Inhibition of GSK3β by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion.

Ischemic Postconditioning

- Brief episodes of ischemia-reperfusion following a prolonged ischemia-reperfusion period
- Attenuate infarct size

Ischemic Postconditioning

- Found in both animal and human hearts
  (Zhao et al., 2003; Argaud et al., 2005; Staat et al., 2005; Heusch et al., 2006; Gomez et al., 2007)
- Angioplasty postconditioning can reduce infarct size in patients with ongoing acute MI. (Staat et al., 2005)
- Pharmacological postconditioning mimetic is needed.

Mitochondrial Permeability Transition Pore (mPTP)

- A nonselective, high conductance channel with multiple macromolecular components, formed at sites where the inner and outer membranes of the mitochondria meet, under certain pathological conditions, e.g. traumatic brain injury, stroke.
- Induction of mPTP can lead to mitochondrial swelling and cell death, and plays an important role in some types of apoptosis.

Mitochondrial Permeability Transition Pore (mPTP)

- Opening is favored by overproduction of reactive oxygen species, ATP depletion, and accumulation of Ca2+ in the matrix.
- Ca2+ stimulates the interaction of cyclophilin D (CypD) with an mPTP component -> permeability transition.
  (Duchen et al., 1993; Crompton, Virji, Ward, 1998; Halestrap et al., 1998)
Mitochondrial Permeability Transition Pore (mPTP)

- mPTP plays a crucial role in lethal reperfusion injury.
- mPTP may be inhibited by ischemic postconditioning.
  (Hausenloy, Duchen, Yellon, 2003; Halestrap, Clarke, Javadov, 2004; Argaud et al., 2005)
- Indirect evidence suggests that opening of the mPTP during reperfusion may be regulated by extramitochondrial activation or inhibition of several kinases, including glycogen synthase kinase-3β (GSK3β).
  (Juhaszova et al., 2004; Hausenloy et al., 2005; Bopassa et al., 2006)

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  (Juhaszova et al., 2004; Hausenloy et al., 2005; Bopassa et al., 2006)

Glycogen synthase kinase-3β (GSK3β)

- Constitutively active
- Important regulator of cell functions
  (Frame & Cohen, 2001; Harwood, 2001)
- Roles in DM, information & cancer
- Negative regulator of myocardial hypertrophy
- Key enzyme in the myocardial response to ischemia-reperfusion injury
  (Harwood, 2001; Hardt & Soshima, 2002; Antos et al., 2002)

Preconditioning-like cardioprotective effect of morphine was mediated by GSK3β.
  (Grosse et al., 2004)

Glycogen synthase kinase-3β (GSK3β)

- GSK3β was involved in preconditioning via its inactivation by phosphorylation at a specific serine residue (Ser9) located in its N-terminal domain.
  (Tong et al., 2002)

mPTP & GSK3β in ischemia-reperfusion

- Ischemia-reperfusion
- incr ROS, decr ATP, incr Ca²⁺ in mitochondrial matrix
- Ca²⁺ stimulates CypD-mPTP interaction
- mPTP opens, dumping Ca²⁺ and others into the cytosol ... etc. mitochondrial swelling, cell death

Hypothesis

- Inctivated GSK3β (phosphorylated at Ser9) favors mPTP closing
- Postconditioning inactivates GSK3β, thus helps alleviating ischemia-reperfusion

Objectives

- To investigate whether phosphorylated GSK3 may protect the heart via the inhibition of mPTP opening during postconditioning
In Vivo Model of Acute Myocardial Ischemia-Reperfusion Injury

WT and transgenic mice (male; 8 to 10 weeks old)

Anesthesia - 1:1 fentanyl citrate : midazolam, 0.3 mL/10 g bw, i.p.

Endotracheal intubation + rodent ventilator; 37 °C

Left thoracotomy, 4th ICS

Left anterior descending coronary artery occlusion by temporary ligation for 60 min.

Confirmed by ST-segment shift and myocardial pallor

Allowed to recover from anesthesia; endotracheal tube removed once spontaneous breathing resumed

Total reperfusion time = 24 hours

Pilot study: Cardiac Phenotype of GSK3β-S9A Mice

A subset of WT and transgenic mice (n=4 to 5 per group)

Myocardial wall thickness and left ventricular (LV) were assessed by echocardiography

Measurements:

- LV end-diastolic diameter
- LV end-systolic diameter
- LV fractional shortening
- Interventricular thickness
- Posterior wall thickness

Methods

Pilot study: Cardiac Phenotype of GSK3β-S9A Mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GSK3β-S9A</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>25±1</td>
<td>25±1</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>76±2</td>
<td>66±2†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>76±18</td>
<td>69±9.5</td>
</tr>
<tr>
<td>PW thickness, mm</td>
<td>0.83±0.04</td>
<td>0.83±0.004</td>
</tr>
<tr>
<td>WS thickness, mm</td>
<td>0.85±0.03</td>
<td>0.51±0.04†</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.93±0.00</td>
<td>2.71±0.23†</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.17±0.06</td>
<td>1.74±0.19†</td>
</tr>
<tr>
<td>LV shortening fraction %</td>
<td>59±2</td>
<td>41±2†</td>
</tr>
</tbody>
</table>

Body and LV weight, heart rate, LV posterior wall (PW) and interventricular septal (IVS) thickness; LV dimensions (end-diastolic [LVEDD] and end-systolic [LVESD] diameters and interventricular septal diameter); LV shortening fraction were measured by echocardiography in WT vs GSK3β-S9A mice (n=4 to 5 per group).

Table 1. Cardiac Phenotype of GSK3β-S9A Mice

*P<0.05 vs respective baseline; †P<0.05 vs baseline WT; ‡P<0.05 vs anesthetized WT; §P=NS vs WT

131 animals

Sham group = no ischemia-reperfusion

PostC = Postconditioning

3 cycles of 1 min reperfusion and 1 min ischemia, immediately after reflow

CsA = 10 mg/kg i.v.

Cyclosporin A, a potent inhibitor of mPTP

70 µg/kg i.v. SB21 = SB 216763

A GSK3β inhibitor

After 24 h reperfusion, mice were euthanized under deep anesthesia. Hearts were excised for determination of infarct size (n=66) or for assessment of calcium-induced mitochondrial permeability transition (n=65).

Pacing also prevented the increase in mortality in S9A mice, strongly suggesting that it was related to heart rate reduction induced arrhythmias.

All subsequent experiments were done with 450 bpm pacing during the prolonged ischemia.
Infarct size determination

- Area at risk (AR) = all the myocardial area supplied by the occluded artery
- 0.5 mg/kg Unisperse blue pigment, i.v.
  to delineate the in vivo area at risk
- The heart was excised and cut into 5 1-mm-thick transverse slices parallel to the A-V groove.
- Area of necrosis (AN) = infarcted area
  - 1% triphenyltetrazolium chloride for 15 min
  - To differentiate infarcted (pale) from viable (brick red) myocardial area.
- AN & AR were quantified by computerized planimetry, corrected for the weight of the tissue slices.

Assessment of mitochondrial functions

- Mitochondria isolation
  - Only AR myocardium were homogenised, centrifuged @ 1,300g x 3 min
  - Supernatant centrifuged @ 10,000g x 10 min -> mitochondrial pellet
  - Protein content assayed (Gornall et al., 1949)
- Mitochondrial Oxidative Phosphorylation
  - Substrates: pyruvate (3 mM), maleate (3 mM), glutamate (3 mM)
  - State 3 (200 µmol/L ADP stimulated)
  - State 4 (ADP limited)
  - Respiratory control ratio were determined
  - Outer membrane intactness = ratio of 8 µM cytochrome c–stimulated respiration on maximally stimulated respiration (2 mmol/L ADP)
- Electron Microscopy

Calcium retention capacity

- The amount of Ca²⁺ required to trigger a massive Ca²⁺ release by isolated cardiac mitochondria
- A measure of mPTP channel resistance
- Monitoring of extramitochondrial Ca²⁺ by calcium green-5N (excitation 500 nm; emission 530 nm)
- Ca²⁺ loading: 5 nmol Ca²⁺ pulse every 60 s
- Abrupt increase in extramitochondrial Ca²⁺
  - Massive release of mitochondrial Ca²⁺
- Assessed with or without additional CsA in vitro

Results: Infarct size in GSK3β-S9A mice

- Figure A: Wild-type
  - Analogous yield in WT vs GSK3β-S9A.

Results: Balanced Isolation Procedure Among Groups

- Table 2. Oxidative Phosphorylation

<table>
<thead>
<tr>
<th></th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
<th>OMI</th>
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</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>66 ± 3</td>
<td>12 ± 1</td>
<td>5.7 ± 3.6</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>Sham-GSK3β-S9A</td>
<td>73 ± 5*</td>
<td>13 ± 1*</td>
<td>5.2 ± 0.2*</td>
<td>0.98 ± 0.01*</td>
</tr>
</tbody>
</table>

OMI indicates outer membrane intactness (cytochrome c/2 mmol/L ADP). States 3 and 4 were measured in isolated mitochondria (nmol O₂ ⋅ min⁻¹ ⋅ mg⁻¹ protein). *P < NS vs WT.
**Results: Calcium retention capacity**

![Graph showing calcium retention capacity for WT and GSK3β-S9A mice.](image)

**Discussion: Inhibition of GSK3β is required for infarct size reduction in postconditioning**

- **Studies**: After prolonged ischemia phosphorylation of certain prosurvival kinases early in reperfusion, e.g., PI3K-Akt, MAPK, extracellular signal-regulated kinases 1 and 2, protects the heart against ischemia-reperfusion injury (Hausenloy et al., 2005).
- **Studies**: GSK3β may be activated downstream of this signaling pathway and play a pivotal role in ischemic preconditioning.
- **Isolated rat heart model** (Tong et al., 2002):
  - Ischemic preconditioning enhances the phosphorylation and decreases the enzymatic activity of GSK3β.
  - Lithium and SB21, which inhibit GSK3β, can both mimic ischemic preconditioning.

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**Discussion: Inhibition of GSK3β is required for infarct size reduction in postconditioning**

- **Studies**: Ischemic preconditioning increases phosphorylation of Akt and GSK3β at 5 minutes of reperfusion and subsequently reduces infarct size in the in vivo rat heart (Nichihara et al., 2006).
- **Results**: Ischemic postconditioning can protect the mouse heart to an extent similar to that previously reported in other species, including humans.
- **Results**: Pharmacological inhibition of GSK3β by SB21 was as efficient as ischemic postconditioning, in agreement with previous reports (Gross et al., 2006; Pagel et al., 2006).

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**Discussion: Postconditioning Inhibits mPTP Opening Through Phosphorylation of GSK3β in a CypD-Independent Manner**

- **Studies**: Postconditioning inhibits mPTP opening, and mPTP inhibitors (e.g., CsA) can mimic postconditioning when administered at the time of reperfusion (Argoud et al., 2005; Hausenloy et al., 2003).
- **Results**: Pharmacological mPTP opening and morphology: the function of mitochondrial permeability transition was comparable in WT & S9A mice under normoxic conditions.
- **Results**: Postconditioning significantly improved the resistance of the mPTP in WT mice.

**Results**: This protection was mimicked by the mPTP inhibitor CsA, which both reduced infarct size and enhanced mPTP resistance in isolated mitochondria.

**Results**: The GSK3β inhibitor SB21 ameliorated mPTP resistance in WT mitochondria to an extent similar to that achieved with postconditioning.

**Studies**: Direct inhibitors of GSK3β, like lithium or SB21, were able to inhibit mPTP opening in WT but not in GSK3β-S9A cardiomyocytes (Juhászová et al., 2004).
Discussion: Postconditioning Inhibits mPTP Opening Through Phosphorylation of GSK3β in a CypD-Independent Manner

Results: In GSK3β-S9A mice, the addition of CsA in vitro improved the resistance of the mPTP to a comparable extent in Control, Postc, and SB21 mice compared with sham group, -> absence of action via CypD.

This result also indicates that GSK3-S9A mitochondria retain the ability of being protected against lethal reperfusion injury by mPTP inhibition and demonstrates that GSK3 is located upstream of the mPTP.

Conclusion

Serine 9 phosphorylation of GSK3 is required for cardioprotection by postconditioning and likely acts by inhibiting opening of the mPTP at the time of reperfusion in a CypD-independent way.

Thank you

Because recent reports indicate that postconditioning can protect the human heart, the present data represent an encouraging background for the search for and future development of pharmacological agents that would attenuate lethal reperfusion injury in patients with ongoing AMI.