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Acetyl-L-carnitine supplementation reverses the age-related decline in carnitine palmitoyltransferase 1 (CPT1) activity in interfibrillar mitochondria without changing the L-carnitine content in the rat heart

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ABSTRACT

The aging heart displays a loss of bioenergetic reserve capacity partially mediated through lower fatty acid utilization. We investigated whether the age-related impairment of cardiac fatty acid catabolism occurs, at least partially, through diminished levels of L-carnitine, which would adversely affect carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme for fatty acyl-CoA uptake into mitochondria for β-oxidation. Old (24–28 mos) Fischer 344 rats were fed ± acetyl-L-carnitine (ALCAR; 1.5% [w/v]) for up to four weeks prior to sacrifice and isolation of cardiac interfibrillar (IFM) and subsarcolemmal (SSM) mitochondria. IFM displayed a 28% ($p < 0.05$) age-related loss of CPT1 activity, which correlated with a decline (41%, $p < 0.05$) in palmitoyl-CoA-driven state 3 respiration. Interestingly, SSM had preserved enzyme function and efficiently utilized palmitate. Analysis of IFM CPT1 kinetics showed both diminished V_{max} and K_m (60% and 49% respectively, $p < 0.05$) when palmitoyl-CoA was the substrate. However, no age-related changes in enzyme kinetics were evident with respect to L-carnitine. ALCAR supplementation restored CPT1 activity in heart IFM, but not apparently through remediation of L-carnitine levels. Rather, ALCAR influenced enzyme activity over time, potentially by modulating conditions in the aging heart that ultimately affect palmitoyl-CoA binding and CPT1 kinetics.

1. Introduction

Aging entails adaptations in energy metabolism to maintain cardiac pump function (Kates et al., 2003; McMillin et al., 1993). For example, fatty acid oxidation, which is typically the primary oxidizable substrate for myocardial bioenergetics, declines with age in rodents (Abu-Erreish et al., 1977; Hyyti et al., 2010; McMillin et al., 1993) and also in humans (Kates et al., 2003). While glucose oxidation appears to compensate this loss (Kates et al., 2003; McMillin et al., 1993), there is increasing evidence that such a shift in metabolism comes at a price, primarily in lower bioenergetic reserve capacity that limits response to heightened energy demands (Davila-Roman et al., 2002; Koonen et al., 2007; van der Meer et al., 2008). Moreover, as myocytes have limited means for exporting fatty acids in the form of triacylglyceride (Lewin and Coleman, 2003), lower fatty acid oxidation may shunt lipids into non-oxidizing metabolic pathways and/or lipid storage in the myocardium (Koonen et al., 2007; Sharma et al., 2004; van der Meer et al., 2008). In its extreme, the age-associated decline in fatty acid-driven mitochondrial bioenergetics may thus initiate a form of myocardial lipotoxicity (Brindley et al., 2010; Slawik and Vidal-Puig, 2006; Wende and Abel, 2010). Therefore, it is important to understand the mechanism for lower fatty acid oxidation in the aging heart muscle.

While age-associated alterations in cardiac energy metabolism are undoubtedly multifactorial, several reports implicate carnitine palmitoyltransferase 1 (CPT1) as a key enzyme in the shift away from fatty acid oxidation (Lee et al., 2002; McMillin et al., 1993; Odiet et al., 1995). CPT1, the rate-controlling enzyme for overall fatty acid β-oxidation, catalyzes the condensation of acyl-CoA with L-carnitine to form acyl-carnitine esters, which are subsequently transported into mitochondria for further catabolism (Bartlett and Eaton, 2004; McGarry and Brown, 1997; Ramsay et al., 2001). There is a consensus that CPT1 activity declines with age in heart and skeletal muscle (Hansford and Castro, 1982; Kim et al., 2009; McMillin et al., 1993; Odiet et al., 1995). Moreover, down-regulation of CPT1 activity correlates with lipid accumulation and insulin resistance in rat skeletal muscle (Dobbins et al., 2001; Kim

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et al., 2009). Therefore, a plausible hypothesis is that lower CPT1activity is an underlying factor in the decline in fatty acid-supported myocardial bioenergetics.

Multiple lines of evidence now indicate that mitochondrial decay is a fundamental factor that leads to alterations in energy metabolism in the aged heart (Hagen et al., 2002; Judge and Leeuwenburgh, 2007; Lesnefsky et al., 2001c). Nevertheless, the exact biochemical events that cause such an alteration in energetics are not completely understood. Furthermore, the existence of two cardiac mitochondrial subpopulations which display different functional features (Palmer et al., 1977; Riva et al., 2005) increases the complexity of a thorough characterization of the molecular events underlying the age-related deterioration of mitochondrial function. Histologically, subsarcolemmal mitochondria (SSM) are associated with the sarcolemma, and appear to provide energy for the regulation of myocellular ion exchange and homeostasis (Kaasik et al., 2001; Sasaki et al., 2001). On the other hand, interfibrillar mitochondria (IFM) are intercalated along the myofibrils, and are believed to supply ATP for the myocardial contraction–relaxation cycle (Hoppel et al., 1982; Kaasik et al., 2001). A number of studies show that IFM functionally deteriorate with age. This subpopulation exclusively displays decreased ADP-stimulated respiration, altered electron transport chain (ETC) components, higher rates of oxidant appearance, and increased susceptibility to both permeability transition and damage during ischemia and reperfusion (Fannin et al., 1999; Hofer et al., 2009; Lesnefsky et al., 2001a,b; Suh et al., 2003).

To our knowledge, no direct characterization as to how myocardial aging affects CPT1 activity and/or substrate utilization in the two mitochondrial subpopulations has been undertaken. Thus, it is not known whether the aging lesion for fatty oxidation lies in a particular mitochondrial subpopulation or more generally results from cardiac decrements in L-carnitine levels which decline with age in humans (Costell et al., 1989; Opalka et al., 2001) and also in rats (Maccari et al., 1990; Tanaka et al., 2004). Thus, the age-related loss of myocardial carnitine levels may diminish overall CPT1 activity, and/or exacerbate enzyme catalytic dysfunction in a particular mitochondrial subpopulation. If carnitine levels indeed contribute to lower CPT1 activity, then it is equally possible that general CPT1-mediated fatty acid oxidation can be remediated by increasing myocardial L-carnitine content. In this regard, cardiac mitochondrial bioenergetics in aged rats has been improved following dietary supplementation with the L-carnitine analogue, acetyl-L-carnitine (ALCAR) (Hagen et al., 2002; Paradies et al., 1994, 1995, 1999).

In the present work, the effects of aging and ALCAR supplementation on the activity of CPT1 were investigated in rat heart SSM and IFM. Our data show that aging selectively decreases CPT1 activity in IFM by reducing enzyme catalytic efficiency for palmitoyl-CoA utilization without inducing significant alterations in the kinetic parameters for L-carnitine. These findings suggest that the decline in IFM CPT1 activity could be a key factor in the mechanism by which fatty acid utilization decreases, and as a consequence, induces lipid toxicity in the aging heart (Slawik and Vidal-Puig, 2006; Wende and Abel, 2010).

2. Materials and methods

2.1. Materials

Sucrose, D-mannitol, MOPS, HEPES, nagarse, palmitoyl-CoA lithium salt, Trizma, L-carnitine hydrochloride, adenosine 5′-diphosphate sodium salt, L-glutamic acid sodium salt, L-malic acid sodium salt, ethylene glycol tetraacetic acid (EGTA), KCl and other salts were from Sigma–Aldrich (St. Louis, MO). All other chemicals were reagent grade or the highest purity obtainable. L-[*methyl*-^{3}H]-carnitine hydrochloride (specific activity 80.0 Ci/mmol) was supplied by Amersham Biosciences (Piscataway, NJ). Bovine serum albumin (fraction V, fatty acid-free) was from EMD Chemicals Inc. (San Diego, CA). ALCAR was a gift of Sigma Tau (Pomezia, Italy).

2.2. Animals

Young (3–4 months) and old (24–28 months) male Fischer 344 rats were obtained from the National Institute on Aging animal colonies. The animals were housed in approved facilities at Oregon State University and maintained by the Laboratory Animal Resources Center. All procedures including diets and animal handling were in keeping with approved institutional animal care and use guidelines.

2.3. ALCAR supplementation

Old rats were given a 1.5% (w/v; pH ~6) solution of ALCAR in their drinking water. Animals were maintained on standard chow diet and provided water ad libitum for one, two or four weeks before sacrifice and isolation of cardiac mitochondria. The average water consumption was ~32 ml/rat per day, corresponding to a daily average ALCAR intake ~1.1 g/kg body weight. ALCAR intake was not found to significantly change body weight in old rats with respect to animals receiving a control diet. At the end of the four week supplementation period, body weight was 391 ± 4 g for animals receiving ALCAR ($n = 4$) and 374 ± 32 g for old control rats ($n = 4$).

2.4. Isolation of cardiac mitochondria

Cardiac subsarcolemmal and interfibrillar mitochondria were isolated from both young and old rats using the method previously described by Palmer et al. (1977). Briefly, SSM were isolated by differential centrifugation from heart homogenates. Proteolytic treatment of the homogenate with Nagarse (3 mg/g tissue) was used to release IFM from myofibrils. All steps of the isolation were performed on ice or at 4 °C. Protein content was determined by the Lowry method using bovine serum albumin as the standard (total protein kit from Sigma–Aldrich, St. Louis, MO).

2.5. Mitochondrial oxygen consumption

Respiratory characteristics of mitochondria were monitored using a SYS-ISO2 dissolved oxygen meter coupled with an OXELP oxygen electrode (World Precision Instruments, Sarasota, FL). Mitochondria were suspended in a buffer composed of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris–HCl and 5.0 mM KH_2PO_4, pH 7.2, supplemented with 0.1% (w/v) bovine serum albumin (fraction V, fatty acid-free). The rate of ADP-stimulated oxygen consumption (i.e. state 3 respiration) was registered using a mixture of 40 μM palmitoyl-CoA, 2 mM L-carnitine and 2.5 mM L-malate. State 3 was monitored as the oxygen consumption rate following the addition of 600 μM ADP. All experiments were performed at 30 °C.

2.6. CPT1 activity

CPT1 enzymatic activity in isolated cardiac mitochondria was measured by monitoring the formation of the palmitoyl-ester of L-[*methyl*-^{3}H]-carnitine (specific activity 80.0 Ci/mmol), as previously described (Grantham and Zammit, 1986, 1988). CPT1 activity was sensitive to inhibition by malonyl-CoA (data not shown).

2.7. Myocardial levels of carnitine

Tissue content of both free L-carnitine and (medium- and long-chain) acyl-carnitine was determined using an enzymatic cycling method with carnitine dehydrogenase, as previously described (Nakamura et al., 1999; Takahashi et al., 1994). Quantitation of total carnitine was performed by determining the content of free L-carnitine upon hydrolysis of acyl-carnitine esters by L-acetylcarnitine hydrolase. Acyl-carnitine levels were calculated as the difference between the content of total carnitine and free L-carnitine. Enzymes and the assay kit for analysis of total and free carnitine levels (Kainos Laboratories, Inc., Tokyo, Japan) were a gift of Dr. Hirohiko Kuramatsu (Osaka University, Japan).

2.8. Statistical analysis

For two-group comparisons, results were analyzed using a two-sided Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. For multi-group comparisons, results were analyzed using the ANOVA *F*-test, together with the Tukey–Kramer procedure, setting the overall family wise confidence level at 95%. Linear regressions were obtained using GraphPad Prism 5 software (GraphPad Software Inc., USA). Additionally, hyperbolic kinetics of CPT1 was established by comparing data fitted to either Michaelis–Menten or allosteric–sigmoidal models, using GraphPad Prism 5.

3. Results

3.1. CPT1 activity selectively declines in interfibrillar mitochondria and impairs fatty acid-driven bioenergetics in the aged rat heart

In order to investigate how age affects fatty-acid supported bioenergetics in the two mitochondrial subpopulations, both fatty acyl-CoA-mediated oxygen consumption characteristics and CPT1

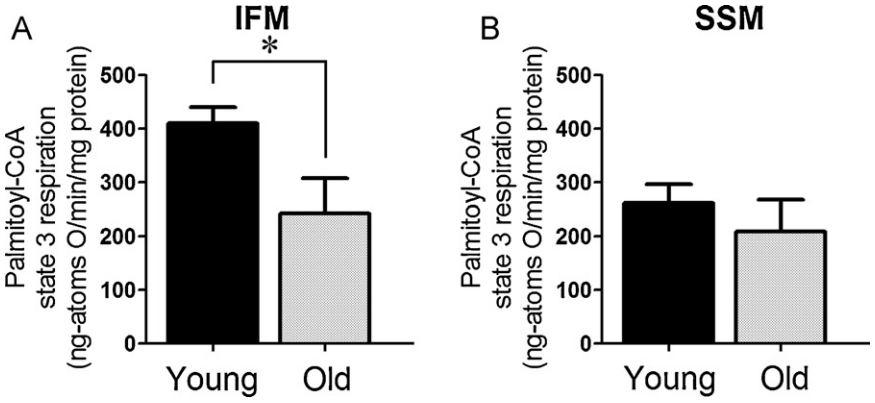

Fig. 1. Fatty acid-driven respiration selectively declines with age in rat heart interfibrillar mitochondria. Palmitoyl-CoA-supported state 3 respiration was determined in two subpopulations of cardiac mitochondria, IFM (A) and SSM (B), isolated from young ($n = 6$) and old ($n = 4$) Fischer 344 rats. Mitochondrial oxygen consumption was measured as described in Section 2.5. All results are presented as the mean ± SEM. $^{*}p < 0.05$ compared to young animals.

activity were monitored. As shown in Fig. 1A, palmitoyl-CoA-supported state 3 respiration significantly declined (41%, $p < 0.05$) in IFM isolated from old versus young animals. However, no significant changes in mitochondrial respiration were observed with age in SSM (Fig. 1B). In concert with these results, there was no significant difference in CPT1 activity in SSM; however, we observed a significant 28% loss in CPT1 activity in the IFM fraction (Table 1). Because release of IFM from the myofibrils during the isolation process requires treating heart homogenates with a protease (see Section 2.4) that could differentially damage CPT1 in IFM, control experiments were performed to investigate whether the apparent decline in IFM CPT1 activity stemmed from an artifact of the isolation procedure or was a result of the aging process. Treatment of myofibrillar fractions with increasing concentrations of protease (i.e. from 0.3 mg/g tissue to 30 mg/g tissue) did not significantly affect CPT1 activity in IFM from old rat hearts. This indicates that CPT1 is not highly sensitive to the brief and relatively mild proteolytic treatment employed for IFM isolation. Moreover, further experiments where tissue homogenates were similarly treated with protease revealed no significant alterations of CPT1 activity in SSM of either young or old rat hearts (data not shown). Therefore, we conclude that the loss of CPT1 activity in IFM is a result of the aging process, and suggests that a differential decline in fatty acid-supported mitochondrial energy transduction occurs with age.

3.2. Aging alters CPT1 enzyme parameters in rat heart interfibrillar mitochondria

The CPT1 enzyme displays a complex mode of catalysis where overall catalytic activity can be affected by substrate affinity for either L-carnitine or fatty acyl-CoA. In order to discern a plausible mechanism as to the age-related decline in IFM CPT1 activity, kinetic parameters for both enzyme substrates were monitored. Lineweaver–Burk plots for palmitoyl-CoA utilization indicated that both V_{max} and K_m decrease with age (Fig. 2A), a kinetic shift which resembles uncompetitive inhibition. Further examination of the data using Eadie–Hofstee plots showed that V_{max} and K_m declined by 60% ($p < 0.05$) and 49% ($p < 0.05$), respectively (Fig. 2B). On the other hand, double-reciprocal plots with respect to L-carnitine did not indicate significant differences in CPT1 kinetic parameters in IFM from old relative to young animals (Fig. 2C). This observation was corroborated by Eadie–Hofstee analysis for both V_{max} and K_m (Fig. 2D). Taken together, these results show that the age-associated loss of CPT1 activity in IFM is mainly caused by a decrease in V_{max} for palmitoyl-CoA without a significant change in enzyme affinity for L-carnitine.

In order to elucidate how the age-associated changes in kinetic parameters with respect to palmitoyl-CoA could regulate the catalytic cycle of IFM CPT1, the data were further analyzed using a theoretical model. Fig. 3A shows that when a decrease in V_{max} predominates over an apparent increase in substrate affinity (i.e. lower K_m), the global result is a loss of catalytic efficiency. Moreover, this dominance is accentuated when substrate concentration is close to the K_m, which is most likely the case for catalysis under physiological conditions. Based on this theoretical construct, our data showing an age-related decrease of 60% and 49% in V_{max} and K_m (Fig. 2B), respectively, suggest that IFM CPT1 is 20% less catalytically efficient with age. This would result in a 45% decline in overall enzyme activity when the concentration of palmitoyl-CoA nears the K_m of the enzyme (i.e. 200 μM) (Fig. 3B). Thus, assuming that there is no age-dependent change in CPT1 levels (a reasonable assumption given no change in carnitine-supported kinetics), this analysis supports the concept that the palmitoyl-CoA-CPT1 complex is destabilized in IFM of aging rat hearts.

Table 1
CPT1 activity in two subpopulations of rat heart mitochondria.

Mitochondrial subpopulation	CPT1 activity[a] (nmol/min/mg protein)	
	Young	Old
Subsarcolemmal mitochondria	7.33 ± 0.42	7.17 ± 0.46
Interfibrillar mitochondria	7.71 ± 0.43	5.59 ± 0.41[b]

Results are presented as the mean ± SEM. In all groups $n = 7$.
[a] Using to a concentration of 70 μM palmitoyl-CoA and 400 μM L-carnitine in the assay mixture.
[b] $p < 0.05$ versus young IFM.

3.3. Carnitine levels decline with age and are partially restored by dietary supplementation with ALCAR

Because substrate limitations per se would adversely affect the formation of palmitoyl–carnitine at the organ level even in the absence of structural alterations of the IFM CPT1 enzyme, we also explored the possibility that lower fatty acid-supported bioenergetics was exacerbated by the age-related decline in myocardial levels of L-carnitine. To this end, both free carnitine and acyl-carnitine levels were measured in cardiac tissue from young and old animals. As shown in Fig. 4, aging leads to a decrease in the content of myocardial carnitine. With respect to young rat hearts, levels of free

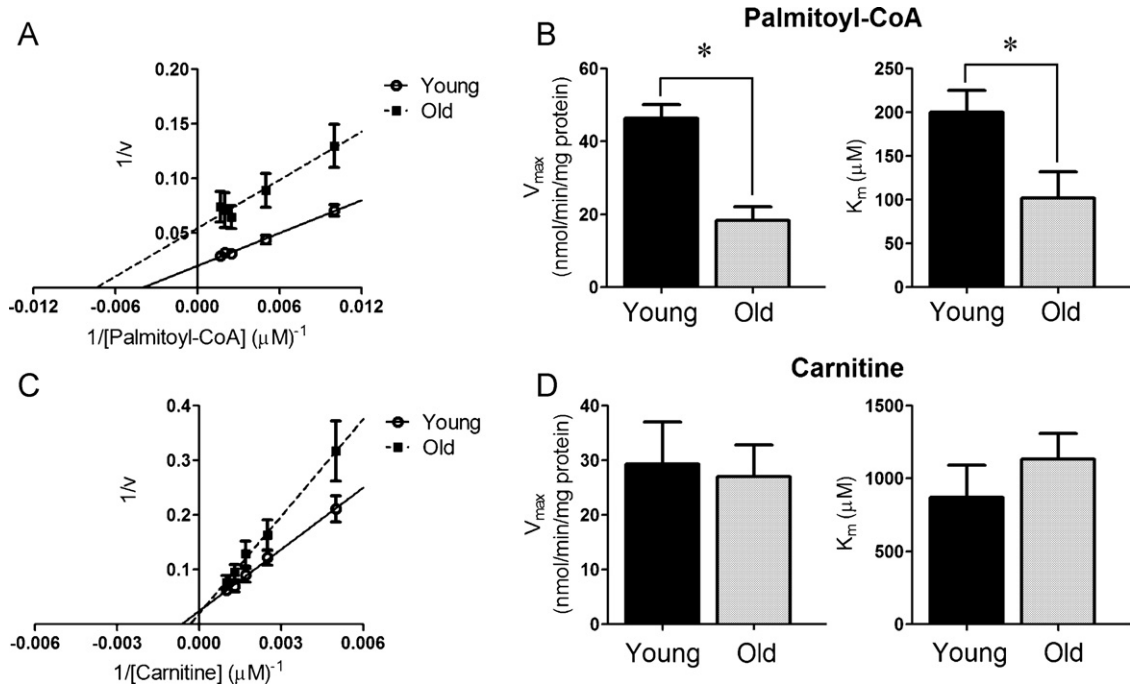

Fig. 2. Age-associated changes in CPT1 enzyme kinetic parameters in rat heart interfibrillar mitochondria. CPT1 activity was measured in IFM as described in Section 2.6. For analysis of palmitoyl-CoA-mediated enzyme kinetics (A and B), CPT1 activity was measured using increasing concentrations of palmitoyl-CoA while maintaining L-carnitine at 400 μM (A and B). Conversely, the effect of increasing L-carnitine levels on CPT1 activity was measured in (C and D) where palmitoyl-CoA levels were kept constant at 70 μM palmitoyl-CoA. Panels (A) and (C) show double reciprocal plots with respect to palmitoyl-CoA and L-carnitine concentrations, respectively. For each concentration of substrate used, independent experiments were done using IFM from both young ($n = 5$) and old ($n = 5$) rats. Eadie–Hofstee plots of the raw data were generated with respect to palmitoyl-CoA (B) and L-carnitine (D) to assess both K_m and V_{max} for each substrate. Reciprocal velocity in (A) and (C) is expressed in $(nmol/min/mg\ protein)^{-1}$. All results are presented as the mean ± SEM. $^*p < 0.05$ compared to young animals.

carnitine (Fig. 4A), acyl-carnitine (Fig. 4B), and total carnitine (Fig. 4C) declined by 38% ($p < 0.05$), 56% ($p < 0.05$) and 42% ($p < 0.05$), respectively, in old animals. Thus, fatty acid-mediated mitochondrial respiration may be attenuated by limited myocardial L-carnitine content.

As previous studies show that carnitine levels increase in cardiac tissue following ALCAR supplementation, we fed ALCAR to aged rats for up to four weeks prior to sacrifice and mitochondrial isolation. ALCAR supplementation failed to remediate the loss of either free- (Fig. 4A) or total-carnitine content (Fig. 4C) over a two-week supplementation period; however, acyl-carnitine levels were restored to those seen in young controls (Fig. 4B). Overall, ALCAR feeding established a new equilibrium in the cardiac acyl-carnitine/free carnitine ratio, resulting in a higher proportion of acyl-carnitine in the aging myocardium (Fig. 4C). These results suggest that CPT1 activity increased after ALCAR treatment, which is shown in Fig. 5.

3.4. Feeding ALCAR to old rats reverses the age-related decline in CPT1 activity in interfibrillar mitochondria

As shown in Fig. 5A, dietary supplementation of old rats with ALCAR increased IFM CPT1 activity over a four-week timecourse. Nevertheless, this increase was gradual and CPT1 activity did not advance to levels evident in young rats until relatively late in the timecourse (Fig. 5A and C). On the other hand, ALCAR did not significantly affect CPT1 activity in SSM (Fig. 5B and D). Thus, our results indicate that feeding ALCAR to old rats preserves fatty acid-supported mitochondrial bioenergetics by restoring the age-related decline in CPT1 activity in heart IFM. ALCAR does not appear to change substrate (i.e. L-carnitine) availability per se as the same dietary intervention had no significant effect on the reaction catalyzed by CPT1 in SSM.

4. Discussion

The present work provides a new perspective on the age-associated decline in fatty acid-supported cardiac bioenergetics. To the best of our knowledge, this is the first side-by-side characterization of CPT1 activity in two sublocalized mitochondrial populations of the aging heart. Our data show that age induces a ~30% decline in CPT1 activity, but only in IFM (Table 1 and Fig. 5). While this activity loss is consistent with previous observations for diminished fatty acyl-CoA supported bioenergetics in hearts from old rats and mice (Abu-Erreish et al., 1977; McMillin et al., 1993; Odiet et al., 1995), our results now indicate that the aging lesion for CPT1 is specific to mitochondria intercalated along the myofibrils. As the IFM supply ATP to the actomyosin complex during the contraction–relaxation cycle (Hoppel et al., 1982; Kaasik et al., 2001), cardiac pump function may therefore be even more adversely affected with age than the subtle loss in overall CPT1 activity would generally indicate. These results thus have important implications for myocardial energy reserve capacity.

Based on both the observational and theoretical data generated in this study, it appears that the mechanism(s) underlying lower IFM CPT1 activity is complex. Modeling enzyme activity versus alterations in catalytic efficiency (Fig. 3A) suggests that the observed age-associated changes in K_m and V_{max} for palmitoyl-CoA account for essentially all of the observed loss of CPT1 activity. Thus, even though there is a general age-dependent decrease in myocardial L-carnitine levels, its decline has little consequence to enzyme activity. These rather surprising results actually reinforce the concept that CPT1 activity loss is localized to a specific mitochondrial subpopulation as diminished L-carnitine content would adversely affect CPT1 activity in the SSM fraction as well. Considering that we also previously showed no age-associated

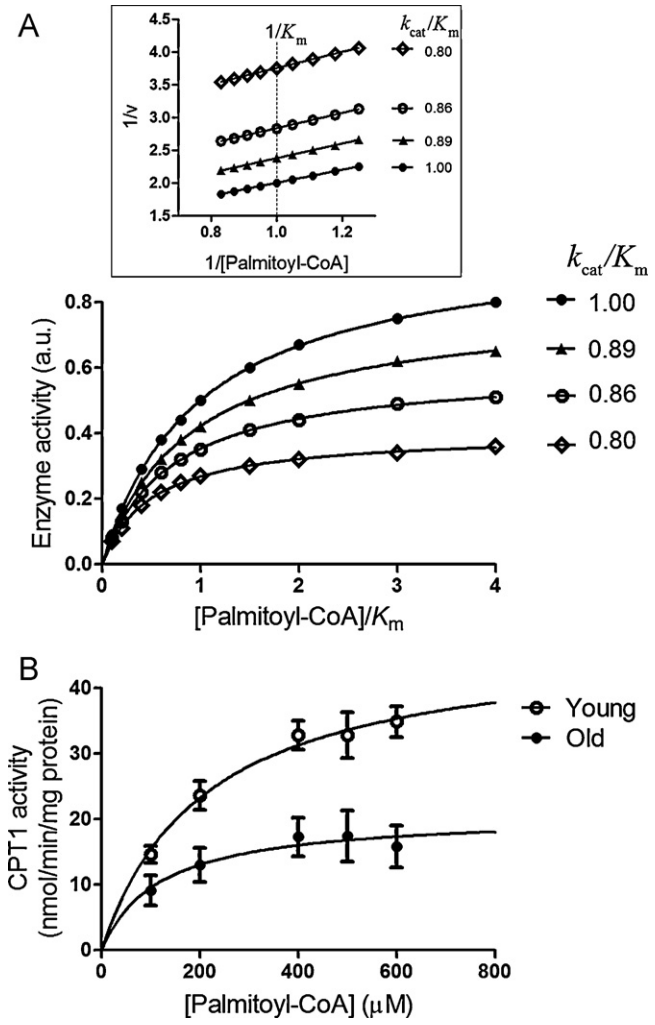

Fig. 3. Theoretical analysis how CPT1 enzyme activity is modulated by fractional changes in V_{max}, K_m, and the turnover number (k_{cat}) for palmitoyl-CoA. Enzyme rate (v) is expressed in terms of total enzyme [E_t] and substrate [S] concentration, as $v = k_{cat}[E_t][S]/(K_m + [S])$ (A). This analysis was performed relative to the reference state where [E_t], V_{max}, K_m, and k_{cat}/K_m were set to 1.0 arbitrary unit (a.u.). A decrease in V_{max} when K_m also declines results in lower CPT1 catalytic efficiency when changes in V_{max} predominate over variations in K_m. Enzyme kinetics follows an uncompetitive inhibition-like behavior and is significant when [palmitoyl-CoA] = $K_m \pm 0.2\ K_m$ (see also inset). Age-associated decline in CPT1 kinetics in rat heart IFM (B).

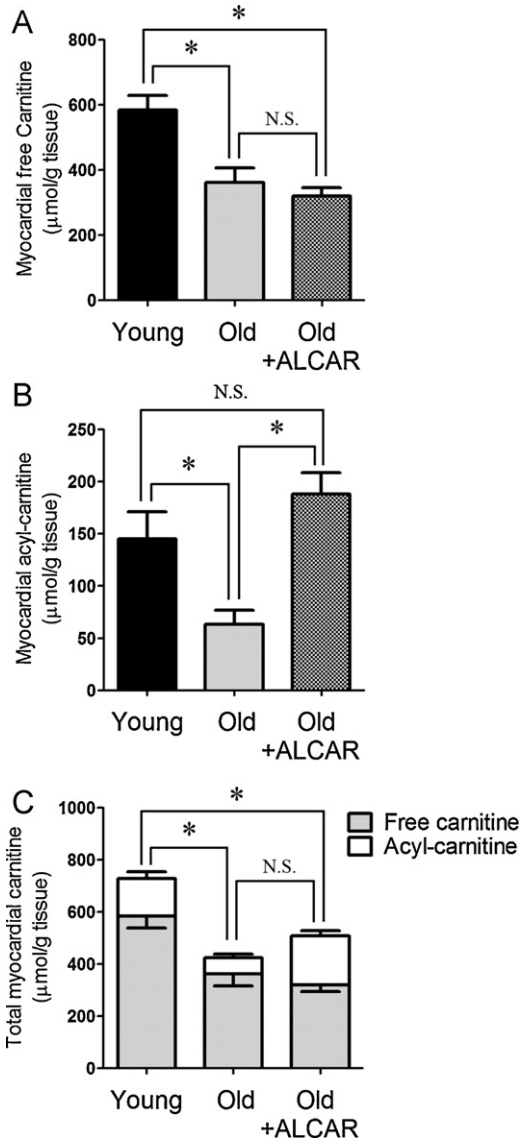

Fig. 4. Myocardial carnitine levels decline with age but can be partially restored by dietary supplementation with acetyl-L-carnitine. Myocardial content of free carnitine and acyl-carnitine was determined as described in Section 2.7. Free carnitine in cardiac tissue from young ($n = 6$), old ($n = 5$) and ALCAR-supplemented old rats (old + ALCAR; $n = 5$) (A). Myocardial acyl-carnitine levels (B). Total carnitine content and its distribution in cardiac tissue (C). For (A) and (B), results are presented as the mean ± SEM. Error bars in (C) correspond to acyl-carnitine (upward) and free carnitine (downward). For simplification, error bars for total carnitine values are not shown. $^*p < 0.05$ compared to young controls.

alterations to malonyl-CoA levels in the aging rat heart (Moreau et al., 2004), it now becomes clear that the age-specific loss of IFM CPT1 catalytic efficiency stems from modifications to the enzyme and is not because of alterations to substrate levels or allosteric effectors.

CPT1 follows a bi–bi ordered mode of catalysis where binding of a long-chain fatty acyl-CoA molecule to the enzyme active site initiates the reaction (McGarry and Brown, 1997; Ramsay et al., 2001). Mutations of glycine residues in human hepatic CPT1 specifically affect the palmitoyl-CoA binding pocket and lead to loss of enzyme function (Gobin et al., 2003; Morillas et al., 2004). Furthermore, enzyme activity was completely abolished in mitochondria from yeast expressing the mutant liver-specific CPT1 protein where Gly^{710} was changed to a Glu residue in the protein hydrophobic core (Gobin et al., 2003). We therefore propose that the loss of catalytic efficiency for palmitoyl-CoA utilization stems from limitations in its binding to the enzyme, thereby lowering overall catalytic activity even when L-carnitine levels are saturating. This type of altered enzyme kinetics is akin to an uncompetitive mode of inhibition where alterations of the enzyme substrate complex adversely affect overall catalytic efficiency of the enzyme (Fig. 6).

The precise cause(s) leading to this uncompetitive mode of IFM CPT1 inhibition was not directly explored in the present study, and may be multifactorial. A plausible hypothesis is that age-associated oxidative modifications that selectively affect IFM CPT1 alter its catalytic activity by destabilization of palmitoyl-CoA, and that long-term supplementation of ALCAR reverses such alterations (see below). This concept is buttressed by previous reports showing that both CPT1 and carnitine octanoyltransferase contain specific binding sites for L-carnitine, acyl-CoA, and CoASH where amino acid residues in or proximal to the acyl-CoA binding region are key to stabilizing the acyl-CoA–enzyme complex during catalysis (Jogl et al., 2005; Morillas et al., 2004). Thus, reactive oxygen and nitrogen species, electrophiles, or small molecule

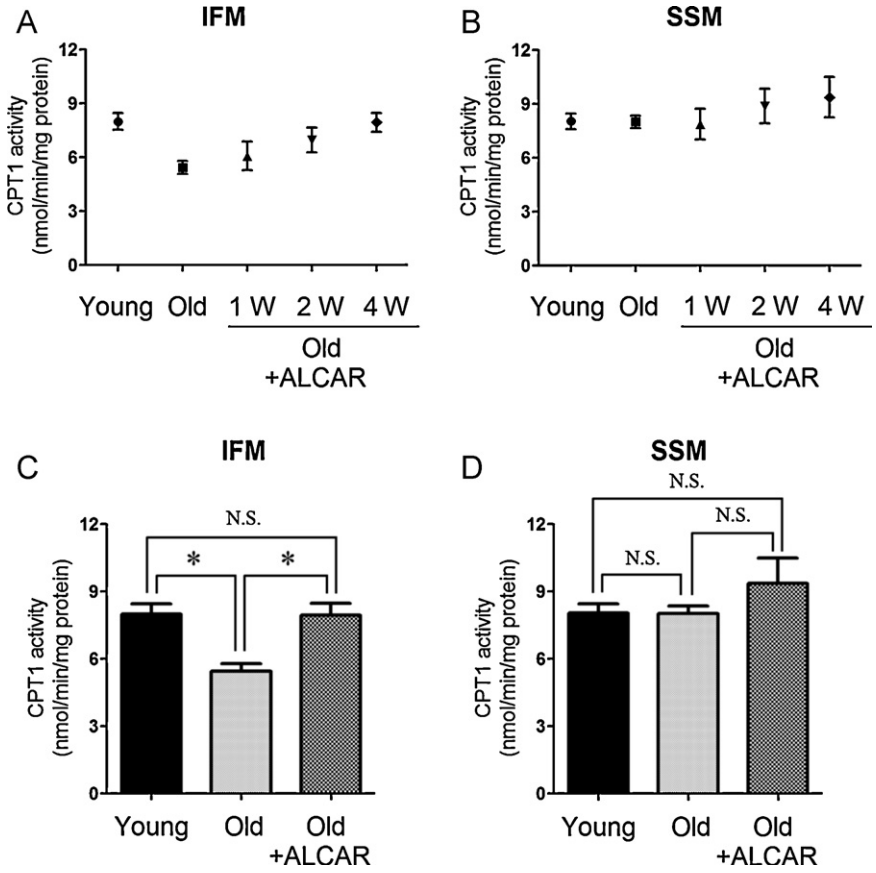

Fig. 5. Dietary supplementation with acetyl-L-carnitine restores the age-related loss of CPT1 activity in rat heart interfibrillar mitochondria. CPT1 activity was measured using a concentration of 70 μM palmitoyl-CoA and 400 μM L-carnitine in the assay mixture, as described in Section 2.6. Enzyme activity in IFM (A and C) and SSM (B and D) isolated from young ($n = 4$), old ($n = 4$) and old + ALCAR ($n = 4$) rat hearts. Old animals were given ALCAR for one (1 W, $n = 4$), two (2 W, $n = 4$) or four (4 W, $n = 4$) weeks, as described in Section 2.3 (A and B). (C) and (D) correspond to four weeks of supplementation with ALCAR. All results are presented as the mean ± SEM. $^*p < 0.05$ compared to young animals.

conjugation, may alter critical amino acid residues at or near the palmitoyl-CoA binding pocket and limit formation of the initial enzyme–palmitoyl-CoA complex (Fig. 6).

While characterization of specific protein modification(s) that lead to the aging lesion in IFM CPT1 catalysis is beyond the scope of the present study, there is literature precedent suggesting that IFM are particularly susceptible to protein modification. First, we previously showed that aging leads to higher rates of IFM oxidant appearance versus SSM (Suh et al., 2003), which could promote oxidative modifications that selectively affect that mitochondrial subpopulation. Second, Lesnefsky et al. (2001b) further reported that the cytochrome *c* binding site of complex III was specifically

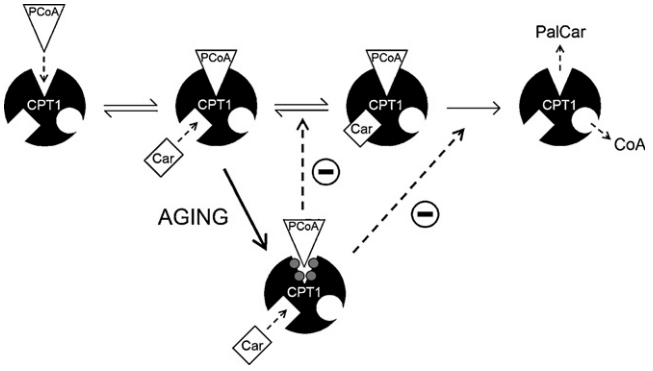

Fig. 6. Schematic representation of the age-associated decline in CPT1 catalytic activity. Alterations of the CPT1–palmitoyl-CoA complex are hypothesized to act as an 'upstream' inhibitor of the formation of palmitoyl-L-carnitine. PCoA: palmitoyl-CoA; Car: L-carnitine; and CoA: coenzyme A.

altered with age, but only in the IFM subpopulation. On the other hand, Sharma et al. (2010) recently showed that incubating cardiac mitochondria with peroxynitrite ($ONOO^-$) initiated post-translational modifications of specific cysteine and tyrosine residues of CPT1, which in turn modulated enzyme activity. Taken together, these results suggest that aging causes an enhanced pro-oxidant milieu that is specific for IFM, thereby initiating conditions that adversely affect CPT1 catalysis in that mitochondrial subpopulation. We are currently examining the linkage between protein modification and the specific alterations to CPT1 activity seen in IFM of aging rat hearts.

We and others have previously found that supplementation of old rats with ALCAR remediates the age-related decay in mitochondrial bioenergetics in liver (Hagen et al., 1998a,b), heart (Paradies et al., 1994, 1999), muscle (Pesce et al., 2010) and brain (Liu et al., 2002). ALCAR has been postulated to mediate this improvement in a straightforward manner, namely, by replenishing L-carnitine levels which otherwise decline with age (Costell and Grisolia, 1993; Costell et al., 1989; Maccari et al., 1990; Tanaka et al., 2004). However, this report shows that even though ALCAR supplementation remediated overall IFM CPT1 activity loss, its mechanism(s) of action may be distinct from its essential role involving mitochondrial fatty acyl-CoA import. This concept is consistent with our current results showing no role for L-carnitine on the age-associated decline in CPT1 catalytic efficiency, and also by the observation that IFM CPT1 catalysis improved slowly over the month-long ALCAR supplementation period. These results suggest a far more complex mechanism of action for this metabolite, which is not consistent with an immediate, direct replenishment of myocardial carnitine levels. Precisely how ALCAR improves IFM CPT1 activity in aged rat hearts is

not currently known; however, a significant body of evidence indicates that it facilitates a number of metabolic changes (Hagen et al., 1998a, 2002; Musicco et al., 2009, 2011; Pesce et al., 2010), which may ultimately improve CPT1 activity specifically in IFM. Even though ALCAR is not a classical free radical terminating molecule, nevertheless, it decreases the formation of nitro-tyrosine protein adducts in alcohol-induced brain damage (Rump et al., 2010), and limits oxidative damage in the heart following ischemia/reperfusion injury (Calvani et al., 2000; Cui et al., 2003). Also, proteomic analysis of aging rat brain shows that ALCAR lowers levels of protein carbonylation (Poon et al., 2006). Furthermore, Gadaleta and coworkers revealed that the mitochondrial isoform of peroxiredoxin III was less oxidatively modified and more active in livers from old rats supplemented with ALCAR (Musicco et al., 2009). Additionally, ALCAR initiates increased mitochondrial biogenesis and turnover (Cassano et al., 2006; Pesce et al., 2010), which would promote clearance of damaged IFM CPT1 proteins. Finally, ALCAR rebalances membrane phospholipid content, and for mitochondria, reverses the age-related decline in cardiolipin levels, a key phospholipid necessary for proper electron transport chain function (Hagen et al., 1998a,b; Paradies and Ruggiero, 1990; Paradies et al., 1995). Taken together, ALCAR may indirectly improve IFM CPT1 activity by maintaining protein integrity through membrane restructuring or limiting oxidative damage, which otherwise specifically increases in the IFM subpopulation with age. This hypothesis is supported by evidence that ALCAR increases cellular stress response, through the regulation of gene expression and the synthesis of important proteins that sustain the oxidative stress defense (Calabrese et al., 2010, 2011). In particular, ALCAR stimulates expression of heme oxygenase-1 (HO-1) and endothelial NO synthase (eNOS) in human endothelial cells during H_2O_2-induced oxidative stress (Calo et al., 2006). Also, ALCAR decreases amyloid beta-mediated oxidative damage in primary cortical neuron cultures by again increasing expression of both HO-1 and heat shock protein 70 (Hsp70) (Calabrese et al., 2010). Furthermore, in rats exposed to γ-radiation, ALCAR reverses the loss of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), restores GSH levels, and prevents the accumulation of malondialdehyde (MDA) in the liver and the lungs (Mansour, 2006). Thus, it is conceivable that ALCAR improves IFM CPT1 activity by preventing oxidative damage to the protein.

Our current data warrants more detailed studies to discern the mechanisms involved in ALCAR-dependent remediation of CPT1 activity. Regardless of the precise nature of its action, however, it is clear that ALCAR improves IFM CPT1 catalysis and thus should be considered as a potential therapy for maintaining cardiac bioenergetics in the aging heart.

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