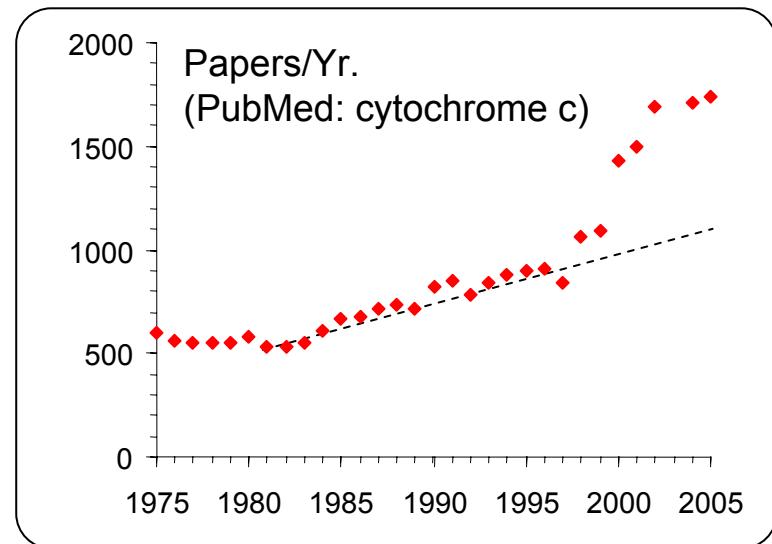
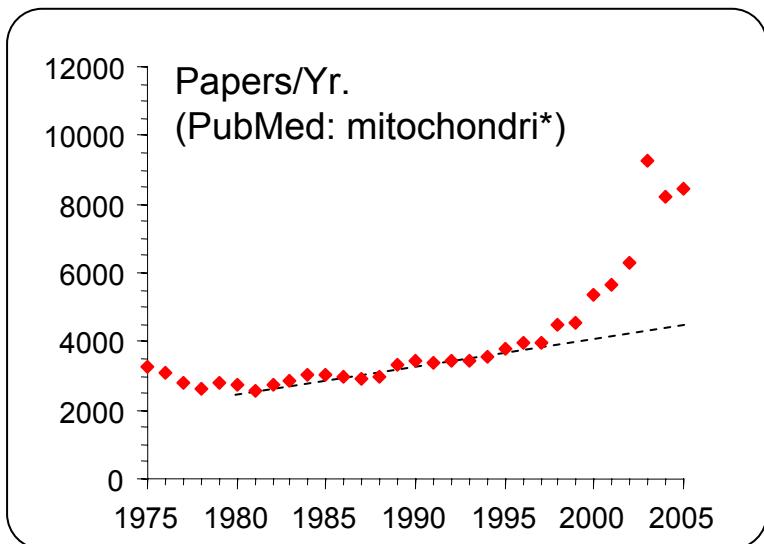


Mitochondria: More Than Just ATP Cows



Paul S. Brookes, PhD.
Anesthesiology, University of Rochester Medical Center

Resurgence in Mitochondrial Research



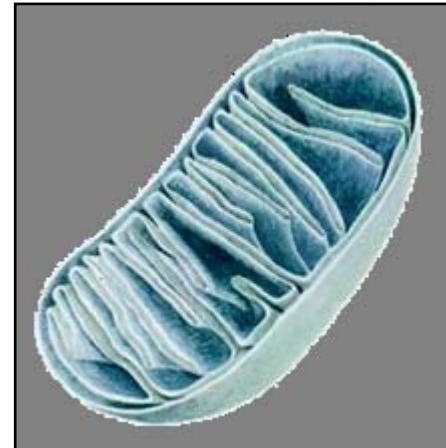
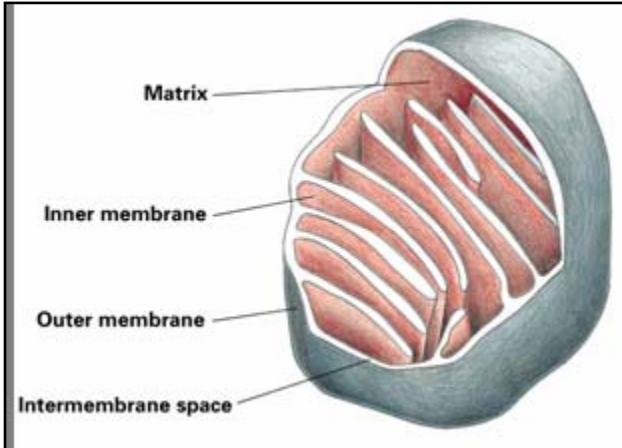
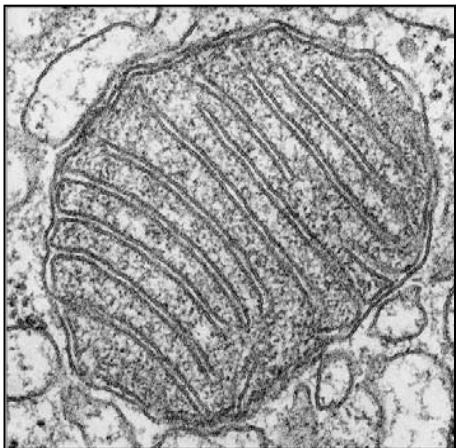
Largely attributable to a single paper...

Liu *et al.* (1996) Induction of apoptotic program in cell-free extracts:
requirement for dATP and cytochrome c. *Cell* **86**: 147-57

But who observed it first ?

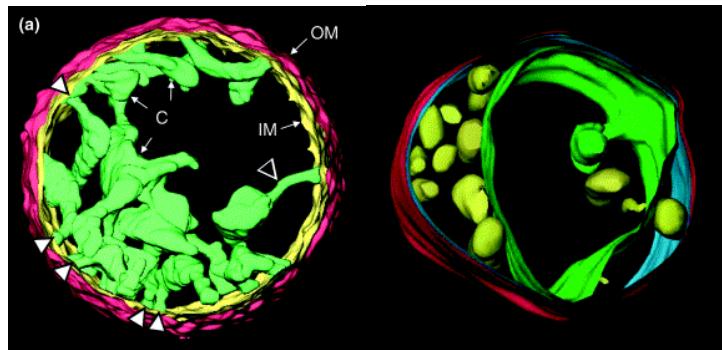
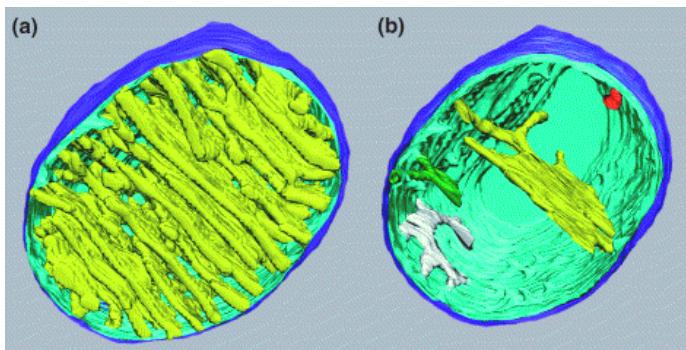
Knyazeva *et al.* (1975) Solubilization of cytochrome c
in ischemic liver tissue. *Vopr Med. Khim.* **21**: 481-85

“Text Book” Views of Mitochondria



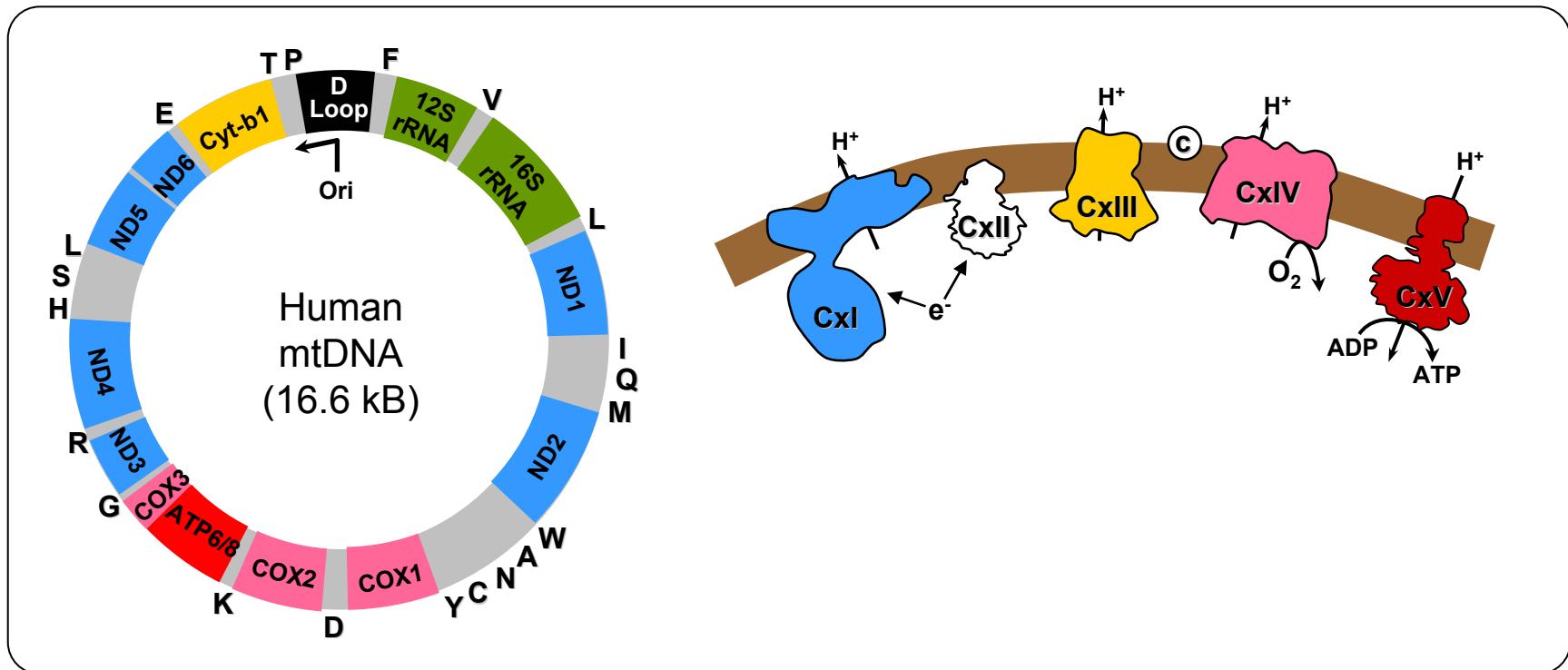
1850: **Mitochondria** coined by microscopists, from Greek *mitos*: thread, *chondros*: grain

Updated Views - 3D electron tomography



Frey & Mannella *TIBS* (2000) **25:** 319-24

Mitochondrial DNA (mtDNA)



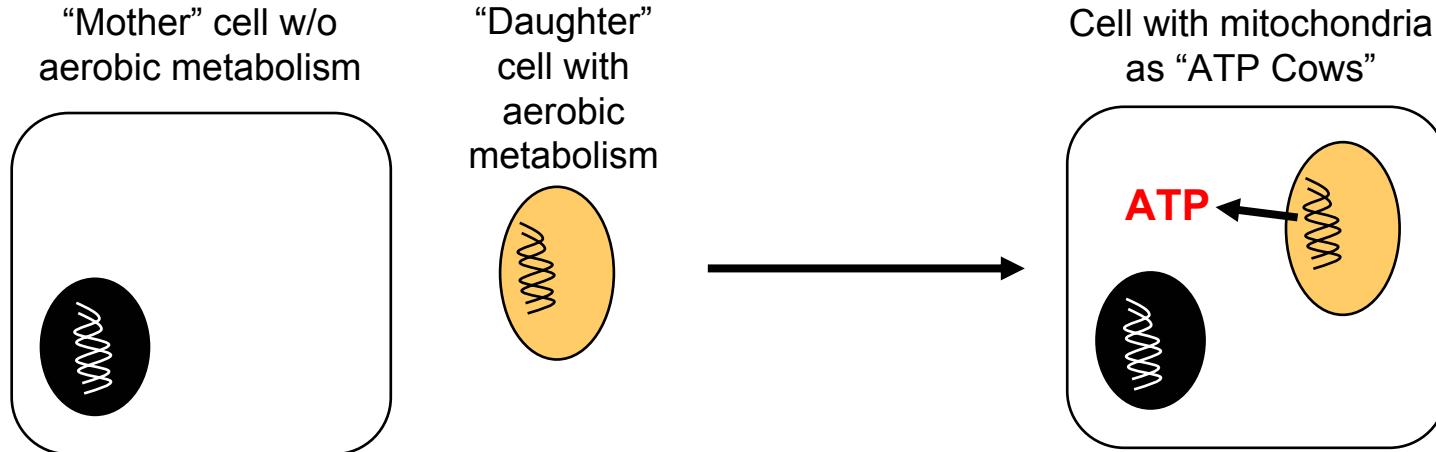
Complex	I	II	III	IV	V
Mw (~kDa)	900	140	250	200	600
Subunits (mtDNA)	46(7)	4(0)	11(1)	13(3)	10(2)

Mitochondria contain histones...

Parseghian & Luhrs (2006) *Biochem. Cell Biol.* **84**: 589-604

The Endosymbiont Theory of Mitochondrial Origin...

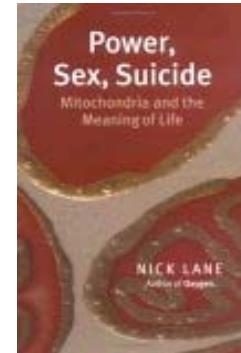
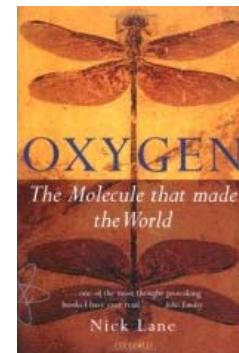
A long, long time ago (about 1.8 billion years)...



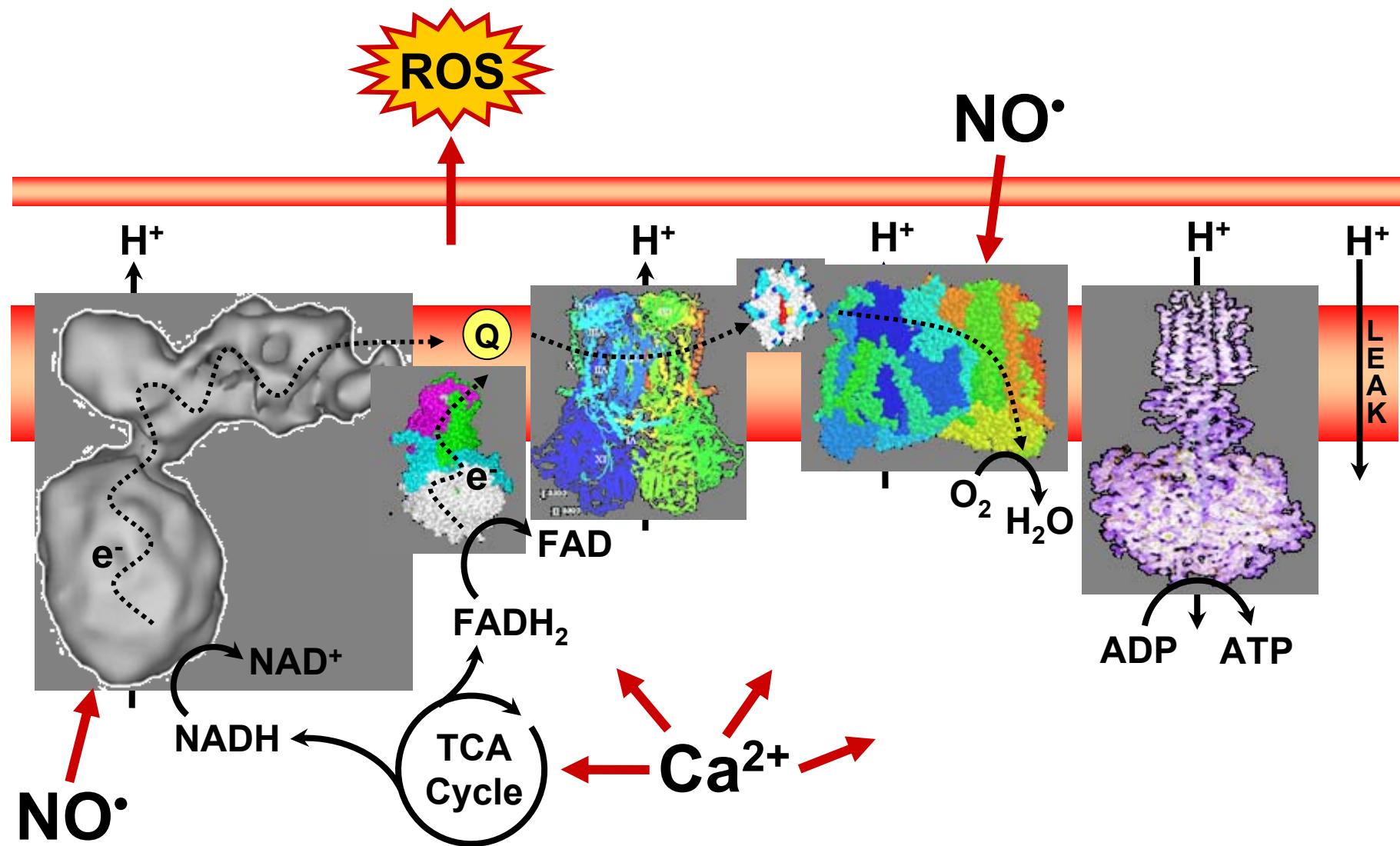
Was it for ATP, or to stop the toxic effects of rising atmospheric $[O_2]$?

2 books by Nick Lane...

- Oxygen: The Molecule that made the World
- Power, Sex, Suicide: Mitochondria & the Meaning of Life

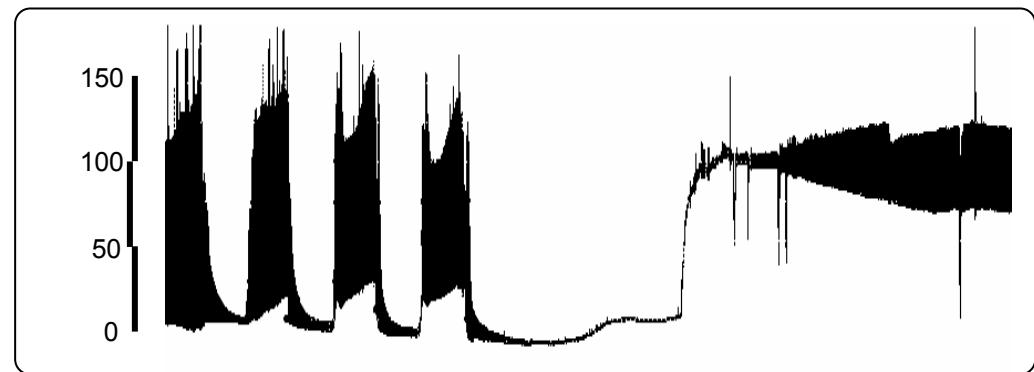
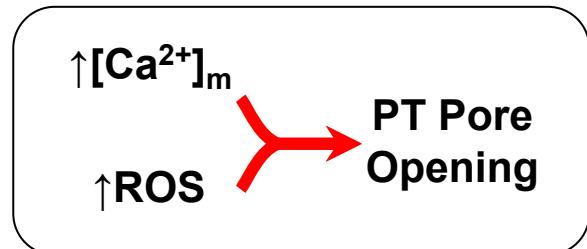
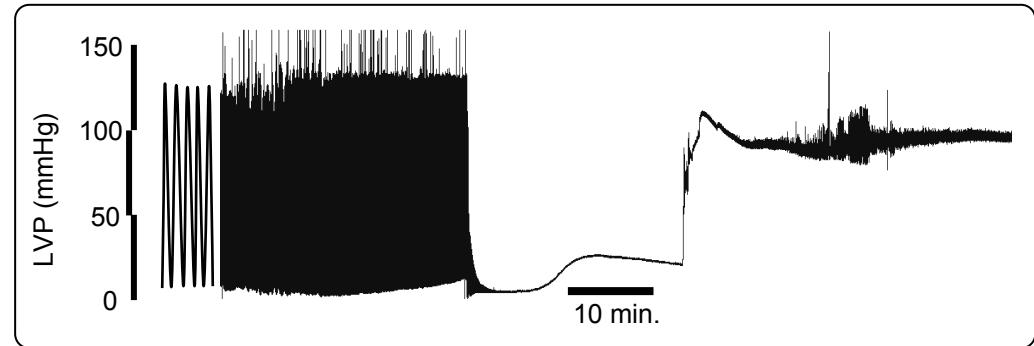
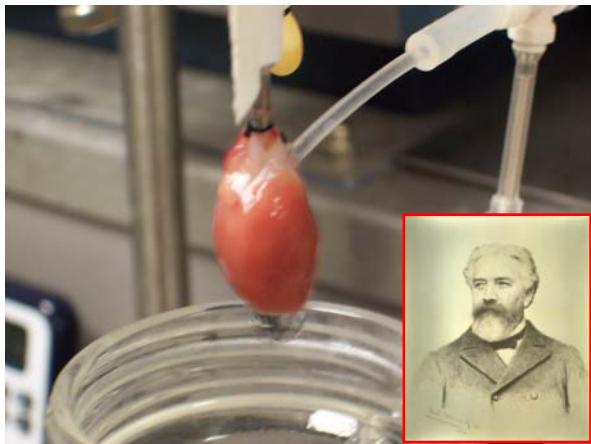


Outline



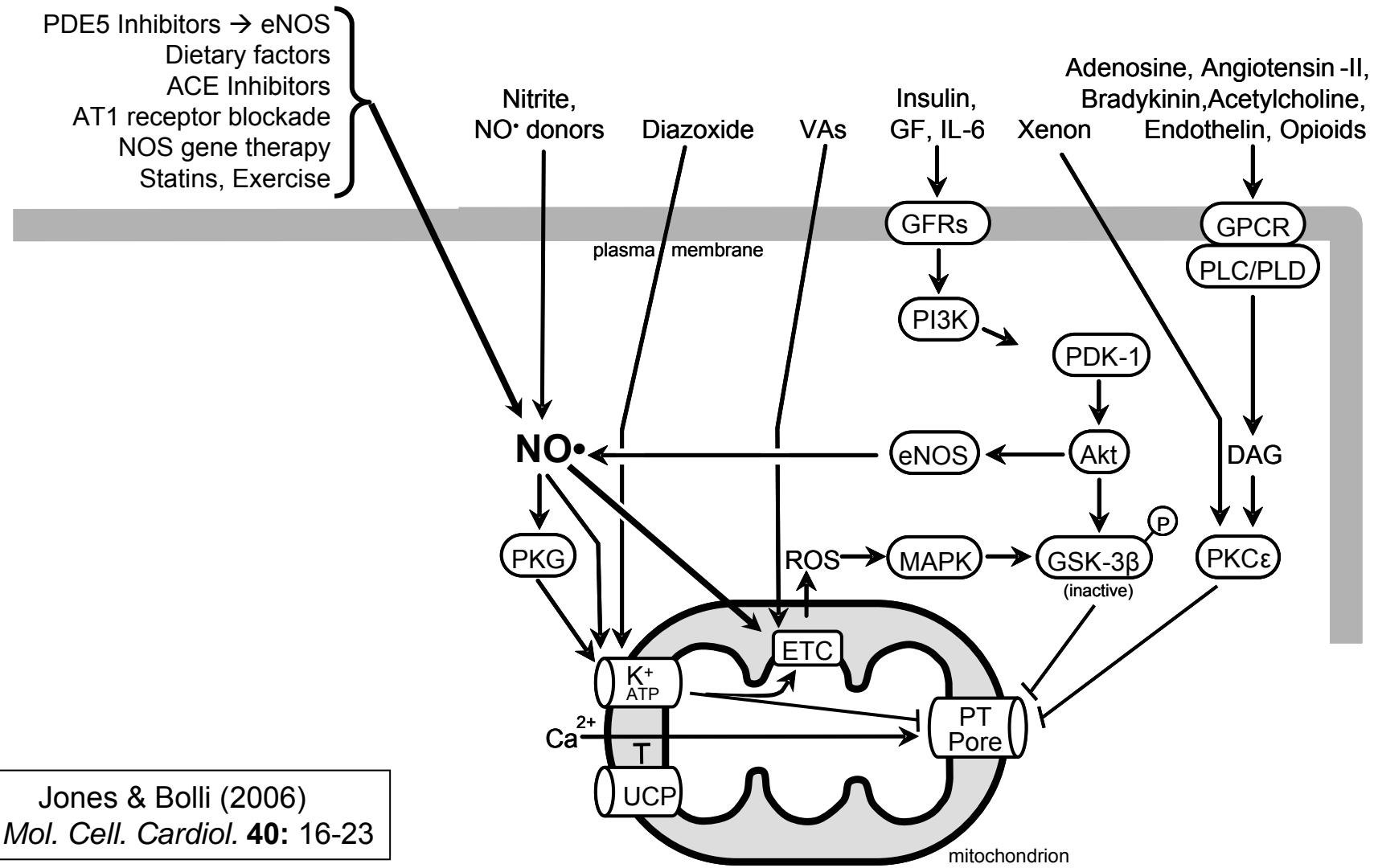
Model System: Cardiac Ischemia-Reperfusion Injury (IR) & Ischemic Preconditioning (IPC)

Langendorff perfused rat heart:



Murry et al. (1986) Circulation 74: 1124-36

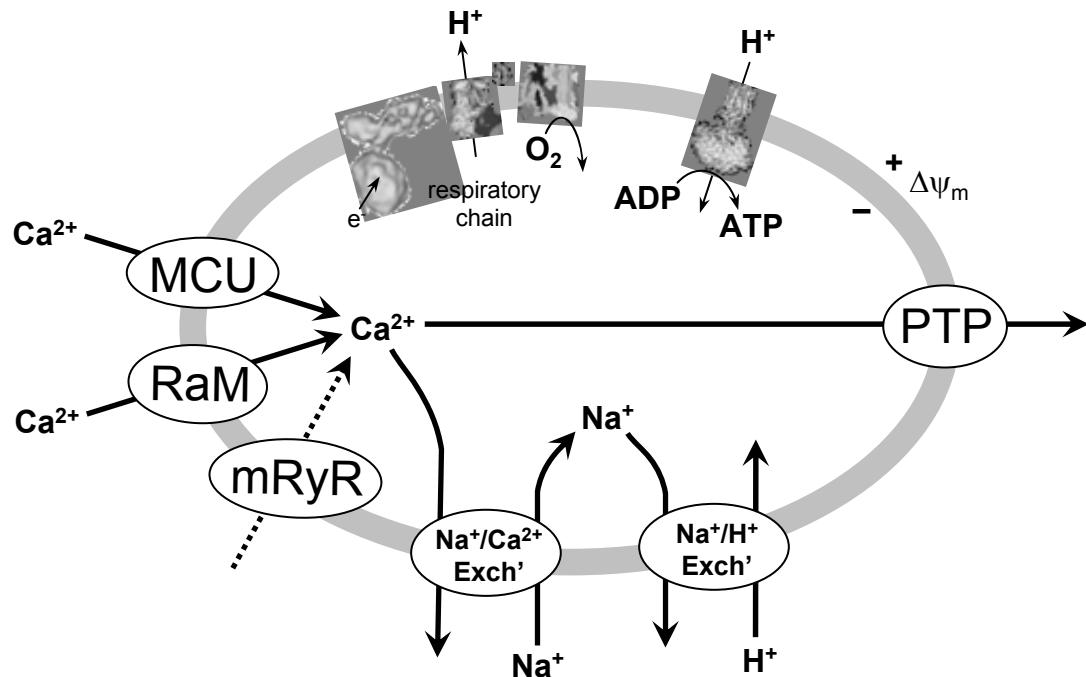
Critical & Convergent Roles of NO[·] & Mitochondria in IPC



Jones & Bolli (2006)
J. Mol. Cell. Cardiol. **40**: 16-23

Zaugg & Schaub (2003)
J. Muscle Res. Cell Motil. **24**: 219-49

Mitochondrial Ca^{2+} Uptake & Efflux Pathways



MCU: Mitochondrial Ca^{2+} Uniporter (high $[\text{Ca}^{2+}]$, quite slow)

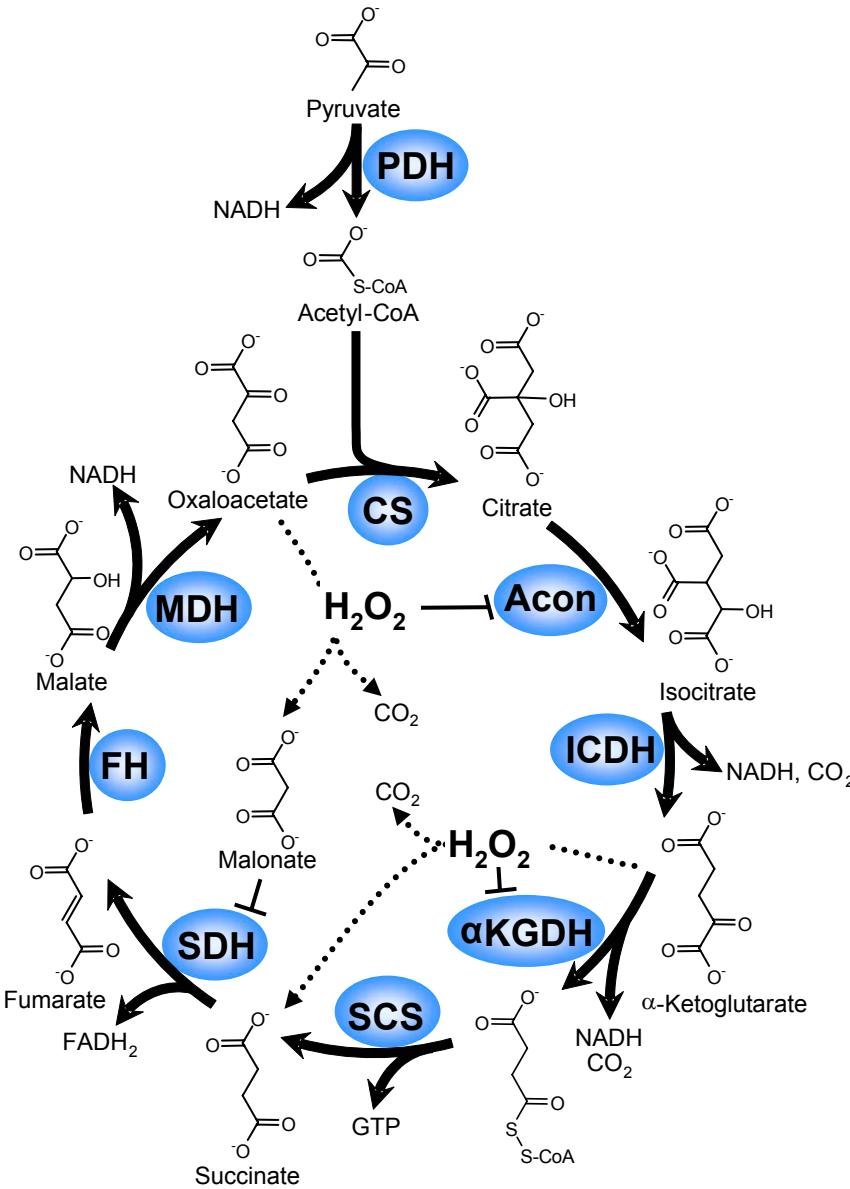
RaM: Rapid Mode Ca^{2+} uptake (low $[\text{Ca}^{2+}]$, very fast)

mRyR: Mitochondrial Ryanodine Receptor

No protein identified

Found in excitable tissues

TCA Cycle... recent insights

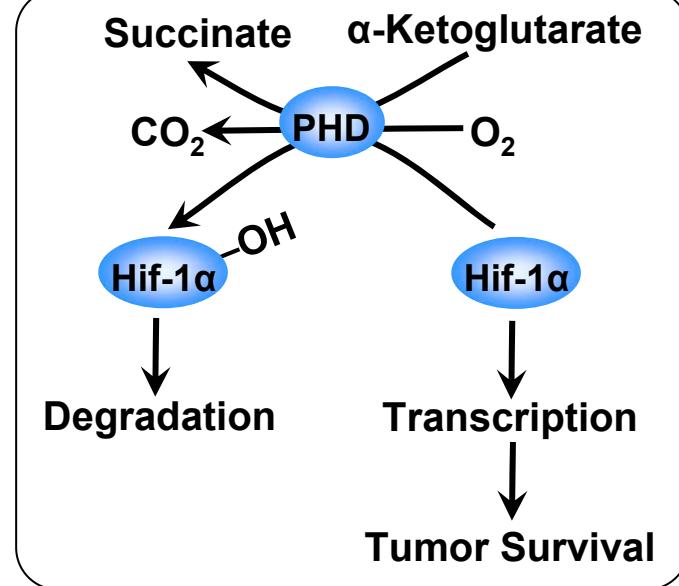


α -keto acids react readily with H_2O_2

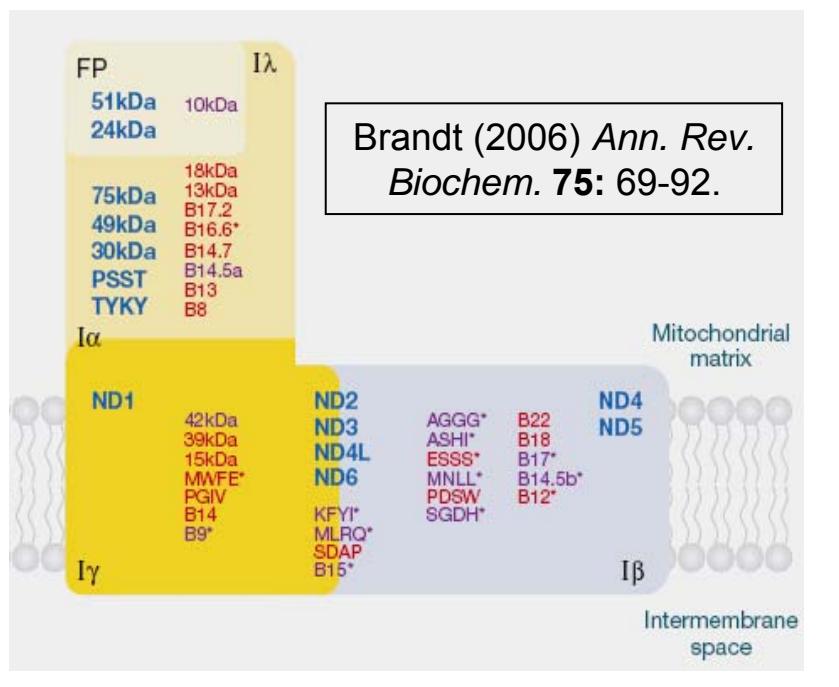
Fedotcheva *et al.* (2006)
Free Rad. Biol. Med. **41**: 56-64

HIF prolyl-hydroxylases use α -KG as a substrate and are inhibited by succinate

Selak *et al.* (2005) *Cancer Cell.* **7**: 77-85



Complex I



Brandt (2006) *Ann. Rev. Biochem.* **75**: 69-92.

Important source of ROS in reverse direction

St-Pierre *et al.* (2002) *JBC* **277**: 44784-44790

Apoptotic protein GRIM-19 is a CxI subunit

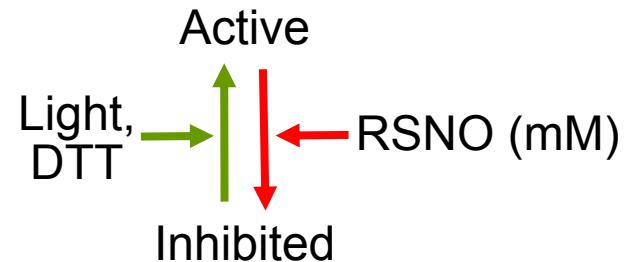
Fearnley *et al.* (2001) *JBC* **276**: 38345-38348

75kDa subunit cleaved by caspases

Ricci *et al.* (2004) *Cell* **117**: 773-786

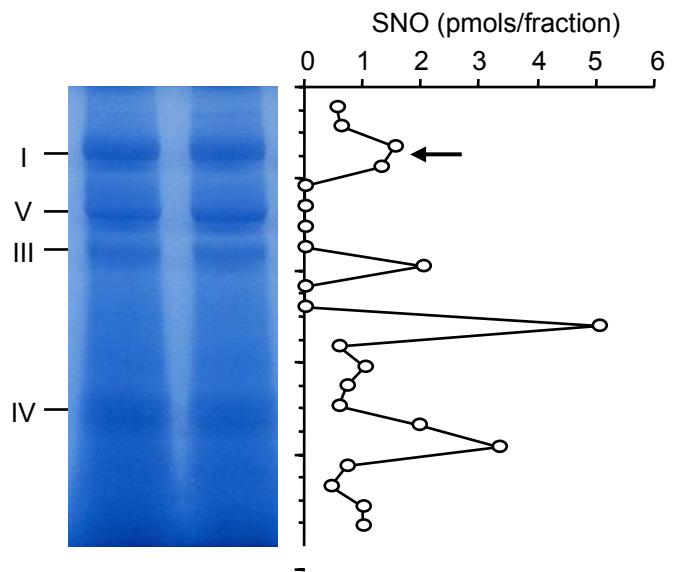
Indirect evidence for S-nitrosation

- Clementi (1998) *PNAS* **95**: 7631-36
Hsu (2005) *J. Neurochem.* **92**: 1091-1103
Brown (2004) *Biochim. Biophys. Acta* **1658**: 44-49
Jekabsone (2003) *J. Mol. Cell. Cardiol.* **35**: 803-09
Riobo (2001) *Biochem. J.* **359**: 139-45
Dahm (2006) *J. Biol. Chem.* **281**: 10056-65
Borutaite (2006) *Biochim. Biophys. Acta* **1757**: 562-6

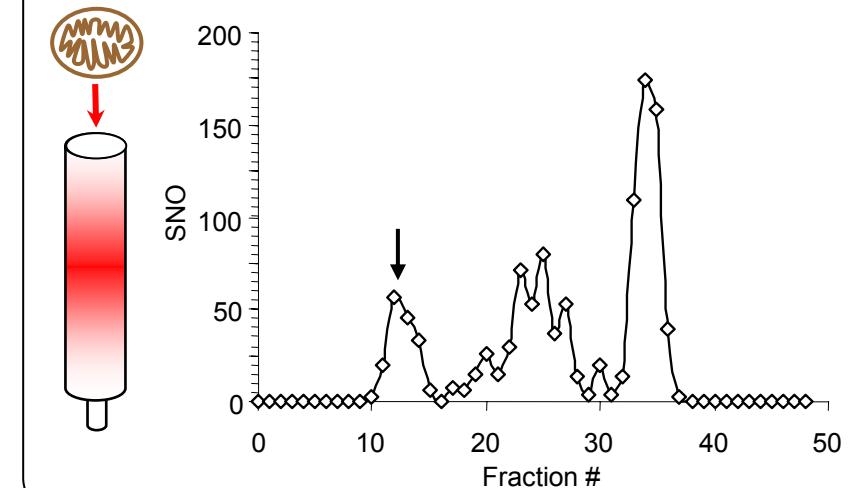


Can Complex I be S-nitrosated ?

Blue-native gel → SNO CL



Superose 6 → SNO CL

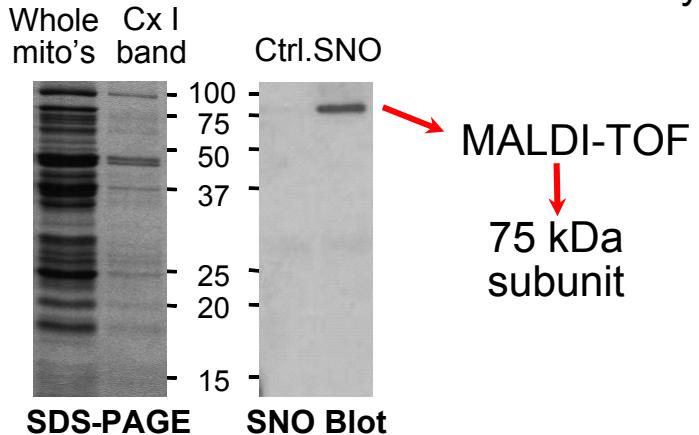


Jaffrey (2005) *Meth. Enzymol.* **396**: 105-18

Yang et al. (2003) *Free Rad. Res.* **37**: 1-10

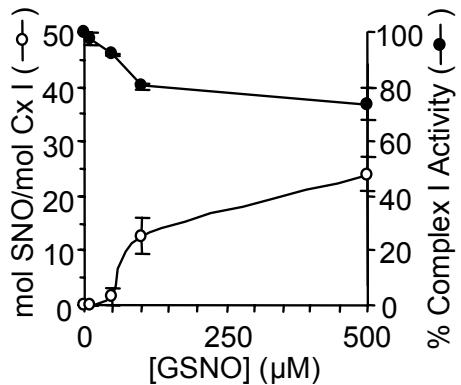
Burwell et al. (2006) *Biochem. J.* **394**: 627-34

Biotin Switch Assay



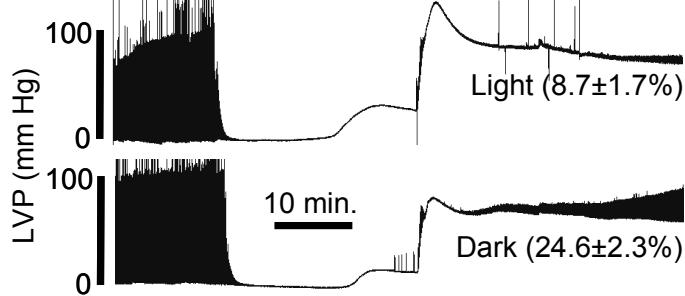
Is Complex I S-nitrosation Physiologically Important?

S-nitrosation vs. Inhibition

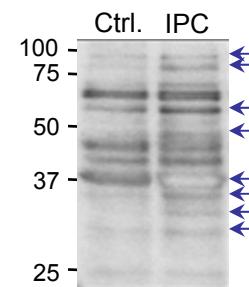
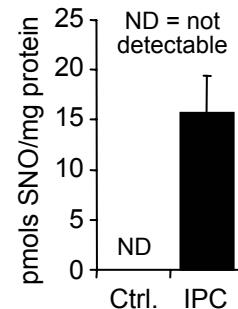


Burwell et al. (2006) *Biochem. J.* **394**: 627-34

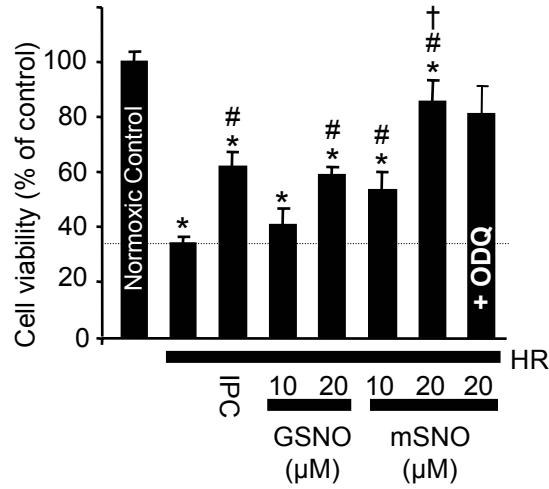
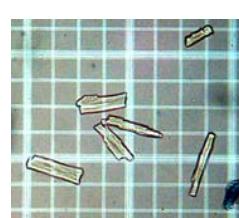
Recovery from IR is enhanced in the dark



Mitochondria from IPC hearts contain SNO



“mito-SNO” protects myocytes from IR injury



Metabolic Shutdown as a Protective Mechanism

Amobarbital

J. Pharmacol. Exp. Therap. (2006) **316**, 200-07

Ranolazine

Biochem. Pharmacol. (1995) **50**, 1599-1606

Capsaicin

Eur. J. Pharm. (1995) **272**, 269-78
BBA (1996) **1273**, 21-30

Nitric Oxide

Biochem. J. (2006) **394**, 627-34

IPC

J. Biol. Chem. (2002) **277**, 24411-9

Nitric Oxide

J. Biol. Chem. (1992) **267**, 24929-32

3-Nitropropionate

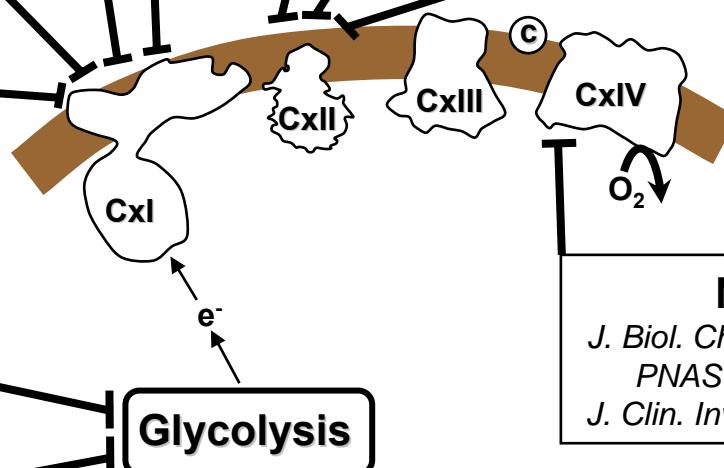
J. Cereb. Blood Flow Metab. (1997) **17**, 257-64

Hydrogen Sulfide

J. Mol. Cell. Cardiol. (2006) **40**, 119-30
Toxicol. App. Pharmacol. (1990) **103**, 482-90

Diazoxide

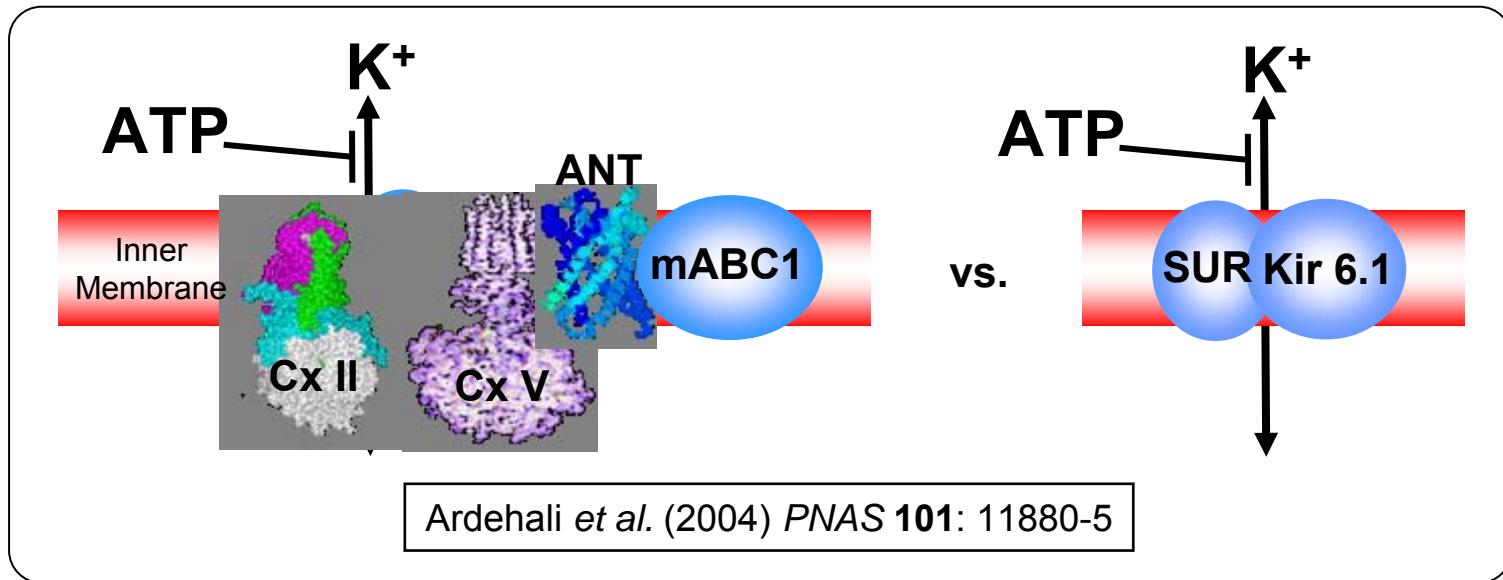
PNAS (2004) **101**, 11880-11885



Nitric Oxide

J. Biol. Chem. (2000) **275**, 20474-9
PNAS (2004) **101**, 13683-88
J. Clin. Invest. (2005) **115**, 1232-40

Complex II



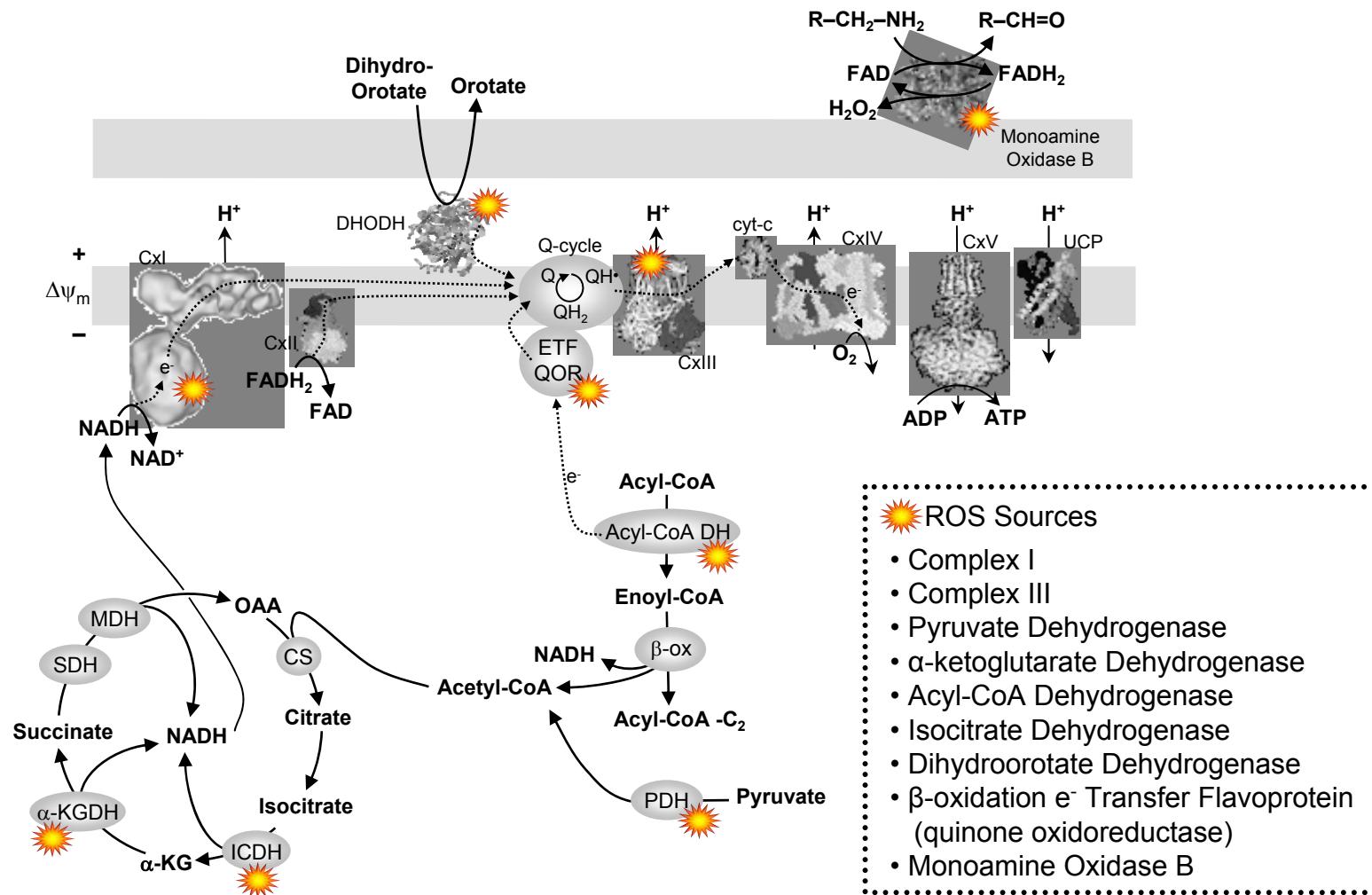
- mitoK⁺_{ATP} channel opening → ischemic preconditioning
- Drugs that open K⁺_{ATP} (e.g. diazoxide) are Cx II inhibitors

Schafer *et al.* (1969) *Biochem. Pharmacol.* **18**: 2678-81

- Cx II inhibitors (e.g. malonate) can mimic preconditioning
- Splice variant of Cx II subunit 3 is within the SUR gene

Wohllk *et al.* (1998) *Mol. Genet. Metab.* **65**: 187-90

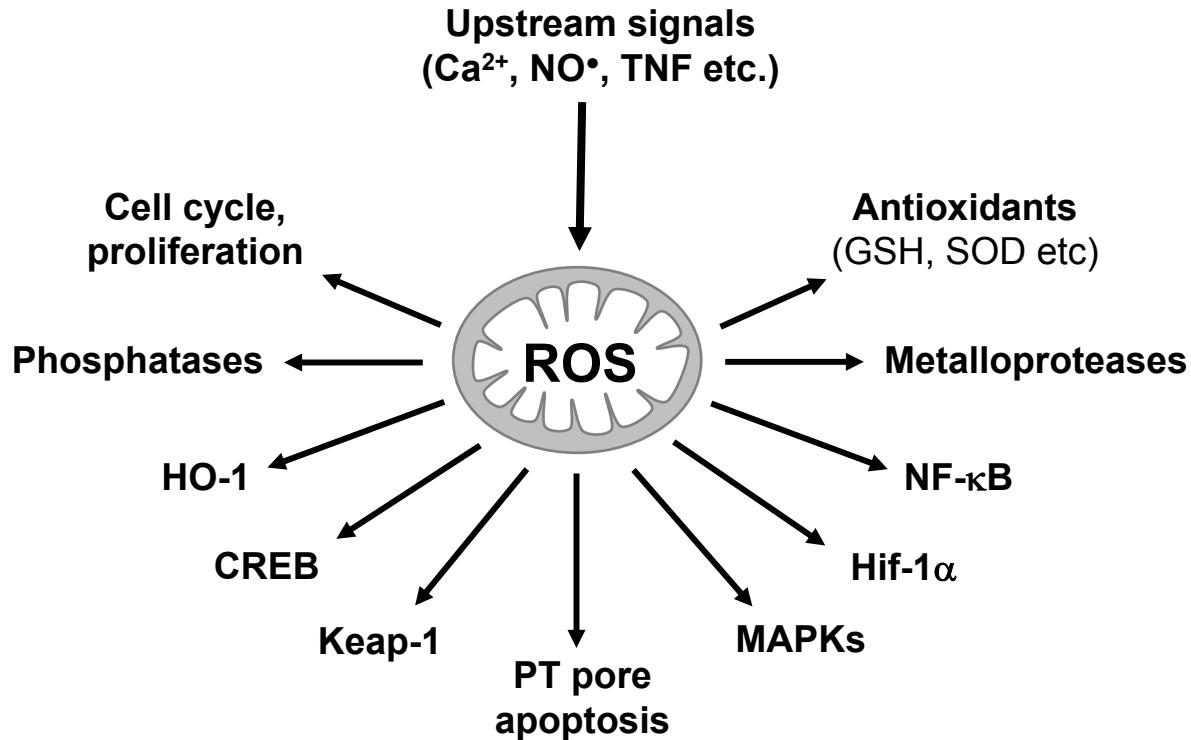
Mito' Sources of ROS – more than just CxI & CxIII



ROS Sources

- Complex I
- Complex III
- Pyruvate Dehydrogenase
- $α$ -ketoglutarate Dehydrogenase
- Acyl-CoA Dehydrogenase
- Isocitrate Dehydrogenase
- Dihydroorotate Dehydrogenase
- $β$ -oxidation e^- Transfer Flavoprotein (quinone oxidoreductase)
- Monoamine Oxidase B

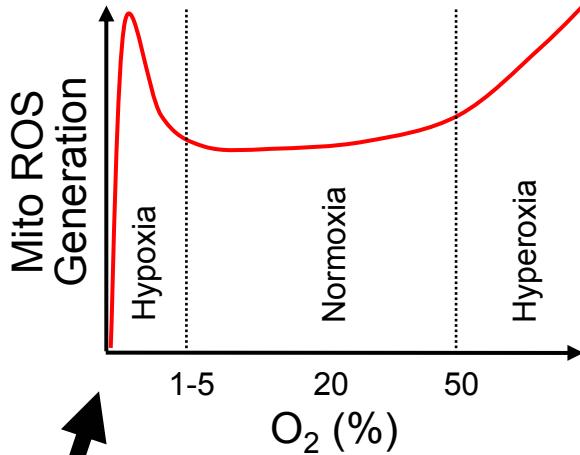
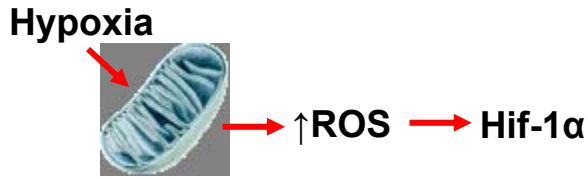
Mitochondrial ROS & Cell Signaling



Mitochondrial ROS Generation & Hypoxia

Waypa & Schumacker (2002) *Respir. Physiol. Neurobiol.* **132**: 81-91

Current Dogma:

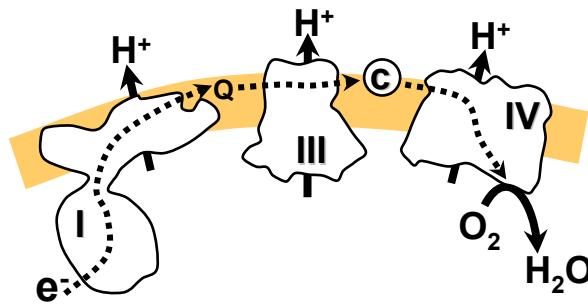


Chandel et al. (2000) *J. Biol. Chem.* **275**: 25130-8

Bell et al. (2005) *Mitochondrion* **5**: 322-32

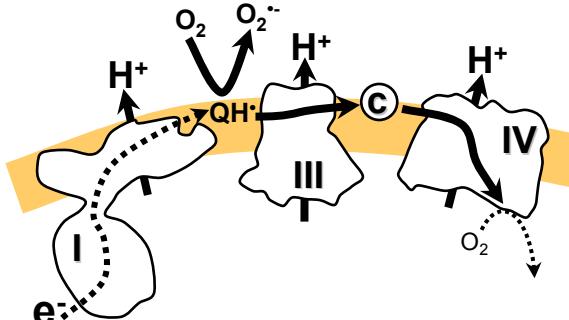
Guzy et al. (2005) *Cell Metab* **1**: 401-8

Proposed Mechanism:



Normoxia

Electrons flow all the way to O_2 at complex IV, and fully reduce it to H_2O

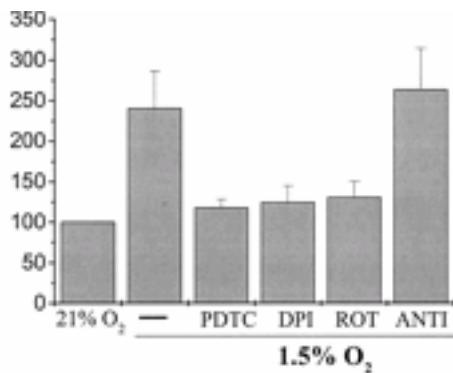


Hypoxia

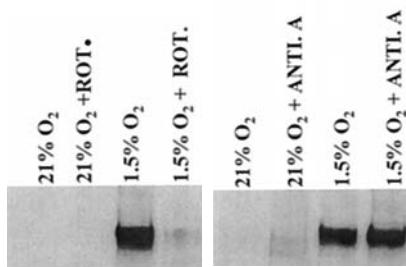
Lack of O_2 at complex IV causes a “back-up” of electrons in the chain, increasing lifetime of semiquinone radical (QH^{\bullet}), which donates electrons to O_2 , forming O_2^-

Mitochondrial ROS Generation & Hypoxia

DCF Fluorescence



Hif-1α Stabilization



(results from Hep-3B cells)

Chandel et al. (2000)
J. Biol. Chem. **275**: 25130-8

Prediction: Mitochondrial hypoxic signaling response is an inherent property of the respiratory chain. Mitochondria function autonomously in hypoxic signaling.

But: ROS generation by isolated mitochondria at hypoxic [O₂] (1% = 10μM) has never been measured.

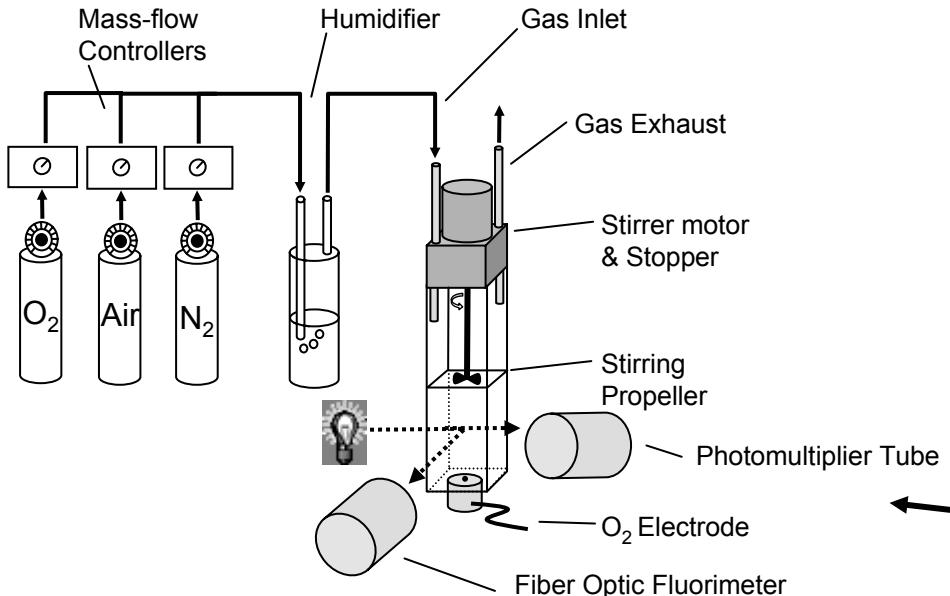
And: k_M of cytochrome c oxidase is ~1μM O₂, but k_M of the ROS generating system is unknown.

Probe specificity (esp. DCF)

Probe location (mitoSOX Red)

Inhibitor specificity (rotenone, AA)

Open Flow Respirometer

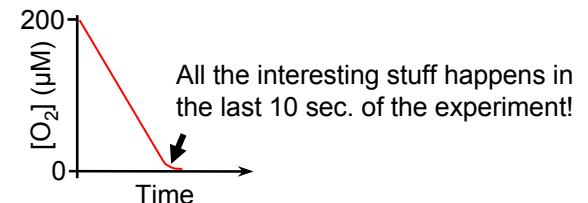


Cole et al. (1982) *J. App. Physiol.* **53**: 1116-24

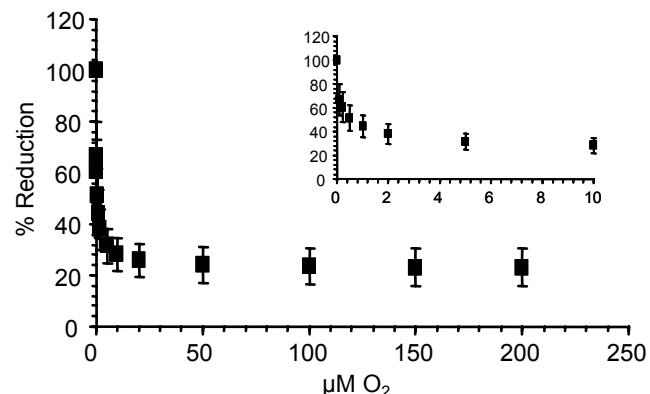
Brookes et al. (2003) *J. Biol. Chem.* **278**: 31603-9

Hoffman et al. (2007) *Am. J. Physiol. Heart.* In-Press

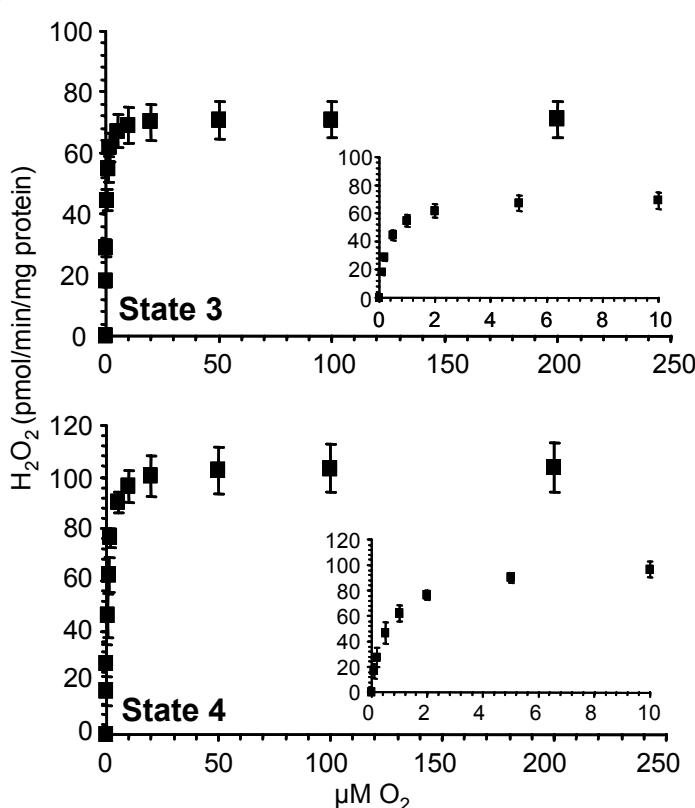
Conventional respirometry chamber: $[O_2]$ is constantly changing, as mitochondria are consuming O_2 . Below 10% O_2 , the only way to maintain a relatively constant $[O_2]$ is to use very small amounts of mitochondria (incompatible with ROS measurements).



Open-flow respirometry chamber: O_2 consumed by mitochondria is replaced by O_2 from headspace gas. This allows incubation at steady-state values of $[O_2]$ as low as 0.1 μM . Simultaneous cytochrome spectral measurements inform on redox state of respiratory chain:



Mitochondrial ROS Generation Decreases in Hypoxia



Isolated liver mitochondria, glutamate plus malate as substrates (i.e. electrons fed into complex I). Amplex red/HRP fluorescent measurement of H₂O₂.

Hoffman et al. (2007)
Am. J. Physiol. Heart. In-Press

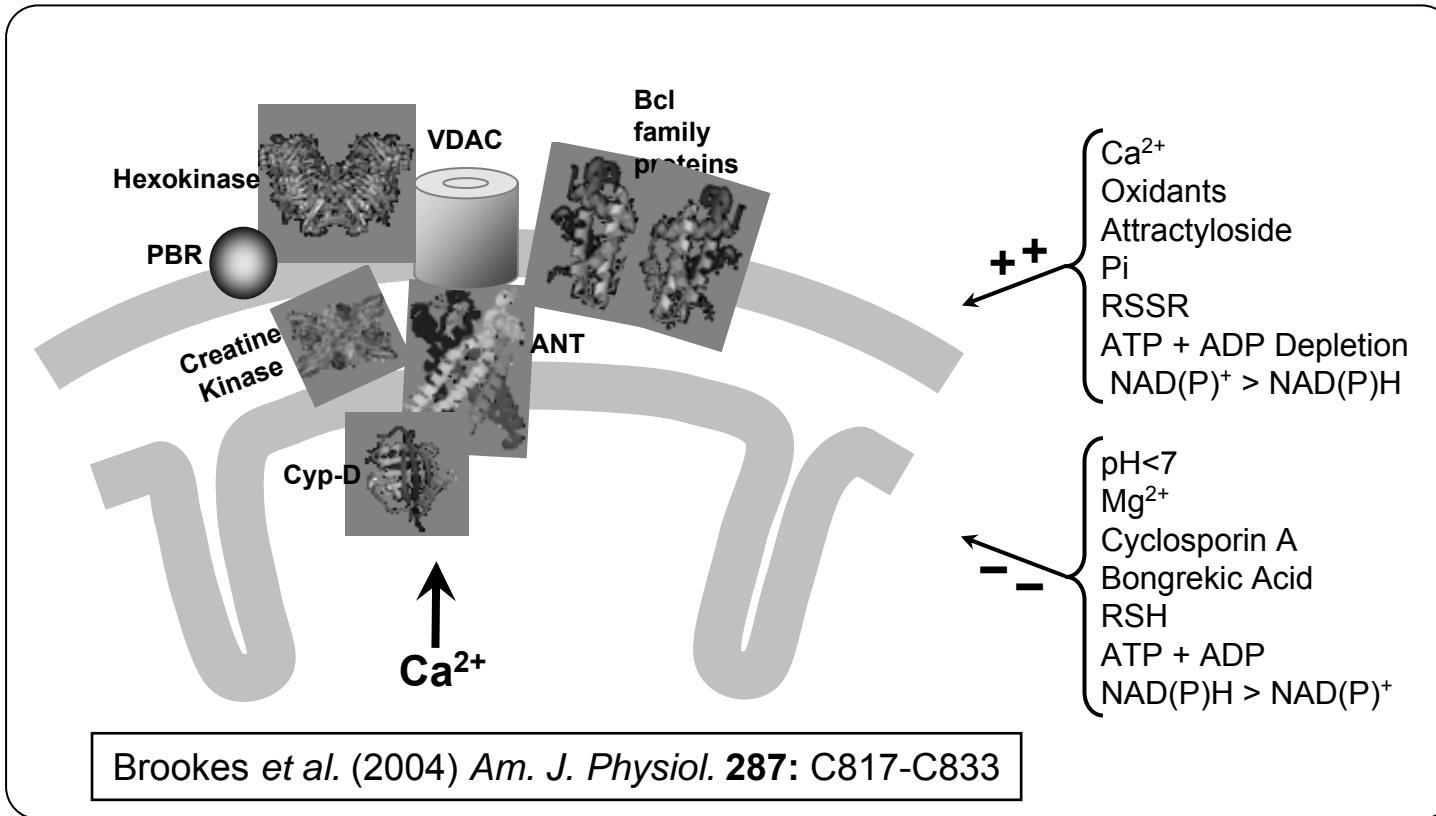
- Apparent k_M of the ROS system ~0.5 μM O₂
- Apparent k_M for respiration ~2 μM O₂
- ROS generation can occur even at [O₂] that inhibits respiration, but ROS cannot increase!

Conclude:

Hypoxic ROS generation is not inherent to the mito' respiratory chain

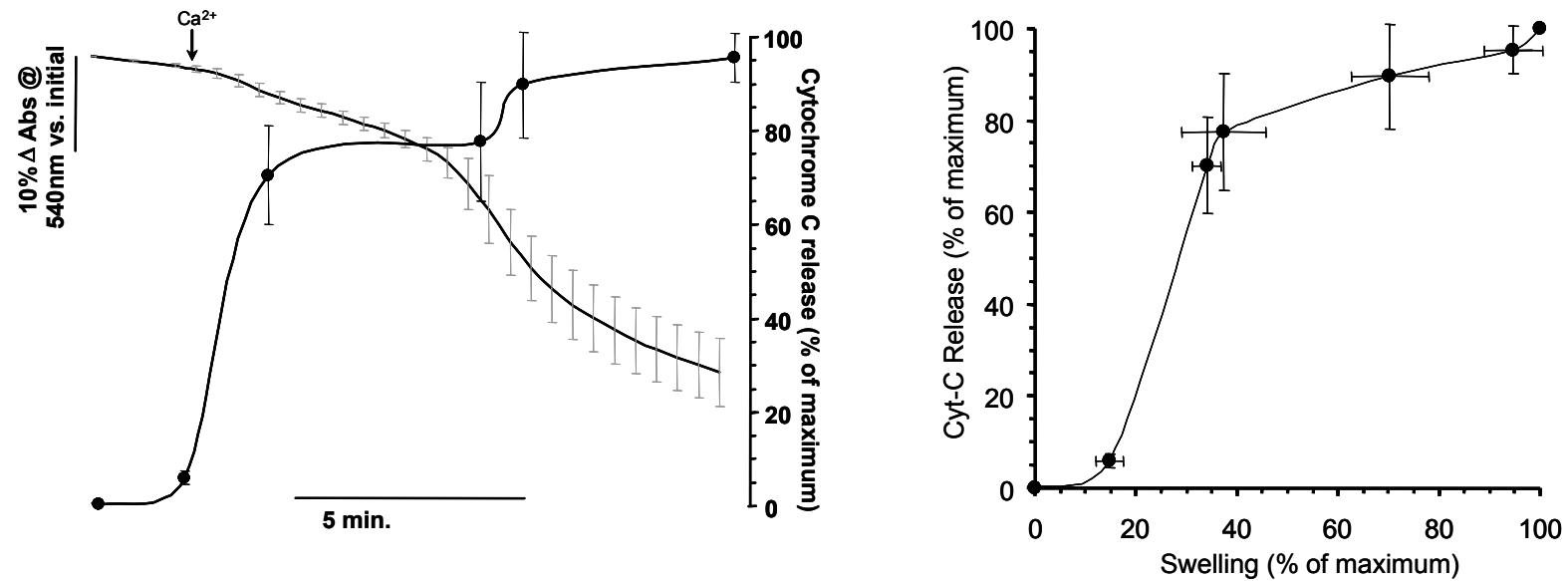
In cells, another signaling factor is probably responsible for elevated ROS:
NO[•], Ca²⁺, Phosphorylation

Permeability Transition Pore (classical view)



Opening of the PT pore is *mechanistically* linked to cytochrome *c* release. Cyt-c release can occur without pore opening, and OMM swelling/rupture does not appear to play a major role

Swelling ≠ Cytochrome *c* Release



Brookes et al. (2000) J. Biol. Chem. 275: 20474-9

Mechanism by which cytochrome *c* exits
Mitochondria during apoptosis is still elusive

Components of the PT Pore: knockout mice data

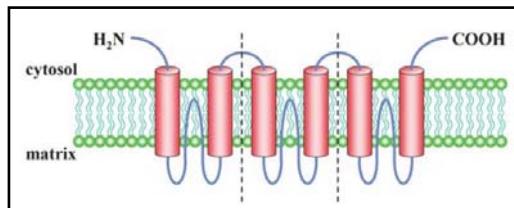
ANT

Kokoszka *et al.* (2004) *Nature* **427**(6973): 461-5 Non-essential

Halestrap (2003) *Curr. Med. Chem.* **10**: 1507-25 Or maybe it is essential?

Palmieri (2004) *Pflugers Arch.* **447**: 689-709

Maybe other MCF proteins can substitute?



VDAC

Krauskopf *et al.* (2006) *Biochim. Biophys. Acta* **1757**: 590-5 Non-essential

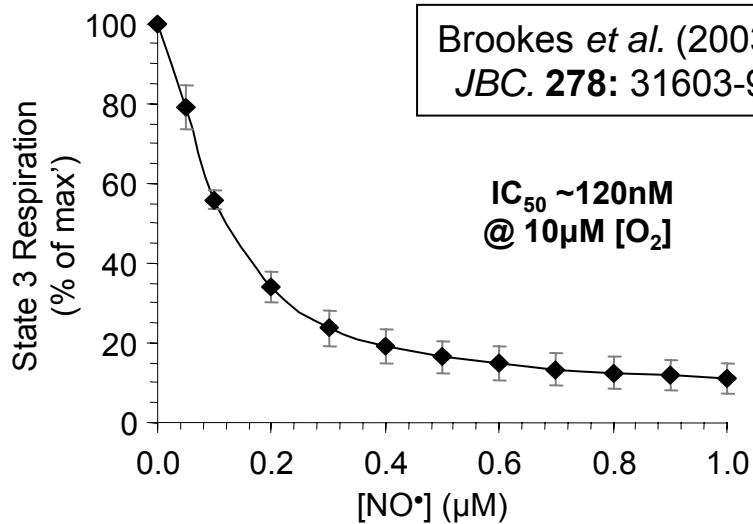
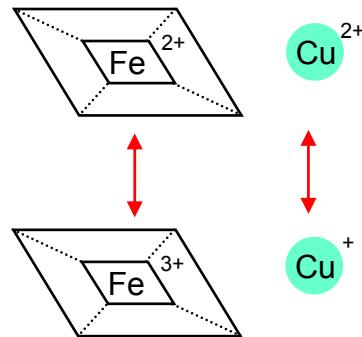
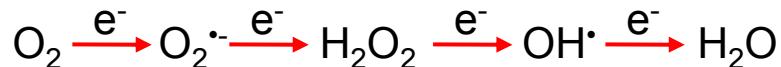
Cyp-D

Nakagawa *et al.* (2005) *Nature* **434**(7033): 652-8

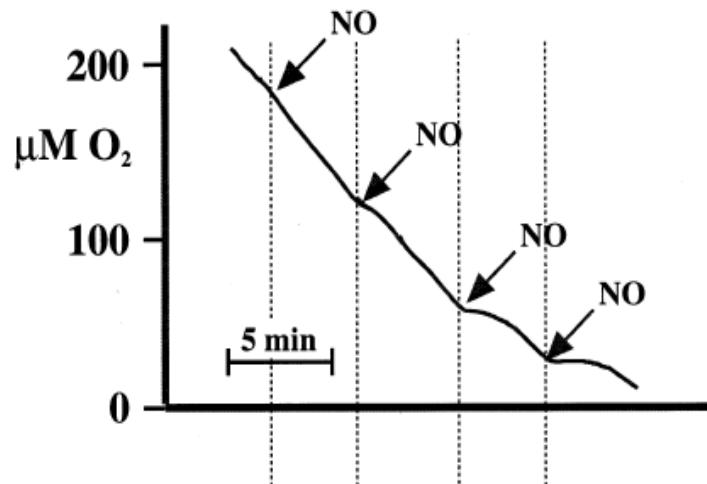
Essential for PT pore, but pore is only important in necrosis, not apoptosis

Baines *et al.* (2005) *Nature* **434**(7033): 658-62

Complex IV (Cytochrome *c* Oxidase) – NO[•] Inhibition



A. Oxygen tension



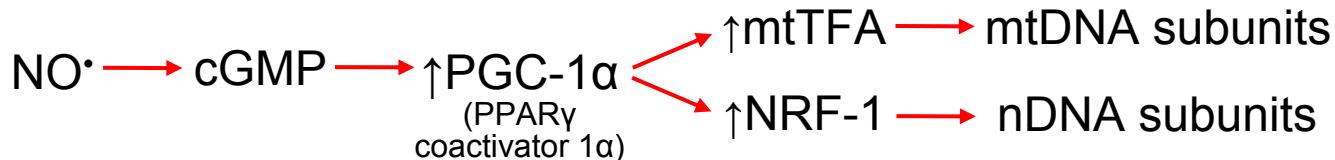
B. NO tension



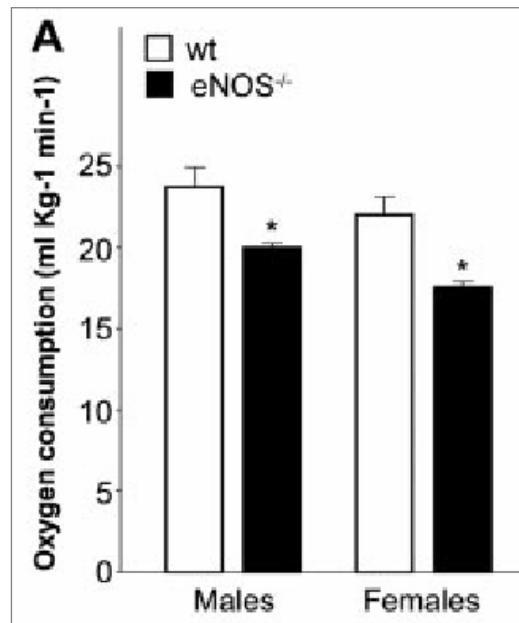
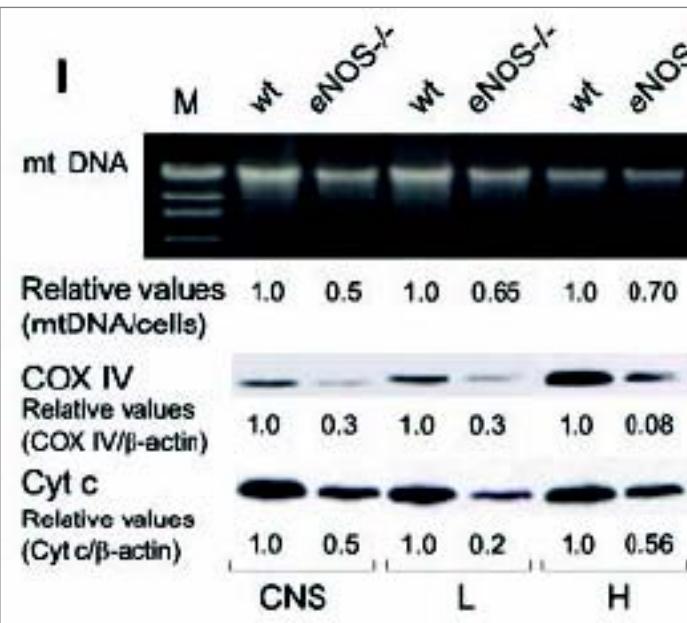
Koivisto et al. (1997)
FEBS Lett. 417: 75-80

NO[•] Stimulates Mitochondrial Biogenesis

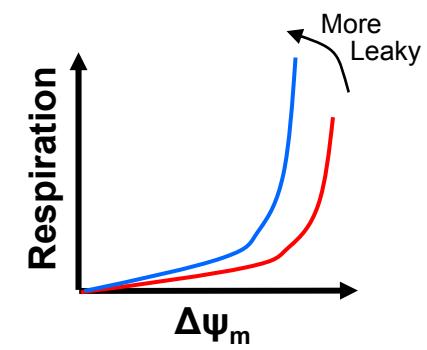
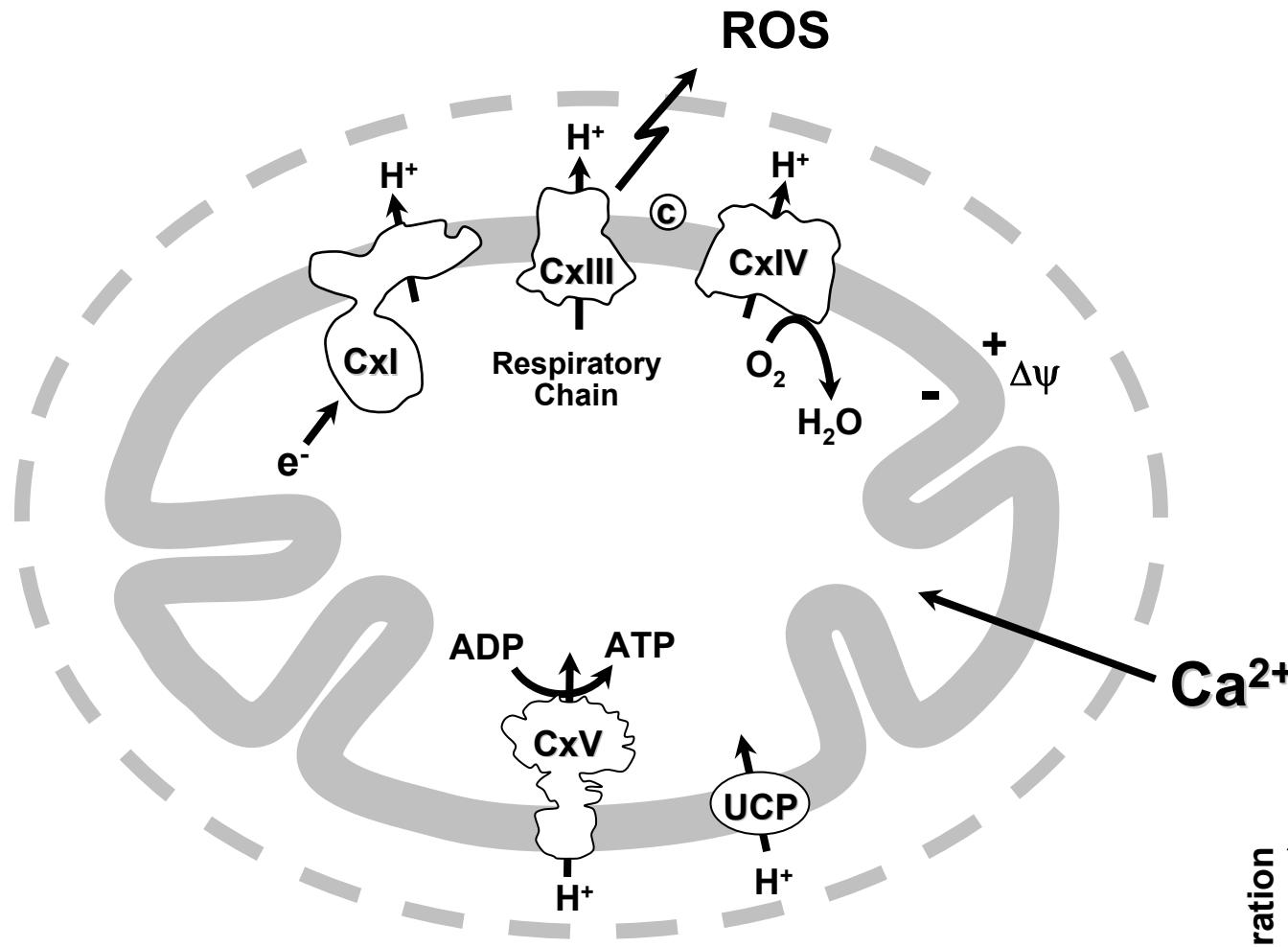
Nisoli et al. (2003) *Science* **299**: 896-9



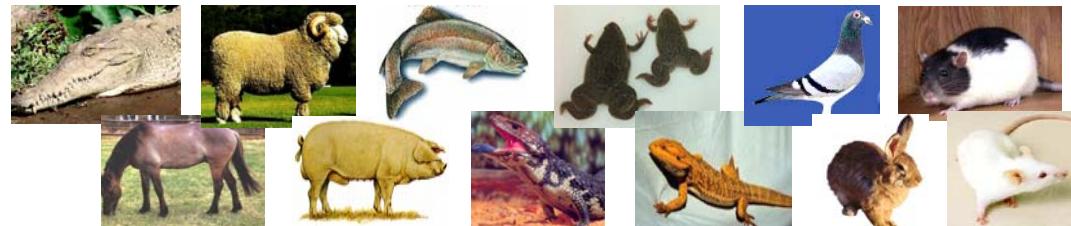
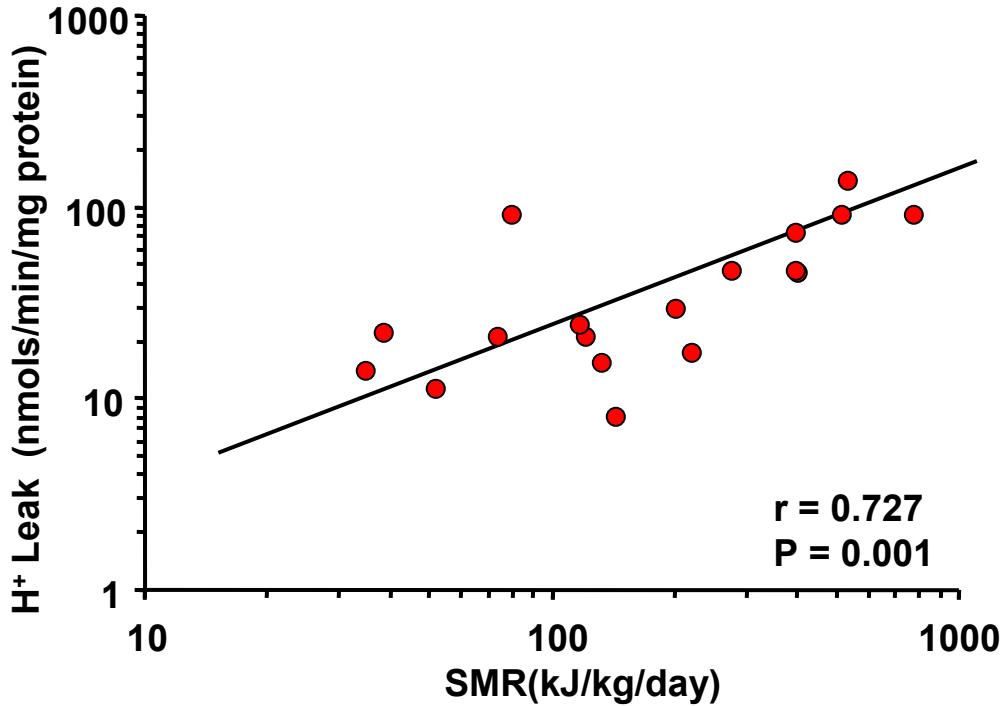
eNOS^{-/-} mice have less mitochondria



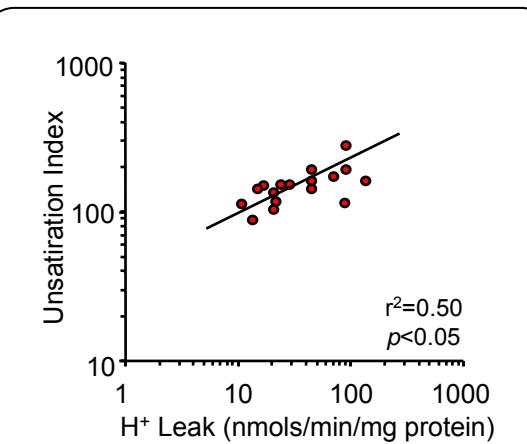
Mitochondrial H⁺ Leak



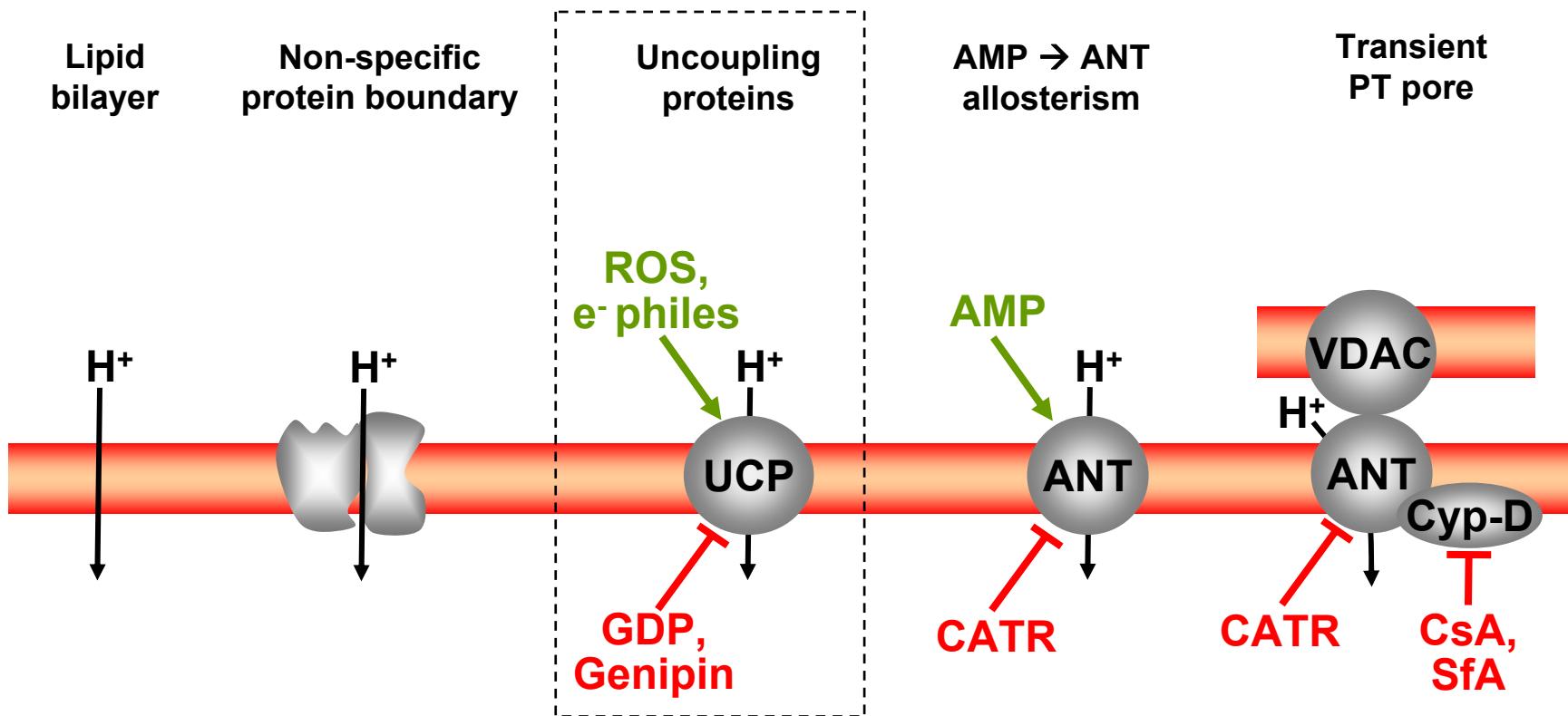
H^+ Leak: a Component of Basal Metabolic Rate



Brookes et al. (1998) *Comp. Biochem. Physiol.* **119B**: 269-72
Brookes et al. (1997) *Biochim. Biophys. Acta* **1330**: 157-64



H^+ Leak Mechanisms & Modulators



Uncoupling Proteins: Location & Homology

UCP-1 Brown adipose “thermogenin”, 10% of protein in BAT mito'

Nicholls & Rial E (1999) *J. Bioenerg. Biomembr.* **31**: 399-406

UCP-2 Leukocytes, Brain, Kidney, Adipose, Sk-M, Heart

Fleury *et al.* (1997) *Nature Genetics* **15**: 269-72 **59% = UCP1**

UCP-3 Sk-M, BAT, T-cells, Macrophages

Boss *et al.* (1997) *FEBS Lett.* **408**: 39-42 **57% = UCP1, 71% = UCP2**

UCP-4 Brain

Bouillaud *et al.* (2001) *Biochim. Biophys. Acta* **1504**: 107-19 **32% = UCP1-3**

UCP-5 Brain

Sanchis *et al.* (1998) *J. Biol. Chem.* **273**: 34611-5 **38% = UCP1-3**

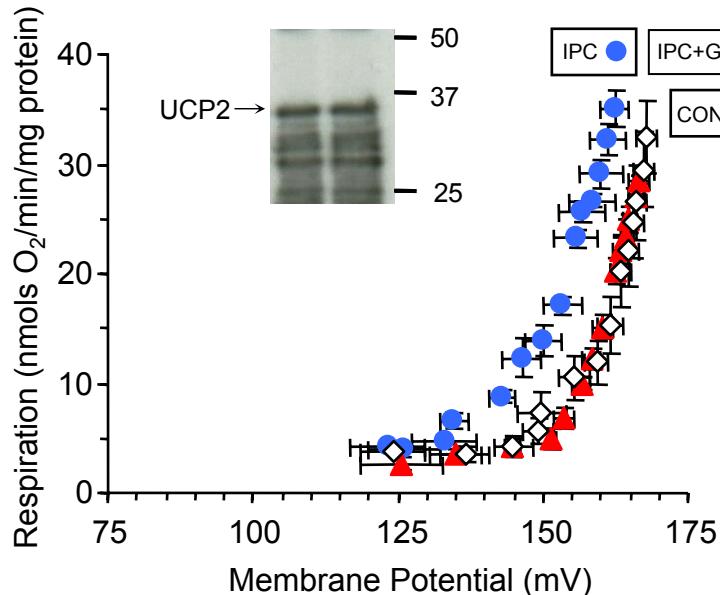
PUMP Plants

Jezek *et al.* (1996) *J. Biol. Chem.* **271**: 32743-8

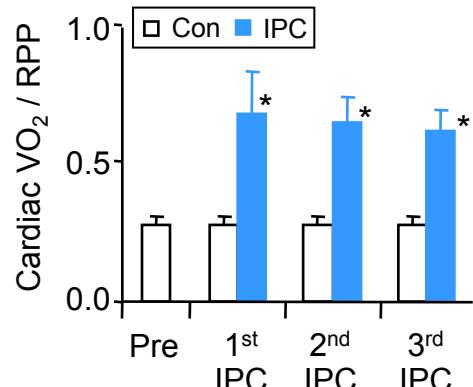
fishUCP

Stuart *et al.* (1999) *Biochim. Biophys. Acta* **1413**: 50-4 **60% = UCP1, 82% = UCP2**

H^+ Leak in IPC



Nadtochiy et al. (2006) *Biochem. J.* **395**: 611-18



UCP2 mRNA↑ in delayed IPC

J. Biol. Chem. (2005) **280**: 33470-6

DNP/FCCP/Tg ↑UCP → cardioprotection

J. Mol. Cell. Cardiol. (2003) **35**: 749-59

Cardiovasc. Res. (2000) **47**: 68-73

Circulation (2004) **110**: 528-33

Circ. Res. (2003) **93**: 192-200

ROS / RNS are essential for IPC

Antiox. Redox. Sig. (2004) **6**: 393-404

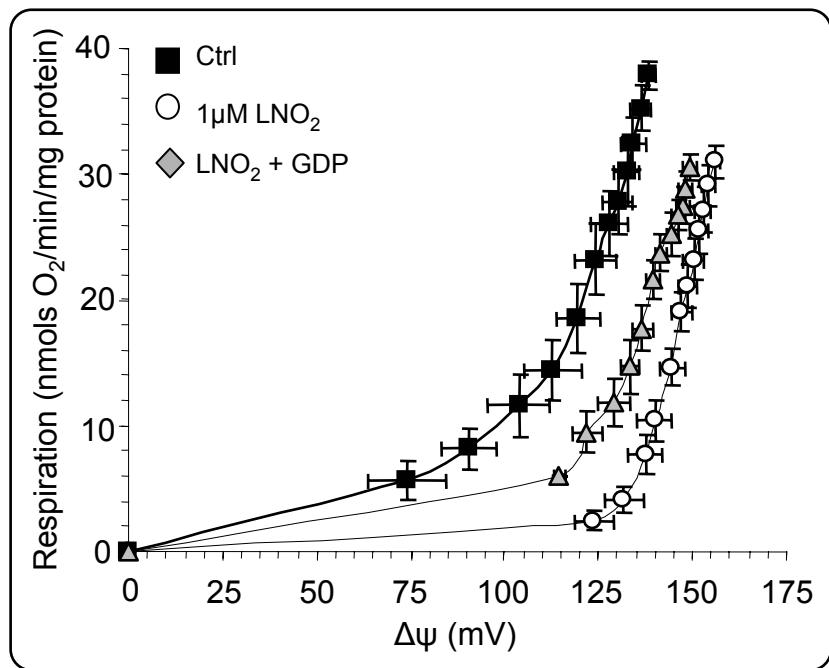
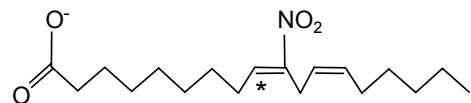
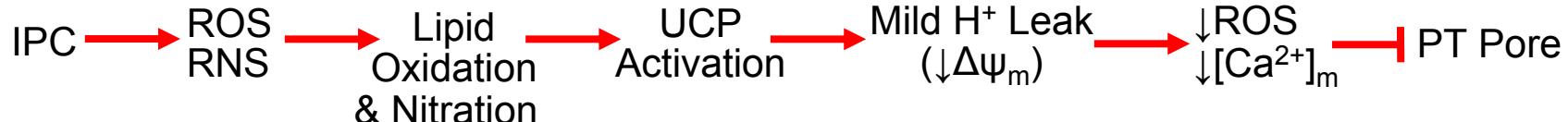
Cardiovasc Res. (2006) **70**: 231-9

ROS, RNS, e-philes ↑H⁺ Leak/UCPs

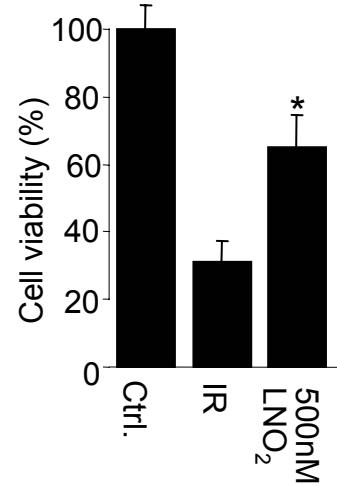
J. Biol. Chem. (2003) **278**: 48534-45

J. Neurochem. (1998) **70**: 2195-202

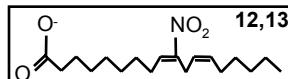
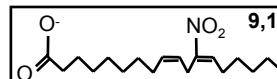
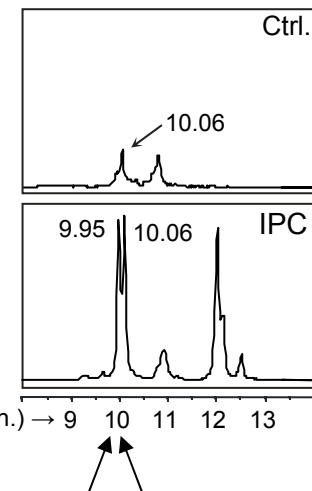
Nature (2002) **415**: 96-9



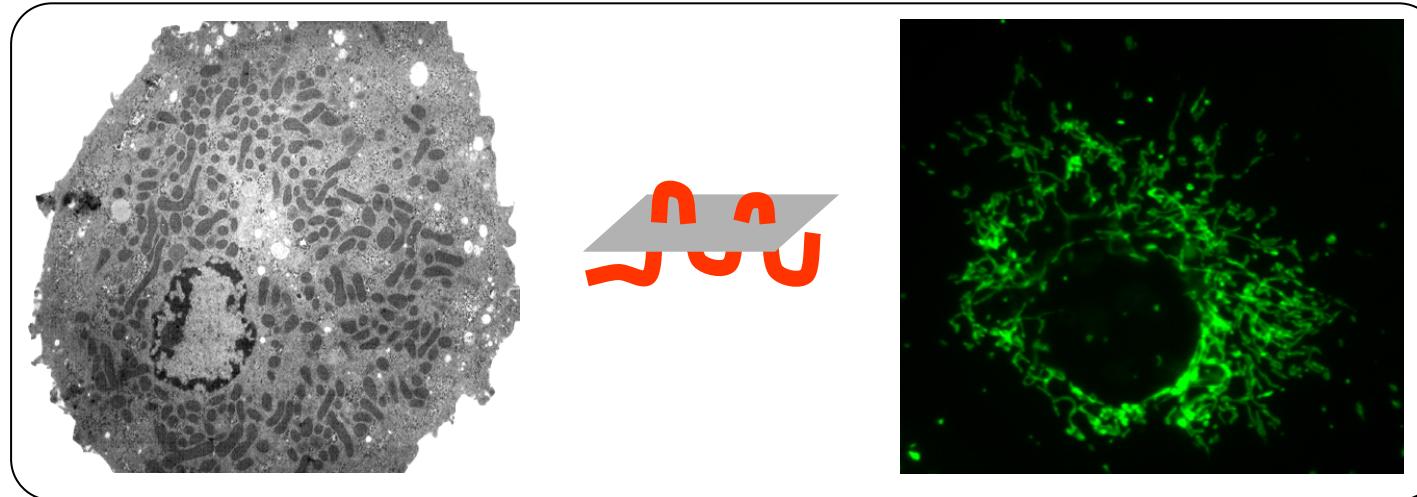
Added LNO₂ protects myocytes from IR



LNO₂ is formed inside mitochondria during IPC



Not complicated enough? Add in Mito Fusion & Fission



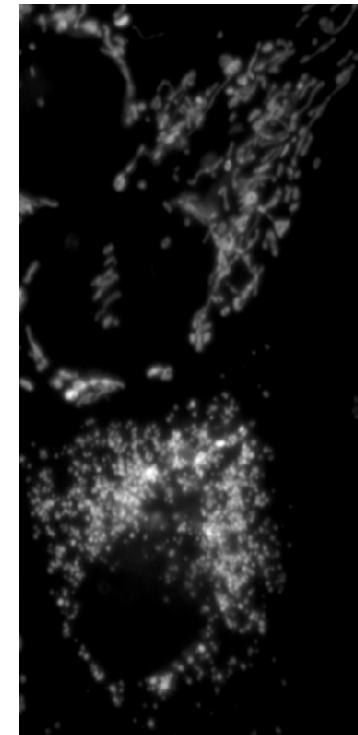
Chan DC (2006) *Cell* **125**: 1241-52

Yoon Y (2005) *Science STKE* Apr 19 2005(280):pe18

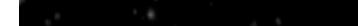
Youle et al. (2005) *Nature Rev. Mol. Cell. Biol.* **6**: 657-63

Functions:

- Required for cell division
- Getting energy (ATP) to the right place
- Regulation of apoptosis
- Exchange of mtDNA
- Dilution/concentration of damaged protein/lipid
- Signaling along tubules?



Tubular



Fragmented

Mitochondrial Morphology: a Tightly Regulated Process

Fragmentation essential for apoptosis

Youle *et al.* (2001) *Dev. Cell* **1**: 515-25

DLP-1 (dynamin-like protein, DRP-1, Dnm1-p) 80 kDa cytosolic GTPase, pinchase

Fragmentation essential for high glucose-induced ROS

Yu *et al.* (2006) *PNAS* **103**: 2653-8

hFis-1 (human homolog of yeast Fis1p) 17 kDa mito' outer membrane protein, recruits DLP1

Overexpress hFis1 → fragmentation → apoptosis

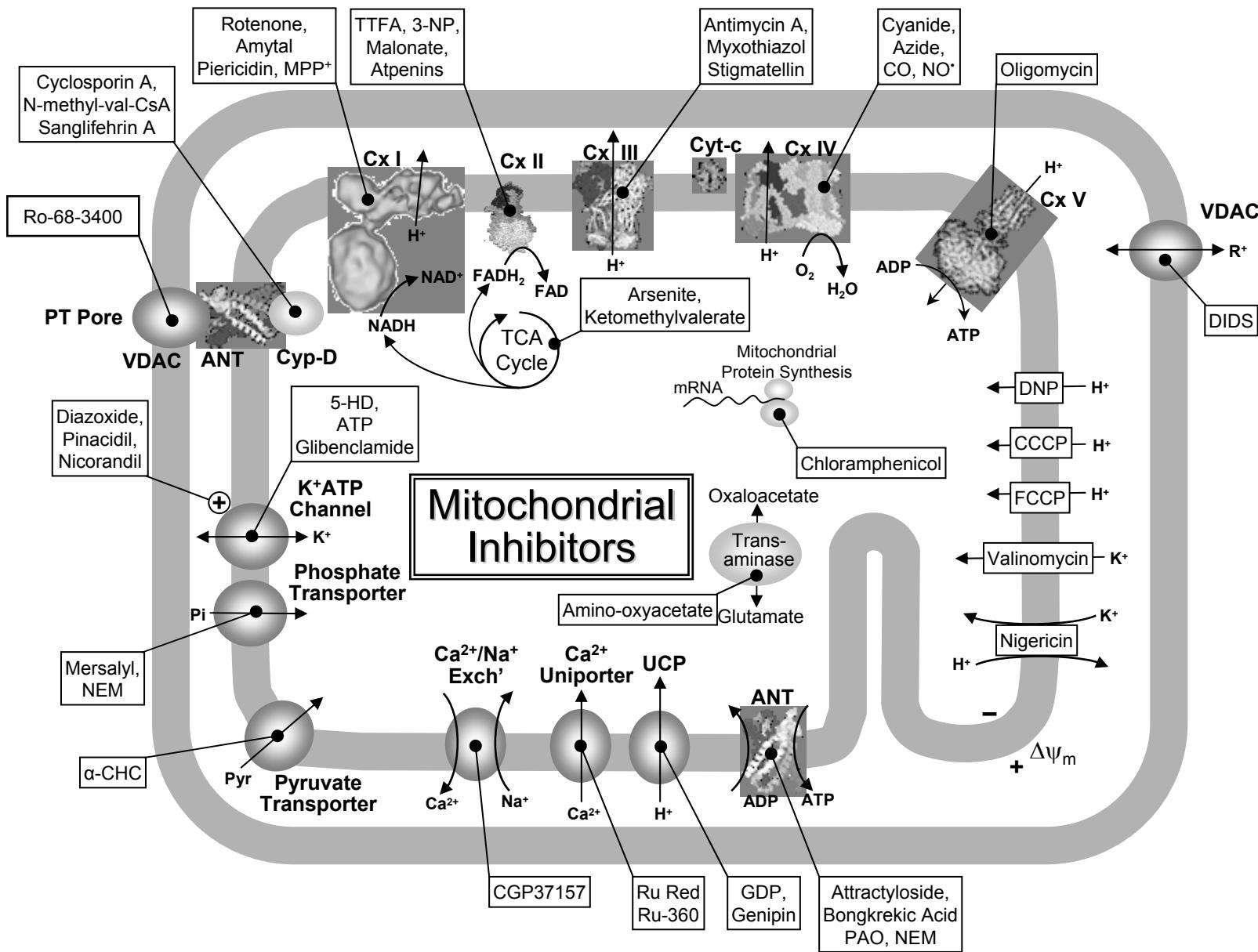
Sugioka *et al.* (2004) *JBC*. **279**: 52726-34

Mfn-1/2 (mitofusin, mammalian homolog of Fzo-1p) 86 kDa GTPase, tethers 2 mitochondria for fusion

Overexpress Fzo-1p → inhibits apoptosis

Yu *et al.* (2005) *J. Cell Sci.* **118**: 4141-51

OPA-1 (mammalian homolog of Mgm-1p) Mito inner membrane GTPase, remodeling of IMM



Abbreviations... 3-NP: 3-nitro propionic acid, 5-HD: 5-hydroxydecanoate, ANT: adenine nucleotide translocase, CCCP: carbonylcyanide m-chlorophenylhydrazone, Cx I: respiratory complex I etc., Cyp-D: cyclophilin D, Cyt-c: cytochrome c, DNP: dinitrophenol, DIDS: 4,4-diisothiocyanato-stilbene-2,2'disulphonate, FCCP: carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone, MPP⁺: 1-methyl-4-phenylpyridinium, NEM: N-ethylmaleimide, PAO: phenylarsine oxide, TTFA: thenoyl-trifluoroacetone, UCP: uncoupling protein, VDAC: volatge dependent anion channel.