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SHORT TAKE

Calorie restriction alters mitochondrial protein acetylation

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Summary

Calorie restriction (CR) increases lifespan in organisms ranging from budding yeast through mammals. Mitochondrial adaptation represents a key component of the response to CR. Molecular mechanisms underlying this adaptation are largely unknown. Here we show that lysine acetylation of mitochondrial proteins is altered during CR in a tissue-specific fashion. Via large-scale mass spectrometry screening, we identify 72 candidate proteins involved in a variety of metabolic pathways with altered acetylation during CR. Mitochondrial acetylation changes may play an important role in the pro-longevity CR response.

Key words: calorie restriction; longevity; mass spectrometry; metabolism; mitochondria; sirtuins.

Calorie restriction (CR) is a robust means of extending lifespan across a wide variety of species (Anderson & Weindruch, 2007). Pharmacomimetics of CR could have far-reaching health benefits in humans. The molecular basis of the CR response remains incompletely understood. Metabolic alterations occurring as part of the adaptation to CR likely underlie the beneficial effects of this intervention (Anderson & Weindruch, 2007). These changes implicate alterations in mitochondrial function in the CR response, since many essential metabolic processes occur in this organelle. Here we report that lysine acetylation of mitochondrial proteins is altered during CR in a tissue-specific manner. Since reversible acetylation is a well-characterized post-translational modification impacting protein biology, this finding offers one potential mechanism of how mitochondrial function may be regulated during CR.

We assessed protein acetylation in purified liver mitochondria from 8-month-old C57BL/6 mice subjected to stepwise CR – 10% restriction initiated at 14 weeks of age, followed by 25% restriction at 15 weeks, and then 40% restriction at 16 weeks and thereafter – as well as from age-matched ad lib fed (AL) isogenic controls. This CR regimen provides robust lifespan extension in C57BL/6 mice (Turturro *et al.*, 1999). Calorie restriction was associated with dramatic changes in acetylation in liver mitochondria (Fig. 1A), with the majority of changes consisting of increases in acetylation. We then screened mitochondria from a panel of tissues for acetylation (Fig. 1B). In heart, kidney, and brain, only subtle changes in mitochondrial protein acetylation occurred during CR. By contrast, in brown adipose tissue (BAT), CR led to markedly decreased mitochondrial acetylation. Thus, mitochondrial acetylation is regulated in liver and BAT during CR.

To identify mitochondrial proteins changing in acetylation during CR, a large-scale proteomics survey was performed. Liver mitochondrial extracts from AL and CR animals were digested with trypsin and immunopurified with acetyl-lysine affinity matrix. Acetylated peptides were subsequently analyzed by mass spectrometry and label-free quantitation (LFQ) (Rush *et al.*, 2005). We identified a total of 287 unique acetylated proteins, of which at least 165 are mitochondrial (see Supplemental Methods section for further details) (Supplementary Table S1). Among this latter group, LFQ predicted 72 candidates as changing in acetylation during CR by at least 2.5-fold (Supplementary Table S2). We also identified a number of potentially significant acetylation changes that did not meet these strict criteria; i.e. acetylation changes from 2.0 up to 2.5-fold (Supplementary Table S3).

To validate the LFQ findings, we performed immunoprecipitation (IP) with acetyl-lysine affinity matrix followed by immunoblot (IB) using commercially available antibodies against candidate proteins. We confirmed hyperacetylation of five of these proteins, which are involved in a variety of biochemical

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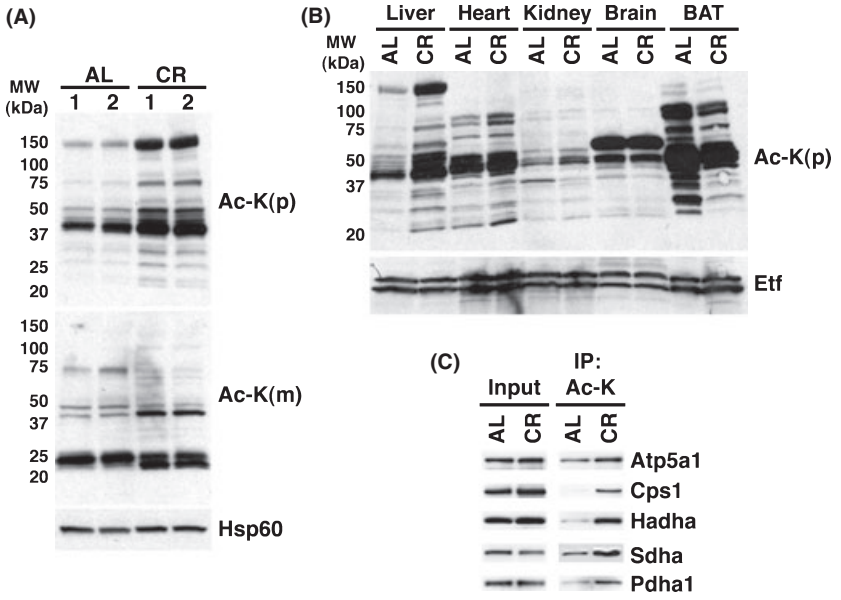

Fig. 1 Calorie restriction alters mitochondrial acetylation. (A) Liver mitochondrial acetylation in CR. Mitochondrial extracts were generated from either CR or AL mice, fractionated by SDS-PAGE, and probed with the indicated antibodies. Ac-K, anti-acetyl-lysine; p, polyclonal; m, monoclonal. (B) Tissue-specific changes in mitochondrial acetylation. Mitochondria were prepared from indicated tissues of CR and AL mice and probed as in panel A. (C) Identification of proteins hyperacetylated in CR. Total acetylated proteins were immunopurified from AL and CR mitochondrial extracts and probed for the indicated proteins. Each panel represents mitochondrial extracts derived from a single AL/CR pair and is representative of at least three independent experiments.

pathways, namely ATP generation (ATP5a1), urea cycle (CPS1), lipid metabolism (HADHA), glycolysis/Krebs cycle (PDHA1), and Krebs cycle/electron transport (SDHA) (Fig. 1C). For 21 other proteins tested, we were unable to obtain immunologic reagents of sufficient quality to definitively assess acetylation.

Of note, in contrast to our findings, a recent study reported that CPS1 acetylation is decreased in mice during CR (Nakagawa *et al.*, 2009). In support of our LFQ and IP results, acetyl-lysine IB of CPS1 purified by IP also revealed hyperacetylation of this enzyme in CR (Supplementary Fig. S1). The cause of the discrepancy between our findings and those of Nakagawa *et al.* is unclear.

Sirtuin family deacetylases are required for increased longevity in response to some CR regimens in invertebrates (Schwer & Verdin, 2008). The three mammalian mitochondrial sirtuins – SIRT3, SIRT4, and SIRT5 – are logical candidates to mediate acetylation changes we observe in liver mitochondria in response to CR (Schwer & Verdin, 2008). We previously showed that SIRT3 plays an important role in deacetylating many mitochondrial proteins (Lombard *et al.*, 2007). Expression levels of mitochondrial sirtuins were compared in liver mitochondria from mice on AL or CR diets (Supplementary Fig. S1). Calorie restriction elicited a moderate increase in SIRT3 protein levels, consistent with mRNA expression profiling results (Han *et al.*, 2000). SIRT4 protein levels were slightly decreased, whereas SIRT5 protein levels did not change during CR, in agreement with previous reports (Supplementary Fig. S1) (Haigis *et al.*, 2006; Nakagawa *et al.*, 2009). It is unlikely that this reduction in SIRT4 levels plays a role in mediating the acetylation increases we observe during CR, as SIRT4-deficient mice show no gross alterations in hepatic mitochondrial acetylation (Lombard *et al.*, 2007). Despite increased SIRT3 expression, SIRT3 activity could be modulated in the context of CR by means other than changes in protein levels: i.e. mitochondrial NAD^+ content, post-translational modifications, and/or interacting partners. Further studies will be required to identify enzymes (deacetylases and acetyltransferases), or putative nonenzymatic processes, that modulate lysine acetylation of mitochondrial proteins during CR.

Here we show that mitochondrial protein acetylation is altered dramatically in a tissue-specific fashion during CR. These acetylation changes may have important functional consequences for activity, complex formation, turnover, or other aspects of protein biology. Recent work indicates that lysine acetylation is present in *Escherichia coli*, and is altered in response to diverse environmental stresses, including starvation (Zhang *et al.*, 2009). In mammals, acute fasting and chronic ethanol ingestion also alter hepatic mitochondrial protein acetylation (Kim *et al.*, 2006; Picklo, 2008), suggesting that changes in acetylation represents an ancient, evolutionarily conserved mechanism for adaptation to metabolic/nutritional stress. Activities of a wide variety of mitochondrial enzymes have been shown to change during CR (Tillman *et al.*, 1996; Dhahbi *et al.*, 2001; Hagopian *et al.*, 2003, 2004, 2005). The targets we validated are involved in a wide range of biological activities. This suggests that lysine acetylation changes may have far-reaching effects on mitochondrial function.

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References

Anderson RM, Weindruch R (2007) Metabolic reprogramming in dietary restriction. *Interdiscip. Top. Gerontol.* **35**, 18–38.

Dhahbi JM, Mote PL, Wingo J, Rowley BC, Cao SX, Walford RL, Spindler SR (2001) Caloric restriction alters the feeding response of key metabolic enzyme genes. *Mech. Ageing Dev.* **122**, 1033–1048.

Hagopian K, Ramsey JJ, Weindruch R (2003) Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. *Exp. Gerontol.* **38**, 267–278.

Hagopian K, Ramsey JJ, Weindruch R (2004) Krebs cycle enzymes from livers of old mice are differentially regulated by caloric restriction. *Exp. Gerontol.* **39**, 1145–1154.

Hagopian K, Harper ME, Ram JJ, Humble SJ, Weindruch R, Ramsey JJ (2005) Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria. *Am. J. Physiol. Endocrinol. Metab.* **288**, E674–E684.

Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G, Wolberger C, Prolla TA, Weindruch R, Alt FW, Guarente L (2006) SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* **126**, 941–954.

Han E, Hilsenbeck SG, Richardson A, Nelson JF (2000) cDNA expression arrays reveal incomplete reversal of age-related changes in gene expression by calorie restriction. *Mech. Ageing Dev.* **115**, 157–174.

Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y (2006) Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol. Cell.* **23**, 607–618.

Lombard DB, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, Mostoslavsky R, Kim J, Yancopoulos G, Valenzuela D, Murphy A, Yang Y, Chen Y, Hirschey MD, Bronson RT, Haigis M, Guarente LP, Farese RV Jr, Weissman S, Verdin E, Schwer B (2007) Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. *Mol. Cell. Biol.* **27**, 8807–8814.

Nakagawa T, Lomb DJ, Haigis MC, Guarente L (2009) SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell* **137**, 560–570.

Picklo MJ Sr (2008) Ethanol intoxication increases hepatic N-lysyl protein acetylation. *Biochem. Biophys. Res. Commun.* **376**, 615–619.

Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **23**, 94–101.

Schwer B, Verdin E (2008) Conserved metabolic regulatory functions of sirtuins. *Cell. Metab.* **7**, 104–112.

Tillman JB, Dhahbi JM, Mote PL, Walford RL, Spindler SR (1996) Dietary calorie restriction in mice induces carbamyl phosphate synthetase I gene transcription tissue specifically. *J. Biol. Chem.* **271**, 3500–3506.

Turturro A, Witt WW, Lewis S, Hass BS, Lipman RD, Hart RW (1999) Growth curves and survival characteristics of the animals used in the biomarkers of aging program. *J. Gerontol. A Biol. Sci. Med. Sci.* **54**, B492–B501.

Zhang J, Sprung R, Pei J, Tan X, Kim S, Zhu H, Liu CF, Grishin NV, Zhao Y (2009) Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*. *Mol. Cell Proteomics* **8**, 215–225.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 (A) Increased CPS1 acetylation in liver mitochondria from mice on CR.

Table S1 Acetylation profile of AL and CR hepatic mitochondrial extracts.

Table S2 Hepatic mitochondrial proteins changing in acetylation during CR.

Table S3 Hepatic mitochondrial proteins potentially changing in acetylation during CR.

Supplemental Methods Materials and Methods.

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