REPUBLIC OF TURKEY
ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

THE EFFECT OF CDP-CHOLINE ON MITOPHAGY AND MITOCHONDRIAL DYNAMICS IN A MONOCYTIC CELL LINE

SÜLEYMAN BOZKURT

MASTER THESIS

MEDICAL BIOTECHNOLOGY PROGRAM

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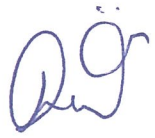
Supervisor of the thesis / Jury Member	Assoc. Prof. Devrim Öz Arslan Acibadem Mehmet Ali Aydinlar University	
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I hereby declare that; this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

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LIST OF ABBREVIATIONS

µg	:	Microgram
µl	:	Microlitre
ADP	:	Adenosine Diphosphate
AMBRA 1	:	Activatng Molecule in Beclin 1-regulated Autophagy Protein 1
ATGs	:	Autophagy Related Genes
ATP	:	Adenosine Triphosphate
BECN1	:	Beclin 1
BLC 2	:	B-cell lymphoma 2
BNIP3	:	BLC2 Interacting Protein 3
CCCP	:	Carbonyl Cyanide 3-chlorophenylhydrazone
CCT	:	Cytidilyltransferase
CDP-choline	:	Cytidine diphosphate 5-choline
CDP-ethanolamine	:	Phosphoethanolamine
CK	:	Cytidine Kinase
CL	:	Cardiolipin
CMA	:	Chaperon Mediated Autophagy
CoQ	:	Coenzyme Q
CPT	:	CDP-choline:1,2- diacylglycerol Choline Pphosphotransferase
DAG	:	1,2 - Dicacylglycerol
DMSO	:	Dimethyl Sulphoxide
DNA	:	Deoxyribo Nucleic Acid
DRP1	:	Dynamin Related Protein
DTT	:	DL-Dithiothreitol
EDTA	:	Ethylediaminetetraacedic acid
ER	:	Endoplasmic Reticulum
ETC	:	Electron Transport System
$FADH_2$	:	Flavin adenine dinucleotide
FBS	:	Fetal Bovine Serum
FIP200	:	RB1-Inducible Coiled Coil Protein
FIS1	:	Fission 1 Homology Protein
FUNDC1	:	FUN14 Domain Containing Protein
H+	:	Hydrogen
Hsc70	:	Heat Shock 70 Cognate
IM	:	Inner Membrane
LIR	:	LC3 - Interacting Region
MAP1LC3	:	Microtubule-Associated 1A/1B LC3
MFF	:	Miitochondrial Fission Factor
MFN 1/2	:	Mitofusion 1/2
mins	:	Minutes

ml	:	mililitre
mM	:	MiliMolar
MMP	:	Mitochondrial Membrane Potential
mtDNA	:	Mitochondrial DNA
mTORC1	:	Mammalian Target of Rapamycin Complex 1
MTT	:	3-(4,5-Dimethylethiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADH	:	Nicotinamide Adenine Dinucleotide
NDP52	:	Nuclear Domain 10 Protein 52
NIX	:	Nip3 Like Protein X
O2	:	Oxygen
OM	:	Outer Membrane
OPA1	:	Optic Atrophy 1
OPTN	:	Optineurin
OXPHOS	:	Oxidative Phosphorylation
P115	:	Vesicular Transport Factor
P13K	:	Phosphatidylinositol-3-kinase
PARKIN	:	PINK1 and E3-Ubiqutin Ligase
PARL	:	Rhomboid-Like Protein
PAS	:	Phagophore Assembly Site
PBS	:	Phosphate Buffer Saline
PC	:	Phosphatidiylcholine
PC	:	Phosphatidlcholine
PE	:	Phosphatidylethanolamine
PE	:	Phosphatidylethanolamine
PHB2	:	Prohibitin 2
PHB2	:	Power of Hydrojen
PI	:	Phosphatidylinositol
PI3KC3	:	Class 3 Phosphatidylinositol 3-Kinase
PINK1	:	PTEN Induced Putative Kinase Protein
PLA2	:	Phospholipase A2 Hydrogen
PMSF	:	Phenylmethanesulfonyl
PPi	:	Pyrophosphate
PS	:	Phosphatidylserine
PTEN	:	Phosphatase and Tensin Homologue
PVDF	:	Polyvinylide Fluoride
RIPA	:	Radioimmunoprecipitation assay
ROS	:	Reactive Oxygen Species
SDS	:	Sodium Dodecylsulfate
SIRT1	:	Sirtunin 1
SM	:	Sphingomyelin
SNARE	:	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
TOMM20	:	Mitochondrial Import Receptor Subunit TOM20

U937	:	Human Macropage Cell Line
ULK 1/2	:	Unc–51 Like Kinase Family 1/2
VDAC	:	Voltage Dependent Anion Selective Channel 1
VPS34	:	Vacuolar Protein Sorting 34

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SUMMARY

Mitophagy is a selective removal of mitochondria by autophagy machinery. Damaged and dysfunctional mitochondria can cause cellular degeneration, which may lead to neurodegenerative, metabolic and cardiovascular diseases. CDP-choline (CDP-ch), an intermediate in the Kennedy pathway for production of phosphatidylcholine (PC), has potential neuroprotective properties. During mitophagy, the level of PC, one of the most abundant phospholipids of cellular membranes, decreases. In this study, we investigated the effect of CDP-ch on mitophagy and mitochondrial dynamics in U937 monocytes. Mitophagy was induced at four hours by CCCP under various conditions of CDP-ch treatment. Mitochondrial dynamics and mitophagy-related proteins were investigated by western blotting. We determined that the levels of PINK1, DRP1, MFN2, COXIV and LC3B proteins were increased during mitophagy. It was observed that the level of DRP1 and MFN2 were changed in response to CDP-ch treatment. We further measured mitochondrial membrane potential, mitochondrial mass and superoxide production by flow cytometry and confocal microscopy using mitochondria-specific dyes. The decrease in mitochondrial mass upon mitophagy induction was reversed by CDP-ch treatment. The changes in mitochondrial fusion or fission and mitochondrial mass in mitophagy-induced U937 cells suggest a modulatory role of CDP-ch in mitochondrial dynamics. Moreover, the enhancement of mitochondrial superoxide levels was reduced by CDP-ch treatment in mitophagy induced U937 cells. We propose that CDP-ch may have protective effects during mitophagy by contributing to the production of mitochondrial membranes in mitophagy-induced cells. Newly synthesized mitochondria can then rescue cells quickly from the stress and maintain cell survival.

Keywords: Phospholipids, CDP-choline, Mitochondria, Mitophagy, Monocytes

ÖZET

Monositik Hücre Hattında CDP-kolinin Mitofaji ve Mitokondriyal Dinamikler Üzerine Etkisi

Mitokondrinin otofaji ile seçici olarak parçalanması mitofaji olarak adlandırılmaktadır. Hasarlı ve işlevsiz mitokondriler hücresel bozukluklara sebep olarak; nörodejeneratif, metabolik ve kardiyovasküler gibi hastalıklara yol açabilmektedirler. Fosfatidilkolin üretimi için Kennedy yolağında bir ara ürün olan CDP-kolin (CDP-k), potansiyel nöroprotektif özelliklere sahiptir. Mitofaji sırasında hücresel membranlarda en çok bulunan fosfatidilkolin seviyesi azalır. Bu çalışmada, CDP-k'nin U937 monosit hücreler üzerinde, mitofajiye olan etkisini araştırdık. Çeşitli CDP-k koşulları kullanılarak CCCP ile mitofaji dört saat boyunca indüklendi. Western blotlama yöntemi ile mitokondrial dinamik ve mitofajiye ile ilişkili proteinlerin seviyesine bakıldı. Mitofaji sırasında PINK1, DRP1, MFN2, COXIV ve LC3B proteinlerin düzeylerinin arttığını belirledik. CDP-k varlığında ise, DRP1 ve MFN2 proteinlerin seviyelerinde değişiklikler olduğunu saptadık. Mitokondriya özgü boyalar kullanılarak, akış sitometrisi ve konfokal mikroskopi ile mitokondriyal membran potansiyeli, kütle ve süperoksit üretimini ölçüldü. Mitofaji indüksiyonu ile azalan mitokondriyal kütle, CDP-k tedavisi sonrasında tersine dönmüştür. Mitofaji uygulanmış U937 hücrelerinde, mitokondriyal füzyon veya fisyon ve mitokondriyal kütlede meydana gelen değişiklikler, CDP-k'nin mitokondrial dinamikler üzerine modülatör bir rolü olduğunu göstermektedir. Ayrıca, mitofaji indüklenmiş U937 hücrelerinde yüksek olan mitokondriyal süperoksit, CDP-k varlığında azalmıştır. Mitofaji indüklenmiş hücrelerde CDP-k mitokondriyal membran oluşumuna katkı sağlayabileceği için bu hücrelerde koruyucu bir özelliği olabileceğini öne sürmekteyiz. Böylece, üretilen yeni mitokondriler ise hücreleri stresten hızlı bir şekilde kurtararak hücrelerin hayatta kalmalarını sağlayabilir.

Anahtar Sözcükler: Fosfolipitler, CDP-kolin, Mitokondri, Mitofaji, Monositler

1. BACKGROUND AND AIM OF STUDY

Phospholipids, type of lipids, are the source of biological membranes. Phosphatidylcholine is one of the amplest phospholipids in the eukaryotic cell and mitochondrial membrane. Phosphatidylcholine is synthesized de novo in the cell by combination of intermediates such as cytidine-5'-diphosphocholine (CDP-choline) and DAG (Diacyl glycerol) via the Kennedy pathway. In recent studies, CDP-choline has been found to accelerate the healing process of cerebral ischemia, tissue and brain injuries (42). Additionally, it has been suggested that it can be used as a protective agent for neurological diseases such as Alzheimer's. Therefore, CDP-choline is also used as supplementary nutrient under the name citicoline (43).

The removal of damaged mitochondria by autophagy machines is called mitophagy (16). Since oxidative phosphorylation takes place in mitochondria, reactive oxygen species are produced, and they lead to mitochondrial and nuclear DNA damage, lipids and proteins which then result in several diseases like cancer and neurodegeneration (19). Damaged mitochondria must be removed via mitophagy. Mitophagy is a relatively new subject and it is needed to be studied in detail by more research on it.

Phosphatidylcholine, which is the most abundant phospholipids in the mitochondrial membrane, has been shown to decrease during mitophagy by Won Lee et al (35). CDP-choline can be used as the source to resynthesize phosphatidylcholine during mitophagy. In this context, we think that increasing phosphatidylcholine via CDP-choline supplementation during exposure to mitochondrial damage, may reduce detrimental effects of the damage on the cells. In addition to this, further investigation on the effect of CDP-choline is investigated. The mechanism of mitophagy on monocytes, one of the innate immune system cells, has not been studied extensively. Therefore, the role of mitochondrial dynamics and mitophagy in monocytes is investigated in this study.

2. INTRODUCTION

2.1. Autophagy

Autophagy is a conserved process from yeast – simple, single-celled organisms to mammalian – highly complex, multicellular organisms. The term autophagy was coined for the first time by Christian de Duve in 1963 and comes from a Greek word meaning as “self-eating” (1, 4). For the last two decades, the researches on autophagy have been expanded. Currently, about 40 autophagy-related genes (ATGs) 15 of which are required for the core machinery have been identified in the yeast (4). Autophagy is a catabolic mechanism that provides recycling of intracellular components. Degradation of intracellular components by the lysosome serves as a new source of energy for maintaining homeostasis of the cells (2). Autophagy is required for the differentiation, development, and survival of the organisms. It plays a very important role in neurodegeneration, aging, cancer, infections along with heart, kidney and liver diseases (5). Autophagy provides continuous regeneration of proteins, lipids, carbohydrates, and organelles during basal conditions. The process of autophagy is initiated for the adaptation of different forms of stresses such as amino acid starvation, pathogenic invaders, hypoxia, glucose deprivation and increased accumulation of damaged organelles or long-lived proteins to protect the cells (3, 6).

The process of regeneration of damaged organelles, cleansing of protein aggregates and eradication of invader pathogens is tightly regulated, and it requires highly selective cargo recognition. There are several different selective types of autophagy depending on their targets. Clearance of protein aggregates, excess or damaged mitochondria, endoplasmic reticulum (ER), peroxisomes, ribosomes, lipid droplets, and pathogens are named as aggrephagy, mitophagy, reiculophagy, pexophagy, ribophagy, lipophagy and xenophagy, respectively (7).

Three different forms of autophagy have been identified so far. These are macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. Macroautophagy is the most studied type of autophagy and generally is simply named as autophagy. Isolation membrane also known as the phagophore starts to enclose a section of cytoplasm containing organelles to generate a double layer membrane called

autophagosome. The autophagosome fuses with the lysosome to degrade its contents in the final step. In macroautophagy, bulk degradation of substrates occurs non-selectively. Microautophagy, on the other hand, is the least studied form of autophagy. In this type, a small portion of the cytoplasm is directly invaginated into the late endosome or lysosome. The third form of autophagy is chaperone-mediated autophagy (CMA). The cargo proteins containing "KFERQ" sequences are recognized by Heat-shock 70 cognate (Hsc70), chaperone and co-chaperone proteins and after that they are bound with Lamp2A protein located on the surface of lysosomes and then cargo proteins are translocated into the lysosomal lumen. The main difference of CMA is the recognition of the cargo in a selective manner. The common feature of these three distinct forms of autophagy is degradation of cargo products into monomers in lysosomes or autolysosome and degraded products can be used later for different cellular needs such as energy production, protein synthesis and gluconeogenesis (Figure 1) (8).

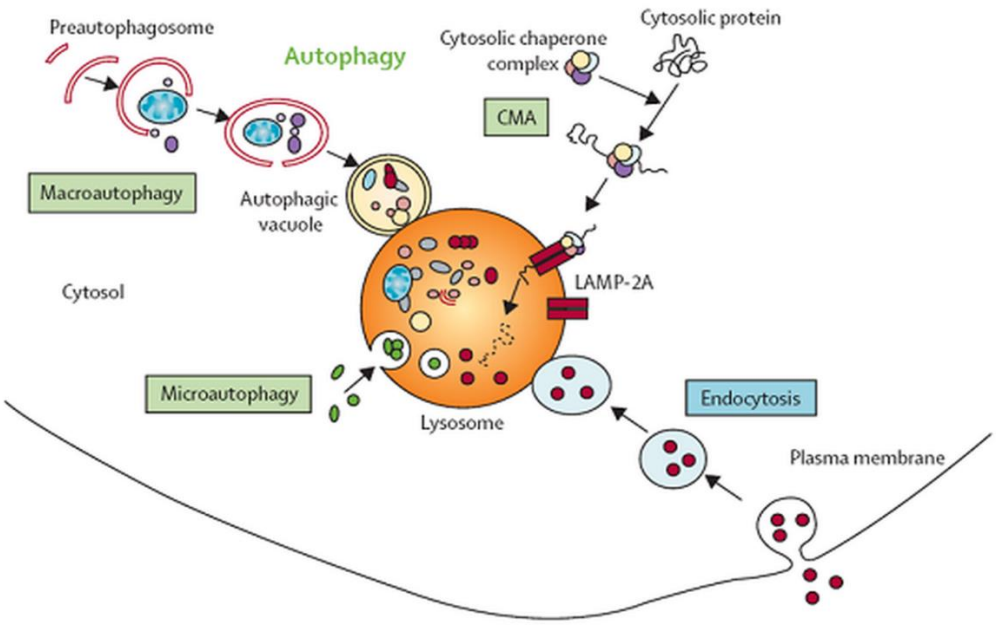


Figure 1. General scheme of different forms of autophagy

Reference: https://www.mdc-berlin.de/research/research_teams/proteomics_and_molecular_mechanisms_of_neurodegenerative_diseases/research/research2, accessed 5th of April 2019

2.1.1. Autophagosome formation and autophagy machinery

Autophagy is a highly complex process which requires more than 30 autophagy-related proteins (ATGs) in mammals. It occurs in five important stages at the molecular level. These are phagophore formation, expansion, maturation, fusion with lysosome and recycling of vesicle (Figure 2) (2).

The induction of autophagy by various stresses recruits ATG proteins to a site called phagophore assembly site (PAS) in yeast however there are multiple start sites in mammals (9, 10). Autophagy is initiated with formation of a phagophore which is also referred to as an isolation membrane. Currently, the exact source of phagophore has not been known in mammalian cells (2). It is high likely originated from ER-mitochondria, ER-plasma membrane contacts sites as well as trans-Golgi apparatus, recycled endosomes and plasma membrane. In basal condition, mTORC1 (mammalian target of rapamycin complex 1), a serine/threonine protein kinase, terminates autophagy by suppression of Unc-51-like kinase family (ULK1 or ULK2) complex through phosphorylation. During stress condtions like starvation, mTORC1 is dissociated by dephosphorylation from ULK1 complex which consists of several proteins; ATG13, ULK1/2, RB1-inducible coiled-coil protein 1 (FIP200) and ATG101, allowing the initiation of the autophagy machinery. Formation and nucleation of phagophore require another complex called ATG14-containing class III phosphatidylinositol 3-kinase (PI3KC3) complex 1. PI3KC3 complex is composed of PI3K, Beclin 1, vacuolar protein sorting 34 (VPS34), ATG14, AMBRA1 (activating molecule in Beclin 1-regulated autophagy protein 1) and p115 (vesicular transport factor). ULK1 complex phosphorylates the PI3KC3 complexes and along with isolation membrane, they go through the expansion step of autophagy (9, 10). In the expansion part of phagophore, formation two ubiquitin-like conjugation systems take place; one of them is ATG5-ATG12-ATG16L1 complex and another one is the modification of MAP1LC3 (microtubule-associated 1A/1B LC3) and GABARAP subfamily proteins. The ATG5-12-16L1 complex is conjugated by E1 and E2- like conjugation enzymes; ATG7 and ATG10, respectively. MAP1LC3 protein is first cleaved by ATG4 to form LC3-I protein after that addition of a phosphatidylethanolamine (PE) to LC3-I follows to form LC3-II by ATG7 and ATG3. ATG5-12-16L1 complex associates with phagophore membrane to facilitate

expansion by acting as an E3 ligase. LC3-II ubiquitin-like protein is embedded into phagophore membrane by ATG5-12-16L1 complex and it is required for expansion and closure of phagophore. During expansion, more membranes from the plasma membrane, ER or Golgi is provided by ATG9 containing vesicles to form a bilayer membrane called autophagosome. Eventually, autophagosome mature and fuse with lysosome producing autolysosomes by SNARE proteins, RAP7. The cargos inside of the autophagosome are then degraded by acidic hydrolysis like cathepsin B, L in the lysosome to produce monomers to be used for other anabolic processes (Figure 2) (9, 10).

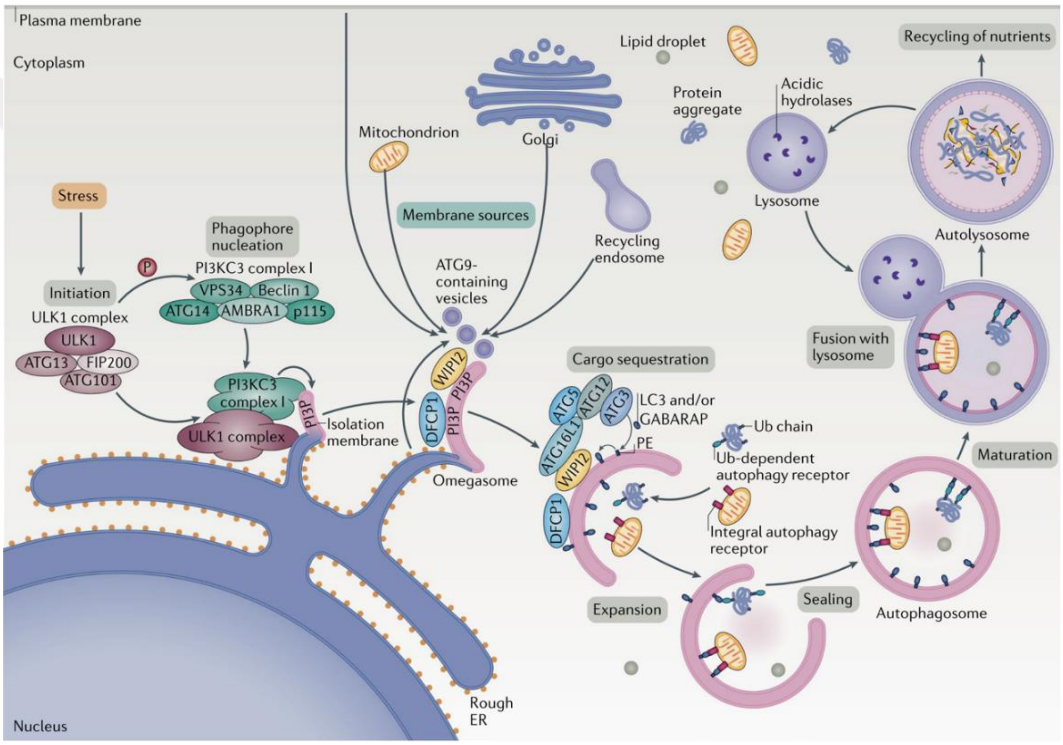


Figure 2. Autophagy machinery

Reference: Parzych KR, Klionsky DJ. An overview of autophagy: Morphology, mechanism, and regulation. Antioxid Redox Signal. 2013;20(3):460–73.

2.2. Mitochondria

Mitochondria are the energy powerhouses of the eukaryotic cells and are the organelles responsible for storing 90% of the chemical energy required for cell survival in the molecule called adenosine triphosphate (ATP). In addition to their role in energy production; they have great importance for various cellular pathways including lipid metabolism, ion homeostasis and apoptotic cell death (11). They are highly dynamic

and multifunctional, double-membrane organelles. Mitochondria are composed of two separate membranes which surround the surface of mitochondria. They are the mitochondrial outer membrane (OMM) and the inner membrane (IMM). The intermembrane space is found between these two membranes. IMM is an elaborated structure with infoldings named as cristae and this provides a great increase on the surface area. The membrane proteins related to ATP production are located in the IMM. In the matrix (cytoplasm of mitochondria), its own circular DNA, enzymes required for oxidative metabolism including the breakdown of fatty acid are located (13, 14). It is postulated that they have originated from the prokaryotic organism, thus they have their own circular DNA, which is important for the synthesis of some mitochondrial proteins. However, nearly 90% of the mitochondrial proteins are encoded in the nuclear genome (11). Approximately several hundreds of mitochondria are found in a typical mammalian cell, but the number may change depending on cell the type and its energy requirements. For example, muscle cells have more mitochondria than other cell types (13, 14). Mitochondrial dysfunction can lead to severe diseases and conditions such as metabolic diseases, neurodegeneration, diabetes, cancer and aging (12). Therefore, a eukaryotic cell has developed some ways for mitochondrial biogenesis and degradation to tackle this problem (11).

2.2.1. Electron transport system and ATP synthesis

In the Krebs cycle, two electron donor molecules are generated, they are NADH and FADH2. They transfer their electrons to electron transport chain (ETC) for initiation of oxidative phosphorylation. Oxidative phosphorylation is a process of production of ATP from adenosine diphosphate (ADP) by a series of electron transfers through protein complexes found in the IMM and it provides the major source of energy for the cell (14, 15).

The process of electron transfer from NADH or FADH2 to O2 is an energy-rich reaction. To utilize this energy, the process must be proceeded in a stepwise manner. There are four large protein complexes I-IV, to shuttle the electrons to oxygen which is the final destination of electrons to form water. Complex I and complex II accept an electron from NADH and FADH2 after that protons are translocated into intermembrane space, creating an electrochemical gradient. This gradient generates an electrical potential across membranes. There are also mobile electron carriers;

coenzyme Q (CoQ) and cytochrome to transport electrons between complexes. The electrical potential drives proton motive force to generate ATP by the fifth complex, ATP synthase. At the final step of ETC, the energy of electrons is very low, thus, it can be donated to oxygen as a final acceptor by last carrier protein and after receiving four electrons, oxygen picks up two protons and produces water (13, 14). The whole process is depicted in figure 3.

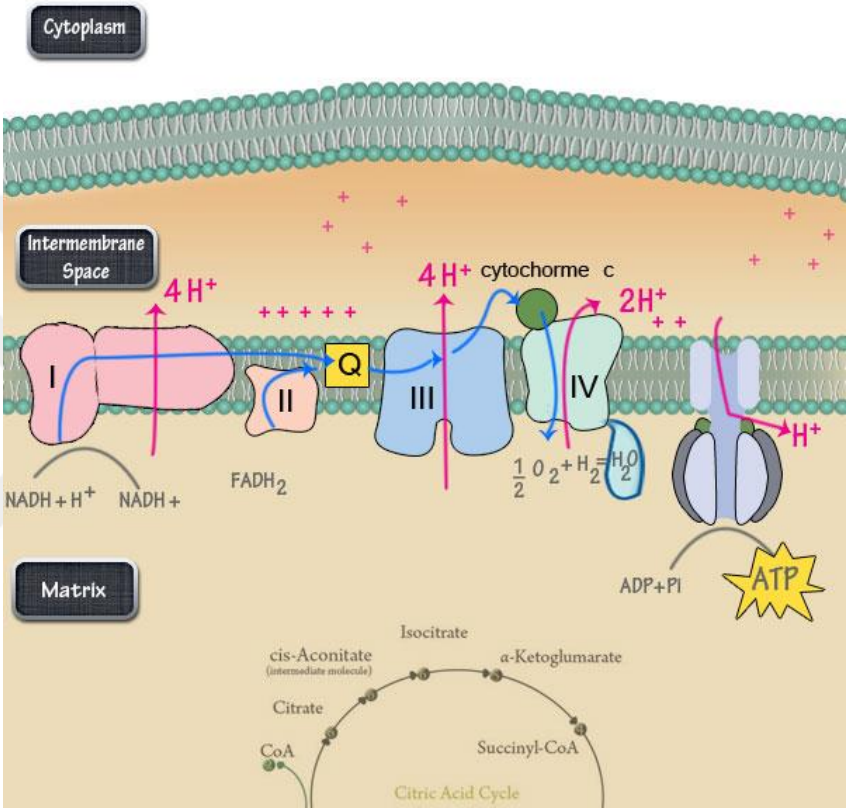


Figure 3. Electron transport chain (ETC)

Reference: http://pinosy.com/media/486318459738359604/ accessed 7th of April 2019.

2.3. Mitophagy

Cell homeostasis is maintained by the cellular quality control system to remove excessive or damaged organelles. One of the organelles which is exposed to the most damage due to reactive oxygen species (ROS) which are the byproducts of oxidative phosphorylation (OXPHOS) is the mitochondrion. Excess amount of ROS can damage lipids, proteins, and DNA in mitochondria causing severe diseases like cancer and neurodegeneration (19). Therefore, removal of damaged mitochondria is crucial for

homeostasis, and this process is carried out by the selective form of autophagy machinery named as mitophagy (16). Two general ways to induce mitophagy is by accumulation of mitochondrial damage which leads to ETC breakdown and loss of mitochondrial membrane potential. If dysfunctional mitochondria accumulate and are failed to be removed by defective mitophagy, this can lead to controlled cell death. For the immune system, toxic accumulation can trigger hyperactivation of inflammation (19). Mitophagy is critical not only for the removal of defective mitochondria but also for the regulation of mitochondrial number (17); because, maintaining proper cellular function requires a balance between division and fusion of mitochondria (18). Detection of damaged mitochondria and distinguishing them from healthy ones is the primary event of the mitophagy process. Identification is accomplished by PTEN-induced putative kinase protein 1 (PINK1) and damaged mitochondria are then engulfed by autophagosomes and processed by fusion with lysosomes (17).

2.3.1. Molecular mechanisms of mitophagy

The mechanism of mitophagy is generally divided into two categories; adaptor protein-mediated and receptor-mediated mitophagy. Several OMM and IMM proteins have been shown to be involved in adaptor protein-mediated mitophagy. There are two key proteins responsible for the detection of defective mitochondria as well as recruitment of autophagosomes to the place where defective mitochondria are engulfed. These proteins are well characterized and studied; PINK1 and E3-Ubiquitin ligase (PARKIN). Both proteins are required for proper mitochondrial function. Mutations in them are associated with Parkinson's disease (20).

In normal physiological conditions, mitochondria membrane potential (MMP) drives PINK1 translocation into IMM trough mitochondrial transport protein complexes; TOM and TIM proteins. After that, the rhomboid-like protein (PARL) which is located in IMM cleaves the PINK1 and it is degraded by proteolytic pathway whereas PARKIN is soluble in the cytoplasm under normal conditions. When mitochondria accumulate damages and MMP is disrupted, PINK1 cannot translocate into IMM and accumulates on OMM as a dimer. After the accumulation of PINK1 on OMM, PARKIN is recruited and phosphorylated by PINK1 (19). PARKIN in OMM then starts polyubiquitination of OMM proteins such as mitofusin 1/2 (MFN 1/2), mitochondrial import receptor subunit TOM20 (TOMM20) and voltage-dependent-

anion-selective channel 1 (VDAC), among them. After polyubiquitination, several adaptor proteins including p62, optineurin (OPTN) and nuclear domain 10 protein 52 (NDP52) bind to ubiquitinated mitochondria. These adaptor proteins have LIR domain where they associate with the autophagosome and mitochondria are engulfed and recycled through autophagy (Figure 4) (20).

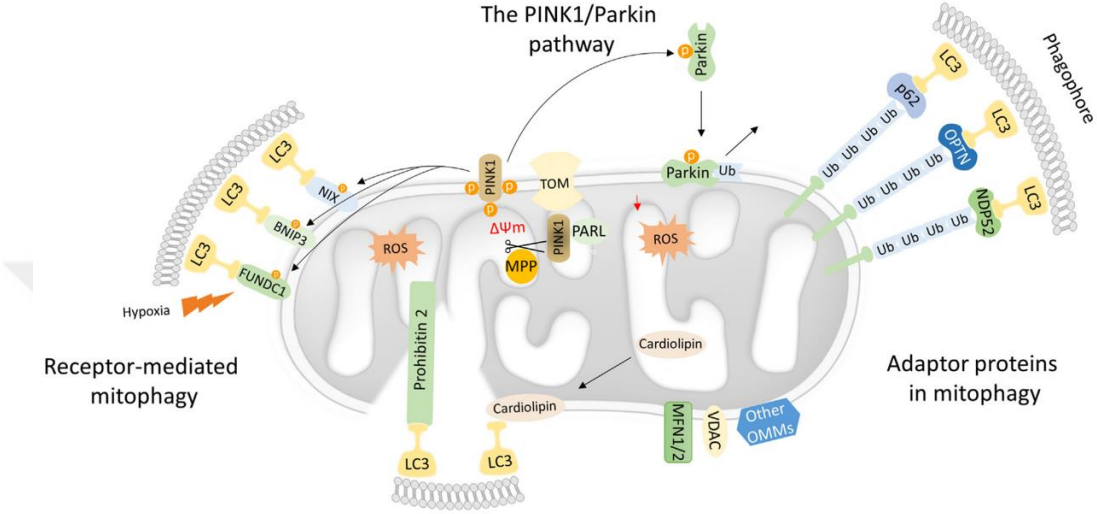


Figure 4. Mitophagy machinery

Reference: Gkikas I, Palikaras K, Tavernarakis N. The role of mitophagy in innate immunity. Front Immunol. 2018;9(JUN):1–15.

The other pathway for mitophagy is receptor-mediated. The receptor proteins are found in OMM and IMM. They have LC3-interacting region (LIR) domain. Therefore, they interact with autophagosome trough this region directly. BCL2 interacting protein 3 (BNIP3), FUN14 domain-containing protein 1 (FUNDC1) and Nip3-like protein X (NIX) are among the OM located ones. For different stress conditions, it has been shown that prohibitin 2 (PHB2) and cardiolipin, which are positioned in IM, also play a role in mitophagy. For this pathway, generally, phosphorylation of receptor takes place to induce mitophagy in response to mitochondrial stress, especially hypoxia. Cardiolipin translocates from IMM to OMM under stress and this translocation is essential for binding to LC3 (Figure 4) (20, 21).

2.4. Mitochondrial Dynamics

Mitochondria are highly dynamic organelles. They continually move, divide and make new connections with other mitochondrial networks while changing mitochondrial architecture (22). Mitochondrial dynamics are essential for mitochondrial quality control. Mitochondria experience fusion and fission events for adaption of changes in the cellular environment. Mitochondria fusion and fission events take place constantly to be able to meet the cellular energy and metabolic needs. Under healthy physiological conditions, mitochondria tend to elongate and continually divide and fuse with other mitochondrial networks in order to provide mitochondrial integrity which is crucial for mitochondrial DNA (mtDNA) and protein quality control (23). Mitochondrial fission provides division of mitochondria as well as the separation of damaged mitochondria from the network. On the other hand, in the mitochondria fusion event, mitochondria are elongated while exchanging their cytoplasm between mitochondria and this may then recover mitochondria having functional defects (23). The proteins responsible for the mitochondrial fusion and fission events are highly regulated through post-translational modifications like phosphorylation, ubiquitination, and sumoylation (26).

Mitochondrial fusion is the process of joining of two mitochondria. Mitochondrial fusion is mediated by three proteins which are mitofusin 1, 2 (MFN1, 2) and optic atrophy 1 (OPA1) in mammalian cells. All of them are dynamin-related large GTPases. MFN1 and 2 are located in the OMM whereas OPA1 is located in the IMM. The fusion process takes place into two steps. The first step is fusion of the outer membrane which is catalyzed by MFN1 and MFN2. Both proteins can be used interchangeably in terms of function. OPA1, on the other hand, is responsible for fusion of the inner membrane in the second step. Mitochondrial fusion is critical for the proper functioning of mitochondria (24). Any obstruction of the fusion process can result in mitochondrial membrane potential loss, which then damages mitochondria fitness. During the fusion process, contents like proteins, lipids, and mtDNA of each mitochondrion are combined. This provides mitochondria to get rid of the accumulation of mitochondrial mutations and ROS. Moreover, it has been suggested that the fusion process can inhibit mitophagy while preventing the removal of damaged mitochondria (23). As a whole,

mitofusin proteins and OPA1 work coordinately, enabling elongation of mitochondrial networks (Figure 5) (23, 24).

Mitochondrial fission is the process in which mitochondrion is separated from the mitochondrial network. This process takes place during mitochondrial division and elimination of damaged mitochondria through mitophagy. In the course of cell division, mitochondria replicate in order to supply enough and equal number of mitochondria for daughter cells. In addition to this, when a mitochondrial network starts to become dysfunctional, damaged mitochondria are eliminated by the fission machinery (23). The fission results in the two distinct mitochondria in terms of mitochondrial membrane potential, one has high MMP and the other one has low MMP. The process occurrs by fission proteins found on the membrane of mitochondria and one GTPase called dynamin-related protein 1 (DRP1). During fission event, recruitment of DRP1 to the OMM takes place by fission 1 homolog protein (FIS1), mitochondrial fission factor (MFF) and mitochondrial dynamics proteins of 49 and 51 kDa (24). When DRP1 is recruited to the mitochondrial surface, it makes dimers and oligomers to surround the mitochondria and divides mitochondria into two by constriction. The process is mediated by GTPase activity of DRP1 (Figure 5) (24, 25).

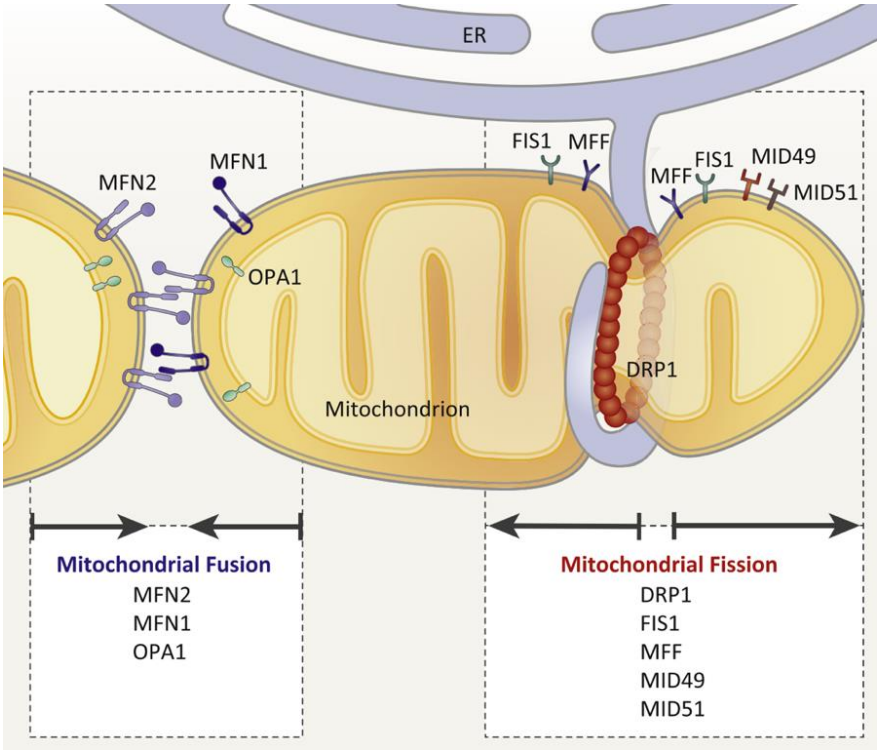


Figure 5. Main proteins responsible for mitochondrial dynamics

Reference: Sebastián D, Palacín M, Zorzano A. Mitochondrial dynamics: Coupling mitochondrial fitness with healthy aging. Trends Mol Med. 2017;23(3):201–15.

2.5. Mitophagy in Immunity

Mitochondria are crucially important for the phenotype and the activity of immune cells' survival and maintenance (60). Maintaining proper mitochondrial function is essential for regulation of immune response and host defense. Improper removal of damaged mitochondria through mitophagy can lead to hyperactivation of the immune system. However, excessive mitophagy due to severe mitochondrial damage can cause programmed cell death. Thus, well-functioning mitophagy process is indispensable to prevent chronic systemic inflammation and tissue collapse (61). Inflammatory response is a cytoprotective effect to preserve tissue homeostasis and keep cells viable upon infection (20). Abnormal inflammatory responses can lead to autoimmune diseases. The inflammatory response is stimulated by mitochondrial defects such as enhanced ROS levels, elevated cytoplasmic calcium and pronounced release of mtDNA (62) and this can be caused by infection as well (20).

Nuclear factor-κB (NF-κB) is a transcription factor that coordinates inflammatory signaling. Mitochondrial antiviral signaling protein (MAVS) is an immune receptor located on the OMM (63). Enhanced ROS levels trigger MAVS oligomerization which activates NF-κB to regulate inflammation and host defense (20).

Macrophages are phagocytic cells that control both pro and anti-inflammatory responses. They provide tissue homeostasis and repair upon infection. They have two distinct subtypes which are M1 and M2 macrophages. M1 macrophages exhibit pro-inflammatory phenotype by releasing pro-inflammatory cytokines like IL-1β and TNFα; whereas M2 macrophages show anti-inflammatory phenotype. M1 macrophages hold tumoricidal and microbicidal activity. M2 macrophages on the other hand are important for wound healing and regulation of host immunity (20). Macrophage polarization towards M1 or M2 is affected by different metabolic conditions such as glycolysis, pentose phosphate pathway (PPP), fatty acid oxidation (FAO), mitochondrial oxidative phosphorylation (OXPHOS), and tricarboxylic cycle (TCA) fluxes (64). M1 macrophages are affected by PPP and glycolysis instead of OXPHOS and TCA. M2 macrophages on the other hand are affected more by FAO, OXPHOS, and TCA instead of PPP and glycolysis (65). Therefore, mitochondrial

activity and mitophagy are crucially important for polarization of M1/M2 macrophages and their functional behavior (Figure 6). Overall, mitochondria and proper regulation of them are essential for the immune system. Well-functioning mitophagy prevents excessive inflammation and keep tissue homeostasis.

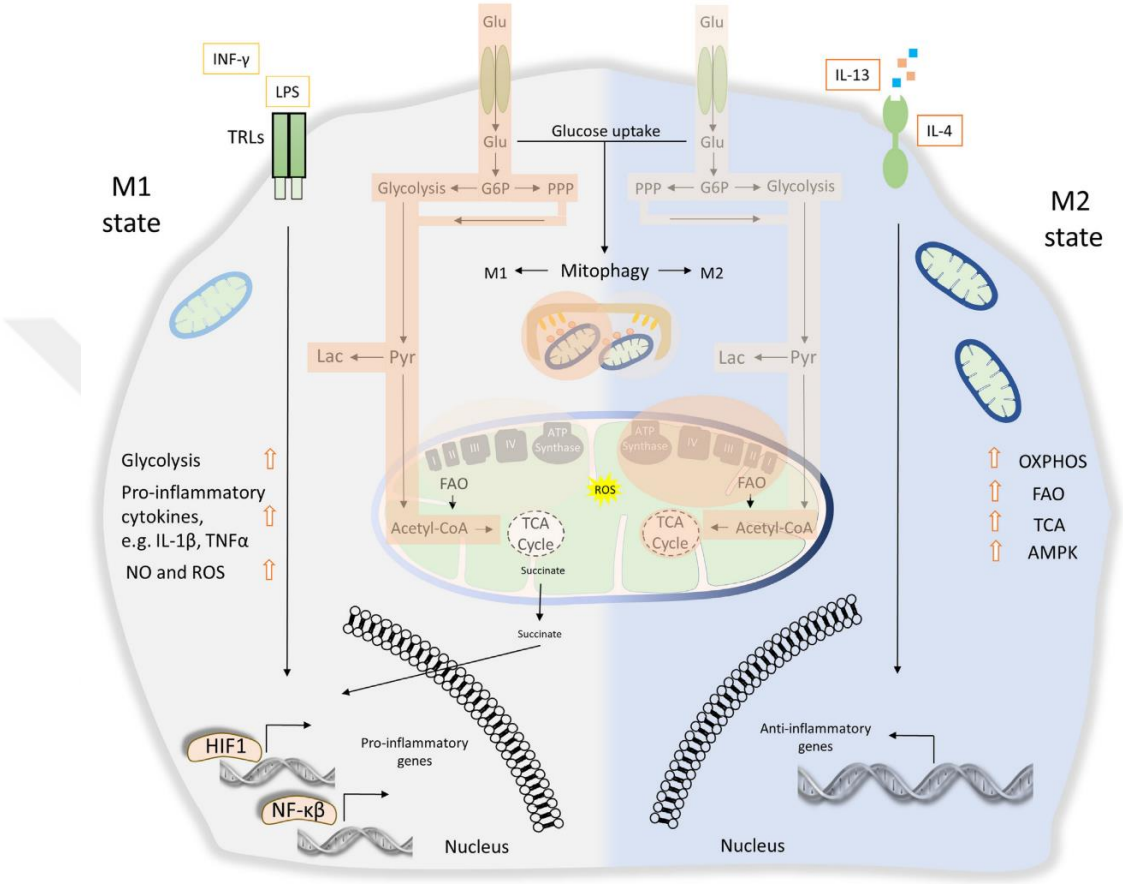


Figure 6. Metabolic signature of M1 and M2 polarization

Reference: Gkikas I, Palikaras K, Tavernarakis N. The role of mitophagy in innate immunity. Front Immunol. 2018;9(JUN):1–15.

2.6. Phospholipids

Phospholipids are types of lipids mainly found in the cellular and organelle membranes with a high turnover rate. Therefore, proper cell function and structure require continuous synthesis of phospholipids (39). Each subcellular organelle is made of different sets of phospholipid compositions Depending on the characteristics of the organelles, protein types are changing. Thus, a defined number of distinct phospholipids are crucial for the proper function of proteins on the membrane of organelles (27). Different cell types and tissues have different class of phospholipid

compositions as well. These different compositions contribute to the proper functioning of the proteins that are embedded in the cellular membranes. Any alteration on these different compositions results in detrimental effects for the survival of cell types (28). Generally, the endoplasmic reticulum (ER) is the organelle responsible for the synthesis of each set of phospholipids, after being synthesized phospholipids are transported from ER to other organelles.

Phosphatidylcholine (PC) is the most abundant phospholipids including 40–50% of total phospholipids in the membrane of mammalian cells. PC maintains membrane integrity by serving a housekeeping function (27, 36). During cell growth, the synthesis of PC is increased as well (36). Phosphatidylethanolamine (PE) is the second most abundant phospholipids in the cellular membrane especially enriched in the mitochondrial inner membrane. Having a high amount of PE in the membrane of mitochondria is another support for the hypothesis of symbiotic origin between bacteria and primitive mammalian cells. Phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), and sphingomyelin (SM) are other types of phospholipids found in the cellular and organelle membranes (Table 1) (27).

Table 1. Lipid composition of averaged mammalian cell membrane

Name of Membrane Lipids	Percentage
Phosphatidylcholine	45–55
Phosphatidylethanolamine	15–25
Phosphatidylinositol	10–15
Phosphatidylserine	5–10
Sphingomyelin	5–10
Cardiolipin	2–5

Reference: Vance JE. Phospholipid synthesis and transport in mammalian cells. Traffic. 2015;16(1):1–18.

The content of PS is much abundant in the plasma membrane, ER, Golgi and nucleus than that in lysosomal and mitochondrial membranes. Cardiolipin (CL) on the other hand is a very special kind of phospholipid that is found only in mitochondria notably in the inner membrane (29). Even though major types of phospholipids are located in the membrane of all organelles, their abundance is different in each organelle. Therefore, there is an unequal distribution of phospholipids among all organelles. Alongside having unequal distribution, phospholipids are distributed

asymmetrically across membrane bilayer as well. In normal physiology, PE and PS are located mostly in the inner of the plasma membrane compared with outer, whereas PC and SM are located in the outer layer (30). This asymmetric distribution of phospholipids serves a very important function for cellular homeostasis. Apoptosis is an important example of this phenomenon. During apoptosis, PS translocates to the outer layer of the plasma membrane to expose itself to macrophages. The PS receptors on the surface of macrophages provide sensing the translocation and engulfment of the apoptotic cells. Another important translocation of PS takes place in platelets, and this initiates the blood clotting cascade (31, 32).

Moreover, phospholipids have different properties other than the contribution to biological membranes. They may serve as receptors to recruit certain proteins to the membranes, secondary messenger molecules, modulators for proper protein function, and aiding for protein folding (36).

2.6.1. Phospholipids and mitochondria

Phospholipids in mitochondrial membranes are critical for proper assembly of ETC and effective responses to mitochondrial damage, mitochondrial protein homeostasis and induction of apoptosis (52). They define the characteristics of outer and inner membranes which creates the shape of mitochondria (50). Outer and inner membrane phospholipids compositions are unique in each membrane. OMM houses metabolic enzymes and transport proteins so that it requires defined phospholipids bilayer to keep them functional. A major phospholipid is PC in both membranes consisting nearly 50%. PE follows the PC in both membranes. The difference between membranes is in the percentage of PI and cardiolipin. IMM has higher cardiolipin ratio and lower PI ratio then OMM (Table 2) (27, 50). The phospholipid compositions of mitochondria are nearly the same among different species; mammals, plants and yeast (50). Even though mitochondria can synthesize some of the required phospholipids, the majority of the required phospholipids are synthesized in the ER in conjunction with the Golgi apparatus (51).

Table 2. Lipid composition of mitochondrial membranes

Lipids	Inner %	Outer %
Phosphatidylcholine	41	49
Phosphatidylethanolamine	38	34
Phosphatidylinositol	2	9
Phosphatidylserine	1	1
Sphingomyelin	2	2
Cardiolipin	16	5
Other	<1	<1

Reference: Vance JE. Phospholipid synthesis and transport in mammalian cells. Traffic. 2015;16(1):1–18.

Cardiolipin is a very special type of phospholipids. It is only found in mitochondria, comprising nearly 20% of the phospholipid found in IMM. In healthy mitochondria, most of the cardiolipin is present in the inner membrane. Cardiolipin provides stabilization of cristae and supports the assembly and proper function of ETC complexes (53). When mitochondria are damaged, cardiolipin translocates from inner to the outer membrane and is exposed to the cytosol where it acts as "eat-me" signal. Then autophagy machinery can recognize and engulf damaged mitochondria (54).

The other important characteristic of phospholipids is that while interacting with dynamin GTPases (etc. DRP1), they may alter biophysical properties of the membranes of mitochondria. Thus, they can play role in mitochondrial dynamics and altered membrane can induce mitophagy as well (33).

Mitsuhashi et al. has shown one particle example of this in which the defect in the synthesis of PC can induce mitophagy in muscle disease (34). In another research conducted by Won Lee et al., the levels of PC, SM and PI were reduced during mitophagy, therefore, phospholipids are crucial for mitophagy (35).

2.6.2. Kennedy pathway

The phosphatidylcholine production pathway is highly conserved from yeast to mammalian cells (36). The Kennedy pathway is de novo synthesis of PC and PE. More than 50 years ago, the pathway was elucidated by Eugene Kennedy and the pathway was named after him. Formation of PC and PE require high energy intermediates, these are CDP-choline, for PC and CDP-ethanolamine, for PE synthesis. Therefore, the Kennedy pathway is also known as CDP-choline and CDP-ethanolamine pathway.

Formation of CDP-choline and CDP-ethanolamine is very similar, the only difference being the requirement of choline instead of ethanolamine during PC synthesis (37).

The Kennedy pathway is made of three enzyme steps which are cytidine kinase (CK), choline phosphate cytidilyltransferase (CCT) and CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT). These three enzymes are localized in the nucleus and ER. Choline, for the pathway, is supplied from an external source by transportation into the cell or turnover of PC via phospholipase D (38). 95% of total choline sources are used for the synthesis of PC. 5% of choline can be free choline, acetylcholine, CDP-choline or other types of phospholipids consisting choline (37). Phosphocholine production from free choline by choline kinase becomes the first step in the pathway. Phosphocholine is then metabolized to form CDP-choline by CTP:phosphocholine cytidylyltransferase which is a rate-limiting enzyme in this pathway. The last step in the production of PC involves condensation of CDP-choline with diacylgecerol (DAG) by CTP:phosphocholine cytidylyltransferase and holinephosphotransferase (Figure 7) (36).

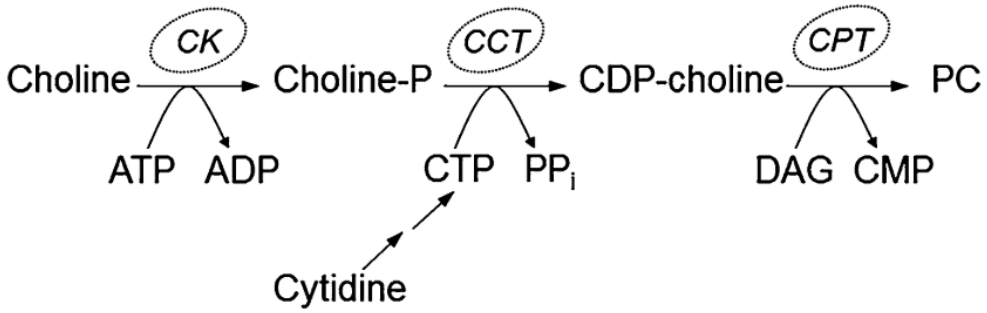


Figure 7. The Kennedy pathway
(CK) cytidine kinase, (CCT) choline phosphate cytidylyltransferase, (CPT) CDP-choline:1,2-diacylglycerol choline phosphotransferase, (ADP) adenosine diphosphate, (ATP) adenosine triphosphate, (CMP) cytidine monophosphate, (CTP) cytidine triphosphate, (DAG) 1,2-dicacylglycerol, (PPi) pyrophosphate, and (PC) phosphatidylcholine.

Reference: Grieb P. Neuroprotective properties of citicoline: Facts, doubts and unresolved issues. CNS Drugs. 2014;185–93.

2.7. CDP-Choline

CDP-choline is a novel intermediate mononucleotide, composed of a ribose sugar, pyrophosphate, cytosine and choline, for the synthesis of PC (39). CDP-choline is synthesized in all mammalian cells. Other than being a key molecule on the production

of PC, it is a choline donor for the synthesis of neurotransmitters such as acetylcholine and dopamine (55, 57). Besides, it is also shown that CDP-choline functions as a membrane stabilizer through the prevention of fatty acid release and it regulates cardiolipin and sphingomyelin levels (58). Exogenous CDP-choline undergoes hydrolysis and absorption as cytidine and choline by membrane-associated phosphodiesterases. These metabolites also facilitate the pharmacological and physiological effects of CDP-choline. Cytidine triphosphate (CTP) and phosphocholine are required for resynthesis of CDP-choline by CTP-phosphocholine cytidylyltransferase (CCT) which is the rate-limiting enzyme in Kennedy pathway (56). Moreover, CDP-choline increases glutathione reductase activity and glutathione synthesis (59). Because of all these beneficial properties of CDP-choline, it has been used for several different diseases such as traumatic brain injury, cerebral ischemia, glaucoma, amblyopia, Alzheimer and Parkinson's diseases (39, 55).

2.7.1. CDP-choline in health and diseases

Citicoline is a generic name of a chemical identical to CDP-choline. Citicoline has started to become a drug since the 1970s. For the first time, Manaka et al. tested the drug on Parkinson's patients in 1974 in Japan (38, 40). Now, the neuroprotective effect of citicoline has been shown on ischemic stroke, glaucoma, amblyopia, Alzheimer and Parkinson's diseases in many studies (39, 41). The cause of these diseases involves changes of phospholipids in the membranes and metabolism of neurons and these changes can trigger an apoptotic cascade (39). CDP-choline in the central nervous system facilitates repair of membranes by accelerating phospholipids synthesis and slowing down the breakdown of phospholipids (38).

The other suggested mechanism of CDP-choline is prevention of phospholipase A2 (PLA2) activation in stroke models (38, 43). This prevention attenuates hydroxyl radical generation and preventing cardiolipin loss which is essential for mitochondrial ETC (38). Additionally, it has been shown that citicoline restores mitochondrial ATPase and membrane Na1/K1ATPase activity (41). Recent studies have shown that citicoline increases the expression of sirtuin-1 (SIRT1) level. This increase suggested that citicoline may have a critical role in neuroprotection in strokes. Combination of resveratrol which is SIRT1 activator and citicoline reduced experimental infarct volume up to 60%, although individual usage of them demonstrated ineffectiveness

(44). Another study of citicoline on ischemia/ reperfusion injury, showed that citicoline protects myocardium by restricting mitochondrial permeability transition. During ischemic reperfusion of organs, oxidative damage is increased which then damages mitochondria and this causes non-specific permeability on mitochondrial membranes. CDP-choline reduced ROS and thus, inhibited mitochondrial permeability transition (45). Another interesting study shows that citicoline can be used for the treatment of cerebral malaria as adjuvant therapy. Citicoline can ameliorate the disease caused by malaria on the membranes as a membrane stabilizer (46).

In a study conducted by Agut et al. (47), acute toxicity of oral and intravenous administration of CDP-choline and choline has been examined. They have concluded that CDP-choline did not induce any cholinergic intoxication whereas choline treatment caused toxic effects with the same dose both orally and intravenously (38). Citicoline is a non-toxic drug. Therefore, citicoline is sold as a food supplement in many countries because of not having any adverse effects (48).

3. MATERIALS AND METHODS

3.1. Materials

Table 3. List of products and suppliers

Product Name	Supplier
0% Fat Skim milk	Regilait
2-mercaptoethanol	Sigma Aldrich
40% Acrylamide/Bis-acrylamide	Serva
Acetic acid	Sigma Aldrich
Amersham Hybond P 0.2 PVDF membrane	GE Healthcare
Amersham Protran 0.2 NC membrane	GE Healthcare
Ammonium persulphate	Sigma Aldrich
Antimycin A from Streptomyces sp.	Sigma Aldrich
Bafilomycin A1 from streptomyces griseus	Sigma Aldrich
Bovine Serum Albumin (BSA) Fraction V	Roche
Bradford Reagent, 5x	Serva
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma Aldrich
CDP-Choline	Fluka
Cell Proliferation Kit I (MTT)	Roche
Counting Slides Duals Chamber for Counter	Biorad
Dimethyl sulphoxide (DMSO) Hybri-Max	Sigma Aldrich
DTT	Applichem
EDTA-free protease inhibitor cocktail	Roche
Ethanol	Sigma Aldrich
Ethylenediaminetetraacedic acid (EDTA)	Sigma Aldrich
Glycerol	Sigma Aldrich
Glycine	Merck
HI Fetal Bovine Serum (FBS)	Gibco
Hydrochloric acid	Sigma Aldrich
IBIDI Slayts μ-Slide VI 0.4	Ibidi

Igepal CA-630	Sigma Aldrich
Methanol	Sigma Aldrich
MitoSOX Red Mitochondrial Superoxide Indicator	ThermoFisher Scientific
MitoTracker Green FM	ThermoFisher Scientific
MitoTracker Red CMXRos	ThermoFisher Scientific
Mouse monoclonal anti-B-Actin Antibody (AC-74)	Sigma Aldrich
Mouse monoclonal anti-DRP1 Antibody (C-5)	Santa Cruz
Oligomycin A	Sigma Aldrich
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	ThermoFisher Scientific
Penicilin-Streptomycin	Gibco
Peroxidase affinipure goat anti-mouse IgG (H+L)	Jackson Immunoresearch
Peroxidase affinipure goat anti-rabbit IgG (H+L)	Jackson Immunoresearch
Phenylmethanesulfonyl (PMSF)	Applichem
Phosphate Buffer Saline (PBS) (1X)	Gibco
Ponceau S	Sigma Aldrich
Potassium chloride	Sigma Aldrich
Potassium dihydrogen phosphate	Sigma Aldrich
Rabbit monoclonal anti-COX IV (3E11)	Cell Signaling Technology
Rabbit monoclonal anti-Mitofusin-2 (D2D10)	Cell Signaling Technology
Rabbit polyclonal anti-LC3B	Sigma Aldrich
Rabbit polyclonal anti-p62/SQSTM1	Novus Biologicals
Rabbit polyclonal PINK1 Antibody	Novus
RPMI 1640 Medium	Gibco
SDS	Sigma Aldrich
Sodium azide	Sigma Aldrich
Sodium chloride	Sigma Aldrich
Sodium fluoride	Sigma Aldrich
Sodium hydroxide	Merck
Sodium orthovanadate	Sigma Aldrich

Sodium phosphate dibasic dodecahydrate	Sigma Aldrich
SuperSignal West Pico Chemiluminescent Substrate	ThermoFisher Scientific
TEMED	Sigma Aldrich
Triton X-100	Sigma Aldrich
Trizma Base	Sigma Aldrich
Trypan Blue Solution	Sigma Aldrich
Tween-20	Sigma Aldrich
Western blotting filter extra thick paper	ThermoFisher Scientific

Table 4. List of Equipment

Equipment	**Brand**
-20°C freezer	Kirsch
-80°C freezer	GLF
4°C fridge	Kirsch
Biosafety cabinet	ThermoFisher Scientific
Cell culture incubator	ESCO CCL-170B-8
ChemiDoc MP Imaging System	BioRad
Confocal Microscope	Zeiss LSM 700
Environmental shaker/incubator	ESCO
Flow cytometer	BD FACSVerse
Inverted Microscope	Zeiss
Micro centrifuge	ThermoFisher Scientific
Mini centrifuge	Biosan
Nitrogen Tank	Thermo 30 Thermo Scientific
Plate reader	BioTek
TC20™ Automated Cell Counter	BioRad (145-0102)
Trans-blot turbo transfer system	BioRad
Varioskan flash	ThermoFisher Scientific
Ventilated micro centrifuge	ThermoFisher Scientific
Water Bath	St 30 Nüve

3.1.1. Preparation of chemicals, buffers and solutions

CDP-choline (100mM)

0.55g of CDP-ch was weighed and dissolved in 10 ml RPMI medium to prepare 100mM main stocks. The stock solution was filtered with 0.22um filter and stored at -80 ^{o}C aliquoted as 100ul aliquots.

CCCP (20mM)

0.005g of CCCP was weighed and dissolved in 1220ul of DMSO to prepare 20mM main stocks. The stock solution was filtered with 0.22um filter and stored at -80 ^{o}C aliquoted as 10ul aliquots.

Bafilomycin A1 (160uM)

Bafilomycin vial contained 2ug and it was dissolved in 20ul of DMSO to prepare 160uM main stocks. The stock solution was filtered with 0.22um filter and stored at -80 ^{o}C aliquoted as 5ul aliquots.

MitoSOX (500uM)

MitoSOX vial contained 50ug and it was dissolved in 130ul of DMSO to prepare 500uM main stocks. The solution aliquoted as 5ul, aliquots were stored at -20 ^{o}C and protected from light.

MitoTracker Green FM (1mM)

MitoTracker Green FM vial contained 50ug and it was dissolved in 75ul of DMSO to prepare 1mM main stocks. The solution aliquoted as 5ul, aliquots were stored at -20 ^{o}C and protected from light.

MitoTracker Red CMXROS (100uM)

MitoTracker Red CMXROS vial contained 50ug and it was dissolved in 941ul of DMSO to prepare 100uM main stocks. The solution aliquoted as 50ul, aliquots were stored at -20 ^{o}C and protected from light.

Protease Inhibitor (PI) (25x)

Roche cOmplete EDTA-free protease inhibitor cocktail was dissolved in 2 ml of ddH2O. 1 tablet of dissolved inhibitors is equivalent to 25x stock. The solution was aliquoted as 100ul, aliquots were stored at -20 ^{o}C. Before protein isolation, PI was diluted with RIPA buffer to make 1x final.

Table 5. 15% polyacrylamide Separating Gel (1x)

30% Acrylamide/Bisacrylamide	2.5 ml
1.5 M Tris (pH 8.8)	1.25 ml
50% Glycerol	375 ul
ddH2O	875 ul
10% APS	50 ul
TEMED	5 ul
Total	5 ml

Table 6. 12% polyacrylamide Separating Gel (1x)

30% Acrylamide/Bisacrylamide	2 ml
1.5 M Tris (pH 8.8)	1.25 ml
50% Glycerol	375 ul
ddH2O	1375 ul
10% APS	50 ul
TEMED	5 ul
Total	5 ml

Table 7. 4% Polyacrylamide Stacking Gel (1x)

30% Acrylamide/Bisacrylamide	325 ul
1 M Tris (pH 6.8)	625 ul
20% SDS	12.5 ul
ddH2O	1512 ul
10% APS	12.5 ul
TEMED	5 ul
Total	2.5 ml

Tris-Glycine running buffer (pH 7.5-8.0), 10x

25 mM Tris

1.92 M Glycine

1% SDS

1x running buffer is diluted from 10x stock with ddH2O

Bjerrum Schafer-Nielsen Transfer buffer, 10x

48 mM Tris

39 mM Glycine

20% methanol

1x transfer buffer is diluted from 10x stock with ddH2O

PBS (pH 7.4), 10x

30 mM KCl

1.37 M NaCl

35 mM KH2PO4

100 mM Na2HPO4.12H2O

1x PBS is diluted from 10x stock with ddH2O

PBST, 1x

1x PBS

% 0.5 Tween-20

RIPA lysis buffer, 1x

50 mM Tris-HCl (pH 7.4)

150 mM NaCl

1 mM NaF

1 mM EDTA

1 mM Na3VO4

0.5 % Igepal CA-630

0.5 % Triton X-100

1 mM DTT

0.5 mM PMSF in EtOH

Skimmed Milk, 5 %

1x PBST is used to prepare 5 % (w/v) skimmed milk

BSA for primary antibodies, 5 %

5 % (w/v) Fraction V BSA

1x PBS

0.02 % NaAzide

Laemmli Sample Buffer, 5X

250mM Tris-HCl (pH 6.8)

5 % SDS

43.5 % Glycerol

0.5 % Bromophenol Blue

Ponceau S

0.1 % (w/v) Ponceau S

5 % Acetic acid

3.2. Methods

3.2.1. Experimental design

In this thesis, we tested the effect of CDP-choline before and after mitophagy induction by the CCCP, uncoupler. After optimization of concentration and timing of the drugs added to U937 cells, we followed figure 8.

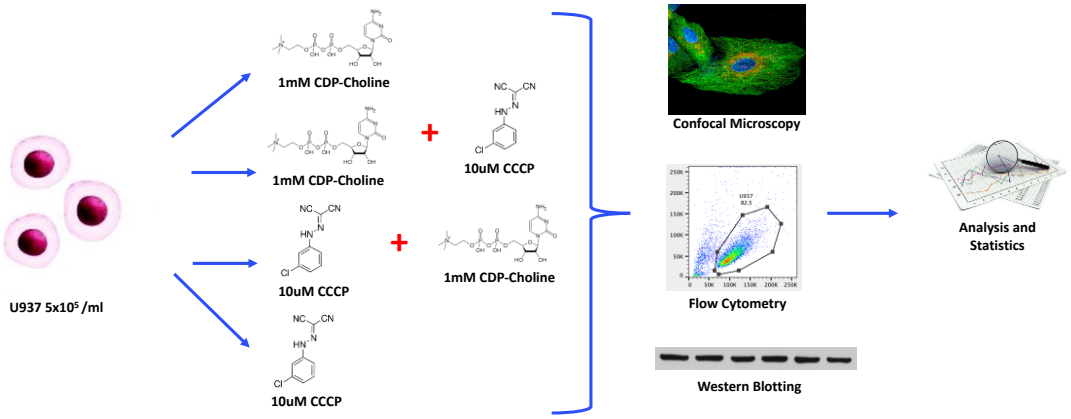


Figure 8. Experimental design

3.2.2. Cell culture

U937 monocyte cells were obtained from ATCC (CRL-1593.2). The cells were cultured in RPMI 1640 medium supplemented with 1% penicillin-streptomycin and 10% heat inactivated and sterile fetal bovine serum (FBS) in a humidified atmosphere

of 5% CO2 at 37 ºC. The medium was changed every 2-3 days. Cells were passaged to $2x10^5$ per ml when they reached to 1x106 per ml.

3.2.3. Cell viability assay

U937 cells were seeded at $1x10^4$ density in a 96-well plate. Cells were administered in triplicate with increasing concentrations of CDP-ch (50 μM, 100 μM, 500 μM, 1mM, 5mM, 10mM) including a control (only medium) for 24 hours at 37°C incubator. Cell viability was measured using the MTT kit I (Roche) following manufacturer's protocol. Briefly, 10μl of MTT labeling reagent was added for 4 hours after that 100ul of solubilization buffer was added for 16h. Absorbance was measured at 570 nm using an ELISA reader with a reference serving as blank. Control cells were taken as 100% and treated cells were calculated according to control.

3.2.4. Trypan blue exclusion assay

U937 cell were seeded $5x10^5$ / ml in a 6 well plate. The cells were treated with or without 10uM CCCP for different time points from 4 to 24h. After each incubation, cells were collected. 10ul of cell population was mixed with 10ul of trypan blue. 10ul from the mix was put into counting slides. Cells were counted by TC20™ automated cell counter. The counter showed the number of dead and live cells. Viability was calculated by division of live cell number with total cell number. Control cells were taken as 100% and treated cells were calculated accordingly.

3.2.5. Protein isolation

Prior to every seeding, the medium of U937 was changed one day before. U937 cells were seeded $5x10^5$ / ml in a 6 well plate. The cells were treated with or without 1mM CDP-ch for 24 hours and 10uM CCCP for 4 hours. For pre-treatment experiments of CDP-ch in the course of mitophagy induction, CDP-ch was administrated for 24h and mitophagy was induced for 4 hours with CCCP. For post-treatment experiments, cells were first incubated with CCCP for 4h and then washed once with PBS to remove CCCP. After that, CDP-ch was added for 24h. Bafilomycin A1 was applied the last 3 hours of incubation. When incubations were done, cells were collected and washed one time with PBS. Then, the cell pellets were digested with 1x RIPA buffer containing 1x protease inhibitors for 10 minutes on ice by pipetting. After 10 minutes, suspensions were pipetted once more and incubated for another 10 minutes

on ice. Afterwards, suspensions were centrifuged at 14000xg for 10 mins at 4 ^{o}C. The supernatants were transferred into new tubes and stored at -20 ^{o}C.

3.2.6. Measurement of protein concentration

Protein quantification was carried out by the Bradford assay. 5x Bradford stock reagent was diluted with ddH2O to make a 1x working stock. Protein concentrations were determined by comparison with BSA known standards. BSA standards were 1.5, 0.75, 0.5, 0,25 and 0.125 μg/ul. Each protein sample was diluted as 1:10 ratio with ddH2O. 10ul of diluted protein samples and BSA standards were plated in triplicate into a 96 well plate. 10 μl of ddH2O was used as blank. On top of 10 μl of each sample, 190 μl of 1x Bradford reagent was added and incubated for 10 minutes at room temperature (RT) and protected from light. After 10 minutes of incubation, the plate was read at 595nm. Triplicate results were averaged, and blank was subtracted from each sample. A standard curve was drawn by the BSA standards. Protein concentration of each sample was determined from the standard curve.

3.2.7. Western blotting

Laemmli sample 5x buffer was diluted 1x with 10 μg of quantified protein samples and boiled at 98 ^{o}C for 5 minutes. After 5 minutes, samples were placed on ice and centrifuged at max speed for 2 minutes. Samples were loaded onto gels. 12% gels were used for higher kDa proteins and 15% gels were used for lower kDa proteins. Samples were run at 80V for 45 minutes and 100V until the samples reached the end of the gels. The 12% gel was transferred onto a nitrocellulose membrane for 45 minutes and the 15% gel was transferred to a 0.2 μm PVDF membrane for 30 minutes in the Biorad trans-blot semi dry system. After transfer, Ponceau S dye was used to stain membrane to check whether proteins were transferred successfully. The membrane was blocked with %5 skimmed milk for 1 hour at RT. Afterwards membranes were cut into pieces, each piece being incubated with the desired primary antibody overnight at 4 ^{o}C. The concentration of primary antibodies was rabbit anti-LC3B (1:4000), mouse anti-p62 (1:1000), mouse anti-DRP1 (1:1000), rabbit anti-MFN2 (1:1000), rabbit anti-PINK1 (1:1000), rabbit anti-COXIV (1.5000) and mouse anti-ACTIN (1:5000). The following day, membranes were washed three times with PBST for 5 mins at RT. Later, membranes were probed with secondary antibodies, peroxidase-conjugated anti-rabbit

(1:1000) or anti-mouse for 1 hour at RT. Membranes were then washed three times with PBST for 5 minutes each. For the final step, the chemiluminescent reagent was applied for 5 minutes onto membranes for imaging. The images were obtained in the ChemiDoc machine and protein bands were analyzed using ImageLab software.

3.2.8. Detection of mitochondrial staining by flow cytometry

U937 cells were seeded at $5x10^5$ per ml into 6 cm plates. The cells were treated with or without 1mM CDP-ch for 24 hours and 10 μM CCCP for 4 hours. For pre-treatment experiments of CDP-ch in the course of mitophagy induction, CDP-ch was administrated for 24h and then mitophagy was induced for 4 hours with CCCP. For post-treatment experiments, cells were first incubated with CCCP for 4h after that washed once with PBS to remove CCCP, after that CDP-ch was given for 24h. After desired incubations, cells were harvested and centrifuged at 500xg for 5mins. and washed with 1x PBS. $3x10^5$ cells from each group were stained with 100nM MitoTracker Red CMXRos, 2.5μM MitoSOX Red and 25nM MitoTracker Green FM for 20min at 37°C to determine mitochondrial membrane potential, mitochondrial superoxide formation and mitochondrial mass, respectively. After 20 minutes, samples washed with 1x PBS once. Pellets were dissolved in 400 μl PBS and transferred to flow tubes to be read by flow cytometry (BD FACSVerse). A total of 10,000 events were acquired, properly gated and analyzed using FlowJo software.

3.2.9. Detection of mitochondrial staining by confocal microscopy

The cells used in confocal microscopy were incubated and prepared in the same manner as in flow cytometry experiments. $5x10^5$ cells from each group were stained with 100 nM MitoTracker Red CMXRos, 5 μM MitoSOX Red and 50 nM MitoTracker Green FM for 20min at 37°C. After 20 minutes, samples washed with 1x PBS, once. Cells were then dissolved in 100 μl PBS and transferred to an IBIDI μ-Slide VI 0.4 chamber. Images were taken with appropriate channels with 63X oil objective in the Zeiss LSM 700 confocal microscope and mean fluorescent intensity (MFI) of more than 50 cells were analyzed by ImageJ software.

3.2.10. Statistical analysis

All the samples were compared with each other using two-tailed ratio or paired student's t-test. GraphPad Prism 6 software was used for statistical analysis and graphical representations. The results were represented as the mean ± SD of at least 3 experimental repeats. P value < 0.05 was considered as statistically significant.

4. RESULTS

4.1. The Effect of CDP-Choline on Cell Viability

U937 is a cell line model isolated from a male histiocytic lymphoma patient. This cell line has myeloid origin and is widely used for studying the functions of monocytes (66). Monocytes are professional phagocytic cells that play an important role in innate immunity. CDP-choline (CDP-ch) is an intermediate in the Kennedy pathway for the synthesis of phosphatidylcholine. CDP-ch is a non-toxic, food supplement having neuroprotective effects on various diseases such as ischemic stroke, glaucoma, amblyopia, Alzheimer and Parkinson (39, 41). The effect of CDP-ch on monocytes is unknown. Moreover, CDP-ch is mostly administrated orally so that CDP-ch can interact with monocytes. Thus, investigation of CDP-ch on monocytes is crucial. In order to determine the effect of CDP-ch on U937 cells, we first aimed to determine the optimal concentration of CDP-ch. MTT is a colorimetric assay that indicates the effect of a drug on cell viability. So, U937 cells were incubated with increased concentrations of CDP-choline (50 µM to 10 mM) for 24 h and then cell viability was measured. We observed that 1mM of CDP-ch was the highest concentration that did not reduce cell viability (Figure 9).

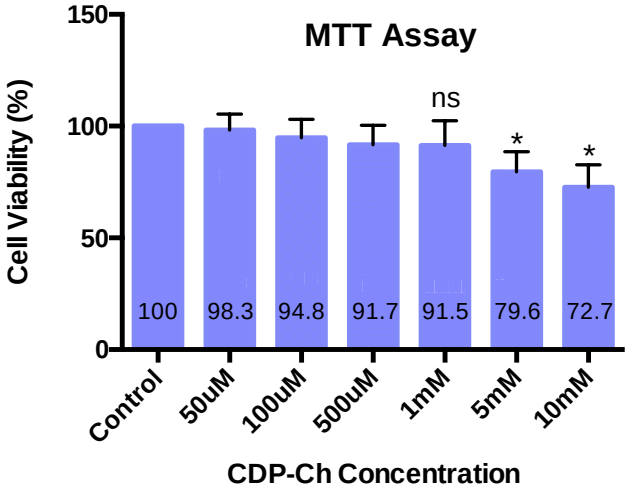


Figure 9. Determination of CDP-ch concentration via MTT Assay
U937 cells were incubated with different concentrations of CDP-ch from 50 µM to 10 mM for 24 h. MTT assay was performed to measure the effect of CDP-ch on cell

viability. CDP-ch treated cells were normalized with untreated control cells. Four independent experiments were represented as mean and standard deviation in the bar graph (ns = not significant, *$p < 0.05$).

U937 cells were also treated with various CDP-ch concentrations at different time points; 4 h, 16 h, 24 h and 48 h (data not shown), and they showed similar effects on cell viability.

4.2. Induction of Mitophagy

Mitophagy is a complex mechanism for removal and recycling of damaged mitochondria. *In vitro* experimental models, biological reagents are used to damage mitochondria and initiate mitophagy via either inhibition of ETC proteins or proton gradient. Rotenone, antimycin A and oligomycin are used for the inhibition of complex I, complex III and complex V, respectively. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) and Carbonyl cyanide-p-trifluoromethoxy phenylhydrazone (FCCP) molecules are uncouplers that diminish mitochondrial membrane potential, leading to inhibition of ATP production and elevated ROS production (67). Mitophagy and mitochondrial inhibitors were not well-studied in U937 monocytes. In a study conducted by Han and Im (68), they observed that U937 cells were sensitive to mitochondrial inhibitors. In our preliminary investigation using different cell lines (SHSY-5Y, PC12 and U937), only the U937 cell line showed mitophagy induction rather than apoptosis in response to mitochondrial inhibitors (Data not shown). Therefore, in our experimental method, we used CCCP and a mix of antimycin A and oligomycin to induce mitophagy in the U937 cell line model.

4.2.1. The effect of mitophagy inducer on cell viability at different time points

In the literature, generally 10 μM concentration of CCCP has been used to induce mitophagy (69). Each cell type shows different sensitivity towards CCCP to induce mitophagy. CCCP can also induce apoptosis for longer incubation times. In order to obtain an optimum time point to induce mitophagy, but not apoptosis, cells were treated with CCCP at different time points; 4, 8, 16, 24 hours and then live/dead cell numbers were counted with trypan blue exclusion assay. It was determined that number of live cells were reduced nearly 20-30% after an 8 h of treatment (Figure 10).

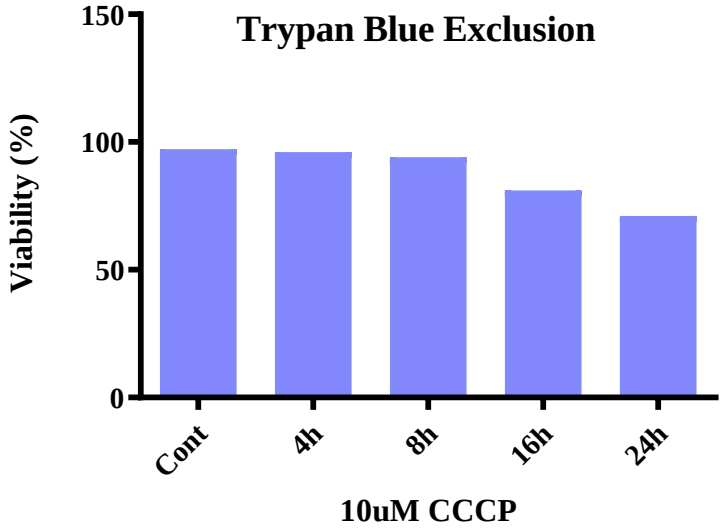


Figure 10. Time dependent effect of CCCP on cell viability
U937 cells were treated with 10 µM CCCP at different time points. The cells were collected and stained with trypan blue and counted in the BioRad automated cell counter. CCCP treated cells were normalized to untreated control cells.

We further monitored cells by flow cytometry to determine the distribution of live/dead U937 cells. After incubation of CCCP at different time points, U937 cells were analyzed by forward and side scatter using flow cytometry. It was determined that increasing time of the CCCP treatment reduced our gated population from 92.4% to 59%. We did not use the live cell detection fluorescein dye but used cell dispersion to show indirectly that cells started to die in response to treatment after 8 h (Figure 11). Thus, combination of trypan blue assay and cell dispersion of CCCP treatment at different time points indicated that 4 or 8 hours would be suitable for the induction of mitophagy in our cell system.

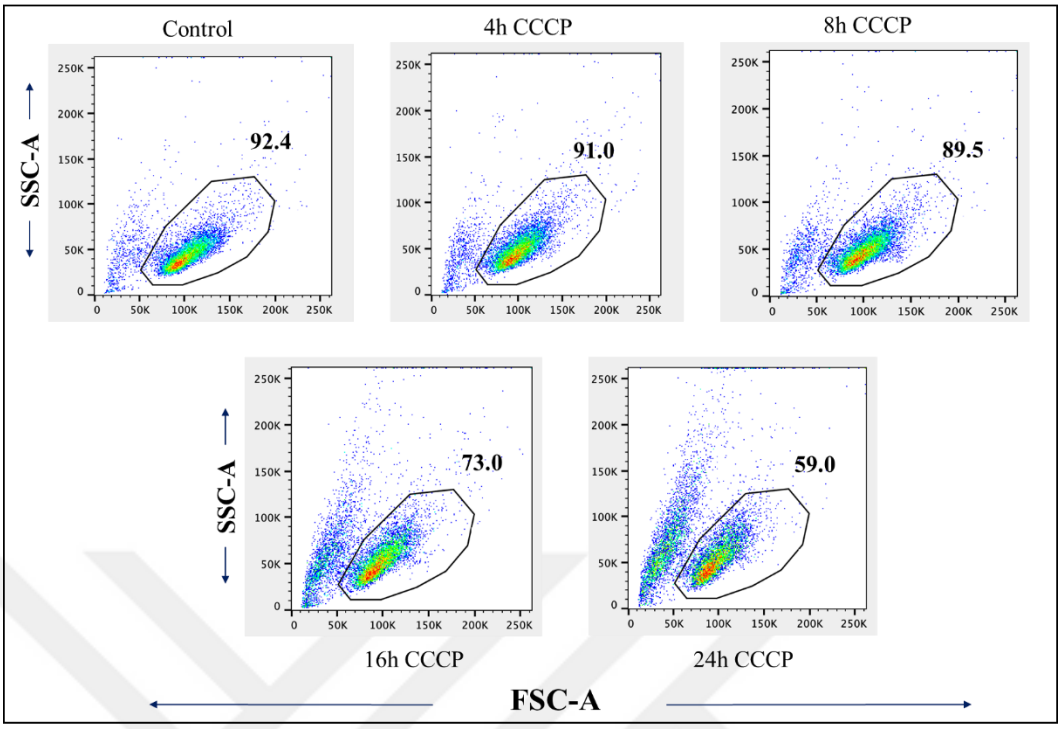


Figure 11. Time dependent effect of CCCP on cell distribution
U937 cells were treated with 10 µM CCCP at different time points and analyzed by flow cytometry. Distribution of U937 cells are indicated as pseudo color dot blot. Living population is gated by forward and side scatter pattern of U937. Percentages of gated cells are represented on the figure.

4.2.2. The effect of mitophagy inducer on mitochondrial dynamics at different time points

Mitochondrial specific dyes were used to observe the changes on mitochondrial dynamics induced by mitophagy inducer. These specific dyes were MitoTracker Sox, MitoTracker Green FM and MitoTracker Red CMXROS and they indicate mitochondrial superoxide, mitochondrial mass and mitochondrial membrane potential (MMP), respectively. These dyes may also be used indirectly for the detection of mitophagy. U937 cells were incubated with 10 µM of CCCP at different time points starting from 30 minutes to 24 hours. 30 minutes incubation of CCCP caused reduction of MMP as expected and also reduced mitochondrial superoxide levels. It is known that CCCP reduces MMP via causing proton leakage from intermembrane space of mitochondria. Uncoupling of the proton gradient affects respiration, thus the level of superoxide is also reduced after 30 minutes (Figure 12A and 12C). Upon 4 h of incubation with CCCP, however, the level of mitochondrial superoxide increased

dramatically with time and reached the highest level at 24 h (Figure 12A). Accumulation of superoxide not only induces mitophagy, but also the excessive level of ROS can damage the cells and induce apoptosis. Thus, the exact time point is important for the induction of mitophagy only but not apoptosis. Although the level of MMP decreased upon CCCP treatment at early time points, further treatment with CCCP (between 4 to 24 h) increased the level of MMP (Figure 12C). Mitochondrial mass level is an indirect indication of the number of mitochondria found in the cells, after 4 hours treatment of CCCP, mitochondrial mass started to decrease throughout mitophagy induction. The reduction of mitochondrial mass at 16 h of CCCP treatment was noticeable and even more pronounced at 24 h of treatment (Figure 12B).

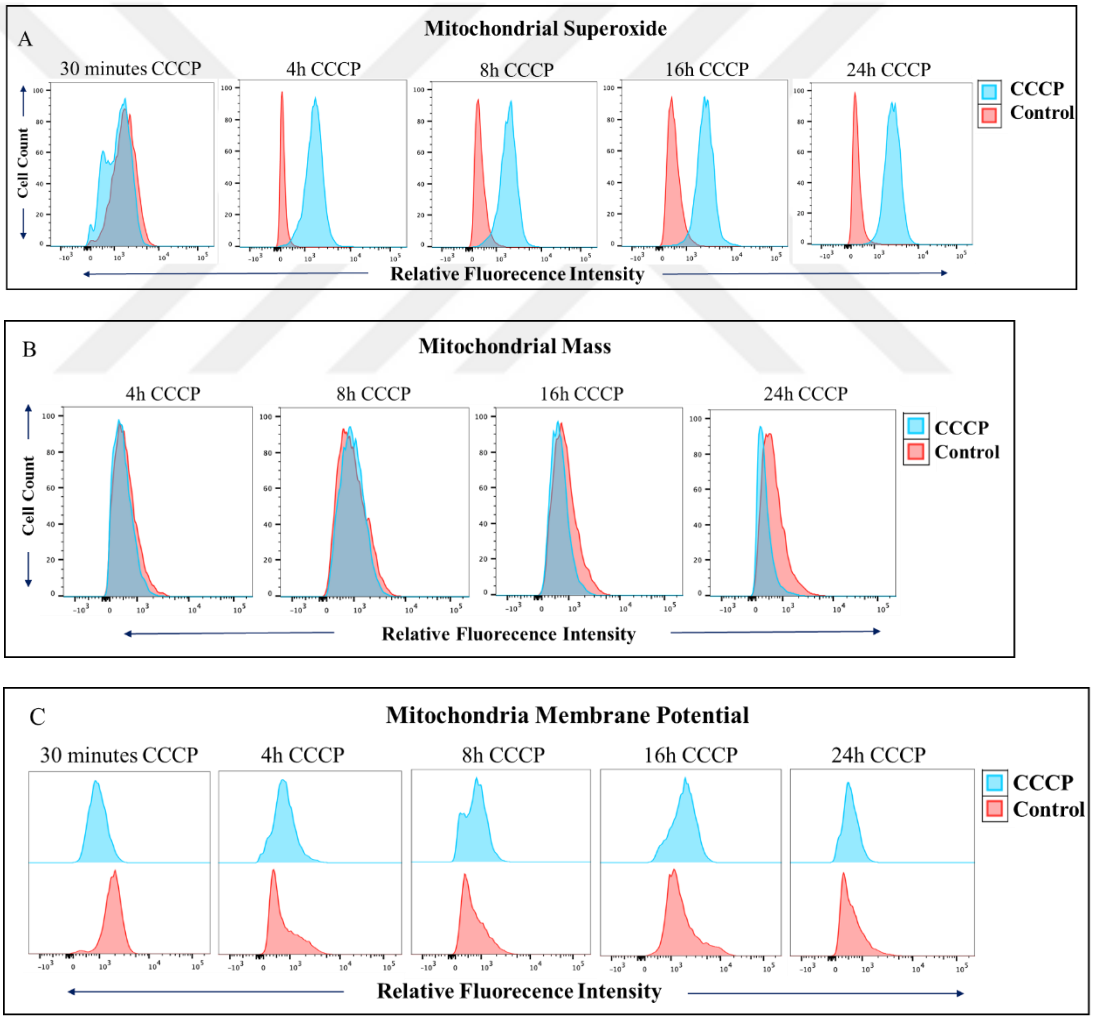


Figure 12. Time dependent analysis of mitophagy inducer on mitochondrial dynamics by flow cytometry

U937 cells were treated with 10 µM of CCCP at different time points starting from 30 minutes to 24 hours. Cells then were collected and stained with different mitochondrial

specific dyes. 2.5 μM MitoSox (A), 25 nM Mitotracker Green FM (B) and 100 nM Mitotracker Red CMXROS (C) were used for detection of mitochondrial superoxides, mitochondrial mass and membrane potential, respectively. Stained cells were read by BD FACSVerse flow cytometry and analyzed by FlowJo software.

4.2.3. The effect of mitophagy inducer on mitochondrial dynamics and mitophagy related proteins at different time points

Time dependent effects of CCCP were investigated on different mitophagy and mitochondrial dynamics related proteins by western blotting. The effect of 10 μM CCCP treatment was carried out at different time points, ranging from 1 hour to 24 hours. DRP1 protein is one of the important proteins which plays role in mitochondrial fission. Its protein level was reduced at the first hour then started to increase with time upon CCCP treatment. It reached the highest value to 1.5-fold of control on the 4th h and then stayed constant close to control (Figure 13A). The other important protein in mitochondrial dynamics is MFN2 which plays role in mitochondrial fusion. It demonstrated a similar pattern with DRP1 protein. At the beginning, the protein quantity of MFN2 was low, but it increased with time. The protein level was doubled at 8 h and then decreased at 24 h approaching to the control level (Figure 13A). PINK1 is an important protein for the detection of damaged mitochondria and provides recognition by autophagy machinery through PARKIN phosphorylation. Its protein level also was low at very first hour then started to increase, reaching the highest level, nearly 1.5-fold of control, at 4 hours after treatment (Figure 13A). LC3B and p62 are also autophagy related proteins. Reduction of p62 levels and increase in the lipidated form of LC3B (LC3B-II) indicate the activation of autophagy machinery. The level of p62 was reduced at 1 h of treatment (Figure 13A) whereas LC3-II increased with time and reached the highest level after 4 hours and remained constant after that. These data demonstrated that autophagy was activated at the very first hour (Figure 13B), to clear out damaged mitochondria.

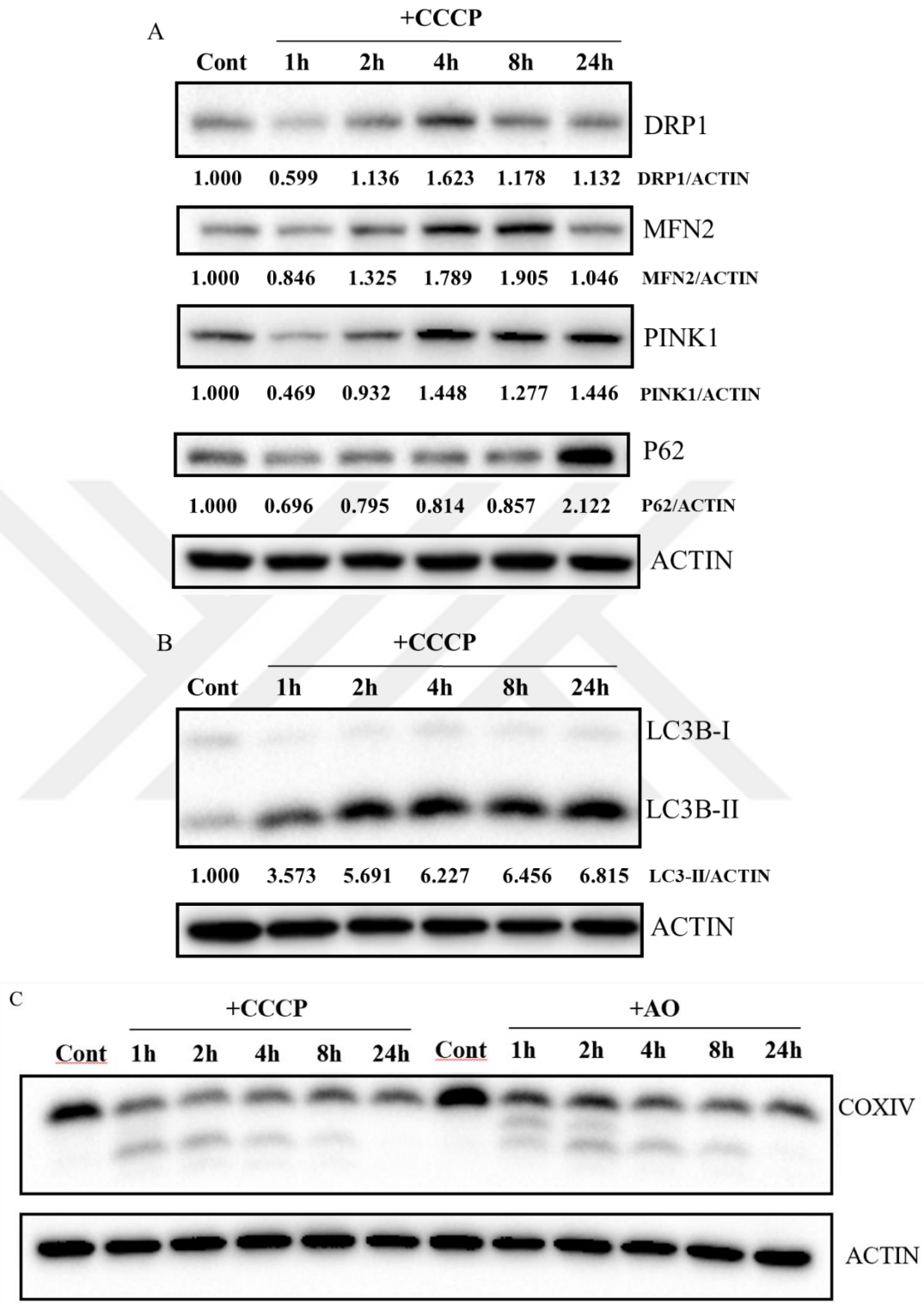


Figure 13. Time dependent effects of mitophagy inducers on mitochondrial dynamics on protein levels

U937 cells were treated with 10 μM of CCCP and 200 nM of antimycin and 2.5 μM of oligomycin (AO) mix separately with different time points ranging from 1h to 24h. Cell lysates were collected and electrophoresed using 12% SDS-PAGE for DRP1,

MFN2, PINK1 and p62. 15% SDS-PAGE was used to separate LC3B and COXIV. Protein bands were quantified by ImageLab software. ACTIN was used for internal loading control. DRP1, MFN2, PINK1, p62, LC3B, COXIV proteins were normalized with ACTIN. DRP1/ACTIN, MFN2/ACTIN, PINK1/ACTIN, p62/ACTIN, LC3B/ACTIN densitometric ratio normalized with untreated control group and marked on the figure.

In our experimental settings, we concluded that induction of mitophagy was achieved by CCCP. It damaged the mitochondria by enhanced mitochondrial superoxide formation and the longer the incubation, the more damage was obtained, eventually leading to cell death. Our aim was to induce mitophagy, not apoptosis; therefore, 4 hours of CCCP treatment was sufficient for mitophagy in U937 cells.

The level of mitochondrial proteins is reduced via removal of damaged mitochondria during mitophagy. One of the mitochondrial proteins is cytochrome c oxidase subunit 4 (COXIV) that is a subunit of a Complex IV protein located in IMM. Although, COXIV is generally used as loading control for mitochondrial experiments, we used as indicator for mitophagy induction through removal of damaged mitochondria. We observed COXIV protein fragmentation on western blotting when mitophagy was induced. This fragmentation was also observed with another mitophagy inducer; antimycin and oligomycin mixture. COXIV protein level reduced and vanished with time (Figure 13C). Moreover, AO mix showed 3 distinct bands at early time points; 30 minutes to 2 hours. Therefore, we decided to use lower concentration of AO and use inhibitors alone to observe the changes on COXIV banding. When the concentration of AO decreased same results were obtained with more time. When inhibitors were used separately, interestingly we did not get the same fragmentation. Therefore, our results on COXIV may indicate that only during mitophagy induction, COXIV fragmentation appears in the U937 cells (Figure 14).

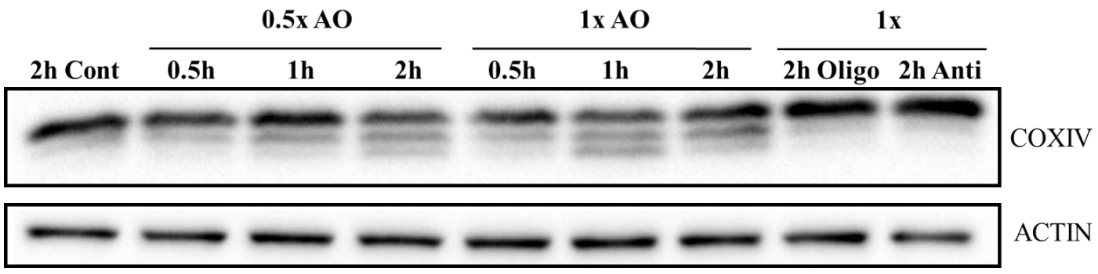


Figure 14. The changes of COXIV protein on early time points of a mitophagy inducer

U937 cells were treated with different concentrations of antimycin and oligomycin (AO) mix as well as antimycin and oligomycin separately at early time points (30 minutes to 2 hours) during mitophagy induction. 1x AO represents 200 nM antimycin and 2.5 μM oligomycin and 0.5x AO represents half the amount of the antimycin and oligomycin. Cell lysates were collected and run using 15% SDS-PAGE. ACTIN was used for internal loading control. Control is the untreated sample.

4.2.4. The effect of mitophagy inducer on COXIV in different cell lines

After we observed this fragmentation of COXIV protein, we decided to test different cell line models to test whether they displayed a similar phenomenon. We tested MDA-MB-231 (triple-negative breast cancer), U87 (glioblastoma), Hek293T (human embryonic kidney), A549 (lung carcinoma), and SHSY-5Y (neuroblastoma) cells. AO mix was used for 2 hours to test fragmentation. As expected, reduction of COXIV level was observed on MDA-MB-231, U87 and SHSY-5Y cell lines. Two hours of CCCP treatment was not enough to reduce COXIV levels in A549 cells. Nevertheless, Hek293T cell line showed the same pattern with U937 which was used as positive control (Figure 15).

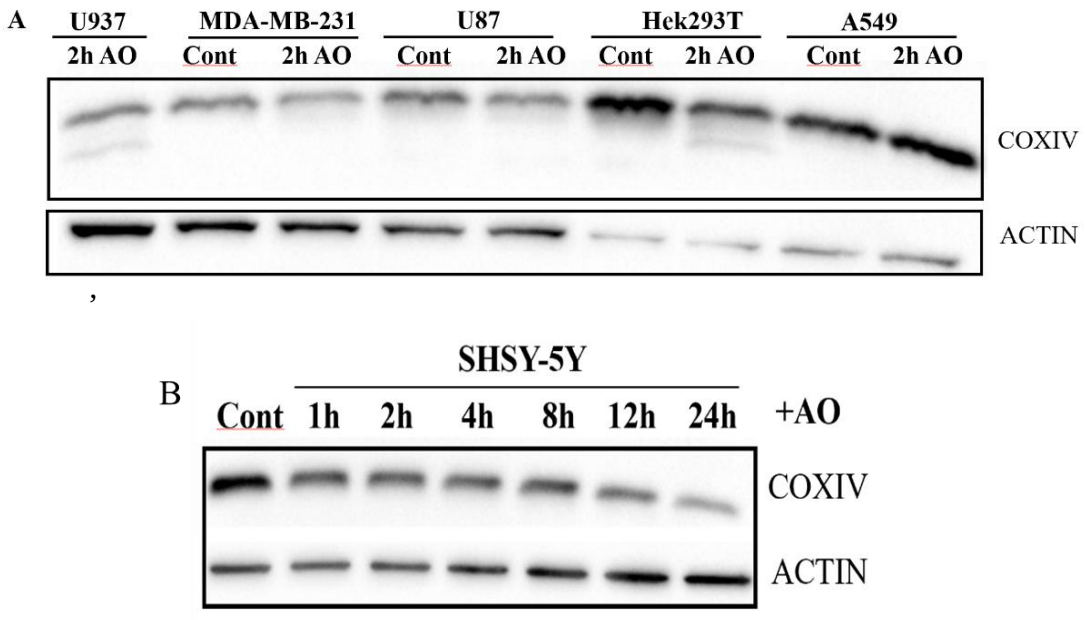


Figure 15. The changes of COXIV protein in the different cell lines after mitophagy induction
U937 (histiocytic lymphoma), MDA-MB-231 (triple-negative breast cancer), U87 (glioblastoma), Hek293T (human embryonic kidney), A549 (lung carcinoma) cells were treated with 200 nM antimycin and 2.5 μM oligomycin (AO) mix for 2 hours. The U937 treated sample was used as positive control (A). SHSY-5Y (neuroblastoma)

cells were treated with AO at different time points (B). Cell lysates were collected and run using 15% SDS-PAGE. Actin was used for internal loading control. Control is the untreated sample.

4.2.5. The effect of bafilomycin A1 during mitophagy induction

To further validate induction of mitophagy, we used bafilomycin A1 (BafA1). BafA1 is a lysosome inhibitor which inhibits fusion of autophagosome with lysosome. During mitophagy, mitochondria are wrapped up by autophagosome and degraded trough lysosome conjugation. When this conjugation is inhibited, one can investigate the effects of CCCP on mitophagy and mitochondrial dynamics proteins more. U937 cells were incubated with 10 μM of CCCP for 4 hours and throughout the last 3 hours, increasing concentrations of BafA1 ranging from 10 to 50 μM were added to the culture medium. We confirmed what we had found previously on protein levels in the experiment displayed in figure 13. DRP1, PINK1, MFN2 were increased more with BafA1 at 4 hours of mitophagy induction. The lipidated form of LC3B was also increased and reduction of p62 remained constant. Fragmentation of COXIV was found to persist (Figure 16). These results also demonstrated that 4 hours of CCCP induction was sufficient to induce mitophagy before carrying out the effect of CDP-ch treatment.

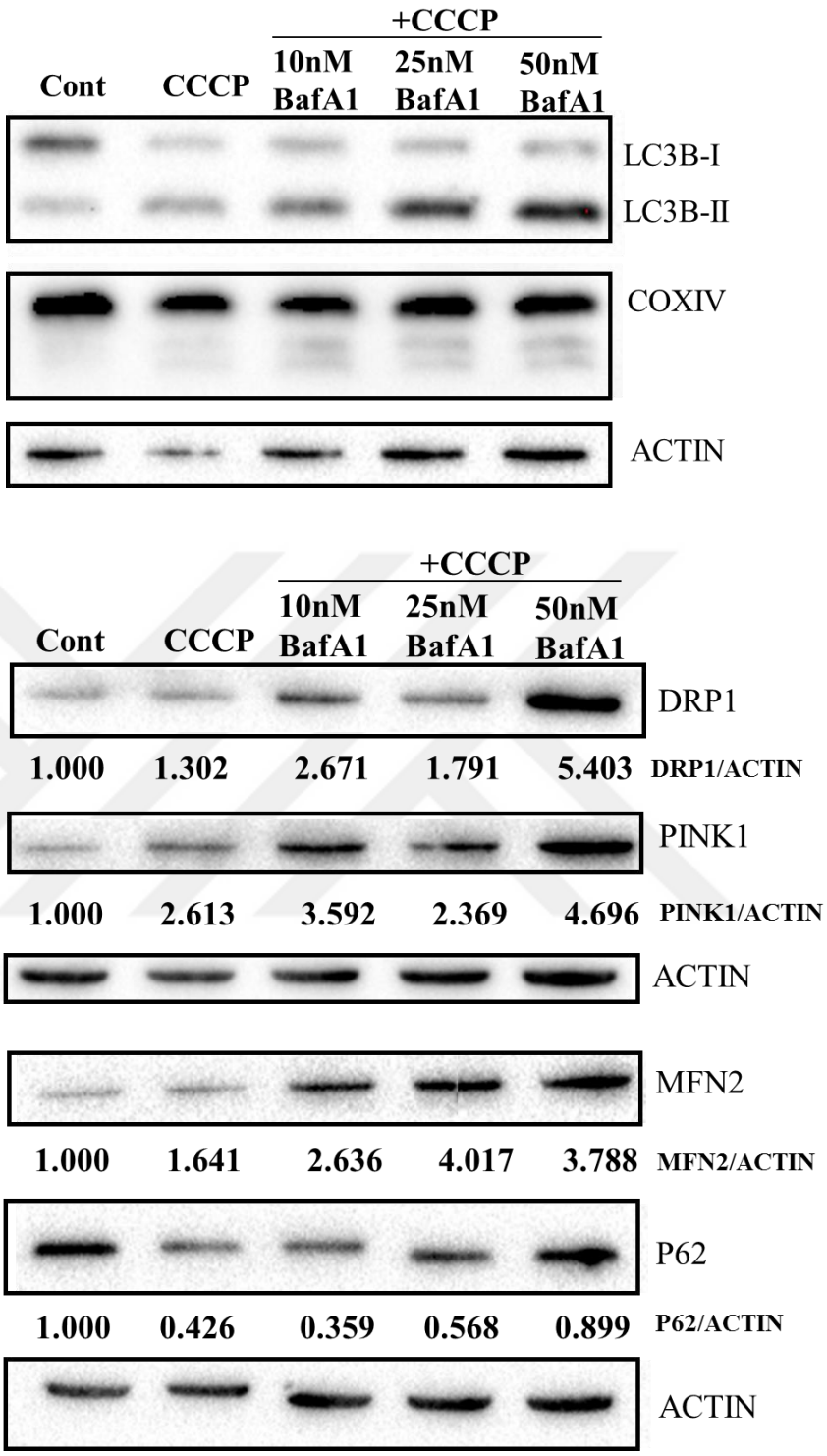


Figure 16. Effects of bafilomycin A1 on mitophagy induced cells.
U937 cells were treated with different concentration (10-50 nM) of bafilomycin A1 throughout of last 3 hour of 4 h treatment of 10 μM of CCCP. Cell lysates were collected and run using 12% SDS-PAGE for DRP1, MFN2, PINK1 and p62; 15% SDS-PAGE was used to separate LC3B and COXIV. Protein bands were quantified by ImageLab software. ACTIN was used for internal loading control. DRP1, MFN2, PINK1, p62 proteins were normalized with ACTIN. DRP1/ACTIN, MFN2/ACTIN,

PINK1/ACTIN, and p62/ACTIN densitometric ratio normalized with untreated control group and marked on the figure.

4.3. The Effect of CDP-Choline on Mitophagy and Mitochondrial Dynamics

This project comprises the first study on the effects of CDP-choline during mitophagy in U937 cells. Prior to testing of the effects of CDP-choline, we determined the optimal concentration and time of CDP-choline and optimized mitophagy induction by western blotting on various proteins related to mitophagy and flow cytometry using mitochondrial specific dyes. We determined that 1mM of CDP-choline treatment for 24 hours was optimal for further investigation. Mitophagy induction was achieved with 10 μM of CCCP for 4 hours. We treated the cells with 1 mM of CDP-ch for 24 hours prior (pre-treatment) and following (post-treatment) mitophagy induction.

The effect of CDP-ch on mitochondrial dynamics and mitophagy was investigated by flow cytometry and confocal microscopy using mitochondrial specific fluorescent dyes that indicate mitochondrial superoxide production, mitochondrial mass and mitochondrial membrane potential. These are important tools for studying mitochondria. In addition to these experiments, we also determined the levels of mitochondrial dynamics- and mitophagy-related proteins using western blotting.

4.3.1. The effect of CDP-ch on mitochondrial dynamics by flow cytometry

We first tested mitochondrial sox with flow cytometry. Four hours of CCCP treatment increased mitochondrial superoxide production significantly ($p < 0.001$), to more than 5-fold of control. 24 h of 1 mM CDP-ch treatment did not change the mitochondrial superoxide level. In the course of mitophagy induction, pretreatment of CDP-ch reduced the superoxide (SOX) level 10% significantly ($p = 0.026$). The post treatment of CDP-ch, on the other hand, increased the SOX level by nearly 10% but this was not found to be significant (Figure 17). This finding shows that pre-treatment of CDP-ch may prevent and reduce damages caused by superoxide and provide longevity of the cells.

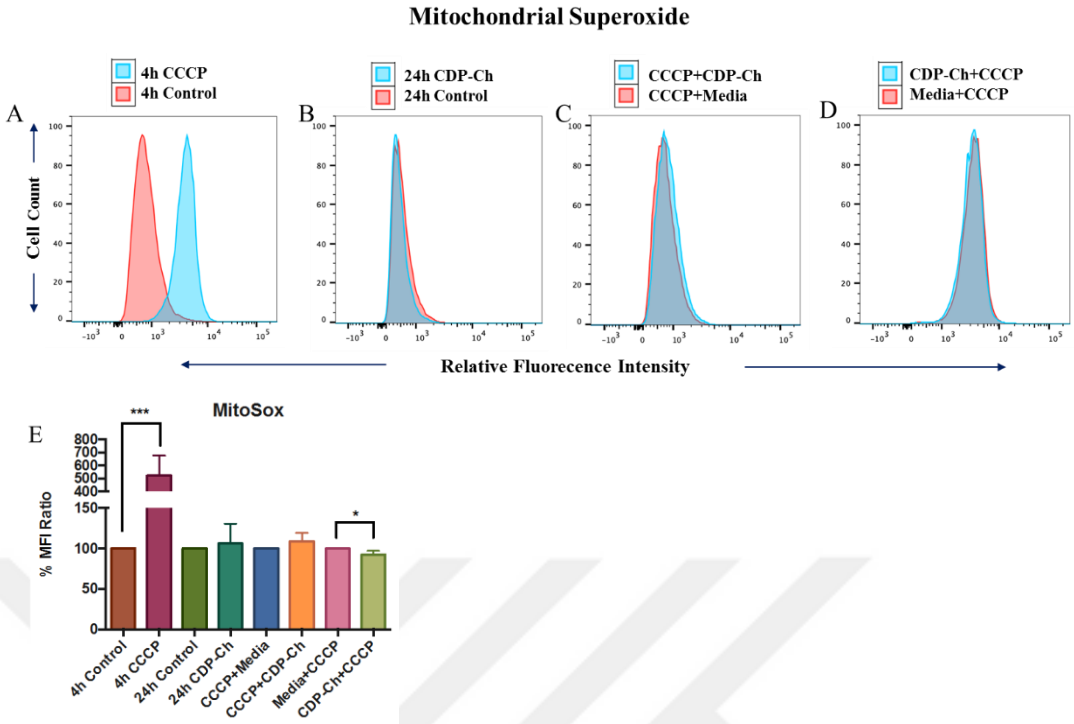


Figure 17. The effect of CDP-ch on mitochondrial superoxide in mitophagy induced cells

Different treatments were performed. (A) Treatment with 10 µM of CCCP for 4 h. (B) Treatment with 1 mM of CDP-ch for 24 h. (C) Treatment with 10 µM of CCCP for 4 h, followed by 1 mM of CDP-ch treatment for 24 h. (D) Treatment with 24 h of 1 mM of CDP-ch, followed by 10 µM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 2.5 µM of MitoSox, a mitochondrial superoxide specific dye. Stained cells were read by BD FACSVerse flow cytometry. Samples were analyzed by FlowJo software and depicted as histogram in the figure. (E) Bar graph represents 4-5 independent repeats as mean and standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

We then tested mitochondrial mass with flow cytometry. CCCP treatment for 4 hours decreased mitochondrial mass significantly ($p < 0.001$) nearly to 15% of control, as expected. 24h of CDP-ch treatment, on the other hand, increased mitochondrial mass by 12% but it was not statistically significant. In the course of mitophagy induction, pretreatment of CDP-ch increased the mass for less than 10%. The post treatment of CDP-ch, however, reduced mitochondria mass level then recovered and increased it significantly ($p = 0.0295$) by 15% (Figure 18). It indicates that post-treatment of CDP-ch recovers mitochondria in mitophagy induced cells by increasing the amount of mitochondria.

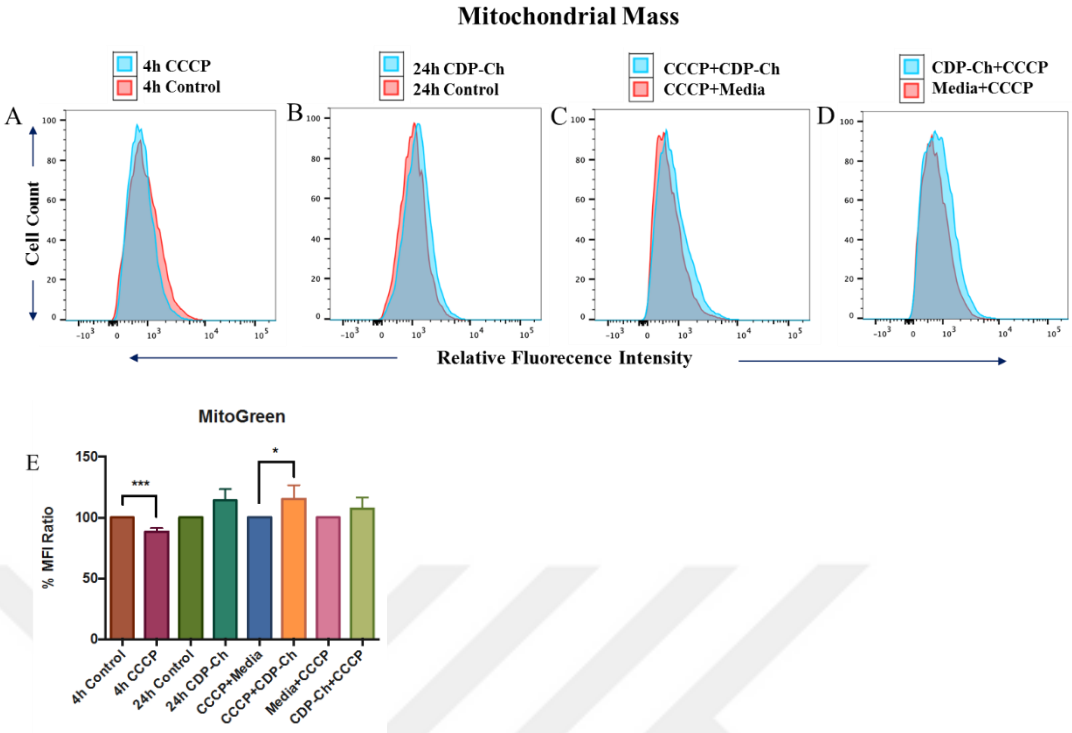


Figure 18. The effect of CDP-ch on mitochondrial mass in mitophagy induced cells

Different treatments were performed. (A) Treatment with 10 µM of CCCP for 4 h. (B) Treatment with 1 mM of CDP-ch for 24 h. (C) Treatment with 10 µM of CCCP for 4 h, followed by 1 mM of CDP-ch treatment for 24 h. (D) Treatment with 24 h of 1 mM of CDP-ch, followed by 10 µM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 50nM of Mitotracker Green FM, a mitochondrial mass specific dye. Stained cells were read by BD FACSVerse flow cytometry. Samples were analyzed by FlowJo software and depicted as histogram in the figure. (E) Bar graph represents 4-5 independent repeats as mean and standard deviation (*p < 0.05, **p < 0.01, ***p < 0.001).

Lastly, we tested mitochondrial membrane potential (MMP) with flow cytometry. CCCP treatment increased MMP by 10% but not significantly (p = 0.0644). CDP-ch treatment did not change the MMP. In the course of mitophagy induction, pretreatment and post treatment of CDP-ch increased MMP for nearly 10% insignificantly (Figure 19). Interestingly, this result indicates that 4h of treatment of CCCP induces mitochondria to produce more protons. The effect of CDP-ch on MMP fluctuates and has very small influence in mitophagy induced U937 cells.

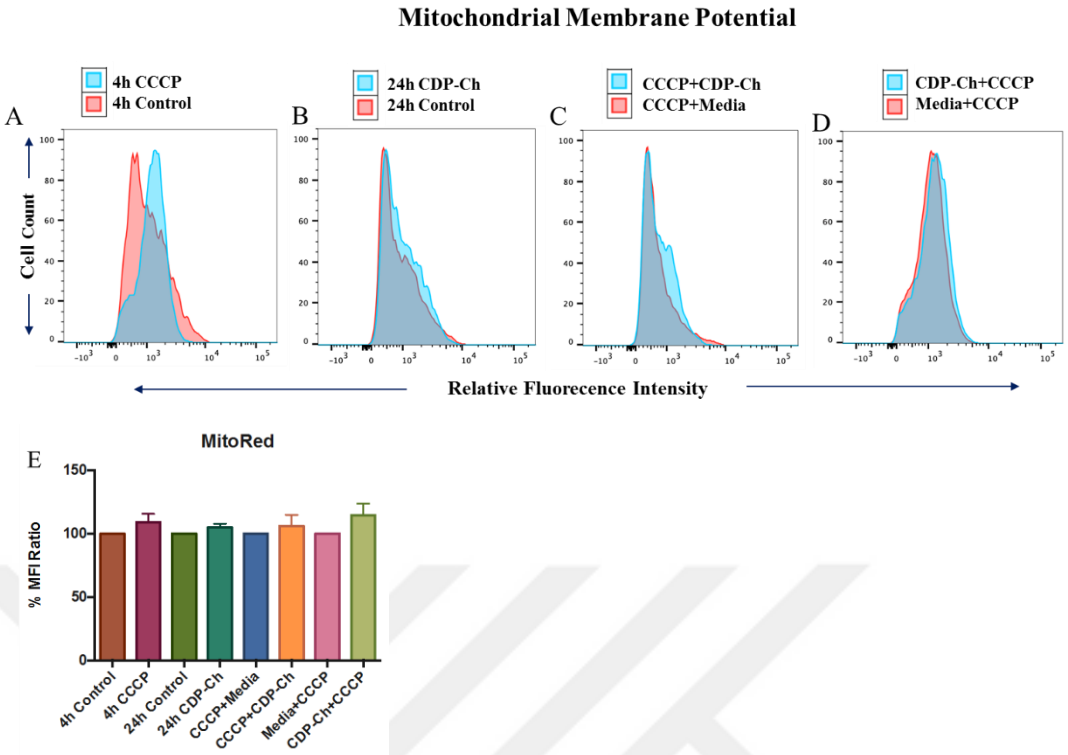


Figure 19. The effect of CDP-ch on mitochondrial membrane potential in mitophagy induced cells

Different treatments had been performed. (A) Treatment with 10 µM of CCCP for 4 h. (B) Treatment with 1 mM of CDP-ch for 24 h. (C) Treatment with 10 µM of CCCP for 4 h, followed by 1 mM of CDP-ch treatment for 24 h. (D) Treatment with 24 h of 1 mM of CDP-ch, followed by 10 µM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 100 nM of Mitotracker Red CMXROS, a mitochondrial membrane potential specific dye. Stained cells were read by BD FACSVerse flow cytometry. Samples were analyzed by FlowJo software and depicted as histogram in the figure. (E) Bar graph represents 4-5 independent repeats as mean and standard deviation (*p < 0.05, **p < 0.01, ***p < 0.001).

4.3.2. The effect of CDP-ch on mitochondrial dynamics by confocal microscopy

In confocal microscopy experiments, we followed the same experimental design with flow cytometry to support our previous findings. We first tested mitochondrial superoxide level. 4 h of CCCP treatment increased mitochondrial superoxide and large accumulation of superoxide was observed under the microscope. Image analysis of mean fluorescent intensity (MFI) of more than 50 distinct cells indicated to more than 3 folds increase. This result is complementary to the findings obtained by the flow cytometry. There is slight increase with 24 h of CDP-ch treatment. Relative observation and quantification of MFI represent that CDP-ch did not alter superoxide production in mitophagy induced cells (Figure 20). We did observe a reduction of

superoxide by flow cytometry but could not obtain the same result with confocal microscopy. This is because flow cytometry is more quantitative, and even small differences can be detected. The reduction in superoxide by CDP-ch was low and the detection of it by confocal microscopy was difficult.

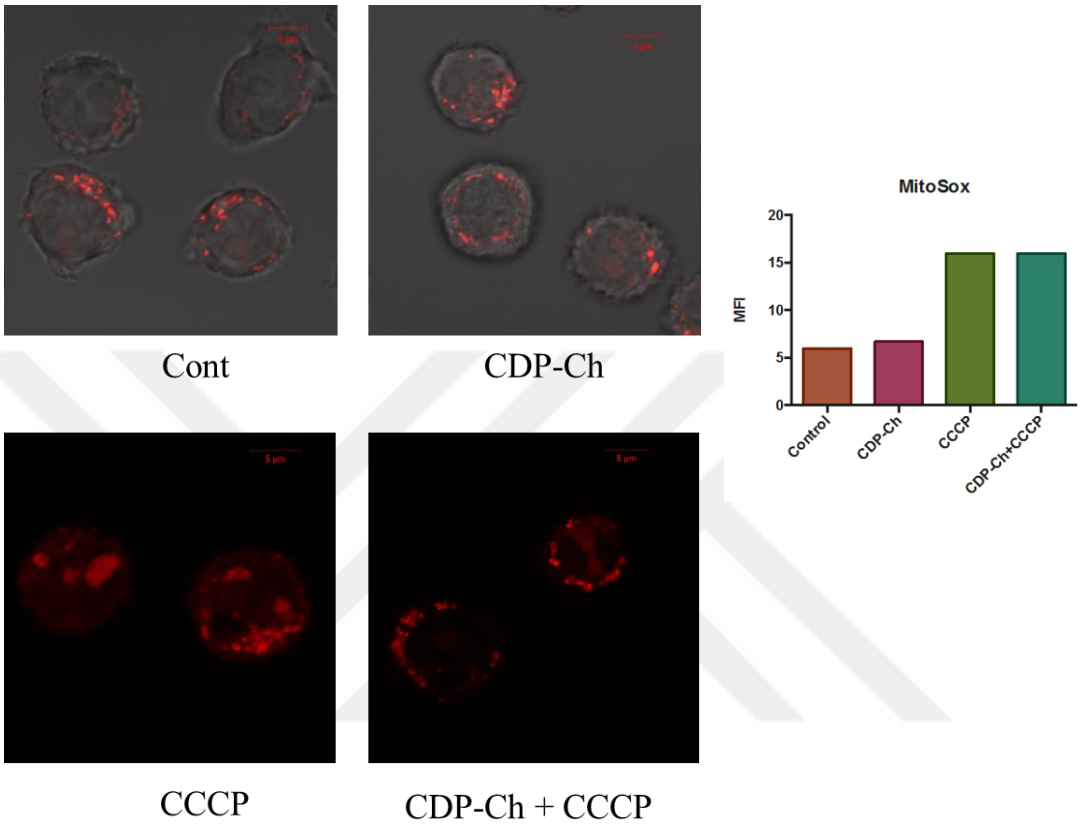


Figure 20. The effect of CDP-ch on mitochondrial superoxide in mitophagy induced cells by confocal microscopy
Different treatments were performed. Treatment with 10 μM of CCCP for 4 h. Treatment with 1 mM of CDP-ch for 24 h. Treatment with 1 mM of CDP-ch for 24 h followed by 10 μM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 2.5 μM MitoSox. Stained cells were put into IBIDI slides and imaged 63x magnification under confocal microscopy. Control and CDP-ch images are representative of merged fluorescent and DIC images. Bar graph represents mean fluorescent intensity (MFI) of 50-70 cells from each group. MFI was analyzed by ImageJ software.

In addition, we tested mitochondrial mass with confocal microscopy. 4h of CCCP treatment decreased mass, as expected and landed support to what was obtained by flow cytometry. Treatment of CDP-ch only did not alter mitochondrial mass. However, the reduction in mitochondrial mass caused by CCCP was reversed by CDP-ch treatment and mitochondrial mass was elevated some more. This result also supports the findings that was obtained by flow cytometry (Figure 21).

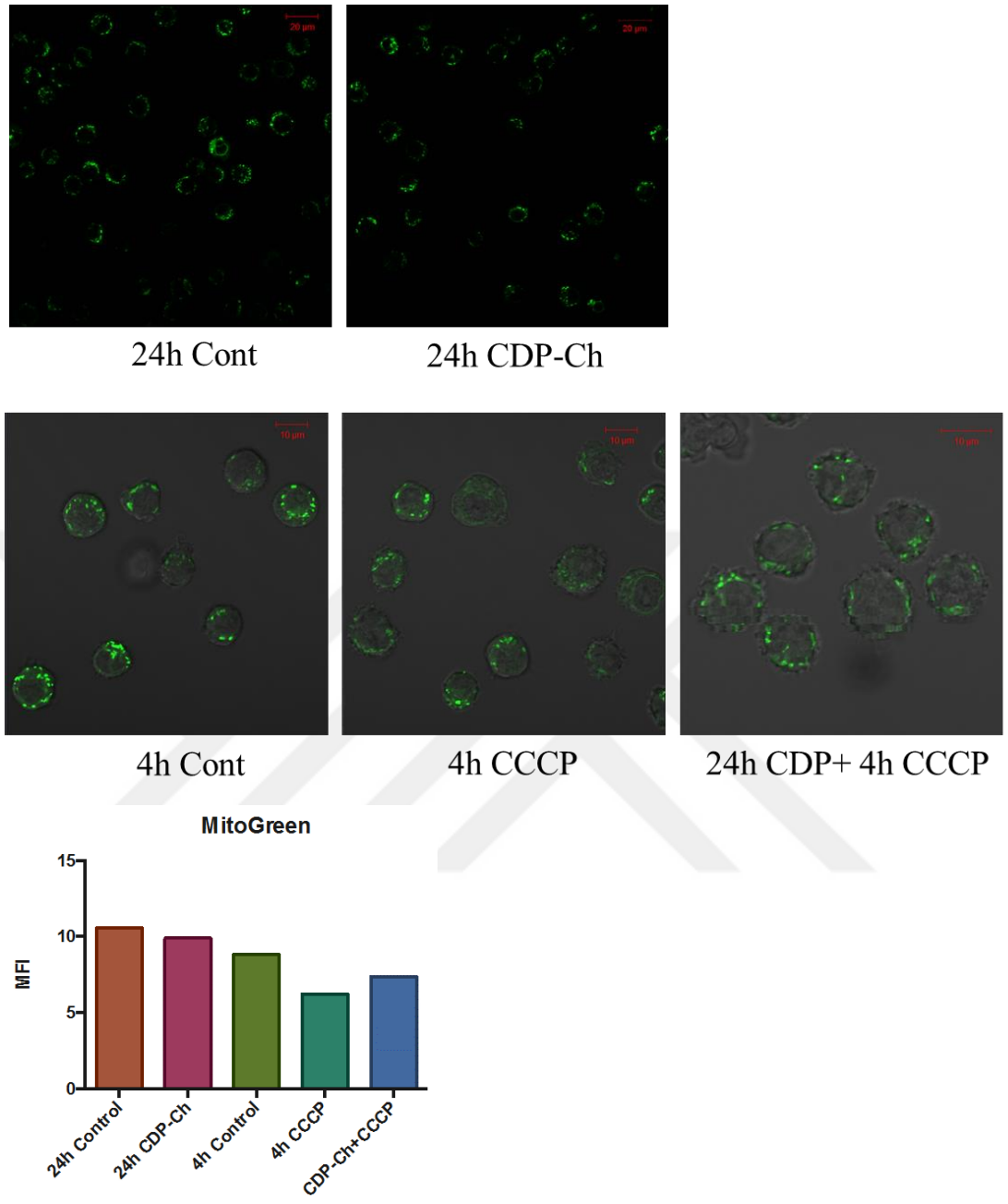


Figure 21. The effect of CDP-ch on mitochondrial mass in mitophagy induced cells by confocal microscopy

Different treatments had been performed. Treatment with 10 μM of CCCP for 4 h. Treatment with 1 mM of CDP-ch for 24 h. Treatment with 1 mM of CDP-ch for 24 h followed by 10 μM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 50nM Mitotracker Green FM. Stained cells were put into IBIDI slides and imaged 63x magnification under confocal microscopy. Control and CDP-ch images are representative of merged fluorescent and DIC images. Bar graph represents mean fluorescent intensity (MFI) of 50-70 cells from each group. MFI was analyzed by ImageJ software.

Furthermore, we tested MMP with confocal microscopy. 4 h of CCCP treatment increased MMP; this is same trend that we found with flow cytometry. Treatment of 24 h of CDP-ch did not alter MMP. The increase in MMP caused by CCCP was observed. MMP was increased further by CDP-ch treatment in mitophagy induced cells (Figure 22).

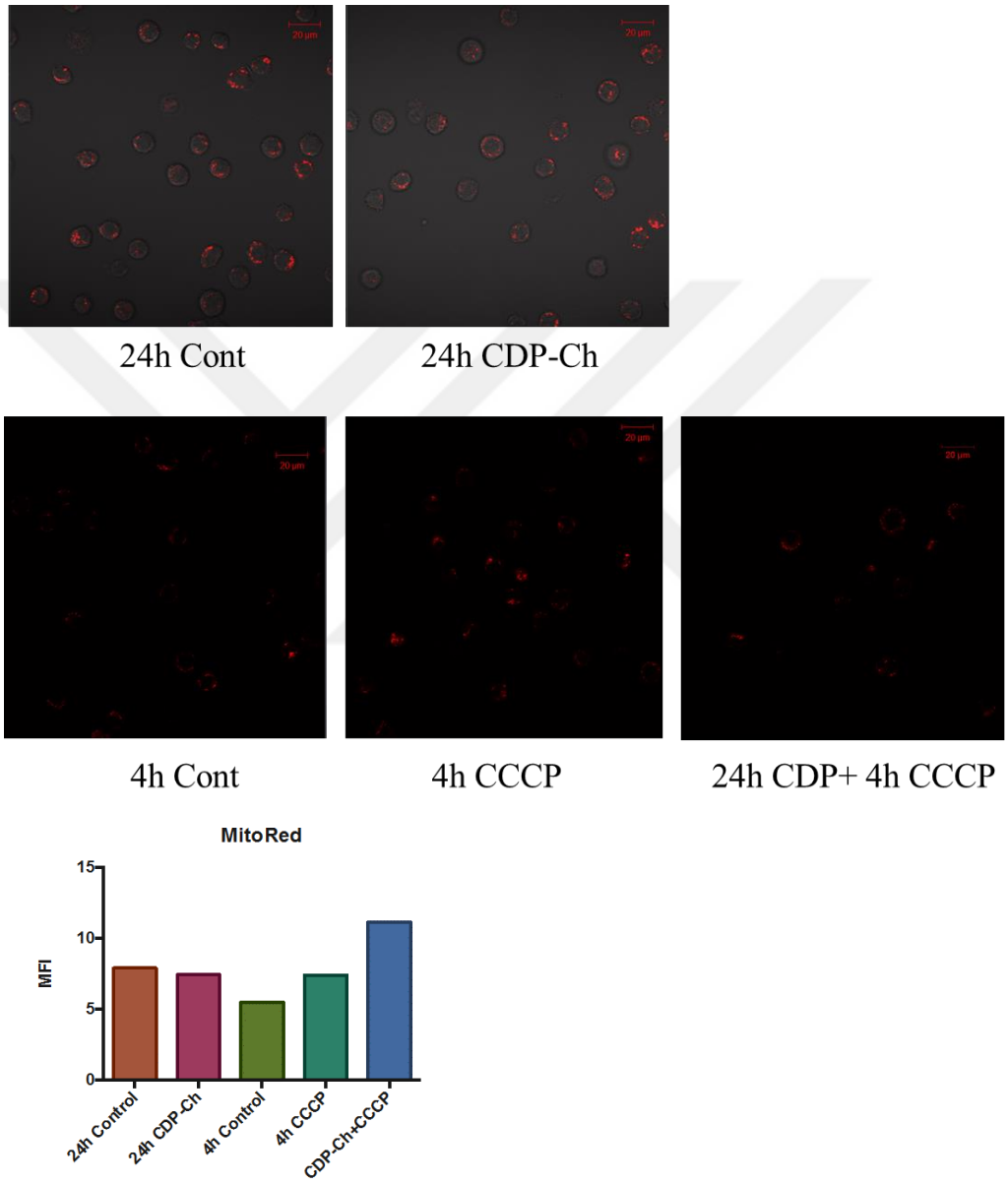


Figure 22. The effect of CDP-ch on mitochondrial membrane potential in mitophagy induced cells by confocal microscopy
Different treatments had been performed. Treatment with 10 µM of CCCP for 4 h. Treatment with 1 mM of CDP-ch for 24 h. Treatment with 1 mM of CDP-ch for 24 h followed by 10 µM of CCCP treatment for 4 h. After treatments, cells were collected and stained with 100 nM Mitotracker Red CMXROS. Stained cells were put into IBIDI slides and imaged 63x magnification under confocal microscopy. Control and CDP-

ch images are representative of merged fluorescent and DIC images. Bar graph represents mean fluorescent intensity (MFI) of 50-70 cells from each group. MFI was analyzed by ImageJ software.

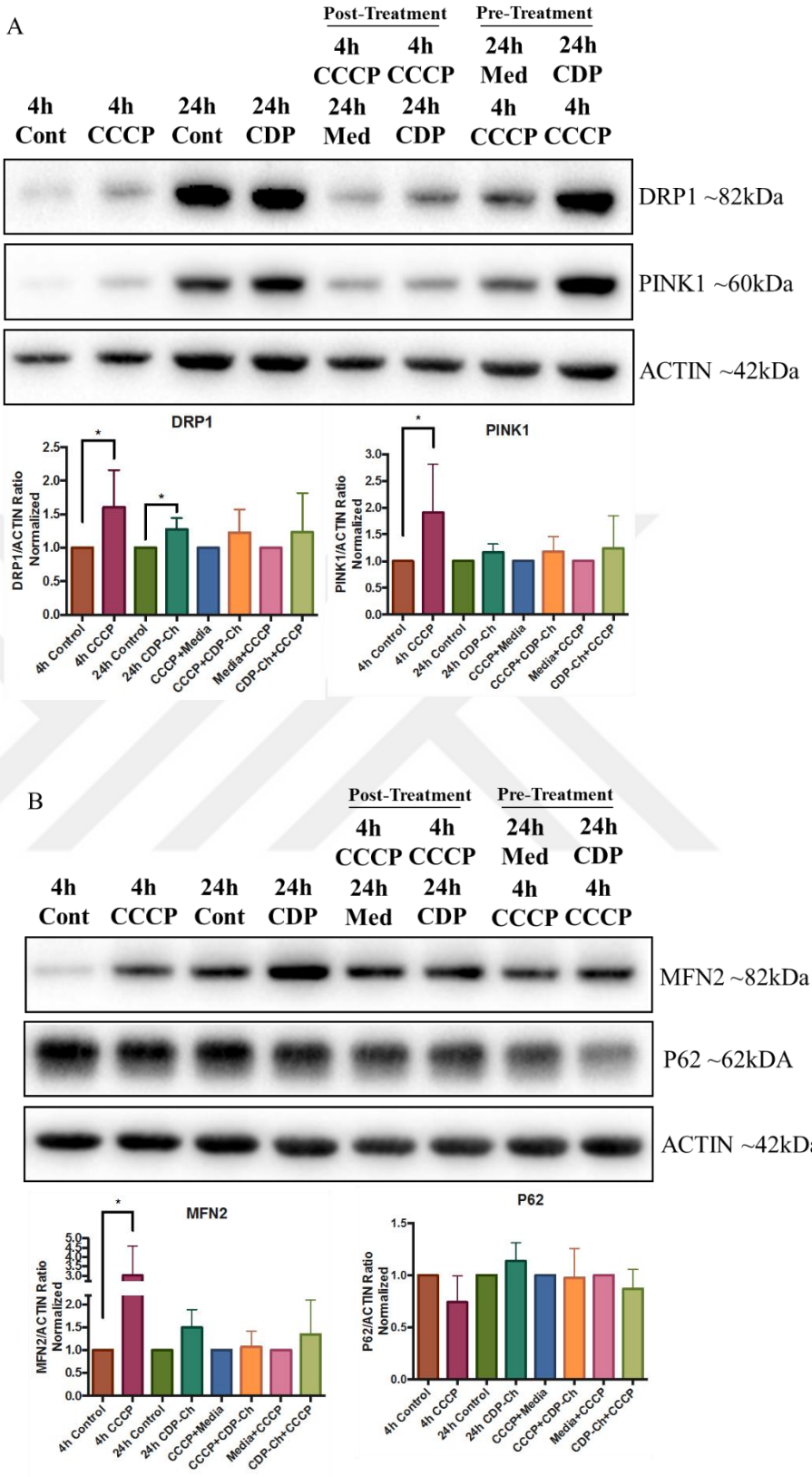
4.3.3. The effect of CDP-ch on levels of mitophagy and mitochondrial dynamics related proteins

To reveal the effects of CDP-ch on mitophagy induced U937 cells and strengthen previous findings, the same experimental design was carried out using western blotting. For this purpose, we looked for the changes the protein levels of DRP1, MFN2, PINK1, COXIV, p62 and LC3B in the course of mitophagy induction. Mitophagy was induced by 10 μM of CCCP treatment for 4 hours and 1mM of CDP-ch was used for 24 hours before and after mitophagy induction.

Upon 4 hours of CCCP treatment, the protein level of DRP1, MFN2, PINK1 and lipidated form of LC3B are increased significantly more than by 1.5-fold ($p = 0.0401$), 3-fold ($p = 0.0106$), PINK1 nearly 2-fold ($p = 0.0404$) and 3-fold ($p = 0.003$) of controls, respectively. On the other hand, the protein level of p62 ($p = 0.1006$) and COXIV ($p = 0.0009$) were reduced nearly by 2-fold (Figure 23 A, B, C). These data also confirmed what was found in time dependent effect of CCCP incubation (Figure 13).

Upon 24 hours of CDP-ch treatment, the protein level of DRP1 protein ($p = 0.0358$) increased significantly by more than 25%. The level of MFN2 was also increased by 1.5-fold. Even though MFN2 levels were increased in every repeat, the increase was not significant ($p = 0.0694$). This can be overcome by the increasing the number of repeats. There were small changes in the protein levels of PINK1, p62, COXIV and LC3B proteins. The results were fluctuating (Figure 23 A, B, C).

In the course of mitophagy induction, both post and pretreatment of CDP-ch increased the levels of DRP1 and PINK1 but not significantly. There were fluctuations on the protein level of MFN2, p62, LC3B and COXIV. Pretreatment of CDP-ch increased MFN2 level and reduced the p62 level of protein. Post treatment of CDP-ch on the other hand decreased lipidated form of LC3B and COXIV levels (Figure 23 A, B, C).


A
Post-Treatment
Pre-Treatment
4h Cont
4h CCCP
24h Cont
24h CDP
4h CCCP 24h Med
4h CCCP 24h CDP
24h Med 4h CCCP
24h CDP 4h CCCP
DRP1 ~82kDa
PINK1 ~60kDa
ACTIN ~42kDa
DRP1
DRP1/ACTIN Ratio Normalized
PINK1
PINK1/ACTIN Ratio Normalized
4h Control
4h CCCP
24h Control
24h CDP-Ch
CCCP+Media
CCCP+CDP-Ch
Media+CCCP
CDP-Ch+CCCP
B
Post-Treatment
Pre-Treatment
4h Cont
4h CCCP
24h Cont
24h CDP
4h CCCP 24h Med
4h CCCP 24h CDP
24h Med 4h CCCP
24h CDP 4h CCCP
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P62
P62/ACTIN Ratio Normalized

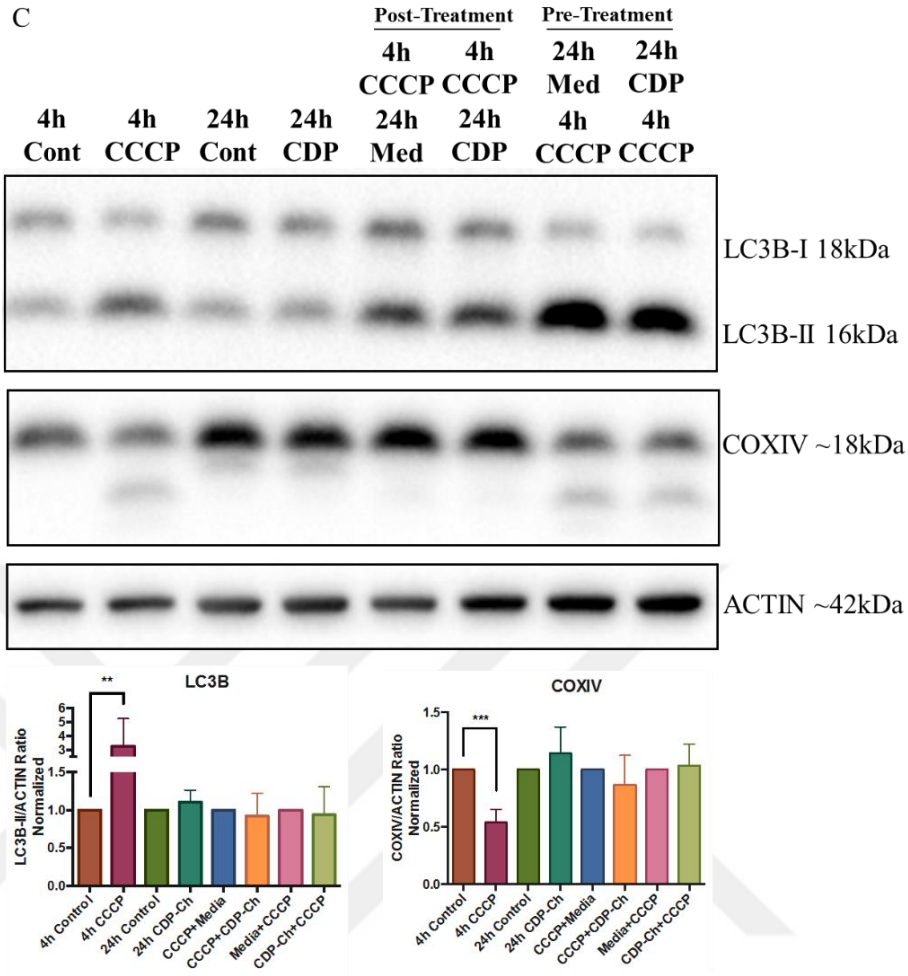

Figure 23. The effect of CDP-ch on mitophagy and mitochondrial related proteins in mitophagy induced cells.

Different treatments were performed. Treatment with 10 μM of CCCP for 4 h. Treatment with 1 mM of CDP-ch for 24 h. Treatment with 10 μM of CCCP for 4 h, followed by 1 mM of CDP-ch treatment for 24 h (Post-treatment). Treatment with 24 h of 1 mM of CDP-ch, followed by 10 μM of CCCP treatment for 4 h (Pre-treatment. After treatments, cells were collected and lysed. Proteins were run using 12% SDS-PAGE for DRP1, MFN2, PINK1 and p62; 15% SDS-PAGE was used to separate LC3B and COXIV. Protein bands were quantified by ImageLab software. ACTIN was used for internal loading control. (A) DRP1 and PINK1, (B) MFN2 and p62, (C) LC3B and COXIV proteins were normalized with ACTIN. Bar graph represents 4-5 independent repeats as mean and standard deviation (*p < 0.05, **p < 0.01, ***p < 0.001).

5. DISCUSSION

The mitochondrion is known as the energy powerhouse of the cells but it is also involved in many survival pathways including lipid metabolism, ion homeostasis and apoptosis (11). Thus, proper functioning of mitochondria is crucial for cell survival. Reactive oxygen species production during aerobic respiration lead to severe damage on mitochondrial and nuclear DNA, lipids and proteins causing diseases such as cancer and neurodegeneration (19). Damaged mitochondria must be removed before irreversible events like apoptosis occurs. The removal of damaged mitochondria by autophagy machinery is called mitophagy (16). Detection and distinction of damaged mitochondria from healthy one is the primary and foremost step for mitophagy and is finalized with fusion with lysosomes (17). Mitochondria are highly dynamic organelles that divide and move constantly while making new connections. Mitochondrial dynamics comprise of fusion and fission events and they are essential for mitochondrial quality control and its health (22).

Phospholipids are a class of lipids mainly found in the cellular membranes. Depending on their head groups, they are varied. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL) are the most abundant among others in the membrane of mammalian cells. Phospholipid composition differs between cell types and organelles and this difference determines the fate of cells and organelles. The most abundant phospholipid in the mitochondrial membrane is PC (27). Chu and Lee showed that U937 cells are composed of more than 50% of PC in their cellular membranes (66).

De novo synthesis of PC and PE is known as Kennedy pathway. It comprises of three enzymatic stems and requires high energy intermediates. CDP-choline is one of the important intermediates in the production of PC (37). Defects in the synthesis of PC can induce mitophagy; therefore, its proper synthesis is crucial for keeping mitochondria healthy (34). CDP-choline is a novel intermediate mononucleotide. It has been used as a drug named as citicoline since 1970s. In addition to its role in the PC synthesis, many studies suggest that it has neuroprotective activity and causes

memory improvement (39). Citicoline is a non-toxic drug and now it sold as a food supplement in many countries because of not having adverse effects (48).

In a study conducted by Won lee mitophagy induction by CCCP reduced the level of PC (35). Damage caused by CCCP on mitochondria induces mitophagy to remove mitochondria by autophagy machinery. Treatment of CDP-choline may facilitate the synthesis of PC in mitophagy-induced cells. Therefore, our main aim in this study was to test the effect of CDP-choline on mitophagy induced cells as a source of PC. In addition to PC synthesis, CDP-choline may affect other pathways as well, through induction of SIRT1 (44) or reduction of oxidative stress (43, 49). We investigated mitophagy and the effects of CDP-choline on U937, a monocytic cell line, for the first time in this study. CDP-choline is an orally administrated food supplement and through the circulatory system, monocytes may metabolize and demonstrate different responses. Therefore, the study of CDP-choline during mitophagy induction in monocytes is worth to investigate.

The effect of CDP-choline in mitophagy induced U937 cells on mitochondrial dynamics were determined by both flow cytometry and confocal microscopy using several mitochondrial specific fluorescent dyes. These specific dyes were used to measure mitochondrial superoxide level, membrane potential (MMP) changes and mitochondrial mass. Additionally, changes in different proteins related to mitophagy and mitochondrial dynamics were determined by western blotting. To test the effects of CDP-choline during mitophagy induction, we first tried to determine the optimal concentration of CDP-choline. Different concentrations and time points of CDP-choline were tested on cell proliferation by MTT and then we decided to use 1mM of CDP-choline for further experiments.

The mitochondrial uncoupler, CCCP, was used to induce mitophagy. CCCP damages the mitochondria leading to reduction of mitochondrial membrane potential and generation of reactive oxygen species (71, 72). We determined that 4 hours of 10 μM of CCCP treatment was sufficient as reported by Youle *et al*. (69). Four hours of treatment of CCCP increased the superoxide level by more than 5-fold significantly ($p < 0.001$). The same result was obtained by both flow cytometry and confocal microscopy. Similarly, enhanced superoxide production by CCCP treatment was previously shown in different studies (71, 72). Even though CDP-choline treatment

alone or post-treatment of CDP-choline during mitophagy did not affect mitochondrial superoxide production, pre-treatment of CDP-choline reduced the level of superoxide significantly ($p = 0.026$). It has been suggested that CDP-choline treatment inhibits the stimulation of phospholipase A2 activity during oxidative stress caused by different agents and increases glutathione synthesis and glutathione reductase activity; and that therefore, the treatment can decrease free radical generation against oxidative stress (42, 43, 49, 56). CCCP also induced superoxide production and CDP-choline was able to reduce this oxidative stress in our study.

In this study, the effect of CDP-choline on mitochondrial mass were investigated for the first time. We hypothesized that CDP-choline may be used as a source to produce PC required for mitochondria biosynthesis upon mitophagy induction. Thus, CDP-choline treatment may then recover mitochondria in U937 cells. Mitophagy induction by CCCP reduced the quantity of mitochondria significantly ($p < 0.001$) in 4 hours. Although, an increase was observed on the mitochondrial mass in the 24 hours pre-treatment and alone of CDP-choline, it was not statistically significant. Whereas, post-treatment of CDP-choline reversed the effect of CCCP and increase the mitochondrial mass significantly ($p = 0.029$).

In a study conducted by Hernández-Esquivel *et al*., it was shown that treatment of CDP-choline inhibited mitochondrial permeability transition (MPT) caused by ischemia/ reperfusion injury (45). Lim *at al.* showed that CCCP treatment induced MPT in another study (73). Therefore, CDP-choline may also protect mitophagy induced U937 cells through the inhibition of MPT under our experimental conditions.

The effect of CDP-choline on mitochondrial membrane potential (MMP) was also investigated. CDP-choline treatment alone did not affect MMP. Although, short incubation time (30 minutes) of CCCP reduced the MMP, longer incubation time (4 hours) increased the MMP. In the literature, the general view is that CCCP reduces the MMP. Even though a lot of studies on MMP suggested that CCCP reduces MMP in short time incubation (74, 75, 76), there are also studies on longer incubation causing MMP reduction (77, 78). This may arise from cell type differences. Pre and post treatment of CDP-choline did not alter MMP significantly in the mitophagy induced U937 cell line.

We further investigated the effect of CDP-choline on different mitochondrial dynamics and mitophagy related proteins which are DRP1, MFN2, PINK1, LC3B, p62 and COXIV. During mitophagy induction with CCCP treatment for 4 hours, DRP1, MFN2, PINK1, lipidated form of LC3B were increased significantly whereas the protein level of COXIV and p62 were reduced. In normal physiology, PINK1 is degraded by PARL enzyme however in the case of mitophagy, it accumulates at OMM. Damage caused by CCCP on mitochondria induced the autophagy machinery to clear damaged mitochondria, therefore, we observed increases in the lipidated form of LC3B and reduction of p62 protein levels. In a study conducted by Park et al. protein level of DRP1 was elevated on the isolated mitochondria during mitophagy in SH-SY5Y cells (77). This finding supports our observations on the protein level of DRP1, however, we obtained DRP1 protein level in whole cell lysates instead of isolated mitochondria. Recent articles suggested that protein level of MFN2 was reduced during mitophagy both in the mitochondrial and cytoplasmic fractions (78, 79, 80). However, in our cellular model we observed increased on the protein level of MFN2 significantly ($p = 0.01$). This increase was even more elevated with bafilomycin A1 treatment. The difference could be due to different cell types. The increase in DRP1 and MFN2 levels during mitophagy induction can be explained as the cells trying to eliminate damaged mitochondria by fission using DRP1 protein or fusing the damaged mitochondria with health network by MFN2. Furthermore, the reduction of mitochondrial protein level, like COXIV, during mitophagy is also used to monitor mitophagy. We observed that the level of COXIV protein reduced significantly in U937 cells upon mitophagy induction.

Although mitophagy related proteins, PINK1, p62 and lipidated form of LC3B were not affected by CDP-choline treatment, the levels of DRP1, MFN2 and COXIV were increased in control U937 cells. Together with these protein levels, increased mitochondrial mass with treatment of CDP-choline indicates that CDP-choline may induce mitochondrial biogenesis in U937 cells.

Another interesting finding in this study was fragmentation of COXIV observed on western blots during mitophagy induction on monocytes. The fragmentation pattern began early as 1 hour, and similar results were obtained by other mitophagy inducers as well. When different cell types were investigated, not all cell types exhibited this

phenomenon. To our knowledge, similar findings have not been reported in the literature, as yet. Bafilomycin A1 is a lysosome inhibitor which inhibits fusion of autophagosome with lysosome. Bafilomycin A1 experiments indicated that this pattern was not caused by lysosome degradation of mitochondria and fragmentation still remained on the blot which indicates that fragmentation of COXIV takes place in mitochondria. We can speculate that this fragmentation may be caused by an enzyme in the mitochondria like PARL which is responsible for cutting PINK1. Since fragmentations was sustained until 8 hours in the cells, the proteasome cannot be responsible for the degradation of COXIV during mitophagy induction.

In summary, the changes in mitochondrial fusion or fission and mitochondrial mass in mitophagy-induced U937 cells suggest a modulatory role of CDP-choline in mitochondrial dynamics. We propose that CDP-choline may have protective effects during mitophagy by contributing to the production of mitochondrial membranes thereby, new mitochondria can rescue cells quickly from the stress and maintain cell survival.

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CURRICULUM VITAE

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Birth Place	Uşak	**Birth Date**	21/02/1992
Nationality	Turkish	**Phone Number**	05432600962
E-mail	Sbozkurt.mbg@gmail.com		

Education

	Institution	**Graduation Year**
BSc	Izmir Institute of Technology, Molecular Biology and Genetics	2015
High School	Uşak Anatolian Teacher High School	2010

Experience

Job Title	**Institution**	**Duration**
Erasmus+ Intern	University of Oxford, Katja Simon Lab	November – March 2019
Erasmus+ Intern	Idibell, bellvitge biomedical research institute, Ballestar Lab	June-September 2014

Languages	**Reading**	**Speaking**	**Writing**
English	Advanced	Advanced	Advanced

Foreign Language Examination Grade									
KPDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE	YÖKDİL
		6.0							80

	Quantitative	**Equally Weighted**	**Verbal**
ALES Note	82,10966	81,11882	70,19957

Computer Skills

Program	Skill degree
Microsoft Office	Very good
Python	Basics

Presentations

- Bilge B., H. Gezmis, S. Bozkurt, D.Yucel, B.Kan, I.H.Ulus, D.Oz-Arslan The effectof CDP-choline on autophagy and mitochondrial dynamics in beta-amyloid treated PC12 cells. 19th IUPAB/11th EBSA/BBS/IoP Congress 16-20 July 2017 Eur. J. Biophys 2017 (Poster Presentation)
- Suleyman Bozkurt, Devrim Oz-Arslan The Effect of CDP-Choline on Mitophagy and Mitochondrial Dynamics in Monocytic Cell Line Molbiyokon'18 5-8 September 2018 Izmir Biomedicine and Genome Center Izmir / Turkey (Poster Presentation)
- Zeynep Aslıhan Durer, Süleyman Bozkurt, Devrim Öz-Arslan, Christina Vizcarra, Abdurrahman Coskun Biochemical characterization of propeptide of pregnancy associated plasma protein A (proPAPP-A) and its cellular effects October 10-13, 2018 30th Annual Biophysics Congress (International) Bodrum / Turkey (Oral Presentation)
- Süleyman Bozkurt, Devrim Öz-Arslan A study on the role of CDP-Choline in mitochondrial dynamics October 10-13, 2018 30th Annual Biophysics Congress (International) Bodrum / Turkey (Oral Presentation)