Medical Microbiology and Immunology (2019) 208:855–868
https://doi.org/10.1007/s00430-019-00631-8

ORIGINAL INVESTIGATION

Pseudomonas aeruginosa quorum-sensing molecule *N*-(3-oxo-dodecanoyl)-L-homoserine lactone triggers mitochondrial dysfunction and apoptosis in neutrophils through calcium signaling

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Received: 26 February 2019 / Accepted: 27 July 2019 / Published online: 3 August 2019

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that utilizes the quorum-sensing (QS) process to regulate the production of different virulence factors and biofilm. *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12) is a key QS molecule of *P. aeruginosa* which interacts with the mammalian immune cells and modulates their function. Here, we investigated the molecular mechanism of C12-induced apoptosis in neutrophils. Our data show that C12 causes apoptosis in neutrophils through an elevation in cytosolic and mitochondrial Ca^{2+} levels. Besides, C12 induces phosphatidylserine (PS) exposure, mitochondrial membrane potential (MMP) depolarization, mitochondrial permeability transition pore (MPTP) formation and mitochondrial reactive oxygen species (mROS) generation. C12-induced rise in intracellular Ca^{2+} level is majorly contributed by endoplasmic reticulum store through the activation of inositol 1, 4, 5-triphosphate receptor. Intracellular calcium chelation inhibited C12-induced mitochondrial dysfunction and apoptosis. Further, inhibition of mitochondrial Ca^{2+} uniporter by ruthenium red or Ru360 abrogated C12-induced mitochondrial Ca^{2+} uptake, MMP loss, MPTP opening, mROS production, and PS exposure. These mechanistic insights are expected to provide a better understanding of the role of C12 in *P. aeruginosa* pathogenesis.

Keywords Neutrophil · Apoptosis · Calcium signaling · Mitochondria · ROS · mCU

Introduction

Pseudomonas aeruginosa, the ubiquitous Gram-negative bacterium, is an opportunistic pathogen that infects individuals with the compromised immune system, patients with burns and cyst fibrosis (CF) [1, 2]. *P. aeruginosa* forms biofilm in airways of CF patients and causes a wide range of acute and chronic infections [3, 4]. *P. aeruginosa*-associated virulence factors, including elastase, rhamnolipids, LasB, pyocyanin, lipase, and hydrogen cyanide, contribute to its pathogenesis, and their expression is regulated by the process of quorum sensing (QS) [5]. QS is a microbial communication system through which bacteria regulate the expression of various genes concerning population density [6]. The majority of Gram-negative bacteria use acyl homoserine lactones (AHLs) as their QS signaling molecules [7]. AHLs are classified based on the length and composition of their acyl side-chain [8]. *P. aeruginosa* has two types of AHL-based QS systems, namely, lasIR and rhlIR, they involve *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12) and *N*-butanoyl-L-homoserine lactone (C4)

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Edited by: Volkhard A.J. Kempf.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00430-019-00631-8) contains supplementary material, which is available to authorized users.

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as their respective QS molecule [7]. These AHL molecules regulate not only the expression of various genes of *P. aeruginosa* but also interact and modulate the functions of several mammalian host cells through inter-kingdom signaling [9–16]. Moreover, C12 induces platelet activation [17], neutrophil chemotaxis [18], and causes the loss of barrier functions in CaCo-2 cells [19, 20]. C12 has also been reported to induce apoptosis in mast cells, airway epithelial cells, fibroblasts, endothelial cells, macrophages, and neutrophils [21–25]. In this study, we have explored the mechanism of C12-induced apoptosis in neutrophils. Neutrophils are the key player of the innate immune system and perform various anti-infectious and pro-inflammatory functions such as phagocytosis, production of antimicrobial peptides, proteolytic enzymes, and reactive intermediate species [26].

Calcium (Ca^{2+}) is a secondary messenger molecule that plays a vital role in the regulation of various cellular processes such as activation, motility, phagocytosis, cell proliferation, cellular senescence and apoptosis [27, 28]. In the homeostatic condition, the cytosolic Ca^{2+} ($[Ca^{2+}]c$) and the extracellular Ca^{2+} concentrations are maintained at ~ 100 nM and ~ 1.3 mM, respectively [29]. Endoplasmic reticulum (ER) is the largest intracellular Ca^{2+} reservoir, where local Ca^{2+} concentration remains up to millimolar level [29]. ER-associated calcium pump and channels such as sarcoplasmic/endoplasmic reticulum calcium-ATPases (SERCAs), inositol-1,4,5-triphosphate receptor (IP3R) and ryanodine receptor (RyR) are involved in the regulation of cytosolic calcium concentration [29]. Mitochondria also contribute to the maintenance of $[Ca^{2+}]c$ level by Ca^{2+} uptake through mitochondrial Ca^{2+} uniporter (mCU) and voltage-dependent anion channel (VDAC) [30]. C12 causes morphological alterations in mitochondria and ER and activates the expression of apoptotic markers in airway epithelial cells [15]. Besides, C12 induces apoptosis in fibroblasts [24, 31] and endothelial cells [23] through intracellular calcium mobilization. C12 has also been reported to elicit intracellular calcium rise in neutrophils [18], but the role of ER–mitochondria Ca^{2+} handling in C12-induced apoptosis has not been investigated in detail. In the present study, we explored the role of ER–mitochondria calcium signaling pathways in C12-induced apoptosis in neutrophils. Our results indicate that $[Ca^{2+}]c$ rise and Ca^{2+} uptake via mitochondrial calcium uniporter contributes to C12-induced mitochondrial dysfunction and apoptosis in neutrophils.

Materials and methods

Reagents

Propidium iodide (PI), 1, 2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM), ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Ca^{2+}-ionophore A23187, Thapsigargin (Tg), Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 2-Aminoethoxydiphenyl borate (2-APB), Phospholipase-C (PLC) inhibitor U73122, Ruthenium red (RuR), C12 and C4 were purchased from Sigma Aldrich; 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Himedia; Xestospongin-C (XeC) from Abcam, Annexin V-FITC and 5,5′,6,6′-tetrachloro-1,1′3,3′-tetra ethyl benzimidazol carbocyanine iodide (JC-1) MitoScreen Kit were procured from BD Biosciences; Calcein acetoxymethyl ester (calcein-AM), Hoechst 33342, Fluo-4-AM, and MitoSOX-Red were purchased from Thermo Fisher Scientific; 4,4′-Diisothiocyano-2.2′-stilbenedisulfonic acid (DIDS) were obtained from Cayman; Digitonin and Cyclosporin A were the products of TCI chemicals, and Ru360 was purchased from Calbiochem. C12 was dissolved in DMSO to make concentrated stock solutions and stored at − 20 °C.

Neutrophil isolation

Blood samples were collected in EDTA-containing tubes from healthy volunteers at Pathology Laboratory Health Center of Motilal Nehru National Institute of Technology (MNNIT) Allahabad, Prayagraj India. Informed written consent was obtained from all volunteers that participated in the study, which was approved by the institutional ethics committee of MNNIT Allahabad, India (Ref. No. IEC/16-17/018). Polymorphonuclear neutrophils (PMN) were isolated using Poly-morph Prep (Axis-shield PoC AS, Oslo, Norway) by centrifugation at 500×*g* for 40 min at room temperature. The PMN layer was collected carefully and washed with HEPES Buffer Saline (HBS; HEPES 10 mM, 0.85% w/v NaCl, pH 7.4) by centrifugation at 400×*g* for 10 min. Erythrocytes were removed by incubation with RBC lysis buffer (HEPES 10 mM, 0.85% w/v NH_4Cl, 0.1 mM EDTA, pH 7.4) for 7 min at 37 °C and the cells were washed twice at 400 × g for 10 min. Finally, cells were re-suspended in an appropriate culture medium or buffer at a final concentration of 1×10^6 cells per ml. Cellular viability was routinely checked using trypan blue exclusion assay, and it was obtained in the range of 91–96%.

MTT assay

Neutrophils at a density of 1×10^6 cells/ml in RPMI were seeded into the 96-well plates and treated with C12, C4, and DMSO separately. Next, cells were incubated at 37 °C for the indicated time period. After that, MTT (50 µg/well) was added and further incubated at 37 °C until the appearance of formazan crystals. The supernatant was removed, and 100 µl

of MTT solvent was added in each well and mixed properly; absorbance was recorded at 570 nm using a microplate reader (Tecan, Sunrise).

Nuclear morphology

Neutrophils were treated with C12 for 3 h, and after incubation, nuclei were stained using Hoechst 33342 (3 µM) for 10 min and visualized under 40 X objective of Olympus CKX53 fluorescence microscope.

Annexin V-FITC/PI assay

Phosphatidylserine (PS) exposure on neutrophils was detected using Annexin V-FITC/PI-based assay. Briefly, 1×10^6 neutrophils in RPMI were treated with C12 and incubated at 37 °C for 1 h. After incubation, cells were stained with Annexin V-FITC and PI for 20 min at RT in the appropriate buffer. Subsequently, samples were acquired in flow cytometer BD Accuri C6 (BD Biosciences), and data were analyzed using FlowJo Software.

Intracellular calcium measurement

Fluo-4-AM, a fluorescent Ca^{2+}-sensitive indicator, was used to measure intracellular calcium level. Neutrophils were pre-loaded with 5 µM of Fluo-4-AM for 45 min at RT in modified Hank's Balanced Salt Solution (HBSS) without Ca^{2+}. The Fluo-4-AM loaded neutrophils were washed and re-suspended in the HBSS at a concentration of 1×10^6 cells/ml. Samples were excited at 494 nm, and emission was recorded at 525 nm in a fluorescence spectrometer (Perkin Elmer LS-45). After 60 s of basal fluorescence recording, samples were exposed to DMSO or AHLs. In these experiments, Cremophor EL was used as an emulsifying agent. Fluorescence (F) was normalized to the basal fluorescence levels (F_0), and fluorescence intensity was expressed as F/F_0.

Mitochondrial membrane potential measurement

The mitochondrial membrane potential (MMP) of neutrophils was assayed using the lipophilic cationic probe JC-1 (Mito Screen Kit) according to the manufacturer's protocol. Briefly, isolated neutrophils were treated with C12 for 30 min and stained with JC-1 for next 30 min at 37 °C; cells were washed twice and resuspended in HBS. The change in MMP was measured using flow cytometer BD Accuri C6 (BD Biosciences), and data analyses were performed using FlowJo software.

Mitochondrial permeability transition pore formation

The Mitochondrial Permeability Transition Pore (MPTP) formation was determined by analyzing the mitochondrial calcein leakage, as previously described [32, 33]. Briefly, neutrophils were loaded with 1 µM calcein-AM for 30 min at RT in the presence of 1 mM $CaCl_2$. Further, 1 mM $CoCl_2$ was added in calcein-AM loaded neutrophils. MPTP opening was evaluated after treatment with C12 for 15 min by measuring mitochondrial calcein quenching. Throughout the experiment, the calcein fluorescence was recorded by flow cytometer BD Accuri C6 (BD Biosciences), and data were analyzed using FlowJo software. The MPTP opening was indicated by a reduction in mitochondrial calcein fluorescence signal.

Measurement of mitochondrial calcium uptake in permeabilized neutrophils

Mitochondrial calcium uptake study in permeabilized neutrophils was performed as described previously [34], with some modifications. Briefly, neutrophils were loaded with Fluo-4-AM (5 µM) for 40 min at 37 °C in modified Tyrode's buffer. Further, neutrophils were treated with thapsigargin (2 µM). Neutrophils were permeabilized with 40 µg/ml of digitonin in intracellular medium (ICM) consisting of (in mM) 135 KCl, 10 NaCl, 20 HEPES, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH_2PO_4, 1 $MgCl_2$, 5 EGTA and 1.86 $CaCl_2$. The Fluo-4 intensity was continuously monitored using a fluorescence spectrophotometer (Perkin Elmer LS-45). C12 (100 µM) or DMSO was added to the incubation before 180 s of $CaCl_2$ (40 µM) pulsing in absence and presence of 2 µM Ru360/RuR (added before 60 s of $CaCl_2$ addition). An increase in the fluorescence intensity of Fluo-4 indicates the Ca^{2+} uptake into the mitochondrial matrix. Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio), which was measured after plasma membrane permeabilization.

Measurement of mitochondrial ROS generation

Neutrophils were loaded with 10 µM of MitoSOX-Red for 40 min at RT and subsequently treated with C12, A23187, or DMSO for 30 min. The samples were acquired in flow cytometer BD Accuri C6 (BD Biosciences), and results were analyzed using FlowJo software.

Statistical analysis

All experimental results were expressed as the mean ± S.E.M of three independent experiments. The data were tested for statistical significance by Student's t test and ANOVA

using GraphPad Prism software and only results at $P < 0.05$ were considered significant. ns = non significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

C12 induces apoptosis in neutrophils

To examine the cytotoxic effect of AHLs; neutrophils were treated with C12 and C4 separately, and metabolic activity was assessed using MTT assay. Neutrophils were treated with different concentrations of C12 (1–200 µM) for 3 h, and a loss of viability was observed in a dose-dependent manner, and this was particularly marked at 100 µM or above (Fig. 1a). C4 did not induce cytotoxic effect even at 200 µM (Fig. 1b). In comparison to DMSO vehicle control, C12 (100 µM) caused a gradual decrease in cell viability in a time-dependent manner and it was marked after 1 h or above, while C4 (100 µM) was unable to affect cell viability significantly (Fig. 1c). Nuclear morphology was examined using Hoechst 33342, a live cell nucleic acid binding dye.

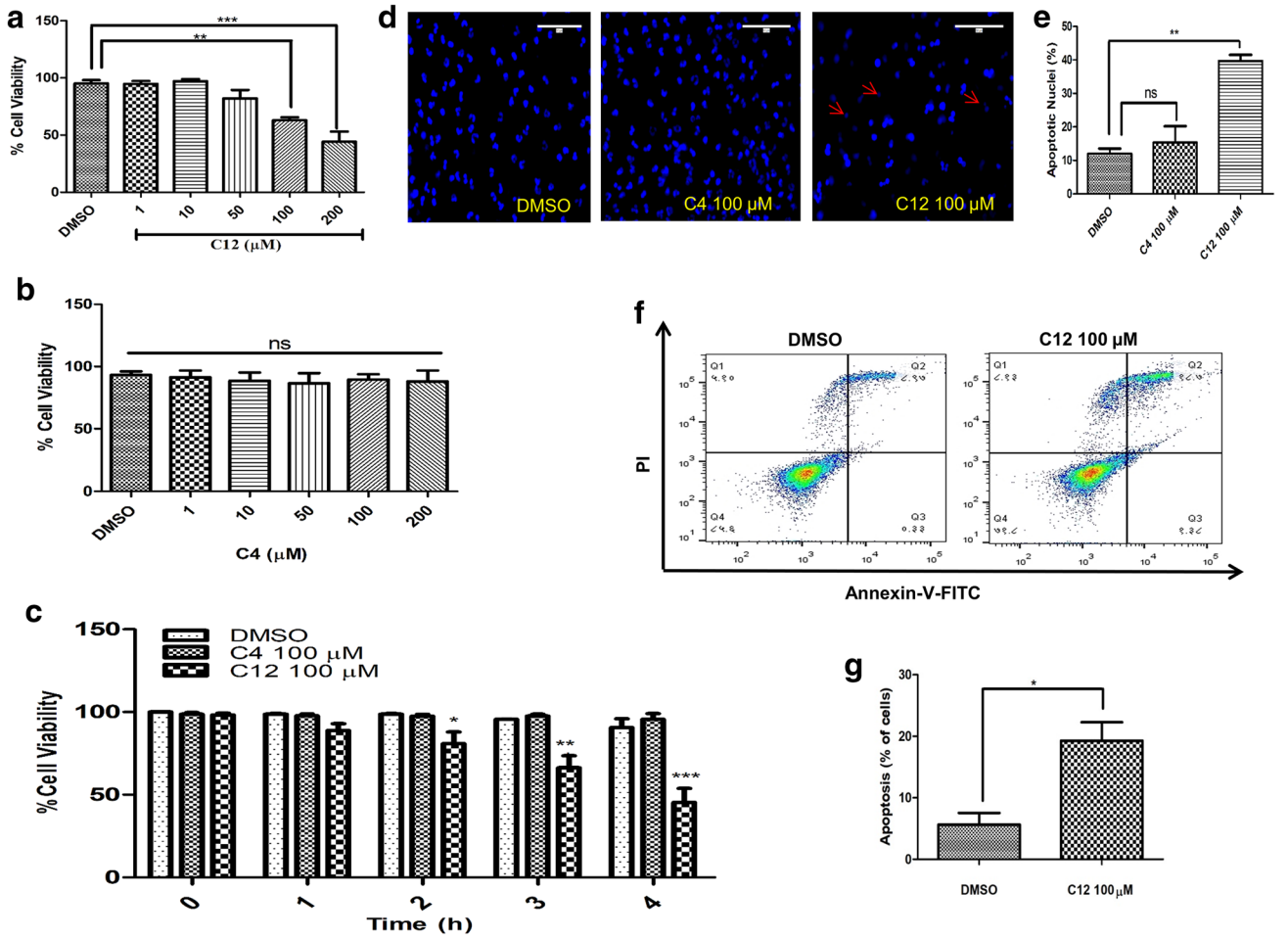

Fig. 1 C12 induces cytotoxic effect, apoptotic nuclei formation, and PS exposure in neutrophils. Cytotoxicity of AHLs was evaluated by MTT assay. **a** Treatment of neutrophils with varying concentrations of C12 (1–200 µM) for 3 h, followed by MTT assay. **b** Treatment of neutrophils with different concentrations of C4 (1–200 µM) for 3 h, followed by MTT assay. **c** C12 and C4 (100 µM each) were applied separately to the neutrophils for the different time periods ranging from 0 to 4 h. C12 has shown significant cytotoxic effect while C4 lacks such activity. **d** Representative fluorescent microscopic image (Hoechst 33342 stained) of neutrophil nuclear morphology after treatment with C12 and C4 separately (100 µM each). **e** Bar graph represents the percentage apoptotic nuclei, from (**d**), C12 treatment increased the number of apoptotic nuclei. **f** Representative flow cytometry picture of Annexin-V FITC/PI stained cells after C12 100 µM treatment. **g** Bar graph represents the apoptosis (% of cells), from (**f**), compared to DMSO diluent control, C12 treatment increased the number of annexin-V FITC/PI stained cells. (Mean ± S.E.M; $n = 3$ independent experiments; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). *AHLs* acyl homoserine lactones, *MTT* 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide, *C12 N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *C4 N*-butyryl-L-homoserine lactone, *PS* phosphatidylserine, *FITC* fluorescein isothiocyanate, *PI* propidium iodide

In comparison to DMSO, treatment of neutrophils with C12 (100 µM) caused an increase in the number of apoptotic nuclei, while no such activity was observed in the sample treated with C4 (100 µM) (Fig. 1d, e). PS exposure, an essential marker of the apoptotic cells, was evaluated by dual staining with annexin V-FITC/PI. It was observed that in comparison to DMSO, treatment of neutrophils with C12 (100 µM) significantly augmented the PS exposure (Fig. 1f, g).

C12 causes mitochondrial dysfunction in neutrophils

Mitochondrial dysfunction, including loss of MMP and the opening of MPTP, occurs during apoptosis. The effect of C12 on mitochondrial membrane potential was assessed using Mito Screen JC-1, a cationic dye that exhibits potential-dependent aggregation in energized mitochondria. JC-1 exists as green color monomer at low MMP, while at high MMP it forms red color J-aggregates. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. CCCP, an uncoupler of oxidative phosphorylation, was used as a positive control for the mitochondrial membrane potential destabilization. In comparison to DMSO, treatment of neutrophils with C12 for 30 min caused a significant decrease in FL2/FL1 (red/green) ratio at 50 µM and 100 µM (Fig. 2a, b). Further, to investigate C12-induced MPTP formation, calcein-AM/cobalt chloride quenching method was used. It was found that C12 (100 µM) significantly induced MPTP formation in neutrophils (Fig. 2c, d).

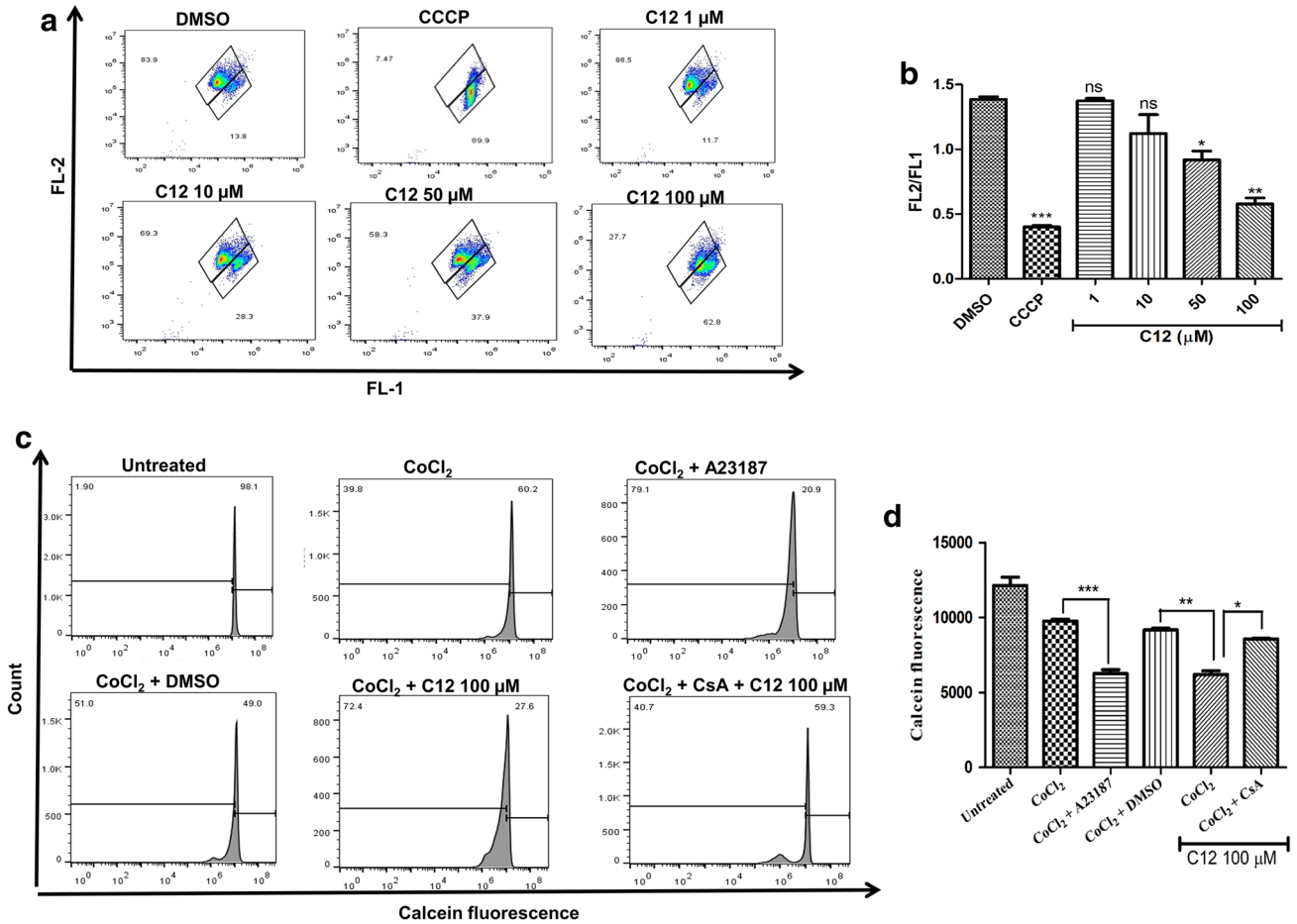

Fig. 2 C12 induces MMP loss and MPTP opening. **a** Representative image of MMP analyzed by flow cytometry after treatment with increasing doses of C12, MMP was measured by red/green (FL2/FL1) fluorescence ratio of JC1. **b** Bar graph represents the mean fluorescence of red/green (FL2/FL1) ratio, from (**a**), C12 causes MMP loss. **c** Flow cytometry-based depiction of MPTP opening in response to C12 100 µM in the absence or presence of CsA, a CyD inhibitor. **d** Bar graph represents the mean calcein fluorescence, from (**c**), C12-induced CyD-dependent MPTP opening. (Mean ± S.E.M; $n = 3$ independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *MMP* mitochondrial membrane potential, *JC-1* 5,5V,6,6V-tetrachloro-1,1V3,3V-tetra ethyl benzimidazol carbocyanine iodide, *CsA* cyclosporine A, *MPTP* mitochondrial permeability transition pore, *CyD* cyclophilin D, *CCCP* carbonyl cyanide m-chlorophenyl hydrazine, *A23187* calcium ionophore, *$CoCl_2$* cobalt chloride

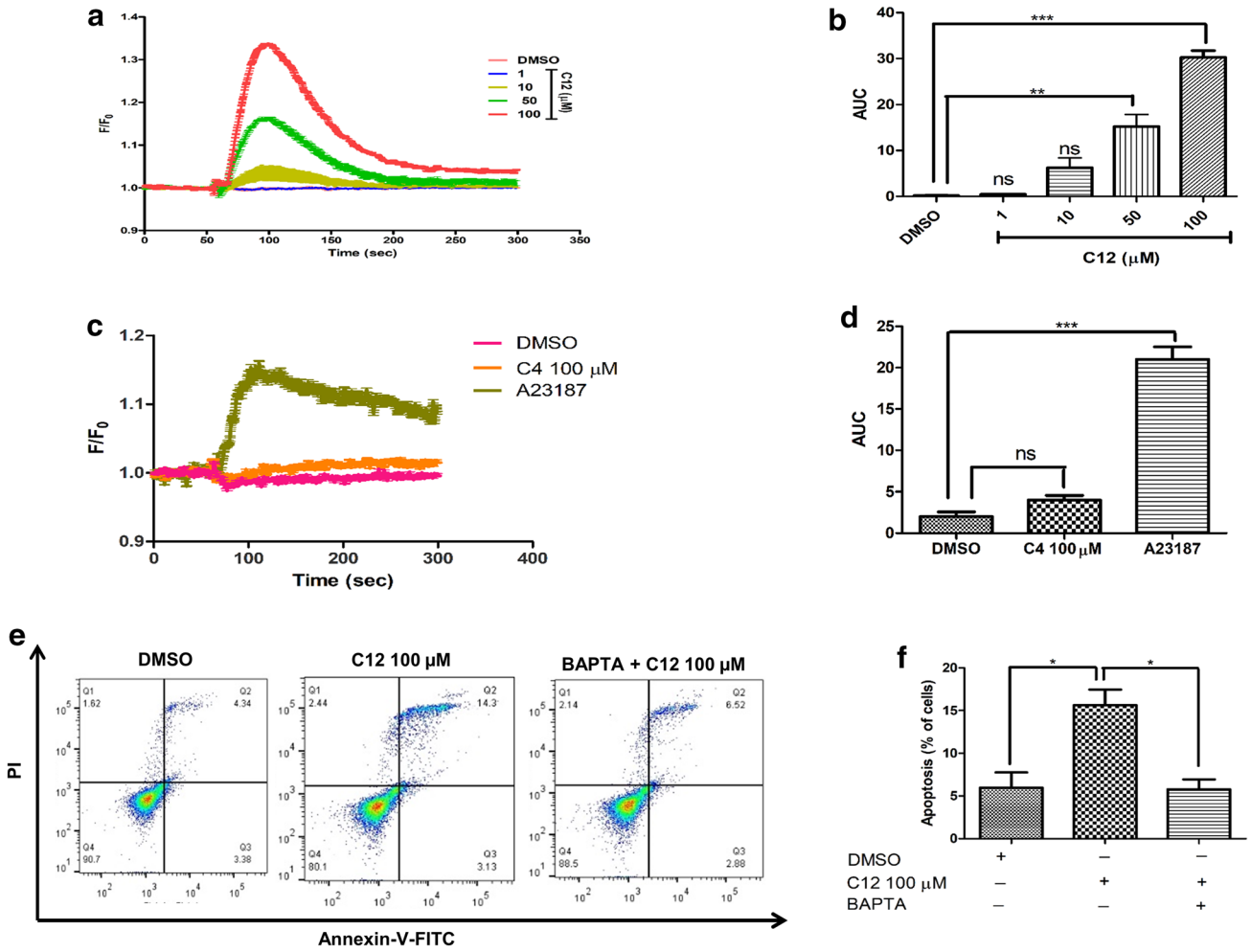

Fig. 3 C12 induces calcium-dependent PS exposure. **a** Representative trace of the intracellular Ca^{2+} rise in response to the increasing doses of C12 (1–100 μM), Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio). **b** Bar graph represents the AUC, from (**a**), C12 induces intracellular Ca^{2+} rise. **c** Representative trace of intracellular calcium rise in the sample treated with C4 (100 μM). DMSO and A23187 were kept as a vehicle and positive control, respectively. Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio). **d** Bar graph represents AUC, from (**c**), C4-treated cells do not have a significant change in the intracellular Ca^{2+} level. **e** Flow cytometry-based analysis of C12 (100 μM) induced PS exposure in the presence or absence of BAPTA-AM. **f** Bar graph represents the apoptosis (% of cells), from (**e**), BAPTA-AM reduced C12-stimulated PS exposure. (Mean ± S.E.M; $n = 3$ independent experiments; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *C4* *N*-butyryl-L-homoserine lactone, *BAPTA-AM* 1, 2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra acetic acid tetrakis acetoxymethyl ester, *AUC* area under curve, *PS* phosphatidylserine, *A23187* calcium ionophore, *FITC* fluorescein isothiocyanate, *PI* propidium iodide, *Fluo-4* calcium indicator

A23187 was used as a positive control for MPTP opening. MPTPs are composed of various components including cyclophilin D (CypD), an essential constituent for MPTP response. Pre-incubation of neutrophils for 20 min with 4 μM cyclosporin A (CsA), an inhibitor of CypD, prevented the C12-induced MPTP formation (Fig. 2c, d).

Cytosolic Ca^{2+} rise contributes to C12-induced apoptosis

Calcium, a ubiquitous secondary messenger, participates in the regulation of various signaling pathways. Calcium homeostasis is required for the normal cellular functions, while its dysregulation is associated with the different forms of cell death, including apoptosis and necrosis. Cytosolic Ca^{2+} level was measured using a calcium-sensitive probe Fluo-4-AM. Neutrophils pre-loaded with Fluo-4-AM were exposed to different concentrations of C12 (1–100 μM). C12 at the concentration of 50 μM and above induces a significant rise in the intracellular calcium level (Fig. 3a, b). However, C4 was unable to induce the intracellular Ca^{2+} mobilization even at a concentration of 100 μM (Fig. 3c, d). A23187, a Ca^{2+} ionophore, was used as a positive control. To investigate the possible link between C12-induced

[Ca^{2+}]c rise and apoptosis; PS exposure was measured in the presence of BAPTA-AM, a cell-permeant intracellular Ca^{2+} chelator. Neutrophils were pre-incubated with 5 µM of BAPTA-AM for 30 min, followed by treatment with 100 µM of C12 and subsequent measurement of PS exposure. BAPTA-AM significantly diminished the C12-induced PS exposure (Fig. 3e, f).

C12 causes calcium-dependent mitochondrial dysfunction

To examine the possible role of cytosolic calcium in the C12-induced mitochondrial dysfunction, MMP alteration and MPTP formation were measured in the presence of BAPTA-AM. It was found that BAPTA-AM significantly prevented the C12-induced MMP loss (Fig. 4a, b) and MPTP opening (Fig. 4c, d).

Role of phospholipase C-IP3R axis in C12-induced Ca^{2+} rise and apoptosis

To investigate the possible role of ER store in C12-induced Ca^{2+} rise, thapsigargin, a non-competitive inhibitor of SERCAs was used. Fluo-4 loaded neutrophils were pre-treated with 2 µM thapsigargin for 10 min in the presence of 1 mM EGTA, followed by addition of C12. It was observed that thapsigargin has almost vanished the intracellular calcium rise caused by C12 (Fig. 5a, b). In non-excitable cells, Ca^{2+} mobilization from ER majorly involves PLC-IP3R axis. Thus, to investigate the possible contribution of PLC-IP3R in C12-induced [Ca^{2+}]c rise, Fluo-4-AM-loaded neutrophils were pre-incubated with 5 µM of 2-APB (an IP3R antagonist) and 10 µM of U73122 (a PLC inhibitor), separately for 10 min, followed by the addition of C12. The results showed that C12-induced intracellular Ca^{2+} elevation was significantly diminished in the presence of both 2-APB and U73122 (Fig. 5c, d). To determine whether C12-induced IP3R activation contributes to PS exposure, cells were treated with C12 in the presence of IP3R antagonist 2-APB

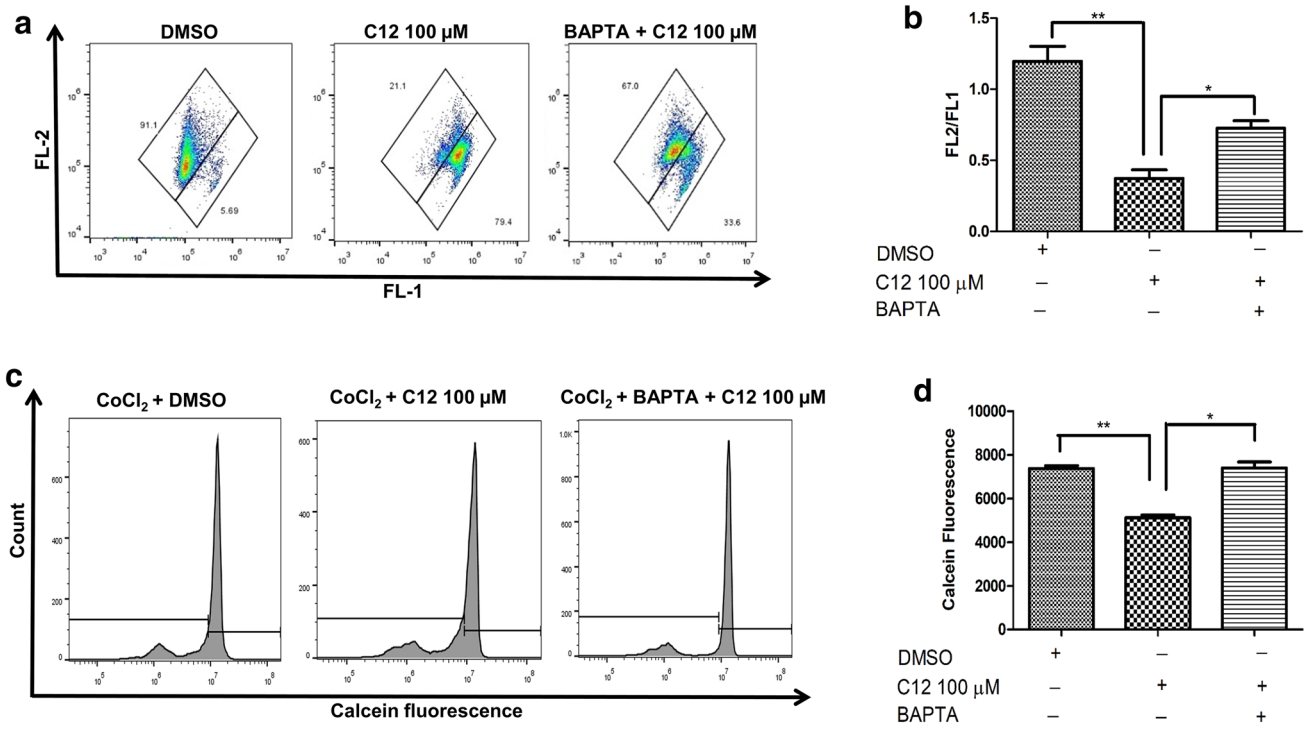

Fig. 4 Intracellular calcium chelation delayed C12-induced mitochondrial dysfunction. **a** Representative trace of C12 (100 µM)-stimulated MMP loss in the absence and presence of BAPTA-AM, MMP was measured by red/green (FL2/FL1) fluorescence ratio of JC1. **b** Bar graph represents the mean FL2/FL1 ratio of JC-1 fluorescence, from (**a**), BAPTA-AM inhibits C12-induced MMP loss. **c** Representative flow cytometry picture of MPTP opening in response to C12 100 µM in the absence and presence of BAPTA-AM. **d** Bar graph represents the mean calcein fluorescence, from (**c**), C12 induced MPTP opening was delayed in the presence of BAPTA-AM. (Mean ± S.E.M; $n = 3$ independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$). *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, MMP; mitochondrial membrane potential, *BAPTA-AM* 1, 2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*,*N*′-tetra acetic acid tetrakis acetoxymethyl ester, *MPTP* mitochondrial permeability transition pore, *JC-1* 5,5V,6,6V-tetrachloro-1,1V3,3V-tetra ethyl benzimidazol carbocyanine iodide

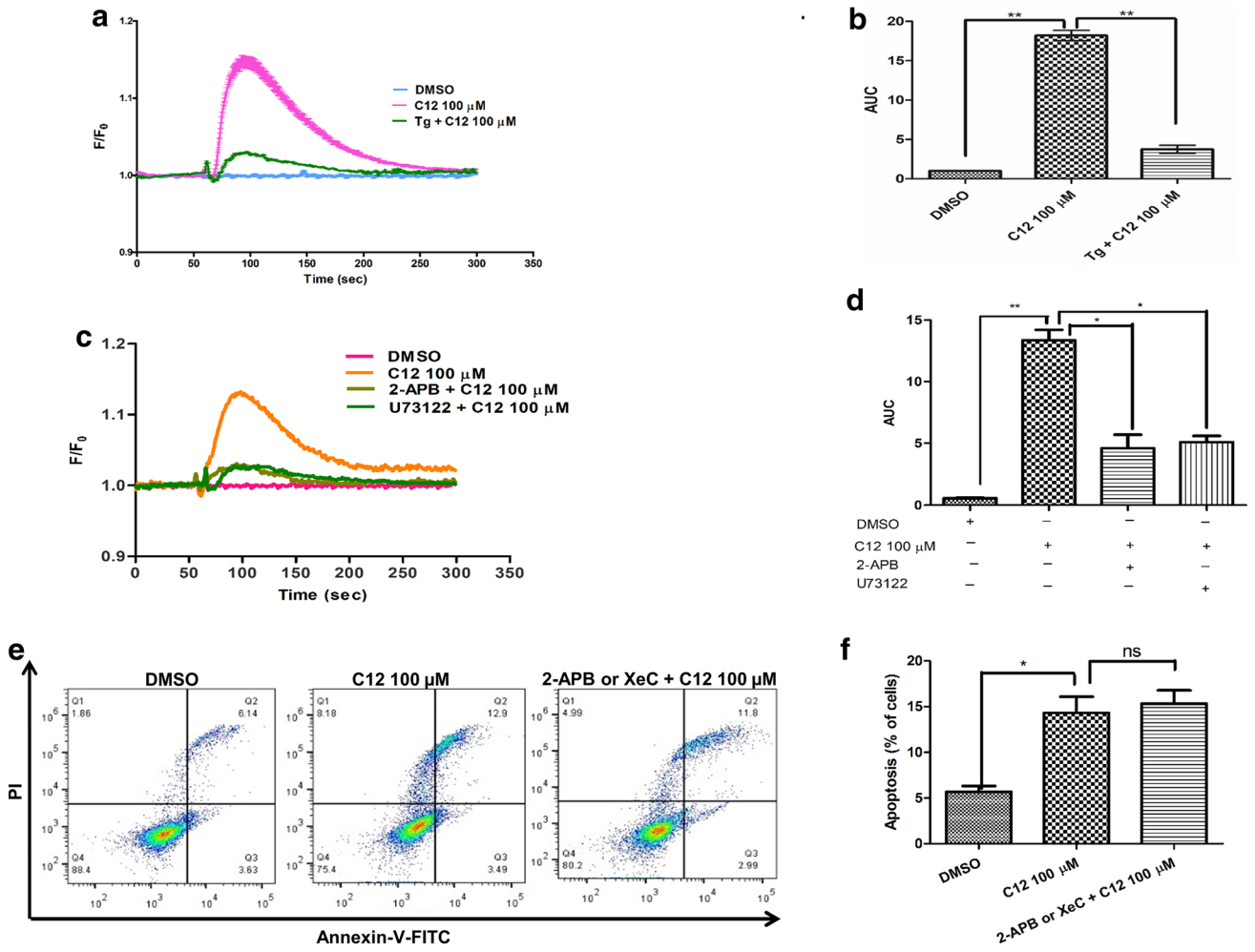

Fig. 5 Role of IP3R in C12-induced calcium rise and apoptosis. **a** Representative trace of C12 100 μM-induced intracellular Ca^{2+} rise in absence and presence of thapsigargin (2 μM), Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio). **b** Bar graph represents the AUC, from (**a**), C12-induced Ca^{2+} rise was abolished in the presence of thapsigargin. **c** Representative trace of C12 (100 μM) induced intracellular calcium rise in the absence or presence of U73122 and 2-APB separately, Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio). **d** Bar graph represents the AUC, from (**c**), C12 (100 μM)-induced intracellular calcium rise was significantly inhibited in the presence of PLC inhibitor U73122, and IP3R blocker 2-APB. **e** Representative flow cytometry pictures of C12 100 μM-induced Annexin-V FITC/PI binding in presence or absence of 2-APB or XeC. **f** Bar graph represents the apoptosis (% of cells), from (**e**), 2-APB, or XeC was unable to reduce C12-induced PS exposure. (Mean ± S.E.M; $n = 3$ independent experiments; $^*P < 0.05$, $^{**}P < 0.01$). *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *AUC* area under curve, *IP3R* inositol triphosphate receptor, *Tg* thapsigargin, *PLC* phospholipase C, *2-APB* 2-aminoethoxydiphenyl borate, *U73122* phospholipase-C inhibitor, *XeC* Xestospongin-C, *Fluo-4-AM* calcium indicator, *PS* phosphatidylserine, *FITC* fluorescein isothiocyanate, *PI* propidium iodide

or Xestospongin-C. Results showed that IP3R inhibition did not prevent C12-induced PS exposure (Fig. 5e, f).

C12 induces mitochondrial calcium uptake via mCU

To study the mitochondrial calcium ($[Ca^{2+}]m$) uptake, Fluo-4 fluorescence was recorded in digitonin-permeabilized neutrophils. Since ER and mitochondria are involved in the cytosolic calcium accumulation, thapsigargin (2 μM) was used to inhibit Ca^{2+} uptake by ER. In this condition, mitochondria are expected to participate majorly in Ca^{2+} uptake. Calcium pulsing (40 μM $CaCl_2$) to the Fluo-4 loaded, digitonin (30 μM) permeabilized cells yielded a transient increase in the Fluo-4 intensity reflecting the $[Ca^{2+}]m$ uptake signal (Fig. 6a). The results showed that $[Ca^{2+}]m$ uptake in the presence of C12 was relatively higher than the DMSO control (Fig. 6a). mCU majorly participates in mitochondrial calcium uptake. To test the possible role of mCU in C12-induced $[Ca^{2+}]m$ uptake, mCU inhibitors Ru360/RuR (2 μM) were used. As shown in Fig. 6b, Ru360/RuR reduces the C12-induced mitochondrial calcium uptake (Fig. 6b).

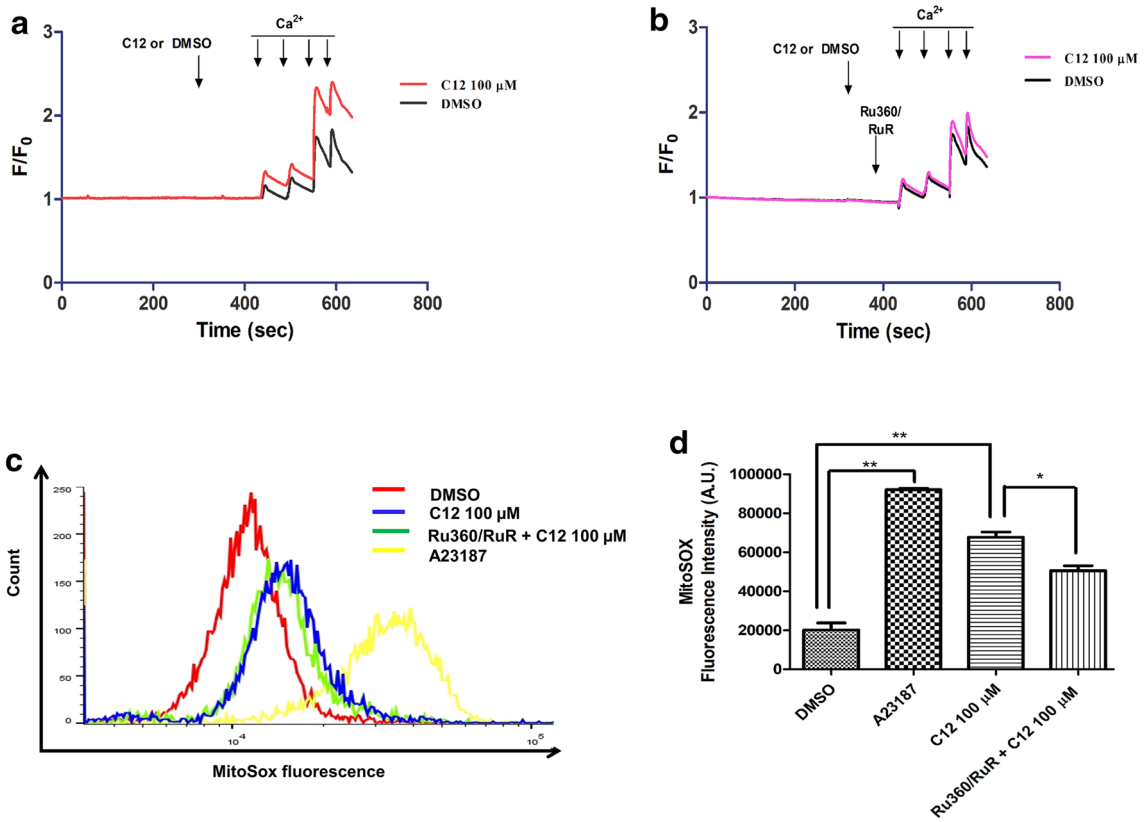

Fig. 6 C12 facilitates mCU-dependent calcium uptake and mROS generation. **a** Effect of C12 on Ca^{2+}-induced mitochondrial Ca^{2+} uptake in digitonin-permeabilized cells. C12 (100 µM) or DMSO, were added 180 s before Ca^{2+} pulsing to the permeabilized cells. C12 facilitates Ca^{2+} loading to mitochondria, Fluo-4 fluorescence intensities were normalized to the baseline (F/F_0 ratio). **b** Effect of C12 on mitochondrial Ca^{2+} uptake in the presence of 2 µM of Ru360/RuR which was added 60 s before Ca^{2+} addition. Ru360/RuR reduced mitochondrial Ca^{2+} uptake **c** Representative picture analyzed by flow cytometry for C12-induced mROS generation using MitoSox-Red in the absence and presence of Ru360/RuR. A23187 used as a positive control. **d** Bar graph represents the mean fluorescence intensity of MitoSox-Red, from (**c**), C12-induced mROS generation was decreased in the presence of Ru360/RuR. (Mean ± S.E.M; $n = 3$ independent experiments; $^*P < 0.05$, $^{**}P < 0.01$). *mCU* mitochondrial calcium uniporter, *mROS* mitochondrial reactive oxygen species, *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *RuR* ruthenium red, *Ru360* mCU inhibitor, *A23187* calcium ionophore, *MitoSox-Red* mitochondria-specific superoxide indicator

C12 causes mitochondrial ROS generation in an mCU-dependent manner

C12-induced mitochondrial ROS (mROS) generation was assessed using MitoSOX-Red, a mitochondria-specific ROS-sensitive probe. MitoSOX-Red permeates live cells and selectively targets mitochondria; it is oxidized by superoxides and produces red fluorescence. In this experiment, A23187 and DMSO were used as positive and vehicle control, respectively. We found that in comparison to DMSO, C12 significantly induced mitochondrial ROS generation (Fig. 6c, d). Further, to investigate the possible role of mCU in C12-induced mitochondrial ROS generation, MitoSOX-Red loaded neutrophils were pre-incubated with 30 µM of Ru360/RuR for 20 min and subsequently treated with C12. It was observed that mCU inhibition significantly prevented C12-induced mitochondrial ROS generation (Fig. 6c, d).

mCU inhibition abrogates C12-induced mitochondrial dysfunction and apoptosis

Calcium overload in mitochondria may result in the loss of mitochondrial membrane potential, opening of mitochondrial permeability transition pores, and apoptosis induction. Further, to investigate the role of mCU in mitochondrial dysfunction and apoptosis, C12-induced MMP loss, MPTP opening, and PS exposure were examined in the presence of Ru360/RuR (30 µM). Results showed that mCU inhibition significantly reduced the C12-induced MMP loss (Fig. 7a, b), MPTP opening (Fig. 7c, d), and PS exposure (Fig. 7e, f).

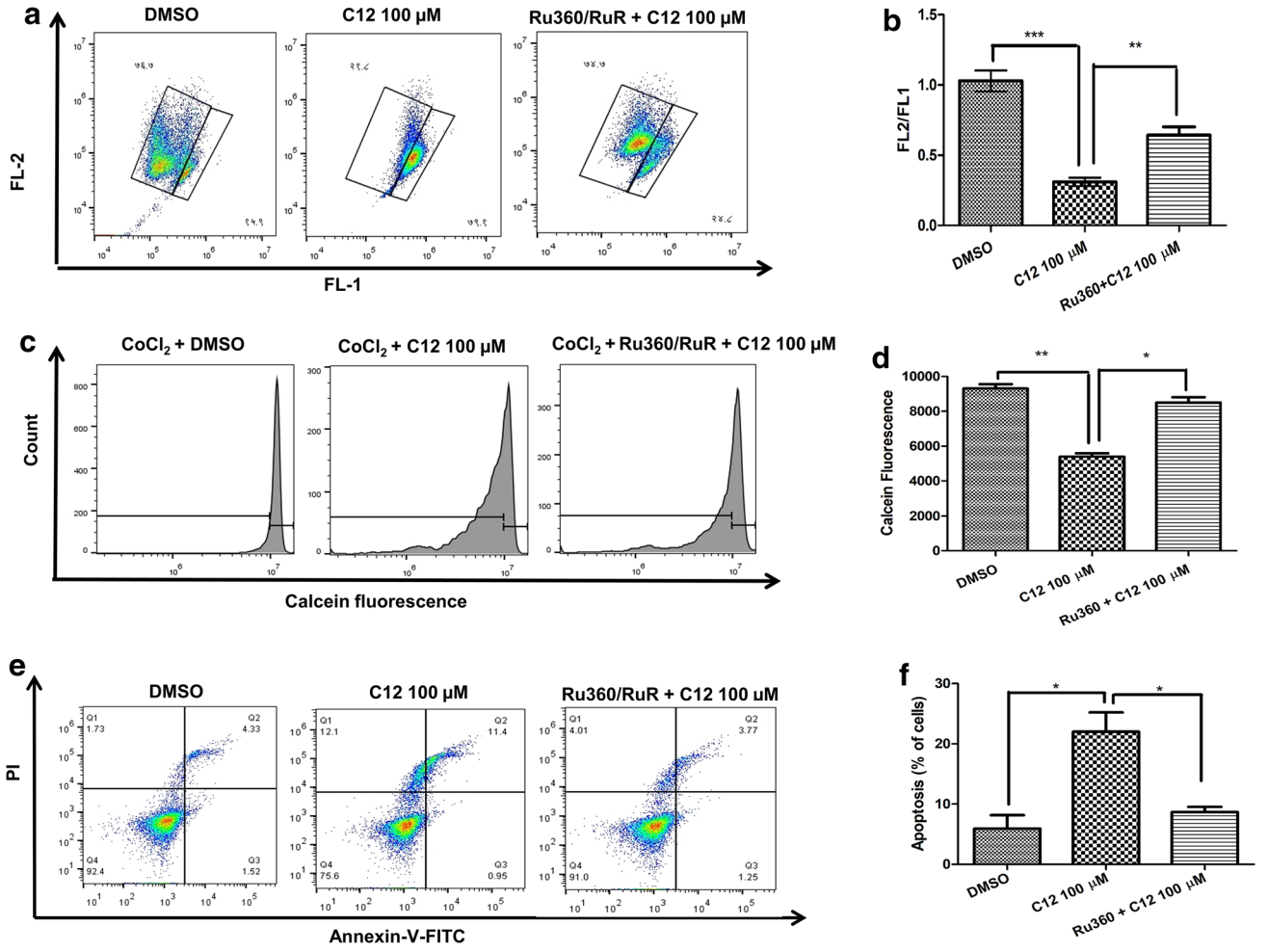

Fig. 7 mCU inhibition abrogates C12-induced mitochondrial dysfunction and PS exposure. **a** Representative trace of C12 (100 µM)-stimulated MMP loss in absence or presence of RuR/Ru360, MMP was measured by red/green (FL2/FL1) fluorescence ratio of JC1. **b** Bar graph represents the mean FL2/FL1 ratio of JC-1 fluorescence, from (**a**), C12-induced MMP loss was delayed in the presence of RuR/Ru360. **c** Representative flow cytometry image of MPTP opening in response to C12 100 µM, in the absence or presence of RuR/Ru360. **d** Bar graph represents the mean calcein fluorescence, from (**c**), C12-induced MPTP opening was delayed in the presence of RuR/Ru360. **e** Representative flow cytometry pictures of Annexin-V FITC/PI binding induced by C12 100 µM in the absence or presence of RuR/Ru360. **f** Bar graph represents the apoptosis (% of cells), from (**e**), RuR/Ru360 treatment has significantly reduced the C12-induced PS exposure. (Mean ± S.E.M; $n = 3$ independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *RuR* ruthenium red, *Ru360* mCU inhibitor, *C12 N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *MMP* mitochondrial membrane potential, *MPTP* mitochondrial permeability transition pore, *PS* phosphatidylserine, *JC-1* 5,5V,6,6V-tetrachloro-1,1V3,3V-tetra ethyl benzimidazol carbocyanine iodide, *FITC* fluorescein isothiocyanate, *PI* propidium iodide

Discussion

P. aeruginosa is an important pulmonary pathogen, and it is a leading cause of morbidity and mortality in CF patients [2]. The interaction between *P. aeruginosa* and host cells not only relies on cell-to-cell contact but also occurs through bacterial secreted products such as QS molecules [35]. *P. aeruginosa*-associated QS molecule (C12) has previously been reported to cause apoptosis in several different eukaryotic cells [21–25]. The present study enhances our understanding of the mechanism linking C12-induced Ca^{2+} signaling and apoptosis in neutrophils. In this study, we found that out of the two major AHL molecules, C12 (100 µM) treatment caused cytotoxicity and induces apoptotic nuclei formation in neutrophils, while C4 was ineffective in these experiments. Differential effect of C12 and C4 has previously been reported in the microbial cells [36, 37] and also in the host cells [17, 25]. These results can be attributed to differences in the length and composition of their acyl chain. Further, we found that C12 (100 µM) caused an increase in intracellular Ca^{2+} level, MMP loss, MPTP opening, and PS exposure. Altogether, these results suggest that C12 induces mitochondrial dysfunction and apoptosis in human neutrophils. In addition to C12, other soluble factors like T3SS

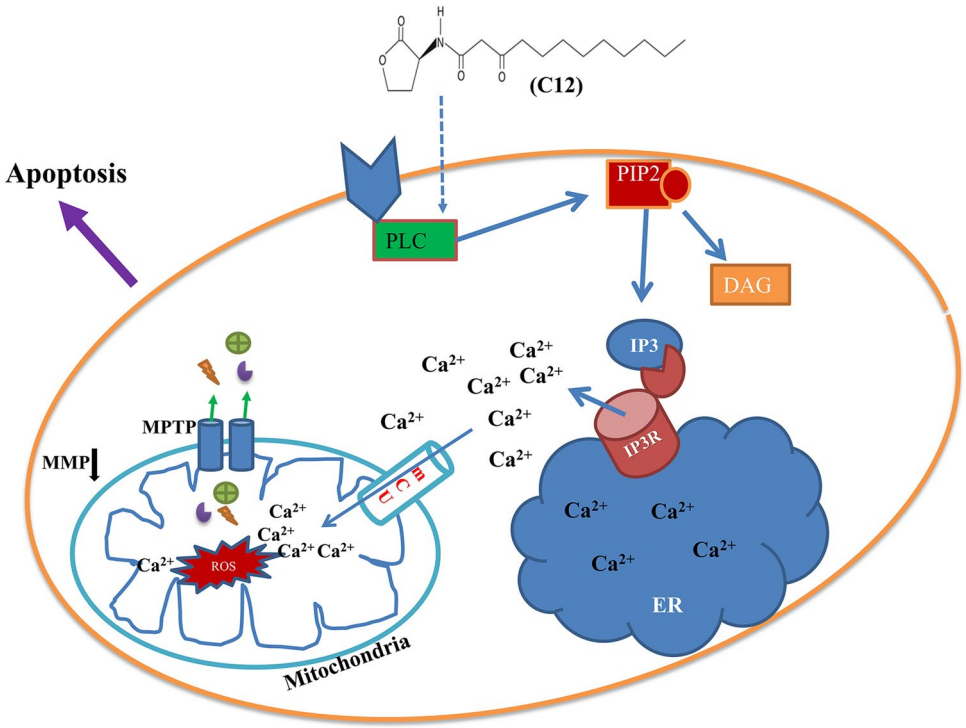

Fig. 8 Proposed mechanism for C12-induced, calcium-mediated mitochondrial dysfunction, and apoptosis in host cells. *P. aeruginosa* produces C12 that interacts with the host cells. C12 causes mitochondrial dysfunction and apoptosis in neutrophils through a rise in cytosolic and mitochondrial calcium levels. Mitochondrial calcium uniporter participates in the C12-induced mitochondrial calcium uptake, mitochondrial ROS generation, MMP loss, MPTP opening, and PS exposure. *C12* *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *mCU* mitochondrial calcium uniporter, *MMP* mitochondrial membrane potential, *MPTP* mitochondrial permeability transition pore, *PS* phosphatidylserine, *ROS* reactive oxygen species, *PLC* phospholipase-C, *PIP2* phosphatidylinositol 4, 5-bisphosphate, *IP3* inositol-1,4,5-triphosphate, *ER* endoplasmic reticulum, *DAG* diacylglycerol, *IP3R* inositol-1,4,5-triphosphate receptor

[38] and pyocyanin [39, 40] of *P.* aeruginosa have been reported to induce apoptosis in neutrophils. Thus, in the case of *P. aeruginosa* infection, along with these soluble factors, C12 may cause a positive synergistic effect on neutrophils.

The concentration of C12 and C4 in *P. aeruginosa*-infected CF patients has been reported in the range of 1–21 nM and 1–5 nM, respectively [41, 42]. Additionally, the concentration of C12 was reported in the range of 1–2 µM in a murine model of *P. aeruginosa* lung infection [43]. However, lower concentrations of AHL could be explained as a consequence of AHL degradation, dilution of sputum sample, reduced efficiency of autoinducer extraction and choice of methods for the quantification of QS molecules [42]. Besides, the in vitro concentration of C12 in the planktonic culture of *P. aeruginosa* was reported in the range of 1–10 µM [44, 45]. Furthermore, a higher concentration of C12 in *P. aeruginosa* biofilm has been reported and it can reach up to 300–600 µM [22, 45–47]. These studies suggest that host cells may get exposure to a higher level of AHL molecules, especially in the near vicinity of *P. aeruginosa* biofilms. AHLs have a half-life in hours under alkaline pH at temperatures > 20 °C, while in acidic conditions, it increases up to days at 4 °C [45]. In many studies, mammalian paraoxonase (PON) enzymes, including PON1, PON2, and PON3, have been reported to possess AHL-degrading activities [45, 48, 49]. However, it has been demonstrated that PON-mediated degradation of C12 is responsible for its apoptotic effect in the host cells [31, 50]. In addition to apoptosis, C12 has also been reported to induce pro-inflammatory responses, for example, production of IL-8, cyclooxygenase-2, and prostaglandin E2 synthase in human lung fibroblasts and epithelial cells [12, 51, 52]. In this regard, Shiner et al., have reported that C12 activates at least two independent signal transduction pathways, including the stimulation of intracellular calcium-mediated apoptosis and calcium-independent modulation of the inflammatory responses [24]. Intracellular calcium mobilization is involved in the regulation of various types of cell death pathways, like apoptosis [53]. Increased cytosolic Ca^{2+} level has been linked with dysregulated mitochondrial homeostasis leading to cell death [54]. We found that intracellular Ca^{2+} chelation using BAPTA-AM abrogated C12-induced MMP loss, MPTP opening, and PS exposure. These findings suggest that intracellular calcium plays an essential role in C12-induced mitochondrial dysfunction

and apoptosis. Various intracellular organelles act as calcium stores, and ER is being considered as a major intracellular calcium reservoir [29, 55]. To investigate the possible role of ER, C12-induced intracellular Ca^{2+} was measured in the presence of thapsigargin (a SERCA inhibitor). We found that inhibition of SERCA by thapsigargin prevented C12-induced Ca^{2+} rise, suggesting that ER significantly contributes to C12-induced elevation in cytosolic Ca^{2+} level. ER is an inositol triphosphate (IP3)-sensitive Ca^{2+} store, and activation of PLC leads to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) to IP3 and diacylglycerol (DAG). IP3 binds with ER surface receptor IP3R and facilitates Ca^{2+} release [56]. Further, we found that IP3R blocker, 2-APB, and PLC inhibitor, U73211, significantly prevented the cytosolic Ca^{2+} rise, suggesting the involvement of PLC-IP3R axis in C12-induced calcium rise. It has been demonstrated that IP3R-mediated calcium release is involved in the process of apoptosis [53]. On the contrary, we observed that the inhibition of IP3R by 2-APB could not prevent the C12-induced PS exposure. 2-APB has been considered as a non-specific inhibitor of IP3R [57], and it may affect other cellular pathways which are involved in the regulation of PS exposure. Further, to confirm the involvement of IP3R in C12- induced PS exposure, we used another IP3R antagonist Xestospongin-C. We found that similar to 2-APB, XeC was unable to prevent C12-induced PS exposure. These results are consistent with the previous study in which intracellular calcium-mediated apoptosis has been shown to progress in IP3R-independent manner [58]. Together, these data suggest that C12-induced calcium-mediated apoptosis is independent of IP3R activation.

Mitochondria act as a Ca^{2+} buffering organelle to regulate cytosolic calcium level [30]. An elevated level of cytosolic Ca^{2+} may lead to mitochondrial Ca^{2+} overload and subsequent disruption of mitochondrial homeostasis resulting in mitochondrial dysfunctions and cell death [30, 54]. Under cell stress conditions, the two major Ca^{2+} channels mCU and VDAC regulate the mitochondrial Ca^{2+} uptake [59, 60]. Further, the involvement of mCU and VDAC in C12-induced apoptosis was investigated. We found that C12 facilitated calcium-induced Ca^{2+} uptake in the mitochondria of permeabilized neutrophils. Inhibition of mCU by RuR/Ru360 prevented mitochondrial Ca^{2+} uptake, indicating that mCU majorly contributes to the C12-induced mitochondrial Ca^{2+} overload. Moreover, mitochondrial Ca^{2+} uptake plays a decisive role in the MMP alteration [30] and MPTP modulation [61]. Next, we examined the role of mCU in C12-induced MPTP opening and MMP loss. Our result showed that inhibition of mCU delayed the MPTP opening and MMP loss, suggesting that mCU is involved in C12-induced mitochondrial dysfunction. Calcium overload leads to mitochondrial ROS generation [62], resulting in mitochondrial damage and cell death [63]. Next, we evaluated the impact of C12 on mROS generation, and it was found that C12 significantly triggers the mROS formation. Further, mCU inhibition diminished mROS production, suggesting that mCU contributes to C12-induced mROS generation. Besides, PS exposure was also diminished in the presence of Ru360/RuR. In addition, inhibition of VDAC by DIDS (10 μM) did not affect PS exposure (Fig S1a, b). Altogether, these results show that mCU contributes significantly to C12-induced mitochondrial dysfunction and apoptosis.

In conclusion, this study shows that C12 causes apoptotic cell death in human neutrophils by increasing the levels of cytosolic and mitochondrial calcium, loss of MMP, generation of mROS, and opening of MPTP. Furthermore, C12-induced mitochondrial dysfunction and apoptosis occur at least partially through mCU-mediated calcium overload in the mitochondria. The findings of this study are summarised in Fig. 8. In addition to mCU and VDAC, various other mitochondrial calcium regulatory channels, including mitochondrial ryanodine receptor (mRyR) and mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) contribute to the $[Ca^{2+}]$ m overload [64]. Therefore, further studies are required to investigate the possible role of these channels in C12-induced mitochondrial dysfunction and apoptosis in neutrophils. Further, in vivo studies are needed to validate these results and to identify mCU as a potential therapeutic target to combat *P. aeruginosa* infections.

Acknowledgements This research work was supported by the Grant from the Department of Biotechnology (DBT) Ministry of Science and Technology (BT/PR6175/GBD/27/367/2012); Science and Engineering Research Board (SERB) (EMR/2014/000496) Government of India. The authors gratefully acknowledge the University Grant Commission (UGC), and the Ministry of Human Resource Development (MHRD) for enabling fellowship. Authors are thankful to the Centre for Interdisciplinary Research (CIR), MNNIT Allahabad for providing the facility of the fluorescence spectrophotometer. Authors are also thankful to the Central Instrumentation Facility (CIF), Department of Biotechnology MNNIT Allahabad for providing the flow cytometry facility.

Author contributions PKS, VKY, and VA conceived and designed the experiments; PKS, VKY, and MK performed the experiments; PKS, VKY, VA, DP, and DS analyzed the data; VA provided comments and technical support; PKS, VKY, MK and VA wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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