Proteomic discovery of functionally important pathways in myocardial ischemia-reperfusion injury

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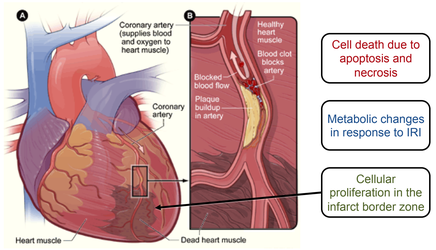
Introduction

- Myocardial ischemia-reperfusion injury (IRI) most commonly manifests as coronary heart disease.
- The World Health Organization estimated that 7.3 million deaths occurred worldwide from coronary heart disease in 2008.
- Coronary heart disease is the leading cause of death and disability worldwide.
- Cardiomyocytes undergo both apoptosis and necrosis and display compensatory metabolic changes during IRI.
- There is increased cell cycle activity in the infarct border zone, which may give rise to new cardiomyocytes.



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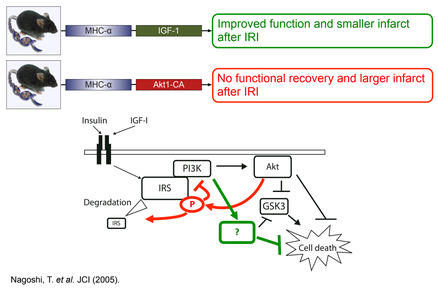
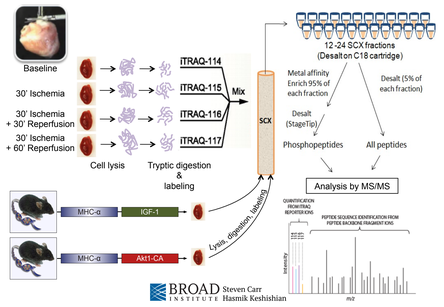


Nagoshi, T. *et al.* JCI (2005).

- This study utilized two transgenic mouse models: cardiac-specific IGF-1 and constitutively active Akt1.
- The differences in response to IRI of these two models is interesting, because Akt is a downstream component of the IGF-1 pathway.
- Components of the IGF-1 pathway that are independent of Akt (highlighted in green) may be mediating the protective effects seen in the IGF-1 transgenic mice.
- The Akt-independent components of the IGF-1 pathway may be inhibited in the Akt-transgenic mice due to chronic feedback inhibition (highlighted in red).

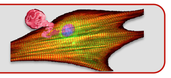
- In order to discover which pathways are functionally important in IRI, we used two experimental approaches: wild-type hearts subjected to IRI on a Langendorff perfusion apparatus and IGF-1 and Akt transgenic mouse hearts at baseline.
- In the wild-type IRI approach, hearts were collected at 4 time points. In the transgenic approach, hearts were collected without IRI.
- Peptides from the hearts were labeled with iTRAQ and fractionated using liquid chromatography.
- Phosphopeptides were separated from peptides using metal affinity chromatography.
- Phosphopeptides and peptides were quantified using tandem mass spectrometry.



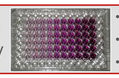
- Our proteomic analyses yielded hundreds of potential candidates as playing a role in IRI.
- In order to narrow down the candidates to the most functionally important proteins, we filtered candidates based on 3 criteria: protein fold change, phosphopeptide fold change, and comparison of the changes seen in the wild-type IRI and transgenic approaches.
- 20 proteins were chosen for *in vitro* studies, which involved siRNA knockdown in neonatal rat ventricular myocytes (NRVMs) followed by simulated IRI.
- The MTT assay, a redox-based viability assay was used, because it detects all of the responses seen in the heart during IRI: survival, metabolic changes, and proliferation.

100s
- Protein fold change
- Phosphopeptide fold change
- Wild-type IRI compared to transgenics

20
- siRNA knockdown
- Simulate ischemia-reperfusion injury



Assay
Redox-based viability assay



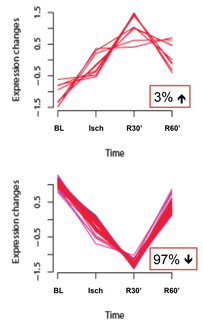
- Survival
- Metabolic changes
- Proliferation

Results

Proteomic results from wild-type IRI approach

		Quantified			Regulated	
		Isch/ BL	R30'/ BL	R60'/ BL	#	%
Proteins	In ≥1 replicate	6403	6403	6403	0	0
	In ≥2 replicates	2506	2506	2506		
	In 3 replicates	1959	1959	1959		
Phosphopeptides	In ≥1 replicate	22833	22783	22822	340	1.5
	In ≥2 replicates	3792	3783	3789		
	In 3 replicates	2041	2034	2038		

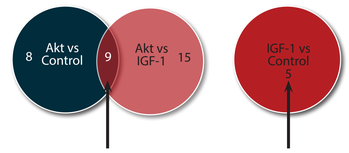
- 3 replicate hearts were analyzed at each time point.
- 6,403 proteins and almost 23,000 phosphopeptides were quantified in at least 1 replicate at each of the time points.
- No proteins showed a change in expression level at any of the time points.
- 340 phosphopeptides showed a significant change in expression level during IRI.



- 97% of the 340 regulated phosphopeptides from the wild-type IRI approach showed a nadir at 30 minutes of reperfusion.
- This may be the result of oxidative stress and energy (ATP) deprivation or phosphatase activation.

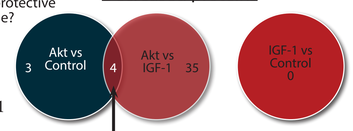
Proteomic results from transgenic approach

Increased proteins



- Proteins upregulated in the Akt transgenic mice may be mediators of adverse outcome.
- Proteins upregulated in the IGF-1 transgenic mice may be mediators of protective outcome.

Decreased proteins

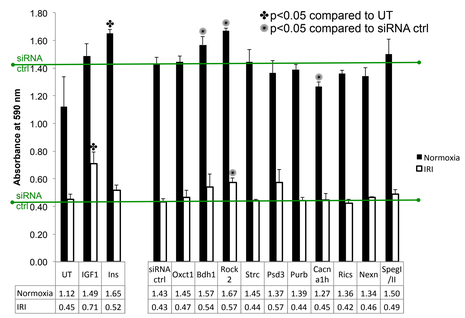


- Proteins downregulated in the Akt transgenic mice may be beneficial effectors that are lost.
- No proteins were decreased in the IGF-1 transgenic mice relative to control.

Increased phosphopeptides

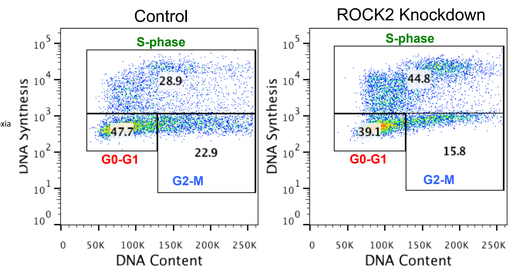
- The Akt transgenic had the largest increase in phosphopeptides relative to control and an even greater number of phosphopeptides were increased relative to the IGF-1 transgenic.

Results from *in vitro* studies

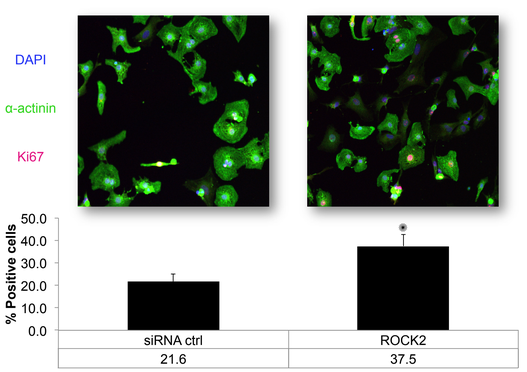


	UT	IGF1	Ins	siRNA ctrl	Oxct1	Bdh1	Rock 2	Strc	Psd3	Purb	Cacn a1h	Rics	Nexn	Spegi /Ii
Normoxia	1.12	1.49	1.65	1.43	1.45	1.57	1.67	1.45	1.37	1.39	1.27	1.36	1.34	1.50
IRI	0.45	0.71	0.52	0.43	0.47	0.54	0.57	0.44	0.57	0.44	0.45	0.42	0.46	0.49

- In this MTT assay using NRVMs, the simulated IRI cells were subjected to 24 hours of hypoxia and serum starvation followed by 2 hours of reoxygenation.
- ROCK2 knockdown increased absorbance at 590 nm in the normoxic and simulated IRI cells, which may be a result of increased survival, proliferation, or metabolic activity.



- In this flow cytometry experiment using NRVMs, DNA synthesis (y-axis) was measured via EdU incorporation, and DNA content (x-axis) was measured with a general DNA dye, APC.
- ROCK2 knockdown increased the progression of cells into S-phase by over 50%.



- Consistent with the flow cytometry results, ROCK2 knockdown increased Ki67 expression, a marker of cell proliferation, in NRVMs by over 70%.

Conclusion

- iTRAQ/LC-MS/MS provides a robust proteomic and phosphoproteomic platform
- Dephosphorylation of the cardiac phosphoproteome is the dominant pattern during IRI
- ROCK2 knockdown increases markers of cell cycle progression

Future Directions

Proteomic analyses
- IRI using transgenic models
- Protein network mapping
- Phosphatase activation during IRI?

More functional assays
- Apoptosis
- Mitochondrial function
- Contribution to IGF1-mediated cardioprotection

ROCK2
- Cell counting
- Examine effects on cell death and metabolism
- Study *in vivo* effects in the adult heart

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