MANIPULATING AND VISUALIZING THE ENERGY METABOLISM IN SINGLE CELLS USING CHEMOGENETIC TOOLS AND GENETICALLY ENCODED BIOSENSORS

by

Zeynep ÇOKLUK

Submitted to the Graduate School of Engineering and Natural Sciences in partial fulfillment of

the requirements for the degree of Master of Science

Sabancı University

December 2021

MANIPULATING AND VISUALIZING THE ENERGY METABOLISM IN SINGLE CELLS USING CHEMOGENETIC TOOLS AND GENETICALLY ENCODED BIOSENSORS

APPROVED BY:

APPROVAL: 17/12/2021

ABSTRACT

MANIPULATING AND VISUALIZING THE ENERGY METABOLISM IN SINGLE CELLS USING CHEMOGENETIC TOOLS AND GENETICALLY ENCODED BIOSENSORS

ZEYNEP ÇOKLUK

Molecular Biology, Genetics and Bioengineering, M.Sc. Thesis, December 2021

Thesis Supervisor: Prof. Dr. Levent Öztürk
Thesis Co-Supervisor: Dr. Emrah Eroğlu

Keywords: Mitochondria, ROS signaling, hydrogen peroxide (H_2O_2), DAAO, cancer metabolism, genetically encoded biosensors, ATP, Glucose

Mitochondria directly or indirectly affect cell fate. These organelles are known as the powerhouse of the cell and are responsible for generating ATP. As a consequence of energy production, highly reactive byproducts such as superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), all collectively known as reactive oxygen species (ROS) or reactive nitrogen species are produced. In recent years it has been established that lower ROS levels drive important cellular signaling pathways, particularly H_2O_2. In a recent study, we developed an H_2O_2 generating chemogenetic tool referred to as D-amino acid oxidase (DAAO) that permits the generation of subcellular H_2O_2 levels with high spatial and temporal resolution. This study exploits multiparametric imaging approaches by combining chemogenetic tools with genetically encoded biosensors HyPer7 for H_2O_2, MaLionR

for ATP, R-GECO for Calcium, and FLII^{12}Pglu-700μδ6 for Glucose, and also chemical sensors such as tetramethylrhodamine methyl ester perchlorate (TMRM) for multispectral live-cell imaging of the membrane potential in cancer cells. These approaches allowed us to simultaneously generate ROS and visualize the downstream effects of H_2O_2 levels on various signaling pathways (subcellular calcium signaling) and the energy metabolism (mitochondrial membrane potential, glucose uptake, and ATP generation) in cancer cells. We observed that chemogenetically produced H_2O_2 did not affect Ca^{2+} and glucose levels while ATP levels were significantly affected. We documented that robust increase in mito-H_2O_2 levels disrupted the mitochondrial membrane potential. We also documented that chronic H_2O_2 production in HeLa cells reversed mitochondrial ATP production upon glucose deprivation. Our results demonstrate that utilizing chemogenetic tools paired with genetically encoded biosensors may serve as an informative approach to investigate the implications of oxidative stress in cancer energy metabolism.

OZET

TEK HÜCRE BAZINDA ENERJİ METABOLİZMASININ KEMOGENETİK ARAÇLAR VE GENETİK OLARAK KODLANMIŞ BİYOSENSÖRLER KULLANARAK MANİPÜLE EDİLMESİ VE GÖRÜNTÜLENMESİ

ZEYNEP ÇOKLUK

Moleküler Biyoloji, Genetik Ve Biyomühendislik, Yüksek Lisans Tezi, Aralık 2021

Tez Danışmanı: Prof. Dr. Levent Öztürk

Tez Eş-Danışmanı: Dr. Emrah Eroğlu

Anahtar Kelimeler: Mitokondri, ROS sinyali, hidrojen peroksit, DAAO, kanser metabolizması, genetik olarak kodlanmış biyosensörler, ATP, Glikoz

Mitokondri doğrudan veya dolaylı olarak hücrelerin karar verme mekanizmasını etkiler. Bu organeller, hücrenin güç merkezi olarak bilinir ve ATP'nin üretilmesinden sorumludur. Enerji üretiminin sonucunda, reaktif oksijen türleri (ROS) olarak bilinen süperoksit anyonu (O^{2-}), hidroksil radikali (OH^{-}), hidrojen peroksit (H_2O_2) ve peroksinitrit ($ONOO^{-}$) gibi oldukça reaktif yan ürünler ve reaktif nitrojen türleri doğal olarak üretilir. Son yıllarda, daha düşük ROS seviyelerinin önemli hücresel sinyal yollarını yönlendirdiği tespit edilmiştir. Bununla birlikte, uygun araçların olmaması nedeniyle, hücre içi ROS seviyelerini manipüle etmek zorlu bir görev olarak kaldı. Yakın tarihli bir çalışmada, D-amino asit oksidaz (DAAO) olarak adlandırılan ve yüksek çözünürlüğe sahip hücre içi H_2O_2 seviyelerinin üretilmesini sağlayan kemogenetik yöntem geliştirdik. Bu çalışma, kemogenetik araçları H_2O_2 için HyPer7, ATP için MaLionR, Kalsiyum için R-GECO ve Glikoz için FLII^{12}Pglu-700μδ6 gibi

genetik olarak kodlanmış biyosensörler ve ayrıca tetrametilrodamin metil ester (TMRM) gibi kimyasal sensörler ile birleştirerek multiparametrik görüntüleme tekniklerinden yararlandık. Bu araçlar, eşzamanlı olarak ROS oluşturmak ve kanser hücrelerinde çeşitli sinyal yolları (hücre içi kalsiyum sinyali) ve enerji metabolizması (mitokondriyal membran potansiyeli, glikoz alımı ve ATP üretimi) üzerinde H_2O_2'nin hücredeki etkilerini görselleştirmek için DAAO'larla birleştirilmiştir.

Kemogenetik olarak üretilen H_2O_2'nin Ca^{2+} ve glukoz düzeylerini etkilemediğini, ATP düzeylerinin ise önemli ölçüde etkilendiğini gözlemledik. H_2O_2 seviyelerindeki güçlü artışın mitokondriyal membran potansiyelini bozduğunu gözlemledik. Ayrıca, HeLa hücrelerinde kronik H_2O_2 üretiminin, glikoz yoksunluğumda mitokondriyal ATP seviyelerinde ters etkiye yol açtığını gözlemledik.

H_2O_2 tarafından hücresel düzeyde aktive edilen sinyal yollarının seçici olarak görüntülenmesi için kemogenetik araç mDAAO ve biyosensörlerin kullanılmasının, redoks sinyalleşmesinde yerel olarak üretilen H_2O_2'nin hücreye ne kadar yararlı veya hücreye zarar verebileceğinin daha iyi anlaşılmasını sağlayacağını düşünüyoruz.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Emrah Eroglu for his guidance and support during my master education.

I would like to thank my jury committee Prof. Dr. Levent Öztürk, Asst. Prof. Dr. Christopher Mayack and Asst. Prof. Dr. Sevde Altuntaş for their helpful comments and accepting to be in my thesis committee.

I would like to thank my lab members Asal, Busra, Gulşah, Melike, Yusuf and Tuba for being with me. I'm also so thankful to Melike for her cell culture helps. I also would like to thanks Ceyhun who help and support me whatever and whenever I need him. I feel lucky to have my friends Asuman, Sezgin, Esra and Mert that always support me during these time. I would like to thank to my lovely friend Seden for her great friendship and support. My master years wouldn’t have been the same without my lovely friends.

I also would like thank my beloved family for always being with me.

to all human beings,

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Mg	Microgram
Ml	Microliter
Mm	Micromolar
mM	Milimolar
a.a	Aminoacid
ATP	Adenosine three phosphate
BP	Binding Protein
$[Ca^{2+}]$	Calcium
CaM	Calmodulin
cpFP	Permuted fluorescent proteins
cpGFP	Circularly permuted GFP
D-PBS	Dulbecco's phosphate-buffered saline
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
ER	Endoplasmic Reticulum
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FP	Fluorescent protein
FRET	Förster resonance energy transfer
$FLII^{12}Pglu\text{-}700\mu\delta6$	FRET-based glucose biosensor
GECO	Genetically encoded calcium biosensor
GFP	Green fluorescent protein
GLUT	Glucose Transporter
GPCR	G protein-coupled receptor
HIF-1	Hypoxia Inducible Factor-1

H_2O_2	Hydrogen peroxide
IP_3R	Inositol 1,4,5-trisphosphate receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
Nrf2	Erythroid 2–related factor 2
O_2^-	Superoxide anion
OH•	Hydroxyl radicals
ONOO	Peroxynitrite
OXPHOS	Oxidative Phosphorylation
ROS	Reactive oxygen species
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
TCA cycle	The Citric Acid Cycle
TMRM	Tetrametilrodaminmetilester
YFP	Yellow fluorescent protein
Ψmito	Mitochondrial membrane potential

1. INTRODUCTION

1.1. ROS and ATP– a chicken-egg problem

Mitochondria play a significant role in cancer. Mutation in mitochondrial DNA, any defect in antioxidant mechanism or damage in oxidative phosphorylation can cause severe or fatal disorders.[1]These organelles are the powerhouse of and responsible for generating ATP, which is the main energy source of the cells. Oxygen dependent process called oxidative phosphorylation synthesize ATP in the mitochondria.[2] As a consequence of energy production, highly reactive byproducts such as superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), all collectively known as reactive oxygen species (ROS) are naturally produced.[3] Antioxidant mechanisms maintain ROS levels in balance and to avoid oxidative distress within the mitochondria.[4] Oxidative distress is often implicated with cellular damage and various pathologies such as cancer, diabetes, cardiovascular, neurological diseases and inflammatory disorders. On the other hand, lower ROS levels, known as oxidative eustress take part in important cellular signaling pathways. Particularly H_2O_2 is a mild oxidant that can modify thiol-residues of proteins to induce intracellular signaling by enzyme (de)activation.[5] Therefore, H_2O_2 can drive intracellular process such as Ca^{2+} signaling, activation of transcription factors, regulation of intracellular and surface receptors, degradation or formation of structural proteins, and phosphorylation of redox-sensitive enzymes.[6,7]

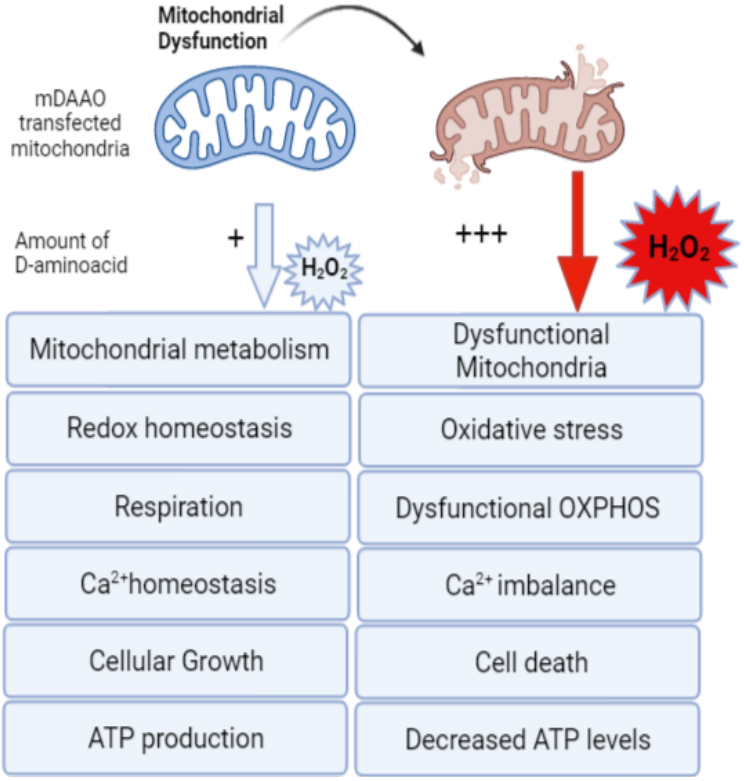


Figure1.1: Redox Balance of Mitochondria. H_2O_2 regulated cellular response of the mitochondria. Dose dependent H_2O_2 responses that are regulated by ROS generators and scavengers are depicted.

Due to the important role of H_2O_2 as a versatile signaling molecule, understanding mitochondria derived H_2O_2 levels, particularly its generation, degradation, and diffusivity among cell organelles is critical. Despite decades of research, H_2O_2 derived signaling and metabolic pathways in cancer cells have been less explored. To tackle this elusive issue, in this study we focus the first time on the energy metabolism of mitochondria in cancer cells using chemogenetic tools to generate site-specifically H_2O_2, and genetically encoded biosensors for real-time imaging of glucose and ATP.

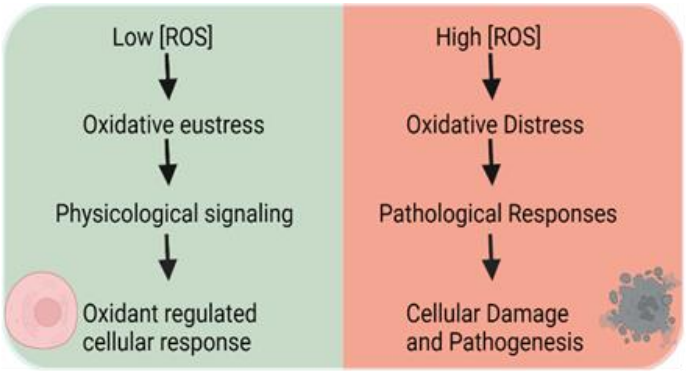


Figure1.3: ROS concentration determines the fate of oxidative stress

1.2.Signaling Role Of Mitochondria

Mitochondria have been accepted as key players for the generation of macromolecules and ATP. Mitochondria also critically take part in ROS mediated cellular signaling in addition to fueling metabolites for biosynthetic and bioenergetics purposes. [8]

H_2O_2 plays a role in a variety of signal transduction pathways that regulate physiological and pathophysiological responses, and it is majorly produced during normal metabolism in the mitochondria.[9]

Intracellular H_2O_2 dependent signaling is primarily associated with oxidative thiol modification of many proteins and molecules.[10,11] Depending on its concentration and location in the cell, it gives the cell the order of growth, survival or death signal.

The balance of ROS and antioxidants under normal conditions is disrupted with the overproduction of free radicals. These excess free radicals can lead to DNA damage, degradation of protein, lipids and result in redox imbalance.

The persistence of uncontrolled ROS production ultimately leads to inappropriate signaling and gives rise to activation of transcriptional factors which induces cancer and neurological diseases. Rapidly growing cells have critical metabolic requirements that extend beyond ATP and these cells have to strictly govern transcriptional factor to demand this requirement.[12]

There are lots of studied transcriptional factors that are considered to be regulated by H_2O_2 as shown Figure1.2. H_2O_2 is required for the activation of multiple cellular pathways that control cell metabolism. H_2O_2 activates hypoxia inducible

factors1 (HIF-1) that regulate angiogenesis, to demand increased metabolic activity of cells.[13] Severely disrupted oxygen balance of cancer cell cause hypoxia and tumor cells in this hypoxic region start adapt to low oxygen conditions, result in activating multiple survival pathways. Activation of the transcription factor HIF1 is one of the most studied pathways that used by hypoxic cells in the tumor microenvironment. For example, transcription factor HIF1 helps hypoxic tumor cells to convert glucose to ATP by activating glycolysis rather than oxidative phosphorylation. When we think about ATP efficiency, oxidative phosphorylation is produce more energy. But shifting from the more efficient oxidative phosphorylation to the less efficient glycolytic pathway give energy generation more rapidly and it is important for tumor cells to fastly expanding in the environment (Warburg effect). HIF1 mediate this metabolic situation of a cell, depend on the oxygen concentration in the environment.[14] To demand this glucose demand, HIF activates glucose transporter (GLUT) which provide uptake of glucose in the cell.[15,16]

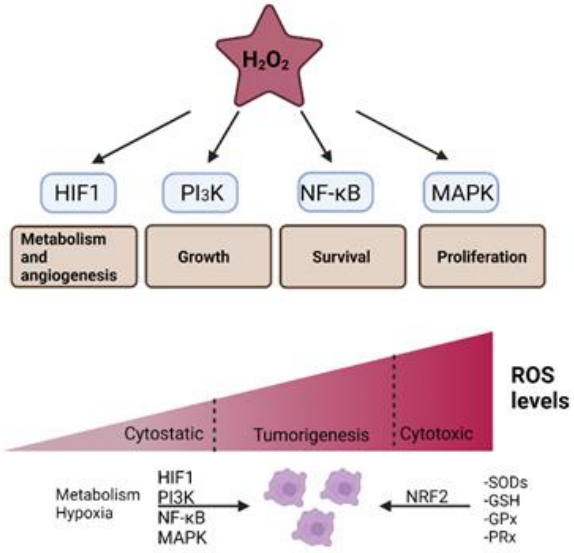


Figure1.2: Signaling Role of H_2O_2. A. Some of the distinct signaling pathways that are activated by H_2O_2 are depicted. **B.** Dose dependent H_2O_2 responses that are regulated by ROS generators and scavengers are depicted.

Furthermore, H_2O_2 activates phoshorylasatide$_3$kinase (PI_3K) pathway that regulates cellular growth. The PI_3K signaling cascade is an important regulatory pathway controlling cell survival, proliferation, and angiogenesis. There are many studies that examine its role in response to alterations in mitochondrial derived H_2O_2.[17]

Abnormal PI_3K activation is common problem in many type of cancer. Misregulation of this pathway is often associated with tumor progression and resistance to cancer treatments.[18]

One of the most important pathway is controlled by H_2O_2 known as nuclear factor-κB (NF-κB). NF-κB pathway prevents apoptosis and regulates cellular survival under normal conditions. Many different types of cancer have misregulated NF-κB signaling and NF-κB become constitutively active.[19] There are two major decisions for cell fate that are dictated from mitochondria to the rest of the cell: proapoptotic and antiapoptotic signaling.[20] Unbalance in redox state of mitochondria directly affect these critical decision of the cells and causes cells to evade from apoptosis.[21]Apoptosis usually happens for a significant reason and is usually beneficial for the organism as clearly seen during biological development. As cells become older or non-functional, they are cleared away and destroyed. This is very important for the cell to stay healthy. Mitochondria determine which cell is going to die. When mitochondria do not function properly due to redox imbalance, they make a lot of free radicals, avoid apoptotic signals and then initiate tumorigenesis. This is very important because some diseases such as cancer involve the break down in normal apoptosis signaling and this is why mitochondria is thought to play a big role in cancer. In fact, many scientists refer to cancer as a mitochondrial and metabolic disease.[21]

Moreover, H_2O_2 involves mitogen activated protein kinase (MAPK) pathway that regulates cellular proliferation. In the cell, there are lots of MAPK enzymes that works in synchronically to mediate proliferation, differentiation, tumor progression, and survival. These kinases are activated in response to external and internal stresses such as bioenergetics stress, depletion of oxygen in the environment and reduction of energy availability.[22–24]

As the mitochondria is a major source of H_2O_2, defects in mitochondrial function cause an excessive production H_2O_2 and these leads to dysregulation of these transcriptional factors and finally results in diseases. However, regulation of normal metabolism is only possible under strictly controlled concentrations of H_2O_2. [25,26]

Due to the important role of H_2O_2 as a versatile signaling molecule, understanding mitochondria derived H_2O_2 levels, particularly its generation, degradation, and diffusivity among cell organelles is very important.

There is also very interesting and important communication that happens from mitochondria out to the rest of the cell, to the nucleus and the cytosol.[27] One of the obvious ways that mitochondria communicate with rest of the cell is through providing metabolites. Several metabolites that are produced by mitochondria have very important signaling and regulatory roles such as Acetyl-coA, alpha-ketoglutarate, ATP which help mitochondria communicate with rest of the cell. [28,29]

Any defect in communication significantly influence the behaviors of cells. When they become aberrant, it is very clear that they have profound impacts on disease progression.[21,30]

1.3.ROS Function in Redox Signaling And Oxidative Stress

Redox signaling is the ability of ROS to activate signaling phosphatases and thereby to initiate biological processes inside the cell. As I previously mentioned above, ROS can function as signaling molecules that regulate physiological processes inside the cell in a very strictly maintained conditions and major molecule that is involved in signal transduction is H_2O_2.[31,32]

It is well understood that cancer cells tend to generate higher levels of ROS than their non-transformed counterparts. This higher levels of ROS disrupt redox balance and signaling inside the cell and cause unrelated biological signals.[33,34]

For this reason, it is important to understand redox signaling in the cell.

The mechanism of redox signaling is based on specific feature of H_2O_2 molecules which is that they can oxidize cysteine residues, thiol groups and within proteins. Cysteine residues at normal condition exist as cystine ions, H_2O_2 molecules can oxidize these ions to sulfanic form and this oxidation changes in protein structure

that result in alteration in protein function and leads to the reversal activation of physiological redox signaling depending on its concentration.[35]

However, everything that is activated needs to be inactivated again, and in order to inactivate the protein, these changes must be reversed. Cysteine residues must be reduced to their initial form.

This reduction is provided by enzymes called disulfide reductases: Thioredoxin and Glutoredoxin.[36,37] However, reversal oxidation of target ions to sulfanic form is only possible under low concentrations of H_2O_2.When H_2O_2 level increase sulfenic acid is further oxidized to sulfinic and sulfonic acid. These change cause permanent alterations in protein structure, disturbing the physiology of the cell and its condition in the tissue.[37,38] Therefore, the level of H_2O_2 must be tightly controlled and should kept within a very narrow range. To do this, cells have antioxidant mechanisms and vary in the type of enzyme regulation so the level of H_2O_2 inside the cell is important and there are two major sources of reactive oxygen species inside the cell. ROS can be produced by NOX2 enzyme or result of oxidative phosphorylation in the mitochondria.[39]

NOX2 enzyme is located on the cell membrane and converts oxygen into superoxide. In mitochondria, these formation occurs during oxidative phosphorylation as a natural byproduct.

Mitochondrial inner membrane located electron transport chain that is composed of four protein complexes, enzyme Q and ATP synthase. The concept is that protein complexes in electron transport chain transport electrons from one complex to another. ATP synthase use this concentration gradient by transportation of hydrogen protons concentration gradient into mitochondrial matrix. In that way ATP synthase complex generates energy that is used for ATP synthesis.[40] However the electron transport chain is not perfectly operate and some electrons may leak out of the ETC. In this situation cells reduce oxygen in another structure. They reduce that leaked out oxygen with formation of superoxide molecules. Here, superoxide dysmutase convert ROS into H_2O_2 and in case of too much converted H_2O_2 in the environment, they are converted into H_2O molecules. Conversion of H_2O_2 to H_2O is provided by peroxiredoxins, glutathione peroxidase and catalase[41–43] (Figure1.4). Peroxidoxin in reduced form reduce hydrogen peroxide and become oxidized with formation of two water molecules. Glutathione peroxidase catalyze reaction where two monomeric

glutathione molecules reduced H_2O_2 with formation of glutathione disulphide and two water molecules. Catalase converts two H_2O_2 molecules to the water molecules and oxygen.[43]

The unconverted mitochodrial H_2O_2 which is produced in this way take part in very important biological signaling. The balance between that conversion is important because this balance determines the level of H_2O_2 inside the cell.

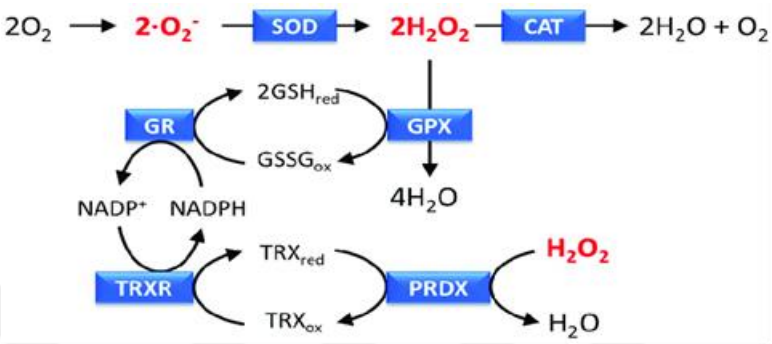


Figure1.4: Neutralization of ROS in the cell.

The level of H_2O_2 molecules must be maintained within strict frames, thereby hydrogen peroxide production must be proportional to hydrogen peroxide reduction. In some point, ROS production exceeds the capacity of antioxidants to reduce superoxide and H_2O_2 molecules and these result in increase in amount of reactive oxygen species inside the cell and this condition is called oxidative stress which cause damage DNA, protein and lipid levels.[5,7]

Therefore, if the H_2O_2 plays such an important signaling role then what would happen when it's produced in a measurable and localized amount inside the cell as signaling molecule? Can we precisely manipulate the redox state of the cell?

1.4. ATP metabolism

ATP Generation in The Mitochondria

The mitochondria can be considered as a major source of ROS production. ROS are naturally produced during process of OXPHOS in the mitochondria. To get energy in usable form, mitochondria convert energy as ATP. ROS are produced

as a normal product of cellular metabolism. During this process, electrons are transferred through the protein that are located in the inner membrane of the mitochondria. They end up as oxygen and they form water. During this electron flows, protons are pumped from matrix to the inner membrane of the mitochondria. Energy of protons gradient are used to make ATP. When glucose is oxidized during glycolysis and the Krebs cycle, coenzymes NADPH and FADH are reduced to NADH and $FADH_2$. Inside the mitochondrial matrix the electrons from NADH are transferred to the electron carrier enzyme Q by NADH dehydrogenase and the protons are transferred across the membrane to the inner membrane space. Coenzyme Q carries the electrons to the cytochrome BC1 complex. As the electrons move from the BC1 to cytochrome c more protons are carried from inside to the outside of the membrane. Electrons are also transferred from FADH2 to the coenzyme Q with the protons being transferred across the membrane. Cytochrome c transfers electrons to the cytochrome c oxidase complex and protons are also transferred to the outside of the membrane by the cytochrome c oxidase complex. Then the cytochrome c complex transfers electrons from cytochrome c to oxygen. The terminal electron acceptor and water are formed as the product(Figure1.5).[43–48]

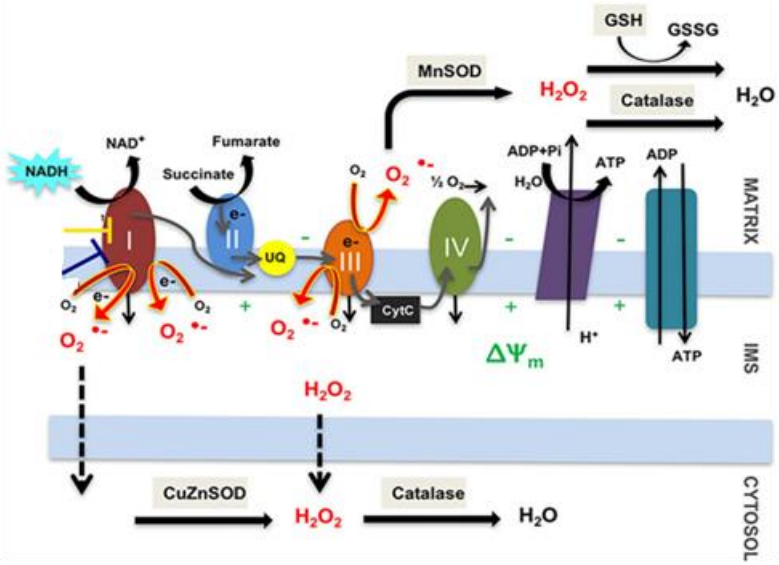


Figure1.5: ATP generation in the mitochondria.

The transfer of the protons to the intermembrane space generates a proton motive force across the inner membrane of the mitochondrion. Since membranes are impermeable to ions, the protons that re-enter the matrix pass through special proton channel proteins called ATP synthase. The energy derived from the movement of these protons is used to synthesize ATP from ADP and phosphate and this occurs at the ATP synthesis complex. Formation of ATP by this mechanism is referred to as oxidative phosphorylation.

When the cell needs energy, this process become active and starts to produce ATP. However, at this point, the cell has two options to generate ATP, it can either go through oxidative phosphorylation or without entering ETC system, it can perform anaerobic phosphorylation. These alternative pathways of the cell is not entirely understood. The switch to anaerobic respiration may be because the lack of oxygen or any defect in mitochondria. In the 1920s a biologist Otto Warburg has claimed that cancer cells make anaerobic phosphorylation despite the presence of oxygen. Based on the current literature, there are lots of ideas that have been used to explain to these phenomena. These include defects in the mitochondria, mutation in mitochondrial DNA, and damage in mitochondrial oxidative phosphorylation enzymes that cause inhibition of oxidative phosphorylation even if there is oxygen. Another idea is that even if there is no mitochondrial defect or lack of enzymes, cancer cells prefer to do anaerobic phosphorylation. According to Warburg observation, cancer cell consume more glucose and make anaerobic

glycolysis. Although this gives them far less efficient ATP yields, this pathway provides the cell some advantages. In this way, cancer cells can produce lactic acid and also can get energy rapidly by making anaerobic respiration. This lactic acid production can give damage neighboring cells and increase competition for glucose uptake. Also it can eliminate oxygen dependent energy generation as they can produce ATP without oxygen. As a result, cancer cells transform the glycolysis end product, from pyruvate, into lactic acid.[49]

1.5. Genetically encoded biosensors and chemogenetic tools

Real-time traces of signaling and metabolite rate within subcellular levels can be imaged easily by the help of genetically encoded biosensors. Genetically encoded biosensors based on fluorescent proteins (FPs) are useful for localization and dynamics of a molecule which is important at the cellular level. It is also important to design an optical sensor that can monitor targeted molecules by applying the best sensing strategy. Genetically encoded biosensors based on FPs have various sensing strategies, for example, translocation, fluorescence resonance energy transfer (FRET), pH sensitivity.

To visualize Ca^{2+} in living cells by fluorescence signals, we have used genetically encoded fluorescent Ca $^{[2+]}$ as an indicator, which is called R-GECO1. It consists of a calmodulin-binding peptide, a circularly permuted red fluorescent protein and calmodulin(CaM).

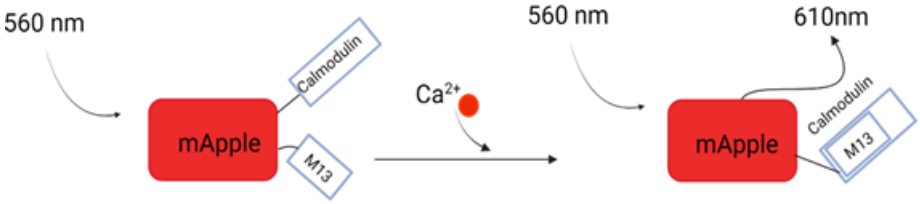


Figure1.6: Conformational change upon Ca^{2+} administration in R-GECOs.

In the case of calcium in the environment, CaM and its peptide M13 undergoes a conformational change. These changes, especially at the interfacial region, where CaM interacts with cpRFP, leads to increased RFP brightness (Figure 1.6).

For real-time glucose imaging, we employed a FRET glucose sensor named FlP-Glu. Molecular interaction can be easily studied by using the tool called FRET. FRET-based biosensors are typically made up of two fluorescent probes (donor and acceptor) coupled to a central metabolite binding protein (BP). This method allow us to investigate protein-protein interactions as a ligand binding to its receptor.

For FRET to occur, two florescent tagged protein are needed with different fluorophore and emission wavelengths where one should overlap with excitation wavelengths of the other ones. The space of interacting molecules must be fewer than 10 nm. When there is a metabolite of interest in the environment, FRET occurs between the two probes. Here, excitation of the donor transfers energy to the acceptor. Due to the conformational changes of the binding protein, the FRET efficiency is either increased or decreased depending on the level of the protein of interest.[50,51]

In the Figure1.7 excitation and absorption spectra of two fluorophores CFP and YFP is shown. Green regions shows emission spectra of CFP (blue peak) with excitation spectra of YFP (green peak). CFP acts as the donor and YFP as the acceptor. When the two fluorophores are move away from each other, there is no protein of interest in the environment, exposing the sample to light with the excitation of CFP results in no contribution to YFP (Figure1.7,right top). When the two fluorophores are near, there is protein of interest in the environment, exposing the sample to the same light results in a energy transfer from CFP to YFP acceptor, causing YFP to emit at its emission wavelength. (Figure1.7,right bottom). [52]

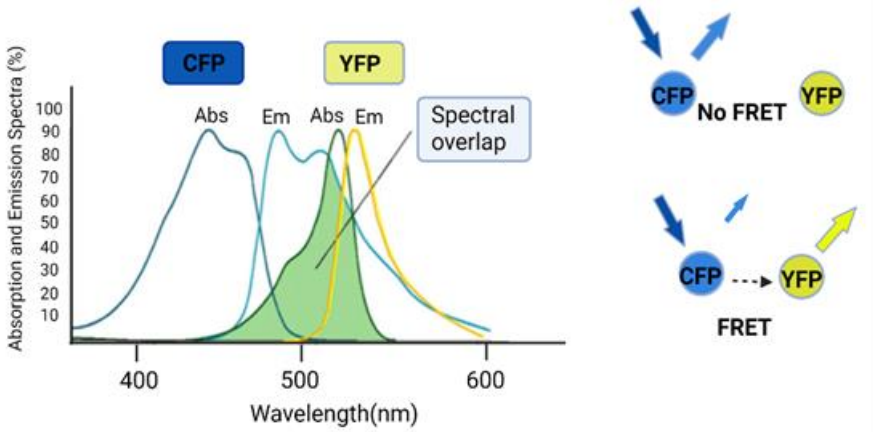


Figure 1.7: Spectral Profiles of CFP and YFP

mDAAO as Chemogenetic Tool

Chemogenetics refers to a tool that powerfully controls the regulation of recombinant proteins with giving or suppressing special biochemical substrates of the protein. It enables critical control of particular signal particles for investigating parts of cell signaling. Improvements in biosensor chemogenetic tools improves the ability to measure signaling in the cell. The use of the biosensor with chemogenetic tools allows the researching of intracellular molecules that take part in critical physiological processes. One of these tools, DAAO, provides a quantifiable and tissue-specific measure of H_2O_2. D-amino acid oxidase (DAAO) is a FAD-dependent enzyme that catalyzes the oxidative deamination of D-amino acids and it produces individual α-keto acids and ammonia. And as a result of this event, H_2O_2 is generated as a by-product (Figure 1.9). As they are genetically encodable, chemogenetic enzymes can be targeted for different subcellular regions as demonstrated in previous studies.[53] In this study, we used mDAAO to selectively produce H_2O_2 in the cytosol, nucleus, and mitochondria of HeLa cells. In addition, we combined mDAAO with various genetic biosensors so we could study energy metabolism under oxidative stress.

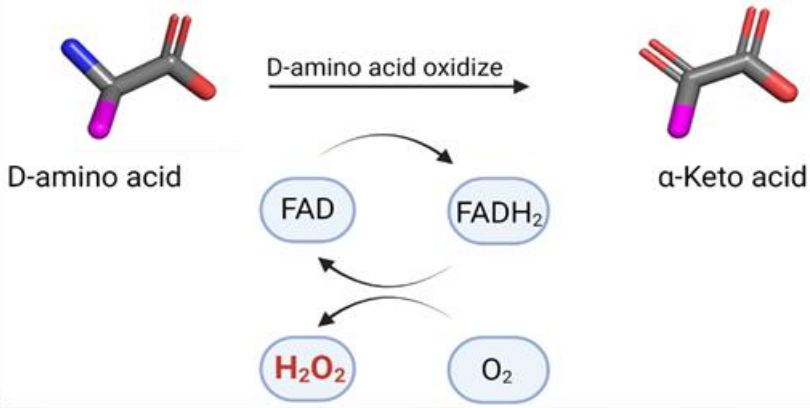


Figure 1.8 DAAO mediated H2O2 generation. D-amino acid (D-alanine), the substrate for DAAO, gets oxidized to imino acid (not shown), which then goes through concomitant hydrolysis to ammonia and corresponding α -keto acid. During this catalysis, FAD gets reduced to FADH2. By utilizing the molecular oxygen, FADH2 is oxidized back to FAD, accompanied by H2O2 formation.

2.AIM OF STUDY

My aim was to generate chemogenetically ROS levels in the mitochondria of cancer cells and simultaneously visualize the downstream effects of H_2O_2 levels on Ca^{2+} signaling pathways and energy metabolism using genetically encoded biosensors employing multiparametric and multispectral imaging approaches.

Thus, the sub aims of this study are as follow:

1. Investigate the role of H_2O_2 and its role on Ca^{2+} signaling
2. Link mitochondrial H_2O_2 with ATP generation in these organelles
3. Visualize glucose uptake in cancer cells upon oxidative stress
4. Probe the role of H_2O_2 and the mitochondrial membrane potential

3. MATERIALS

3.1 Chemicals

Chemicals used during this thesis study are tabulated in **Appendix A.**

3.2 Equipment

Equipment used during this thesis study is tabulated in **Appendix B.**

4.METHODS

Cell culture

HEK231 and HeLa cells were used in this study. Cell were grown in 1% penicillin/streptomycin (complete medium). Cells were incubated in 5% CO_2 at 37°C and were passaged when reach approximately 90% confluency in 10cm cell culture plates. For the passaging process, firstly, cells washed with Dulbecco's phosphate-buffered saline (D-PBS) and incubated in 1 ml tyripsin with 5% CO_2 at 37°C for 5 min. Then 9 ml DMEM was added into the plate. 300.000 cells were seeded that glass placed 1x6 well plate and sub-cultured with either 1:5 or 1:10, then cells were seeded into the new sterile 10 cm cell culture plates.

Transient Transfection

After 24 h incubation, cells were transfected transiently. To do that, 1 ng DNA and 2uL polyget were used for each well. For 1x6 well, 300 uL colorless DMEM was added into a eppendorf tube and 6 ng DNA added in that eppendorf tube. 300 uL colorless DMEM was added in a another eppendorf tube and 12 uL polyget was added into the these eppendorf. These two eppendorf tube were mixed and incubated for 15 min. After incubation, 100 ul DMEM from these mixture was added into the each well. After 3 hour incubation in 5% CO_2 at 37°C, media was changed. Cells were incubated within new media for overnight.

MTT assay

Cell viability was assessed using the MTT assay. 10.000 cells were seeded in 96-well plates and the cells were treated 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, 30 mM,
100 mM D-alanine for 24 hours. After incubation, 10 µL MTT(Sigma) was added into the each well for 4 hours. Then 50 µL DMSO was added to each well for 10 min and incubated 5% CO_2 at 37°C. The absorbance measured at 540nm by spectrophotometer.

TMRM Staining

Prior to experiment, the cells were stained 10 nM tetramethylrhodamine ethyl ester (TMRM; ThermoFisher) for 30 minutes. After 30 min, cells were washed once w DPBS. 5 µM Antimycine/Oligomycin was used as positive control for mitochondrial depolarization.

Imaging Buffers

Before the experiment, cells were incubated in EHL that included: 2 mM $CaCl_2$, 135 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1% of MEM of amino acid and vitamins. Before the incubation, pH of the EHL were adjusted to 7.4.
Buffer that is used in the experiment which is called 2CaNa includes: 10 mM HEPES, 2 mM $CaCl_2$, 138 mM NaCl, 1 mM $MgCl_2$, 10 mM D-Glucose, 5 mM KCl and its pH was adjusted to 7.4.

Statistical Analysis

MS Excel 2011 or with Graphpad Prism version 5.0 were used for analyze the result. The two-tailed Student t-test was applied to compare two groups. One-way ANOVA and Benferroni post test were applied to compare multiple groups and ($p<0.05$) were considered statistically significant.

5.RESULTS

Visualizing the relationship of intracellular Ca^{2+} and H_2O_2 signals

We first investigated intracellular Ca^{2+} profiles in different cell locales including the cell cytosol, mitochondria, and nucleus. For this purpose we employed the well-established Ca^{2+} indicator GECO, which stands for genetically encoded optical calcium indicators. Out of a range of differently colored GECOs, we choose the red-shifted R-GECO, which has the advantage for multichromatic dual-imaging with other spectrally distinct and compatible biosensors. For this purpose we utilized HeLa cells and transfected this easy-to-transfect cells with the mitochondria-, nucleus-, and cytosolic-targeted R-GECOs. This approach yielded a high number of positively transfected Hela cells imaged on a high-resolution wide-field microscope (Figure 5.1A). For intracellular Ca^{2+} mobilization we used the G-protein coupled receptor (GPCR) agonist adenosine triphosphate (ATP). As expected, cell treatment with ATP instantly caused a robust R-GECO signal in all three cell compartments including in the cytosol, mitochondria and nucleus (Figure 5.1B). Notably, the Ca^{2+} signal profiles in the cytosol and mitochondria were comparable yet the signal amplitude was significantly lower in the mitochondria indicating a lower Ca^{2+} entry into this organelle. However, as the GECOs are intensiometric biosensors the amplitude is not reliably reporting Ca^{2+} quantity. In contrast the nuclear Ca^{2+} profiles appeared to be flatter compared to the cytosol and mitochondria. Overall, these observations indicate that GECOs are suitable biosensors to visualize intracellular Ca^{2+} signals in single cancer cells and subcellular locales.

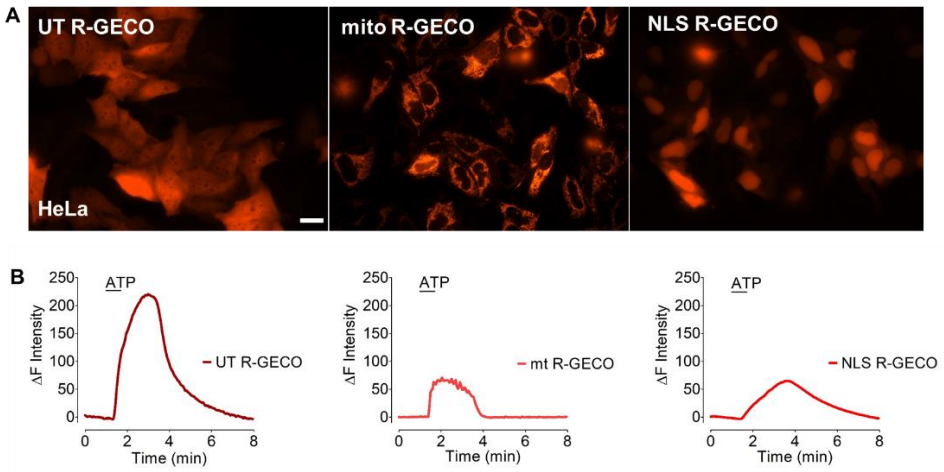


Figure 5.1. Real-time imaging of Ca^{2+} signals in HeLa cells using GECOs in response to ATP. (A) Representative widefield images HeLa cells transfected with nucleus-localized R-GECO1, cytoplasmic R-GECO1, and mitochondria-localized R-GECO1 as indicated in the wide field images. Scale bar represents 20 μm.(B)Representative real-time traces of Ca^{2+} signals in the mitochondria (left panel) ,nucleus (middle panel) and cytosol (right panel) , in the HeLa cells.

We next tested whether exogenous H_2O_2 administration affects intracellular Ca^{2+} levels in different cell locales. For this purpose we applied co-imaging experiments in HeLa cells that were co-expressing R-GECOs with the H_2O_2 biosensor HyPer7 differentially targeted to the mitochondria, cytosol or nucleus. Alternately exciting the R-GECO and HyPer biosensors we recorded in two channels, measuring the change of fluorescence over time in HeLa cells upon treatment with ATP. Cell treatment with this agonist evoked a robust intracellular Ca^{2+} increase in all three compartments (Figure 5.2 A, B). Yet HyPer7 signals remained in all three compartments unresponsive, indicating that intracellular Ca^{2+} signals do not affect H_2O_2 generation in these cell types. Subsequent administration of low levels of exogenous H_2O_2 caused and instant and robust HyPer7 signal indicating the functionality of the biosensor. Overall, our observations indicate that in Hela cells acute intracellular Ca^{2+} mobilization is not sufficient to trigger H_2O_2 generation.

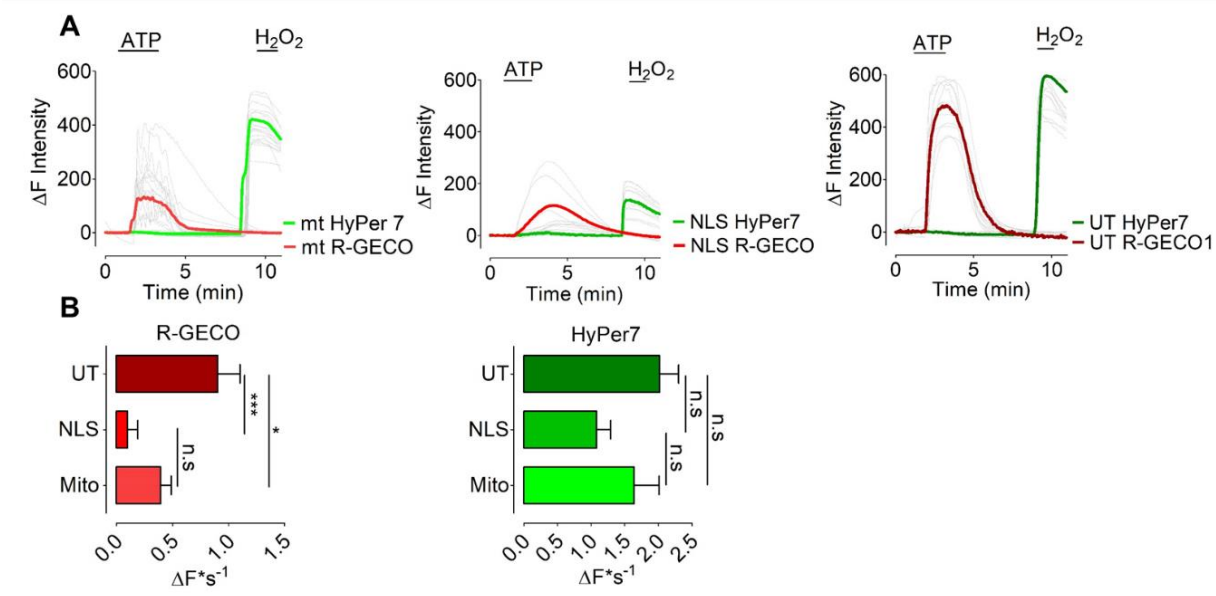
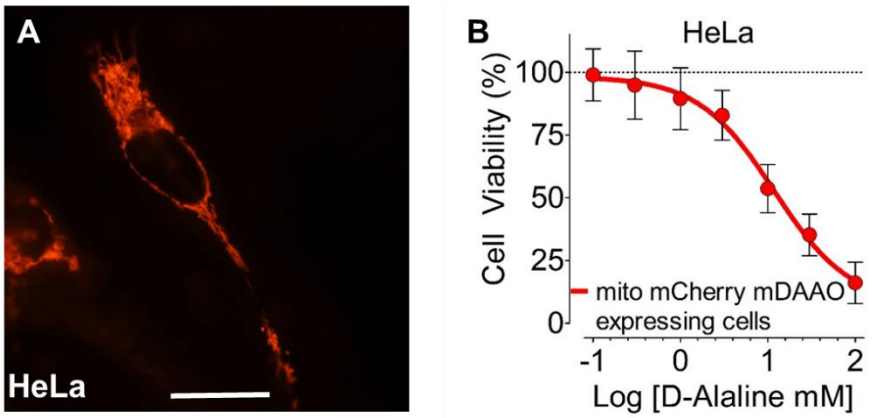


Figure 5.2. ATP treatment did not cause H_2O_2 rise in cytosol, mitochondria and nucleus. (A) HeLa cells transfected with mitochondria-localized R-GECO1, HyPer7.1, nucleus-localized R-GECO1, HyPer7 and cytoplasmic R-GECO1, HyPer7.Average curve (n=20 cells) of real-time HyPer traces shows results cytosol(left), mitochondria(middle) and nucleus(right) that stimulated subsequently with 100 µM ATP and 50 µM H_2O_2 (B) Kinetic changes [H_2O_2] out during 60 s in the presence of H_2O_2 (right panel) and [Ca^{2+}] out during 120s in the presence of ATP (left panel).

Implications of chemogenetic generation of H_2O_2 on intracellular Ca^{2+} signals

As shown in Figure 5.2, exogenous administration of low concentrations of H_2O_2 instantly evoked a robust signal in any cellular compartment indicating that H_2O_2 can readily diffuse into all cell compartments. These observations also demonstrate that exogenous H_2O_2 is less suitable to control local H_2O_2 levels. Methods that permit the generation of H_2O_2 with high spatial and temporal resolution might be more informative in comparison to the exogenous administration of physiologically irrelevant levels of H_2O_2. Thus, we employed a novel chemogenetic tool which we recently developed in our laboratory to generate H_2O_2 in cellular compartments such as the nucleus, cytosol or mitochondria. To selectively generate H_2O_2 in the mitochondria and we first generated HeLa cells stably expressing mCherry-mDAAO in mitochondria (Figure 5.3A). Exploiting these cells we tested the toxic doses of D-alanine employing an MTT assay for cell viability and proliferation in response to different ROS doses. For this purposes we incubated HeLa cells for 24h with increasing concentrations of D-Alanine ranging from 0.1mM, 0.3mM, 1mM, 3mM, 10mM, 30mM, 100mM in the mitochondria. As

shown in Figure 5.3B cell death significantly occurred at 10 mM D-alanine treatment after 24 hours incubation. Therefore we used 10 mM D-alanine treatment for further experiments in order to achieve a robust H_2O_2 challenge in the mitochondria.



Figures 5.3. HeLa cells show distinct decreases in cell viability at mitochondria in response to increasing D-Alanine concentrations. (A)Representative widefield image show HeLa cell stably-expressing mCherry-mDAAO-mito in RFP channel. Scale bar represents 20 μm. (B) Traces represent the percentage of viable cells, with respect to control cells (Ctrl, 100%), after treated with: 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, 30 mM, 100 mM D-alanine for 24 h in HeLa cells stably expressing mCherry-mDAAO-mito. Results shown are average of three different experiments.

Next we sought to co-image H_2O_2 and Ca^{2+} signals in response to chemogenetically induced ROS levels. However, due to the spectral overlap, we utilized a mDAAO chimera that was fused to a blue fluorescent protein (EBFP-mDAAO-mito). Co-expression of this chimera with the HyPer7 biosensor yielded a robust expression levels and correct targeting(Figure5.4A,B). Administration of 10 mM D-alanine to cells instantly evoked a robust HyPer7 signal demonstrating that we can selectively and robustly generate H_2O_2 in the mitochondria of Hela cells (Figure5.4C).

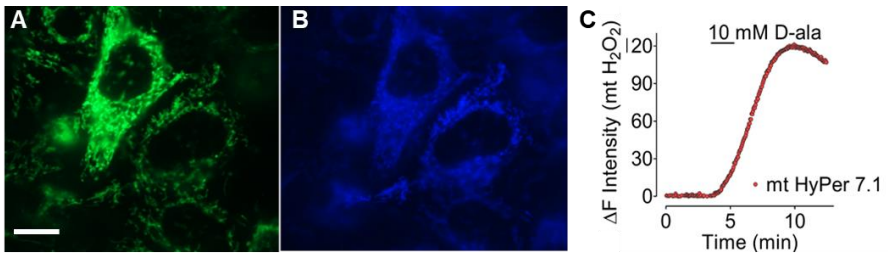


Figure 5.4.Functionality test of mDAAO with Hyper7. Hela cells transfected with (A) mt-HyPer7 and (B) EBFP-mDAAO- mito, then treated with (C) 10 mM D-ala at indicated time point. Scale bar represents 20 μm.

We next tested how chemogenetically produced H_2O_2 affects mitochondrial Ca^{2+} signaling. For this purpose we applied multispectral co-imaging experiments. We again used HeLa cells co-expressing the mitochondria targeted EBFP-mDAAO, the

mitochondria localized R-GECO1, and the mitochondria targeted mito-HyPer7 (Figure5.5A). We again used ATP as an agonist to induce intracellular Ca^{2+} mobilization. In control experiments cell treatment with ATP triggered an instant and robust increase in mitochondrial Ca^{2+} levels, as expected (Figure 5.5B). To generate instant H_2O_2 elevations in the mitochondria we used D-Phenylalanine as a substrate as this substrate causes a significantly faster ROS increase in cells compared to D-alanine. As shown in Figure 5.5C, cell treatment with D-phenylalanine caused an instant H_2O_2 accumulation in the mitochondria, however, co-imaging of GECO signals unveiled that this treatment did not affect intracellular Ca^{2+} signals. Overall, in contrast to several published articles, our observations indicate that intracellular Ca^{2+} does not affect ROS generation and intracellular H_2O_2 generation does not affect Ca^{2+} signals in response to acute ROS changes.[54] These findings raise the question about the role of the relationship between acute oxidative stress and mitochondrial Ca^{2+} signaling in cancer cells.

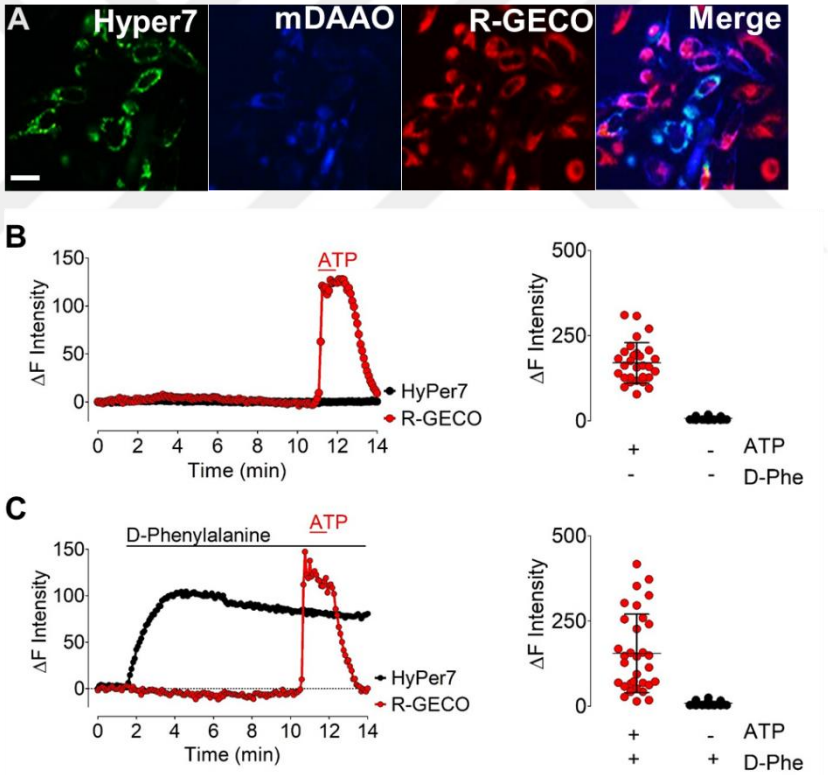


Figure 5.5. Real-time imaging of Ca^{2+} signals in HeLa cells using GECOs in response to ATP acute oxidative stress. (A) HeLa cells transfected with mito-localized R-GECO1, BFP- mDAAO -mito and mito-HyPer7. Scale bar represents 20 µm. **(B)**Real-time traces of Ca^{2+} signals in the mitochondria stimulated with 100 µM ATP(left panel) and Scatter dot blot(left panel) shows the distribution of maximum responses of HeLa cells upon administration of ATP. Red dots show mitochondrial Ca^{2+} responses, black dots represent mitochondrial HyPer7 signals **(C)** Ca^{2+} signal in the mitochondria of HeLa cell stimulated with 100 µM ATP, under pretreatment of 10mM D-Phenylalanine (left panel) and Scatter dot blot (left panel) shows the distribution of maximum responses of HeLa cells upon administration of ATP. Red dots show mitochondrial Ca^{2+} responses, black dots represent mitochondrial Hyper7.1 signals.

Manipulating the mitochondrial membrane potential in cancer and non-cancer cells using chemogenetic tools

Mitochondrial health can be evaluated by checking changes in mitochondrial membrane potential (MMP). Cationic fluorescent dyes are commonly used tools to assess MMP. TMRM is a well-established dye which accumulates in the negatively charged mitochondria. The schematic in Figure 5.6A shows the working mechanism of the TMRM sensor, which has been used in this study to monitor changing mitochondrial membrane depolarization upon chemogenetically induced mitochondrial H_2O_2 generation. It has been reported that the mitochondrial membrane potential is sensitive to H_2O_2 and can be depolarized via oxidative stress further causing mitochondrial Ca^{2+} release and apoptosis (Figure 5.6B). First, to test the functionality of the probe TMRM, we loaded HeLa cells and applied a cocktail of the complex III inhibitor Antimycin A and the complex V inhibitor Oligomycin to HeLa cells loaded with TMRM. As shown in Figure 5.6C, the mitochondrial membrane potential instantly collapsed from this cell treatment indicating the functionality of our experimental setup.

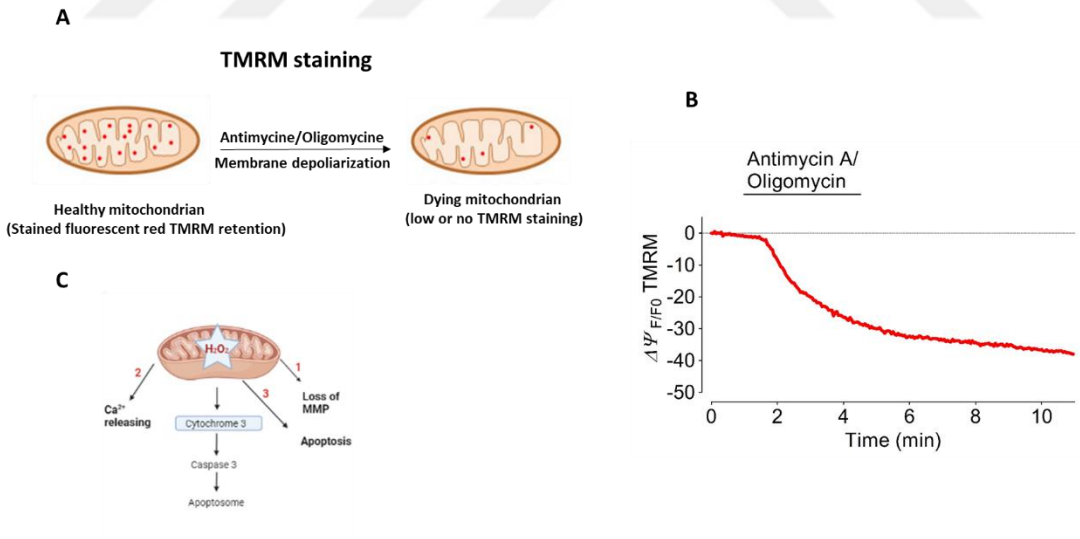


Figure5.6: (A) TMRM, Tetramethylrhodamine, is a cationic,cell permenant dye that accumulates in negatively charged mitochondria and it quantifies changes in mitochondrial membrane potential of only live cells **(B)** Representative real-time traces of TMRM dyed HeLa cells in response to mixture of 5 uM antimycin/oligomycin. **(C)** H_2O_2 depolarizes mitochondrial membrane potential and result in apoptosis.

We next sought to compare the effects of Antimycin/Oligomycin induced mitochondrial membrane potential collapse with endogenously produced H_2O_2, using our chemogenetic approaches. Thus, we next tested the stability of the mitochondrial membrane potential

in two different cell lines: HEK293 and HeLa, as a model system for non-cancer cells and cancer cells, respectively. We again applied the same protocol as shown in Figure 5.3 using 10 mM D-amino acids in Hela cells expressing the mitochondria targeted EBFP-mDAAO loaded with TMRM. Cell treatment with the D-amino acid instantly caused a robust collapse in the mitochondrial membrane potential within minutes indicating the redox sensitivity of mitochondria in HeLa cells (Figure 5.7). Mostly to our surprise, the same treatment in HEK293 cells did not affect the membrane potential of mitochondria. Overall, these findings rise important questions about the mitochondrial integrity in response to oxidative stress between cancer and non-cancer cells and makes the mitochondria a promising anticancer target for antioxidants and oxidant-based therapies.

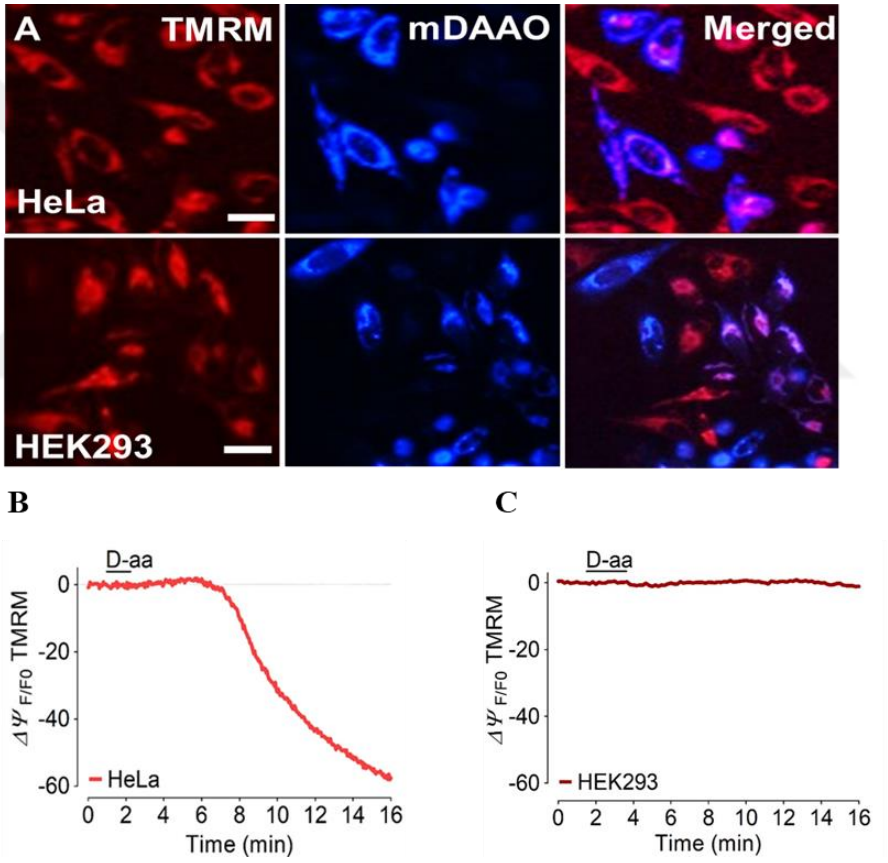


Figure 5.7. Effects of chemogeneticacally produced mitochondrial H_2O_2 on mitochondrial membrane potential. Representative widefield images HeLa and HEK293 cells that were expressing EBFP-mDAAO-mito and stained with TMRM dye. Scale bar represents 20 µm for HeLa and . Representative real-time traces of TMRM dyed (B) HeLa and (C) HEK cells in response to 10 mM D-aminoacid. All experiments have been repeated at least 4 times and similar results were obtained with individual experiments.

Relationship between oxidative stress and glucose uptake in (cancer) cells

Cellular glucose uptake is known to be affected by many stressors including H_2O_2. The schematic in Figure 5.8A shows effect of H_2O_2 and the GLUTs stimulation. Uptake of glucose regulated by H_2O_2 either by change in expression level transporter of glucose with activating HIF1 or by enhancing the translocation of GLUTs from intracellular vesicles to the plasma membrane. To test this hypothesis whether or not cancer cells are responsive to oxidative stress in terms of glucose uptake, we utilized a cytosolic targeted and FRET-based glucose biosensor termed FLII^{12}Pglu-700μδ6. To tackle this issue, we established a standard protocol for real-time imaging of glucose uptake. As shown in Figure5.8B intracellular glucose levels instantly dropped in response to glucose withdrawal from the imaging media using a gravity-based super-fusion system. Re-addition of glucose instantly recovered intracellular glucose levels. For further investigations this approach was used as a standard protocol.

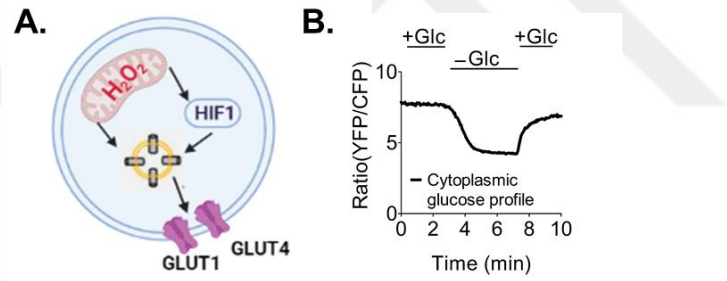


Figure 5.8. (A)Schematic shows how mitochondria sourced H_2O_2 affect glucose uptake through HIF1 activation and GLUT expression. (B) Standard protocol using the FRET-based ratiometric genetically encoded glucose sensor, *FLIPglu* for real-time imaging of glucose levels in HeLa cells.

In order to investigate how chemogenetically produced H_2O_2 affects the glucose homeostasis we again used HeLa cells that were stably expressing mCherry-mDAAO-mito (Figure5.9A) or mCherry-mDAAO-NES (Figure 5.9B) and were transiently transfected with FLII^{12}Pglu-700μδ6. Figure5.9 C, D shows the changes in glucose levels upon glucose removal and the qualitative comparison of basal glucose levels of H_2O_2 challenged cells that were treated with either no D-Alanine, 10 mM D-alanine or 30 mM D-alanine for 24 hours. This experiment document that chemogenetic H_2O_2 generation in the mitochondrial matrix and cytosol did not cause any significant alterations in glucose levels, neither under basal or starvation conditions.
(Figure 5.9C-D, right panels)

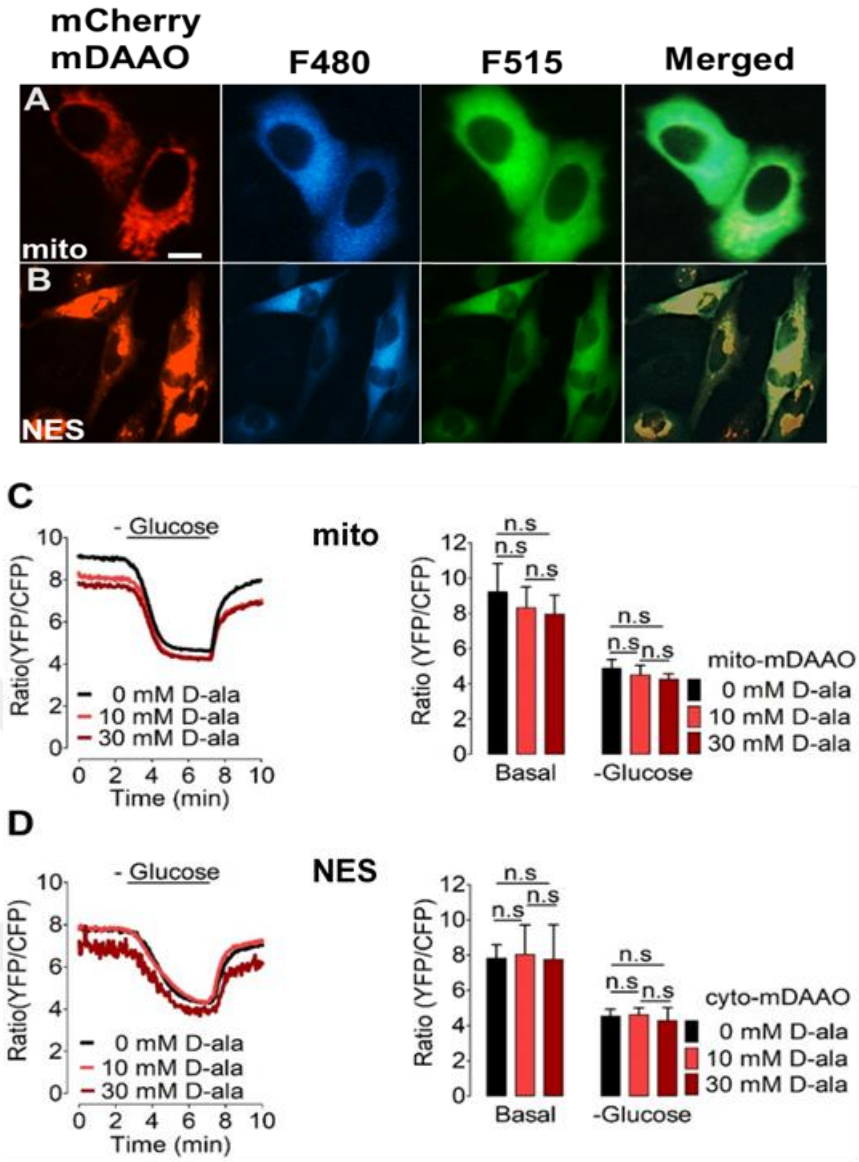


Figure 5.9. Real time changes of glucose levels upon glucose removal in HeLa cells that were differentially H_2O_2 challenged at cytosol or mitochondria. Representative widefield images HeLa cells that were stably expressing mCherry-mDAAO-mito (A) or mCherry-mDAAO-NES (B) and were transiently transfected with *FLIPGlu* as indicated in the wide field images. The changes in glucose levels of these cells upon glucose removal has been shown in mito (C) and NES (D). Glucose levels basal and under starvation condition were analyzed (C-D left). Scale bar represents 20 µm. One way Annova test was performed and post analyzed using bonferroni test to compare each column with another.

Visualizing and manipulating mitochondrial ATP levels under normal and oxidative stress conditions

Following the results from the previous experiments, which demonstrated that H_2O_2 challenged cells remained unaffected upon glucose starvation. Thus, we next sought to visualize mitochondrial ATP levels in HeLa cells under the same experimental conditions as shown in Figure5.9. For imaging of mitochondrial ATP we utilized the novel ATP

biosensor termed Malion (Figure5.10A). Malions are single FP-based intensiometric biosensors that are either GFP- or RFP-based. In this study we utilized the green-shifted MalionG. HeLa cells that stably expressing mCherry mDAAO transfected with mito MalionG and were treated with 30 mM D-ala for 24 h. Cells were again subjected to glucose starvation as shown in Figure 5.10B. Control cells displayed ATP profiles as expected and dropped upon glucose starvation instantly and recovered in response to glucose re-administration. However, cells that have been exposed to 30mM D-Alanine for 24 hours, showed clearly different ATP profiles to the same cell treatment. Single cell traces show that some cells even increased there ATP levels in response to glucose starvation. The drop in ATP levels in the majority of cells were clearly delayed in cells under oxidative stress compared to regular cells. Overall, this experiment might indicate a metabolic transition in cancer cells and their energy metabolism upon oxidative stress.

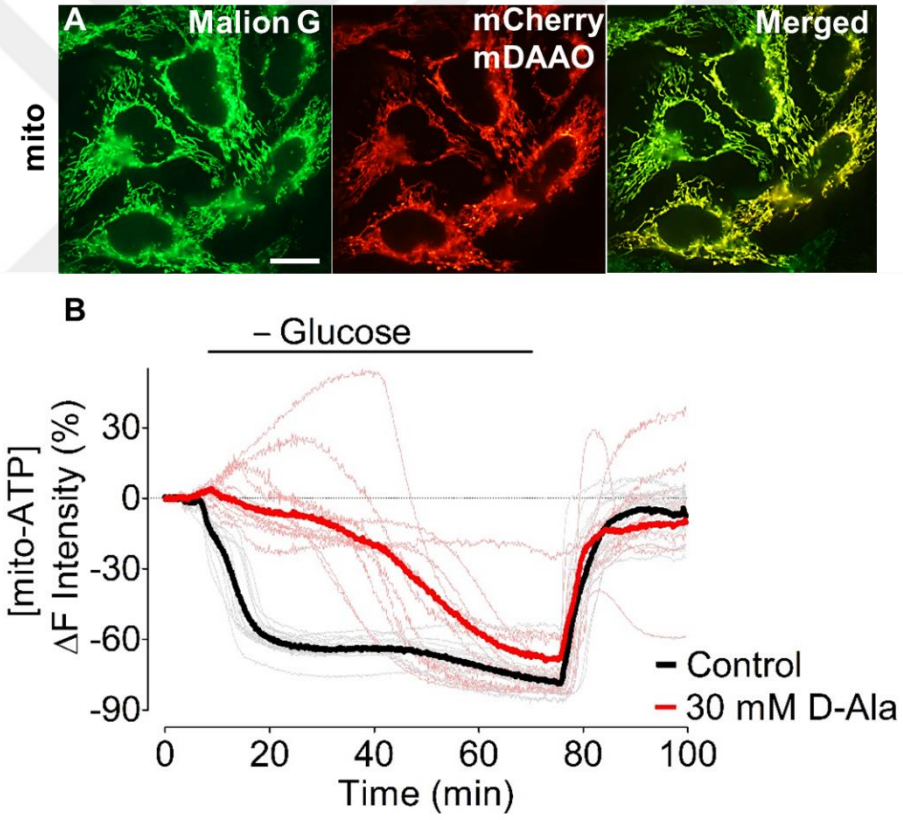


Figure 5.10. Mitochondrial ATP changes upon glucose deprivation after chronic oxidative stress. Representative widefield images show HeLa cells (A) mito-mCherry-mDAAO (stable expression) in RFP channel, (B) MalionG (transient expression) in GFP channel and (C) the overlayed image of two channels. (D)At the indicated time points, glucose was removed from the imaging media. Mean curves shows mitochondrial ATP levels of these cells either non-treated. Scale bar represents 20 μm.

DISCUSSION

In this work, we have investigated the effects of chemogenetically produced H_2O_2 on the mitochondria in terms of Ca^{2+}, mitochondrial membrane potential and energy metabolism.

H_2O_2 and Ca^{2+} are important signaling messengers in the cell. H_2O_2-depended Ca^{2+} signaling are essential signals for many biological responses, including bioenergetic stress, apoptosis and activation many type of protein kinases. However, it is still not clear how these two compounds can crosstalk within each other in signaling pathways.

Calcium can act directly on the activation of signal transduction. To prevent inappropriate signaling, it should always kept under the control in the cytosol. To maintain this low concentration, Ca^{2+} is actively pumped from the cytosol to the ER and the mitochondria.

In Figure5.1, we have observed that cells show different Ca^{2+} signals at different locales in response to ATP, which is the agonist of Ca^{2+} in the cell. Cytoplasm shows the most abundant amplitude of Ca^{2+} compared to mitochondria and NLS. Ca^{2+} amplitude in the cytoplasm can be high due to Ca^{2+} release from the mitochondrial matrix and / or ER.

We next wanted to understand relationship of Ca^{2+} and exogenously and chemogenetically introduced H_2O_2 in Figure5.2 and Figure 5.5.

In the co-imaging experiment at Figure 5.2, we documented that ATP treatment did not cause any changes in H_2O_2 levels in cytosol, mitochondria or nucleus, while Ca^{2+} levels increased at each locale with the highest amplitude being in the cytosol. We have also observed that extracellular H_2O_2 provision did not alter the intracellular Ca^{2+} profiles at mitochondria, nucleus and cytosol. Therefore, our observations can be concluded as there is no acute interplay between Ca^{2+} release and H_2O_2.

Then, we wanted to see the effect of mitochondria sourced chemogenetic H_2O_2 generation on the Ca^{2+} levels. At higher doses, H_2O_2 is known to act as a cytotoxic

agent. Recently, we have published an article, which shows thorough characterization of mDAAO as a chemogenetic tool to induce intracellular H_2O_2 generation at different subcellular locales. In this study by Erdogan et. al, it has been observed that mDAAO can generate H_2O_2 in a concentration dependent manner.[53] In order to determine the toxic doses of D-alanine for the chemogenetic studies, we used a cell toxicity assay called MTT. We have incubated HeLa cells for 24h with increasing concentrations of D-Alanine starting from 1mM to 100 mM as can be seen in Figure5.3. As the cell death increases after 10 mM, we decided to use 10 mM D-Alanine for further experiments in order to achieve a robust H_2O_2 challenge.

In Figure5.5, we have tested endogenous effect of H_2O_2 in intracellular Ca^{2+} levels. In order to induce a robust chemogenetic H_2O_2 generation, we used D-Phenylalanine, which is reported to show faster H_2O_2 generation compared to D-alanine. The effect of endogenous H_2O_2 was analogous to that of exogenous H_2O_2 elevations in Figure 5.2, that is, they both do not affect intracellular Ca^{2+} dynamics. Taken together, these findings indicate that the acute rise of exogenously or endogenously produced H_2O_2 did not affect intracellular Ca^{2+} levels.

It is known that membrane of mitochondria serve an essential role in keeping up cellular calcium levels inside the cell. Moreover, mitochondrial membrane potential is really vulnerable to H_2O_2 as ROS can depolarize this membrane. So far, we have not observed any alterations in Ca^{2+} levels when H_2O_2 levels were increased either endogenously or exogenously. Therefore, we were interested to see whether chemogenetic H_2O_2 production in mitochondria would affect mitochondrial membrane potential or not. Although intracellular Ca^{2+} levels were not affected by H_2O_2, there was a similar induction of H_2O_2 that depolarized the mitochondrial membrane. This was an interesting outcome as mitochondrial membrane potential is known to be tightly related to intracellular calcium levels. But we observed very interesting effects of chemogenetacilly produced mitochondrial H_2O_2 on mitochondrial membrane potential. The chemogenetically produced H_2O_2 directly diminished membrane potential even though it did not altered Ca^{2+} levels with the same amount of D-Phenylalanine (Figure5.6). These processes are meticulously regulated because calcium acts as a trigger of regulatory enzyme that affect the mitochondria and the rest of the cell and, in

consequence, metabolism, migration and cell survival. As this was a very interesting finding and we wanted further investigate how H_2O_2 can dramatically affect membrane potential of HeLa cells yet there was no significant alteration in the Ca^{2+} levels.

We have decided to try effects of H_2O_2 on mitochondrial membrane potential in a non-cancer cells such as HEK cells. We stimulated membrane potential again with chemogenetically produced mitochondrial H_2O_2 and we observed no decrease in acute treatment. Here, we thought that cancer cells may have damaged mitochondria and this may make them more vulnerable to H_2O_2 than mitochondria of normal cells. In 1920s biologist Otto Warburg has explained this phenomena that cancer cells make anaerobic phosphorylation despite presence of oxygen, since they have damaged mitochondria.[55] Based on the current literature, there are lots of ideas that have explained this phenomenon. These include defect in mitochondria, mutation in mitochondrial DNA, and damage in mitochondrial oxidative phosphorylation enzymes which may cause inhibition of oxidative phosphorylation even under the presence of oxygen.[56–58] All together with such observations it is thought that mitochondria of cancer are more vulnerable to the environment in comparison to a normal cell's mitochondria. Within observations of Figure 5.7, we also can conclude that acutely produced H_2O_2 diminished mitochondrial membrane potential in only mitochondria of cancer cells.

Glucose is an integral player of most of the energy production pathways, we wanted to check how endogenously produced H_2O_2 will affect glucose levels. In Figure 5.9, we observed that there is no significant change in their glucose level in 24 h H_2O_2 challenged cells. As glucose levels are strongly linked with intracellular ATP levels, we wanted to check how ATP levels in H_2O_2 challenged cells will change.

In Figure 5.10, interestingly, we observed slower decrease upon glucose starvation in 24h H_2O_2 challenged cell. In this experiment, we observed significant morphologic changes in their mitochondria. We have hypothesized that this change may somehow cause activation of the TCA cycle by H_2O_2 , make their mitochondria usable and because of that these cells may continue generating ATP when there is no glucose in the environment. In addition to continue generation of ATP in absence of glucose, they can adapt and change their energy source by activating metabolic signaling pathways, including the glutamine uptake

pathway.[59] [60] Under glucose-starvation conditions, cells, which are able to use TCA cycle can metabolize glutamine. Glutamine can be metabolized as an energy source and cells can continue generating ATP for a while. When we consider cancer cell metabolism, we can say that cancer cells compete to reach the available glucose. Many cancer cells make vascularization and rapid glucose uptake so they have to develop a strategy to uptake energy and survive. Shifting from oxidative phosphorylation to anaerobic phosphorylation, metabolizing another energy source under glucose-starvation condition, and having the flexibility of which way is more beneficial, even in non-starvation conditions, are the vital adaptations of the cancer cells to survive under unfavorable conditions. [9,55–58,60–62]

We have concluded that; H_2O_2 may activate mitochondrial oxidative phosphorylation through an unidentified pathway. Moreover, when there is no glucose in the environment, cells may be able to use another energy source that can metabolize in the TCA cycle. This experiment might indicate a metabolic transition in cancer cells and their energy metabolism upon oxidative stress.

In Figure 5.10, cell culture media that we used contains glucose and glutamine, thus, the media may influence decision of energy metabolism. Even if there is no energy source to use in starvation experiments, cells may have stored glutamine in their cytoplasm, coming from media that they had been grown in.

This experiment showed us how mDAAO driven H_2O_2 may activate oxidative phosphorylation in a way that allows HeLa cells to metabolize micronutrients that they normally cannot use.

In Figure5.10, the increase in ATP levels that starts with the removal of glucose in H_2O_2 challenged cells may imply these possible scenario:

These HeLa cells might utilize glutamine, fatty acid or do micropinocytosis for other available nutrients in their microenvironment to metabolize them in TCA cycle. If chemogenetic mitochondrial H_2O_2 generation led to an activation of some pathways, such as the growth signaling PI_3K, cancer cells then may promote more cellular growth in an uncontrolled manner. It can be suggested that metabolizing fuels in the TCA cycle is easier in the H_2O_2 challenged cells. It may be because of too much stress that comes from H_2O_2 incubation and under such a stress condition cell may be forced to use the TCA cycle to survive. Nevertheless, when glucose was removed, there was nothing to make ATP generation other than these metabolites and cell may start to use these metabolites to make ATP. At that

point, cells have to make oxidative phosphorylation because these metabolites such as glutamine, fatty acid can only be utilized in TCA cycle to make ATP. This is the reason why we see higher increase when there is no glucose and why they continue to generate ATP at a higher basal level. When we compare with anaerobic respiration, during this time, they use oxidative phosphorylation and they generate 18 times more ATP.

In order to investigate this scenario, to test preference in ATP generation using either oxidative phosphorylation or anaerobic phosphorylation, we can use genetically encoded fluorescent biosensors for metabolic chemical screening of OXPHOS such as NAD^+ /NADH ratio. SoNar is a biosensor that detects changes in NAD^+ /NADH ratio.[63,64]NAD^+ and its reduced form, NADH, are the most important cofactors involved in energy metabolism. NAD^+ is oxidizing agent which involves in redox reactions, accepts electrons from one reaction to another in oxidative phosphorylation and reduced to form, NADH.[63,65] So, when ETC becomes active, we can monitor the transfer of energy from NAD^+ to NADH as it is a FRET sensor. Therefore, changes in cell metabolic status can be tracked by the help of SoNar biosensor.

Another way to perform a metabolic screening, one can perform a seahorse assay. This assay basically measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The major oxygen-consuming pathway in cells is oxidative phosphorylation and it can be easily detected by OCR, as an indicator of mitochondrial respiration. ECAR also measures glycolysis as it measures acidification of the cell as well. Therefore, the seahorse assay will provide us critical information that relates to the cell's preferred metabolic pathway.

H_2O_2-challenged cells have a different response to a limitation of glucose than normal cells; therefore, targeting parallel nutrient acquisition pathways has the potential to serve as a therapeutic strategy for the restriction of cancer cell growth. This study demonstrates that a simultaneous blocking of nutrient access pathways with mDAAO may lead to a novel approach to overcome the resistance in cancer metabolism. Thus, by elucidating some of these altered utilizations of nutrients, we may exploit this phenomenon in cancer therapy.

In the literature, cancer metabolism has been characterized to be robust numerous times[56,61,66–70]. This means that there are many ways to generate the same metabolic end product. In conventional experimental setups, inhibiting one process in metabolism can be bypassed by another alternative pathway as a compensation mechanism, which is the biggest challenge in cancer research. We need to have a better understanding of that circuit so that we can overcome aforementioned challenges. The chemogenetic enzyme, mDAAO, may serve as a tool to manipulate subcellular locale-specific H_2O_2 levels and help understand energy metabolism better upon manipulations of redox states and signaling pathways, while targeting mitochondria on the underlying metabolic vulnerabilities of that cell setting.

The other important conclusion that there is a lot of reports claiming glucose starvation induces autophagy and finally results in apoptosis of cancer cells. [7273] However, starvation of cells from glucose may not actually starve cancer cells regardless the stress state of mitochondria, although the mechanism is unclear now according to our findings. Also, this type of starvation may make (H_2O_2-challenged) tumor cells more aggressive as normal redox balance was disrupted. As we see in Figure 5.10, they can generate ATP to survive in a glucose-independent way, while their normal counterpart (non-treated) cells showed diminished ATP generation under starvation of glucose (Figure 5.10). Moreover, certain dietary interventions might improve the activity of existing therapies.

In order to better understand these result, future studies should investigate how tumor cells differ from normal cells in their metabolism of fuels. From understanding how all of these influences interact with each other, we may exploit unique metabolic properties and vulnerabilities of cancers in order to improve cancer therapy.

CONCLUSION

Our results demonstrate that utilizing chemogenetic tools paired with genetically encoded biosensors may serve as an informative approach to investigate the implications of oxidative stress in cancer energy metabolism.

REFERENCES

1. Weinhouse S. On Respiratory Impairment in Cancer Cells. *Science*. 1956;124(3215):267-269. doi:10.1126/science.124.3215.267
2. Neupane P, Bhuju S, Thapa N, Bhattarai HK. ATP Synthase: Structure, Function and Inhibition. *Biomolecular Concepts*. 2019;10(1):1-10. doi:10.1515/bmc-2019-0001
3. Turrens JF. Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*. 2003;552(2):335-344. doi:10.1113/jphysiol.2003.049478
4. Foo BJA, Eu JQ, Hirpara JL, Pervaiz S. Interplay between Mitochondrial Metabolism and Cellular Redox State Dictates Cancer Cell Survival. *Oxidative Medicine and Cellular Longevity*. 2021;2021:1-20. doi:10.1155/2021/1341604
5. Paulsen CE, Carroll KS. Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery. *Chemical Reviews*. 2013;113(7):4633-4679. doi:10.1021/cr300163e
6. Poole LB, Nelson KJ. Discovering mechanisms of signaling-mediated cysteine oxidation. *Current Opinion in Chemical Biology*. 2008;12(1):18-24. doi:10.1016/j.cbpa.2008.01.021
7. Zhu L, Lu Y, Zhang J, Hu Q. Subcellular Redox Signaling. In: ; 2017:385-398. doi:10.1007/978-3-319-63245-2_25

8. Murphy MP. How mitochondria produce reactive oxygen species. *Biochemical Journal*. 2009;417(1):1-13. doi:10.1042/BJ20081386
9. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metabolism*. 2016;23(1):27-47. doi:10.1016/j.cmet.2015.12.006
10. Schieber M, Chandel NS. ROS Function in Redox Signaling and Oxidative Stress. *Current Biology*. 2014;24(10):R453-R462. doi:10.1016/j.cub.2014.03.034
11. Finkel T. Signal transduction by reactive oxygen species. *Journal of Cell Biology*. 2011;194(1):7-15. doi:10.1083/jcb.201102095
12. Porporato PE, Filigheddu N, Pedro JMBS, Kroemer G, Galluzzi L. Mitochondrial metabolism and cancer. *Cell Research*. 2018;28(3):265-280. doi:10.1038/cr.2017.155
13. Masoud GN, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. *Acta Pharmaceutica Sinica B*. 2015;5(5):378-389. doi:10.1016/j.apsb.2015.05.007
14. Infantino V, Santarsiero A, Convertini P, Todisco S, Iacobazzi V. Cancer cell metabolism in hypoxia: Role of HIF-1 as key regulator and therapeutic target. *International Journal of Molecular Sciences*. 2021;22(11). doi:10.3390/ijms22115703
15. Ismail-Beigi F. *Topical Review Metabolic Regulation of Glucose Transport*. Vol 135.; 1993.
16. Mamun A al, Hayashi H, Yamamura A, Nayeem MJ, Sato M. Hypoxia induces the translocation of glucose transporter 1 to the plasma membrane in vascular endothelial cells. *Journal of Physiological Sciences*. 2020;70(1). doi:10.1186/s12576-020-00773-y
17. Arcaro A, Guerreiro A. The Phosphoinositide 3-Kinase Pathway in Human Cancer: Genetic Alterations and Therapeutic Implications. *Current Genomics*. 2007;8(5):271-306. doi:10.2174/138920207782446160
18. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Molecular Cancer*. 2019;18(1):26. doi:10.1186/s12943-019-0954-x

19. Park M, Hong J. Roles of NF-κB in Cancer and Inflammatory Diseases and Their Therapeutic Approaches. *Cells*. 2016;5(2):15. doi:10.3390/cells5020015
20. Shamas-Din A, Kale J, Leber B, Andrews DW. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harbor Perspectives in Biology*. 2013;5(4):1-21. doi:10.1101/cshperspect.a008714
21. Wallace DC. A Mitochondrial Paradigm of Metabolic and Degenerative Diseases, Aging, and Cancer: A Dawn for Evolutionary Medicine. *Annual Review of Genetics*. 2005;39(1):359-407. doi:10.1146/annurev.genet.39.110304.095751
22. Cargnello M, Roux PP. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiology and Molecular Biology Reviews*. 2012;76(2):496-496. doi:10.1128/MMBR.00013-12
23. Soni S, Anand P, Padwad YS. MAPKAPK2: The master regulator of RNA-binding proteins modulates transcript stability and tumor progression. *Journal of Experimental and Clinical Cancer Research*. 2019;38(1). doi:10.1186/s13046-019-1115-1
24. Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. Activation of mitogen-activated protein kinase by H2O2: Role in cell survival following oxidant injury. *Journal of Biological Chemistry*. 1996;271(8):4138-4142. doi:10.1074/jbc.271.8.4138
25. Cenini G, Lloret A, Cascella R. Oxidative Stress and Mitochondrial Damage in Neurodegenerative Diseases: From Molecular Mechanisms to Targeted Therapies. *Oxidative Medicine and Cellular Longevity*. 2020;2020. doi:10.1155/2020/1270256
26. Singh A, Kukreti R, Saso L, Kukreti S. Oxidative stress: A key modulator in neurodegenerative diseases. *Molecules*. 2019;24(8). doi:10.3390/molecules24081583
27. Nakhle J, Rodriguez AM, Vignais ML. Multifaceted Roles of Mitochondrial Components and Metabolites in Metabolic Diseases and Cancer. *International Journal of Molecular Sciences*. 2020;21(12):4405. doi:10.3390/ijms21124405

28. F. C. Lopes A. Mitochondrial metabolism and DNA methylation: a review of the interaction between two genomes. *Clinical Epigenetics*. 2020;12(1):182. doi:10.1186/s13148-020-00976-5
29. Dong Z, Pu L, Cui H. Mitoepigenetics and Its Emerging Roles in Cancer. *Frontiers in Cell and Developmental Biology*. 2020;8. doi:10.3389/fcell.2020.00004
30. van der Wijst MGP, Rots MG. Mitochondrial epigenetics: an overlooked layer of regulation? *Trends in Genetics*. 2015;31(7):353-356. doi:10.1016/j.tig.2015.03.009
31. Zeida A, González Lebrero MC, Radi R, Trujillo M, Estrin DA. Mechanism of cysteine oxidation by peroxynitrite: An integrated experimental and theoretical study. *Archives of Biochemistry and Biophysics*. 2013;539(1):81-86. doi:10.1016/J.ABB.2013.08.016
32. Poole LB, Nelson KJ. Discovering mechanisms of signaling-mediated cysteine oxidation. *Current Opinion in Chemical Biology*. 2008;12(1):18-24. doi:10.1016/J.CBPA.2008.01.021
33. Zong WX, Rabinowitz JD, White E. Mitochondria and Cancer. *Molecular Cell*. 2016;61(5):667-676. doi:10.1016/j.molcel.2016.02.011
34. Weinberg F, Hamanaka R, Wheaton WW, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences*. 2010;107(19):8788-8793. doi:10.1073/pnas.1003428107
35. Poole LB. The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology and Medicine*. 2015;80:148-157. doi:10.1016/j.freeradbiomed.2014.11.013
36. Klomsiri C, Karplus PA, Poole LB. Cysteine-Based Redox Switches in Enzymes. *Antioxidants & Redox Signaling*. 2011;14(6):1065-1077. doi:10.1089/ars.2010.3376
37. Hossain MF, Bodnar Y, Klein C, et al. Molecular Basis for the Interactions of Human Thioredoxins with Their Respective Reductases. *Oxidative Medicine and Cellular Longevity*. 2021;2021:1-17. doi:10.1155/2021/6621292

38. Biswas S, Chida AS, Rahman I. Redox modifications of protein–thiols: Emerging roles in cell signaling. *Biochemical Pharmacology*. 2006;71(5):551-564. doi:10.1016/J.BCP.2005.10.044
39. Grivennikova VG, Vinogradov AD. Partitioning of superoxide and hydrogen peroxide production by mitochondrial respiratory complex I. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2013;1827(3):446-454. doi:10.1016/j.bbabio.2013.01.002
40. Vercellino I, Sazanov LA. The assembly, regulation and function of the mitochondrial respiratory chain. *Nature Reviews Molecular Cell Biology*. Published online 2021. doi:10.1038/s41580-021-00415-0
41. Veal EA, Day AM, Morgan BA. Hydrogen Peroxide Sensing and Signaling. *Molecular Cell*. 2007;26(1):1-14. doi:10.1016/j.molcel.2007.03.016
42. Sullivan-Gunn MJ, Lewandowski PA. Elevated hydrogen peroxide and decreased catalase and glutathione peroxidase protection are associated with aging sarcopenia. *BMC Geriatrics*. 2013;13(1). doi:10.1186/1471-2318-13-104
43. Napolitano G, Fasciolo G, Venditti P. Mitochondrial management of reactive oxygen species. *Antioxidants*. 2021;10(11). doi:10.3390/antiox10111824
44. Napolitano G, Fasciolo G, Venditti P. Mitochondrial management of reactive oxygen species. *Antioxidants*. 2021;10(11). doi:10.3390/antiox10111824
45. Cecchini G. Function and structure of complex II of the respiratory chain. *Annual Review of Biochemistry*. 2003;72:77-109. doi:10.1146/annurev.biochem.72.121801.161700
46. Maranzana E, Barbero G, Falasca AI, Lenaz G, Genova ML. Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex i. *Antioxidants and Redox Signaling*. 2013;19(13):1469-1480. doi:10.1089/ars.2012.4845
47. Cecchini G. Function and structure of complex II of the respiratory chain. *Annual Review of Biochemistry*. 2003;72:77-109. doi:10.1146/annurev.biochem.72.121801.161700

48. Desa DE, Nichols MG, Smith HJ. Aminoglycosides rapidly inhibit NAD(P)H metabolism increasing reactive oxygen species and cochlear cell demise. *Journal of Biomedical Optics*. 2018;24(05):1. doi:10.1117/1.jbo.24.5.051403
49. da Veiga Moreira J, Hamraz M, Abolhassani M, et al. The redox status of cancer cells supports mechanisms behind the Warburg effect. *Metabolites*. 2016;6(4). doi:10.3390/metabo6040033
50. Stephens DJ, Allan VJ. *Light Microscopy Techniques for Live Cell Imaging*. www.sciencemag.org
51. van Rheenen J, Langeslag M, Jalink K. Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission. *Biophysical Journal*. 2004;86(4):2517-2529. doi:10.1016/S0006-3495(04)74307-6
52. Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophysical Journal*. 1998;74(5):2702-2713. doi:10.1016/S0006-3495(98)77976-7
53. Erdogan YC, Altun HY, Secilmis M, et al. Complexities of the chemogenetic toolkit: Differential mDAAO activation by d-amino substrates and subcellular targeting. *Free Radical Biology and Medicine*. 2021;177:132-142. doi:10.1016/j.freeradbiomed.2021.10.023
54. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. *Physiological Reviews*. 2014;94(3):909-950. doi:10.1152/physrev.00026.2013
55. vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*. 2009;324(5930):1029-1033. doi:10.1126/science.1160809
56. Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA. Energy Substrate Modulates Mitochondrial Structure and Oxidative Capacity in Cancer Cells. *Cancer Research*. 2004;64(3):985-993. doi:10.1158/0008-5472.CAN-03-1101
57. Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. *Journal of General Physiology*. 1927;8(6):519-530. doi:10.1085/jgp.8.6.519

58. DeBerardinis RJ, Mancuso A, Daikhin E, et al. Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences*. 2007;104(49):19345-19350. doi:10.1073/pnas.0709747104
59. Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *Journal of Biological Chemistry*. 1979;254(8):2669-2676. doi:10.1016/S0021-9258(17)30124-2
60. Altman BJ, Stine ZE, Dang C v. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nature Reviews Cancer*. 2016;16(10):619-634. doi:10.1038/nrc.2016.71
61. Bellance N, Benard G, Furt F, et al. Bioenergetics of lung tumors: Alteration of mitochondrial biogenesis and respiratory capacity. *The International Journal of Biochemistry & Cell Biology*. 2009;41(12):2566-2577. doi:10.1016/j.biocel.2009.08.012
62. Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. *Journal of General Physiology*. 1927;8(6):519-530. doi:10.1085/jgp.8.6.519
63. Zhao Y, Wang A, Zou Y, Su N, Loscalzo J, Yang Y. In vivo monitoring of cellular energy metabolism using SoNar, a highly responsive sensor for NAD+/NADH redox state. *Nature Protocols*. 2016;11(8):1345-1359. doi:10.1038/nprot.2016.074
64. Zhao Y, Hu Q, Cheng F, et al. SoNar, a Highly Responsive NAD+/NADH Sensor, Allows High-Throughput Metabolic Screening of Anti-tumor Agents. *Cell Metabolism*. 2015;21(5):777-789. doi:10.1016/j.cmet.2015.04.009
65. Moreira J, Hamraz M, Abolhassani M, et al. The Redox Status of Cancer Cells Supports Mechanisms behind the Warburg Effect. *Metabolites*. 2016;6(4):33. doi:10.3390/metabo6040033
66. Kroemer G, Pouyssegur J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell*. 2008;13(6):472-482. doi:10.1016/j.ccr.2008.05.005
67. Gao P, Tchernyshyov I, Chang TC, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*. 2009;458(7239):762-765. doi:10.1038/nature07823

68. Gaglio D, Metallo CM, Gameiro PA, et al. Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Molecular Systems Biology*. 2011;7(1):523. doi:10.1038/msb.2011.56
69. Shields JM, Pruitt K, McFall A, Shaub A, Der CJ. Understanding Ras: 'it ain't over 'til it's over'. *Trends in Cell Biology*. 2000;10(4):147-154. doi:10.1016/S0962-8924(00)01740-2
70. Commisso C, Davidson SM, Soydaner-Azeloglu RG, et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature*. 2013;497(7451):633-637. doi:10.1038/nature12138
71. DeNicola GM, Cantley LC. Cancer's Fuel Choice: New Flavors for a Picky Eater. *Molecular Cell*. 2015;60(4):514-523. doi:10.1016/j.molcel.2015.10.018
72. Wu F, Liu Y, Cheng H, et al. Enhanced Cancer Starvation Therapy Based on Glucose Oxidase/3-Methyladenine-Loaded Dendritic Mesoporous OrganoSilicon Nanoparticles. *Biomolecules*. 2021;11(9):1363. doi:10.3390/biom11091363
73. Shanmugam M, McBrayer SK, Qian J, et al. Targeting Glucose Consumption and Autophagy in Myeloma with the Novel Nucleoside Analogue 8-Aminoadenosine. *Journal of Biological Chemistry*. 2009;284(39):26816-26830. doi:10.1074/jbc.M109.000646

APPENDIX A – CHEMICALS

1.1 Chemicals	Manufacturing Company
Ampicillin	Sigma Aldrich, Turkey
Antimycin	Sigma Aldrich, Turkey
ATP	Sigma Aldrich, Turkey
DMEM	PAN-Biotech, Germany
DMSO	Sigma Aldrich, Turkey
D-Alanine	Alfa Aesar, Germany
D-Phenylalanine	Alfa Aesar, Germany
Fetal Bovine Serum	PAN-Biotech, Germany
Glass coverslips	Asistent, Germany
Glucose	Sigma Aldrich, USA
MTT	Sigma Aldrich, USA
TMRM	Thermo-Fischer, USA
Oligomycin	Sigma Aldrich, USA
Penicillin/Streptomycin	PAN-Biotech, Germany
Phosphate Buffered Saline	PAN-Biotech, Germany
PolyJet	SignaGen Laboratories, USA

APPENDIX B – EQUIPMENT

1.2 Equipment	Manufacturing Company
Axio Observer.Z1/7	Zeiss, Germany

Axio Vert. A1	Zeiss, Germany
Axiocam 503 mono	Zeiss, Germany
Perfusion Chamber	NGFI, Austria
Plan-Apochromat 40x/1.4 Oil	Zeiss, Germany
Waterbath	Nüve, Turkey
Zeiss Primovert phase-contrast microscope	Zeiss, Germany
Hemocytometer	Isolab, Germany
ThermoCycler	BioRad, USA