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## Life-long caloric restriction elicits pronounced protection of the aged myocardium: a role for AMPK

Andrew G. Edwards<sup>1</sup>, Anthony J. Donato<sup>1</sup>, Lisa A. Lesniewski<sup>1</sup>, Rachel A. Gioscia<sup>1</sup>, Douglas R. Seals<sup>1</sup>, and Russell L. Moore<sup>1</sup>

<sup>1</sup>Department of Integrative Physiology, University of Colorado Cardiovascular Institute, University of Colorado at Boulder, Boulder CO 80309, USA

### Abstract

Short-term caloric restriction (CR) protects the young myocardium against ischemia/reperfusion (I/R) injury through a mechanism involving AMP-activated protein kinase (AMPK). Here we ask whether a life-long CR intervention can extend this protection to the aged myocardium, and whether AMP-activated protein kinase (AMPK) plays a role in that protection. Hearts from ad libitum fed (AL) and life-long calorically restricted (LCR) mice were examined at 30 months of age by 25/90 minute global I/R, with and without AMPK inhibition (AraA). LCR hearts were protected from infarction (AL, 28±4 % vs. LCR, 10±1 %,  $p < 0.01$ ) and post-ischemic functional deficit (LVDP recovery: AL, 65±8 % vs. LCR, 93±7 %,  $p < 0.01$ ). Pre-ischemic AraA impaired both of these protective effects (Infarct size: LCR+AraA, 22±4 %; LVDP recovery: LCR+AraA, 82±9 %, both  $p$  vs. AL  $> 0.1$ ). AMPK $\alpha$  phosphorylation was dramatically increased in LCR hearts prior to I/R (AL, 1.18±0.01 vs. LCR, 1.68±0.04, ratio,  $p < 0.0001$ ), and accompanied by a more modest increase in total AMPK $\alpha$  (AL, 2.18±0.03 vs. LCR, 2.39±0.08 ratio,  $p < 0.05$ ). These results indicate that life-long caloric restriction profoundly protects the aged heart against I/R injury, and suggest that AMPK may play a role in that protection.

### Keywords

Diet; nutrition; preconditioning; ischemia; myocardial infarction

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Recent estimates of global disease burden indicate that ischemic heart disease (IHD) accounts for 12.2 % of deaths worldwide (WHO, 2004), and among high-income nations, 89.8 % of IHD mortality occurs in individuals 60 years of age or older (WHO, 2004).

One biological contributor to this age-association of IHD mortality is a progressive impairment of myocardial resistance to ischemia/reperfusion (I/R) injury<sup>1</sup>. This impairment not only affects basal myocardial resistance to I/R, but also diminishes induction of I/R resistance by a variety of preconditioning strategies<sup>2–5</sup>. Together, these effects describe an

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Address for Correspondence: Russell L. Moore University of Colorado at Boulder 354 UCB Boulder, CO 80309-0354 Phone: (303)-492-5209/1087 Fax: (303)-735-4125 RMoore@colorado.edu.

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aged myocardium that is basally susceptible to metabolic challenge, and less able to recruit cellular defenses after exposure to known protective treatments.

Caloric restriction (CR) is associated with enhanced physiological function in many species<sup>6,7</sup>, and recent evidence indicates that life-long CR can reduce cardiovascular disease (CVD) incidence by 50% in aged primates<sup>8</sup>. Shorter-term CR paradigms elicit protection against infarction<sup>5,9</sup>, post-ischemic functional deficit<sup>5,9,10</sup>, and susceptibility to arrhythmia<sup>3</sup>. However, none of these effects have been studied after life-long CR, or in an age range comparable to that at which human IHD manifests. To that end, we employ a well-described mouse model<sup>11</sup> to test the primary hypothesis that life-long CR remains cardioprotective in animals aged to point comparable to that at which most human IHD occurs. Secondly, we examine the role played by AMP-activated protein kinase (AMPK) in the I/R responses of the myocardium after life-long CR or *ad libitum* feeding. While this kinase is known to be involved in protection due to short-term CR<sup>9</sup>, some evidence suggests that its potential to protect the heart wanes over longer term interventions<sup>5</sup>.

B6D2F1 mice were purchased from the NIA at 24 months of age. At the time of delivery, all mice had undergone caloric restriction from 14 weeks of age (*LCR*), or been *ad libitum* (*AL*) fed according to the model developed for the Biomarkers of Aging Program (BAP)<sup>11</sup>. These treatments were replicated on-site until 30 months of age when mice were killed<sup>11</sup>. Global I/R (25/90 minute) was performed conventionally by the constant-pressure Langendorff method under normothermic conditions, as described previously<sup>12–14</sup>. The perfusate was modified slightly to better represent metabolic conditions *in vivo* through inclusion of 0.4 mM oleate and 10  $\mu\text{U}\cdot\text{mL}^{-1}$  insulin<sup>9,15</sup>. Hearts undergoing AMPK blockade were exposed to 100  $\mu\text{M}$  9- $\beta$ -D-arabinofuranoside (*AraA*) for 20 minutes prior to ischemia, which has previously been shown to reduce myocardial AMPK phosphorylation<sup>9</sup>. Left ventricular pressure waveforms and terminal infarct size were measured by a pressure-transducing catheter and tetrazolium staining, respectively, as described previously by our laboratory<sup>12–14</sup>. Hearts from separate cohorts of *AL* and *LCR* were used for Western immunoblotting. Upon excision they were briefly rinsed, then immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Further steps of tissue preparation and blotting were performed as previously described<sup>9</sup>. Sample sizes for I/R were: *AL*,  $n = 6$ ; *AL + AraA*,  $n = 5$ ; *LCR*,  $n = 6$ ; *LCR + AraA*,  $n = 5$ . Blot data include six hearts from each group of animals. Post-ischemic cardiac function was statistically assessed by linear mixed model, where diet and *AraA* were included as a fixed factors and time was the sole random factor. Infarct size was treated by factorial ANOVA, and immunoblot data by Student's t-tests. An alpha level of 0.05 was assumed for all analyses.

Anticipated effects of CR were observed for morphological variables at the time of death (Table 1). While LV mass was higher in *AL* hearts ( $p < 0.001$ ), *in vivo* cardiac dimension (measured by short-axis M-mode echocardiography one week prior to death), was unchanged by *LCR* (Table 1). *LCR* hearts were considerably more resistant (66 %) to infarction than *AL* hearts ( $p < 0.005$ ; *LCR* vs. *AL*,  $p < 0.005$ ), and this protection was susceptible to inhibition of AMPK (*LCR* vs. *LCR + AraA*,  $p < 0.05$ , Figure 1). Similar protection was observed in terms of post-ischemic recovery of left ventricular developed pressure (LVDP), which approached pre-ischemic levels among *LCR* hearts during minutes 30 – 75 of reperfusion. During the same time period *AL* hearts recovered to only 73 % ( $p < 0.05$  vs. *LCR*, Figure 2) of pre-ischemic LVDP, and *AraA* treated *LCR* hearts recovered similarly to *AL* at 79 %, which was significantly less than *LCR* alone ( $p < 0.05$  vs. *LCR*, Figure 2). Western blot data suggest that, after excision, *LCR* hearts exhibited much higher phosphorylation of the AMPK alpha subunit ( $p < 0.001$ , Figure 3), and that this catalytic subunit was also expressed at slightly higher levels compared to *AL* ( $p < 0.05$ , Figure 3).

The data presented here provide evidence that life-long CR elicits substantial myocardial resistance to I/R injury. This finding extends previous work by establishing CR as a strategy that remains cardioprotective after life-long intervention, and most importantly, that the conferred protection is pronounced in the otherwise I/R-susceptible aged murine heart. While the mechanisms underlying this protection are likely to involve many genes and substantial pleiotropy, our results suggest that AMPK is one active constituent of the protected myocardial phenotype accompanying life-long CR. Inhibition of AMPK by AraA markedly reduced infarct resistance and functional recovery in our *LCR* mice, and AraA has previously been shown to simultaneously decrease AMPK $\alpha$  phosphorylation and remove myocardial protection due to short-term CR<sup>9</sup>.

The means by which AMPK exerts its cardioprotective effects are unclear, in large part because the many roles that AMPK plays during I/R have not been reconciled in combination. Certainly AMPK orchestrates responses that are protective in isolation, such as translocation of GLUT4 and sarcolemmal K<sub>ATP</sub> channels<sup>16,17</sup>, but it has also been proposed that AMPK contributes to I/R injury by shifting myocardial metabolism to favor less efficient fatty acid oxidation<sup>18</sup>. We cannot comment on these possibilities directly, but have attempted to permit for the latter by including physiologically representative concentrations of fatty acid and insulin in our perfusion system.

In conclusion, life-long caloric restriction elicits a myocardial phenotype that is profoundly protected against I/R injury, even at an age comparable to that at which most human ischemic events occur. AMPK activation may contribute to the mechanism by which life-long CR protects the myocardium against ischemia late in life.

## Supplementary Material

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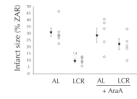
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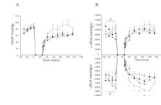
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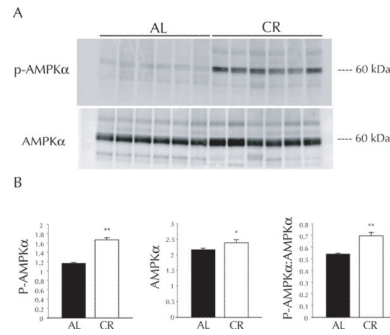


**Figure 1.**

Post I/R infarct size (% ZAR mass) among AL and LCR, in the absence and presence (+AraA) of pre-ischemic 9- $\beta$ -d-arabinofuranoside (100  $\mu$ M). Open circles are individual data, filled circles are means  $\pm$  SEM. \* $p$  < 0.05 compared to AL, # $p$  < 0.05 compared to LCR + AraA.

**Figure 2.**

Left ventricular function during 25/90 min global I/R, by diet (LCR, open circles; AL, open squares), and preischemic AraA (LCR + AraA, *filled circles*; AL + AraA, *filled squares*). Data are means  $\pm$  SEM. Panel A) LV developed pressure (mmHg). Panel B) Rates of left ventricular pressure development ( $dP/dt$ ) and decay ( $-dP/dt$ ). Data are means  $\pm$  SEM. #  $p < 0.05$  diet  $\times$  drug (fixed effect), \*  $p < 0.05$  CR vs. CR + AraA (pairwise contrast), \*\*  $p < 0.05$  (diet, diet  $\times$  drug).



**Figure 3.**

Panel A: Representative immunoblot for life-long calorically restricted (LCR) and ad-libitum (AL) hearts: P-AMPKα (top) and total AMPKα (bottom). Panel B: Densitometry for immunoblot data, presented as means  $\pm$  SEM. \*  $p < 0.05$  vs. AL, \*\*  $p < 0.001$  vs. AL.

**Table 1**

Tissue masses and echocardiographic dimensions for CR and AL animals at time of sacrifice.

	AL	CR
<b>Heart (mg)</b>	270 ± 6	190 ± 6
<b>Body (g)</b>	38 ± 2	25 ± 1**
<b>WAT (g)</b>	1.2 ± 0.3	0.1 ± 0.0**
<b>Soleus (mg)</b>	18 ± 6	13 ± 1
<b>TA (mg)</b>	74 ± 12	45 ± 4*
<b>Heart: Body</b>	7.3 ± 0.4	7.4 ± 0.1
<b>TA:Body</b>	1.8 ± 0.5	1.8 ± 0.2
<b>WAT:Body</b>	29.2 ± 6.0	5.8 ± 0.5**
<b>LVIDs</b>	2.0 ± 0.2	1.8 ± 1.7
<b>LVIDd</b>	3.8 ± 1.4	3.5 ± 1.8
<b>FS</b>	0.47 ± 0.04	0.49 ± 0.03

TA: Tibialis anterior, WAT: White Adipose Tissue, LVIDs: end systole Left Ventricular Internal Diameter; LVIDd: end diastole Left Ventricular Internal Diameter, FS: Fractional Shortening.