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Research article

Short-term caloric restriction and regulatory proteins of apoptosis in heart, skeletal muscle and kidney of Fischer 344 rats

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Abstract

Long-term caloric restriction reduces oxidative stress, increases mean and maximum lifespan in rodents and tends to enhance apoptosis, particularly in the liver. We investigated the effect of short-term (2 months) caloric restriction (40% reduction) in 6-month-old male Fischer 344 rats on various indicators of apoptosis (caspase-3, -7, -12, the inhibitor of apoptosis protein XIAP and cytoplasmic histone-associated DNA fragments) in the post-mitotic heart and gastrocnemius muscle, and the kidney that contains mitotic cells. Short-term caloric restriction significantly reduced body mass (30%), gastrocnemius muscle mass (22%), heart mass (25%) and kidney mass (32%) compared to *ad libitum* controls. The levels of procaspase-3 in gastrocnemius muscle and caspase-3 in kidney were significantly lower in the caloric restricted than in the *ad libitum* fed group. While caloric restriction did not alter DNA fragmentation levels (indicative of apoptosis), differences did exist amongst tissues with significantly elevated levels of fragmentation in the kidney compared to the heart and gastrocnemius muscle and significantly higher levels in the heart compared to gastrocnemius muscle. No differences were observed between groups in the levels of procaspase-7 or -12 or in XIAP (an endogenous inhibitor of apoptosis, particularly of caspase-3 and -7) in any tissue. The active forms of caspase-7 and -12 were present only in the kidney. These findings suggest that while the rate of apoptosis was higher in the kidney, which contains mitotic cells, compared to the post-mitotic heart and gastrocnemius muscle, short-term caloric restriction did not enhance the apoptosis rate in any tissue measured.

Abbreviations: CR – caloric restriction; calpains – calcium-dependent neutral cysteine proteases; caspases – cysteine-dependent aspartic acid specific endoproteases; XIAP – inhibitor of apoptosis protein

Introduction

Caloric restriction (CR), encompassing a reduction in energy intake without malnutrition, is the most reproducible experimental treatment that increases both mean and maximum lifespan in a phylogenetically wide-ranging group of animals (Barja 2002; Merry 2002; McCarter 1995; Sohal and Weindruch 1996; Zainal et al. 2000). CR has been shown to delay the incidence and severity of various age-associated pathologies including cardiomyopathy, nephropathy and spontaneous and chemically induced tumors (Shimokawa et al. 1996). However, the exact mechanism by which CR extends lifespan is unknown, although several lines of evidence support the role of a reduction in oxidative stress, probably via reduced mitochondrial free radical generation (Gabbita et al. 1997; Gredilla 2001a, b; Lopez-Torres et al. 2002; Sohal et al. 1994; Sohal and Weindruch 1996).

The reduction in the incidence of age-associated pathologies and tumors by life-long CR is well established (James et al. 1994; Shimokawa et al. 1996; Frame et al. 1998). One proposed mechanism involved is an enhanced rate of apoptosis or programmed cell death (Wachsman 1996). Apoptosis appears central to the removal of cells that may affect homeostasis, including pre-neoplastic, senescent or developmentally obsolete cells, and can be stimulated via several signals including reactive oxygen and nitrogen species, TNF-α, glucocorticoid levels, a rise in intracellular Ca^{2+} levels, various proteases (e.g., granzyme B), and cell-death receptors (Papa and Skulachev 1997; Phaneuf and Leeuwenburgh 2002; Pollack and Leeuwenburgh 2001; Wachsman 1996). Caspases (cysteine-dependent aspartic acid specific endoproteases) are thought to play an integral role in cell death. The mitochondrial mediated apoptotic pathway is initiated by cytochrome-c release from the mitochondria into the cytosol, followed by the Apaf-1 oligomerisation, procaspase-9 activation and the formation of the apoptosome. This initiates proteolytic cleavage, switching precursor procaspases to active enzyme proteases, such as caspase-3 (Cregan et al. 2002; Pollack and Leeuwenburgh 2001; Ravagnan et al. 2002; Troy and Salvesen 2002). Caspase-independent apoptosis also occurs and may involve apoptosis-inducing factor (Cregan et al. 2002; Joza et al. 2001), or calpain activation (calcium-dependent neutral cysteine proteases, Mathiasen et al. 2002).

Studies examining the effect of CR on apoptosis have concentrated primarily on the liver and used long-term CR as the paradigm (Grasl-Kraupp et al. 1994; Higami et al. 2000; James and Muskhelishvili 1994; James et al. 1998; Muskhelishvili et al. 1995). A 40% reduction in food intake over a 3-month period, resulted in a 20–30% decrease in normal liver cells and an 85% reduction in the incidence of putative preneoplastic foci in rats (Grasl-Kraupp et al. 1994). CR also increased apoptosis in mouse T-lymphocytes (Luan et al. 1995; Spaulding et al. 1997), mouse brain CT-2A synergistic malignant astrocytomas (Mukherjee et al. 2002) and in the bladder of heterozygous p53-deficient mice (Dunn et al. 1997). In C57BL/6 × C3H F1 mice, a strain highly susceptible to hepatic tumors, CR increased the rate of apoptosis, decreased cellular proliferation rate and reduced the incidence of spontaneous hepatoma (James et al. 1998; James and Muskhelishvili 1994; Muskhelishvili et al. 1995). Conversely, however, a decreased apoptosis rate (alteration in Fas, Fas-L and Bcl-2 expression) was observed in splenic lymphocyte of lupus-prone B/W mice (Reddy Avula et al. 2002), during life-long CR (30% restriction) in the kidney of Fischer 344 rats (Fas antigen, Razzaque 1999) and a significant lowering in hepatocyte proliferation rate without an enhancement in apoptosis was observed in 3-month Fischer 344 rats (Higami et al. 2000).

The effects of short-term CR are less clear with respect to various parameters of oxidative stress and radical production, with decreases detected in the liver (Gredilla et al. 2001a) and heart in long-term but not short-term CR (Gredilla et al. 2001b), and the effects of short-term CR on apoptosis are not well established. In the following study, we examined various parameters of apoptosis in the heart, skeletal muscle (gastrocnemius) and kidney of 6-month-old Fischer 344 rats, after 8 weeks CR (40% reduction compared to *ad libitum* controls). We hypothesized that short-term CR would enhance apoptosis and measured the levels of caspase-3, -7 and -12 involved in the apoptotic cascade and XIAP, an endogenous inhibitor of apoptosis protein (specifically of caspase-3 and -7) by Western analysis. The levels of cytoplasmic histone-associated-DNA fragments (mono- and oligo-nucleosome), indicative of apoptosis, were also quantified in each tissue.

Experimental procedures

Animals

Both *ad libitum* and caloric restricted male Fischer 344 rats were obtained from the National Institute of Aging (NIA) colony (Indianapolis, Indiana, USA) at 4 months of age. Caloric restriction commenced at the NIA at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months of age and maintained from 4 months at 40% restriction until the termination of the experiment at 6 months of age. From 4 months onwards, all animals were housed individually at the University of Florida Animal Care Services (Gainesville, Florida, USA), under a photoperiod of 12L:12D and an ambient temperature of 18–20 °C. The 40% restriction regime employed at the University of Florida followed exactly that of the NIA, with the food provided for the caloric restricted rats being purchased directly from the NIA, in order that the feeding regime and diet composition of food did not alter between the two institutions. After eight weeks of acclimation, two animals per day (one

ad libitum and one caloric restricted) were weighed and then anaesthetized by an intraperitoneal injection of pentobarbital sodium solution (Abbot Laboratories, Illinois, USA; 5 mg/100g body weight). The heart, both gastrocnemius muscles and kidneys were rapidly removed and using differential centrifugation, the cytosolic and mitochondrial protein fractions were obtained. In brief, the tissues were homogenized (on ice) in 1:10 wt/vol of an ice-cold buffer (0.225 M mannitol, 0.075 sucrose, 1 mM EDTA and 0.2% BSA) using a Potter–Elvehjem glass–glass homogeniser. The homogenates were centrifuged (Eppendorf, 5810R, Brinkmann Instruments Inc., New York, USA) at 700 *g* (4 °C) for 10 min, the resulting supernatant centrifuged for 8000 *g* (4 °C) for 10 minutes and the cytosolic fractions then subsequently frozen at –80 °C until use. This experiment received local institutional animal care and use committee approval.

Caspase-3, -7, -12 and XIAP levels

For Western analysis, gastrocnemius muscle, heart and kidney cytosolic proteins were separated using 4–20% PAGEr® Gold precast Tris-glycine gels (BioWittaker Molecular Applications, Rockland, Maine, USA) under denaturing conditions and transferred to nitrocellulose membranes (0.2 μm, Trans-Blot® Transfer Medium, Bio-Rad Laboratories, California, USA) and blocked overnight using a 5% milk solution. Protein content was determined using the Bradford method and subsequently normalized so that the protein content of each sample, within a gel, was equal. Subsequently, 15 μl of sample was loaded to each lane. A 10 μl HeLa cell lysate sample (Stressgen, British Columbia, Canada) was used as a housekeeping control. In this study, we only compared enzyme bands within an individual gel; therefore, we did not make any across gel comparisons. Membranes were incubated and rocked at room temperature for 90 min in the 5% blocking solution containing either the polyclonal primary antibody: caspase-3 (1:1000, NeoMarkers, California, USA), caspase-7 (1:1000, NeoMarkers, California, USA), caspase-12 (1:1000, Oncogene Research Products, Massachusetts, USA) or XIAP (1:200, Medical and Biological Laboratories Co., Japan). Membranes were finally incubated and rocked with anti-rabbit Ig horseradish peroxidase-linked whole secondary antibody (Amersham Biosciences UK Ltd, Amersham, UK) for 90 min at room temperature. The resulting Western blots were exposed to film (Hyperfilm™ ECI™, Amersham Pharmacia Biotech, UK) and analysed using the NIH Apple-J program. The values (expressed as arbitrary OD units) were calculated by multiplying the area of each band by its optical density, repeated three times and the resulting mean used for analysis. The inactivated/precursor form of each caspase is described from this point forward as ‘procaspase’ and the activated/cleaved protease form described as ‘caspase’.

Induced cell death levels

Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), markers of cell death were measured in the gastrocnemius, heart and kidney cytosolic fractions using a ‘Cell Death Detection ELISA’ (Roche Diagnostics, GmbH, Germany). Apoptosis-activated endogenous endonucleases cleave double stranded DNA in the linker region between nucleosomes to produce mono- and oligonucleosomes of 180 base pairs or multiples. Samples were run in triplicate and the means expressed as arbitrary OD units normalized to mg cytosolic protein, with protein values determined using the Bradford method.

Statistical analysis

All analyses were performed in triplicate and the mean values obtained used for independent *t*-tests. All data was tested for normality and if not normal was log-transformed. Statistical analyses were carried out using MINITAB 13 statistical package (Minitab Inc., State College, Pennsylvania, USA). Statistical significance was considered where $P < 0.05$. All data are reported as mean ± SEM.

Results

Body and organ mass

Body ($t_{17} = 16.71$, $P < 0.001$), heart ($t_{16} = 10.78$, $P = 0.001$), gastrocnemius muscle ($t_{16} = 10.20$, $P = 0.001$) and kidney ($t_{16} = 5.59$, $P = 0.001$) mass were all significantly lower in the CR group compared to the *ad libitum* group (Table 1). However, these reductions in organ mass were in proportion to the differences seen in body mass, as when employing a general linear model with body mass as a covariate, no significant differences were observed between groups ($F_{1,19} = 0.54$, $P = 0.471$; $F_{1,18} = 0.85$, $P = 0.370$; $F_{1,19} =$

Table 1. Body and organ masses.

	Ad libitum (g)	CR (g)	% Decline in mass
Body mass	390.5 ± 5.2	**272.5 ± 4.7***	30.2
Gastrocnemius	3.591 ± 0.05	**2.810 ± 0.05***	21.8
Heart	1.034 ± 0.02	**0.772 ± 0.02***	25.3
Kidney	2.480 ± 0.06	**1.684 ± 0.13***	32.1

Mean ± SEM body mass, heart, gastrocnemius and kidney mass in *ad libitum* and short-term caloric restricted (CR) rats.
n = 10 in each group.
*(Bold text) denotes significant difference ($P < 0.05$) to *ad libitum* levels % decline in mass compared to *ad libitum* levels.

0.95, $P = 0.343$; for heart, gastrocnemius and kidney, respectively).

Levels of caspase-3, -7 and -12 in heart, gastrocnemius muscle and kidney

Procaspase and caspase-3, -7 and -12 levels were measured in heart, gastrocnemius muscle and kidney of the CR and *ad libitum* groups. No statistical differences were observed between groups in heart or kidney procaspase-3 levels ($P > 0.05$; Table 2), although a significantly lower procaspase-3 level was seen in the gastrocnemius muscle of the CR rats ($T_{10} = 2.620$, $P = 0.047$; Table 2). Caspase-3 levels did not differ between groups in heart or gastrocnemius muscle but were significantly lower in the kidney of the CR rats compared to the *ad libitum* rats ($T_{10} = 2.470$, $P = 0.043$; Table 2). The levels of procaspase-7 and -12 did not alter between groups in any tissue (Table 2). No significant difference was observed between experimental groups in the levels of caspase-7 or -12 in the kidney. However, there was no evidence of either caspase-7 or -12 in either the heart or the gastrocnemius muscle (Table 2).

XIAP levels, an inhibitor of apoptosis

XIAP, an inhibitor of apoptosis specifically caspase-3 and -7, was measured by Western blotting and did not differ significantly between the CR and *ad libitum* group in any of the tissues (Table 2).

Cell death activity

Endogenous endonucleases, activated by apoptosis, cleave double stranded DNA, resulting in cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes). These fragments were quantified in heart, gastrocnemius muscle and kidney. No significant differences in mono-and oligonucleosome levels were observed (Figure 1) between experimental groups in the heart ($T_{11} = 0.890$, $P = 0.396$), gastrocnemius muscle ($T_{11} = 0.590$, $P = 0.570$) or kidney ($T_{11} = 0.050$, $P = 0.960$). However, there were significant tissue differences ($F_{1,17} = 48.43$, $P < 0.001$; *post-hoc* Tukey test $P < 0.05$) in DNA fragmentation levels (data from groups pooled), with the mitotic kidney having significantly higher levels compared to the heart and gastrocnemius muscle. The heart also had significantly higher levels of DNA fragmentation compared to the gastrocnemius muscle (Figure 1).

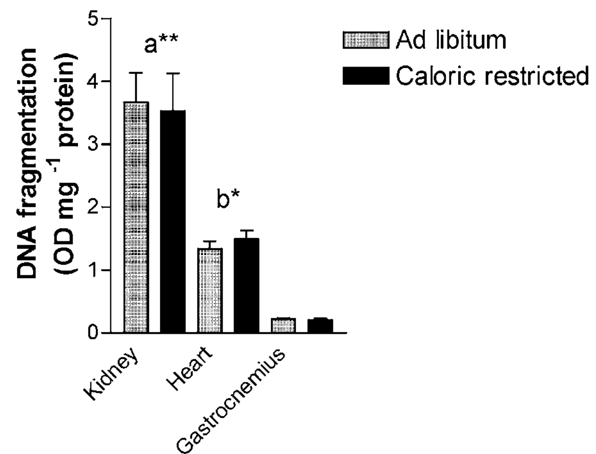

Figure 1. Levels (OD mg^{-1} protein) of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes) quantified in the heart, gastrocnemius muscle and kidney. No difference was observed between the short-term caloric restricted group and the *ad libitum* controls in the level of DNA fragmentation in the heart ($P = 0.396$), gastrocnemius muscle ($P = 0.570$) or kidney ($P = 0.960$). However, when data was pooled across groups, a significant difference between tissues was observed, with the kidney having significantly greater DNA fragmentation levels than both the heart and gastrocnemius muscle (a**), and the heart having significantly higher DNA fragmentation than the gastrocnemius muscle (b*).

Discussion

In this study, we examined the effect of short-term (2-month) caloric restriction (CR), compared to *ad libitum* controls, on various indicators of apoptosis, in the post-mitotic heart and gastrocnemius muscle and the kidney, which is capable of cell division. The ability to balance cellular proliferation and removal appears central to cellular homeostasis and the preven-

Table 2. Levels of various caspases and XIAP in gastrocnemius, heart, and kidney.

	Gastroc.	Gastroc.	Heart	Heart	Kidney	Kidney
	AD	CR	AD	CR	AD	CR
Procaspase-7	32054 ± 2656	28915 ± 3129	56290 ± 7655	62979 ± 6676	32470 ± 2041	23228 ± 4544
Caspase-7	–	–	–	–	29937 ± 3262	26381 ± 3704
Procaspase-12	21664 ± 1675	28098 ± 2673	21447 ± 1041	23043 ± 1430	5235 ± 1168	5422 ± 1432
Caspase-12	–	–	–	–	32054 ± 2656	28917 ± 3129
Procaspase-3	15601 ± 103	**4551 ± 387***	10658 ± 525	10503 ± 542	50327 ± 1431	46901 ± 2451
Caspase-3	11643 ± 3341	7944 ± 1079	19465 ± 2735	13011 ± 1678	25876 ± 2561	**18902 ± 1201***
XIAP	41702 ± 3440	35454 ± 4289	86216 ± 8169	96631 ± 4303	56943 ± 2264	53222 ± 2823

Mean (± SEM) levels of procaspase-7, -12, -3; caspase-7, -12, -3 and XIAP (an inhibitor of apoptosis) in heart, kidney and gastrocnemius (gastroc.) of *ad libitum* (AD) and short-term caloric restriction (CR) rats. No evidence of caspase-7 or -12 was seen in either heart or gastrocnemius muscle. $n = 6$ in each group.
*(Bold text) denotes significant difference ($P < 0.05$) to *ad libitum* levels. Values expressed in arbitrary OD units.

tion of carcinogenesis (Ravagnan et al. 2002), and it has been estimated that approximately 10^{11} cells are removed daily from an adult human, primarily through programmed cell death or apoptosis (Troy and Salvesen 2002). CR is an established paradigm to extend both mean and maximum lifespan, and probably works through reducing mitochondrial free radical generation and subsequent oxidative stress (Barja, 2002; Gabbita et al. 1997; Gredilla 2001a, b; Lopez-Torres et al. 2002; Sohal et al. 1994; Sohal and Weindruch 1996). CR also reduces both the incidence and intensity of age-associated and chemically induced tumors (Shimokawa et al. 1996), and in several studies has been shown to enhance apoptosis, particularly in the liver and in lymphocytes (James and Muskhelishvili 1994; James et al. 1998; Luan et al. 1995; Muskhelishvili et al. 1995).

During short-term CR, a significant reduction in overall body mass, heart, gastrocnemius muscle and kidney mass was observed, although we found no evidence to suggest that the rate of apoptosis was enhanced by CR in either the heart, gastrocnemius muscle or kidney. Indeed, contrary to our initial prediction, the general trend of our data was a decline in various apoptotic parameters after short-term CR (including the levels of the caspases-3, -7 and -12) and a significant decrease in both gastrocnemius muscle procaspase-3 and kidney caspase-3 levels after short-term CR. In accordance with our findings, a reduction in apoptosis, as indicated by the Fas antigen, was previously reported in the kidney of male Fischer 344 rats, after 6- and 12-month CR (Razzaque 1999) and in splenic lymphocytes of lupus-prone B/W mice during CR (Reddy Avula et al. 2002).

There are several possible reasons as to why we did not see an increase in apoptosis after short-term CR, as originally hypothesized. Firstly, the majority of studies that have examined the effects of CR on apoptosis directed their focus on restriction protocols longer than the two-month period of this study (James and Muskhelishvili 1994; James et al. 1998; Muskhelishvili et al. 1995). It has been proposed that the life extending effects of CR occur in a time-dependent fashion (Gredilla et al. 2002; Gredilla et al. 2001b), and that the effects of short-term CR are tissue dependent, in that it reduced mitochondrial radical production in the liver (Gredilla et al. 2001a) but not in the heart (Gredilla et al. 2001b), while long-term CR reduced mitochondrial radical production in both tissues (Gredilla et al. 2001b; López-Torres et al. 2002). The liver is central to mammalian metabolism, appears particularly sensitive to changes in diet (Lopez-Torres et al. 2002) and has the capacity for cellular proliferation, which may be one reason why apoptosis is generally enhanced in the liver after CR (James and Muskhelishvili 1994; James et al. 1998; Luan et al. 1995; Muskhelishvili et al. 1995), although caspase levels were not measured in those studies. Using microarrays, it was shown in CR mice that various genes related to apoptosis were altered in the liver (Cao et al. 2001) but not in the gastrocnemius muscle, neocortex or cerebellum (Lee et al. 1999; Lee et al. 2000). This suggests a tissue specific response to CR. Indeed, apoptosis in multinucleated cells, such as myocytes, may initiate a multicomplex process of proteolytic activity, causing atrophy rather than wholesale cell death (Agusti et al. 2002; Selman and Leeuwenburgh 2003). Zhang et al. (2002)

suggested that CR might aid rapid removal of damaged but replaceable cells such as hepatocytes but reduce the loss of irreplaceable post-mitotic cells such as neurons, cardiomyocytes or myocytes. This, however, does not satisfactorily explain why no increase in apoptosis was observed in the kidney during our study.

Many previous studies examining the effect of CR on apoptosis have used rodent strains highly susceptible to various types of cancer (James and Muskhelishvili 1994; James et al. 1998; Luan et al. 1995; Muskhelishvili et al. 1995). Interestingly, mice heterozygous for a p53 mutation had enhanced resistance to spontaneous tumors but an accelerated aging phenotype (Tyner et al. 2002). It has been suggested that apoptosis increases in tissues with age (Zang et al. 2002) and it is possible that we observed no increase in apoptosis rate in our CR rats because they were only six months of age and any changes in caspase levels encountered were too low to be detected. However, while there was no increase in apoptosis in the liver of three-month old (Higami et al. 2000) or six-month old (Razzanque at al. 1999) Fischer 344 rats after CR and a decrease in four-month old CR mice (Reddy Avula et al. 2002), increases in apoptosis were observed in other studies using animals under six months of age (e.g., Grasl-Kraupp et al. 1994; Luan et al. 1995), indicating an absence of consistent age-related changes.

Short-term CR may have resulted in an inhibition of apoptosis compared to *ad libitum* controls, as in the study of Reddy Avula et al. (2002), resulting in the reduced levels of the various caspases. However, the level of apoptosis inhibitor protein XIAP between groups was not different in our study. It is feasible that the increased apoptosis rate observed in certain studies (e.g., James and Muskhelishvili 1994; James et al. 1998; Luan et al. 1995; Muskhelishvili et al. 1995, 1996) may have occurred in a caspase-independent manner (Cregan et al. 2002; Mathiasen et al. 2002). We failed to observe any increases, in any tissue, of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosome), in the short-term caloric restricted rats when compared to the *ad libitum* controls. This parameter would indicate any increase in apoptotic index irrespective of the apoptosis-initiating mechanism, suggesting that there was an overall diminishing of the rate of apoptosis during short-term CR in the tissues measured. However, we did observe significant tissue differences in the levels of DNA fragmentation, with significantly higher levels in the mitotic kidney compared to the post-mitotic heart or gastrocnemius muscle, and the heart having significantly higher levels than the gastrocnemius muscle. This, in addition to the presence of cleaved caspase-7 and -12 only in the kidney, suggests a higher rate of apoptosis in this tissue, giving credence to the suggested tissue-specific nature of apoptosis, with rapid removal of damaged but replaceable cells occurring in mitotic tissues but reduction or inhibition of apoptosis in post-mitotic tissues to reduce the loss of irreplaceable cells, as suggested by Zhang et al. (2002).

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