# Mitochondria in ischemia and reperfusion

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CBCS Summer School on Cardiovascular Sciences
Basic Mechanisms translated to the Clinic
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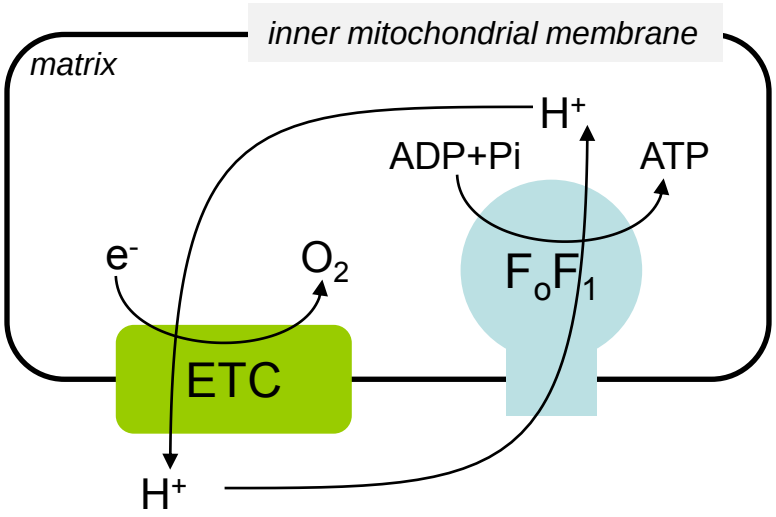
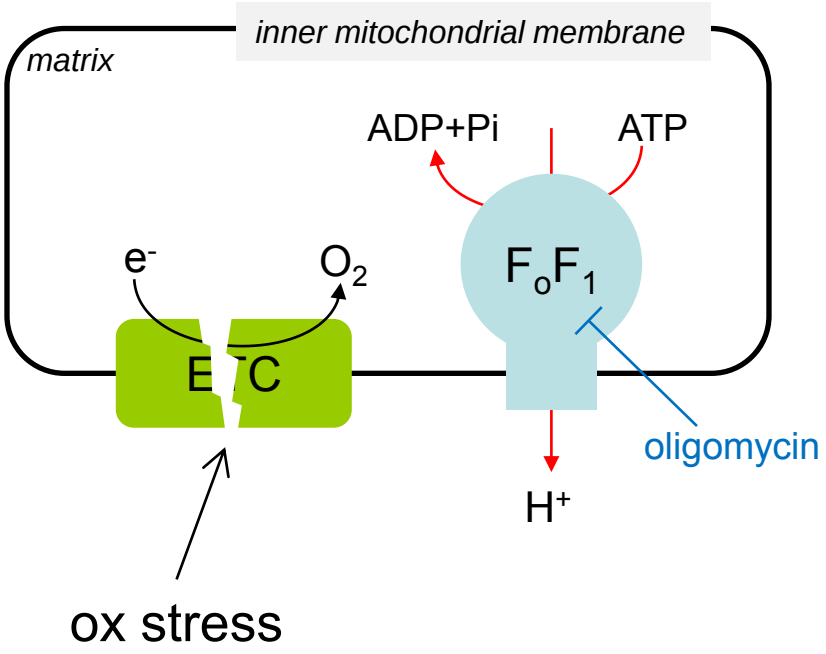
# Mitochondria in ischemia and reperfusion

- (boring) background
- $Ca^{2+}$ and permeability transition
- ROS formation


inner mitochondrial membrane
matrix
H+
ADP+Pi
ATP
e-
O2
FoF1
ETC
H+


inner mitochondrial membrane
matrix
ADP+Pi
ATP
e-
O2
FoF1
ETC
oligomycin
H+
ox stress

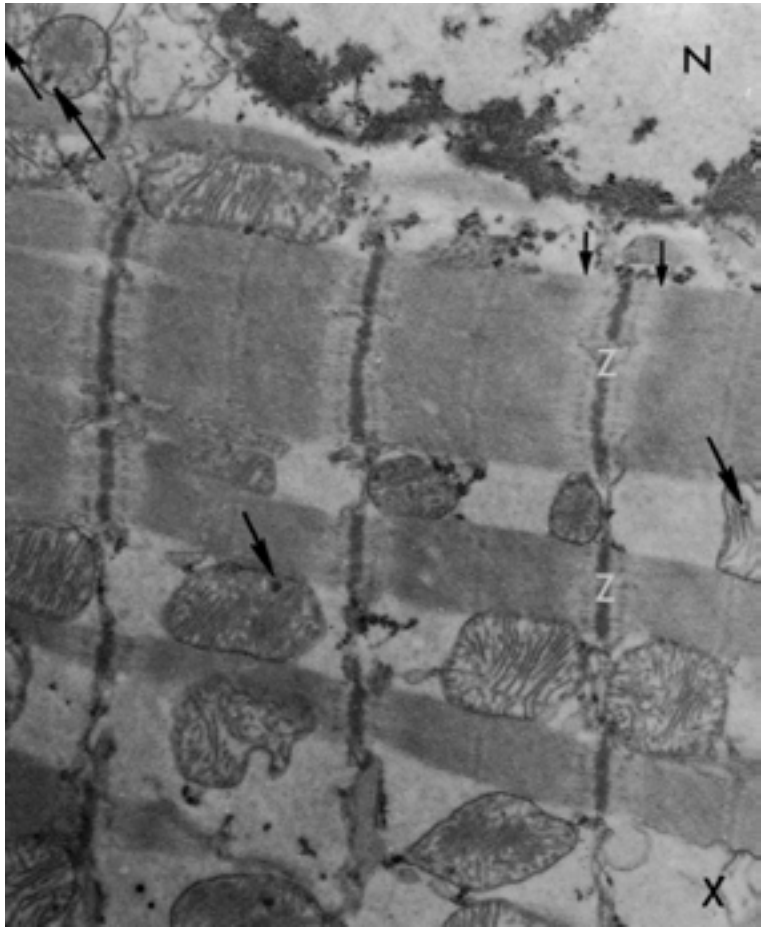
- Structural and functional abnormalities of mitochondria are caused by ischemia/reperfusion

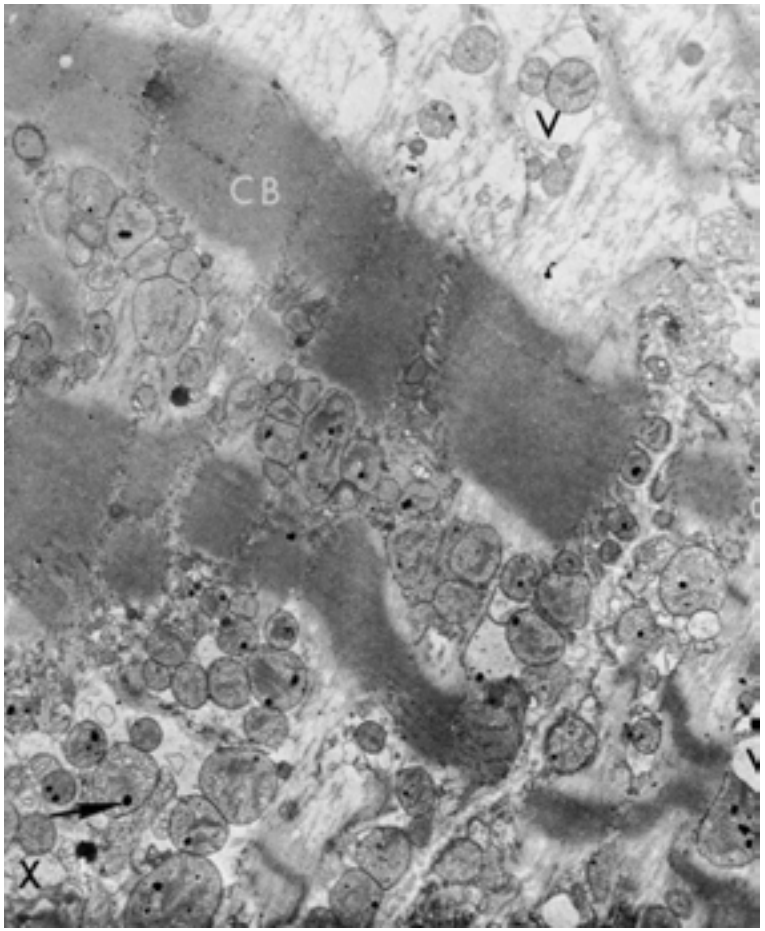
- Mitochondrial dysfunction might result in myocardial protection

# Calcium accumulatiom within mitochondrial matrix of cardiomyocytes induced by post-ischemic reperfusion in dog hearts

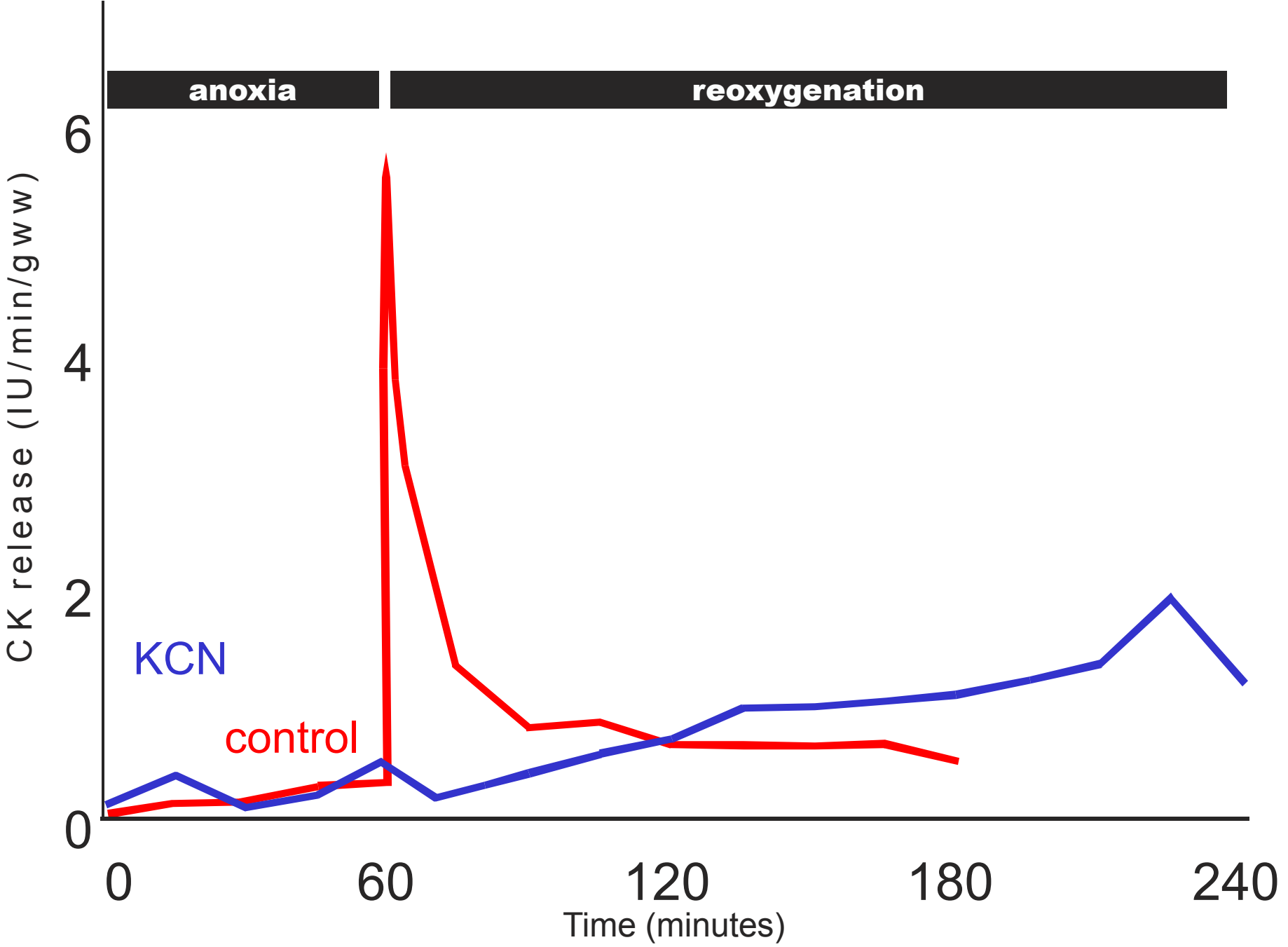
60 min ischemia

40 min ischemia +
20 min reperfusion

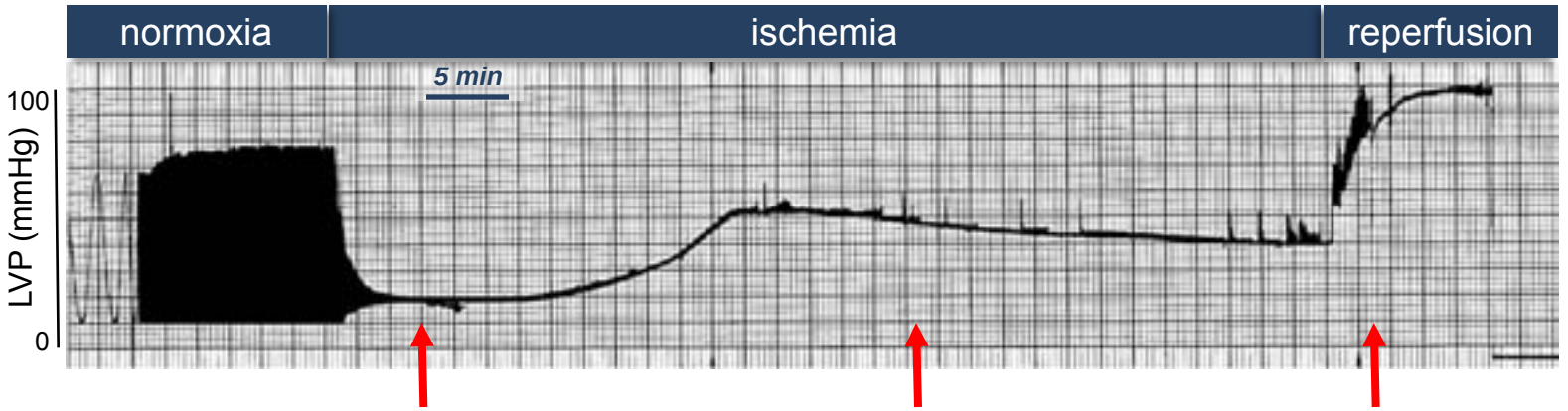



AC Shen and RB Jennings, *Am.J.Pathol.*, 67:417-440, 1972




*redrawn from Ganote et al., 1976*




lack of contraction

rigor contracture

hypercontracture and loss of viabilility

*acidosis*

*increased anaerobic glycolysis*

*Pi increase*

*ATP depletion*

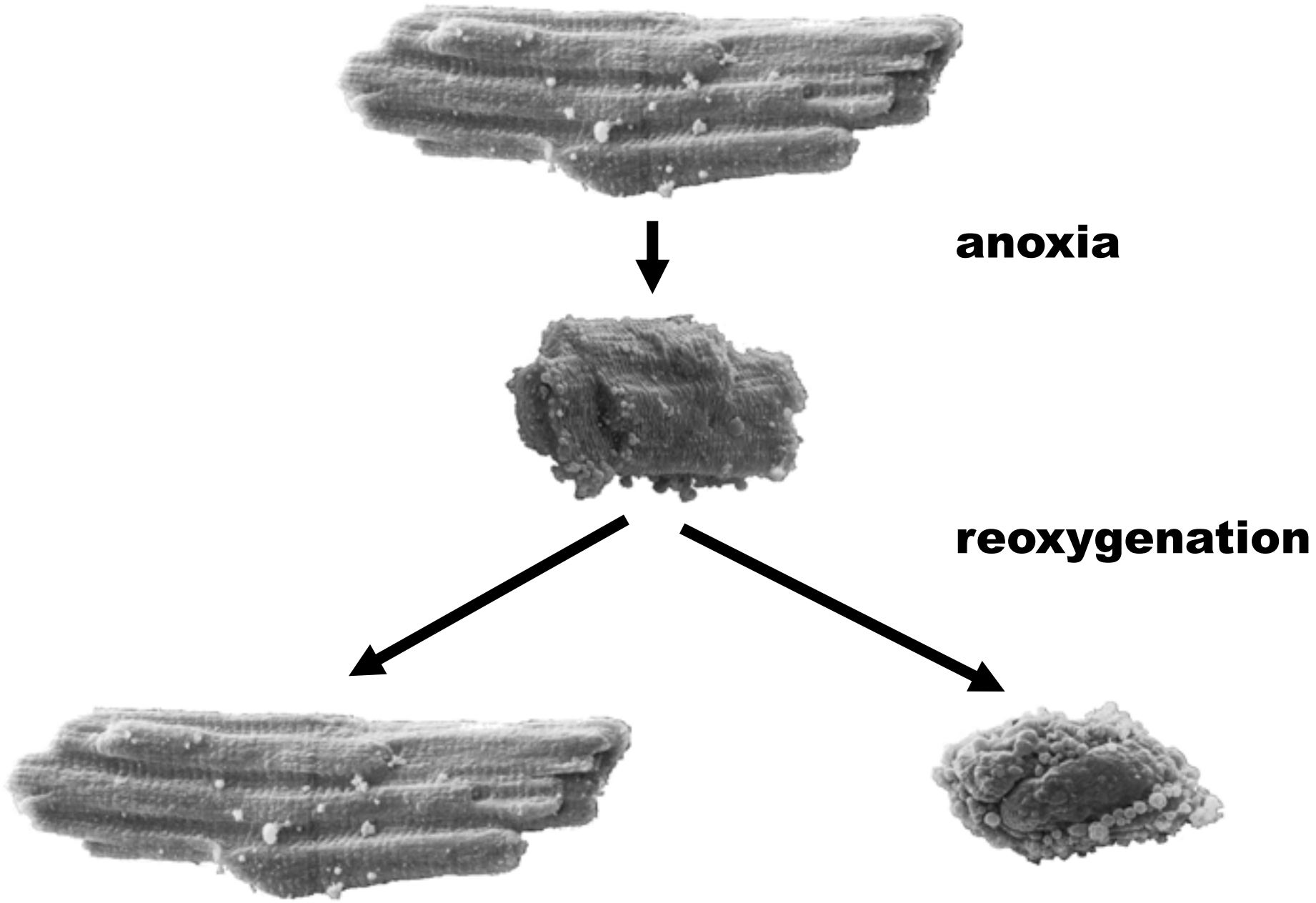
*Suboptimal recovery of mitochondrial function (low ATP) in the presence of elevated [$Ca^{2+}$]*

*decreased ATP synthesis and increased ATP hydrolysis*

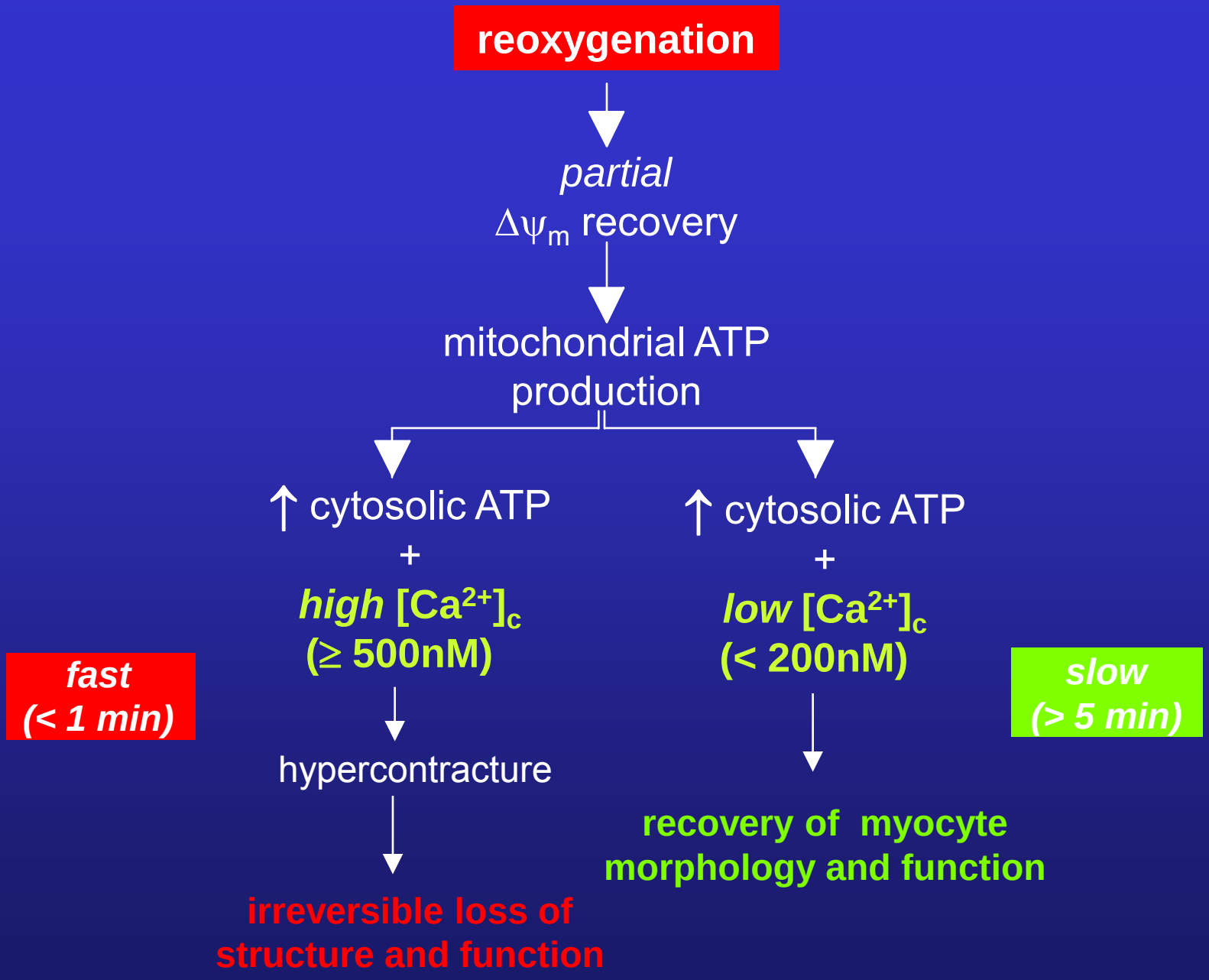
*inhibition of oxidative phosphorylation*

*lack of oxygen*

*readmission of oxygen*

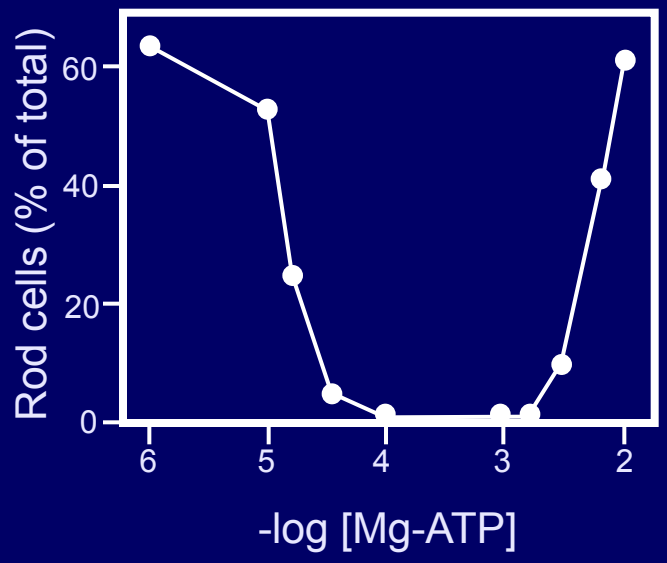


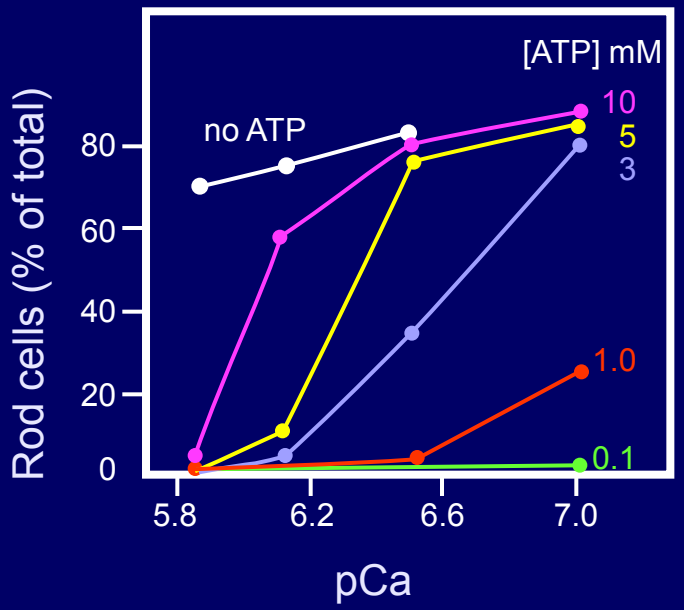

*adapted from Siegmund et al., Am. J. Physiol, 1991*


reoxygenation
partial
$\Delta\psi_m$ recovery
mitochondrial ATP production
↑ cytosolic ATP
+
high $[Ca^{2+}]_c$
(≥ 500nM)
hypercontracture
irreversible loss of structure and function
fast
(< 1 min)
↑ cytosolic ATP
+
low $[Ca^{2+}]_c$
(< 200nM)
recovery of myocyte morphology and function
slow
(> 5 min)

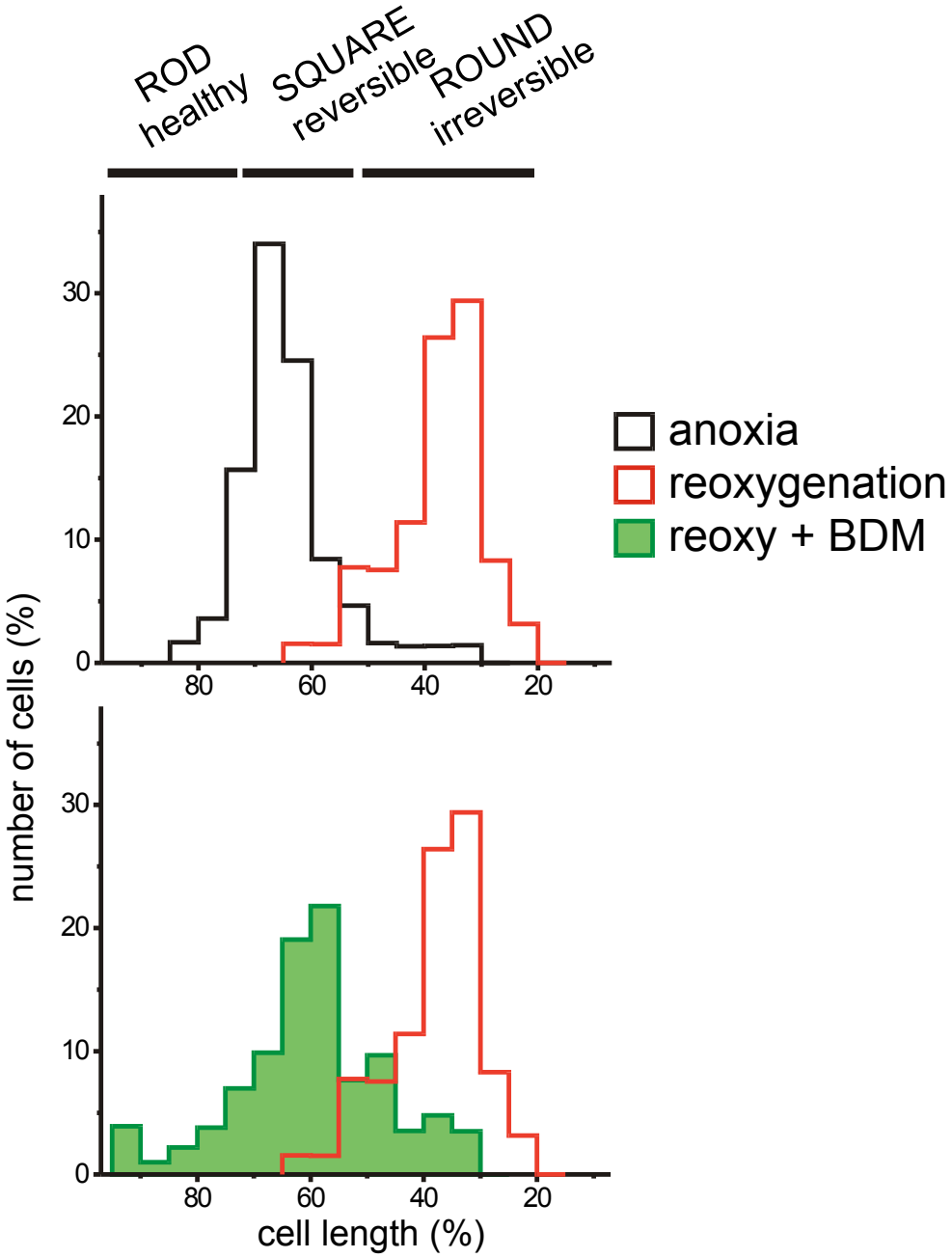
# Relaxation and maintenance of rod-shaped morphology require [ATP] in the millimolar range

## Submillimolar [ATP] results in hypercontracture, a process facilitated by high [$Ca^{2+}$]

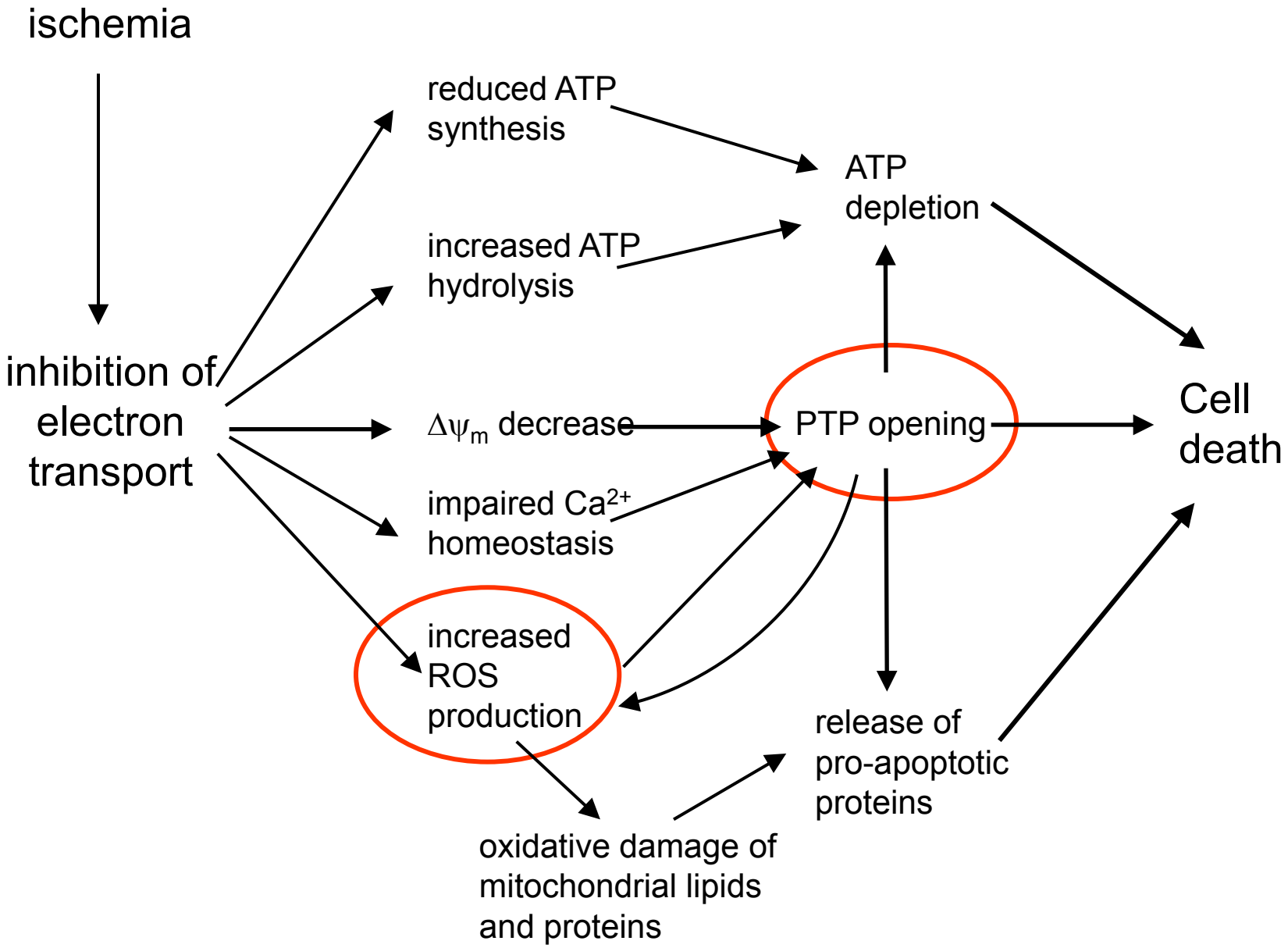



*R.A. Altschuld et al., J.Biol. Chem., 260:14325-30, 1985*



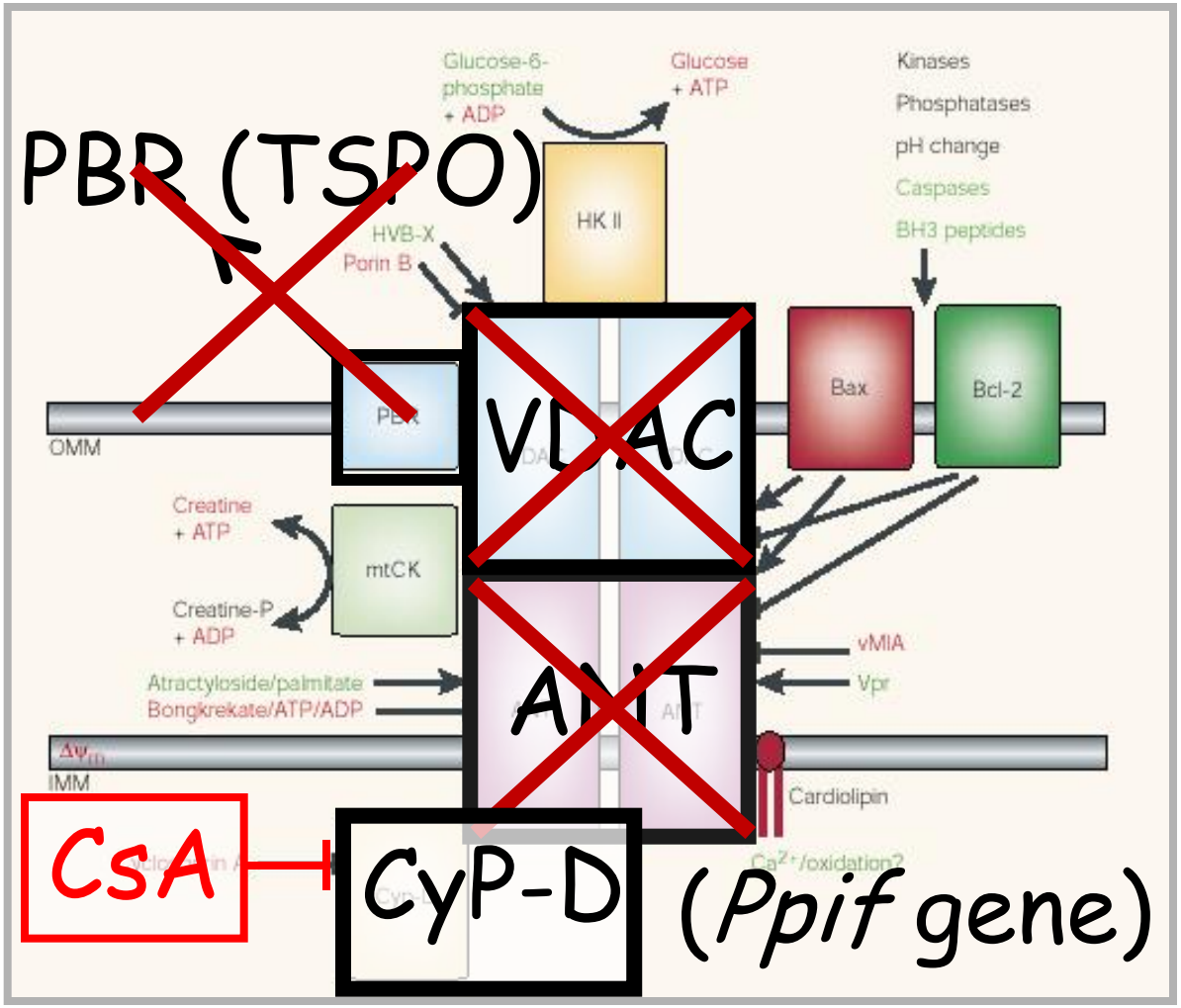

*redrawn from Siegmund et a., Am.J.Physiol., 1991*


ischemia
inhibition of electron transport
reduced ATP synthesis
increased ATP hydrolysis
$\Delta\psi_m$ decrease
impaired $Ca^{2+}$ homeostasis
increased ROS production
oxidative damage of mitochondrial lipids and proteins
ATP depletion
PTP opening
release of pro-apoptotic proteins
Cell death

# The Permeability Transition Pore

The mitochondrial permeability transition (PT) defines a sudden increase in the permeability of the inner mitochondrial membrane to solutes with molecular masses up to 1500 Da.

This process is attributed to the opening of a voltage- and $Ca^{2+}$-dependent, cyclosporin A (CsA)-sensitive, high-conductance channel that is termed permeability transition pore (PTP)


Glucose-6-phosphate + ADP
Glucose + ATP
Kinases
Phosphatases
pH change
Caspases
BH3 peptides
PBR (TSPO)
HK II
HVB-X
Porin B
PBR
VDAC
Bax
Bcl-2
OMM
Creatine + ATP
mtCK
Creatine-P + ADP
vMIA
Vpr
Atractyloside/palmitate
Bongkrekate/ATP/ADP
ANT
IMM
Cardiolipin
CsA
CyP-D
(Ppif gene)

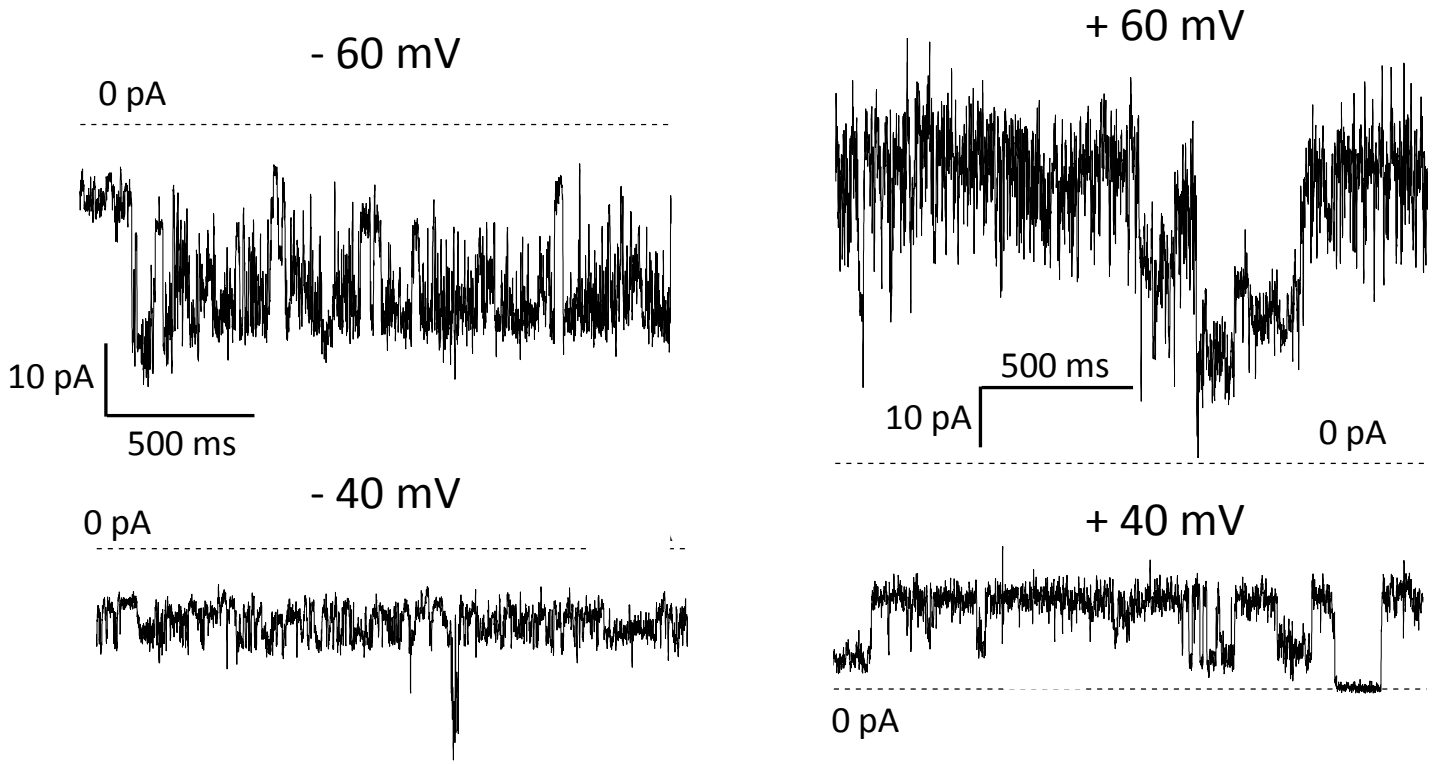
# Dimers of Mitochondrial ATP Synthase form the Permeability Transition Pore

Valentina Giorgio[a], Sophia von Stockum[a], Manuela Antoniel[b], Astrid Fabbro[b], Federico Fogolari[c], Michael Forte[d], Gary D. Glick[e], Valeria Petronilli[a], Mario Zoratti[a], Ildikó Szabó[f], Giovanna Lippe[b]*and Paolo Bernardi[a]*

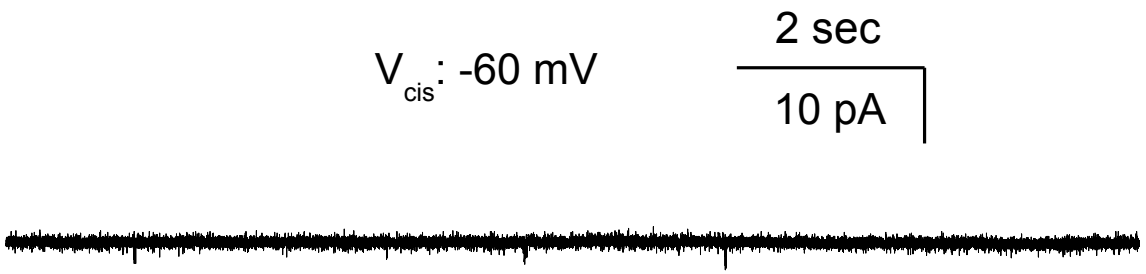
[a]Consiglio Nazionale delle Ricerche Institute of Neuroscience and Department of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, 35121 Padova Italy. [b]Department of Food Science, University of Udine, Via Sondrio 2, 33100 Udine, Italy. [c]Department of Medical and Biological Sciences, University of Udine, Piazzale Kolbe 4, 33100 Udine, Italy.

~~Submitted~~ *in press* to Proceedings of the National Academy of Sciences of the United States of America

# Currents can be elicited by $Ca^{2+}$ and Pi alone



# No currents are seen with the monomer


or with gel-purified complex I

## Soluble factors

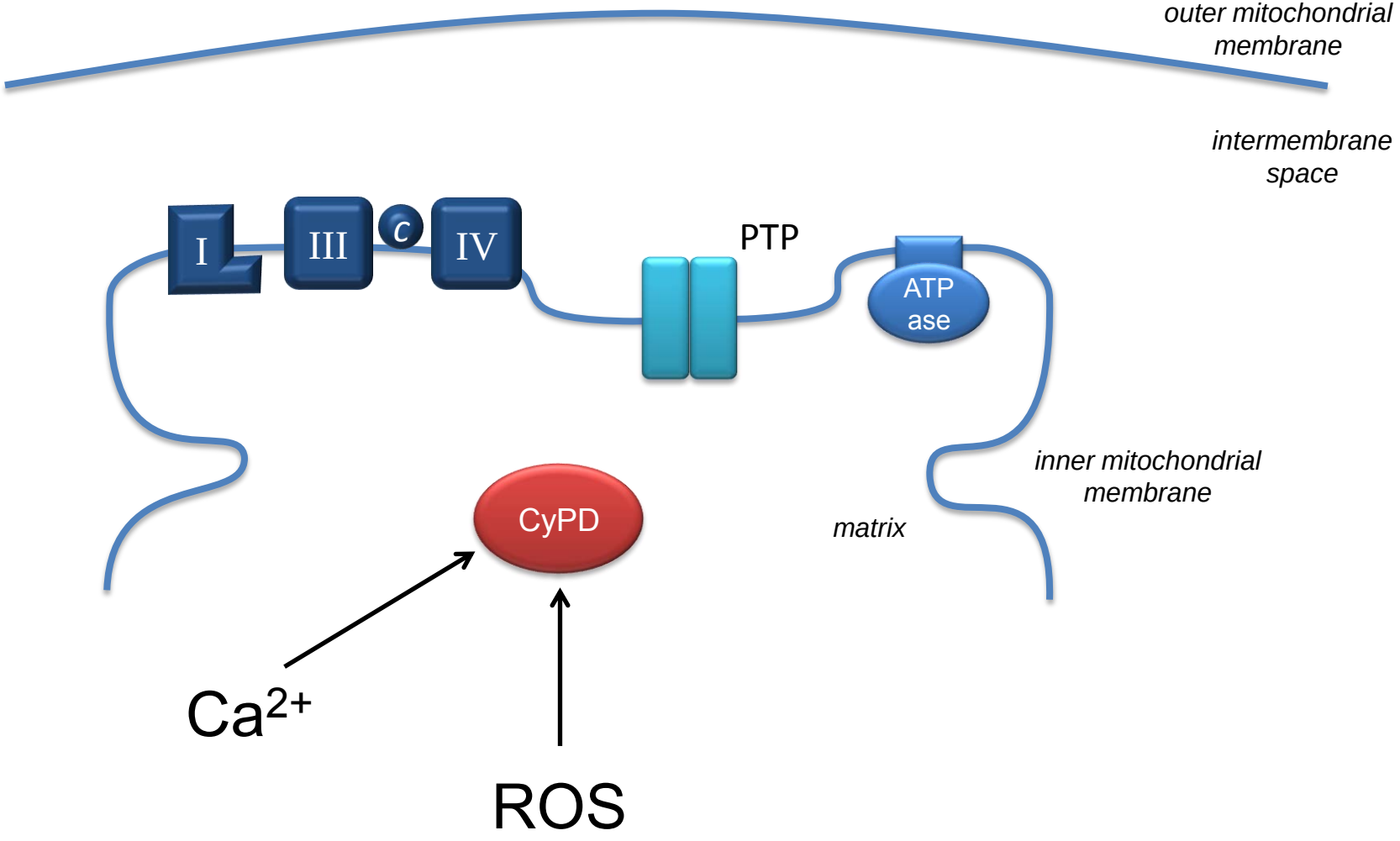
- $Ca^{2+}$
- Pi
- ADP
- $Mg^{2+}$
- pH
- RNS
- ROS
- $NAD(P)H/NAD(P)^{+}$
- GSH/GSSG

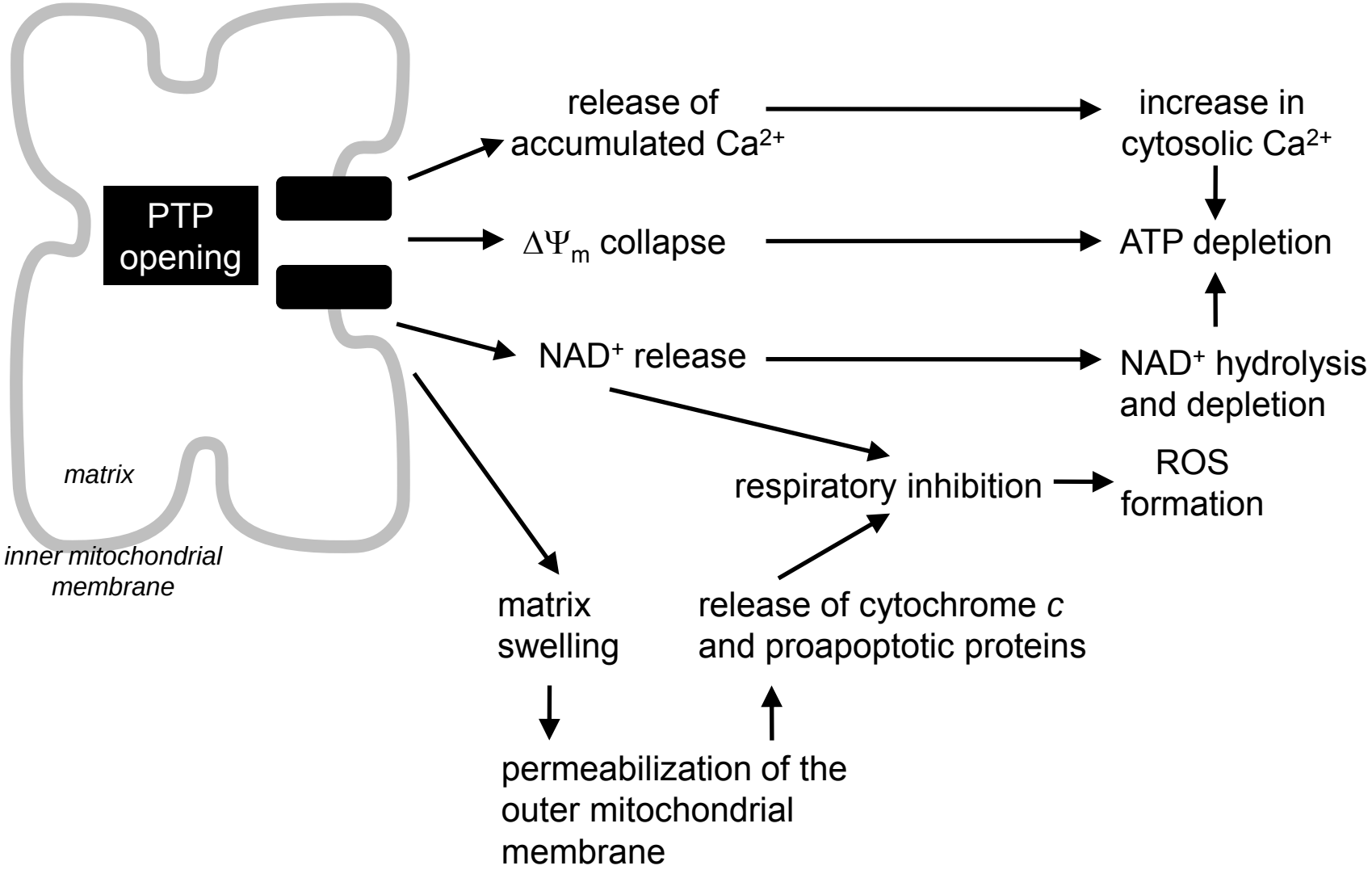
## IMM function and lipids

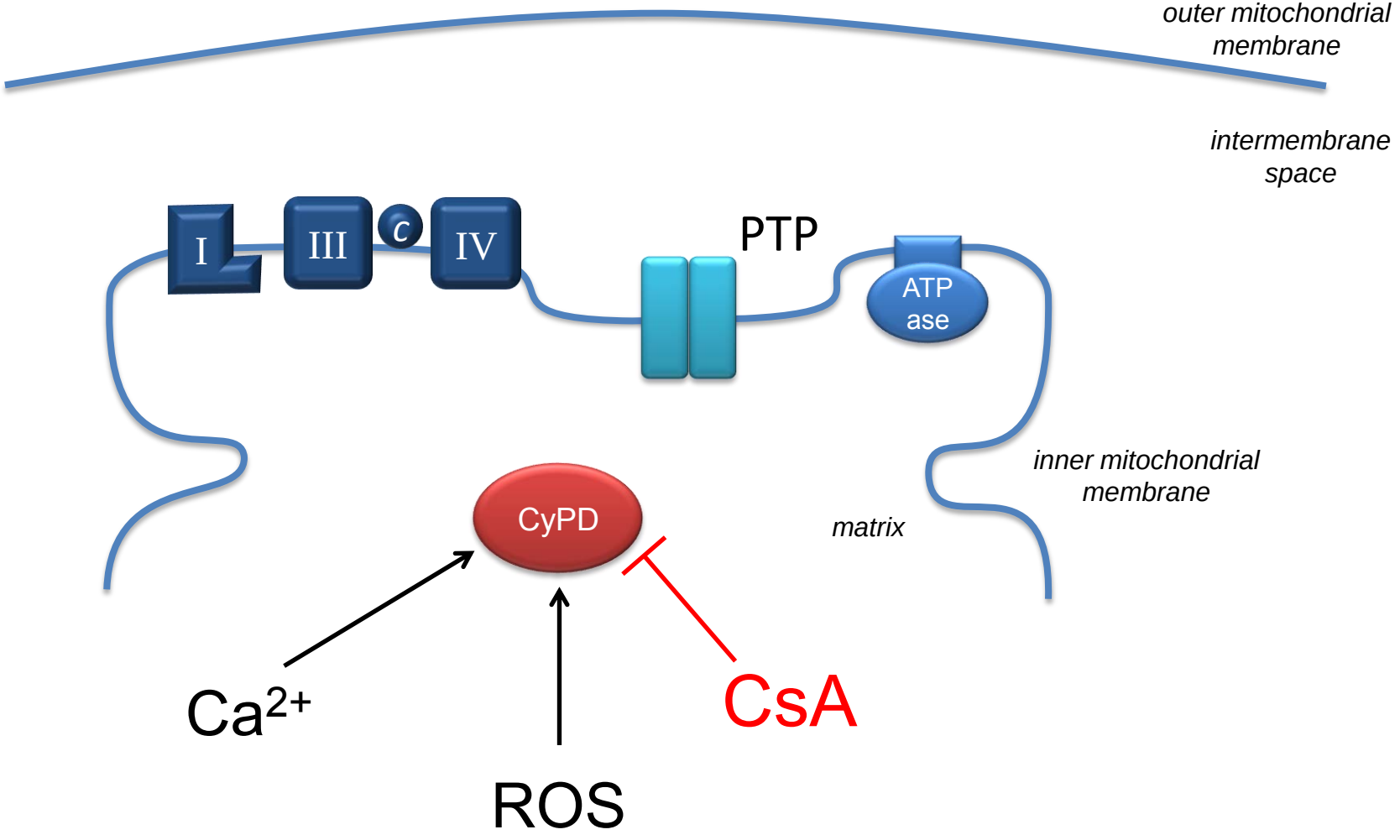
- $\Delta\Psi_m$
- DHA
- Arachidonate
- Surface potential

## Proteins

- CyPD
- PiC
- ANT
- VDAC
- HK
- GSK3β
- PKCε
- Protein unfolding


outer mitochondrial membrane
intermembrane space
I
III
c
IV
PTP
ATP ase
inner mitochondrial membrane
matrix
CyPD
Ca2+
ROS


PTP opening
matrix
inner mitochondrial membrane
release of accumulated $Ca^{2+}$
increase in cytosolic $Ca^{2+}$
$\Delta\Psi_m$ collapse
ATP depletion
$NAD^+$ release
$NAD^+$ hydrolysis and depletion
respiratory inhibition
ROS formation
matrix swelling
release of cytochrome *c* and proapoptotic proteins
permeabilization of the outer mitochondrial membrane


outer mitochondrial membrane
intermembrane space
I
III
c
IV
PTP
ATP ase
inner mitochondrial membrane
matrix
CyPD
Ca2+
ROS
CsA

# Effect of Cyclosporine on Reperfusion Injury in Acute Myocardial Infarction

C. Piot, P. Croisille, P. Staat, H. Thibault, G. Rioufol, N. Mewton, R. Elbelghiti, T. Tri Cung, E. Bonnefoy, D. Angoulvant, C. Macia, F. Raczka, C. Sportouch, G. Gahide, G. Finet, X. André-Fouët, D. Revel, G. Kirkorian, J-P. Monassier, G. Derumeaux and M. Ovize

N Engl J Med 2008;359:473-81.

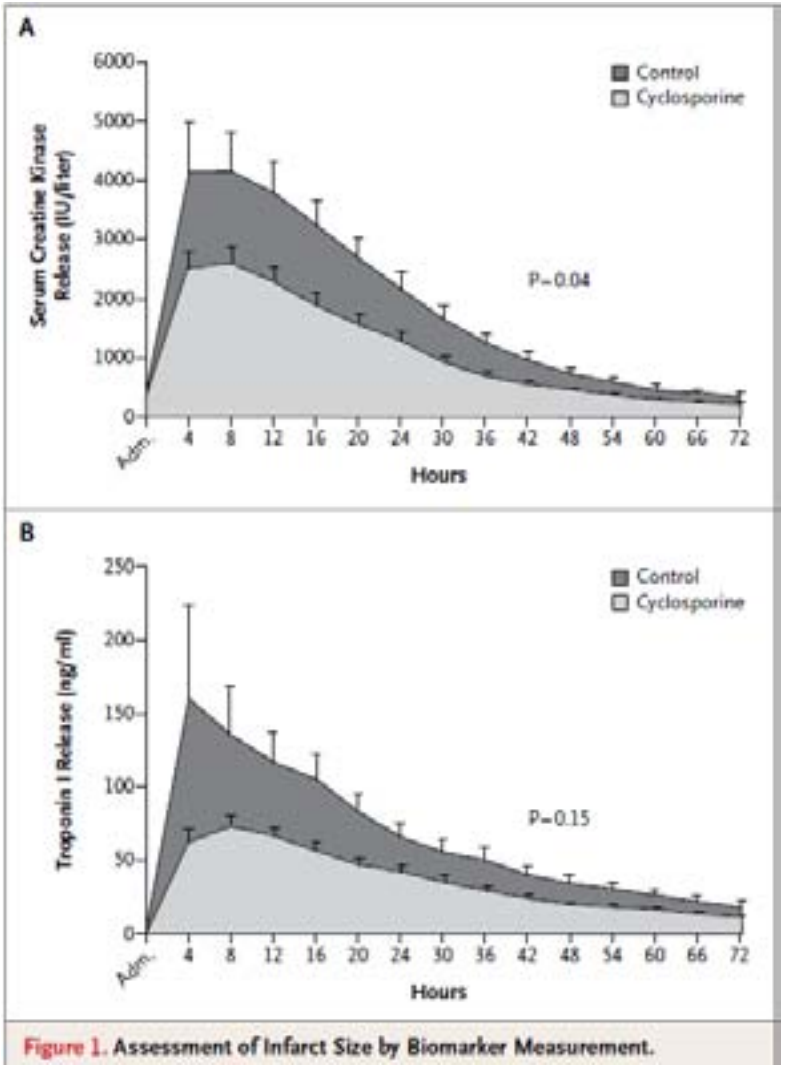



**Figure 1.** Assessment of Infarct Size by Biomarker Measurement.

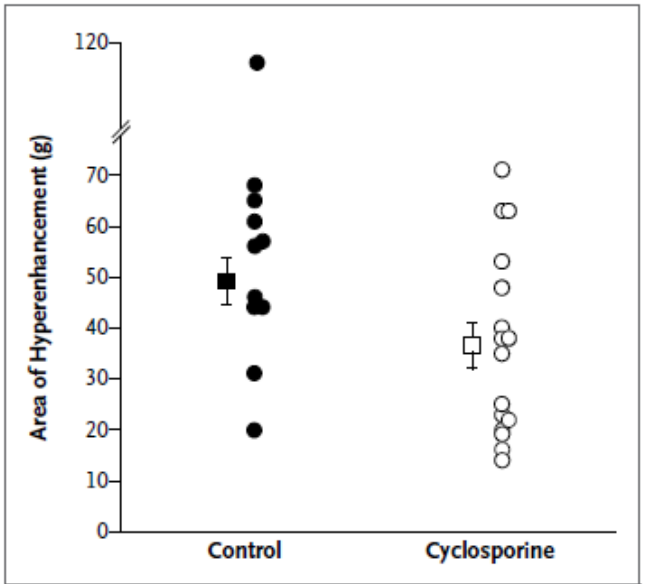
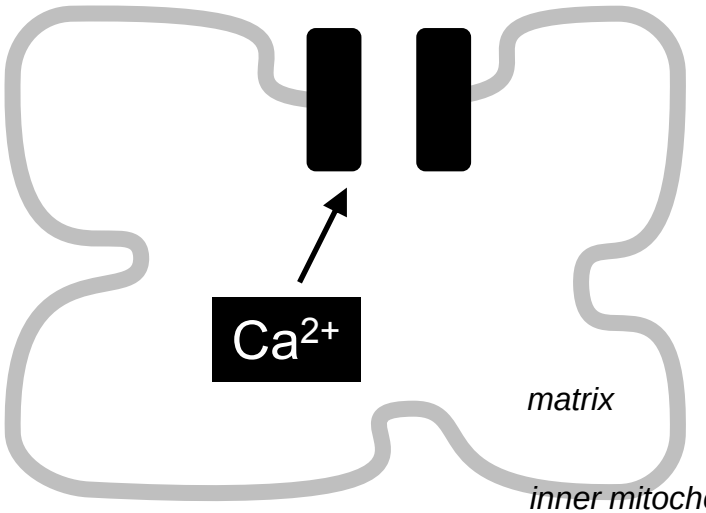
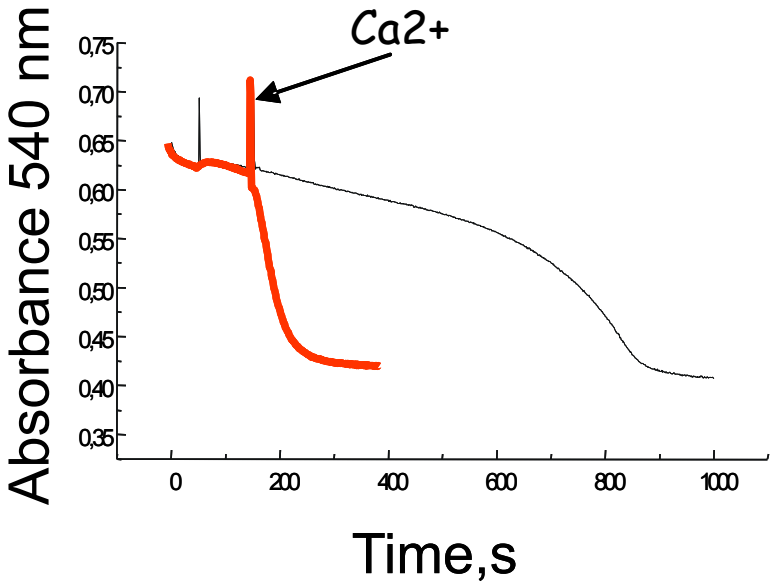


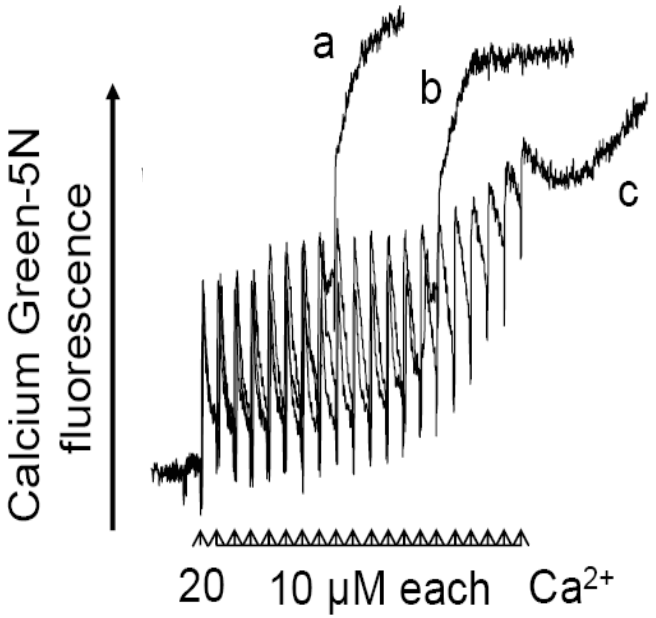

**Figure 3.** Assessment of Infarct Size by Magnetic Resonance Imaging (MRI).



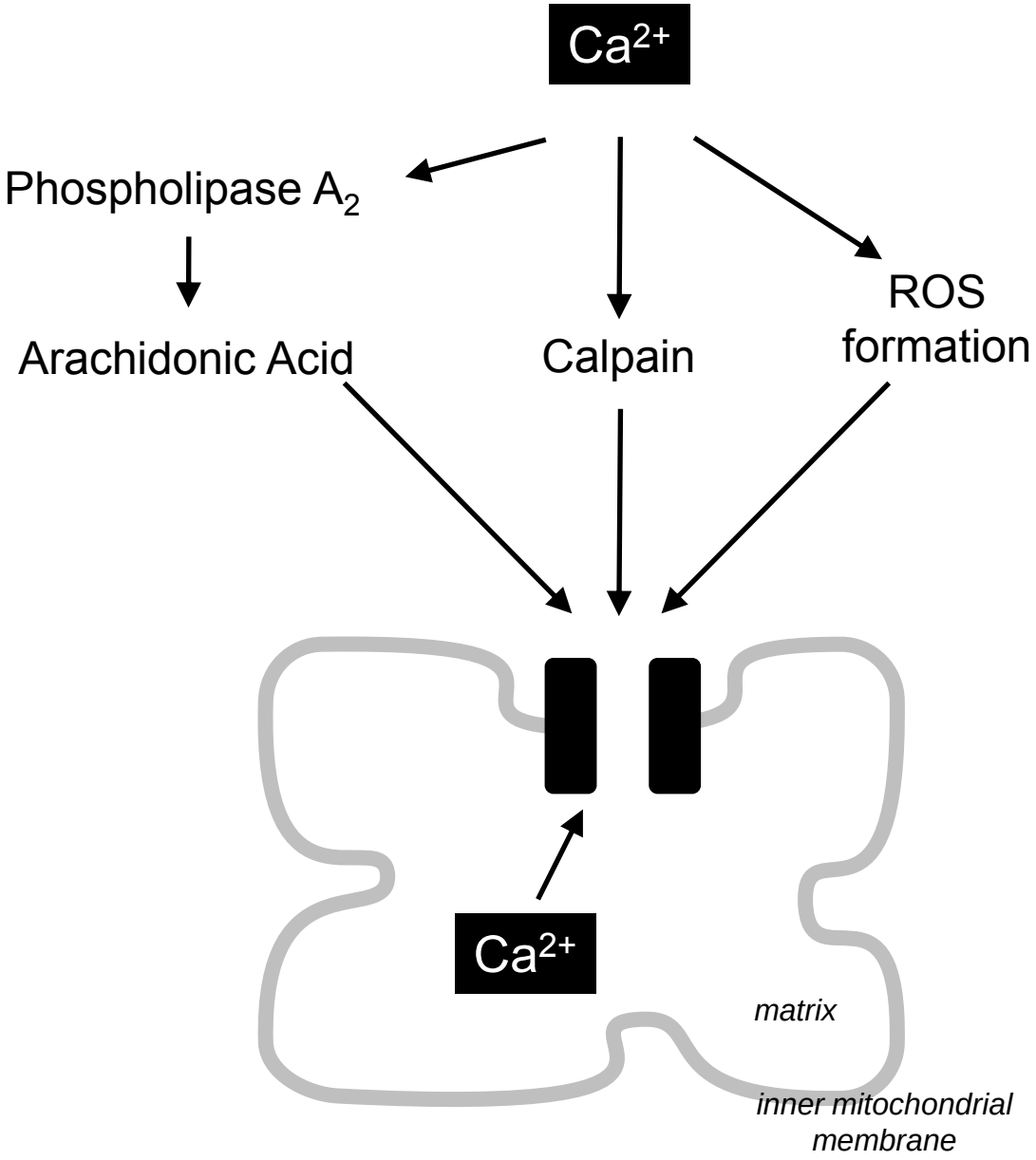

DIRECT

# Spectrophotometric assessments of PTP opening

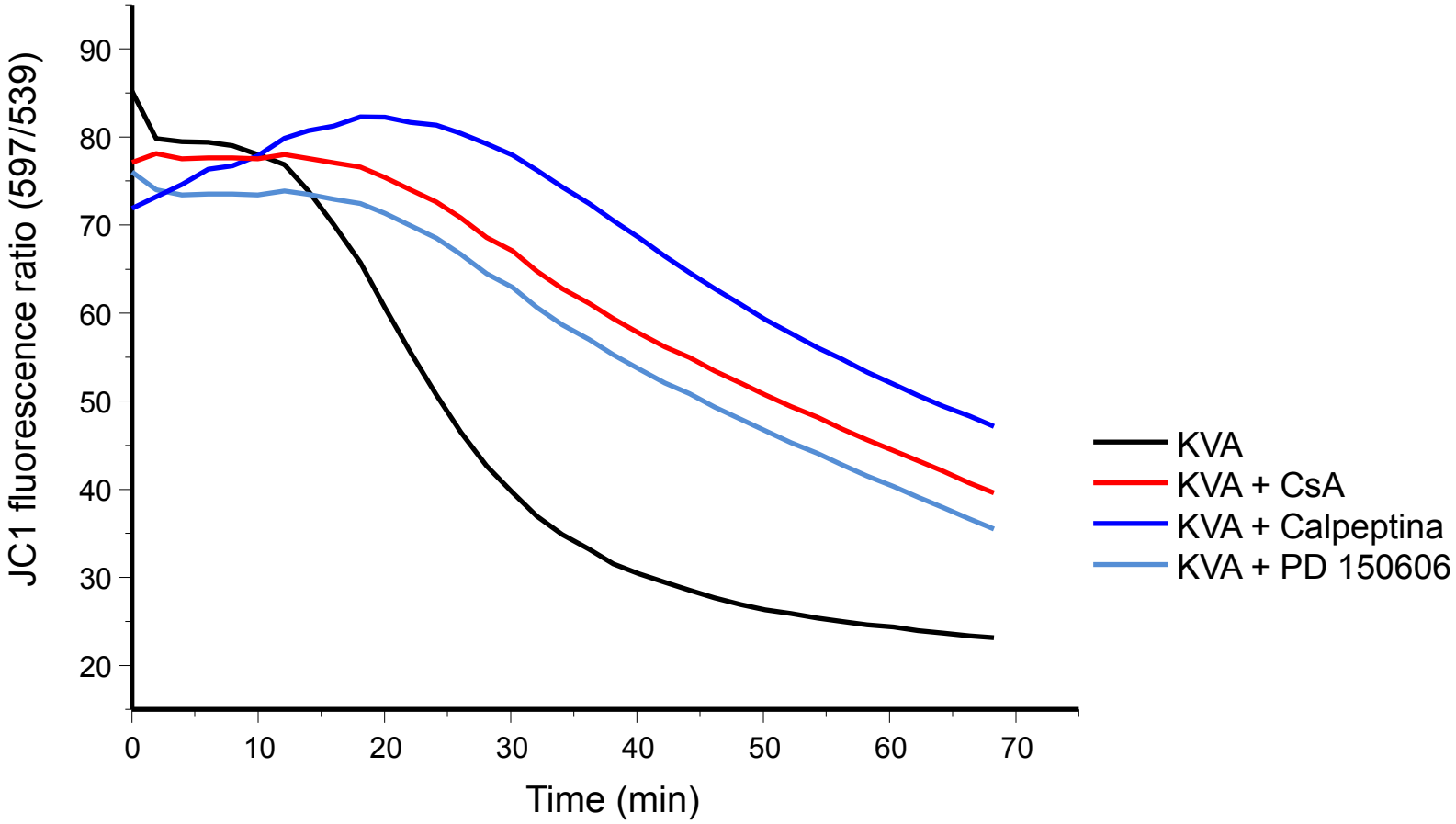



In isolated mitochondria PTP opening requires the addition of high (i.e., non physiological) $Ca^{2+}$

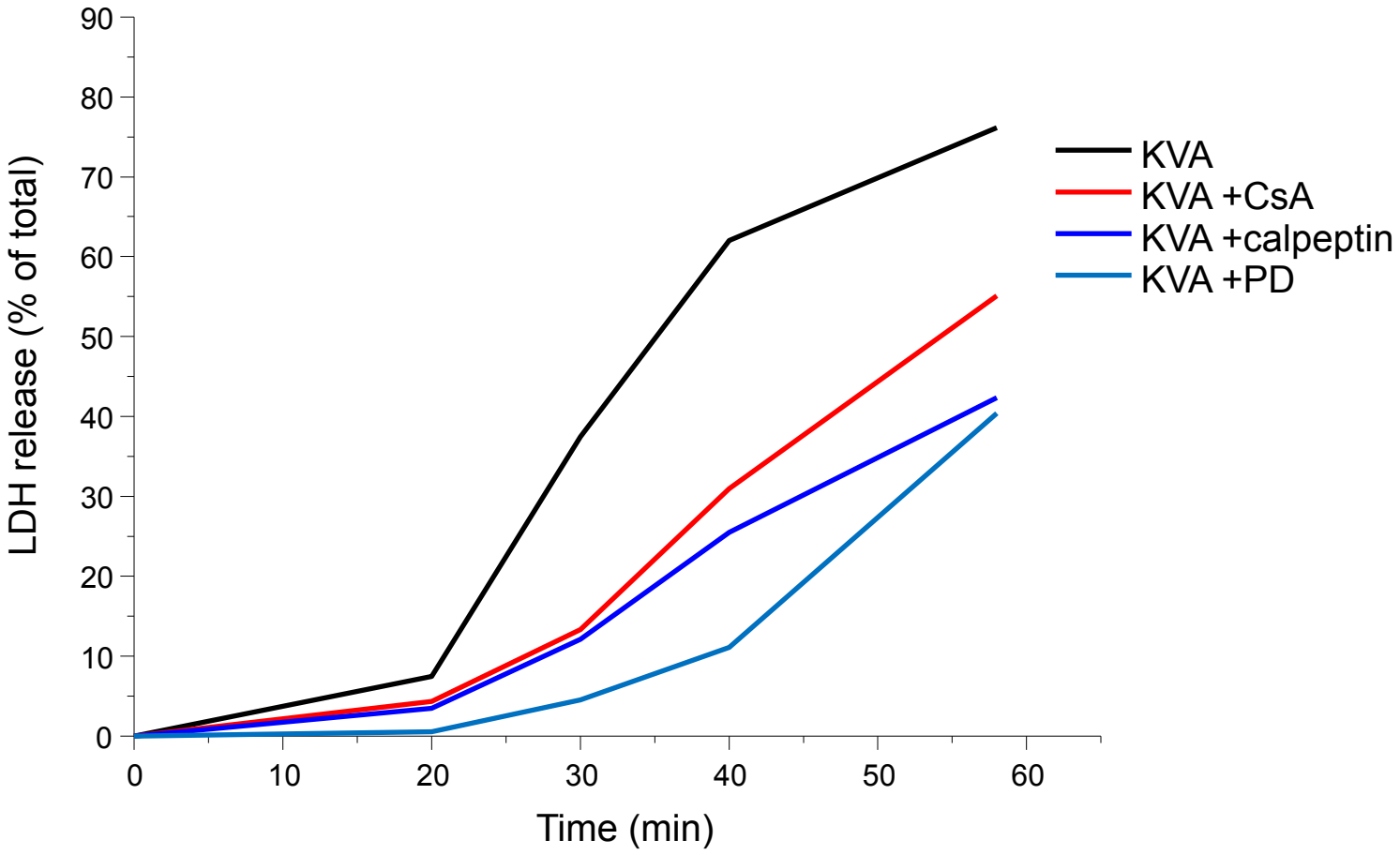

Ca2+
Phospholipase A2
Arachidonic Acid
Calpain
ROS formation
INDIRECT
DIRECT
Ca2+
matrix
inner mitochondrial membrane

# Mitochondrial dysfunction induced by intracellular Ca2+ overload is decreased by calpain or PTP inhibition

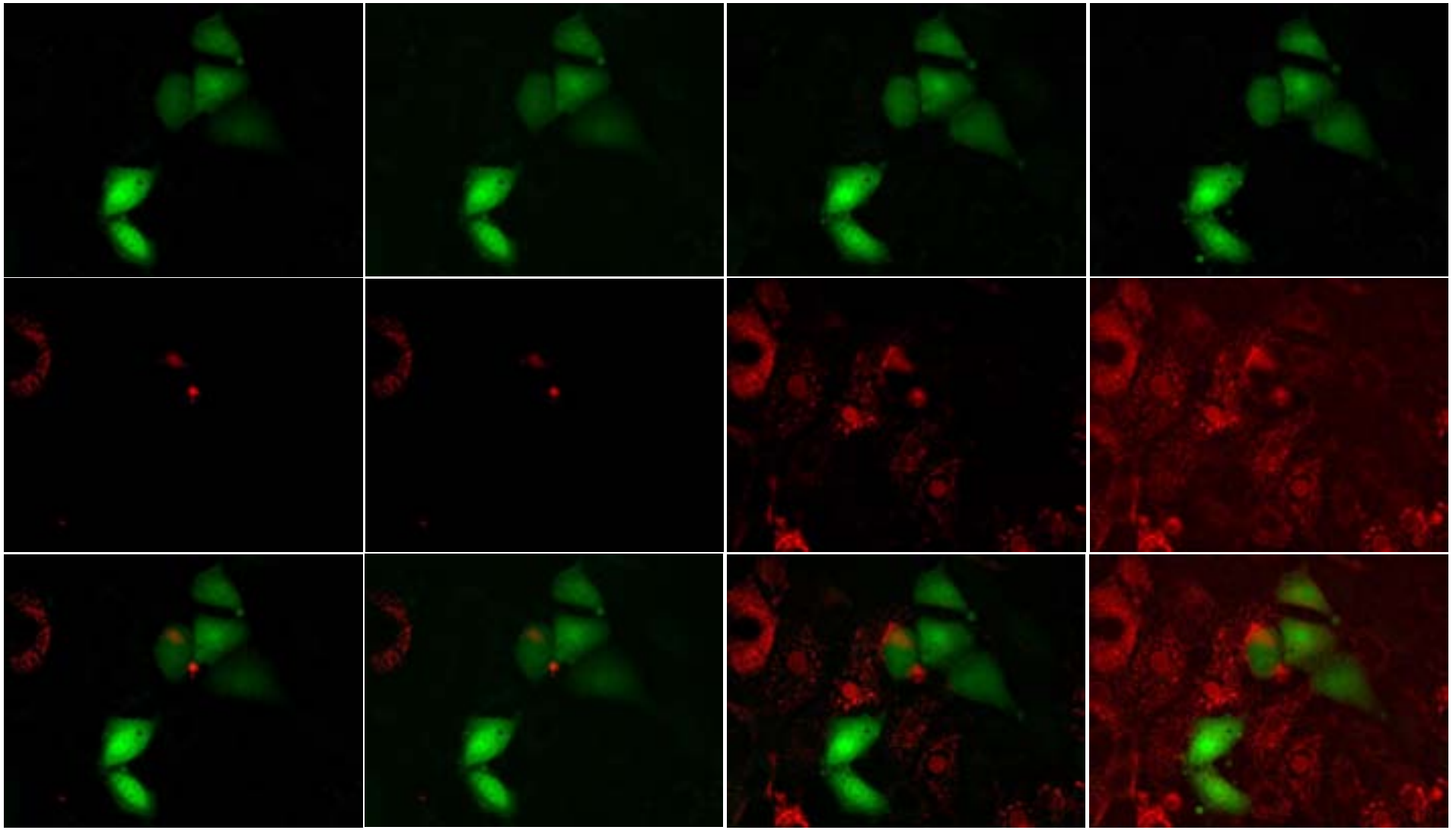



KVA: KCl, vanadate, A23187

# Cell death induced by intracellular Ca2+ overload is decreased by calpain or PTP inhibition




KVA: KCl, vanadate, A23187



T=0min KV T=10min KV T=30min KV T=60min KV

+ A23

GREEN = Calpastatin-GFP expressing cells
RED = Trypan blue

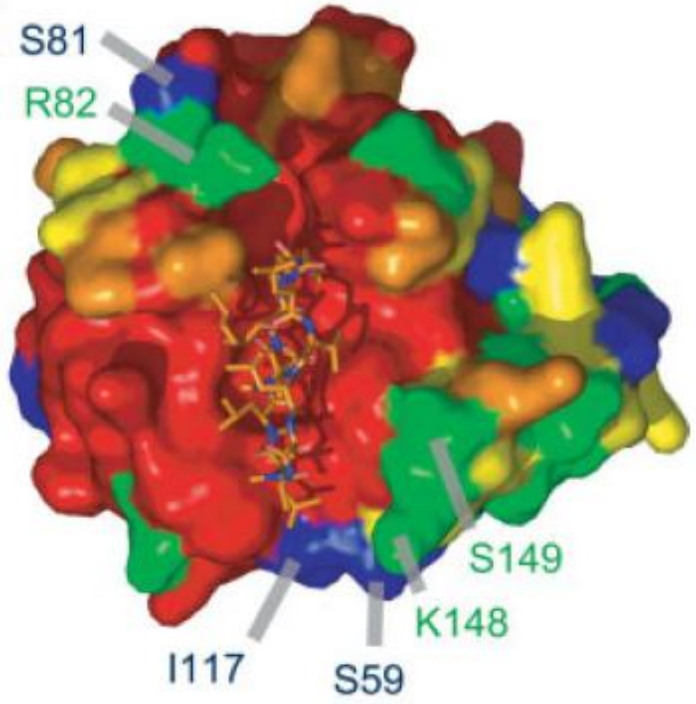
Does CsA protection depend only on PTP inhibition?

# Crystal structure of human cyclophilin D in complex with its inhibitor, cyclosporin A at 0.96-Å resolution

Kenji Kajitani,[1] Masahiro Fujihashi,[1] Yukiko Kobayashi,[1] Shigeomi Shimizu,[2] Yoshihide Tsujimoto,[2] and Kunio Miki[1*]

**Conserved between Cyp A,B,C,D,E**

**Red:** 5 out of 5
**Orange:** 3 out of 4
**Yellow:** 2 out of 4
**Green:** 1 out of 4
**Blue:** unique



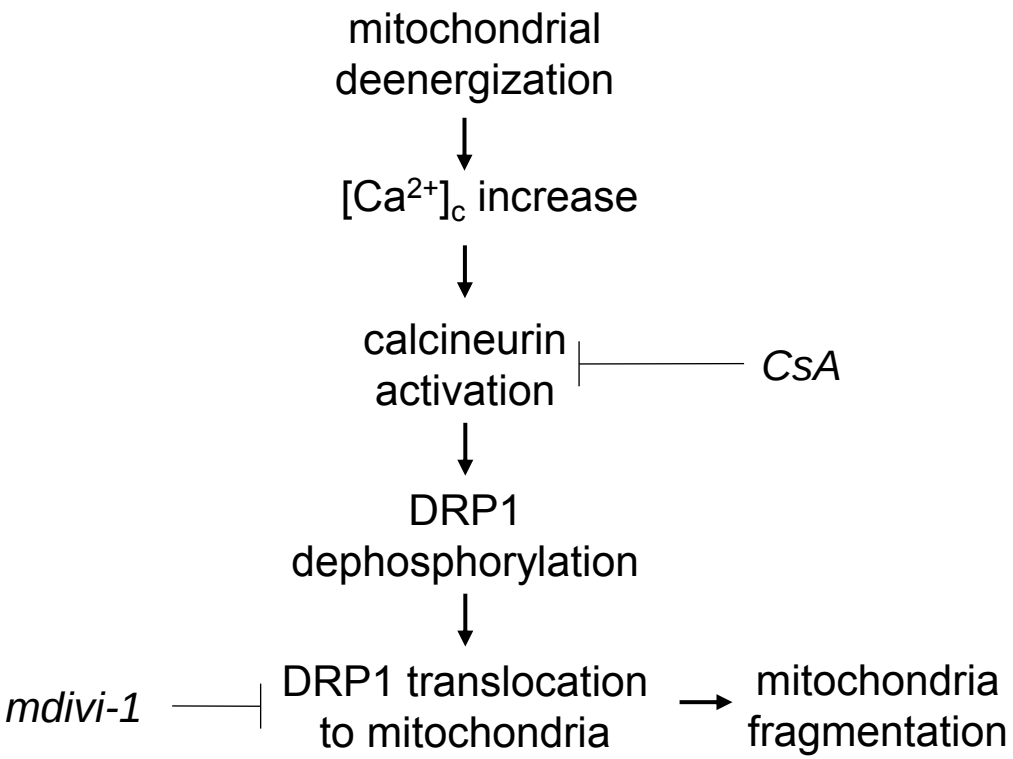

Proteins (2008) 70, 1635-1639

# Does CsA protection depend also on sites other than CypD?

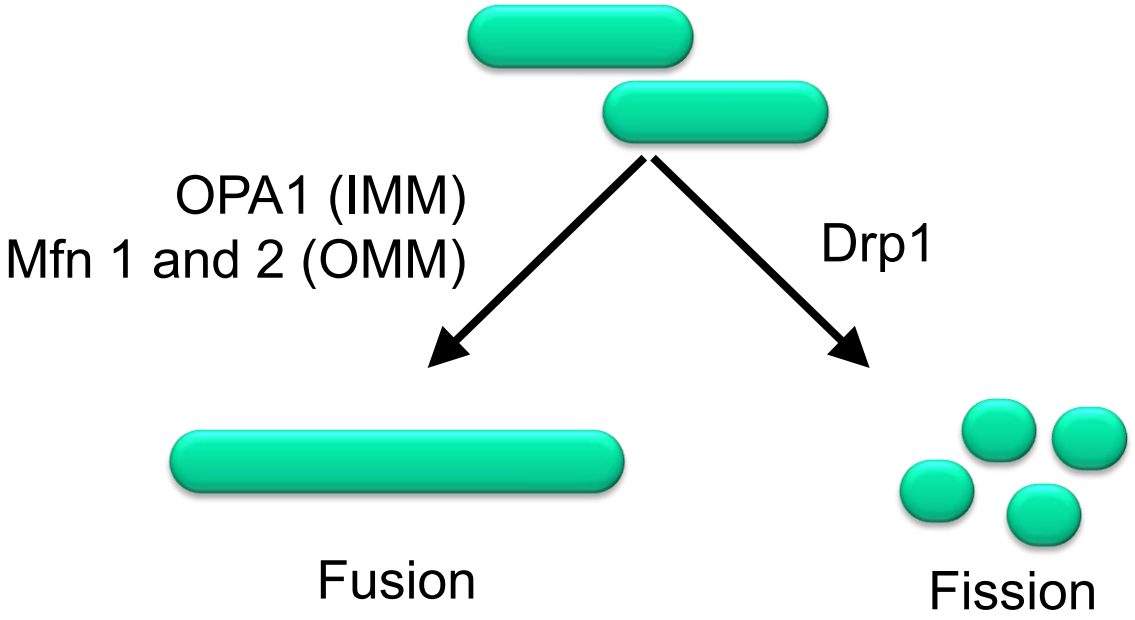
## Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria

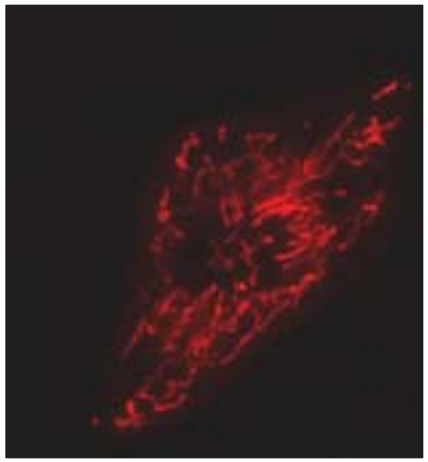
G. M. Cereghetti*†‡, A. Stangherlin*†, O. Martins de Brito*†, C. R. Chang§, C. Blackstone§, P. Bernardi†¶, and L. Scorrano*†‖

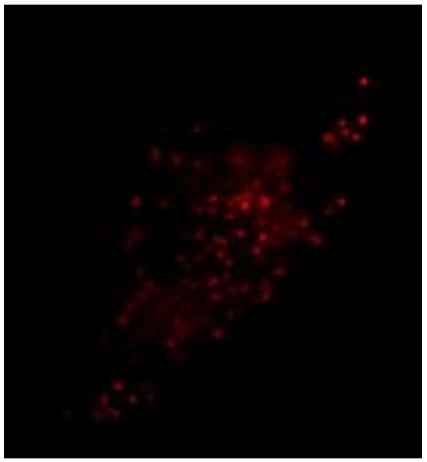
PNAS | October 14, 2008 | vol. 105 | no. 41 | 15803–15808



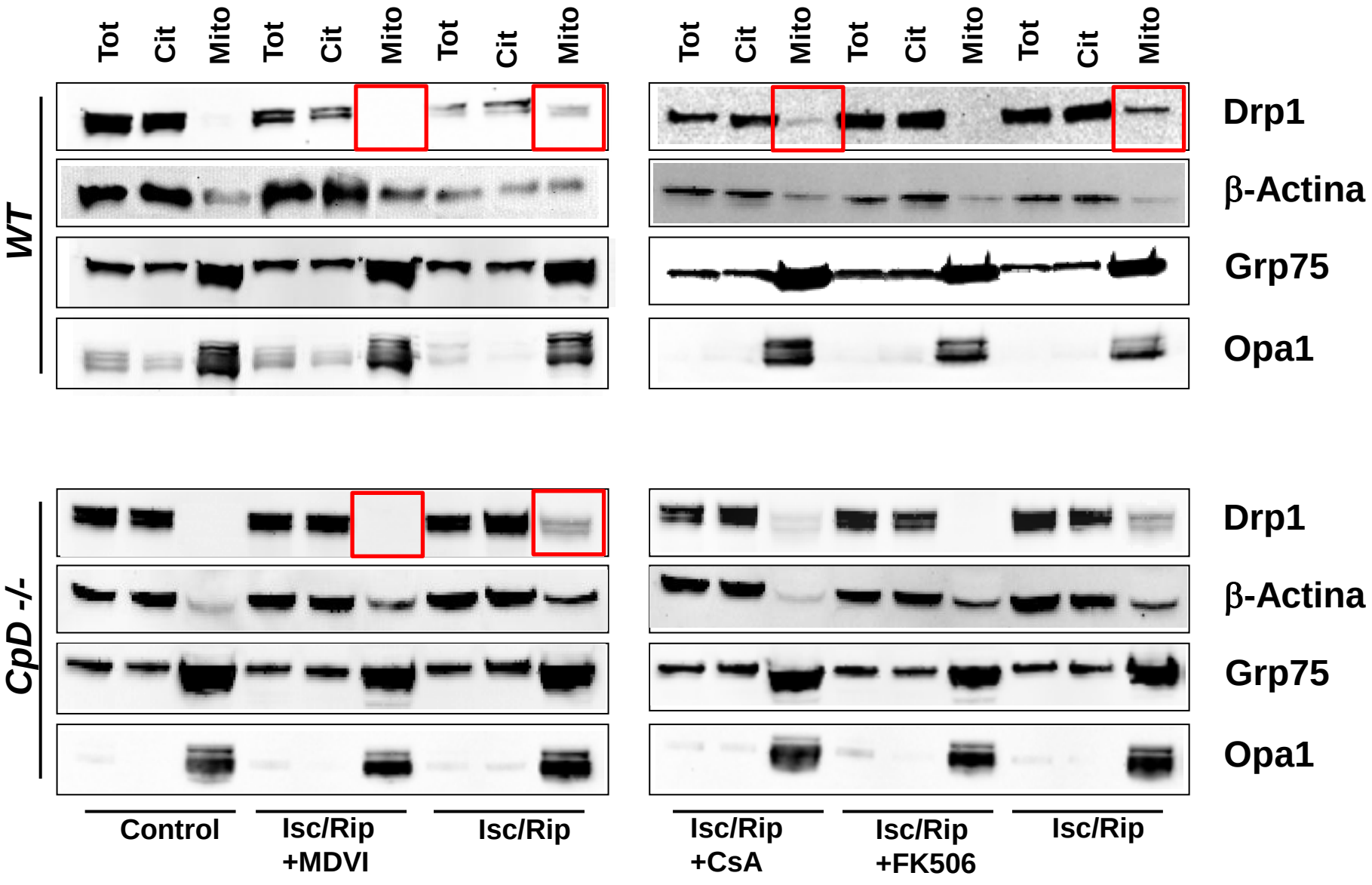
# Mitochondrial dynamics



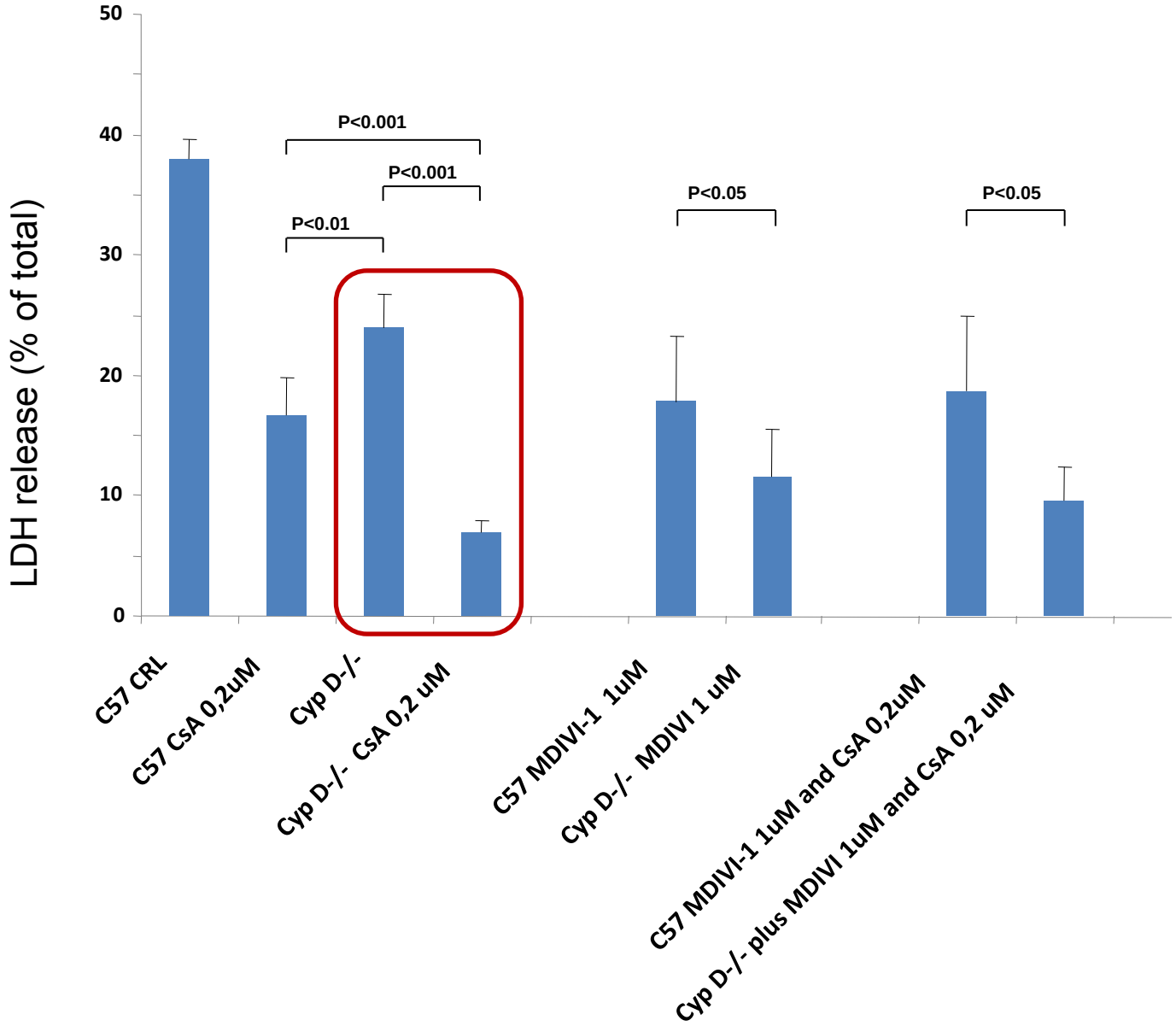



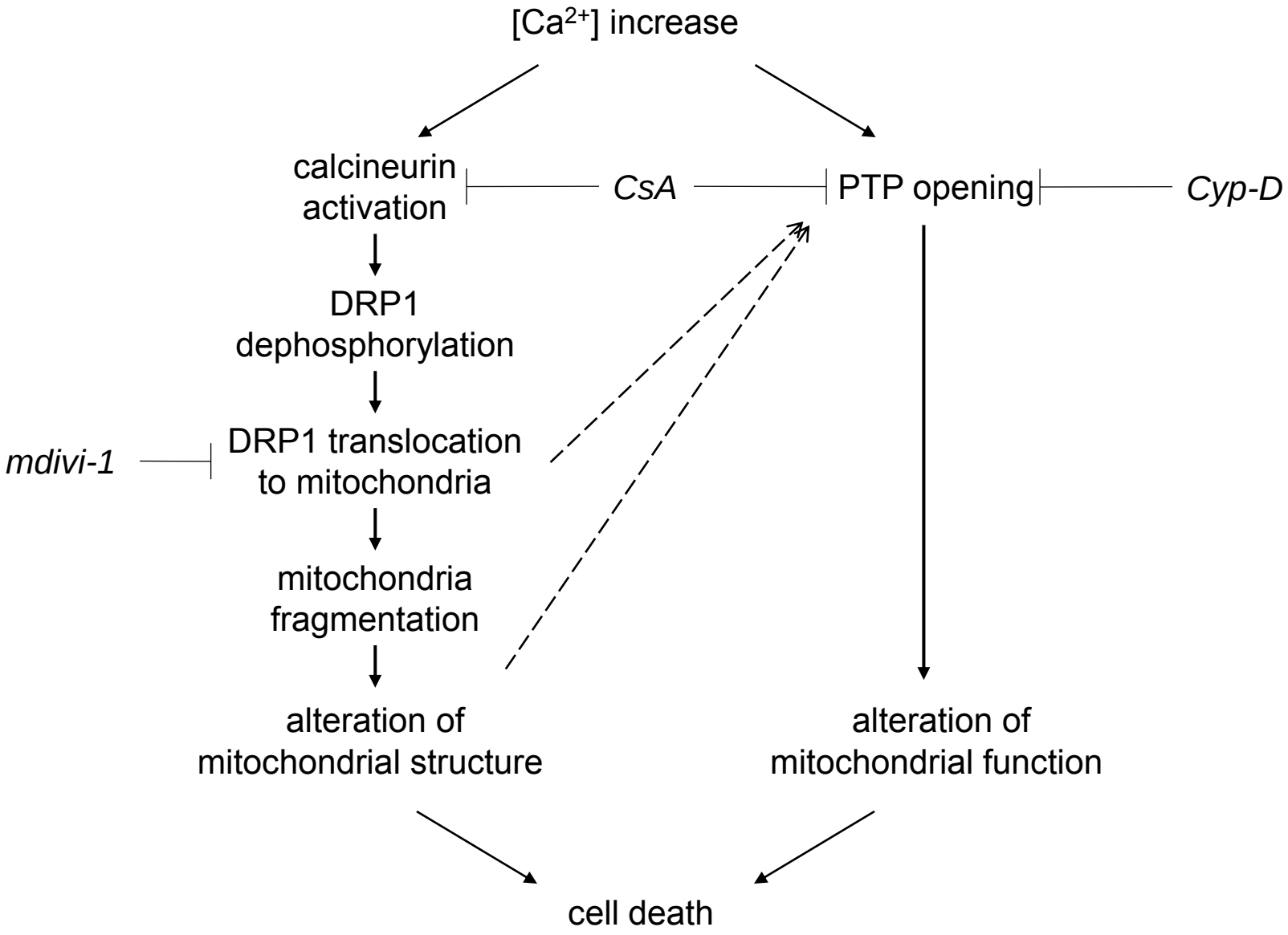


# Mdivi-1 prevents I/R-induced translocation of DRP-1 to mitochondria in perfused mouse hearts



# CsA-induced protection against I/R injury results from adding PTP desensitization with DRP-1 inhibition




[Ca2+] increase
calcineurin activation
CsA
PTP opening
Cyp-D
DRP1 dephosphorylation
mdivi-1
DRP1 translocation to mitochondria
mitochondria fragmentation
alteration of mitochondrial structure
alteration of mitochondrial function
cell death

# Is Cyp-D inhibition/deletion always beneficial?

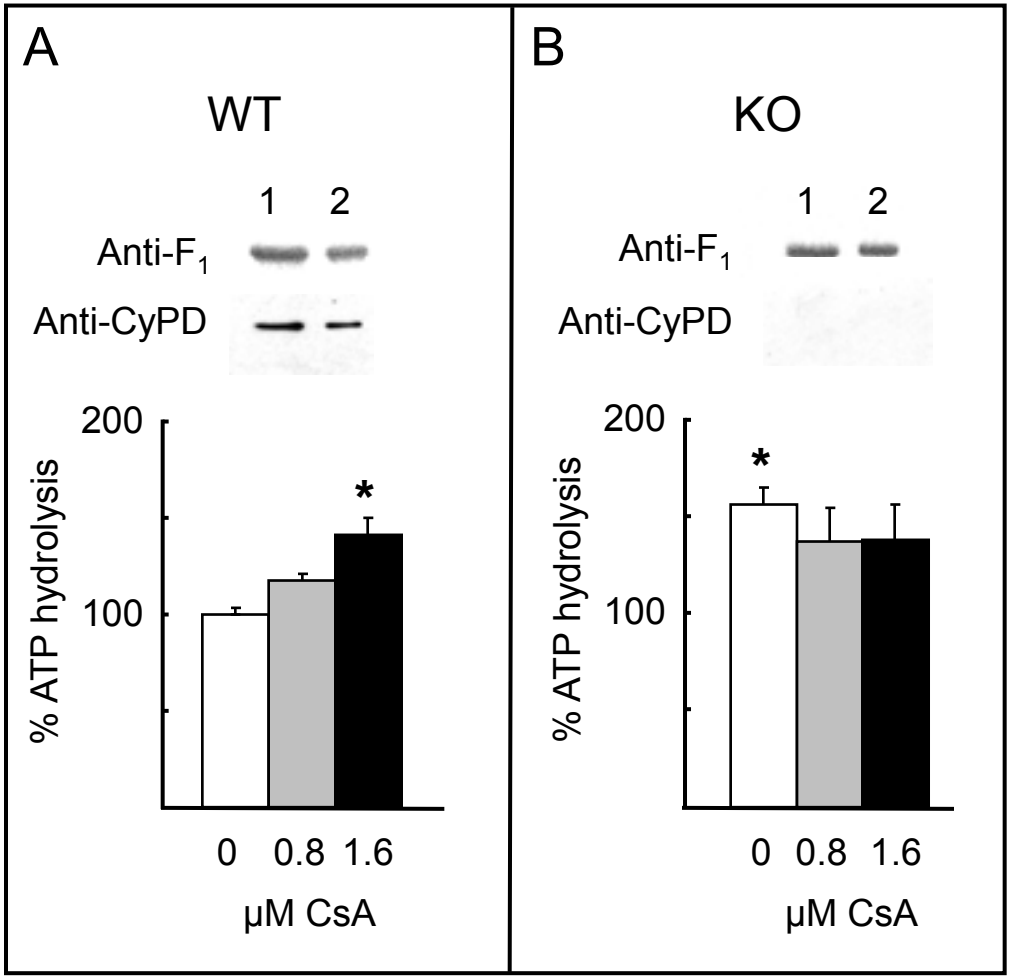
**Advantages**

- Decreased susceptibility to PTP opening ➔ decreased cell death (necrosis)

**Disadvantages**

- Increased activity of FoF1 ATPase ➔ possible increase in ATP hydrolysis
- Inceased matrix [$Ca^{2+}$] content ➔ deranged substrate oxidation
- Decreased interaction with Bcl2 ➔ increased apoptosis

- CyP-D binds to $F_OF_1$ ATPase
- Diplacement of CyPD by CsA increases ATP synthesis and hydrolysis
- **Mitochondria lacking CyPD display a higher $F_OF_1$ ATPase activity**




V. Giorgio et al., JBC 284:33982-8, 2009

# Cyclophilin D controls mitochondrial pore–dependent $Ca^{2+}$ exchange, metabolic flexibility, and propensity for heart failure in mice

John W. Elrod,[1] Renee Wong,[2] Shikha Mishra,[3] Ronald J. Vagnozzi,[4] Bhuvana Sakthievel,[5] Sanjeewa A. Goonasekera,[1] Jason Karch,[1] Scott Gabel,[2] John Farber,[5] Thomas Force,[4] Joan Heller Brown,[3] Elizabeth Murphy,[2] and Jeffery D. Molkentin[1]

*J. Clin. Invest. 120:3680-7, 2010*

Cyp-D KO mice exhibit substantially greater cardiac hypertrophy, fibrosis, and reduction in myocardial function in response to pressure overload and sustained exercise than control mice.

The maladaptive phenotype in the hearts of Cyp-D KO mice was associated with an alteration in PTP-mediated $Ca^{2+}$ efflux resulting in elevated levels of mitochondrial matrix $Ca^{2+}$.

PTP appears to maintain homeostatic mitochondrial $Ca^{2+}$ levels to match metabolism with alterations in myocardial workload.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 15, pp. 9692–9699, April 10, 2009


# Cyclophilin D Interacts with Bcl2 and Exerts an Anti-apoptotic Effect*

Received for publication, November 18, 2008, and in revised form, February 5, 2009 Published, JBC Papers in Press, February 19, 2009, DOI 10.1074/jbc.M808750200

**Roman A. Eliseev[‡1], Jonathan Malecki[§], Tobias Lester[§], Yu Zhang[‡], John Humphrey[‡], and Thomas E. Gunter[§]**

*From the [‡]Center for Musculoskeletal Research and [§]Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*

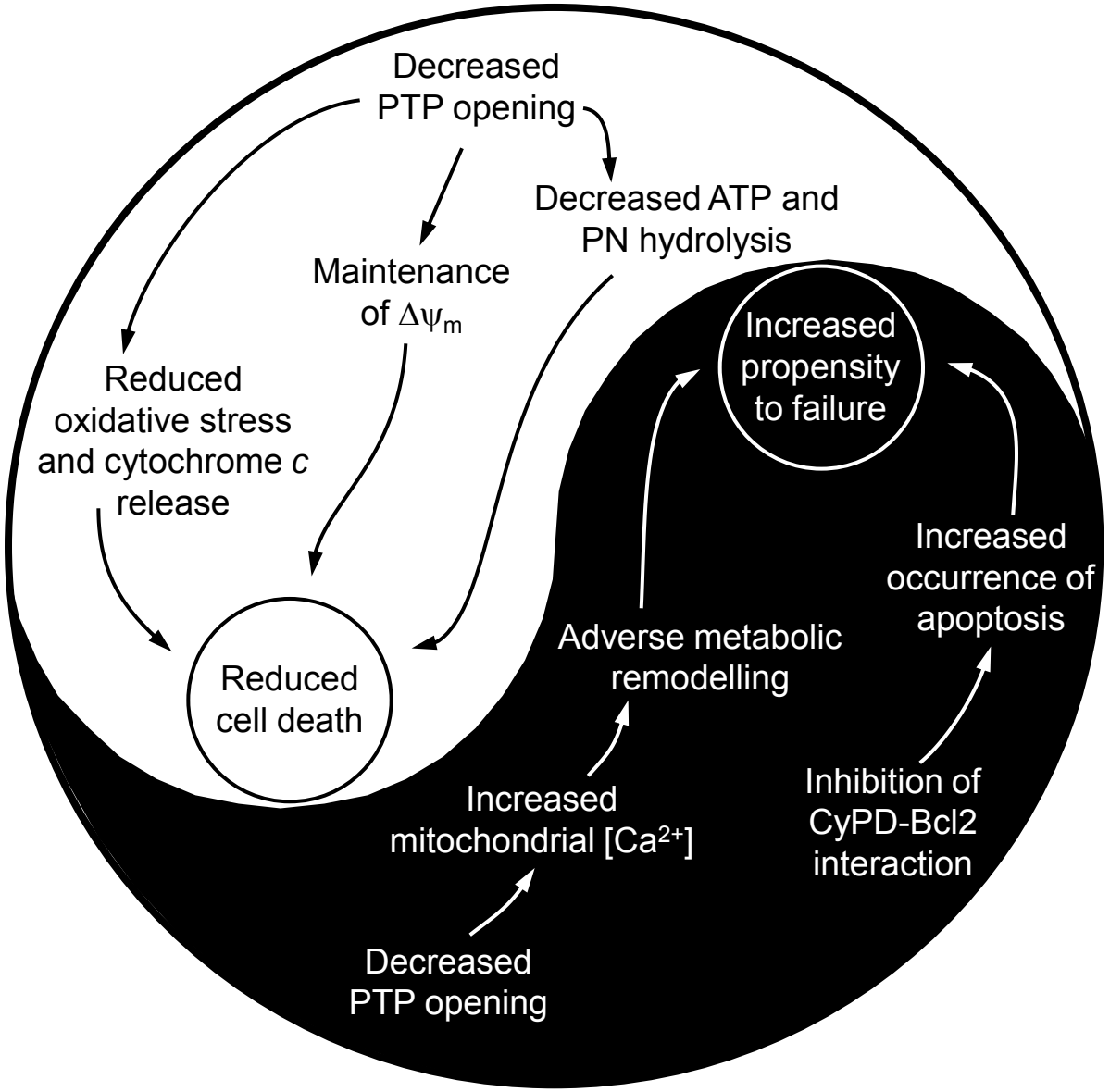
CypD interacts with Bcl2 as confirmed with co-immunoprecipitation, pulldown, and mammalian two-hybrid assays.

Cyclosporine A, disrupts the CypD-Bcl2 interaction.

CypD has a limiting effect on cytochrome *c* release from mitochondria. Such an effect of CypD is cyclosporine A- and Bcl2-dependent.

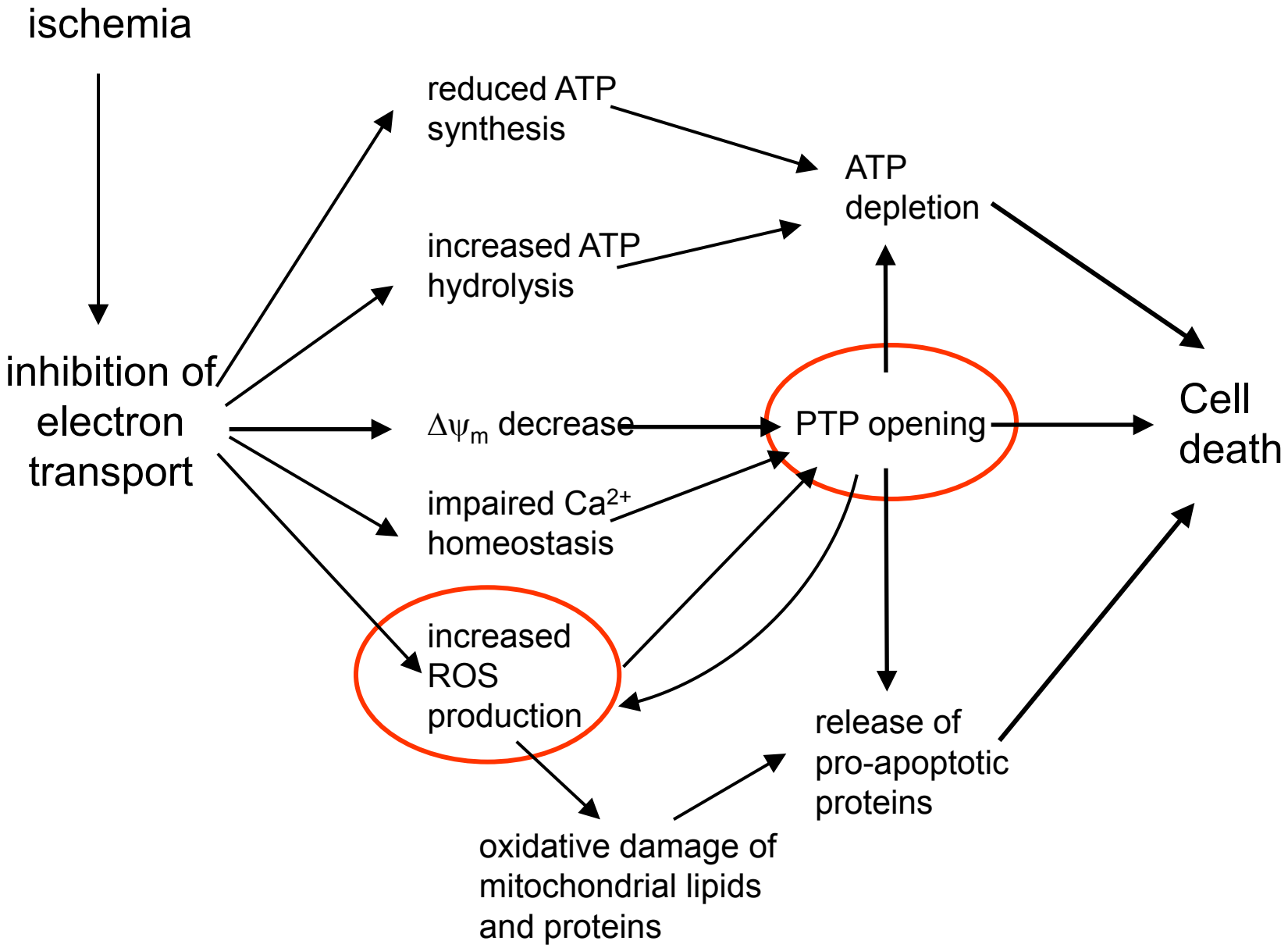
Overexpression or knockdown of CypD respectively decreases or increases cytochrome *c* release from mitochondria.

# The yinyang of PTP inhibition

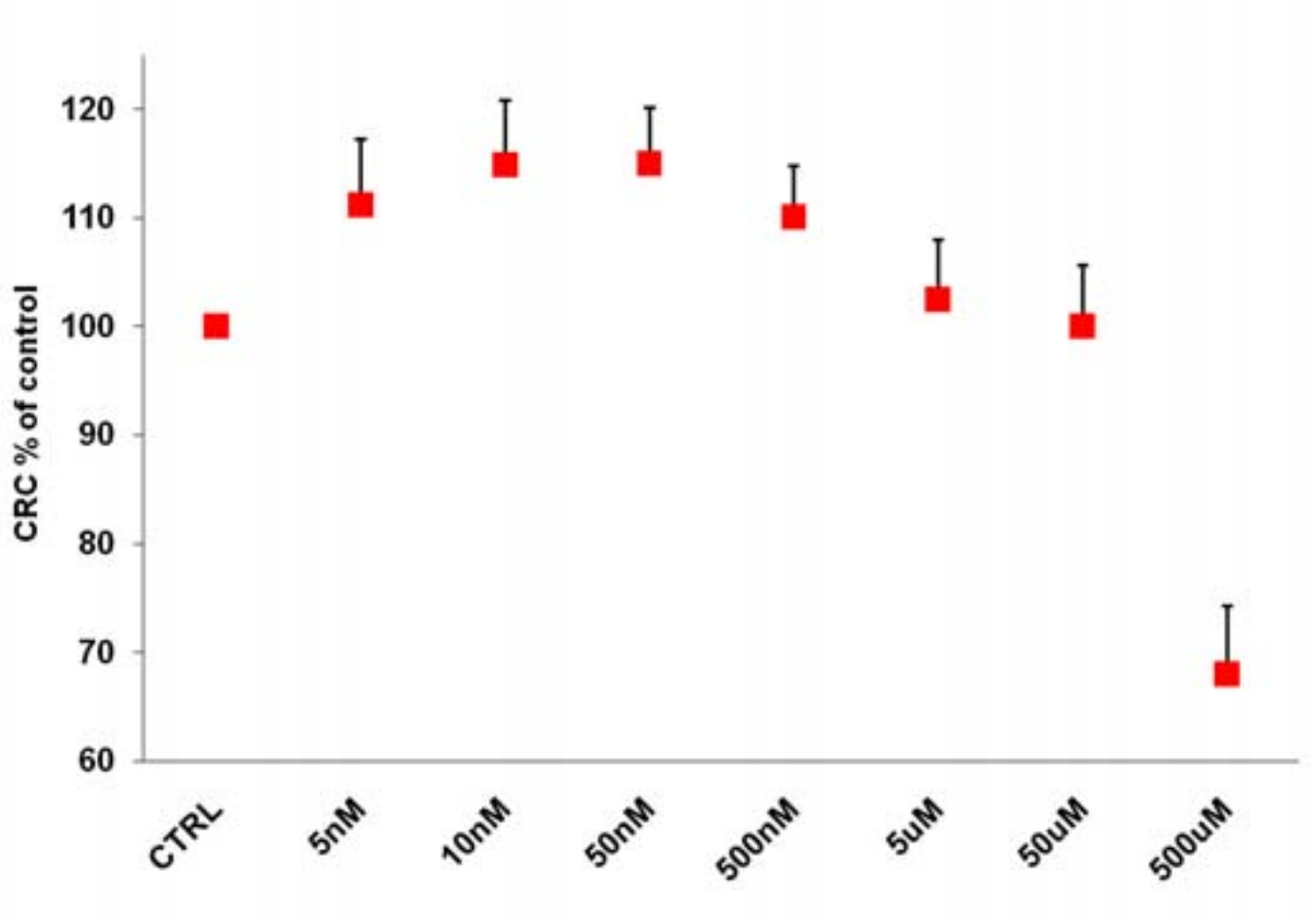


Cardioprotection afforded by CsA might be contributed by actions at sites other than CypD.

CypD (and PTP) inhibition is likely to be not always beneficial.


ischemia
inhibition of electron transport
reduced ATP synthesis
increased ATP hydrolysis
$\Delta\psi_m$ decrease
impaired $Ca^{2+}$ homeostasis
increased ROS production
oxidative damage of mitochondrial lipids and proteins
ATP depletion
PTP opening
release of pro-apoptotic proteins
Cell death

# $H_2O_2$-dependent changes in CRC of mouse heart mitochondria



THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 46, pp. 40184–40192, November 18, 2011
Printed in the U.S.A.

# Cysteine 203 of Cyclophilin D Is Critical for Cyclophilin D Activation of the Mitochondrial Permeability Transition Pore*

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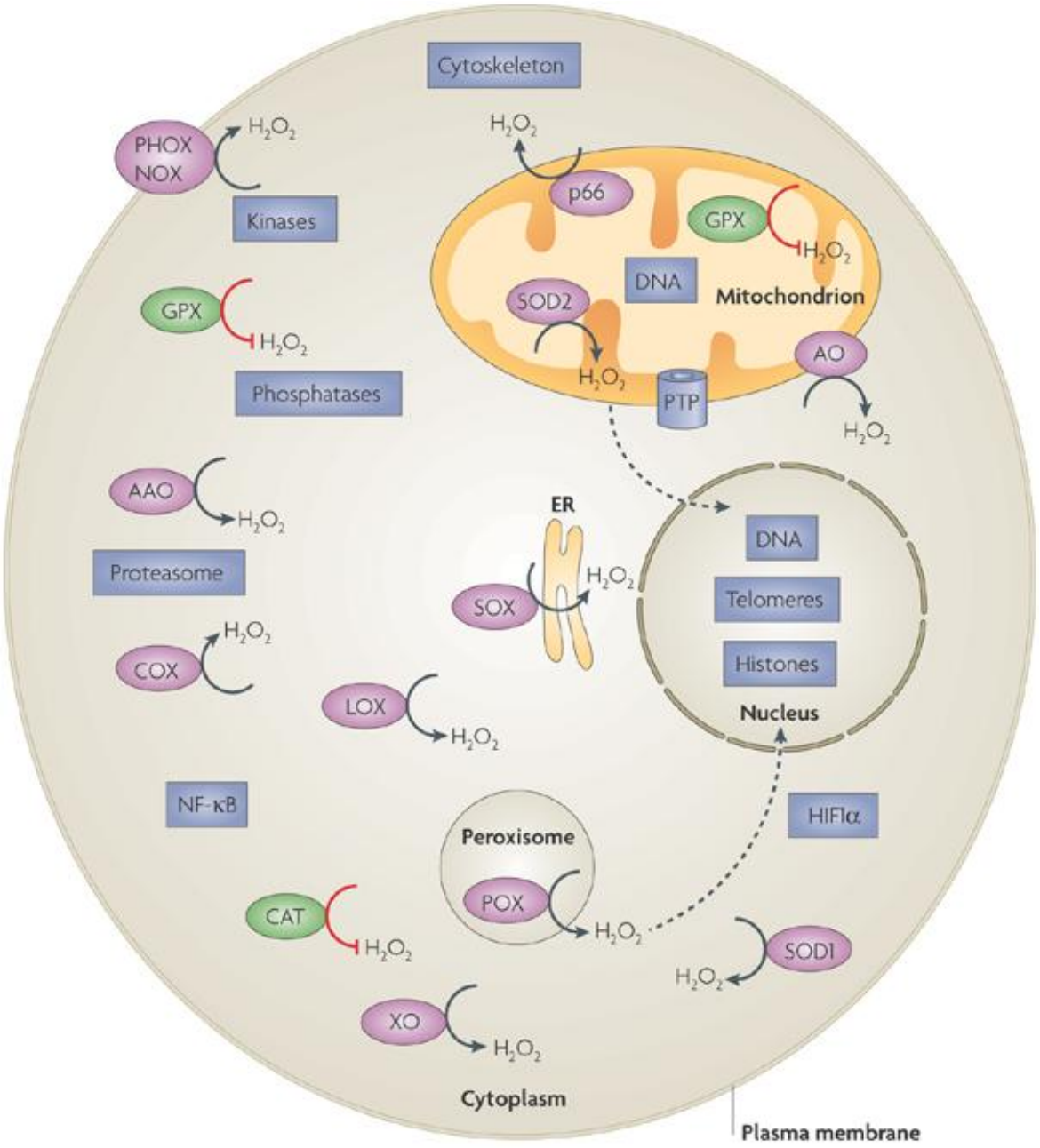
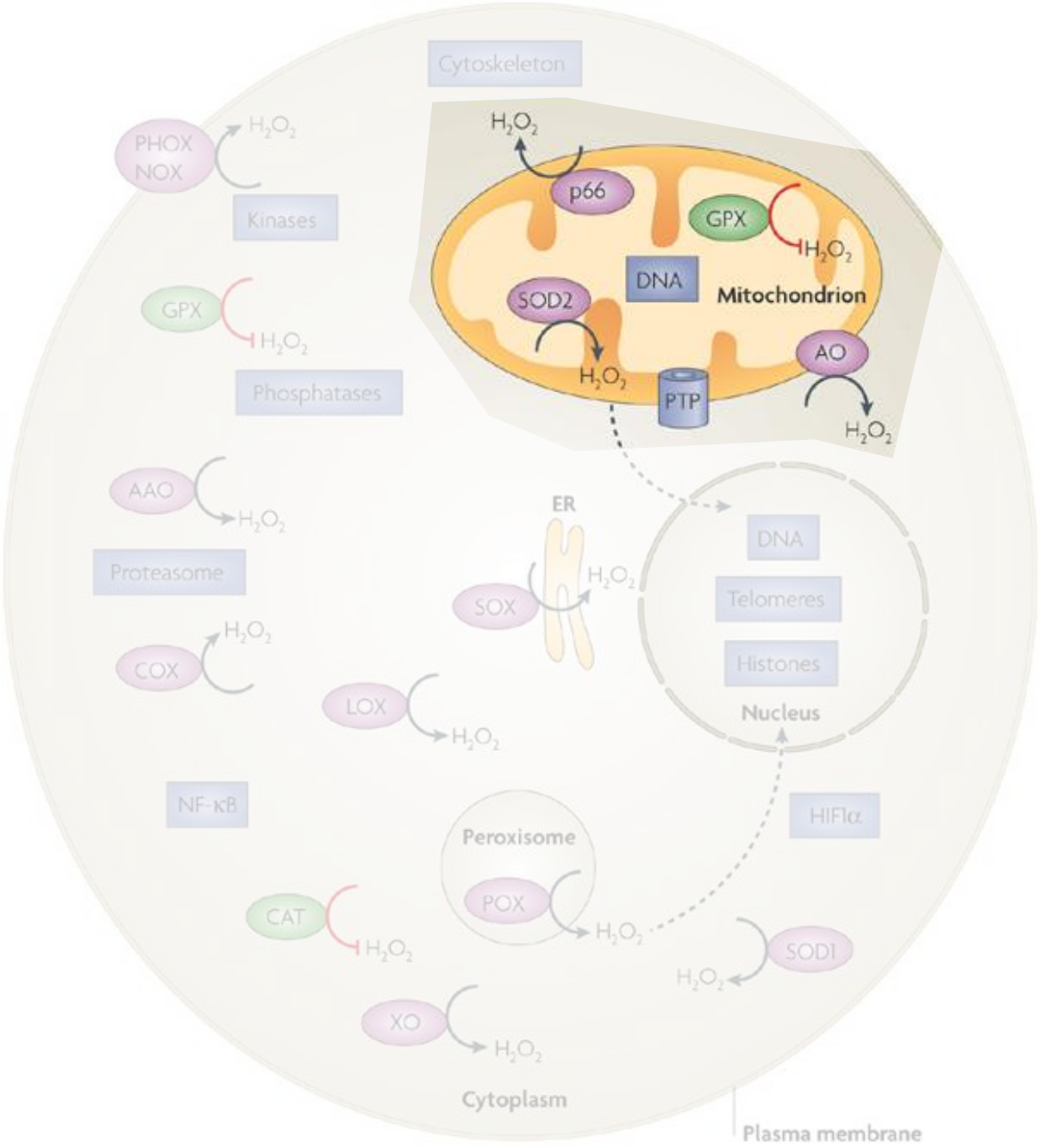
**Background:** Cyclophilin D, a known mitochondrial permeability transition pore (mPTP) regulator, is associated with cellular protection.
**Results:** Mutation of cysteine 203 of cyclophilin D inhibits mPTP opening and improves cell viability.
**Conclusion:** Cysteine 203 of cyclophilin D is a critical residue for mPTP activation.
**Significance:** This work provides novel mechanistic insights into mPTP regulation.

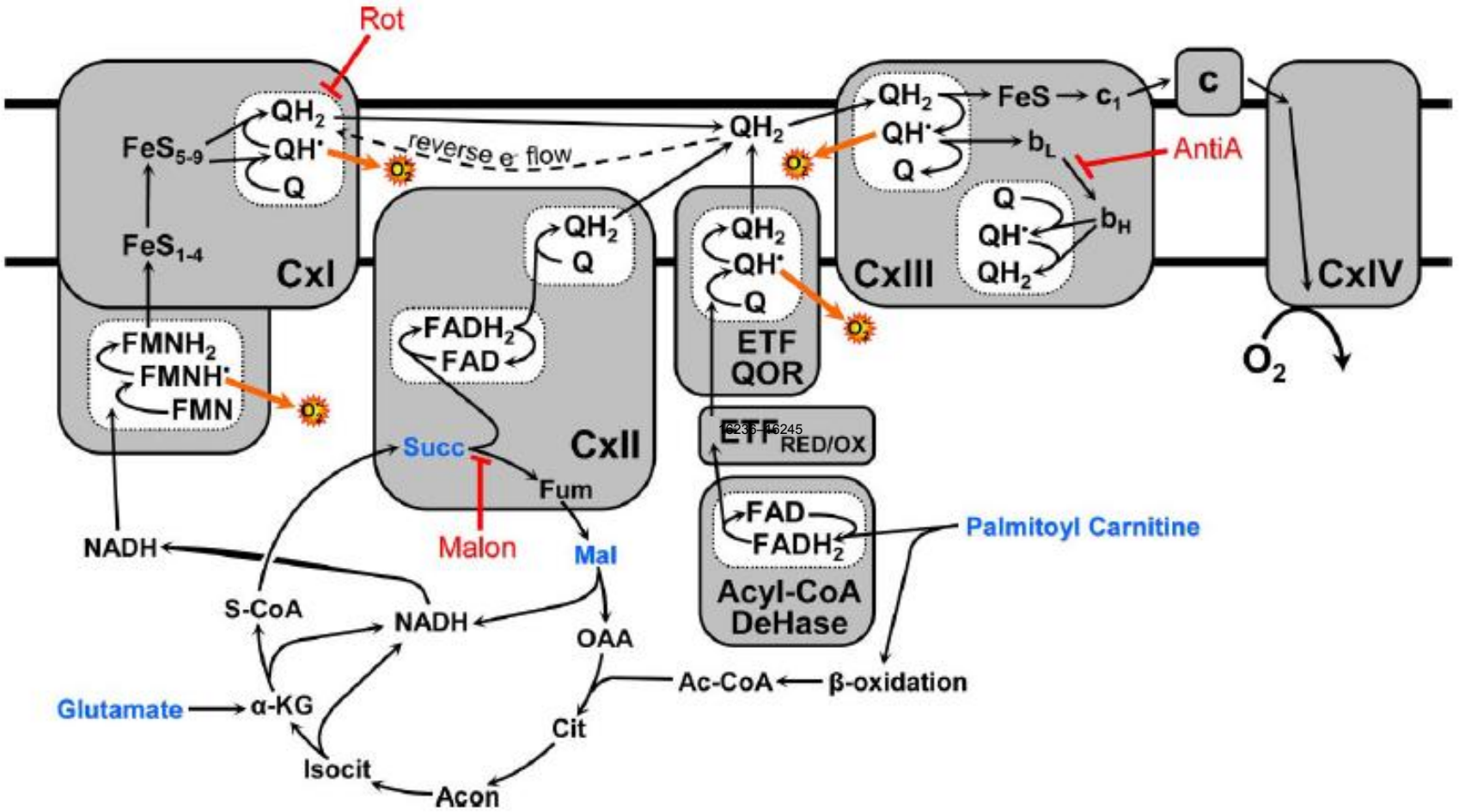
Mutation of cysteine 203 of cyclophilin D inhibits mPTP opening and improves cell viability

*i.e., oxidation of Cys203 in CyPD is likely to be required for PTP opening*




M. Giorgio et al., *Nat Rev Mol Cell Biol*, 8:722-728, 2007


Cytoskeleton
PHOX
NOX
$H_2O_2$
Kinases
GPX
$H_2O_2$
Phosphatases
AAO
$H_2O_2$
Proteasome
COX
$H_2O_2$
LOX
$H_2O_2$
NF-κB
CAT
$H_2O_2$
XO
$H_2O_2$
Cytoplasm
$H_2O_2$
p66
GPX
$H_2O_2$
DNA
Mitochondrion
SOD2
$H_2O_2$
AO
$H_2O_2$
PTP
ER
SOX
$H_2O_2$
DNA
Telomeres
Histones
Nucleus
Peroxisome
POX
$H_2O_2$
HIF1α
SOD1
$H_2O_2$
Plasma membrane

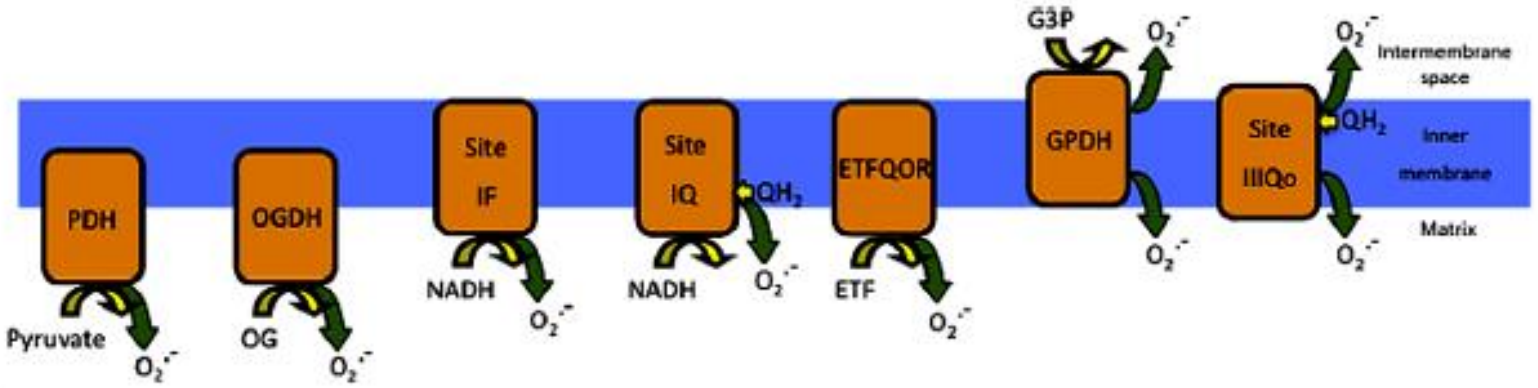


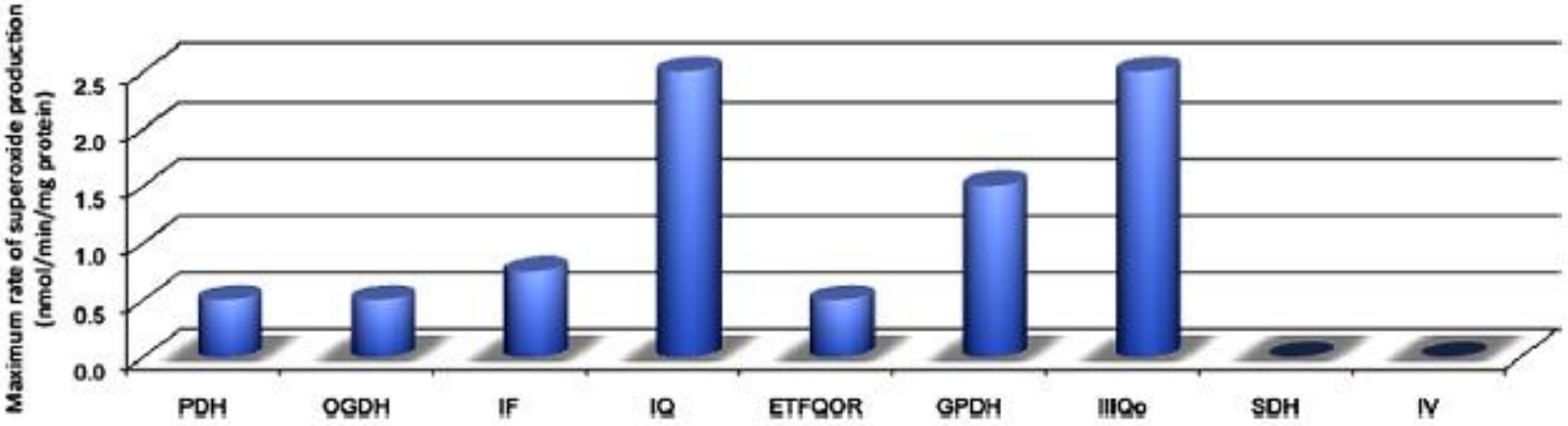

D.L Hoffman, P.S. Brookes, JBC, 284:16236–16245, 2009

# The sites and topology of mitochondrial superoxide production

Martin D. Brand*

Experimental Gerontology 45 (2010) 466–472



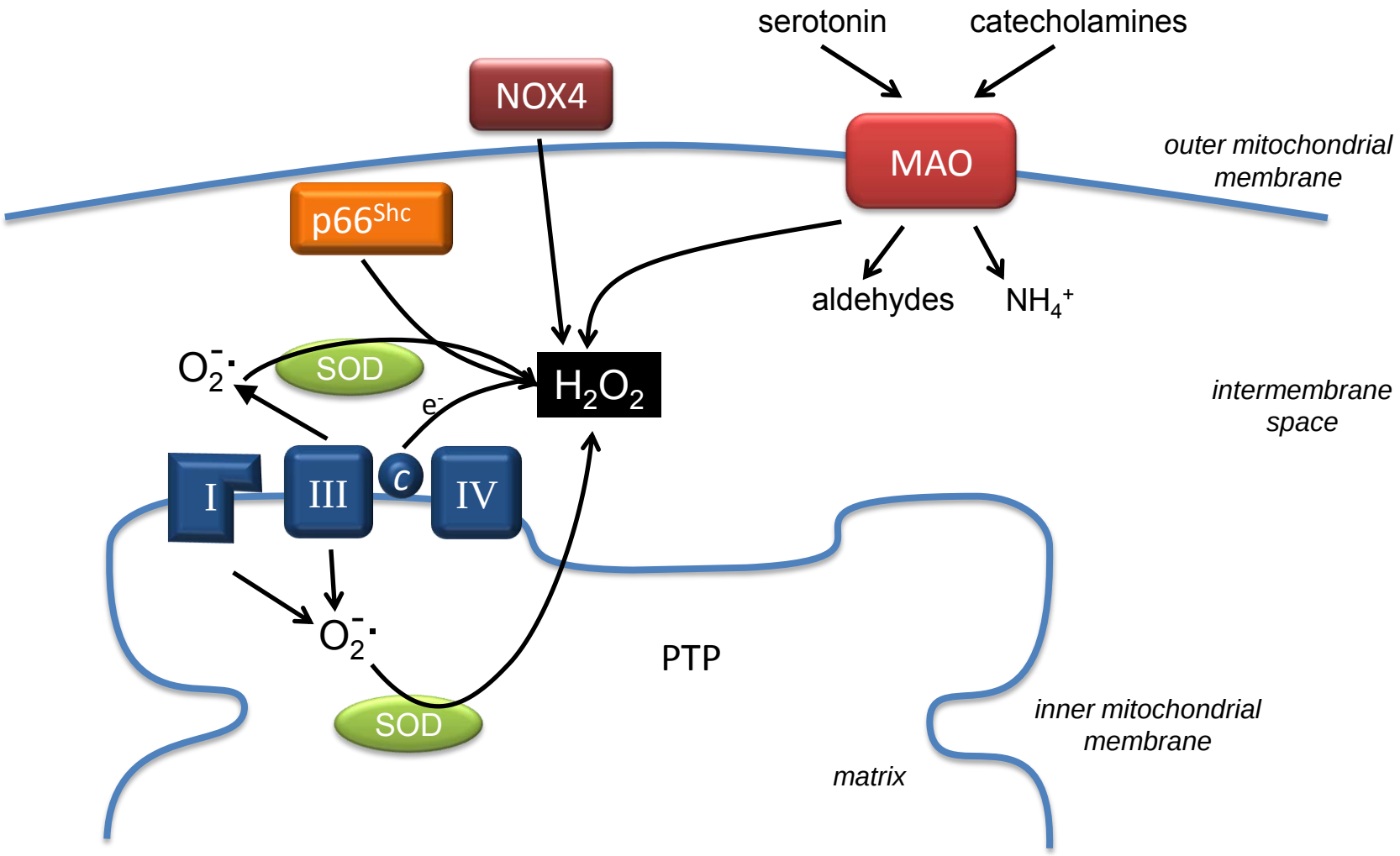



Reviewer's argument

Experimental data showing that mitochondria from aged animals produce more ROS should be presented.

(weak) response

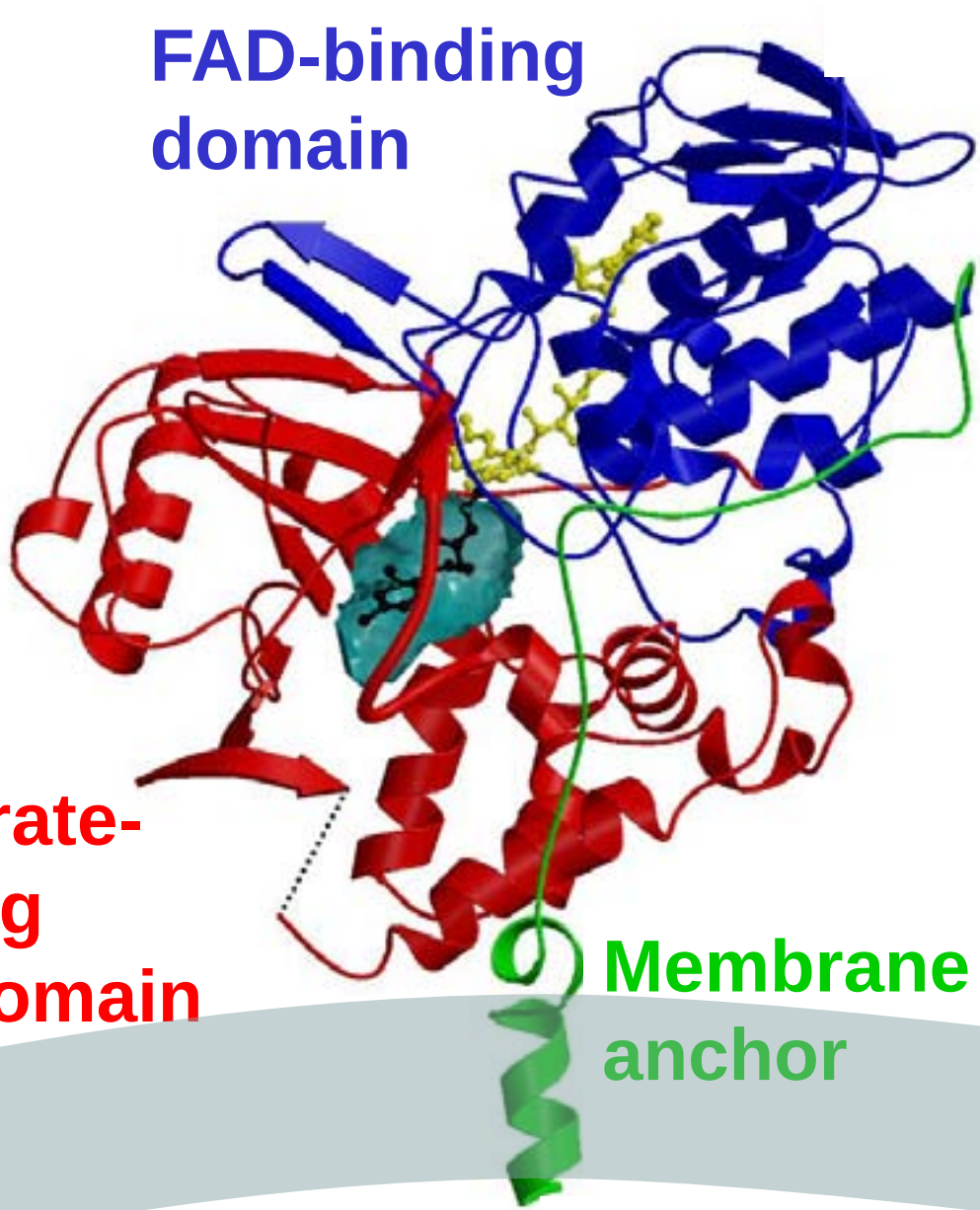
*Direct evidence is not available. The increased formation of ROS in mitochondria from aged animals is indirectly supported by mtDNA oxidation, lipoperoxidation and increased susceptibility to ischemic damage.*


serotonin
catecholamines
NOX4
MAO
outer mitochondrial membrane
p66Shc
aldehydes
NH4+
O2-·
SOD
H2O2
e-
intermembrane space
I
III
c
IV
O2-·
PTP
SOD
inner mitochondrial membrane
matrix

"It is noteworthy that the brain intramitochondrial $[H_2O_2]_{ss}$ obtained during the monoamine oxidase-catalyzed oxidative deamination of tyramine is **48-fold higher** than that originating during the oxidation of substrates via complex II of the electron transfer chain in the presence of Antimycin A."

*Cadenas and Davies, Free Radic Biol Med. 2000 Aug;29(3-4):222-30*

# Overall Structure of Human MAO A



# Advantages with studying MAO and its inhibition

- Molecular structure identified
- Specific substrates
- Clinically available inhibitors

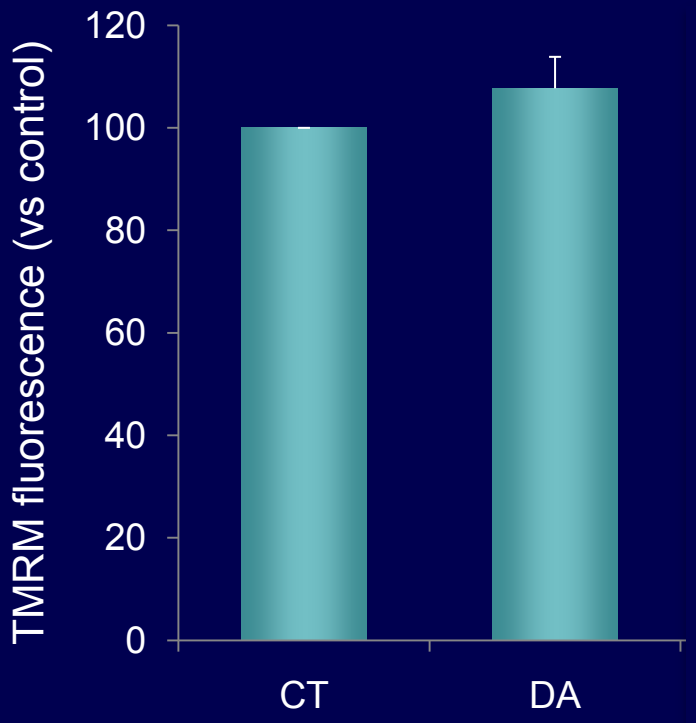
Two isoforms: MAO A and MAO B

In rat heart mitochondria MAO A is the prevailing isoform

| | |
|---|---|
| Substrates: | MAO A, serotonine, norepinephrine |
| | MAO B, dopamine |
| | MAO and B, tyramine |
| Inihibitors: | MAO A, Clorgyline |
| | MAO B, Deprenyl |
| | MAO A and B, Pargyline |

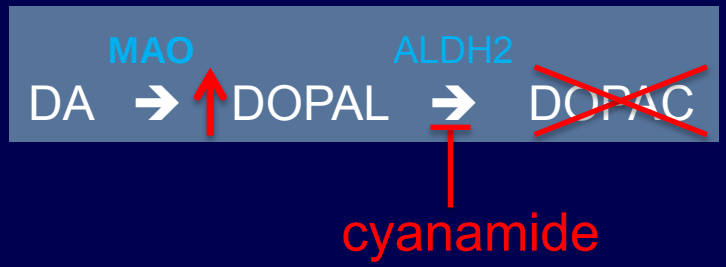
Can MAO activity directly target mitochondrial function?

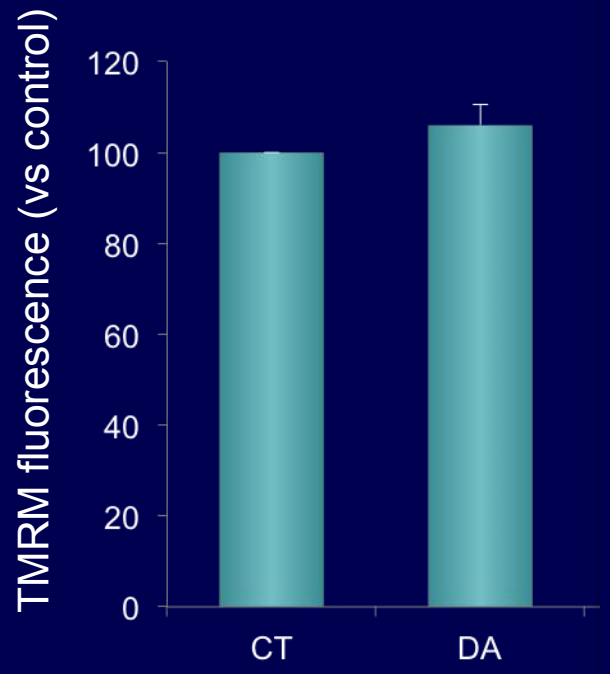
# MAO activation and mitochondrial function



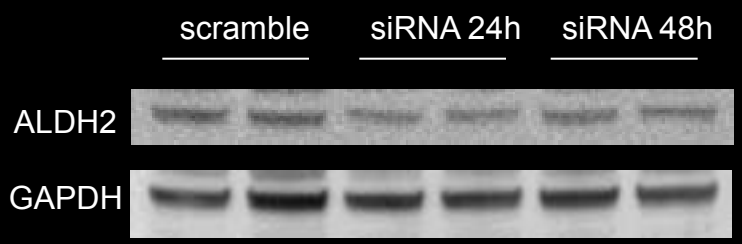

*N. Kaludrcic et al., ARS 2013*

# Products of MAO activity and mitochondrial function



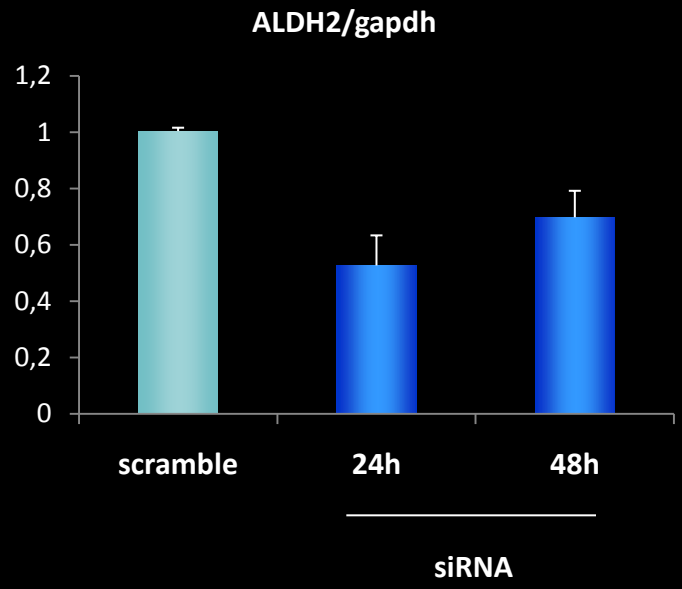



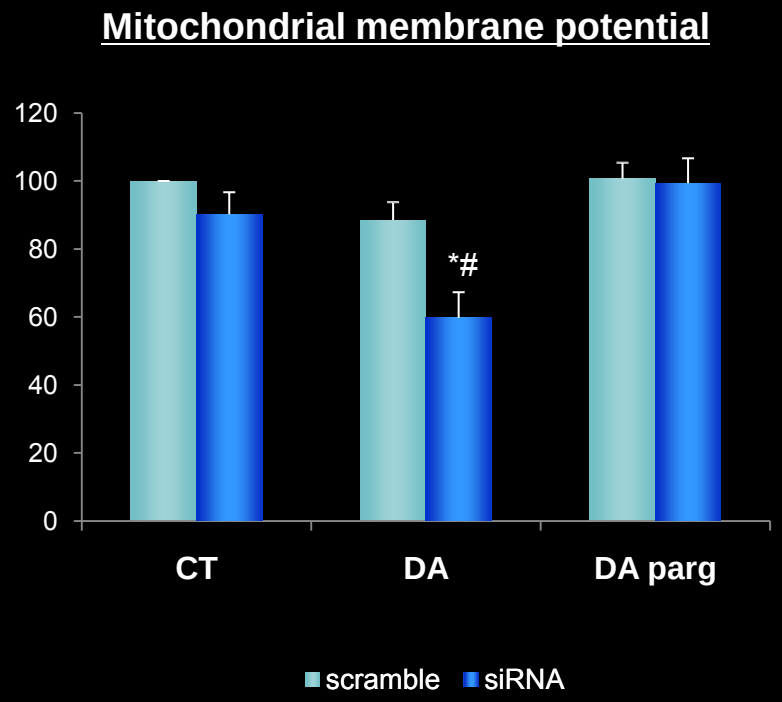
# Aldehyde generation and mitochondrial function




MAO ALDH2

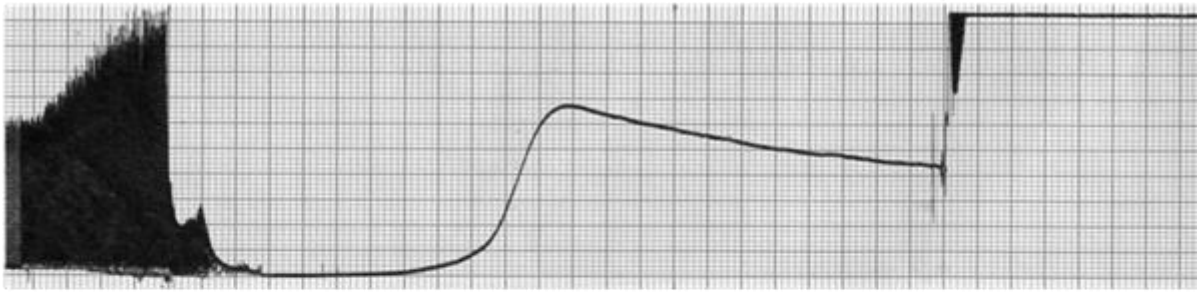
DA → DOPAL → DOPAC

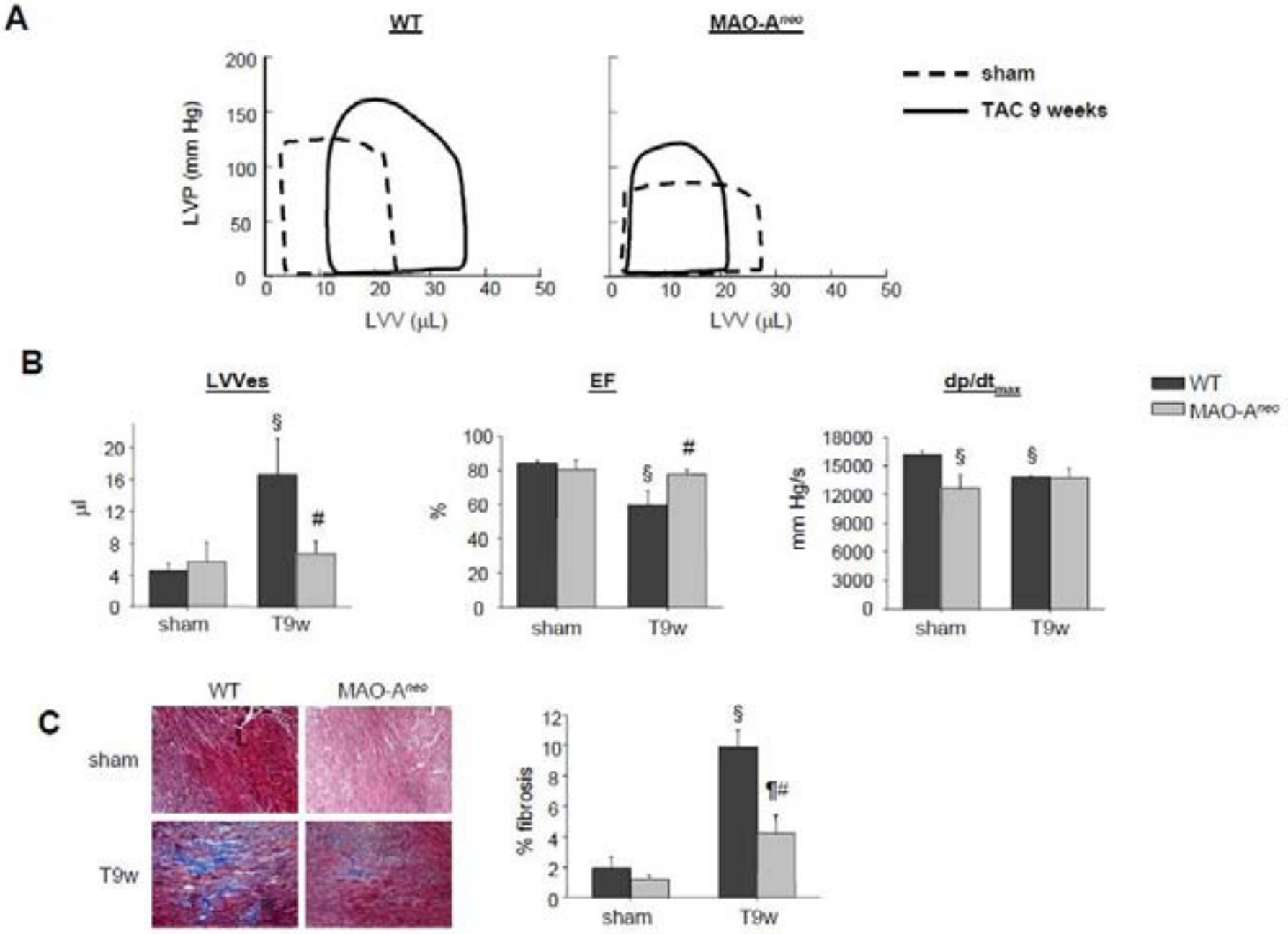


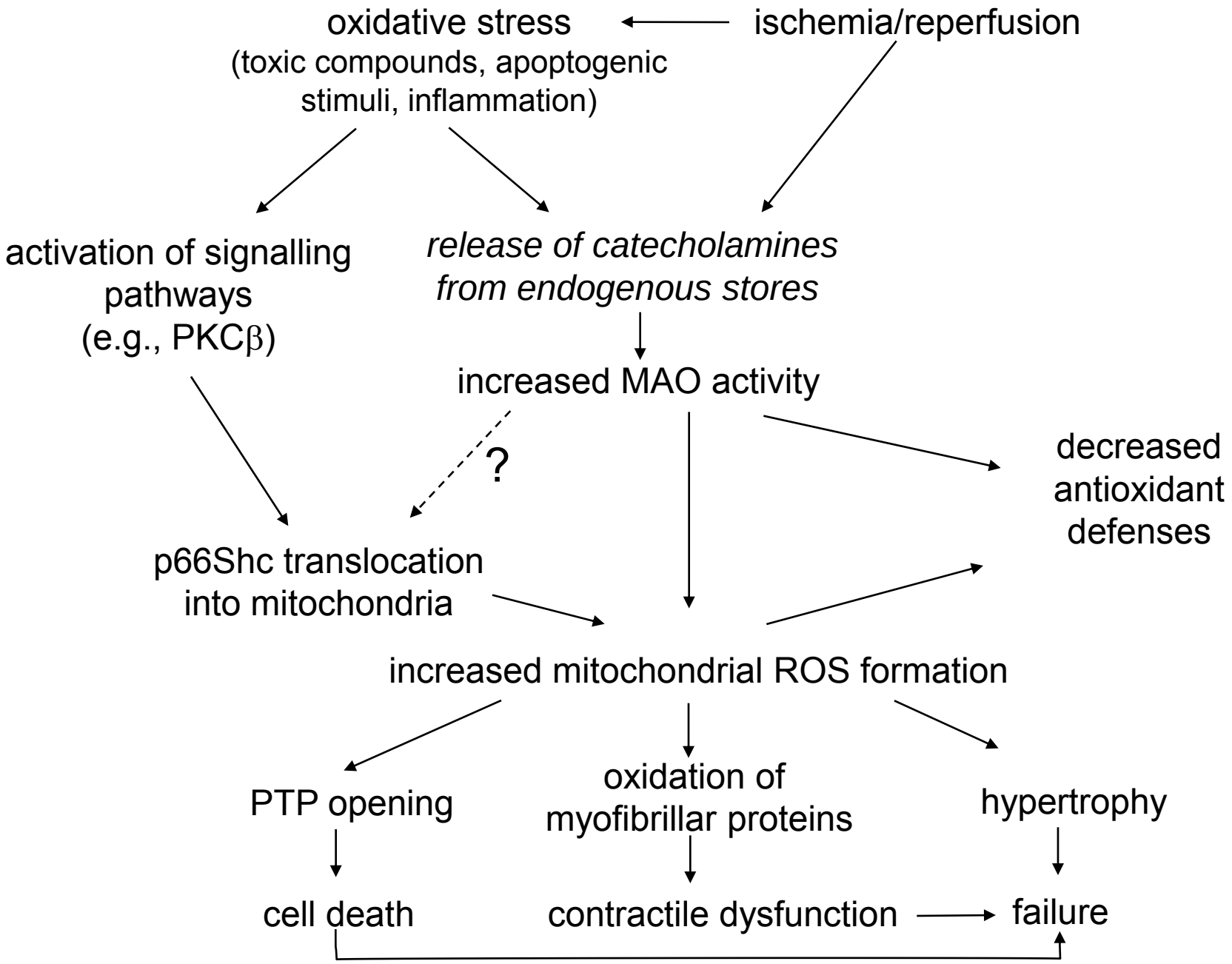
ISCHEMIA

Control






N. Kaludercic et al., Circ Res 2010


oxidative stress
(toxic compounds, apoptogenic stimuli, inflammation)
ischemia/reperfusion
activation of signalling pathways (e.g., PKCβ)
release of catecholamines from endogenous stores
increased MAO activity
?
decreased antioxidant defenses
p66Shc translocation into mitochondria
increased mitochondrial ROS formation
PTP opening
oxidation of myofibrillar proteins
hypertrophy
cell death
contractile dysfunction
failure

Mitochondria (by means of MAO) amplify ROS formation

DOXORUBICIN administration

↓

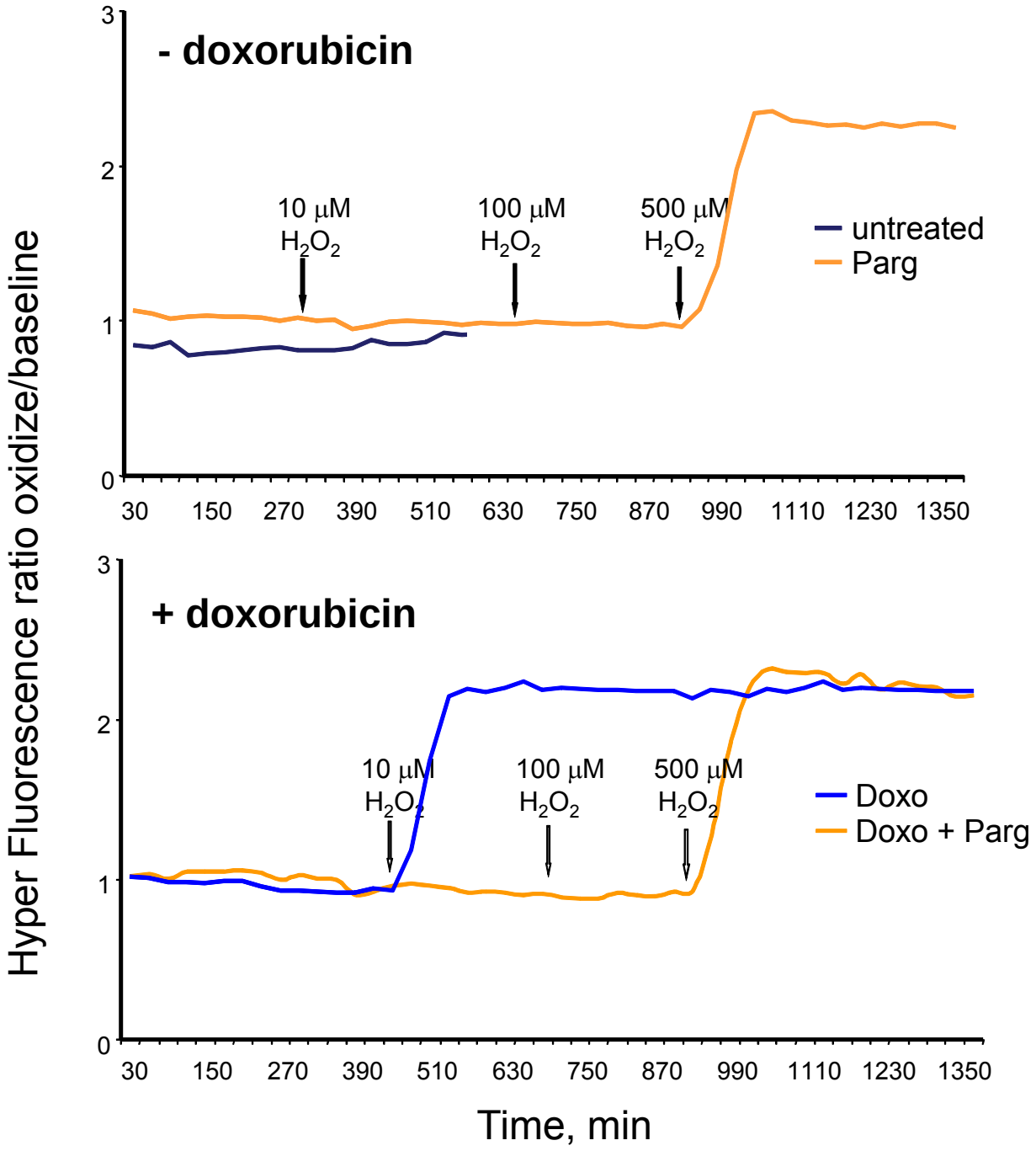
Mitochondrial localization
and accumulation

↓

Redox cycling by means
of interaction with complex I

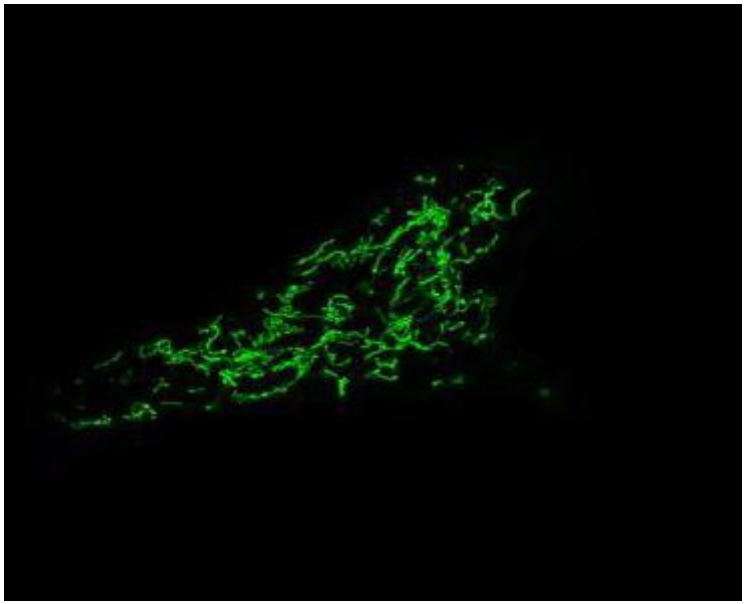
↓

Increased mitochondrial
ROS generation and/or
decreased mitochondrial antioxidant defenses

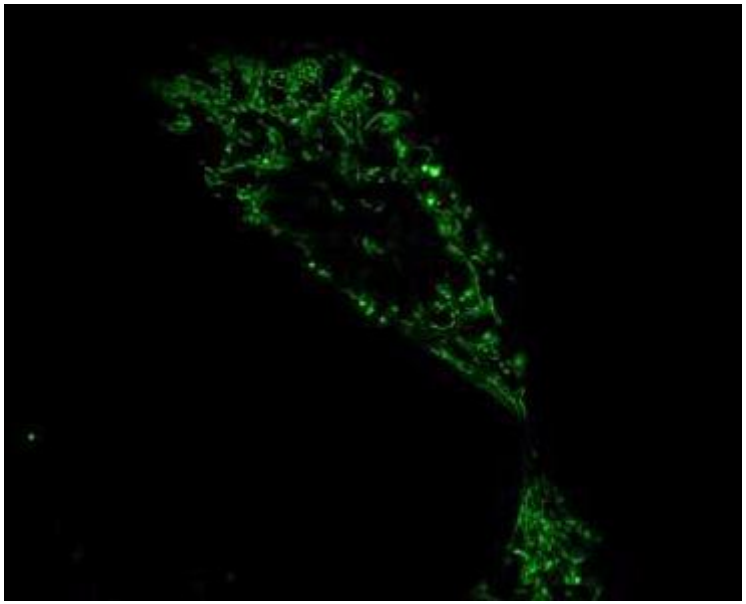



Doxorubicin-treated cells are more susceptible to $H_2O_2$-induced oxidative stress and are protected by pargyline (i.e., MAO amplifies oxidative stress)

# Doxorubicin-induced increase in mitochondrial ROS formation monitored by means of mitoHyPer. Protective effect by MAO inhibition (pargyline)

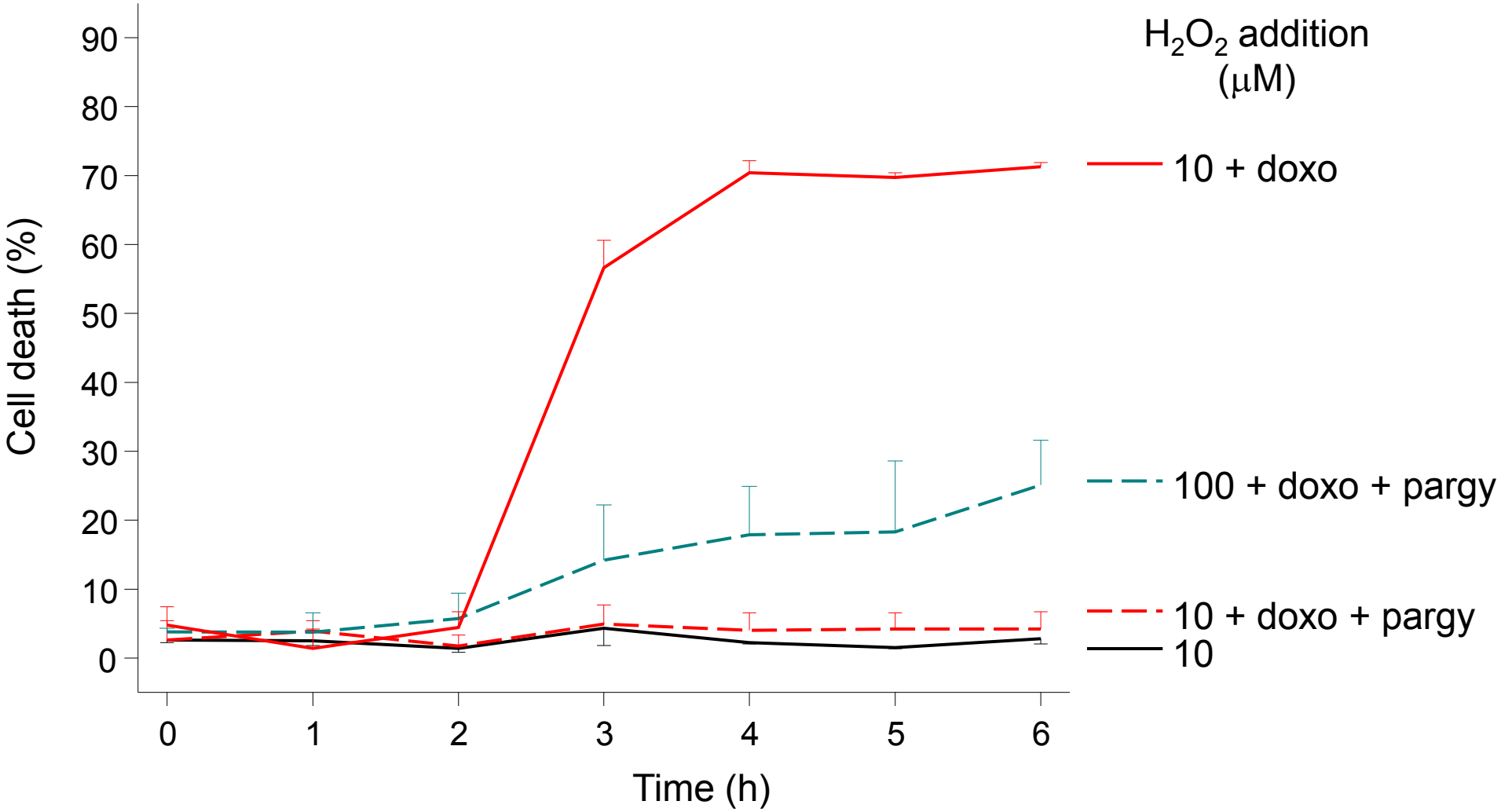


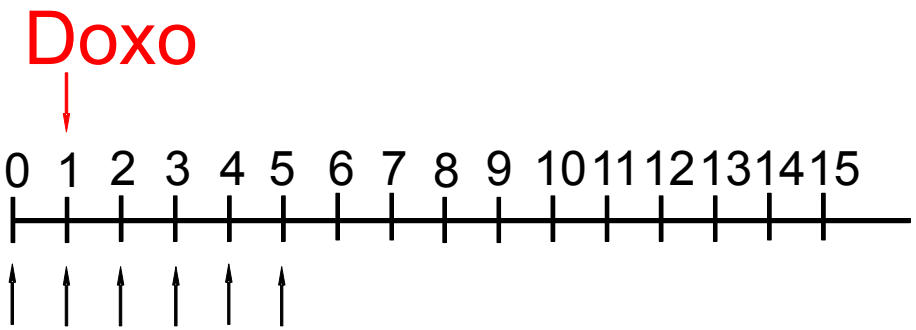
- pargyline



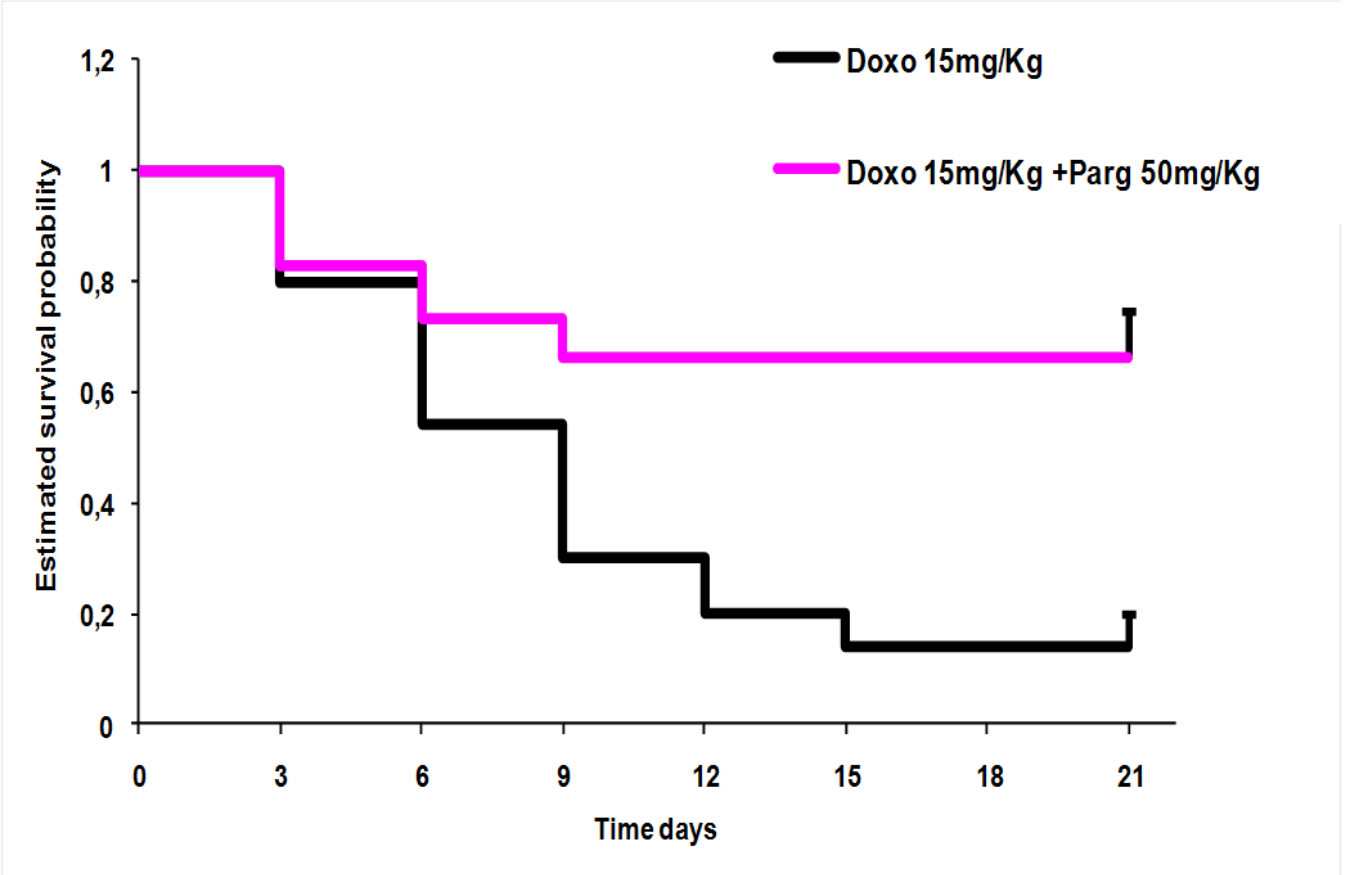
+ pargyline

# $H_2O_2$-induced loss of viability of HL-1 cardiomyocytes




Doxo
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Parg


Doxo 15mg/Kg
Doxo 15mg/Kg +Parg 50mg/Kg
Estimated survival probability
1,2
1
0,8
0,6
0,4
0,2
0
0
3
6
9
12
15
18
21
Time days

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Edon Melloni

IEO Milan
Marco Giorgio
Simon Plyte

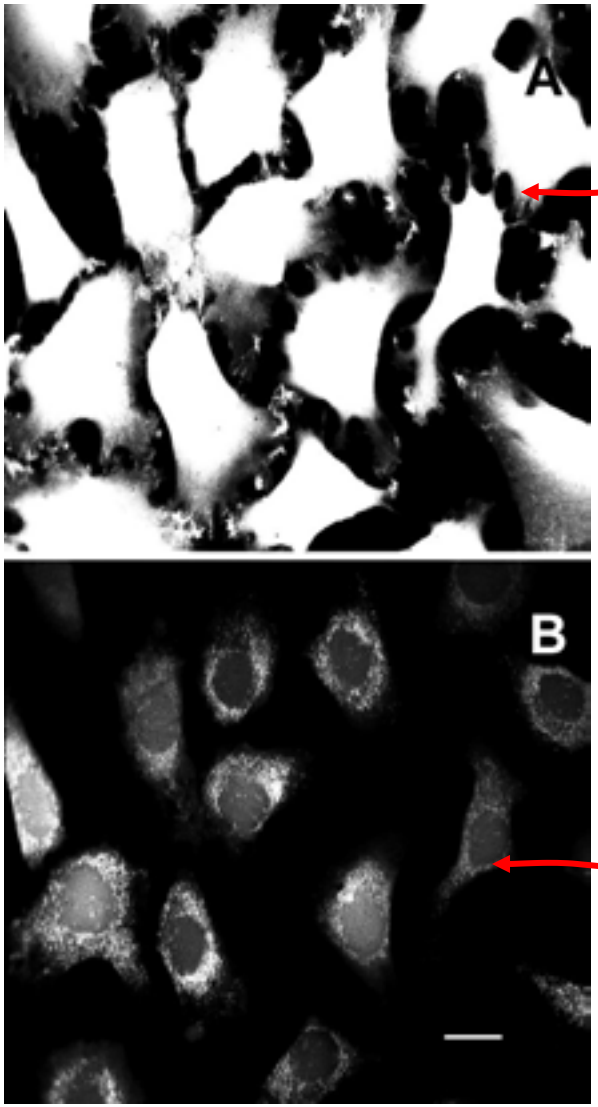
# DETECTABLE PARAMETERS OF MITOCHONDRIAL FUNCTION

| | |
|---|---|
| ISOLATED MITOCHONDRIA | Oxygen consumption<br>ATP synthesis<br>Redox changes (NAD and FAD)<br>$\Delta\Psi_m$ (quantitative)<br>Matrix volume<br>Ion movements |
| ISOLATED CELLS | Oxygen consumption<br>Redox changes (NAD and FAD)<br>$\Delta\Psi_m$ (semiquantitative)<br>*Matrix volume*<br>*Ion movements* |
| ISOLATED HEART | Oxygen consumption<br>*ATP content* |
| IN SITU HEART | Oxygen consumption<br>*ATP content* |

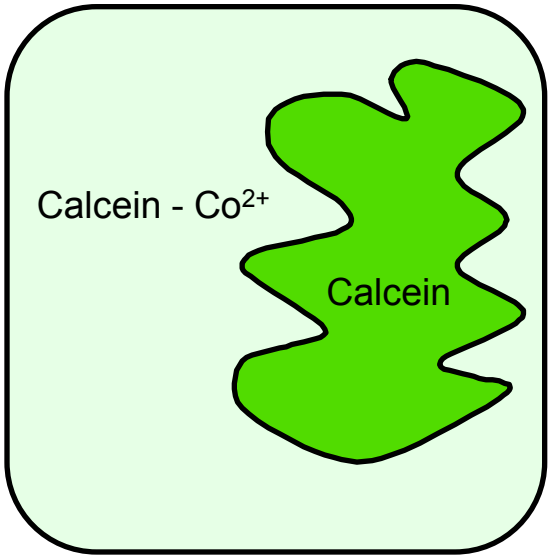
# METHODS FOR DETECTING THE OPENING OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE (PTP)

| | |
|---|---|
| ISOLATED MITOCHONDRIA | Swelling |
| | $Ca^{2+}$ retention capacity (CRC) |
| | Permeability to solutes |
| | *CsA inhibitable changes* |
| ISOLATED CELLS | Calcein redistribution |
| | *Swelling* |
| | *CsA inhibitable changes* |
| INTACT HEART | Mitochondrial NAD depletion |
| | Mitochondrial accumulation of deoxyglucose |
| | *CsA inhibitable changes* |

## $Co^{2+}$ addition after calcein loading

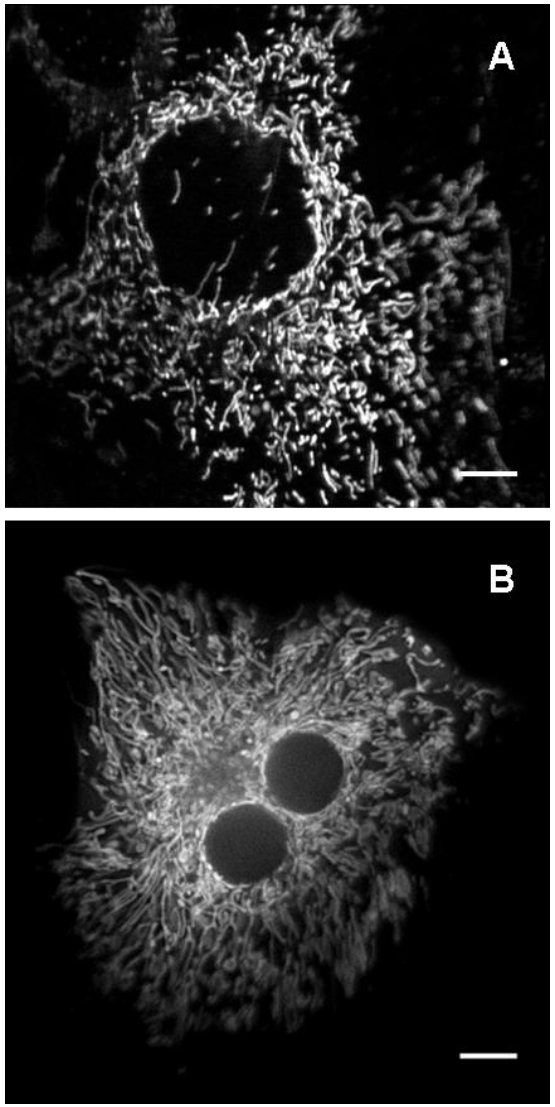



Calcein loading in the absence of $Co^{2+}$



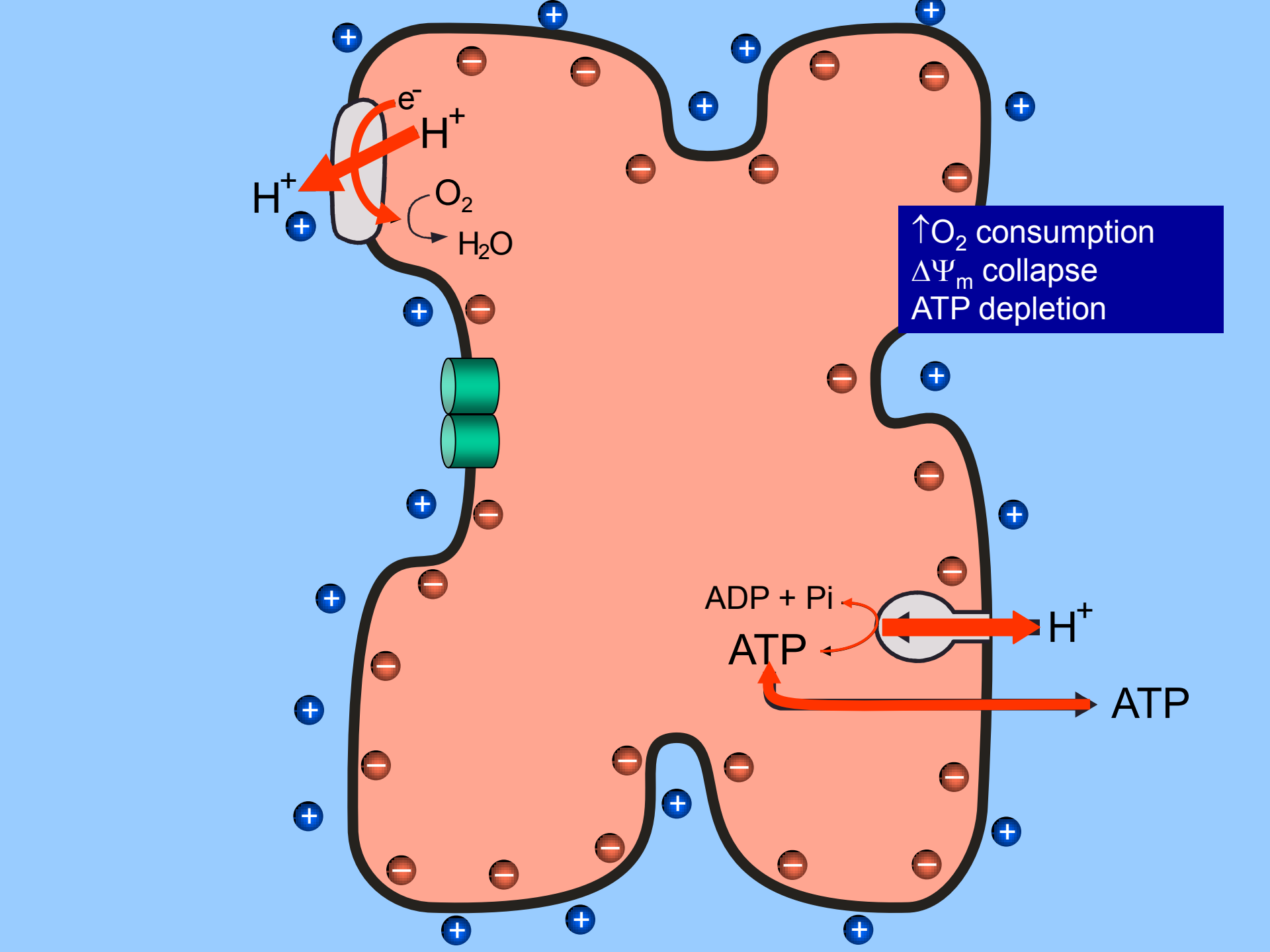

$Co^{2+}$ addition to calcein loaded cells

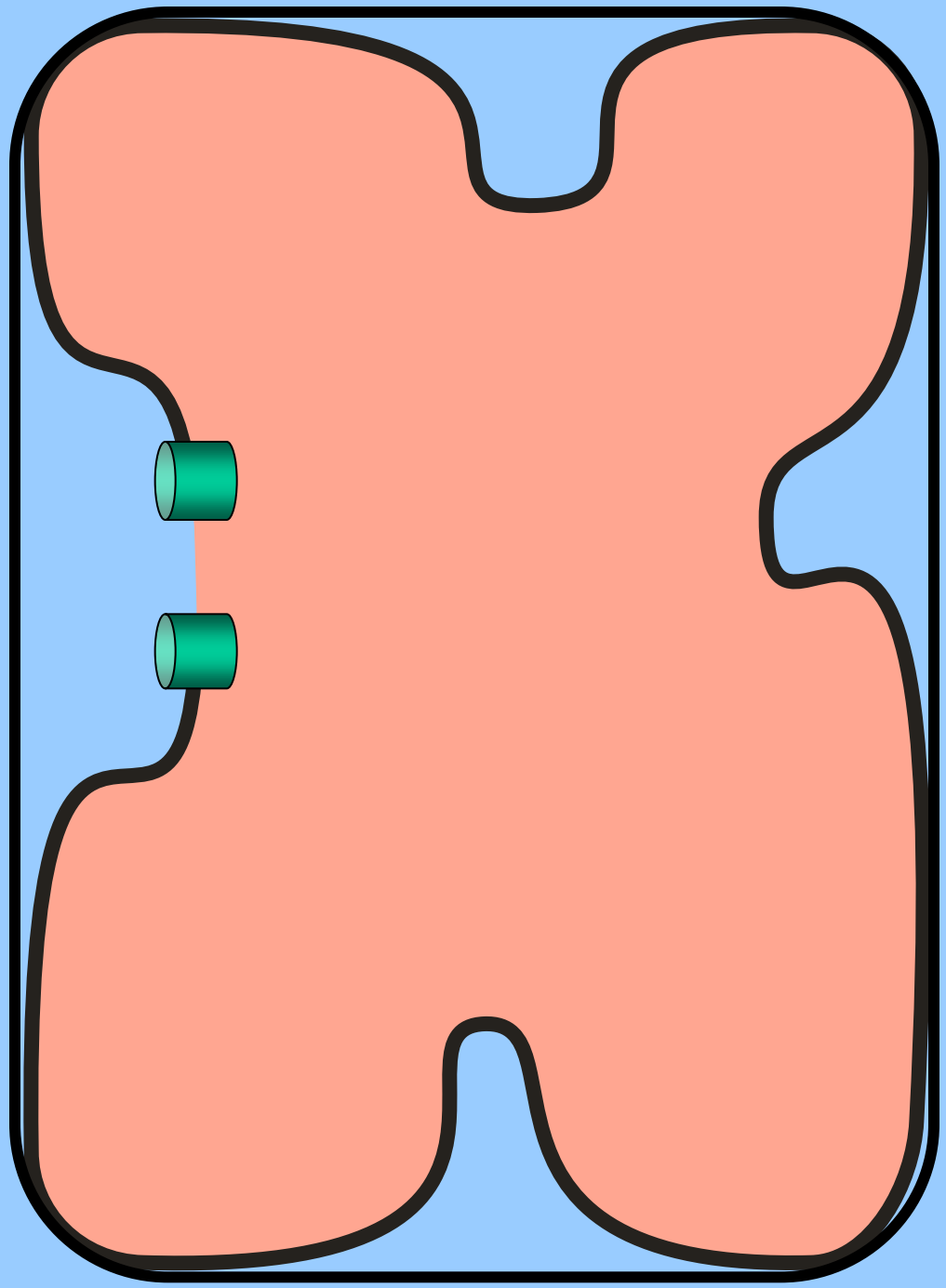
## Coloading

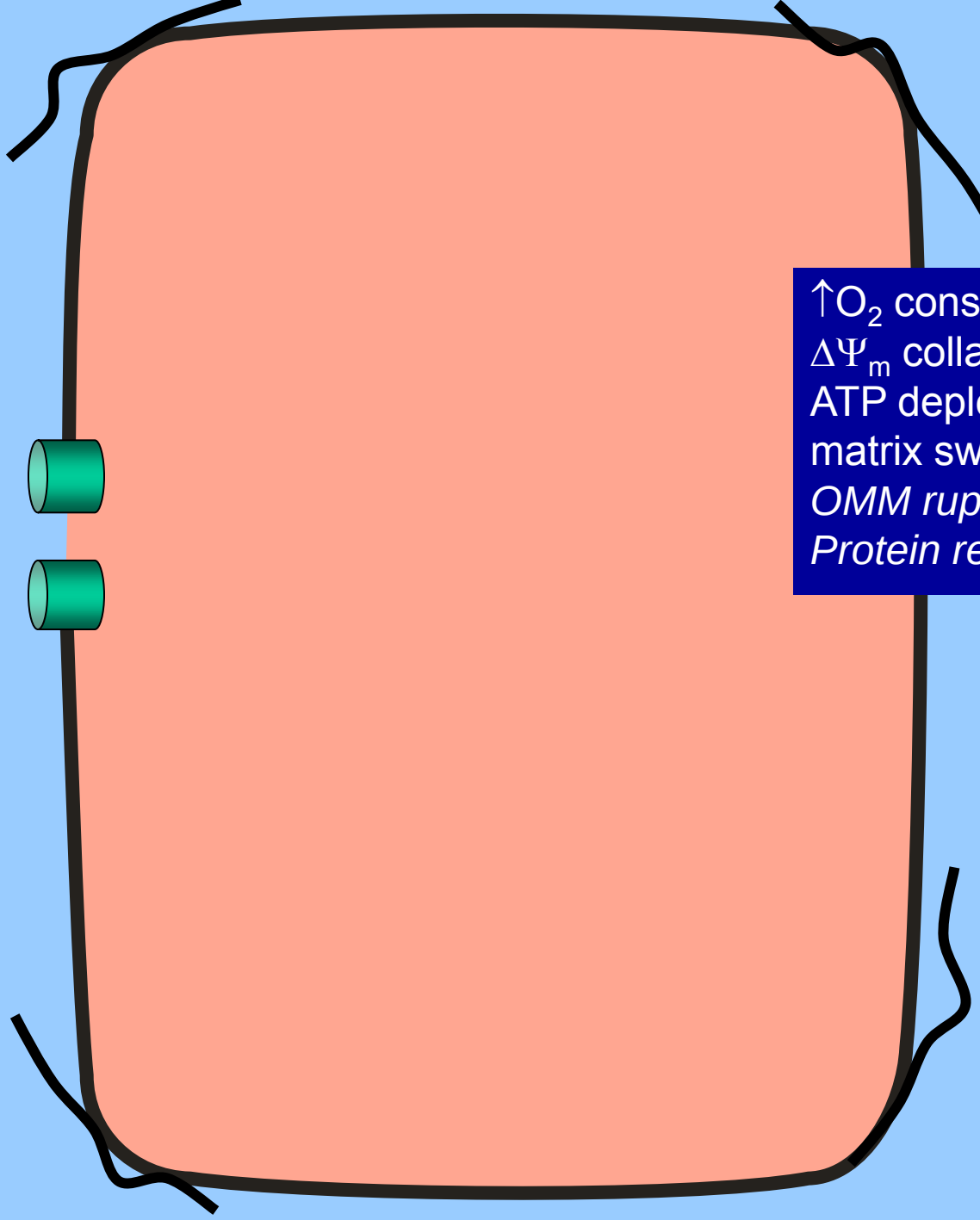


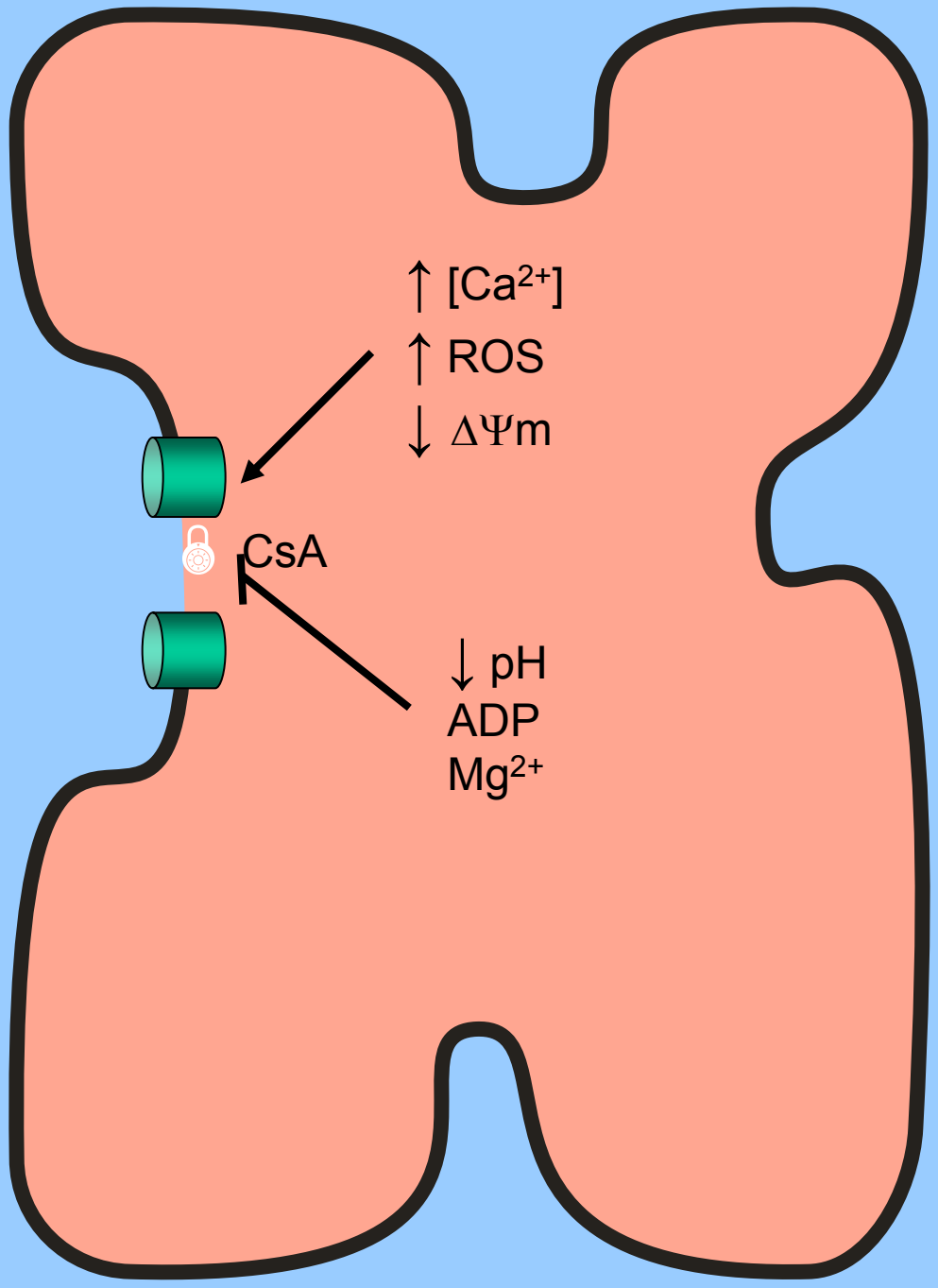
- **Short PTP openings are detected only by trapped calcein and may have little impact on cell viability, while changes of TMRM distribution require longer PTP openings, which cause release of cytochrome *c* and may result in cell death.**
- **Modulation of PTP open time appears to be a key element in determining the outcome of stimuli that converge on the PTP.**

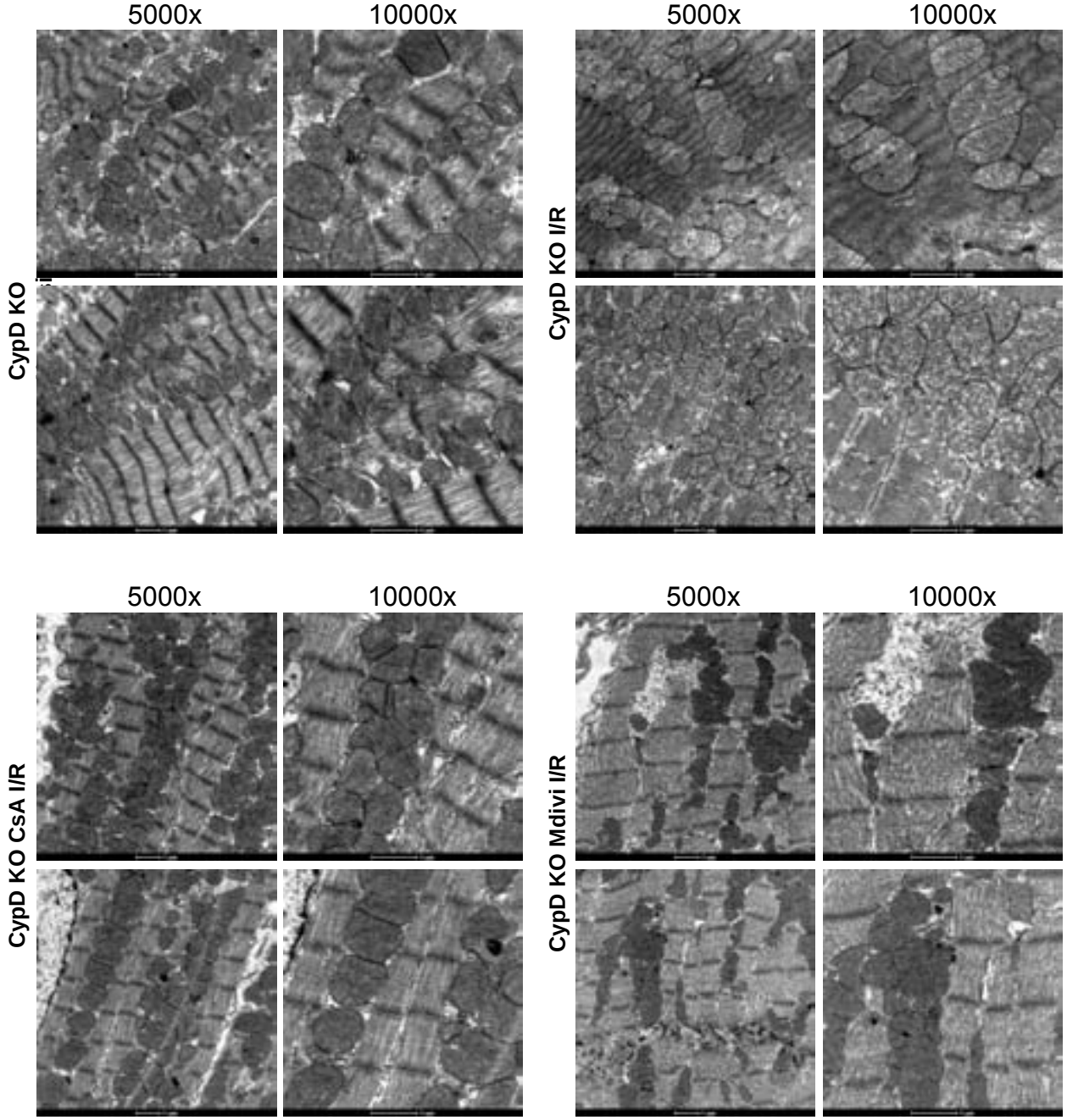
Petronilli et al., J. Biol. Chem. 276:12030-4, 2001


e-
H+
H+
O2
H2O
↑O2 consumption
ΔΨm collapse
ATP depletion
ADP + Pi
ATP
H+
ATP



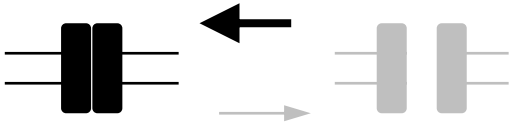

↑$O_2$ consumption
$\Delta\Psi_m$ collapse
ATP depletion
matrix swelling
*OMM rupture*
*Protein release*


↑ [Ca2+]
↑ ROS
↓ ΔΨm
CsA
↓ pH
ADP
Mg2+


5000x
10000x
CypD KO
5000x
10000x
CypD KO I/R
5000x
10000x
CypD KO CsA I/R
5000x
10000x
CypD KO Mdivi I/R

## ISCHEMIA

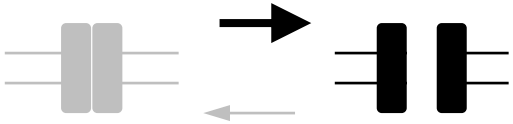
low pH
high [$Mg^{2+}$ ]
low ROS



$\Delta\psi_m$ fall
high Pi

## REPERFUSION

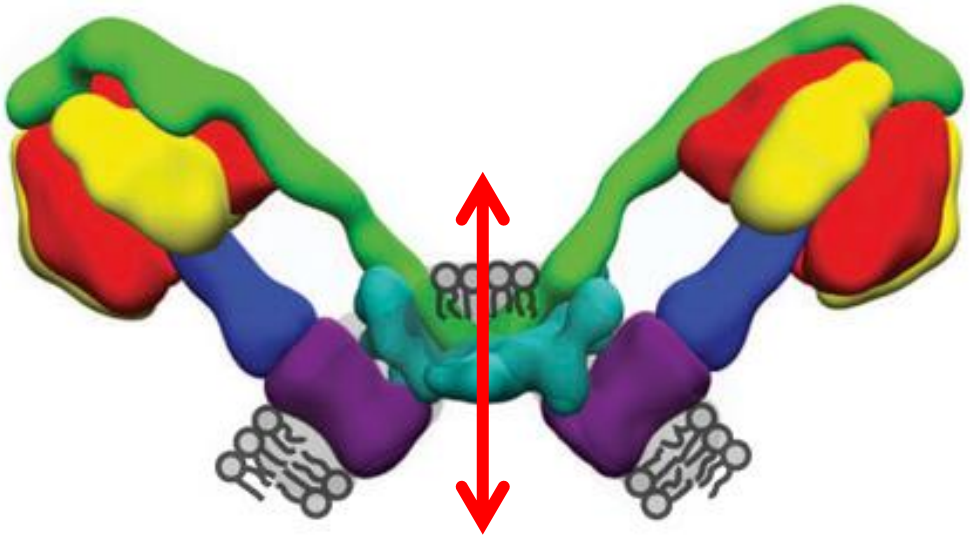
pH increase
[$Ca^{2+}$] increase
high ROS
[$Mg^{2+}$] decrease



$\Delta\psi_m$ recovery
low Pi

# Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM

Lindsay A. Baker[a,b], Ian N. Watt[c], Michael J. Runswick[c], John E. Walker[c], and John L. Rubinstein[a,b,d,1]



PNAS | July 17, 2012 | vol. 109 | no. 29 | 11675–11680

# Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM

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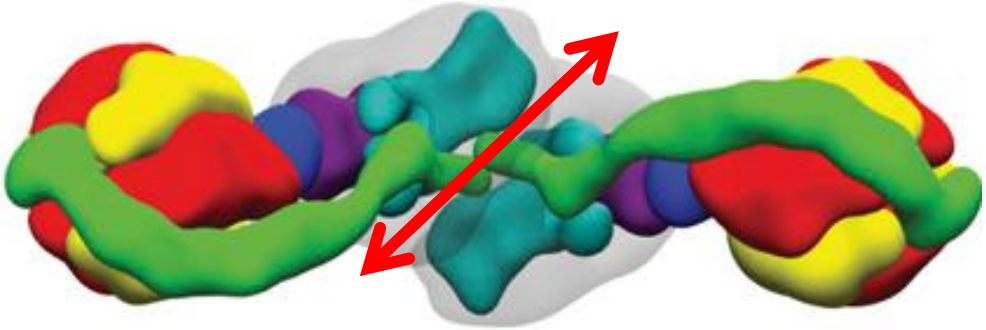
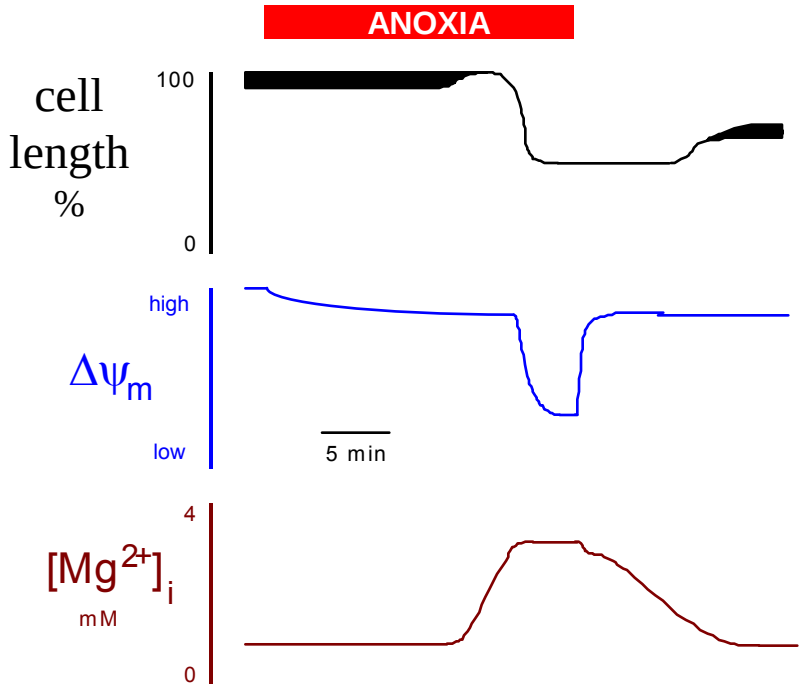
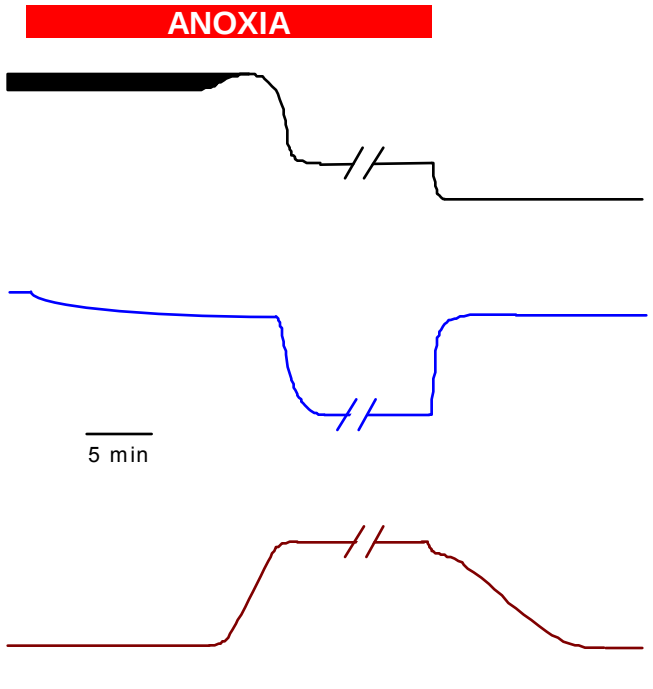


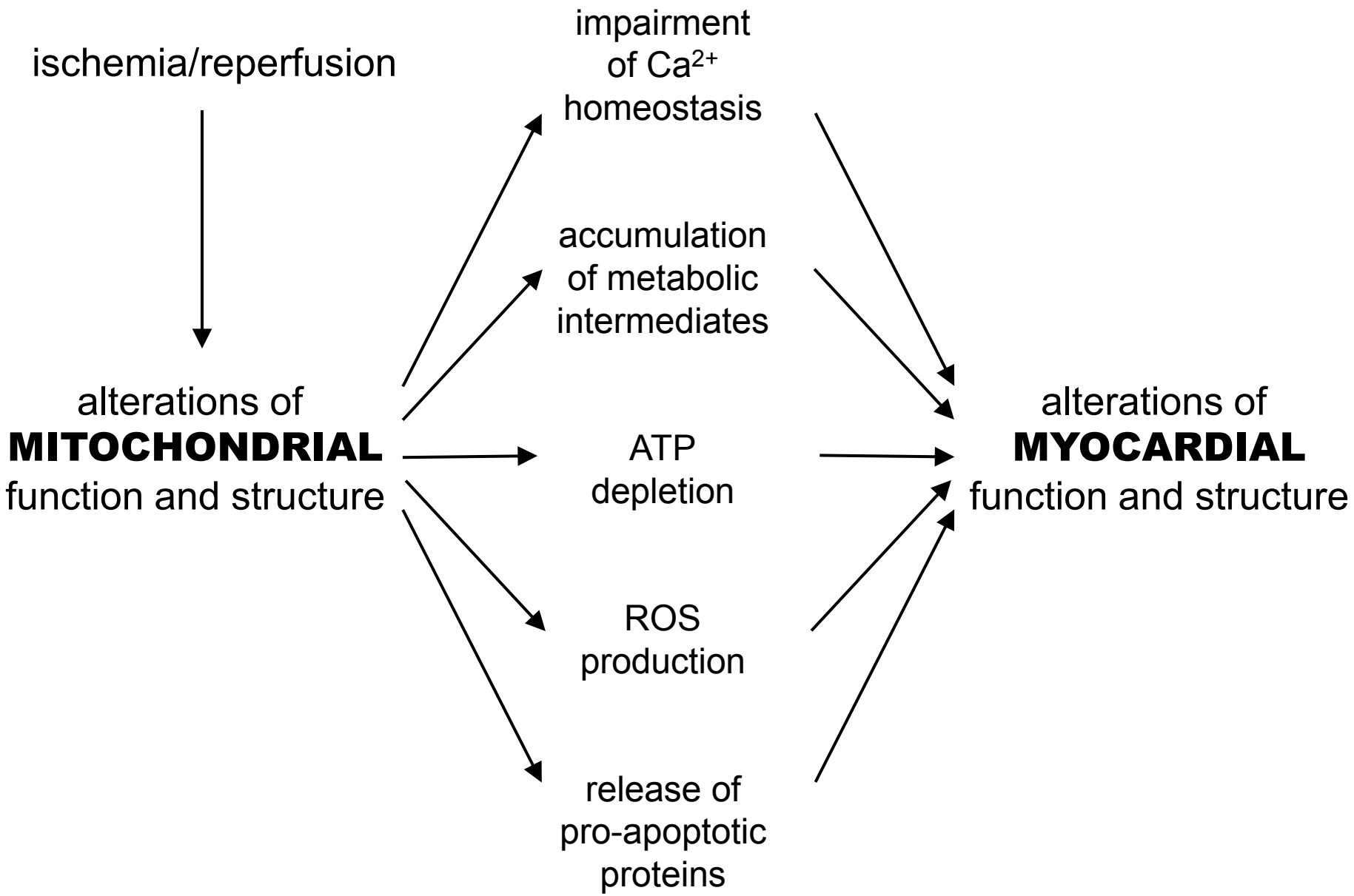
PNAS | July 17, 2012 | vol. 109 | no. 29 | 11675–11680

# Reversible damage

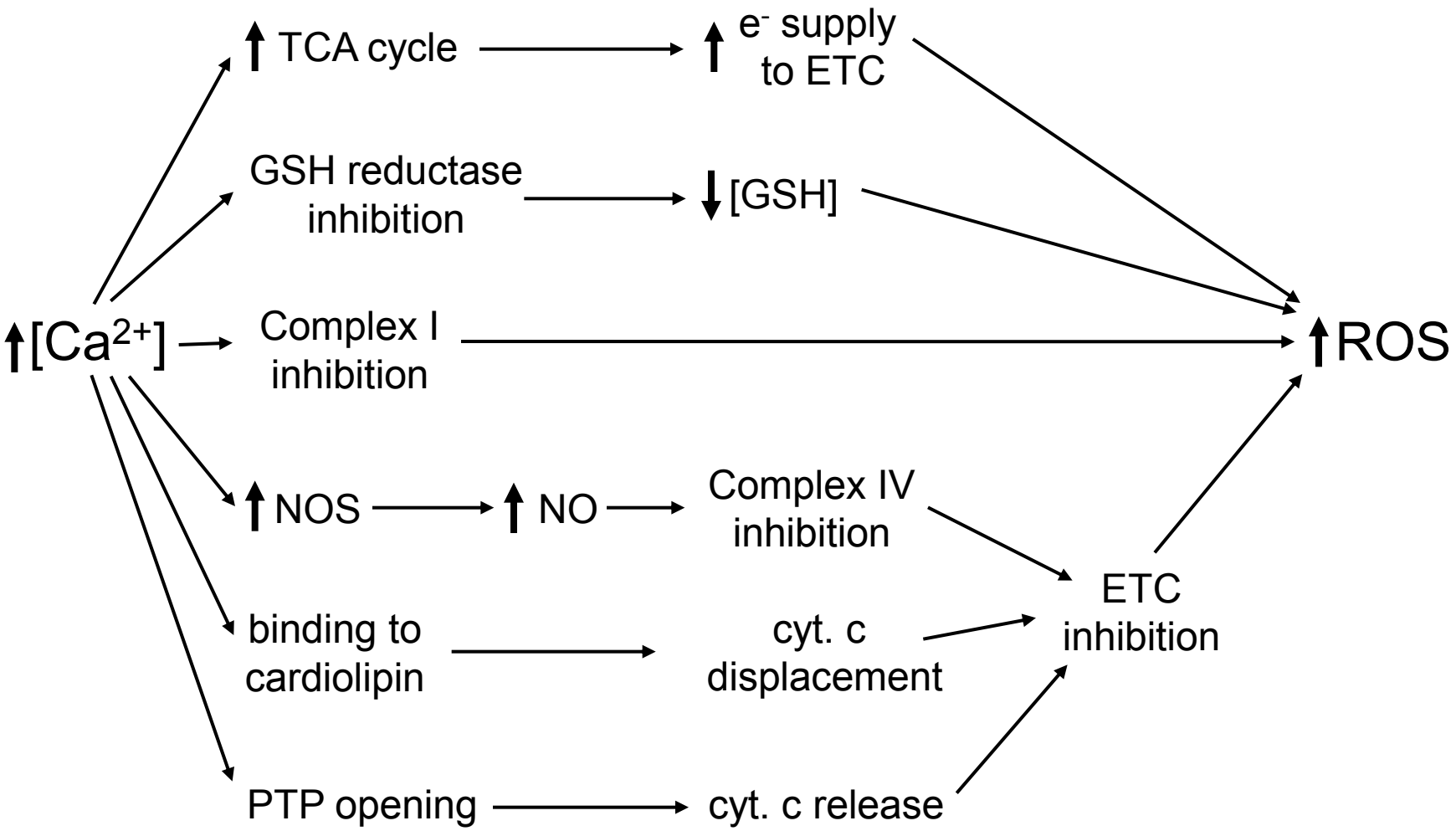



# Irreversible damage




ischemia/reperfusion
alterations of
MITOCHONDRIAL
function and structure
impairment
of $Ca^{2+}$
homeostasis
accumulation
of metabolic
intermediates
ATP
depletion
ROS
production
release of
pro-apoptotic
proteins
alterations of
MYOCARDIAL
function and structure

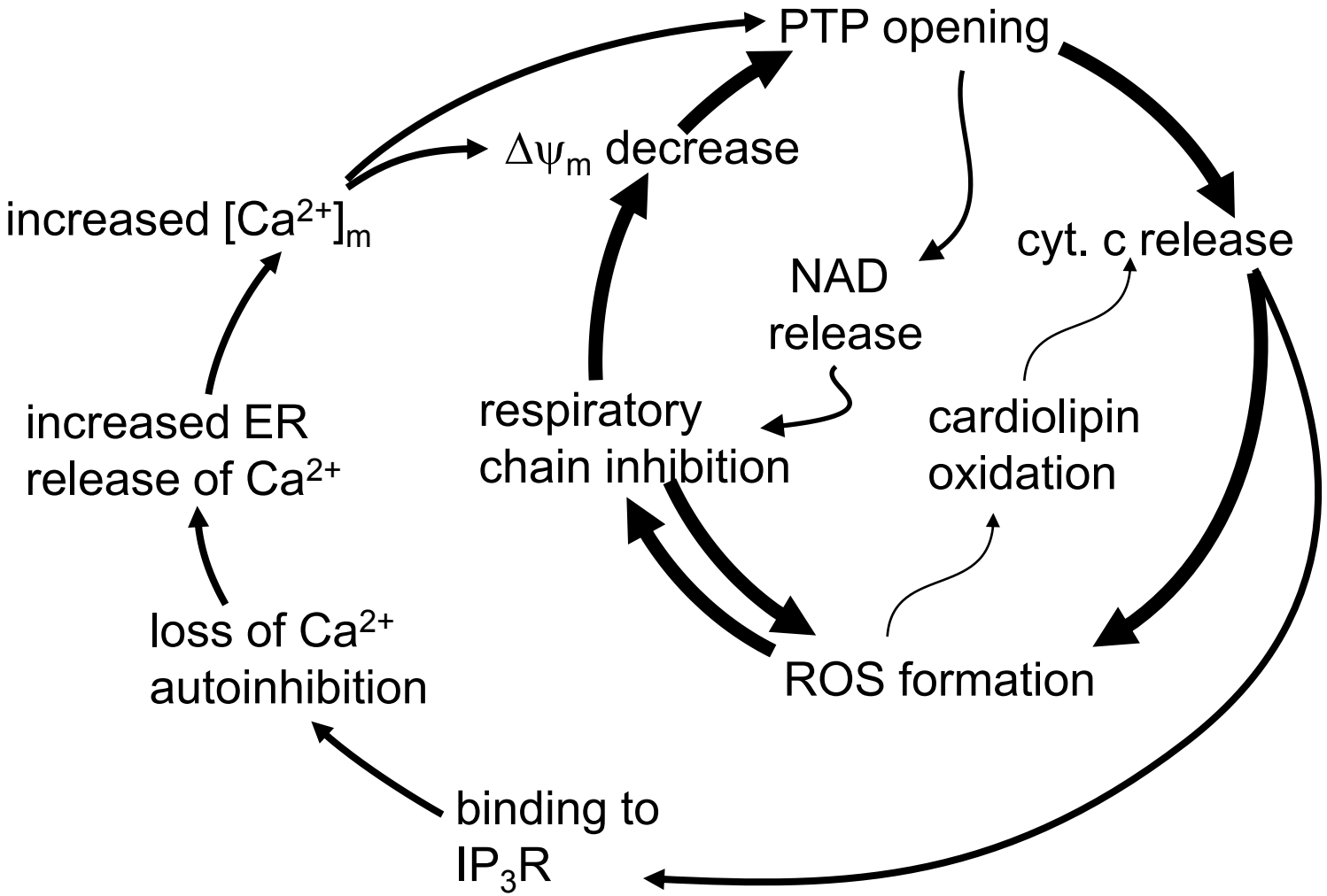
# Relationships between $[Ca^{2+}]_i$ increase and increased ROS formation in mitochondria




*adapted from P.S. Brookes et al., Am J Physiol 287:C817-C833, 2004*

**OXIDATIVE STRESS IS UPTREAM AND DOWNSTREAM OF PTP OPENING**

Amplification loops linking $[Ca^{2+}]_m$ with ROS formation and PTP opening



# The oxidation of tropomyosin ocurring upon post-ischemic reperfusion is largely reduced in mice lacking CypD