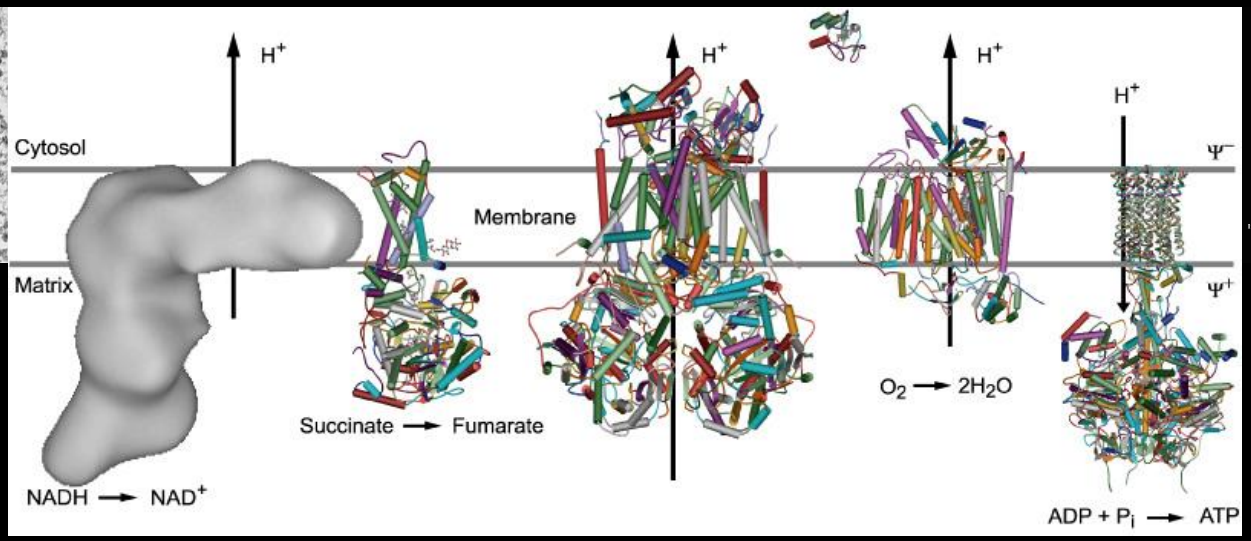
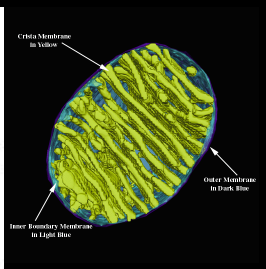




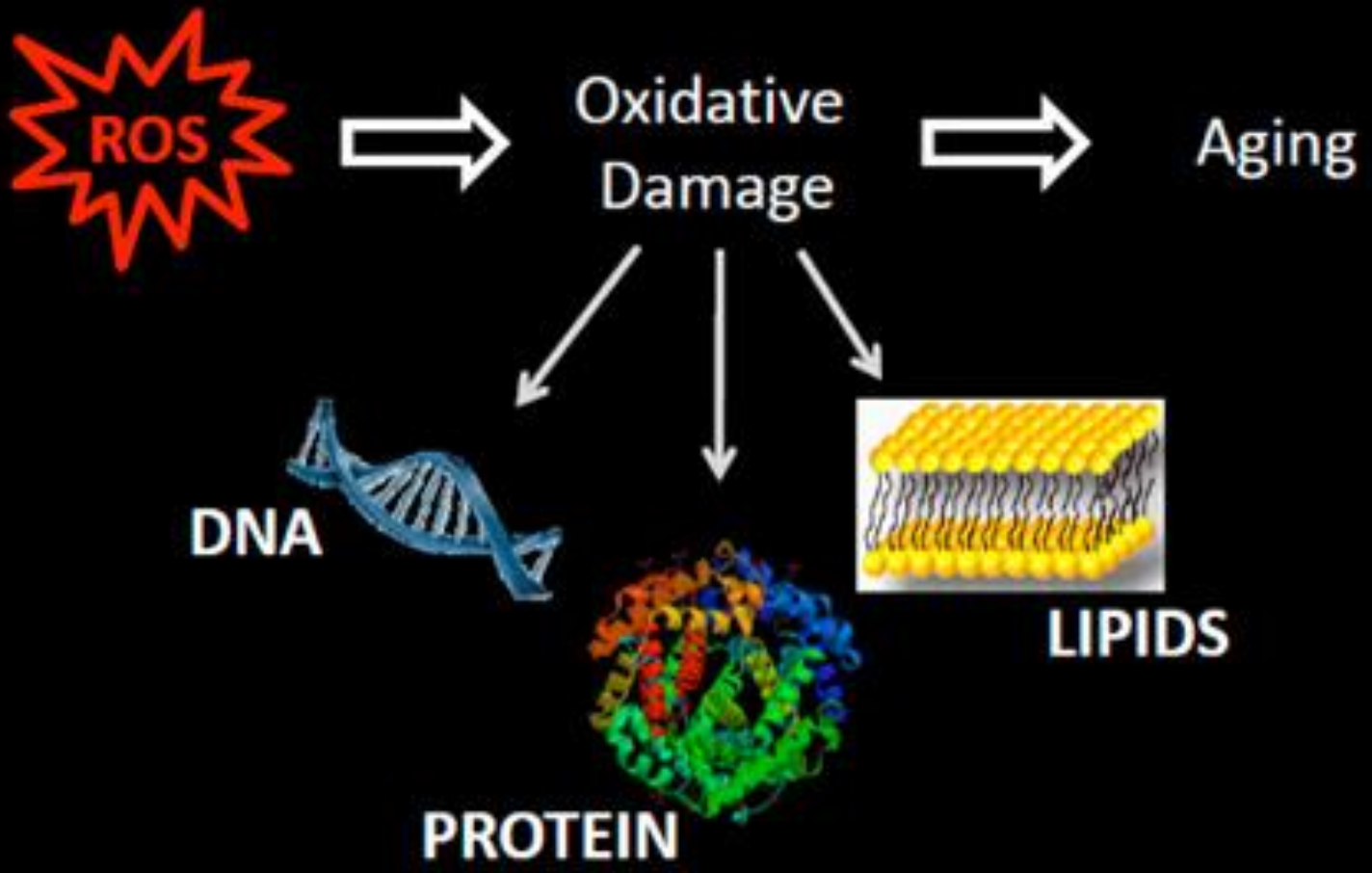
Oxygen Consumption and ROS Production: Challenges for Evaluation of Experimental Protocols.

Sameh Saad Ali, Ph.D.
Zewail City of Science and Technology
Egypt

مدينة زويل
ZEWAIL CITY
ESTABLISHED 2000
INAUGURATED 2011

IBRO-Qatar, Dec 2014

Inflammation in neuronal injury and degeneration

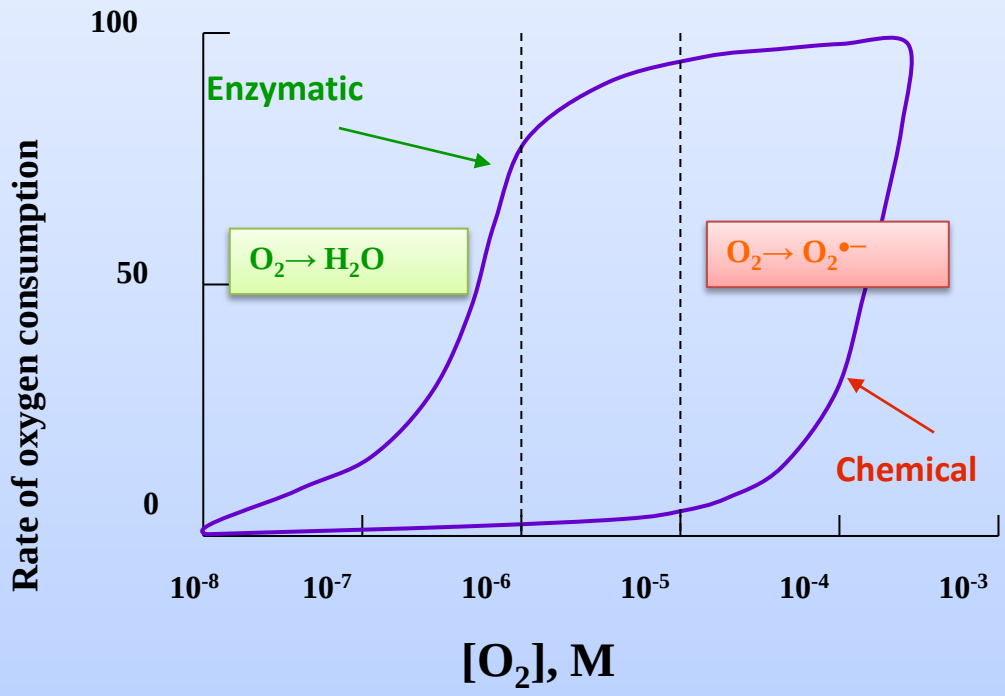


ROS; what are they and where are they coming from?

Two most important reactions to our existence and survival:

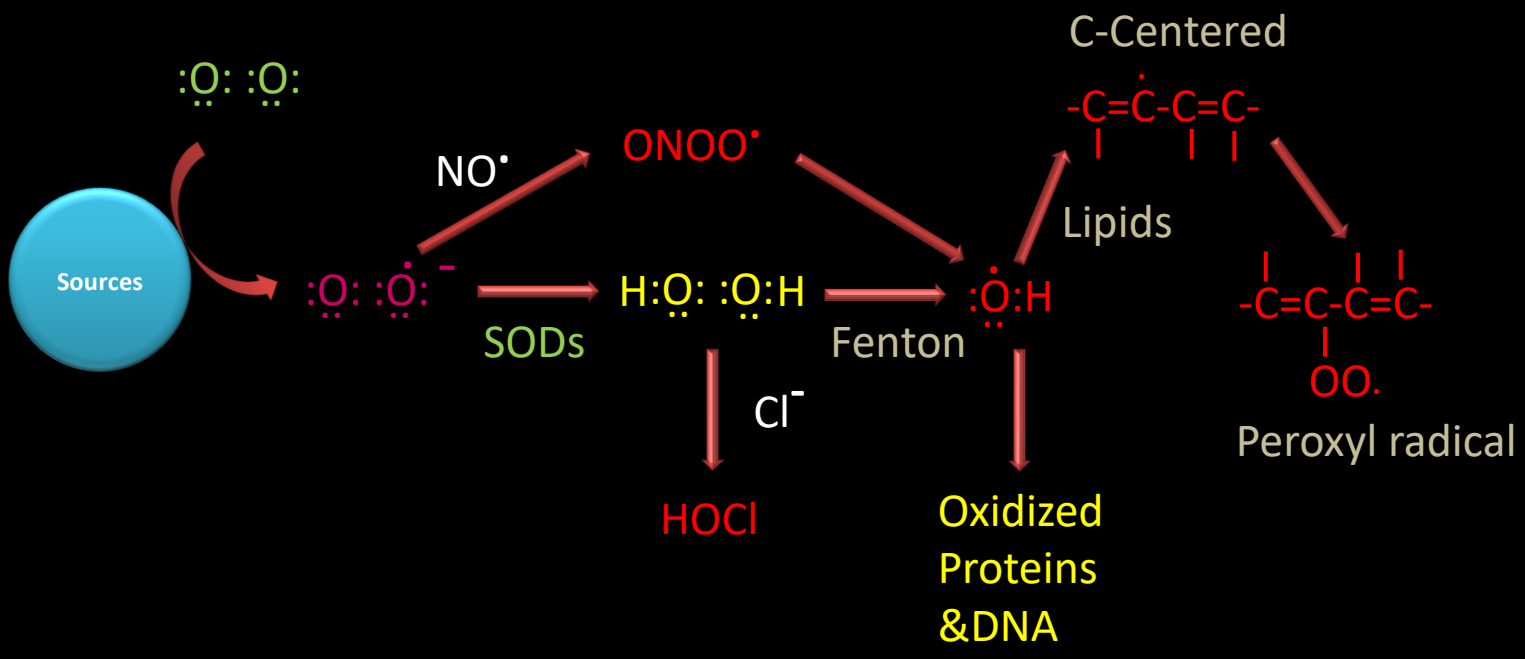
$\frac{1}{2} O_2 + 2 H^+ + 2 e^- \rightarrow H_2O$ ΔG^o = -37.8 kcal/mol (+0.82 V)

$O_2 + e^- \rightarrow O_2^{\bullet -}$ ΔG^o = +3.45 kcal/mol (-0.15 V)



Enzymatic (four electrons) and non-enzymatic (one electron) reductions of O_2 as a function of oxygen concentration. Oxygen availability in the range between the two dashed vertical lines are optimum for maximum physiological performance with least superoxide formation.

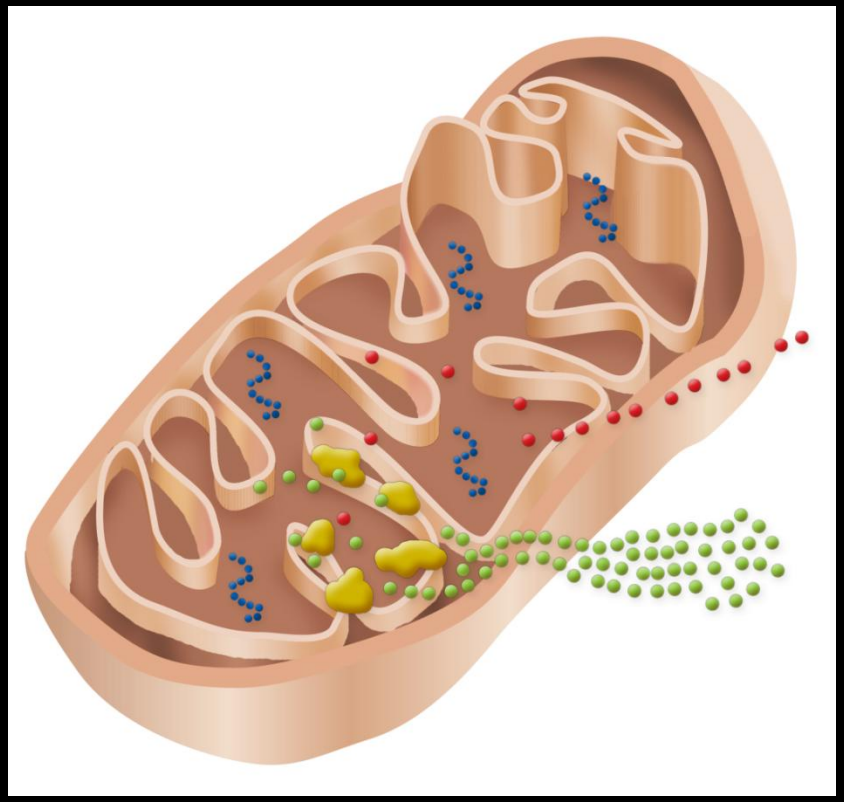
ROS; what are they?

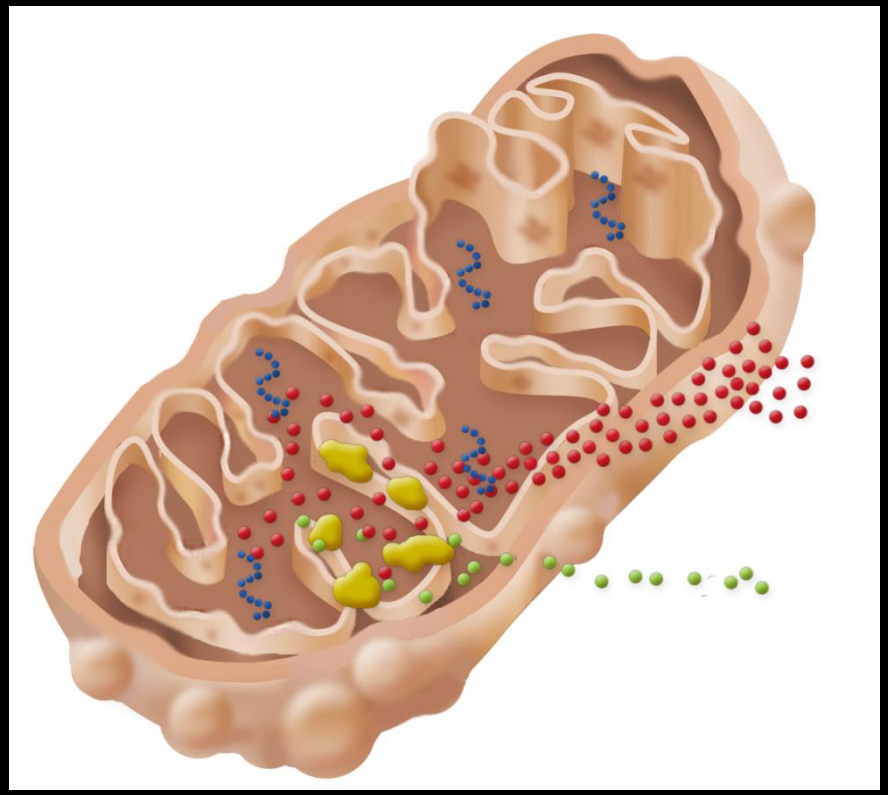


ROS; where are they coming from?

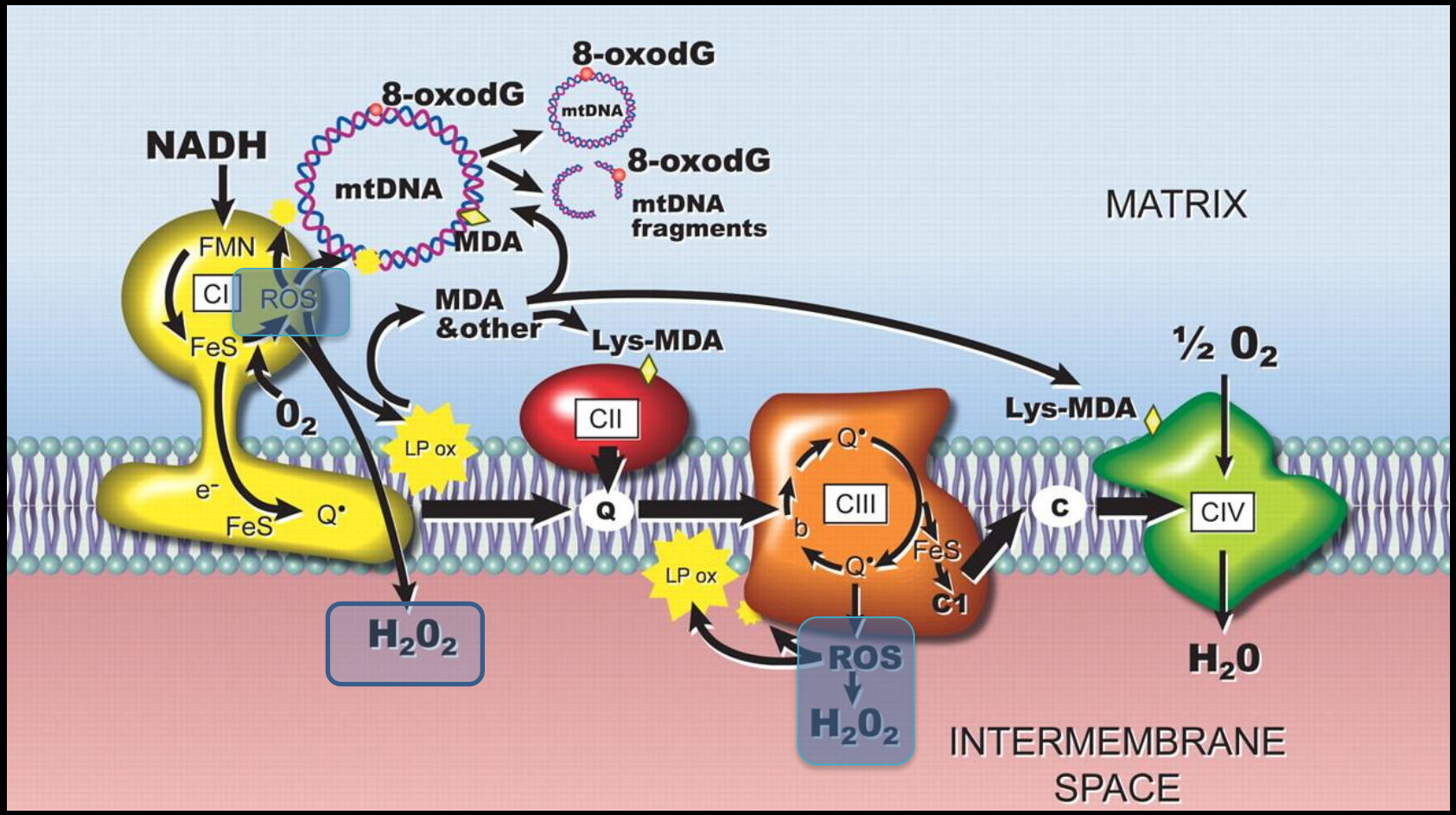
Aging is viewed as a state of increased chronic inflammation!

The mitochondria theory of Aging



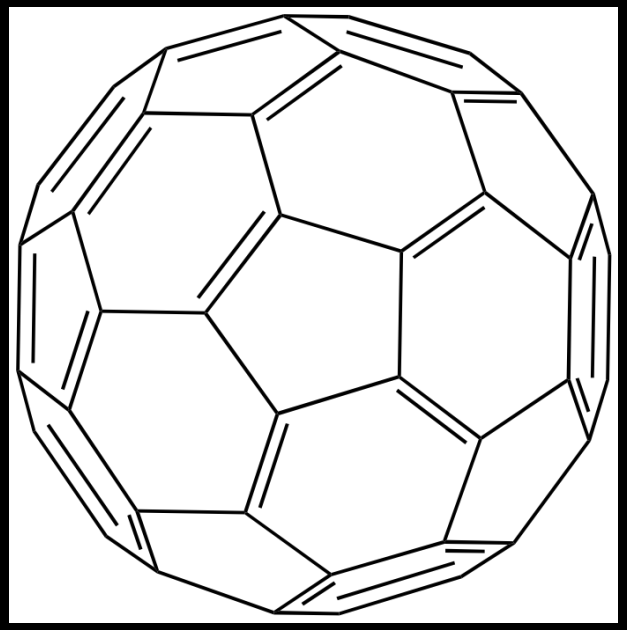


ROS; where are they coming from?

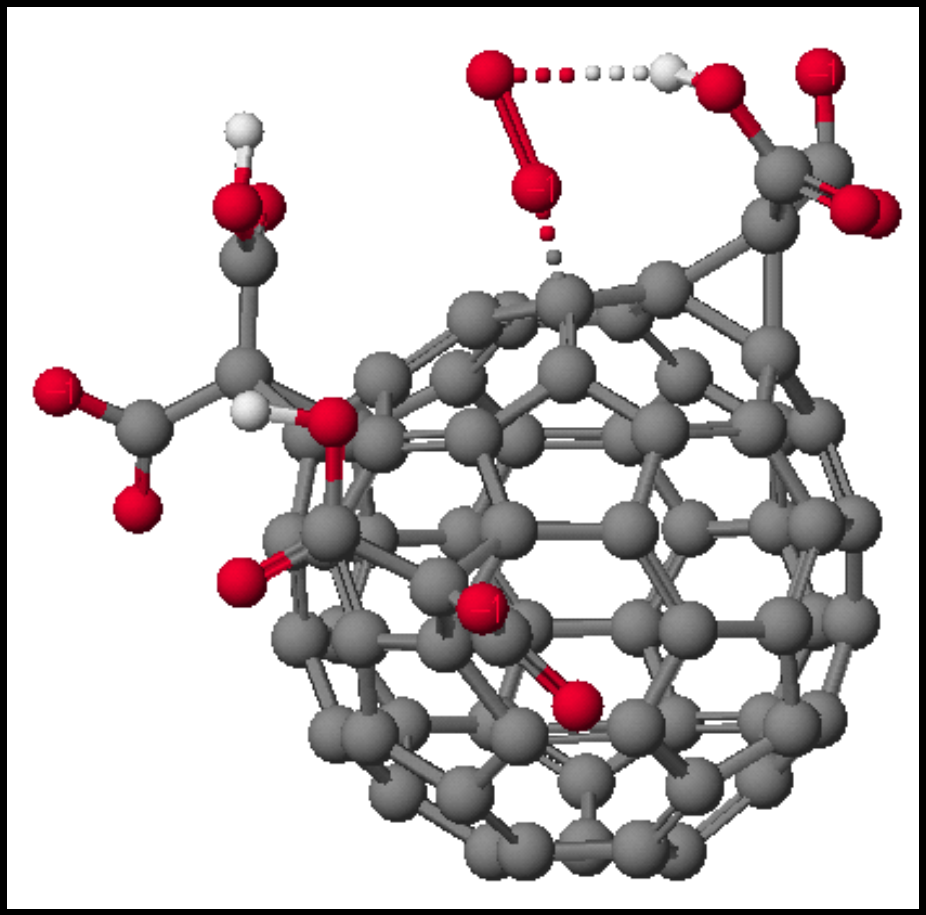


Conclusion so far:
Mitochondrial ROS are major players in the aging process.

Solutions!?
Antioxidants → total failure
Unconventional antioxidants …..

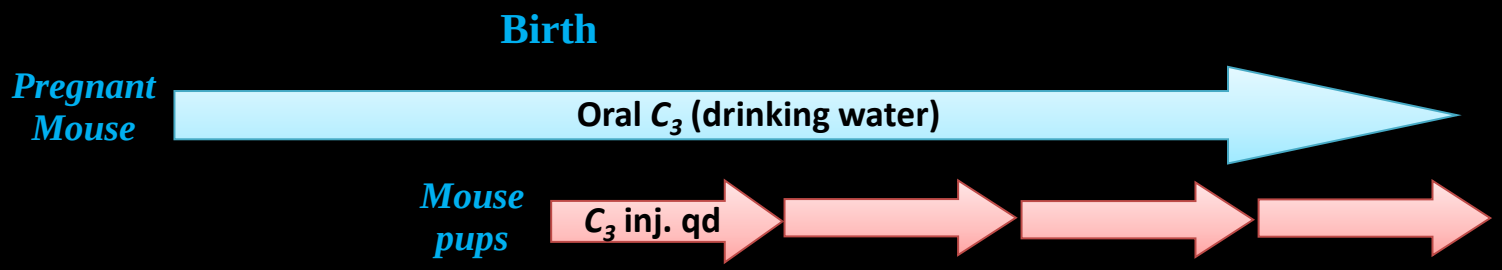


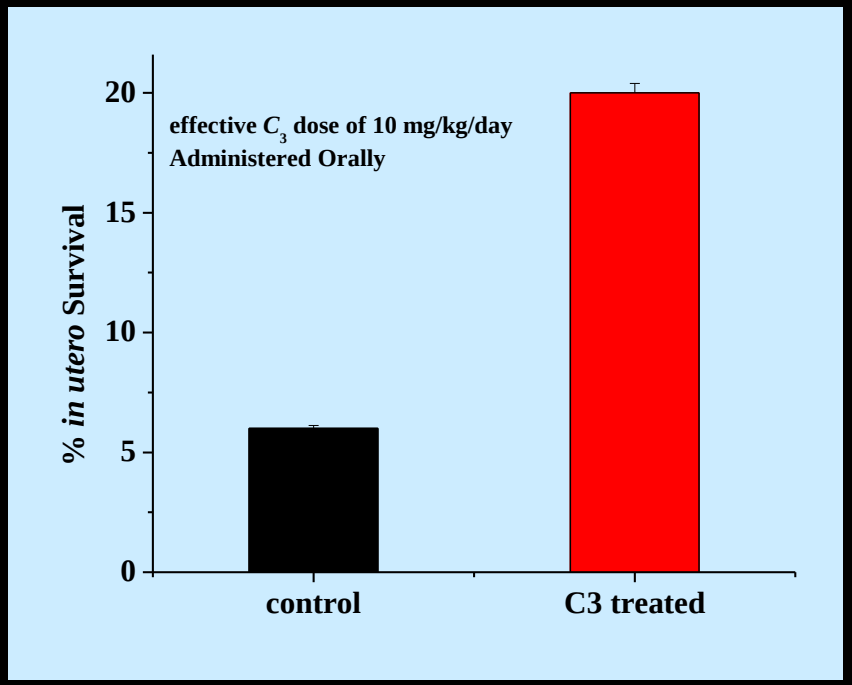
Free radical “sponge”
Adds > 30 radicals/C_{60}!

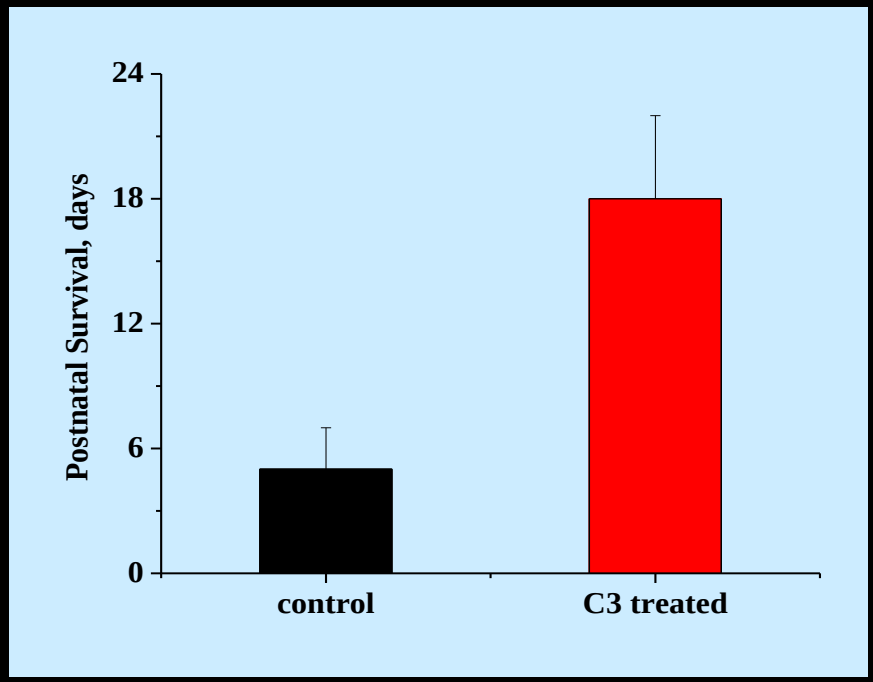


Water-soluble C_3

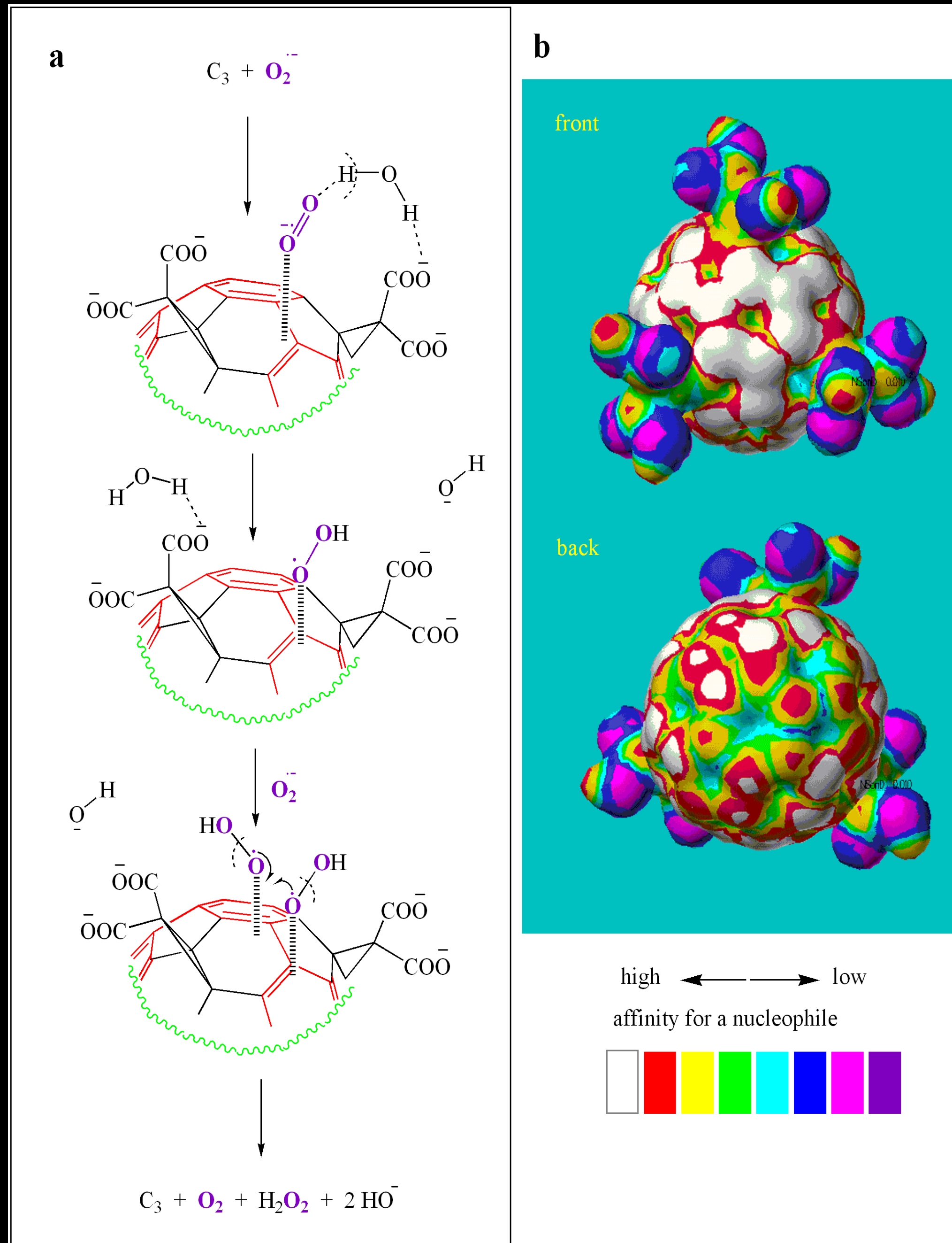
Extension of the life-span of SOD2 knock-out mice.

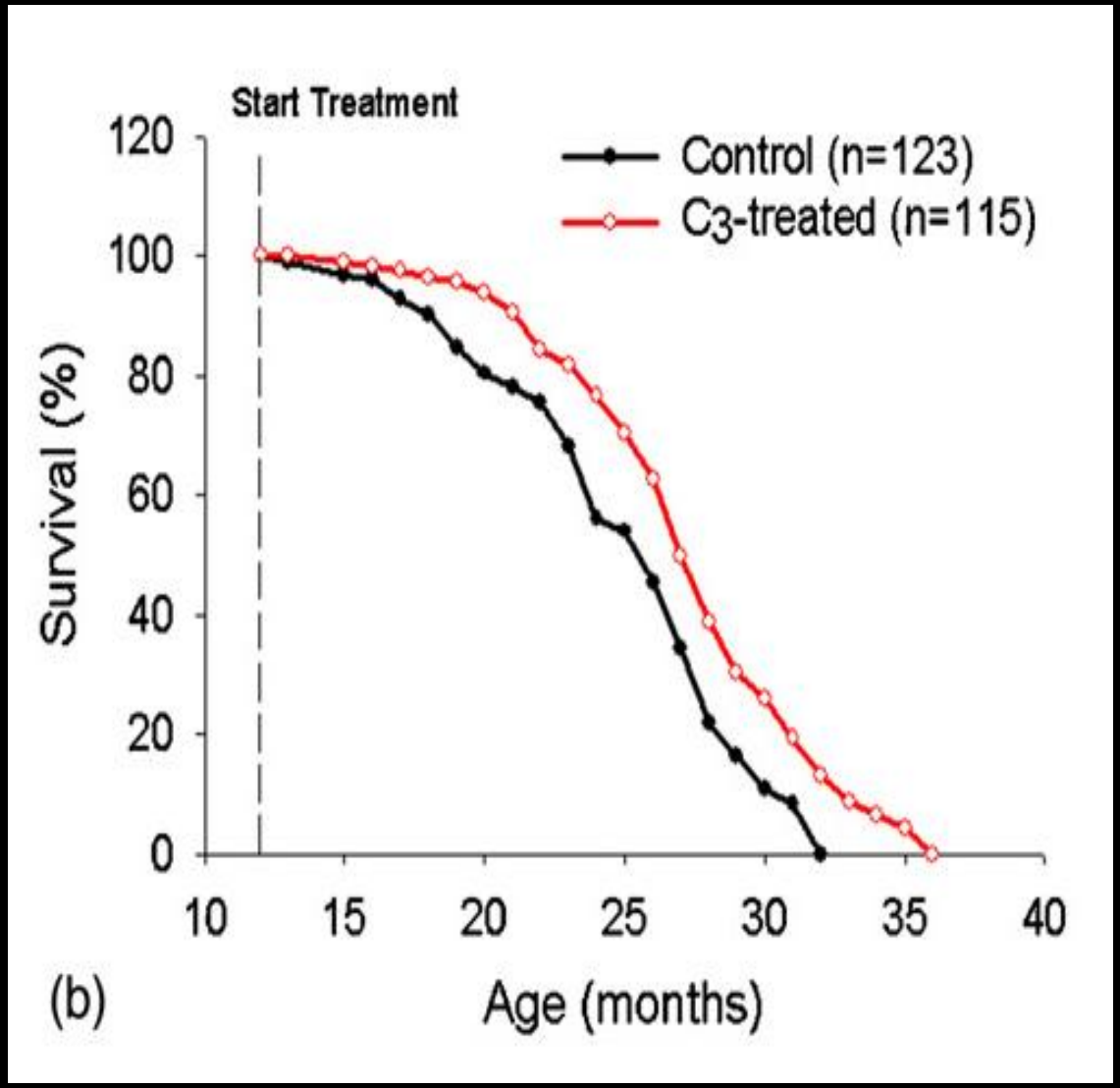






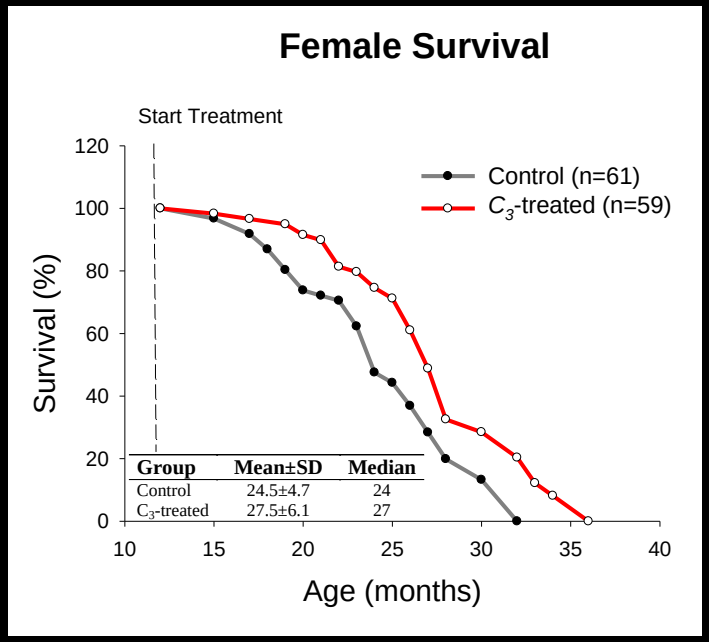
Ali et al. (2004) *Free Radical Biology and Medicine*

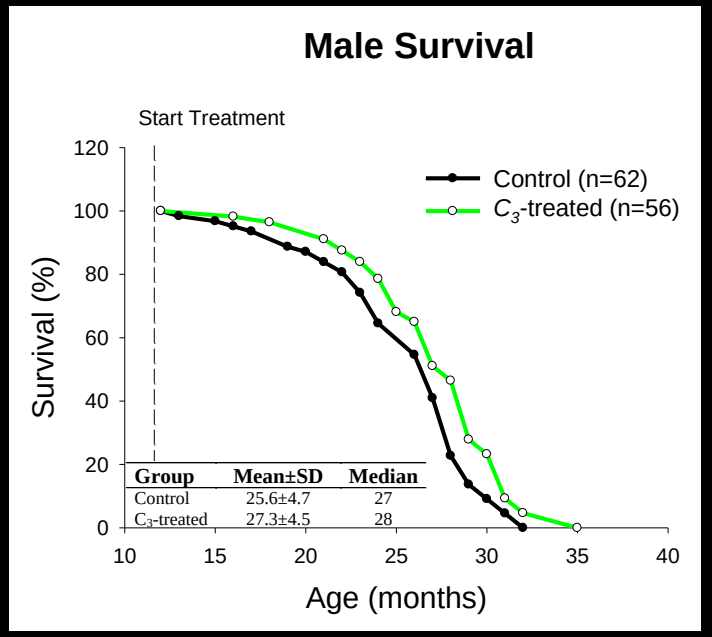

a
C3 + O2·−
COO−
−OOC
COO−
COO−
H—O
H
O
OH
O·
O
HO
OH
O2·−
C3 + O2 + H2O2 + 2 HO−
b
front
back
high
low
affinity for a nucleophile

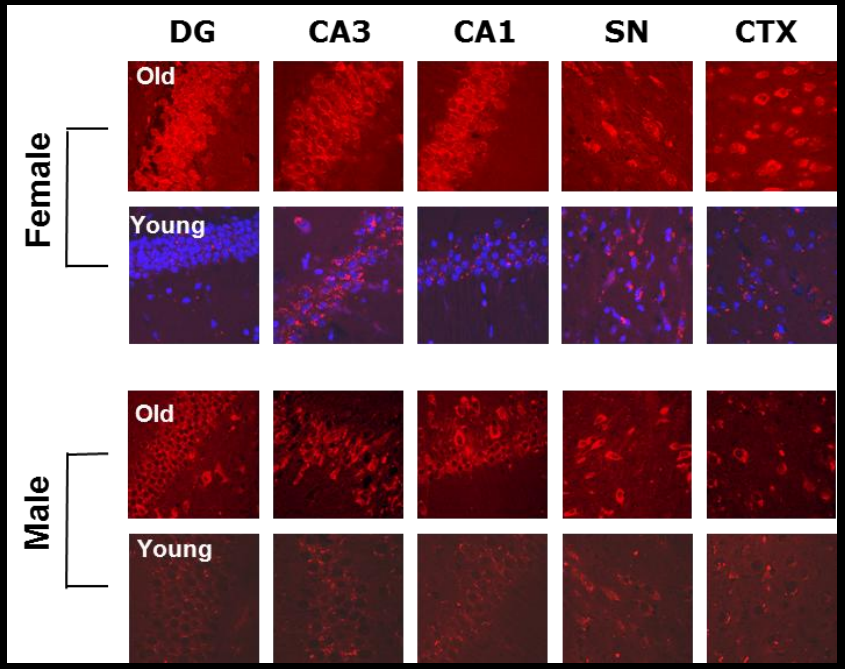


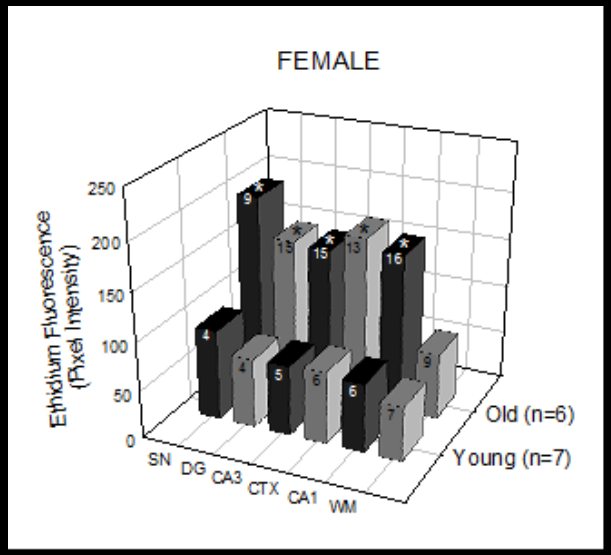
Quick, Ali et al. (2008) *Neurobiology of Aging*

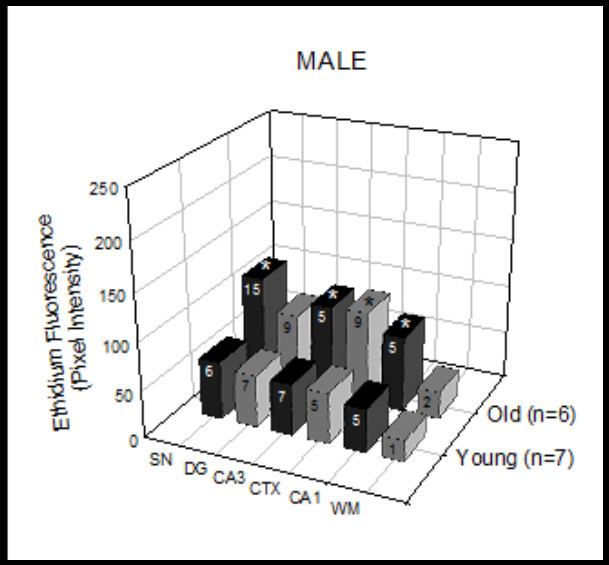
All is good!?
However ….

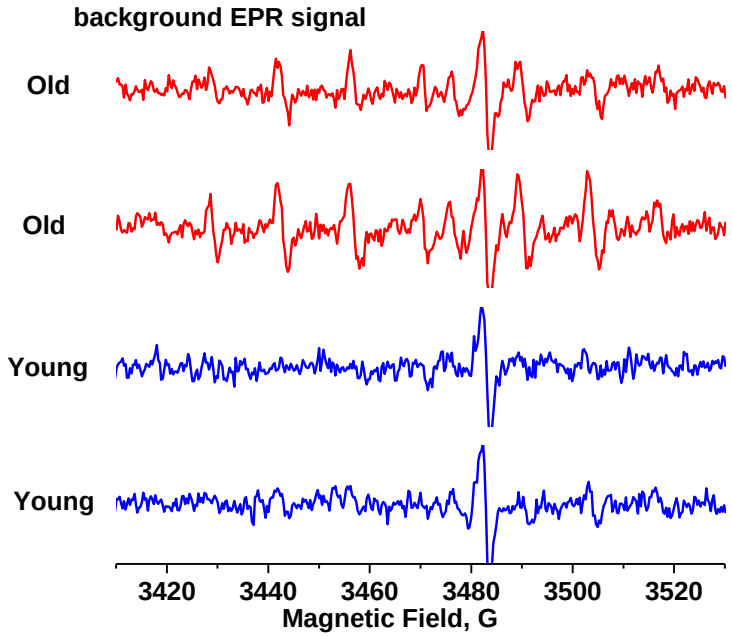

Female Survival
Start Treatment
Control (n=61)
C3-treated (n=59)
Survival (%)
Age (months)
Group | Mean±SD | Median
Control | 24.5±4.7 | 24
C3-treated | 27.5±6.1 | 27

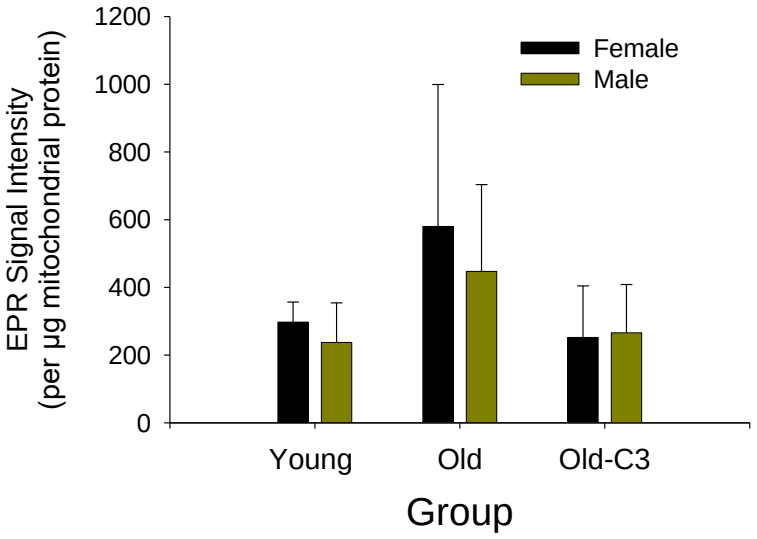

Male Survival
Start Treatment
Control (n=62)
C3-treated (n=56)
Survival (%)
Age (months)
Group | Mean±SD | Median
Control | 25.6±4.7 | 27
C3-treated | 27.3±4.5 | 28


DG
CA3
CA1
SN
CTX
Female
Old
Young
Male
Old
Young


FEMALE
Ethidium Fluorescence (Pixel Intensity)
250
200
150
100
50
0
SN
DG
CA3
CTX
CA1
WM
Old (n=6)
Young (n=7)


MALE
Ethidium Fluorescence (Pixel Intensity)
250
200
150
100
50
0
SN
DG
CA3
CTX
CA1
WM
Old (n=6)
Young (n=7)



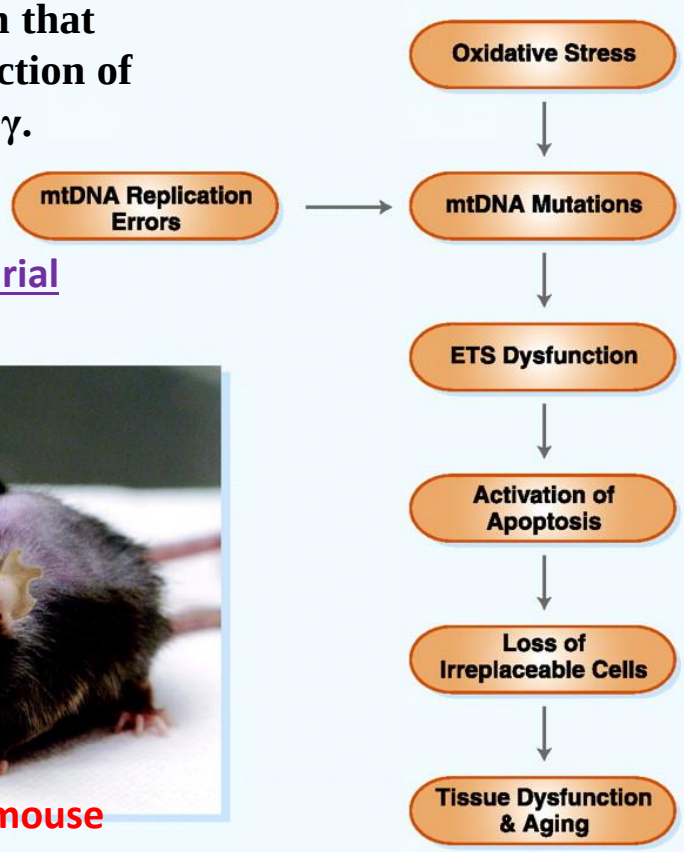


Although cellular ROS level is increased, mitochondria are not fully responsible!!

mtDNA mutations accumulate in natural aging potentially as a result of free radical-induced oxidative damage or nucleotide misincorporation during replication, the latter being particularly important in the D257A mice.

D257A mouse: knockin mutation that inactivates the proofreading function of mitochondrial DNA polymerase γ.

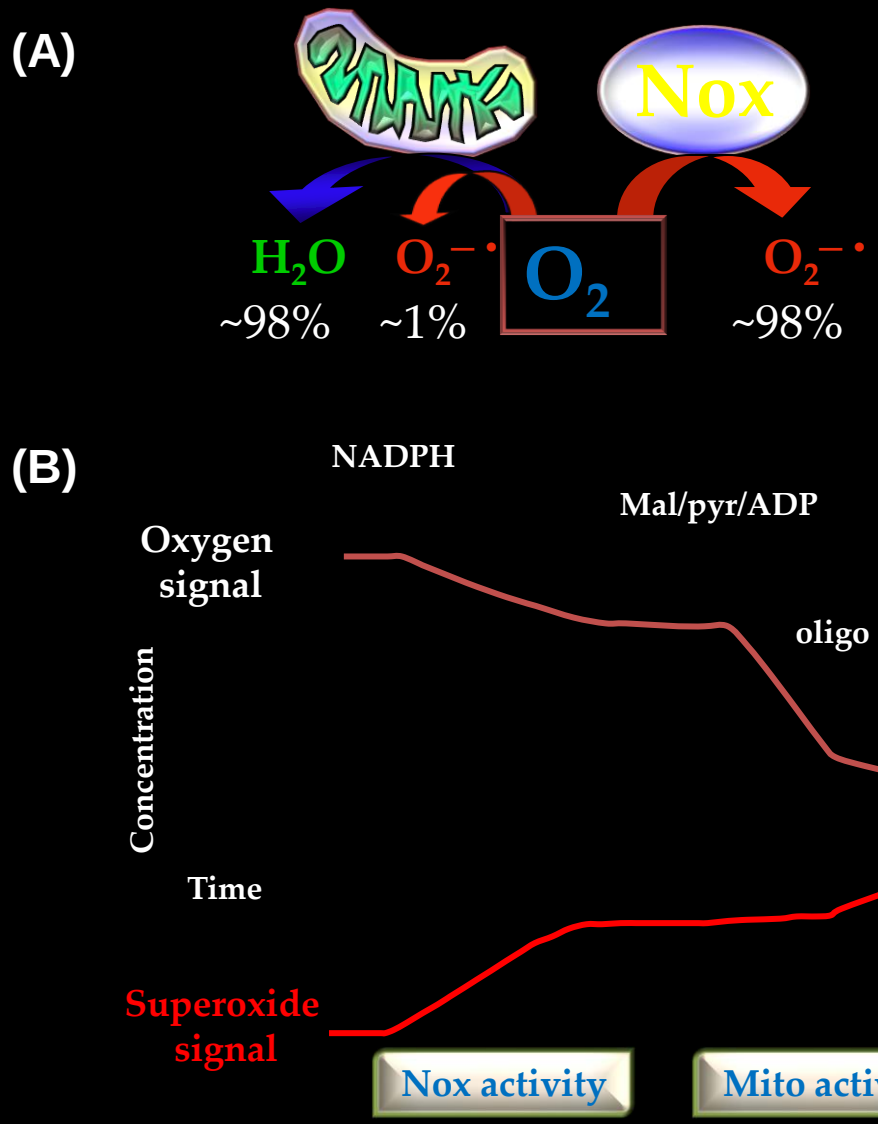
No signs for increased mitochondrial oxidative stress in D257A mice.



Kujoth G C et al. Cancer Res 2006;66:7386-7389

If mitochondria are NOT the main sources of ROS, what is it?!

Mitochondria are not the major ROS source!



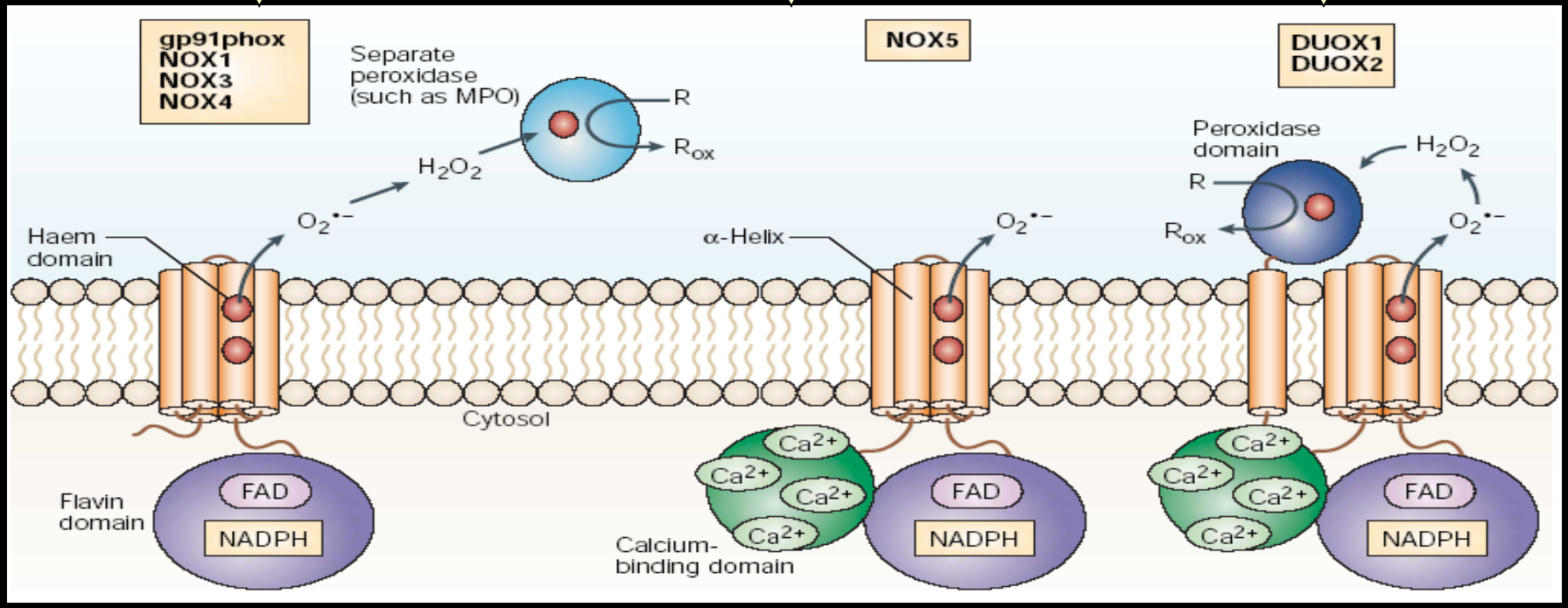
Dugan, Behrens, and Ali, *Oxidative Stress in Hypoxic-Ischemic Brain Injury* 2009

Known 3-Types of NADPH oxidases

- NOX2 (gp91^{phox}) family.
- Almost identical in size and structure.
- The enzymes differ in their regulatory factors.
- NOX1 : Colon and vascular smooth muscle.
- NOX2 : Phagocytes.
- NOX3 : Fetal Kidney.
- NOX4 : Widespread; e.g. kidney, ovary, eye, etc.

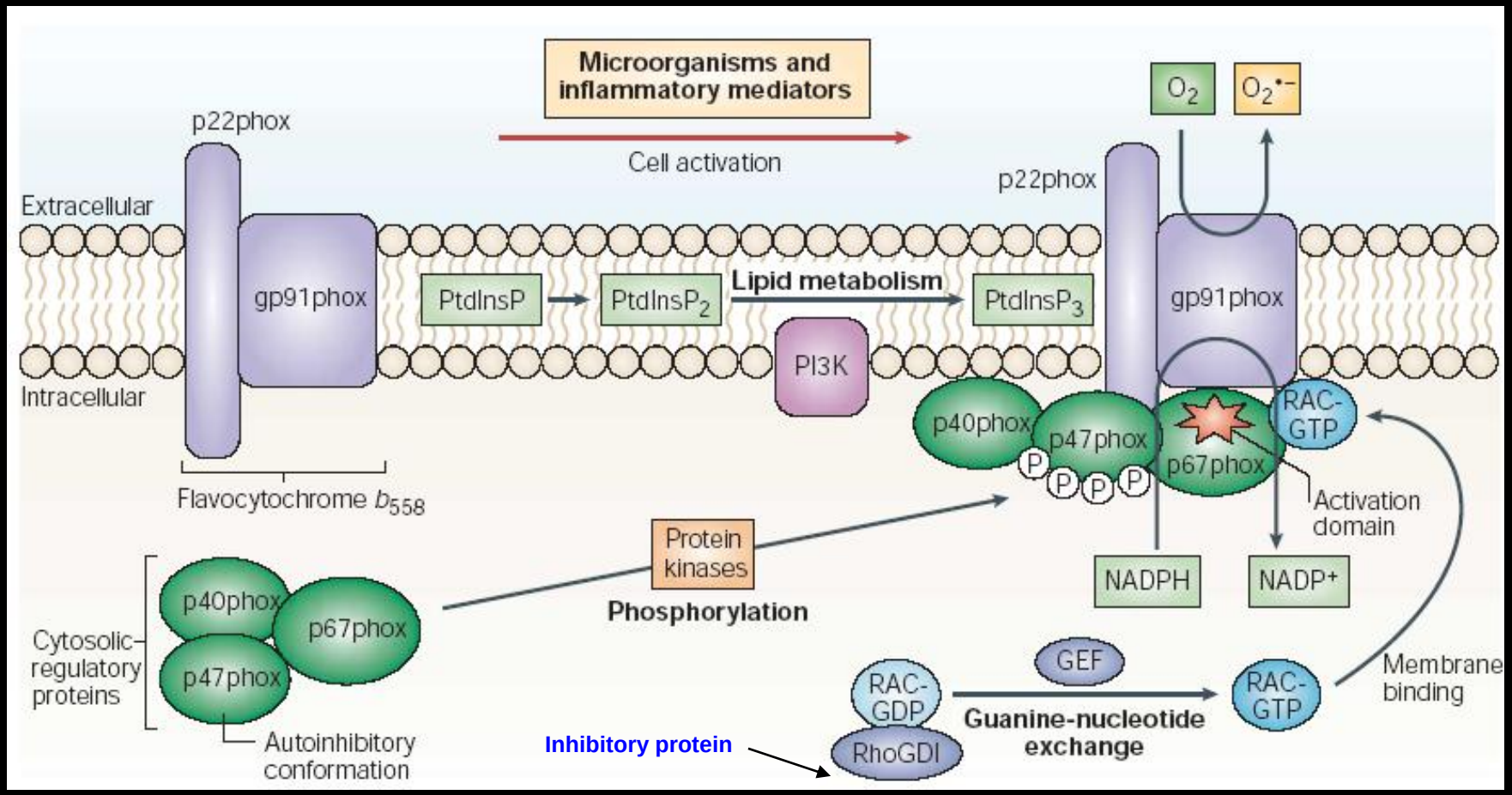
- NOX5 builds on the basic structure of gp91^{phox}, adding an amino terminal calcium binding unit.
- The Ca binding exposes the hydrophobic domains that bind to and regulate the activity of NOX5.
- NOX5 : Spleen, sperm, mammary glands, and cerebrum.

- DUOX enzyme family further extends the NO5 structure by adapting a peroxidase domain on the extracellular face of the plasma membrane.
- It appears that the dual, and paradoxically contrasting functions of DUOX enzymes is to oxidize an extracellular co-substrate such as extracellular matrix proteins.
- DUOX : Colon, pancreatic islets and prostate

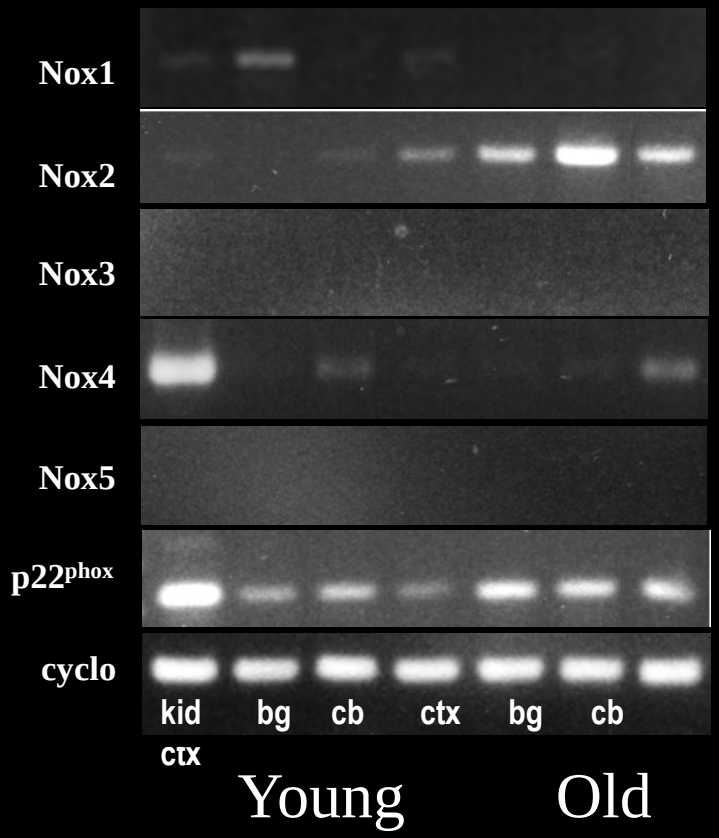


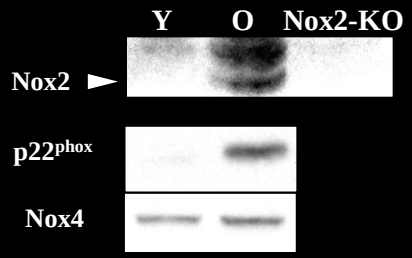
Lambeth, Nature Reviews 2004

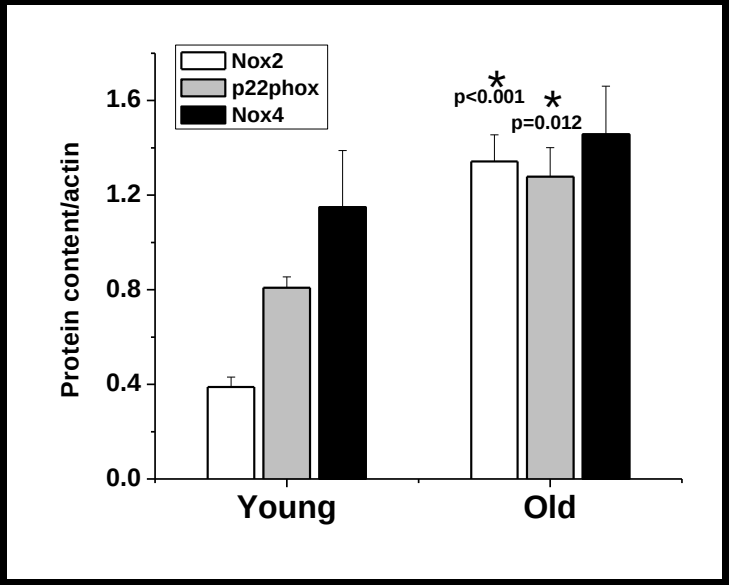
Activation of phagocytic NOX

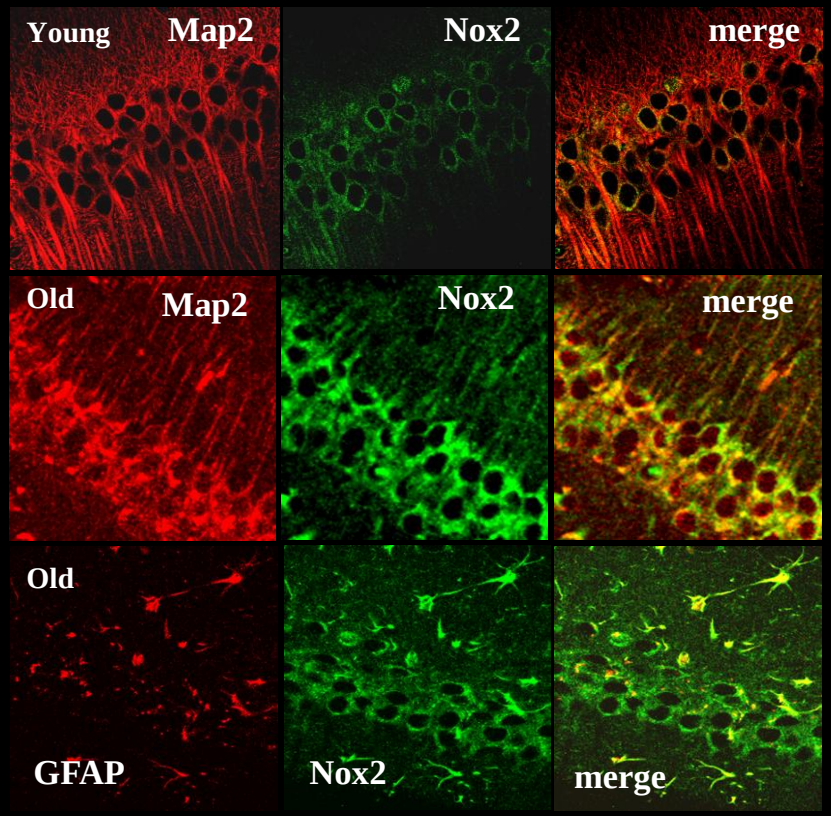


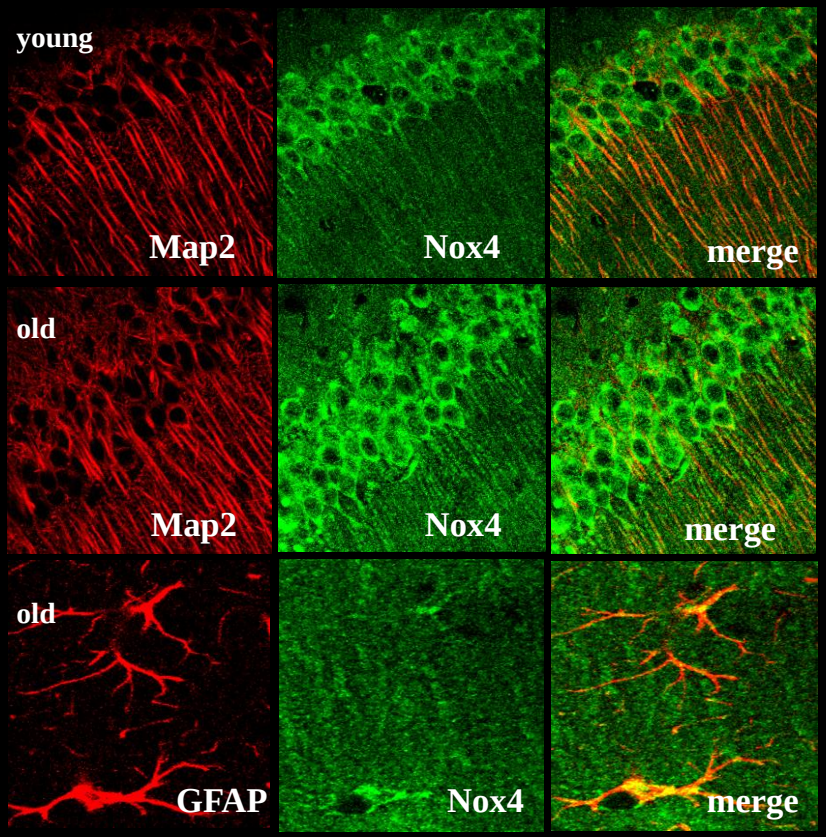
- **The flavocytochrome b558 is inactive in unstimulated phagocytes, but becomes activated after exposure of cells to microorganisms or inflammatory mediators as a result of assembly of cytosolic components.**
- **Exposure of cells to microorganisms or inflammatory mediators initiates three molecular triggers: Protein phosphorylation, lipid metabolism, and guanine-nucleotide exchange.**
- **NOX4 is constitutively active and may not require subunits for further activation.**


Nox1
Nox2
Nox3
Nox4
Nox5
p22phox
cyclo
kid
bg
cb
ctx
bg
cb
ctx
Young
Old


Y
O
Nox2-KO
Nox2
p22phox
Nox4

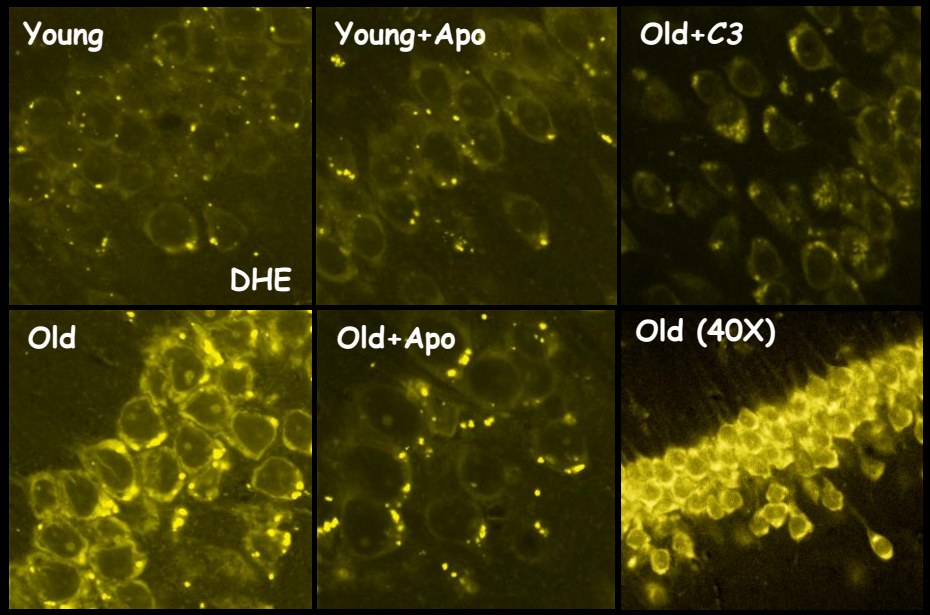

Nox2
p22phox
Nox4
Protein content/actin
1.6
1.2
0.8
0.4
0.0
*
p<0.001
*
p=0.012
Young
Old

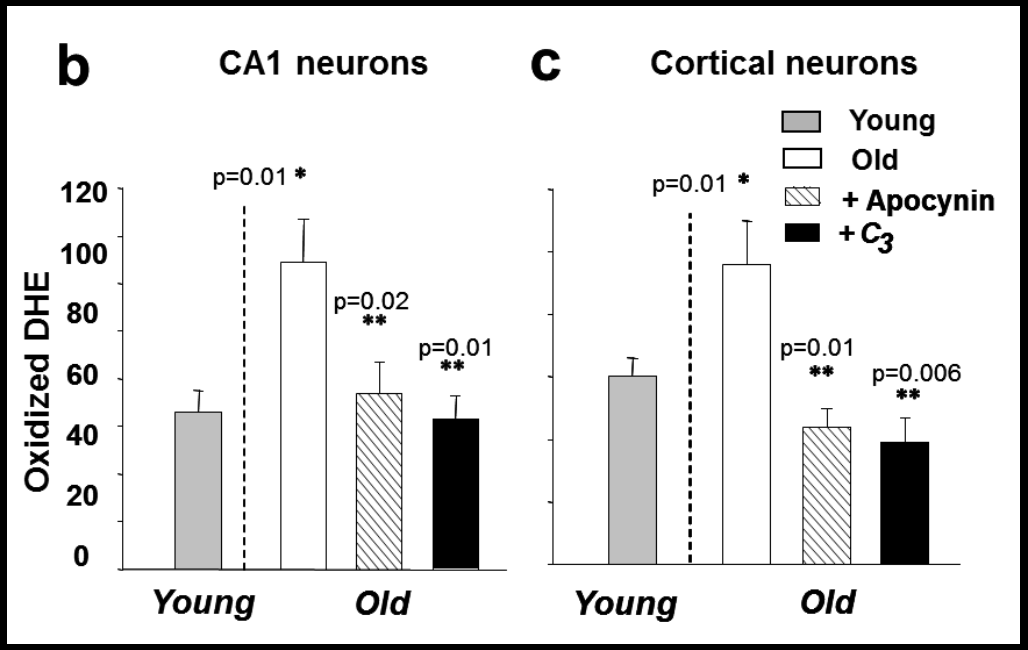

Young
Map2
Nox2
merge
Old
Map2
Nox2
merge
Old
GFAP
Nox2
merge


young
Map2
Nox4
merge
old
Map2
Nox4
merge
old
GFAP
Nox4
merge

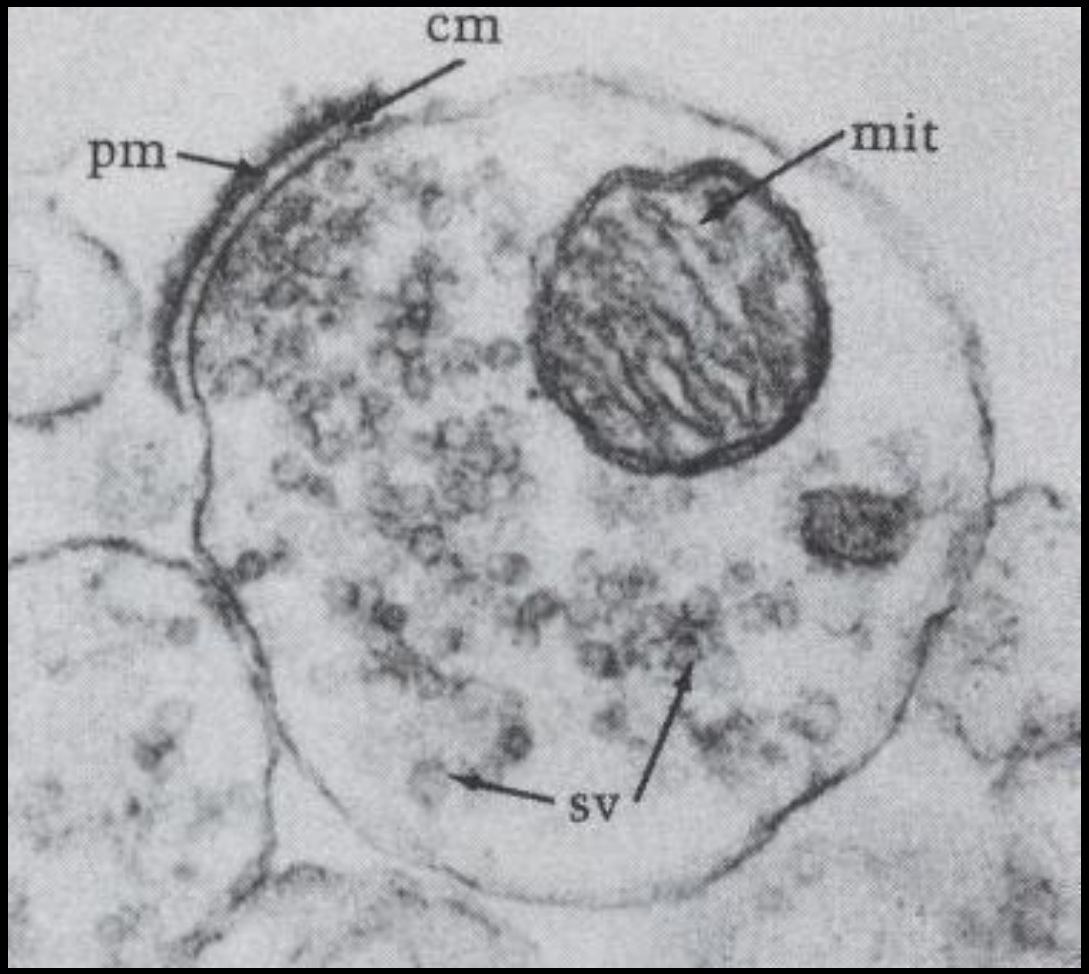
Nox2

Nox4

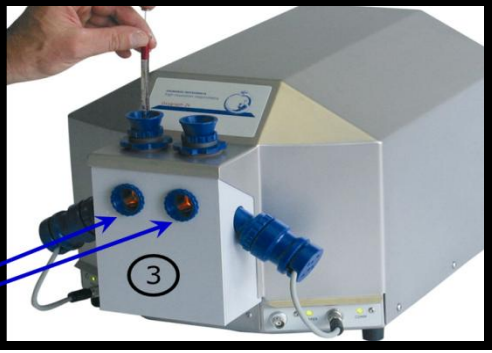

Young
Young+Apo
Old+C3
DHE
Old
Old+Apo
Old (40X)


b
CA1 neurons
c
Cortical neurons
Young
Old
+ Apocynin
+ C3
Oxidized DHE
120
100
80
60
40
20
0
p=0.01 *
p=0.02
**
p=0.01
**
p=0.01 *
p=0.01
**
p=0.006
**
Young
Old
Young
Old

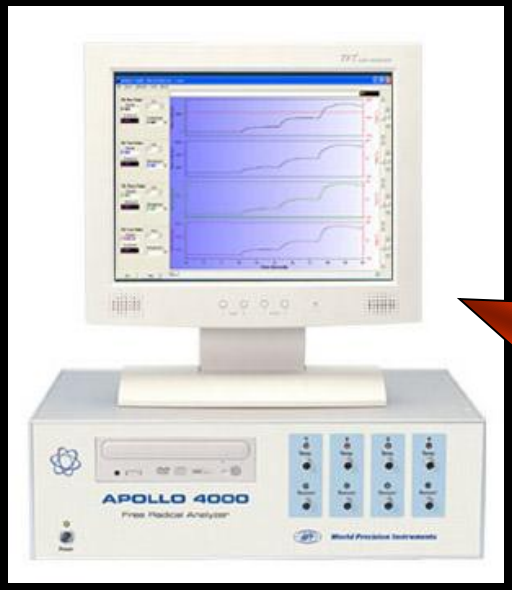
Synaptosomes as neuronal model



Techniques to study metabolism and ROS



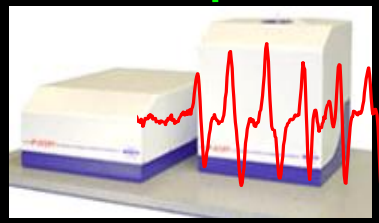
Mitochondria or Synaptosomes



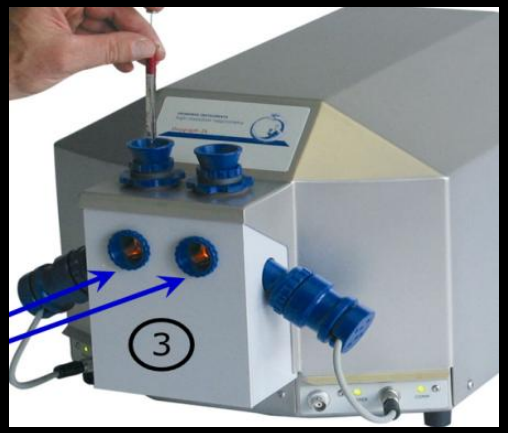
The WPI ROS analyzer
Real-time detection of:

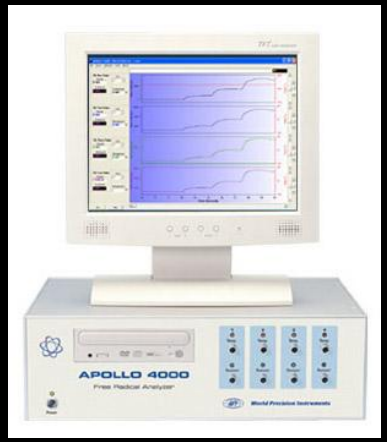
- H_2O_2
- ATP
- O_2
- NO

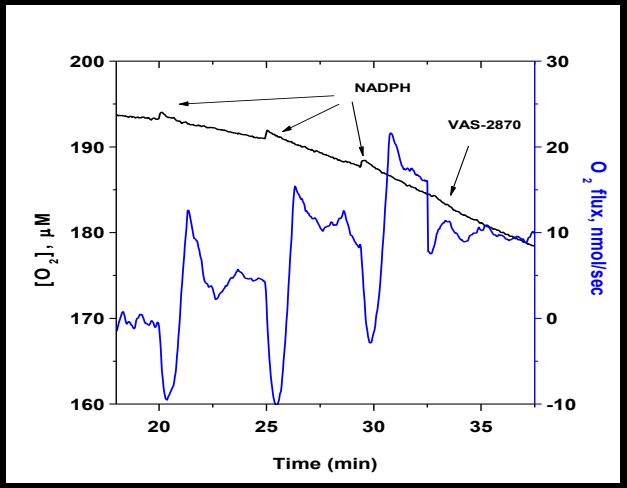
Oxygraph

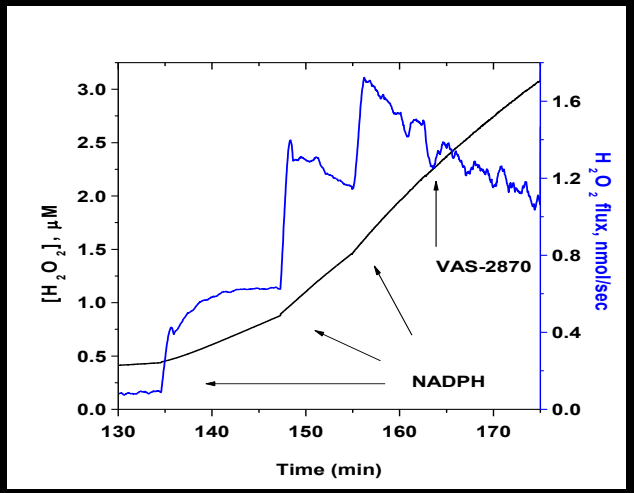


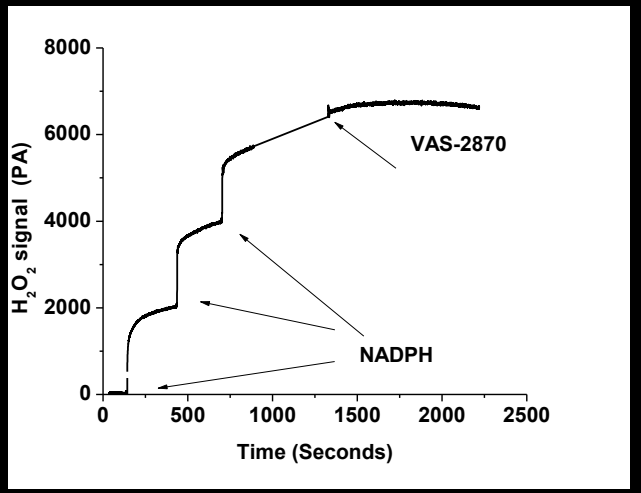
EPR

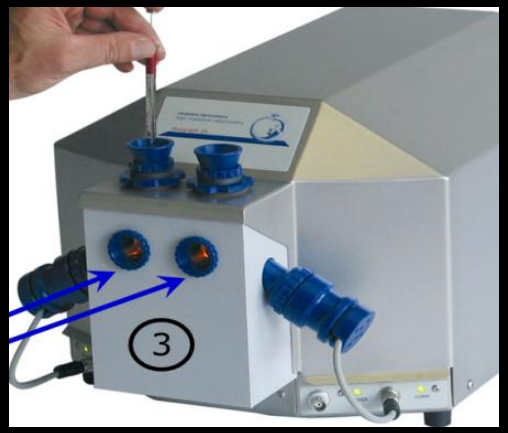

3

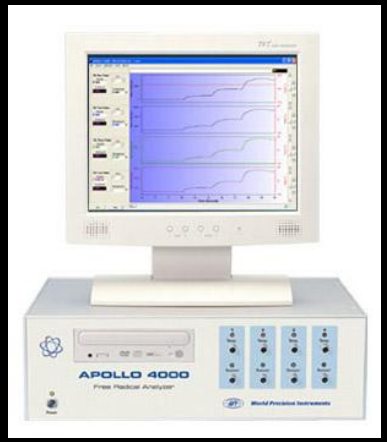

APOLLO 4000
Free Radical Analyzer

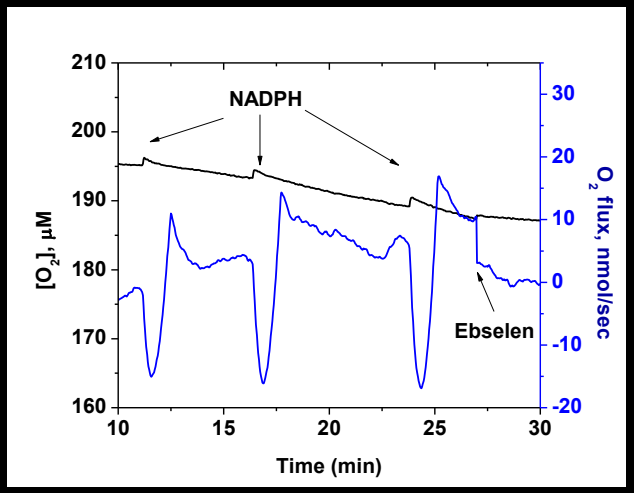

NADPH
VAS-2870
[O2], µM
O2 flux, nmol/sec
Time (min)

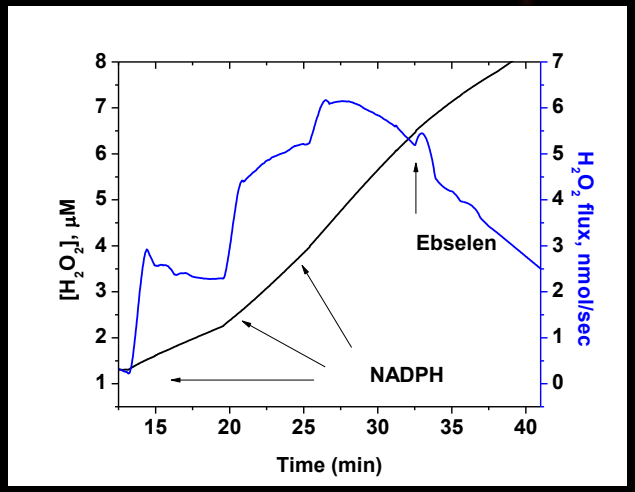

VAS-2870
NADPH
[H2O2], µM
H2O2 flux, nmol/sec
Time (min)

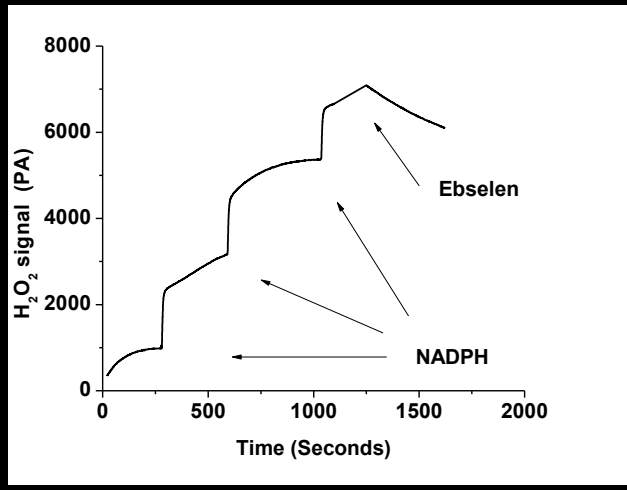

VAS-2870
NADPH
H2O2 signal (PA)
Time (Seconds)

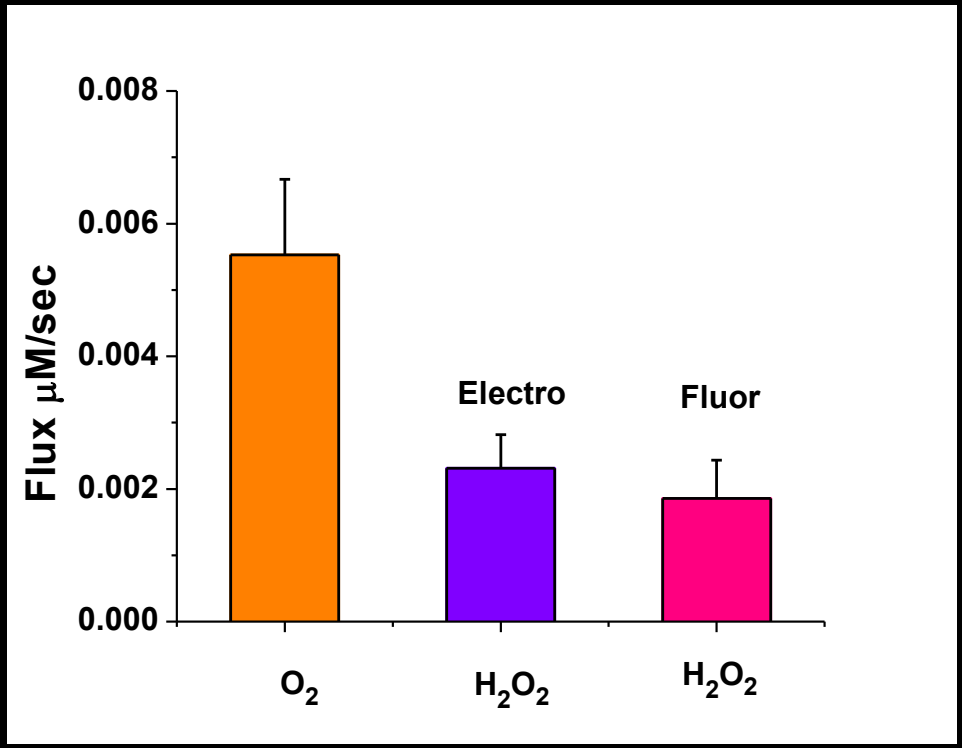

3


APOLLO 4000
Free Radical Analyzer

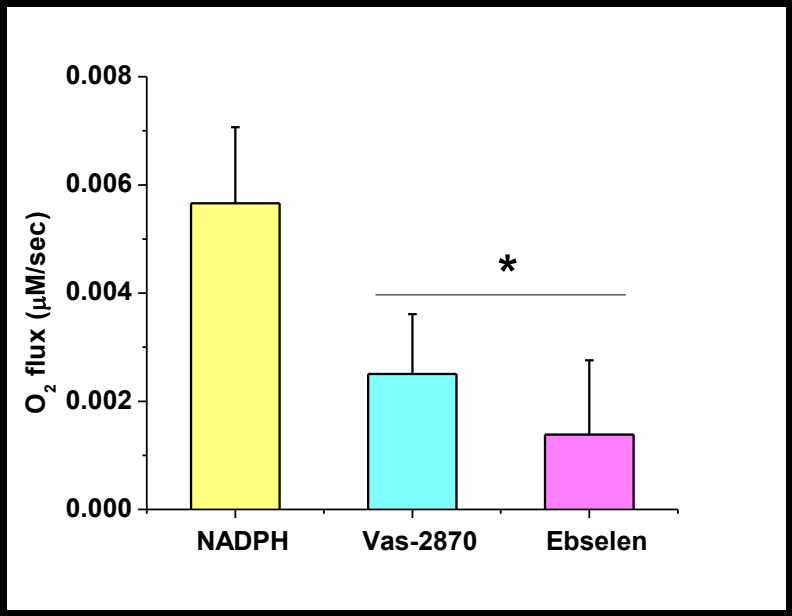

NADPH
Ebselen
[O2], µM
O2 flux, nmol/sec
Time (min)
210
200
190
180
170
160
30
20
10
0
-10
-20
10
15
20
25
30

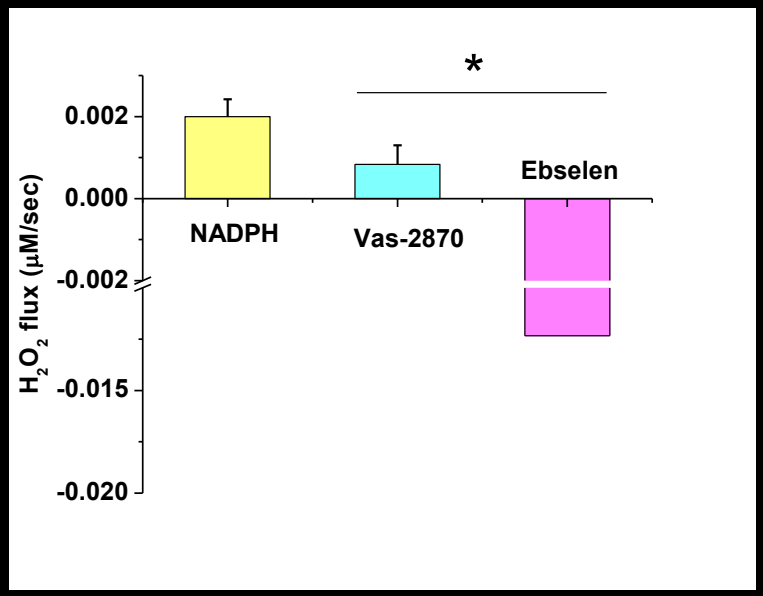

Ebselen
NADPH
[H2O2], µM
H2O2 flux, nmol/sec
Time (min)
8
7
6
5
4
3
2
1
0
15
20
25
30
35
40


Ebselen
NADPH
H2O2 signal (PA)
Time (Seconds)
8000
6000
4000
2000
0
500
1000
1500
2000



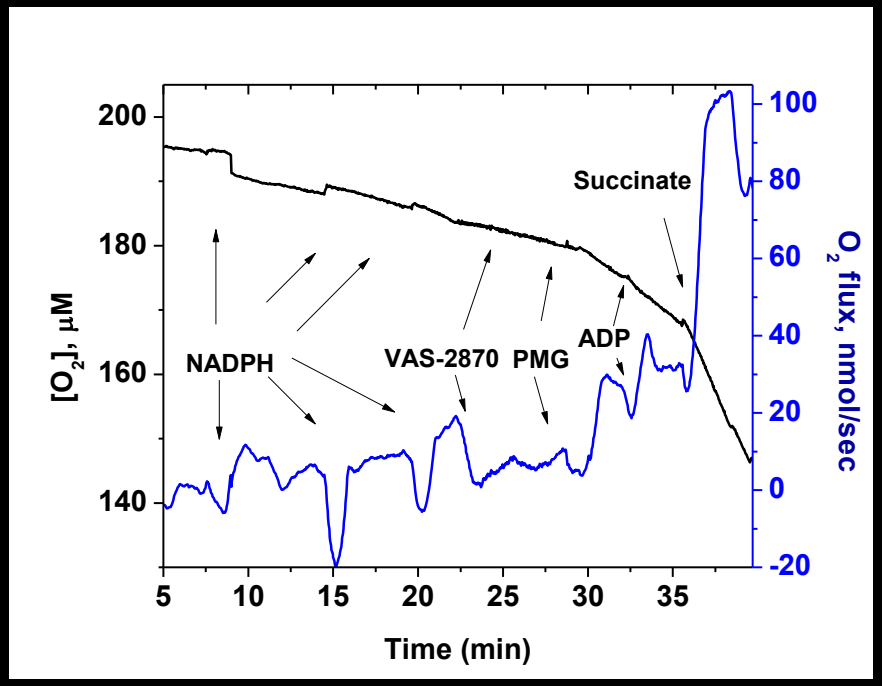
1. **The hydrogen peroxide production by synaptosomal NOX didn't significantly differ between the two experimental approaches.**
2. **38.85 % of oxygen consumed by synaptic NOX is converted to hydrogen peroxide.**

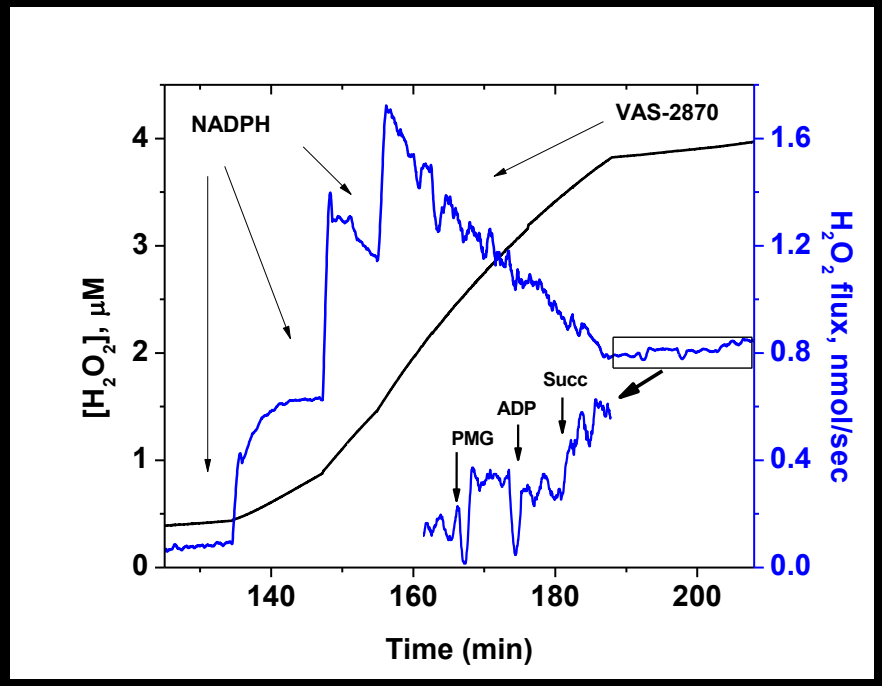


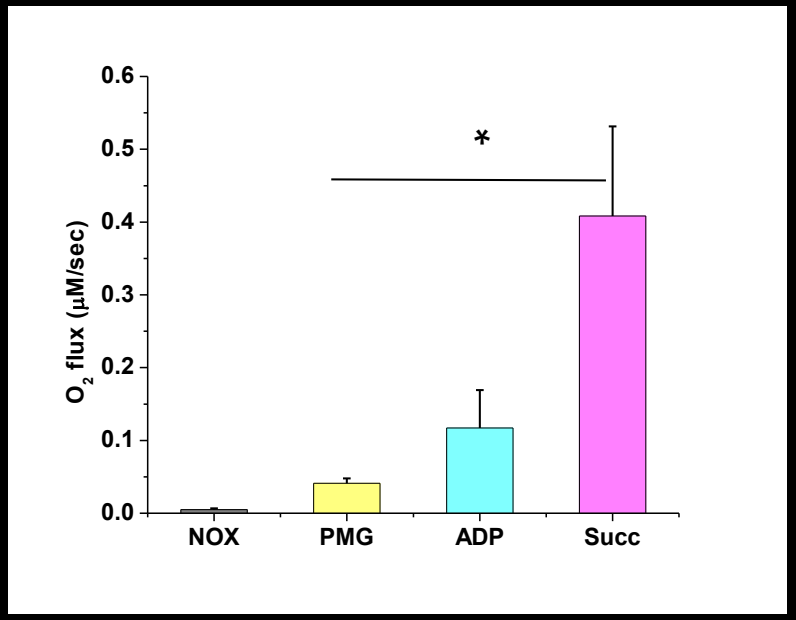


1. 10-μM VAS-2870 inhibited ~50% of NADPH-induced activity whether recorded as oxygen consumption or as H_2O_2 production.
2. Meanwhile, 10 μM ebselen quenched ~75% of NADPH-induced oxygen consumption while completely reversing H2O2 signal

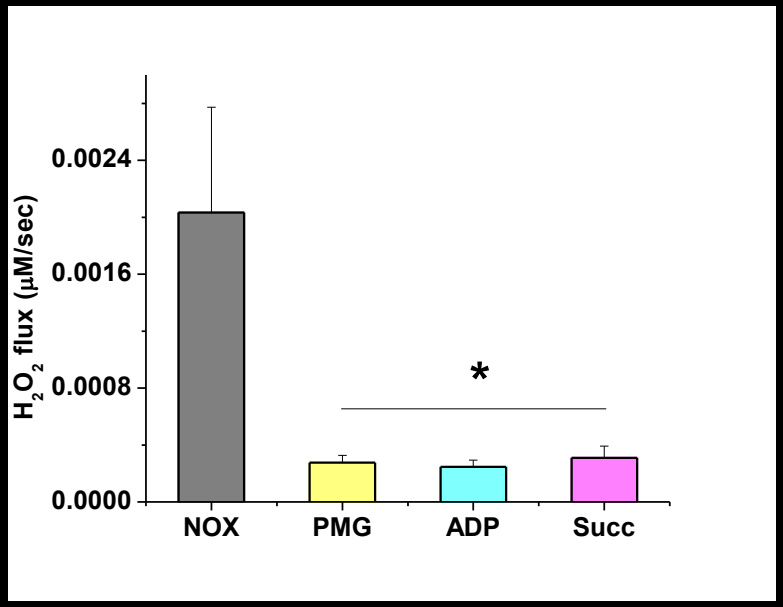
Synaptic NOX vs. Synaptic mitochondria

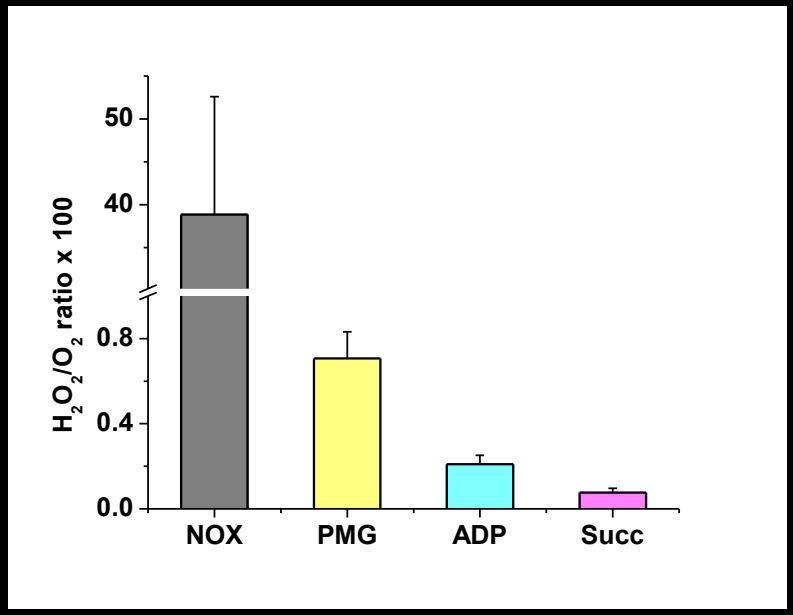






NADPH oxidases are minor oxygen consumers but major hydrogen peroxide producers in synaptosomes



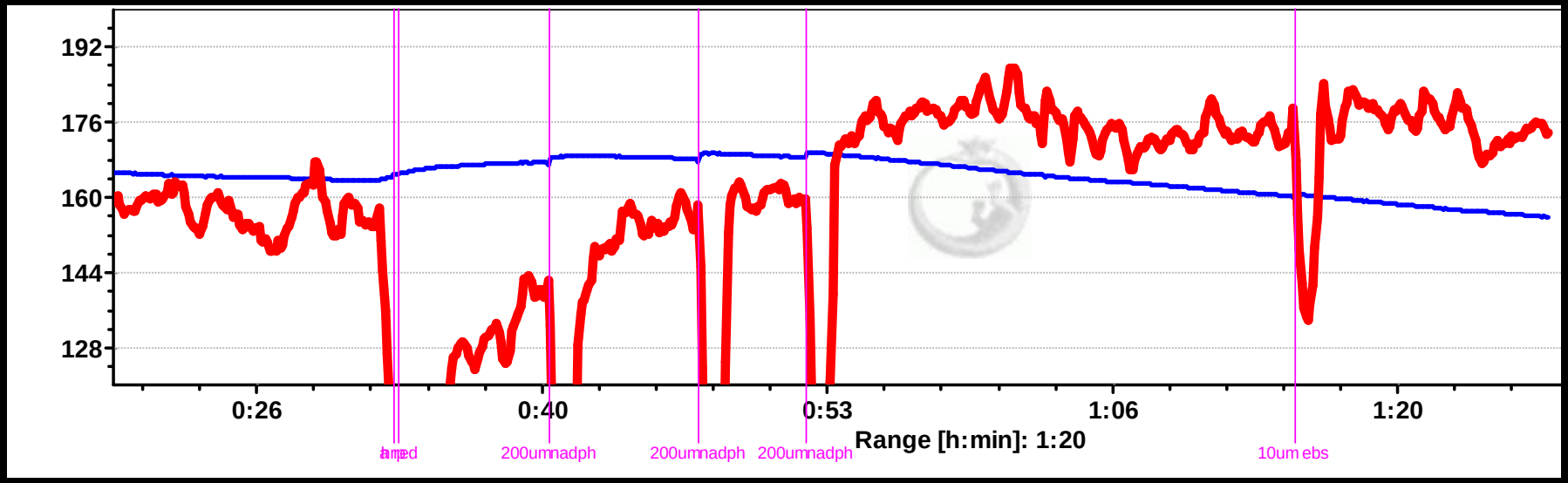


Challenges for Evaluation of Experimental Protocols

- **Fluorescence background and residual oxygen consumption due to NAD(P)H interaction with the HRP/Amplex Red.**
- **In the absence of added synaptosomes, the addition of NADPH alone in our AR/HRP assay resulted in enhanced fluorescence as well as oxygen consumption.**

Solution:

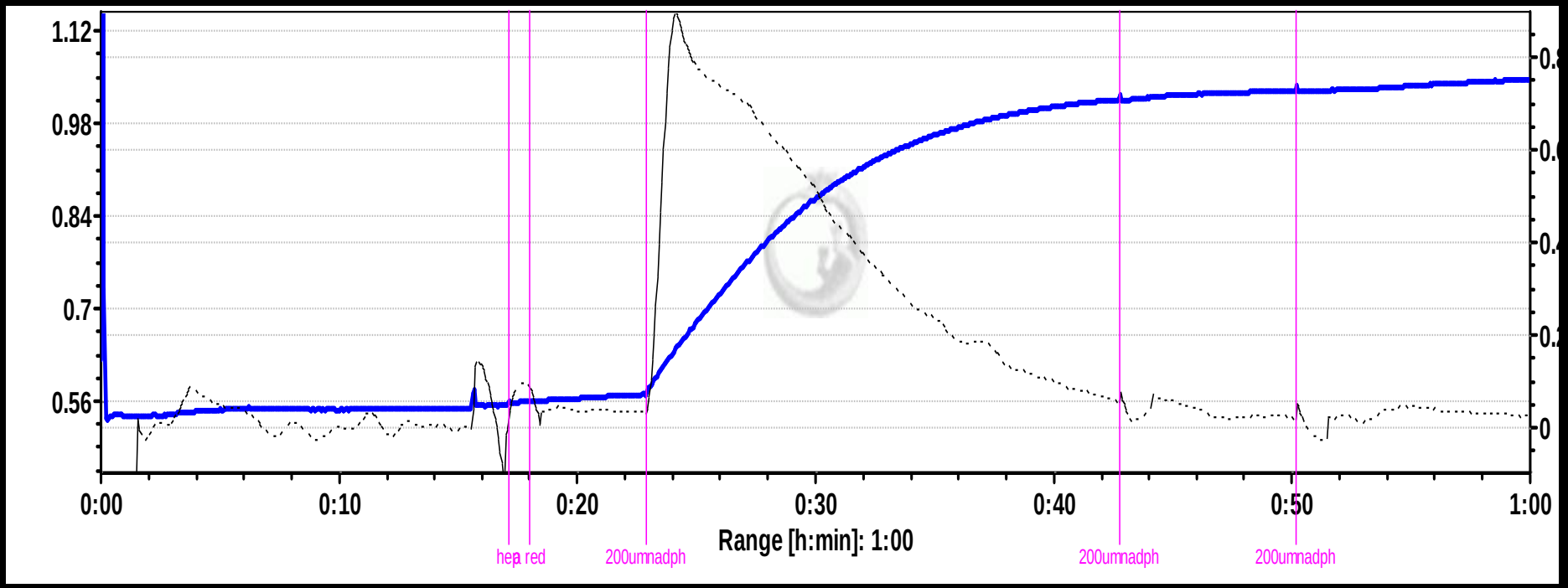
- **Reduce NADPH concentration from 5 mM to 200 µM!**



This increase in background fluorescence was far less than the resorufin fluorescence detected in the presence of synaptosomes.

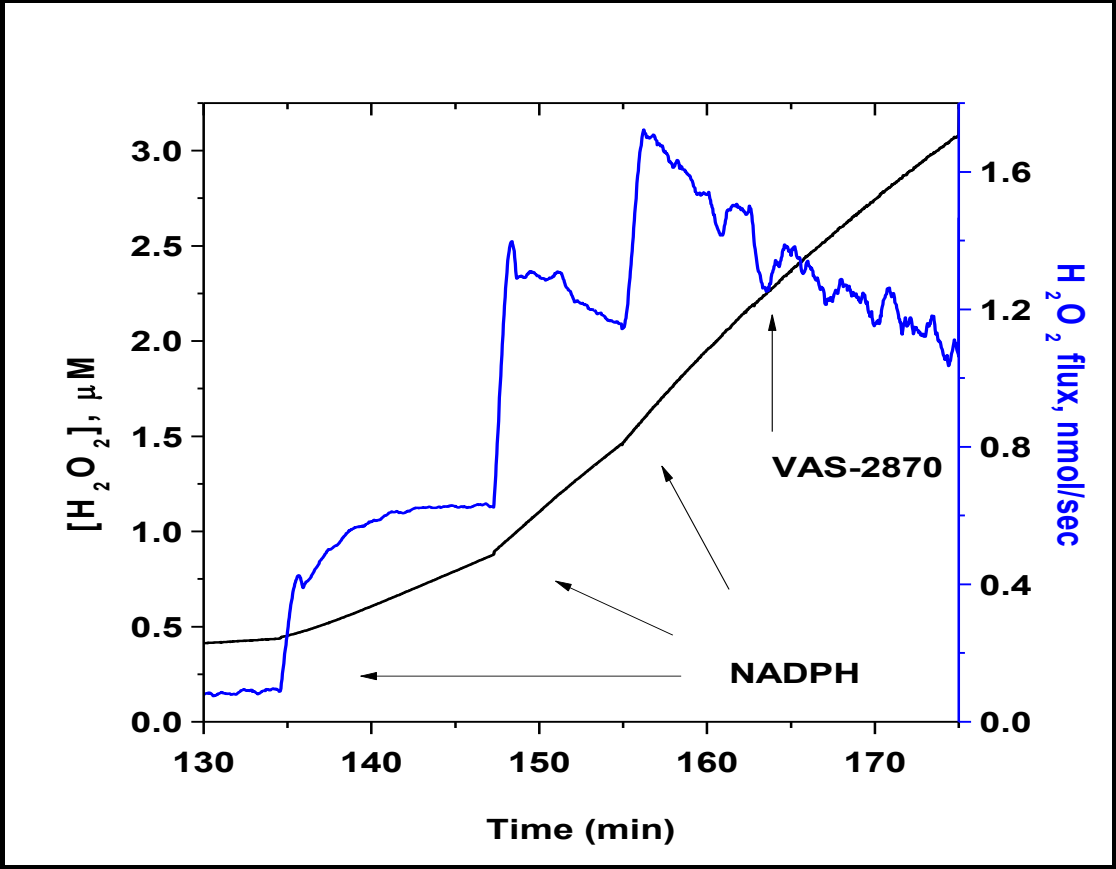
Challenges for Evaluation of Experimental Protocols

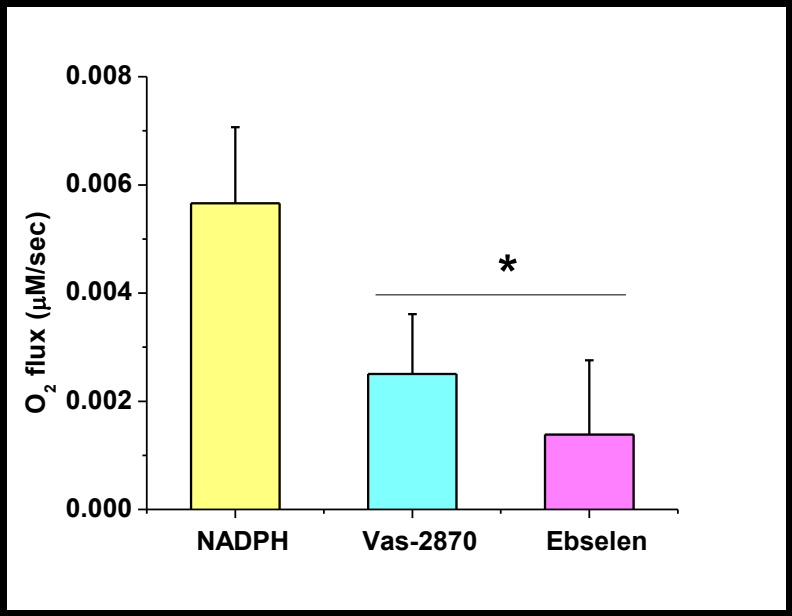
- Fast consumption the Amplex Red dye in the presence of NADPH.

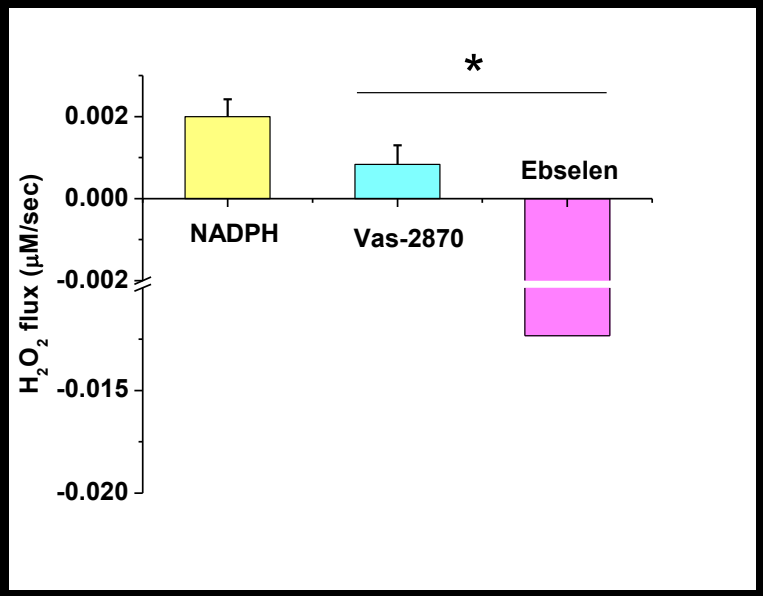


Solution:

- Replace Amplex Red with Amplex Ultra Red dye.







1. Ebselen quenched ~75% of NADPH-induced oxygen consumption while **completely reversing H2O2 signal** → in tune with reports showing that EBSELEN EXHIBITS **glutathione peroxidase activity**

Solution: Use VAS-2870 to confirm NOX-related activity.

Acknowledgments

- Dr. Ahmed Zewail

- Dr. Engy Abdel-Rahman
- Ali Mokhtar
- Abdullah Aaliya
- Yasmine Radwan
- Basma Yasseen
- Abdelrahman Al-Okda
- Ahmed Atwa
- Eslam Elhanafy
- Moaaz Habashy

- Egypt Science and Technology Development Fund