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Signaling ROS in Cardioprotection

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(No financial interests to disclose)
### Are we making progress in CVD?

<table>
<thead>
<tr>
<th>THE GOOD NEWS:</th>
<th>CVD Death Rate (age-adjusted)</th>
<th>Cancer Death Rate (age-adjusted)</th>
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<tbody>
<tr>
<td>1955: 589/100,000</td>
<td>2015: 192/100,000</td>
<td>1955: 194/100,000</td>
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<td>(-67%)</td>
<td>2015: 161/100,000</td>
<td>(-17%)</td>
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<table>
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<tr>
<th>THE BAD NEWS:</th>
<th>Total CVD Deaths</th>
<th>Total Cancer Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955: 848,000</td>
<td>2015: 787,000*</td>
<td>1955: 322,000</td>
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<tr>
<td>2015: 787,000*</td>
<td>2015: 586,000</td>
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*48% of CVD deaths are due to CAD

(note that US population has doubled since 1955)
Protecting the Heart from Ischemia/Reperfusion Injury

Control ischemia

45 min

ROS

Ca

Ischemic Preconditioning (IPC)

Ischemic Post-conditioning (IPostC)

Pharmacologic Preconditioning (PPC)

Adenosine ACh, NE, Epi, Opioids, NO, diazoxide, PMA, TNF, H₂O₂, Ca

The Mitochondrial Permeability Transition Pore (PTP)

- **ROS-, Ca-, voltage-, pH-sensitive pore** (MWt cut-off 1500) which immediately dissipates mitochondrial membrane potential $\Delta \Psi_m$, converts ATP synthesis to ATP consumption by reversing $F_1-F_0$ ATPase, and releases matrix Ca stores

- **Long-lasting high conductance PTP opening** leads to mitochondrial swelling, OMM rupture, irreversible mitochondrial injury and cell death (apoptosis/necrosis)

- However, **transient low conductance PTP openings** function as a Ca release mechanism and generate signaling ROS (superoxide flashes) (Wang et al, Cell. 2008;134:279-90; Korge et al. J Biol Chem. 2011;286:34851-7)

**Key components:**
- $F_0F_1$-ATPase dimers
- CyPD

**IMM Modulators:**
- ANT, PiC

**OMM Modulators:**
- VDAC, TSPO,
- Bax/Bcl, CK, HK

Dual Roles of ROS in Ischemia/Reperfusion Injury & Cardioprotection

- **Control ischemia**: ROS in large amounts cause damage (by inducing MPT)
- **Preconditioning**: ROS in small amounts trigger cardioprotective signaling by activating the **RISK pathway** (*PKCɛ / adenosine receptors → PI3 kinase → Akt → GSK-3β*) and/or the **STAT pathway** (*TNFα / Stat 3*), which suppresses a massive ROS burst during reperfusion, thereby inhibiting large conductance long-lasting MPT & cell death.
Cardioprotective Signaling in Heart

ROS production by mito K$_{\text{ATP}}$ and/or transient PTP opening

Inhibition of long lasting PTP opening & cell death

Current cardioprotection paradigms focus on identifying signaling pathways that directly inhibit PTP opening (e.g. preventing CyPD phosphorylation).
Cardioprotective Signaling in Heart

does cardioprotective signaling suppress MPT & cell death by:

- decreasing the massive ROS burst to indirectly suppress PTP opening?
- making PTP opening intrinsically more resistant to ROS or other inducing factors?
- both?

production by mito $K_{ATP}$ or transient PTP activation

$\downarrow$ Damaging ROS burst

Direct inhibition of PTP opening

SAFE pathway activation

Sources of mitochondrial ROS generation

**Signaling ROS:**
- **MitoK\textsubscript{ATP} activation** causing matrix alkalinization
- Succinate-driven reverse electron transport (RET) through complex I
  - **Glutamate dehydrogenase (GDH)**

**Damaging ROS:**
- **Succinate-driven RET through complex I** (Chouchani et al, *Nature* 2014;515:431-5)
- Damaged ETC complexes (I, II and III)
- Matrix dehydrogenases (\(\alpha\)KGDH, PDH, acyl-CoA-DH, G3PDH) & reductases (GR, TR2)
- Matrix MAO and dihydroorotate dehydrogenase
- Matrix NOX4 (?)
How redox sites generate ROS – Electron donors transfer electrons to electron acceptors

If the usual electron acceptor is not available, and O\textsubscript{2} has access to the redox site, the electron can be donated to O\textsubscript{2} to form \textit{O}_2^- or H\textsubscript{2}O\textsubscript{2}
Electron Transport Chain (ETC)

Succinate-driven RET through complex I with intact $\Delta \Psi$

Limited NAD$^+$ available, e.g. high NADH/NAD$^+$ ratio

ETC inhibitors blocking forward electron flow, e.g. rotenone, antimycin, cyanide

Reductive stress, e.g. no GSSG or oxidized Trx2 available

Molecular mechanisms of mitochondrial ROS production
NADH/NAD\(^+\) ratio during brief I/R

- During ischemia, NADH/NAD\(^+\) ratio (normally \(\approx 0.1\)) becomes \(>1\)\(^*\), but no \(O_2\) is present to generate \(\cdot O_2^-\).
- During reoxgenation, unless prolonged ischemia has damaged ETC complexes to impede electron flow, the NADH/NAD\(^+\) ratio very rapidly decreases to \(<<1\), so there is only a brief window with both high NADH and \(O_2\) present to generate ROS by inhibited complex I, dehydrogenases or reductases.
- Is the window long enough to generate sufficient ROS to activate the RISK and/or SAFE pathway?

\(^*\)From 0.11 to 1.2 after 10 min of global ischemia in rabbit ventricle (Ferrari R et al Circulation. 1996;94:2587-96)
Is there another ROS-producing matrix enzyme with appropriate properties?

*Ideal requirements:*

- Inactive during normoxia
- Activated during brief anoxia, e.g. by the fall in matrix ATP/ADP ratio
- Generates ROS transiently during reoxygenation *despite a low NADH/NAD ratio*, and then inactivates again after the matrix ATP/ADP ratio has recovered
- The ROS generated are sufficient activate the RISK and/or SAFE pathways
Glutamate Dehydrogenase (GDH)

- Mainly expressed in liver, kidney, brain & pancreas
- Generates NH$_3$ for the urea cycle
- Generates $\alpha$-KG for the TCA cycle
- Low activity in heart
**PDH or α–KGDH**

- Single redox site (E3 subunit)
- Single NAD(H) binding site

**GDH**

- Two redox - NAD(H) binding sites
- 1\(^{st}\) site is like PDH or α-KGDH,
- However, substrate oxidation & NADH release from the 1\(^{st}\) site is inhibited by ATP/GTP and relieved by ADP/GDP
- When activated by ADP/GDP, NADH is released from the 1\(^{st}\) site and binds to the 2\(^{nd}\) site, reducing its redox state and donating the electron to O\(_2\)
H$_2$O$_2$ generation by purified GDH with NADH/NAD ratio $<<1$

- **Purified PDH**
  - ±Pyr
  - NADH
  - NAD$^+$
  - H$_2$O$_2$
  - 50 a.u.
  - 2 min

- **Purified GDH**
  - KG+NH$_4$Cl
  - NAD$^+$
  - Glut
  - H$_2$O$_2$
  - 50 a.u.
  - 5 min

$\text{NAD}^+ = 0.25 \text{ mM}$

$\text{Glut} = 2.5 \text{ mM}$
NADH and H$_2$O$_2$ production by GDH in the presence of GTP or ATP is activated by ADP.

- **NADH production**
  - Glut → ADP → NAD$^+$
  - GTP 1 mM
  - $F_{NADH}$ 20 a.u.
  - 3 min

- **H$_2$O$_2$ production**
  - Cat → Glut → NAD$^+$
  - GTP 1 mM
  - $H_2O_2$ 15 a.u.
  - 5 min

- **GTP, ATP = 1 mM**
- **NAD$^+$ = 0.5 mM**
- **Glut = 2.5 mM**
- **ADP = 0.25 mM**
H$_2$O$_2$ production by GDH in permeabilized rabbit cardiac mitochondria

Glut = 2.5 mM  NAD$^+$ = 0.5 mM  
KG = 10 mM  ADP = 0.5 mM
Cardiac-specific GDH KO mouse

(\textit{Glud1} encodes GDH1)

\textit{Glud1}^{lox/lox} kindly provided by Dr. Pierre Maechler
MyhCre^{+/+} mice kindly provided by Dr. Reza Ardehali
Glutamate-stimulated $\text{H}_2\text{O}_2$ production in isolated permeabilized cardiac mitochondria

Glut = 5 mM
$\text{NAD}^+$ = 0.5 mM
$\text{ADP}$ = 0.5 mM
Experimental protocols

(1) Control
- Stab. | Ischemia | Reperfusion
- 20min | 25min   | 120min

(2) IPC
- Stab. | IPC | Ischemia | Reperfusion
- 20min | 3x[5/5]min | 25min | 120min

(3) $H_2O_2$
- Stab. | $H_2O_2$-PC | Ischemia | Reperfusion
- 20min | 3x[3/3]min | 25min | 120min

(4) IPoC
- Stab. | Ischemia | IPoC | Reperfusion
- 20min | 25min | 3x[5/5]sec | 120min
Ischemic PC fails to activate the RISK/SAFE pathways in GDH KO mice.

The diagram shows protein phosphorylation levels normalized to controls for WT and MyhGlud1−/− mice under different conditions: Ref, IPC, H2O2. The proteins analyzed include Gsk3β, p-Gsk3β, Akt, p-Akt, Erk 1/2, p-Erk 1/2, Stat3, and p-Stat3.
In progress – is the signaling ROS burst absent in GDH KO mice?

(In collaboration with Linda Cai & Ji Youn)
Conclusions

• GDH is **required** for cardioprotection by IPC in mouse hearts

• The finding that exogenous ROS protect GDH KO hearts suggest that GDH may be a critical source of ROS triggering cardioprotective signaling via RISK and/or SAFE pathways
  ✷ Inactive during normoxia
  ✷ Activated during brief hypoxia by the fall in matrix ATP/ADP ratio
  ✷ Generates ROS transiently during reoxygenation **despite a low NADH/NAD ratio**, and then inactivates again after the matrix ATP/ADP ratio has recovered

• GDH is **not required** for cardioprotection by IPoC in mouse hearts (not unexpected, since damaged ETC complexes can generate ROS during ischemic IPoC in addition to GDH)
Questions we are investigating:

Is GDH the cause of ROS production during transient PTP opening?

- Transient PTP opening depolarizes $\Delta \Psi$, stimulating reverse ATP synthase to elevate ADP & activate GDH to generate ROS
- Would explain why CsA blocks cardioprotection by IPC
- Currently being tested in isolated mitochondria from GDH KO mice

Is GDH the cause of ROS production by mitoK\textsubscript{ATP} channel activation during IPC?

- MitoK\textsubscript{ATP} channel opening does not depolarize $\Delta\Psi$ sufficiently to stimulate reverse ATP synthase and raise ADP to activate GDH

- Is another mechanism responsible?
  - Matrix alkalinization $\rightarrow$ transient PTP opening $\rightarrow$ $\Delta\Psi$ depolarization $\rightarrow$ reverse ATP synthase activity $\rightarrow$ GDH activation
  - Direct effect of mitoK\textsubscript{ATP} channel openers on GDH

- Currently being explored in isolated mitochondria from GDH KO mice & with purified GDH
A Further Prediction: If GDH is the source of signaling ROS, then pharmacologic PC interventions upstream of signaling ROS generation by mitoK$_{ATP}$ channel or transient PTP opening will be ineffective in GDH KO mice, whereas downstream interventions will remain cardioprotective.
Ischemic PC
GPCR stimulation, NO
Phorbol esters, KCO

\[ \text{mitoK}_{\text{ATP}} \text{ opening} \]

\[ [\text{Ca}]_o \]

TNF\( \alpha \)R stimulation

Preconditioning

\[ 5\text{HD} \rightarrow \]

\[ \text{PKG, PKC} \]

\[ \text{Matrix alkalinization} \]

\[ \text{Transient PTP opening} \]

\[ \text{CsA} \]

SAFE pathway

\[ \text{JAK, STAT3} \]

RISK pathway

\[ \text{PI3K, ERK1/2, Akt, GSK3}\beta \]

\[ \downarrow \text{Long-lasting PTP Opening} \]

\[ \downarrow \text{Damaging ROS Burst} \]

\[ \downarrow \text{Cell Death} \]
The Two-Edged Sword of ROS

Paracelsus
(Phillipus Theophratus Bombastus von Hohenheim)
1493-1541

“All things are poison, and nothing is without poison. The right dose differentiates a poison from a remedy.”

http://www.levity.com/alchemy/paracelsus_portraits.html
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