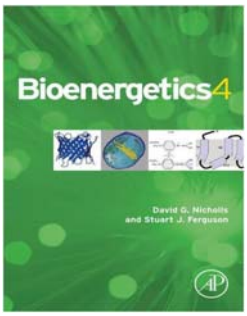
Mitochondrial ROS Generation

UNIVERSITY of ROCHESTER MEDICAL CENTER

Paul S. Brookes, PhD.
www.psblab.org



Sections
4.7 & 9.10

Biochem. J. (2009) **417**, 1–13 (Printed in Great Britain) doi:10.1042/BJ20081386

REVIEW ARTICLE

How mitochondria produce reactive oxygen species

Michael P. MURPHY[1]

Basic Free Radical / ROS Nomenclature

Free Radical: Species with one or more unpaired electrons that is free to partake in chemical bonds. Denoted by $^{\bullet}$ before the charge:

e.g. $NO^{\bullet}$ $O_2^{\bullet -}$

Reactive oxygen species (ROS): Species derived from reduction of molecular dioxygen (O_2), which may or may not be radicals:

Step-wise reduction of O_2

$$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow OH^{\bullet} \rightarrow H_2O$$

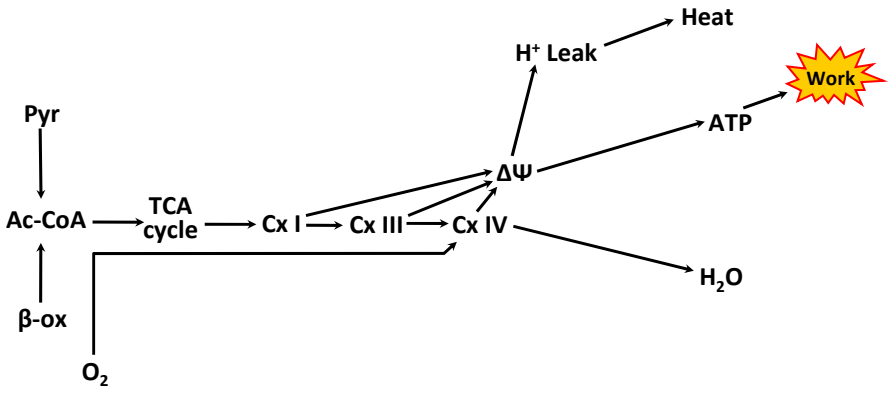
Superoxide anion radical — Hydrogen peroxide — Hydroxyl radical

pKa~5 ⇌ $HO_2^{\bullet}$ (Hydroperoxyl radical)

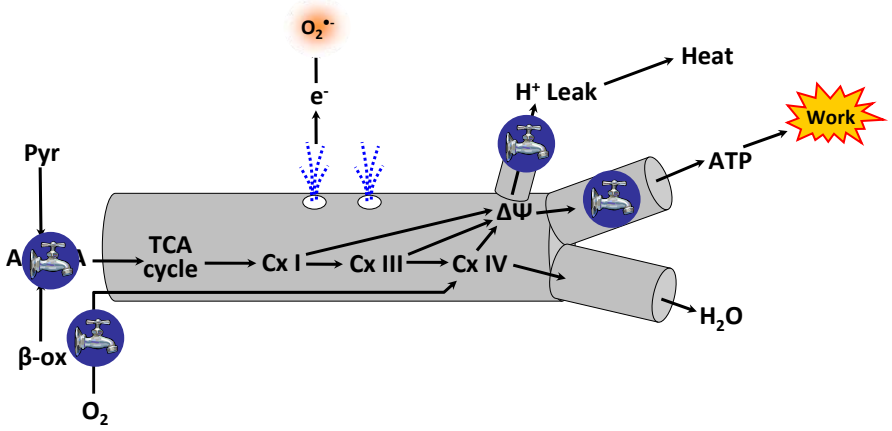
A note about **OXYGEN**

Atmospheric pO_2	~ 160mmHg (21% of 760)
Arterial	~ 100
Tissue	~ 10
Intracellular	~ 0.5-1.0

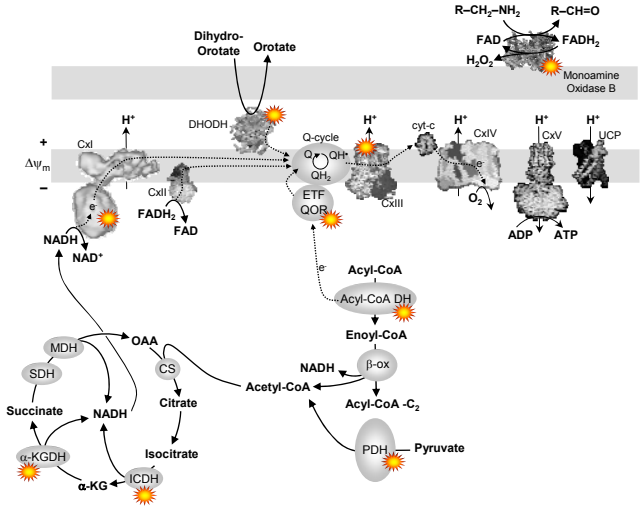
Mito' pathways of electron (energy) flow



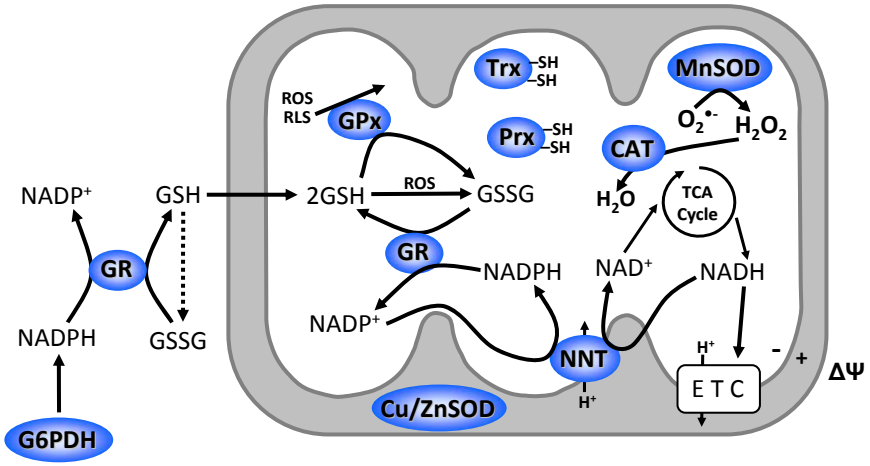
Leaky Electron Pipes



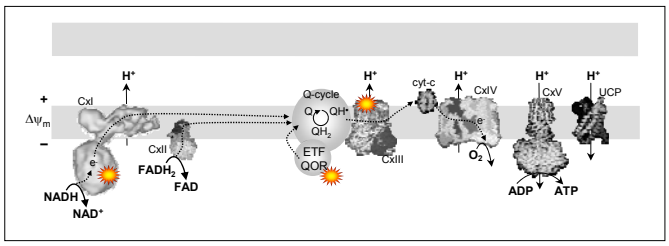
Mitochondrial Sources of ROS

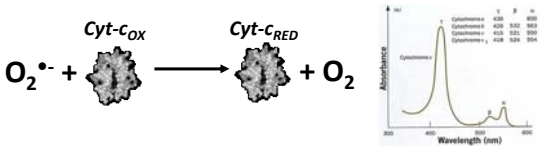


Mitochondrial Antioxidants



$O_2^{\bullet-}$ reduces cytochrome *c*





1312 Biochemical Society Transactions (2003) Volume 31, part 6

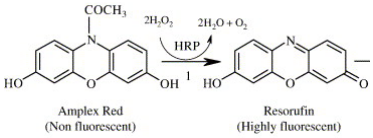
Cytochrome *c*, an ideal antioxidant

M.O. Pereverzev, T.V. Vygodina, A.A. Konstantinov and V.P. Skulachev[1]

How can we measure mito' ROS

Small molecule ROS probes (DCF, DHE, Amplex red, mitoSOX etc.)

- Accumulation of product
- Kinetic information



HUGELY artifact prone…

- DCF fluo' is dependent on free iron (Fenton chemistry?)
- DHE → HE, fluo' enhanced upon DNA binding
- Many other reactive species (e.g. ONOO-) can convert DHE>>HE

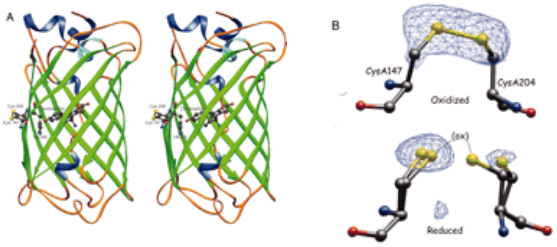
Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations

Balaraman Kalyanaraman [a,*], Victor Darley-Usmar [b], Kelvin J.A. Davies [c,d], Phyllis A. Dennery [e], Henry Jay Forman [c,f], Matthew B. Grisham [g], Giovanni E. Mann [h], Kevin Moore [i], L. Jackson Roberts II [j], Harry Ischiropoulos [e]

Free Radical Biology & Medicine 52 (2012) 1–6

Genetically encoded probes (roGFP, cpYFP etc.)

JBC (2004) **279:**13044-13053



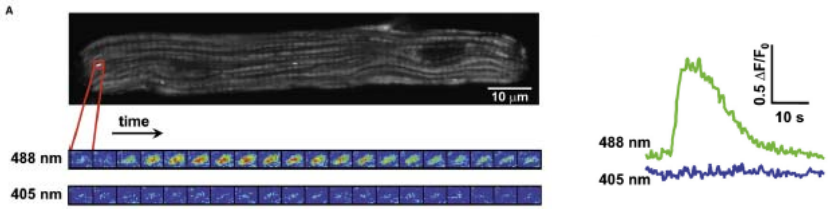
- Steady-state reduction status of cysteine couple
- No kinetics
- Does not "measure ROS"

"Superoxide Flashes"

Superoxide Flashes in Single Mitochondria

Wang Wang,[1,*] Huaqiang Fang,[4] Linda Groom,[5] Aiwu Cheng,[2] Wanrui Zhang,[4] Jie Liu,[4] Xianhua Wang,[4] Kaitao Li,[4] Peidong Han,[4] Ming Zheng,[4] Jinhu Yin,[3] Weidong Wang,[3] Mark P. Mattson,[2] Joseph P.Y. Kao,[6] Edward G. Lakatta,[1] Shey-Shing Sheu,[5] Kunfu Ouyang,[7] Ju Chen,[7] Robert T. Dirksen,[5] and Heping Cheng[4,*]

Cell *134*, 279–290, July 25, 2008



Letter to the Editor

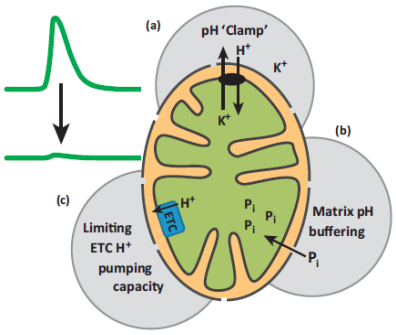
A critical evaluation of cpYFP as a probe for superoxide

Florian L. Muller Free Radical Biology & Medicine 47 (2009) 1779–1780

Mitochondrial 'flashes': a radical concept repHined

Trends Cell Biol. (2012) **22:** 503-508

Markus Schwarzländer[1], Michael P. Murphy[2], Michael R. Duchen[3], David C. Logan[4], Mark D. Fricker[5], Andrew P. Halestrap[6], Florian L. Müller[7], Rosario Rizzuto[8], Tobias P. Dick[9], Andreas J. Meyer[1] and Lee J. Sweetlove[5]



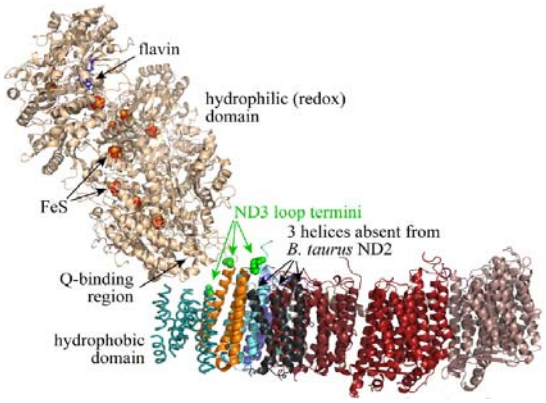
- Entire signal can be accounted for by an alkaline pH shift of 0.2-0.4 units.
- Limiting pH shifts (by pH clamp, enhancing buffering, or limiting ETC activity) eliminates flashes.
- "Superoxide flashes" more likely transient uncoupling/PTP opening.

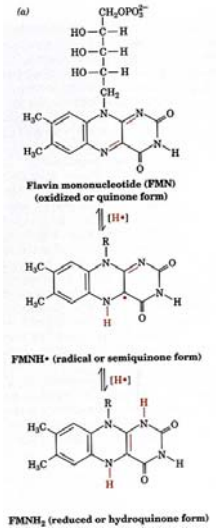
The 'mitoflash' probe cpYFP does not respond to superoxide

E12 | NATURE | VOL 514 | 23 OCTOBER 2014

Markus Schwarzländer[1], Stephan Wagner[1], Yulia G. Ermakova[2], Vsevolod V. Belousov[2], Rafael Radi[3], Joseph S. Beckman[4], Garry R. Buettner[5], Nicolas Demaurex[6], Michael R. Duchen[7], Henry J. Forman[8,9], Mark D. Fricker[10], David Gems[11], Andrew P. Halestrap[12], Barry Halliwell[13], Ursula Jakob[14], Iain G. Johnston[15], Nick S. Jones[15], David C. Logan[16], Bruce Morgan[17], Florian L. Müller[18], David G. Nicholls[19], S. James Remington[20], Paul T. Schumacker[21], Christine C. Winterbourn[22], Lee J. Sweetlove[10], Andreas J. Meyer[1], Tobias P. Dick[17] & Michael P. Murphy[23]

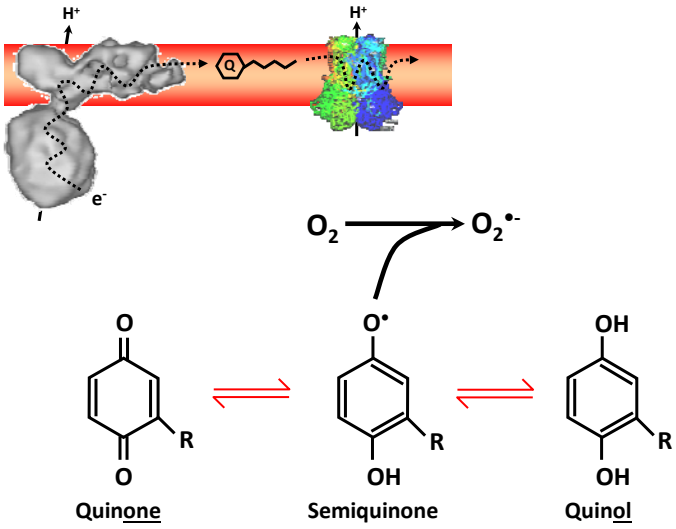
ROS from Complex I - Flavin

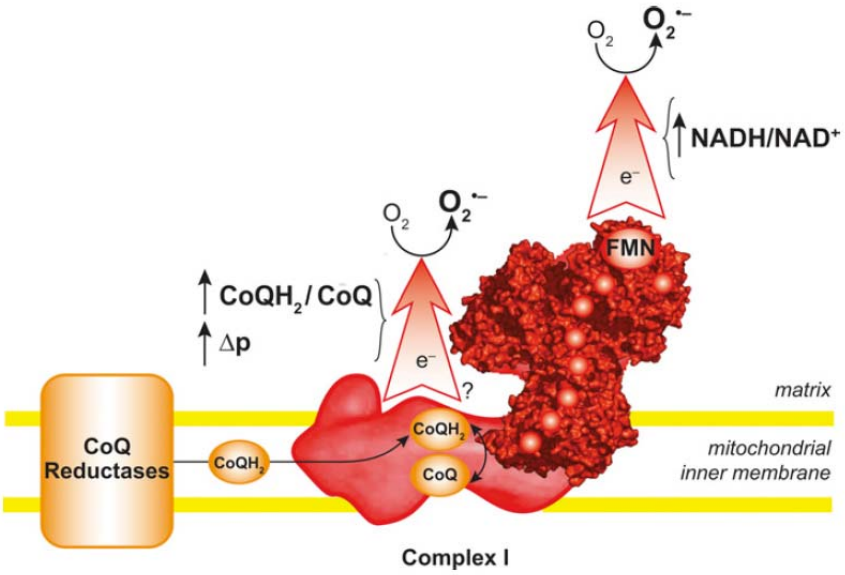




Hirst, JBC 2012

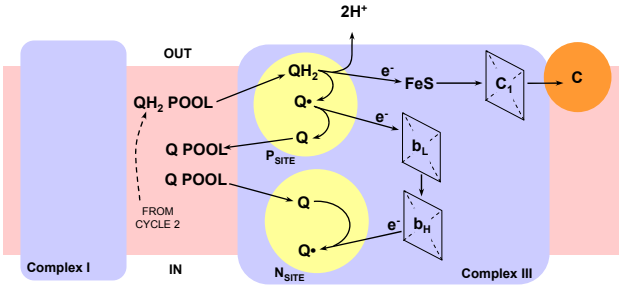
ROS at Complex III (& CxI?) from Q_{10}





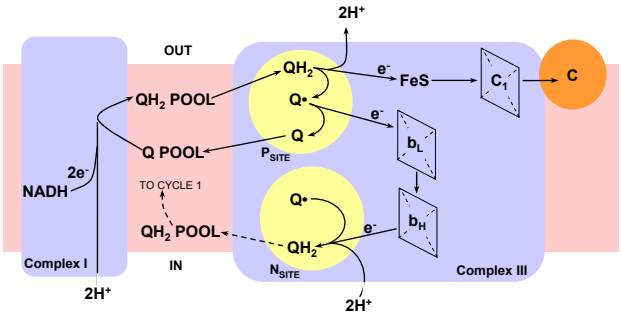
Murphy Biochem J 2009

The Q Cycle @ Complex III



Cycle 1

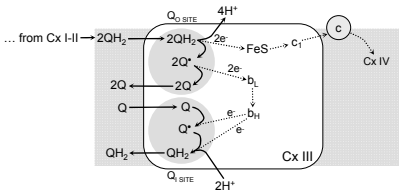
- QH_2 in pool goes to P site, gives $2H^+$ to outside & 1 e^- to the Fe-S, making Q•
- Q• gives 2nd e^- to cyt-b_L , making Q, which leaves P site
- e^- passes from cyt-b_L to cyt-b_H
- Q from pool receives e^- from cyt-b_H at N site, making Q•



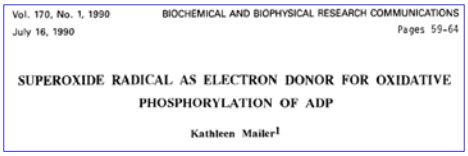
Cycle 2

- Cx I uses NADH to add $2e^-$ & $2H^+$ to Q from pool, making QH_2
- QH_2 enters P site as in cycle 1
- At N site, Q• leftover from cycle 1 receives 2nd e^- from P site via cyt-b
- $2H^+$ taken up at N site to make QH_2, which migrates to pool, used in cycle 1

ROS at CxIII Qo site = Uncoupling



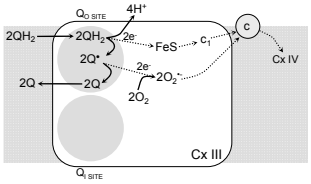
Normal Q cycle



Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 59-64

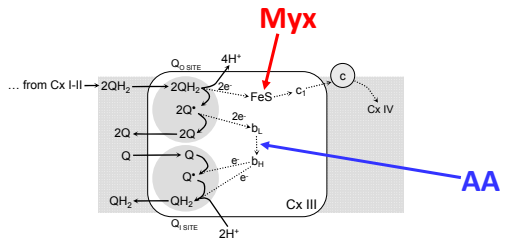
SUPEROXIDE RADICAL AS ELECTRON DONOR FOR OXIDATIVE PHOSPHORYLATION OF ADP

Kathleen Mailer[1]



$O_2^{\bullet-}$ Bypass

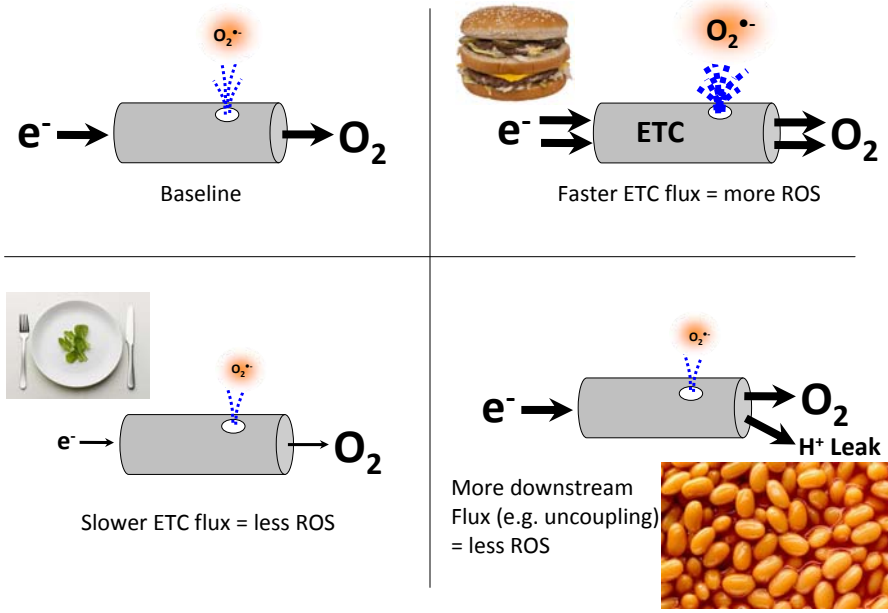
Complex III – different inhibitors



AA – Antimycin A, blocks at b-hemes, prevents ubisemiquinone at Qo site from passing on 2nd electron, so stabilizes radical at Qo → more ROS

Myx – Myxothiazol (N.B. Also inhibits CxI at higher doses), blocks Fe-S center, prevents passage of 1st electron and the generation of ubisemiquinone radical → less ROS

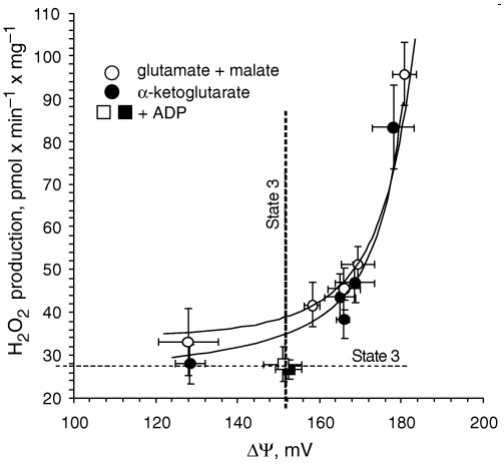
Regulation of Mito' ROS Generation



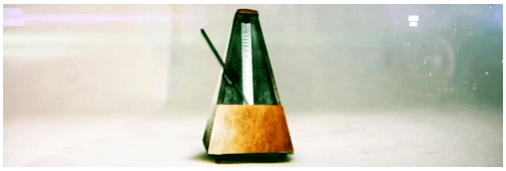
Regulation of Mito' ROS Generation

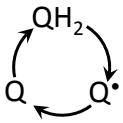
Transmembrane Proton Gradient

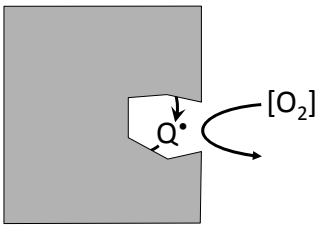
J. Neurochem. (2003) **86:** 1101-7



"Dwell Time" of Ubisemiquinone Radical







Faster flux, Q• appears more frequently, but for less time

Slower flux, Q• appears less often, but for longer

Slow… likelihood of one car falling off in loop-de-loop is greater, but fewer cars go through overall

Fast… each car less likely to fall off in loop, but more cars go through overall

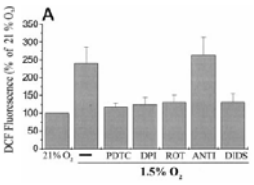
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 33, Issue of August 18, pp. 25130–25138, 2000
Printed in U.S.A.

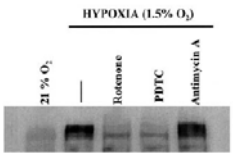
Reactive Oxygen Species Generated at Mitochondrial Complex III Stabilize Hypoxia-inducible Factor-1α during Hypoxia

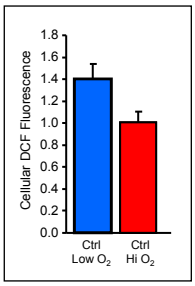
A MECHANISM OF O_2 SENSING*

Received for publication, March 7, 2000, and in revised form, May 22, 2000
Published, JBC Papers in Press, May 31, 2000, DOI 10.1074/jbc.M001914200

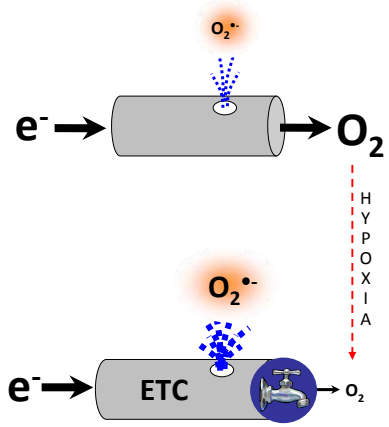
Navdeep S. Chandel‡, David S. McClintock, Carlos E. Feliciano, Teresa M. Wood, J. Andres Melendez, Ana M. Rodriguez, and Paul T. Schumacker§







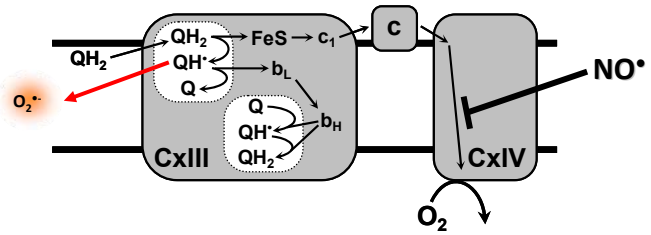
Mitochondrial ROS Elevation in Hypoxia?



How can there be not enough O_2 for ETC to work, but still enough O_2 to make ROS?

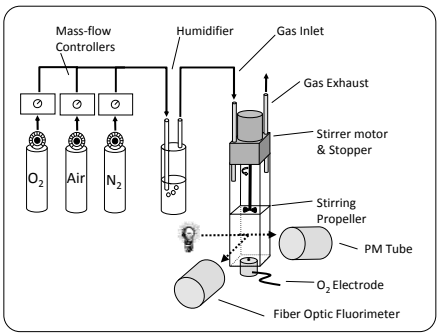
What is the K_M for O_2 of mito' ROS generating systems?

Also… NO• Doesn't Elevate ROS



- Inhibition at CxIV → Reduced cyt-c & c_1 → Less $QH^{\bullet}$ → Less ROS
- CN inhibition does not elevate ROS
- Knocking down cyt-c_1 or Fe-S lowers hypoxic ROS *Cell Metab* (2005) **1:** 401-8

Open Flow Respirometry

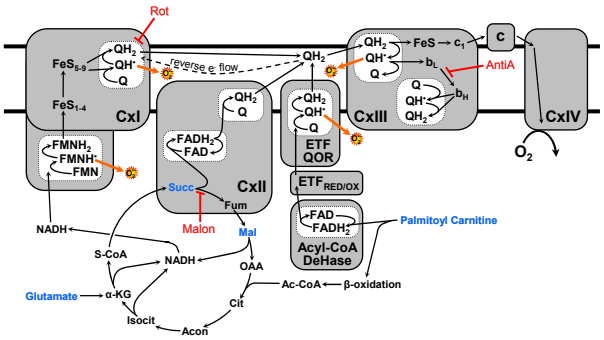


Cole (1982) *J. App. Physiol.* **53:** 1116-24
Brookes (2003) *J. Biol. Chem.* **278:** 31603-9
Hoffman (2007) *Am. J. Physiol.* **292:** H101-108
Hoffman (2009) JBC **284:** 16236-45

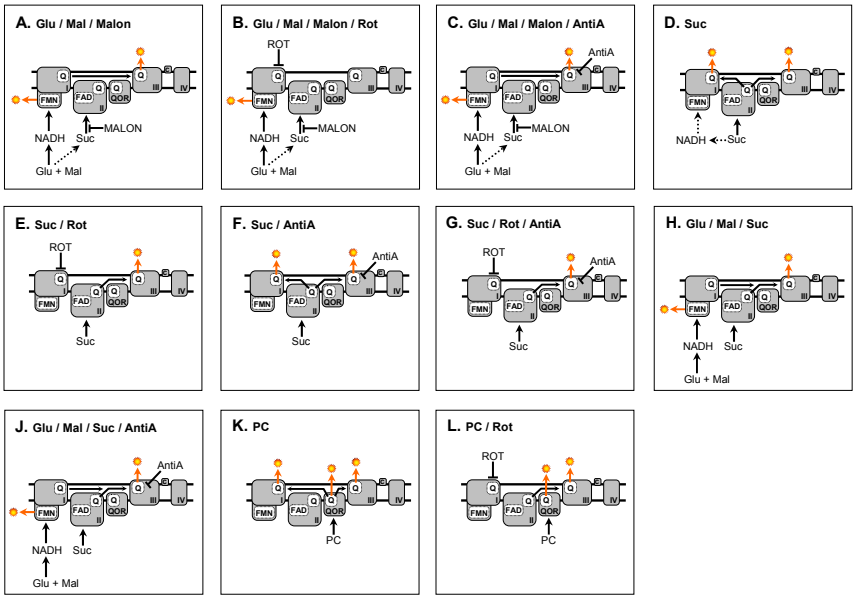
- **Enables prolonged (1 hr.) incubation of mitochondrial suspensions at defined steady-state O_2 tensions**
- **Monitoring of cytochrome redox state throughout**
- **Amplex Red/HRP to measure mito' H_2O_2 generation**
- **11 different combinations of respiratory substrates, to determine ROS from 4 different sites in ETC**

Model of Ox-Phos

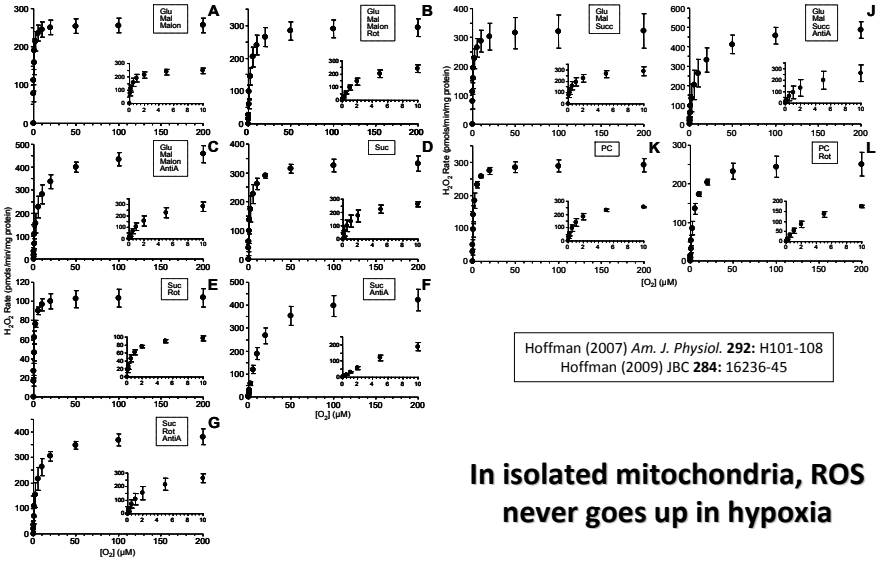
(to determine ROS from different sites)



11 Experimental Conditions



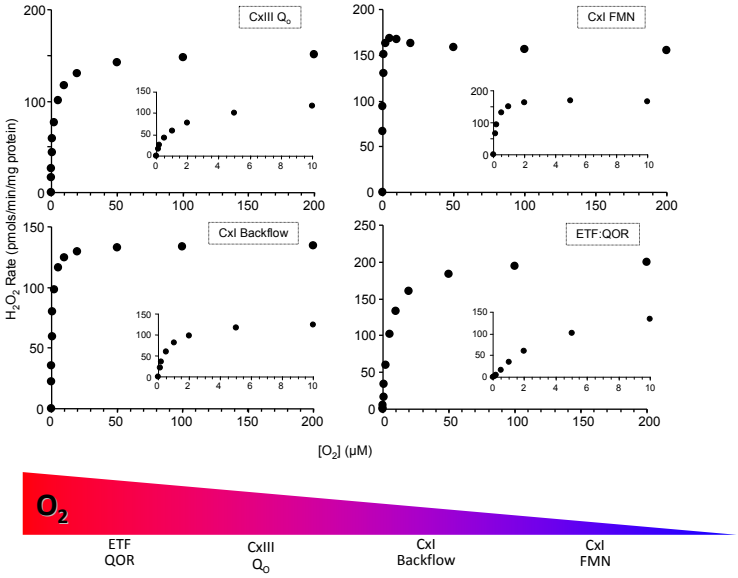
Data !



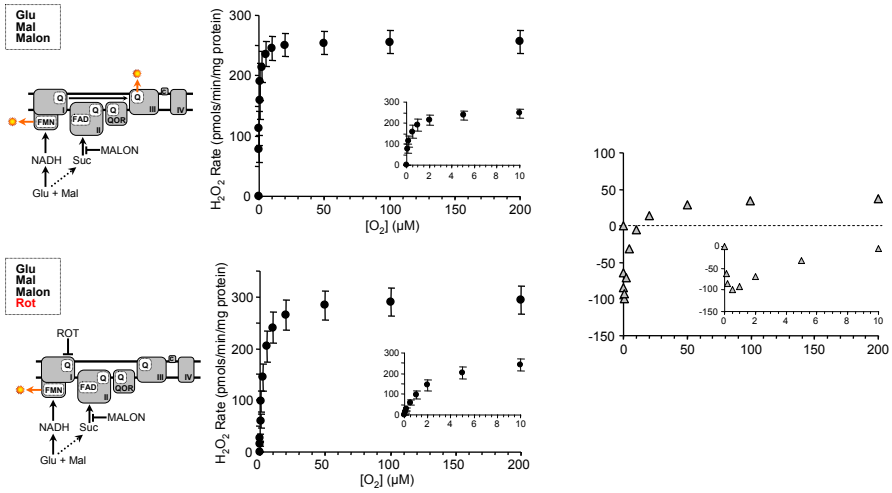
Hoffman (2007) *Am. J. Physiol.* **292:** H101-108
Hoffman (2009) JBC **284:** 16236-45

In isolated mitochondria, ROS never goes up in hypoxia

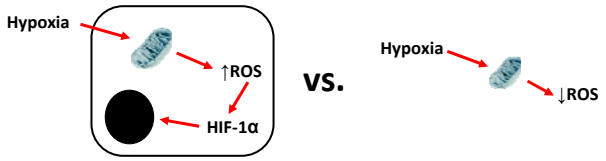
Derived Data



Novel Insights… Effect of Rotenone

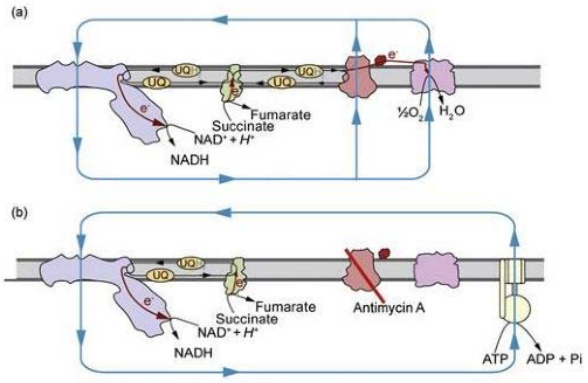


Cells vs. Mito's



Missing signal remains to be identified

Reversed Electron Transfer



CxI is "thermodynamically weak", so high Δp or reduced Co-Q could drive it backwards to reduce NAD^+

But…

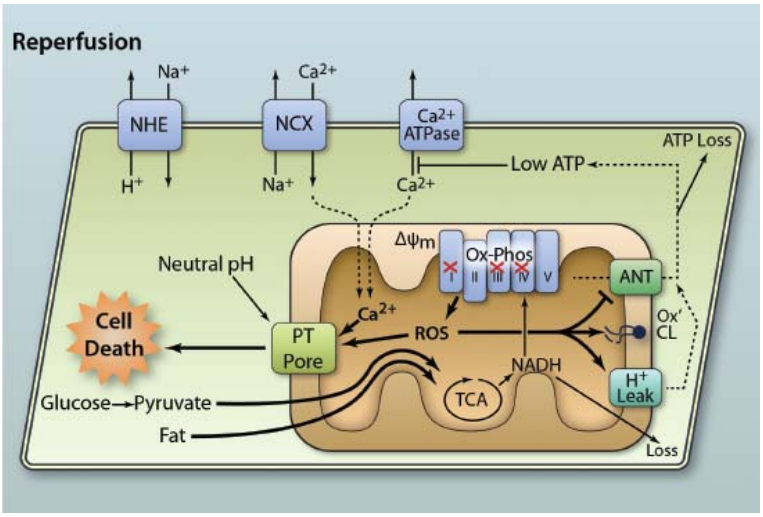
[NAD^+] is usually low under normal conditions

Succinate to drive RET would normally come from upstream dehydrogenases (αKGDH) that consume NAD^+

So… if RET occurs, where could those backward flowing electrons go, if not onto NAD^+?

What about a complex pathologic situation?

Cardiac Ischemia-Reperfusion Injury



Circ. Res. (2012) 111: 1222-36.

Lots of Complex I inhibitors are protective against cardiac IR injury

Rotenone, Amobarbital, Volatile Anesthetics,
S-nitrosothiols, Ranolazine, Capsaicin,
Metformin, Ischemic Preconditioning

Original idea…. Slow reversal of inhibition at reperfusion allows more "gradual wake up" of respiration, avoids surge of ROS

BUT… lots of Complex II inhibitors are also protective against cardiac IR injury

Diazoxide, 3-NP, Nitro-linoleate, HNO,
Atpenin A5, Malonate, Methyl-malonate

Complex II doesn't contribute much to respiration, especially in the heart which is mostly reliant on β-oxidation

Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS

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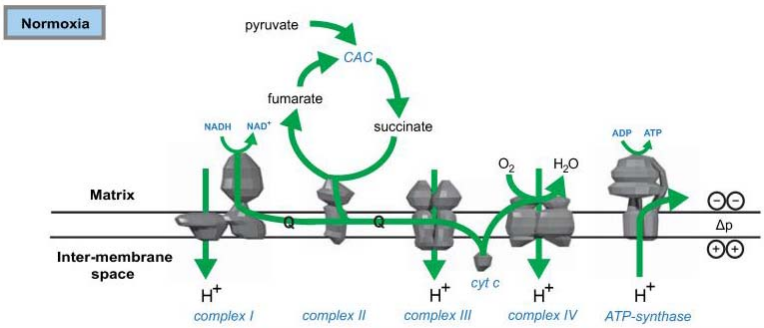

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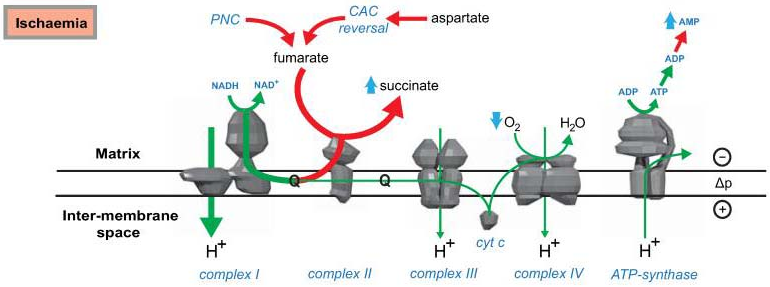

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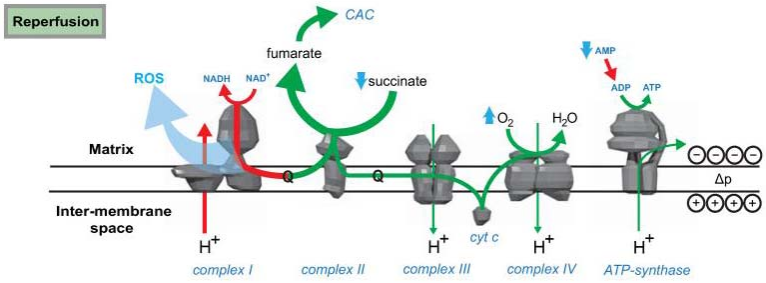

Summary / Conclusions

There is no simple relationship between rate of ROS generation & status/activity of the respiratory chain.

So, what does matter?

- **Dwell time of reactive intermediates**
- **Degree of "electron pressure" in the ETC**
- **pH and ΔpH**
- **$[O_2]$**
- **Status of antioxidants (GSH, SOD etc.)**

Resources

SFRBM - Society for Free Radical Biology & Medicine (www.sfrbm.org)

- Virtual Free Radical School down-loadable lecture notes on >100 topics
- $25 student membership
- Annual conference ~700 attendees
- Free webinars
- $2500 mini-fellowships, for trainees to visit another lab and learn a new method
- 2 Journals (FRBM, Redox Biology)