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Bardoxolone methyl induces mitochondrial dysfunction, activation of the cytosolic stress response, and increased chaperone capacity in SH-SY5Y cells. Focus on mitochondrial protease LONP1

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Abstract. Our work aimed to study the impact of bardoxolone methyl, 2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oic acid methyl ester (CDDO-Me), on mitochondrial function and morphology in SH-SY5Y cells, molecular responses, with a focus on the expression of proteins of mitochondria-specific unfolded protein response (mtUPR), the endoplasmic reticulum-specific UPR (UPR^{ER}), and the cytosolic stress response. Treatment of SH-SY5Y cells with CDDO-Me is associated with decreased relative cell survival associated with gasdermin E cleavage, compatible with pyroptotic cell death features. Treatment of SH-SY5Y cells with CDDO-Me results in decreased ROUTINE respiration, maximal respiration, succinate-driven maximal respiration, ATP-coupled respiration, and spare respiratory capacity. We have not observed significant changes in the expression of proteins that play important roles in both mtUPR (LONP1) and UPR^{ER} (HRD1 and SEL1L), whereas expression of cytosolic chaperone HSP70 was significantly increased in response to CDDO-Me. In addition, we have observed increased expression of mitochondrial chaperones HSP60 and GRP75 as well as ER chaperone GRP78. Finally, we have observed the formation of donut-like mitochondria induced by CDDO-Me. Our results, together with previously published data, indicate that mitochondrial dysfunction, activation of cytosolic stress response, and an increase in chaperone capacity elicited by CDDO-Me could be attributed to CDDO-Me's ability to inhibit LONP1 protease.

Key words: LONP1 — Mitochondria — Endoplasmic reticulum — Unfolded protein response — Cytosolic stress

Introduction

Bardoxolone methyl, the C-28 methyl ester of naturally occurring 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) known as CDDO-Me, is one of the orally bioavailable derivatives of synthetic triterpenoids that was tested for the treatment of chronic kidney disease, cancer (including lymphoma and some solid tumours), and pulmonary arterial hypertension. CDDO-Me exhibits pleiotropic effects across various intracellular pathways (Borella et al. 2019). At nanomolar concentrations, CDDO-Me binds to Keap1, leading to the release of Nrf2 and further induction of an antioxidant and anti-inflammatory response. At micromolar concentrations, CDDO-Me binds to IKKβ and prevents the release of NF-κB from its complex with IκB in the cytosol,

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thereby inhibiting NF-κB activation and the downstream proinflammatory signalling cascade. CDDO-Me has also been shown to inhibit JAK/STAT3 pathways in different cancer cells. Other effects of CDDO-Me at the micromolar level include induction of extrinsic or intrinsic apoptotic pathways, autophagic cell death, inhibition of telomerase activity, and the deubiquitylating enzyme USP7 (Borella et al. 2019). CDDO-Me is also well characterised (Lee et al. 2022) and widely used as a potent inhibitor of mitochondrial protease LONP1 (Bernstein et al. 2012; Shin M et al. 2021; Li et al. 2025). With respect to mitochondrial proteostasis, the results of LONP1 knockdown experiments and LONP1 inhibition by CDDO-Me were almost identical regardless of the method of LONP1 downregulation or inhibition (Li et al. 2025).

LONP1 is a primary mitochondrial protein quality control protease involved in maintaining mitochondrial proteostasis (Lebeau et al. 2018; Song et al. 2021). It is also an essential protein of mitochondria-specific unfolded protein response (mtUPR) that represents a cytoprotective mechanism to cope with stress-inducing conditions and to restore mitochondrial functions (Fiorese et al. 2016; Shpilka and Haynes 2018). In mammals, LONP1 function involves proteolytic degradation of misfolded and damaged proteins, components of the electron transport chain, and mtDNA regulatory proteins such as DNA polymerase subunit gamma and mitochondrial transcription A (Fu and Markovitz 1998; Bota and Davies 2002; Fukuda et al. 2007; Matsushima et al. 2010; Lu et 2013). In addition, LONP1 functions as an ATP-dependent, protease-independent chaperone that works in concert with the mtHSP70-DNAJA3 chaperone system to stabilize a significant fraction of imported mitochondrial proteins, including mitochondrial inner membrane insertase, NADH ubiquinone oxidoreductase subunit A9, and caseinolytic mitochondrial matrix peptidase chaperone subunit X (Rep et al. 1996; Cheng et al. 2013; Shin CS et al. 2021). Homozygous elimination of the *Lonp1* gene in mice is embryonically lethal, indicating an essential role of LONP1 in regulating mitochondrial functions during the development of the organism (Quiros et al. 2014), while embryonic fibroblasts from *Lonp1*$^{wt/-}$ mice showed impairment of mitochondrial ultrastructure and functions (De Gaetano et al. 2020). Silencing of LONP1 in RKO colon cancer cells causes significant alterations of mitochondrial proteome, function, and morphology, resulting in cell death (Gibellini et al. 2014). In addition, inhibition of LONP1 activity is associated with cell cycle arrest and cell death (Alabran et al. 2008; Gibellini et al. 2015; Ju et al. 2021; Lee et al. 2022). Finally, disturbances of both activities of LONP1 are implicated in mitochondrial dysfunction associated with aging and diverse pathologic conditions, including cancer, and numerous other human diseases (Ngo and Davies 2007; Quiros et al. 2014, 2015; Pinti et al. 2015; Bota and Davies 2016; Lu 2017; Peter et al. 2018; Besse et al. 2020). A recent study revealed that reduced LONP1 expression in β cells of donors with type 2 diabetes is associated with mitochondrial protein misfolding and reduced respiratory functions, leading to β cell apoptosis and hyperglycaemia (Li et al. 2025).

This study aimed to examine the impact of CDDO-Me on the relative viability of SH-SY5Y cells, mitochondrial respiration, and molecular responses of the cells with a focus on the expression of proteins that play essential roles in mtUPR, endoplasmic reticulum-specific UPR (UPR^{ER}), and cytosolic stress response, as well as on the morphology of both mitochondria and ER.

Materials and Methods

DPBS (Sigma), Trypsine (EC 3.4.21.4, Sigma), ADP (Merck Millipore, 117105), antimycine A (Sigma, A8674), carbonyl cyanide 3-chlorophenylhydrazone (Sigma, C2759), cytochrome c (Sigma, C3131), digitonin (Wako Chemicals, 043-21371), EGTA (Sigma, 03779), HCl (Sigma, 339253), Hepes (Sigma, 54457), KCl (Merck Millipore, 104938), K_2HPO_4 (Sigma, 17835), KOH (Sigma, P5958), MiR05-Kit (Oroboros AT), mannitol (Sigma, M9546), $MgCl_2$ (Sigma, M1028), NaCl (Sigma, 9888), sodium pyruvate (Sigma, P2256), oligomycin (Sigma, O4876), rotenone (Sigma, R885), succinate (Sigma, 14080), CDDO-Me (Merck Millipore, SMB00376), thapsigargin (Calbiochem, Sigma-Aldrich, 586005), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (AppliChem), mouse monoclonal antibodies against HSP60 (SC-271215, Santa Cruz Biotechnology), HSP70 (SC-66048, Santa Cruz Biotechnology), GRP75 (SC-133137, Santa Cruz Biotechnology) and β-actin (3700, Cell Signaling); rabbit polyclonal antibody against HRD1 (13473-1-AP, Proteintech), DNAJA1 (11713-1-AP, Proteintech), SSBP1 (12212-1-AP, Proteintech), GRP78 (11587-1-AP, Proteintech), Gasdermin E (13075-1-AP, Proteintech), SEL1L (PA5-88333, Invitrogen), LONP1 (PA5-51692, Invitrogen); rabbit monoclonal antibody against Gasdermin D (HL1334, Novus Biologicals); rat monoclonal antibody against HSF1 (A305101, Antibodies.com); goat anti-rabbit (A0545, Sigma-Aldrich), donkey anti-rat (SA00001-10, Proteintech) and goat anti-mouse (A0168, Sigma-Aldrich) secondary antibodies conjugated with horse radish peroxidase.

Culturing, treating, and harvesting of SH-SY5Y cells

Undifferentiated human neuroblastoma SH-SY5Y cells (ATCC) were cultured at 37°C, 5% CO_2 humidified atmosphere maintained in medium DMEM:F12 (1:1) (Dulbecco's Modified Eagle's Medium and Ham's *F-12* Nutrient Mixture, Sigma) supplemented with 10% fetal

bovine serum (FBS) and 1% penicillin streptomycin stock (all PAA). The medium was changed every 2–3 days until 80% confluence was achieved. SH-SY5Y cells were treated with the indicated concentrations of CDDO-Me for 24 and 48 h at 37°C and under a 5% CO_2 humidified atmosphere. In some experiments, heat shock was induced by cultivation of the cells at 42°C for 15 min. The cells were further cultured at 37°C, 5% CO_2 humidified atmosphere for 1, 3, 6, and 24 h. At the end of the treatments, the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and then resuspended in a lysis buffer (30 mM Tris-HCl, 150 mM NaCl, 1% CHAPS, 1× protease inhibitor cocktail, pH = 7.6) for total protein extraction. Protein concentrations were determined using a protein DC assay kit (Bio-Rad) with BSA as a standard. On the day of high-resolution respirometry analysis, control and treated cells were trypsinised, washed twice with DPBS, and the Trypan blue exclusion test was used to determine the number of viable cells present in a cell suspension using a Countess™ 3 automated cell counter (Invitrogen/Thermo Fisher Scientific). For high-resolution respirometry analysis, an aliquot of 2×10^6 of viable cells was used.

Cell viability

The cells were seeded in 96-well plates at concentrations of 0.1×10^6 cells *per* ml. Control cells and cells treated with CDDO-Me were incubated for the indicated time intervals at 37°C under a 5% CO_2 humidified atmosphere. At the end of incubation, 0.01 ml MTT solution (5 mg/ml) was added to each well, and the cells were further incubated for 4 h at 37°C and under a 5% CO_2 humidified atmosphere. The insoluble formazan, which resulted from the oxidation of added MTT by vital cells, was dissolved by the addition of 0.1 ml of SDS solution (100 mg/ml) and overnight incubation at 37°C under a 5% CO_2 humidified atmosphere. The absorbance of formazan was determined spectrophotometrically by using a Synergy H4 microplate reader (Agilent). The relative viability of the cells was determined as the ratio of the optical density of formazan produced by treated cells to the optical density of the formazan produced by untreated control cells and was expressed as a percentage of the control. For each treatment time, the optical density values of untreated control cells were considered 100% of viable cells.

High resolution respirometry (HRR)

Mitochondrial respiration was analysed using HRR measurements of intact cells using the O2k-FluoRespirometer two-chamber system (Oroboros Instruments) and the Oroboros coupling control protocol for intact cells as previously described in detail (Evinova et al. 2020, 2022; Liskova et al. 2025).

Western blotting

Isolated proteins (30 µg proteins loaded *per* lane) were separated on 10% SDS-polyacrylamide gels (PAGE) under reducing conditions. Separated proteins were transferred to nitrocellulose membranes by using semi-dry transfer. Membranes were probed with antibodies specific to HRD1 (1:1000), GRP78 (1:1000), GRP75 (1:1000), DNAJA1 (1:1000), SSBP1 (1:1000), HSF1 (1:1000), SEL1L (1:1000), HSP60 (1:1000), HSP70 (1:1000), LONP1 (1:1000), Gasdermin E (1:1000), Gasdermin D (1:1000) and β-actin (1:2000). Further incubation of the membranes with particular secondary antibodies (1:10 000 mouse, 1:20 000 rabbit) was followed by the visualisation of immunopositive bands by using the chemiluminiscent substrate SuperSignal West Pico (Thermo Fisher Scientific) and the Chemidoc XRS system (Bio-Rad). Intensities of specific bands were quantified by Quantity One software (Bio-Rad). The intensities of the bands of interest were normalised to the corresponding intensities of bands of β-actin. They were expressed as the intensity of the band of the particular protein in treated cells relative to the intensity of the band in control non-treated cells.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from harvested cells treated with CDDO-Me or thapsigargin using Tri reagent (Sigma-Aldrich) following the manufacturer's protocol. Total RNA (1 µg) was reversely transcribed to cDNA using mRNA-MAXIMA First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to the protocol supplied by the manufacturer.

Reverse-transcription polymerase chain reaction RT-PCR

Aliquots of the resulting cDNA corresponding to 50 ng of total RNA were used in a PCR reaction. Sequences of primers used for amplification of XBP1 mRNA (XBP1 forward primer: CCTGGTTGCTGAAGAGGAGG and XBP1 reverse primer: CCATGGGGAGATGTTCTGGA) were designed and verified using the nucleotide database of the National Centre for Biotechnology Information. Amplification of the cDNAs was initiated by denaturation at 95°C for 3 min, followed by 35 PCR cycles (denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s) and final extension at 72°C for 7 min in a DNA thermal cycler (Biometra). The PCR products were separated by electrophoresis in 3% agarose gel and then visualised with ethidium bromide staining.

Confocal microscopy

Both mitochondrial and ER morphology were evaluated using a Zeiss LSM 880 scanning confocal microscope (LSM

AxioExaminer platform) with a W Plan-Apochromat 40×/1.0 DIC M27 water-immersion objective. The Mitotracker Red FM staining (final concentration 500 nM, Thermo Fisher Scientific) and ER-Tracker Blue-White DPX staining (final concentration 1 μM, Thermo Fisher Scientific) were performed according to the manufacturer's protocol. Confocal images were acquired using a wet objective with 40× magnification at a resolution of 1024×1024 pixels, with a scaling factor of 0.07×0.07 μm *per* pixel, resulting in a field of view of ~71.68×71.68 μm. The scanner zoom was set to X:3.0, Y:3.0, with a pixel dwell time of 8.24 μs. Fluorescence excitation was performed at 561 nm, and emission was detected at 629 nm, ensuring optimal signal capture for Mitotracker Red FM-labelled mitochondria. ER-Tracker Blue-White DPX-labelled ER was visualized using excitation at 405 nm and emission at 540 nm.

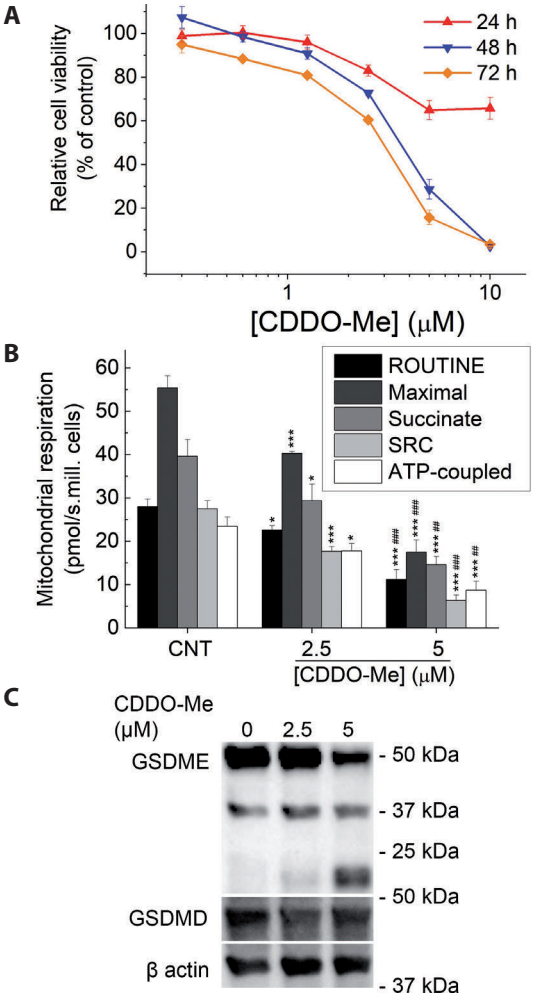

Figure 1. Impact of CDDO-Me on relative viability of SH-SY5Y cells, mitochondrial respiration, and cell death induction. **A.** SH-SY5Y cells were incubated with indicated concentrations of CDDO-Me for 24, 48, and 72 h, and then the relative cell viability was determined by MTT test as described in Materials and Methods. Data are presented as means ± SD (4 independent experiments). **B.** SH-SY5Y cells were treated with CDDO-Me at concentrations of 2.5 and 5 μM for 24 h. Mitochondrial respiration was assessed using HRR as described in Materials and Methods. Data are presented as mean ± SD (4 independent experiments per treatment). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA, followed by Tukey's test to determine differences in mitochondrial respiration of treated cells compared to control non-treated cells). CNT, control, untreated cells. **C.** The representative images of 4 independent experiments that were cropped from the same blot. The impact of CDDO-Me on GSDME and GSDMD was evaluated by Western blot analysis of total cell extracts prepared from SH-SY5Y cells treated with CDDO-Me for 24 h at concentrations of 2.5 and 5 μM as described in Materials and Methods.

Statistical analysis

One-way ANOVA (GraphPad Prism V8.0.2, GraphPad Software) was first performed to test for differences among the experimental groups. In addition, Tukey's test was used to determine the differences among individual groups. Data are presented as mean ± SD. A $p < 0.05$ was considered significant.

Results

Impact of CDDO-Me on relative cell viability, mitochondrial respiration, and induction of cell death

We first analysed the impact of CDDO-Me on the relative viability of SH-SY5Y cells. The testing of relative cell viability with MTT assay at 24, 48, and 72 h after cell treatment with different concentrations of CDDO-Me revealed both concentration- and time-dependent reduction of the relative viability of SH-SY5Y cells (Fig. 1A).

We have further examined mitochondrial respiration after the treatment of the cells with CDDO-Me at concentrations of 2.5 μM and 5 μM for 24 h (Fig. 1B). Based on MTT assay results, we have selected concentrations of CDDO-Me that would elicit clear mitochondrial and stress-response effects while maintaining sufficient cell viability at 24 h. Treatment of the cells with CDDO-Me at a concentration 2.5 μM for 24 h was associated with a significant decrease of ROUTINE respiration (80.7% of control, $p < 0.05$), maximal respiration (72.7 % of control, $p < 0.001$), succinate driven maximal respiration (74.2% of control, $p < 0.05$), ATP-coupled respiration (75.7% of control, $p < 0.05$) and spare respiratory capacity (SRC) (64.4% of control, $p < 0.001$) (Fig. 1B). Treatment of the cells with CDDO-Me at a concentration 5 μM for 24 h was associated with a further significant decrease of ROUTINE respiration (40% of control, $p < 0.001$), maximal respiration (31.6% of control, $p < 0.001$), succinate driven maximal respiration (36.9% of control, $p < 0.001$),

ATP-coupled respiration (37% of control, $p < 0.001$) and SRC (22.94% of control, $p < 0.001$) (Fig. 1B).

To unveil the mechanism of cell death induced by CDDO-Me, we have performed Western blot analysis of the expression of gasdermin D (GSDMD) and gasdermin E (GSDME). As shown in Figure 1C, treatment of the cells with CDDO-Me did not affect expression of GSDMD. In cells treated with CDDO-Me at a concentration of 5 μM, we have observed decreased signal corresponding to the full-length GSDME and increased signal most probably corresponding to the C-terminal fragment of GSDME. In addition, we have observed a signal with an apparent molecular mass of approximately 37 kDa. Since the intensity of the 37 kDa signal was not significantly changed in the cells treated with CDDO-Me at a concentration of 5 μM, we presume that this signal is not specific to the N-terminal fragment of GSDME. Although the C-terminal fragment of GSDME is not a pore-forming polypeptide, its appearance is an important marker of cell death called pyroptosis (Bai et al. 2025).

Impact of CDDO-Me on expression of mtUPR- and UPR^{ER}-related proteins

To examine the impact of CDDO-Me on the induction of mtUPR, we have performed Western blot analysis of HSP60 and LONP1 expression. Both proteins are considered important molecular components of mtUPR (Fiorese et al. 2016). Treatment of the cells for 24 h with CDDO-Me at a concentration of 5 μM (Fig. 2) was associated with a significant increase in HSP60 expression (151.9% of control, $p < 0.05$) while LONP1 expression was not significantly altered. In addition to mtUPR proteins, we have examined the impact of CDDO-Me on the expression of mitochondrial chaperone GRP75 that promotes mitochondrial protein folding in cooperation with LONP1 (Shin CS et al. 2021). Treatment of the cells for 24 h with CDDO-Me at a concentration of 5 μM (Fig. 3) was associated with a significant increase in GRP75 expression (144.1% of control, $p < 0.05$).

Contacts and communications of intracellular organelles were increasingly identified to play an essential role in cellular functions and homeostasis (Zhang et al. 2023). Therefore, in addition to mtUPR, we have focused our interest on the proteins involved in UPR^{ER}. GRP78 is a master protein involved in the activation of UPR^{ER} that represents the main cellular response to ER stress (Luo et al. 2003; Almanza et al. 2019; Acosta-Alvear et al. 2025). HRD1, which is upregulated after ER stress (Dibdiakova et al. 2019), is an E3 ubiquitin ligase involved in the process of ER-associated degradation (ERAD) (Kikkert et al. 2004) that includes retro-translocation of aberrant proteins from the ER to the cytosol through the membrane-spanning retro-translocon (Hampton et al. 2012), polyubiquitinylation of aberrant proteins by means of HRD1, and final degradation of aberrant

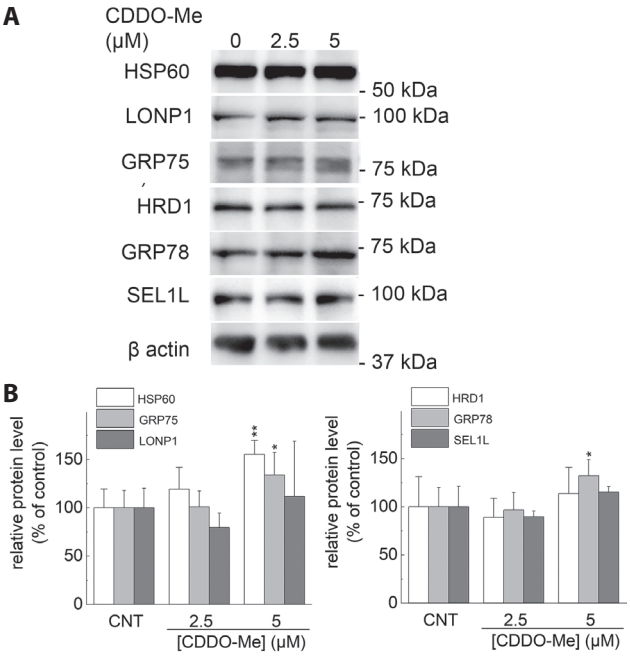

Figure 2. Impact of CDDO-Me on the expression of mtUPR- and UPR^{ER}-related proteins. **A.** Representative images of 4 independent experiments were cropped from different blots. For quantitative evaluation of the impact of CDDO-Me on expression of HSP60, LONP1, GRP75, GRP78, HRD1, and SEL1L, total cell extracts were prepared from SH-SY5Y cells that were treated with CDDO-Me for 24 h at concentrations of 2.5 and 5 μM. The levels of indicated proteins were evaluated by Western blot analysis of total cell extracts as described in Materials and Methods. **B.** Levels of indicated proteins were normalized to β-actin levels and are expressed relative to non-treated controls. Data are presented as means ± SD (4 independent experiments *per* treatment). * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA, followed by Tukey's test to determine differences between the levels of analysed proteins in control non-treated cells and treated cells).

proteins by the 26S proteasome (Preston and Brodsky 2017). The interaction of HRD1 with SEL1L is a prerequisite for the formation of a functional HRD1-ERAD complex (Lin et al. 2024; Wang et al. 2025). Therefore, we have also analysed the SEL1L expression. Treatment of the cells for 24 h with CDDO-Me at a concentration of 5 μM (Fig. 2) was associated with a significant increase in GRP78 expression (132.2% of control, $p < 0.05$) while expression of both HRD1 and SEL1L was not significantly altered.

Impact of CDDO-Me on induction of cytosolic HSP70 and XBP1 mRNA splicing

Since mitochondria are considered important regulators of cytosolic proteostasis (Williams et al. 2020), we have also examined the impact of CDDO-Me on the expression of

cytosolic chaperone HSP70, the main protein of cytosolic response to proteasomal stress (Bush et al. 1997). In the cells treated with CDDO-Me for 24 h at concentrations of 2.5 and 5 μM (Fig. 3A), the expression of HSP70 was significantly increased to 281% of control ($p < 0.05$) and to 460.6% of control ($p < 0.001$), respectively.

In addition to examination of the expression of key proteins involved in UPR^{ER}, we have also examined the impact of CDDO-Me inhibition on the splicing of XBP1 mRNA mediated by active IRE1α. IRE1α is the most evolutionarily conserved protein involved in the induction of UPR^{ER} (Siwecka et al. 2021). Activation of IRE1α triggers splicing of XBP1 mRNA. As shown in Figure 3B, splicing of XBP1 mRNA was observed after 14 to 20 h in cells treated with CDDO-Me for 24 h at a concentration of 5 μM.

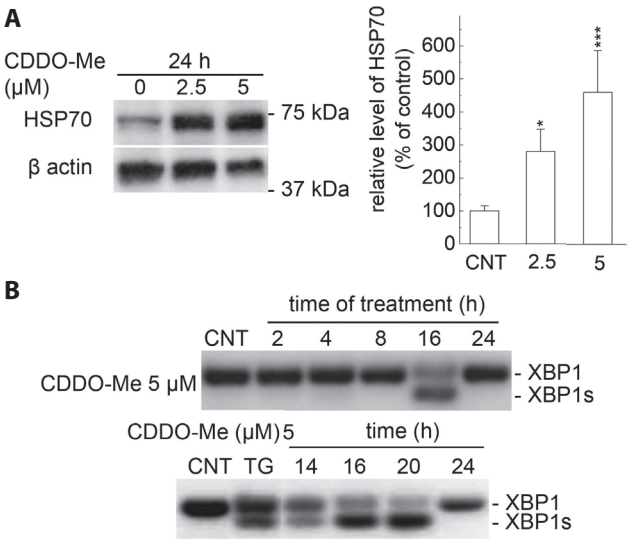

Figure 3. Impact of CDDO-Me on the expression of HSP70 and splicing of XBP1 mRNA. **A.** The representative images of 4 independent experiments that were cropped from the same blot. For quantitative evaluation of the impact of CDDO-Me on expression of HSP70, total cell extracts were prepared from SH-SY5Y cells that were treated with CDDO-Me for 24 at concentrations of 2.5 and 5 μM. The effect of the treatment on HSP70 level was evaluated by Western blot analysis of total cell extracts as described in Materials and Methods. HSP70 levels were normalized to β-actin levels and are expressed relative to non-treated controls. Data are presented as means ± SD (4 independent experiments *per* treatment). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA, followed by Tukey's test to determine differences between the levels of analysed proteins in control non-treated cells and treated cells). **B.** The cells were treated with CDDO-Me at a concentration of 5 μM for the indicated time intervals. At the end of the treatments, total RNA was isolated, cDNA synthesis, RT-PCR, and agarose gel electrophoresis were performed as described in Materials and Methods. CNT, control non-treated cells; TG, cells treated with thapsigargin at a concentration of 800 nM for 6 h.

Impact of CDDO-Me on expression of HSF1, DNAJA1, and SSBP1

To identify the mechanism of induction of HSP70 expression, we have analysed the expression of proteins that could drive transcription of the *Hsp70* gene, namely HSF1, DNAJA1 (Sutandy et al. 2023), and SSBP1 (Tan et al. 2015). We have treated the cells with CDDO-Me at a concentration of 5 μM for the indicated time intervals. As shown in Figure 4A, the expression of HSF1 was decreased in parallel with the increase in HSP70 expression that was observed after 4 h of treatment of the cells with CDDO-Me. Expression of both DNAJA1 and SSBP1 was not changed. We have also performed heat shock of SH-SY5Y cells followed by analysis of the expression of HSF1, DNAJA1, SSBP1, and HSP70. As shown in Figure 4B, the level of HSF1 was elevated immediately after the heat shock, and it remained elevated 1 h after heat shock. Expression of SSBP1 protein exhibited a similar pattern to that of HSF1. Expression of DNAJA1, which works as a co-chaperone of HSP70 (Faust et al. 2020), was increased 1, 3, and 6 h after heat shock,

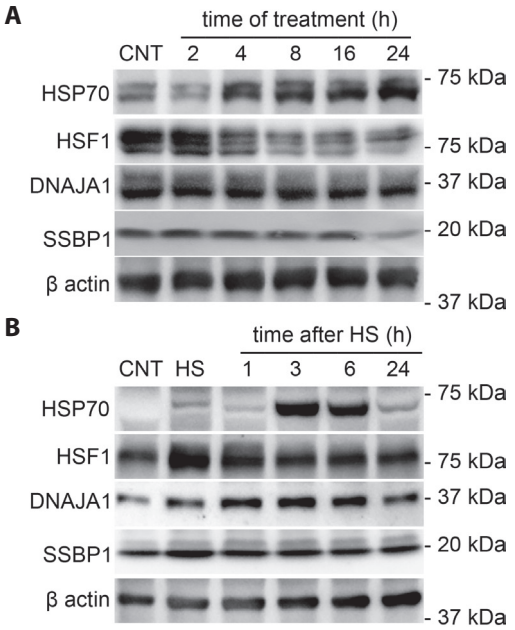

Figure 4. Impact of CDDO-Me and heat shock on expression of HSP70, HSF1, DNAJA1, and SSBP1. **A.** The cells were treated with CDDO-Me at a concentration of 5 μM for the indicated time intervals. At the end of the treatments, total proteins were extracted, and Western blot analysis was performed as described in Materials and Methods. **B.** The heat shock was induced by cultivation of the cells at 42°C for 15 min. The cells were further cultured for the indicated time intervals. Then, total proteins were extracted, and Western blot analysis was performed as described in Materials and Methods. Representative images of 3 independent experiments that were cropped from different blots.

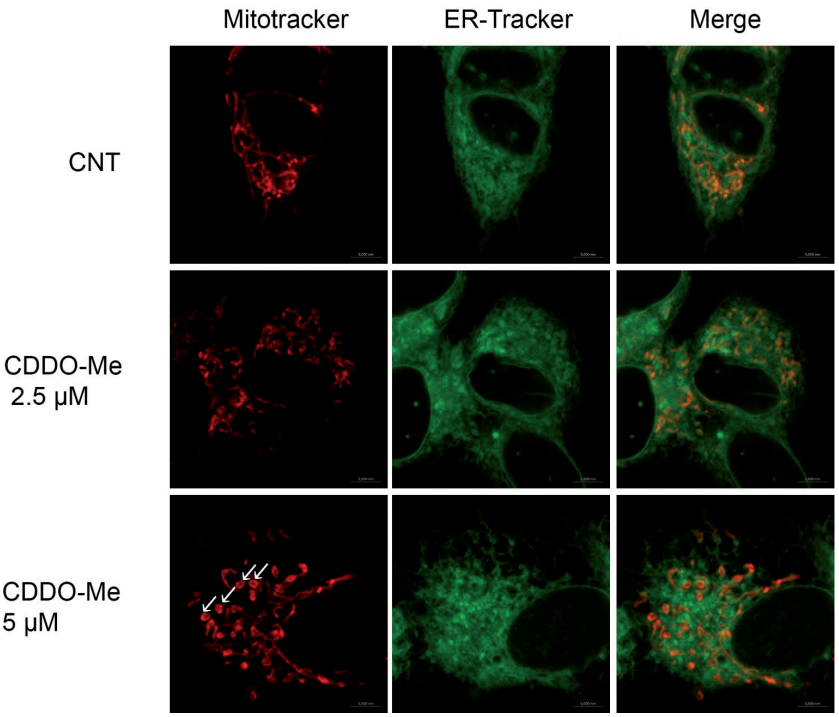

Figure 5. Impact of CDDO-Me on the mitochondrial and ER network. The cells were treated with CDDO-Me at concentrations of 2.5 and 5 μM for 24 h. At the end of the treatment, the cells were stained with Mitotracker and ER-Tracker as described in Materials and Methods. Representative images of the cells stained with Mitotracker and ER-Tracker (scale bar – 5 μm). Donut-like mitochondria are indicated by arrows.

while expression of HSP70 was increased 3 and 6 h after heat shock.

Impact of CDDO-Me on mitochondrial and ER network

We have further analysed the impact of CDDO-Me on the mitochondrial and ER networks (Fig. 5). As shown in Figure 5, treatment of cells with CDDO-Me for 24 h at a concentration of 5 μM is associated with the formation of donut-like mitochondria. The morphology of the ER network did not exhibit distinct changes after treatment of the cells with CDDO-Me for 24 h at a concentration of 5 μM (Fig. 5) mouse embryonic fibroblasts

Discussion

Decreased mitochondrial respiration, activation of cytosolic stress response, and increased chaperone capacity induced by CDDO-Me represent the main findings of our study. We have also shown that CDDO-Me has a significant negative impact on relative cell viability as a result of cell death associated with gasdermin E cleavage, compatible with pyroptotic features. In addition to mitochondrial dysfunction, the production of donut-like mitochondria was observed in response to CDDO-Me. Although we have observed increased expression of HSP60 and GRP78, other examined proteins of either mtUPR (LONP1) or UPR^{ER} (HRD1 and SEL1L) were not elevated.

In our study, treatment of the cells with CDDO-Me resulted in activation of the cytosolic stress response associated with increased expression of cytosolic HSP70 as well as increased expression of organelle-specific chaperones (mitochondrial HSP60 and GRP75, and ER resident GRP78). HSP70 represents the main protein of the proteasomal stress response (Bush et al. 1997). In addition to its chaperone function (Rosenzweig et al. 2017), HSP70 is a master regulator in protein degradation (Fernández-Fernández et al. 2017) involved in UPS-mediated protein degradation (Grune et al. 2011; Tian et al. 2021) and translocation of aberrant proteins to lysosomes for final proteolytic degradation (Chiang et al. 1989). Elevated expression of HSP70 was observed in fibrosarcoma HT1080 cells and metastatic melanoma WM266-4 cells after the treatment with CDDO-Me (Taouktsi et al. 2022). In addition, increased expression of HSP70 was documented in HeLa cells after inhibition of mitochondrial chaperone HSP90 by gamitrinib-triphenylphosphonium (GTPP) (Sutandy et al. 2023) and in C. elegans after RNAi-mediated downregulation of mitochondrial chaperone GRP75 (Kim et al. 2016). Thus, increased expression of HSP70 was observed after inhibition of proteins that play an important role in maintaining mitochondrial proteostasis, and agrees with previous findings that mitochondria also play an important role in the regulation of HSF1 activity and cytosolic proteostasis (Kim et al. 2016; Labbadia et al. 2017; Boos et al. 2019). The mechanism of HSP70 induction by CDDO-Me seems to be different from the mechanism described after heat shock, since we did not observe the same pattern of expression

of HSF1 and other investigated proteins after treatment of the cells with CDDO-Me and heat shock. In addition, the expression of HSP70 after heat shock was temporal; the level of HSP70 24 h after heat shock was comparable to the HSP70 level in control non-treated cells, while expression of HSP70 remained elevated even 24 h after treatment of the cells with CDDO-Me. Increased HSP70 expression documented after inhibition of mitochondrial chaperone HSP90 by GTPP was associated with HSF1 translocation to the nucleus without changes in HSF1 expression (Sutandy et al. 2023). Nuclear translocation of HSF1, along with HSF1 phosphorylation and elevated HSP70 expression, was documented after treatment of the mouse embryonic fibroblasts (MEF) with both GTPP and CDDO, a less potent inhibitor of LONP1 than CDDO-Me (Katiyar et al. 2020). Interestingly, in response to the respiratory chain impairment, HSF-1 is partially dephosphorylated *via* protein phosphatase 2 and further translocated to the nucleus. This results in the selective induction of small heat shock proteins and HSP70 (Williams et al. 2020).

Increased expression of HSP60, along with increased expression of GRP75 and HSP70, was documented after the treatment of MEF cells with both GTTP and CDDO (Katiyar et al. 2020). The same study documented important involvement of HSF1 in the CDDO-induced overexpression of mitochondrial chaperones HSP60 and GRP75 that was considered to be a specific form of mtUPR dependent on HSF1 (Katiyar et al. 2020). Another study showed increased GRP78 expression after upregulation of HSF1 (Kim et al. 2017). Increased expression of GRP78, HSP60, and GRP75, documented in our study, indicates an increase in cell chaperone capacity in response to CDDO-Me, due to LONP1 inhibition, and emphasizes the importance of proper cellular proteostasis regardless of the origin of proteostasis disturbance.

Although GRP78 and HSP60 are essential molecular components of UPR^{ER} and mtUPR, respectively, expression of other proteins like HRD1 or LONP1 that are involved in organelle-specific UPR was not elevated after the treatment of the cells with CDDO-Me. With respect to LONP1 expression, our results agree with previous studies that did not document increased LONP1 expression after treatment of different cells with CDDO-Me (Bernstein et al. 2012; Lee et al. 2022; Taouktsi et al. 2022). With respect to UPR^{ER}, activation of both PERK and IRE1α as well as increased expression of GRP78 was documented after treatment of lymphoblast K562 cells with CDDO-Me (Wang et al. 2017). A previous study has also documented that genetic deletion of LONP1 induced UPR^{ER} before mtUPR (Li et al. 2023). Although we have previously documented the correlation between XBP1 splicing, as a result of IRE1α activation, and expression of HRD1 after either ER stress in SH-SY5Y cells (Dibdiakova et al. 2019) or inhibition of HRD1 enzymatic activity in glioblastoma T98G cells (Guzikova et al. 2025), HRD1 was not observed to be overexpressed after the treatment of SH-SY5Y cells with CDDO-Me despite documented splicing of XBP1 mRNA. We have also observed an unaltered level of HRD1 despite XBP1 mRNA splicing in astrocyte K1884 cells treated with LS-102, an inhibitor of HRD1 enzymatic activity. In glioblastoma U87 cells treated with LS-102, HRD1 was elevated without XBP1 mRNA splicing (Guzikova et al. 2025). These results indicate complex regulation of HRD1 expression that is not solely dependent on XBP1-mediated transcription of *HRD1* gene.

Despite the rapid induction of cytosolic HSP70, which is considered cytoprotective (Rosenzweig et al. 2017), treatment of the cells with CDDO-Me culminates in cell death that was most prominent 48 h after treatment of cells with CDDO-Me at concentrations 5 μM and higher. In our previous study, we have also documented induction of HSP70 simultaneously with death of SH-SY5Y cells treated with proteasome inhibitor bortezomib (Pilchova et al. 2017). However, pre-treatment of SH-SY5Y cells with $CoCl_2$, which was associated with the induction of HSP70 expression, resulted in increased cell resistance to bortezomib and inhibition of caspase 3 activation (Saksonová et al. 2018). Negative impact of CDDO-Me on relative cell viability was studied previously using different cell lines derived from cancer cells (Alabran et al. 2008; Gibellini et al. 2015; Borella et al. 2019; Ju et al. 2021; Lee et al. 2022) that are more sensitive to CDDO-Me, and cell death induction is faster in comparison to what was observed in this study for SH-SY5Y cells. Previous studies have documented several mechanisms of cell death, but the majority of the studies referred to mitochondrial apoptosis (Borella et al. 2019; Lee et al. 2022). Results presented in this study are more consistent with the view that treatment of the cells with CDDO-Me is associated with GSDME cleavage compatible with features of pyroptosis, a specific form of cell death (Bai et al. 2025).

Treatment of the cells with CDDO-Me resulted in severe mitochondrial dysfunction, documented by depressed ROUTINE, maximal, and ATP-coupled respirations as well as decreased SRC. Decreased mitochondrial respiration was documented in leukaemia cells (Samudio et al. 2006) and pancreatic ductal adenocarcinoma cells treated with CDDO-Me (Akuetteh et al. 2022). Decreased basal respiration was also documented in MEF cells of *Lonp1*$^{wt/-}$ mice (De Gaetano et al. 2020). Interestingly, basal and maximal respirations were decreased in B16F10 melanoma cells with both knockdown and overexpression of LONP1 (Quiros et al. 2014). In prostate adenocarcinoma PC3 cells, LONP1 knockdown resulted in a significant reduction in both basal and maximal respiration as well as SRC compared to control cells (Yao et al. 2025). In contrast to the results of Quiros and colleagues (Quiros et al. 2014), an apparent increase in mitochondrial respiration in cells that overexpressed LONP1

was not observed (Yao et al. 2025). Depressed respirations were explained by decreased expression of mitochondrial chain complexes (Gibellini et al. 2014; Quiros et al. 2014; De Gaetano et al. 2020; Li et al. 2025).

In addition to mitochondrial dysfunction, we have observed the formation of donut-like mitochondria, considered a sign of incomplete fission and cellular stress (Jenkins et al. 2024). Previously published data indicate that changes in mitochondrial morphology in response to CDDO-Me are not a result of LONP1 inhibition (Kim et al. 2019; Lee et al. 2023). Fragmentation of mitochondria was documented in cardiomyocytes after deletion of LONP1 (Li et al. 2023). On the contrary, mitochondrial elongation relative to control cells was observed in PC3 cells with LONP1 knockdown, whereas fragmented mitochondria were observed in cells that overexpress LONP1 (Yao et al. 2025). Our results are in agreement with the current view that fragmented mitochondria, which are encountered in quiescent and metabolically inactive cells, exhibit reduced mitochondrial respiration and are associated with different pathological conditions, while fused mitochondria, prevalent in healthy metabolically active cells, exhibit higher mitochondrial respiration and ATP production (Chen et al. 2023; Tábara et al. 2025). In our recent study, we documented a more developed mitochondrial network in differentiated SH-SY5Y cells, attributable to mitochondrial fusion, compared with non-differentiated SH-SY5Y cells (Evinova et al. 2024). Changes in the mitochondrial network were accompanied by significantly increased mitochondrial respiration in differentiated cells. In turn, the mitochondrial network was changed toward more fragmented mitochondria after treatment of the cells with rotenone (Evinova et al. 2024).

In conclusion, we have shown that CDDO-Me has an important impact on cell survival, induction of cell death compatible with pyroptotic features, cytosolic stress response induction, increased chaperone capacity, mitochondrial dysfunction, and formation of donut-like mitochondria. Based on our results and previously published data, we presume that mitochondrial dysfunction, activation of cytosolic stress response, and an increase in chaperone capacity elicited by CDDO-Me could be attributed to the ability of CDDO-Me to inhibit LONP1 protease.

Conflicts of interest. The authors declare that they have no competing interests.

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Availability of data and materials. The data are available on request.

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