of the TPP-based compounds SkQ1 and C_{12}TPP on antigen-dependent degranulation of mast cells is associated with the inhibition of Erk1/2-dependent mitochondrial fragmentation. These data expand the fundamental understanding of the role of mitochondria in the activation of MC. It also contributes to the rationale for the development of mitochondrial-targeted drugs for the treatment of allergic diseases.

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CRediT authorship contribution statement

Anastasia N. Pavlyuchenkova: Investigation, Formal analysis, Visualization, Writing – original draft. **Roman A. Zinovkin:** Methodology, Writing – review & editing. **Ciara I. Makievskaya:** Methodology. **Ivan I. Galkin:** Investigation. **Maria A. Chelombitko:** Investigation, Formal analysis, Visualization, Writing – original draft, Supervision.

Declaration of competing interest

The authors have no conflict of interest in relation to this work.

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