

# Caloric restriction delays cardiac ageing in rats: role of mitochondria

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**Aims** We tested whether long-term caloric restriction (CR) corrects pre-existing manifestations of cardiac ageing in rats.

**Methods and results** The age-specific effects of CR (−40%, 6 months) on mortality, left ventricular (LV) function, mitochondrial function, oxidative damage, and apoptosis were analysed in young (6 + 6 months) and senescent rats (24 + 6 months). CR in senescent rats significantly reduced mortality. LV and cardiomyocyte hypertrophy were reduced together with the mRNA expression and plasma concentrations of overload indicators BNP/ANP. Mitochondrial function was improved, resulting in lower oxidative damage and apoptotic activation. In particular, the pro-apoptotic Bcl-xS/Bcl-xL isoform pattern, mitochondrial translocation of Bax, release of cytochrome C into cytosol, and caspase-9 activation were reduced in comparison to age-matched rats on the control diet. However, CR resulted only in minor changes in young rats. Serum obtained from old control or CR rats was used for *in vitro* experiments. H9C2 cardiomyoblasts and adult rat ventricular cardiomyocytes preconditioned with CR serum demonstrated a low Bcl-xS/Bcl-xL ratio. H9C2 cells were resistant against H<sub>2</sub>O<sub>2</sub>-mediated loss of mitochondrial membrane potential, apoptosis activation, and reduced cell viability. Thus, beneficial effects of CR are mediated through circulating factors and can be mimicked with CR serum. However, this protection critically depended on a high Bcl-xL protein expression as seen after siRNA-mediated Bcl-xL knockdown.

**Conclusion** CR is cardioprotective in senescent myocardium by correcting pre-existing mitochondrial dysfunction and apoptotic activation and by preventing deterioration in LV function. Therefore, interventions that mimic these effects of CR may represent an additional therapeutic option for the aged or failing heart.

**Keywords** Ageing • Mitochondria • Caloric restriction • Cardiac function • Apoptosis

## 1. Introduction

Long-term caloric restriction (CR) is known to retard the ageing process in many organisms including mammals, but the basic mechanisms of its efficacy remain unclear and its actions in different organs are remarkably heterogeneous.<sup>1</sup> Previous data from our laboratory have shown that the protective effects of moderate CR on muscular tissues differ between young and senescent animals.<sup>2</sup> A global analysis of gene expression in murine hearts indicates (among others) that long-term CR protects cardiomyocytes from age-associated apoptosis by reducing endogenous DNA damage, enhancing DNA repair capacity, reducing the expression of pro-apoptotic genes, and inducing apoptosis-inhibitory genes.<sup>3</sup> This fits nicely to histological data on reduced numbers of cardiomyocytes, enlargement of myocytes, and signs of activated cell death in myocytes as hallmarks of aged hearts.<sup>4</sup>

We hypothesized that a basic mechanism for the cardioprotective effects of CR is the attenuation of age-associated enhancement of mitochondrial susceptibility for cell death activation. Bcl-2 family proteins are known to affect cell survival by regulating the permeability of mitochondria. The ratio of pro- and anti-apoptotic Bcl-2 family proteins can determine cell fate also in the heart (recent review in ref.<sup>5</sup>). Previous data from our laboratory are supportive for a role of pro- and anti-apoptotic splice isoforms of the *Bcl-x* gene in cardiomyocytes: experimental augmentation of pro- vs. anti-apoptotic *Bcl-x*-isoform enhances apoptotic susceptibility and induces signs of mitochondrial dysfunction.<sup>6</sup>

In the present study, we performed 6 months of CR (−40%) in young and senescent animals to address the following questions: (i) whether CR will result in changes in cardiac and mitochondrial function of young and senescent rats; (ii) whether a modulation of

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apoptotic activation can be induced by CR; and (iii) whether CR started late in life for a limited time is sufficient to reverse pre-existing alterations. Furthermore, we tested whether the physiological effects observed in CR animals including increased tolerance to oxidative stress can be simulated at the cellular level in H9C2 cardiomyoblasts cultured with media supplemented with serum obtained from animals fed a CR diet.

## 2. Methods

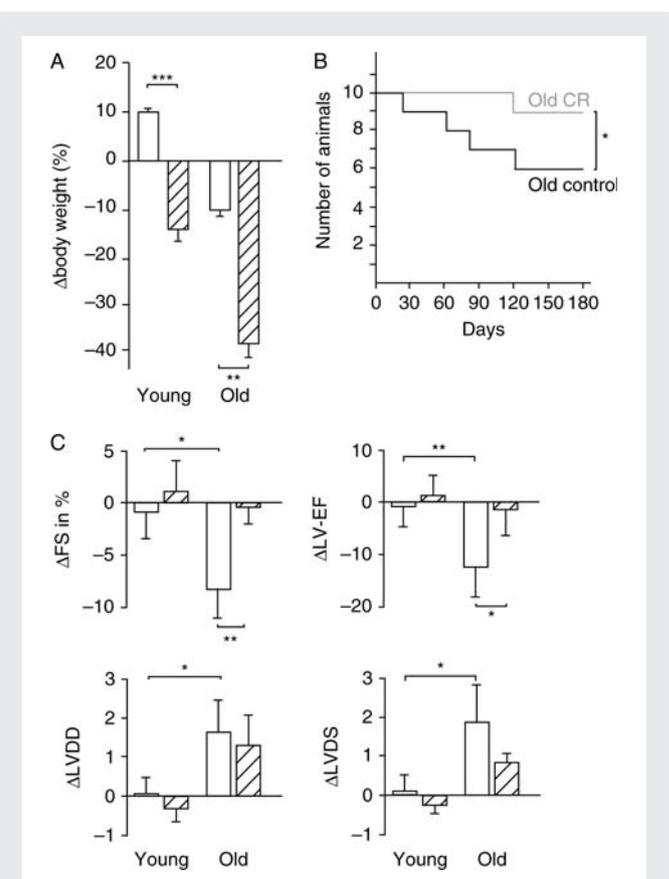
### 2.1 Animals and diet protocol

Sprague–Dawley rats at the age of 6 months (young animals) or at the age of 24 months (old animals) were randomly assigned to one of the following diets for the next 6 months: rats on 'control diet' (CD) received their individual pre-diet average of Altromin<sup>R</sup> 1244 (2550 cal/g). Rats subjected to CR received also their pre-diet average, but of a calorically reduced, fibre-rich diet (Altromin<sup>R</sup> 1344/1500; 1550 cal/g) as described before.<sup>7</sup> The animals were assigned to one of the diets as matched pairs according to their left ventricular (LV) function. In addition to these rats, two groups of male rats (6 months or 24 months old, each  $n = 6$ ), kept on control diet, were sacrificed and characterized for gene expression and mitochondrial function in order to define the basic status at this age prior to diet. All animals were handled in accordance with a protocol approved by the animal care and use committee of the Martin Luther University Halle-Wittenberg (#2-677 MLU) and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996). Details on all other methods including echocardiography, real-time PCR, western blotting, analyses of mitochondrial function, cell culture and treatment of H9C2 cardiomyoblasts, isolation and treatment of adult rat cardiomyocytes, cell-based assays (caspase activity, mitochondrial membrane potential, cell death detection, cell viability), histological analyses of LV sections [cardiomyocyte cross-sectional area (CSA), TUNEL assay, Sirius Red staining, senescence-associated beta-galactosidase activity (SA  $\beta$ -gal)], tissue collagen content, or plasma analyses are provided in the Supplementary material online.

## 3. Results

### 3.1 Systemic effects of caloric restriction

In old rats on control diet surviving the entire protocol (24 + 6 months,  $n = 6$ ), body weight (BW) declined during the protocol. This decline was further augmented in old CR animals (24 + 6 months,  $n = 9$ , Figure 1A). In young growing rats under control diet (6 + 6 months,  $n = 8$ ), BW increased, while 40% CR (6 + 6 months,  $n = 8$ ) resulted in a reduction (Figure 1A). None of the young animals died during the study (not shown), but survival was significantly increased in old CR animals compared with old controls (Figure 1B). Ventricular weights were higher in old control rats than in young controls and were significantly decreased in young and old animals after 6 months CR (Table 1). This results in increased LV/BW ratio (Table 2) in young CR rats (12%), but reduced LV/BW in old CR rats (−8%). Insulin, leptin, and triglyceride plasma levels demonstrated a significant reduction together with a strong induction of plasma adiponectin in young CR animals (Table 2), indicating a change in insulin sensitivity. Minor changes in these plasma parameters were also observed in old CR rats (Table 2).



**Figure 1** Effects of 6 months CR on body weight, survival, and LV function. (A) Body weight alterations during 6 months of control diet and 6 months of CR in young rats ( $n = 8$  for each diet) and in old rats ( $n = 6$  for control diet and  $n = 9$  for caloric restriction). (B) Survival in old animals under 40% CR (depicted in grey) compared with old animals under control diet (depicted in black). (C) Changes in LV fractional shortening ( $\Delta$ FS%, upper left panel), LV ejection fraction ( $\Delta$ EF%, upper right panel), LV end-diastolic diameter ( $\Delta$ LVDD, lower left panel), and LV end-systolic diameter ( $\Delta$ LVDS, lower right panel) during 6 months of the study in young and old rats under 40% CR (hatched columns) and age-matched animals under control diet (open columns). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Table 1** Effects of caloric restriction on LV and body weight

	Diet	Young rats	$n$	Old rats	$n$
Left ventricle (g)	CD	1.19 $\pm$ 0.03	8	1.75 $\pm$ 0.06 <sup>†</sup>	6
	CR	1.02 $\pm$ 0.01**	8	1.14 $\pm$ 0.05**	9
Body weight (g)	CD	500 $\pm$ 15		494 $\pm$ 21	
	CR	382 $\pm$ 9***		351 $\pm$ 13***	
LV/BW (g/kg)	CD	2.39 $\pm$ 0.04		3.54 $\pm$ 0.08 <sup>‡</sup>	
	CR	2.67 $\pm$ 0.04*		3.27 $\pm$ 0.02	

CD, rat with control diet; CR, rat with caloric restriction; LV/BW, left ventricular weight/body weight.

\* $P < 0.05$  vs. respective control diet.

\*\* $P < 0.01$  vs. respective control diet.

\*\*\* $P < 0.001$  vs. respective control diet.

<sup>†</sup> $P < 0.001$  vs. control diet in young rats.

<sup>‡</sup> $P < 0.01$  vs. control diet in young rats.

**Table 2** Effects of caloric restriction on left ventricular function and biochemical parameters

Systolic parameters	Young CD (n = 8)	Young CR (n = 8)	Old CD (n = 6)	Old CR (n = 9)
% FS				
Beginning of diet	44.6 ± 4.1	43.2 ± 5.5	32.0 ± 0.2 <sup>†</sup>	29.9 ± 2.6
End of diet	43.7 ± 1.6	44.3 ± 2.7	24.7 ± 3.3 <sup>‡</sup>	29.6 ± 1.6*
% EF				
Beginning of diet	81.8 ± 2.3	80.4 ± 2.6	67.2 ± 1.4 <sup>†</sup>	64.9 ± 3.8
End of diet	78.4 ± 3.1	81.8 ± 1.6	54.1 ± 6.1 <sup>‡</sup>	63.2 ± 3.1*
Serum parameters				
Glucose (mmol/L)	7.4 ± 0.6	6.9 ± 0.9	7.5 ± 1.1	6.8 ± 0.7
Insulin (pM)	219 ± 16	106 ± 23*	375 ± 38 <sup>‡</sup>	246 ± 25*
Triglycerides (mg/dL)	148.4 ± 31.2	95.9 ± 16.8*	198.1 ± 22.3	152.6 ± 16.2*
Adiponectin (ng/mL)	16.6 ± 2.9	41.2 ± 7.8***	5.1 ± 1.5 <sup>‡</sup>	8.4 ± 2.6*
Leptin (pg/mL)	23.1 ± 3.8	3.7 ± 1.1***	30.3 ± 5.2	23.1 ± 3.7*
BNP (ng/mL)	0.01 ± 0.005	0.01 ± 0.004	1.92 ± 0.29 <sup>‡</sup>	0.35 ± 0.18***

CD, rat with control diet; CR, rat with caloric restriction, % FS, mid-wall-fractional shortening, % EF, ejection fraction.

\*P < 0.05 vs. respective control.

\*\*\*P < 0.001 vs. respective control.

<sup>†</sup>P < 0.05 vs. young control.

<sup>‡</sup>P < 0.001 vs. young control.

### 3.2 Effects of caloric restriction on left ventricular systolic function

LV function was not altered during the course of the study in young animals (Table 2 and Figure 1C). However, LV fractional shortening and ejection fraction declined significantly during the period of control diet in old rats (Table 2 and Figure 1C). This age-associated decline in LV fractional shortening was prevented by CR in old rats (Table 2 and Figure 1C). Similar alterations were calculated for the ejection fraction (Table 2 and Figure 1C). Besides the beneficial effects of CR on fractional shortening and ejection fraction (Table 2), these old animals also demonstrated a trend for diminished progression of LV dilatation (Figure 1C) and left atrial distension under CR (data not shown). Accordingly, plasma BNP as a clinically established marker for heart failure was significantly increased in old control animals (Table 2). CR resulted in a significantly lower level in old animals, but did not alter plasma BNP in young animals (Table 2). LV ANP and BNP mRNA expression were both significantly increased in senescent rats on control diet (Table 3). Whereas ANP mRNA was reduced by CR in young and in old animals (Table 3), BNP mRNA responded only in old animals with a significant reduction after 6 months of 40% CR (Table 3). The resulting BNP mRNA expression was as low as in both groups of young animals (Table 3). The mean CSA of LV cardiomyocytes of old control rats was significantly higher than in all other groups. Six months of CR result in a reduction of CSA in old animals, resulting in a value comparable to young animals (Figure 2B).

### 3.3 Effects of caloric restriction on mitochondria

Oxygraphic measurements of freshly isolated, permeabilized cardiac fibres demonstrated a decrease in pyruvate-/malate- and succinate-dependent respiration in old rats (Figure 3A). CR partially renormalized complex I-dependent pyruvate respiration, while no alteration was observed when mitochondria were respiring under the

**Table 3** mRNA expression data in left ventricle after 6 months 40% caloric restriction

mRNA/18S	Diet	Young rats	n	Old rats	n
ANP	CD	0.85 ± 0.12	8	3.83 ± 0.13 <sup>†</sup>	6
	CR	0.48 ± 0.09*	8	1.71 ± 0.22**	9
BNP	CD	0.05 ± 0.011		1.22 ± 0.02 <sup>†</sup>	
	CR	0.05 ± 0.003		0.12 ± 0.008***	
Bcl-xS	CD	0.19 ± 0.07		1.09 ± 0.06 <sup>†</sup>	
	CR	0.18 ± 0.02		0.66 ± 0.06*	
Bcl-xL	CD	0.34 ± 0.18		0.68 ± 0.24	
	CR	0.43 ± 0.22		1.38 ± 0.18**	
Bcl-xS/Bcl-xL	CD	0.64 ± 0.26		2.05 ± 0.68 <sup>‡</sup>	
	CR	0.45 ± 0.17*		0.51 ± 0.11*	
Bcl-2	CD	0.52 ± 0.12		0.49 ± 0.16	
	CR	0.50 ± 0.17		0.53 ± 0.07	
Bax	CD	1.54 ± 0.09		1.64 ± 0.06	
	CR	1.55 ± 0.11		1.65 ± 0.07	

CD, rat with control diet; CR, rat with caloric restriction.

\*P < 0.05 vs. respective control.

\*\*P < 0.01 vs. respective control.

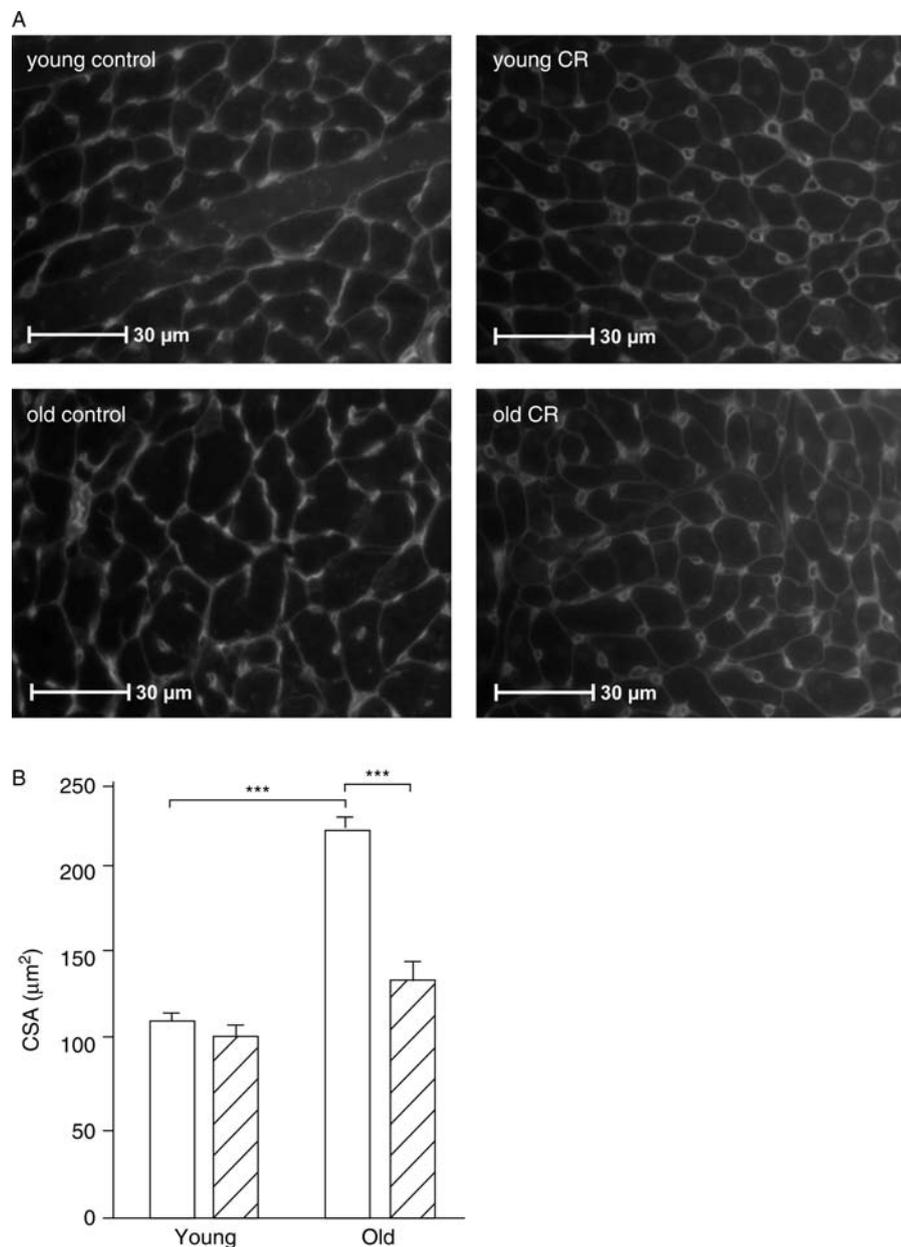
\*\*\*P < 0.001 vs. respective control.

<sup>†</sup>P < 0.001 vs. young control.

<sup>‡</sup>P < 0.05 vs. young control.

complex II substrate succinate (Figure 3A). At the same order of magnitude as observed for the coupled respiration, respiration uncoupled by dinitrophenol with pyruvate/malate as the substrate was significantly decreased in old control animals and CR resulted in a mildly increased uncoupled respiration in old animals (235 ± 17 vs. 240 ± 15 in young control vs. young CR; 158 ± 26 vs. 193 ± 19 in old control vs. old CR).

Similar to these measurements, CR enhanced the enzymatic activity of respiratory chain complex I in old rats when compared with the



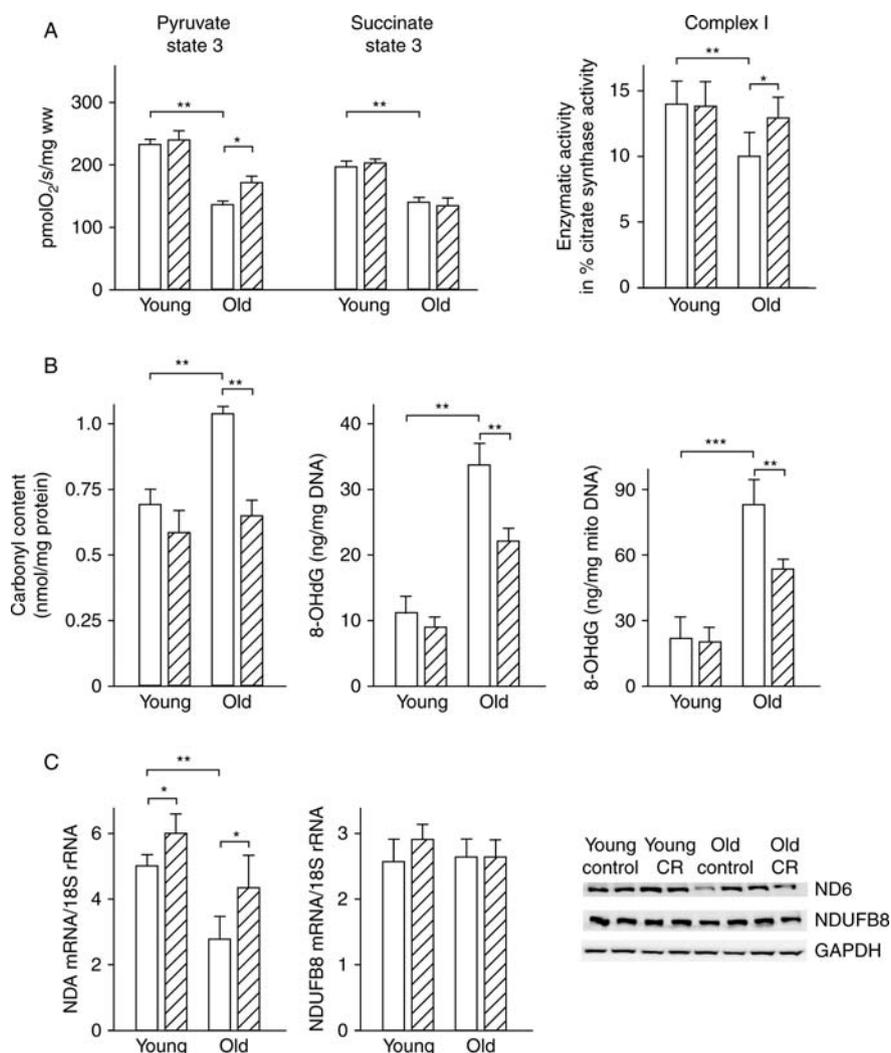
**Figure 2** Effects of 6 months CR on cardiac hypertrophy. (A) Representative images and (B) summarized data on cardiomyocyte CSA (FITC-labelled lectin) from at least 400 myocytes per LV in randomly selected fields in young and old rats under 40% CR (hatched columns) and age-matched animals under control diet (open columns). \*\*\* $P < 0.001$ .

depressed activity under control diet in old rats (Figure 3A). The activity of respiratory chain complexes II, III, and IV was not altered in any group (Supplementary material online, Figure S1A and B). In young rats, neither the enzymatic activity of complexes I–IV nor mitochondrial respiration (Figure 3A) was modified. However, CR resulted in a minor but significant increase in citrate synthase activity in young and old animals, suggesting an increase in mitochondrial mass in these animals (Supplementary material online, Figure S1A). Differences in respiratory chain activities remained largely unaffected by this (absolute vs. CS referenced enzymatic activities, Supplementary material online, Figure S1A and B). The respiratory control index was not significantly different among the groups. Markers of oxidative stress [protein carbonyl content; oxidative damage in total and even more pronounced in

mitochondrial DNA (8-OHdG)] were significantly higher in the LV of old control rats and CR resulted in reduced oxidative damage (Figure 3B). Ageing resulted in a significantly decreased expression of the mtDNA-encoded component of complex I (ND6), while protein and mRNA expression of the nuclear-encoded component of complex I (NDUFB8) were not altered (Figure 3C). CR induced higher ND6 expression in young and old animals (Figure 3C).

### 3.4 Effects of caloric restriction on mitochondrial apoptosis

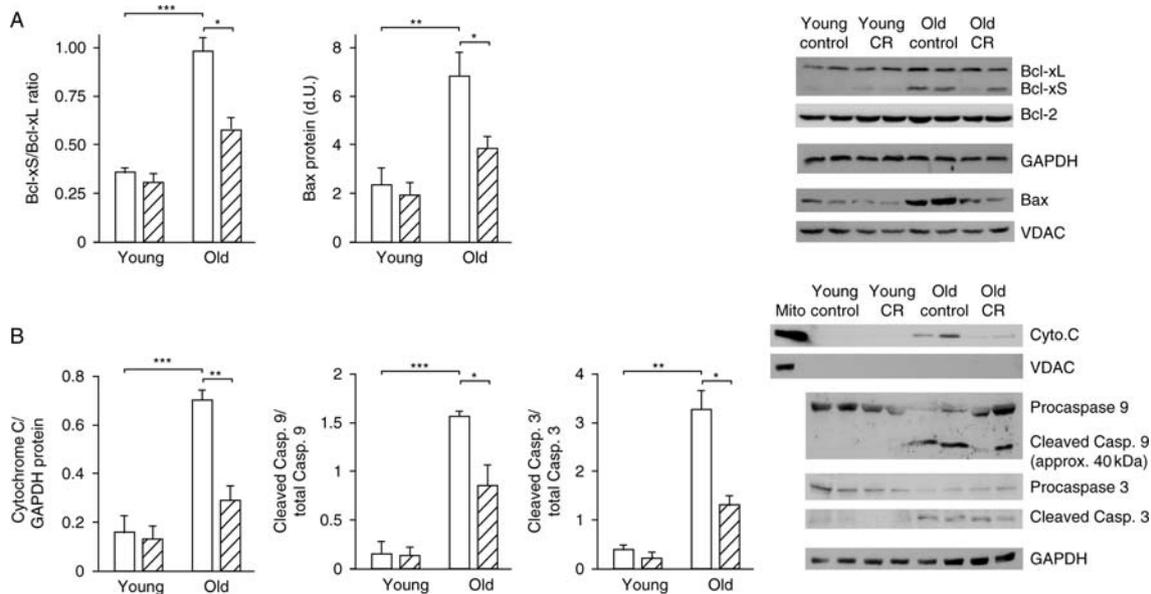
The pro-apoptotic Bcl-xS protein was enhanced and the Bcl-xS/Bcl-xL protein ratio was elevated in the myocardium of old rats under



**Figure 3** Effects of 6 months CR on mitochondrial function, oxidative stress markers, and expression of complex I proteins. (A) Effects on mitochondrial respiration (state 3) as measured in saponin-skinned fibres in the presence of 5 mM ADP and either 10 mM pyruvate/2 mM malate or 10 mM succinate (left panel) or citrate synthase normalized LV complex I activity (right panel) in hearts of young and old rats under 40% CR (hatched columns) and age-matched animals under control diet (open columns). (B) Quantification of oxidative protein damage (left panel) and oxidative DNA damage (8-OHdG) of total DNA (middle panel) and mitochondrial DNA (right panel). (C) Expression of complex I proteins. Left panel: NDA mRNA (mitochondrial-encoded); middle panel: NDUFB8 mRNA (nuclear-encoded); and right panel: ND6 and NDUFB8 protein expression in left ventricles. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

control diet compared with young rats (Figure 4A). After 6 months of CR, the Bcl-xS mRNA (Table 3) and protein and the Bcl-xS/Bcl-xL protein ratio (Figure 4A) were lowered in old rats, almost back to the levels in young rats (Figure 4A). Bcl-2 mRNA (Table 3) or protein (Figure 4A) and Bax mRNA (Table 3) were not different between young and old animals and were not modified by CR. However, ageing was associated with an increased amount of Bax protein localized at mitochondria (Figure 4A), which is normalized to the level of young animals after 6 months of CR. Several other parameters indicated apoptotic activation in old control rats: release of cytochrome C into cytosol, caspase 9 and caspase 3 cleavage (Figure 4B). Although nucleosomal DNA fragmentation was not detectable by agarose gel electrophoresis, an ELISA-based measurement of histone-associated DNA fragmentation demonstrated an

increase in this apoptotic parameter in old control rats (Supplementary material online, Figure S2B). Similarly, in old animals, some cells showed evidence of DNA fragmentation by TUNEL (Supplementary material online, Figure S3). CR significantly reduced these myocardial signs of apoptotic activation in senescent rats: cytosolic cytochrome C, caspase 9 cleavage products (Figure 4B), and nucleosomal DNA fragments (Supplementary material online, Figure S2B). None of the signs of apoptotic activation was detected in ventricles of young rats (Figure 4A and B). Cardiac fibrosis, as deduced from the fibrotic area (Supplementary material online, Figure S4A and B) as well as the tissue collagen content (Supplementary material online, Figure S4C), was significantly reduced in young and in old calorically restricted rats. However, both parameters remained significantly higher in old animals (Supplementary material online, Figure S4A–C).



**Figure 4** Effects of 6 months CR on apoptosis activation. (A) Quantification and representative western blots of Bcl-xS/L and Bcl-2 in homogenates of left ventricles from young and old rats with control diet or 40% CR. Mitochondrial fraction was isolated to detect Bax translocation to mitochondria. GAPDH (total lysate) and VDAC (mitochondrial fraction) were used to demonstrate equal loading. (B) Quantification and representative western blots of cytosolic cytochrome C, caspase 9, and caspase 3 in left ventricles from young and old rats with control diet (open columns) or 40% CR (hatched columns). Mitochondria (Mito) served as a positive control. Blots were also probed with an antibody detecting VDAC to demonstrate purity of the cytosolic fraction. Blots were probed with GAPDH as a loading control. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

### 3.5 Correction of mitochondrial ageing by caloric restriction

For identification of pre-existing dysfunctions existing before the onset of CR, left ventricles from 24 months old rats kept on control diet throughout their life were compared with those from 6 months old rats on control diet (Supplementary material online, Table S2). Left ventricles of these old animals had an enhanced LV/BW ratio, augmented expression of ANP and BNP mRNA (Supplementary material online, Table S2), appearance of cytochrome C in the cytosol (data not shown) together with caspase 9 cleavage, and increase in the pro-apoptotic Bcl-xS and mitochondrial Bax protein (Supplementary material online, Table S2). Besides this pro-apoptotic shift, the hearts were also characterized by a depression of the mitochondrial respiration (Supplementary material online, Table S2) and a 30% reduction in the maximal enzyme activity of the complex I of the respiratory chain (data not shown). The SA  $\beta$ -gal, a widely used biomarker of cellular senescence, was barely detectable in young animals (Supplementary material online, Figure S5). CR strongly reduced SA  $\beta$ -gal in old CR animals compared with controls (Supplementary material online, Figure S5) or 24 months old rats (data not shown).

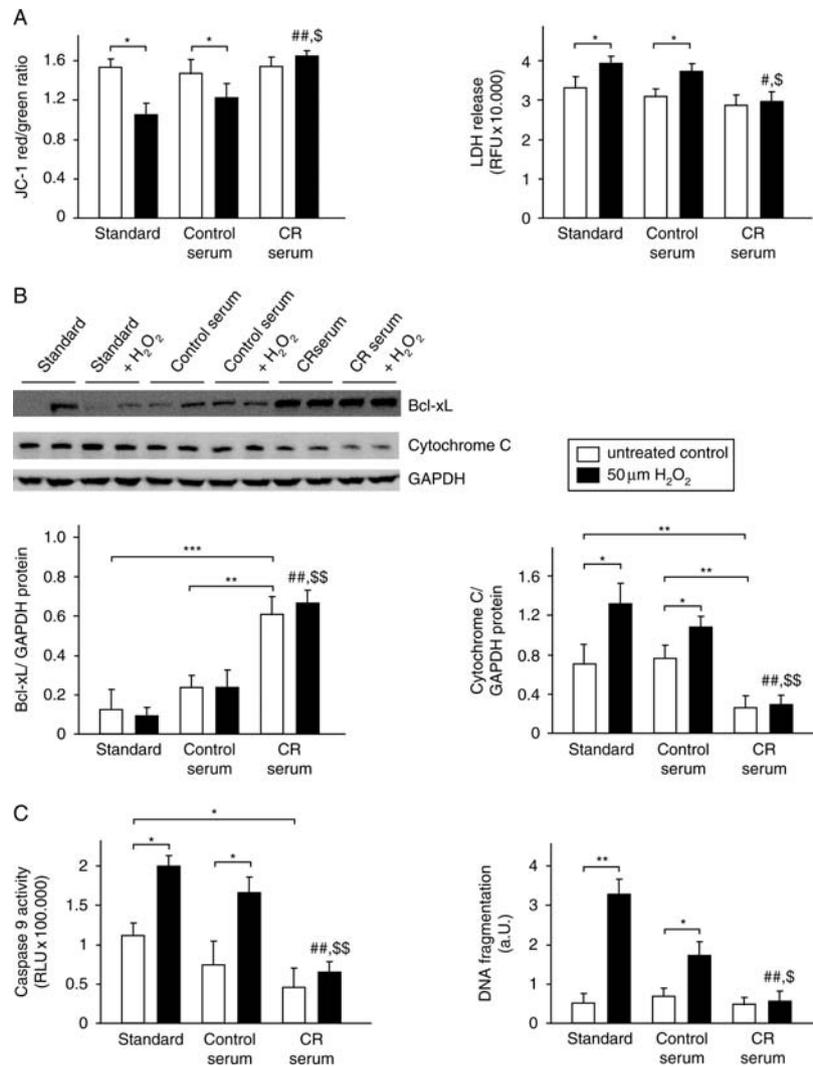
### 3.6 Protective effects of caloric restriction can be mimicked *in vitro*

We used serum obtained from old rats under control diet or CR to culture H9C2 cells as shown in other cell types recently.<sup>8</sup> Twenty-four hours before pro-oxidative stimulation with  $H_2O_2$ , cells were preconditioned with the respective medium supplemented with serum from old controls or old CR rats. The acute loss in mitochondrial membrane potential in response to pro-oxidative stimulation with  $H_2O_2$  was

prevented in cells preconditioned with CR serum but not with serum from control rats (Figure 5A). CR serum resulted in a robust induction of the anti-apoptotic Bcl-xL (Figure 5B) without altering Bcl-xS (data not shown). Following  $H_2O_2$  treatment, a significant reduction in cytochrome C release into cytosol (Figure 5B), in caspase 3 (data not shown) and caspase 9 activation (Figure 5C) as well as in nucleosomal DNA cleavage (Figure 5C) was achieved by preconditioning with CR serum compared with control cells (standard medium with 10% FBS) or cells preconditioned with serum from control rats. Accordingly, cell viability as deduced from LDH release into the culture medium was significantly reduced by  $H_2O_2$  in H9C2 cells cultured under standard conditions or cultured with serum from control rats (Figure 5A). Cell viability of H9C2 cells preconditioned with CR serum, however, remained totally unaffected by  $50 \mu M H_2O_2$  (Figure 5A). siRNA-mediated reduction of Bcl-xL expression in CR serum preconditioned cells abolished the protective effects of CR serum on cytochrome C release and caspase 9 activation (Figure 6B). Although cardiac cell lines such as H9C2 cells are a well-accepted model in cardiovascular research, we sought to validate the effects of CR serum on Bcl-x expression in adult ventricular cardiomyocyte. Similar to the changes observed in LV tissue, treatment of adult rat cardiomyocytes with CR serum resulted in a significantly reduced Bcl-xS but increased Bcl-xL protein expression compared with cells cultured with control serum (Supplementary material online, Figure S6). Heat-inactivation or protease treatment of CR serum abolished this effect.

## 4. Discussion

CR has been considered hitherto as preventive against the progression of ageing, when started early in life of mammals. Here we show that 6 months of 40% CR, started at the senescent stage,

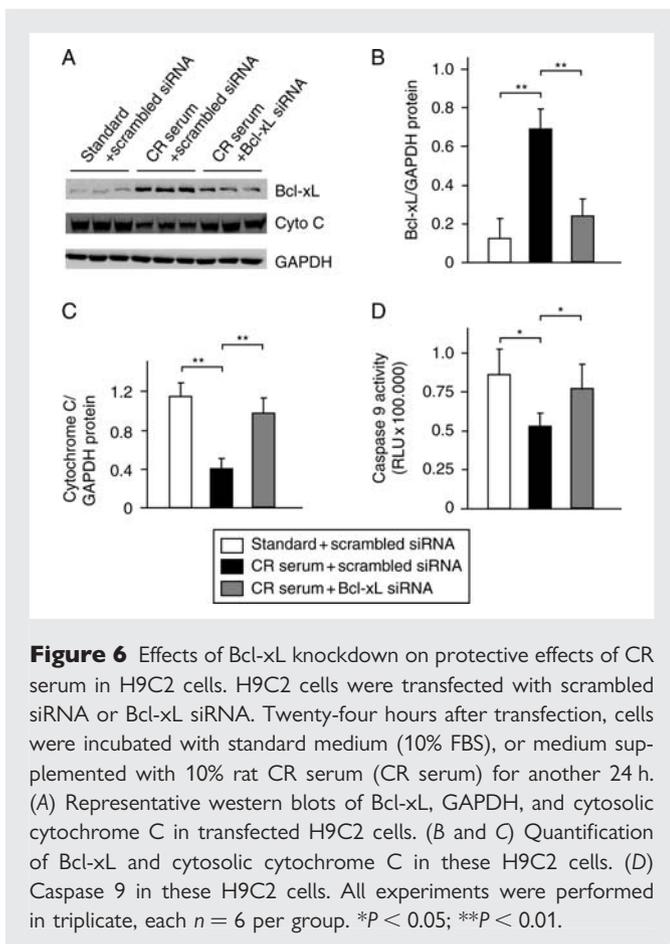


**Figure 5** Effects of CR serum on mitochondrial membrane potential, apoptosis activation, and cell death in H9C2 cells. (A) Changes in mitochondrial membrane potential as indicated by a loss of J-aggregates (red fluorescence) and accumulation of JC-1 monomers (green fluorescence) were measured 30 min after exposure to  $H_2O_2$ . Cell viability (release of LDH) was measured 12 h after exposure to  $H_2O_2$ . (B) Representative western blots and quantification of Bcl-xL and cytosolic cytochrome C 3 h after exposure to  $H_2O_2$ . (C) Caspase 9 activity (relative light units  $\times$  100,000) and oligosomal DNA fragments (artificial units) were analysed 6 h after treatment with  $H_2O_2$ . All experiments were performed in triplicate at least, each  $n = 8-12$  per group. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . # $P < 0.05$  vs. standard and  $H_2O_2$ ; ## $P < 0.01$  vs. standard and  $H_2O_2$ ; \$ $P < 0.05$  vs. control serum and  $H_2O_2$ ; \$\$ $P < 0.01$  vs. control serum and  $H_2O_2$ .

results in improved survival and preservation of LV function, attenuation of mitochondrial dysfunction and oxidative stress, and repression of the mitochondrial death pathway.

The mitochondrial respiratory chain is generally assumed as the main source of reactive oxygen species (ROS) in the mitochondrial theory of ageing. ROS formation by the respiratory chain, mainly at complexes I and III, is increasing when electron flow slows down.<sup>9</sup> The significant reduction in complex I activity in old rats could therefore be a likely source of increased ROS. Such a reduced complex I activity is a common feature in heart failure<sup>10</sup> or brain ageing.<sup>11</sup> Furthermore, mitochondrial function of cardiac and skeletal muscle is severely impaired in obesity and diabetes.<sup>12,13</sup> The observed metabolic changes in response to CR (serum insulin and triglycerides) in our animals may also have contributed to the changes in mitochondrial and LV function.

Respiratory chain function decreases with age, while oxidative damage and mutations in mtDNA increase (review in ref.<sup>14</sup>). The mitochondrial theory of ageing is based on the idea of a vicious cycle, in which mtDNA mutations cause respiratory chain dysfunction, enhancing the production of DNA-damaging ROS, finally resulting in cell death and an ageing phenotype. The notion that premature ageing is generated by this vicious cycle was challenged recently.<sup>15</sup> It was proposed that the respiratory chain dysfunction *per se* not increased ROS is the primary inducer of premature ageing in mice expressing an error-prone version of the mtDNA polymerase.<sup>15</sup> This dysfunctional respiratory chain accelerates ageing by provoking an energy deficit or decreasing the signal threshold for cell death. However, the unique role of mitochondria-derived ROS in the ageing process has been clearly shown by others: only mice with an overexpression of catalase localized to the mitochondria but not to



**Figure 6** Effects of Bcl-xL knockdown on protective effects of CR serum in H9C2 cells. H9C2 cells were transfected with scrambled siRNA or Bcl-xL siRNA. Twenty-four hours after transfection, cells were incubated with standard medium (10% FBS), or medium supplemented with 10% rat CR serum (CR serum) for another 24 h. (A) Representative western blots of Bcl-xL, GAPDH, and cytosolic cytochrome C in transfected H9C2 cells. (B and C) Quantification of Bcl-xL and cytosolic cytochrome C in these H9C2 cells. (D) Caspase 9 in these H9C2 cells. All experiments were performed in triplicate, each  $n = 6$  per group. \* $P < 0.05$ ; \*\* $P < 0.01$ .

the peroxisome or the nucleus demonstrated an increase in lifespan together with a reduction in oxidative DNA damage and age-associated cardiac pathology.<sup>16</sup> Furthermore, mice expressing a proofreading-deficient mitochondrial DNA polymerase gamma accumulate mtDNA mutations and show accelerated ageing with increased apoptosis in various organs.<sup>17</sup> These data suggest that mitochondrial dysfunction and accumulation of oxidative damage are central mechanisms for the induction of apoptosis and mammalian ageing. The close proximity to the production site of ROS, the limited repair capacity, and lack of histones render the mtDNA highly susceptible to oxidative stress. The observed significant decrease in the mtDNA-encoded gene transcript of complex I (ND6) may have resulted from increased ROS release from the respiratory chain. Although no direct ROS measurements were performed, markers of increased oxidative stress indicate a higher ROS release in aged myocardium. The significantly higher 8-OHdG values per mg DNA in mtDNA compared with total DNA are suggestive for higher oxidative damage at the mitochondrial level. Accordingly, it has been shown that 8-oxodG in mitochondrial DNA but not in nuclear DNA is inversely correlated with maximum lifespan in the heart and brain of mammals.<sup>18</sup>

Important properties of mitochondria differ in the intact heart *in situ* and in isolated mitochondria. Therefore, we did not analyse mitochondrial respiration in isolated mitochondria but used functional mitochondria *in situ* in saponin-permeabilized cardiac fibres. Uncoupled and coupled mitochondrial respiration was similarly impaired in aged myocardium. Thus, although inhibition or damage of ANT or ATPase may occur, this does not appear to contribute

significantly to the observed age-associated declines in the rate of mitochondrial respiration. Therefore, age-related declines in mitochondrial respiration reflect damage to electron transport chain components or a reduction in the supply of reducing equivalents. The observed decrease in ND6 may represent one mechanism underlying the decreased function of complex I. Our study demonstrates an age-associated impairment of mitochondrial respiration and function, mainly affecting complex I of the respiratory chain, which is abolished after 6 months of CR. Thus, ageing induces alterations in mitochondrial composition and function which are suitable for the induction of mitochondrially mediated cell death. According to the results from mtDNA mutator mice,<sup>15,17</sup> these changes may lead to a higher susceptibility for cell death thereby accelerating the ageing process.

Remodelling of the aged heart involves a loss of cardiomyocytes due to apoptosis and necrosis,<sup>4</sup> reactive hypertrophy of the remaining myocytes, and increased fibrosis.<sup>19</sup> The significant reductions in ANP/BNP, in cardiac fibrosis as well as cardiomyocyte CSA are suggestive for a reverse remodelling induced by CR in the aged myocardium. Our study also shows that CR significantly attenuates the age-induced increase of LV apoptosis as evidenced by lower cytochrome C release, caspase 9 activation, and nucleosomal DNA cleavage when compared with age-matched animals under control diet. The levels of nucleosomal DNA cleavage (DNA laddering) and TUNEL-positive cells in aged myocardium were low. However, chronic, even low levels of cardiac myocyte apoptosis can contribute to the development of heart failure. Pro-survival Bcl-2 family proteins such as Bcl-2 or Bcl-xL have been shown to antagonize mitochondrial membrane permeabilization, cytochrome C release, and cell death by binding pro-apoptotic factors such as Bax or Bak.<sup>20</sup> Indeed, CR also resulted in a significant reduction in mitochondrial Bax translocation in senescent rats, reduced the pro-apoptotic Bcl-xS, and enhanced the anti-apoptotic Bcl-xL in the ageing hearts. Experimental interference with Bcl-x-splicing towards enhanced Bcl-xS and reduced Bcl-xL expression is sufficient to cause mitochondrial dysfunction, enhance ROS release, and trigger apoptosis in cardiomyocytes in culture.<sup>6,21</sup> Furthermore, our experiments in H9C2 cardiomyoblasts preconditioned with CR serum suggest that Bcl-xL is indispensable for some of the beneficial effects of CR. Cardioprotection mediated by several survival signal transduction pathways acts through multiple mechanisms including preservation of mitochondrial integrity. Bcl-xL inserted into mitochondrial membranes appears to interact with a variety of other molecules such as VDAC or mTOR involved in regulating apoptosis as well as cellular metabolism.<sup>22</sup> Bcl-xL may thus represent an important nodal point, integrating various signalling inputs with cardioprotective potency.

Ischaemic preconditioning and postconditioning, both cardioprotective by reducing infarct size in young animals, are less effective in aged myocardium.<sup>23</sup> However, following CR ischaemic preconditioning reduces postischaemic dysfunction in hearts from food-restricted senescent rats but not in *ad libitum*-fed senescent rats.<sup>24</sup> Accordingly, short-term CR has been shown to improve myocardial ischaemic tolerance in young and old animals.<sup>25</sup> In addition, increased resistance to severe ischaemia after CR is strongly related to changes in mitochondrial respiration.<sup>26</sup> Therefore, mechanisms activated by CR itself or by CR mimetics may represent powerful tools to preserve cardiac function also in aged human myocardium. In the present study, we show that 40% CR resulted in a significant preservation of LV function in old rats compared with animals under control diet. The

echocardiographic data on preserved ventricular function in old CR animals are supported by the reductions of plasma BNP and ANP/BNP mRNA expression, sensitive and clinically established markers for cardiac dysfunction. While ANP mRNA was reduced by CR in young and in old animals, BNP mRNA responded only in old animals. A similar dissociation between ANP and BNP mRNA in rats was shown before suggesting that BNP may serve as a marker for the transition from compensated to overt heart failure.<sup>27</sup> Lifelong CR has been shown to improve the age-related impairments in diastolic function in mice<sup>28</sup> as well as in Dahl salt-sensitive rats.<sup>29</sup> Our study is, to our knowledge, the first report of the protective effects of CR on LV systolic function when CR was started late in life. There are no controlled trials on the effects of long-term CR on cardiac function in humans for obvious reasons including unresolved safety issues or difficulties in lifelong observation of participants. A recent study<sup>30</sup> described for the first time the protective effects of long-term CR on diastolic dysfunction in humans practicing self-imposed CR for 3 to 15 years ( $n = 25$ ). However, it is difficult to compare the data from this human study<sup>30</sup> with our experimental model. First, the diet conditions were much less well defined: the age of the subjects ( $53 \pm 12$  years), the duration of the diet ( $6.5 \pm 4.6$  years), and the degree of diet differed among the participants. Second, while the human study investigated middle-aged humans on a diet for about 6 years (7–8% of their lifetime) our old rats had all reached senescence at the beginning of the study, and it lasted for approximately 20% of their lifetime. The animal diets used in the present study did not exclusively differ in calories but also in composition, mainly fibre content. Thus, we cannot totally exclude that some of the observed protective effects were induced by the higher fibre content via altered gut hormone production, overall absorption of fat and protein, or toxin detoxification and absorption.

Progress in elucidating the mechanisms underlying the protective effects of CR has been slow due to the complexities of using animals for CR studies. It is therefore of great interest to develop *in vitro* models of CR. In the presents study, we used H9C2 cardiomyoblasts preconditioned with serum from old CR rats to test whether this cell culture model recapitulates phenotypic features observed in the LV of old CR rats. We show that the H<sub>2</sub>O<sub>2</sub>-induced acute loss in mitochondrial membrane potential and cell viability was prevented by preconditioning with CR serum. Thus, treatment with CR serum results in enhanced stress responsiveness *in vitro*. It also resulted in reduced apoptotic activation as deduced from reduced cytochrome C release, caspase 9 activation, and nucleosomal DNA cleavage after H<sub>2</sub>O<sub>2</sub> treatment. Thus, CR serum contains factors which promote survival of cultured cells. The protective effects of CR serum appear to be related to a proteinaceous factor since protease treatment and heat-inactivation abolished this effect. The anti-apoptotic effects of CR serum were also abolished in cells with siRNA-mediated Bcl-xL knockdown. This implies that Bcl-xL is indispensable to maintain at least some of the beneficial effects of CR serum. Our data suggest that several effects of CR are mediated through circulating factors in the serum of CR animals. However, the exact mechanisms resulting in enhanced stress resistance and the induction of Bcl-xL in cells preconditioned with CR serum remains to be elucidated in future studies. CR as well as CR serum resulted in a significant reduction of the pro-apoptotic Bcl-xS in the heart and in adult rat cardiomyocytes, respectively. In H9C2 cardiomyoblasts, CR serum induced mainly a splicing shift towards the anti-apoptotic Bcl-xL. These differences in Bcl-x splicing, both resulting in a shift towards

anti-apoptosis, may be related to the differentiation status of this cell line. They also underline the need to validate major findings in adult cardiomyocytes.

In summary, CR stabilizes mitochondrial respiratory function, reduces ROS release and pro-apoptotic activation in aged LV, thereby retarding the age-associated deterioration of cardiac function. CR is protective even when started late in life and basic cardioprotective mechanisms of CR are preserved in the senescent heart. Their activation results in improved survival and preserved LV function in old animals. Therefore, drugs activating these cardioprotective mechanisms of CR may also yield a promising strategy to improve cardiac function in older patients.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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