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Caloric Restriction in Saccharomyces cerevisiae as a Model for Intracellular Changes During Aging

Marina Hendrickson
University of Colorado Boulder

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Caloric Restriction in *Saccharomyces cerevisiae* as a Model for Intracellular Changes During Aging

Marina Hendrickson

Department of Molecular, Cellular, and Developmental Biology

University of Colorado at Boulder

April 3, 2014

*Thesis Committee:*

*Thesis Advisor and Principle Investigator:* Dr. Robert Poyton, Department of Molecular, Cellular, and Developmental Biology

*Member:* Dr. Christy Fillman, Department of Molecular, Cellular, and Developmental Biology

*Member:* Dr. Christine Kelly, Department of Chemistry and Biochemistry
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Abstract

Aging is a universal experience that is accompanied by increased risk for diseases like cancer and diabetes. Understanding the cellular basis of aging will therefore aid our understanding of these diseases and will allow us to better prevent and treat them. Caloric restriction is a proven method of elongating lifespan not only in lower eukaryotes, like *Saccharomyces cerevisiae* (yeast), but also in mammals. As there are conserved pathways that mediate the cellular adaptations to caloric restriction, model organisms are quite useful in elucidating the mechanisms involved. This study utilized a yeast model system, due to ease of manipulation, genetic knowledge, and short lifespan. One key adaptation to caloric restriction involves the nitrite dependent production of nitric oxide (NO) by the terminal enzyme in the electron transport chain, Cytochrome *c* oxidase (Cco). Until recently, it was thought Cco only produced NO under conditions of hypoxia. This study explores changes in Cco isoform switching, Cco subunit phosphorylation, and adenine nucleotide levels during caloric restriction, all of which could alter Cco’s oxygen sensitivity to allow for NO production. Phosphorylation changes in subunit II of Cco and changes in ATP:ADP observed during caloric restriction appear to be regulatory mechanisms utilized by the cell to regulate Cco’s NO production, while isoform switching is not. These findings help to solidify the role of AMP-activated protein kinase in mediating cellular adaptations to caloric restriction.
Introduction

When Benjamin Franklin stated only death and taxes were certain, he overlooked another universal experience- aging. Aging is defined by a time-dependent increase in cellular senescence beginning at reproductive maturity that will ultimately lead to the death of the organism, if uninterrupted.[1] Every organism ages, and they age in very similar ways, as characterized by an increasing mortality risk with age (Fig. 1).[2][3] While the effect of neuronal or endocrine systems on mammalian aging are not applicable in the aging of single-celled organisms like yeast, the intracellular changes seen during aging all have their counterparts in yeast.[4] This, coupled with easy genetic manipulation, vast genetic knowledge, and short lifespan, make *Saccharomyces cerevisiae* (yeast), a fantastic model organism with which to study aging. As to how it is best to study aging and its intracellular effects, one method has been shown to be especially amenable- caloric (or dietary) restriction (CR). CR is the only intervention that extends lifespan in yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Macaca mulatta* (rhesus monkey), where it was also seen to decrease the occurrence of age-related diseases, such as cancer and cardiovascular disease, the top two causes of death in the US.[5][6][7] Elucidating the changes elicited by CR will provide insight to the pathways responsible for aging and age-related diseases, and yeast CR studies are directly relevant, more cost-effective, and less time-intensive.

Theories about aging have long abounded. Half a century ago, Denham Harman proposed that that which gives us energy, the mitochondrion, also produces free radicals, primarily superoxide, that ultimately lead to our aging and demise, thus introducing the Free Radical Theory of Aging.[8] This fit well with the understanding of aging being defined as a decrease in
Figure 1. A. Survival curve of humans in US in 2006 (adapted from National Vital Statistics Report, 2010). B. Survival curve of yeast (adapted from Li et al., 2011).
cell functionality, which could readily be brought about by oxidative damage to cellular structures. It was also in accordance with observations about lifespan in different organisms as it relates to respiratory rates, i.e. those with a higher cellular metabolic rate and therefore increased free radical production have a correspondingly shorter lifespan.[9] Finally, it also made sense in the context of CR. CR is defined differently based on the organism being studied, however moderate CR normally consists of a 25-50% decrease in caloric consumption without nutritional deficiencies, with a typical yeast model using a decrease from 2% glucose media to 0.5% glucose media.[10] It was hypothesized that a decrease in caloric intake would then also result in a decrease in respiration, which would lower the amount of damaging free radicals produced and elongate lifespan.[9]

As our understanding of aging has evolved, so have the theories of aging. When it was discovered that there are numerous agents, like peroxides and aldehydes, capable of producing oxidative stress besides free radicals, the theory shifted (in name only) to the Oxidative Stress Theory of Aging.[11] Then it was found that mitochondrial DNA is especially vulnerable to oxidative damage, and mutations could decrease energy production while increasing free radical production, and the Mitochondrial Theory of Aging was born.[12] While these theories of aging cast oxidative stress resulting from reactive oxygen species (ROS) and reactive nitrogen species (RNS) as the cause of aging, the mechanistic details emerging from studies of CR were painting a more nuanced picture.

In yeast, high glucose levels repress respiration and instead push metabolism towards fermentation. Conversely, low glucose levels result in higher respiration levels.[13] This finding directly countered the previous theory that CR was leading to decreased respiration and therefore ROS production. Altered rate of electron transfer then arose as a new explanation for the
decreased oxidative damage seen in CR.[14] ROS production primarily occurs in electron transport chain complexes I and III, and the rate of production increases with oxygen levels and the concentration of reduced electron donors. When there are more reduced electron donors, there is slower electron flow through the complexes, which gives increased opportunity for electrons to escape and form superoxide. The amount of reduced electron donors, such as NADH, is increased when there is high glucose availability.[15] Therefore, although CR may increase respiration, it decreases the amount of ROS produced as a by-product. Even with this in mind, doubt is being cast on the relevance of ROS production as a mediator of lifespan, with evidence that both under and over expression of cellular antioxidants does not change lifespan, and even more contradictory, an abundance of antioxidants can reverse CR lifespan elongation.[16]

To better understand the role of respiration and ROS in aging, further knowledge of the respiratory complexes was sought. Complex IV, or Cytochrome c Oxidase (Cco), of the respiratory chain is responsible for the reduction of O$_2$ to H$_2$O. Cco is a multimeric membrane protein, comprised of three mitochondrially-encoded subunits and up to ten additional nuclearly encoded subunits.[17] The binuclear reaction center composed of heme $a_3$ and a copper B (Cu$_B$) is housed in subunit I, while a copper A (Cu$_A$) is bound by subunit II (Fig 2). These are the sites of primary activity within the enzyme, however other subunits are involved in regulation.[18] In yeast and other organisms, Cco also has a nitrite reductase activity under conditions of hypoxia, thereby producing nitric oxide (NO). NO acts as a signaling molecule to induce a hypoxic response, primarily by stabilizing the transcription factor HIF-1$\alpha$. Another response to hypoxia is an isoform switch, where subunit Va in Cco is switched for isoform Vb. Vb has a higher NO
Figure 2. A labeled ribbon figure of yeast Cco’s structure, with bovine subunits superimposed (provided by Maréchal et al.)
turnover rate, and is thus a means of amplifying the hypoxic NO-signaling.[19] Although the conversion of nitrite to NO by Cco is favored by hypoxia, it is now clear that this pathway is modulated and can function under normoxic conditions as well, such as during CR. Even more astounding, the NO-production was found to be necessary for maximal lifespan elongation from CR.[3] Is it really possible that the very reactive species initially proposed to cause aging are actually fundamental agents in preventing it?

Out of these insights, the concept of mitohormesis emerged, where a small amount of ROS/RNS initiates a protective effect, while large amounts are damaging. In this framework, the slight increase in ROS/RNS production seen in CR would precondition the cell and activate stress responses, such as increased superoxide dismutase expression. When significant ROS production occurs as a result of further stress, the cells are now better able to handle it, so the damage is minimized.[20] ROS are so fundamental to the process of CR-induced lifespan extension that low levels of ROS production alone was sufficient to increase lifespan in yeast and C. elegans.[21]

From here, the pathways upstream and downstream of the increased respiration and ROS production take the spotlight. Upstream, nutrient sensing pathways like Tor have shown to be of import. Tor is a serine/threonine kinase that responds to changes in nutrient availability. Decreased signaling results in increased autophagy of damaged mitochondria, increased mitochondrial biogenesis, increased stress-response signaling via the transcription factors Msn2 and Msn4, and increased respiration.[4] Decreasing Tor signaling will also prolong lifespan in yeast, in a manner dependent on ROS/RNS signaling.[22] However, decreased activity in Tor alone is not equivalent to the changes brought about by CR, where there is a more global metabolic reprogramming of the cell that occurs which involves numerous pathways.[4]
One downstream pathway involves the deacetylase silent information regulator 2 (Sir2) in yeast, or Sirt1 in mammals. Sir2 is involved in cellular stress and survival mechanisms, and therefore has an obvious place in CR-mediated lifespan increases. Sir2 is NAD$^+$-dependent and is activated by an increase in the ratio of NAD$^+$ to NADH, which is seen when cells shift away from glycolysis, as in CR. Sir2 is also inhibited by nicotinamide, and catabolism of nicotinamide increases during CR. As to how increased Sir2 activity leads to increased lifespan, one mechanism in yeast involves decreased formation of extra chromosomal rDNA circles that directly limit replicative lifespan. Although this mechanism is unique to yeast, a similar process involving Sirt1 maintaining DNA integrity in humans is quite possible.[5] Sir2 has also been shown to increase the activity of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), which in turn increases mitochondrial biogenesis.[16]

Decreased insulin-like signaling has also been shown to increase lifespan in mice. Although the insulin-like pathway is not present in single-celled organisms like yeast, there are downstream factors of the insulin-like pathway that have homologs in yeast. AKT is a kinase involved in transducing insulin signaling and also in cellular survival mechanisms and it has a homolog in yeast, Sch9. Increased Sch9 signaling has been shown to inhibit CR-mediated lifespan elongation, thus showing a conserved function for Sch9/AKT in CR.[5] Sir2 has also been implicated in the insulin secretory pathway, highlighting the importance of the molecular mechanisms of CR elucidated in model organisms for understanding the role of complex pathways in CR and aging in mammals.[23]

While the role of NO in reprogramming cells during CR has yet to be established, it is clear that mitochondrial NO synthesis and respiration do play key roles. Also unknown is how CR results in an increase in NO production by Cco, however there are several possibilities. As
mentioned, there is a difference in activity between the two isoforms of subunit V in yeast (subunit IV in mammals). Va is the aerobic isoform that produces NO only in conditions of low oxygen, while Vb is the anaerobic isoform that produces much higher levels of NO and shows less oxygen sensitivity.[19] Therefore, it is possible that an isoform switch from Va to Vb is responsible for the increased NO production seen during CR.

Another possibility is a change in allosteric regulation. Cco is a key modulator of electron flow rate through the electron transport chain, so it is the main site of regulation.[24] Purified Cco has been shown to have variable oxygen reductase activity based on the concentration of adenosine triphosphate (ATP) and adenosine diphosphate (ADP), where high levels of ATP inhibit its activity.[25] It is therefore quite possible that a change in ATP and/or ADP concentration could also alter its nitrite reductase activity. As levels of ATP and ADP are responsive to nutrient availability, this seems a very plausible means of regulation.[26]

Finally, there could be post-translational modifications to Cco, most likely through phosphorylation. Phosphorylation of mammalian subunit IV-1 (homologous to yeast Va) in response to metabolic fluctuations has been established, as has phosphorylation of several other Cco subunits, including subunits I and II. These modifications can cause changes in respiration and allosteric regulation by ATP and ADP.[25] Therefore, it is possible that the increased respiration associated with CR is the result of an activating phosphorylation, or the removal of an inhibitory phosphorylation. Whether this change in phosphorylation state could also cause an increase in NO production has yet to be determined. The location of the active site for nitrite reduction has not been fully determined, but has been assumed to be the binuclear reaction site responsible for O₂ reduction. However, there is another copper reaction center (Cu₄) present in Cco subunit II so it is possible that the nitrite reductase activity occurs there.[27] Ultimately,
**Figure 3.** A summary of the known and proposed pathways involved in CR-mediated lifespan elongation.
there are dozens of possibilities for how phosphorylation could affect the activity of Cco and mediate the cellular response to CR and aging. In this study, isoform switching, phosphorylation, and allosteric regulation are explored as possible explanations for the increase in NO production by Cco during CR (Fig. 3).

**Methods and Materials**

*Yeast and media*

Yeast strains JM43 (MATα his4-580 trp1-289 leu2-3, 112 ura3-52 [ρ+]), DW27, and DW29 were used. DW27 and DW29 are both isochromosomal with JM43, but DW27 only expresses subunit Va of Cco, and DW29 only expresses subunit Vb.[28][19] YPD media was composed of 1% Bacto-yeast extract, 2% Bacto-peptone, and either 2% glucose (non-CR) or 0.5% glucose (CR) and adjusted to pH 6.2. The media was sterilized via autoclaving at 121°C for 15 minutes. Yeast were grown in a New Brunswick Scientific Excella E24 Incubator Shaker at 30°C and 200 rpm. Growth was measured using a Klett-Summerson Photoelectric Colorimeter with the assumption that a Klett of 100 corresponded to a cell density of 2.25 x 10⁷ cells per mL, and that the relation between the Klett and cell density was linear.

*Mitochondrial Isolutions*

Mitochondrial isolations were performed as previously described.[29] Briefly, cells were harvested in mid-exponential phase, as determined by growth rates, and centrifuged at 4,000 rpm for 10 minutes. The pellets were then resuspended in 20 mL 4°C dH₂O and centrifuged at 5,000 rpm for 10 minutes. Prespheroplast buffer (1.21% Tris base and 0.04% dithiothreitol (DTT), pH
9.3) was added at 1 mL per 0.2 g cells, and this mixture was then incubated in a 28°C water bath at 100 rpm for 20 minutes. The mixture was then centrifuged at 5,000 rpm for 5 minutes and washed twice with 20 mL 4°C dH₂O, centrifuging at 5,000 rpm for 5 minutes each time. Zymolyase in spheroplast buffer (0.14% NaH₂PO₄·H₂O, 24.6% sorbitol, and 1 mM EDTA, pH 7.5, with 5 mg zymolyase added per mL spheroplast buffer) was added at 0.6 mL per 1 g of cells. The mixture was incubated in a 35°C water bath at 100 rpm until the yeast were spheroplasted, as checked by their lysis upon immersion in dH₂O and 2% N-lauroyl sarcosine and by microscopy. Spheroplasted cells were then centrifuged at 5,000 rpm for 5 minutes, and washed twice with 20 mL post-spheroplast buffer (27.3% sorbitol, 1 mM EDTA, and 0.1% bovine serum albumin (BSA), pH 7.0). Cells were resuspended in 10 mL lysis buffer with BSA (10.92% mannitol, 2 mM EDTA, and 0.1% BSA, pH 7.4) and mixed in an Omnimixer (Omni International) for 3 seconds at 1,000 rpm and 25 seconds at 20,000 rpm. The mixture was then centrifuged at 4,000 rpm for 5 minutes. The supernatant was decanted into new tubes and centrifuged at 10,000 rpm for 10 minutes. Again, the supernatant was decanted, and the pellet resuspend in 20 mL lysis buffer without BSA (10.92% mannitol and 2 mM EDTA, pH 7.0), and centrifuged at 3,700 rpm for 5 minutes. The supernatant was then removed and centrifuged at 14,000 rpm for 10 minutes. The final pellet was resuspended in mannitol buffer (10.9% mannitol and 10 mM H₃PO₄, pH 7.0) and stored at -20°C until use.

**Protein Determination**

One part BSA standard or sample was combined with 15 parts 660 nm Pierce Protein Assay Reagent (Thermo Scientific) and incubated for 5 minutes. The absorbance was measured
at 660 nm using a Molecular Devices SpectraMax M5 plate reader and the absorbance of the BSA standards was used to determine the unknown protein concentration.

**SDS-PAGE**

Isolated mitochondria were thawed and diluted into protein dissociation buffer (0.01 M NaPO₄, 2% SDS, 4% glycerol, and 20 mM DTT). The samples were then heated at 37°C for 30 minutes and boiled at 100°C for 2 minutes. Bromophenol blue (0.05%) was then added at 5 µL per 100 µL dissociated sample. A separating gel (2 mL glycerol, 5.76 g Urea, 500 µL dH₂O, 5 mL 1.5 M Tris-Cl, pH 8.8, 200 µL 10% SDS, 8 mL 40% polyacrylamide, 100 µL 10% ammonium persulfate (APS) and 6 µL TEMED) was poured and allowed to polymerize for 45 minutes, followed by a stacking gel (3.25 mL dH₂O, 50 µL 10% SDS, 1.25 mL 0.5 M Tris-Cl, pH 6.8, 438 µL 40% polyacrylamide, 50 µL APS, and 3 µL TEMED), which polymerized for 30 minutes. Ten µg of each sample was loaded into each well and the gel was run at 75 V until the dye front reached the top of the separating gel and then at 140 V until the dye front reached the bottom of the separating gel. 1X Tris-Glycine-SDS running buffer was used.

**Western Blot**

SDS-PAGE gels were transferred to PVDF membranes (wetted in methanol, then soaked in 1X Semi-Dry Buffer (Thermo)) using a Pierce Semi-Dry Blotter at 25V for 8 minutes. Membranes were then rinsed with dH₂O and air-dried. They were then rewet in methanol and blocked overnight in 5% non-fat milk in 1X PBS-T (1.418% Na₂HPO₄, 0.345% NaH₂PO₄ H₂O, 0.731% NaCl, and 1 mL 80% Tween per 1 L). Membranes were then washed 4 times, 7 minutes each in 1X PBS-T and transferred to a solution containing 1:1,500 of primary antibody (rabbit or
mouse) in 5% non-fat milk in 1X PBS-T and incubated for 1.5 hours. Again, membranes were washed 4 times, 7 minutes each in 1X PBS-T and transferred to a solution containing 1:20,000 of secondary anti-rabbit goat antibody or 1:10,000 of secondary anti-mouse goat antibody, conjugated to horseradish peroxidase, in 5% non-fat milk in 1X PBS-T and incubated for 1.5 hours. Membranes were washed 4 times, 7 minutes each in 1X PBS-T then incubated in a solution containing 10 mL Western Lightning luminol reagent, 10 mL Western Lightning Oxidizing reagent (PerkinElmer), and 20 mL dH₂O for 30 seconds. Membranes were then sealed airtight in plastic and exposed to film for varying amounts of time and developed. Antibodies used include one to a conserved region in subunit Va and Vb (DD-7), an anti- subunit II antibody (11-1B), and an anti-phospho-Ser58 (PhosphoSolutions). Quantification of bands was performed using ImageJ Software (National Institutes of Health).

Sequence Alignment, Phosphorylation Site Prediction, and Protein Imaging

Sequences were aligned using Uniprot (European Bioinformatics Institute, the SIB Swiss Institute of Bioinformatics, and the Protein Information Resource) and phosphorylation sites predicted using the Eukaryotic Linear Motif.[30][31] Protein structures were imaged using DeepView.[32]

Nitric Oxide Production Measurements

NO measurements were made using the inNOII Nitric Oxide Detector and an amino-700 electrode (Innovative Instruments, Inc.). For NO measurements, JM43 yeast were grown in either 2% or 0.5% YPD. Samples were spun down at 10,000 rpm for 10 minutes, and then washed in water and spun down again at 14,000 rpm for 5 minutes. Whole cells were suspended
in a thermostated chamber set to 30°C at a concentration of 85 mg/mL in 1X-PBS pH 6.5, for a total volume of 1 to 2 mL.

Ascorbate-TMPD Assay

The Ascorbate-TMPD assay was completed as previously described.[33] Briefly, 50 µg of solubilized mitochondria were suspended in a thermostated chamber set to 30°C containing 2 mL of assay buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM KH2PO4, 10 mM Tris, 50 µM EDTA, pH 7.4). After 10 minutes of incubation, 5 kU of superoxide dismutase (SOD) was added. One minute later, 10 uM of yeast cytochrome c (Sigma) was added. After another minute, ascorbate was added to a final concentration of 0.5 mM. Finally, TMPD was added to a final concentration of 0.5 mM. The chamber was allowed to reach anoxia, and then 40 µL of nitrite (NO₂⁻) was added and NO production measured using an animo-700 electrode and the inNOII Nitric Oxide Detector. To measure the effects of phosphorylation on nitrite reductase activity either alkaline phosphatase (Sigma) or a phosphatase inhibitor cocktail (Sigma) was added to the assay buffer prior to adding the mitochondria.

Glucose Assay

JM43 yeast were grown in 2% or 0.5% YPD and cell density was determined using a Klett-Summerson Photoelectric Colorimeter. Samples were taken immediately after inoculation and then subsequently every 30 minutes for 6.5 hours. Samples were centrifuged at 5,000 rpm for 10 minutes and the cells were removed. Media was stored at -20°C until use. Media glucose concentrations were assessed using the Sigma Glucose (Glucose Oxidase) Assay Kit, as
described. Briefly, samples were diluted 1:511 in dH₂O and mixed with the assay reagent, which consisted of Glucose Oxidase/Peroxidase and o-Dianoside. The assay was allowed to incubate for 30 minutes and then the reaction was halted using 12 N H₂SO₄. Absorbance was measured at 540 nm using a Molecular Devices SpectraMax M5 plate reader. Glucose concentrations were quantified based off the absorbance of the glucose standards.

**ATP and ADP Effects on Cco/NO Activity**

Fifty µg of isolated mitochondria were solubilized in 200 mM Tris pH 7.0 and 0.5 mM dodecyl-ß-D-Maltoside, with a final reaction volume of 2 mL. Differential concentrations of ATP or ADP were then added, with the concentration of ATP being held constant by a regenerating system (10 mM phosphoenolpyruvate, 10 U/mL pyruvate kinase, 5 mM MgSO₄, and varying amounts of MgATP). NO production was then measured using the Ascorbate-TMPD assay described above.

**Nucleotide Extraction**

JM43 yeast were grown in 2% or 0.5% YPD. Samples containing 5x10⁷ cells were taken and centrifuged at 14,000 rpm for 5 minutes and the media removed. The cells were then washed three times with dH₂O at 14,000 rpm for 5 minutes and either flash frozen in dry ice and stored at -80°C or stored at -20°C until use. Cells were then lysed by various methods. One extraction method involved boiling the cells to release the adenine nucleotides.[34] Briefly, 1.5 mL 100°C dH₂O or 0.1 M Tris-EDTA buffer pH 7.75 (TEB) was added to samples. Samples were then placed in boiling water for 10 minutes with vortexing every minute. Cellular debris was then
spun out at 14,000 rpm for 5 minutes and the supernatant saved. Samples were stored on ice or frozen at -20°C until assayed. A separate method used trichloroacetic acid (TCA) to denature the cells.[35] An ice-cold 10% TCA and 4mM EDTA solution was added to the frozen samples in equal parts (5.7 µL) for a final concentration of 5% TCA and 2 mM EDTA. Cells were then vortexed and after 5 minutes, the extraction diluted to a final volume of 1.5 mL either using dH₂O or TEB. Again, cellular debris was then spun out at 14,000 rpm for 5 minutes and the supernatant saved. Samples were then stored on ice or frozen at -20°C until assayed. To determine ADP concentrations, a 200 µL aliquot of cell extract was removed. The aliquot received 200 µL of a solution of 200 mM triethanolamine pH 7.6, 2 mM MgCl₂, 240 mM KCl, 4.6 mM phosphoenolpyruvate (Sigma), and 5 U/mL pyruvate kinase (Sigma). The mixture was incubated at 37°C for 10 minutes and then placed on ice or frozen at -20°C until use.[34]

ATP and ADP Assay

ATP levels were assessed using the Molecular Probes’ ATP Determination Kit (A22066) as per manufacturer instructions. Briefly, a standard reaction solution comprised of 8.9 mL dH₂O, 0.5 mL 20X Reaction Buffer (500 mM Tricine buffer pH 7.8, 100 mM MgSO₄, 2 mM EDTA, and 2 mM sodium azide), 0.1 mL 10 mM DTT, 0.5 mL 10 mM D-luciferin in 1X Reaction Buffer, and 2.5 µL firefly luciferase (5 mg/mL) was prepared and kept on ice and protected from light. ATP standards of 0.25 µM, 0.5 µM, 0.75 µM, and 1 µM were prepared in dH₂O. For ATP determination, 10 µL sample or standard and 90 µL standard reaction solution was added to each well and the luminescence was measured using a Molecular Devices SpectraMax M5 plate reader over a 5-minute period. For ADP determination, 10 µL of each sample incubated with pyruvate kinase was then added to a well with 90 µL standard reaction
solution and the luminescence was again measured over a 5-minute period. The measured luminescence of the unreacted samples was then subtracted from that of the reacted samples in order to determine the ADP concentration. Final extraction volumes were considered when analyzing the raw data to account for unintended volume variations, and a cell count of 5 x 10^7 and cell radius of 3 µm were assumed.

**Results**

*Isoform Switching Does Not Occur During CR*

Previously, it has been reported that the two isoforms of subunit V have differential effects on both the activity and oxygen sensitivity of NO synthesis by Cco.[19] The subunit Vb isoform increases Cco nitrite reductase activity (Cco/NO) and decreases oxygen sensitivity, allowing NO production in normoxic conditions. Subunit Vb is expressed predominantly during hypoxia, has a higher NO turnover rate, and its increased expression during CR could account for the increased NO production seen. To test for a change in subunit V isoforms during CR, SDS-PAGE was performed using isolated mitochondria from mid-exponential phase CR and non-CR JM43, with DW27 and DW29, strains that only express Va or Vb, as controls. The gels were transferred to a membrane and blotted with an anti-subunit V antibody that recognizes both Va and Vb. No expression of subunit Vb was observed in JM43 yeast grown in 2% or 0.5% media (Fig. 4a). A 3.3-fold average increase in expression of subunit Va was seen in JM43 yeast grown in 0.5% YPD as opposed to yeast grown in 2% YPD (Fig. 4b). To see whether this increase was also seen for other Cco subunits, levels of subunit II were quantified. A 3.4 average increase in subunit II levels was seen in CR JM43 yeast as opposed to non-CR JM43 yeast (Fig.
A. Western Blots comparing the relative levels of Va and Vb in CR and non-CR yeast (1- DW27, 2-DW29, 3- non-CR JM43, 4- CR JM43) show no increase in Vb expression during CR.

B. A comparison of relative levels of subunit Va in CR (0.50% glucose) and non-CR (2% glucose) JM43 yeast shows expression of Va increases 3.3-fold during CR.

**Figure 4.**
Figure 5. Western Blots comparing relative amounts of subunit II in CR (0.5% glucose) and non-CR (2% glucose) JM43 yeast show that expression of subunit II increases 3.4-fold during CR.
5). Given that roughly the same relative increase was seen for subunit Va and subunit II expression (3.3 vs 3.4), this suggests an overall increase in Cco expression during CR.

NO Production Decreases with Added Phosphatases and Increases with Phosphatase Inhibitors

Using the ascorbate- TMPD assay to measure Cco’s nitrite reductase activity, either alkaline phosphatases or phosphatase inhibitors were added to JM43 mitochondria isolated during mid-exponential phase. The addition of phosphatase inhibitors led to a 7.5-fold increase in NO production compared to the addition of phosphatases in non-CR mitochondria and a 8.75-fold increase in NO production in CR mitochondria (Fig. 6). This suggests that a key site of regulation in increasing nitrite reductase activity (or decrease oxygen sensitivity) is regulated via phosphorylation. This change would happen quite rapidly in response to CR, and thus changes in cells recently switched to CR should be evaluated, as opposed to those in mid-exponential phase.

Phosphorylation of Subunit II Changes in CR

Numerous phosphorylation sites have been identified in bovine Cco subunits. As there is a large degree of homology in subunits I and II, there is a good possibility that key regulatory phosphorylation sites will be conserved in yeast. An alignment of yeast subunit I with bovine subunit I showed high levels of conservation. Phosphorylation of mammalian subunit I has been established at serine 115 and 116, and there are corresponding serines/threonines in yeast. The next known phosphorylation site on subunit I is tyrosine 304, which not only is conserved in
Figure 6. NO production by Cco in CR (0.50% glucose) and non-CR (2% glucose) JM43 yeast, with added phosphatases or phosphatase inhibitors. NO production in the presence of phosphatase inhibitors increases 8.75-fold above levels in the presence of phosphatases in CR JM43 and increase 7.5-fold in non-CR JM43 (provided by Andrew Nelson).

Figure 6. NO production by Cco in CR (0.50% glucose) and non-CR (2% glucose) JM43 yeast, with added phosphatases or phosphatase inhibitors. NO production in the presence of phosphatase inhibitors increases 8.75-fold above levels in the presence of phosphatases in CR JM43 and increase 7.5-fold in non-CR JM43 (provided by Andrew Nelson).
yeast, but the surrounding amino acids are also highly conserved, suggesting this could be an important point of regulation (Fig. 7a). In an alignment of bovine subunit II with yeast subunit II, the only known site of phosphorylation in bovine subunit II, serine 126, does not have a conserved serine in yeast. There is a site of serine conservation at the -6 site from serine 126, however that is not a known phosphorylation site. A predicted phosphorylation site on yeast subunit II falls at threonine 15 (Fig. 7b). It is predicted that a proline directed kinase, such as MAP Kinase, could phosphorylate this site. This corresponds to serine 14 in both bovine subunit II and human subunit II. Importantly, they also all retain the +1 proline, which is required for MAPK phosphorylation and are all located in the mitochondrial intermembrane space, and therefore accessible to MAPK. An alignment of subunit IV-1 in humans with subunit Va in yeast shows possible conserved sites of phosphorylation. Serine 58 is a key site of regulation for IV-1, and when it is phosphorylated, ATP is less capable of inhibiting Cco’s activity.[33] There is a nearby serine 51 in yeast Va that could be a corresponding site of regulation in yeast. There is also a phosphorylation site at serine 136 in IV-1 that has a corresponding serine 141 in yeast Va, which could be another site for phosphorylation (Fig. 7c).[36] Using an anti-phosphoserine antibody, phosphoproteins were identified in CR and non-CR yeast. The bands were identified based on comparison with antibodies to specific subunits and then relative levels were quantified. The only bands readily identifiable corresponded to subunit II. CR JM43 mitochondria showed 1.6-fold higher levels of phosphorylated subunit II (Fig. 8). The differences between CR and non-CR mitochondria did not initially appear significant, as there were fairly similar relative levels. However, given the different levels of Cco, if phosphorylation patterns were consistent in CR and non-CR mitochondria, then there should be an increased
A.

59 TAHAFVMIFFMVMPIMIGGFNWLVPMIGAPDMFPRMNNSFWLPPSFLLLASSMV 118 I (bovine)
60 VGHAVLMIFFLVMAPALIGGGFNYLPLLMIAGTDTAFPRINIAF/WLPMGLVCLVTSSTLV 119 I (yeast)

B.

1 MLDLRLQTTFTIMDVPTYACYFQDSATPNQEGILEHLNDHYNMYLFYLLVLGLVSWMLYT 60 II (yeast)

C.

1 MLATRVSLSVKGRAISTSVCKRAEHSVVKSED puls paymdr-DRDHLPEVAH/VKHLSAS 58 IV-1 (human)
1 ML-RNTFTRAGLSRITSVRFAQ----THALSNAAVMDLQSRWENMPSTEQ----QDI 49 Va (yeast)

Figure 7. A. Excerpts from an alignment of bovine subunit I and yeast subunit I shows a conserved site of phosphorylation at serine 115 and 116 and tyrosine 304. B. Excerpts from an alignment of bovine subunit II and yeast subunit II shows no conservation of the phosphorylation site serine 126 in yeast. Threonine 15, which was identified as a target for MAPK phosphorylation, is highly conserved. C. An alignment of human subunit IV-1 and its homolog in yeast, Va shows some conservation of both serine 58 and 136 in yeast. Known phosphorylation sites are in green, potential conserved phosphorylation sites in yeast are in red, and predicted phosphorylation sites are in blue. (*-fully conserved, :-highly conserved, .-semi conserved)
**Figure 8.** Western blotting indicates subunit II is phosphorylated both during CR (2% glucose) and non-CR (0.50% glucose). Levels of phosphorylated subunit II are 1.6-fold higher in CR JM43 (0.50%) than non-CR JM43 (2%).
signal (roughly 2x of what was seen) of phosphorylated subunit II in CR mitochondria corresponding to the increased expression of subunit II (Fig. 5).

**ATP and ADP Both Affect Cco/NO Activity**

It has been established that adenine nucleotides can allosterically affect the rate of respiration.[37] It is therefore possible that Cco/NO is also subject to allosteric modulation by ATP and ADP. To explore this, NO production by Cco was measured in the presence of added ATP or ADP. When ATP levels increased, Cco/NO activity decreased, as measured by the ascorbate-TMPD assay. The decrease plateaued at roughly 65% baseline activity beginning at 12.5 mM ATP. Conversely, when ADP levels increased, Cco/NO activity did too. Again, activity responded to changes up until roughly 260% activation above baseline at 17.5 mM ADP (Fig. 9). As Cco’s ability to reduce O₂ is also affected by high ATP levels, this introduces a plausible mechanism by which both O₂ consumption and NO production increase during CR. This also suggests that changes in response to CR would occur quite rapidly within the cell, and that earlier time points should be analyzed to observe initial changes.

**Early Events in the Activation of Cco/NO during CR**

As indicated by previous results, the cellular adaptations to CR are rapidly inducible. Therefore, NO production and ATP and ADP levels were assessed immediately following a switch from stationary phase and no glucose to 2% or 0.5% media. This methodology allowed for the time frame of NO production and the response to CR to be established.
**Figure 9.** Cco/NO activity is affected by both ATP and ADP levels. ATP maximally inhibits baseline Cco/NO by 35%, while ADP maximally activates Cco/NO by 260% (adapted from Pablo Castello).
I. Changes in Glucose Levels During the Growth of CR and non-CR JM43

First, glucose concentrations during growth were measured to ensure that there was a sufficient difference in glucose concentrations between CR and non-CR media during subsequent measurements of NO production and ATP/ADP levels. Media glucose levels were determined using a colorimetric assay. In 2% (non-CR) media, glucose levels remained above 80% of their initial value at a cell density below $9.9 \times 10^6$ cells/mL (Fig. 10a). In 0.5% (CR) media, glucose levels remained above 80% of their initial value at a cell density below $6.98 \times 10^6$ cells/mL (Fig. 10b).

II. Changes in Mitochondrial NO Synthesis During the Growth of CR and non-CR JM43

While NO production in mid-exponential phase cells has been established, the time point at which NO production increases has yet to be determined. Cells were harvested at various time points after a switch to 0.5% media. NO production was then measured in the presence and absence of added nitrite. Exogenous nitrite was added to observe whether NO production was limited by Cco/NO’s rate of reaction or by a lack of nitrite. At 30 minutes after the shift, NO was produced at a rate of 0.176 nM per second per gram of cells (wet weight) with and without nitrite in CR JM43. In CR JM43 at 90 minutes, NO was produced at a rate of 0.147 nM s$^{-1}$ g$^{-1}$ without nitrite and 0.157 nM s$^{-1}$ g$^{-1}$ with added 1 mM nitrite. At 120 minutes, NO was produced at a rate 0.072 nM s$^{-1}$ g$^{-1}$ with and without nitrite in CR JM43. At 210 minutes, CR JM43 produced 0.07 nM NO s$^{-1}$ g$^{-1}$ without nitrite and 0.08 nM NO s$^{-1}$ g$^{-1}$ with 1 mM added nitrite (Fig. 11). Preliminary data of NO production in non-CR JM43 shows no NO production during these early time points, even with added nitrite. However, they will produce NO during anoxia, indicating the necessary components are in place, but the oxygen sensitivity is much higher than in CR JM43 (data not shown).
Figure 10. A. Glucose concentration (x) and cell density (●) as a function of time for JM43 yeast in 2% YPD (non-CR). Glucose concentrations do not depreciate considerably until 200 minutes. 
B. Glucose concentration (x) and cell density (●) as a function of time for JM43 yeast in 0.50% YPD (CR). Glucose concentrations do not fall as rapidly as seen in non-CR growth, with non-CR yeast taking half the time of CR yeast to consume 5 mg/mL glucose.
Figure 11. NO production in CR JM43 both without nitrite (■) and with 1mM added nitrite (□) shows a rapid induction that declines over time, suggesting transient NO production as a means of signaling.
III. Changes in Adenine Nucleotide Levels During the Growth of CR and non-CR JM43

A. Boiling Samples in Buffer Results in the Highest Adenine Nucleotide Extraction

Numerous methods have been published pertaining to extracting free nucleotides from whole cells.[34][35] Each method involves a delicate balance of lysing cells and inactivating enzymes either via physical or acid disintegration, while ensuring ATP and ADP are not destroyed in the process. This problem is compounded in cells containing a cell wall, such as yeast, as even more vigorous methods of disintegration are required to extract ATP. For this reason, several sample storage methods and extraction preparation techniques found in the literature were tested. For storage, cell samples were either stored at -20°C or frozen on dry ice and then stored at -80°C until extraction. For preparations, samples were either boiled in 1.5 mL dH₂O or TEB or extracted in 5% TCA and 2 mM EDTA and then diluted to 1.5 mL with dH₂O or TEB. Extraction/dilution in dH₂O resulted in much higher yields of ADP than ATP, which is physiologically inconsistent, as the amount of ATP in the cell must be higher than ADP when cells are viable, therefore extraction/dilution in dH₂O was leading to preferential degradation of ATP. Extraction/dilution in TEB led to a much higher yield of ATP, and suggested more physiologically plausible ratios, indicating buffer be used (Fig. 12a). Samples that were stored at -20°C showed higher yields, specifically of ATP, than did samples frozen on dry ice and then transferred to -80°C (Fig. 12b). This is an interesting discovery, as it was expected that cells frozen at -20°C would be able to respond to the stress of the cold more so than the cells frozen in dry ice, and would therefore have relatively lower ATP levels. Ultimately, boiling samples in TEB resulted in the highest extraction, and was therefore the method used henceforth (Fig. 12c).
Figure 12. A. Comparison of ATP extraction in dH$_2$O (■) or in Tris-EDTA buffer (TEB) (■) when boiling or using 5% TCA showed TEB to be more effective. B. Comparison of ATP extraction when freezing cells and storing them at -20°C (■) or freezing them on dry ice and storing them at -80°C (■) when boiling in TEB or using 5% TCA and diluting into TEB. Freezing at -20°C produced the highest yields of ATP. C. Comparison of ATP and ADP extractions when boiled in TEB (■) or using 5% TCA and diluting into TEB(■). Boiling in TEB resulted in higher extraction of ATP, and both methods showed equal ADP extraction.
B. Increases in ATP, ADP Levels Persist Longer in CR JM43

The ratio of ATP to ADP in yeast has previously been found to be close to 2, though it can change rapidly in response to multiple factors, including nutrient availability.[38] To test what the effect of CR is on the concentration of ATP and ADP, JM43 yeast were grown in 2% or 0.5% media and 5x10^7 cells taken per sample, with samples taken over the course of 6 hours. Both 2% and 0.5% yeast showed an immediate rise in ATP levels (Fig. 13a and 13b). The increase quickly subsided and ATP levels fell below their starting values for non-CR yeast. Non-CR yeast also showed a brief, rapid increase in ADP levels followed by a depression (Fig. 13a). In CR yeast, the increase in ATP was slower to reverse and ADP levels held much more constant in the initial 2 hours, though there was an initial decrease (Fig. 13b). After 6 hours, ATP and ADP levels were very similar in CR and non-CR yeast (Fig. 13a and 13b). The ratio of ATP to ADP was found to oscillate, with an average value slightly above 2 for both CR and non-CR yeast. Immediately after the switch, ATP:ADP increased 2-fold in CR JM43, while a much smaller increase was observed in non-CR JM43 (Fig. 14).
**Figure 13.**

**A.** Time-dependent concentrations of ATP (•) and ADP (□) in JM43 switched to 2% media (non-CR). After a brief peak at 10 minutes, both ATP and ADP levels declined before returning to close to their starting values.

**B.** Time-dependent concentrations of ATP (•) and ADP (□) in JM43 switched to 0.50% media (CR). ATP levels had a more sustained peak beginning at 10 minutes than seen in non-CR media, but also eventually declined below its initial value and then returned to close to its starting value. ADP concentration briefly dipped at 10 minutes but then remained very close to its starting value until 200 minutes, when it declined. After 200 minutes ADP values rose again and returned to around its starting value.
Figure 14. The ratio of ATP to ADP in both non-CR (*/) and CR (■) JM43 in highly variable, but averages to slightly above 2 in both groups. Immediately after the switch, CR JM43 had a 2-fold increase in ATP:ADP, which was 1.6 times higher than the increase seen in non-CR JM43.
Discussion

This study focused on answering how Cco/NO’s oxygen sensitivity is altered in CR to allow NO production during normoxia. An isoform switch of Cco subunit V, phosphorylation of Cco, and allosteric regulation of Cco by adenine nucleotides were all explored as possible mechanisms.

Isoform Switching

Though an isoform switch between Va and Vb makes sense based on previous observations about their different oxygen sensitivities, there is no increase in Vb expression during CR.[3] Rather, an increase in Va levels was seen during CR. A similar increase was seen in subunit II expression, indicating a rise in Cco subunits relative to other proteins. Although mitochondrial biogenesis does increase during CR, isolated mitochondria were run, so the increase of Cco is in relation to mitochondrial protein levels. Even considering the increased expression, Va has high oxygen sensitivity, so some other change must occur that causes the increase in NO production in normoxia during CR.[19]

ATP and ADP Allosteric Modulation of Cco/NO

The data on ATP and ADP regulation of Cco/NO shows that, much like respiration, its activity was indeed affected by ATP and ADP concentrations. ATP has a mild inhibitory effect on Cco/NO, while ADP can activate it 2.6-fold. Concentrations of ATP had to be above 2.5 mM in order to see any inhibition, while there was an immediate, rapid rise in activation based on ADP. This contrasts the data on ATP and ADP’s effects on respiration, where there was a larger
inhibitory effect of ATP than an activating effect of ADP on respiration, though they were fairly close.[38] Also, considering the highest ATP concentration seen during the ATP and ADP Assay was 0.25 mM, it is possible that allosteric regulation of Cco/NO is more of activation by ADP than inhibition by ATP, as ATP would not be high enough intracellularly to inhibit Cco’s activity. However, it is also important to note the limitations of the ATP/ADP assay; it assumed a certain cellular size and uniform ATP and ADP distribution within the cell. In reality, vacuoles and endosomes, devoid of any ATP or ADP, can take up variable amounts of space within yeast, which would mean concentrations of ATP and ADP were higher intracellularly than reported here.[39]

Even considering the problems inherent in precise quantification of ATP and ADP concentrations, the data obtained on ATP and ADP levels six hours following a switch from stationary phase to high or restricted glucose show the relative changes of these nucleotides. The relative change seems to counter the hypothesis that ATP:ADP should decrease during CR. Instead an immediate peak in ATP is seen both in CR and non-CR yeast. The peak rapidly subsides in non-CR yeast and after a dip, ATP levels return to roughly their starting value. For CR yeast, the peak in ATP levels is less quick to subside, and instead slowly tapers off, followed by a brief dip and then a return to their initial value. For ADP, the pattern in non-CR yeast is very similar to that of ATP. In CR yeast, ADP has a brief dip, but then remains relatively stable for an hour, before taking the dip taken by all the others and then stabilizing near initial values. In some way, this is the expected response, as respiration yields higher ATP than does glycolysis.[40] If ATP inhibits both Cco’s oxygen and nitrite reductase activity, why then do ATP levels increase in CR cells that also have higher levels of respiration and NO production?
The likely answer to this question involves phosphorylation that could abolish Cco’s ATP sensitivity in CR.

*Phosphorylation Effects on Cco/NO*

The difference in NO production between JM43 mitochondria treated with phosphatases and mitochondria treated with phosphatase inhibitors was quite large for both CR and non-CR mitochondria, with CR mitochondria showing a 8.75-fold average increase and non-CR mitochondria showing a 7.5-fold average increase. This difference indicates an important role for phosphorylation in modulating the NO production of Cco. Although subunit Va is an obvious site of such phosphorylation, the results did not show any phosphorylation of Va, and in fact only showed phosphorylation of subunit II.

*Potential Phosphorylation Sites*

Based on a western blot using an anti-phosphoserine antibody, subunit II appeared to be differentially phosphorylated in CR and non-CR mitochondria. Therefore, it is possible that the decrease of phosphorylation seen on subunit II during CR plays an important role in the oxygen sensing of Cco/NO. Subunit II houses copper A (CuA), which accepts electrons from cytochrome c and then passes them to heme a. They are then transferred to heme $a_3$ and copper B (CuB), which together compromise the binuclear reaction center that ultimately reduces O$_2$ to H$_2$O.[41] Subunit II is therefore the interface between the intermembrane space and the binuclear reaction center in subunit I (Fig. 2).[42] Given this role, a site of regulation via phosphorylation on subunit II makes good sense.
As indicated in figure 7b, there is a known phosphorylation site on subunit II, serine 126. The group that initially identified this phosphorylation site on subunit II proposed it could be involved in regulation of ATP’s allosteric effects, as it is proximal to subunit Va in yeast, which is known to house the ATP/ADP binding site. Here, it is important to note that although phosphorylation is required for ATP to inhibit respiration, with serine 126 on subunit II proposed to enable it, phosphorylation at serine 58 on subunit IV (mammalian) abolishes ATP’s ability to bind Cco.[43] Therefore, a careful balance of phosphorylation and dephosphorylation coupled with allosteric effects regulate Cco’s activity in respect to respiration. Is this also the case with Cco’s nitrite reductase activity? The evidence for allosteric regulation of Cco/NO by ATP and ADP, increased NO production upon inhibition of phosphatases, and decreased phosphorylation of subunit II during CR indicate that it is. As with most cellular processes, there is no one phosphate group or allosteric effect that accounts for all changes seen, and the changes in respiration and NO production could be the result of a phosphate added to one site, like Va, and lost from another, like subunit II.

The question again returns to where these key phosphorylation sites are within Cco. Although the data here do not show any phosphorylation of subunit Va, it is an established site of Cco regulation and should be afforded due consideration. Subunit Va has a key role in establishing oxygen sensitivity of Cco’s nitrite reductase activity and modulating allosteric effects of ATP and ADP, as organisms that lack a subunit V homolog, such as prokaryotes, do not show inhibition by ATP.[44] Phosphorylation of serine 58 on subunit IV-1 in mammals abolishes allosteric inhibition by ATP, however it still allows allosteric modulation by ADP. Serine 58 is located in the mitochondrial matrix, and thus requires a matrix kinase for phosphorylation. Mitochondrial adenylyl cyclase generates cAMP, which activates
mitochondrial PKA, and PKA then phosphorylates serine 58. Once phosphorylated, subunit IV-1 can no longer bind ATP. This phosphorylation allows for increase oxidative phosphorylation during periods of high growth, as demonstrated by serine 58 mutants that show decreased growth.[25] This appears to be a key phosphorylation site in the response to CR, as it would allow Cco to ignore the high ATP levels but respond to the increase in ADP with an increase in respiration and NO production. It is possible that this phosphorylation slightly lowers Va’s oxygen sensitivity and that this, coupled with overall higher levels of Cco, leads to the increase in NO production. This becomes even more plausible considering that ATP levels drop and ADP levels rise during hypoxia, as glycolysis produces less ATP per glucose.[45] Although serine 58 is not conserved in yeast, there is a proximal serine 52 that could potentially serve the same purpose in yeast.

Subunit II is another potential site for regulation. As mentioned, serine 126 on subunit II is an identified phosphorylation site. This site is not conserved in yeast, however. A search for potential phosphorylation sites identified a mitogen activated protein kinase (MAPK) consensus sequence at threonine 15 in yeast or serine 14 in mammals. Without further analysis, it cannot be determined whether the difference observed in phosphorylation on subunit II was due to threonine 15 or another phosphorylation site. If it was threonine 15, and if it was phosphorylated by MAPK, the mechanism would be in accord with other known pathways in CR. In yeast, MAPK regulates several stress responses, such as starvation. In mammals, MAPK’s function is similar as it is involved in stress responses, like cell survival and apoptosis. It is also involved in insulin-like signaling.[46] Therefore, decreased phosphorylation by MAPK during CR would be assumed, as there is lower insulin-like signaling via Sch9 in yeast. As to what effect the
phosphorylation of threonine 15 could have on Cco activity, it would make the most sense for it to allow ATP inhibition or otherwise decrease Cco activity.

Finally, there is the phosphorylation site on tyrosine 304 in subunit I. Like with subunit Va, although subunit I was not identified as phosphorylated via western blotting, it has a known phosphorylation site that could play a role in CR adaptation. When phosphorylated, tyrosine 304 decreases respiration. Tyrosine 304 is close to the binuclear reaction site in subunit I, therefore it could interact directly with active site kinetics in order to slow the reaction rate. Interestingly, tyrosine 304, like serine 58, is phosphorylated in the presence of high cAMP levels. So, both an inhibitory and activating phosphorylation are caused by an increase in cAMP. One potential explanation is the different compartments involved in the phosphorylations. Serine 58 is located in the matrix and is regulated by mitochondrial cAMP levels and PKA, while tyrosine 304 is in the intermembrane space, and thus would be under the control of cytosolic cAMP levels and kinases. Therefore, the two responses could be separated.

Connections with Other Pathways in CR

More research needs to be done to elucidate the exact phosphorylation sites changed during CR and their effects on respiration and NO production. This is a path with large pharmacological relevance, for if these changes could be induced with a small molecule, it could delay aging and onset of age-related disorders. Such attempts have already been made, most prominently with the compound resveratrol. Resveratrol is a polyphenol found in the skins of certain fruits, including grapes, that was found to increase the lifespan of C. elegans and other lower eukaryotes, and induce intracellular changes reminiscent of CR. In mice, it was found to delay the aging phenotype, although it did not extend lifespan. It was initially thought
that resveratrol was an activator of Sir2, as increased Sir2 activity followed resveratrol administration.[50] However, evidence now indicates that resveratrol instead inhibits cAMP phosphodiesterases, which indirectly activates Sir2, PGC-1α, and AMP-activated protein kinase (AMPK). Once evidence shifted away from resveratrol directly acting on Sir2, evidence also started to emerge that undermined Sir2’s central role in CR and nutrient sensing abilities. Overexpression of Sir2 alone did not increase lifespan in mice, and Sir2 activation by rising NAD⁺ concentration alone has been questioned.[51]

As Sir2’s role fell, AMPK’s began to rise, with mounting evidence supporting its role in CR adaptations. Resveratrol’s positive effects on mice were abolished in those lacking AMPK and AMPK appears to be a key energy regulator, as it responds to AMP, ADP, and ATP levels in relation to one another as a means of regulating cellular energy charge.[52][51] This again brings up the changes in ATP and ADP seen during a shift to CR, as ATP:ADP increased 1.6-fold higher in CR yeast than non-CR yeast. Although previous focus was on the effect of ATP and ADP on Cco regulation, this is another mechanism of energy sensing within the cell that could mediate the adaptation to CR.

**NO’s Role in Caloric Restriction**

Another missing piece of the puzzle involves what role NO plays in CR. During hypoxia, NO helps stabilize HIF-1α, which allows it to induce hypoxic gene induction.[53] Such a mechanism is equally plausible in CR. Although a separate nitric oxide synthase in yeast has yet to be identified, they do exist in mammals, and CR has been found to also increase expression of the endothelial nitric oxide synthase (eNOS) in mice, which results in increased mitochondrial biosynthesis. Many of the effects associated with CR, like increased respiration,
were lost when eNOS activity was eradicated.[54] So NO evidently plays a key role in mediating the changes induced by CR. Elucidating the precise mechanism by which NO acts would greatly increase understanding of CR.

The vital role of NO in CR also introduces a new site of pharmacologic intervention. Does increasing NO by added NO donors during conditions of non-CR also lead to increases in lifespan? The answer is not straightforward. In certain conditions, addition of NO donors induced some aspects reminiscent of CR and led to slight lifespan increases in yeast.[3] In mammals, L-arginine is a substrate for NOS and administration increases NO production.[55] In humans, L-arginine supplementation has only been tested in patients who suffered myocardial infarctions, and it was found to increase mortality.[56] Whether L-arginine supplementation will have any effect, positive or negative, on degenerative diseases and aging has yet to be explored. Given the important role of NO, and the numerous cellular mechanisms found in this study to regulate its production, it is a question worth exploring.

Conclusion

Ultimately, the intracellular changes in metabolites, phosphorylation, and protein levels each paint part of the picture. As CR induces a metabolic reprogramming of the cell, it fits that there are numerous pathways and mechanisms responsible for the adaptations. Though it is appealing to find a singular causative agent for how CR delays aging, like Sir2 or AMPK, such a quest undermines the high complexity and regulation of the cell. Future research examining the relationship between dynamic phosphorylation and allosteric regulation of respiration and NO production will provide vital insights into cellular adaptations to CR, and into the degenerative changes seen in aging and diseased cells.
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