Quantitative Proteomics And Functional Consequences Of Mitochondrial Protein Carbonylation

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Seahorse Bioscience Travel Award Winner

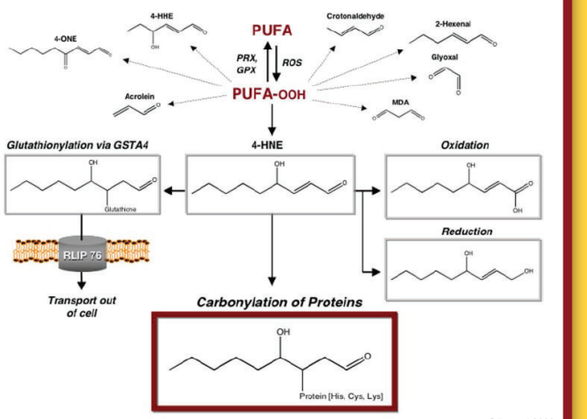
Abstract

Carbonylation is the modification of proteins by lipid peroxidation end products. Glutathione S-transferase (GST) A4 is the primary detoxification method of these reactive species. With obesity and insulin resistance, GSTA4 expression is downregulated specifically in the adipocyte, leading to elevations of protein carbonylation and subsequent alterations of glucose uptake, mitochondrial respiration, superoxide production, TCA cycle flux, and b-oxidation.

The goal of this study was to identify mitochondrial targets of protein carbonylation in the adipocyte and to establish the pathological role of protein carbonylation in mitochondrial dysfunction associated with insulin resistance and Type II Diabetes. To this aim, mitochondria were isolated from GSTA4 silenced or overexpressing 3T3-L1 adipocytes and respective control cell lines. Free carbonyls were labeled with biotin hydrazide and captured on avidin-affinity chromatography columns. Equal amounts of protein were trypsin digested, labeled with iTRAQ reagents, and combined. Peptides were analyzed with a LTQ Orbitrap mass spectrometer and resultant spectra were identified with the UniProt *Mus musculus* database.

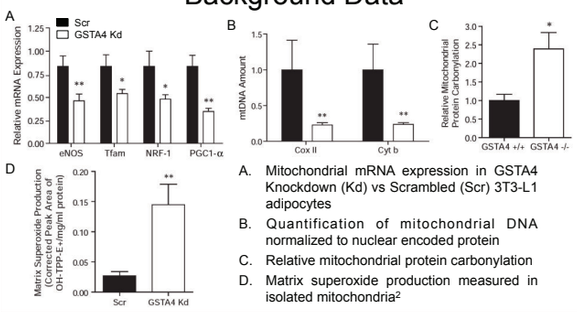
Relative to GSTA4 overexpressing adipocytes, GSTA4 silenced adipocytes displayed elevations of carbonylation on several key metabolic proteins, including the phosphate carrier protein, mitochondrial adenylate kinase, aconitase, isocitrate dehydrogenase, succinate dehydrogenase, cytochrome c oxidase, and multiple subunits of electron transport complexes I, III, and V (F_OF_1 ATP Synthase). Functional consequences of protein carbonylation include a 25% reduction in oxygen consumption rate and 85% less F_OF_1 ATP Synthase activity in GSTA4 silenced adipocyte mitochondria relative to GSTA4 overexpressing cells. This data suggests that protein carbonylation may be responsible for mitochondrial dysfunction associated with insulin resistance.

Mechanism of Protein Carbonylation



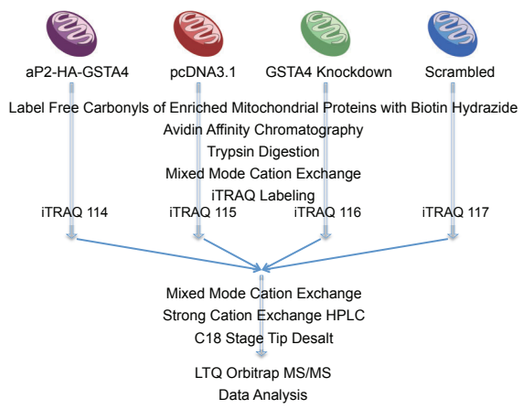
Grimsrud 2008

Background Data



A. Mitochondrial mRNA expression in GSTA4 Knockdown (Kd) vs Scrambled (Scr) 3T3-L1 adipocytes
B. Quantification of mitochondrial DNA normalized to nuclear encoded protein
C. Relative mitochondrial protein carbonylation
D. Matrix superoxide production measured in isolated mitochondria[2]

Quantitative Proteomic Strategy for Identification of Carbonylated Mitochondrial Proteins



Top Targets of Carbonylation

Protein Name	Protein Symbol	Uniprot ID	Assigned Spectra
60 kDa heat shock protein, mitochondrial	CH60	P63038	143
Stress-70 protein, mitochondrial	GRP75	P38647	110
ATP synthase subunit beta, mitochondrial	ATPB	P56480	104
ATP synthase subunit alpha, mitochondrial	ATPA	Q03265	103
Glutamate dehydrogenase 1, mitochondrial	DHE3	P26443	89
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDH3A	Q9D6R2	89
Aconitate hydratase, mitochondrial	ACON	Q99KI0	85
Protein disulfide-isomerase	PDIA1	P09103	85
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM	P45952	80
Long-chain-fatty-acid--CoA ligase 1	ACSL1	P41216	79
Dihydrolipoyl dehydrogenase, mitochondrial	DLDH	O08749	72
2,4-dienoyl-CoA reductase, mitochondrial	DECR	Q9CQ62	68
78 kDa glucose-regulated protein	GRP78	P20029	61
ADP/ATP translocase 1	ADT1	P48962	59
Malate dehydrogenase, mitochondrial	MDHM	P08249	49
Methylmalonate-semialdehyde dehydrogenase, mitochondrial	MMSA	Q9EQ20	45
Trifunctional enzyme subunit alpha, mitochondrial	ECHA	Q8BMS1	45
Apoptosis-inducing factor 1, mitochondrial	AIFM1	Q9ZOX1	40
Adenylate kinase 2, mitochondrial	KAD2	Q9WTP6	37
Citrate synthase, mitochondrial	CISY	Q9CZU6	32

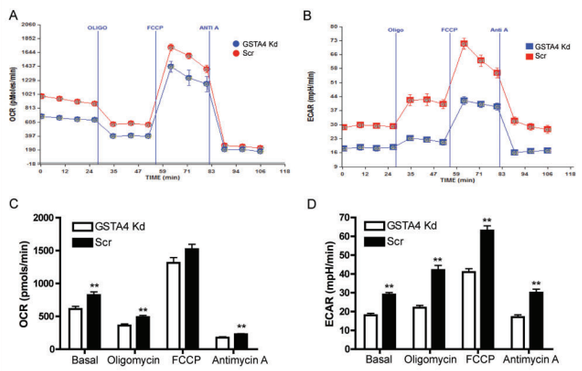
328 Total Proteins Identified

Relative Protein Carbonylation: GSTA4 Knockdown vs. aP2-HA-GSTA4

Fold Change	p-value	Protein Name	Symbol	Uniprot ID
5.331	> 10E-15	Mitochondrial Phosphate Carrier	PiC	Q8VEM8
4.770	3.30E-06	NADH Dehydrogenase, Complex I - B9	NDUFA3	Q9CQ91
3.886	1.96E-02	PAST Homolog 1	EHD1	Q9WVK4
2.558	5.77E-04	Ribophorin II	RPN2	Q9DBG6
2.557	3.98E-02	Mitochondrial inner membrane Translocase	TIMM50	Q9D880
2.512	1.97E-09	Protein transport protein Sec23	SEC23A	Q01405
2.271	2.64E-03	Erlin-1	ERLIN1	Q91X78
2.172	9.29E-06	NADH Dehydrogenase, Complex I - B8	NDUFA2	Q9CQ75
2.130	5.00E-15	Ras-related protein RAP1A	RAP1A	P62835
1.851	4.72E-02	Valyl-tRNA ligase, mitochondrial	VARS2	Q3U2A8
1.740	1.95E-06	Transitional endoplasmic reticulum ATPase	VCP	Q01853

Experimental values of Fold Change normalized to respective controls

Extracellular Flux Analysis

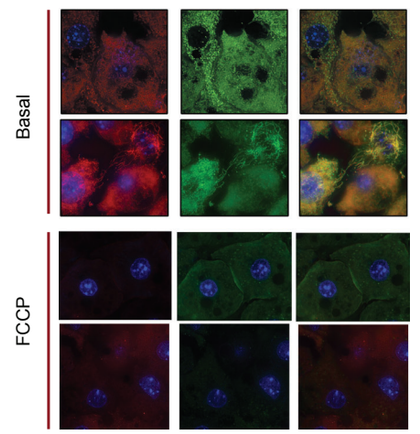


A. Oxygen consumption rate (OCR) for GSTA4 Knockdown (Kd) and Scrambled (Scr) control 3T3-L1 adipocytes over time with Oligomycin (2 µg/ml), FCCP (2.5 µM), and Antimycin A (4 µM) injections.
B. Extracellular acidification rate (ECAR) measurements taken in parallel with panel A.
C-D. Quantification of panels A & B (** = $p < 0.01$).

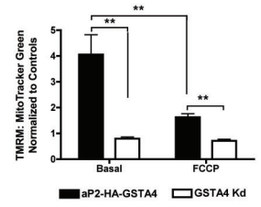
Methods:

3T3-L1 adipocytes were cultured in XF24 V7 microplates from Seahorse Biosciences. Eight days post differentiation, cell monolayers were washed and incubated with XF Assay Media supplemented with 25 mM glucose, 1 mM sodium pyruvate, and 2 mM Glutamax, pH 7.5, for 1 hr without CO_2. Then the XF24 plate was loaded into the XF24 Extracellular Flux Analyzer from Seahorse Biosciences and data collected. (n=10 per experiment; data representative of three separate experiments)

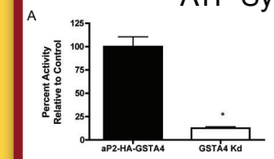
Mitochondrial Membrane Potential Imaging



- Tetramethyl rhodamine methyl ester (TMRM) is a cationic mitochondrial probe. Staining is indicative of membrane potential.
- Live cells were labeled and imaged at 37°C.
- The fluorescence intensity was calculated for each cell using ImageJ and normalized to respective controls.



ATP Synthase Activity



A. GSTA4 Knockdown 3T3-L1 adipocyte mitochondria have 85% less ATP Synthase activity than GSTA4-overexpressing cells.

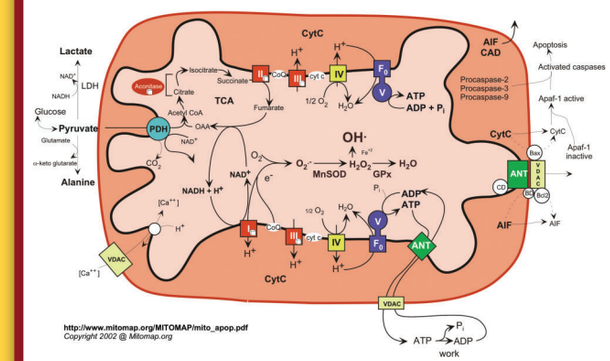
Methods:

F_OF_1 ATPase activity was assayed in isolated mitochondria spectrophotometrically at 340 nm by coupling the production of ADP to NADH oxidation with pyruvate kinase and lactate dehydrogenase.

Conclusions

- Silencing GSTA4 in adipocytes leads to elevated protein carbonylation relative to control and GSTA4 overexpressing cells
- GSTA4 Knockdown adipocytes have impaired oxygen consumption and altered glycolysis
- GSTA4 overexpressing adipocytes have 4 fold more membrane potential than GSTA4 knockdown cells
- ATP Synthase activity is reduced 85% in GSTA4 Knockdown adipocytes compared to overexpressing cells
  - Other studies have implicated the Mitochondrial Phosphate Carrier (PiC) as part of the ATP Synthasome, consisting of Complex V, ANT, and PiC and in the formation of the permeability transition pore.
- Our studies show that carbonylation may be responsible for dysfunction of several mitochondrial proteins, which leads to global alterations of cellular metabolism

Mitochondrial Energetics



References

1. Grimsrud, PA., et al. (2008) *JBC*, 238:32.
2. Curtis, JM., et al. (2010) *Diabetes*, 59: 5.

Research & Travel Funding:

NIH-DK084669

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