Case Study 3: Mitochondria

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Redox Biology Course
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Background Questions
1. Which metals are the major contributors to hydroxyl radical formation in cells? What are the intracellular concentrations of these metals in mammals? Show the reaction by which these metals catalyze the formation of hydroxyl radicals.

Short life-time: 1ns
Very reactive, reason for oxidative damage of most biomolecules
HABER WEISS REACTION

\[
\begin{align*}
Cr^{5+} + H_2O_2 & \rightarrow Cr^{6+} + OH^- + \cdot HO \\
Fe^{3+} + O_2^- & \rightarrow Fe^{2+} + O_2 \\
Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + OH^- \\
Ni^{2+} & \rightarrow Ni^{3+}
\end{align*}
\]

(Fenton-like reaction, Coudray et al., 1992 Biological Trace Element Research)

Intracellular concentrations:
Fe: unbound 0.001 mM; Ferritin: 0.7 mM - 3.6 mM
Cu: unbound: $<10^{-18}$ M

Ni as a temporary catalyst (Toreilles et al., 1990, FEBS letters)
2. Describe DNA and lipid modifications that are found in cells exposed to oxidative stress agents?

**DNA modifications:**
- Mutations, deletions, crosslinking with protein moieties, single or double stranded breaks, degradation of bases.
- Nucleotide modifications: glycol, dTG, and 8-hydroxy-2-deoxyguanosine glycol, 8-hydroxy-2-deoxyguanosine glycol is a common known oxidative stress marker.

**Lipid modifications:**
- The phospholipids are most sensitive to peroxidation, damaging the membrane destructing functions like permeability and fluidity.
- ROS cause lipid peroxidation, generating hydroperoxides and endoperoxides, which degrade to reactive carbonyl species and aldehydes, such as 4-hydroxynonenal (HNE).
- The diverse carbonyl species and aldehydes can induce modifications/damage to proteins and DNA bases, for example by creating covalent adducts.
How do these modifications affect cell viability and contribute to disease progression?

- Modifications of proteins and DNA → dysfunction → metabolic problems, apoptosis, cancer
- DNA damage recruits the repair machinery which can trigger inflammation (e.g. OGG1 (8-oxoG glycosylase) is involved in inflammation)
- 8-hydroxy-2-deoxyguanosine is also a marker for carcinogenesis. Formation of these nucleotides in promoter regions can alter the binding site of transcription factors and alter gene expression
- Lipid modifications on membranes can alter or inactivate enzyme or receptors bound to membranes
- Products of lipid peroxidation can crosslink to proteins and inactivate them.
- Alkanes, malonaldehyde, and isoprotanes are biomarkers for lipid peroxidation. These markers are found in high levels in patients with neurogenerative diseases, ischemic reperfusion injury.
- Products of lipid peroxidation, decreases GSH levels, stimulates epidermal growth factor activation and induces fibronectin production.
Background

Endogenous sources
- Mitochondrial oxidative phosphorylation
- Lipid peroxidation chain reactions
- Fenton reaction
- Haber-Weiss reaction
- and etc. (see Table 1)

Exogenous sources
- Radiation
- Pathogens
- Chemicals
- Pollutants
- Inflammation
- Food and nutrients
- Smoking

Mitochondrial dysfunction

ROS

Oxidative stress
- lipid peroxidation of membranes, nDNA and mtDNA oxidation,
  modification of proteins, lipids and nucleic acids
- dysregulation of critical pathways
  (e.g., NF-kB, MAPK, Fas/Fadd, mTOR, mitochondria dependent apoptosis)
  DNA damage and mutations, chromosomal abnormalities and instability,
  accumulation of insoluble protein adducts, RNA damage

Cancer

Senescence

Neurodegenerative disorders

Apoptosis

Initiation

Unsaturated lipid

+ \( \text{H}_2\text{O} \)

Lipid radical

Propagation

O

Lipid peroxide

Lipid peroxyl radical
3. What are some of the most common protein modifications that occur under oxidative stress? Describe how oxidative modification of SOD is associated with a disease.

Common protein modifications under oxidative stress:

1. **Protein carbonylation** (oxidation of protein side chains)
   - Target amino acid side chains:
     - Cysteine (Cys)
     - Lysine (Lys)
     - Histidine (His)
   - Cause inactivation of enzymes and membrane transporters
   - Mechanisms:
     - Oxidation of amino acid side chains with metals and $\text{H}_2\text{O}_2$
     - Lipid peroxidation
       - Products such as aldehyde/lipid hydroperoxide modify proteins
       - Aldehydes diffuse easily and can modify local and distant proteins

Abbreviations: NRP, nonradical product; LOOH, lipid hydroperoxide; $\alpha$-TOH, $\alpha$-tocopherol; $\alpha$-TO·, $\alpha$-TOH radical; LH, lipid substrate; LOO·, lipid peroxyl radical.

Adapted from Waldeck and Stocke (1996)
3. What are some of the most common protein modifications that occur under oxidative stress? Describe how oxidative modification of SOD is associated with a disease.

2. **Protein glutathionylation**-is the posttranslational modification of protein cysteine residues by the addition of glutathione.

![Glutathione (GSH) structure](image)

Hossam et al., 2013

Background

Hossam et al., 2013
3. What are some of the most common protein modifications that occur under oxidative stress? Describe how oxidative modification of SOD is associated with a disease.

**Superoxide dismutase (SOD)**
- Catalyze conversion of superoxide into oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$)

**Oxidized modification of SOD and disease**
- Hydrogen peroxide oxidizes amino acid residues around the active site (copper)

Wang, Branicky, Noe et al. (2018)

Oxidative modifications of human EC-SOD induced by hydrogen peroxide.

Randi H. Gottfredsen et al., 2013
4. How do cells repair oxidatively damaged DNA?

DNA repair pathways:

- **base excision repair (BER)** – majority of modifications by ROS
- **mismatch repair (MMR)**
- **nucleotide excision repair (NER)**

Nowadays, more than 100 DNA repair enzymes have been described.
4. How do cells repair oxidatively damaged proteins?

- **Oxidative damage** affects the **structure and activity of proteins** and can induce cell death.
- Carbonylation: covalent modification that causes protein inactivation. Arg, Lys, Pro, Thr
- His to oxo-histidine: covalent modification
- Protein containing sulfur amino acid (**cysteine and methionine**) are **more susceptible to ROS**, which will damage proteins. Due to presence of an electron-rich sulfur atom.
4. How do cells repair oxidatively damaged proteins?

1. Oxidized Cys repair:
   a. Oxidoreductases (thioredoxins (Trxs) and glutaredoxins (Grxs)) – Cytoplasm

2. Oxidized Met repair:
   b. Methionine sulfoxide reductases (Msrs) – Cytoplasm

In e coli 4 Msrs were identified: MsrA, B and C and biotin sulfoxide reductase (BisC). MrsA and MrsB can reduce free Met-O and Met-O in proteins. MrsA reduces Met-S-O and MrsB Met-R-O.
Protein oxidative damage repair in the cytoplasm – Oxidized Cys repair

Case Study
The AAA ATPase Afg1 preserves mitochondrial fidelity and cellular health by maintaining mitochondrial matrix proteostasis

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Mitochondrion

- ATP through respiration: driving force in evolution
- mtDNA more vulnerable because of the lack of histones
- Defect in mitochondrial proteins required for aerobic ATP production → mitochondrial diseases
- High bioenergetic demand: vulnerable to mitochondrial dysfunction

'Everything that can possibly go wrong will go wrong'
(Murphy's Law)
Solution:

- Evolutionary conserved dedicated mechanisms and factors (MQC system)
  - Organellar level
  - Molecular level

Sugiura et al., The EMBO Journal (2014) 33, 2142-2156
Case Study: Introduction

AAA proteases (ATPases associated with various cellular activities)

- oligomeric motors
- ATP hydrolysis
- substrate remodeling
- translocation of a polypeptide through a central pore

Case Study: Introduction

Mitochondrial AAA ATPases

- Diverse roles
- Highly specialized and broader as well
- E.g. Bcs1, Msp1/ATAD1, Afg1/LACE1

Previous studies:
- role of Afg1 in degradation of CcO subunits
- … in stress induced translocation of p53 into mitochondria
- Suggestion of Afg1 in MQC
Case Study: Introduction

The AAA ATPase Afg1 preserves mitochondrial fidelity and cellular health by maintaining mitochondrial matrix proteostasis

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Afg1 suppresses mitochondrial defects

- Involvement of Afg1 as a mitochondrial quality control protein
- Yeast Oma1 and m-AAA double KO
- Yeast growth under restrictive temperature with glucose
  - Afg1 overexpression in oma1Δ yta10Δ mutant allows growth
- Flow cytometry analysis of the membrane potential of mitochondria by JC-1 dye
Mitochondrial structure was assessed by confocal fluorescence microscopy
- MTG: MitoTracker Green
- Su9-RFP: mitochondrial marker protein
- WT and oma1Δ cells: normal
- Yta10Δ: colocalization and network morphology partly compromised
- oma1Δ yta10Δ cells: little colocalization
  - Resemble morphology of mitochondrial import mutants
  - Authors suggest double mutant, under temperature shift, have depolarized mitochondria
Loss of Afg1 promotes mitochondrial dysfunction

- Evaluating the role of Afg1 in mitochondrial function
- Growth test of synchronized WT and afg1Δ cells
- Mitochondrial depolarization in exponential and stationary cells
- O2 consumption rates of cells
  - Afg1Δ sees reduced oxygen consumption

Case Study: Results - Figure 2
Does impairment Afg1 cause oxidative damage?

Approach:
Flow cytometry analysis with superoxide radical sensitive dye, assessment of aconitase-activity and growth test in presence of paraquat.
Study of the functional determinants of Afg1 by studying how different variants can rescue the oxidative damage phenotype of afg1\(\Delta\)

Generation of C-terminal 6xHis-tagged Afg1 variants which are impaired in different ways of ATPase function:

- Catalytic mutant – not able to hydrolyze ATP → E206Q
- Substrate translocation mutant → Y160A
- Oligomerization mutants → L225D, L226D

None of the variants alleviated the aging-associated impairment of aconitase activity in these cells

1. ATP hydrolyzing
2. Protein unfolding activities
3. Ability to oligomerize

Critical for oxidative stress tolerance and normal aging in yeast
Genetic interactions between Afg1 and MQC components Oma1 and Pim1/LON proteases

Deficiency of MQC components associated with intolerance to oxidative insults

Generation of double deletion mutants and survival study after $H_2O_2$ exposure

- afg1Δ oma1Δ
- afg1Δ pim1Δ

- Marked impairment in the viable colonies formation after $H_2O_2$ exposure in lack of Afg1, Oma1 or Pim1
- Reduction of the colony formation capacity after peroxidase stress in Afg1Δ oma1Δ and afg1Δ pim1Δ – Genetic interaction between AFG1 and OMA1 and PIM1

Afg1 functions with MQC components → promotion of mitochondrial protein homeostasis
Case Study: Results - Figure 5

Afg1 is Required for normal protein homeostasis in the mitochondrial matrix

Looking at how Afg1 might be working with MQC pathways to maintain protein homeostasis

A)
- SSC1-2 (heat shock protein chaperone), which is a temperature sensitive strain of cells. When transfected with AFG1 over expression growth is rescued in raised temperatures.
- When E206Q, a catalytic mutant cell strain that is unable to hydrolyze ATP, and Y106A were transfected and unable to keep them alive in high temperatures.
Afg1 is Required for normal protein homeostasis in the mitochondrial matrix

DHFR is dihydrofolate reductase which is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid. Tetrahydrofolic acid is important for cell proliferation and growth. mx-DHFR is the properly folding enzyme vector and mx-DHFR mut. is the improperly folding enzyme.
Afg1 Function is Evolutionarily Conserved

A) In ssc1 deficient cell strain you can see that CeLACE-1, an ortholog of Afg1, is able to protect cell propagation even in higher temperatures. LACE-1 protects equally as well as AFG1 overexpression.

B) Aconitase specific activity is sensitive to superoxide oxidative stress. In cells where afg1 gene is KO, CeLACE-1 is able to rescue aconitase activity or bring oxidative stress down.
C. elegans LACE1 KD are more affected by ox. stress in mitochondria
Case Study: Results - Figure 7

C. elegans LACE1 KD impairs development

https://www.wormatlas.org/dauer/introduction/Images/dintrofig1leg.htm
Whole body LACE1 KD leads to mtUPR but not ER-UPR

hsp-60 mtUPR
hsp-6 mtUPR
hsp-4 ER-UPR

and reduces the life-span
Typical phenotypic changes in C. elegans with mitochondria dysfunction:
- Retardation of developmental rates
- Disrupted germline development
- Disrupted body wall muscle integrity
- Neurodegeneration

Results: No significant phenotypic changes
- Normal motility
- No significant degeneration of dopaminergic neurons

Next step: Examine whether neurons indeed undergo oxidative stress in the absence of LACE-1
- Label specific cell groups:
  - \( P^{dat-1}:GFP \), dopaminergic neurons
  - \( P^{unc-17}:GFP \), cholinergic neurons
  - \( P^{myo-3}:GFP \), muscle neurons
- Homogenize
- Separate GFP-expressing cells:
  - fluorescence-assisted cell sorting (FASC)
  - Anti-GFP magnetic beads
- qRT-PCR analysis of RNA extractions

Case Study: Results - Figure 8
Increase in expression of oxidative stress-responsive genes, significantly in mutants

- Sod-2: Superoxide dismutase
- Mtl-1: Metallothionein
- Hsp-16.2: Small heat shock protein

Significant increase expression of UPRmt genes in aged mutants compared to young mutants and WT

- Dopaminergic neurons undergo oxidative stress which were enhanced in absence of LACE-1
- Day 9: no sign of neuron degeneration suggests that physiological effects of LACE-1 is complex and may be cell type-specific
No gross phenotypic changes: Normal motility on surfaces

However, mutants have significant impairment in “thrashing” capacity
  ○ Unable to increase motility rates required to sustain movement in liquid media
  ○ Due to decrease acetylcholine neurotransmission in LACE-1 deficient animals
  ○ Mutants enhanced resistance to aldicarb neurotoxin
    ■ All WT worms paralyzed after 2h treatment
    ■ Most mutant worms remained active
      ● 1.7x more resistant

Oxidative stress regulation and maintaining mitochondrial protein homeostasis is important, but functionality of specific cell type must also be maintained under conditions of high activity.
Discussion & Conclusion

● Previously defined roles for Afg1 were supported
  ○ CcO subunit turnover in yeast and mammalian cells

● New functions beyond Cytochrome c Oxidase (CcO) maintenance identified among models
  ○ Afg1 AAA ATPase role in establishing matrix proteostasis and oxidative stress tolerance in yeast & metazoans

● Afg1 orthologue (LACE-1) deficiency in C. elegans yields neuromuscular phenotypes related to shorter lifespan and increased vulnerability to oxidative stress
  ○ Connect Afg1 function and physiological role
THANK YOU